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I. Nomenclature

ROBERT S. HARRIS

Accepted name: p-Aminobenzoic acid

Obsolete names: Vitamin B_x
B_x factor
Chromotrichia factor
Anticanitic vitamin
Bacterial vitamin H

Empirical formula: C_7H_7NO_2
Chemical name: 4-Aminobenzoic acid

Structure:

\[
\begin{align*}
\text{H}_2\text{N} & \quad \text{\(\text{C} \quad \text{O} \quad \text{H}\)}
\end{align*}
\]

II. Chemistry

LEMUEL D. WRIGHT and PETER A. TAVORMINA

A. PREPARATION

Both reductive and oxidative methods have been used in the synthesis of p-aminobenzoic acid (PABA).

The chemical reduction of p-nitrobenzoic acid has been effected with ammonium sulfide,\(^1\) tin and hydrochloric acid,\(^2,3\) ferrous sulfate and am-

\(^3\) E. Widnmann, *Ann.* 193, 202 (1878).
monia, or sodium hydrosulfide. Catalytic hydrogenation has been carried out with platinum oxide as catalyst. More recently, Ravenscroft and his associates employed electrolytic reduction of the nitro compound to prepare PABA in very high yields.

The oxidation of p-acetotoluide offers an alternative route for the preparation of PABA. Potassium or calcium permanganate has been used for this purpose. Hydrolysis of the p-acetamidobenzoic acid with mineral acid yields the amino compound.

Of special interest are the syntheses leading to isotope-labeled PABA. Murray and his collaborators have described a reaction whereby C$^{14}$ is introduced in the carboxyl group:

\[
\text{H}_2\text{N}\text{-Br} \xrightarrow{\text{n-C}_6\text{H}_5\text{Li}} \left[\text{(Li)}_2\text{N}\text{-Li}\right] \xrightarrow{1, \text{CuO}_2, 2, \text{H}_2\text{O}} \text{H}_2\text{N}\text{-C}^{14}\text{OOH}
\]

PABA labeled with N$^{15}$ has been synthesized by Lustig et al. by heating a solution of p-bromobenzoic acid in a sealed tube with N$^{15}$-labeled ammonia.

B. PHYSICAL PROPERTIES

PABA crystallizes in the form of monoclinic prisms which have a melting point of 186 to 187°. Andrews and his coworkers reported a melting point of 188 to 188.5°. The molal heat capacity of the solid was determined to be 42.5 calories at 25° and 62.2 calories at the melting point, with a heat of crystallization of 5000 calories per mole.

PABA is soluble in water, aqueous alcohol, methanol, isopropanol, butanol, ether, glacial acetic acid, chloroform, and ethyl acetate; sparingly

---

5 J. Hirata, Japanese Pat. 109,708 (Feb. 21, 1935) [C.A. 29, 4776 (1935)].
8 A. W. Hofmann, Ber. 9, 1299 (1876).
9 F. Ullmann and J. B. Uzbachian, Ber. 36, 1797 (1903).
soluble in benzene and carbon disulfide; and insoluble in petroleum ether.\(^{14, 15}\)

The pH of a water solution of PABA at 25° has been reported\(^{16}\) to be 3.79 with the hydrogen electrode, and 3.63 with the antimony electrode. The latter value agrees more closely with the calculated value of 3.60.

The acid and base dissociation constants of PABA have been determined by a number of investigators\(^{17-22}\) to be of the order of \(K_a = 1.2 \times 10^{-5}\) and \(K_b = 1.5 \times 10^{-12}\).

PABA in its isoelectric state in aqueous solution exists in the neutral rather than the zwitterionic form. Evidence for this behavior was first presented by Harris\(^{23, 24}\) in his classic papers on the formol titration of various amino acids. A different approach to the problem was made by Klotz and Gruen.\(^{25}\) By calculating the ionization constants of PABA and of its methyl and ethyl esters from the ultraviolet absorption spectra of the compounds, these authors reached the same conclusion as did Harris. It is interesting to note that sulfanilamide, too, exhibits this behavior at its isoelectric point.

The ultraviolet spectrum of PABA is characterized by two bands, one of high extinction at long wavelengths, the other of low extinction at short wavelengths. For the primary band Kumler\(^{26}\) reports a molar extinction coefficient, in 95 % alcohol, of 17,400 at the wavelength of maximum absorption, 288 m\(\mu\), and an extinction coefficient of 8220, for the secondary band, at 220 m\(\mu\). Kern and his associates\(^{15}\) found the wavelength of peak absorption to be 288 m\(\mu\) in 95 % alcohol or 99 % isopropanol, with extinction coefficients ranging, for several experiments, from 15,760 to 16,250 and from 18,400 to 18,900 for the respective solvents. In aqueous solution, values for \(\lambda_{\text{max}}\) of 266 m\(\mu\),\(^{15}\) 271 m\(\mu\),\(^{27}\) and 284 m\(\mu\)\(^{28}\) have been reported, with extinction coefficients of the order of 14,000.


\(^{16}\) F. Fenwick and E. Gilman, J. Biol. Chem. 84, 605 (1929).


\(^{19}\) A. Albert and R. Goldacre, Nature 149, 245 (1942).


\(^{21}\) K. Winkelblech, Z. physik. Chem. 36, 546 (1901).

\(^{22}\) B. Holmberg, Z. physik. Chem. 62, 726 (1908).

\(^{23}\) L. J. Harris, Biochem. J. 24, 1080 (1930).

\(^{24}\) T. W. Birch and L. J. Harris, Biochem. J. 24, 1080 (1930).


Sodium \( p \)-aminobenzoate exhibits maximum absorption at 266 \( \text{m} \mu \) with an extinction coefficient of 14,700 to 14,900.\(^{15,28}\)

The hydrochloride of PABA has an extinction coefficient of only 970 at the wavelength of maximum absorption, 270 \( \text{m} \mu \).\(^{28}\) In this connection may be mentioned the rule proposed by Kumler and Strait\(^{29}\) that an aromatic amine, in a solution sufficiently acid to produce the salt, will have an absorption spectrum similar to the compound which one would have if the amino (or alkylamino) group were replaced by hydrogen (or an alkyl group). Kumler and Strait found that the spectrum of PABA hydrochloride was remarkably similar to that of benzoic acid\(^{28}\) which exhibits an absorption maximum at 273 \( \text{m} \mu \) with an extinction coefficient of 980.

Riegel and Buchwald\(^{27}\) proposed that the high degree of absorption which is characteristic of neutral PABA-type molecules \((\epsilon = \text{ca. 15,000})\) can be attributed to the hemiquinoid form in which such compounds can exist.

\[
\text{HN=}[\text{C}][\text{OH}][\text{OH}]
\]

The low degree of absorption of aniline in the vicinity of 270 \( \text{m} \mu \) was accounted for on the basis that this substance cannot assume such a structure.

Kumler,\(^{26}\) however, demonstrated that the absorption of \( p \)-dimethylaminobenzoic acid is actually greater than that of PABA but occurs in a region of longer wavelength \((\epsilon = 25,400 \text{ at } 308 \text{ m} \mu )\). Kumler was able to show that, with every increase in basicity of the amino group, peak absorption is shifted to a longer wavelength, and the extinction coefficient becomes greater. These phenomena were explained\(^{26,29}\) on the basis of the resonant forms of this type of molecule, a high degree of absorption being attributable to a structure such as

\[
\text{H}_2\text{N}^+=[\text{C}][\text{OH}][\text{O}^-]
\]

or, in the case of the dimethylamino derivative,

\[
\text{CH}_3\text{N}^+=[\text{C}][\text{OH}][\text{O}^-]\]

This theory can be extended to account for the low absorption of the hydrochloride of PABA, since in the salt form the amino group can no longer resonate with the benzene nucleus or with any group on the ring, so that only the resonance within the ring itself, or between the ring and the carboxyl group can function in absorption.

The high degree of absorption exhibited by the N-alkyl analogs of PABA and their esters occurs at those wavelengths of light which are responsible for sunburn. The investigation of these compounds as potential sun-screening agents has received considerable attention in recent years.

C. CHEMICAL PROPERTIES

PABA is decomposed by prolonged boiling with water to give aniline and carbon dioxide. Concentrated hydriodic acid at elevated temperatures degrades PABA to benzoic acid. Potassium chlorate and hydrochloric acid convert the amino acid to chloranil. Mild oxidation of PABA with sodium hypobromite yields p-azobenzoic acid.

In those reactions involving the amino or carboxyl group PABA exhibits the behavior usually associated with arylamines or benzoic acid-type molecules. Alkylation of the amine function has been carried out with methyl, ethyl, and allyl iodides and a base as well as with dimethyl sulfate and base. By the proper choice of conditions the reaction can be controlled to yield the mono- or dialkylated amine or the trialkyl-p-benzobetaine.

PABA shows no exception to the ease with which aromatic amines and phenols, which are not sterically hindered, can be brominated. Beilstein and Geitner used bromine water to produce a mixture of 3,5-dibromo-4-aminobenzoic acid and 2,4,6-tribromoaniline. Francis and Hill, using a mixture of potassium bromide and potassium bromate in acid medium to generate bromine, found that at 0°C formation of the dibromo compound is favored, whereas above 40°C the tribromoaniline is obtained. The use of iodine monochloride in cold acid solution leads to the formation of 3-ido-

---

35 A. Kwisda, Monatsh. 12, 419 (1891).
36 W. Meigen and E. Nottebohm, Ber. 39, 744 (1906).
38 M. Jaffé, Ber. 38, 1208 (1905).
40 F. Beilstein and P. Geitner, Ann. 189, 1 (1866).
and 3,5-diiodo-4-aminobenzoic acid, whereas iodine and the potassium salt of PABA react to produce p-iodoaniline.42, 43

Diazotization of PABA has been used in the estimation of the vitamin (see section on determination). Saunders44 recommends use of the "inverted" method for the diazotization of monoamino sulfonic or carboxylic acids which otherwise are not completely transformed to the diazonium compound because of their low solubility. The "inverted" method consists in adding the nitrite to the alkaline solution of the amino acid and then running this mixture steadily into the chilled mineral acid. Diazotization takes place instantly, and the insoluble material which forms is the diazo compound.

The formation of such PABA salts as the hydroxylamine salt,45 the triethyl lead salt,46 and the chlorostannate47 has been described. The acid chloride can be prepared in the usual manner using thionyl chloride.48 Esterification can be carried out in the conventional manner, using anhydrous alcohols and dry hydrogen chloride.39 In practice, the preparation of PABA esters is usually accomplished by reduction of the previously esterified nitro compound.49-50

Esters of PABA have long been known to possess valuable properties as local anesthetics.60 Although it is beyond the scope of this review to discuss in great detail this aspect of the properties of PABA, it seems desirable to summarize some of the more important considerations in this

46 H. Gilman and J. D. Robinson, Rec. trav. chim. 49, 766 (1930).
58 J. L. Regnier, British Pats. 477,822-3 (Jan. 3, 1938).
60 A. Einhorn, Ann. 371, 125 (1909).
field. Most local anesthetics derived from PABA have the structure

\[
\begin{array}{c}
\text{H}_2\text{N} - \text{COO} - \text{X} \\
\text{R}_1 \\
\text{R}_2
\end{array}
\]

Table I lists some of the more common anesthetics of this type. The specific substituents for the variables in the general formula are indicated.

<table>
<thead>
<tr>
<th>Compound</th>
<th>X =</th>
<th>R₁ =</th>
<th>R₂ =</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzocaine (anesthesine)</td>
<td>−CH₃</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propesin</td>
<td>−n-C₃H₇</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycloform</td>
<td>−CH(CH₃)₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butesin</td>
<td>−n-C₄H₉</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monocaine</td>
<td>−CH₂CH₃−</td>
<td></td>
<td>−CH⁻</td>
</tr>
<tr>
<td>Amylecaine</td>
<td>−CH₂CH₃−</td>
<td></td>
<td>−H</td>
</tr>
<tr>
<td>Novocaine (procaine)</td>
<td>−CH₃CH₂−</td>
<td></td>
<td>−C₂H₅</td>
</tr>
<tr>
<td>Tutocaine</td>
<td>−CH−CH−CH₂−</td>
<td></td>
<td>−CH₃</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larecaine</td>
<td>−CH₂C−CH₂−</td>
<td></td>
<td>−C₂H₅</td>
</tr>
<tr>
<td>Butyn</td>
<td>−CH₂−CH₂−CH₂−</td>
<td></td>
<td>−n-C₃H₉</td>
</tr>
<tr>
<td>Panthesine</td>
<td>−CH₂−CH⁻</td>
<td></td>
<td>−C₂H₅</td>
</tr>
<tr>
<td>Pontocaine (pantocaine,</td>
<td>−CH₂CH₂−</td>
<td></td>
<td>−CH₃</td>
</tr>
<tr>
<td>tetraecaine) (a p-(N-butyl-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>amino)benzoate)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The nature of the substituents X, R₁, R₂ determines to a large extent the degree of toxicity as well as of anesthetic efficiency of the particular ester. In general, the longer the group X (up to n-butyl), the greater is the anes-
thetic value, a normal carbon chain being more effective than a branched chain. Again, increasing the size of the $R_1$, $R_2$ groups produces compounds of enhanced toxicity and activity, the anesthetic value, however, increasing more rapidly than does the toxic effect.

Hansen and Fosdick have described a sulfur analog of novocaine, "thiocaine":

$$
\text{H}_2\text{N}\begin{array}{c}
\text{COSCH}_2\text{CH}_2\text{N}(	ext{C}_2\text{H}_5)_{2}
\end{array}
$$

This substance has an activity several times that of procaine, and only one-half the toxicity of cocaine.

Fellows has tested some substituted phenyl $p$-aminobenzoates. The hydrochloride of 2-(piperidinomethyl)-4,6-dimethylphenyl-$p$-aminobenzoate was found to be more active topically than cocaine, without producing as high a toxic reaction as does the natural alkaloid.

The preparation and pharmacology of many other anesthetic esters of PABA have been described in the chemical and patent literatures, to which the reader is referred for more detailed information on this subject.

In recent years a number of derivatives of PABA of interest as potential biochemical intermediates have been synthesized. Auhagen has prepared and described the properties of the $N$-(p-aminobenzoyl) derivatives of D-, L-, and DL-leucine, of D-, L-, and DL-glutamic acid, of L-aspartic acid, of glycine, and of glycylglycine.

King and Spensley have synthesized the $N$-(p-aminobenzoyl) derivatives of DL-isoglutamine, of DL-glutamine, and of L-glutamine. Lampen and

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Peterson have obtained the N-D-ribosido- and N-L-arabinosido-p-aminobenzoic acids. N-Glucosido-p-aminobenzoic acid has also been reported. Cherbuliez and Mori mercerated PABA by means of mercuric acetate. The reaction results in the formation of an inner salt in which the mercury atom is linked to the ring in the 2 position. Support for this structure is to be found in the fact that when the internal salt is made to react with iodine the mercury is displaced, and 2-iodo-4-aminobenzoic acid is obtained.

D. DERIVATIVES

A number of solid derivatives useful for the identification of PABA are listed in Table II.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Melting point</th>
<th>Reference</th>
<th>Compound</th>
<th>Melting point</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl</td>
<td>250–251</td>
<td>8, 72</td>
<td>3,5-Dinitrobenzoyl</td>
<td>&gt;290</td>
<td>76</td>
</tr>
<tr>
<td>Benzoyl</td>
<td>278</td>
<td>73</td>
<td>3,5-Dinitrobenzoate salt</td>
<td>195</td>
<td>76</td>
</tr>
<tr>
<td>Benzylamide</td>
<td>89–90</td>
<td>74</td>
<td>Pieryl</td>
<td>287–288</td>
<td>77</td>
</tr>
<tr>
<td>m-Nitrophenylthiourea</td>
<td>221–222</td>
<td>75</td>
<td>s-Trinitrobenzene add’tion epd.</td>
<td>151</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>s-Trinitrobenzene add’n 111(d)</td>
<td>epd. (K salt)</td>
<td>78</td>
</tr>
</tbody>
</table>

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70 A. Dansi, *Farm. sci. e tcc. (Pavia)* 2, 195 (1947) [C.A. 42, 639 (1948)].
72 A. Kaiser, *Ber.* 18, 2942 (1885).
E. COLOR REACTIONS

A number of color reactions have been described with reference to PABA. One such reaction, which may be used as a qualitative test for the acid, is to be found in Mulliken.\textsuperscript{7} The addition of one drop of a saturated solution of bleaching powder to an aqueous solution of the acid produces a red to violet coloration which changes to an opaque orange after 5 minutes.

The colorimetric method for the determination of sulfanilamide developed by Marshall and his colleagues\textsuperscript{73-81} and subsequently modified by Bratton and Marshall\textsuperscript{82} has since been applied to the estimation of PABA.\textsuperscript{83} The basis for the method is the formation of a red to purple dye which results when diazotized PABA is made to couple with a suitable arylamine.

When PABA in glacial acetic acid solution is made to react with a 1% solution of p-dimethylaminobenzaldehyde in the same solvent, a deep-yellow color is produced. Tauber and Laufer\textsuperscript{84} have described a quantitative assay for PABA based on this reaction. The test is not given by the aliphatic amino acids or by tyrosine, phenylalanine, glutathione, urea, pantothenic acid, niacin, niacinamide, or thiamine. Substances which were found to give rise to a similar coloration (e.g., the o- and m- aminobenzoic acids and their esters, aniline and some of its derivatives) are not normally present in biological materials.

Kirch and Bergeim\textsuperscript{85,86} observed that diazotized thiamine will couple with PABA to yield a red-colored dye.

Nakahara and his associates\textsuperscript{87} have devised a scheme based on color tests:

\[
\begin{align*}
\text{CH}_3 & \text{N} \begin{array}{c} \text{CHO} \end{array} + \text{H}_2\text{N} \begin{array}{c} \text{COOH} \end{array} \rightarrow \\
\text{CH}_3 & \text{N} \begin{array}{c} \text{CH=NM} \end{array} \begin{array}{c} \text{COOH} \end{array}
\end{align*}
\]

assay for PABA based on this reaction. The test is not given by the aliphatic amino acids or by tyrosine, phenylalanine, glutathione, urea, pantothenic acid, niacin, niacinamide, or thiamine. Substances which were found to give rise to a similar coloration (e.g., the o- and m- aminobenzoic acids and their esters, aniline and some of its derivatives) are not normally present in biological materials.

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\text{CH}_3 & \text{N} \begin{array}{c} \text{CH=NM} \end{array} \begin{array}{c} \text{COOH} \end{array}
\end{align*}
\]
for the differentiation of the isomeric aminobenzoic acids. Three samples of the solution to be examined are treated individually with potassium ferrocyanide, potassium ferricyanide, and sodium nitrosylopentacyanoferrate (II), and the three mixtures are exposed to sunlight. Table III indicates the colors formed in this test. These colored complexes can be extracted with butanol from the acidified aqueous solutions.

Other reactions in which the production of color has been attributed to the presence of PABA have appeared in the literature. Mayer\(^8\) found that Myobacterium tuberculosis (human strain) when grown on a medium containing PABA elaborates a yellow pigment. The process is enzymatic and requires ions of magnesium and iron. Glucose, ascorbic acid, or sodium cyanide represses formation of the pigment. Little is known concerning the nature of this material, other than that it appears to be some oxidation product of PABA.

### TABLE III

<table>
<thead>
<tr>
<th>Acid</th>
<th>K(_4)Fe(CN)(_6)</th>
<th>K(_3)Fe(CN)(_6)</th>
<th>Na(_2)Fe(CN)(_5)NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>(o)-Aminobenzoic</td>
<td>Brown</td>
<td>Blue-green</td>
<td>Brown-yellow</td>
</tr>
<tr>
<td>(m)-Aminobenzoic</td>
<td>Violet</td>
<td>(Colorless)</td>
<td>Brown-yellow</td>
</tr>
<tr>
<td>(p)-Aminobenzoic</td>
<td>(Colorless)</td>
<td>(Colorless)</td>
<td>Brown-red</td>
</tr>
</tbody>
</table>

### III. Industrial Preparation

H. M. WUEST

The role of \(p\)-aminobenzoic acid in human nutrition has not been established, nor is a deficiency disease in higher animals known that is caused by its absence in the feed. The industrial importance of the compound as a vitamin is therefore very limited. PABA and its salts, however, are recommended in ricketsial diseases, including Rocky Mountain spotted fever and scrub typhus (4 to 6 g., daily).\(^1\)

The U. S. Pharmacopeia describes PABA as a reagent (not as a pharmaceutical) and gives criteria for its purity.\(^2\) The industrial preparation starts from \(p\)-nitrotoluene, which is oxidized (e.g., with dilute nitric acid) to \(p\)-nitrobenzoic acid; the reduction to the amino acid is done with tin or iron and hydrochloric acid or by catalytic hydrogenation.

IV. BIOCHEMICAL SYSTEMS

Production, sales, and value of sales have decreased considerably in 1952 over 1951, as the following figures show: 2

<table>
<thead>
<tr>
<th></th>
<th>1951</th>
<th>1952</th>
</tr>
</thead>
<tbody>
<tr>
<td>Production, lb.</td>
<td>191,000</td>
<td>122,000</td>
</tr>
<tr>
<td>Sales, lb.</td>
<td>169,000</td>
<td>90,000</td>
</tr>
<tr>
<td>Value of sales</td>
<td>$595,000</td>
<td>$276,000</td>
</tr>
</tbody>
</table>

The price of the acid (U.S.P.) in September, 1953, was $2.50, and $1.77 for 1 lb. technical grade. 3

The important role of \( p \)-aminobenzoic acid in the production of folic acid (pteroylglutamic acid) is mentioned on p. 33.

IV. Biochemical Systems

LEMUEL D. WRIGHT and PETER A. TAVORMINA

The demonstration that a substance is an essential component of a system which functions as a biological catalyst is perhaps one of the most useful criteria for a vitamin. Such a relationship has been shown for many of the B vitamins. The relative difficulty of freeing PABA from some of its conjugate forms suggested to Miller and his associates 1 that PABA may exist in nature in combination with a protein, that is, that it may function as the prosthetic group of some enzyme. A number of crystalline enzymes were examined for their PABA content, which was found to range from 13 \( \gamma \) per gram of phosphorylase to 130 \( \gamma \) per gram of yeast polypeptidase. It is quite apparent from a consideration of the molecular weights usually assigned to these proteins that the vitamin was present as an impurity, rather than as an integral part of the enzymes studied.

Williams and his coworkers 2 investigated a wide variety of proteins, hormones, and viruses and found that in all cases their PABA content was too low to be a constituent part of the molecule.

Despite the negative results obtained thus far in the search for a specific enzyme with which PABA is associated, a number of interesting publications have appeared concerning enzymatic systems which involve PABA or its conjugates. Cohen and McGilvery 3-5 investigated the enzymatic con-

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2 U. S. Tariff Comm. Repts. 175, 36 (1951); 190, 33 (1952).
3 Oil, Paint and Drug Repr. September 14, 1953.
jugation of PABA and glycine as a model system for the study of peptide bond formation. Rat liver and kidney cortex slices (but not heart, testis, muscle, brain, or spleen) were found to carry out the synthesis of p-aminobenzonic acid (PAHA). The reaction is aerobic and will not proceed in the presence of cyanide, arsenite, iodoacetate, azide, fluoride, or malonate.\(^5\) Adenosinetriphosphate, however, will support the synthesis anaerobically. Potassium and magnesium ions stimulate the conjugation, phosphate is without effect, and calcium is inhibitory.\(^4\) In order to maintain the reaction at low tissue concentrations the addition of members of the citric acid cycle was found necessary. Under anaerobic conditions the addition of diphosphopyridine nucleotide (DPN) inhibits the synthesis, presumably by diverting to other reactions the energy furnished by ATP. The enzyme system responsible for PAHA synthesis was found associated with the large insoluble particles of the liver cells and is unstable to freezing, acetone-drying, and non-isotonic concentrations of salt.\(^6\) Cohen and McGilvery suggest the name “synthetase” for enzymes involved in the formation of amides and esters exclusive of phosphate esters.

The findings of Cohen and McGilvery regarding PAHA formation were confirmed by Beyer and his associates.\(^6\) The latter workers, employing guinea pig liver and renal cortex, observed that conjugation is essentially complete in 1 hour. Beyer \textit{et al.}\(^6\) report that “benemid” produces 70\% in-

\[
\begin{align*}
\text{CH}_3\text{CH}_2\text{CH}_2 & \quad \text{NSO}_2 \quad \text{COOH} \\
\text{CH}_3\text{CH}_2\text{CH}_2 & \quad \text{“Benemid”}
\end{align*}
\]

hibition of the synthesis at a concentration of 25 \times 10^{-4} \text{ M}, without, however, affecting the oxygen uptake in the aerobic system. PAHA formation in the ATP-anaerobic system is also inhibited by “benemid.” The compound has no effect, however, on the \textit{in vitro} phosphorylation of glucose. The authors interpret the data to mean that “benemid” exerts its inhibitory action specifically by obstructing the utilization of energy by the conjugase, rather than by affecting the production of high energy phosphates.

The inhibitory action of a nitrogen mustard (methylbis-\(\beta\)-chloroethylamine hydrochloride) on PAHA formation in rat liver slices is similarly interpreted by McKinney\(^7\) as being directed specifically toward the “synthetase” rather than involving the reactions which produce or utilize energy.


Recently, Woolley\(^8\) reported that the thiaminase found in an extract of carp viscera when incubated with synthetic 2-amino-1-hydroxy-6-pteridylmethyl-(4'-methyl-5'-hydroxyethylthiazolium) bromide and PABA will yield pteroic acid. Substitution of \(p\)-aminobenzoylglutamic acid for PABA gives rise to folic acid. Woolley points out that the specificity of the enzyme appears to be for the thiazolium group, and that this is not necessarily the mechanism whereby PABA is transformed into folic acid. The experiment is especially worthy of note in that a new biosynthetic mechanism is presented, i.e., one in which the energy for the reaction is derived from a quaternary ammonium ion rather than from a high-energy phosphate bond.

Baur and Rüf\(^9\) were able to demonstrate that low concentrations of PABA, or of sulfanilic acid, sulfathiazole, sulfanilamide, and sulfapyridine, inhibit to a considerable extent the air oxidation of hydroquinone as well as the degradation of tyrosine by potato tyrosinase.

Another indication that PABA exerts some influence on an enzyme system is to be found in the work of Mayer,\(^10\) who observed that the growth of *Mycobacterium tuberculosis* in a medium containing PABA is attended by the formation of a yellow pigment. The pigment appears to consist, in part at least, of some oxidation product of PABA, produced by a specific oxidase which requires ions of magnesium and iron for activity. As might well be anticipated, glucose, ascorbic acid and cyanide repress the enzymatic action, and pigment is not produced.

According to Makino and Yamamoto\(^11\) rabbit and cattle livers contain an enzyme which cleaves folic acid. The authors, using the methylene blue


technique, found that the enzyme can oxidize L-glutamic acid or p-aminobenzoylglutamic acid in essentially the same length of time as is required for pteroylglutamic acid oxidation, which occurs without any detectable formation of either PABA or p-aminobenzoylglutamic acid. They interpret this to mean that folic acid is not oxidized as such but is hydrolyzed to pteroyl acid and glutamic acid, the latter being the agent responsible for the reduction of the methylene blue. Accordingly, the enzyme must attack the amide bond of folic acid. It would appear interesting, when one considers the specificity of enzyme action, to investigate the behavior of this enzyme toward other PABA conjugates, such as PAHA or the bound PABA reported by Ratner.¹² ¹³

V. Biogenesis

LEMUEL D. WRIGHT and PETER A. TAVORMINA

The biogenesis of PABA is only one aspect of a much larger subject, that of the biogenesis of aromatic compounds in general.

Quinic acid has been suggested as a precursor of aromatic compounds. By the loss of three molecules of water this compound could yield p-hydroxybenzoic acid. Quinic acid when administered to man does indeed yield urinary hippuric acid.¹ ² Phenolic compounds are produced from quinic acid by a variety of microorganisms including molds, yeast, and bacteria.³

Quinic acid will promote growth of a Neurospora mutant with a requirement for an aromatic compound, although it is only about 10% as active for this purpose as tryptophan.⁴ On the other hand, quinic acid is inactive in promoting growth of any one of a variety of Escherichia coli mutants with nutritive requirements for two to five aromatic compounds.³

meso-Inositol has been suggested by Fischer⁵ as a precursor of the aromatic ring. Aromatization conceivably could result from a loss of three molecules of water. No actual experimental evidence indicating that PABA originates from inositol has been published.

¹ E. Lautemann, Ann. 125, 9 (1863).
² A. J. Quick, J. Biol. Chem. 92, 65 (1931).
⁵ H. O. L. Fischer, Harvey Lectures 40, 156 (1944-1945).
Phloroglucinol has been implicated by Shive\(^\text{6}\) as a possible intermediate in the biogenesis of the aromatic ring. These studies involved a system where a strain of *Leuconostoc mesenteroides* that requires pantothenic acid and either acetate or aromatic amino acids for growth is inhibited by dl-N-pantoyl-\(n\)-butylamine. Pantothenic acid reverses the inhibitor with an index of about 300 in the presence of acetate. In the presence of phenylalanine, tryptophan, or tyrosine the inhibition index is increased to 3000. Phloroglucinol, particularly in the presence of increased phosphate, is just as effective as the aromatic amino acids. These data were interpreted to indicate that pantothenic acid functions in the conversion of acetate to an intermediate, such as phloroglucinol, common to the biogenesis of the aromatic amino acids and presumably of PABA. Phloroglucinol is inactive in promoting growth of certain *Escherichia coli* mutants\(^3\) with a nutritive requirement for aromatic amino acids.

A scheme for the synthesis of aromatic compounds, including PABA, has been advanced by Davis\(^4\) as a result of the study of a variety of mutants of *Escherichia coli* selected by the penicillin method.\(^7-\text{9}\) Mutants were obtained in this manner with requirements for two or more of the aromatic compounds tyrosine, phenylalanine, tryptophan, and PABA. For certain mutants with a requirement for the above four aromatic compounds, shikimic acid was found to duplicate the growth-promoting effect of a combina-

---


tion of phenylalanine, tyrosine, tryptophan, and PABA. For rapid growth one such strain requires, in addition, \textit{p}-hydroxybenzoic acid (POB).\textsuperscript{10} Certain other mutants, blocked further along in the pathway of aromatic synthesis so that they require tyrosine and phenylalanine for growth, accumulate shikimic acid and another compound (compound X) in the medium.\textsuperscript{3, 10, 11}

Compound X has been isolated in crystalline form and is characterized as follows: m.p. 150 to 152°; \([\alpha]^{28}_D = 52.1 \pm 1.0°; \lambda_{\text{max}} \text{(in ethanol)} 233 \text{ m}\mu;\]

\[
\begin{align*}
3\text{CH}_3\text{COOH} &\xrightarrow{\text{pantothenic acid}} \text{HO-\text{OH} } \rightarrow \text{ [Unknown intermediates]} \\
&\text{HO-\text{OH}} \rightarrow \text{Phenylalanine} \\
&\text{HO-\text{OH}} \rightarrow \text{Tryptophan} \\
&\text{HO-\text{OH}} \rightarrow \text{Tyrosine} \\
&\text{HO-\text{OH}} \rightarrow \text{PABA}
\end{align*}
\]

log, 4.0; pK 3.2; C, 48.83; H, 4.85; molecular weight 162; neutralization equivalent 162, 179; reduces Fehling's and Tollen's reagents; forms crystalline semicarbazone, phenyllosazone, methyl ester, and acetate. Compound X has recently been identified as a 5-dehydroshikimic acid.\textsuperscript{11}

A tentative and necessarily incomplete outline of aromatic biosynthesis in \textit{Escherichia coli} may be summarized according to Davis as follows:

Evidence has been presented by Davis\textsuperscript{12} indicating that PABA participates in the synthesis of vitamin B\textsubscript{12}, since PABA has a sparing effect on the B\textsubscript{12} requirement of certain \textit{Escherichia coli} mutants. Although a cata-

\textsuperscript{10} B. D. Davis, \textit{Nature} \textbf{166}, 1120 (1950).
lytic function for PABA was not excluded, structural origin of the benzeno
ing of vitamin B\textsubscript{12} from PABA was the favored hypothesis.

VI. Estimation

LEMUEL D. WRIGHT and PETER A. TAVORMINA

A. CHEMICAL METHODS

PABA, being a primary aromatic amine, diazotizes with nitrous acid, and the resulting diazo compound then couples with various other aromatic compounds to yield colored products that may be determined spectrophotometrically. The Bratton-Marshall method\textsuperscript{1} for the determination of sulfanilamide as applied to the determination of PABA entails diazotization, removal of excess nitrous acid with ammonium sulfamate,\textsuperscript{2} and coupling of the diazo product with N-(1-naphthyl)ethylenediamine dihydrochloride. This affords the basis of the preferred chemical method for PABA deter-

\begin{align*}
H_2N&\text{-}\begin{array}{c}
\text{COOH}
\end{array} \xrightarrow{\text{NaNO}_2/\text{HCl}} \text{HOOC}-\begin{array}{c}
\text{-N}_2^+\text{Cl}^-
\end{array} \\
\text{H} \quad & N-\text{CH}_2\text{CH}_2\text{NH}_2 \\
\text{HOOC}-\begin{array}{c}
\text{-N}_2^+\text{Cl}^-
\end{array} & + \begin{array}{c}
\text{N=CH}_2\text{CH}_2\text{NH}_2
\end{array} \xrightarrow{\text{H}} \\
\quad & \begin{array}{c}
\text{N=CH}_2\text{CH}_2\text{NH}_2
\end{array}
\end{align*}

mination. The colored compound formed by coupling diazotized sulfanila-

Several advantages derive from the use of this amine: ease of purification of the diamine, rapid coupling, greater solubility of the dye, increased sensitivity, elimination of the use of buffer, and stability of the color.

Prior to the use of N-(1-naphthyl)ethylene diamine as the coupling agent, N,N-dimethyl-1-naphthylamine and N-ethyl-1-naphthylamine had been advocated as coupling agents. The determination of PABA in which N,N-dimethyl-1-naphthylamine is the coupling agent has been described by Eckert.

\[
\begin{align*}
\text{N,N-Dimethyl-1-naphthylamine} & \\
\text{N-Ethyl-1-naphthylamine} & \\
\end{align*}
\]

In the original Marshall method in which the coupling agent is N,N-dimethyl-1-naphthylamine, metals and thiocyanate interfere with color production. Presumably these objections also apply to the improved method where the coupling agent is N-(1-naphthyl)ethylene diamine.

A disadvantage in the Bratton-Marshall method for the determination of PABA is that the color reaction is not specific and a variety of biological compounds all give essentially the same color reaction. Among the known biological compounds that interfere are 5(4)-amino-4(5)-imidazolecarboxamide, kynurenine, anthranilic acid, tryptophan, and indole.

The Bratton-Marshall method has been applied extensively in studies of PABA and/or other ary lamines formed by sulfonamide-sensitive and -resistant strains of staphylococci.

11 M. G. Sevag and M. N. Green, J. Bacteriol. 48, 615 (1944).
PABA also may be determined chemically by a procedure in which diazotized thiamine couples with PABA to yield a colored product that is measured quantitatively in the spectrophotometer after extraction into isoamyl alcohol.

*p*-Dimethylaminobenzaldehyde reacts with aromatic amines to give a yellow color. This reaction has been suggested as a method for the determination of sulfonamides and PABA. The compound responsible for the color with sulfanilamide is *p*-dimethylaminobenzylidene-*p*-aminobenzene-sulfonamide. Presumably the compound responsible for the color with PABA is the following:

\[
CH_3 \begin{array}{c} \text{N} \\ \text{CH} = \text{N} \end{array} \begin{array}{c} \text{COOH} \\ \text{CH}_3 \end{array}
\]

\[\text{p-Dimethylaminobenzylidene-}p\text{-aminobenzoic acid}\]

*R* values for *o*-, *m*-, and *p*-aminobenzoic acid by paper chromatography in the system butanol saturated with 5 N NH₄OH were found to be 0.38, 0.19, and 0.12, respectively, by Lederer. *R* values by paper chromatography for PABA, PAHA (*p*-aminohippuric acid), and PASA (*p*-aminosalicylic acid) in the system *n*-butanol-water have been published by Kelemen et al. These values may be of aid in the quantitative determination of PABA and related compounds, particularly in urine.

B. MICROBIOLOGICAL METHODS

Prior to the discovery of microorganisms with a growth factor requirement for PABA, a number of investigators had examined natural material microbiologically for sulfonamide inhibitor. A test for "sulfonamide inhibitor" that depends upon the activity of natural material in reversing the bacteriostatic activity of sulfapyridine for *Escherichia coli* was employed.
by MacLeod\textsuperscript{26} in a survey of a wide variety of substances of animal and bacterial origin. "Sulfonamide inhibitor" is found in many tissue extracts. The amount found is greatly increased by prior autolysis or acid hydrolysis. In certain species of bacteria the inhibitor is found in the cells only and is not demonstrable in the culture medium, whereas in other species the inhibitor is found in the culture supernatant, and the cells themselves are relatively free. Sulfonamide resistance (fastness) in a strain of pneumococcus is accompanied by greatly increased production of "sulfonamide inhibitor."

MacLeod was quite aware of the limitations of a microbiological assay that depends upon the reversal of sulfonamides for the determination of PABA (see section on sulfonamides). He found that the "sulfonamide inhibitor" present in some natural materials is not extractable by ether at an acid pH as would be expected if it were PABA.

The first essentially specific microbiological method for the determination of PABA was proposed by Landy and Dicken.\textsuperscript{26} Their method was based on the primary observations of Underkoffler \textit{et al.}\textsuperscript{31} and Lampen \textit{et al.}\textsuperscript{32} that \textit{Acetobacter suboxydans} requires PABA as a growth factor. Their original basal medium contained a purified acid hydrolyzate of casein, plus tryptophan and cystine as nitrogen sources, glycerol as an energy source, inorganic salts, and the only other growth factors required by the organism, namely, pantothenic and nicotinic acids. With this basal medium the organism responds linearly to PABA over a concentration range of 0 to 0.03 γ of PABA per 10 ml. of medium. A variety of compounds with some relationship to PABA in structure were examined for activity, but no compound examined had more than 2% of the activity of PABA. Water extracts or solutions of natural materials were examined for apparent PABA content. Yeast and yeast extract were by far the best sources of PABA studied.

Landy and Streightoff\textsuperscript{33} subsequently have shown that the incorporation of purines (adenine alone in relatively large amounts or a mixture of adenine, guanine, and xanthine) in the original basal medium greatly increases the sensitivity of response of \textit{Acetobacter suboxydans} to PABA. In the absence of purines (original medium) a response to PABA could be detected at a level of 0.01 γ per 10 ml. of culture. The inclusion of purines permits a detectable response to PABA at a level of 0.001 γ per 10 ml. Cheldelin and Bennett\textsuperscript{34} have modified the Landy-Streightoff medium by the further inclusion of glucose, Norit-treated peptone, Norit-treated liver, and addi-

\textsuperscript{34} V. H. Cheldelin and M. J. Bennett, \textit{J. Biol. Chem.} \textbf{161}, 751 (1945).
tional Norit-treated hydrolyzed casein. These modifications were found to promote more growth of *Acetobacter suboxydans* in response to PABA than had previously been obtained.

The *Acetobacter suboxydans* method for the determination of PABA has been applied by Landy *et al.*\(^{11}\) and by Spink *et al.*\(^{15}\) to a study of PABA production by sulfonamide-sensitive and -resistant bacteria.

A microbiological method for PABA determination was proposed by Lewis\(^{35}\) that depends upon the essential nature of PABA for *Lactobacillus arabinosus*.\(^{36}\) The strain employed, the basal medium, and the cultural details are quite similar to those outlined by Snell and Wright\(^{37}\) for the microbiological determination of nicotinic acid. It is essential that the acid-hydrolyzed, vitamin-free casein used as the main source of nitrogen be vigorously Norit-treated to remove traces of PABA or other contaminating, microbiologically active material. The *Lactobacillus arabinosus* method is an extremely sensitive method for the determination of PABA with a response range to the compound of about 0 to 0.0005 \(\gamma\) per 10 ml.

Considerable difficulty has been experienced in a number of laboratories in the use of this method for the determination of PABA because of excess growth in the "blanks" (no added PABA). Although contamination of the glassware and the medium with PABA appears to be the most plausible explanation for the difficulty, it is now the conclusion of a number of investigators,\(^{38}\) including also the reviewers, that spontaneous mutants of *Lactobacillus arabinosus* are produced with such frequency that selection of these mutants readily occurs. For this reason most laboratories have abandoned the use of the *Lactobacillus arabinosus* method for the determination of PABA.

The *Lactobacillus arabinosus* method has been applied by Spink *et al.*\(^{15}\) to a study of PABA synthesis by sulfonamide-sensitive and -resistant strains of staphylococci.

A microbiological assay for PABA has been developed by Mitchell *et al.*\(^{39}\) and by Thompson *et al.*\(^{40}\) that depends upon the essential nature of PABA for an x-ray induced mutant of *Neurospora crassa* discovered by Tatum and Beadle.\(^{41,42}\) The latter investigators determined that a variety of compounds related in structure to PABA do not promote growth of the mutant, that both the mutant and the normal parent strain are inhibited by sulfanil-

---


amide, that PABA reverses sulfanilamide inhibition of both strains, and that the presence of benzoic acid, \( p \)-hydroxybenzoic acid, or tyrosine does not increase the amount of PABA synthesized by the parent strain.

In the development of an assay method for PABA with the \textit{Neurospora crassa} mutant it was observed by Thompson \textit{et al.}\textsuperscript{39, 40} that water extraction, autolysis, or enzymatic digestion of natural materials is not always sufficient to release all the PABA contained in the sample. Autoclaving at 120° for 1 hour with 6 \( N \) \( H_2SO_4 \) was the procedure finally recommended for release of "bound" PABA.

As originally described, the \textit{Neurospora crassa} method involves the preparation of a number of petri plates, each containing basal medium supplemented with a definite amount of PABA solution or material to be assayed. An inoculum block is then placed on each plate and, following a 20-hour period of growth, the diameter of the mold growth surrounding the inoculum block is measured with calipers and is dependent on the amount of PABA in the culture plate. From a response curve obtained with PABA the potency of unknown samples may be calculated. Although the authors claim a number of advantages for this method of assay, including rapidity and freedom from contamination due to the short incubation period employed, it would appear that the method is subject to a number of possible sources of error owing to the cumbersome method of inoculating the test plates.

Agarwala and Peterson\textsuperscript{43} have described procedures for the determination of PABA with the PABA-less mutant of \textit{Neurospora crassa} in which the mold is grown in liquid culture. Following growth of the organism in response to PABA or unknown material, the mycelial pads are removed from the flasks in which they grew and are dried and weighed in the conventional manner. A variety of compounds (see Table IV) related to PABA in structure, including folic acid, were found to be devoid of microbiological activity. Acid and alkaline hydrolysis were studied as methods for the liberation of "bound PABA." Although either method of hydrolysis gives higher apparent PABA values than are obtained without hydrolysis, the authors point out that the increments observed probably represent destruction of folic acid. Since the structure of folic acid was unknown at the time that most of the microbiological methods for the determination of PABA were worked out, many of the data concerning the distribution of "bound PABA" (see Table V, p. 30) obtained by microbiological methods of assay are subject to re-evaluation.

The essential nature of PABA (along with biotin) for \textit{Clostridium acetobutylicum} forms the basis for the microbiological methods for the determination of PABA with this organism.\textsuperscript{44-47}


Lampen and Peterson have described a rapid turbidimetric method involving a basal medium containing Norit-treated casein as the only semipurified component. Anaerobic conditions for the growth of Clostridium acetobutylicum were established by the use of sodium hydrosulfite (Na₂S₂O₄) and reduced iron as components of the basal medium, and by incubating the assays in an "oat jar," where respiration of the oats establishes a partial pressure of CO₂. The assay range is approximately 0.3 to 1.5 mg of PABA per 10-ml culture tube. Lampen and Peterson, confirming other investigators, found PABA to exist in many natural materials, including water-soluble products in microbiologically unavailable forms. They reported that combined PABA may be released by alkaline hydrolysis but that strong acid hydrolysis destroys PABA.

Housewright and Koser utilized Clostridium acetobutylicum for PABA determination with a basal medium containing all purified components. Anaerobic conditions for the assay were established by alkaline pyrogallol. A number of compounds related in structure were examined by Housewright and Koser for microbiological activity. p-Nitrobenzoic acid, p-aminobenzoylglycine and p-nitrobenzoylglycine are essentially as active as PABA. Certain other related compounds are considerably less active. As with Acetobacter suboxydans, less PABA is required for maximum growth when the basal medium contains added purines (adenine, guanine, and xanthine). The microbiological method was applied to a study of PABA synthesis by sulfonamide-sensitive and sulfonamide-resistant strains of Staphylococcus aureus. The findings of Landy et al. and Spink et al. that resistant strains produce considerably more PABA were confirmed.

Mirick has proposed the use of a pseudomonas organism isolated from soil for the highly specific determination of PABA. A specific adaptive enzyme is formed by this organism which oxidizes PABA presumably to CO₂, H₂O, and NH₃. Diazotizable amine is determined on samples before and after the action of the enzyme. The difference obtained is attributed to PABA. A few related compounds are attacked by the enzyme, but none of the compounds thus oxidized undergoes the diazo reaction. The main limitation of the method is primarily a matter of sensitivity of the diazo

### TABLE IV

**Specificity of Microbiological Methods for the Determination of PABA**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Clostridium acetobutylicum</th>
<th>Acetobacter suboxydans</th>
<th>Neurospora crassa&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Lactobacillus arabinosus</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Aminobenzoic acid (PABA)</td>
<td>100 (49)</td>
<td>100 (30)</td>
<td>100 (40)</td>
<td>100 (35)</td>
</tr>
<tr>
<td>o-Aminobenzoic acid</td>
<td>0.01-0.1 (49); 0.001 (51)</td>
<td>0 (30)</td>
<td>0 (41, 42)</td>
<td>0.00005 (35)</td>
</tr>
<tr>
<td>m-Aminobenzoic acid</td>
<td>0.01-0.1 (49); 0.001 (51)</td>
<td>0 (30)</td>
<td>0 (41, 42)</td>
<td>0.009 (35)</td>
</tr>
<tr>
<td>p-Nitrobenzoic acid</td>
<td>90-100 (49)</td>
<td></td>
<td>1.0-1.2 (41, 42)</td>
<td>0 (40)</td>
</tr>
<tr>
<td>p-Aminophenylacetic acid</td>
<td>0.01 (49)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.6 (30)</td>
<td>0.08 (52)</td>
<td></td>
</tr>
<tr>
<td>Sulfanilic acid</td>
<td>0 (49)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Nitroaniline</td>
<td>0 (49)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Nitrobenzaldehyde</td>
<td>1.0 (49)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Dimethylaminobenzalde-</td>
<td>0.1 (49)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>hyde</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Amino-o-Aminophenylacetic acid</td>
<td>0.1 (49)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl-p-aminobenzoate</td>
<td>10 (49)</td>
<td>0.06 (30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methyl-p-aminobenzoate</td>
<td>8-10 (49)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>10 (49); 1 (51)</td>
<td>2.0 (30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Aminobenzoylglycine</td>
<td>100 (49)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Nitrobenzoylglycine</td>
<td>100 (49)</td>
<td>0.35 (30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Chloroacetetylbenzoylglycine</td>
<td>10 (49)</td>
<td>0.2 (30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Aminobenzaldehyde</td>
<td>100 (44, 51)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Aminobenzamide</td>
<td>0.001 (51)</td>
<td>3 (40)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Aminobenzyl alcohol</td>
<td>0 (51)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>p-Aminophenol</td>
<td>0 (51)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Benzamide</td>
<td>0 (51)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aniline</td>
<td>0 (51)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>0 (51)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Acetamidobenzoic acid</td>
<td>0.04 (53)</td>
<td>0 (30)</td>
<td>0 (40); 2.6 (41, 42)</td>
<td></td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>0.8 (53)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-d-Ribosido-p-aminobenzoic acid</td>
<td>87 (53)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Hydroxybenzoic acid</td>
<td>0 (44, 51)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Benjamidobenzoic acid</td>
<td>10 (51)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Fluoro-4-aminobenzoic acid</td>
<td>38 (52)</td>
<td></td>
<td>38 (52)</td>
<td></td>
</tr>
<tr>
<td>2-Bromo-4-aminobenzoic acid</td>
<td>1.05 (52)</td>
<td>1.05 (52)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Jodo-4-aminobenzoic acid</td>
<td>0.10 (52)</td>
<td>0.19 (52)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Aminoisophthalic acid</td>
<td>0.13 (52)</td>
<td>0.13 (52)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Glycylaminobenzoic acid</td>
<td>9.0 (39)</td>
<td></td>
<td></td>
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</tbody>
</table>

**Folic acid and related compounds**

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<tr>
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<tbody>
<tr>
<td>See text</td>
<td>See text</td>
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<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Each of the following compounds also has been found to be inactive as a source for Neurospora crassa: p-aminobenzyol-β-alanine, p-aminobenzyol-L-aspartic acid, p-aminobenzyol-D-aspartic acid, p-aminomuramic acid, p-aminobenzoylglutamic acid, p-aminobenzoyl-α-glutamyl-γ-glutamylglutamic acid, tetrachloroethyl ester (also the corresponding γ,α and α,α compounds), p-nitrobenzoyl-α-glutamyl-γ-glutamic acid tetrachloroethyl ester, p-nitrobenzoyl-L-a-minoethyl acid, p-nitrobenzoyl-γ-glutamyl-β-alanine, p-nitrobenzoyl-L-threonine, p-nitrobenzoyl-L-methionine, p-nitrobenzoyl-α-glutamylglutamic acid, sodium fumarate, pteroyl-γ-glutamyl-γ-glutamylglutamic acid, pteroylmonoglutamic acid.  

<sup>b</sup> Stated by Rubbo and Gillespie to be ten times as active as PABA.
reaction which requires the presence of PABA to the extent of at least one part per million.

VII. Occurrence in Foods

LEMUEL D. WRIGHT and PETER A. TAVORMINA

A variety of natural materials of both plant and animal origin have been found to contain PABA (Table V). The vitamin occurs not only as such but also in conjugated form. Thompson and his colleagues\(^1\) have reported that approximately 80% of the PABA content of animal tissues is in the bound form, whereas in plants the bound form represents only 44% of the total PABA.

Several procedures have been utilized for the liberation of the PABA from the conjugate form. Autolysis,\(^2,4\) enzymes,\(^3,8\) and acid\(^1,3,5\) and alkaline\(^1,3,6,7\) hydrolysis all have been employed. Although it is generally accepted that autolytic and enzymatic methods free only part of the bound PABA,\(^2,3,8\) there is little agreement concerning which of the two alternative procedures is the more desirable. Thompson et al.\(^1\) reported that pure PABA is destroyed in part by either acid or basic hydrolysis. Lampen and Peterson\(^2\) obtained maximum values for liver PABA, using 5 N alkali. Pure PABA added to a liver sample prior to hydrolysis is recovered in 92 to 100% yields. Lampen and Peterson suggest that part of the bound PABA in natural materials is in a form which resists acid hydrolysis. On the other hand, Pennington\(^5\) reports that tissue PABA, but not pure PABA, is virtually completely destroyed by alkaline hydrolysis.

The first indication that not all of the PABA of yeast is in the free state was the report by Loomis and his associates\(^9\) of an antisulfanilamide fraction from yeast which was ether-insoluble, not diazotizable, and not inactivated by acetylation. Blanchard\(^1\) observed that yeast has a higher PABA content after autolysis, and he attributes this to the presence in yeast of a

bound PABA. The conjugated form was thought to be a peptide involving the arylamino group, since diazotization was found to occur only after autolysis.

A second form of bound PABA was discovered by Ratner and her collaborators.\textsuperscript{10,11} This conjugate is believed to consist of one PABA moiety associated with ten or eleven glutamic acid residues and one other acidic amino acid. In contrast to the complex reported by Loomis\textsuperscript{9} and by Blanchard,\textsuperscript{4} the PABA in the peptide described by Ratner is linked to the rest of the molecule through the carboxyl group. The amino group is free, as shown by its positive response to the Bratton-Marshall test.

In recent years PABA has been shown to occur as a component of several biologically important entities. These include the group of factors that comprise the folic acid family, namely, folic acid (the liver \textit{L. casei} factor),\textsuperscript{12} pteroyl-\(\gamma\)-glutamyl-\(\gamma\)-glutamylglutamic acid (the fermentation \textit{L. casei} factor),\textsuperscript{13-15} pteroylhexaglutamylglutamic acid (Be conjugate),\textsuperscript{16} rhizopterin (the \textit{S. faecalis} R factor),\textsuperscript{17} and folinic acid (the \textit{L. citrovorum} factor).\textsuperscript{18}

\[ \text{Folic acid} \]

\[ \text{Fermentation \textit{L. casei} factor} \]


VII. OCCURRENCE IN FOODS

Rhizopterin

Be conjugate

Folinic acid


<table>
<thead>
<tr>
<th>Source</th>
<th>PABA, (γ/g, wet weight)</th>
<th>Method of hydrolysis</th>
<th>Method of assay</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free</td>
<td>Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alfalfa meal</td>
<td>2.0</td>
<td>2.00</td>
<td>Alkaline</td>
<td>5</td>
</tr>
<tr>
<td>Asparagus juice concentrate</td>
<td>1.15</td>
<td>2.00</td>
<td>Acid</td>
<td>1, 2</td>
</tr>
<tr>
<td>(dry weight)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Banana</td>
<td></td>
<td>0.43-0.46</td>
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<td>Cabbage, dried</td>
<td>9.7</td>
<td>13.9</td>
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<td>Carrots</td>
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<td>0.1-0.22</td>
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<tr>
<td>Carrots, dried</td>
<td>0.178</td>
<td>0.43</td>
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<td>Corn meal</td>
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<td>Molasses, γ/ml.</td>
<td>0.01-0.2</td>
<td>0.52</td>
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<td>0.5</td>
<td>1.3</td>
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<td>0.33</td>
<td></td>
<td>Acid</td>
<td>5</td>
</tr>
<tr>
<td>Oats, seed</td>
<td>0.13</td>
<td>0.5</td>
<td>Acid</td>
<td>4</td>
</tr>
<tr>
<td>Peanuts, raw</td>
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<td>1.6-1.7</td>
<td>Acid</td>
<td>1, 2</td>
</tr>
<tr>
<td>Pineapple juice (solid portion)</td>
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<td>0.17-0.22</td>
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<tr>
<td>Potato, Irish</td>
<td>0.34</td>
<td>0.36-0.5</td>
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<td>0.06-0.12</td>
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<td>1.1</td>
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<td></td>
<td>5</td>
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<tr>
<td>Rice bran concentrate (Galen &quot;B&quot;)</td>
<td>2.0</td>
<td>16.2</td>
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<tr>
<td>Rice polish concentrate (Labco)</td>
<td>2.95</td>
<td>9.2</td>
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<tr>
<td>Spinach</td>
<td>0.12</td>
<td>0.6-1.3</td>
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<td>1, 2, 4</td>
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<tr>
<td>Wheat germ</td>
<td>0.5-1.0</td>
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<td>1, 2, 4, 5</td>
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<td>Wheat middlings</td>
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<td>Whole wheat</td>
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<td>0.49-0.63</td>
<td>Acid</td>
<td>1, 2, 4</td>
</tr>
<tr>
<td>Yeast</td>
<td>3.6-4.7</td>
<td>4.0-7.9</td>
<td>Acid, autolysis</td>
<td>3, 4</td>
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<td>Yeast, brewer's</td>
<td>102.</td>
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<td>Yeast, brewer's, dried (3 samples)</td>
<td>6.6-91.</td>
<td>9.3-50.</td>
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<td>Yeast extract (Bacto)</td>
<td>40</td>
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<tr>
<td>Yeast extract (Difeo)</td>
<td>157.</td>
<td>156</td>
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<tr>
<td>Yeast, autolyzed, dried (Difeo)</td>
<td>7.1</td>
<td>12.0</td>
<td>Alkaline</td>
<td>2</td>
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<td>Yeast torula, autolyzed (fresh</td>
<td>1.74</td>
<td>2.04</td>
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</tr>
<tr>
<td>basis)</td>
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<tr>
<td>Spent torula culture medium</td>
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<td>0.235</td>
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<tr>
<td>(fresh basis)</td>
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<td>Brain, rat</td>
<td>0.14</td>
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<td>Heart, rat</td>
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<td>Kidney, rat</td>
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<tr>
<td>Liver, beef</td>
<td>&lt;0.1-0.2</td>
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<td>1, 2, 4</td>
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<td>0.2</td>
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<td>Liver extract, Lilly 343</td>
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<td>Liver A (dried powdered sample)</td>
<td>1.6</td>
<td>0.8-11.0</td>
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<td>2.3-6.7</td>
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<tr>
<td>original A</td>
<td></td>
<td></td>
<td>enzymatic</td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>0.07</td>
<td>0.25-0.4</td>
<td>Acid</td>
<td>1, 2, 4</td>
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<tr>
<td>Egg, whole, dried (2 samples)</td>
<td>0.475-0.247</td>
<td>0.238-0.30</td>
<td>Alkaline</td>
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<tr>
<td>Egg, whole, dried (Difeo)</td>
<td>0.36</td>
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<td>Egg yolk, dried (Difeo)</td>
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<td>Egg albumin, dried, (Difeo)</td>
<td>0.055</td>
<td>2.4</td>
<td>Acid</td>
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</table>
VII. OCCURRENCE IN FOODS

TABLE V Concluded

<table>
<thead>
<tr>
<th>Source</th>
<th>PABA, (γ/g. wet weight)</th>
<th>Method of hydrolysis</th>
<th>Method of assay</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free</td>
<td>Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epidermis, mouse, methyl cholanthrene treated</td>
<td>2.4</td>
<td>Acid</td>
<td>4</td>
<td>20, 21</td>
</tr>
<tr>
<td>Epidermis, mouse, transplanted carcinoma</td>
<td>3.69</td>
<td>Acid</td>
<td>4</td>
<td>20, 21</td>
</tr>
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<td>Muscle, rat</td>
<td>0.15</td>
<td>1.7</td>
<td>Acid</td>
<td>1</td>
</tr>
<tr>
<td>Muscle, beef</td>
<td>0.3</td>
<td>0.04-0.64</td>
<td>Acid</td>
<td>1, 2, 4</td>
</tr>
<tr>
<td>Meat extract</td>
<td>1.3</td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Beef extract (Difco)</td>
<td>0.673</td>
<td>0.157</td>
<td>Alkaline</td>
<td>2</td>
</tr>
<tr>
<td>Neopeptone (Difco)</td>
<td>0.11</td>
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<td></td>
<td>2</td>
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<td>Peptone</td>
<td>0.4-0.45</td>
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<td>Peptone (Difco)</td>
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<td>0.204</td>
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</tr>
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<td>Proteose-peptone (Difco)</td>
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<td></td>
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</tr>
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<td>Protone (Difco)</td>
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<td>2</td>
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<td>Tryptone (Difco)</td>
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<td></td>
<td>2</td>
</tr>
<tr>
<td>Gelatin (Difco)</td>
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<td>Casein &quot;PABA-free&quot;</td>
<td>0.24-2.6</td>
<td>Alkaline</td>
<td>6</td>
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<td>Pork</td>
<td>0.3</td>
<td>0.8</td>
<td>Acid</td>
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</tr>
<tr>
<td>Pork chop</td>
<td>0.26-0.34</td>
<td></td>
<td></td>
<td>1, 2</td>
</tr>
<tr>
<td>Nucieic acid (Eastman)</td>
<td>0.40</td>
<td>0.64</td>
<td>Alkaline</td>
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</tr>
<tr>
<td>Milk, γ/ml</td>
<td>0.08-0.15</td>
<td>0.03-0.10</td>
<td>Acid</td>
<td>1, 2, 4, 5</td>
</tr>
<tr>
<td>Milk, skim, fresh, γ/ml</td>
<td>0.0046</td>
<td>0.0043</td>
<td>Alkaline</td>
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</tr>
<tr>
<td>Milk, skim, slightly sour, γ/ml</td>
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<td>Milk, skim, sour, γ/ml</td>
<td>0.0075-0.019</td>
<td>0.0098</td>
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<tr>
<td>Blood, human, γ/ml</td>
<td>0.055</td>
<td>0.03-0.04</td>
<td>Acid</td>
<td>1, 2</td>
</tr>
<tr>
<td>Blood, mouse, dried, γ/g.</td>
<td>1.49</td>
<td></td>
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<tr>
<td>Blood, rat, γ/ml</td>
<td>0.06</td>
<td>0.27</td>
<td>Acid</td>
<td>4</td>
</tr>
<tr>
<td>Blood, ox, whole, γ/ml</td>
<td>0.0004</td>
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<td>Urine, human, γ/ml</td>
<td>0.004-0.021</td>
<td>0.325-0.5</td>
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<td>2, 4, 5, 6</td>
</tr>
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<td>Sweat, human, γ/ml</td>
<td>0.0024</td>
<td></td>
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<td>5</td>
</tr>
<tr>
<td>Spinal fluid, human, γ/ml</td>
<td>0.25</td>
<td></td>
<td></td>
<td>5</td>
</tr>
</tbody>
</table>

*a Method of assay: 1. Leuconostoc mesenteroides
2. Lactobacillus arabinosus
4. Neurospora crassa mutant
5. Acetobacter suboxydans
6. Clostridium acetobutylicum

VIII. Effects of Deficiency

LEMUEL D. WRIGHT and PETER A. TAVORMINA

A. IN BACTERIA

1. Nutrition

Investigations in bacterial nutrition have uncovered a number of microorganisms with growth factor requirements for PABA. These species together with their approximate requirements for PABA are summarized in Table VI. Note in particular the wide range in PABA requirement from about 0.00003 \( \gamma \) per milliliter for one-half maximum growth of *Lactobacillus arabinosus* to about 0.001 \( \gamma \) per milliliter for one-half maximum growth of *Acetobacter suboxydans*.

The requirement of certain microorganisms for PABA forms the basis for several microbiological methods for the determination of PABA. This subject is discussed in considerable detail under that heading.

Wyss *et al.*\(^1\) have shown that the growth factor requirement of a PABA-less mutant of *Neurospora crassa*\(^2\) is a function of the pH of the medium. With increase in the pH of the medium from 4 to 7, larger amounts of PABA are required for equivalent growth in a given length of time. Wyss *et al.* point out that PABA has a dissociation constant of about \( 2 \times 10^{-5} \). At pH 4.8 PABA exists in solution as equal amounts of molecules and ions. At pH 5.8 the molecular form decreases from 50 to 10 %. The portion present as the molecule drops almost tenfold with each unit rise in pH above 5.8. They conclude on the basis of the above data that the efficiency of the vitamin in the nutrition of *Neurospora crassa* is a function of the molecular form rather than of the ion. Although the reviewers cannot subscribe to this explanation as the only possible one, the establishment of the pH optimum for the response of the organism to PABA is of significance in the use of the PABA-less mutant for the microbiological determination of the factor.

The specificity of the growth factor requirement for PABA has been examined with a number of species. These data are summarized in Table IV. As might be expected, the \( \text{o} \) and \( \text{m} \) isomers of PABA are essentially inactive for all species studied. Rubbo and Gillespie\(^4\) have reported that \( p \)-aminophenylacetic acid is ten times as active as PABA as a growth factor for *Clostridium acetobutylicum*.

It is reasonable to assume from the structure of folic acid as well as other

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relationships (see section on sulfonamides) that PABA is a precursor of this vitamin. Mayer\textsuperscript{5} observed that *Mycobacterium tuberculosis* produces a yellow compound in the presence of high concentrations of PABA. Mills *et al.*\textsuperscript{6} showed that in the presence of high concentrations of PABA *Mycobacterium tuberculosis* produces increased amounts of vitamins B\textsubscript{10} and B\textsubscript{11} (folic acid or its conjugates). Possibly these compounds are responsible for the yellow color originally described by Mayer.

Sarett\textsuperscript{7} demonstrated that *Lactobacillus arabinosus* synthesizes folic acid in proportion to the PABA content of the basal medium. The bulk of the folic acid synthesized occurs free in the medium, only a small fraction remaining in the bacterial cells. The synthesis of folic acid from PABA is largely completed at the end of the log growth period (20 hours) and is greater in acid-hydrolyzed casein medium than in enzymatically digested casein or amino acid medium. Addition of L-glutamic acid does not increase folic acid synthesis.

The extent to which folic acid can satisfy the PABA requirement of bacteria is a controversial subject. Sarett\textsuperscript{7} found that folic acid or pteroic acid has some activity in replacing PABA as a growth factor for *Lactobacillus arabinosus*. Similarly, Lampen and coworkers\textsuperscript{8, 9} reported that folic acid, pteroylglutamic acid, pteroic acid, or *p*-aminobenzoylglutamic acid is less active on a molecular basis than is PABA as a growth factor for *Lactobacillus arabinosus*. Koft *et al.*\textsuperscript{10, 11} demonstrated the unstable nature of folic acid and related compounds in solution. It would appear from the data of these investigators that the PABA activity of folic acid for *Lactobacillus arabinosus* is due to the presence of PABA in the compound as an impurity or to the unstable nature of folic acid in solution.

Folic acid was found by Lampen *et al.*\textsuperscript{12} to be inactive as a source of PABA for a PABA-less mutant of *Escherichia coli*. As might be expected (see section on sulfonamides) the PABA requirement of this mutant may be satisfied by a combination of amino acids, a purine, and thymine. Under these conditions, however, growth is not optimal.

Zalokar\textsuperscript{13} found that the PABA requirement of the PABA-less mutant of *Neurospora crassa* described by Tatum and Beadle\textsuperscript{2} cannot be satisfied by

\textsuperscript{7} H. P. Sarett, *J. Biol. Chem.* 171, 265 (1947).
folic acid, pteric acid, pteroylglutamic acid, or p-aminobenzoylglutamic acid.

More recent studies by Strehler\textsuperscript{14} have shown that methionine satisfies the PABA requirement of a PABA-less mutant of \textit{Neurospora crassa}\textsuperscript{15} produced with nitrogen mustard. Apparently methionine has not been tested for PABA activity with the x-ray induced mutant of Tatum and Beadle\textsuperscript{2, 3} the mutant more commonly employed in microbiological assays. If active, it would be a source of interference to be contended with in the conduct or interpretation of assays carried out with \textit{Neurospora crassa}.

2. Synthesis

The synthesis of PABA by representatives of a great number of bacterial species has been investigated by Landy \textit{et al.}\textsuperscript{16} using his \textit{Acetobacter suboxydans} assay.\textsuperscript{17, 18} Prior to PABA assay, half of each bacterial culture was filtered through a Seitz filter while the other half was adjusted to pH 5.0 and hydrolyzed by autoclaving at 120° for one-half hour. The PABA values for cells were calculated from the difference in assay between that found for the whole culture and that found for the medium. A summary of the data obtained is contained in Table VII. Note that in most instances the bulk of the PABA produced is found in the culture medium rather than in the cells. The method used by Landy \textit{et al.}\textsuperscript{16} in preparing samples for assay is not sufficient to break down folic acid or related compounds, so that it would appear that the data obtained are reasonably valid.

PABA synthesis by \textit{Torula utilis} is adversely affected by a deficiency of iron according to Lewis.\textsuperscript{19} Normal yeast cells contain about 60 μg of PABA per gram, whereas yeast suffering from iron deficiency contains about 3 μg per gram. Lewis \textit{et al.}\textsuperscript{20} showed that immediate separation of yeast cells from medium is essential for retention of PABA activity within the cells. In one experiment 18.3 μg per gram was found on immediate separation, and 12.0 μg per gram after separation was delayed for 18 hours while the fermentation mixture was held at 2°.

Eppwright and Williams\textsuperscript{21} found that the addition of thiamine, pyridoxine, alanine, and inositol to a synthetic medium is accompanied by a

\begin{itemize}
\item\textsuperscript{11} B. L. Strehler, \textit{J. Bacteriol.} 59, 105 (1950).
\item\textsuperscript{15} W. D. McElroy, J. E. Cushing, and H. Miller, \textit{J. Cellular Comp. Physiol.} 30, 331 (1947).
\item\textsuperscript{17} M. Landy and D. M. Dicken, \textit{J. Biol. Chem.} 146, 109 (1942).
\item\textsuperscript{19} J. C. Lewis, \textit{Arch. Biochem.} 4, 217 (1944).
\item\textsuperscript{20} J. C. Lewis, J. J. Stubbs, and W. M. Noble, \textit{Arch. Biochem.} 4, 389 (1944).
\item\textsuperscript{21} M. A. Eppright and R. J. Williams, \textit{J. Gen. Physiol.} 30, 61 (1946).
\end{itemize}
decrease in PABA synthesis by *Saccharomyces cerevisiae* from 42 γ per liter to 0.83 γ per liter. The specific vitamin concerned with this effect on PABA synthesis was not established.

Agarwala and Peterson\(^{22}\) found a depression of PABA synthesis with four yeasts when the vitamins thiamine, pyridoxine, biotin, pantothenic acid, nicotinic acid, and inositol were added to a synthetic medium. Thiamine appears to be the factor most concerned in influencing PABA synthesis, since reductions from 124 γ per gram to 41 γ per gram and from 11.8 γ per gram to 2.7 γ per gram were observed with *Candida krusoides* and *Torula utilis*, respectively.

### TABLE VI

**Microorganisms with a Growth Factor Requirement for PABA**

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Approximate PABA requirement, γ/ml.</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetobacter suboxydans</td>
<td>0.001</td>
<td>17, 18, 23-25</td>
</tr>
<tr>
<td>Chlamydomonas Moewusii mutant</td>
<td>a</td>
<td>26</td>
</tr>
<tr>
<td>Clostridium acetobutylicum</td>
<td>0.0001</td>
<td>27-33</td>
</tr>
<tr>
<td>Clostridium butylicum</td>
<td>a</td>
<td>27</td>
</tr>
<tr>
<td>Clostridium felsineum</td>
<td>a</td>
<td>27</td>
</tr>
<tr>
<td>Clostridium sporogenes</td>
<td>0.0001</td>
<td>34</td>
</tr>
<tr>
<td>Clostridium thermosaccharolyticum</td>
<td>0.001</td>
<td>35, 36</td>
</tr>
<tr>
<td>Corynebacterium diphtheriae graves (Dundee)</td>
<td>a</td>
<td>37</td>
</tr>
<tr>
<td>Escherichia coli mutants</td>
<td>0.00011</td>
<td>8, 12, 38, 39</td>
</tr>
<tr>
<td>Lactobacillus arabinosus</td>
<td>0.00003</td>
<td>7, 40, 41</td>
</tr>
<tr>
<td>Leuconostoc mesenteroides P-60</td>
<td>0.0001</td>
<td>42</td>
</tr>
<tr>
<td>Neurospora crassa mutant</td>
<td>0.004</td>
<td>1-3, 15, 43-46</td>
</tr>
<tr>
<td>Ophiostoma multiannulatum mutant</td>
<td>a</td>
<td>47</td>
</tr>
<tr>
<td>Plasmidium knowlesi</td>
<td>a</td>
<td>48</td>
</tr>
<tr>
<td>Propionibacterium sps.</td>
<td>a</td>
<td>49</td>
</tr>
<tr>
<td>Rhizobium trifolii</td>
<td>Stim.</td>
<td>50</td>
</tr>
<tr>
<td>Rhodopseudomonas palustris</td>
<td>a</td>
<td>51</td>
</tr>
<tr>
<td>Rhodotorula aurantiaca</td>
<td>0.001</td>
<td>52</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>0.007</td>
<td>53-55</td>
</tr>
</tbody>
</table>

\(^a\) Not specified.


<table>
<thead>
<tr>
<th>Organism</th>
<th>Supernatant</th>
<th>Whole culture</th>
<th>Reference and method of assay&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobacter aerogenes</td>
<td>0.028</td>
<td>0.045</td>
<td>16, a</td>
</tr>
<tr>
<td>Alkaligenes fecalis</td>
<td>0.005</td>
<td>0.006</td>
<td>16, a</td>
</tr>
<tr>
<td>Bacillus megatherium</td>
<td>0.007</td>
<td>0.007</td>
<td>16, a</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>0.019</td>
<td>0.019</td>
<td>16, a</td>
</tr>
<tr>
<td>Bacillus vulgatus</td>
<td>0.030</td>
<td>0.071</td>
<td>16, a</td>
</tr>
<tr>
<td>Brucella abortus</td>
<td>0.013</td>
<td>0.013</td>
<td>16, a</td>
</tr>
<tr>
<td>Clostridium botulinum</td>
<td>—</td>
<td>0.004</td>
<td>16, a</td>
</tr>
<tr>
<td>Clostridium sporogenes</td>
<td>—</td>
<td>0.003</td>
<td>16, a</td>
</tr>
<tr>
<td>Clostridium tetani</td>
<td>—</td>
<td>0.008</td>
<td>16, a</td>
</tr>
<tr>
<td>Corynebacterium diphtheriae</td>
<td>0.171</td>
<td>0.241</td>
<td>16, a</td>
</tr>
<tr>
<td>Diplococcus pneumoniae—I</td>
<td>0.0027-0.004</td>
<td>0.0029-0.005</td>
<td>16, 28 a, b</td>
</tr>
<tr>
<td>Diplococcus pneumoniae—I, sulfonamide-resistant</td>
<td>—</td>
<td>0.0026</td>
<td>28, b</td>
</tr>
<tr>
<td>Diplococcus pneumoniae—II</td>
<td>0.003</td>
<td>0.004</td>
<td>16, a</td>
</tr>
<tr>
<td>Diplococcus pneumoniae—III</td>
<td>0.003</td>
<td>0.005</td>
<td>16, a</td>
</tr>
<tr>
<td>Eberthella typhosa</td>
<td>0.018</td>
<td>0.018</td>
<td>16, a</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>0.007</td>
<td>0.045</td>
<td>16, a</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>0.032</td>
<td>0.042</td>
<td>16, a</td>
</tr>
<tr>
<td>Lactobacillus arabinosus</td>
<td>0</td>
<td>0</td>
<td>16, a</td>
</tr>
<tr>
<td>Lactobacillus casei</td>
<td>0.016</td>
<td>0.016</td>
<td>16, a</td>
</tr>
<tr>
<td>Mycobacterium smegma</td>
<td>0.052</td>
<td>0.059</td>
<td>16, a</td>
</tr>
<tr>
<td>Mycobacterium stercoritis</td>
<td>0.019</td>
<td>0.022</td>
<td>16, a</td>
</tr>
<tr>
<td>Mycobacterium tuberculosis</td>
<td>0.200</td>
<td>—</td>
<td>16, a</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>0.019</td>
<td>0.020</td>
<td>16, a</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>0.050</td>
<td>0.060</td>
<td>16, a</td>
</tr>
<tr>
<td>Salmonella paratyphi</td>
<td>0.019</td>
<td>0.020</td>
<td>16, a</td>
</tr>
<tr>
<td>Salmonella schottmuelleri</td>
<td>0.029</td>
<td>0.050</td>
<td>16, a</td>
</tr>
<tr>
<td>Serratia marcescens</td>
<td>0.007</td>
<td>0.014</td>
<td>16, a</td>
</tr>
<tr>
<td>Shigella dysenteriae</td>
<td>0.018</td>
<td>0.018</td>
<td>16, a</td>
</tr>
<tr>
<td>Shigella paradysenteriae</td>
<td>0.011</td>
<td>0.017</td>
<td>16, a</td>
</tr>
<tr>
<td>Shigella paradysenteriae Sonne</td>
<td>0.057</td>
<td>0.059</td>
<td>28, b</td>
</tr>
<tr>
<td>Shigella paradysenteriae Sonne, sulfonamide-resistant</td>
<td>0.080</td>
<td>0.084</td>
<td>28, b</td>
</tr>
<tr>
<td>Staphylococcus albus</td>
<td>0.047</td>
<td>0.061</td>
<td>16, a</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>0.023-0.063</td>
<td>0.026-0.067</td>
<td>28, 58, a, b</td>
</tr>
<tr>
<td>Staphylococcus aureus, sulfonamide-resistant</td>
<td>0.347-3.40</td>
<td>0.361-2.40</td>
<td>28, 58, a, b</td>
</tr>
<tr>
<td>Streptococcus hemolyticus</td>
<td>0.004</td>
<td>0.012</td>
<td>16, a</td>
</tr>
<tr>
<td>Streptococcus salivarius</td>
<td>0.005</td>
<td>0.010</td>
<td>16, a</td>
</tr>
<tr>
<td>Streptococcus scharlattae</td>
<td>0.030</td>
<td>0.050</td>
<td>16, a</td>
</tr>
<tr>
<td>Tubercle bacillus, II37 (human)</td>
<td>0.0-0.033</td>
<td>0.0216</td>
<td>59-61, a, c</td>
</tr>
<tr>
<td>Tubercle bacillus, Ravanel (bovine)</td>
<td>0.002</td>
<td>—</td>
<td>61, a</td>
</tr>
<tr>
<td>Vibrio cholerae</td>
<td>0.022</td>
<td>—</td>
<td>16, a</td>
</tr>
<tr>
<td>Yeasts</td>
<td>0.02-8.5</td>
<td>0.04-8.5</td>
<td>16, 62, a, d</td>
</tr>
</tbody>
</table>

<sup>a</sup> Method of assay: a. Acetobacter suboxydans  
b. Clostridium acetobutyllicum  
c. Colorimetric assay of Tauber and Laufer<sup>c</sup>  
d. Neurospora crassa mutant
B. IN ANIMALS

1. Rats

The discovery by Woods and Fildes\(^4\) that PABA is somehow concerned in bacterial metabolism served as the impetus for studies on the significance, if any, of this compound in animal nutrition. Ansbacher\(^5\) in 1941 concluded that PABA is a "vitamin," since its oral administration at a level of 3 mg. per day to black or piebald rats that had become gray on a purified basal diet containing pantothenic acid was accompanied by a return of the fur to normal pigmentation. Untreated controls continued to show typical achromotrichia. These observations appeared to contradict much contemporary evidence that pantothenic acid deficiency alone is responsible for nu-

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40 J. C. Lewis, J. Biol. Chem. 146, 441 (1942).
42 D. Pennington, Science 103, 397 (1946).
46 G. W. Beadle, Physiol. Revs. 25, 643 (1945).
47 N. Fries, Nature 155, 757 (1945).
52 W. J. Robbins and R. Ma, Science 100, 85 (1943).
56 M. Landy, N. W. Larkum, E. J. Oswald, and F. Streightoff, Science 97, 265 (1943).
63 S. Ansbacher, Science 93, 164 (1941).
tritional achromotrichia.\textsuperscript{66-70} It should be mentioned, however, that Williams\textsuperscript{71} was unable to show any effect of pantothenic acid on gray hair in rats, and Frost et al.\textsuperscript{72} were able to protect only a small percentage of their rats against achromotrichia with pantothenic acid alone, whereas liver concentrates were much more effective.

Martin\textsuperscript{73} in further studies produced evidence that PABA is essential for growth and prevention of achromotrichia in the rat only when inositol is present in the diet. Conversely, the presence of PABA was reported to provoke a need for inositol. Thus the six B vitamins—thiamine, riboflavin, pyridoxine, choline, nicotinic acid, and calcium pantothenate—or these six plus PABA and inositol afford normal nutrition, but the omission of either one of the last two factors induces a requirement for the other.

The forementioned findings of Anscher and Martin with respect to the significance of PABA in animal nutrition instigated one of the most protracted polemics in the nutrition field. Thus Unna et al.,\textsuperscript{74} Emerson,\textsuperscript{75} and Henderson et al.\textsuperscript{76} confirmed the activity of pantothenic acid as an achromotrichial factor and reported only negative results from their studies with PABA. Ershoff\textsuperscript{77} failed to confirm Martin\textsuperscript{73} by showing that no adverse effects in rats are encountered when either PABA or inositol alone is incorporated in their diet. On the other hand, a number of investigators have reported beneficial effects from the feeding of PABA. Sure\textsuperscript{78,79} reported that, on a purified diet containing thiamine, riboflavin, pyridoxine, choline, calcium pantothenate, nicotinic acid, and factor W concentrate from liver extract, female rats are unable to nurse their young. When the diet is supplemented further with PABA and inositol most of the young born to mothers consuming the supplemented diet are raised successfully. Climenko and McChesney\textsuperscript{80} confirmed the essential nature of inositol for lactation.

\textsuperscript{66} P. György and C. E. Poling, \textit{Science} \textbf{92}, 202 (1940).
\textsuperscript{69} K. Unna, \textit{Am. J. Physiol.} \textbf{133}, 473 (1941).
\textsuperscript{71} R. R. Williams, \textit{Science} \textbf{92}, 561 (1940).
\textsuperscript{73} G. J. Martin, \textit{Am. J. Physiol.} \textbf{136}, 124 (1942).
\textsuperscript{74} K. Unna, G. V. Richards, and W. L. Sampson, \textit{J. Nutrition} \textbf{22}, 553 (1941).
\textsuperscript{78} B. Sure, \textit{Science} \textbf{94}, 167 (1941).
\textsuperscript{79} B. Sure, \textit{J. Nutrition} \textbf{26}, 275 (1943).
in the rat and also demonstrated a favorable effect of PABA on the mortality rate of the newborn.

The etiology of achromotrichia in the rat appeared to be further complicated when Martin\(^{51,52}\) reported that black rats receiving a purified sulfaguanidine-containing diet adequate in calcium pantothenate and PABA become gray. The graying could be cured with folic acid concentrates. Similar findings were reported by Wright and Welch\(^{53}\) with black rats receiving succinylsulfathiazole. Accompanying the achromotrichia seen in the latter studies were low hepatic stores of pantothenic acid. The achromotrichia responded favorably, and the hepatic stores of pantothenic acid were restored to normal with the daily oral administration of folic acid and biotin. It was concluded that folic acid and biotin are concerned with "utilization of pantothenic acid in the rat."

Some clarification with respect to the role of PABA in nutrition resulted when it was announced by Angier and coworkers\(^{54}\) that PABA is a structural moiety of folic acid. There seems little doubt but that the beneficial effect observed from the feeding of PABA, as, for example, in the studies of Briggs and coworkers,\(^{55}\) may be directly attributable to an increased synthesis of folic acid on the part of intestinal microorganisms (and possibly even tissue cells\(^{56}\)) in the presence of the structural component PABA.

A plausible explanation for the occurrence of achromotrichia in rats receiving diets containing both pantothenic acid and PABA, and its cure with folic acid and biotin, is possible as a result of three converging lines of investigation. Firstly, it has been demonstrated by Lipmann and coworkers\(^{57}\) that pantothenic acid is present in tissues largely, if not entirely, in the form of coenzyme A, a "modified" nucleotide containing adenine as the purine component. Secondly, free pantothenic acid has been found by Wright et al.\(^{58}\) to have a high renal clearance so that pantothenic acid probably does not remain long in the animal body without some mechanism for retention by tissue cells. Thirdly, PABA in the form of folic acid,

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\(^{52}\) G. J. Martin, Federation Proc. 1, 58 (1942).

\(^{53}\) L. D. Wright and A. D. Welch, Science 97, 426 (1943).


or probably more correctly folinic acid, as recently reviewed by Shive,\textsuperscript{89} is concerned with purine synthesis. Thus it would appear that dietary PABA is a precursor of folic or folinic acid which is essential for the synthesis of the purine portion of coenzyme A. Coenzyme A is visualized as the form in which pantothenic acid is retained. Pantothenic acid, then, rather than PABA emerges as the factor most directly concerned with normal pigmentation of fur or hair. Obviously, any break in the postulated sequence of events, such as dietary restriction of pantothenic acid, dietary restriction of folic acid combined with the administration of a poorly absorbed sulfonamide, the feeding of hydroquinone, or other bacteriostatic agent, as well as dietary imbalance of B vitamins (possibly by inositol), may be expected to cause achromotrichia. The condition then will respond to whatever measure will restore the normal sequence of events. Some suggestions with respect to practical means by which ample tissue stores of pantothenic acid (as coenzyme A or other combined form) might be achieved as a possible means of combating achromotrichia involve (1) administration of LBF (\textit{Lactobacillus bulgaricus} factor,\textsuperscript{90}) a compound intermediate in structure between pantothenic acid and coenzyme A; (2) administration of large amounts of pantothenic acid along with "caronamide"\textsuperscript{91} or "benemide"\textsuperscript{92} to decrease the renal elimination of the vitamin along the lines studied by Roholt and Schmidt;\textsuperscript{93} and (3) administration of repository forms of pantothenic acid alone or as in (2).

In rats fed certain sulfonamides such as sulfaguanidine (but not sulfanilic acid or granine or succinylsulfathiazole) hyperemia and enlargement of the thyroid occur, according to MacKenzie \textit{et al.}\textsuperscript{94} The thyroid hyperplasia is accompanied by a decreased food intake, a lessened rate of body growth and development, and a lessened basal oxygen consumption. This thyroid effect of sulfaguanidine is not increased with PABA, but, on the contrary, PABA actually enhances the effect.\textsuperscript{95} The effect of PABA in inhibiting the thyroid was confirmed by Astwood,\textsuperscript{96} who found that of the three isomeric aminobenzoic acids PABA, the only one known to occur naturally, is the most active. Thiouraëil and PABA were compared as antithyroid

\textsuperscript{89} W. Shive, \textit{in Vitamins and Hormones}, 9, 76 (1951).
agents by Gordon et al. Both thiouracil and PABA (0.2% and 3.0% of the food, respectively) administered for 19 to 45 days cause marked thyroid hyperplasia, basophilia, and appearance of "thyroidectomy" cells in the anterior hypophysis, and increased resistance of rats to lowered barometric pressures (190 mm. Hg.). These effects, however, are more pronounced with thiouracil than with PABA. Both drugs reduce the basal metabolic rate; neither alters the resistance of the rats to the degree of lung hemorrhage or extent of eye cataract encountered at the reduced pressures. Thiouracil inhibits normal weight gains and, after 38 days, induces slight anemia and granulocytopenia. PABA, on the other hand, does not retard body growth and exerts no effect on the blood picture.

The effect of PABA on uptake of radioactive iodine by surviving slices of sheep thyroid has been studied by Franklin et al. This compound as well as p-aminophenylacetic acid has a pronounced inhibitory effect on the rate of conversion of inorganic iodide to thyroxine and diiodothyrosine.

2. Mice

Martin and Ansbacher have reported that PABA is essential for the prevention of achromotrichia in the black mouse fed a purified diet adequate with respect to pantothenic acid. In the black mouse the administration of hydroquinone causes an achromotrichia that may be cured, according to Martin and Ansbacher, with PABA.

On the other hand, Fenton et al., employing a number of purified diets and working with two strains of mice (C57 and A) could find no evidence for the essential nature of PABA in the diet of this species. Growth was just as good in the absence of PABA and inositol as in their presence. Inositol did not provoke a requirement for PABA.

Keresztesy et al. reported that the inhibition of tumor growth by inositol previously noted by Laszlo and Leuchtenberger may be reversed with PABA. Their chemotherapeutic experiments involved the use of groups of seven or eight female Rockland mice bearing transplanted tumors of similar size (sarcoma 180, 7 to 10 days after transplantation) and maintained on a normal diet. Each mouse of a group was injected intravenously twice a day for 2 consecutive days with the substance or mixture of substances under test. Forty-eight hours after the first injection the animals

100 G. J. Martin and S. Ansbacher, J. Biol. Chem. 138, 441 (1941).
103 D. Laszlo and C. Leuchtenberger, Science 97, 515 (1943).
were killed with ether and the tumors were removed, freed from surrounding tissue, and weighed. The reversal of the tumor-inhibiting effect of 100 \( \gamma \) of inositol was found to be complete with 100 \( \gamma \) of injected PABA. Fifty micrograms of PABA was described as reversing only partially the 100 \( \gamma \) of inositol. \( \alpha \)- and \( \alpha \)-Aminobenzoic acids were described as much less effective antagonists of inositol than PABA.

Although the tumor-inhibiting effect of inositol against mammary adenocarcinoma of mice has been confirmed by Hesselbach and Burk,\(^{104}\) Harris et al.\(^{105}\) found that neither inositol nor PABA has any effect on \( p \)-dimethylaminoazobenzene carcinogenesis in rats. It may be pointed out that others have reported that PABA has a retarding effect in chronic myelogenous leukemia (see separate section) which is just the reverse of its reported effect in antagonizing a tumor-inhibiting compound.

3. Hamsters

Cooperman et al.\(^{106}\) reported that weanling hamsters fail to grow and die when receiving purified diets containing the six B vitamins—thiamine, riboflavin, calcium pantothenate, pyridoxine, nicotinic acid, and choline plus biotin. When the diet is supplemented further with PABA and inositol, there are fewer deaths. They conclude that PABA and/or inositol is required by the growing hamster. Here, again, PABA may be functioning as a source stimulant for the synthesis of folic acid.

4. Chicks

Ansbacher\(^{65}\) reported that chicks fed a ration consisting largely of wheat middlings and yellow corn that had been heated at 110\(^\circ\) for about one week are stimulated in growth when the diet is supplemented with 300 \( \gamma \) of PABA per gram of diet. Similarly Briggs et al.\(^{55}\) found that PABA at levels of 5 to 15 mg. per 10 g. of diet produces growth responses in chicks receiving purified diets low in folic acid but complete in all other respects.

5. Swine

Cunha et al.\(^{107}\) found that during a seven-week feeding experiment with growing pigs no beneficial effect on external appearance, growth, or efficiency of feed utilization is obtained when either PABA or folic acid is added alone or in combination with inositol and biotin to a purified basal ration which contains the six B complex vitamins—thiamine, riboflavin, niacin, vitamin B12, pantothenic acid, pyridoxine, and biotin.

\(^{104}\) M. L. Hesselbach and D. Burk, Record Chem. Prog. 5, 37 (1944).


pyridoxine, pantothenic acid, and choline. The addition of PABA or folic acid alone to the basal purified ration did, however, stimulate hemoglobin formation to a small extent.

Johnson and James\(^{108}\) could find no definite evidence for a requirement for PABA or inositol when their combined deficiencies were superimposed on a choline deficiency, although their omission from the diet of growing pigs appeared to accentuate the degree of fatty infiltration of the liver.

6. Fish

McLaren \textit{et al.}\(^{109}\) found that a deficiency of PABA in the diet of trout results in fatty livers, so that PABA is considered to be a vitamin for this species. The requirement of trout for PABA is placed at 10 to 20 mg. per 100 g. of ration.

### IX. Metabolism

**LEMUEL D. WRIGHT and PETER A. TAVORMINA**

#### A. EXCRETION IN LOWER ANIMALS

The fate of intraperitoneally administered N\(^{15}\)-labeled PABA has been studied in the mouse by Lustig \textit{et al.}\(^{1}\) A male mouse weighing 22 g. received three subcutaneous doses of 10 mg. within a 24-hour period. Nineteen hours after the last injection, however, only traces were found in the organs, but 227 $\gamma$ of N\(^{15}\), corresponding to 82\% of that injected, was present in the excreta.

Similar results with respect to the rapidity with which administered PABA is excreted by mice were reported by Tabor \textit{et al.}\(^{2}\) One gram of PABA per kilogram as the sodium salt was administered either orally or intraperitoneally to mice. At intervals after administration of the PABA, animals were homogenized in a Waring Blender with water. Aliquots of each homogenate were assayed for total amine (Bratton-Marshall method, hydrolysis in 6 $\text{N}$ HCl at 100$^\circ$ for 30 minutes). In mice killed immediately after administration of the drug, all the PABA is recovered in the tissues. After oral or intraperitoneal administration the total amine disappears rapidly, so that only 50\% is present in the tissues after 3 hours, and little, or none at all, after 8 hours.


The distribution of PABA in the tissues of rats 2 hours after the oral administration of the sodium salt at a level of 5 g. per kilogram of body weight was studied by Tabor et al. Free amine is found in greatest concentration in lung, liver, and kidney (ca. 1.3 mg. per gram), and in a lower concentration in brain and muscle (ca. 0.4 mg. per gram). The per cent conjugated is low and probably insignificant. The concentration in plasma (1.35 mg. per gram) is twice that in red blood cells.

The acetylation of PABA as a method of "detoxification" prior to elimination in the urine appears to have been observed first in the rabbit by Ellinger and Hensel and subsequently in man by Muenzen et al.

Increased amounts of acetylated PABA were found by Harrow et al. to occur in the urine of rabbits as a result of insulin injections. The same group observed that acetylation of PABA by the rabbit is increased by the simultaneous administration of a variety of compounds that presumably function as a source of acetyl donors.

Increased acetylation of PABA (0.2 g. per kilogram per os) was observed in rabbits by Venkataraman et al. when glycine or DL-malic acid was fed along with the compound. The excretion of PABA as the glucuronide was also observed. When excretion of acetylated PABA was increased by the simultaneous administration of glycine or DL-malic acid, there was a decrease in that portion of PABA excreted in the acetylated form. Charalampous and Hegsted point out that the administration of PABA in large amounts imposes a stress on the animal, so that increases in acetylation thus observed represent a measure of the effectiveness of such compounds as acetyl donors.

Acetylation of PABA is impaired in the rat made diabetic with alloxan. Acetylation in such animals is normal again after the administration of insulin, dicarboxylic acids of the tricarboxylic acid cycle, ATP, acetyl phosphate, or diacetyl. A return to normal is seen in the fasting animal as well as in animals on a high fat diet. Such an effect is not apparent, however, when acetate, acetyl methyl acetate, pyruvate, lactate, glyceral diacetate, acetoin, butylene glycol, phosphate salt, adenylic acid, or pantothetic acid is fed to the animal together with PABA.

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4 J. B. Muenzen, L. R. Cereccdo, and C. P. Sherwin, J. Biol. Chem. 67, 469 (1926).
5 B. Harrow, A. Mazur, and C. P. Sherwin, J. Biol. Chem. 102, 35 (1933).
7 B. Harrow, A. Mazur, E. Borek, and C. P. Sherwin, Biochem. Z. 293, 302 (1937).
8 E. A. Doisy, Jr., and W. W. Westerfeld, J. Biol. Chem. 149, 229 (1943).
Pantothenic acid is administered. Charalampous and Hegsted\textsuperscript{13} note that of the compounds effective in increasing acetylation of PABA by the alloxan-treated rat only ATP, or compounds expected to stimulate ATP synthesis, are effective. Reduced acetylation of PABA in the alloxan-treated rat, then, is due to a relative deficiency of ATP, the immediate energy source of the reaction.

As might be expected from the fact that pantothenic acid, as coenzyme A, is concerned with the acetylation of such compounds as sulfanilamide\textsuperscript{12}, pantothenic acid deficiency is accompanied by impaired acetylation of PABA in the rat. Riggs and Hegsted\textsuperscript{15} found that normal rats acetylate 70\% of the amount of PABA excreted in 24 hours after a 1-mg. or 2.5-mg. dose of PABA administered intraperitoneally. On the other hand, pantothenic acid-deficient rats acetylate only 50\% of a 1-mg. dose or 37\% of a 2.5-mg. dose similarly administered. Acetylation of PABA is less than normal in thiamine-deficient and riboflavin-deficient rats, but the magnitude of the effect is not so great as in pantothenic acid deficiency.\textsuperscript{16} Acetylation by riboflavin-deficient rats is not influenced by the administration of pantothenic acid. Differences in acetylating ability as a function of diet are only apparent following the administration of amounts of PABA of the order of 1 to 2 mg. At a dose of 10 mg. or more, normal, thiamine-, riboflavin-, or pantothenic acid-deficient rats all acetylate about the same percentage of injected PABA, namely, about 40\%.

Dumm and Ralli\textsuperscript{17} confirm the effect of pantothenic acid deficiency as a factor in reducing the ability of the rat to acetylate PABA. Male rats on a normal diet, a pantothenic acid-deficient diet, or a pantothenic acid-high diet were found to acetylate a greater percentage of peritoneally injected PABA than female rats on corresponding diets.

Riggs and Hegsted\textsuperscript{18} have studied the acetylation of PABA by normal and pantothenic acid-deficient rats of various body weights after the intraperitoneal injection of 1 mg. of the amine. Rats weighing about 300 g. acetylate a greater percentage of the PABA administered than do weanling rats of about 40 g. body weight. Young rats show decreased acetylation almost immediately upon withdrawal of pantothenate from the diet. Older animals continue to acetylate PABA normally for at least two months. These latter findings are correlated with the well-known difficulties encountered in producing vitamin deficiencies in older animals.\textsuperscript{19}

\textsuperscript{13} F. Lipmann and N. O. Kaplan, \textit{Federation Proc.} 5, 145 (1946).
\textsuperscript{14} F. Lipmann and N. O. Kaplan, \textit{J. Biol. Chem.} 162, 743 (1946).
\textsuperscript{17} M. E. Dumm and E. P. Ralli, \textit{J. Nutrition} 44, 265 (1951).
Bray and his associates$^{20}$ have studied in detail the chemical nature of the metabolites of PABA that occur in the urine of the rabbit. In the light of their experiments it appears that PABA may be excreted by the rabbit in a variety of forms: as free PABA, as the ester-type glucuronide, as the glycine conjugate (PAHA), as acetylated PABA, as acetylated PAHA, and as acetylated glucuronide. Although the various metabolites were not estimated individually, Bray et al. found that only 0 to 21% of the administered dose (0.25 to 1.0 g. per kilogram) is excreted as the acetylamino derivatives (acetylated PABA, acetylated PAHA, and acetylated glucuronide of PABA), whereas 44 to 87% of the dose is excreted as "free diazotizable material" (PABA, PAHA, and glucuronide of PABA).

These findings render difficult the proper interpretation of any studies on the in vivo acetylation of PABA, either in humans or in lower species, unless such studies have been conducted with reference to the specific form(s) in which the PABA conjugate under investigation has been excreted.

B. EXCRETION IN HUMAN BEINGS

PABA has been determined in urine by Lewis$^{21}$ with his _Lactobacillus arabinosus_ microbiological method. Normal urine was found to contain about 0.01 to 0.02 \( \gamma \) of "free" PABA per milliliter, and about 0.3 to 0.5 \( \gamma \) of "combined" PABA per milliliter. When these values are recalculated in terms of probable daily output, about 10 to 20 \( \gamma \) of "free" PABA and about 300 to 500 \( \gamma \) of "combined" PABA appear to be excreted.

Landy and Dicken,$^{22}$ using the _Acetobacter suboxydans_ assay, found about 0.01 to 0.02 \( \gamma \) of "free" PABA per milliliter of normal urine or, on recalculating to daily output, about 10 to 20 \( \gamma \) of "free" PABA per day.

Thompson et al.$^{23}$ with the _Neurospora crassa_ \( p \)-aminobenzoic-less mutant found 0.02 \( \gamma \) of "free" PABA or about 0.5 \( \gamma \) of "combined" PABA per milliliter of normal urine. In agreement with Lewis and with Landy and Dicken, this indicates a daily output of about 500 \( \gamma \) of "combined" PABA per day.

PABA excretion in normal adults has been studied by Denko et al.$^{24}$ using the _Neurospora crassa_ \( p \)-aminobenzoic-less assay of Thompson et al.$^{23}$ No mention is made in their paper of hydrolysis of samples prior to determination, so it may be presumed that the values reported represent "free"


PABA. Urinary excretion of PABA was found to be of the order of 150 γ per day. This value for "free" PABA is much higher than that reported by Lewis\textsuperscript{21} or by Landy and Dicken.\textsuperscript{22} Fecal excretion of PABA, also determined without prior hydrolysis of the samples, was found by Denko \textit{et al.} to average about 250 γ per day.

PABA excretion by normal adults has been studied by Bloomberg,\textsuperscript{25} using the microbiological assay of MacLeod\textsuperscript{26} that measures PABA in terms of sulfonamide-reversing activity. Like others, Bloomberg found PABA to occur in the urine both in the "free" and in the "combined" state. Less than 0.002 γ of "free" PABA per milliliter was found, while the "combined" PABA, determined after acid hydrolysis, was found to be about 0.02 γ per milliliter. From a study of the behavior of pure solutions of derivatives of PABA to acid hydrolysis, Bloomberg concluded that the "combined" form of PABA present in urine is mainly \( p \)-acetylaminobenzoic acid. In the opinion of the reviewers, the approach of Bloomberg is subject to criticism in that the presence of other derivatives of PABA of the same acid lability is not precluded. When the results reported by Bloomberg are recalculated in terms of probably daily output, less than 2 γ of "free" and about 20 γ of "combined" PABA appear to be excreted. These values are considerably lower than those found by Lewis,\textsuperscript{21} Landy and Dicken,\textsuperscript{22} Thompson \textit{et al.}\textsuperscript{23} and Denko \textit{et al.}\textsuperscript{24}

The investigations of Bray \textit{et al.}\textsuperscript{29} (see Excretion in Lower Animals) on the nature of the metabolic forms of PABA found in the urine of rabbits have been extended by Tabor \textit{et al.},\textsuperscript{2} who made a similar study in normal human beings. Using the countercurrent distribution technique, Tabor and her collaborators concluded that very little of the administered PABA (single 6.2-g. dose) is eliminated as such, and only a small fraction is excreted as acetylated PABA. The bulk of the PABA is excreted either as the glycine conjugate or as the glucuronide. Additional evidence for the excretion of PABA in the form of the glucuronide is to be found in the work of Zarafonetis and Chandler,\textsuperscript{27} who suggested that this was the reducing substance\textsuperscript{28},\textsuperscript{29} found in the urine of patients receiving PABA, rather than glucose as had been earlier presumed.\textsuperscript{30}

Gershberg and Kuhl\textsuperscript{31} report that 95 % of a small dose (100 mg.) of PABA

\textsuperscript{26} C. M. McLeod, \textit{J. Exptl. Med.} \textbf{72}, 217 (1940).
is acetylated by the normal human. Although this high percentage may appear at first sight to be at variance with the results of Tabor et al., two points must be kept in mind: (1) the per cent of administered PABA acetylated decreases with increase in the size of the dose (the small percentage of acetylated PABA reported by Tabor et al. is associated with a dose of 6.2 g.); (2) Gershberg and Kuhl used the Bratton-Marshall reaction as their method of assay, so that in actuality the "amount acetylated" represents not only acetyl PABA, but also acetyl PAHA, acetyl PABA glucuronide, and any other PABA derivatives in which the amino group is involved.

Gershberg and Kuhl included in their studies the "acetylation" of PABA in subjects suffering from various metabolic disorders. A standard dose of 500 mg. of PABA was used in most experiments. Normal (control) individuals were found to "acetylate" 88 ± 1.9% of the standard dose. In patients with severe liver disease acetylation is normal. The authors infer that acetylation does not occur exclusively or necessarily primarily in the liver but that other tissues also participate in acetylation. Acetylation is normal in patients with rheumatoid arthritis, diabetes mellitus, hypothyroidism, sprue, and leukemia. Patients with hyperthyroidism have a diminished capacity to acetylate. This usually returns to within normal limits as the hyperthyroidism is controlled. In two patients with hyperthyroidism the administration of sodium acetate was found to increase the capacity to acetylate. The authors suggest that in hyperthyroidism there is a decrease in available acetate, owing to its rapid utilization, which may contribute to the decreased ability to acetylate PABA.

Zilli and Di Ferrante found very little difference between normal subjects and patients with icterus and cirrhosis of the liver, in the rate at which 1 g. of injected PABA is excreted, in the total excretion, or in the proportions of free and acetylated PABA excreted.

Although PABA has been reported to inhibit the in vitro conversion of tyrosine and melanine by tyrosinase, White et al. found that PABA (20 g. daily) is without effect on the excretion of homogentisic acid by an alcaptonuric.

No data could be found in the literature concerning acetylation of PABA in individuals in whom a pantothenic acid deficiency might be suspected from a study of their nutritional history. It would appear to the present reviewers that such a study could be instrumental in demonstrating the essential nature of pantothenic acid in human nutrition.

The fate of PABA in the kidney has been studied by Lundquist, using the techniques of modern renal physiology. It was concluded that PABA is secreted by the tubules in addition to being filtered by the glomerulus. Unfortunately, here, too, PABA was determined by the Bratton-Marshall colorimetric method which does not differentiate between PABA and p-aminobipirinic acid (PAHA). Since it is well known that PAHA is secreted by the tubules, the significance of Lundquist's conclusions may be questioned. Similarly, Terp's conclusions that PABA is eliminated by tubular secretion in the dog and rabbit is open to question because the method employed in the determination of PABA does not differentiate PABA from PAHA.

Beyer et al. have studied PABA clearance in the dog, utilizing the Cohen and McGilvery method involving ether extraction at pH 3.95 for the separation of PABA from PAHA. Extremely low clearance ratios for PABA were found, thus indicating tubular reabsorption. Although comparisons between the human being and the dog with respect to renal physiology are not always warranted, it would be surprising, indeed, if clearance studies of PABA in the human subject, utilizing a specific method for the determination of PABA, did not show low renal clearance for PABA, indicative of tubular reabsorption.

PABA is excreted dermally to the extent of about 0.24 γ per 100 ml. of sweat, according to Johnson et al. Their results were based on microbiological assays with Acetobacter suboxydans.

X. Toxicity

LEMUEL D. WRIGHT and PETER A. TAVORMINA

A. IN LABORATORY ANIMALS

The acute toxicity of PABA and its sodium salt has been determined in animals by Scott and Robbins. The results are summarized in Table VIII.

Orally, acute toxic signs in mice consist at first only of weakness and loss of normal posture, death occurring in several hours. Mild chronic

convulsions with death in 5 to 10 minutes result from intravenous lethal doses in mice and rats. In dogs, 1 to 2 days elapse before death occurs. Toxic signs are tremor, listlessness, and weakness. Toxic and chronic convulsions were observed in one dog receiving 3.0 g. per kilogram. Autopsies in the animals which received lethal doses show acute gastroenteritis with hemorrhages apparently of capillary origin in the small intestine. Acute necrosis of the liver occurred in the dogs given the two highest dosages. In chronic toxicity trials of 28 days' duration, growing rats receiving up to 1.4 g. of PABA per kilogram orally per day made as good gains as the controls. At autopsy none of the rats receiving the drug showed evidence of toxicity.

Richards\(^2\) studied the toxicity of sodium p-aminobenzoate at pH 6.0 in a number of species. Only 30% of a group of rats died after intravenous injections of 4 g. per kilogram. The symptoms consisted of convulsions and respiratory paralysis that might be due to the high osmotic pressure of the solution. On a dose of 4 g. per kilogram given intraperitoneally, 40% died within a few hours. No deaths occurred after oral dosages unless at least 6 g. per kilogram was fed daily for 3 successive days. The LD\(_{50}\) in rabbits is approximately 2 g. per kilogram, given intravenously. The blood pressure of dogs and cats is raised slightly by doses of 100 mg. of PABA per kilogram, but this rise was found to depend upon the rate of injection of the drug. No change in respiration occurs.

Chronic intraperitoneal toxicity of PABA in growing rats at the single level of 7.5 mg. per day has been studied by Sullivan and Archdeacon.\(^3\) After 48 days of treatment, rats receiving PABA show distinct symptoms of toxicity. Prominent evidences of toxicity are depressed growth, bloated abdomens, diarrhea, and increased weight of the adrenals.

Chronic oral toxicity of PABA in growing rats at a level of 3% of the diet was studied by Gordon \(et\ al.\)\.\(^4\) PABA feeding causes a significant in-

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crease in the size of the thyroid gland and a reduced oxygen consumption. It does not cause significant alterations in the blood picture.

The chronic oral toxicity of PABA fed in the diet in an amount calculated to furnish 2 g. per kilogram of body weight has been studied in rats by Upton and Zarafonetis.\(^5\) This dose is approximately five times that ordinarily administered as a therapeutic dose in man. After the animals had received the PABA-containing diet for 6 months, complete autopsies were done and tissue sections examined. No significant pathological changes were observed with the exception of histological alterations in the thyroid. These thyroid changes consisted of a reduction in the amount and cosinophilia of intrafollicular colloid associated with epithelial hypertrophy and hyperplasia. No hepatotoxic effects were observed.

Ershoff\(^6\) has fed purified diets containing 1 or 2% of PABA or 1% of PABA and 1% of inositol to female rats. No adverse effects on growth or reproduction were noted in the animals fed these diets for a number of months. Lactation was poor, but this was observed on unsupplemented control diets as well and could not be ascribed, therefore, to PABA.

**B. IN HUMAN BEINGS**

Studies by Strauss and Finland\(^7\) have shown that large amounts of PABA may be administered orally to human beings without untoward effects. One patient received 2 g. of PABA at 3-hour intervals for 12 hours. Another patient was given PABA at 2-hour intervals over a 52-hour period during which time 29 g. was ingested. Later the same patient received 33 g. of PABA over a 66-hour period. Thus in less than two weeks this patient received over 62 g. of PABA.

With the advent of PABA for the treatment of ricketsial diseases, amounts of this compound of the order of 25 to 30 g. per day have been administered orally to hundreds of patients. Concerning the toxicity of PABA in the treatment of ricketsial diseases, Cruickshank and Mitchell\(^8\) state: "No record of pathological changes caused by the drug in human subjects has been found, although the pathology of two fatal cases of typhus, treated with it, is said to have been studied".\(^9\)

On the other hand, Cruickshank and Mitchell\(^8\) were the first to note toxicity when they observed two cases of acute rheumatic fever and one of arthritis in children who were treated with PABA where the outcome was

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P-AMINOBENZOIC ACID

fatal in each instance. Each patient received about 20 g. per day for about a week. At autopsy these cases showed marked to extreme deposits of fat in the epithelial cells of the liver, kidney and myocardium. Similar fatty changes in liver, kidney, and heart of rabbits were produced experimentally with PABA.

XI. Pharmacology
CHARLES C. SCOTT

The literature reveals little of pharmacologic interest concerning PABA prior to recognition of its vitamin activity. Early works dealt with acetylation processes by the body, toxicity, and relation to certain local anesthetics. Obviously, PABA seemed relatively weak in ordinary pharmacologic actions, which no doubt accounts for lack of interest in the compound by early investigators.

Ansbacher in 1944 and Molitor and Emerson in 1948 have published extensive reviews on the pharmacology of this substance.

A. ABSORPTION, FATE, AND EXCRETION

PABA is readily absorbed by usual routes of administration. For example, oral and subcutaneous doses resulted in maximal blood concentrations in 1 hour when given to mice and rabbits. Elimination of the substance occurs mainly in the urine. Ellinger and Hensel noted that PABA was partially acetylated (approximately 30%) by rabbits before excretion. Man and rabbit were found to acetylate the substance before excretion, but acetylation did not occur in dogs. According to Harrow and co-workers, rabbits acetylated 25% of PABA, and this process was greatly increased by insulin. Conversely, acetylation of PABA was significantly lower than normal in alloxan-diabetic rats.

Recent work, however, has shown that in man, at least, PABA is excreted predominantly in other forms. Smith et al. found PABA to be

1 S. Ansbacher, Vitamins and Hormones 2, 215 (1944).
6 J. B. Muenzen, L. R. Cerecedo, and C. P. Sherwin, J. Biol. Chem. 63, xvi (1925).
7 B. Harrow, A. Mazur, and C. P. Sherwin, J. Biol. Chem. 102, 35 (1933).
converted to \( p \)-aminohippuric acid and excreted in this form in the urine. Conversion of PABA occurred in the liver, normally being rapid, but was depressed in primary liver disease.\(^8\) Apparently the \( p \)-aminohippurate is the main excretory form, but there is still another. According to Tabor and coworkers,\(^11\) man excreted very little PABA in either the free or acetylated forms, excretion occurring mainly as a conjugation product with glycine or glucuronic acid. Zarafonetis and Chandler\(^12\) also noted the reducing substance, glucuronic acid, in urine of patients receiving large doses of PABA. Dogs were found to excrete \( p \)-aminohippuric acid when PABA administration was prolonged.\(^3\)

Acetylation of PABA in various species of cold-blooded animals is likewise variable. Chen and associates\(^15\) found that the spadefoot toad and the nebulous toad were able to acetylate PABA, whereas acetylation did not occur in the leopard frog or the turtle.

Renal clearance studies have shown that excretion of PABA occurs partly through secretion by the renal tubules as well as glomerular filtration.\(^14\), \(^15\) Secretion may be blocked by \( o \)-iodohippurate.

Excretion is accomplished by routes other than the urinary tract. In fact, Denko et al.\(^16\) have found that fecal excretion is greater than urinary, but this is probably owing to synthesis of PABA by intestinal flora. Johnson et al.\(^17\) noted a concentration of 0.24 \( \gamma \) per 100 ml. in the sweat of four male humans.

Studies on the entire animal have shown that mice metabolize PABA rapidly.\(^11\) No significant amount of the substance can be detected after 8 hours. It appears to penetrate freely into the cells of liver, lung, and kidney, but only slowly into muscle, brain, and erythrocytes.\(^15\) Studies by Lustig et al.\(^18\) likewise have revealed no measurable amount of PABA accumulating in organs and tissues of injected mice.

**B. TOXICITY**

Gibbs and Hare\(^19\) in 1889 concluded that PABA was without effect on the animal organism. Doses of 500 mg. to 2 g. in dogs weighing 5 to 7 kg.

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caused no toxic reactions when the substance was administered as a single
dose by stomach tube. In mice, 50 mg. subcutaneously was without effect,
although larger amounts caused temporary excitation followed by par-
alysis.\textsuperscript{20} Ellinger and Hensel\textsuperscript{4} noted that 1 g. subcutaneously produced
death in some rabbits, but Hensel\textsuperscript{21} subsequently found that doses up to
2 g. subcutaneously per day for 4 days were apparently non-toxic.

Tadpoles could withstand a 0.5\% solution for 3 days, when death oc-
curred.\textsuperscript{22} A dose of 1 g. per kilogram was tolerated by dogs but 10 g. per
kilogram caused emesis. In humans, 5 g. produced nausea and vomiting,
although this dose is now commonly exceeded. Toxic symptoms in mam-
mals were similar to those following aniline, namely, fall of body tempera-
ture, marked muscular weakness, accelerated respirations, cardiac depres-
sion, tetanic cramps, and finally death from respiratory standstill.

According to Selbie,\textsuperscript{23} the oral lethal dose in mice was 100 to 200 mg.
Scott and Robbins\textsuperscript{24} found PABA to be more toxic to mice and dogs than
to rats. In dogs, single oral doses larger than 1 g. per kilogram caused death
in some instances, with acute gastroenteritis and hemorrhage into the
small bowel. Acute necrosis of the liver resulted from doses of 2 g. per kil-
ogram. Rats, however, tolerated a daily oral dose of 1.4 g. per kilogram for
4 weeks without inhibition of growth or appearance of pathologic changes.
Intravenous injection of 4 g. per kilogram was lethal to 30\% of rats, mani-
festations of toxicity being convulsions and respiratory paralysis.\textsuperscript{25} Given
intraperitoneally, this dose killed 40\% of the animals within 4 hours.
Given orally, no deaths occurred unless at least 6 g. per kilogram was fed
daily for 3 consecutive days. The LD\textsubscript{50} intravenously in rabbits was ap-
proximately 2 g. per kilogram. Young rats injected with 200 to 500 mg.
per kilogram daily for 21 days grew slightly faster than controls. No ab-
normailties in the blood or urine were noted. After receiving 600 mg. per
kilogram intraperitoneally, rats showed a slight increase of oxygen con-
sumption.

On feeding 1\% PABA in the diet of female rats, Ershoff\textsuperscript{26} could find no
adverse effect on growth, reproduction, or lactation. Adult male rats which
ate a diet containing 3\% PABA gained weight normally, and the blood
picture did not change.\textsuperscript{27} On the other hand, Sullivan and Archdeacon\textsuperscript{28}

\begin{itemize}
\item \textsuperscript{20} H. Hildebrandt, \textit{Beitr. Chem. Physiol. Pathol.} 3, 365 (1903).
\item \textsuperscript{21} M. Hensel, Z. physiol. Chem. 93, 401 (1915).
\item \textsuperscript{22} A. Oswald, Chemische Konstitution und Pharmakologische Wirkung. Gebrüder
   Bornträger, Berlin, 1924.
\item \textsuperscript{23} F. R. Selbie, \textit{Brit. J. Exppl. Pathol.} 21, 90 (1940).
\item \textsuperscript{25} R. K. Richards, \textit{Federation Proc.} 1, 71 (1942).
\item \textsuperscript{27} A. S. Gordon, E. D. Goldsmith, and H. A. Charriper, \textit{Endocrinology} 37, 223 (1945).
\item \textsuperscript{28} C. D. Sullivan and J. W. Archdeacon, \textit{Endocrinology} 41, 325 (1947).
\end{itemize}
found depression of growth in young rats injected intraperitoneally with only 7.5 mg. daily for 48 days. Marked accumulation of intestinal gas occurred, along with diarrhea. Young human patients who received up to 148 g. of PABA over a period of days in treatment of acute rheumatic fever on autopsy showed deposits of fat in epithelial cells of liver, kidney, and myocardium, according to Cruickshank and Mitchell. These authors noted similar changes in rabbits.

It is obvious from the above that the toxicity of PABA is relatively of a very low grade, either in acute or chronic administration.

C. PHARMACOLOGICAL ACTIONS

1. Circulatory System

Like procaine, intravenous injection of PABA reduced the incidence of ventricular fibrillation induced by epinephrine in dogs under cyclopropane anesthesia.

According to Gibbs and Hare, PABA given intravenously in doses of 0.5 to 1 g. per 8 kg. had no effect on pulse rate or blood pressure of dogs. This was confirmed by Richards, who noted only slight elevation of blood pressure of dogs or cats injected intravenously with doses of 100 mg. per kilogram.

2. Blood

PABA was found to increase blood and tissue iron of liver and muscle of rabbits treated simultaneously with reduced iron. Likewise, Annoni found that certain cases of anemia responded to PABA, with a rise of hemoglobin level amounting to 8 to 12% and a small increase of red blood cells. PABA was capable of restoring prothrombin time to normal when depressed by sodium salicylate.

3. Gastrointestinal Tract

Baldini and Ceir reported that PABA temporarily stimulated gastric motility and gastric secretion but had no effect on gastric acidity of humans. Merlo and Ercoli confirmed the gastric secretory effect except in

33 F. Muratore and T. Putignano, Boll. soc. ital. biol. sper. 24, 270 (1948).
36 P. Merlo and G. Ercoli, Vitaminologica 1946, 104. [C. A. 41, 5629 (1947)].
histamine-resistant patients. This effect occurred following either oral or parenteral administration of PABA.

4. **Nervous System**

PABA may exert a mild sedative effect in certain autonomic nervous, extrapyramidal, Parkinsonian, and choreic syndromes, tics, and epilepsy, according to a recent report by Morpurgo and Fachini.\(^{37}\)

5. **Muscle**

Torda and Wolff\(^{38}\) observed no effect of PABA on acetylcholine sensitivity of muscle. Survival of frog muscle and nerve after somatic death was found by Sollman\(^{39}\) to be prolonged by PABA.

6. **Enzyme Systems**

**a. Cholinesterase**

In physiologic concentrations, PABA inhibited serum cholinesterase by 10 to 30\%, according to Zeller.\(^{40}\) This may account for the acetylcholinergic action of PABA reported by Danielopolu.\(^{41}\) The weak effect of PABA on serum cholinesterase of humans was confirmed by Paget and Dhellemmes.\(^{42}\) However, Koelle\(^{43}\) reported no protective action by PABA against inactivation of brain cholinesterase by diisopropyl fluorophosphate.

**b. Tyrosinase**

The *in vitro* destruction of epinephrine by tyrosinase was shown by Martin *et al.*\(^ {44}\) to be inhibited by PABA.

**c. Phosphatase**

PABA apparently depresses phosphatase in certain locations but stimulates in others. Tamayo and Cuellar\(^ {45}\) and Javier de Elio and Felix Sanz Sánchez\(^ {46}\) reported inhibition of serum phosphatase, and Silver and Gold-
ing noted depression of bone phos-phatase by this substance. On the other hand, alkaline phosphatase of renal distal tubuli of rats was increased by PABA and glycosuria was said to result.\(^{48}\) The significance of these findings is not clear. Sulfonamides in general were not antagonistic but exerted the same effect as PABA.

7. Endocrine System

a. Thyroid

The MacKenzie\(^{49}\) first noted that PABA caused enlargement of the thyroid gland of rats, and this observation was confirmed almost simultaneously by Astwood.\(^{50}\) The substance likewise produced this effect in mice and dogs.\(^{51}\) \textit{In vitro} studies by Franklin et al.\(^{52}\) showed that PABA in a concentration of \(10^{-3}\) M depressed the rate of conversion of inorganic iodide to thyroxine and diiodotyrosine. Berman\(^{53}\) studied the effect of PABA in six human patients with thyrotoxicosis and obtained a good antithyroid response in each case. Dosage was 1 to 1.5 g. parenterally six times weekly for 3 to 9 months.

Adult male rats fed large doses of PABA for 19 to 45 days\(^{57}\) responded with an increase in size of the thyroid gland from 17 mg. to 41 mg. and a fall of oxygen consumption of 30%. However, the goiterogenic activity of PABA was relatively weak.\(^{54}\) According to McGinty and Bywater,\(^{55}\) the activity of PABA is 0.3, with thiouracil taken as 100. Vanderlaan and Bissell\(^{56}\) also noted the goiterogenic action of PABA to be slight but definite. In large doses (2 g.) to human subjects, Gualco et al.\(^{57}\) claimed antithyroid activity of PABA to be nearly comparable to that of thiouracil and methylthiouracil. PABA was found to resemble thiouracil in its action rather than sulfaguanidine in that both its goiterogenic and hyperplastic actions were inhibited by iodide.\(^{58}\) Both PABA and thiouracil inhibited the \textit{in vitro} formation of acetylthryroxine from acetyldiiodotyrosine.\(^{59}\)


\(^{51}\) G. J. Martin, \textit{Arch. Biochem.} 3, 61 (1943).


\(^{56}\) W. P. Vanderlaan and A. Bissell, \textit{Endocrinology} 38, 308 (1946).

\(^{57}\) S. Gualco and V. Patrono, \textit{Minerva med.} 2, 353 (1947) [\textit{C. A.} 42, 1657 (1948)].


b. Miscellaneous

In studies utilizing the Warburg technique and bioassay in ovariectomized rats, Ansbacher et al. found that PABA inhibited the destruction of stilbesterol. Possibly a similar effect was noted recently by Wiesel et al. in that PABA made possible a marked reduction in the cortisone requirements of rheumatoid arthritic patients.

8. Detoxicating Action

In a series of papers, Sandground and Hamilton reported that PABA was highly effective in affording protection against high lethal doses of certain pentavalent arsenicals and antimonials. This protective action of PABA did not interfere with the trypanocidal activity of the metalloids. If injected before the arsenical, PABA protection was maximal. The effect was distinctly reduced when PABA was administered after the toxic agent, and protection was further decreased with increasing time interval. This would suggest a competitive effect for action on some receptor, but no chemical similarity was noted. Protection was not afforded against the trivalent arsenicals, inorganic arsenious acid, mapharsen and arsphenamine, or tartar emetic (trivalent antimonial). However, PABA did protect against neoarsphenamine, and this was confirmed by McChesney et al. Peters also found no detoxicant action by PABA against mapharsen. The mechanism of protection was studied by Harris, who showed a decrease in extent and severity of renal damage of rats treated with PABA as compared with animals which received only the toxic drug.

Inhibition of the convulsive action of procaine by PABA was reported by Richards and Kueter. However, the peripheral local anesthetic effect of procaine was not inhibited. The chemical similarity here is obvious.

Voss and Tatum found a definite, although low-grade, protection by PABA against certain organic bismuth preparations in rats.

69 P. N. Harris, J. Pharmacol Exptl. Therap. 82, 251 (1941).
D. THERAPEUTIC USES

1. GRAY HAIR

Early in the work on PABA as a vitamin-like substance, Ansbaecher\(^{73}\) reported the substance to be effective against nutritional achromotrichia of rats. Subsequently, Martin and Ansbaecher found that PABA cured the gray hair of mice caused by hydroquinone\(^{74}\) and nutritional deficiency.\(^{75}\) Emerson,\(^{76}\) however, could obtain no chromotrichial effect with PABA in grayed rats, although pantothenic acid was effective. Her work was confirmed by Unna et al.,\(^{77}\) Henderson et al.,\(^{78}\) and Brown and Sturtevant.\(^{79}\) Ershoff,\(^{80}\) who also was unable to confirm the work of Martin, stated that the latter employed near-marginal levels of some of the B-complex vitamins.

Early clinical trials by Sieve\(^{81}\) and Eller and Diaz\(^{82}\) were said to show evidence of darkening of gray hair in human beings, even though the gray color was of many years' duration. Friedgood\(^{83}\) also noted a few patients in whom PABA seemed to have chromotrichial activity. Photographs showing darkening of hair were presented by DeVilbiss\(^{84}\) for a series of sixteen patients who received the substance. Stimulation of libido was noted, and this was also claimed by Sieve\(^{85}\) in a series of 600 patients. Also reported in this large series were darkening of gray hair and increased pigmentation of normally pigmented areas as well as of areas lacking pigmentation. Nevi and freckles almost disappeared. Lactation was increased, and amenorrhea was relieved in some cases. Of twenty-two women sterile for over 5 years, PABA therapy was followed by conception in twelve cases. Male impotency was said to be relieved and some cases of bronchial asthma were improved. Confirmation of these extensive claims has been lacking.

The effect on gray hair of human beings has not been verified by subsequent work. Martino and Fabiano\(^{86}\) found no effect on white hair when

\(^{73}\) S. Ansbaecher, *Science* 93, 164 (1941).


\(^{86}\) L. Martino and A. Fabiano, *Boll. soc. ital. biol. sper.* 17, 702 (1942) [C. A. 40, 7389 (1946)].
PABA was given by intramuscular injection or by direct application to the scalp. In carefully controlled observations, Brandaleone et al. 87, 88 found PABA to be valueless in treatment of gray hair of human beings. These authors pointed out the difficulty of ascertaining darkening of hair when objective methods are not used. At present, the consensus is that vitamin therapy is ineffective in treatment of ordinary gray hair of human beings.

2. Dermatology

According to Strauss et al., 89, 90 PABA in amounts sufficient to nullify the bacteriostatic effects of sulfathiazole had no beneficial action on rash or fever caused by the latter. Rothman et al. 91 observed that PABA in an ointment base protected the skin against sunburn. Irradiated solutions of PABA caused erythema when injected intradermally, whereas non-irradiated solutions did not. A 15% ointment of PABA protected against fifty to one hundred times the usual erythema dose of ultraviolet light. 92 These findings support the concept that ultraviolet erythema is due in part to photochemical reaction of PABA in the skin. Costello 93 reported a case of vitiligo successfully treated with PABA, which agreed with the findings of Sieve. 95

3. Rickettsial Diseases

Snyder et al. 94 first noted that PABA in large doses was effective therapy for experimental rickettsial infections. This was confirmed by Greiff et al. 95 Human tests were first made on twenty cases of typhus fever by Yeomans et al., 96 who found that when PABA was administered in large doses during the first week of the disease the course was much less severe. Subsequently, these findings have been substantiated in experimental rickettsial infections by Murray et al., 97 Snyder and Zarafonetis, 98 Hamilton, 99 and Hamilton

et al., Clinical verification has been reported by Anigstein and Bader, Rose et al., Tierney, Faust, Smith, Levy and Arnold, Maroney et al., Ravenel, Snyder et al., and Hendricks and Peters.

Faust states that PABA may be considered as relatively specific in the treatment of the typhus group of diseases when given in the first week following onset of infection. Dosage is very large. Ravenel specifies that blood levels of 30 to 60 mg. per 100 ml. are desirable and that 60 to 80 mg. per 100 ml. may be necessary in some cases. Levels above 80 mg. per 100 ml. of blood are dangerous. Alkalis also are necessary to prevent acidosis. Under this regimen, a practical guarantee may be given that fatality will not occur.

4. Neoplastic Diseases

Burk et al. found that PABA exerted an anticarcinogenic action on the production of hepatomas of rats fed p-dimethylaminobenzene and excess biotin. Large doses of PABA produced a striking lowering of leucocyte counts of six patients with chronic or subacute myelogenous leukemia. Less definite decreases in leucocyte counts occurred in cases of chronic lymphatic leukemia. Prompt rise in counts followed discontinuation of therapy. The use of PABA did not appear to be a practical form of treatment.

5. Other Diseases

Dry et al. reported the use of PABA to raise and maintain the blood level of salicylates in patients with acute rheumatic fever. PABA alone

seemed to be without therapeutic value. The mechanism by which PABA elevated salicylate blood level was shown to be a depression of conjugation of glycine with salicylate. After PABA, only small amounts of salicyluric acid appeared in the urine. In addition, the urinary acidity was increased, and this decreased the renal clearance of free salicylate. The above holds true in man, but not in dog.

In experimental allergic encephalomyelitis of guinea pigs, Good et al. found that large doses of PABA plus salicylates had prophylactic value, but no therapeutic action. Large doses of salicylates were less effective, and PABA alone was actionless.

No therapeutic or prophylactic value could be demonstrated with PABA against myxoma or vaccinia viruses in eggs.

Rosenblum and Fraser, in contrast to Dry, noted that PABA definitely relieved both fever and joint pains in a series of nine patients with acute rheumatic fever. Dosage was comparable to that used in rickettsial diseases.

Recently, Wiesel and associates reported the use of PABA with cortisone in treatment of fifteen patients with severe rheumatoid arthritis. A completely ineffective daily dose of cortisone (25 mg.) plus 12 g. of sodium p-aminobenzoate daily brought about suboptimal control of the disease. PABA alone was ineffective. The authors believed the action of PABA was related to its ability to suppress hepatic inactivation of estrogens.

XII. Detoxication of Arsenicals

LEMUEL D. WRIGHT and PETER A. TAVORMINA

The concept of competitive inhibition stimulated a host of investigators to attempt a demonstration of this phenomenon in a variety of systems. In an experiment designed to explore the behavior of PABA as an inhibitor of the trypanocidal activity of carbarsone, Sandground made the observation that PABA confers an exceedingly high degree of protection in rats infected with Trypanosoma equiperdum against the toxic properties of the arsenical, without decreasing its trypanocidal effect. The adminis-

tration of PABA in conjunction with lethal doses of the pentavalent arsenicals tryparsamide, acearsonone, arsenilic acid, and benzenearsonic acid was reported by Sandground\(^2\) to be attended by similar protective action, and this was found to be true regardless of the route of administration of either the arsenical or the PABA. The protection is evident within 24 hours, and the usual symptoms of arsenic poisoning, such as tremor, diarrhea, paralysis, and emaciation, are minimized. Histologically, the most significant manifestation of the beneficial action of PABA is in the considerable reduction, both in extent and in severity, of the renal lesions produced by the arsenicals.\(^3\)

It had long been known that the toxicity of trivalent arsenicals could be substantially reduced by means of various agents. The injection of sulfhydryl-containing compounds (e.g., reduced glutathione) simultaneously with, or prior to, the administration of the arsenical was used with success by Voegtlin et al.\(^4\) to offset to a large degree the toxic effects in the rat of mapharside (3-amino-4-hydroxyphenylarsenoxide). Similarly, the suppression of the toxic manifestations of neoarsphenamine was accomplished by Durel\(^5\) and by McChesney and his associates,\(^6\) by means of ascorbic acid. In contrast to these results with the trivalent arsenicals, no effective means of counteracting the ill effects of the pentavalent compounds had been demonstrated, and the elucidation by Sandground of this particular function of PABA was attractive in its implication of potential clinical significance.

In a series of papers devoted to a more detailed study of this detoxication effect, Sandground and his associates reported that essentially all rats are protected against a LD\(_{50}\) of carbarsone (1000 mg. per kilogram) or of arsenilic acid (400 mg. per kilogram) by the prior administration of 500 mg. per kilogram for the latter. The use of 750 mg. per kilogram of the vitamin protects all animals against a dose of carbarsone of 1500 mg. per kilogram, a dose which is not tolerated by a single unprotected rat. As little as 15 mg. of PABA per kilogram protects 50% of animals subjected to a LD\(_{50}\) of arsenilic acid.\(^7\)

In addition to establishing the amount of the vitamin required for a specific measure of protection, Sandground demonstrated\(^8\),\(^9\) the vital


\(^5\) P. Durel, _Bull. soc. franç. dermatol. syphiligr._ **44**, 1077 (1937) [C. A. **32**, 3816 (1938)].


role of the time element in the detoxication of pentavalent arsenicals. Injection of PABA up to 3 hours before the administration of the arsenical affords protection for nearly all animals. On the other hand, when the animals receive the vitamin subsequent to the administration of the arsenical, the detoxicating effect is less pronounced and continues to decrease with increasing time interval. Sandground suggests\(^5\) that this time interval coincides with the time required for the \textit{in vivo} reduction of the pentavalent arsenicals (or for the oxidation of the arsenobenzenes) to the corresponding arsinoxides, the toxicity of which is not reduced by PABA.\(^9\), \(^10\) In support of this theory the author points to the relatively

\[
\frac{1}{2} \text{RAs} = \text{AsR} \rightarrow^{[\text{O}]} \text{RAsO} \leftarrow^{[\text{H}]} \text{RAsO}_2\text{H}_2
\]

slow development of host toxicity and of parasiticidal activity of the pentavalent compounds, as compared with the rapid appearance of both these effects in the case of the arsinoxides. The fact that PABA does not decrease the trypanocidal effect of the pentavalent arsenicals is interpreted by Sandground as indicating that the vitamin does not interfere in the reduction of the arsanic acid to the arsioxide.

The reviewers find some difficulty in subscribing to this view. If the toxicity of arsanic acids can be ascribed to their conversion to the arsioxide (detoxication of which is not effected by PABA) and if PABA offers no appreciable obstruction to the conversion, it would seem unlikely that PABA could materially reduce the toxicity of the pentavalent arsenical.

In further studies Sandground\(^11\) showed that the three isomeric amino-benzoic acids, the hydroxy and nitro analogs of PABA, phenylacetic acid, phenylpropionic acid, and benzoic acid, itself, all confer some degree of protection. This observation led him to consider unlikely the direct combination of arsenical and detoxicant to produce a non-toxic complex as a mechanism of action. Similarly, the possibility of competition for a specific enzyme site was excluded in view of the protective activity of compounds so unrelated structurally to the arsanic acids as phenylglycine or benzyl succinate. The only structural arrangement which appears to be a prerequisite for activity is a carboyclic system of five or six members, associated with a free carboxyl group. Esterification or amidation of the acid group reduces activity.

The apparent lack of specificity of PABA in this connection is supported by the data of Martin,\(^12\) who found that the administration of thyroxine along with the vitamin nullifies to a great extent its detoxicating property. The theory was advanced that PABA functions by virtue of its ability to

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inhibit the thyroid, thus decreasing tissue metabolism and, concomitantly, arsensoxide formation.

It has been mentioned that PABA does not influence the lethal action of the more usual type of arsencals such as mapharside, neoarsphenamine, etc. In contrast to this, the trypanocidal action of \( \gamma \)-\((p\text{-arsenosophenyl})\)-butyric acid is completely inhibited by the vitamin. Williamson and Lourie\(^{13, 14} \) demonstrated this interference both in vivo and in vitro. The authors noted that lethal quantities of arsenic are absorbed by trypanosome cells in a matter of seconds. Furthermore, the arsencal, when injected, disappears from the blood stream in one-half hour, with or without the prior administration of PABA. The rapidity with which the arsencal takes effect is believed responsible for the inability of PABA to interfere in the “cidal” activity when the vitamin is administered subsequently to the arsencal.

In vitro, PABA immobilizes *Trypanosoma rhodesiense* reversibly and innocuously and protects the organism for at least 24 hours from the action of \( \gamma \)-\((p\text{-arsenosophenyl})\)butyric acid. Williamson and Lourie express the opinion that PABA immobilizes the parasite cell by absorption into, or adsorption on the surface of, the organism. Presumably, this process inhibits the lethal action of the arsencal by impeding its penetration into the cell or its fixation on the surface of the cell.

The inhibition of the trypanocidal activity of \( \gamma \)-\((p\text{-arsenosophenyl})\)-butyric acid by PABA, a property which this vitamin does not exercise against the usual arsencals, is attributed by Williamson and Lourie to the different process by which this arsencal permeates the cell. In support of this view the authors cite the activity of this compound against tryparsamide-resistant strains of the trypanosome, as well as the presence of the acetic acid configuration, a structure which has been found to be common to the group of arsencals active against atoxyl-resistant trypanosomes. These compounds are rendered more water-soluble by the presence of the more polar groups and would be expected to penetrate the cell in a fashion different from that of compounds devoid of hydrophilic groups. The latter type of arsencal ostensibly enters via the lipid components of the organism.

It has been mentioned that except for its anomalous behavior in the case of \( \gamma \)-\((p\text{-arsenosophenyl})\)butyric acid PABA does not reduce the trypanocidal action of any of the arsencals. Schleyer and Schnitzer\(^{15} \) have found, however, that the methyl and ethyl esters and the amides of benzoic, \( p \)-hydroxybenzoic, \( p \)-aminobenzoic, 2,4- and 2,5-dihydroxybenzoic, and nicotinic acid do reduce the parasiticidal activity of mapharside or acriflavin.


on *T. equiperdum*. No such effect could be demonstrated with the corresponding free acids.

The observations reported by Sandground on the protective action of PABA against the toxicity of arsenicals have been supplemented by analogous findings with respect to the detoxication of other organo-metallic substances. Sandground\(^1^6\) extended his studies to the antimonial, "stibosan"

\[
\text{CH}_3\text{CONH—}——\text{SbO}_3\text{HNa}
\]

"Stibosan"

and found PABA to afford a high order of protection against the toxic action in rats of this drug, without a reduction of its trypanocidal potency. Hardcastle and Foster\(^1^7\) found that PABA appears to have promise as a detoxicant for borax. Voss and Tatum\(^1^8\) have reported that PABA exerts a protective action in rats against lethal doses of sodium bismuthyl citrate, mannonate, or tartrate. Similar findings have been published by Sala and Borasi\(^1^9\) concerning the detoxication by PABA of both organic and inorganic mercury compounds.

### XIII. Sulfonamide Reversal

**LEMUEL D. WRIGHT AND PETER A. TAVORMINA**

The advent of the sulfonamides (1935–1940) stimulated considerable research into the mechanism of action of these highly useful chemotherapeutic agents. Lockwood in 1938 showed\(^1\) that sulfonamide activity *in vitro* can be reversed by peptone. Heat-killed bacterial cells or their extracts were shown by Stamp\(^2\) to reverse sulfonamide action. He showed also that the sulfonamide-neutralizing activity of such bacterial extracts is stable to heat, acid, and alkali. MacLeod\(^3\) confirmed the antisulfonamide activity of peptone and tissue extracts. He further demonstrated that the production of sulfonamide-neutralizing material by the pneumococcus is markedly increased as the organism becomes resistant to sulfonamides.

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\(^{19}\) G. Sala and M. Borasi, *Atti soc. lombarda sci. med. e biol.* 3, 70 (1948) [C. A. 45, 3946 (1951)].

\(^1\) J. S. Lockwood, *J. Immunol.* 35, 155 (1938).


\(^3\) C. M. MacLeod, *J. Exptl. Med.* 72, 217 (1940).
Woods and Fildes, and subsequently Woods in more detail, showed that the sulfonamide-reversing agent present in yeast in addition to being stable to heat, acid, and alkali, as reported by Stamp, is inactivated by nitrous acid, acetylation, or esterification. These data together with a consideration of the solubility and acid-dissociation constant of the sulfonamide-reversing agent led to a trial of synthetic PABA. This compound proved highly active as a sulfonamide-neutralizing compound not only in vitro but also in vivo against sulfanilamide in the treatment of a hemolytic streptococcal infection of mice. Although the sulfonamide-neutralizing activity of natural material was not isolated and identified as PABA by Woods, this was accomplished subsequently by Rubbo and Gillespie and by Blanchard.

Woods' observation that PABA is highly active in reversing the bacteriostatic action of the sulfonamides has been confirmed in many laboratories and against many strains of bacteria. PABA does not, however, reverse sulfonamide action in all species, Bacterium tularense being the best-known exception.

Since PABA is an effective reversing agent for the sulfonamides, at least in the case of the majority of microorganisms, it was apparent to Woods that the development of sulfonamide resistance in bacteria may be associated with increased synthesis of PABA. For a critical test of this hypothesis, a specific test for PABA was essential. With the development by Landy and Dicken of a microbiological assay for PABA based on the essential nature of this compound for Acetobacter suboxydans, the synthesis of PABA by sulfonamide-sensitive and sulfonamide-resistant bacteria could be examined. Landy and co-workers observed in culture filtrates from sulfonamide-resistant staphylococci fifty to one hundred times as much PABA as was encountered in culture filtrates from sulfonamide-sensitive strains. Increased PABA synthesis occurs even when the strains of staphylococci are grown for many generations in the absence of sulfonamides. Similar findings with respect to the synthesis of large amounts of PABA by sulfonamide-resistant staphylococci were recorded by Spink et al., using both the Acetobacter suboxydans and the Lactobacillus arabino-
sus^{15} methods for PABA determination. Increased synthesis of PABA by sulfonamide-resistant strains of staphylococci was noted by Housewright and Koser,^{14} using the Clostridium acetobutylicum assay. Increased PABA production was subsequently found by Landy and Gerstung^{15, 16} to be associated with the development of sulfonamide resistance in Neisseria gonorrhoeae. Less conclusive evidence that PABA synthesis is correlated directly with sulfonamide resistance in Diplococcus pneumoniae,^{3, 17, 18} strains of Clostridia,^{19} Brucella paramelitensis,^{20} and Shigella sonnei^{21} has been presented. On the other hand, Landy et al.^{11} did not find increased PABA synthesis associated with sulfonamide resistance in strains of Escherichia coli, Vibrio cholerae, Shigella dysenteriae, or Diplococcus pneumoniae, and Housewright and Koser^{14} concluded as a result of microbiological assays with Clostridium acetobutylicum that sulfonamide resistance in Shigella paradysenteriae and Diplococcus pneumoniae is not associated with increased PABA production. It must be concluded that with certain species of bacteria, particularly the staphylococci, sulfonamide resistance is explainable in terms of increased PABA synthesis, but that in other species, for example, Escherichia coli, sulfonamide resistance is not associated with the same phenomenon.

Of equal interest with respect to the functions of PABA in metabolism is a consideration of the reversal of sulfonamide action by compounds unrelated to PABA in structure.

Soon after Woods observed that PABA reverses sulfonamide action, it was observed that methionine exhibits a similar activity.^{21-24} Adenine or hypoxanthine is reportedly as active as PABA in negating the effect of sulfanilamide against Streptococcus hemolyticus infection in mice,^{25} and various purines reverse the effect of sulfanilamides on lactic acid bacteria.^{26} More recently the sulfonamide-reversing activities of thymine,^{27-29} folic

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23 J. S. Harris and H. I. Kohn, J. Biol. Chem. 141, 989 (1941).
acids, arginine, histidine, lysine, glutamic acid, aspartic acid, isoleucine, tryptophan, and valine have been reported.

Considerable clarification with respect to the activities of the above apparently unrelated compounds in reversing the action of sulfonamides has come about from the use of inhibition analysis as developed by Shive and co-workers. Inhibition analysis has been explained in detail a number of times by Shive, and these references should be consulted for a full appreciation of the possibilities inherent in the procedure as well as the significance of the terms used to describe the results obtained.

Sulfonamides inhibit the functioning of PABA in Escherichia coli as determined by Shive and coworkers in a synthetic salts-glucose medium with an inhibition index of about 3000 (1 part of PABA will reverse the effect of 3000 parts of sulfanilamide). In the presence of methionine, this inhibition ratio is increased to 10,000 (which indicates that more sulfanilamide is required for inhibition in the presence of methionine). Thus it would appear that one function of PABA, not necessarily as such but in a form whose synthesis is inhibited by sulfanilamide, is concerned with methionine synthesis. The reaction affected is postulated by Shive and coworkers as follows:

\[
\begin{align*}
\text{NH}_2 & \quad \text{HSC} \text{H}_2\text{CH}_2\text{CHCOOH} \\
\text{inhibition} & \quad \text{index, 3000} \\
\text{Homocysteine (or related precursor)} & \quad \text{CH}_3\text{S—CH}_3\text{H}_2\text{CHCOOH} \\
\text{Methionine} & \end{align*}
\]

In the presence of methionine the inhibition index of sulfanilamide for Escherichia coli may be raised from 10,000 to about 30,000 by purines. Purines and their related compounds effective in reversing sulfanilamide under these conditions include adenosine, xanthine, guanine, and inosine. Adenine not only is ineffective in reversing sulfanilamide but acts synergistically as an inhibitor.

An increase in the inhibition index from 10,000 to 30,000 with addition of purines to the medium indicates that PABA functions in the synthesis of purines. In this case the precursor of the reaction blocked by the failure of PABA, in the presence of sulfanilamide, to be converted to a metabolically active form has been elucidated. There accumulate in Escherichia

coli cultures grown in the presence of just sub-bacteriostatic amounts of sulfanilamide, and, preferably, increased amounts of glycine, an amine that has been isolated and identified as 5(1)-amino-4(5)-imidazolecarboxamide. The reaction blocked indirectly by sulfanilamide in the synthesis of purines is as follows:

\[
\begin{align*}
\text{H}_2\text{N} & \text{—C} = \text{O} \\
\text{C} & \text{—NH} \\
\text{H}_2\text{N} & \text{—C} \equiv \text{N} \\
\end{align*}
\]

\[
\begin{align*}
\text{inhibition} & \text{, index, 30,000} \\
\end{align*}
\]

5(4)-Amino-4(5)-imidazolecarboxamide (or riboside)

\[
\begin{align*}
\text{H}_2\text{N} & \text{—C} = \text{O} \\
\text{C} & \text{—NH} \\
\text{H}_2\text{N} & \text{—C} \equiv \text{N} \\
\end{align*}
\]

\[
\begin{align*}
\text{inhibition} & \text{, index, 50,000–100,000} \\
\end{align*}
\]

Hypoxanthine (or ribose)

A increase in the inhibition index from 30,000 to 50,000–100,000 with addition to the Escherichia coli medium now containing methionine and purines may be obtained by addition of serine. These data lead to the conclusion (considerably strengthened by unrelated experiments from other sources) that PABA functions indirectly in the synthesis of serine.

\[
\begin{align*}
\text{CH}_2\text{COOH} & \text{ —NH}_2 \\
\text{inhibition} & \text{, index, 50,000–100,000} \\
\text{CH}_3 & \text{—CHCOOH} \\
\text{OH} & \text{—NH}_2 \\
\end{align*}
\]

Glycine

Serine

An increase in the inhibition index from 50,000–100,000 to 200,000–300,000 may now be obtained in the presence of methionine, purines, and serine with either relatively large amounts of thymine or much smaller amounts of folic acid. In this instance the precursor of thymine is unknown.

\[
\begin{align*}
\text{HN} & \text{—C} = \text{O} \\
\text{O} & \text{—C} \equiv \text{CH}_2 \\
\text{HN} & \text{—CH} \\
\end{align*}
\]

Thymine (or derivative such as thymidine)

Although a study of the various compounds that reverse sulfonamide inhibition of microorganisms leads to considerable clarification of the reactions carried out by a metabolically active derivative of PABA whose

synthesis is inhibited by sulfonamides, these studies were not in themselves instrumental in describing the derivative.

The site of action of the sulfonamides has been elucidated from several other lines of investigation that appeared at the time to be unrelated.

When certain sulfonamides are incorporated into *highly purified* diets adequate with respect to thiamine, riboflavin, pyridoxine, nicotinic acid, and pantothenic acid, typical signs of dietary deficiency are produced in rats consuming such diets.\(^{39-43}\) Nielsen and Elvehjem\(^ {44}\) were the first to report that the deficiency thus induced could be alleviated with folic acid concentrates and biotin. The effectiveness of folic acid was soon confirmed in a number of laboratories.\(^ {45-48}\) These animal studies were the first to show that folic acid could be a derivative of PABA whose synthesis, in this case by intestinal bacteria, is inhibited by sulfonamides.

A remarkably specific effect of sulfonamides in inhibiting folic acid synthesis *in vitro* was pointed out by Miller\(^ {49}\) in tests with *Escherichia coli*. In her studies folic acid synthesis was 5 to 10\% of that observed in controls, while synthesis of other B vitamins was not significantly altered.

Further evidence that the primary mode of action of the sulfonamides is in inhibiting synthesis of folic acid from PABA is contained in experiments of Lampen and Jones\(^ {27,28}\) with folic acid-dependent and -independent strains of lactobacilli. These investigators observed that lactobacilli with a growth factor requirement for folic acid are relatively non-susceptible to inhibition by sulfonamides, whereas those strains that can make their own folic acid are readily inhibited.

The structure of evidence that sulfonamides act primarily by inhibiting the synthesis of folic acid from PABA was essentially complete when it was finally announced by Angier *et al.*\(^ {50}\) that folic acid is a derivative of PABA.

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\(^{43}\) A. D. Welch, *Federation Proc.* 1, 171 (1942).


## TABLE IX

Sulfonamide Reversal by Derivatives of p-Aminobenzoic Acid

<table>
<thead>
<tr>
<th>Compound</th>
<th>Species</th>
<th>Activity, of PABA, %</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Aminobenzoic acid</td>
<td>Str. hemolyticus, <em>Diplococcus pneumoniae</em>, <em>Staph. aureus</em>, <em>Lactobacillus arabinosus</em></td>
<td>100</td>
<td>5, 13, 51</td>
</tr>
<tr>
<td>p- and m-Aminobenzoic acid</td>
<td>Str. hemolyticus, <em>Diplococcus pneumoniae</em>, <em>Staph. aureus</em>, <em>Lactobacillus arabinosus</em></td>
<td>0</td>
<td>52</td>
</tr>
<tr>
<td>Ethyl p-aminobenzoate</td>
<td>Str. hemolyticus</td>
<td>0.033</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Str. pyogenes</td>
<td>+</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td>0</td>
<td>53</td>
</tr>
<tr>
<td>Diethylaminoethyl p-aminobenzoate (procaine)</td>
<td>Str. hemolyticus</td>
<td>ca 21</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td>ca 1</td>
<td>53</td>
</tr>
<tr>
<td>Dimethylaminoethyl p-(n-butylamino)-benzoic acid (pantocaine)</td>
<td>E. coli</td>
<td>&gt;0.01</td>
<td>53</td>
</tr>
<tr>
<td>p-Aminobenzamide</td>
<td>Str. hemolyticus</td>
<td>0.86</td>
<td>5</td>
</tr>
<tr>
<td>p-Aminobenzooylglycine</td>
<td>E. coli</td>
<td>&lt;0.02</td>
<td>54</td>
</tr>
<tr>
<td>p-Nitrobenzoic acid</td>
<td>Str. hemolyticus</td>
<td>0.0007</td>
<td>5</td>
</tr>
<tr>
<td>p-Hydroxylaminobenzoic acid</td>
<td>Str. hemolyticus</td>
<td>ca 21</td>
<td>5</td>
</tr>
<tr>
<td>p-Hydroxybenzoic acid (POB)</td>
<td>Str. hemolyticus</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>p-Acetamidobenzoic acid</td>
<td>Str. hemolyticus</td>
<td>0.0007</td>
<td>5</td>
</tr>
<tr>
<td>p-Aminophenylacetic acid</td>
<td>Str. hemolyticus, <em>Diplococcus pneumoniae</em>, <em>E. coli</em>, <em>Staph. aureus</em>, <em>Clostridium acetobutylicum</em></td>
<td>0</td>
<td>53</td>
</tr>
<tr>
<td>2-Chloro-4-aminobenzoic acid</td>
<td>Str. hemolyticus</td>
<td>ca 0.1</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Str. pyogenes</td>
<td>+</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td><em>Diplococcus pneumoniae</em></td>
<td>+</td>
<td>56, 57</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td>+</td>
<td>56</td>
</tr>
<tr>
<td>2-Fluoro-4-aminobenzoic acid</td>
<td>E. coli</td>
<td>38</td>
<td>55</td>
</tr>
<tr>
<td>2-Bromo-4-aminobenzoic acid</td>
<td>E. coli</td>
<td>0.79</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td><em>Diplococcus pneumoniae</em></td>
<td>+</td>
<td>57</td>
</tr>
<tr>
<td>2-Iodo-4-aminobenzoic acid</td>
<td>E. coli</td>
<td>0.13</td>
<td>55</td>
</tr>
<tr>
<td>2-Hydroxy-4-aminobenzoic acid (PASA)</td>
<td>E. coli</td>
<td>4-16</td>
<td>58</td>
</tr>
<tr>
<td>4-Aminoisophthalic acid</td>
<td>Str. pyogenes</td>
<td>+</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td>0.07</td>
<td>55</td>
</tr>
<tr>
<td>2-(p-Aminobenzamido)pyridine</td>
<td>Str. hemolyticus</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>2-Aminopyrimidine-5-carboxylic acid</td>
<td>Str. pyogenes</td>
<td>0.05</td>
<td>59</td>
</tr>
<tr>
<td>3-Methyl-4-aminobenzoic acid</td>
<td>Str. pyogenes</td>
<td>+</td>
<td>52</td>
</tr>
<tr>
<td>p-(p-Amidobenzamido)benzoic acid</td>
<td>Str. pyogenes</td>
<td>+</td>
<td>52</td>
</tr>
<tr>
<td>p-Aminocinnamic acid</td>
<td><em>Streptobacterium plantarum</em></td>
<td>0.03</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Str. hemolyticus</td>
<td>+</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td><em>Diplococcus pneumoniae</em></td>
<td>+</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td>+</td>
<td>61</td>
</tr>
<tr>
<td>4-(p-Hydroxyphenyl)azobenzoic acid</td>
<td>Str. hemolyticus, <em>E. coli</em></td>
<td>+</td>
<td>62</td>
</tr>
<tr>
<td>p-Aminobenzoic hydrazide</td>
<td>Str. hemolyticus, <em>E. coli</em></td>
<td>+</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>Str. hemolyticus, <em>E. coli</em></td>
<td>+</td>
<td>63</td>
</tr>
<tr>
<td>N-Glucosido-p-aminobenzoic acid</td>
<td>Str. hemolyticus, <em>E. coli</em></td>
<td>+</td>
<td>63</td>
</tr>
<tr>
<td>Ethyl p-(N-acetylbuthanilamido)benzoate</td>
<td>Str. hemolyticus, <em>E. coli</em></td>
<td>+</td>
<td>63</td>
</tr>
<tr>
<td>p-Aminophenylglycine</td>
<td>Str. hemolyticus, <em>Diplococcus pneumoniae</em>, <em>Staph. aureus</em></td>
<td>0</td>
<td>51</td>
</tr>
<tr>
<td>4-Aminocyclohexancarboxylic acid</td>
<td><em>Staph. aureus</em></td>
<td>77</td>
<td>64</td>
</tr>
<tr>
<td>p-Toluic acid</td>
<td>Str. hemolyticus</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>p-Ethylaminobenzoic acid</td>
<td>Str. hemolyticus</td>
<td>0.06</td>
<td>65</td>
</tr>
<tr>
<td>p-n-Butylaminobenzoic acid</td>
<td>Str. hemolyticus</td>
<td>&lt;0.07</td>
<td>65</td>
</tr>
<tr>
<td>3-Dimethylamino-1,2-dimethylpropyl-p-aminobenzoate (tutncaine)</td>
<td><em>Streptobacterium plantarum</em></td>
<td>3.5</td>
<td>60</td>
</tr>
<tr>
<td>5-(p-Aminobenzoxyethyl)-1-methylthiazole</td>
<td><em>Streptobacterium plantarum</em></td>
<td>0.48</td>
<td>60</td>
</tr>
<tr>
<td>Ethyl N-(p-aminobenzoxy)alanine</td>
<td><em>Streptobacterium plantarum</em></td>
<td>0.17</td>
<td>60</td>
</tr>
<tr>
<td>p-Aminobenzenesulfonic acid</td>
<td><em>Streptobacterium plantarum</em></td>
<td>0.003</td>
<td>60</td>
</tr>
<tr>
<td>Trimethylbetaine of p-aminobenzoic acid</td>
<td><em>Streptobacterium plantarum</em></td>
<td>0.02</td>
<td>60</td>
</tr>
<tr>
<td>Trimethylbetaine of sulfanilic acid</td>
<td><em>Streptobacterium plantarum</em></td>
<td>0</td>
<td>60</td>
</tr>
</tbody>
</table>
The extent to which compounds related to PABA in structure reverse sulfonamide inhibition of bacteria is summarized in Table IX.

With the information of the preceding paragraphs forming the essential "framework" for a "working hypothesis" of the fate of PABA within the cell as revealed by studies employing sulfonamides, some of the more controversial aspects of the subject as well as further details of the scheme may be examined.

Although it has been almost universally agreed for some time that sulfonamides act by inhibiting the conversion of PABA to folic acid, the exact locus of this effect has not been established.

\[
\text{Folic acid}
\]

A logical point for interference is the conjugation of PABA with glutamic acid. If this condensation is interfered with by sulfonamides, then sulfonamides should be ineffective in the presence of \(p\)-aminobenzoylglutamic acid. With the exception of the results of Auhagen, who reported that


70. R. Kuhn, E. F. Möller, G. Wendt, and H. Beinert, Ber. 75B, 711 (1942).


73. A. Dansi, Farm. sci. e tec. (Pavia) 2, 195 (1947) [C. A. 42, 639 (1948)].


\[ \text{P-AMINOBENZOIC ACID} \]

\[
\begin{align*}
\text{COOH} \\
\text{H}_2\text{N} & \text{CONHCHCH}_2\text{CH}_2\text{COOH} \\
\end{align*}
\]

\(p\)-Aminobenzoylglutamic acid

\(p\)-aminobenzoylglutamic acid is eight to ten times as active as PABA in reversing sulfonamide inhibition of *Streptobacterium plantarum*, \(p\)-aminobenzoylglutamic acid otherwise has been found to be less active than PABA, and the reversal obtained is competitive, indicating according to *inhibition analysis* that \(p\)-aminobenzoylglutamic acid is not a product of the reaction inhibited by sulfonamides.\(^{27, 28, 67}\)

The synthesis of pteroic acid is a possible site for sulfonamide inhibition.

\[ \text{Pterois acid} \]

As with \(p\)-aminobenzoylglutamic acid, pteroic acid is less active than PABA in reversing sulfonamides, and the reversal so obtained is competitive, indicating that pteroic acid is not a product of the reaction inhibited by sulfonamides.\(^{27, 28, 67}\) Utilization of pteroic acid by *Streptococcus faecalis* whose "folic acid" requirement can be satisfied with pteroic acid is not inhibited by sulfonamides.\(^{28, 67}\)

O’Meara *et al.*\(^{65}\) have suggested that sulfonamides may prevent a combination of PABA with reductone, \(\alpha,\beta\)-dihydroxyacrolein, by combining irreversibly with it instead. They picture reductone as being either toxic to the cell or essential at only certain phases of development. It would seem more plausible to consider reductone as an essential intermediate in the synthesis of folic acid and that sulfonamides can interfere with this process.

\[ \text{2,4,5-Triamino-6-hydroxypyrimidine} \]

\[ \text{Reductone} \]

\[ \text{p-Aminobenzoylglutamic acid} \]


It has been suggested by Tschesche\(^69\) that sulfonamides interfere with the conjugation of 2-amino-4-hydroxypteridine-6-aldehyde with PABA, but strong experimental evidence for the hypothesis is lacking.

\[
\begin{align*}
\text{OH} & \quad \text{CHO} \\
\text{H}_2\text{N} & \\
\end{align*}
\]

2-Amino-4-hydroxypteridine-6-aldehyde

Recent studies indicate that folinic acid, the *Leuconostoc citrovorum* factor or 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid, is nearer to the form in which folic acid functions in metabolism. It is not impossible that folic acid is an artifact that does not actually occur in the normal sequence of PABA utilization.

That sulfonamides actually interfere with the uptake of PABA by bacterial cells has been shown by Noll *et al.*\(^70\) in studies with *Escherichia coli* and S\(^85\)-labeled sulfathiazole. Competitive interference as determined by radioactivity counts was demonstrated between sulfathiazole and PABA.

A consideration of the deficiencies produced in animals by the feeding of a poorly absorbed sulfonamide in conjunction with highly purified diets containing only thiamine, riboflavin, pantothenic acid, vitamin B\(_6\), niacin, and choline as a source of B vitamins is most logically presented in the chapter on folic acid. Mention is made here of the extent to which PABA has been found to reverse the growth-inhibiting effect of the sulfonamides.

Black *et al.*\(^39,71\) found that PABA reverses the growth-inhibiting effects of sulfaguanidine (sulfanilyl guanidine) when this compound is fed to rats to the extent of 0.5% of a highly purified diet, provided that the PABA is given from the start of the experiment. If PABA is given after a nutritional deficiency has been developed by the feeding of the drug, some lag period is observed before the beneficial effects of the PABA become apparent. The growth-promoting effect of PABA in purified diets containing sulfaguanidine first observed by Black *et al.*\(^39,71\) has been confirmed by Martin\(^47\) and by MacKenzie *et al.*\(^40\).

Welch\(^43\) reported that PABA does not antagonize the growth inhibition produced by sulfasuxidine (succinylsulfathiazole) when this compound is


included in a purified diet to the extent of 1%. Other more extended studies from the same and other laboratories have shown that PABA does have a certain small effect in reversing sulfasuxidine, especially with respect to reversing the incidence of the induced hypoprothrombinemia. The failure to observe complete reversal with PABA of the growth inhibition produced by the incorporation of succinylsulfathiazole in *highly purified* diets, as contrasted with the reversal of soluble sulfonamides *in vitro*, probably is attributable to absorption of PABA early in the gut so that the compound does not reach the cecum and large intestine in effective concentration.

Emerson and Cushing have described a unique mutant of *Neurospora crassa* that requires sulfanilamide or other similar sulfonamide for growth at 35\(^\circ\). At 30\(^\circ\) or lower, sulfonamides are not strictly essential, but growth rates are lower in their absence. For this strain PABA is an inhibitor that is competitively antagonized by sulfanilamide with an inhibition ratio for one-half maximum growth of about 100. Another mutant obtained by crossing the sulfonamide-requiring mutant with a PABA-requiring mutant requires both sulfanilamide and PABA for growth. The molar ratio giving maximum growth at 35\(^\circ\) is about 1000 of sulfanilamide to 1 of PABA. The possibility that sulfanilamide is utilized as a metabolite by the sulfonamide-requiring strain was entertained, but it has been ruled out by Zalokar, who showed that the sulfonamide-requiring mutant may be made to grow by any one of the following ways: (1) by the presence of sulfanilamide; (2) by reducing available PABA by genetic means, that is, by crossing the sulfonamide-requiring mutant with a PABA-requiring mutant to give a strain that does not synthesize PABA in large amounts; (3) by reducing available methionine by genetic means, that is, by similarly crossing the sulfonamide-requiring strain with a methionine-requiring strain to give a new strain that does not synthesize methionine in large amounts; (4) by the addition to the medium of threonine, which acts as an inhibitor; or (5) by making free ammonia available as a nitrogen source. As summarized by Zalokar the data are best interpreted as follows: The

gene that is altered in the sulfonamide-requiring strain produces a deleterious reaction in *Neurospora crassa* with the following characteristics: (1) it requires a precursor of methionine as substrate, and the amount required is greater than that needed for essential reactions normally using the same substrate; (2) it requires PABA as a catalyst, and in much larger amounts than are required by other reactions using the same catalyst; (3) at the same time sulfanilamide is a much stronger competitor of PABA in this reaction than in other reactions requiring PABA as a catalyst; and (4) the reaction, or a product of the deleterious reaction, interferes with the normal utilization of threonine. That the step in threonine utilization interfered with may involve amination is suggested by the growth-promoting activity of free ammonia.

As demonstrated by the content of the foregoing paragraphs, most of the studies that have involved relationships between PABA and the sulfonamides have been carried out on bacteria. That antagonism between PABA and the sulfonamides may be demonstrated in a variety of plant and animal systems has been demonstrated. Terzian\(^{81}\) has reported that sulfanilamide or sulfadiazine in the diet of the mosquito *Aedes aegypti* will increase the insect’s susceptibility to the malarial parasite *Plasmodium gallinaceum*. PABA fed along with the sulfonamide nullifies its effect on susceptibility of the host. Hindmarsh\(^{82}\) has shown with onion rootlets that sulfanilamide prevents the nuclei from coming into prophase and upsets the spindle mechanism so that anaphase rarely occurs. These effects are reversed by PABA. Schopfer and Anker\(^{83}\) found that PABA, guanine, adenylie acid, and riboflavin exert an antagonistic effect on sulfonamide inhibition of the growth of pea roots in aseptic culture. The growth-inhibiting action of the sulfonamides on growth of pepper grass (*Lepidium sativum*) roots was found by Andus and Quastel\(^{84}\) to be neutralized by PABA. Sulfonamide inhibition of blood carbonic anhydrase is reversed with PABA according to van Goor.\(^{85}\) PABA is an antagonist of the inhibiting action of sulfanilamide on germination and growth of flax seed in the experiments of Hazard.\(^{86}\) Maier and Riley\(^{87}\) found that the therapeutic effect of the sulfonamides on *Plasmodium gallinaceum* infections of chicks is negated by administration of PABA. Sulfonamide inhibition of yeast growth is reversed by PABA.\(^{88}\) Growth of isolated tomato roots is inhibited by sul-


\(^{88}\) M. Landy and D. M. Dicken, *Nature* 149, 244 (1942).
fonamides, and the inhibition is reversed by PABA in the experiments of Bonner.\textsuperscript{89} Sulfanilamide inhibition of the growth of onion (\textit{Allium capa}) rootlets is reversed with PABA, according to Stoll.\textsuperscript{90} Germination of rice seeds is retarded by sulfanilamide, and the effect is reversed with PABA in the experiments of Ribeiro.\textsuperscript{91} Chodat and Soloweitchik\textsuperscript{92} found that PABA reduces the cytostatic influence of sulfanilamide on various species of green algae. Growth of a fresh-water diatom (\textit{Nitzschia palea}) is inhibited by sulfonamides and the inhibition is reversed with PABA, according to Wiedling.\textsuperscript{93}, \textsuperscript{94} In experiments of Efimov,\textsuperscript{95} PABA was reported to counteract the depressant effect of sulfanilamide on isolated frog hearts. PABA has been reported to reverse the effect of sulfanilamide in inhibiting the adsorption of methylene blue by charcoal.\textsuperscript{96} The foregoing enumeration of antagonisms between PABA and the sulfonamides that have been demonstrated in non-bacterial systems is by no means exhaustive.

Although the aromatic sulfones are quite different from the sulfonamides in structure and have a different spectrum of antibacterial activity, such as might be expected if their mechanism of action were different, it has been shown by Steenken and Heise\textsuperscript{97} that PABA antagonizes \textit{in vitro} the antitubercular activity of promin.

\textit{p}-Aminosalicylic acid, a compound resembling both sulfanilamide and PABA in structure, was found by Lehmann\textsuperscript{98}, \textsuperscript{99} to be a remarkably specific chemotherapeutic agent against \textit{Mycobacterium tuberculosis} both \textit{in vitro} and \textit{in vivo}.

\begin{center}
\begin{tabular}{ccc}
\text{SO}_2\text{NH}_2 & \text{COOH} & \text{COOH} \\
\text{NH}_2 & \text{NH}_2 & \text{OH} \\
\text{Sulfanilamide} & \text{PABA} & \text{p-\textit{Aminosalicylic acid}} \\
\end{tabular}
\end{center}

\textsuperscript{91} F. Ribeiro, \textit{J. Biol. Chem.} \textbf{152}, 665 (1944).
\textsuperscript{93} S. Wiedling, \textit{Nature} \textbf{150}, 290 (1942).
\textsuperscript{94} S. Wiedling, \textit{Science} \textbf{94}, 389 (1941).
\textsuperscript{95} N. I. Efimov, \textit{Farmakol. i Toksikol.} \textbf{8}, No. 5, 28 (1945) [\textit{C. A.} \textbf{40}, 7409 (1946)].
The compound does not have appreciable bacteriostatic activity against microorganisms other than the tubercle bacillus. The bacteriostatic activity of PASA against *Mycobacterium tuberculosis* is reversed by PABA and to a small extent by salicylic acid. PASA replaces PABA as a growth factor for two mutant strains of *Escherichia coli*.

The antibacterial activities determined *in vitro* of thirty compounds related in structure to PABA have been summarized by Shive.

**XIV. Therapeutic Activity**

**LEMUEL D. WRIGHT and PETER A. TAVORMINA**

**A. RICKETTSIAL DISEASES**

PABA was found to be an antirickettsial compound when Snyder *et al.* observed that mortality in mice infected with yolk sac suspension of swine typhus is markedly reduced by oral administration of PABA as a component of the diet. Their findings were confirmed in mice by Andrews *et al.* Quite independently, Greiff *et al.* found PABA to be rickettsiostatic in typhus-infected yolk sacs and in swine typhus infection in mice.

The effectiveness of PABA as a practical chemotherapeutic agent was established by Yeomans *et al.*, who found the drug useful in the treatment of louse-borne typhus in humans. PABA was subsequently shown to be of value in the treatment not only of louse-borne typhus but of other rickettsial diseases as well, including tsutsugamuchi disease (scrub typhus) and Rocky Mountain spotted fever. PABA continued to be the drug of

choice in the treatment of rickettsial diseases until the advent of chlor-
amphenicol (chloromycetin) and aureomycin. PABA was usually ad-

39 F. Sanchez Ruiz, Medicina mex. 30, 165 (1950).
ministered orally at 2- to 3-hour intervals for a total daily dose of 25 to 30 g.

The mechanism by which PABA acts to inhibit rickettsia is not known, but recent work of Davis and colleagues has thrown some light on the subject. For the W strain of Escherichia coli Davis showed\textsuperscript{17, 48} that PABA is a bacteriostatic compound. The bacteriostatic activity of PABA is reversed by p-hydroxybenzoic acid (POB). Separate studies by Davis showed previously\textsuperscript{19} that for certain mutants of Escherichia coli with a requirement for five aromatic compounds (quintuple aromatic auxotrophs) POB is a bacterial vitamin essential for rapid growth. Shikimic acid and compound X, previously shown by Davis \textit{et al.} to be precursors of the aromatic ring in \textit{Escherichia coli}, similarly are active in the reversal of PABA inhibition. Thus it would appear that POB is an essential metabolite for which PABA is a naturally occurring antimetabolite. This concept is slightly complicated but not necessarily vitiated by the fact that POB may be derived from PABA at a slow rate. As summarized by Davis,\textsuperscript{48} it would appear that PABA exerts three metabolic effects on \textit{Escherichia coli}: it acts as a normal vitamin at low concentrations, as a source of another vitamin, POB, at moderate concentrations, and as a growth inhibitor at high concentrations.

Snyder and Davis have published a preliminary note\textsuperscript{50} concerning the extent to which the metabolite-antimetabolite relationship between POB and PABA established in \textit{Escherichia coli} can explain the rickettsiostatic effect of PABA. POB was found to reverse PABA in two tests with the Breinl strain of epidemic typhus in chick embryos, in one test with murine typhus in white mice, and in one test with Rocky Mountain spotted fever in chick embryos. For reversal of rickettsiostasis in chick embryos, the effective ratio of POB to PABA (on the basis of the amounts inoculated into each egg as a single dose at zero time) appears to be in the range of 1:1 to 1:10.

B. RHEUMATOID DISEASES

PABA has shown some promise in the treatment of acute rheumatic fever. Dry \textit{et al.}\textsuperscript{51} encountered a case of acute rheumatic fever in an adult

\textsuperscript{44} N. A. Tierney, \textit{Southern Med. J.} \textbf{40}, 81 (1947).
\textsuperscript{47} B. D. Davis, \textit{Federation Proc.} \textbf{10}, 406 (1951).
\textsuperscript{49} B. D. Davis, \textit{Nature} \textbf{166}, 1120 (1950).
\textsuperscript{50} J. C. Snyder and B. D. Davis, \textit{Federation Proc.} \textbf{10}, 419 (1951).
man that did not respond to salicylate therapy (10 g. per day). On more or less empiric grounds the simultaneous oral administration of PABA was instituted (initial dose of 4 g. followed by 2 g. every 2 hours). Prior to the use of PABA the salicylate blood level varied between 12.5 and 15 mg. per 100 ml. With PABA there was a steady increase in salicylate level from 12.5 to 34.5 mg. per 100 ml. There was a dramatic and complete clinical response as the content of salicylate reached 37.5 mg. per 100 ml. of blood. Separate experiments with healthy men indicated that salicylates and PABA appear to have a reciprocal effect in increasing their separate concentrations in the blood when they are administered together. Increased blood levels of salicylate attained by the simultaneous administration of PABA are accompanied by less salicylate appearing in the urine.

The findings of Dry et al.51 have been confirmed by Hoaglund,52 who reported that a case of rheumatic fever with refractoriness to 10 g. of aspirin daily, manifested by an uninterrupted fever for 5 weeks, showed dramatic improvement after the salicylate therapy was supplemented with PABA in a daily dose of 24 g.

Smith53 has compared the combination PABA and salicylate with salicylate alone as a treatment for a variety of rheumatic diseases exclusive of acute rheumatic fever. The pain relief with the PABA-salicylate combination was found to be superior to that with sodium salicylate alone in 64 patients with rheumatoid arthritis and fibrositis. In 60 patients with osteoarthritis or with cervical osteoarthritis with radicular involvement and chronic painful shoulder, the superiority was less marked. The combination therapy gives longer pain relief than sodium salicylate alone. Significant toxic reactions to the combinations were not observed. Sodium salicylate alone, however, produced some toxic effects in 69 (55.2%) of the 125 patients.

The mechanism of action of PABA as an adjunct to salicylates has been investigated by Salassa et al.,54 who found that PABA produces the following effects. First, it alters the detoxication of salicylate by interrupting or greatly depressing the conjugation of glycine with salicylate so that only very small quantities of salicyluric acid appear in the urine after ingestion of salicylate. Second, it tends to lower the pH of the urine and thus decreases the renal clearance of the free salicylate fraction. Third, it causes a decrease in the urinary excretion of total salicylate and a rise in the plasma salicylate level as a result of the foregoing effects. In dogs, the administration of PABA does not alter the excretion of salicylate and does not increase the plasma salicylate levels.

PABA was used alone by Rosenblum and Fraser\textsuperscript{55} in the treatment of acute rheumatic fever in nine children. The dosage schedule involved an immediate dose of 3 to 4 g. followed by a maintenance dose of 1 to 3 g. given at 2- to 3-hour intervals. That PABA has some effect alone in the treatment of acute rheumatic fever was indicated by a decrease in temperature, a decrease in severity of joint pains, and the attainment of a sense of well being.

PABA and cortisone have been found by Wiesel \textit{et al.}\textsuperscript{56} to function synergistically in the treatment of rheumatoid arthritis. A trial of the combination appeared warranted for two reasons. Firstly, PABA alone or cortisone alone is active in rheumatoid arthritis. Secondly, the inactivation of estrogens by the liver is inhibited by PABA,\textsuperscript{57} and it might be reasoned that the inactivation of cortisone, a compound closely related to the estrogens in structure, might be similarly prevented, or at least inhibited, by PABA. Although the preliminary paper of Wiesel \textit{et al.} was limited to a study of only 15 patients, the results obtained indicated that a combination of the two drugs at levels where neither is active alone produces satisfactory control of rheumatoid arthritis. The euphoria and subsequent depression frequently observed in patients receiving cortisone alone were not observed on the combination.

C. ACHROMOTRICHIA

The reported chromotrichial effect of PABA observed by Ansbacher\textsuperscript{58} in the rat and by Martin and Ansbacher\textsuperscript{59} in the mouse stimulated a number of clinical investigators to try PABA against human achromotrichia.

Sieve\textsuperscript{60} reported that, in 30 patients from a group ranging in age from 20 to 55 in which PABA was the sole therapy, marked darkening of the hair occurred in \textit{all} cases. The dose of PABA administered was 100 mg. twice a day. Subsequently, Sieve\textsuperscript{61} reported the beneficial effect of PABA in achromotrichia in 82 out of 460 patients. Another paper by Sieve\textsuperscript{62} reports a variety of favorable effects, including darkening of the hair, in a group of 800 patients.

Brandaleone \textit{et al.}\textsuperscript{63} have studied achromotrichia in a group of 19 elderly

\textsuperscript{57} S. Ansbacher, W. A. Wisansky, and G. J. Martin, \textit{Federation Proc.} 1, 98 (1942).
\textsuperscript{58} S. Ansbacher, \textit{Science} 93, 164 (1941).
\textsuperscript{60} B. F. Sieve, \textit{Science} 94, 257 (1941).
men and women hospitalized for various chronic diseases common to old age. Seven received 100 mg. of calcium pantothenate, 200 mg. of PABA and 50 g. of brewer's yeast daily, five received yeast and PABA, and seven received yeast and calcium pantothenate. The treatment was continued for eight months. Significant improvement was observed in only two individuals, both of whom were men receiving the combination calcium pantothenate, PABA, and yeast. Only questionable improvement occurred in those patients receiving one or the other compound along with the yeast.

Eller and Diaz\(^64\) administered PABA to 88 persons of different ages and both sexes in various stages of achromotrichia. Tablets of 100 mg. were given three or four times daily over periods ranging from three to five months. A few of the patients showed definite changes in the color of their hair.

Visual darkening of the hair was reported by DeVilbiss\(^65\) in all of 16 patients treated with PABA at a recommended dose of 100 mg. four times daily. Photos taken before and after treatment, in color as well as in black and white, supported the patients' own opinion that PABA is of value in the treatment of premature graying.

Friedgood\(^66\) observed a few cases of human achromotrichia that appeared to respond to PABA, but he was inclined to minimize the extravagant claims of Sieve.\(^62\)

**D. THYROTOXICOSIS**

PABA has been used clinically with some success as an antithyroid compound by a number of investigators. Berman\(^67\) claimed good results in the treatment of six cases of hyperthyroidism with parenteral PABA. Papp\(^68\) gave PABA by mouth with success in four patients with hyperthyroidism. Williams\(^69\) was not enthusiastic about the use of PABA in thyrotoxicosis, since he was able to reduce the basal metabolic rate in only two out of eight patients by use of PABA. 3,5-Diiodo-\(p\)-aminobenzoic acid showed some promise in his series, presumably because of the affinity of the thyroid for iodine. Goodwin *et al.*\(^70\) treated ten thyrotoxic patients with PABA and obtained full control in only one, and six did not respond at all. They conclude that PABA has a slight but definite antithyroid action in safe dosage but that, to obtain an action comparable to that of the

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thiouracil group, toxic doses would be necessary. It is concluded that PABA is suitable only for the treatment of mild cases of thyroidism.

E. MISCELLANEOUS DISEASES

The effectiveness of PABA in the treatment of rickettsial diseases, rheumatic fever, and related rheumatic diseases is unequivocal. Results that may be summarized as encouraging, at best, have been obtained in the treatment of the following conditions with PABA: lymphoblastoma cutis, lupus erythematosus, active dermatomyositis, scleroderma, dermatitis herpetiformis, chronic myelogenous leukemia, and experimental allergic encephalomyelitis.

PABA has been found by a number of investigators to have a low order of fungistic activity.

# Chapter 13

**PTEROYLGLUTAMIC ACID**

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I. Nomenclature and Formula

E. L. R. Stokstad*

There are few examples in biochemistry where a single compound has provided the key to as many nutritional phenomena as has pteroylglutamic acid. Deficiency states of this vitamin had been described in man, monkeys, chicks, rats, guinea pigs, insects, and microorganisms before pteroylglutamic acid was identified and its structure determined.

In view of the many phases of biological work already in progress by the time pteroylglutamic acid was synthesized and made available, it is not surprising that a large number of papers soon appeared on its role in clinical medicine, animal nutrition, bacterial metabolism, enzyme chemistry, and tumor research.

Since work on this vitamin has proceeded independently in many laboratories employing different species, the literature is replete with the various names used to designate it. A summary of the names which have been given to various biologically active materials whose activity can be ascribed to pteroylglutamic acid is presented in Table I.

The nomenclature in this field is also complicated by the existence of different chemical forms which were subsequently shown to be conjugates of the vitamin with varying numbers of glutamic acid residues. Thus the name "fermentation L. casei factor" was given to a compound isolated from a fermentation product and which later was shown to be pteroyltri-glutamic acid. The name "vitamin B₁₂ conjugate" was used to designate the compound isolated from yeast and which was shown to contain seven glutamic acid residues. This will be referred to as pteroylheptaglutamic acid. The name "folic acid" has found the most universal acceptance, largely because of its brevity. The term "folacin," recommended by a joint nomenclature committee of the American Institute of Nutrition and the Society of Biological Chemists, has not as yet received wide usage. In this chapter the name pteroylglutamic acid, abbreviated PGA, will be used except in accounts of original literature where the investigator used a special name to designate a biologically active factor, the exact chemical identity of which cannot be established.

One of the first published observations describing a clinical syndrome which was the result of PGA deficiency was made by Wills¹ in 1931. This disease was found to have a blood picture similar to that in pernicious anemia, except that some of the other symptoms, such as achlorhydria and nervous lesions observed in pernicious anemia, were absent. This condition

* It is a pleasure to acknowledge the assistance of M. J. Fahrenbach and H. P. Broquist in writing portions of this review and the services of Carol Gunderson in the preparation of the manuscript.
was observed in women patients in India and was associated with pregnancy. The anemia was relieved by feeding large doses of Marmite, which is a concentrated extract of autolyzed yeast.

A deficiency disease similar to this clinical syndrome was experimentally induced in monkeys by Wills and Stewart\(^\text{11}\) by feeding diets similar to those consumed by the women in Bombay who developed the tropical macrocytic anemia. This anemia in monkeys could be cured by autolyzed yeast extract.

### TABLE I

**Nomenclature of Preparations Having Biological Activity Presumably Due to Pteroethylamino Acid or Related Compounds**

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Date</th>
<th>Ref.</th>
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<td>Wills factor</td>
<td>Yeast extract, effective in the treatment of tropical macrocytic anemia</td>
<td>1931</td>
<td>1</td>
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<tr>
<td>Vitamin M</td>
<td>Yeast and liver extract, effective against nutritional cytopenia in monkeys</td>
<td>1938</td>
<td>2</td>
</tr>
<tr>
<td>Factor U</td>
<td>Growth factor for chicks, present in yeast extract</td>
<td>1938</td>
<td>3</td>
</tr>
<tr>
<td>Vitamin (B_e)</td>
<td>Adsorbed on fuller's earth, prevented nutritional anemia in chicks</td>
<td>1939</td>
<td>4</td>
</tr>
<tr>
<td>Norit eluate factor</td>
<td>Growth factor for (L. casei) from yeast and liver</td>
<td>1940</td>
<td>5</td>
</tr>
<tr>
<td>Folic acid</td>
<td>Active for (S. lactis) R; concentrates prepared from spinach</td>
<td>1941</td>
<td>6</td>
</tr>
<tr>
<td>(L. casei) factor</td>
<td>Isolated from liver and yeast</td>
<td>1943</td>
<td>7</td>
</tr>
<tr>
<td>SLR factor (rhizopterin)</td>
<td>Growth factor for (S. lactis) R, inactive for (L. casei); obtained from (Rhizopus nigricans) fermentation product</td>
<td>1943</td>
<td>8</td>
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*Note: In addition to the above, less clearly defined fractions have received separate names, including vitamins \(B_1\) and \(B_2\) and factors R and S.*\(^\text{10}\)

and certain types of liver extracts which were also effective in the treatment of tropical macrocytic anemia in human patients. However, two liver...

---

preparations were found which were effective in the treatment of human pernicious anemia but which produced no response in the anemic monkeys on the experimental diets. These observations thus served to distinguish the anti-pernicious anemia factor of liver from the factor effective against tropical macrocytic anemia.

Day and coworkers\(^2\) gave the name “vitamin M” to a factor essential for hematopoiesis in monkeys. This factor was later shown to be pteroylglutamic acid by Day et al.,\(^3\) who also found that this deficiency disease could be corrected by pteroyltriglutamic acid. Stokstad and Manning\(^2\) observed that chicks receiving a diet consisting essentially of washed fish meal, polished rice, riboflavin, thiamine, and a pantothenic acid concentrate responded to an unknown growth factor in yeast, alfalfa, and wheat bran. This factor was designated “factor U” and was later shown to be identical with PGA. Hogan and Parrott\(^4\) gave the name “vitamin B\(_6\)” to a factor present in liver which prevented macrocytic anemia in chicks.

Shortly after the publication of these experiments with animals, Snell and Peterson\(^5\) reported that \textit{Lactobacillus casei} required an unknown growth factor which they termed the “Norit eluate factor.” The term “folic acid” was given by Mitchell et al.\(^6\) to a substance obtained from spinach, which stimulated the growth of \textit{Streptococcus faecalis} R and was active for \textit{L. casei}. It appeared to have the same microbiological properties as the Norit eluate factor.

II. Chemistry

E. L. R. STOKSTAD

A. ISOLATION

1. Of Pteroylglutamic Acid (PGA)

Many papers have appeared describing the concentration and chemical properties of PGA. In the first publication by the Wisconsin workers, Snell and Peterson\(^1\) reported that this factor was adsorbed by activated charcoal and Lloyd’s reagent, was stable to acid and alkali, could be precipitated by basic lead acetate, and was extracted from aqueous acid solutions by butanol. A later publication by Hutchings et al.\(^2\) from this same laboratory described additional methods of purification by adsorption on Super Filtrol and formation of a zinc salt. The isolation of a highly active folic acid


preparation from spinach was described in a series of papers by Mitchell and coworkers at Texas.\textsuperscript{3-6} These workers employed repeated adsorption and elution from activated charcoal, precipitation with heavy metals, and chromatographic adsorption on alumina. Since folic acid from spinach has never been crystallized, it is not possible to establish its identity with PGA. However, comparison of samples of folic acid concentrates of known potencies with pure PGA\textsuperscript{7,8} indicates that the most active preparation obtained by the Texas workers had essentially the same biological potency as pure PGA.

The isolation of PGA from liver has been reported from two laboratories. Pfiffner \textit{et al.}\textsuperscript{9} obtained this compound by starting with liver in which the conjugates had been converted into free PGA by autolysis.\textsuperscript{10} The isolation process involved extraction with boiling water, adsorption and elution using first the ion exchange resin Amberlite 4R and later activated charcoal, extraction of an aqueous solution of the free acid with butanol at pH 3 to 4, formation of a barium salt and a zinc salt, and finally crystallization of the free acid from water.

Stokstad\textsuperscript{11} reported the isolation of the methyl ester of PGA and later of the free acid.\textsuperscript{12} The starting material was an 80\% alcohol precipitate of an aqueous extract of liver. The method involved adsorption and elution, first with Norit and then with Super Filtrol, precipitation of the barium salt with methanol, esterification in acid methanol, extraction of the methyl ester from aqueous solution with butanol, chromatographic adsorption of the methyl ester on Super Filtrol, and fractional precipitation of the ester from water and methanol. The free acid was obtained by saponification of the ester and crystallization from hot aqueous dilute acetic acid solution.

Pterooylglutamic acid has also been isolated from yeast extracts by Pfiffner \textit{et al.}\textsuperscript{10} after treatment with hog kidney enzyme which hydrolyzed the conjugated vitamin to free PGA.

a. Existence of an Acid-Labile Chick Anti-Anemic Factor in Liver

In the isolation of vitamin $B_6$ (PGA) from horse liver, Pfiffner et al.\textsuperscript{10} described certain fractions whose activity for chicks and $L$. casei was inactivated by acid conditions which had no effect on pure PGA. Concentrates containing this acid-labile factor contained twice as much PGA activity by $S$. faecalis as by $L$. casei assay. After acid treatment in 1.5\% methanol-hydrogen chloride, the $L$. casei activity decreased to 20 to 30\% of its original value and the activity as measured by the two organisms became the same. A similar treatment of pure PGA produces insignificant losses in activity. The zinc salt of the acid-labile factor is more soluble than that of PGA. There has been some speculation that this acid-labile form may be the citrovorum factor or a related compound. The acid lability properties of the citrovorum factor correspond roughly with those of the acid-labile form. However, the acid lability of citrovorum factor is less than that of the acid-labile form of PGA obtained by Pfiffner. Until further evidence is available, no definite conclusions can be drawn regarding the identity of these two compounds.

2. Of PGA Conjugates

Soon after it became apparent that the growth factor for $L$. casei was also involved in animal nutrition, evidence was obtained by Welch and Wright\textsuperscript{12} which showed that milk possessed more activity for animals than could be accounted for on the basis of its microbiological activity. Similarly Mallory et al.\textsuperscript{13} noted that yeast concentrates were more active in promoting growth and preventing leucopenia in rats fed sulfasuxidine (succinylsulfathiazole) than liver preparations containing up to fifteen times as much "Streptococcus lactis R-stimulating factor." These workers also pointed out the parallelism between the activity of liver and yeast preparations for the sulfonamide-fed rat and for the vitamin M-deficient monkey, and the lack of parallelism with the microbiological activity. They also suggested that these yeast preparations contained potential "Streptococcus lactis R-stimulating factors" which could be enzymatically converted to microbiologically active compounds.

A conjugated form of PGA which is much more active for $L$. casei than for $S$. faecalis was described by Hutchings et al.\textsuperscript{15} This compound, which was

\textsuperscript{10} A. D. Welch and L. D. Wright, Science 100, 153 (1944).
later shown to be pteroyltriglutamic acid, was isolated by Hutchings et al.\textsuperscript{16} from a cell-free filtrate obtained from the aerobic fermentation of a Corynebacterium. The method involved adsorption and elution, esterification of the barium salt to give the methyl ester, and extraction of the ester from an aqueous solution with butanol. The methyl ester at this stage could be dissolved in hot methanol and reprecipitated on cooling in the presence of 0.05 N sodium chloride. In the absence of an electrolyte a gel was formed instead. By repeated reprecipitation of this ester from methanol in the presence of an electrolyte, it was obtained in a microcrystalline form. The free acid was obtained by hydrolysis of the ester and crystallization at pH 2.8 in the presence of electrolytes such as sodium or calcium chloride.

Although pteroylglutamic acid can be precipitated from water and methanol in the absence of electrolytes, pteroyltriglutamic acid and its ester require the presence of electrolytes for their precipitation. The solubility of pteroyltriglutamic acid in the presence of calcium chloride at pH 2.8 is 3.0 mg. per milliliter at 80° and 0.10 mg. per milliliter at 5°.

Binkley et al.\textsuperscript{17, 18} isolated in crystalline form a microbiologically inactive conjugate of PGA (vitamin B\textsubscript{6} conjugate) from yeast. These workers found that a yeast extract rich in anti-anemic activity for the chick was relatively inactive for L. casei or S. faecalis. Digestion of this yeast concentrate with a suitable enzyme from hog kidney increased the microbiological activity.

This conjugate which crystallized from 5% sodium chloride solution was 0.4% as active as PGA for L. casei and 0.2% as active for S. faecalis assay. This conjugate was later found by Pfiffner et al.\textsuperscript{18} to contain seven glutamic acid residues. Enzymes which split this conjugate have no effect on its methyl ester, showing that the conjugases may be classified as carboxypeptidases. Although PGA exists in a microbiologically inactive form in liver, no conjugates have yet been isolated from this source. A more detailed discussion of the various types of PGA precursors occurring in liver will be given in a subsequent portion of this review.

\textit{a. Pteroylglutamic Acid–Protein Complex in Yeast}

Specific and reversible combinations of proteins and vitamins have been noted in many instances in which the vitamin forms the basic part of the coenzyme. This has been demonstrated for thiamine, pyridoxal, riboflavin, and nicotinamide. Attempts have been made to find similar combinations\textsuperscript{16} B. L. Hutchings, E. L. R. Stokstad, N. Bohonos, N. H. Sloane, and Y. SubbaRow, \textit{J. Am. Chem. Soc.} 70, 1 (1948).
\textsuperscript{18} J. J. Pfiffner, D. G. Calkins, B. L. O'Dell, E. S. Bloom, R. A. Brown, C. J. Campbell, and O. D. Bird, \textit{Science} 102, 228 (1945).
between PGA and proteins. Allfrey and King\textsuperscript{20} found that during fractional precipitation of a yeast autolysate with ammonium sulfate there was a correlation between the amounts of PGA conjugate and protein that were precipitated. The PGA conjugate was measured microbiologically after appropriate enzyme digestion. The linkage between PGA conjugate and protein is essentially salt-like or highly dissociable. The bond is broken by the addition of organic solvents such as ethanol, acetone, or dioxane to protein fractions prepared by salting-out procedures. Heating for 1 minute at 80\textdegree{} or for 5 minutes at 60\textdegree{} also released the PGA conjugate from the protein. During dialysis of freshly prepared yeast autolysates in viscous casing the PGA conjugate was rapidly released from the protein. By the use of fractional precipitation with ammonium sulfate between 2.4 \textit{M} and 3.1 \textit{M} at varying pH values, protein preparations were obtained which contained 160 \textgamma{} of PGA per gram in the form of the microbiologically inactive conjugate. Pteroylheptaglutamic acid by itself in low concentrations is not precipitated under these conditions.

Six crystalline proteins were obtained from this ammonium sulfate precipitate. Most contained little or no PGA activity, the highest having a value of 75 \textgamma{} of PGA per gram.

3. Degradation Reactions

\textit{a. Degradation of Pteroyltetraglutamic Acid}

The relationship between pteroyltetraglutamic acid isolated from a fermentation product and PGA isolated from liver was established by Stokstad \textit{et al.}\textsuperscript{21} by the use of anaerobic alkaline hydrolysis. The tetruglutamic acid derivative, which is active for \textit{L. casei} but only slightly active for \textit{S. faecalis} R, was rapidly inactivated for both organisms by aerobic alkaline hydrolysis. Anaerobic hydrolysis, however, produced only a slight decrease in the activity for \textit{L. casei} and greatly increased the activity for \textit{S. faecalis} R. On anaerobic alkaline hydrolysis the ratio of the activity for these two organisms approached that of pteroyltetraglutamic acid isolated from liver. Two moles of \textalpha{}-amino acid nitrogen were liberated during anaerobic alkaline hydrolysis, and the active compound which was formed was approximately half as active as pteroyltetraglutamic acid by both \textit{L. casei} and \textit{S. faecalis} R assay. This compound was later identified as racemic pteroyltetraglutamic acid by comparison with the synthetic material. The pteroyltetraglutamic acid had apparently been racemized by the anaerobic alkaline hydrolysis.

Stokstad \textit{et al.}\textsuperscript{21} found that aerobic alkaline hydrolysis of pteroyltetruglutamic acid or of racemic PGA resulted in the formation of a fluorescent pig-


ment and a diazotizable aromatic amine which could be estimated by the method of Bratton and Marshall. In the absence of oxygen no diazotizable amine or fluorescent pigment was produced by alkaline hydrolysis. The fluorescent pigment proved to be a dibasic acid having pKa values of 3.9 and 7.7. Elementary analysis suggested the empirical formula C7H5N5O5. Decarboxylation of the fluorescent dibasic pigment at 300° resulted in the liberation of approximately 1 mole of carbon dioxide and the formation of a fluorescent monobasic acid with a pKa of 8.0. Oxidation of the original dibasic acid with chlorine water, followed by hydrolysis with 0.1 N HCl at 140°, yielded a compound which gave positive color tests for guanidine. The formation of guanidine under such conditions constitutes evidence for a pyrimidine ring with an amino group in the 2 position. The fluorescent dibasic acid showed characteristic absorption spectra in 0.1 N sodium hydroxide with maxima at 253 and 365 mU. The empirical formula C7H5N5O5, the titration data, and formation of guanidine suggested a 2-aminopurine or a 2-aminopteridine. The absorption spectra, however, eliminated the possibility of a purine, because purines do not have absorption maxima above 300 mU. Thus the available evidence pointed toward a 2-aminopteridine with an enolic and a carboxy group. With this evidence available, attempts were made to synthesize pteridines having these functional groups. The compound was identified as 2-amino-4-hydroxypteridine-6-carboxylic acid by comparison with the synthetic compound, and the monobasic fluorescent pigment produced by decarboxylation was identified as 2-amino-4-hydroxypteridine.

The structure of these two pteridines was established by Mowat et al. by the following series of reactions. Diethylmesoxalate was condensed with 2,4,5-triamino-6-hydroxypyrimidine (II) to yield isoanthopterin-carboxylic acid (III). The structure of isoanthopterin-carboxylic acid (III) is shown by formula III, although it had not previously been definitely established whether the carboxyl group occupied the 6 or 7 position. On chlorination of isoanthopterin-carboxylic acid (III) and subsequent reduction with hydrogen iodide, one of the hydroxyl groups was removed to give compound V, which was identical with the dibasic fluorescent pigment. Presumably either the 4- or the 7-hydroxyl could have been removed by this procedure. The presence of the 4-hydroxyl in compound V was shown in two ways. First decarboxylation of 2-amino-4-hydroxypteridine-6-carboxylic acid (V) gave 2-amino-4-hydroxypteridine (VI), the structure of which was established by its synthesis from glyoxal and 2,4,5-triamino-6-hydroxypyrimidine (II). Its formation by this method demands a hydroxyl group in

the 4 position. Second, the synthesis of 2-amino-1-hydroxypteridine-6-carboxylic acid (V) was accomplished by condensation of 2,4,5-triamino-6-hydroxyuracil (II) and ethyl-β,β-diethoxy-α-bromopropionate. This reaction also established the presence of a hydroxyl group in the 4 position of the pteridine.

Unequivocal proof establishing the 6 position of the carboxyl group in compound V was obtained by degrading the corresponding methyl derivative, 2-amino-4-hydroxy-6-methylpteridine (VII) to give a compound identical with 2-amino-5-methylpyrazine (VIII). The corresponding 7-methylpteridine would have yielded 2-amino-6-methylpyrazine instead. Oxidation of 2-amino-4-hydroxy-6-methylpteridine (VII) by alkaline permanganate gave the corresponding 2-amino-4-hydroxypteridine-6-carboxylic acid (V). These reactions are outlined in Fig. 1.

Hydrolysis with 0.5 N sulfurous acid at room temperature was shown by Hutchings et al.\textsuperscript{24} to rapidly inactivate pteroylglutamic acid and give an

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aromatic amine and a fluorescent pigment. This pigment reacted rapidly with typical aldehyde reagents, indicating the presence of an aldehyde group. This fluorescent pigment did not possess a carboxyl group, as evidenced by the fact that its distribution coefficient between water and butanol was the same at pH 3.0 as at pH 7.0. When the pigment obtained by sulfurous acid hydrolysis was treated anaerobically with dilute sodium hydroxide, approximately equal amounts of 2-amino-4-hydroxypteridine 6-carboxylic acid (V) and 2-amino-4-hydroxy-6-methylpteridine (VII) were formed. The formation of approximately equal molal quantities of carboxy and methyl derivatives from what is apparently an aldehyde probably involves a Cannizzaro type of reaction, although the mechanism of this reaction is obscure.

Prolonged aqueous hydrolysis of pteroyltriglutamic acid at pH 4 yielded 1-pyrrolidonecarboxylic acid, which on hydrolysis with alkali yielded L(+)-glutamic acid.24

The aromatic amine which was produced during sulfurous acid hydrolysis was isolated as the barium salt. This compound when diazotized and coupled with N-(1-naphthyl)ethylenediamine dihydrochloride yielded a red pigment,22 which indicated a primary aromatic amine with a highly negative substituent group. The aromatic amine nitrogen as measured by the method of Bratton and Marshall22 constituted approximately 25% of the total nitrogen. The remaining 75% of the nitrogen could be converted into α-amino acid nitrogen by alkaline hydrolysis. From such hydrolyzates the aromatic amine was isolated and identified as p-aminobenzoic acid. Microbiological assay of the hydrolyzate indicated the presence of 3 moles of glutamic acid. The peptide linkage of the glutamic acid to p-aminobenzoic acid must involve the carboxyl group of the latter as a primary aromatic amine is required for reaction in the Bratton and Marshall test.22

The diazotizable aromatic amine obtained by aerobic alkaline hydrolysis of racemic pteroylglutamic acid was found to contain 2.1 atoms of nitrogen for each atom of aromatic amino nitrogen. On hydrolysis with 2 N sulfuric acid it yielded p-aminobenzoic acid and 45% of the total nitrogen appeared as α-amino acid nitrogen. This was later identified as glutamic acid, and thus the diazotizable amine was identified as p-aminobenzoylglutamic acid.

Evidence regarding the mode of linkage is furnished by the results of alkaline hydrolysis.24 The absence of fluorescence and of the free aromatic amine in the original pteroylglutamic acid, and the simultaneous appearance of these two during aerobic alkaline hydrolysis, suggested that the pteridine is linked to the aromatic amine nitrogen. As hydrolysis proceeded, the liberation of pteridine and aromatic amine appeared at approximately the same rate.

Reduction in acid solution, either catalytically or with zinc dust, yielded
the aromatic amine and a reduced pteridine.\textsuperscript{21} After reoxidation with manganese dioxide, the pteridine obtained by zinc reduction was identified as 2-amino-4-hydroxy-6-methylpteridine.

The foregoing evidence indicated the following conclusions regarding the structure of pteroylglutamic acid.

1. Aerobic alkaline hydrolysis, sulfurous acid cleavage, and chemical or catalytic reduction each yielded a pteridine and a primary aromatic amine. This indicated the linkage of the pteridine to the nitrogen of the aromatic amine.

2. The aromatic amine formed during sulfurous acid cleavage of pteroylglutamic acid was a tetrapeptide, \textit{p}-aminobenzoylglutamylglutamic acid. The aromatic amine from pteroylglutamic acid was \textit{p}-aminobenzoylglutamic acid.

3. There was a single-carbon atom linkage between the pteridine and the aromatic amine. This was indicated by the fact that only pteridines with a single-carbon atom side chain were obtained and that no other 2-carbon fragments could be detected in the two degradation reaction products. The evidence also indicated that this single-carbon atom is present in a methylene link. If this carbon atom were present in an amide linkage, the cleavage would be hydrolytic and would not require oxygen. The formation of 2-amino-4-hydroxy-6-methylpteridine by reduction also constitutes evidence for the methylene linkage.

b. Degradation of Vitamin B\textsubscript{6} (Pteroylglutamic Acid)

Wittle \textit{et al.}\textsuperscript{25} described the oxidative degradation of vitamin B\textsubscript{6} (pteroylglutamic acid) which had been isolated from liver. Oxidation with alkaline permanganate or chloric acid gave a fluorescing pigment with a characteristic absorption spectrum which was identified as 2-amino-4-hydroxy-6-carboxypteridine. The chloric acid oxidation mixture also yielded an ethyl acetate-soluble crystalline material which proved to be 3,5-dichloro-4-aminobenzoylglutamic acid. This compound decomposed at a temperature slightly above the melting point to yield two lower molecular weight fragments which were identified as 3,5-dichloro-4-aminobenzoic acid and \textit{dl}-pyrrolidoncarboxylic acid.

**B. PHYSICAL AND CHEMICAL PROPERTIES**

Pteroylglutamic acid when crystallized from water forms yellow, spear-shaped leaflets (Fig. 2). On heating it darkens, and it chars at around 250° without melting (Pfiffner \textit{et al.}\textsuperscript{10}). Its solubility in water as the free acid is 10 mg. per liter at 0° and over 500 mg. per liter at 100°. The disodium salt

has a solubility of over 15 g. per liter. It forms highly insoluble salts with zinc, lead, and silver (Pfiffner et al.). The alkali metal salts are soluble in water but can be precipitated by the addition of alcohol. Pteroylglutamic acid is practically insoluble in most organic solvents but is slightly soluble in acetic acid (Pfiffner et al.).

The optical rotation $\left[ \alpha \right]_D^{20}$ is $+16^\circ$ in 0.1 N sodium hydroxide solution at a concentration of 7.6 g. per liter (Weygand et al.).

Pteroylglutamic acid dried at moderate temperatures and at atmospheric

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Fig. 2. Crystals of pteroylglutamic acid.

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pressure contains an amount of water corresponding roughly to a dihydrate. Samples dried at 145° in high vacuum lose this water of crystallization and become hygroscopic.

Pteroylglutamic acid has a characteristic ultraviolet absorption spectrum (Waller et al.\textsuperscript{27}) which is markedly influenced by pH (Fig. 3). In 0.1 N sodium hydroxide it exhibits maxima at 256, 282, and 365 m\(\mu\) with \(E_{1\text{cm}}^1\) values of 585, 570, and 206. The peaks at 256 and 365 m\(\mu\) are due to the pteridine portion of the molecule; the peak at 282 is due to the \(p\)-amino-benzoic acid moiety. The glutamic acid exerts very little effect on the ultraviolet absorption spectrum. The absorption spectrum of pteroic acid (Fig. 3) is slightly different from that of PGA. However, addition of more than one glutamic acid does not change the position of the absorption peaks, and the extinction coefficients of PGA conjugates are in inverse proportion to their molecular weights.

1. HYDROGENATION

Pteroylglutamic acid and related pterins are readily hydrogenated and can be reoxidized by atmospheric oxygen. O'Dell \textit{et al.}\textsuperscript{28} found that pteroyl-


glutamic acid in alkaline solution with platinum oxide as a catalyst takes up 1 mole of hydrogen to form a colorless dihydro derivative with an absorption maxima at 284 mμ in alkaline solution. In glacial acetic acid over platinum 2 moles of hydrogen are taken up to form the tetrahydro derivative which has an absorption spectrum similar to that of the dihydro form. Alkaline solutions of the dihydro derivative readily absorb oxygen to yield PGA, whereas in acid solution reoxidation is less rapid. The tetrahydro derivative is reoxidized to PGA by oxygen over platinum in glacial acetic acid. The ultraviolet absorption spectra of PGA, dihydro and tetrahydro PGA, and reoxidized dihydro PGA are shown in Fig. 4 (O'Dell et al.28).

Reduction of 2-amino-4-hydroxypteridine-6-carboxylic acid in alkaline solution with hydrogen over platinum oxide yields the tetrahydro form. The reduced solutions rapidly absorb oxygen from the air to yield the parent compound. It is interesting to note that the 365-mμ absorption band of pteroylglutamic acid disappears during formation of the di- or tetrahydro derivatives, whereas the same band is shifted to longer wavelengths in the tetrahydro derivatives of 2-amino-4-hydroxypteridine-6-carboxylic acid and 2-amino-4-hydroxy-7-methylpteridine (Fig. 5, O'Dell et al.28).

II. CHEMISTRY

Hydrogenation of pteroyltriglutamic acid with a palladium catalyst at pH 1 results in rapid biological inactivation and liberation of \( p \)-amino-benzoyltriglutamic acid (Hutchings et al.\textsuperscript{24}). Reduction of PGA with zinc in acid solution also cleaves the bond between the nitrogen of the \( p \)-amino-benzoic acid and the methylene carbon attached to the pteridine ring. Thus the reduced form of PGA is unstable in acid solution and rapidly splits to give the free aromatic amine.

![Figure 5](https://example.com/image.png)

**Fig. 5.** Ultraviolet absorption spectra of pterins: ——— 2-amino-4-hydroxy-6-carboxyppteridine at pH 11.0; ——— tetrahydro-2-amino-4-hydroxy-6-carboxyppteridine at pH 11.0; ——— tetrahydro-2-amino-4-hydroxy-6-carboxyppteridine at pH 3.0; ——— ——— 2-amino-4-hydroxy-6-carboxy-7-methyltetrahydropteridine at pH 11. Courtesy *J. Am. Chem. Soc.* (O’Dell et al.\textsuperscript{28}).

2. Other Reactions

Cosulich and Smith\textsuperscript{29} observed that treatment of PGA with cold nitrous acid yields \( N^{10} \)-nitrosopteroylglutamic acid with biological activity approximately equal to that of PGA for the chick and for *S. faecalis* R. It will be recalled that nitrous acid has also been used to remove the 2-amino group of \( N^{10} \)-formylpteramid acid (rhizopterin) (Wolf et al.\textsuperscript{30}). Heating PGA with formic acid and acetic anhydride gives \( N^{10} \)-formylpteroylglutamic acid (Gordon et al.\textsuperscript{31}). Stokstad et al.\textsuperscript{32} found that irradiation of PGA with sun-


light rapidly destroyed the biological activity. The liberation of p-aminobenzoylglutamic acid paralleled that of biological inactivation which showed that the first step in the reaction consists in a rupture in the bond between the C2-methylene group and the N10 atom of aromatic amino group.

C. CONSTITUTION

\[
\begin{align*}
\text{HOOC} & \quad \text{O} \\
\text{CHNH} & \quad \text{C} \quad \text{-} \\
\text{CH2} & \quad \text{NH} \quad \text{CH2} \\
\text{CH2} & \quad \text{NH2} \\
\text{HOOC}
\end{align*}
\]

Pteroylglutamic acid: N-[4-\{[2-amino-4-hydroxy-6-pteridyl]methyl\}amino]-benzoyl]glutamic acid

D. SYNTHESIS

The final proof of the structure of this compound was obtained by synthesis of pteroylglutamic acid by different methods. The method of Waller et al.\textsuperscript{33} utilizes the simultaneous condensation of 2,4,5-triamino-6-hydroxypyrimidine (II), p-aminobenzoylglutamic acid (X), and \(\alpha,\beta\)-dibromopropionaldehyde in aqueous solution. Pterolic acid was obtained by the corresponding reaction, except that p-aminobenzoic acid was used instead of p-aminobenzoylglutamic acid. The procedure of Hultquist et al.\textsuperscript{33} involved the reaction of \(\alpha,\beta\)-dibromopropionaldehyde with pyridine, 2,4,5-triamino-6-hydroxypyrimidine (II), and potassium iodide to yield N-\{(2-amino-4-hydroxy-6-pteridyl)methyl\}pyridinium iodide (XI). This was then treated with p-aminobenzoylglutamic acid (X) in ethylene glycol to yield pteroylglutamic acid. The position of the methylpyridinium group on the 6 position of the pteridine was established by oxidation with alkaline permanganate to yield 2-amino-1-hydroxypteridine-6-carboxylic acid (V). These two methods of synthesis are schematically outlined in Fig. 6.

Angier et al.\textsuperscript{34} condensed diethyl N-(p-aminobenzoyl)glutamate with re-
duct (2,3-dihydroxyacrylaldehyde) to give diethyl N-\([p-(2,3-dihydroxy-2-ene-propylideneamino)benzoyl]\) glutamate. This was then condensed with 2,4,5-triamino-6-hydroxypyrimidine (II) in hot ethylene glycol to give pteroylglutamic acid diethyl ester in 5 to 20% yield. A number of derivatives of pteroic acid, including the ethyl ester, amide, and pteroylglycine, were prepared by this procedure.

A fourth method, described by Boothe et al.,\(^{35}\) consists in attaching a pre-

\[
\begin{align*}
\text{HOOC-} & \text{C-N-C-} \text{H} \text{N} \text{H}_2 + \text{Br-CH}_2\text{-C-Br} + \text{H}_2\text{N} \text{N} \text{H}_2 \\
\text{HOOC-} & \text{CH}_2 \\
\text{X} \\
p-\text{Aminobenzyol glutamic acid} \\
\text{HOOC-} & \text{C-N-C-} \text{H} \text{N} \text{H}_2 \\
\text{HOOC-} & \text{CH}_2 \\
P\text{teroyl glutamic acid} \\
\text{X in ethylene glycol} \\
\end{align*}
\]

Fig. 6. Two syntheses of pteroylglutamic acid.

formed pteridine to a derivative of \(p\)-aminobenzoic acid. 2-Amino-4-hydroxy-6-methyl-pteridine (VII) was prepared by the reduction of 2-amino-4-hydroxy-6-pteridylmethylpyridinium iodide (XI) with zinc in alkaline solution and subsequent oxidation by iodine of the resulting dihydro-2-amino-4-hydroxy-6-methylpteridine. This was brominated at 150°, and the resulting crude 2-amino-4-hydroxy-6-bromomethylpteridine was condensed with \(p\)-aminobenzyol glutamic acid diethyl ester in ethylene glycol at 100°.

Numerous synthetic methods have subsequently been developed, the large number of which is reflected in the many publications and patents in this field. These procedures may be divided into two main categories: first, those in which 2,4,5-triamino-6-hydroxypprymidine (II) is condensed simultaneously with a 3-carbon intermediate and p-aminobenzoylglutamic acid; second, those in which a substituted pterin is first formed and then coupled with an aromatic amine to give PGA.

1. **Simultaneous Condensation of Triaminopyrimidine, 3-Carbon Intermediate, and Aromatic Amine**

The first method of synthesis, that of Waller et al.\(^\text{37}\) in which 2,3-dibromo-propionaldehyde is used as the 3-carbon fragment, has already been described. Examples of other 3-carbon systems which have been used are given below. (X in the formula denotes chlorine or bromine. Reference numbers are given in parentheses.)

\[
\begin{align*}
\text{O} & \quad \text{H} \\
\text{CH}_2\text{X} & \quad \text{C} \quad \text{C--CHO} \quad (36) \\
\text{O=\text{C}--\text{C=}\text{C}--\text{COOH}} & \quad \text{X} \quad \text{X} \\
& \quad \text{(followed by decarboxylation)} \\
\text{HO--\text{C=}\text{C}--\text{CHO}} & \quad \text{X--\text{CH}_2--\text{C--CHX}_2} \quad (34, 38-40) \\
\text{X--\text{CH}_2--\text{CX}_2--\text{CHO}} & \quad \text{\text{ClCH}_2--\text{C}--\text{CHO}} \quad (41-43) \\
& \quad \text{\text{NOH}}
\end{align*}
\]


\(^{37}\) C. W. Waller and J. H. Boothe (to American Cyanamid Co.), U. S. Pat. 2,442,867 (June 8, 1948).

\(^{38}\) R. B. Angier (to American Cyanamid Co.), U. S. Pat. 2,442,836 (June 8, 1948).

\(^{39}\) R. B. Angier (to American Cyanamid Co.), U. S. Pat. 2,442,837 (June 8, 1948).

\(^{40}\) R. B. Angier (to American Cyanamid Co.), U. S. Pat. 2,466,670 (Apr. 12, 1949).


\(^{42}\) M. E. Hultquist and P. F. Dreisbach (to American Cyanamid Co.), U. S. Pat. 2,443,165 (June 8, 1948).


\(^{44}\) J. H. Boothe (to American Cyanamid Co.), U. S. Pat. 2,444,002 (June 22, 1948).

\(^{45}\) D. B. Cosulich (to American Cyanamid Co.), U. S. Pat. 2,444,005 (June 22, 1948).
II. CHEMISTRY

\[
\begin{align*}
\text{H} & \quad \text{O} \\
\text{X} & \quad \text{C}=\text{CH} \quad \text{CH}_2 \\
X \quad \text{C} & \quad \text{C} \quad \text{H} \quad \text{CH}_2 \quad \text{C} \quad \text{H} \\
\text{X} & \quad \text{H} \quad \text{O} \\
X & \quad \text{C} \quad \text{H} \quad \text{CH}_2 \\
\text{CH}_2 & \quad \text{CH} \quad \text{CHO} + \text{NaI} \quad (50, 51) \\
\text{O} & \quad \text{O} \\
\text{tosyl} & \quad \text{tosyl} \\
\text{Cl} & \quad \text{CH}_2 \quad \text{C} \quad \text{CH}_2 \text{Cl} \\
\end{align*}
\]

2. CONDENSATION OF PREFORMED PTERIN WITH AROMATIC AMINE

These methods involve formation of a 2-amino-4-hydroxy-6-methylpteridine with a suitable substituent in the 6-methyl group for reaction with \( p \)-aminobenzoic acid. Examples of these are \((R = 2\text{-amino-4-hydroxy-6-substituted pteridine})\):

\[
\begin{align*}
\text{R} & \quad \text{CH}_2 \text{Cl} + p\text{-aminobenzoylglutamic acid (PABG)} \quad (54, 55) \\
\text{R} & \quad \text{CHO} + \text{PABG} \quad \text{in formic acid, followed by hydrolysis} \quad (56, 57)
\end{align*}
\]

\[\text{R} = \text{2-amino-4-hydroxy-6-substituted pteridine}\]

46 M. E. Hultquist and P. F. Dreisbach (to American Cyanamid Co.), U. S. Pat. 2,472,482 (June 7, 1949).
47 J. H. Boothe (to American Cyanamid Co.), U. S. Pat. 2,472,462 (June 7, 1949).
48 M. E. Hultquist and D. R. Seeger (to American Cyanamid Co.), U. S. Pat. 2,472,481 (June 7, 1949).
52 J. Geraei (to American Cyanamid Co.), U. S. Pat. 2,501,168 (March 21, 1950).
53 D. I. Weisblat and A. R. Hanze (to The Upjohn Company), U. S. Pat. 2,560,616 (July 17, 1951).
54 Roche Products, Ltd. Brit. Pat. 624,394 (June 7, 1949).
57 H. Lindlar and H. Klaeui (to Hoffmann-La Roche, Inc.), U. S. Pat. 2,520,156 (August 29, 1950).
Dihydro R—CHO + PGA in formic acid, followed by hydrolysis\textsuperscript{58} 
R—CHO + PABG with catalytic hydrogenation\textsuperscript{26, 59}
R—CH\textsubscript{2}OH + p-nitrobenzoylglutamic acid with catalytic hydrogenation\textsuperscript{66}
R—CH\textsubscript{2}OH + p-aminobenzoylglutamic acid\textsuperscript{61}
R—CH\textsubscript{3} brominated + p-aminobenzoylglutamic acid\textsuperscript{35}
R—CH\textsubscript{2}—N— + p-aminobenzoylglutamic acid\textsuperscript{33, 62}
R—CH—COOH + p-aminobenzoylglutamic acid\textsuperscript{63}

\[
\text{Br}
\]

3. Synthesis of Pteroylpolyglutamic Acid Derivatives

The isolation of pteroyltriglutamic acid from a fermentation product and the existence of pteroylheptaglutamic acid in yeast made the synthesis of various polyglutamic acid derivatives of pteroic acid a problem of interest. The findings of Lewisohn \textit{et al.}\textsuperscript{64} that pteroyltriglutamic acid from a fermentation product caused spontaneous regression of mammary tumors in mice also attached a special interest to the synthesis of this type of compound. Pteroyl-α-glutamyldiglutamic acid was prepared by Mowat \textit{et al.}\textsuperscript{65} and found to be 0.5% as active as pteroylglutamic acid by \textit{S. faecalis} assay and 0.8% by \textit{L. casei}, and fully active for chicks. Pteroyl-α,γ-glutamyldiglutamic acid was 0.14% active by \textit{S. faecalis} and 0.17% by \textit{L. casei}. Pteroyl-γ-glutamyldiglutamic acid (Boothe \textit{et al.}\textsuperscript{66}) is 71% active by \textit{S. faecalis} and 63% by \textit{L. casei} assay. In both cases pteroylglutamic acid was used as a standard. Pteroyl-γ-glutamyl-γ-glutamylglutamic acid had an activity of 2.5% by \textit{S. faecalis} and 71% by \textit{L. casei}. These activities are very similar to those observed for pteroyltriglutamic acid isolated from a fermentation product (Hutchings \textit{et al.}\textsuperscript{13}).

\textsuperscript{59}Roche Products, Ltd., Brit. Pat. 628,305 (March 15, 1948).
\textsuperscript{60}H. Spiegelberg (to Hoffmann-LaRoche, Inc.), U. S. Pat. 2,487,363 (Nov. 8, 1949).
\textsuperscript{61}J. Semb (to American Cyanamid Co.), U. S. Pat. 2,491,285 (Dec. 13, 1949).
\textsuperscript{62}M. E. Hultquist (to American Cyanamid Co.), U. S. Pat. 2,473,796 (June 21, 1949).
E. SPECIFICITY

1. Chemistry of Rhizopterin (Formyl Pteroic Acid)

Keresztesy et al.\textsuperscript{67, 68} reported the isolation of a compound which was highly active for \textit{S. faecalis} but relatively inactive for \textit{L. casei}. This ratio of activity for these two organisms is in contrast to that of pteroylglutamic acid which is more active for \textit{L. casei} than for \textit{S. faecalis}. Rhizopterin was obtained from a charcoal adsorbate derived from a fumaric acid fermentation product of \textit{Rhizopus nigricans}. It was isolated by adsorption on Norit and fuller's earth and by chromatographic adsorption on alumina.

Wolf \textit{et al.}\textsuperscript{30} described a novel method for recrystallizing rhizopterin by forming a double salt with lutecethylenediamine cobaltic chloride. This double salt was recrystallized from hot water and reconverted back to rhizopterin by treatment with acetic acid.

Alkaline treatment of rhizopterin was shown by Rickes \textit{et al.}\textsuperscript{69} to decrease its activity for \textit{S. faecalis}, to have no effect on its \textit{L. casei} activity, and to change the adsorption spectrum. This alkaline degradation product was shown by Wolf \textit{et al.}\textsuperscript{30} to be pteroic acid. Acid hydrolysis of rhizopterin yielded formic acid. The position of the formyl group on the N\textsuperscript{10} position of the p-aminobenzoic acid was established by the following reactions. Benzoylrhizopterin was prepared which yielded benzoylguanidine on oxidation with potassium chlorate and hydrochloric acid. This showed that the 2-amino group of rhizopterin was free and that benzoylation occurred on this nitrogen atom. Oxidation of rhizopterin with potassium chlorate and hydrochloric acid yielded tetrachloro-p-benzoquinone, an oxidation product of p-aminobenzoic acid and oxalogenuanidine which in turn yielded guanidine on acid hydrolysis. p-Aminobenzoic acid was obtained by sublimation of the alkaline hydrolysis product (pteroic acid) at 220 to 360° at very low pressure and by acid or alkaline hydrolysis. A number of different acyl derivatives were prepared by heating rhizopterin with the corresponding acid anhydrides. These acyl groups were attached to the 2-amino group. The 2-amino group could be removed by nitrous acid to give desimino-rhizopterin.

Rhizopterin was synthesized by treatment of synthetic pteroic acid, obtained by the method of Waller,\textsuperscript{27} with 98% formic acid on a steam bath

2. Chemistry of Citrovorum Factor (Leucovorin, Folinic Acid)

a. Biochemical Relations between Citrovorum Factor and PGA

In 1948 Sauberlich and Baumann\(^7^0\) noted that the organism *Leuconostoc citrovorum* failed to grow in a chemically defined medium containing all the known amino acids and vitamins, but the addition of small amounts of natural materials such as liver extract or yeast to the medium promoted luxuriant growth. The substance(s) in these natural materials necessary for growth of *Le. citrovorum* was termed the citrovorum factor (CF). It was also noted by these workers that the organism responded to thymidine or to massive doses of PGA, but because the response to these substances was delayed and submaximum, it appeared that these substances could not be the citrovorum factor. It soon became apparent, however, that a close relationship existed between PGA and CF. Thus Sauberlich\(^7^1\) noted that rats on a PGA-deficient diet excreted very little CF in the urine, but the administration of PGA resulted in large increases in the CF content of the urine. Subsequently it was noted by Sauberlich\(^7^2\) and confirmed by Broquist *et al.*\(^7^3\) that concentrates of CF can competitively overcome the toxicity of aminopterin (4-aminopteroylglutamic acid), an inhibitory analog of PGA, for *Le. citrovorum*. These latter workers also showed that CF had approximately the same distribution coefficient as PGA between butanol and water at pH 2. It appeared then that PGA might be a biological precursor of CF and that a close chemical relationship existed between the two substances.

In a seemingly unrelated series of investigations, Bond *et al.*\(^7^4\) provided evidence from microbiological studies for the existence of an unrecognized form of PGA in natural materials. These workers studied the toxic effect of "x methyl PGA" for growth of *L. casei* and observed that liver extract was approximately fifteen times more active than PGA in counteracting the toxicity of "x methyl PGA" than could be accounted for on the basis of its PGA content when assayed in the absence of inhibitor. With the use of this inhibition assay highly refined concentrates of the factor from liver, termed folinic acid by the Texas group, were prepared and found to be highly active for *Leuconostoc citrovorum*. It thus seems very likely that folinic acid and CF are identical.

Gordon *et al.*\(^7^5\) had previously reported the preparation of 10-formyl-pteroylglutamic acid and observed that it was more active than PGA in counteracting the inhibitory action of "x methyl PGA" for *S. faecalis* R.

\(^7^0\) H. E. Sauberlich and C. A. Baumann, *J. Biol. Chem.* 176, 165 (1948).
\(^7^1\) H. E. Sauberlich, *J. Biol. Chem.* 181, 467 (1949).
This observation suggested that CF might be a formyl derivative of PGA in view of the similar behavior of CF in counteracting PGA antagonists. This supposition was confirmed by the finding that catalytic hydrogenation of formyl PGA produced a mixture of compounds with high growth-promoting activity for CF.75-77

b. Synthesis and Purification

The synthesis of leucovorin (XII) appears to consist in two essential steps (cf. Fig. 7):

1. Reduction of either PGA (I) or 10-formyl PGA (XV) in 87 to 100% formic acid at 0° to 30° over a platinum catalyst, whereby 2 moles of hydrogen are absorbed to form 10-formyl-5,6,7,8-tetrahydro PGA (XIV).

2. Rearrangement of the neutralized reduction mixture under anaerobic conditions by long standing at room temperature or by heating the alkaline solution at pH 10 to 12. This treatment results in the rearrangement of 10-formyl-5,6,7,8-tetrahydro PGA to leucovorin in a 40 to 50% over-all yield from either PGA or 10-formyl PGA. During this process the formyl group shifts from the N10 to the N6 position of the molecule. The leucovorin is isolated by chromatographic adsorption and purification of the neutral calcium or barium salt. The free acid of leucovorin is crystallized as the free acid after acidification of the calcium salt to pH 3.5.

c. Stage of Reduction and Location of Hydrogen Atoms

Elemental analyses of crystalline leucovorin (XII) are in agreement with the formula C20H23N7O7, and the presence of one formyl group has been demonstrated. Thus from this analytical data and the method of preparation described above, the conclusion seemed justified that leucovorin is a tetrahydroformyl PGA.

Allen and coworkers75 studied the stage of reduction of pteridines by means of the potential at the dropping mercury electrode in the polarograph. They demonstrated that it is possible to distinguish between PGA, 10-formyl PGA, and dihydro and tetrahydro PGA by means of the potential at pH 9.0. Thus, with PGA (and pteridines in general) the polarograph gives three inflection points in the curve corresponding to the tetrahydro, dihydro, and aromatic forms; the quantity of a particular reduction state

Fig. 7. Chemical transformations in the synthesis of leucovorin from pteroylglutamic acid.
can be readily determined, since it is a function of the height of the wave between inflection points. Control experiments showed that neither the pyrimidine ring nor p-aminobenzoylglutamic acid is reduced under the same polarographic conditions. In this manner it was demonstrated that the crude product formed by the reduction of PGA or 10-formyl PGA under the conditions used in the synthesis\(^7\) is a tetrahydropteridine derivative (XIV) because it produces the typical tetrahydropteridine wave in the polarograph at pH 9.0. Leucovorin was found to give no wave in the polarograph at pH 9.0, but after the mild acid treatment which destroyed activity for \textit{Lc. citrovorum} but not for \textit{S. faecalis} \(R\) the typical tetrahydropteridine wave was found. This observation, discussed in greater detail below, not only adds to the proof that leucovorin is a tetrahydropteridine derivative but serves as the basis for a polarographic assay of leucovorin. Thus, determinations are made before and after mild acid treatment, and the difference in height of the tetrahydro wave forms a measure of the concentration of leucovorin.

Available evidence indicates that it is the pyrazine ring which undergoes reduction during hydrogenation of pteridines. Hydrogen and platinum readily reduce pyrazine derivatives to the corresponding piperazines,\(^8\) whereas the pyrimidine nucleus is more resistant to catalytic hydrogenation.\(^9\)

It has been shown by Waller \textit{et al}.\(^{10}\) that treatment of PGA with sulfurous acid results in cleavage to 2-amino-4-hydroxypteridine-6-carboxaldehyde, probably through the corresponding intermediate dihydropteridine. Under similar conditions leucovorin is partially destroyed.\(^11\) Since unsaturation of the pyrazine ring appears necessary for the occurrence of this reaction, the stability of leucovorin is a further indication of the 5,6,7,8-tetrahydro structure (XII).

Additional evidence that the four hydrogen atoms of leucovorin reside in the 5, 6, 7, and 8 positions of the pyrazine ring and not the pyrimidine ring is provided by electrometric titration data.\(^12\) Thus, leucovorin exhibits a phenolic hydroxyl group with a \(pK_a\) of 10.4 which is comparable to the value of 8.2 found for the hydroxyl group in the 4 position of PGA. If reduction of the pyrimidine ring had occurred, no enolic group would be found.


The ultraviolet spectra of a number of PGA derivatives,\(^8^5\) pyrimidines,\(^8^5,\) \(^8^6\) and their reduction products provides further support for the hypothesis that all three double bonds in the pyrimidine ring of PGA remain intact in its conversion to leucovorin. Thus, dihydropyrimidines such as dihydouracil or thymine glycol demonstrate little if any absorption in the ultraviolet region.\(^8^6\) On the other hand, the ultraviolet absorption of tetrahydro PGA (XVI), 1-formyldihydro PGA (XIV), leucovorin (XII), and 5,10-diformyltetrahydro PGA (XVII) closely resembles those of certain model pyrimidines. Furthermore, it is highly improbable that the benzene ring of the p-aminobenzoyl portion of PGA would be reduced under the relatively mild conditions employed in the synthesis of leucovorin.

d. Location of the Formyl Group

Since the reduction of 10-formyl PGA (XV) to a tetrahydro derivative (XIV) does not result in appreciable leucovorin activity until the reaction mixture has been allowed to stand or is poured into dilute alkali and heated, the development of activity appears to depend on a subsequent rearrangement. The resulting leucovorin (XII) has several unique chemical properties. Thus, it is quite stable in dilute alkali but is quickly inactivated in dilute acid;\(^7^3,\) \(^7^4,\) \(^8^7-\) \(^8^9\) the acid inactivated product, however, retains activity for \(S.\) \(faecalis\) R.

Many possibilities have been considered to explain the pronounced changes in biological activity and stability exhibited by leucovorin and the various intermediates between PGA and leucovorin which have been isolated. These include the formation of a new ring linking the \(N^{10}\) position by a 1-carbon bridge to position 5, 7, or 8 in the tetrahydropyrazine ring; a shift of the \(N^{10}\)-formyl group to the pyrazine ring; and the introduction of an additional formyl group (or groups) in the tetrahydropyrazine ring with subsequent removal of the \(N^{10}\)-formyl group, by hydrolysis in the alkaline treatment.

The possibility that the formyl group in leucovorin exists as a bridge linking the 10 position with the 7 position of the tetrahydropteridine ring was investigated\(^9^0\) by means of oxidation with alkaline permanganate. Such treatment converts 2-amino-4-hydroxy-6- or 7-alkylpteridines, including PGA and its analogs, to the corresponding 6- or 7-carboxypteridines.\(^2^3\)

\(^{8^5}\) L. F. Cavalieri, A. Bendich, J. F. Tinker, and G. B. Brown, \(J.\) \(Am.\) \(Chem.\) \(Soc.\) \(70,\) 3875 (1948)

\(^{8^6}\) L. F. Cavalieri and A. Bendich, \(J.\) \(Am.\) \(Chem.\) \(Soc.\) \(72,\) 2587 (1950).

\(^{8^7}\) T. H. Jukes, H. P. Broquist, and E. L. R. Stokstad, \(Arch.\) \(Biochem.\) \(26,\) 157 (1950).

\(^{8^8}\) J. C. Keresztesy and M. Silverman, \(J.\) \(Biol.\) \(Chem.\) \(183,\) 473 (1950).

\(^{8^9}\) M. Silverman and J. C. Keresztesy, \(J.\) \(Am.\) \(Chem.\) \(Soc.\) \(73,\) 1897 (1951).

\(^{9^0}\) D. B. Cosulich, B. Roth, J. M. Smith, Jr., M. E. Hultquist, and R. P. Parker, \(J.\) \(Am.\) \(Chem.\) \(Soc.\) \(74,\) 3252 (1952).
Treatment of leucovorin with mineral acid followed by oxidation with alkaline permanganate gave 2-amino-4-hydroxypteridine-6-carboxylic acid in 68% yield. Since no 2-amino-4-hydroxypteridine-6,7-dicarboxylic acid was found, the existence of a carbon-to-carbon linkage to the 7 position of the tetrahydropteridine ring seems quite unlikely. This narrowed the investigation to the remaining possibilities of a shift of the formyl group, or a new ring formation involving the 5 or 8 position. From inspection of molecular models it seems more logical to choose the 5 position because of its proximity to the 10-formyl group.

Further evidence for the location of the formyl group on the 5 position has been provided by the study of numerous model compounds. The properties exhibited by 2,4-diamino-6-hydroxy-5-formamidopyrimidine\(^{81, 92}\) are found\(^ {90}\) to be strikingly similar to leucovorin and other monoformyltetrahydropteridines, some of which are discussed below in more detail. Thus, the 5-formamidopyrimidine is stable to hydrolysis upon heating for 1 hour in 0.1 N sodium hydroxide solution. It does not react with nitrous acid in the cold, but, after hydrolysis of the formyl group with mineral acid at room temperature, reaction with 1 mole of nitrous acid occurs. Since the 2- and 4-amino groups in this model pyrimidine are inert to formylation and nitrosation, it is logical to assume that the 2 and 8 positions of the tetrahydropteridine are similarly unreactive; this again points to the 5 position of leucovorin as the most probable site for the formyl group.

Both 2-amino-4-hydroxy-6-methylpteridine (XX)\(^{23}\) and 2-amino-4-hydroxy-6,7-dimethylpteridine (XXI) can be reduced and formylated to give the corresponding 5-formyl-5,6,7,8-tetrahydropteridines (XXII\(^ {90}\) and XXIII\(^ {84}\)). As in the case of 2,4-diamino-6-hydroxy-5-formamidopyrimidine, XXII is stable in 0.1 N sodium hydroxide solution for 1 hour at 95° and shows no wave in the polarograph at pH 9; however, after treatment with mineral acid, the characteristic tetrahydropteridine wave is found. There is also no reaction with nitrous acid until the formyl group has been removed by acid hydrolysis, at which time 1 mole is consumed. Reaction of the 2-amino group in this instance appears unlikely, since it has been shown\(^ {29}\) that XX does not react with nitrous acid under similar conditions. The ultraviolet absorption curves of both XXII and XXIII are also similar to that of leucovorin, and, as in the conversion of 5,6,7,8-tetrahydro PGA to leucovorin, XXII and XXIII exhibit a much higher intensity of absorption than the corresponding unformylated tetrahydropteridines.

Another experiment which presents almost unequivocal proof that the formyl group is located in the 5 rather than the 8 position involves the


preparation of 2-amino-4-hydroxy-5-formyl-6,7-diphenyl-8-ethyl-5,6,7,8-tetrahydropteridine (XXV) by reduction and formylation of 2-amino-4-hydroxy-6,7-diphenyl-8-ethyl-7,8-dihydropteridine (XXIV). The reduction in 98 to 100 % formic acid and formylation in the presence of acetic anhydride are carried out under the same conditions as those used to prepare the 6-methylpteridine model (XXII). The resulting product (XXV) reacts similarly to XXII; thus, it has the same polarographic behavior, the formyl group is stable in 0.1 N sodium hydroxide, and 1 mole of nitrous acid is consumed only after treatment with mineral acid at pH 2. In this case, the original dihydropteridine (XXIV) was isolated after reaction with nitrous acid.

acid, which indicates that the action of nitrite on tetrahydropteridines is one of oxidation rather than nitrosation. Furthermore, since XXV is blocked in the S position with an ethyl group and yet has properties which are similar to those of leucovorin, the possibility of a 5,8-linked ring system in leucovorin can also be eliminated.

Because of the unique stability of the formyl group in 5-formyltetrahydropteridines to dilute alkali, it seemed probable that there might be hydrogen bonding or even actual linkage of the formyl to the 4-hydroxyl group. This question was investigated by the preparation of 2-amino-4-methyl-6,7-diphenylpteridine (XXVI),\(^4\) followed by the usual hydrogenation and formylation to give 2-amino-4-methyl-5-formyl-6,7-diphenyl-5,6,7,8-tetrahydropteridine (XXVII). As with the reduced and formylated 4-hydroxypteridines, XXVII is stable to hot 0.1 N sodium hydroxide and surprisingly stable to acid, requiring hot 2.5 N hydrochloric acid to remove the formyl group. Such behavior virtually eliminates the possibility of bonding between the 4-hydroxyl and the formyl group and also presents evidence that the 4-oxygen of pteridines in acid solution exists mainly as the ketone form, since the 5-formyl group is more labile in the 4-hydroxypteridines than it is in XXVII.

As additional evidence that the formyl group of leucovorin is not involved in a new ring linking the 5 and 10 positions, reduced 10-methyl PGA\(^5\) has been found to formylate under the usual conditions in the presence of acetic anhydride to give 2-amino-4-hydroxy-5-formyl-10-methyl-5,6,7,8-tetrahydro PGA (XXVIII).\(^6\) This analog has the same type of alkali-stable, acid-labile formyl group as leucovorin and exhibits the same behavior in the polarograph. When titrated immediately with nitrous acid, XXVIII does not react, but after removal of the formyl group by standing in dilute acid, nitrous acid is readily absorbed. The preparation


\(^5\) D. B. Cosulich.

\(^6\) D. B. Cosulich and J. M. Smith, Jr.
of 10-nitroso PGA by treatment of PGA with nitrous acid in cold mineral acid has been reported. This is evidence that the nitrogen atom in the 10 position of PGA is capable of reacting as a secondary aromatic amine. Under similar conditions, 10-formyl PGA (XV) does not react with nitrous acid, which is reasonable proof that the formyl group is in the 10 position in this compound. Furthermore, immediate and rapid addition of 1 mole of nitrous acid to leucovorin in cold dilute hydrochloric acid results in the formation of the 10-nitroso derivative. This shows that the 10 position, as in PGA, is free to act as a secondary amine, and thus the formyl group in leucovorin must be attached only at the 5 position. However, when leucovorin is allowed to stand in dilute mineral acid for a short time at room temperature, there is no reaction with nitrous acid in the cold. This indicates that both the 5 and 10 positions had become blocked.

The above experiments suggest that under mild acid conditions the formyl group of leucovorin forms a new ring linking the 5 and 10 positions. This assumption was proved by the subsequent isolation of an imidazolinium salt, isoleucovorin chloride (XIII), obtained by treatment of leucovorin with hydrochloric acid at pH 1.3 or below. Isoleucovorin chloride does not react with nitrous acid; furthermore, it contains ionic chlorine and a potential formyl group, since 1 mole of formic acid is formed during the drastic acid hydrolysis employed in the usual formyl analysis.

Leucovorin can be considered a formyl derivative of a N,N'-aromatic disubstituted ethylene diamine. As a model of such a system, the monoformaldehyde derivative of N',N'-diphenylethylenediamine, N-(2-anilinoethyl)-formanilide (XXIX), was prepared in order to compare its behavior to

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\[ \text{XXVIII} \]

(2-Amino-4-hydroxy-5-formyl-10-methyl-5,6,7,8-tetrahydro PGA)

---

acid with that of leucovorin. A study of the ultraviolet absorption of XXIX under acid and basic conditions shows that it behaves quite similarly to

\[
\text{O} \\
\text{CH} \quad \text{H} \\
\text{CH}_2-\text{CH}_2
\]

XXIX

\[N-(2-\text{Anilinoethyl})\text{formanilide}\]

leucovorin; thus, in acid the maximum shifts in a manner which suggests a more highly conjugated system (XXX). Furthermore, a rapid change from strongly acidic to strongly basic solutions results in an equally rapid regeneration of XXIX. At intermediate pH values, however, the interconversion of the two forms occurs much more slowly, which shows that the shift of the maximum is not an immediate effect of pH but represents a more drastic change in the molecular structure.

It is readily apparent that opening the imidazolinium ring of XXX to form XXIX can give a product with the formyl group on either nitrogen; but, because the molecule is symmetrical, only one product is formed. However, treatment of isoleucovorin chloride with alkali may give either the 10- or 5-formyl derivative. The major product appears to be 10-formyl-5,6,7,8-tetrahydro PGA (XIV), although some regeneration to leucovorin also occurs. Thus, when isoleucovorin chloride is treated with alkali aerobically, one-half of a molecular equivalent of oxygen is rapidly consumed to give a product which slowly oxidizes in acidic solution to 10-formyl PGA (XV). Since 10-formyl PGA can be hydrolyzed readily to PGA with alkali, it is therefore possible to reconvert leucovorin to PGA.

As can be seen from Fig. 7, several other products have been isolated during investigations on the chemistry of leucovorin. One of these is anhydroleucovorin-A (XVIII),\textsuperscript{90, 95} which is formed by dissolving isoleucovorin chloride in boiling water, then cooling slowly. This crystalline substance is thought to be an imidazolinium "betaine-type" derivative of leucovorin. By treatment of anhydroleucovorin-A or isoleucovorin chloride
with boiling pH 4.0 buffer, anhydroleucovorin-B (XIX)\textsuperscript{90, 95} is formed; the exact structure of this derivative has not been completely elucidated. Both anhydroleucovorin-A and anhydroleucovorin-B can be converted to isoleucovorin chloride with 0.1 \textit{N} hydrochloric acid and to leucovorin with alkali under anaerobic conditions. Another derivative is 5,10-diformyl-5,6,7,8-tetrahydro PGA (XVII),\textsuperscript{79, 84} which can be formed from either 5,6,7,8-tetrahydro PGA (XVI), 10-formyl-5,6,7,8-tetrahydro PGA (XIV), or leucovorin (XII) by formylation with 98 to 100\% formic acid in the presence of acetic anhydride; this substance has an activity for \textit{Lc. citrovorum} which is equivalent to 0.5\% of pure leucovorin. Alkaline hydrolysis removes the 10-formyl group to give leucovorin.

It is conceivable that, during migration of the formyl group which occurs in the synthesis of leucovorin and its transformation products, an intermediate hydroxymethylene bridge might result. Two stereoisomeric configurations are possible for such a bridge. Although many have claimed\textsuperscript{97-99} that the formyl and other acyl derivatives of aromatic \(\beta\)-secondary diamines exist as ring structures with hydroxymethylene bridges, recent results obtained by infra-red absorption studies of some acyl compounds of similar structure indicate that the open amide form exists at least to some extent.\textsuperscript{100}

c. Relation of Natural Citrovorum Factor to Leucovorin

Recently Keresztesy and Silverman\textsuperscript{101} and Sauberlich\textsuperscript{102} have succeeded in isolating crystalline citrovorum factor from liver which has permitted comparison of the properties of the naturally occurring vitamin with 5-formyl-5,6,7,8-tetrahydro PGA. The product obtained from horse liver by Keresztesy and Silverman was found to be approximately twice as active as synthetic 5-formyl-5,6,7,8-tetrahydro PGA for growth of \textit{Leuconostoc citrovorum}. In 0.1 \textit{N} NaOH solution (at a concentration of 10 mg. per liter) leucovorin exhibits a maximum at 282 m\(\mu\) (optical density = 0.631) and a minimum at 243 m\(\mu\) (optical density = 0.123). When the two compounds are examined spectrophotometrically under identical conditions, they are indistinguishable. Since the natural factor is twice as active microbiologically as the synthetic product, and since there appears to be no spectral difference between the compounds, it is possible that the synthetic factor is a mixture of diastereoisomers, one of which is the biologically active, naturally occurring form. Examination of the structure of 5-formyl-5,6,7,8-tetrahydro PGA indicates the possibility of diastereoisomers. In the synthe-

\textsuperscript{97} O. Fischer, \textit{Ber.} \textbf{34}, 930 (1901).
\textsuperscript{98} St. Niementowski, \textit{Ber.} \textbf{20}, 1874 (1887).
\textsuperscript{99} O. Fischer and M. Rigaud, \textit{Ber.} \textbf{34}, 4203 (1901); \textbf{35}, 1258 (1902).
sis of this compound a new asymmetric center has been created at position 6 in the pyrazine ring. Another point of difference between the natural and synthetic citrovorum factor is that, when the two substances are exposed to the action of dilute acid, the natural material loses about one-third of its potency as a source of folic acid for S. faecalis whereas the synthetic material following acid treatment increases 11% in activity for S. faecalis. 

Synthetic leucovorin has been separated into its two diastereoisomers by Cosulich et al.\(^{104}\) by utilizing the difference in solubility of the calcium salts. The \(L_{L}\) form is the less soluble form, and it has an \([\alpha]_D\) of \(-15.1\) compared with \(+15.3\) for the original mixture of the two diastereoisomers. The calcium \(L_{L}\)-leucovorin has biological activities for S. faecalis and Leuconostoc citrovorum strictly comparable to those for the natural material and is decreased in activity by acid treatment. The more soluble calcium \(L_{L}\)-leucovorin has not been obtained in a pure state.

The existence of different forms of the citrovorum factor in nature was first clearly demonstrated by Wisten and Eigen.\(^{103}\) When various crude materials such as yeast extract or liver were chromatographed on paper and the strips placed on an agar medium deficient only in CF and seeded with Leuconostoc citrovorum, several distinct zones of growth appeared. It was suggested that the chromatographically slower factors represent conjugated forms of the faster forms. Different forms of the synthetic factor have also been prepared corresponding to the mono-, di-, and triglutamic acid derivatives of PGA.

### III. Industrial Preparation

ROBERT S. HARRIS

Because of the intense activity of commercial groups in the development of inexpensive and effective methods for the synthesis of pteroylglutamic acid, it is not possible at this time to describe the methods now being used in the industrial preparation of this vitamin. Instead it is necessary to describe some of the synthetic methods first reported in the scientific literature and to list a few of the patents which have been issued.

The chemical synthesis of PGA was first reported by Angier et al.,\(^{1}\) who

\(^{103}\) W. A. Wisten and F. Eigen, *J. Biol. Chem.* 184, 155 (1950).


reported success by four different methods. Method I\textsuperscript{1,2} consisted in reacting equimolecular amounts of 2,4,5-triamino-6-hydroxypyrimidine, p-aminobenzoyl-L-glutamic acid, and 2,3-dibromopropionaldehyde in the presence of an acetate buffer. The resulting product was 15% active. The dihydro compound was formed first, and this oxidized to the aromatic compound during the course of the reaction. The crude mixture was dissolved in dilute alkali and the impurities removed by precipitation with barium chloride, adjusting to pH 7.0, filtering, and extracting three times with 10 volumes of butanol. The aqueous phase was concentrated, acidified to pH 3.0, and cooled to 0.5\degree. The precipitate was redisolved in dilute alkali, the solution was treated with charcoal and acidified to pH 3.0, and the active compound was recovered by crystallization from hot water.

In Method II\textsuperscript{3} 2,3-dibromopropionaldehyde was reacted with pyridine, and the product was condensed with 2,4,5-triamino-6-hydroxypyrimidine and KI to yield N-[(2-amino-4-hydroxy-6-pteridyl)methyl]-pyridinium iodide. By heating with Na methoxide in ethylene glycol at 140\degree, this compound was condensed and the product was found to contain 15% of the active compound.

Method III\textsuperscript{4} involved the reaction of reductone (2,3-dihydroxyacrylaldehyde) with p-aminobenzoylglutamic acid to form p-(2,3-dihydroxy-2-ene-propylideneamino)benzoylglutamic acid, then esterification and condensation of the ester with 2,4,5-triamino-6-hydroxypyrimidine.

Method IV\textsuperscript{5} involved the reduction of 2-amino-4-hydroxy-6-pteridylmethylpyridinium iodide to 2-amino-4-hydroxy-6-methylpteridine, bromination or chlorination, then condensation of the product with the diethyl ester of p-aminobenzoylglutamic acid.

These and similar methods have been patented by the American Cyanamid Company.\textsuperscript{6}


\textsuperscript{5} American Cyanamid Co., U. S. Pats. 2,442,836, 2,442,837, 2,442,867, 2,443,165,
Karrer and Schwzyer\textsuperscript{7} condensed 2,4,5-triamino-6-hydroxypyrimidine with glyceraldehyde or dihydroxyacetone, giving a mixture of 2-amino-4-hydroxy-6-hydroxymethylpteridine and 2-amino-4-hydroxy-7-hydroxymethylpteridine. Reacted with \textit{p}-aminobenzoylglutamic acid, the former compound yielded PGA.

Roche Products Ltd.\textsuperscript{8} obtained a patent for PGA synthesis by reacting 2-amino-4-hydroxy-6-hydroxymethylpteridine with thionyl chloride and then treating the product with \textit{p}-aminobenzoylglutamic acid, or hydrogenating a mixture of pteridine and \textit{p}-nitrobenzoylglutamic acid. This company subsequently obtained a series of patents on the synthesis of PGA and intermediate compounds, a few of which are listed.\textsuperscript{9}

Hoffman-LaRoche Co.\textsuperscript{10} condensed 2,4,5-triamino-6-hydroxypyrimidine with a ketohexose and oxidized the 2-amino-4-hydroxy-6-tetrahydroxybutylpteridine which resulted with lead tetraacetate or another agent capable of producing the glycol cleavage. The 2-amino-4-hydroxy-6-pteridylaldehyde that was formed was hydrogenated in an inert solvent or in formic acid in the presence of \textit{p}-aminobenzoylglutamic acid and a catalyst, and PGA was obtained. Some formyldpteroylglutamic acid was produced, and this was converted to PGA by treatment with ammonia. Several of the patents issued to this company are listed.\textsuperscript{11}

Forrest and Walker\textsuperscript{12} reacted glucose and fructose with 2,4,5-triamino-6-hydroxypyrimidine in the presence of hydrazine and obtained 2-amino-4-hydroxy-6-\textit{d}-arabotetrahydroxybutylpteridine; in the absence of hydrazine he found 2-amino-4-hydroxy-7-\textit{d}-arabotetrahydroxybutylpteridine.

Uyeo and Mizukami\textsuperscript{13} reported better yields when mercuric acetate was used as a dehydation agent in Waller's method\textsuperscript{9} of synthesis. In a more recent paper Uyeo \textit{et al.}\textsuperscript{14} described a further modification of this method of PGA synthesis.

\begin{itemize}
\item 2,444,002, 2,444,005, 2,472,520, 2,500,206, 2,517,530, 2,520,479, 2,537,006, 2,547,519, 2,547,520, 2,568,597, 2,570,391, 2,570,392; British Pats. 12,491, 14,216, 24,564, 25,001, 25,002 (1948); 3,413, 631,494 (1949); 638,411, 638,480, 640,092 (1950); 644,913, 648,896, 650,276, 654,512, 655,771, 656,403, 657,902 (1951); 258,538 (1952).
\item 8. Roche Products Ltd., British Pats. 264,394, 630,751.
\item 9. Roche Products Ltd., British Pats. 624,394, 628,305, 626,171 (1949); Belgium Pat. 481,230 (1948).
\item 10. Hoffman-LaRoche and Co., British Pats. 626,171, 628,305; Roche Products Ltd., British Pat. 631,516.
\item 11. Hoffman-LaRoche and Co., U. S. Pats. 2,487,393, 2,520,882; British Pats. 629,440 (1949); 657,254 (1951); 664,721 (1952); Swiss Pats. 253,838 (1948); 255,409, 258,141, 263,147 (1949); 263,281 (1950); 263,147 (1951); 268,328 (1952).
\end{itemize}
Weygand et al.\textsuperscript{15} described a new synthesis of PGA by the reaction of the condensation products of \( p \)-tolyl-\( D \)-isoglucoasamine and sugars, with 6-hydroxy-\( 2,4,5 \)-triaminopyrimidine. Later Weygand and Schmied-Kowarzik\textsuperscript{16} described a series of syntheses of PGA by other reactions.

Kirsanova and Trufanov\textsuperscript{17} described the synthesis of PGA by reaction of \( p \)-aminobenzoyl-\( D \)-(1)-glutamic acid, 2,3-dibromopropionaldehyde, and 2,4,5-triamino-6-hydroxypyrimidine. This synthesis is similar to that reported by Angier\textsuperscript{1} and others. They described an improved purification process by which there was no loss in activity, whereas the method of Angier involved a loss of 85\% or more.

Hultquist and Dreisbach\textsuperscript{18} have patented a method for the synthesis of PGA.

Haehner et al.\textsuperscript{19} reported PGA synthesis by condensing 2-amino-5,5-dibromobarbituric acid with \( p \)-[2,3-diaminopropyl]amino benzoyl glutamic acid to give an unstable intermediate product which rearranged to dihydro PGA. On oxidation it gave a good yield of relatively pure PGA.

Petering and Schmitt\textsuperscript{20} have patented methods for synthesizing 2,4-diamino-6-(3-carboxy-1,2,3-trihydroxypropyl)pteridine and 2-amino-4-hydroxy-6-(3-carboxy-1,2,3-trihydroxypropyl)pterines.

Merck and Co.\textsuperscript{21} and Parke, Davis & Co.\textsuperscript{22} have patented other methods for synthesizing this vitamin.

### IV. Biochemical Systems

#### A. COENZYMES AND ENZYMES

The primary biochemical role of PGA appears to be the synthesis of compounds such as purines, pyrimidines, and certain amino acids which involve the incorporation of a single carbon fragment.

1. **Function of PGA in Synthesis of Purines and Pyrimidines**

The relationship between PGA and purines in the nutrition of lactic acid organisms was noted early in the work on this vitamin. Snell and Mitchell\textsuperscript{1} described a new synthesis of PGA by the reaction of the condensation products of \( p \)-tolyl-\( D \)-isoglucoasamine and sugars, with 6-hydroxy-\( 2,4,5 \)-triaminopyrimidine. Later Weygand and Schmied-Kowarzik described a series of syntheses of PGA by other reactions.

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\textsuperscript{17} V. A. Kirsanova and A. V. Trufanov, Biokhimiya 14, 413 (1949).
\textsuperscript{18} M. E. Hultquist and P. F. Dreisbach, British Pat. 657,831 (1951).
\textsuperscript{21} Merck and Co., U. S. Pat. 2,510,274 (1951); British Pat. 653,068 (1951).
\textsuperscript{22} Parke, Davis & Co., U. S. Pat. 2,476,360 (1949); British Pat. 639,154 (1950).
reported that adenine or guanine stimulated the growth of *Streptococcus faecalis* R and that a further response was produced by thymine. Stokstad found that the PGA requirement of *Lactobacillus casei* can be partially replaced by a combination of 0.5 mg of thymine and 5.0 mg of guanine per milliliter of media. However, the maximum growth obtained by a combination of purine and pyrimidines is only about half that produced by concentrates of *L. casei* factor (PGA). Thymine could not be replaced by other pyrimidines such as uracil or cytosine. The purine requirements were less specific and could be met by guanine, xanthine, adenine, or hypoxanthine. A number of pyrimidines, purines, and pterins have been tested by Stokes as possible substitutes for thymine in the nutrition of *S. faecalis* R. Of these, thymidine (thymine desoxyriboside) is the only substance which has thymine-like activity equal to that of thymine on a molar basis. Thymus nucleic acid is inactive, which demonstrates the inability of *S. faecalis* R to hydrolyze this nucleic acid to the riboside form. No information is available on the activity of thymidine desoxynucleotide. A study of pyrimidine analogs reveals that a methyl group in the 5 position is essential for activity. Shifting the methyl group to the 1, 3 or 4 position, or replacing it by an ethyl, amino, or nitro group, results in complete loss in activity.

A possible role of thymine in serving as a substitute for PGA was suggested on the basis of experiments with these two compounds in the nutrition of *S. faecalis* R and *L. casei*. It was found that, whereas PGA alone would give a response with *S. faecalis* R, the maximum effect was not obtained unless a purine also was added. In the presence of a purine five thousand times as much thymine as PGA is needed for maximum growth. With *S. faecalis* R, thymine gives the same maximum growth rate as can be obtained with PGA, although with *L. casei* only half-maximum growth is achieved.

The possibility of formation of PGA by *S. faecalis* R cells during growth

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on thymine was tested by autoclaving the cells with dilute hydrochloric acid and assaying with L. casei. A "plateauing" of the L. casei assay response at approximately half-maximum growth was observed. This response is characteristic of that obtained with thymine and suggests that the material in the S. faecalis R cells giving the response is thymine and not PGA. These facts led to the suggestion that PGA functioned directly or indirectly as a coenzyme in the synthesis of thymine by S. faecalis R.

Hitchings et al. studied the effect of a large number of pyrimidines on the growth of L. casei and found that only those compounds which retain a methyl group in the 5 position possess any activity. Substitution of an imino group for one oxygen such as in 5-methylcytosine or 5-methylisocytosine yields compounds one-tenth as active as thymine. Replacement of both oxygens by imino groups as in 5-methyl-2,4-diaminopurine results in a still further decrease of activity. Replacement of the 5-methyl group by oxygen, amino groups, or halides produces inhibitory compounds. Of special interest is 5-bromouracil, which inhibits completely the growth of L. casei with thymine as the nutrient but which produces slight growth stimulation when a "folic acid concentrate" (PGA) is used as a nutrient.

Further evidence concerning the function of PGA in purine and pyrimidine synthesis has been provided by the work of Rogers and Shive using the method of inhibition analysis. The antagonist employed was "x methyl PGA," which was prepared from α,β-dibromobutyaldehyde, and which had been shown by Franklin et al. to function competitively as a PGA antagonist for rats and microorganisms. The inhibition ratios (ratio of inhibitor to PGA for complete inhibition) for this antagonist were determined in the presence of various purines and thymine. Thymine was found to have no effect on the toxicity of "x methyl PGA" in the absence of a purine but did counteract it when a purine was present. The inhibition ratio in the absence of a purine was approximately 30. The addition of 10 γ of hypoxanthine per milliliter increased it to 100. The further addition of 3 γ of thymine per milliliter further increased the antibacterial ratio to about 1000. These results also show that the PGA requirement for purine synthesis is larger than that for thymine synthesis, since the synthesis of purines is blocked at a lower concentration of antagonist. Thus at certain critical levels of antagonist it is possible effectually to block one enzyme function of a vitamin without seriously impairing another. Inhibition of growth by high levels of the antagonist in the presence of adenine and thymine shows that PGA has still another function. This is to be expected, since with L. casei a combination of thymine plus purine gives only half-maximum

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growth. Similar results were obtained with *S. faecalis* using both "N methyl PGA" and N\(^{10}\)-methylpterolic acid as antagonists (Stokstad *et al.*\(^7\)). In this case the antagonist had no effect, even at high concentrations when both adenine and thymine were supplied. These results with N\(^{10}\)-methylpterolic acid are shown in Table II. This indicates that the only function of PGA in *S. faecalis* is purine and pyrimidine synthesis and is in contrast to the situation in *L. casei* where PGA has a third additional role.

<table>
<thead>
<tr>
<th>TABLE II</th>
</tr>
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<tbody>
<tr>
<td><strong>Effect of Adenine and Thymine on Inhibition Ratio of N-Methyl Pteroic Acid—Organism: <em>S. faecalis</em> R (Stokstad <em>et al.</em>(^7))</strong></td>
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<tr>
<td>Pteroylglutamic acid, γ/10 ml.</td>
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<td>0.00</td>
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2. **Sulfonamide, p-Aminobenzoic Acid, and Purine Relationships**

An interesting relationship between *p*-aminobenzoic acid and the metabolism of purines, amino acids, and pyrimidines has been shown in *E. coli* by inhibition studies with sulfanilamide.

Shive and his associates\(^8\), \(^9\) and Winkler and de Haan\(^10\) have shown that sulfanilamide, in progressively higher concentrations, inhibits successively

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the synthesis of methionine, purine, serine, and thymidine by E. coli. The inhibition ratio for sulfanilamide to \( p \)-aminobenzoic acid is 3000. In the presence of methionine it becomes 10,000; in the presence of methionine and purine it is 30,000; and in the presence of methionine, purine, and serine it becomes 100,000. Further addition of thymine increases it to 200,000. Thymine may be partially replaced by PGA. This indicates that the synthesis of methionine, purine, serine, and thymine are mediated directly or indirectly by \( p \)-aminobenzoic acid and that the synthesis of each of these end products can be blocked in succession by progressively increasing the amounts of sulfanilamide. In order for any end product to be effective, the other end products, whose production is blocked at lower antagonist concentrations, must be present in the medium. In the presence of methionine, xanthine, and serine and with 2 mg. of sulfanilamide per milliliter and approximately 3 \( \text{mg} \) of \( p \)-aminobenzoic acid per milliliter, growth is inhibited. Growth can be restored at this point by the addition of either 30 \( \gamma \) of thymine, 0.3 \( \gamma \) of PGA, or 0.010 \( \gamma \) of \( p \)-aminobenzoic acid per milliliter.\(^{10}\) The fact that such a large amount of PGA is required relative to the \( p \)-aminobenzoic acid is difficult to explain if one assumes that the former is synthesized from the latter. It is possible, however, that the conversion of preformed PGA to a more active intermediate may be limiting.

Valine synthesis is the next limiting factor after thymine requirements are met. The growth produced by thymine or PGA reaches the maximum in 72 hours, whereas with \( p \)-aminobenzoic acid it is reached in 32 hours. Addition of valine at this point permits early growth, showing that this amino acid becomes the limiting factor after inhibition of thymine synthesis.\(^{10}\)

A similar series of products reverse the toxicity of sulfanilamide for \textit{Salmonella typhimurium},\(^{10}\) and methionine, xanthine, thymine, and valine have been reported to function similarly in sulfonamide reversal in \textit{Staphylococcus aureus}.\(^{11}\)

The relationship of these metabolites to sulfonamide inhibition and the possible precursors are shown in Fig. 8.

**B. MECHANISM OF ACTION**

1. **ROLE OF 4-AMINO-5-IMIDAZOLECARBOXAMIDE IN PURINE SYNTHESIS**

If PGA blocks the synthesis of purine, one might expect an intermediate product to accumulate in a system in which purine synthesis is blocked by a minimum amount of antagonist and in which growth is permitted by the addition of a purine. This has not been demonstrated with lactic acid or-

organisms either in cells grown on vitamin-deficient media or with PGA antagonists. However, in studies with sulfanilamide inhibition of \textit{E. coli} a compound, 4-amino-5-imidazolecarboxamide, was obtained which appears to be an intermediate in purine synthesis. The existence of this compound was first noted by Stetten and Fox,\textsuperscript{12} who observed that \textit{E. coli} grown in a synthetic medium containing amino acids and a bacteriostatic concentration of sulfadiazone produce a diazotizable amine. The formation of the amine is prevented by addition of \textit{p}-aminobenzoic acid in concentrations sufficient to block the action of the sulfonamide. Other bacteriostatic agents such as atebrin or penicillin do not produce this diazotizable amine.

The fact that this amine forms under conditions of sulfanilamide inhibition where purine synthesis is inhibited, together with the fact that the empirical analysis corresponded to that of a purine minus one carbon atom, suggested that it might well be an intermediate in purine synthesis. On this basis Shive \textit{et al.}\textsuperscript{13} synthesized 4-amino-5-imidazolecarboxamide and

\textsuperscript{12} M. R. Stetten and C. L. Fox, Jr., \textit{J. Biol. Chem.} \textbf{161}, 333 (1945).

found that it had the same properties as the compound isolated by Stetten and Fox. Subsequently it was observed that the synthesis of this amine by *E. coli* is increased by addition of glycine and to a lesser extent by threonine but not by serine.

PGA has not been shown to be directly involved in the synthesis of purines in the sulfonamide-inhibited *E. coli* system. Addition of PGA to this system at the points where methionine, purine, or serine synthesis is critical has no effect on the inhibition ratio. The inactivity of added PGA, however, does not exclude the possibility that a more highly active metabolic form or conjugate of PGA may be formed from *p*-aminobenzoic acid which cannot be replaced by PGA itself. Support for such a view comes from the observations of Woolley and Pringle, who found that the aminoimidazolecarboxamide accumulates in the medium when *E. coli* is grown in the presence of sufficient 4-aminopteroylglutamic acid to inhibit growth slightly. In the presence of larger amounts of 4-aminopteroylglutamic acid (250 γ per milliliter) the growth of *E. coli* is inhibited in a medium containing purines. Growth can be restored by the addition of thymidine but not by thymine or the deoxyribosides of hypoxanthine or guanine (Franklin et al). This evidence favors the view that *p*-aminobenzoic acid acts in the synthesis of purines and pyrimidines by way of an intermediate synthesis through PGA. This is in accord with the hypothesis of Woods that the primary action of sulfonamides is the inhibition of PGA formation. The only place where PGA has thus far been shown to be directly involved in *E. coli* metabolism is in the synthesis of thymine where either thymine or PGA affects the inhibition index. However, the role of PGA in purine and thymine synthesis in both *L. casci* and *S. faecalis*, the presence of *p*-aminobenzoic acid in PGA, and the action of 4-aminopteroylglutamic acid in stimulating production of the amine in *E. coli* certainly invite the view that PGA may be an intermediary in the synthesis of purine by *p*-aminobenzoic acid.

Aminoimidazolecarboxamide can replace purine in the nutrition of certain organisms. Shive reported that the amine promotes growth of *L. arabinosus* in a manner similar to purines and disappears from the medium. It also replaces hypoxanthine or adenine in stimulating growth of *Ophiostoma multiformatum* if a large inoculum is employed or if suboptimal concentrations of these purines are added to the medium.
It is interesting, however, that no 4-amino-5-imidazolecarboxamide has been detected in cultures of *L. casei* grown with inhibitory concentrations of “x methyl PGA” in the presence of some purine. This is not at all surprising when one considers that both the C2 and the C8 atoms of the purine ring are introduced as 1-carbon fragments. If the introduction of the C8-carbon happens to be the first limiting reaction, then the imidazolecarboxamide would not be expected to accumulate. It is ineffective as a growth promoter for nine purine-less mutants of *E. coli* but is effective for one mutant at the high level of 100 to 200 γ per milliliter, which is about fifteen times as high as the level of purine required to give the same effect. Another mutant was found which accumulates the amine when grown in the presence of a purine. With 5 γ of xanthine per milliliter, growth is approximately half of maximum, and 11.3 γ of aminoimidazolecarboxamide accumulates per milliliter of medium.

A synergistic effect was observed between purine and aminoimidazolecarboxamide. In the presence of only 4 γ of any purine per milliliter, which by itself gives only 30% growth, 30 γ of aminoimidazolecarboxamide per milliliter gives maximum growth. Formylaminoimidazolecarboxamide which contains the “missing” carbon atom, but not in the form of the closed pyrimidine ring, is more active than the parent amine. Vitamin B12 (0.2 mg per milliliter) increased growth of the purine-less *E. coli* mutant and increased utilization of added aminoimidazolecarboxamide. *p*-Aminobenzoic acid (0.1 γ per milliliter) increased growth but did not increase utilization of the imidazole derivative.

The activity of vitamin B12 in increasing the utilization of amine is of special interest. Shive has found that vitamin B12 increases the inhibition ratio of sulfanilamide with *E. coli* at the inhibition levels for methionine, purine, and serine synthesis. The non-specific effect of vitamin B12 in this case was explained on the basis that B12 aids in the conversion of *p*-aminobenzoic acid to the active coenzyme.

An alternate explanation for the activity of vitamin B12 is that it facilitates the conversion of the imidazole derivative to a deoxyriboside. Certain lactic acid bacteria requiring vitamin B12 are able to utilize thymine deoxyriboside or purine deoxyribosides but not free thymine. Under these circumstances the function of vitamin B12 appears to be synthesis of a deoxyriboside derivative capable of attachment to thymine. The recent work of Greenberg has shown that ring closure in a pigeon liver system does not

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occur with the free imidazole derivative. The latter may represent a breakdown product of an intermediate which may be a ribotide. This ribotide is converted into inosinic acid (hypoxanthine ribose-5-phosphate). Evidence for this is the acceleration in rate of incorporation of radioactive formate into inosinic acid by the addition of ribose phosphate.

This hypothesis fits the observations of Gots\textsuperscript{24} that in sulfonamide-inhibited \textit{E. coli} the \textit{p}-aminobenzoic acid added at intervals after inoculation is able to prevent the accumulation of imidazole derivative and to increase growth but is unable to decrease the amount already formed. This shows that imidazole derivative lies outside the active metabolic pool and that the enzyme system formed from \textit{p}-aminobenzoic acid is unable to reutilize the imidazole once it has been converted to the free state. Greenberg has recently obtained evidence that the aminomidazolecarboxamide as formed by sulfonamide-inhibited \textit{E. coli} is originally formed as the desoxyriboside.\textsuperscript{25}

Although aminomidazolecarboxamide is not an intermediate \textit{per se} in the synthesis of hypoxanthine, Schulman \textit{et al.}\textsuperscript{26} found that there was appreciable disappearance of C\textsuperscript{14}-labeled aminimidazolecarboxamide when incubated with pigeon liver homogenates and that radioactive hypoxanthine was formed.

Buchanan\textsuperscript{27} also found that the conversion of the imidazole derivative to hypoxanthine is catalyzed by soluble proteins. The presence of insoluble proteins appears unnecessary for optimal activity. When non-isotopic inosinic acid and hypoxanthine are incubated with either isotopic glycine or the isotopic imidazole derivative in pigeon liver homogenate, the inosinic acid contains a concentration of C\textsuperscript{14} three to five times greater than that of hypoxanthine. These results were interpreted to mean that the element of ribose is added to the imidazole derivative prior to ring closure with formic acid.

2. Inosinic Transformylase

Buchanan\textsuperscript{28} has observed that incubation of formate with inosinate in a pigeon liver system results in rapid exchange of the carbon in position 2 of the hypoxanthine with the carbon of formic acid. This enzyme system was termed “inosinic transformylase” and was found to be catalyzed by the citrovorum factor. This represents the first partially purified enzyme system in which the function of a PGA derivative can be clearly recognized.

3. Effect of Aminopterin on Purine Synthesis in Mice

Skipper et al.\textsuperscript{29} found that administration of 4-aminopteroylglutamic acid (A-aminopterin) or 4-amino-N\textsuperscript{10}-methylpteroylglutamic acid (A-methopterin) decreases the incorporation of radioactive formate into the nucleic acids and purine fractions to one-fifteenth the normal rate. The antagonists were administered for 7 days prior to the injection of formate.

4. Relation of p-Aminobenzoic Acid to PGA

The presence of p-aminobenzoic acid in PGA and the similarity in function has fostered the attractive hypothesis that the p-aminobenzoic acid functions as a precursor of PGA. There is no doubt that under certain conditions this is true. Whether it is always the case remains a disputed point in spite of the large mass of data brought to bear on this subject.

If the only function of p-aminobenzoic acid is for synthesis of PGA, then the following conditions might be expected: (a) PGA should promote growth in organisms which require p-aminobenzoic acid; (b) PGA should reverse sulfonamide non-competitively in all susceptible organisms; (c) organisms which require PGA should be resistant to sulfonamides.

There are organisms whose nutritional requirements for p-aminobenzoic acid can be met by PGA; in other cases this is not true. Pteroylglutamic acid counteracts the toxicity of sulfonamide in only a few organisms. However, those organisms which do require PGA are resistant to sulfonamide. In considering the data pertinent to this subject it might be well to divide them into two categories: first, those in which PGA and p-aminobenzoic acid have a common function; second, those in which they are not interchangeable.

a. Biochemical Systems Where p-Aminobenzoic Acid Functions via PGA

1. Sulfanilamide inhibits the synthesis of PGA by both susceptible and resistant strains of \textit{E. coli}.\textsuperscript{30} Levels of sulfanilamide (0.005 \textit{M}) which produce only partial inhibition of growth decrease the PGA synthesis to less than 1\% of the control level. In resistant strains inhibition of PGA synthesis is less marked, but no reduction in growth occurs. This effect is not due to a general lowering of cellular activity, as no marked effect on biotin synthesis occurs.

2. Increasing concentrations of p-aminobenzoic acid increase the production of PGA by growing cultures of \textit{L. arabinosus}, the yield being approximately 5\% on a molar basis.\textsuperscript{31}


3. Resting cell suspensions of *Streptobacterium plantarum* synthesize PGA (measured by *L. casei* assay) in a system containing only buffer, glucose, glutamic acid, and p-aminobenzoic acid. A quantitative relationship exists between the amount of PGA synthesized and the concentration of p-aminobenzoic acid used. It is also significant that synthesis of PGA begins at that level at which p-aminobenzoic acid becomes limiting for the growth of organism. Synthesis of PGA is inhibited by sulfonamide and reversed in a competitive manner by p-aminobenzoic acid.

4. The p-aminobenzoic acid requirement (0.1 γ per milliliter) of *L. arabinosus* can be replaced by approximately thirty times as much PGA (Lampen and Jones) or by much larger quantities of thymine (50 γ per milliliter) in the presence of a purine. The amount of PGA needed is thus approximately 3.0 mγ per milliliter or about five times the requirement of *S. faecalis* for this vitamin. The ability of a combination of thymine plus a purine to produce maximum growth with *L. arabinosus* in the absence of PGA parallels that of *S. faecalis*. In *L. arabinosus* the function of p-aminobenzoic acid seems solely that of PGA synthesis which in turn mediates the synthesis of thymine plus purine. The low activity of PGA compared to p-aminobenzoic acid suggests that PGA is not the active biological form synthesized by the organism from p-aminobenzoic acid. Citrovorum factor is no more active than PGA in this system.

Sulfapyridine inhibits the growth of *L. arabinosus* and is competitively reversed by p-aminobenzoic acid and non-competitively by both PGA (10 mγ per milliliter) and thymine (30 γ per milliliter). The amount of PGA required to induce growth in the presence of a sulfonamide approximates that which is effective in its absence. Similar sulfonamide–PGA relationship results are obtained with *Streptobacterium plantarum* and with a strain of *L. arabinosus* trained to grow in the absence of p-aminobenzoic acid.

5. Lampen and Jones observed that *L. casei* and *S. faecalis* R which require preformed PGA are relatively insensitive to sulfonamides. *S. faecalis* Raltson, however, which requires PGA for rapid early growth, is sensitive to sulfonamides, and this inhibition is reversed non-competitively by PGA or by thymine. p-Aminobenzoic acid at a level equal to that of PGA has only a small effect on sulfonamide toxicity. The amount of PGA required to reverse the effect of sulfonamide corresponds to that required for growth of *S. faecalis* R.

Three pairs of different strains of enterococci (*S. faecalis*, *S. zymogenes*,

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and *S. durans*), one member of which required PGA and the other did not, were compared for their sulfonamide sensitivity by Lampen and Jones.\(^5\) None of the cultures requiring preformed molecule were inhibited by 1280 \(\gamma\) of sulfadiazine per milliliter. Those members of strains which were able to synthesize their own PGA were inhibited by 0.3 \(\mu\)g. of sulfonamide per milliliter but became insensitive when supplied with an amount of pteroyltetrahydrofolic acid equal to that used to promote growth of PGA-requiring members of the same strain.

Thus in the case of the lactic acid bacteria and the *Streptococci* the evidence is in harmony with the thesis that *p*-aminobenzoic functions solely in the synthesis of PGA and that sulfonamide inhibits this conversion.

6. PGA behaves as a non-competitive sulfonamide antagonist for *Cl. acetobutylicum* (two strains) and *Streptobacterium plantarum* (three strains). These organisms, which require *p*-aminobenzoic acid, will also respond to ten to one hundred times as much PGA. The amount of PGA necessary to reverse the sulfonamide is approximately the same as that required to promote growth in its absence and is independent of the amount of sulfonamide.\(^6\) This is similar to results obtained with *Streptobacterium plantarum.*\(^3\)

7. The *Le. citrovorum* factor (folinic acid) is able to replace *p*-aminobenzoic acid for growth of *L. mesenteroides* and overcomes sulfonamide inhibition in a non-competitive manner. Neither PGA nor N\(^{10}\)-formyl PGA is active in this respect. These observations of Lascelles *et al.*\(^3\) lend support to the view that the inactivity of PGA in replacing *p*-aminobenzoic acid may reflect the inability of the organism to convert preformed PGA to the more metabolically active form.

### b. Biochemical Systems Where PGA Cannot Replace *p*-Aminobenzoic Acid

1. A *p*-aminobenzoic acid-less mutant strain of *E. coli* was observed which would not respond to PGA.\(^3\) Growth could be induced either by low concentrations of *p*-aminobenzoic acid (0.2 \(\gamma\) per milliliter) or by a combination of amino acids, purine, and thymine. This mutant strain, when grown in the presence of 0.01 \(\gamma\) of *p*-aminobenzoic acid per milliliter, produces a material giving a growth response with *S. faecalis* on a PGA-free medium.

With other organisms, such as *Acetobacter suboxydans* 621 and a *Neurospora* mutant, which require *p*-aminobenzoic acid, PGA reverses the action of sulfanamide in a non-competitive manner.\(^3\)

2. PGA is unable to affect the inhibition ratio of sulfanilamide for *E. coli* at the levels where methionine, purine, or serine synthesis is affected. At the level of thymine synthesis, PGA is effective.\(^3\) The lack of activity


of PGA in sulfanilamide-inhibited E. coli is similar to that observed with E. coli mutants (Lampen et al.\textsuperscript{35}).

In summary, it can be said that in many organisms PGA can replace the requirement for \( p \)-aminobenzoic acid. This especially seems true where the function of \( p \)-aminobenzoic acid is concerned only with synthesis of purines and pyrimidines. In E. coli, where \( p \)-aminobenzoic acid is concerned in the synthesis of methionine, serine, purine, and pyrimidine, PGA has been implicated directly only in the synthesis of thymine and indirectly in that of adenine. How then does \( p \)-aminobenzoic acid function in the synthesis of methionine and serine? Three hypotheses seem probable: (a) \( p \)-Aminobenzoic acid is converted efficiently to a PGA-like type of coenzyme, but the conversion of preformed PGA to this coenzyme is very inefficient; (b) \( p \)-aminobenzoic acid functions as a coenzyme without being incorporated into a PGA-like type of molecule; (c) \( p \)-aminobenzoic acid functions via vitamin \( B_12 \) in the synthesis of methionine and serine.

Unfortunately little direct experimental data can be brought to bear on this point. However, it is significant that the synthesis of methionine, serine, and adenine, which cannot be taken over by PGA in E. coli, can be ascribed to PGA function in other types of organisms. This will be discussed in greater detail in the next section.

5. Role of PGA in Synthesis of Amino Acids

PGA promotes serine synthesis in the growth of S. faecalis R.\textsuperscript{39, 40} It has been observed\textsuperscript{37} that pteroylglutamic acid is essential for the synthesis of serine from glycine and formate by resting washed cells of S. faecalis R. In this particular system \( N^10 \)-formyl PGA is 30\% more active than PGA. Tetrahydro PGA and citrovorum factor are no more active than PGA when added simultaneously with other components. However, when the cells are first incubated with citrovorum factor in glucose-phosphate, and the glycine and formate added subsequently, serine synthesis is more rapid than when PGA is similarly used. Also, in the above systems formation of citrovorum factor accompanies the synthesis of serine promoted by PGA. These same workers\textsuperscript{37} also found that the serine requirement for growth by Leuconostoc mesenteroides can be replaced by high concentrations of glycine plus a high carbon dioxide tension and that the citrovorum factor greatly reduces the carbon dioxide requirement. It thus appears that carbon dioxide may serve as the source of the single-carbon fragment in this experimental system. The very small amount of citrovorum factor used precludes the possibility of its serving as a stoichiometric source of the single-carbon fragment, and this factor must function instead as a more efficient catalyst.


\textsuperscript{40} B. R. Holland and W. W. Meinke, \textit{J. Biol. Chem.} \textbf{178}, 7 (1949).
which reduces the need for mass action pressure of large amounts of carbon dioxide.

Serine synthesis in the rat from isotopic formate is decreased in PGA deficiency.41

PGA has been similarly shown to be involved in methionine synthesis from homocystine in animals,42 and methionine synthesis in yeast can be inhibited by 4-aminopteroylglutamic acid and the action of the antagonist reversed by the citrovorum factor.43

6. PGA in Single-Carbon Transfer in Animals

Pteroylglutamic acid has been shown to be involved in the synthesis of serine, the conversion of serine to glycine, the synthesis of methionine, and the synthesis of purines in the rat. Both vitamin B₁₂ and PGA are concerned in the synthesis of choline and methionine.

a. Serine-Glycine Relationships

Incorporation of radioactive formate into serine is greatly depressed in PGA deficiency induced by sulfonamide feeding (Plant et al.44). Treatment with PGA for 4 days prior to formate administration increases C¹⁴ fixation into liver protein tenfold. Serine synthesis is most markedly affected; glutamic acid, arginine, and glycine are less affected. The carbon of formate was predominantly incorporated into the β-carbon of serine.

Heme from the blood of PGA-treated rats also contains an appreciable quantity of C¹⁴, whereas that from deficient rats contains none. Biotin deficiency has no influence on incorporation of C¹⁴ from formate into β-carbon of serine, showing that this effect of PGA deficiency is a specific one and is not the result of a general manifestation of vitamin deficiency such as ananition.

Pteroylglutamic acid is also concerned in the conversion of serine to glycine for hippuric acid synthesis in the rat (Elwyn and Sprinson45). From the lower utilization of serine and the greater utilization of glycine in the PGA-deficient rat, it has been estimated that the rate of conversion of serine to glycine is reduced to one-sixth of the normal value in PGA deficiency.

A comparison has also been made of the rates of incorporation of α-carbon of glycine and β-carbon of serine into choline.45 B₁₂ is concerned mainly with the utilization of α-carbon of glycine but not the utilization of β-carbon.

bon of serine. Conversely PGA deficiency reduces the utilization of β-carbon of serine and to a smaller extent that of the α-carbon of glycine. These relationships are expressed schematically in Fig. 9.

The in vitro incorporation of glycine, containing C14 in the carboxyl position, by chick liver homogenates is decreased in PGA deficiency (Totter et al.46). This effect was observed both in the liver proteins and the phospholipid fractions and was not influenced by in vitro addition of PGA to the deficient liver homogenates.

Elwyn and Sprinson47 found that the methyl group of thymine is derived from the α-carbon of glycine and the β-carbon of serine. This indicates that the methyl carbon of thymine is derived from a one-carbon intermediate by methylation of a pyrimidine nucleus.

b. Counteraction of Glycine Toxicity by PGA

Feeding large amounts of glycine depresses the growth of chicks and rats. The growth depression induced by 10% glycine in rats can be prevented by supplementation with PGA whereas a liver extract containing vitamin B12 is ineffective (Dinning et al.48). Similarly Machlin et al.49 found that 6% glycine depresses the growth of chicks and that additional quantities of PGA prevent the growth depression. It is thus apparent that the PGA requirements are increased by high levels of glycine.

c. Effect on Purine Synthesis in Animals

Drysdale et al.50 studied the effect of PGA depletion in rats on the incorporation of formate into purines. The rats were rendered deficient in PGA by maintenance on a sulfasuxidine-containing diet until they exhibited symptoms of leucopenia. The control animals were given PGA for 4 days, which induced a remission of the leucopenia. This procedure of depleting

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both groups of animals and then administering PGA to one group for a short time makes the experiment more specific for PGA and eliminates general effects of inanition which might occur. Under these conditions, incorporation of formate into adenine and guanine in the liver is depressed to approximately 30% of that in the PGA-supplemented. No difference between deficient and repleted animals was noted in visceral purines. PGA deficiency decreases the entrance of formate into the C-2 and C-8 positions of liver purines equally, showing that PGA is involved in the incorporation of both these atoms.

In the formation of 4-amino-5-imidazolecarboxamide during 4-aminopteroylglutamic acid inhibition of E. coli the incorporation of the 2-carbon is the only one affected. Apparently in this microorganism the incorporation of the 8-carbon is not inhibited, as this is a part of the imidazole molecule. If PGA is concerned equally with both C-2 and C-8, it is possible to understand why the imidazole derivative has not been observed during growth of any lactic acid organism on a medium devoid of PGA and supplemented with purine.

d. Thymine and Purines in Nutrition of Animals

The ability of thymine to replace PGA partially or completely in the nutrition of certain bacteria has prompted interest in whether thymine can replace PGA in the nutrition of animals. In the rat (Daft) and in the chick (Stokstad et al.) thymine is inactive both in growth stimulation and hematopoietically. Thymine is ineffective for rats both on a PGA-free diet containing sulfasuxidine and on a diet containing crude "x-methyl PGA" antagonist. Adenine is also ineffective in promoting growth in these experiments. More recently Daft has found that a combination of adenine and thymine, each at a level of 1%, produces a marked leucocyte and granulocyte response in rats rendered deficient in PGA by the feeding of sulfamamide. No growth response is obtained, however. The animals remain or become anemic, and kidney damage develops. Adenine appears to account for most of the activity of the mixture. It was further observed that the agranulocytosis symptoms of PGA deficiency which had previously been observed on low protein diets can be corrected by a mixture of methionine, tryptophan, and threonine plus either adenine, adenosine, or yeast extract. Previously, mixtures of the three amino acids had been found ineffective unless supplemented with PGA. This suggests that PGA-deficient rats are unable to synthesize amounts of adenine needed for white cell formation. The apparent inactivity of thymine is in accord with the information gained

by isotope studies that adult rats cannot utilize free thymine for nucleic acid synthesis.\textsuperscript{53}

Pigs, however, have been observed to give a hematological response to thymine in doses of 10 g. daily (Welch and Heinle\textsuperscript{54}).

Of special interest is the fact that patients with pernicious anemia respond hematologically to thymine, uracil, and thymidine. Spies \textit{et al.}\textsuperscript{55} found that 15 g. of thymine is capable of producing a hematological response similar to that induced by PGA. About five thousand times as much thymine as PGA is required. This ratio is similar to the relative effectiveness of these two compounds in promoting the growth of \textit{S. faecalis} (Stokes\textsuperscript{3}). In view of the ineffectiveness of uracil in replacing thymine in the nutrition of PGA-requiring organisms, the observations of Vilter \textit{et al.}\textsuperscript{56} that uracil in doses of 15 to 30 g. per day often causes hematological remission are of special interest. Quite surprising also is the fact that thymine proved effective in a pernicious anemia patient who had become refractory to PGA.

Thymidine, which is able to substitute for vitamin B\textsubscript{12} in the nutrition of certain lactic acid bacteria, has been reported by Hausmann\textsuperscript{57} to be effective in \textit{total} doses of 2.0 and 2.8 g., respectively, and to produce a response comparable to that elicited by PGA or vitamin B\textsubscript{12} in pernicious anemia.

e. Influence of PGA and Vitamin B\textsubscript{12} on Methionine and Choline Synthesis

Both vitamin B\textsubscript{12} and PGA have been shown to have a role in choline and methionine metabolism in chicks and rats. This was first shown by the work of Bennett\textsuperscript{58} who found that rats with a certain previous dietary history were able to utilize homocystine for growth in the absence of labile methyl donors when supplemented with PGA and with purified liver extract containing vitamin B\textsubscript{12}. This requirement for PGA was observed both in the absence and presence of sulfasuxidine. Since rats normally do not develop PGA deficiency on a purified type of diet unless supplemented with sulfonamide, the results of Bennett suggest that the PGA requirements of the rat are increased on a "labile methyl"-deficient diet. Both vitamin B\textsubscript{12} and PGA are required for synthesis of methionine from homocystine under this regimen.

On a methionine-deficient diet (containing alcohol-extracted peanut meal


\textsuperscript{58} M. A. Bennett, \textit{Science} \textbf{110}, 589 (1949).
and oxidized casein) supplemented with homocystine, there is a relationship between homocystine, choline, PGA, and vitamin B₁₂ requirements (Schaefer and Knowles⁵⁹). On such a diet no growth takes place, and there is a 100% incidence of renal hemorrhage. The effects of methyl donors, vitamin B₁₂, and PGA are shown in Table III. The results clearly indicate that choline alone or a combination of betaine plus aminoethanol will protect against renal hemorrhage in the absence of both vitamin B₁₂ and PGA. On diets marginal in choline or betaine neither B₁₂ nor PGA has any effect on renal damage. Complete protection can be achieved with a combination

<table>
<thead>
<tr>
<th>TABLE III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effect of Vitamin B₁₂ and PGA on the Utilization of Betaine, Choline, and Aminoethanol</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Choline, g./kg.</th>
<th>Betaine, g./kg.</th>
<th>Aminoethanol, g./kg.</th>
<th>Vitamin B₁₂, γ/kg.</th>
<th>PGA, mg./kg.</th>
<th>Gain in body wt. in 2 wk., g.</th>
<th>Incidence of renal hemorrhage, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>...</td>
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<td>28</td>
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<td>...</td>
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<td>50</td>
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</tr>
<tr>
<td>...</td>
<td>6.0</td>
<td>0.6</td>
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<td>...</td>
<td>33</td>
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<td>60</td>
<td>2</td>
<td>43</td>
<td>0</td>
</tr>
</tbody>
</table>

of these two vitamins or by increasing the level of betaine or choline. This clearly shows that the requirements for PGA are higher on diets marginal in choline.

Although a combination of vitamin B₁₂ and PGA affords complete protection against renal damage on diets marginal in choline, it does not produce a maximum growth response. Maximum growth is given only by higher levels of choline supplemented with vitamin B₁₂, PGA having little effect on growth at these levels of choline.

A similar effect of PGA and B₁₂ on lipotropism has been noted in rats (Schaefer et al.⁶⁰). Fatty livers were noted in rats receiving 0.1% of choline.

which prevented kidney hemorrhage. Protection against lipotropism could not be obtained by vitamin B₁₂ or PGA alone but was achieved with a combination of B₁₂ and PGA or with a higher level of choline.

The influence of PGA on utilization of methanol as a source of methyl groups in choline has been demonstrated by Verly et al.⁶¹ C¹⁴-Deuteriomethanol was injected into rats deficient in PGA and into similar rats treated with PGA prior to methanol administration. PGA caused a two- to threefold increase in utilization of methanol as measured by the C¹⁴ and deuterium content of isolated choline. Vitamin B₁₂ under similar conditions was without effect.

f. In Vitro Studies on Methionine Formation

Livers from PGA-deficient chicks exhibit reduced ability to form methionine from homocystine in the presence of choline or betaine (Dinning et al.⁶²). Choline oxidase is also decreased by PGA deficiency as evidenced by slower oxidation of choline. In vitro addition of PGA to the deficient liver does not affect methionine synthesis but addition of PGA to repleted livers does. This effect of added PGA in vitro is difficult to understand. The effect of PGA on choline oxidase offers an explanation for the effect of this vitamin on methionine synthesis as it has been suggested that choline is first oxidized to betaine before transmethylation can occur.

V. Specificity of Action

E. L. R. STOKSTAD

A large number of PGA analogs have been made. Most of these behave as antagonists and will be considered in another section of this chapter. Certain modifications can be made in the molecule without nullifying the biological activity, but they may modify the activity for different species. This information, which can best be presented in tabular form, is shown in Table IV.

It is apparent from these data that the higher animals, with the exception of the fox, are capable of utilizing derivatives of PGA containing more than one glutamic acid molecule. It is of special interest that the diglutamic acid derivative containing an α linkage is utilized efficiently by the chick even though it has low biological activity for L. casei and S. faecalis. Chicken liver conjugase has been shown to split α-glutamic acid linkages more slowly

### TABLE IV

**Specificity of the Pteroylglutamic Acid Derivatives—Relative Activities for Various Species**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Man</th>
<th>Monkey</th>
<th>Fox</th>
<th>Rat</th>
<th>Chick</th>
<th>Tetrahymena geleei W</th>
<th>L. casei</th>
<th>S. faecalis R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pteroylglutamic acid</td>
<td>100</td>
<td>100</td>
<td>+</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Pteroyl-α-glutamic acid³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100 ca. (3)</td>
<td>100</td>
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<tr>
<td>Pteroyl-γ-glutamylglutamic acid⁴</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.9 (5)</td>
<td>55 (2)</td>
<td>57 (2)</td>
</tr>
<tr>
<td>Pteroyl-α,γ-glutamylglutamic acid⁵</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.2 (5)</td>
<td></td>
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<tr>
<td>Pteroyl-α-glutamyl-α-glutamylglutamic acid⁶</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0.6 (5)</td>
<td></td>
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</tr>
<tr>
<td>Pteroyl-α-glutamyl-γ-glutamylglutamic acid⁷</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.5 (5)</td>
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<tr>
<td>Pteroyl-γ-glutamyl-α-glutamylglutamic acid⁸</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>3.6 (5)</td>
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<tr>
<td>Pteroyl-γ-glutamyl-γ-glutamylglutamic acid⁹</td>
<td></td>
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<td></td>
<td>0.5-1.6 (14)</td>
<td>50-100 (5, 19)</td>
<td>50-100 (5, 19)</td>
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<tr>
<td>(pteroyltriglutamic acid)</td>
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<td></td>
<td></td>
<td></td>
<td>0.5 (5)</td>
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<tr>
<td>Pteroylheptaglutamic acid</td>
<td></td>
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<td></td>
<td></td>
<td>100 (11)</td>
<td>100 (23)</td>
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<tr>
<td>Pter ic acid¹⁵,¹⁶</td>
<td>0 (17)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.01 (23)</td>
<td>100 (24)</td>
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<tr>
<td>N¹⁵-Formylpterolic acid (rhizopterin)²⁰</td>
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<td></td>
<td></td>
<td></td>
<td>100 (24)</td>
<td>100 (24)</td>
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</tr>
</tbody>
</table>

*¹⁴ Taken from Biochemistry of B vitamins.²⁵
than the γ linkages (Dabrowska et al.). Both pteroic acid and its N\textsuperscript{10}-formyl derivative are inactive for the higher animals, *Tetrahymena geleii* and *L. casei*. They are active only for *S. faecalis* and other Enterococci.

A. RELATION OF XANTHOPTERIN TO PGA

The role of xanthopterin in nutrition is an enigma in the PGA problem. From the beginning, xanthopterin has crossed and recrossed the trail, sometimes guiding, sometimes serving only to confuse those intent on learning the nature and function of PGA. This yellow pigment, one of a class of compounds found in the wings of butterflies, has a structure closely related to the pteridine portion of PGA.

Although our knowledge of xanthopterin originated in 1891 when Hopkins began his investigations on the wing pigments of the English brimstone butterfly, it was not until 1941 that Wieland and his collaborators completed the degradation work with xanthopterin and leucopterin, and Purrman finally accomplished their synthesis. The problem of obtaining sufficient material for chemical degradation studies was an acute one, and altogether over 1,000,000 butterflies of various types were used by different workers in this field.

Xanthopterin has also been isolated from human and animal urine and designated uropterin by Koschara. It occurs in insects other than the lepidoptera; it has been detected in the crab but apparently not in plants.

Rominger et al. found that rats 3 to 4 weeks old developed macrocytic anemia when fed exclusively on goat’s milk. The erythrocyte count fell to around 1 million, and the animals did not respond to supplementation with iron. Typical reticulocyte responses were obtained with liver preparations. Tschesche and Wolf found that injection of uropterin (xanthopterin) obtained from Koschara produced a rise in the erythrocyte count. A daily dose of 1.0 g produced an elevation of 2 to 3 million in the erythrocyte count in 14 days. Ten micrograms produced no greater response than 1.0

26 R. Purrman, Ann. 548, 284 (1941).
γ. In contrast to the preceding report\(^ {31} \) concerning the ineffectiveness of iron, the anemia was found to respond to iron and copper as well as to uropterin. These results with xanthopterin were repeated by the same authors\(^ {34} \) with other samples of xanthopterin. However, Koschara reported that Rominger was unable to get any response with the same sample of uropterin that Tschesche had used in his experiments.\(^ {35} \)

Xanthopterin was also found to have a hemapoietic effect when injected into fingerling salmon with nutritional anemia.\(^ {36} \) The anemia was produced by feeding the salmon a high protein diet containing yeast as a source of the vitamin B complex. The erythrocyte counts of the control fish varied between 501,000 and 916,000 per cubic millimeter, whereas in those injected with 50 γ of xanthopterin the counts varied between 944,000 and 1,305,000. In a subsequent study these same investigators\(^ {37} \) observed a similar anemia on salmon raised on diets of 20% liver and 80% spawned-out salmon. The ability of salmon to become anemic on a diet containing 20% liver certainly indicates that PGA is ineffective in this blood dyscrasia. A larger variability existed in the erythrocyte count of these fish, but by selection a group of uniform experimental fish having an average count of 800,000 cells per cubic millimeter was obtained. Injection of a single dose of xanthopterin at a level of 10 mg. per kilogram of body weight increased the RBC to 1,300,000 per cubic millimeter; 20 and 40 mg. per kilogram of body weight increased it to approximately 1,580,000, with the peak on the third to fifth day. The erythrocyte count returned to its original level within 10 to 14 days. The erythrocyte counts of normal wild salmon caught in fresh or salt water were in the range of 1,500,000 to 1,600,000. Comparison of different species of salmon raised on the experimental diet and having the same initial erythrocyte count showed that different species varied in their response to xanthopterin.

Variable results have been reported with xanthopterin in sulfonamide-fed rats. Totter and Day\(^ {38} \) observed that 20 γ of xanthopterin administered daily to rats receiving 1% sulfasuxidine produced an immediate weight gain and increased the leucocytes to 9400 cells per cubic millimeter, compared with 3420 for the controls. The growth rate and the white blood cells were not completely restored to normal, which showed that the response produced by xanthopterin was qualitatively different from that produced by liver fractions. These results with xanthopterin have not been confirmed.


\(^ {34} \) E. R. Norris and R. W. Simmons, J. Biol. Chem. 168, 449 (1945).

by others,\textsuperscript{9, 39, 40} or by the same workers.\textsuperscript{38} O'Dell and Hogan\textsuperscript{41} have stated that xanthopterin is inactive in chicks fed on a diet low in PGA.

In vitamin M deficiency in the monkey, xanthopterin has produced more consistent effects. Totter and coworkers\textsuperscript{42} found that administration of 2.5 to 10 mg. of xanthopterin to vitamin M-deficient monkeys produced a reticulocytosis in 3 to 6 days. The white and red cell counts made transient increases to normal levels in 3 to 13 days. Xanthopterin alone given prophylactically failed to protect completely but did delay the onset of nutritional cytopenia. One animal given xanthopterin plus heated liver (shown to be ineffective by itself) maintained a normal white and red cell count for 71 days. Removal of xanthopterin at this point resulted in prompt return of cytopenia, and reinstatement of xanthopterin therapy produced a response similar to the first. Cerecedo and his associates found that xanthopterin, like PGA, produced significant improvement in the lactation performance of mice\textsuperscript{43} and of rats.\textsuperscript{44} Definite but small hematological responses have been noted by Heinle \textit{et al.}\textsuperscript{45} in two PGA-deficient pigs given 10 mg. of xanthopterin parenterally for 10 days. A third pig responded to a single dose of xanthopterin.\textsuperscript{46} Cartwright \textit{et al.}\textsuperscript{47} observed a reticulocyte response of 9% in one similarly deficient pig given a single injection of 20 mg. of xanthopterin.

The larva of the insect \textit{Ephestia kuehneola} is able to utilize xanthopterin in place of PGA but at levels one thousand times that of the vitamin.\textsuperscript{48}

Another curious and still largely unexplained effect of xanthopterin was noted early by Wright and Welch.\textsuperscript{39} When fresh rat liver is incubated with xanthopterin a marked increase in the folic acid content of the preparation is observed as compared with rat liver incubated either alone or with leucoprotein or certain purines or pyrimidines. In a more detailed investigation of this phenomena the same workers\textsuperscript{49} found that the folic acid content of rat liver and muscle was influenced during digestion by factors such as

\textsuperscript{39} L. D. Wright and A. D. Welch, \textit{Science} \textbf{98}, 179 (1943).


\textsuperscript{41} B. L. O'Dell and A. G. Hogan, \textit{J. Biol. Chem.} \textbf{149}, 323 (1943).


\textsuperscript{43} L. Mirono and L. R. Cerecedo, \textit{Arch. Biochem.} \textbf{15}, 324 (1947).

\textsuperscript{44} A. J. Sica, A. M. Allgeier, and L. R. Cerecedo, \textit{Arch. Biochem.} \textbf{18}, 119 (1948).


xanthopterin, salts, cyanide, degree of dispersion of the tissue, pH of the tissue, and the addition of taka-diastase. This observation was confirmed by Totter et al.,\textsuperscript{50} and it was found that the addition of xanthopterin increased the content of microbiologically active PGA during incubation of liver from vitamin M-deficient monkeys but not of liver tissue from normal chickens. This species difference in response of liver tissue to xanthopterin is further emphasized by the fact that addition of this pterin to hog liver produces tenfold increases of PGA activity\textsuperscript{51} in contrast with the earlier reports of two- to threefold increases with rat liver.

As an explanation of this phenomenon, it was suggested that liver contains a precursor of pteroylheptaglutamic acid which is susceptible to attack competitively by two types of enzymes.\textsuperscript{46} One reaction at pH 7.0 leads to the formation of a compound, presumably pteroylheptaglutamic acid, which is susceptible to further enzymic conversion at pH 4.7 to give PGA. A second reaction at pH 7.0 leads to inactivation of this precursor and may be inhibited by xanthopterin. This hypothesis rests on the following facts:

1. Incubation of pig liver with xanthopterin at pH 7.0 increases the PGA from an original of 0.25 to a final value of 3.0 \( \gamma \) per gram.
2. Incubation at pH 4.7 gives 0.65 \( \gamma \) with or without xanthopterin.
3. Incubation for 4 hours at pH 7.0 followed by 14 hours at pH 4.7 gives 18.5 \( \gamma \) with xanthopterin.
4. At pH 7.0 pteroylheptaglutamic acid is only partially converted to PGA; at pH 4.7 the conversion is nearly complete.

If this explanation of the effect of xanthopterin is correct, then the true amount of PGA derivatives in liver is much higher than that observed with any combination of autolysis and digestion on unheated liver tissue.

If one accepts this explanation of the effect of xanthopterin, one might account for the transient and partial responses observed with xanthopterin in rats. It may be that in PGA-deficient animals there are small amounts of PGA conjugates in the liver whose major pathway of metabolism would normally cross that of inactivation. Xanthopterin, by inhibiting this inactivation, increases the utilizable quantity of the vitamin, and as soon as the PGA precursor is completely exhausted, no further response can be elicited by xanthopterin.

The possibility that xanthopterin may be converted to PGA has been entertained, but recent work with xanthopterin labeled with C\textsuperscript{14} in the 6 and 7 positions makes such a view unlikely.\textsuperscript{46} Administration of this pterin to a PGA-deficient pig did give a small hematological response, but no radioactivity could be detected in PGA isolated from the liver by use of the carrier vitamin. In addition, incubation of the liver in vitro with isotopic

\textsuperscript{51} A. D. Welch, E. M. Nelson, and M. F. Wilson, Federation Proc. 8, 346 (1949).
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xanthopterin did not lead to the appearance of any labeled carbon in the PGA isolated from the homogenate.

B. PGA ANTAGONISTS

The study of PGA antagonists has proved to have both academic and clinical interest. These compounds have facilitated the study of the mechan-

<table>
<thead>
<tr>
<th>TABLE V</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TYPES OF PGA ANTAGONISTS</strong></td>
</tr>
</tbody>
</table>

1. Pyrimidine derivatives
   - Example: 5-Nitouracil (Hitchings et al.\(^52\))

2. Purine derivatives
   - Example: 2,4-Diaminopurine (Elion\(^51\))

3. Pteridine derivatives
   - Example: 2,4-Diamino-6,7-diphenylpterdine (Daniel et al.\(^52\))

4. Modification of pterin nucleus
   - Example: Quinoxaline-2-carboxyl-p-aminobenzoylglutamic acid (Woolley and Pringle\(^56\))

5. Pteroyl derivatives of different amino acids
   - Example: Pteroylaspartic acid (Hutchings et al.\(^57\))

6. Alkyl derivatives of pteroylglutamic acid
   - Example: N\(^{10}\)-Methylpteroylglutamic acid (Cosulich and Smith\(^48\))

7. Substitution on the pterin nucleus
   - Example: 4-Aminopteroylglutamic acid (Seeger et al.\(^59\))

8. Sulfur analogs with a sulfonoyl group in place of the carboxyl of p-aminobenzoic acid
   - Example: Benzimidazole-2-methyl-p-aminobenzene sulfonoylglutamic acid (Edwards *et al.*\(^60\))

anism of PGA function in microorganisms and in tissues of higher animals. They have also provided new tools for the study of leukemic processes and have proved to have some clinical application in the therapy of leukemia.

A complete cataloguing of the antagonists which have been synthesized is well outside the scope of this review. For more detailed information the reader is referred to the excellent reviews by Shive\(^25\) and Martin.\(^62\) The


discussion will be confined to representatives of the main types of antagonists and to a description of their more important biological effects.

The principle classes of compounds which function as PGA antagonists or in some cases possess PGA-like activity are shown in Table V.

1. Substituted Pyrimidines

Since pyrimidines are formed by PGA-catalyzed reactions, it is not surprising that substituted pyrimidines function as PGA antagonists under certain conditions. A large number of pyrimidines have been prepared and their growth-promoting or growth-inhibiting actions determined. In many cases the antagonistic action is not reversed by PGA and must therefore be regarded primarily as an inhibition of pyrimidine or purine function.

Detailed information on pyrimidine and purine antagonists has been compiled in the excellent reviews of Hitchings et al.\(^{61}\) and Wright.\(^{62}\)

Replacement of the 5-methyl group of thymine (5-methyluracil) by hydroxy, bromine, amino, or nitro groups produces compounds which inhibit the growth of \(L.\) \(casei\) (Hitchings et al.\(^{63}\)). Of these compounds, 5-nitro-uracil was reversed competitively by PGA, 5-aminouracil and 5-bromo-uracil were competitively reversed by thymine, and 5-hydroxyuracil by uracil.

Of special interest are the 2,4-diaminopyrimidines and their substituted ring systems which include the 2,4-diaminopurines and pteridines. Hitchings et al.\(^{64}\) found that nearly all 2,4-diaminopyrimidines inhibited \(L.\) \(casei\) in the presence of PGA and in the absence of a purine. A possible structural similarity between the PGA antagonist 2,4-diamino-5-\(p\)-chlorophenoxypyrimidine and the antimalarial paludrine suggested that the former compound may have antimalarial properties and the latter may have anti-PGA activity (Falco et al.\(^{65}\)).

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2,4-Diamino-5-p-chlorophenoxy pyrimidine

The action of 2,4-diamino-5-p-chlorophenoxy pyrimidine was found to inhibit the growth of *L. casei* at levels of 0.5 γ per milliliter and to be competitively reversed by PGA. Paludrine produced partial growth inhibition at levels of 100 γ per milliliter which could be reversed by PGA. Higher levels of paludrine which produced complete growth inhibition could not be reversed by an excess of PGA. The 2,4-diamino-5-p-chlorophenoxy pyrimidine was found to be as effective as quinine against *Plasmodium gallinaceum*.66

Burchenal *et al.*,66 found twenty-three pyrimidine analogs to be ineffective against transmitted leukemia Ak4 in mice.

2. PURINE DERIVATIVES

Purines with amino groups in the 2,6-positions have been found to be potent PGA antagonists (Elion and Hitchings64). The 2,6-positions of the purine correspond to the 2,4-positions of pyrimidines and pteridines.

2,6-Diaminopurine inhibits growth of *L. casei* at a level of 0.1 γ per milliliter. Pteroylglutamic acid is capable of reversing the growth inhibition produced by low levels of this compound but not that produced by high levels. Adenine is also capable of reversing the antagonist at low levels of the inhibitor. In contrast, the inhibitory action of 2-aminopurine is reversed by either PGA or purines over a considerable range of concentration.

---

Burchenal et al.\textsuperscript{66} studied the action of six purines against Ak4 leukemia in mice and found 2,6-diaminopurine effective. The corresponding methyl derivative, 2,6-diamino-7-methylpurine, was ineffective.

3. Pteridine Derivatives

A large series of 2,4-diaminopteridines and 2-amino-4-hydroxypteridines have been prepared\textsuperscript{67} and their biological effects studied.

\textbf{TABLE VI}

\textbf{Antagonistic Action of Substituted Pteridines}

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition Index</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-Diaminopteridine</td>
<td>3,000</td>
<td>55</td>
</tr>
<tr>
<td>2,4-Diamino-7-methylpteridine</td>
<td>20,000</td>
<td>55</td>
</tr>
<tr>
<td>2,4-Diamino-6,7-dimethylpteridine</td>
<td>8,000</td>
<td>55, 68</td>
</tr>
<tr>
<td>2,4-Diamino-6,7-dicarboxypteridine</td>
<td>400,000</td>
<td>55</td>
</tr>
<tr>
<td>2,4-Diamino-6,7-diphenylpteridine</td>
<td>8</td>
<td>55, 68</td>
</tr>
<tr>
<td>2,4-Diamino-6,7-phenanthro-(9,10-(e))-pteridine</td>
<td>3</td>
<td>55</td>
</tr>
<tr>
<td>2-Amino-4-hydroxy-6,7-diphenylpteridine</td>
<td>40,000</td>
<td>68</td>
</tr>
</tbody>
</table>

![Diagram of 2,4-Diaminopteridine](image)

![Diagram of 2-Amino-4-hydroxy-pteridine](image)

The microbiological effects of some of these are shown in Table VI. One interesting fact which is apparent from this data is the much higher activity of the inhibitors for \textit{S. faecalis} than for \textit{L. casei}. Thus 2,4-diamino-6,7-diphenylpteridine has an inhibition index (ratio of antagonist to metabolite) of 8 for \textit{S. faecalis} and 100,000 for \textit{L. casei}. However, when the 2,4-diaminopteridine is incorporated into the PGA molecule to give 4-aminopteroylglutamic acid, the inhibition index for the two organisms is approximately the same (Swendseid\textsuperscript{68}). The inhibitory activity of the 2,4-diaminopteridines increases as the size of the substitution groups in-


increases; the 6,7-diphenyl and the 6,7-phenanthro derivatives are much more active than those containing hydrogen, methyl, or carboxyl groups.

A synergism between 2,4-diaminopteridine and sulfathiazole has been observed by Daniel and Norris\textsuperscript{69} with \textit{E. coli} and \textit{Staphylococcus aureus}. For example, 150 $\gamma$ of 2,4-diamino-6,7-dimethylpteridine per milliliter or 100 $\gamma$ of sulfathiazole is required to prevent growth of \textit{Staphylococcus aureus}, but a combination of 2 $\gamma$ of each of these gives the same degree of inhibition. With \textit{E. coli} 100 $\gamma$ of this same pteridine or 5 $\gamma$ of sulfathiazole is required to give the same degree of inhibition that can be attained with 10 $\gamma$ of pteridine plus 1.0 $\gamma$ of sulfathiazole. This synergistic effect suggests that the two antagonists are acting on two enzyme systems functioning in series.

The biological effect of a large number of pteridines has been determined for chicks (Daniel \textit{et al.}\textsuperscript{69}) and rats (Swendseid \textit{et al.}\textsuperscript{68}). The 2,4-diamino-6,7-diphenylpteridine which is highly inhibitory for \textit{S..faecalis} has no effect on chick growth and hemoglobin formation\textsuperscript{70} Swendseid \textit{et al.}\textsuperscript{68} however, observed that this same compound at a level of 500 mg. per kilogram of diet produces leucopenia and a marked granulocytopenia in the rat but has no effect on hemoglobin formation. This leucopenia can be prevented by simultaneous feeding of 500 mg. of PGA per kilogram. The corresponding 2,4-diamino-6,7-dimethylpteridine also produces a leukemia, but higher levels of antagonist are required. The 2-amino-4-hydroxy-6,7-diphenylpteridine is less active than the corresponding 2,4-diamino derivative in producing leucopenia and granulocytopenia in the rat\textsuperscript{68} but is more active in depressing growth and hemoglobin formation in the chick.\textsuperscript{70}

4. Modification of Pterin Nucleus

Replacement of the pteridine ring by benzimidazole\textsuperscript{60} and quinazoline\textsuperscript{71} has yielded growth-promoting compounds rather than inhibitors. The substitution of a sulfonyl group for the carboxyl of \textit{p}-aminobenzoic acid changes the weak growth-promoting activity of benzimidazole-2-methyl-\textit{p}-aminobenzoylglutamic acid to a weak antagonist.\textsuperscript{60}

Quinoxaline-2-carboxyl-\textit{p}-aminobenzoarylglutamic acid is a weak antagonist for \textit{S. faecalis}.$^{56}$ This may, however, be partially due to the fact that there is an amide linkage between the aromatic amine and the quinoxaline group instead of the methylene group present in PGA. The biological activity of these compounds shows that major modifications of the PGA molecule frequently yield growth promoters rather than inhibitors. The formulas of these compounds are shown in Fig. 10.

5. PTEROYL DERIVATIVES OF DIFFERENT AMINO ACIDS

A large number of pteroyl derivatives of different amino acids have been prepared; the only one that functions as an antagonist is the aspartic acid analog. The inhibition index is 40 for *S. faecalis* and 2000 for *L. casei* and is constant over relatively large ranges of concentration. The inhibition ratio for *S. faecalis* in the presence of pteroic acid is decreased to 2. Pteroyl-

\[ HOOC \quad HC-N-C(\text{Benzimidazole-2-methyl-p-aminobenzoylglutamic acid}^{69}) \]

Growth promoter 0.01% as active as PGA for *S. faecalis* R

\[ HOOC \quad HC-N-S(\text{Benzimidazole-2-methyl-p-aminobenzene sulfonylglutamic acid}^{60}) \]

Growth inhibitor inhibition ratio for *S. faecalis* R : 8000

\[ HOOC \quad HC-N-C(\text{Quinazoline-4-p-aminobenzoylglutamic acid}^{70}) \]

Growth promoter 1 to 10% as active as PGA

\[ HOOC \quad HC-N-C(\text{Quinazoline-2-carboxyl-p-aminobenzoylglutamic acid}^{59}) \]

Antagonist inhibition ratio for *L. casei* : 300,000

Fig. 10. Modification of the pteridine nucleus of pteroylglutamic acid.
aspartic acid inhibits growth and hemoglobin formation in the chick, the
inhibition ratio being approximately 500, but has no effect on rats at a
level of 1.5 mg. per day.

Kirsanova and Trufanov\(^2\) found that the corresponding 6-carbon analog,
pteroylaminoadipic acid, was 5% as active as PGA for the chick and 10 to
20% as active as PGA for the rat. Similarly, the 7-carbon analog, pteroyl-
aminopimelic acid,\(^3\) was about 10% as active as PGA for \(L.\) \(casei\), 20% as
active for rats, and 50% as active for chicks.

Wright \textit{et al.}\(^4\) found that the pteroyl derivatives of alanine, \(\epsilon\)-amino-
caproic, cystine, phenylalanine, serine, and valine were neither stimulatory
nor inhibitory activity. The \(\beta\)-alanine, methionine, and sarcasine derivatives
had slight growth-promoting activity for \(S.\) \(faecalis\) R.

6. 

\textbf{Alkyl Derivatives}

Alkyl derivatives of PGA have been prepared in which the methyl group
has been added to the pteridine ring,\(^5\) \(^6\) to the C\(^9\) position\(^7\) and the N\(^10\)
position,\(^8\) and to the 2-amino group.\(^9\)

\textit{a. Biological Experiments with "x Methyl PGA"}

Martin \textit{et al.}\(^6\) first described a PGA antagonist designated 7-methyl-
pteroyl-d-glutamic acid which had an inhibition index of 150 for \(S.\) \(faecalis\) R. This was prepared by the condensation of 2,4,5- triamino-6-hydroxy-
pyrimidine with \(\alpha,\beta\)-dibromobutyaldehyde and \(p\)-aminobenzoyl-d-gluta-
mic acid. The methyl group was ascribed to the 7 position of the pteridine
ring, although this fact has not yet been definitely established.

The biological properties of a similar compound have been described by
Franklin \textit{et al.}\(^7\) in which the natural \(L\) isomer of \(p\)-aminobenzoylelglutamic
acid was used. The inhibition index of the crude reaction product designated
 crude "x methyl PGA" is 30 for \(S.\) \(faecalis\), 1000 for \(L.\) \(casei\), and 3000
for rats. The inhibition indices for microorganisms are relatively constant
over large ranges of concentration, and the inhibition produced in rats is
completely reversible by PGA. The syndrome produced in rats by the
antagonist is similar to, but more acute, than that induced by feeding a
purified diet plus sulfasuxidine and was accompanied by oral lesions. Reversal


\(^4\) W. B. Wright, Jr., D. B. Cosulich, M. J. Fahrenbach, C. W. Waller, J. M. Smith,


\(^7\) M. E. Hultquist, J. M. Smith, Jr., D. R. Seeger, D. B. Cosulich, and E. Kuh,

of the syndrome by PGA in the presence of the antagonist was accompanied by temporarily overcompensatory increases in total white blood cell and granulocyte counts.

This antagonist has produced symptoms of PGA deficiency in chicks, mice, \(^79\) pigs, \(^80\) and dogs. \(^81\) The dog does not develop deficiency symptoms on a purified diet devoid of PGA even when a sulfonamide is added. \(^82\) The deficiency syndrome which develops with the antagonist consists of loss in appetite, skin changes, uticarious dermatitis, ulceration of bony prominences, mild macrocytic anemia, and slight leucopenia. These symptoms develop much more slowly than those in the rat or chick, and no diarrhea or gingivitis occurs, which is characteristic of the antagonist-induced syndrome in the rat. Complete and rapid reversal of the deficiency disease is produced by feeding of PGA with the continued feeding of the antagonist.

The effects of "x methyl PGA" in affecting the action of the sex hormones have already been described.

Shive et al. \(^84\) found thymidine to be more effective than PGA in reversing the action of "x methyl PGA" for Leuconostoc mesenteroides. Thymine is inactive.

**b. 9- and 10-Methyl PGA derivatives**

The biological effects of a series of methyl derivatives of PGA are shown in Table VII. It should be noted that the inhibition indices reported for 9-methyl PGA and for 9,10-dimethyl PGA are lower than those reported elsewhere. \(^77\) It should be borne in mind that these values are not absolute but will vary from one experiment to another, depending on the time of incubation, etc. Of special interest is the fact that 10-methyl PGA, which is a very potent antagonist for S. faecalis, has slight pro-PGA activity for chicks. The 9-methyl derivative is an antagonist for chicks and can be completely reversed by PGA. The inhibitory effect of 4-amino PGA and the 9- and 10-methyl derivatives of 4-amino PGA on chicks is only partially reversed by PGA over a narrow range of concentration. The inhibition ratios of these compounds tend to decrease with increasing concentration.


which shows that PGA is becoming less effective in reversing the action of the antagonist. This is characteristic of systems in which inhibition occurs at more than one step in the reaction series.

7. 4-Aminopteroylglutamic Acid Derivatives

This class of compounds is unique among the PGA antagonists. They are highly potent antagonists for both microorganisms and animals and have proved to be the most effective of the PGA antagonists in treatment of experimental and clinical leukemias.

**TABLE VII**

**Some Derivatives of Pteroylglutamic Acid and Their Biological Effects**

<table>
<thead>
<tr>
<th>PGA derivative</th>
<th>Inhibition of <em>S. faecalis</em> R at three PGA levels, γ/ml of culture medium</th>
<th>Toxicity for animals, p.p.m. of purified PGA-deficient diet for LD50</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude “x-methyl”</td>
<td>30 20 30</td>
<td>1000 1000 1000</td>
<td>+</td>
</tr>
<tr>
<td>9-Methyl</td>
<td>300 400 400</td>
<td>30-1000</td>
<td>+</td>
</tr>
<tr>
<td>10-Methyl</td>
<td>1 1 0.8</td>
<td>Pro4</td>
<td></td>
</tr>
<tr>
<td>9,10-Dimethyl</td>
<td>3 2 2</td>
<td>100 30 30</td>
<td></td>
</tr>
<tr>
<td>4-Amino</td>
<td>6 3 2</td>
<td>1 3-3</td>
<td>-</td>
</tr>
<tr>
<td>4-Amino-9-methyl</td>
<td>2 2 2</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>4-Amino-10-methyl</td>
<td>2 0.5 0.3</td>
<td>1 3</td>
<td>5 -</td>
</tr>
<tr>
<td>4-Amino-9,10-dimethyl</td>
<td>0.4 0.2 0.2</td>
<td>3 3</td>
<td>3 -</td>
</tr>
</tbody>
</table>


b Inhibition ratio to PGA for half-maximum growth.

< Reversible by PGA over a wide range.

< Slight PGA-like effect.

A number of derivatives containing an amino group in place of the 4-hydroxy group of the pteridine ring have been prepared (Seeger et al.66). The replacement of the glutamic acid by alanine in 4-amino PGA results in almost complete loss of antagonist activity (Wright et al.74). Introduction of methyl groups in the 9 and 10 positions increases the activity for *S. faecalis* R.55

In contrast to the effects of “x methyl PGA” and 9-methyl PGA, which are readily reversible by PGA, the inhibitory action of 4-amino PGA and related compounds cannot be completely counteracted by PGA. In *S. faecalis* the ratio of 4-amino PGA to PGA for half-maximum inhibition is 266 at 0.003 γ of PGA and 0.07 at 0.3 γ per milliliter. This shows that PGA becomes less effective at higher concentrations of the antagonist, a feature


which is characteristic of systems in which the antagonist blocks the enzyme system at more than one point. The growth of chicks is retarded by 5 mg. of 4-amino PGA per kilogram of diet. The toxicity is partially counteracted by 5.0 mg. of PGA per kilogram of diet, but complete reversal is not attained by as much as 25 mg. (Oleson et al.\textsuperscript{87}). In the rat, 10 γ of 4-amino PGA per day or 1 mg. per kilogram of diet is lethal in about 3 to 4 days.\textsuperscript{87} The symptoms produced in the rat are severe diarrhea, weight loss, and porphyrin-stained whiskers. Large amounts of PGA (20 mg. per kilogram of diet) produced partial reversal of toxic symptoms. One milligram of 4-amino PGA per kilogram of diet produces high mortality in the mouse which cannot be reversed by one hundred times as much PGA (Franklin et al.)\textsuperscript{88}. Guinea pigs given 0.5 to 5.0 mg. of 4-amino PGA subcutaneously daily lose weight and die within 11 to 28 days. The symptoms of toxicity include normocytic anemia, leucopenia, agranulocytosis, thrombocytopenia, and a hypoplastic bone marrow. PGA at levels of twenty-five to one hundred times that of the analog prevents the development of leucopenia but not the anemia (Minnich and Moore\textsuperscript{89}).

Other animals which are susceptible to the action of 4-amino PGA are the dog,\textsuperscript{90} the monkey,\textsuperscript{85} and Drosophila.\textsuperscript{91} Early embryonic death has been induced in the dog and the mouse and rat.\textsuperscript{92} The level of 4-amino PGA (0.1 mg. per kilogram of body weight) which was used to induce fetal death produced a temporary depletion of the bone marrow and a transient loss in body weight but did not make the animals seriously ill.

The chick egg embryo is inhibited by 3 to 5 γ of 4-amino PGA.\textsuperscript{93} The inhibitor becomes relatively less toxic as embryonic development progresses.\textsuperscript{94} Although PGA is almost completely ineffective in reversing the action of the antagonist,\textsuperscript{93, 94} a mixture of thymidine and hypoxanthine desoxyriboside produced\textsuperscript{94} a partial reversal. Thymine plus hypoxanthine desoxyriboside was ineffective. This provides a parallel to the action of thymidine in reversing the action of 4-amino PGA in E. coli. and Le. citrovorum and is the only example outside of microorganisms where thymidine is capable of reversing the action of a PGA antagonist. The citro-

\textsuperscript{87} J. J. Oleson, B. L. Hutchings, and Y. SubbaRow, J. Biol. Chem. \textbf{175}, 359 (1948).
vororum factor is also effective in counteracting the toxicity of 4-amine PGA for the egg embryo.95

*Tetrahymena geleii* is unique among the PGA-requiring organisms in that it is not inhibited by either 4-amine PGA or \( N^{10} \)-methyl PGA. It has been reported that 4-amine PGA functions as a growth promoter, but this was later shown to be due to appreciable amounts of PGA which were present in the sample of 4-amine PGA (Heinrich et al.96). Although \( N^{10} \)-methyl PGA functions as a growth promoter and 4-amine PGA neither stimulates nor inhibits growth, 4-amine-10-methyl PGA, as well as 4-amine-9-methyl PGA, functions as an antagonist (Dewey et al.97).

The resistance of *T. geleii* to 4-amine PGA is similar to that of a strain of *S. faecalis* R which had become resistant to 4-amine-10-methyl PGA by repeated culture in the presence of this antagonist (Burchenal et al.98). Broquist et al.99 has found that the resistance of this organism to 4-amine PGA is due to an increased ability to convert PGA to the citrovorum factor.

8. Effect of Antagonists on Experimental Tumors

The striking effect of PGA in stimulating growth of white cells in PGA-deficient animals provided a basis for the suggestion that PGA antagonists might be able to reduce the large number of white cell blood elements which form in leukemia. Clinical work has borne out the validity of the assumption100 and a large amount of data has accumulated on the effects of these antagonists in experimentally induced tumors of various types. Interesting in this connection is the observation that the PGA and citrovorum factor content of leucocytes is higher in leukemia than in normal blood. In advanced stages of the disease, leucocytes may contain as high as 300 \( \mu \gamma \) of citrovorum factor per milliliter as compared with normal levels of 30 to 80 \( \mu \gamma \) per milliliter (Swendseid et al.101).

The growth of Rous chicken sarcoma, which is a virus disease and thus not necessarily directly related to other types of tumors, is inhibited by a PGA deficiency. The deficiency can be induced either by feeding a diet

low in PGA or by the administration of 4-amino PGA (Little et al.\textsuperscript{102}). The growth of this tumor is also reduced by deficiencies of other vitamins such as nicotinic acid, pantothenic acid, riboflavin,\textsuperscript{103} and vitamin B\textsubscript{12}.\textsuperscript{104} PGA exerted the greatest effect and was the only vitamin, a deficiency of which completely prevented tumor growth.

Similar to the effect of PGA antagonists on virus-induced tumors in animals is their inhibiting action on growth of plant tumors. 4-amino PGA and three other methylated derivatives of this antagonist were effective in suppressing the growth of tumorous tissue induced on carrots by inoculation with crown gall bacteria (de Ropp\textsuperscript{105}).

The action of 4-amino PGA in normal and in leukemic animals has been studied in great detail by the workers at Sloan-Kettering Institute.\textsuperscript{106, 107} In a study of ninety compounds related to PGA only four showed definite chemotherapeutic effect as measured by doubling the survival time of leukemic Ak4 mice. These compounds were 4-amino-10-methyl PGA, 4-amino-9-methyl PGA, 4-amino-9,10-methyl PGA, and 2,6-diaminopurine. 4-amino-PGA and 4-aminopteroylaspartic acid were less effective (Burchenal et al.\textsuperscript{66}). In each case the compounds were given at the maximum tolerated dose. In the treatment of a solid tumor, sarcoma 180, 4-amino PGA and 4-amino-10-methyl PGA were the most effective, 4-aminopteroylaspartic acid, pteroylaspartic, and pteroyltriglutamic acid being ineffective (Stock et al.\textsuperscript{107}). The narrow margin between toxic and therapeutic dose is reflected in the fact that in the case of 4-amino-10-methyl PGA 1.0 mg. per kilogram of body weight per day shows no activity, 2.0 mg. is markedly toxic, and 1.5 mg. gives marked inhibition of tumor activity with little evidence of toxicity.

One of the problems associated with clinical use of the PGA antagonists in the treatment of leukemia is the eventual failure of therapy due to development of resistance to this agent. If this resistance could be overcome, the leukemia could conceivably be held indefinitely in check by use of the therapeutic agent. This problem of resistance has been studied by the development of a resistant strain of lymphoid leukemia, Ak4, by repeated passage through mice treated with 4-amino-10-methyl PGA (Burchenal


\textsuperscript{105} R. S. de Ropp, Nature 164, 954 (1949).


et al.\textsuperscript{108}). This strain was also resistant to other 4-amino PGA derivatives but was not resistant to 2,6-diaminopurine or to "x methyl PGA" with a PGA-deficient diet containing a sulfonamide.\textsuperscript{108} This therapeutic effect of "x methyl PGA" on a strain of transplanted mouse leukemia in Ak mice had previously been demonstrated by Weir et al.\textsuperscript{109} An interesting metabolic difference between the normal strain, Ak4, and the resistant strain, Ak4R, lies in their relative ability to incorporate radioactive formate into the nucleic acid purines. Both strains have the same ability in this respect in the absence of antagonist. On the administration of 4-amino-10-methyl PGA the incorporation of radioactive formate was reduced to 4\% of its original level in the normal Ak4 strain but was reduced to only 25\% in the resistant Ak4R strain (Skipper and Burchenal\textsuperscript{109}).

Pteroyltriglutamic acid has been reported by Leuchtenberger, Lewisohn, and associates\textsuperscript{111, 112} to produce complete regressions in spontaneous breast tumors in mice. Intravenous injection of 5 \( \gamma \) per day led to complete regressions in 43\% of a group of eighty-nine animals. No regressions occurred in sixty control animals. In contrast, PGA had no inhibitory effect, and it was observed that primary tumors in mice receiving 100 \( \gamma \) of PGA grew more rapidly than the untreated controls. The effect of pteroyltriglutamic acid on transplanted sarcoma 180 has not been confirmed in investigations by Burchenal et al.\textsuperscript{66} and Schoenbach et al.\textsuperscript{113}

VI. Estimation

E. L. R. STOKSTAD

A. CHEMICAL METHODS

Pteroylglutamic acid on reduction in acid solution is split to yield a methylpteridine and \textit{p}-aminobenzoylglutamatic acid.\textsuperscript{1} This reaction has been made the basis of a quantitative procedure\textsuperscript{2} in which the sample is reduced


\textsuperscript{113} E. B. Schoenbach, A. Goldin, B. Goldberg, and L. G. Ortega, \textit{Cancer} 2, 57 (1949).


with zinc in 0.5 N hydrochloric acid and the aromatic amine estimated by
the method of Bratton and Marshall.\(^3\) In crude reaction products contain-
ing free \(p\)-aminobenzoic acid or its derivatives, the aromatic amine is de-
termined before and after reduction with zinc. The increase resulting from
reduction is a measure of the amount of PGA. \(p\)-Aminobenzoic acid and
its peptides with other amino acids give the same amount of color on a
molar basis when diazotized and coupled with \(N\)-(1-naphthyl)ethylen-
diamine dihydrochloride by the method of Bratton and Marshall. Thus the
method can be used for pteroic acid, its amide, ester, or peptide with any
amino acid. Any derivative which contains a substitution on the nitrogen
of the \(p\)-aminobenzoic acid cannot be estimated by this method because
the presence of a primary amino group is necessary for the diazotization
and coupling reaction.

Titanous chloride has been used by Glazko and Wolf\(^4\) to reduce ptero-
ylglutamic acid. They found that, in the assay of tissue homogenates by the
zinc reduction methods, adenine and nucleic acid products interfered by
producing diazotizable substances on reduction with zinc. The use of
titanous chloride eliminates this difficulty and permits the analysis of
PGA in the presence of adenine.

A micromethod has been proposed by Allfrey et al.\(^5\) which is based on
the oxidation of pteroylglutamic acid by alkaline permanganate solution
to give 2-amino-4-hydroxypteridine-6-carboxylic acid. This pterin is then
estimated by fluorometric methods. In the presence of extraneous flou-
rescent pigments, such as xanthopterin, which are affected by permanganate,
the 2-amino-4-hydroxypteridine-6-carboxylic acid is adsorbed on Florisil,
eluted, and then measured fluorometrically. This procedure has been
adopted to use with natural products and gives results which are com-
parable to, although slightly higher than, those obtained by microbiological
methods. Such a chemical procedure has the advantage that it measures
total soluble pteroylglutamic acid in free and in conjugated form.

B. MICROBIOLOGICAL METHODS

Two main problems are involved in the microbiological estimation of
pteroylglutamic acid in natural products. The first involves extraction and
hydrolysis of pteroylglutamic acid conjugates which may be present; the
other, assay of the microbiologically active compounds. Detailed descrip-
tions of these methods in review form have been published by Stokstad
and Hutchings\(^6\) and Snell.\(^7\)

\(^6\) E. L. R. Stokstad and B. L. Hutchings in Biological Symposia, The Microbiolog-
1. Extraction from Tissues and Hydrolysis of Conjugates

A number of experiments have shown that microbiologically inactive conjugates exist in natural products. Vitamin B₆ conjugate (pteroylhexaglutamylglutamic acid) found in yeast is inactive both for *S. faecalis* and *L. casei*. Pteroyltriglutamic acid isolated from a fermentation material is active for *L. casei* but only slightly active for *S. faecalis*. Pteroyl-γ-glutamylglutamic acid, which has been prepared synthetically but not yet observed in natural products, is active for both *S. faecalis* and *L. casei*. It is thus apparent that *L. casei* is capable of responding to larger conjugates than *S. faecalis*. This offers a possible explanation for the fact that many natural products give a higher result by *L. casei* assay than by *S. faecalis* assay. Rhizopterin and pteroic acid are active for *S. faecalis* but inactive for *L. casei*. Thus the presence of a high activity for *S. faecalis* compared to *L. casei* might indicate the presence of rhizopterin or pteroic acid in the sample.

A number of methods have been proposed for the release of pteroylglutamic acid from tissues. Cheldelin *et al.* investigated the effect of a number of commercially available enzymes on the liberation of folic acid from tissues. This hydrolysis presumably consisted in two parts: first, the release of pteroylglutamic acid from the tissue to give a soluble form; second, the hydrolysis of the soluble but inactive conjugates to yield active compounds. These workers found taka-diastase to be more effective than papain, pepsin, trypsin, pancreatin, malt diastase, or pancreatic amylase. Digestion of hog heart with taka-diastase gave about ten times as much pteroylglutamic acid activity as autolysis alone. However, subsequent work indicated that, although taka-diastase may be effective in releasing the nutrilite from the tissues, it does not hydrolyze all the conjugates present. Mims *et al.* demonstrated the existence of an enzyme capable of activating a microbiologically inactive conjugate in yeast. Cell-free extracts of plants and tissues which had been previously digested with taka-diastase could be further activated by this enzyme which was obtained from liver. This distinguished the action of the conjugate-splitting enzyme from that of taka-diastase.

*a. Conjugases*

Shortly after the observation that certain natural materials possessed more activity by animal assay than could be accounted for by microbiological Assay of *Lactobacillus casei* Factor (Vitamin B₆, Folic Acid), Volume XII, p. 339. Jaques Cattell Press, Lancaster, Pa., 1947.


logical test, it was observed that the activity for microorganisms could be increased by appropriate enzymatic digestion. The name "vitamin $B_6$ conjugase" was given by Bird et al.\textsuperscript{11} to this enzyme because it was capable of splitting vitamin $B_6$ conjugate (pteroylheptaglutamic acid).

Two main types of conjugase have been described, one present in hog liver and having a pH optimum of 4.5,\textsuperscript{11, 12} the other in chicken pancreas with a pH optimum of 7 to 8.\textsuperscript{13} Chicken pancreas conjugase is inactivated at temperatures above 45\degree C and is relatively stable to the action of crystalline trypsin. Autolysis of chicken pancreas tissue increases the amount of extractable enzyme activity a thousandfold. Crystalline chicken pancreas conjugase has not been obtained, but concentrates 2800 times as active as the crude starting material have been prepared by Mims and Laskowski\textsuperscript{14} using adsorption on tricalcium phosphate gel, precipitation with alcohol, and fractional precipitation with sodium sulfate. Calcium ions were found to be a component of the enzyme system, which accounted for the apparent loss of activity which sometimes occurred during dialysis and the increase in activity observed on purification by adsorption on calcium phosphate.

Hog kidney conjugase has a pH optimum of 4.5. This enzyme does not attack the methyl ester of pteroylheptaglutamate, a fact which led to its classification as a carboxypeptidase. However, neither of the conjugases is identical with carboxypeptidase, since the purified carboxypeptidase has no conjugase activity.\textsuperscript{15}

Studies on the specificity of chicken pancreas conjugase by Dabrowska et al.\textsuperscript{16} show that $\gamma$-peptides of pteroylglutamic acid are attacked much more readily than $\alpha$-peptides. In the action of the enzyme on pteroyl-$\gamma$-glutamyl-$\gamma$-glutamylglutamic acid only one mole of glutamic acid is removed. This presumably is the terminal glutamic acid group, since pteroyl-$\gamma$-glutamylglutamic acid is not attacked by chicken pancreas conjugase. Thus it appears that the final product resulting from the cleavage of pteroylheptaglutamic acid by chicken pancreas conjugase is pteroyl-$\gamma$-glutamylglutamic acid rather than the monoglutamate.

Hog kidney conjugase is capable of splitting pteroylheptaglutamate all the way down to the monoglutamate. This is shown by the fact that pteroylglutamic acid has been isolated from a hog kidney conjugase digest of the

heptaglutamates derived from yeast (Pfiffner et al.17). Evidence that hog kidney conjugase consists of two enzymes has been obtained by Mims and Bird.18 The hydrolysis of pteroylheptaglutamic acid was found to require both components, whereas the hydrolysis of pteroyltriglutamic acid could be effected by only one. Calcium ion has not been found to be a part of the hog kidney conjugase system.

Conjugases have been observed by Simpson and Schweigert19 in the blood of several animal species, including man. When the conjugase in blood is allowed to act on the conjugates in blood, the pH optimum is observed to be 7. However, when the substrate is either pteroylheptaglutamic acid or the conjugates present in taka-diastase, equal activity is obtained with blood conjugase at pH 4.5 and at pH 7. This suggests that the conjugases in blood have different pH optima, depending on the substrate. These observations point out the hazards associated with the use of crude enzyme preparations in the hydrolysis of conjugates prior to microbiological assay. Incubation of taka-diastase with blood produces an increase in pteroylglutamic acid activity which has been interpreted as indicating the presence of conjugates in the blood which are hydrolyzed by conjugases in the taka-diastase. Actually, the converse has proved to be true, and the conjugases in the blood are really hydrolyzing the conjugates present in the blood and in the taka-diastase.

Complete hydrolysis of the pteroylheptaglutamate cannot be accomplished by either chicken pancreas or hog kidney conjugase alone. Thus Sreenivasan et al.20 found that complete hydrolysis of conjugates for assay purposes could be attained only by the stepwise use of chicken pancreas and hog kidney conjugase. Rat liver contains two enzymes with pH optima at 4.5 and 7. Autolysis of liver or digestion of liver by hog kidney conjugase at pH 4.5 liberates very little PGA. Since hog kidney conjugase produces approximately 75% hydrolysis of the pteroylheptaglutamate, it is apparent that this conjugate is not present in liver. Autolysis or chicken pancreas digestion of liver at pH 7.0 produces an increase in activity. Further evidence for the differences in the behavior of these two conjugases lies in the fact that pteroyltriglutamic acid is completely split by both chicken pancreas and hog kidney conjugases where microbiological activity is used as a criterion of hydrolysis. The pteroyldiglutamic acid formed by chicken pan-

creas digestion apparently possesses the same activity for the assay organism as PGA. Pteroylheptaglutamate is only 30% hydrolyzed by chicken pancreas and 75% by hog kidney conjugase. A stepwise combination of the two enzymes accomplishes complete hydrolysis of heptaglutamate compound.

Hog kidney conjugase is readily inhibited by thymus nucleic, yeast nucleic acid, and extracts of yeast and molasses (Mims et al.21). A number of proteins such as casein, gelatin, egg albumin, and crystalline bovine plasma albumin also inhibit hog kidney conjugase.22

Thymus and yeast nucleic acids which inhibit hog kidney conjugase have no effect on chicken pancreas conjugase.21 This enzyme is, however, inhibited by a glutamic acid polypeptide of p-aminobenzoic acid derived from yeast.23 p-Aminobenzoyl-γ-glutamyl-γ-glutamylylglutamic acid also inhibits chicken pancreas conjugase.24 The tetraethyl ester of this peptide gives no inhibition, and the p-nitro derivative of the acid peptide is half as active as the peptide with an amino group.

b. Digestion Procedures for Assay Purposes

Bird et al.25 described four methods for the enzymatic hydrolysis of pteroylglutamic acid conjugates and compared the results with those obtained by assay with chicks. These results, presented in Table VIII show that enzymatic hydrolysis followed by microbiological assay gave essentially the same results as assay with chicks except in two samples. One sample of liver extract, which gave only half as much pteroylglutamic acid by digestion with acetone-desiccated hog kidney and microbiological assay as it did with chick assay, was found to give comparable results when digested with an extract of unheated almond. Certain natural materials were found to contain enzyme inhibitors, and in these cases very large amounts of enzyme were necessary to secure maximum hydrolysis of the conjugates present.

Digestion with acetone-dried or fresh chicken pancreas at pH 7.2 is the recommended procedure of the Association of Official Agricultural Chemists.26 Enzyme digestion is preceded by autoclaving the finely ground sample at the same pH to extract the factor from the tissue.

22 A. Z. Hodson, Arch. Biochem. 16, 309 (1948).
It is apparent that no one procedure can be recommended for digestion of the conjugates in all types of natural materials. In order to determine whether complete hydrolysis has been obtained, it may be necessary to employ different enzyme systems and to vary the amounts of enzyme.

2. Microbiological Procedures

Although a number of organisms have been found which will respond to pteroylglutamic acid, only two organisms, *L. casei* and *S. faecalis*, have been used for the assay of this vitamin. *L. casei* has the advantage in that it is the more sensitive of the two organisms. It gives half-maximum growth with approximately 0.05 m\(\gamma\) of pteroylglutamic acid per milliliter of medium, whereas *S. faecalis* requires 0.3 m\(\gamma\). *L. casci* does not give maximum growth with purines and thymine, whereas *S. faecalis* does. Thus *L. casci* is better suited to the assay of low-potency materials and those products which may contain thymine. Approximately five thousand times as much thymine is required to produce the same response as PGA both for *S. faecalis* and *L. casci*. *L. casei* is a slower growing organism than *S. faecalis*, and the assays with the former organism are usually carried out 72 hours and the

<table>
<thead>
<tr>
<th>Substance assayed</th>
<th>PGA content (\gamma/g.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enzyme method(^b)</td>
</tr>
<tr>
<td>Yeast extract(^c)</td>
<td>1</td>
</tr>
<tr>
<td>Concentrate from yeast</td>
<td>1</td>
</tr>
<tr>
<td>Yeast extract(^c)</td>
<td>2</td>
</tr>
<tr>
<td>Yeast extract(^c)</td>
<td>3</td>
</tr>
<tr>
<td>Bacto-yeast extract(^d)</td>
<td>1</td>
</tr>
<tr>
<td>Concentrate from yeast</td>
<td>2</td>
</tr>
<tr>
<td>Liver extract</td>
<td>1</td>
</tr>
<tr>
<td>Liver extract</td>
<td>2</td>
</tr>
<tr>
<td>Concentrate from liver extract</td>
<td>2</td>
</tr>
<tr>
<td>Asparagus juice concentrate</td>
<td>4</td>
</tr>
<tr>
<td>Concentrate from yeast</td>
<td>4</td>
</tr>
</tbody>
</table>

\(^a\) Courtesy *J. Biol. Chem.* (Bird et al.\(^5\)).

\(^b\) Enzyme method: 1. Acetone desiccated hog kidney, pH 4.2-4.5.

\(^c\) Extract of unheated almonds, pH 6.0-7.0.

\(^d\) Extract of commercial almond meal, pH 6.0-7.0.

\(^4\) Extract of autolyzed hog kidney, pH 4.5.

\(^5\) An aqueous extract of plasmolyzed yeast.

\(^6\) Difco Laboratories, Detroit.
growth measured by titration of the acid produced. Assays with \emph{S. faecalis} can be made in 16 hours, and the growth measured turbidimetrically or by titration after 40 hours incubation. A method has been developed by Roberts and Snell\textsuperscript{27} by using \emph{L. casei} with a 16 to 24-hour incubation period. A trypsin digest of casein is used in the basal medium which serves as a source of strepogenin and permits maximum growth of the organism to be reached in a short time.

\section*{VII. Standardization of Activity}

\textbf{E. L. R. STOKSTAD}

The ease of microbiological assay has prevented extensive use of animal assays for the measurement of pteroylglutamic acid. Chicks and rats are used on occasion to determine the total pteroylglutamic acid activity for animals. This affords a partial answer to the problem of whether the microbiological methods are a true measure of the pteroylglutamic acid activity for animals.

Two methods have been proposed for chick assay. One is a curative test\textsuperscript{1} in which the sample is administered orally in six doses given every other day. The increase in hematocrit is used as a measure of the response. A 4-week prophylactic test using a purified type of diet of chemically defined ingredients has been also used.\textsuperscript{2, 3}

Rats cannot be made deficient in pteroylglutamic acid by the simple exclusion of this factor from the diet. By the use of sulfonamides, such as sulfaguanidine or succinylsulfathiazole, in the diet it is possible to produce a pteroylglutamic deficiency in this species. An assay method based on this type of diet has been proposed in which growth is used as the criterion of response in rats.

\section*{VIII. Occurrence in Foods}

\textbf{E. L. R. STOKSTAD}

The distribution of PGA in foods is shown in Table IX. The data present a summary of information compiled by the Bureau of Human Nutrition


\textsuperscript{1} B. L. O'Dell and A. G. Hogan, \textit{J. Biol. Chem.} \textbf{149}, 323 (1943).


TABLE IX
Pteroylglutamic Acid Content of Foods

<table>
<thead>
<tr>
<th>Food</th>
<th>Moisture, %</th>
<th>Total PGA content, g/g.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Meats and Eggs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef, round steak</td>
<td>70</td>
<td>0.06-0.14</td>
</tr>
<tr>
<td>Beef, heart</td>
<td>76</td>
<td>0.03</td>
</tr>
<tr>
<td>Beef, kidney</td>
<td>76</td>
<td>0.58</td>
</tr>
<tr>
<td>Beef, sweetbreads</td>
<td>78</td>
<td>0.23</td>
</tr>
<tr>
<td>Beef, liver</td>
<td>68</td>
<td>2.9</td>
</tr>
<tr>
<td>Lamb, stew meat</td>
<td>59</td>
<td>0.03</td>
</tr>
<tr>
<td>Lamb, liver</td>
<td>69</td>
<td>2.76</td>
</tr>
<tr>
<td>Pork, loin</td>
<td>66</td>
<td>0.03</td>
</tr>
<tr>
<td>Pork, liver</td>
<td>68</td>
<td>2.2</td>
</tr>
<tr>
<td>Pork, ham (smoked)</td>
<td>67</td>
<td>0.10</td>
</tr>
<tr>
<td>Chicken, dark meat</td>
<td>77</td>
<td>0.03</td>
</tr>
<tr>
<td>Chicken, white meat</td>
<td>77</td>
<td>0.03</td>
</tr>
<tr>
<td>Chicken, liver</td>
<td>72</td>
<td>3.77</td>
</tr>
<tr>
<td>Eggs, whole</td>
<td>74</td>
<td>0.03-0.08</td>
</tr>
<tr>
<td>Egg white</td>
<td>87</td>
<td>0.004</td>
</tr>
<tr>
<td>Egg yolk</td>
<td>50</td>
<td>0.13</td>
</tr>
<tr>
<td><strong>Nuts</strong></td>
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<td></td>
</tr>
<tr>
<td>Peanuts</td>
<td>1</td>
<td>0.51</td>
</tr>
<tr>
<td>Almonds</td>
<td>4</td>
<td>0.45</td>
</tr>
<tr>
<td>Coconuts</td>
<td>44</td>
<td>0.27</td>
</tr>
<tr>
<td><strong>Vegetables, fresh</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asparagus</td>
<td>93</td>
<td>0.89-1.42</td>
</tr>
<tr>
<td>Beans, lima</td>
<td>70</td>
<td>0.10-0.40</td>
</tr>
<tr>
<td>Beans, green snap</td>
<td>90</td>
<td>0.14-0.41</td>
</tr>
<tr>
<td>Beets</td>
<td>86</td>
<td>0.10-0.15</td>
</tr>
<tr>
<td>Broccoli</td>
<td>91</td>
<td>0.21-0.35</td>
</tr>
<tr>
<td>Brussels sprouts</td>
<td>84</td>
<td>0.20-0.34</td>
</tr>
<tr>
<td>Cabbage</td>
<td>92</td>
<td>0.11-0.75</td>
</tr>
<tr>
<td>Carrots</td>
<td>88</td>
<td>0.07-0.15</td>
</tr>
<tr>
<td>Cauliflower</td>
<td>91</td>
<td>0.17-0.29</td>
</tr>
<tr>
<td>Corn, sweet</td>
<td>61-81</td>
<td>0.09-0.70</td>
</tr>
<tr>
<td>Cucumbers</td>
<td>96</td>
<td>0.97-2.07</td>
</tr>
<tr>
<td>Greens, beet</td>
<td>90</td>
<td>0.31-0.39</td>
</tr>
<tr>
<td>Greens, spinach</td>
<td>92</td>
<td>0.48-1.15</td>
</tr>
<tr>
<td>Lettuce</td>
<td>96</td>
<td>0.03-0.54</td>
</tr>
<tr>
<td>Peas, fresh</td>
<td>76</td>
<td>0.12-0.35</td>
</tr>
<tr>
<td>Peppers, green</td>
<td>94</td>
<td>0.04-0.09</td>
</tr>
<tr>
<td>Potatoes, peeled</td>
<td>80</td>
<td>0.04-0.06</td>
</tr>
<tr>
<td>Potatoes, peel</td>
<td>79</td>
<td>0.08-0.20</td>
</tr>
<tr>
<td>Pumpkin</td>
<td>92</td>
<td>0.05-0.10</td>
</tr>
<tr>
<td>Sweet potatoes</td>
<td>70</td>
<td>0.05-0.19</td>
</tr>
<tr>
<td>Tomatoes</td>
<td>92-94</td>
<td>0.02-0.15</td>
</tr>
<tr>
<td><strong>Vegetables, dried</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beans, kidney</td>
<td>9</td>
<td>1.90</td>
</tr>
<tr>
<td>Food</td>
<td>Moisture, %</td>
<td>Total PGA content, fresh basis, g/100g</td>
</tr>
<tr>
<td>------------------------------</td>
<td>-------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>Beans, navy</td>
<td>11</td>
<td>1.28</td>
</tr>
<tr>
<td>Beans, soybeans</td>
<td>8</td>
<td>2.08</td>
</tr>
<tr>
<td>Peas, green split</td>
<td>9</td>
<td>0.24</td>
</tr>
<tr>
<td>Peas, yellow split</td>
<td></td>
<td>0.24</td>
</tr>
<tr>
<td><strong>Fruits, fresh</strong></td>
<td></td>
<td></td>
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<tr>
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and Home Economics and the Texas Agricultural Experiment Station. In addition to values for total PGA content measured by the *L. casei* assays, as listed in the table, assays were also made with *S. faecalis* for both free and conjugated forms. Bound PGA was measured after digestion with chicken pancreas enzyme.

IX. Effects of Deficiency

A. IN ANIMALS

E. L. R. STOKSTAD

Various animal species differ markedly in their requirement for PGA. This reflects in part their ability to utilize bacterial intestinal synthesis as a source of the vitamin. The chick, the monkey, and the guinea pig develop deficiencies on a purified diet low in the vitamin. The rat, on the other hand, constitutes an excellent example of an animal that is capable of meeting its dietary requirements for PGA by intestinal synthesis; consequently, deficiencies of this vitamin were not recognized in the rat until intestinal antiseptics such as sulfonamides were employed. Other species, such as the dog, do not develop deficiencies on a purified diet even with the use of intestinal antiseptics, and PGA antagonists are necessary to produce a deficiency syndrome.

1. Rats

a. Production of PGA Deficiency by Sulfonamide Feeding

Rats can be grown on a diet free of PGA without development of any deficiency symptoms unless an intestinal antiseptic is added or a stress is imposed which increases the requirement of the vitamin. Black et al. showed that addition of a sulfonamide depressed the growth rate which could then be restored in the presence of the sulfonamide by the feeding of liver extracts. Shortly after this, Spicer and coworkers at the National Institutes of Health observed hematological changes including leucopenia, agranulocytosis, hypocellularity of the bone marrow, and less frequently anemia, on diets containing sulfaguanidine or sulfasuxidine (succinylsulfathiazole). These symptoms could be prevented or cured by the feeding of

liver extracts. Ransone and Elvehjem\textsuperscript{3} later showed that "folic acid concentrates" prepared from liver were effective in increasing growth on the sulfonamide diet.

The activity of pure PGA in correcting the blood dyscrasia was reported by Daft and Sebrell\textsuperscript{4} and Wright and Welch.\textsuperscript{5} The blood of normal rats contains 10,000 to 15,000 total leucocytes and 2000 to 4000 granulocytes. These counts were reduced, respectively, to 700 to 2000 leucocytes and 10 to 500 granulocytes after 30 to 90 days feeding on a diet containing sulfaguanidine. The daily feeding of 10 $\gamma$ of PGA or 20 $\gamma$ of pteroyltriglutamic acid for 4 days increased the leucocytes and the granulocytes to normal. In some cases there was a surge of leucocytes and especially the granulocytes to higher than the normal levels.

Definite evidence that PGA is actually synthesized in the intestinal tract comes from the observation that the depressed growth resulting from sulfonamide feeding can be restored by the feeding of rat feces as well as yeast and yeast extract (Light \textit{et al.}\textsuperscript{6}).

A thorough investigation of the effects of sulfonamide feeding to rats has been made by Daft, Sebrell, and coworkers at the National Institutes of Health, and their work has been excellently reviewed in a Harvey Lecture.\textsuperscript{7} One of these symptoms which has been frequently observed in rats receiving sulfonamides is hypoprothrombinemia. This accounts for the subcutaneous hemorrhages observed on these types of diets.\textsuperscript{7} This hypoprothrombinemia can be prevented or cured by the feeding of vitamin K, folic acid concentrates,\textsuperscript{8} and $p$-aminobenzoic acid.\textsuperscript{9} The effect of $p$-aminobenzoic acid can be readily accounted for by its ability to reverse the bacteriostatic effect of the sulfonamide in the intestine and thus permit resumption of bacterial synthesis of vitamin K. The effect of the folic acid concentrates cannot be as readily accounted for. Their method used in preparation of the folic acid concentrate precludes the presence of a fat-soluble form of vitamin K. Unfortunately, the effect of pteroylglutamic acid on hypoprothrombinemia under conditions employed by Welch and Wright\textsuperscript{8} has not yet been reported.

Rats receiving a sulfonamide have an increased susceptibility to anemia induced by frequent bleeding.\textsuperscript{10} Administration of pteroylglutamic acid has

\textsuperscript{5} L. D. Wright and A. D. Welch, \textit{Science} \textbf{98}, 179 (1943).
\textsuperscript{7} W. H. Sebrell, \textit{Harvey Lectures Ser.} \textbf{39}, 288 (1943-44).
\textsuperscript{8} A. D. Welch and L. D. Wright, \textit{J. Nutrition} \textbf{25}, 555 (1943).
a corrective and preventive effect on this anemia as measured by hemoglobin concentration, hematocrit reading, and white count. The occurrence of hydrocephalus in about 2% of the young offspring of rats on a purified ration has been noted.\textsuperscript{11} This abnormality can be largely prevented by the addition of pteroylglutamic acid to the maternal diet. A high incidence of infarction of the spleen has been noted in PGA deficiency in young rats but not in control rats receiving 5\(\gamma\) of this factor per day.\textsuperscript{12}

\textit{b. Production of PGA Deficiency by Stress of Other Deficiencies}

Deficiencies of PGA can be induced by the stress of other vitamin deficiencies as well as by inhibition of intestinal synthesis by use of sulfonamides. In a series of studies by Kornberg \textit{et al.},\textsuperscript{13-16} granulocytopenia was observed in a small percentage of animals fed diets not containing sulfonamides. This dyscrasia was also produced by feeding diets low in pantothenic acid, and under these conditions the symptoms of anemia, leukopenia, granulocytopenia, and hypoplasia of the bone marrow which are characteristic of PGA deficiency could be corrected by pantothenic acid alone. The similarity of the blood dyscrasia observed in pantothenic acid deficiency with that found in pteroylglutamic acid deficiency is evidence of a relationship between these two factors.

Folic acid concentrates have been reported to be effective in restoring the color of black rats which had turned grey on diets containing sulfaguanidine.\textsuperscript{17} The well-established role of pantothenic acid in the control of hair color in the rat suggests a possible relationship in the metabolism of PGA and pantothenic acid. Addition of sulfasuxidine to a purified diet containing adequate amounts of the vitamin B complex produces a reduction in the hepatic storage of PGA and pantothenic acid. No change occurs in the riboflavin and nicotinic acid contents of the liver. Increasing the dietary intake of pantothenic acid failed to increase growth or to alleviate the typical pantothenic acid deficiency symptoms such as alopecia, spectacled eye, porphyrin-caked whiskers, and achromotrichia. Administration of crystalline biotin and a folic acid concentrate did produce resumption of growth, alleviate the symptoms, and increase the pantothenic and PGA contents of the liver.

Riboflavin deficiency also produced hematological changes resembling those of PGA insufficiency. The leucopenia but not the anemia could in some cases be corrected by administration of PGA, or by riboflavin, or by a combination of both.\textsuperscript{15} Restriction of intake of a diet containing riboflavin also produced the typical blood dyscrasia which suggests that limitation of other dietary essentials by low feed consumption may be an important factor.

Severe granulocytopenia and anemia were produced in rats fed proteinfree diets.\textsuperscript{16} The condition was prevented by casein alone but not by PGA. The blood dyscrasia could not be cured by either casein or pteroylglutamic acid alone but did respond to a combination of the two. When weanling rats were given a 4% casein diet, the animals again became anemic, leucopenic, and granulocytopenic. In this case the leucocyte count was increased by PGA alone or by parenteral 15-unit liver extract. The activity of the liver extract could not be accounted for on the basis of its PGA content as determined by microbiological assay. The simultaneous administration of methionine and threonine with the PGA increased the incidence and magnitude of the response. This observation foreshadowed subsequent work which established the role of PGA in the metabolism of these compounds.

The interchangeability of pteroylglutamic acid and liver extract in correcting the agranulocytosis has been confirmed both on a normal and on a low protein diet.\textsuperscript{18} In these experiments it was impossible to account for the activity of the liver extract on the basis of PGA or its conjugates as measured by microbiological assay after enzymatic hydrolysis.

Anemia, leucopenia, and hemorrhage and necrosis of the adrenals were observed by Daft et al. in rats fed thiourea in a purified diet.\textsuperscript{19} The blood dyscrasia was not prevented by thyroid powder or thyroxine but was corrected by administration of PGA. These results are in contrast to the reported ineffectiveness of PGA in preventing the appearance of agranulocytosis in two patients receiving thiouracil.\textsuperscript{20} Higgins\textsuperscript{21} observed that pteroylglutamic acid at a level of 18 \(\gamma\) per day counteracted the hypochromic anemia induced by feeding of promin and promizole to young rats on a purified diet.

c. PGA and Reproduction in the Rat

Reproduction performance in rats during lactation is improved by the addition of PGA to a purified diet. The maximum improvement is obtained when the supplement is given to the parent animals from the time of wean-

ing rather than during gestation and lactation only (Sica et al.,22). Under the same conditions xanthopterin has only a small effect. "Laetation leucopenia" in rats on a purified diet was found by Nelson et al.23 to be partially prevented by addition of brewer's yeast or liver extract. Subsequently, PGA at a level of 1 mg. per kilogram of diet was observed to increase maternal weight and the total leucocytes and granulocytes although the effect was not as complete as that produced by a liver fraction.24 In view of subsequent information it seems probable that the additional improvement noted with liver extract may have been due to its vitamin B₁₂ content. The severity of the lactation leucopenia was increased and occasional anemia was induced by addition of sulfasuxidine to a purified diet. Pteroyl-glutamic acid was effective in preventing these symptoms.25

2. Chicks

A dietary deficiency of PGA is readily produced in chicks on purified diets. Chicks appear to derive very little of their water-soluble vitamins by intestinal synthesis and, in marked contrast to the rat, readily develop a PGA deficiency by simple exclusion of this vitamin from the diet.

Beginning in 1938 several lines of investigation were initiated which led to the recognition of PGA deficiency in chicks. Stokstad and Manning26 found that chicks grew slowly on a diet composed primarily of polished rice and water-washed fish meal and supplemented with sources of thiamine, riboflavin, and pantothenic acid. Growth was increased by the addition of yeast or alfalfa or their corresponding water extracts. This growth factor, which was designated factor U, was adsorbed by fuller's earth and was stable to autoclaving with acid and alkali.

A macrocytic anemia and reduction of growth rate on a semipurified type of diet were noted by Hogan and Parrott.27,28 Growth was restored and anemia prevented by inclusion of liver extract in the diet. Evidence was presented to show that the anti-anemia principle was distinct from any of the vitamins previously described, and the name vitamin B₉ was given to this factor. A third group of investigators, Hutchings et al.,29 presented strong evidence that the "Norit eluate factor" required by Lactobacillus

casei was essential for the chick. Concentrates of this factor were effective at levels of 100 mg. per kilogram of diet. The "Norit eluate factor" is now known to be PGA.

Vitamin B<sub>12</sub> from liver (PGA) was first reported to be active for the chick by Pfiffner <em>et al.</em>,<sup>30</sup> who found that addition of 2.5 mg. of vitamin B<sub>12</sub> per kilogram of purified diet prevented anemia and permitted normal growth.

<em>a. Feather Growth and Pigmentation</em>

Pteroylglutamic acid is necessary for feather growth and pigmentation. Mills <em>et al.</em><sup>31</sup> reported that a concentrate of the "Norit eluate factor" for <em>L. casei</em> promoted feathering, growth, and hemoglobin formation in chicks. Frost <em>et al.</em><sup>32</sup> raised black leghorn chicks on purified diets containing pure forms of the water-soluble vitamins. The black breed of bird has the advantage that it readily shows the effect of PGA on pigmentation as well as feather growth. Preliminary experiments with concentrates of PGA showed that 0.400 mg. per kilogram of diet gave good feather growth and pigmentation. It is interesting to note that chicks which received the supplement for the first 2 weeks only showed normal feathering and pigmentation up to 10 weeks even though they grew at a decreasing rate and finally began to lose weight after 7 weeks. When pure PGA was injected at levels of 1, 2.5, 5, and 10 γ per day, it was found that the highest level gave normal feathering and pigmentation. The growth rate, however, was not as high as that produced by 10% of brewer's yeast added to the diet. Most marked depigmentation occurred at the 5-γ level, presumably because feather growth at the lower vitamin levels was so poor that true pigmentation was difficult to observe.

Depigmentation became most pronounced in the proximal portions after the feathers were fairly well developed. The wing and tail feathers were most affected but nearly all feathers showed some depigmentation.

<em>b. Requirements of the Chick</em>

The requirements of this vitamin have been found to be between 0.25 and 2.0 mg. per kilogram of diet, depending on the criteria of adequacy. Campbell <em>et al.</em><sup>33</sup> noted that 0.4 mg. per kilogram of diet was sufficient for maintenance for normal hemoglobin, hematoctit, red cell count, and thrombocyte values, but 4 mg. per kilogram was required for maintenance of normal leucocyte levels.

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<sup>33</sup> C. J. Campbell, R. A. Brown, and A. D. Emmett, <em>J. Biol. Chem.</em> 152, 483 (1944).
Comparison of different methods of administration\textsuperscript{31} showed that subcutaneous injection was slightly more effective than feeding by pipette. In further studies Pfiffner et al.\textsuperscript{35} reported that either PGA or its conjugate at a level of 0.25 mg. per kilogram of diet gave maximum growth but submaximal hemoglobin response. Briggs and Lillie\textsuperscript{36} have shown that 1 mg. per kilogram of diet is necessary for normal feather pigmentation and 2 mg. for maximum growth. Oleson et al.\textsuperscript{37} reported that PGA at a level of 1 to 5 mg. per kilogram of diet gave normal growth and pigmentation of feathers in chicks. The requirement was not appreciably altered by addition of sulfamerazine or certain other intestinal antiseptics.

Oleson et al.\textsuperscript{38} found that the requirements for maximum growth up to 4 weeks were 0.4 mg. per kilogram of diet in one experiment and 1.0 mg. in another. In both cases 1.0 mg. was required for optimum hemoglobin response. Pteroyltriglutamic acid was found to be as effective on a molar basis as the monoglutamate. The addition of 0.7\% of either sulfasuxidine, sulfaguanidine, or carboxysulfathiazole did not affect the response on marginal levels of pteroyltriglutamic acid. Robertson et al.\textsuperscript{39} found the requirements to be 0.45 mg. per kilogram of diet for growth and hemoglobin up to 4 weeks of age. The requirement at 6 weeks for optimum hemoglobin was stated to be 0.35 mg. and 0.55 mg. for normal feathersing. In these experiments the PGA requirements were not affected by addition of 1 and 2\% sulfasuxidine to a purified diet. It thus appears that sulfonamides do not have as marked an effect on the PGA requirements in chicks as they do in rats.

Perosis has been observed by Hill et al.\textsuperscript{40} in chicks on a purified-type of diet adequately substituted with choline, biotin, and manganese but deficient in PGA. The incidence was increased by addition of 2\% sulfasuxidine. The perosis was prevented by addition of 0.2 mg. per kilogram of PGA without the sulfonamide and by 0.3 mg. in the presence of the sulfonamide.

Pteroyltriglutamic acid has been shown to be effective for the chick.\textsuperscript{41} It has also been reported by Scott et al.\textsuperscript{42} that the effectiveness of

\begin{footnotesize}
\end{footnotesize}
Pteroylglutamic acid is increased by the addition of 5-pyridoxic acid (2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxypyridine) or 4-pyridoxic acid (2-methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine). This supplementary effect has been observed both for growth and hemoglobin formation in the young chick\(^{42}\) and for regeneration of hemoglobin in hens rendered anemic by bleeding.\(^{43}\)

Jukes and Stokstad\(^{44}\) failed to observe any effect of the pyridoxic acids in increasing the effectiveness of pteroylglutamic acid. In a similar way pteroylheptaglutamic acid was found by Binkley et al.\(^{45}\) to be as effective on a molar basis as the monoglutamate without the addition of pyridoxic acid.

c. Requirements of Turkeys and Ducks

Richardson et al.\(^{46}\) reported that a deficiency of this factor in the diet of the turkey produces cervical paralysis (Fig. 11) in addition to a reduction in growth rate. No marked anemia was observed. Cervical paralysis was also observed on a practical diet containing corn, wheat bran, and wheat middlings, which indicates that a deficiency of PGA can occur on diets composed primarily of natural ingredients. Spontaneous remissions of cervical lesions were not observed. Complete recovery was obtained in 4 to 6 hours by injection of 100 \(\gamma\) of PGA. Thus far cervical paralysis has not been reported in chicks deficient in PGA. Jukes et al.\(^{47}\) found the requirement of this factor for maximum growth of the turkey to be approximately 1.0 mg. per kilogram of diet.

Although large variations exist, the PGA requirement of the turkey for growth appears to be higher than that of the chick. Schweigert\(^{48}\) reported PGA and pteroyltriglutamic acid to be equally effective in increasing PGA blood levels and preventing the symptoms of mild anemia to severe anemia, poor feather condition, and weakened legs. After 8 weeks of the un-supplemented diet, the hematocrit had dropped to 28\%, compared to 40\% for those receiving 2 mg. of PGA per kilogram of diet. Curative treatment with the vitamin increased the hematocrit to normal in 2 weeks.

Ducks grown on a purified type of diet give a marked growth response to the addition of 100 \(\gamma\) per day of pteroyltriglutamic acid.\(^{49}\)

\(^{47}\) B. S. Schweigert, Arch. Biochem. 20, 41 (1949).
d. Requirements for Reproduction

The requirements for egg production are low. A diet composed primarily of polished rice and sardine meal and containing 0.12 mg. of PGA per kilogram of diet by microbiological assay was found by Taylor\textsuperscript{50} to be adequate for egg production. Further supplementation with 1.0 mg. of PGA per kilogram yielded no increase in egg production although the hatchability of fertile eggs was increased. These results are in agreement with those of Schweigert \textit{et al.},\textsuperscript{51} who reported that egg production and hatchability on a basal diet containing 0.42 mg. of PGA per kilogram (determined by microbiological assay) and composed primarily of corn and casein were not improved by the addition of 2.0 mg. of PGA. This was observed with both turkeys and hens. The average PGA content of the eggs before and after supplementation of the hen diet was 0.12 and 0.21 γ per gram in the case of the turkey and 0.099 and 0.133 γ, respectively, in the case of the chicken. For pouls from hens adequately supplemented with PGA, 0.8 mg. of the vitamin per kilogram appeared to be adequate. The requirement was higher for pouls from hens which received no supplementation. Thus, although 0.45 mg. of PGA per kilogram of diet (measured by

microbiological assay in the basal diet) appeared adequate for egg production and hatchability, it was not adequate for optimum livability of the poult and storage of this vitamin in the egg.

3. Monkeys

The rhesus monkey is peculiarly susceptible to a deficiency of PGA, and the symptoms are more similar to those of the human than are those of any other species. They include anemia, leucopenia, oral lesions, diarrhea, ulcerations of the colon, and increased susceptibility to infections of the intestinal tract. Re-examination of the literature shows that certain of these symptoms had been described and credited to unknown nutritional factors long before PGA was recognized as a vitamin. Day has termed this factor vitamin M and in an excellent review on the early work in this field52 points out that "this syndrome, or certain manifestations of it, at least, have been rediscovered and described every few years since 1919. The work has been approached from many angles; the diets used have been very different, but what appears to be the same syndrome has been found in each."

Wills and coworkers53 were able to distinguish between the anti-pernicious factor in liver and the anti-anemic principle for monkeys, the anti-pernicious anemia factor being precipitated by ammonium sulfate and the monkey anti-anemia factor remaining in solution.

A wide variety of diets have been used for the production of vitamin M deficiency (PGA deficiency) in the monkey. That used by Day and his collaborators at Arkansas was composed of Labco vitamin-free casein, polished rice, wheat, salt mixture, and ascorbic acid, and the known water- and fat-soluble vitamins.54

a. Blood Changes

The following symptoms have been described by Day52 and credited to PGA deficiency. There is a progressive decrease in all types of peripheral blood cells. The normal values for the rhesus monkey are 5.0 million RBC, 10,000 to 20,000 total leucocytes. A profound leucopenia involving all white cell types is the most striking aspect of the deficiency. There is an almost absolute neutropenia and lymphopenia. Total white cells drop to 2000 to 3000, and there is a marked agranulocytosis. This agranulocytosis is thus similar to that observed in rats fed a purified diet containing sulfonamide. Limited data indicate a thrombocytopenia. Some animals show a pronounced anemia, but others will develop a marked leucopenia which is rapidly fatal without showing any severe anemia. Minimum counts of 2

and 3 million are common, and counts of 1 million or slightly less have been observed. The anemia is slightly macrocytic, the volume index and mean cell diameter being slightly increased.

The addition of PGA, or concentrates of this vitamin, effects a reticulocyte crisis which may be as high as 40%, a dramatic increase in white cells,

![Graph](image)

Fig. 12. Typical responses of the cytopenic monkey to natural pteroyltriglutamic acid (*L. casei* factor). In each of the two experiments on this monkey the total dose of vitamin was approximately 3 mg., given intramuscularly. Courtesy *J. Biol. Chem.* (Day et al.,\(^5\)).

and a slower return of red cell and hemoglobin values.\(^5\) A typical response of a deficient monkey to administration of pteroyltriglutamic acid (fermentation *L. casei* factor) is shown in Fig. 12.

A megaloblastic bone marrow is found in monkeys with nutritional cytopenia. Wilson\(^5\) reported that there is a relative hypoplasia in the myeloid elements in monkeys in the terminal stages of peripheral leucopenia.


Further details of the blood picture may be obtained from the papers of Wills and Stewart,\(^5\) Langston \textit{et al.},\(^6\) and Wilson \textit{et al.}\(^7\)

\textbf{b. Oral Lesions}

Oral lesions, particularly of the margins of the gums, are one of the most frequently observed signs of nutritional cytopenia in the rhesus monkey. These have been described in detail by Day \textit{et al.},\(^8\) Langston \textit{et al.},\(^9\) Chapman and Harris,\(^10\) and Saslaw \textit{et al.}\(^11\) The oral lesions develop shortly after the onset of leucopenia and begin as a recession of the gums which develops into a yellowish ulceration. The ulceration usually develops among the in-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{gum-lesions}
\caption{Gum lesions in vitamin M deficiency in monkey. Courtesy Dr. P. L. Day.}
\end{figure}

\(^{9}\) O. D. Chapman and A. E. Harris, \textit{J. Infectious Diseases} \textbf{69}, 7 (1941).
lesions of the oral mucous membranes often become infected with *Strep-
tococci, Staphylococci*, or the flora of Vincent’s angina. In monkeys on normal
diets these lesions do not occur spontaneously, and where accidental trauma
of the gum or buccal mucosa does occur no secondary infection appears.
This illustrates the increased susceptibility of the monkey to bacterial in-
fection during a deficiency of PGA.

c. Diarrhea and Susceptibility to Bacillary Dysentery

Diarrhea is one of the most frequent and striking characteristics of the
vitamin M deficiency syndrome. The stools become watery in a short time,
and in some cases blood and mucus appear. Administration of PGA has a
rapid and dramatic effect. In a few days formed stools appear which almost
suggest that this vitamin has an effect on water balance in the intestine.
The diarrhea in the monkey and the marked effect of PGA are very similar
to that observed in sprue in clinical patients. When blood and mucus ap-
ppear, the monkey becomes ill with the clinical symptoms of bacillary dysen-
tery. Stool cultures usually reveal the presence of *Shigella* type of organism.

Janota and Dack\(^6^3\) demonstrated convincingly the relation between vita-
min M deficiency and dysentery in the monkey. They found that monkeys
which have been kept in the laboratory on a stock diet do not develop bacil-
lar dysentery, even though animals are received with the disease from the
dealers. If the resistance of the animal is lowered by vitamin M deficiency,
some of the animals develop bacillary dysentery. This suggests that *Bact.
dysenteriae* (Flexner) may live in a saprophytic existence in the intestine.

"The fact that control monkeys do not develop dysentery when kept in
the same room with animals on a vitamin M-deficient diet indicates that
the disease occurs as a result of lowered resistance due to vitamin deficiency
and not to contact infection." These workers also found "that monkeys
deficient in vitamins C, A and D in addition to vitamin M did not show
any increased incidence of lesions of the mouth or bowel over those monkeys
deficient in vitamin M alone."

Day,\(^6^2\) in his excellent review of this subject, reported that the feeding of
large numbers of living dysentery bacilli to a monkey receiving a normal
diet (a vitamin M-deficient diet supplemented with liver extract) did not
produce diarrhea, clinical dysentery, or any changes in the blood picture.
A year later this monkey was made deficient in vitamin M and spontane-
ously developed an acute clinical dysentery and died.

Saslaw *et al.*\(^6^2\) found that monkeys on a vitamin M-deficient diet mani-
fested a "markedly lowered clinical resistance to spontaneous infections
with high mortality. The susceptibility to experimental infections with

**Streptococcus hemolyticus** Group C and to influenza virus A administered intranasally was likewise increased in contrast with controls on normal diets."

d. Requirements of the Monkey

Daily injections of 0.1 to 0.2 mg. of PGA produced a reticulocyte response of about 7%. This apparently represents the minimum amount that will elicit a response. In a separate investigation Day et al. found that totals of 4 and 4.5 mg. of pteroyltriglutamic acid given in divided doses over 4 to 5 days produced reticulocyte responses of 10 and 8% with prompt elevation of the total white cells and granulocytes.

Cooperman et al. reported that daily administration of 100 γ of PGA produced a rise in white cells and a gain in weight in deficient monkeys. No marked rise in hemoglobin occurred. Two hundred micrograms of vitamin B₁ conjugate (pteroylheptaglutamate) produced less of a response in one animal than 100 γ of PGA. The monkey is obviously able, however, to utilize some of the conjugate in yeast, since a sample of yeast which was found to be low in free PGA (measured by *S. faecalis* R) was a good source of vitamin M for monkeys. This same sample of yeast was subsequently shown to contain PGA conjugate because its activity for *S. faecalis* was increased by digestion with rat liver enzyme. Day and Totter have reported the minimum daily protective dose of 100 γ on their basal diet which furnished an additional 20 γ per day. However, with a purified type of diet Smith and Elvehjem obtained normal growth and blood cell formation with 100 γ per day. The minimum protective dose was not established.

It is of interest to compare the PGA requirements of the monkey and the human. In pernicious anemia the minimal effective curative dose is approximately 5 mg. per day. On a body weight basis this would correspond to approximately 0.2 mg. per day for a 3-kg. monkey.

e. Monkey Anti-Anemia Factor

The existence of an anti-anemia factor for monkeys has been postulated by Cooperman et al. The evidence is based largely on the failure of PGA at levels of 100 γ per day to completely restore the blood picture in monkeys which have been depleted on a PGA-deficient diet containing the

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other known B vitamins and ascorbic acid. These levels of PGA produce a rise in white cells and an increase in growth, but the hemoglobin does not rise to normal. Feeding of whole liver produces complete restoration of hemoglobin. One marked characteristic of this syndrome is a reversal of the normal ratio of neutrophiles to lymphocytes. It seems possible that the levels of PGA used in these experiments were near the marginal level and that the response produced by the liver may have been due to its PGA content. This is supported by the observations of Smith and Elvehjem\textsuperscript{69} that 0.5 to 1.0 mg. of PGA per day completely eliminated the need for the monkey anti-anemia factor. This was about five to ten times the amount needed for maintenance. Once the animals had been “cured” by 1.0 mg. of PGA per day, they could be maintained with 0.10 mg. per day. A methanol extract of liver which furnished only 4 \gamma of PGA per day produced a transient response. This amount of liver extract was able to maintain the blood picture when supplemented with 50 \gamma of PGA. Although the assayable amounts of PGA in the methanol extract were low, the possible presence of PGA conjugates cannot be ruled out. Other liver extracts have been reported which gave PGA-like responses in excess of their assayable PGA content. Thus the chick factors vitamins B\textsubscript{10} and B\textsubscript{11} were liver fractions which contained only traces of assayable B\textsubscript{12}. However, it was reported that in the presence of sufficient PGA vitamins B\textsubscript{10} and B\textsubscript{11} were no longer required by the chick.\textsuperscript{70} Certain parenteral liver extracts have also been found which have a greater effect on the granulocyte count of sulfonamide-fed rats than can be accounted for by their PGA content.\textsuperscript{13}

It is apparent that the monkey does not need the monkey anti-anemia factor for maintenance, since purified basal diet supplemented with 100 \gamma of PGA per day and without any additional source of the monkey anti-anemia factor is adequate for growth and maintenance of a normal blood picture. The requirement for this has been shown only during the stress of a deficiency of PGA or of riboflavin.\textsuperscript{71}

The possibility exists that the monkey anti-anemia factor is the citrovorum factor, since the latter has been shown to be more active than PGA for the monkey in the anemia induced by ascorbic acid deficiency.\textsuperscript{72} Smith and Elvehjem point out that it is unlikely that the monkey anti-anemia factor is “identical with the citrovorum factor of Sauberlich and Baumann since reticulogen (injectable liver extract) which is a good source of CF is inactive in the monkey” as a source of the anti-anemia factor.\textsuperscript{73} However,

\textsuperscript{70} T. D. Luckey, P. R. Moore, C. A. Elvehjem, and E. B. Hart, \textit{Science} 103, 682 (1946).
although reticulogen is active for *Lc. citrovorum* at very low concentrations, the actual amount of citrovorum factor supplied by the 45 U.S.P. units of reticulogen would correspond to only about 5 \( \gamma \) of CF per day. It would indeed be interesting to see whether the citrovorum factor (folic acid) would be more effective than PGA under the conditions of this test.

4. Pigs

PGA deficiencies in swine have not been observed on either purified or natural diets without the use of intestinal antisepsics. Baby pigs which had been given colostrum for 4 days and then placed on a diet of synthetic milk plus 2\% sulfathalidine (calculated on solids content of the milk) did not develop any signs of PGA deficiency (Johnson et al.\(^{74}\)). The baby pig diet contained 30\% of Labco vitamin-free casein and 27\% lard. Since subsequent work with swine and previous work with rats have shown that PGA requirements are decreased at higher protein levels, it seems possible that the failure to produce any PGA deficiency as measured by growth or blood picture may be a reflection of the high protein level used.

With adult pigs blood changes have been produced by feeding of purified diets containing sulfonamides. Cartwright et al.\(^{75}\) fed a diet containing 26\% of Labco vitamin-free casein and 2\% of sulfasuxidine. After 120 days growth almost stopped and marked alopecia developed, especially over the flanks and rump. A normocytic anemia developed, and the hematocrit dropped to 21\%. Administration of biotin produced no change in the skin condition. Administration of 1 ml. of purified liver extract for 9 days produced a reticulocyte response with a peak of 9.4\%, a marked growth response of 30 kg. in 50 days, and restoration of a normal hair coat. Four animals which received sulfasuxidine and crude casein in place of the vitamin-free casein were normal in all respects. In this particular experiment it appears that vitamin \( B_12 \) was producing a response on a diet supposedly deficient in PGA.

In a subsequent paper these same workers,\(^{76}\) using a larger number of animals, found that pigs on this same diet containing Labco vitamin-free casein and sulfasuxidine would respond only partially to refined liver extract but completely to PGA. The reticulocyte peak with PGA varied between 4 and 16\% and that with liver extract between 5 and 8\%. Substitution of crude casein for vitamin-free casein reduced the severity of the anemia, and spontaneous remissions with broad reticulocyte responses sometimes occurred. Reduction of the crude casein level from 26 to 10\% made the anemia more severe.

\(^{74}\) B. C. Johnson, M. F. James, and J. L. Krider, *J. Animal Sci.* 7, 486 (1948).


Cunha et al., found that on a diet containing 26% of "Vitamin-Test Casein" and 2% sulfasuxidine no effect of PGA could be observed on growth rate. However, a normocytic anemia developed in which the hemoglobin content was raised from 8.6 g. to 14.4 g. % by the addition of 0.5 mg. of PGA per kilogram of diet. Refined liver extract containing the anti-pernicious anemia factor produced a smaller hemoglobin response. It seems probable that the diets employed were deficient both in PGA and in vitamin B₁₂. Crude casein in the amounts used in the diets constitutes a good source of B₁₂. Since pigs have been shown to require B₁₂, it seems very likely that these pigs which received vitamin-free casein were deficient both in vitamin B₁₂ and in PGA.

Pteroylglutamic acid deficiency has also been induced in the pig by the feeding of a crude "x methyl PGA" antagonist. This antagonist has the advantage that its action can be completely reversed by pteroylglutamic acid. The feeding of antagonist accentuates the anemia observed on a purified diet containing sulfasuxidine. The anemia produced with the sulfonamide ration is normocytic. The hematological manifestations observed with sulfasuxidine plus antagonist include (a) severe macrocytic anemia, (b) leucopenia, with a greater proportional reduction of polymorphonuclear than of mononuclear cells, (c) mild thrombocytopenia, and (d) hyperplastic bone marrow with an increase in immature nucleated red cells which resemble the megoblasts observed in pernicious anemia. The blood and bone marrow are rapidly restored to normal by the injection of 10 to 20 mg. of either PGA or the corresponding diglutamate, triglutamate, or heptaglutamate. Thus, all the known conjugates appear active. A partial response was given by injection of a single dose of 0.10 mg. of PGA which produced a reticulocyte peak of 10% and an increase in hematocrit reading of 10% in 9 days. With larger amounts of PGA, reticulocyte responses varied between 11 and 42% and there was a corresponding rapid increase in the hematocrit value between 10 and 20%. When 1 unit (corresponding to approximately 1 γ of vitamin B₁₂) of U.S.P. injectable liver extract was given per day, the blood and bone marrow changes were neither prevented nor was their onset postponed. When used curatively, liver extract, 30 to 150 units total, and vitamin B₁₂, 150 γ total, produced some hemopoietic response although less than that elicited by PGA.

Thymine, which produces a hematological response in pernicious anemia, was inactive; xanthopterin, tyrosine, adenine, and uracil were also ineffective.

In confirmation with observations previously made by these same workers, on a sulfasuxidine diet the severity of the anemia was greater on a 10% casein diet than on a 26% casein diet even when the "x methyl PGA" was present in the ration.

The urinary excretion of tyrosyl derivatives (hydroxyphenol) was abnormal in the PGA-deficient pigs and was not altered by therapy with PGA or liver extract. This is an interesting observation in view of the findings that the failure of ascorbutic guinea pigs to metabolize tyrosyl compounds can be corrected by administration of PGA. 80

Heinle et al. 81 have found that pigs made deficient on a diet containing sulfasuxidine and "x methyl PGA" respond well to PGA initially, but as the deficiency is maintained the ability to respond diminishes. At this point purified liver extract gives a response. Conversely, they also observed that when a pig kept on this PGA-deficient diet for several months was given large amounts of liver extract and later vitamin B12, there was an initial partial response. The blood values were maintained at an improved level but never at a normal level. Very marked macrocytosis developed and persisted, but was promptly corrected by administration of PGA. A vitamin B12 deficiency was produced by feeding a diet containing purified soybean protein. On this regimen anemia developed which was less macrocytic than that observed in PGA deficiency and no megaloblasts were found in the bone marrow. The animals responded to vitamin B12 therapy.

Johnson et al. 82 studied the effects of PGA and vitamin B12 deficiency in the baby pig using synthetic milk diets containing purified soybean protein. This protein is lower in vitamin B12 than casein and permitted the simultaneous production of PGA and vitamin B12 deficiencies. When pigs were placed on a 20% protein diet without vitamin B12 or PGA and with 0.6% of sulfathalidine, the growth rate decreased and a mild anemia developed. After a 7-week depletion period, administration of vitamin B12 produced an increase in growth and a 7% reticulocyte peak on the fifth day. After 4 weeks of continued injection of 2 γ of vitamin B12 daily, the oral administration of PGA produced a second sharp reticulocyte peak of 7% on the second day.

The pigs were also given a diet deficient in PGA and vitamin B12 but without sulfathalidine for 3 weeks which was followed by the addition of "x methyl PGA" for the next 2 weeks. At this point either PGA or vitamin B12 provoked the maximum hematological and bone marrow responses but only B12 elicited a growth response.

IX. Effects of Deficiency

These results show that the pig requires both vitamin B₁₂ and PGA. The basic requirement for blood cell formation seems to be that of PGA, but in some cases of double deficiency either one may provoke a response. Continued administration of vitamin B₁₂ does not give complete protection against anemia whereas continued administration of PGA apparently does. The growth-stimulating function of vitamin B₁₂ cannot be substituted by PGA.

5. Mice

Evidence has been obtained that mice require PGA for growth and reproduction. Nielsen and Black⁸³ observed that mice grown on a purified diet containing sulfa-suxidine give a growth response to folic acid concentrates prepared from liver extract. The extent of the deficiency was less when the sulfonamide was omitted from the diet. Cerecedo and Mirone⁸⁴ investigated the nutritive requirements of lactation and found that addition of 10 mg. of PGA per kilogram of a purified diet doubled the average number of mice weaned and increased the weight gain of the lactating mother. No significant effect on litter size was observed.

6. Guinea Pigs

A series of studies with guinea pigs on purified diets indicated that three unidentified dietary factors are required, two of which were found present in linseed oil meal. It was later shown by Woolley and Sprince that PGA could replace one of the factors.⁸⁵ The deficiency was characterized by lethargy, salivation, loss in weight, terminal convulsions, and death.

7. Mink

The effects of pteroylglutamic acid deficiency in mink have been studied by Schaefer et al.⁸⁶ This was characterized by loss of body weight, diarrhea, anorexia and leucopenia. The deficiency symptoms were rapidly corrected by administration of pteroylglutamic acid. Some evidence was obtained which indicated that an unidentified factor in liver was also needed.⁸⁶

8. Dogs

Dogs are very refractory to pteroylglutamic acid deficiency. Michaud et al.⁸⁷ found that a purified ration supplemented with the water-soluble vitamins will support excellent growth even in the presence of 4% sulfas-

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suxidine. Repeated production of anemia by bleeding failed to show any difference between the control dogs and those receiving sulfasuxidine. Regeneration of hemoglobin after hemorrhage was rapid even in the presence of 4% sulfasuxidine. This is in marked contrast to the rat, which develops an impaired capacity to regenerate hemoglobin on a sulfonamide-containing diet. Deficiencies of PGA were obtained by Krehl et al. by imposing the stress of another vitamin deficiency. Dogs kept on a niacin-deficient diet were found to respond to a few successive single doses of niacin but then finally failed to respond and died even though niacin was administered. In each case the animal was allowed to become deficient again before the next dose of niacin was administered. If PGA was included in the diet, the response to niacin was improved and the dog could be alternately treated with niacin and allowed to become deficient again a large number of times. This production of deficiency of PGA by a concomitant nicotinic acid deficiency is reminiscent of the relationship observed between PGA and riboflavin in the rat.

9. Foxes

Schaefer et al. found that foxes kept on a purified type of diet develop critical symptoms of PGA deficiency in 7 to 14 weeks. These include anorexia, rapid loss of body weight, and reduction of hemoglobin. Total white cell and erythrocyte counts were reduced to approximately 50% of the normal value. Administration of 0.25 to 1.0 mg. of PGA per kilogram of diet resulted in an increase in hemoglobin, white cells, and growth rate. A yeast preparation containing pteroylglutamic acid conjugate, when fed at a level calculated to give 0.60 mg. of PGA in the form of the conjugate, failed to produce any hematological or growth response. A similar yeast preparation hydrolyzed with kidney enzyme produced a typical increase in hemoglobin level. This shows that the fox, in contrast to all other animals thus far observed, is unable to utilize the PGA conjugates present in yeast.

It is also interesting that the fox, although taxonomically related to the dog, is so susceptible to PGA deficiency, whereas the dog is quite resistant. Adult foxes as well as growing pups developed PGA deficiency symptoms on the purified diet. There is evidence that an unidentified factor present in liver is also required by foxes in addition to PGA.

10. Insects

Several insects have been observed which require PGA at some stage of their development. Larvae of the mosquito Aedes aegypti were found by


Goldberg et al.\textsuperscript{90} to require PGA for pupation, xanthopterin or thymine being ineffective. PGA also increases growth and survival rates, body pigmentation, and size of the larvae. PGA has the most marked effect during the third stage of larval life. Fraenkel et al.\textsuperscript{91} showed the need for PGA in the nutrition of the meal worm Tenebrio molitor. Growth of the larvae of the carpet beetle was increased by concentrates of PGA (Moore\textsuperscript{92}). Grob et al.\textsuperscript{93} reported that the larvae of rice flour beetle Tribolium confusum requires two unknown factors, one of which could be replaced by PGA concentrates.

11. Relation Between PGA and Ascorbic Acid

a. PGA and Tyrosine Metabolism

An interesting relationship has been observed between PGA and ascorbic acid in the metabolism of tyrosine. Ingestion of large amounts of tyrosine by scorbutic guinea pigs produces a large increase in the excretion of \( p \)-hydroxyphenylpyruvic acid, \( p \)-hydroxyphenyllactic acid, and small amounts of tyrosine. This apparent defect in tyrosine metabolism disappears on the administration of small amounts of ascorbic acid (Sealock and Silberstein\textsuperscript{94}). The scorbutic guinea pig is thus unable to oxidatively rupture the benzene ring. The failure of \( D \)-isoascorbic acid to prevent the tyrosyluria except in doses twenty times that of ascorbic acid indicates that the activity of the latter is specifically related to its vitamin activity and not simply to its reducing properties. Painter and Zilva\textsuperscript{95} found that the excretion of abnormal metabolites began as early as 24 to 48 hours after the ascorbic acid was withdrawn from the diet and before the tissues became depleted of this vitamin. It is especially interesting that 3 hours after the administration of 0.5 g. of tyrosine to guinea pigs on a scorbutic diet an amount of hydroxyphenyl compounds equivalent to half the tyrosine appeared, mainly in the large intestine. Twenty-four hours later none remained in the tissues or the intestine but instead had appeared in the urine.

Reports that the high excretion of phenolic compounds by patients having pernicious anemia in relapse was reduced by liver therapy\textsuperscript{96} prompted the study of the effect of PGA on the metabolism of tyrosine in the scorbutic guinea pig. Woodruff et al.\textsuperscript{97} observed that guinea pigs on a scorbutic diet and receiving 5% tyrosine excreted 30 to 60% of the dietary tyrosine in the form of hydroxyphenyl derivatives. Administration of either 5 mg. of

\textsuperscript{90} L. Golberg, B. De Meillon, and L. Lavoipierre, J. Exptl. Biol. 21, 90 (1945).
\textsuperscript{91} G. Fraenkel, M. Blewett, and M. Coles, Physiol. Zoöl. 23, 92 (1950).
\textsuperscript{92} W. Moore, Ann. Entomol. Soc. Amer. 39, 513 (1946).
\textsuperscript{93} C. A. Grob, T. Reichstein, and H. Rosenthal, Experientia 1, 275 (1945).
\textsuperscript{94} R. R. Sealock and H. E. Silberstein, J. Biol. Chem. 135, 251 (1940).
\textsuperscript{95} H. A. Painter and S. S. Zilva, Biochem. J. 41, 511 (1948).
PGA subcutaneously per day or 25 mg. of ascorbic acid orally or a combination of the two was equally effective in reducing the excretion of hydroxyphenyl derivatives. Only ascorbic acid prevented weight loss and increased serum ascorbic acid. Pteroyltriglutamic acid was effective, but the corresponding diglutamate was ineffective in reducing the excretion of hydroxyphenyl compounds in two animals on each compound tested. Administration of 5 U.S.P. units of anti-pernicious anemia liver extracts (approximately 5 γ of vitamin B₁₂) was without effect.

The response of scorbutic monkeys to a test load of tyrosine is similar to that of the guinea pig. Salmon and May⁹⁷ found that PGA was ineffective in reducing the excretion of hydroxyphenyl compounds even in doses as high as 95 mg. (40 mg. per kilogram of body weight), half being given orally and the other half subcutaneously. Subsequently, these same investigators⁹⁸ found that 1.2 mg. of folic acid (citrovorum factor) daily given intramuscularly was ineffective. Ten milligrams of cortisone, however, injected intramuscularly for 10 days prevented the excretion of large amounts of hydroxyphenyl compounds.

An effect of PGA in clinical hydroxyphenyluria of infantile scurvy after tyrosine ingestion has been observed in approximately half the cases studied by Govan and Gordon.⁹⁹ In some cases 5 mg. per day produced an effect. In another case 10 mg. per day orally was ineffective but 30 mg. intramuscularly produced a striking reduction of hydroxyphenyluria. Other cases, in which 10 to 30 mg. intramuscularly was ineffective, promptly responded to the administration of ascorbic acid.

Morris et al.¹⁰⁰ also observed hydroxyphenyluria in infants with scurvy after tyrosine ingestion. Injection of 45 mg. of PGA to one patient was ineffective in abolishing the abnormal excretion, but 45 mg. twice daily for several days (corresponding to 13.7 mg. per kilogram per day) did decrease the hydroxyphenyluria. A single dose of 30 γ of vitamin B₁₂ was ineffective, a finding which is parallel to the ineffectiveness of parenteral liver extract in the scorbutic guinea pig.

Woodruff¹⁰¹ reported on four cases of tyrosine-induced hydroxyphenyluria in scorbutic infants which were given PGA intramuscularly in doses of 2 mg. per kilogram of body weight without any effect on tyrosine metabolism. Apparently the amounts of PGA necessary to reduce hydroxyphenyluria are very large compared to those for growth of the guinea pig and hematological response in scorbutic infants. In the guinea pig 20 mg. per kilogram

of body weight per day is effective in controlling hydroxyphenyluria, and a definite but suboptimal response is procured with 4 mg.\textsuperscript{101} In Woodruff's study the three patients studied in whom no effect was produced received between 1.3 and 2.2 mg. per kilogram per day. In cases reported by other workers the effective doses varied between 3 and 13.7 mg. per kilogram of body weight per day. Woodruff\textsuperscript{101} has also observed that in megaloblastic anemia of infancy as little as 0.2 mg. of PGA will effect a maximal hematological response. These experiments dramatically illustrate the fact that vitamin requirements for different functions may vary and that requirements can be enormously altered by imposing a metabolic load on the organism.

\textit{b. Relation of PGA and Ascorbic Acid in Megaloblastic Anemia}

The role of PGA in megaloblastic anemia of infancy provides additional information on the relations between ascorbic acid, PGA, and vitamin B\textsubscript{12}. The effectiveness of PGA in curing the megaloblastic condition of the bone marrow was demonstrated by Zuelzer and Ogden.\textsuperscript{102} May et al.\textsuperscript{103} has reviewed this work and focused attention on the role of ascorbic acid deficiency in the etiology of this disease. In megaloblastic anemia of infancy the most frequent symptom is a megaloblastic bone marrow. Examination of the peripheral blood alone will not always suffice for the recognition of this syndrome because the circulating red cells are not always macrocytic and not all macrocytic anemias are associated with megaloblastic marrow. The majority of the cases occur in patients between the ages of 5 and 12 months, which corresponds roughly to the period of most frequent development of clinical signs of ascorbic acid deficiency. Megaloblastic anemia has been frequently reported as a complication of scurvy.

May and co-workers\textsuperscript{103} experimentally reproduced the condition of megaloblastic anemia of infancy in monkeys on milk diets used in infant feeding. Their salient experimental findings are:

1. When ascorbic acid is provided, the diets do not lead to megaloblastic anemia.
2. If vitamin C is inadequate, all the diets result in megaloblastic anemia.
3. Megaloblastosis of the marrow is quickly eliminated by PGA without addition of vitamin C.
4. Ascorbic acid alone might permit \textit{gradual} return of the marrow to normal.
5. Vitamin B\textsubscript{12} administered intramuscularly does not significantly alter the megaloblastic bone marrow within 72 hours.

\textsuperscript{102} W. W. Zuelzer and F. N. Ogden, \textit{Am. J. Diseases Children} 71, 211 (1946).
6. Vitamin B₁₂ plus ascorbic acid administered simultaneously intramuscularly effects a prompt correction of the megaloblastic marrow.

7. Vitamin B₁₂ given intramuscularly prophylactically does not prevent characteristic development of the megaloblastic marrow.

8. PGA given prophylactically prevents the development of the megaloblastic marrow on the vitamin-deficient diets even though scurvy and anemia develop.

9. Marked reduction in food intake alone leading to severe malnutrition does not cause anemia or megaloblastic marrow.

The role of vitamin B₁₂ in the experimental anemia of monkeys just described and in megaloblastic anemia of infancy seems secondary to that of PGA. Pure vitamin B₁₂ and liver extracts containing vitamin B₁₂ but only small amounts of PGA are sometimes able to completely cure megaloblastic anemia in infants. Pteroylglutamic acid is almost always effective. In the monkey vitamin B₁₂ is ineffective in preventing the megaloblastosis, but it is effective when given together with ascorbic acid. It should be borne in mind that the diets contained milk and hence some PGA. Thus, the vitamin B₁₂ and ascorbic acid together may have increased the effectiveness of the PGA in the basal diet used.

The basal diets used by the Arkansas workers to produce nutritional cytopenia in the monkey contained orange juice. Liver extract was unable to cure the macrocytic anemia in these experiments, which shows that vitamin B₁₂ in the presence of vitamin C is ineffective when no PGA is present in the basal diet. The failure of May to observe megaloblastic anemia on his milk diet supplemented with ascorbic acid, whereas Day observed anemia on his cereal diet with orange, is undoubtedly due to the presence of some PGA in the milk rations. Thus it appears that the PGA requirements of the monkey are increased by a deficiency of ascorbic acid.

The most unique aspect of the scorbutically induced anemia in the monkey is its capacity to respond to small quantities of “folic acid” (citrovorum factor). Fifteen micrograms of the factor per day in the form of 50 γ of concentrate produced the maximum hematological response, whereas 750 γ of PGA produced a suboptimal effect. The folic acid produced a prompt reticulocytosis followed by regeneration of the bone marrow to a normal cellular pattern. Nichol and Welch have found that ascorbic acid functions in the conversion of PGA to citrovorum factor. These data, together with observations on the high activity of citrovorum factor in the monkey, suggest that in the absence of adequate levels of ascorbic acid the


"activation" of pteroylglutamic acid is performed inefficiently. This can be corrected by (a) administering excess precursor which in this case is PGA, (b) giving small amounts of the end product of the reaction, or (c) correcting the scurvy. If this theory is valid, one must assume that the conversion of PGA to citrovorum factor is inefficient even in the presence of adequate ascorbic acid, since 100 γ of PGA produces a submaximal hematological response on a diet which contains orange juice. It would indeed be interesting to compare the activities of PGA and CF on nutritional cytopenia in monkeys which are receiving adequate ascorbic acid.

12. ENDOCRINE RELATIONSHIPS OF PGA

A fundamental observation regarding the role of PGA in endocrine function was the finding by Hertz and coworkers\textsuperscript{106, 107} that a deficiency of PGA interfered with the response of chicks to stilbestrol. The normal oviduct weight of a 100- to 150-g. chick is approximately 25 mg. Injection of 0.5 mg. of stilbestrol per day for 6 days to chicks on a normal stock diet increases the size of the oviduct to approximately 1000 mg. The oviduct weights of PGA-deficient chicks treated with stilbestrol were 66 to 100 mg., whereas in those supplemented with 20 or 50 γ of PGA and treated with stilbestrol they were 637 to 777 mg. It is worthy of note that the oviduct weights produced by stilbestrol on the stock diet were larger (1090 to 1295 mg.) than those (777 mg.) which could be achieved by large amounts of PGA on a purified type of diet. This indicates that there are elements in the stock diet in addition to PGA which mediate the function of stilbestrol. The function of PGA in the metabolism of stilbestrol is a specific one, as deficiencies of pantothenic acid, riboflavin, and pyridoxine do not interfere with the oviduct-stimulating action of stilbestrol on birds of comparable body weight.

Production of a PGA deficiency by the use of an antagonist "x methyl PGA" also interferes with the effect of stilbestrol in stimulating growth of the female reproductive tract.\textsuperscript{108, 109} The effect of the antagonist could be completely reversed by PGA. A reduction in the amount of alkaline phosphatase in the oviduct of chicks after treatment with estradiol or stilbestrol has been observed in PGA deficiencies produced by "x methyl PGA."\textsuperscript{110}

Similar results have been observed in monkeys on PGA-deficient diets. Hertz\textsuperscript{111} kept sexually immature monkeys on a purified type of diet for

\textsuperscript{106} R. Hertz and W. H. Sebrell, \textit{Science} 100, 293 (1941).

\textsuperscript{107} R. Hertz, \textit{Endocrinology} 37, 1 (1945).

\textsuperscript{108} R. Hertz, \textit{Science} 107, 300 (1948).


periods of 43 to 66 days until typical signs of PGA deficiency developed. They were then given 66 $\gamma$ of estradiol benzoate. Six of the eight deficient monkeys failed to show the characteristic normal estrogenic response in the external genitalia. Simultaneous administration of a liver extract containing PGA permitted the normal response to estrogen although pure PGA was not employed in this experiment. A similar relationship exists in the estradiol-treated ovariectomized rat.\textsuperscript{112} The weight of the uterus of the untreated ovariectomized rat was found to be 25 mg., which was increased to 84 mg. by injection with estradiol. In the presence of 12.5 $\gamma$ of the antagonist 4-amino PGA, the uterine weight was 32 mg. after estradiol injection. The effect of 12.5 $\gamma$ of antagonist in decreasing the response to estradiol was reversed by PGA. Corresponding results have been observed in frogs. Goldsmith \textit{et al.}\textsuperscript{113} showed that the oviducts of newly metamorphosed frogs exhibited a marked response to weekly injections of 0.1 mg. of estradiol benzoate. Administration of PGA increased the response, whereas 4-amino PGA decreased it. The effect of 4-amino PGA was not negated by simultaneous administration of PGA.

Evidence regarding the role of PGA in endocrine responses have not been as clearly defined in the male as they have in the female. Goldsmith \textit{et al.}\textsuperscript{114} measured the increase in weight of the seminal vesicles and coagulating glands of testosterone-treated mice. Ten per cent of crude "x methyl PGA" in the diet prevented the effect of testosterone, but 6% did not. Thus a higher level of antagonist is necessary to prevent the testosterone effect in mice than is required to inhibit the action of stilbestrol in chicks.\textsuperscript{108, 109} In the rat 4-amino PGA did not interfere with stimulation of the prostate by testosterone in either the intact immature rat or in the castrate animal (Brendler\textsuperscript{115}). It was also observed that 4-amino PGA did prevent the depressing effect of $\alpha$-estradiol on the prostate in the intact adult animal. Thus this PGA antagonist interferes with the tissue response to estradiol, whether it be the growth stimulation of the oviduct or uterus in the female, or the depressing effect of estradiol on the prostate in the male.

No effect of PGA has thus far been observed on the increased comb growth produced by androgen in the chick. Haque \textit{et al.}\textsuperscript{116} found that deficiency of PGA, pantothenic acid, nicotinic acid, choline, or vitamin A had no effect on the increase in comb growth produced by testosterone pro-

\textsuperscript{112} R. Hertz and W. W. Tullner, \textit{Endocrinology} 44, 278 (1949).
\textsuperscript{115} H. Brendler, \textit{Science} 110, 119 (1949).
pionate. It was also found in confirmation of the work of Hertz that PGA deficiency was the only one that interfered with the oviduct weight produced by estradiol. Zarrow et al. observed that a PGA deficiency produced either by feeding a purified diet or a commercial chick mash containing 2% of crude "x methyl PGA" had no effect on the comb stimulation produced by testosterone. Four per cent of this antagonist actually enhanced the effect of androgen on comb growth.

Dehydroisoandrosterone acetate in relatively large amounts has been found by Gaines and Totter to stimulate the growth of S. faecalis and L. casei in the absence of PGA. Cortisone acetate has been found to support the growth of Leuconostoc citrovorum in the absence of the citrovorum factor.

13. Role of Citrovorum Factor in Animal Nutrition

Just as leucovorin (synthetic citrovorum factor) can meet the requirement of certain microorganisms for PGA, it has also been found that leucovorin is active in promoting growth and hemopoieses in PGA-deficient chicks and turkeys. Experiments with crystalline leucovorin have shown that, when it is mixed with the ration of chicks or turkeys, it is considerably less active than PGA; but the two substances have similar activities when injected. Experiments with leucovorin in chicks may be compared with numerous studies that have been conducted with leucovorin in anemia in which it has been shown that leucovorin, like PGA, is a potent hemopoietic agent in pernicious anemia, sprue, and nutritional macrocytic anemia.

The discovery that leucovorin is more active than PGA in reversing the toxicity of 4-amino PGA for S. faecalis is paralleled by experiments with mice in which the same relationship was found. There are now many instances of the ability of leucovorin to counteract the action of 4-amino PGA or its close chemical derivatives. Thus Burchenal and coworkers found that leucovorin when administered prior to 4-amino-10-methyl PGA completely counteracted the antileukemic effect of this antagonist in mice.

with Ak₄ leukemia. The toxic effect of 4-amino PGA for rats,¹²⁵ the chick embryo,¹²⁶ Drosophila,¹²⁷ and human beings¹²⁸ has also been demonstrated to be effectively blocked by CF. It is apparent, then, that 4-amino PGA may more correctly be termed a CF antagonist than an antagonist of PGA.

a. Enzymatic Conversion of PGA to Citrovorum Factor

From evidence so far published it appears that, before PGA can carry out its catalytic functions, the organisms must first convert PGA to CF, and that CF either represents the catalytically active form of PGA or is more closely related to this form than PGA itself. Nichol and Welch¹⁰⁵ have presented evidence for the existence in liver of an enzyme(s) that is involved in the conversion of PGA to CF; of particular interest was their finding that a greater amount of CF was formed when ascorbic acid was present. Apparently ascorbic acid brings about favorable reducing conditions essential for the conversion. These workers also found that 4-amino PGA was markedly effective in blocking the conversion of PGA to CF by liver slices;¹²⁸ thus it is apparent that 4-amino PGA has two sites of action in the cell: (1) to block the formation of CF and (2) to compete with CF for essential reactions in the cell.

The knowledge that CF contains a formyl group is of considerable interest from a biochemical standpoint in that it provides a structural basis for the concept that CF is concerned with the transfer of the 1-carbon unit in metabolic reactions. If CF functions by shuttling a formyl group from one substrate to another, it might be predicted that tetrahydro PGA might also have activity comparable to CF. Indeed such has been found to be the case,¹²⁹ for it has been reported that tetrahydro PGA has about 2.5% of the activity of leucovorin for Le. citrovorum and about one-third the activity of leucovorin in counteracting 4-amino PGA toxicity in the mouse. However, Elwyn et al.¹³⁰ found that the β-hydrogen atoms of serine accompanied the β-carbon atom during the conversion to the methyl groups of choline and thymine. Such a finding precludes the oxidation of the β-carbon to the formate oxidation level unless the β-hydrogen atoms are held in escrow by the citrovorum factor and returned to the β-carbon atom when the formyl group of CF is reduced back to the methyl group to be incorporated into choline or thymine.

IX. EFFECTS OF DEFICIENCY

B. IN MICROORGANISMS

E. L. R. STOKSTAD

The distribution of organisms requiring PGA is not as widespread as that of those requiring other growth factors such as biotin, nicotinic acid, or pantothenic acid. The development of PGA-less mutants is much less frequent than that of other vitamins requiring mutants. Certain classes of microorganisms, particularly the lactic acid bacteria, have a number of members which require an external source of PGA. Peterson and Peterson,\(^1\) in a comprehensive review of the vitamin requirements of bacteria, list 10 bacteria out of a total of 136 as requiring "follic acid," "eluate factor," "vitamin B₆," or "L. casci factor."

Although recognition of the need of animals for PGA antedates that of microorganisms, it was the discovery of the latter that provided a rapid assay and hastened the isolation of this vitamin. The nutritive requirements of lactic acid bacteria for PGA were first pointed out by Snell and Peterson.\(^2\) They found that an unidentified factor was required which later proved to be PGA. Later work from this same laboratory showed that other lactic and propionic acids formed bacteria such as Streptococcus faecalis R, Lactobacillus delbrueckii, and Propionibacterium pentosaceum also required this factor.\(^3\)

In an investigation of the growth factor requirements of a large number of Enterococci, Niven and Sherman\(^4\) found 9 organisms requiring PGA out of a total of 43 studied. Out of 21 strains of Streptococcus lactis, none required PGA.\(^5\)

Tetrahymena geleii is unique among the microorganisms in that it has the ability to utilize large PGA conjugates which are unavailable to bacteria. The nutrition of the protozoan has been thoroughly investigated by Kidder and associates,\(^6\) who showed that this organism requires 0.00065 \(\gamma\) PGA per milliliter of culture media for half-maximum growth which is about three times that required by S. faecalis R.\(^7\) Rhizopterin, also known as the "S. L. R. factor," (\(N^{10}\) formylpteroic acid) which is active for S. faecalis R but relatively inactive for L. casci was found to be about 0.2% as active as PGA for T. geleii.\(^8\) Pteroylglutamic acid and pteroylglutamate-

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glutamic acid were reported by Kidder\textsuperscript{139} to be approximately as active as PGA when compared on a molar basis. Thus the ability of this ciliated protozoan to utilize PGA conjugates and its inability to use pteroic acid and rhizopterin parallels that of the rat, the chick, and the human.

_Clostridium tetani_ was early reported by Mueller and Miller\textsuperscript{140, 141} to require an unknown growth factor which could be replaced by concentrates of PGA prepared from liver or by folic acid preparations obtained from spinach.

1. Metabolism of Rhizopterin

A survey of the rhizopterin and PGA requirements of a number of lactic acid bacteria was made by Stokes _et al._\textsuperscript{142} This showed that all _Streptococci_ which were stimulated by rhizopterin (N\textsuperscript{10}-formylpteroic acid) also responded to PGA. A certain number of organisms, including both _Lactobacilli_ and _Streptococci_, responded only to PGA, and a third group responded to neither. They also found that different strains of the same organism vary widely in their requirements. Thus one strain of _S. faecalis_ can use either rhizopterin or PGA, another responds to PGA only, and a third requires neither. The strains that do not require PGA were found to synthesize this factor when grown on a medium devoid of the vitamin.

An interesting observation made by these same investigators was the finding that certain _Enterococci_ are able to convert rhizopterin into a form active for _L. casei_. Presumably this conversion involves the addition of glutamic acid to rhizopterin to give either PGA or N\textsuperscript{10}-formylpteroylglutamic acid. Resting cell suspensions are also able to effect this conversion.\textsuperscript{143} A 10-ml. resting cell suspension of _S. lactis_ R or _S. zymogenes_ converts 5 \(\gamma\) of rhizopterin to about 1 \(\gamma\) of PGA\textsuperscript{144} in 3 hours. Two other strains formed only 0.18 \(\gamma\) under similar conditions which shows the wide variation in the ability of various organisms to effect this conversion. The conversion rate is increased by the addition of carbohydrates which were able to act as hydrogen acceptors as evidenced by reduction of methylene blue.

Some interesting facts regarding the assay of PGA in microbial cells were revealed in this same study.\textsuperscript{143} In the assay of PGA formed from conversion of rhizopterin by resting cells, the entire cell suspensions were added directly to the assay medium without any preliminary treatment. The bulk of the activity was found to reside in the cell. When the cells were auto-


\textsuperscript{140} J. H. Mueller, P. A. Miller, _J. Biol. Chem._ 140, 933 (1941).


\textsuperscript{142} J. L. Stokes, J. C. Keresztesy, and J. W. Foster, _Science_ 100, 522 (1944).

\textsuperscript{143} J. L. Stokes and A. Larsen, _J. Bacteriol._ 50, 210 (1945).

\textsuperscript{144} The data have been recalculated on the basis of PGA’s having the same activity as folic acid of potency 137,000.
claved with water before being added to the assay medium, only 12% of the activity was recovered. However, if the cells were autoclaved with reducing agents such as 0.5% sodium glycollate, 5% ascorbic acid, or in a small amount of assay medium, complete recovery was obtained. In each case the residual PGA remaining in the liquid phase after autoclaving was resistant to further autoclaving which showed that a labile form existed in the bacterial cells. This suggests that reducing substances exert a protective influence during extraction from the cell and emphasizes the difficulties that may be encountered in the assay of PGA in microbial cells. This lability of PGA under certain conditions is reminiscent of the acid-labile PGA found in horse liver by Pfiffner et al.\textsuperscript{145} This behavior might also be explained partially by the known properties of the citrovorum factor which during its conversion to PGA in slightly acid conditions and in the presence of oxygen goes through certain intermediates, which in turn are converted to PGA in varying yields, depending on the reducing potential and pH of the solution.

2. Microbiological Activity of Citrovorum Factor

A summary of the microbiological activity of leucovorin, 5-formyl-5,6,7,8-tetrahydro PGA (synthetic citrovorum factor), and related compounds for several lactic acid bacteria is shown in Table X. It has been found from a number of microbiological assays that 0.15 mg of the anhydrous free acid of leucovorin is required for half-maximum growth of \textit{Le. citrovorum};\textsuperscript{121} however, when the vitamin is exposed to mildly acidic conditions as is indicated in the table, only 2% of the activity remains for \textit{Le. citrovorum}. About 200,000 times as much PGA as leucovorin is required by \textit{Le. citrovorum} for a comparable growth response; apparently this organism has lost the ability to convert PGA to CF. It is apparent from the table that leucovorin has PGA activity for \textit{S. faecalis} and \textit{L. casei}, although

\begin{table}
\centering
\begin{tabular}{|l|c|c|c|}
\hline
Organism & Amount of compound required per ml. culture medium for half-maximum growth in 24 hr., mg & & \\
\hline
\textit{Le. citrovorum} & Leucovorin & Acid-treated leucovorin\textsuperscript{a} & Pteroylglutamic acid \\
\hline
\textit{S. faecalis} & 0.15 & 7.5 & 30,000 \\
\textit{L. casei} & 0.17 & 0.38 & 0.18 \\
\hline
\end{tabular}
\caption{Comparative Growth Response of \textit{Le. citrovorum}, \textit{S. faecalis}, and \textit{L. casei} to Leucovorin and Related Compounds}
\end{table}

\textsuperscript{a} Prepared by allowing a leucovorin solution (4 mg./ml.) to stand at pH 2 for 24 hours at 25°.

twice as much leucovorin as PGA is required to produce half-maximum growth.

It is evident, then, that in the past when \textit{S. faecalis} or \textit{L. casei} has been employed as the assay organism to determine the pteroylglutamic acid content of natural materials, these assays have given a figure representing both PGA and CF. It should be possible to determine with \textit{Le. citrovorum} that proportion of the “free PGA” which exists as CF, since this organism for all practical purposes does not respond to PGA. Before such differential assays are performed, consideration should be given to the existence of bound forms of PGA. There is now considerable evidence that the CF in fresh liver, and presumably in most other tissues, exists as a conjugate which behaves in all respects as a polyglutamate analogous to that of pteroylglutamic acid. Thus Hill and Scott\textsuperscript{146} reported that a sample of dried brewer’s yeast which contained initially 1200 CF units per gram was found to contain 15,300 CF units per gram after incubation overnight at 37° with a hog kidney enzyme preparation. Moreover this hog kidney preparation was found to have similar properties to the PGA conjugase known to be present in hog kidney. Other sources of “CF conjugase” include chicken pancreas and the enzymes normally present in certain tissues that are active during autolysis.\textsuperscript{147, 148} There is no evidence at present to indicate whether “CF conjugase” is identical with PGA conjugase, although it seems logical that it would be.

Another interesting microbiological property of leucovorin is that, although it appears to be only half as active as PGA for growth of \textit{S. faecalis} and \textit{L. casei} (cf. Table X), when a folic acid antagonist such as 4-amino PGA is incorporated in the medium, leucovorin is markedly more effective than PGA in reversing growth inhibition.\textsuperscript{121} In the presence of an equimolar amount of leucovorin or PGA about eight times as much 4-amino PGA is required to reduce growth of \textit{S. faecalis} to half-maximum in the presence of leucovorin as is needed in the presence of PGA. Such data imply that CF may be more closely related to the biologically active coenzyme than PGA itself.

C. IN MAN

FRANK H. BETHELL

The most conspicuous effect of PGA deficiency in man is macrocytic anemia associated with megaloblastic erythropoiesis. Normal growth and


differentiation of other hemic elements, the leucocytes and thrombocytes, are also dependent upon adequate supply of PGA. The integrity of mucosal surfaces may be impaired in PGA deficiency states with secondary infection and ulceration. Glossitis, disturbances in gastrointestinal function, and peripheral neuropathies are sometimes associated with lack of this vitamin. The increase in appetite, strength, and sense of well-being which attends the administration of PGA to a severely deficient patient precedes any appreciable change in peripheral blood values and suggests that the vitamin participates in many metabolic processes. The predominance of hematologic manifestations is presumably attributable to the continuous production and rapid turnover of the cells of the blood.

A wide variety of more or less well-defined clinical entities are associated with apparent need for PGA. Although lack of this vitamin may constitute but one facet of a complex dietary deficiency state, many of the disorders in question are wholly correctible by the administration of PGA alone, whereas in others, notably addisonian pernicious anemia, PGA has proved to be an imperfect or secondary form of replacement therapy.

From what is known and has been discussed earlier regarding the naturally occurring forms of PGA, the mode of PGA absorption by the human being, its metabolic function, and its relationship to other nutritional factors, a number of theoretical mechanisms may be postulated by which PGA deficiency states might be presumed to occur. These potential modus operandi include: (1) primary dietary lack of PGA and CF and their conjugates; (2) incomplete breakdown of conjugates to the free and absorbable forms within the alimentary tract, due, perhaps, to lack of intestinal conjugates; (3) impaired absorption due to intestinal pathology, such as chronic inflammatory disease, hypermotility, and diminished absorptive surface; (4) non-utilization of PGA after absorption, which may depend upon lack of other nutritional factors such as vitamin B12, ascorbic acid, or nitrogen and carbon donors, or which may result from the presence of competitive or inhibitor substances such as PGA antimitabolites and antagonists. Playing accessory roles which may serve to convert latent or relative PGA deficiency to overt clinical manifestations are situations in which there is an increased demand for the vitamin such as pregnancy and the rapid growth of infancy.

1. Nutritional Macrocytic Anemia

By this designation is meant the presence of macrocytic anemia with megaloblastic bone marrow in persons who have a history of dietary inadequacy without gastric, intestinal, or hepatic disease. The specific entities listed below are excluded from this classification. Although the pathogenesis of such anemia would appear obvious, its interpretation is rendered some-
what unsatisfactory by a number of considerations. In the first place, most of the available food analyses for PGA were performed before the existence of CF was recognized so that the values reported represent summations of the growth-stimulating properties of both PGA and CF. Moreover, in the case of \textit{S. faecalis} R assays, there may have been incomplete liberation of PGA and CF from their conjugates. A disturbing feature of PGA dietary analyses is the apparent great loss of the vitamin after cooking, amounting to approximately 50 to 95\% of the original activity. PGA determinations on canned foods, except for spinach, were especially low. These differences between PGA content of fresh raw foods and that of their cooked or canned counterparts have been attributed to a possible binding of the vitamin by the tissues. It may be stated, however, that diets deficient in animal protein and green vegetables are low in PGA and CF. It is true, of course, that such diets are also low in vitamin B$_{12}$ and in amino acids which are concerned in PGA metabolism, so that a number of factors may contribute to the manifest deficiency state.

Therapeutic responses to the administration of PGA in cases of nutritional macrocytic anemia were first reported in 1945 and 1946. Of interest is the report of a patient who responded to PGA, relapsed after discontinuing treatment, and subsequently showed a favorable response to pteroylheptaglutamyl acid (hexaglutamyl conjugate of PGA). The significance of this event lies in the reported observations, to be discussed later, indicating that pernicious anemia patients may be unable to utilize effectively the naturally occurring hexaglutamyl conjugate and that the metabolic defect may be corrected by vitamin B$_{12}$ administration. It seems probable, although not clearly proved, that the "Wills factor" is identical with PGA including its conjugates. This hemopoietic factor, as supplied by a preparation of autolyzed yeast (Marmite) was shown by Wills and her associates to produce responses in cases of tropical macrocytic anemia which failed to benefit from the prior administration of a purified liver extract (Anahaemin). Comparable results were obtained in monkeys.

Although many of the patients treated by Wills were pregnant and so do not fall within the classification of uncomplicated nutritional macrocytic anemia, it seems proper to regard her cases as primarily attributable to dietary lack, and to assign to her priority in demonstrating a clear distinction between the anti-pernicious anemia factor of liver and a hemopoietic factor which we now recognize as probably PGA. It must, however, be borne in mind that nutritional macrocytic anemia varies greatly throughout the world and that the isolated instances of the condition seen in temperate zones are quite different in associated clinical manifestations and probably in pathogenesis from endemic deficiency states. Moreover, in most of the instances reported, no attempt has been made to establish the primary or major deficiency, whether PGA or vitamin B₁₂.

2. Idiopathic Refractory Megaloblastic Anemia or Achreistic Anemia

These terms, coined, respectively, by Davis and Davidson¹ and Israëls and Wilkinson,² have been applied to a form of macrocytic anemia with megaloblastic bone marrow for which no dietary or other etiologic explanation was discoverable and which failed to respond to purified extracts of liver. The condition has not been generally recognized in the United States or on the European continent, and its occurrence in Great Britain may possibly be attributed to unknown factors affecting nutrition if not to direct dietary deficiency. The pathogenesis of this type of anemia is probably closely related to the macrocytic anemia of pregnancy on the one hand, and to that associated with idiopathic steatorrhea or the sprue syndrome on the other. As a rule, such patients respond favorably to the administration of PGA although they may later relapse while continuing with the medication. In some instances, liver extract injections, although initially ineffective, were later successful in maintaining remission.³

3. Megaloblastic Anemia of Pregnancy and the Puerperium

Mention has already been made of the cases of tropical macrocytic anemia complicated by pregnancy observed by Wills and her associates. In temperate climates such anemia, manifested in severe degree, is uncommon, although a comparatively high incidence of mild macrocytic anemia has been observed in pregnant women whose diets were habitually low in protein of good quality and presumably also in PGA and vitamin B₁₂.⁴ Yet opinion is divided on the question of the importance of dietary inadequacy

in the pathogenesis of megaloblastic anemia of pregnancy. Ungley states that "Several of our patients had excellent diets . . ." His further argument that "In others, the diet, although not good, was at least no worse than that of other women who did not develop anaemia" cannot be given much weight, since such an observation is common to all clinical nutritional deficiency states. Certainly, in most of the reported instances of this condition pre-existing dietary inadequacy has been noted and malnutrition has characterized every case within the writer's experience. Such nutritional abnormalities may well include deficiencies other than PGA itself, such as amino acids and ascorbic acid which play a role in PGA and CF metabolism. On the other hand, contributing factors, such as the increased demands of gestation, possible digestive disturbances, and alterations in the output of sex hormones, as suggested by Ungley,\textsuperscript{166} may play a part in the actual development of severe macrocytic anemia.

Diminished secretion of intrinsic factor during gestation was proposed as a cause of macrocytic anemia of pregnancy, on the basis of clinical experiments by Strauss and Castle.\textsuperscript{167} In the light of present knowledge, such an explanation involves a defect in the absorption and utilization of vitamin B\textsubscript{12}. The same result might, as suggested by these authors, follow a low dietary intake of extrinsic factor (B\textsubscript{12}). However, before the isolation of PGA and B\textsubscript{12}, numerous observers reported the ineffectiveness of purified liver extract injections in the treatment of pregnancy macrocytic anemia, followed by good results obtained from yeast, whole liver, or crude liver extracts. The earlier therapeutic observations were summarized and analyzed critically by Watson and Castle.\textsuperscript{168} Since PGA and B\textsubscript{12} became available, it has been universal experience that the former constitutes effective and complete therapy for this type of anemia.\textsuperscript{164, 169} whereas vitamin B\textsubscript{12} is essentially valueless and may even be detrimental.\textsuperscript{170-174}

Patients with macrocytic anemia of pregnancy may exhibit striking clinical and hematological manifestations in addition to anemia. In a case observed by the author,\textsuperscript{170} spiking fever, glossitis, vulvovaginitis, and diarrhea were conspicuous. During a period of vitamin B\textsubscript{12} administration,

\begin{itemize}
  \item \textsuperscript{166} C. C. Ungley, Brit. J. Nutrition 6, 299 (1952).
  \item \textsuperscript{167} M. B. Strauss and W. B. Castle, Am. J. Med. Sci. 185, 539 (1933).
  \item \textsuperscript{169} L. S. P. Davidson, R. H. Girdwood, and J. R. Clark, Brit. Med. J. 1, 819 (1948).
  \item \textsuperscript{172} V. Ginsberg, J. Watson, and H. Lichtman, J. Lab. Clin. Med. 36, 238 (1950).
  \item \textsuperscript{173} R. H. Furman, W. B. Daniels, L. L. Hefner, E. Jones, and W. J. Darby, Am. Practitioner and Dig. Treatment 1, 146 (1950).
  \item \textsuperscript{174} C. C. Ungley and R. B. Thompson, Brit. Med. J. 1, 919 (1950).
\end{itemize}
these changes were aggravated and pre-existing leucopenia progressed to a leucocyte count of less than 500 per cubic millimeter. Dramatic symptomatic improvement and return of temperature to normal occurred within 48 hours after institution of PGA therapy, followed by rise in the leucocyte count and subsequent return of all hematologic values to normal. The evidences of severe illness seen in this patient are similar to those observed in cases of advanced sprue or idiopathic steatorrhea and bear a close resemblance to the toxic effects of PGA antagonists, such as aminopterin.

4. Megaloblastic Anemia of Infancy

For some reason, perhaps related to the wider use of processed baby foods, this condition has been seen more commonly in the United States than in Great Britain or the European continent. It is to be distinguished from the normoblastic anemia occurring in marasmic infants and, in fact, children afflicted with megaloblastic anemia are usually well nourished. The age of development of the blood changes, which include leucopenia and thrombocytopenia as well as anemia, is usually between 6 months and 2 years. Apparently the condition may result from direct or "conditioned" deficiency of either PGA or vitamin B₁₂. Sporadic case reports of this type of anemia and its response to liver extract injection appear in the literature prior to the advent of PGA and B₁₂. In the writer's experience with several cases seen before PGA or B₁₂ became available, all responded to liver injection, whether of crude or purified type.

The first clear demonstration of the efficacy of PGA in the treatment of megaloblastic anemia of infancy was made by Zuelzer and Zuelzer and Ogden. These authors stated that the effect of PGA was indistinguishable from that produced by liver extract in certain cases, but that in others PGA was effective after failure of response to liver injection. Similar observations have been made with reference to vitamin B₁₂ by Luhby and associates who obtained responses to PGA or to institution of a diet of whole milk and vegetables after failure to respond to vitamin B₁₂, and by Woodruff and associates, who reported three instances of megaloblastic anemia of infancy, one of which responded to PGA after no effect from B₁₂, whereas the other two were treated successfully with B₁₂ by injection.

177 A. L. Luhby, Health Center J. (Ohio State University) 2, 35 (1948).
McPherson and associates\textsuperscript{181} and Sturgeon and Carpenter\textsuperscript{182} have reported good remissions after administration of $B_{12}$, although the latter authors obtained only "equivocal responses" in two of their five cases. There have been no instances reported of failure of response to PGA after adequate trial.

The existence of a relationship between lack of ascorbic acid and megaloblastic anemia of infancy\textsuperscript{129} receives support from the observation of the role of this vitamin in the conversion of PGA to CF,\textsuperscript{133} from the occurrence of megaloblastic anemia in scrobutic and PGA-deficient monkeys,\textsuperscript{184-186} and from the apparent decrease in incidence of megaloblastic anemia in infancy after the addition of ascorbic acid to a popular brand of processed milk food. However, unqualified application of the findings of $in$ $vitro$ studies and animal experiments to the problem of infantile megaloblastic anemia is unjustifiable, and moreover it has not been shown that CF is more effective than PGA in the treatment of this form of anemia in humans.

In the present state of our knowledge, it must be concluded that dietary lack of PGA, vitamin $B_{12}$, or ascorbic acid may play a role in the development of megaloblastic anemia of infancy and that it is probable that most cases are due to a combination of these deficiencies. In any event, PGA appears to be completely efficacious in the treatment of this form of megaloblastic anemia.

5. Sprue and Idiopathic Steatorrhea

These conditions are considered together, since the biochemical defects are indistinguishable although etiologic factors are undoubtedly different. In the so-called tropical sprue which was formerly endemic in the southern part of the United States and parts of Latin America, malnutrition was almost invariably the rule. Tropical sprue as described by British authors commonly affects European residents in the tropics and is attributed to a peculiar environmental effect. Non-tropical sprue or idiopathic steatorrhea is a sporadic disease of unknown etiology in which there is generally no history of dietary abnormality or unusual environment. Megaloblastic anemia is of common occurrence in each of these related conditions, but the factors responsible for its development may be presumed to vary and the effects of therapy are known to differ, at least in degree.

Soon after synthetic PGA became available, its effectiveness was demon-


\textsuperscript{182} P. Sturgeon and G. Carpenter, Blood 5, 458 (1950).


strated in the treatment of endemic American sprue.\textsuperscript{187-189} PGA therapy in three patients with this form of sprue resulted in complete reversal of the clinical manifestations, including glossitis, atrophy of the lingual papillae, anorexia, weight loss, and diarrhea with high fecal fat.\textsuperscript{189} Maximum reticuloocyte responses were noted in 6 to 8 days, erythrocyte, leucocyte, and platelet values returned to normal, and megaloblasts and abnormal granulocytes disappeared from the bone marrow. A therapeutic response was obtained in one case of sprue after the administration of the pure PGA conjugate, pteroyltriglutamic acid, in a daily dose of 4.9 mg., whereas another patient responded to the administration of a concentrate of pteroylheptaglutamic acid containing in each daily dose 8.4 mg. of pteroylheptaglutamic acid and about 0.3 mg. of PGA.\textsuperscript{191} The therapeutic efficacy of thymine in daily doses of 15 g. has been reported in four cases of sprue, although the results were less striking than those obtained with PGA.\textsuperscript{192}

PGA may be regarded as almost universally effective in the treatment of the megaloblastic anemia of sprue and idiopathic steatorrhea, although exceptions to this general rule have been reported. Jones \textit{et al.}\textsuperscript{193} include in a series of cases responding to vitamin B\textsubscript{12} one patient with sprue and megaloblastic anemia who had been receiving PGA, and Girdwood\textsuperscript{163} mentions cases of idiopathic megaloblastic anemia which became refractory to PGA and developed manifestations of sprue in spite of its continued administration. It has been common experience that the dramatic curative effects of PGA in the syndrome of endemic sprue are rarely observed in the sporadic form of the disease, idiopathic steatorrhea. In the latter group megaloblastic anemia is less common and, when present, generally less severe than in endemic sprue. Furthermore, since there is evidence of associated vitamin B\textsubscript{12} deficiency in many cases of both endemic and sporadic sprue, it is to be expected that relapse may occur after long-continued administration of PGA, just as is true of addisonian pernicious anemia.

6. MEGALOBLASTIC ANEMIAS BELIEVED TO BE DUE PRIMARILY TO LACK OF VITAMIN B\textsubscript{12}

This group of conditions includes certain nutritional anemias as well as pernicious anemia, and megaloblastic anemias associated with fish tape-
worm infestation, and those following gastrectomy, intestinal strictures, blind loops, and short-circuiting operations or fistulae. Megaloblastic anemia sometimes occurring in cases of chronic liver disease is also probably due to a "conditioned" deficiency of vitamin B₁₂. These anemias are discussed in connection with the pathology of vitamin B₁₂, but it is pertinent at this time to consider certain disturbances of PGA metabolism which may be encountered in them. Soon after PGA and certain of its conjugates became available for clinical trial their effect was observed in pernicious anemia. For a more detailed account of the early therapeutic experiences with PGA and related compounds, with complete bibliography, reference should be made to the review of Jukes and Stokstad.¹⁹⁴ A reticulocyte response, a rise in erythrocyte values, a conversion of marrow megaloblastosis to normoblastosis, and symptomatic improvement occurred in essentially every case of pernicious anemia in relapse treated with PGA. The conjugates of PGA, pterooyldiglutamic and pteroyltrim glutamic acids, were also found to be effective, although the number of patients receiving these compounds was much smaller than that treated with PGA itself. On the other hand, the "hexaglutamyl conjugate," also known as pteroylheptaglutamic acid, failed to produce responses in several patients treated by two independent groups of investigators.¹⁹⁵, ¹⁹⁶ This is not invariably true, since a few patients have subsequently been observed who responded in some degree to the administration of this conjugate.¹⁹⁷ The impaired utilization of pteroylheptaglutamic acid by patients with pernicious anemia, which appears to be a quantitative defect, gains special significance from the fact that a large part of the dietary PGA is in the form of this conjugate.

Demonstration of the effectiveness of PGA in the treatment of pernicious anemia raised the question of the relationship of the compound to the active principle in liver known as the erythrocyte maturing factor (EMF). It was at once apparent that the two substances could not be identical for the following reasons. First, although liver is a rich source of PGA or related compounds, the processes of fractionation and purification yielding the most active anti-pernicious anemia extracts eliminate almost all the PGA originally present. Second, the weight of the total nitrogenous solids present in a volume of concentrated parenteral liver extract sufficient to treat a pernicious anemia patient for 15 days (15 U.S.P. units per milliliter) is far less than the quantity of PGA required for the same purpose (50 to 75 mg.). Third, the relative efficacy of liver extract given by intra-

muscular injection is about fifty times as great as when administered by mouth, whereas PGA is almost as effective by the oral as by the parenteral route of administration. It was not long before even more convincing evidence was at hand clearly distinguishing between PGA and EMF and demonstrating that the former constituted incomplete therapy for pernicious anemia. The occurrence and progression of subacute combined degeneration of the spinal cord in pernicious anemia patients receiving PGA was first reported by Vilter et al.198 Additional reports of similar observations soon appeared in the literature.199-201 The accumulated experience of these authors as well as that of many other clinical observers enable the following conclusions to be drawn with respect to the effects of PGA therapy in pernicious anemia. Most, if not all, patients receiving this medication as the only form of specific treatment ultimately develop some degree of relapse. The time required for hematologic abnormalities to occur varies from a few weeks to one year or longer. Glossitis may appear during PGA administration, even when the blood values are normal. Paresthesias and evidence of combined system disease may develop insidiously months after PGA therapy is instituted, or rapidly progressive nervous system damage may take place during the initial treatment period while the erythrocyte count is rising. Some patients experience glossitis or a fall in blood values after long-continued PGA administration without ever showing evidence of peripheral or central neuropathy. The mode of action of PGA with reference to nervous system injury is not well understood. It is the opinion of some observers that the complication is more likely to develop in patients whose diets have been poor. In the writer's experience, no such correlation has been apparent, and the most rapidly progressive cases of nervous system involvement occurred in patients who were not severely ill with anemia and whose dietary histories were good. It is a widely held impression, which the writer shares, that relatively large daily doses of PGA, 10 to 20 mg. or more, are especially likely to be associated with the development of neuropathy. Institution of liver extract or vitamin B₁₂ therapy arrests the progress of the condition and, if begun early, reverses much of the functional impairment associated with peripheral and central nervous system disease. There is no evidence that PGA is toxic to nerve

tissue or that it is capable of producing nervous system damage in persons other than patients with pernicious anemia or with related vitamin B\textsubscript{12} deficiency states. The explanation of the effect of PGA in these situations may lie in the aggravation of the B\textsubscript{12} deficiency through exhaustion of the small amount of available material. This theory is based in part on the supposition that vitamin B\textsubscript{12} functions in a number of metabolic pathways which involve nervous as well as hemopoietic tissue, and it gains credence from observations on the relationship of vitamin B\textsubscript{12} to PGA metabolism. These are discussed in the chapter on vitamin B\textsubscript{12}.

There remains to be considered the manner in which PGA deficiency may develop in pernicious anemia and other conditions associated with a lack of vitamin B\textsubscript{12}. In the first place, it should be emphasized that the deficiency of PGA may be more apparent than real, since much of the difficulty may lie in the inability to utilize effectively PGA and its derivatives which are present in the tissues in "bound" or conjugated forms. It is known that PGA or related compounds, such as conjugates of PGA or CF are present in the tissues of patients with pernicious anemia in relapse.\textsuperscript{203}

The impaired utilization of PGA in pernicious anemia may be due to the presence of inhibiting substances, and one of the functions of vitamin B\textsubscript{12} may be to eliminate such inhibitors or overcome their influence.\textsuperscript{203} The presence of a substance which inhibits the breakdown of pteroylheptaglutamic acid to free PGA has been demonstrated in certain yeast concentrates, liver, and spinach.\textsuperscript{205, 206} In accordance with this theory, the therapeutic efficacy of PGA may be due to a mass action effect overcoming the influence of inhibitors.

On the other hand, some degree of actual PGA deficiency is almost certainly present in patients with untreated pernicious anemia. The amount of PGA contained in average diets is small, probably less than 1 mg. daily, and most of it is in conjugated form. Reference has already been made to the impaired utilization of pteroylheptaglutamic acid by pernicious anemia patients. It has been shown that such persons on standard diets excrete less PGA in the urine than is the case with normal subjects, and that, in contrast to normal subjects, the administration of yeast concentrates containing pteroylheptaglutamic acid is followed by little or no increase in excretion of PGA.\textsuperscript{195, 207} Following a short period of therapy with concentrated parenteral liver extract, pernicious anemia patients excrete free PGA after oral administration of its hexaglutamyl conjugate in amounts com-


IX. EFFECTS OF DEFICIENCY

parable to the normal rate of excretion.\textsuperscript{195, 205} These observations may be interpreted as evidence of a defect in PGA metabolism, characteristic of pernicious anemia, which is corrected by supplying a constituent of liver extract, presumably vitamin B\textsubscript{12}.

Is there reason to suppose that any degree of PGA deficiency may be present in pernicious anemia patients who have been receiving, by injection, adequate amounts of liver extract or vitamin B\textsubscript{12} for a considerable period of time and whose diets are good? In other words, do these therapeutic agents completely counteract the factors responsible for impaired utilization of dietary PGA in untreated pernicious anemia? Lack of information prevents categorical replies to these questions. It is true that liver extracts or vitamin B\textsubscript{12} constitute apparently complete therapy for pernicious anemia as measured by usual clinical and hematologic criteria. On the other hand, the fact that the dietary supply of PGA is almost never large, and the persistent changes in the alimentary tract characteristic of pernicious anemia, including achlorhydria, altered upper intestinal flora, and sometimes motility disturbances, provide grounds for believing that absorption of food PGA may not be optimal in every case. This conclusion is supported by the impression of some clinicians, including the writer, that supplementing adequate liver extract or vitamin B\textsubscript{12} therapy with PGA in daily oral doses of 5 mg. may lead to an increase in sense of well-being and to slight rises in erythrocyte values.\textsuperscript{208} However, evidence to the contrary has also been presented.\textsuperscript{209}

In cases of macrocytic anemia associated with chronic partial intestinal obstruction, or anastomoses of the intestine with diminished absorptive surface and possible blind loops, there may be clear evidence of further improvement following administration of PGA after initial response to B\textsubscript{12} therapy.\textsuperscript{210}

7. INDUCED PGA DEFICIENCY IN MAN

The administration of PGA antagonists for therapeutic purposes would appear to provide a means of observing the manifestations of pure PGA deficiency under controlled conditions. However, although some clinical entities which respond to PGA therapy bear certain resemblances to the clinical and hematologic picture associated with antagonist administration, there is no close similarity between the two types of deficiency. The explanation of these differences undoubtedly lies, in part, in the rapidity with which the effects of such antagonists as 4-aminopteroylglutamic acid


\textsuperscript{209} R. B. Chodos and J. F. Ross, Blood 6, 1213 (1951).

\textsuperscript{210} F. H. Bethell, M. F. Swendseid, S. Miller, and A. A. Cintron-Rivera, Ann. Internal Med. 35, 518 (1951).
(Aminopterin) are induced. Thus the much more rapid turnover of leucocytes and thromboocytes than of circulating erythrocytes is reflected by the early development of profound leucopenia and thrombocytopenia during antagonist administration. These changes, if not arrested, lead to the fatal complications of infection and hemorrhage before the effects of erythropoietic depression have time to become manifest in great degree. That alterations in red cell production, similar to those seen in megaloblastic anemias, do occur after antagonist administration was first pointed out by Thiersch and Philips.\(^{211}\) However, the extensive changes in tissues other than those concerned with hemopoiesis, especially mucosal ulceration and hemorrhage, may be attributed to the predominant mode of action of the 4-amino analogs of PGA. The virtual impossibility of reversing the action of aminopterin and similar antagonists by any amount of PGA which is feasible to administer had impressed many observers as an argument against the concept of such compounds being true PGA antimetabolites. The problem was solved by Nichol and Welch,\(^ {212}\) who showed that the conversion of PGA to CF \textit{in vitro} and \textit{in vivo} is blocked by the presence of aminopterin. The major metabolic effect of this compound is, therefore, inactivation of an enzyme catalyst concerned with the biologically essential PGA \(\rightarrow\) CF reaction. These authors also showed that the effects of such antagonists could readily be reversed by the administration of CF, an observation soon confirmed by others.\(^ {213-215}\)

Since the prevention or reversal of the action of aminopterin by CF is accomplished when the compounds are given in fairly well-defined ratios, it may be concluded that aminopterin competes with CF in biological systems which require this factor.

In the words of Welch and Heinle:\(^ {197}\) "It is evident, therefore, that aminopterin exerts two quite different types of antagonism. That which is related to the citrovorum factor is competitive and readily reversible, while that concerned with folic acid is essentially non-competitive and only reversible under conditions where very high concentrations of folic acid are attainable. Obviously, therefore, the practical means of attempting to reverse the toxic actions of the 4-amino analogues of folic acid involve the use of citrovorum factor, but the principal reason for the development of the toxic effects of the analogues when folic acid is administered is the blockade of the biological utilization of the vitamin."

IX. EFFECTS OF DEFICIENCY

It is of interest to mention in connection with the discussion of PGA antagonists that pernicious anemia patients are unusually susceptible to the action of the 4-amino analogs, which is presumably attributable to their already defective metabolism or limited supply of PGA. Furthermore, administration of aminopterin prevents therapeutic response in pernicious anemia to vitamin B₁₂. This observation suggests that in the growth of blood cells PGA functions secondarily to vitamin B₁₂, a topic which is discussed further in the section dealing with vitamin B₁₂.

8. SUMMARY

The pathology of PGA deficiency in man is expressed in a number of clinical manifestations but especially in altered hemopoiesis, resulting in anemia, granulocytopenia, and thrombocytopenia. The characteristic changes in the bone marrow are megaloblastic erythropoiesis, qualitative abnormalities in granulocytic and megakaryocytic development, and quantitative changes in the absolute number and relative proportions of erythrocyte and granulocytic elements.

The factors which may contribute to the development of PGA deficiency states are multiple and complex. Decreased dietary intake of PGA is probably rarely the sole cause of clinically apparent deficiency, although the etiologic importance of a poor diet is evident in a group of clinical categories including nutritional megaloblastic anemia, megaloblastic anemias of pregnancy and of infancy, and certain cases of the sprue syndrome. Lack of other dietary constituents may play important roles in contributing to disturbances of PGA metabolism. Among such substances are vitamin B₁₂, ascorbic acid, and amino acids.

Deficiency of PGA may result from non-absorption or non-utilization of the naturally occurring conjugated forms of the vitamin, owing to inability to liberate the free material. The mechanism involved in this process is an enzymatic breakdown of a peptide linkage. The enzymes, or conjugases, responsible for the liberation of free PGA may be quantitatively deficient, or they may be rendered inert by the presence of inhibitory substances, which have been demonstrated in animal and plant materials. One of the metabolic defects present in pernicious anemia and some other related conditions results in relatively poor absorption of dietary PGA, and probably also in impaired utilization of such amounts of the vitamin as are absorbed and stored in the tissues.

The metabolic function of PGA involves its enzymatic conversion to a formyl derivative, the citrovorum factor. The most extreme degrees of PGA deficiency may be rapidly produced by the administration of the

4-amino analogs of PGA which have the double effect of blocking the conversion of PGA to CF and of functioning competitively as antagonists of CF itself. Such therapeutically induced PGA deficiency is employed in the treatment of selected cases of acute leukemia.

X. Pharmacology
FRANK H. BETHELL

A. ACTIONS AND USES

Pteroylglutamic acid and its analog, the citrovorum factor, play a role in the synthesis of nucleoproteins. PGA has been termed a hemopoietic vitamin because its presence in a metabolically active form is required for the normal development of blood cells. Its therapeutic use is largely confined to certain megaloblastic anemias and nutritional disorders which may be associated with megaloblastic anemia. It is the most efficacious form of therapy available for the megaloblastic anemias of pregnancy and of infancy, as well as most cases of nutritional megaloblastic anemia and tropical sprue. It is also employed, with variable degrees of success, in the management of idiopathic steatorrhea, coeliac disease, and other chronic intestinal disorders. It should not be used in the treatment of pernicious anemia, except possibly as an adjunct to adequate liver extract or vitamin B₁₂ therapy. The value of PGA as a dietary supplement in the maintenance of optimal nutrition has not been established.

B. DOSE

Five to twenty milligrams daily is the usual range of dosage. Ten milligrams daily probably produces a maximal effect in most cases, although in certain refractory cases of megaloblastic anemia much larger doses have reportedly been required. Parenteral administration of PGA has no advantage over the oral route except in the presence of persistent diarrhea or greatly diminished intestinal absorptive surface.

C. TOXICITY

Pteroylglutamic acid has an extremely low acute and chronic toxicity by animal tests and produces almost no side reactions even when the dose is far greater than the therapeutic range. In man, no unfavorable effects have been observed except in pernicious anemia when it was employed as

the sole form of anti-anemia therapy. In such cases, the neurologic damage following its use is probably attributable to extreme depletion of vitamin B₁₂.³

XI. Requirements and Factors Influencing Them

A. OF ANIMALS

(See Section IX.A.)

B. OF MAN

FRANK H. BETHELL

The daily requirement of PGA for maintenance of normal nutrition is unknown, and data applicable to this problem are difficult to obtain. It would appear, on the basis of food analyses,¹-⁴ that the daily dietary supply of PGA is probably less than 1 mg. and, owing to losses in cooking, the figure may be only a fraction of a milligram. The accuracy and reliability of food assays for PGA may be impaired by a number of circumstances: (1) Free PGA may not be released from naturally occurring conjugated forms which do not support the growth of the test organisms. (2) Interfering or inhibiting agents may be present. (3) Substances other than PGA which support the growth of the organisms, such as thymine, may be responsible for erroneously high values. (4) Of special importance is the fact that most of the reported food values for PGA were obtained before the existence of the citrovorum factor (CF) was recognized and its microbial growth-stimulating properties described. Finally, intestinal bacterial synthesis of PGA or CF may constitute an important source of the vitamin.

On the basis of therapeutic experience in PGA deficiency states, it appears probable that the total daily requirement of PGA and its analogs may be supplied by the equivalent of 1 to 2 mg. of the free vitamin. The demand is presumably greater in pregnancy, in conditions associated with hypermetabolism, and in disorders characterized by excessive tissue breakdown and regeneration.

Chapter 11
PYRIDOXINE AND RELATED COMPOUNDS
(Vitamin B₆ Group)

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I. Nomenclature

ROBERT S. HARRIS

Accepted group name: Vitamin B₆
Accepted names: Pyridoxine,¹ pyridoxal,² pyridoxamine²

² E. E. Snell, B. M. Guirard, and R. J. Williams *J. Biol. Chem.* 143, 519 (1942).
I. NOMENCLATURE

Obsolete names:  
Adermin
Antiaerodynia rat factor
Antidermatitis rat factor
Yeast eluate factor
Factor I
Factor Y
Vitamin H
Complementary factor

Empirical formulas:  
Pyridoxine: $C_8H_{14}O_5N$  
Pyridoxal: $C_8H_{14}O_2N$  
Pyridoxamine: $C_8H_{16}O_5N_2$

Chemical names:  
Pyrodoxine: 3-hydroxy-4,5-dihydroxymethyl-2-methylpyridine  
Pyridoxal: 3-hydroxy-4-formyl-5-hydroxymethyl-2-methylpyridine  
Pyridoxamine: 3-hydroxy-4-aminomethyl-5-hydroxy-methyl-2-methylpyridine

Structural formulas:

3 R. Kuhn and G. Wendt, Ber. 61, 1534 (1938).
8 P. György, R. Kuhn, and T. Wagner-Jauregg, Naturwiss. 21, 561 (1933).
II. Chemistry

JOHN C. KERESZTESY

A. ISOLATION

1. Introduction

The apparent and specific chemical properties of vitamin B₆ (pyridoxine) as present in crude concentrates were definitely established by Birch and György.¹ Within a few years of their publication the nitrogen base postulated as the active vitamin by these authors was isolated in several laboratories almost simultaneously. There was no long lapse between the recognition of the existence of this vitamin and its separation in pure form as had been the case with the first of the B vitamins. To be sure, pyridoxine is a much more stable substance than thiamine. Within approximately two years of the original observations of Birch and György, five independent reports appeared announcing its isolation. Lepkovsky,² Keresztesy and Stevens,³ György,⁴ Kuhn and Wendt,⁵ and Ichiba and Michi⁶ had succeeded in isolating pyridoxine from various natural materials. The compound was now available for the study and the elucidation of its chemistry and structure which lead directly to its synthesis. It must be noted here that as early as 1932, Ohdake⁷ in his systematic study of the constituents of rice polishings extract had isolated the hydrochloride of an unknown nitrogen base to which he had assigned the improbable empirical formula C₅H₁₀N₂O₃·HCl. Its identity with pyridoxine was pointed out later by Wiardi.⁸ At this stage in the development of the vitamin, animal assays were required to follow the biological activities of fractions obtained in the respective isolation procedures. Such assays are costly in both time and material as compared with the microbiological assay methods now employed to follow the fractionation procedures for growth factors. Since pyridoxal and pyridoxamine are the microbiologically active forms, the question arises whether pyridoxine itself would have been isolated if the latter assay methods had been used.

² S. Lepkovsky, Science 87, 169 (1938).
⁵ R. Kuhn and G. Wendt, Ber. 71, 780 (1938).
2. Methods of Isolation

a. General

The methods of isolation reported by the various investigators depended upon the use of some of the following properties of pyridoxine: (1) adsorption on fuller’s earth, or charcoal; (2) solubility in neutral ethanol or acetone; (3) stability to acid and alkaline hydrolysis; (4) failure to precipitate with heavy metal salts; (5) formation of an acetyl derivative; and (6) precipitation with phosphotungstic acid. The sources used were rice polishings or bran and yeast.

b. Kuhn and Wendt

The method used by Kuhn and Wendt involved partial purification of a heat- and alkali-sensitive protein complex in freshly prepared Lebedew juice from that unique source, Müncher Lowenbrau yeast. Low molecular weight impurities could be dialyzed away at temperatures below 3°, leaving behind the vitamin-protein complex. After further purification of the complex as a protein, the prosthetic group of the protein was split off by heating. Treatment with acetic anhydride yielded a chloroform-soluble acyl derivative which then was crystallized. After hydrolysis the hydrochloride was obtained in crystalline form.

c. Lepkovsky

Using a barium hydroxide extract of a fuller’s earth adsorbate prepared from rice bran extract, Lepkovsky effected further purification of his factor I concentrate by removal first of alcohol-insoluble mercury salts, and then of the water-insoluble lead salts followed by precipitation of the vitamin with phosphotungstic acid. The phosphotungstate was then crystallized. The vitamin was recovered by decomposing this product with barium hydroxide and final crystallization of pyridoxine (or factor I, as then named by this investigator) as presumably the sulfate.

d. György

Peter’s eluate, a yeast concentrate of thiamine widely used before commercial thiamine chloride was available, served as the starting material for György in his isolation of pyridoxine. From this he prepared a barium hydroxide eluate of fuller’s earth adsorbate. Then this was freed of neutral alcohol-ethyl acetate-insoluble impurities. Further purification was effected

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with aqueous platinic chloride to precipitate impurities. The concentrate was further treated with phosphotungstic acid, and the vitamin was precipitated. From the decomposed phosphotungstate pyridoxine was precipitated from alcoholic solution with ether. The vitamin was isolated in crystalline form, presumably as the free base.

e. Keresztesy and Stevens

An eluate of a fuller’s earth adsorbate prepared from rice bran extract was hydrolyzed successively with strong hydrochloric acid and sodium hydroxide in the method used by Keresztesy and Stevens. After removal of acetone-insoluble impurities, the concentrate was acetylated and the hydrolyzed acetyl derivatives were fractionated with phosphotungstic acid. The hydrochloride of pyridoxine was recovered from the decomposed phosphotungstates with acetone from alcoholic solution.

f. Ichiba and Michi

Isolation was accomplished by Ichiba and Michi by removing impurities with lead acetate followed by adsorption on acid clay and charcoal. Fractional precipitation of the alcoholic mercuric chloride-soluble fraction resulted in crystals which could be fractionated with alcohol.

B. CHEMICAL AND PHYSICAL PROPERTIES

1. Chemical Properties

Pyridoxine exhibits the properties of a stable hydroxylated weak nitrogen base. Birch and György found that it was not precipitated from solution by the heavy metal salts such as those of lead, mercury, silver, or platinum and not by picric acid but was precipitated by phosphotungstic acid. It was not inactivated by nitrous acid. Hydrolytic agents such as mineral acids or aqueous alkali, hot or cold, do not affect the vitamin. It is stable to the action of agents such as ethyl nitrite and Fehling’s solution. With ferric chloride, pyridoxine reacts as a phenolic substance giving a reddish-brown coloration. In alkaline solution, pyridoxine on treatment with 2,6-dichloroquinone chlorimide gives an immediate blue color fading to reddish-brown. This last reaction was used as the basis of a color test for the vitamin.

15 J. V. Scudi, J. Biol. Chem. 139, 707 (1941).
2. Physical Properties

Pyridoxine hydrochloride, C₇H₆NO₂·HCl, occurs as white platelets, melting point 204 to 206° with decomposition. The free base, C₇H₆NO₂, melts at 160°. The compound is optically inactive. Both base and hydrochloride readily sublime without decomposition.

The hydrochloride is freely soluble in water but sparingly in alcohol and acetone. The base is soluble in methanol and is not precipitated from methanol solution by ethyl ether.

![Fig. 1. Absorption spectra of vitamin B₆ at: O, pH 4; ●, pH 5.1; ▼, pH 6.75 (Stiller et al.)](image)

Rapid destruction of pyridoxine by light occurs in neutral and alkaline solutions. In 0.1 N HCl there is very little destruction.

The tautomeric properties of pyridoxine are well illustrated by the changes in its ultraviolet absorption produced by varying the hydrogen ion concentration. The single maximum at 2925 Å at pH 2 diminishes in intensity at pH 4.5, and concomitantly a new maximum appears at 3275 Å. This latter band increases in intensity when the pH is changed to 6.75, and the 2925 Å maximum disappears but a new band appears at 2560 Å. When the pH is further raised to 10.2 both bands increase in intensity and

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shift to shorter wave lengths. Figure 1 illustrates the absorption spectra of pyridoxine at various pH values.\[^{13}\]

The pK (base) value of pyridoxine was found to be $6.2 \times 10^{-10}$.\[^{13}\]

C. CONSTITUTION

1. Introduction

Only a short time elapsed between the isolation of the vitamin in crystalline form and the recognition of its chemical constitution by two independent groups of chemists, Stiller, Keresztesy, and Stevens in the United States, and Kuhn, Wendt, and Westphal in Germany.\[^{17-19}\]

2. Proof of Structure

a. Stiller, Keresztesy, and Stevens

The establishment of the structure of pyridoxine by Stiller, Keresztesy, and Stevens\[^{18}\] was based upon the study of the properties of the vitamin as isolated and its methoxy derivative. The elementary analysis of pyridoxine (as the free base) gave the empirical formula of $C_9H_{11}NO_3$. The electrometric titration curve of the hydrochloride showed only one break, indicating that the salt is a monohydrochloride and that its formula must be $C_9H_{11}NO_3 \cdot HCl$.\[^{12}\] Analysis showed it contained one C-methyl group. O-methyl and N-methyl groups were absent. Its failure to react with nitrous acid, its phenolic reaction with ferric chloride, and its ultraviolet adsorption characteristics pointed to the probability that the vitamin was a derivative of $\beta$-hydroxypyridine. Comparison of the ultraviolet absorption of pyridoxine with that of 2-methyl-3-hydroxy-5-ethylpyridine confirmed this point.\[^{18}\]

Methylation of the base with diazomethane resulted in the formation of the methyl ether, $C_9H_{13}O_3N$, melting point 101 to 102°. The absorption spectrum showed a single maximum at 2800 Å, which was not altered by pH changes. Oxidation with permanganate yielded two products. One was a dibasic acid which crystallized with one molecule of water, $C_9H_9O_5N$, melting point 208 to 209° (dec.). The other was a lactone, $C_9H_9O_4N$, melting point 209 to 210° (dec.).

Since the dibasic acid gave a negative test with ferrous sulfate, it appeared that neither of the carboxyl groups was in the $\alpha$-position of the pyridine ring. Furthermore, the test with resorcinol was positive, and therefore the two carboxyl groups must be attached to adjacent carbon atoms. Thus the dibasic acid could be represented by either of two structures.

Since the Gibbs test with 2,6-dichloroquinone chlorimide was positive for pyridoxine, the compound could not be para substituted as regards the hydroxyl, and therefore position 6 in the ring was unsubstituted. The structure of the methoxydicarboxylic acid should then be structure 1, which was confirmed by synthesis by Harris et al.\textsuperscript{20}

\[
\text{CH}_3\text{C}-\text{CH}_2\text{C}-\text{CH}_2\text{OC}_2\text{H}_5 + \text{CNCH}_2\text{C}-\text{NH}_2 \xrightarrow{\text{C}_2\text{H}_5\text{OH}} \text{pyridine as catalyst}
\]

\[
\begin{align*}
\text{H}_3\text{C} &\quad \text{HCl or H}_2\text{SO}_4 \text{ at } 120^\circ \\
\sqrt{\text{N}} &\quad \text{O}_3
\end{align*}
\]

\[
\begin{align*}
\text{H}_3\text{C} &\quad \text{H}_2\text{Pt} \\
\sqrt{\text{N}} &\quad \text{H}_2\text{Pd on BaCO}_3 \\
\sqrt{\text{N}} &\quad \text{NaNO}_3 \text{ in H}_2\text{SO}_4
\end{align*}
\]

\[
\begin{align*}
\text{H}_3\text{C} &\quad \text{CH}_2\text{N}_2 \\
\sqrt{\text{N}} &\quad \text{Ba(MnO}_4)_2 \\
\sqrt{\text{N}} &\quad \text{COOH}
\end{align*}
\]

Synthesis of Dicarboxy Acid derived from Pyridoxine Methyl Ether\textsuperscript{20}

The vitamin was therefore 2-methyl-3-hydroxy-4,5-di(hydroxymethyl) pyridine.

\[
\begin{align*}
\text{H}_3\text{C} &\quad \text{CH}_2\text{OH} \\
\sqrt{\text{N}} &\quad \text{CH}_2\text{OH}
\end{align*}
\]

\textit{b. Kuhn, Wendt, and Westphal}

In a series of three papers, Kuhn, Wendt, and Westphal\textsuperscript{17-19} independently established the structure of pyridoxine. The line of attack was also

through the oxidative degradation of the methyl ether of pyridoxine or adermin, the name used by the German workers. On oxidation of the methyl ether, the lactone, the same as that of Stiller, Keresztesy, and Stevens, was obtained. On more vigorous oxidation with hot permanganate, a tricarboxylic acid resulted which could be degraded to the dicarboxylic acid. Since the tricarboxylic acid gave a red color with ferrous sulfate, a test for pyridine-α-carboxylic acids, and since the dicarboxylic acid did not give a color with the reagent, it was concluded that the CO₂ had been split off from the α position on the pyridine ring. Pyridoxine gave a deep blue color with the Folin-Dennis phenol reagent. On the other hand, this test with the methyl ether was negative. Thus the compound was a β-hydroxy pyridine derivative. Furthermore, the formation of a lactone of the partially oxidized methyl ether of pyridoxine indicated that in the vitamin two of the original hydroxymethyl groups were located on adjacent carbon atoms in the pyridine nucleus. The final proof that the dicarboxylic acid oxidation product of the methyl ether of pyridoxine was identical with synthetic 2-methyl-3-methoxy pyridine-4,5-dicarboxylic acid was accomplished by Kuhn et al.²¹ by its preparation from 3-methyl-4-methoxy isoquinoline.

c. Ichiba and Michi

Ichiba and Michi²²-²⁴ in studying the chemistry of pyridoxine were led to believe at first that the vitamin was either an α- or γ-hydroxy pyridine derivative by the enolic nature of the hydroxyl group. On methylation of the vitamin with diazomethane they obtained the N-methyl compound which still gave a red coloration with ferric chloride. The formation of the N-methyl derivative substantiated the claim of Stiller, Keresztesy, and Stevens²³ that the vitamin is amphoteric in nature as opposed to the concept of Kuhn and Wendt,¹⁷ who considered changes in the ultraviolet absorption spectra as merely reversible shifting of the maxima.

Ichiba and Michi²³ also synthesized the key compound, i.e., 2-methyl-3-methoxy pyridine-4,5-dicarboxylic acid. After preparing 2-methyl-3-methoxy-1-chloro isoquinoline, they reduced this compound to 2-methyl-3-methoxy isoquinoline, which on oxidation yielded the desired dicarboxylic acid which they found to be identical with that obtained by the oxidation of the methyl ether of pyridoxine.

D. SYNTHESIS

a. Kuhn, Westphal, Wendt, and Westphal

With the structure well established in 1939 by the synthesis of the methoxydicarboxylic acid derivative of pyridoxine, the synthesis of the vitamin was accomplished without delay in the same year. The simplest approach to the synthesis of pyridoxine was by reconverting this degradation product to pyridoxine. Kuhn, Westphal, Wendt, and Westphal\textsuperscript{21} reported their method for accomplishing this by first converting the dicarboxylic acid to 2-methyl-3-methoxy-4,5-dicyanopyridine. On catalytic hydrogenation in which eight hydrogens were taken up, 2-methyl-3-methoxy-4,5-di(aminomethyl)pyridine was obtained. Nitrous acid converted the aminomethyl groups to hydroxymethyl groups, the resulting compound being identical.
with the methoxy vitamin. Kuhn and Wendt\textsuperscript{25} had previously shown that treatment of the methyl ether of pyridoxine with HBr not only hydrolyzes the ether but also replaces the hydroxyl groups in the hydroxymethyl groups with Br which could be replaced by hydroxyl groups by use of silver acetate. Pyridoxine as its hydrochloride was crystallized from dilute hydrochloric acid by the addition of acetone.

\textit{b. Harris and Folkers}

Following their announcement in 1939\textsuperscript{26} of their synthesis of pyridoxine, Harris and Folkers published their original method of synthesis.\textsuperscript{27} They later described an improved and more practical synthesis.\textsuperscript{28}

The original synthesis started with the condensation of ethoxyacetylacetone with cyanoacetamide to yield 3-cyano-4-ethoxymethyl-6-methyl-2-pyridone. The following is the graphic representation of the various steps of the synthesis.

\begin{center}
\begin{tikzpicture}
\node at (0,0) {CH\textsubscript{3}C\textsubscript{2}H\textsubscript{4} - C - CH\textsubscript{2}OC\textsubscript{2}H\textsubscript{5} + CNCH\textsubscript{2} - C - NH\textsubscript{2}};
\node at (3,0) {\textcolor{red}{\textbf{3-cyano-4-ethoxymethyl-6-methyl-2-pyridone}}};
\node at (6,0) {\textcolor{red}{\textbf{Synthesis of Pyridoxine}}};
\end{tikzpicture}
\end{center}

\textit{c. Other Syntheses}

Morii and Makino\textsuperscript{29} reported their synthesis of pyridoxine by practically the same method used by Harris and Folkers. Later Mowat \textit{et al.}\textsuperscript{30, 31} also

\textsuperscript{25} R. Kuhn and G. Wendt, \textit{Ber.} \textbf{72B}, 311 (1939).
\textsuperscript{26} S. A. Harris and K. Folkers, \textit{Science} \textbf{89}, 347 (1939).
\textsuperscript{29} S. Morii and K. Makino, \textit{Enzymologia} \textbf{7}, 385 (1939).
\textsuperscript{31} G. H. Carlson, U. S. Pat. 2,310,167 (1943).
published their synthesis in which 2-methyl-4-carboxy-5-cyano-6-pyridone amide served as the starting compound. The synthesis of Szabo\textsuperscript{32} consists in oxidizing 3-methyl-4-methoxyquinoline to the dicarboxylic derivative of the methoxypyridoxine. This was then converted to the dicyano compound through intermediates. Reduction to the diamino compound followed by hydrolysis of the ether and treatment with nitrous acid yielded the vitamin.

More recently applying the method developed for reducing the esters of heterocyclic carboxylic acids to the corresponding hydroxymethyl compounds, Jones and Kornfeld\textsuperscript{35} directly reduced the 4,5-dicarboxylic ester of pyridoxine to pyridoxine with lithium aluminum hydride. Excellent yields were obtained. By this step the complicated conversion of the carboxylic groups used by others was neatly overcome.

E. SPECIFICITY

1. General

Since the recognition that only a part of the vitamin B\textsubscript{6} activity of natural products can be attributed to pyridoxine, vitamin B\textsubscript{6} is now used as a class name to include all compounds having vitamin B\textsubscript{6} activity. On the adoption of the report of the Committee on Biochemical Nomenclature of the American Society of Biological Chemists, pyridoxine is correctly applied only to the single substance, 2-methyl-3-hydroxy-4,5-di(hydroxymethyl)pyridine. The two other important naturally occurring substances with vitamin B\textsubscript{6} activity are pyridoxal, 2-methyl-3-hydroxy-4-formyl-5-hydroxymethylpyridine, and pyridoxamine, 2-methyl-3-hydroxy-4-aminomethyl-5-hydroxymethylpyridine.

2. Pyridoxal and Pyridoxamine

The existence of other forms of pyridoxine was recognized by Snell \textit{et al.}\textsuperscript{34} as the result of the comparison of microbiological assays on extracts of natural materials with the values based on chemical and animal assay. In some instances the discrepancies were as much as several thousandfold. This highly active form of pyridoxine was given the trivial name, pseudo-pyridoxine. When pyridoxine was treated with mild oxidizing agents, Carpenter and Strong\textsuperscript{35} observed a marked increase in activity for the microorganism \textit{Lactobacillus casei}. Snell\textsuperscript{36-38} extended his observation that

\begin{itemize}
  \item J. L. Szabo, U. S. Pat. 2,359,260 (1944).
  \item E. E. Snell, B. M. Guirard, and R. J. Williams, \textit{J. Biol. Chem.} 143, 519 (1942).
  \item L. E. Carpenter and F. M. Strong, \textit{Arch. Biochem.} 3, 375 (1944).
  \item E. E. Snell, \textit{J. Biol. Chem.} 154, 313 (1944).
\end{itemize}
autoclaving of pyridoxine with the assay medium or amino acids greatly increased the activity of pyridoxine for the test organism Streptococcus faecalis R. and concluded that the products formed by treating pyridoxine with aminating agents and mild oxidizing agents were, respectively, the amino and aldehyde derivatives of pyridoxine. By deduction it appeared that one of the hydroxymethylene groups in either the 4 or 5 position had been modified. Tests of the compounds synthesized by Harris et al. showed that the compounds were 2-methyl-3-hydroxy-4-aminomethyl-5-hydroxymethylpyridine and 2-methyl-3-hydroxy-4-formyl-5-hydroxymethylpyridine.

Pyridoxamine was prepared by aminating either the acylated pyridoxine or, in better yield, by aminating 2-methyl-3-hydroxy-4-methoxymethyl-5-hydroxymethylpyridine.

Owing to the greater reactivity of the methylene group in the 4 position, this reaction was possible. The isomeric 5-aminomethylpyridine was also synthesized.

Careful oxidation of pyridoxine with potassium permanganate yielded an aldehyde which could be separated from the reaction mixture as its oxime. This was decomposed with nitrous acid and then treated with ethanol and hydrochloric acid to yield a cyclic acetal which easily hydrolyzed to the desired aldehyde. The proof that the formyl group was in the 4 position was accomplished by converting the oxime to the 4-amino compound by catalytic reduction.

III. INDUSTRIAL PREPARATION

JOHN C. KERESZTESY

A. SYNTHESIS

At this writing at least one commercial method for the synthesis of pyridoxine hydrochloride is based on the method used by Harris and Folkers. In this method an alkoxyacetylacetone is condensed with cyanoacetamide to give 3-cyano-4-alkoxymethyl-6 methyl-2-pyridone. This compound on nitration, chlorination, and subsequent reduction yields 2-methyl-3-amino-4-alkoxymethyl-5-aminomethylpyridine dihydrochloride. On hydrolysis and diazotization, pyridoxine hydrochloride (2-methyl-3-hydroxy-4,5-di(hydroxymethyl)pyridine hydrochloride) is obtained.

The major producers of pyridoxine hydrochloride, the only form in which pyridoxine is commercially available, are the American Cyanamid Co., Hoffman-LaRoche, and Merck & Co., Inc.

B. PURITY STANDARDS, U.S.P.

Commercial pyridoxine hydrochloride is produced to meet the following purity standards.  

C₈H₁₁NO₃·HCl: a white crystalline powder, stable in air and slowly affected by sunlight. Melts with some decomposition between 204 and 208°; 1 g. dissolves in 5 ml. of H₂O and in about 90 ml. of ethanol; pH about 3.0.

Loss of weight on drying over concentrated sulfuric acid in vacuum desiccator should not exceed 0.5%. Residue on ignition should not exceed 0.1%. No ammonia odor when heated at 100° with dilute aqueous caustic and less than 40 p.p.m. of heavy metals, as Pb.

C. U.S. PRODUCTION

<table>
<thead>
<tr>
<th>Year</th>
<th>Production, lb.</th>
<th>Average value per pound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1940</td>
<td>—</td>
<td>$3450 ($5450 initially)</td>
</tr>
<tr>
<td>1941</td>
<td>—</td>
<td>1418</td>
</tr>
<tr>
<td>1942</td>
<td>—</td>
<td>1022</td>
</tr>
<tr>
<td>1943</td>
<td>—</td>
<td>622</td>
</tr>
<tr>
<td>1944</td>
<td>4,200</td>
<td>455</td>
</tr>
<tr>
<td>1945</td>
<td>2,700</td>
<td>457</td>
</tr>
<tr>
<td>1946</td>
<td>10,300</td>
<td>381</td>
</tr>
<tr>
<td>1947</td>
<td>12,000</td>
<td>304</td>
</tr>
<tr>
<td>1948</td>
<td>10,000</td>
<td>246</td>
</tr>
<tr>
<td>1949</td>
<td>10,000</td>
<td>239</td>
</tr>
<tr>
<td>1950</td>
<td>17,500</td>
<td>234</td>
</tr>
</tbody>
</table>

1 The author wishes to express his appreciation to Dr. Max Tishler for his assistance in assembling of the information of this section.

2 U. S. Pat. 2,122,617 (1941).


4 U. S. Pharmacopeia, 14th revision, p. 900, 1950.
IV. Biochemical Systems

W. W. UMBREIT

A. GENERAL

Pyridoxine, like other vitamins, functions in the form of a coenzyme. This coenzyme, which has been variously termed codecarboxylase, cotransaminase, etc., is the 5-phosphate of pyridoxal. The various members of the vitamin B_6 group—pyridoxine, pyridoxal, pyridoxamine, and their respective phosphates—owe their vitamin activity to the ability of the organism to convert them into the enzymatically active form, pyridoxal-5-phosphate. Recently several papers have appeared which further extend our information. In addition to chemical studies on structure,\(^1\) the crystalline pyridoxal and pyridoxamine phosphates\(^{1a}\) have been prepared.

Pyridoxine is concerned with the activity of a wide variety of enzyme systems. These catalyze reactions which have apparently very little in common, except that all are characterized by action of one sort or another upon amino acids. The known enzyme systems are listed in the following paragraphs.

B. AMINO ACID DECARBOXYLASES

The properties of the amino acid decarboxylases have been so well described in reviews\(^2\text{–}^6\) that no great detail is necessary here. Their distribution and their relation to pyridoxal phosphate is given in Table I, from which it is evident that they occur predominantly in bacteria and to a lesser extent in other organisms. However, even among the bacteria, their distribution varies greatly from one strain to another. Their physiological significance is at present unknown. The enzymes decarboxylating aspartic acid to β-alanine are probably of nutritional value,\(^{43}\text{,}^{44}\) and those decarboxyl-

---


\(^7\) E. F. Gale, *Biochem. J.* 34, 392 (1940).

\(^8\) E. S. Taylor and E. F. Gale, *Biochem. J.* 39, 52 (1945).


\(^10\) S. R. Mardashen and R. N. Etingof, *Biokhimia* 13, 469 (1948) [C. A. 43, 3065 (1949)].
### TABLE I

**Summary of the Distribution of the Amino Acid Decarboxylases**

<table>
<thead>
<tr>
<th>Decarboxylase</th>
<th>Bacteria</th>
<th>Plants</th>
<th>Animals</th>
<th>Pyridoxal phosphate&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>+ (7, 8, 37)</td>
<td>-</td>
<td>-</td>
<td>+ B</td>
</tr>
<tr>
<td>Histidine</td>
<td>+ (7, 9-11)</td>
<td>? (12)</td>
<td>+ (6, 13-20)</td>
<td>-&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>+ (21-31)</td>
<td>-</td>
<td>+ (17, 32-34)</td>
<td>+ B</td>
</tr>
<tr>
<td>Ornithine</td>
<td>+ (7, 8, 22, 35)</td>
<td>-</td>
<td>-</td>
<td>+ B</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>+ (22, 35, 37)</td>
<td>+ (38, 39)</td>
<td>+ (40, 41)</td>
<td>+ B P A</td>
</tr>
<tr>
<td>Aspartic to β-alanine</td>
<td>+ (42-44)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Aspartic to α-alanine</td>
<td>+ (45)</td>
<td>-</td>
<td>-</td>
<td>+ B</td>
</tr>
<tr>
<td>Lysine</td>
<td>+ (7, 46)</td>
<td>-</td>
<td>-</td>
<td>+ B</td>
</tr>
<tr>
<td>Dihydroxyphenylalanine</td>
<td>-</td>
<td>? (12)</td>
<td>+ (2, 17, 47-)</td>
<td>+ A</td>
</tr>
</tbody>
</table>

<sup>a</sup> The following decarboxylases are alleged to occur in animal tissues: tryptophan<sup>24</sup>, cysteic acid<sup>5, 57-59</sup>, hydroxyphenylserine<sup>6</sup> (but not phenylserine), and phenylalanine<sup>60</sup>. However, in the case of the last two enzymes, at least, it is quite possible that these are further reactions of tyrosine decarboxylase (which has been shown to decarboxylate phenylalanine<sup>60</sup>) or dihydroxyphenylalanine decarboxylase.

<sup>b</sup> Indicates whether pyridoxal phosphate has been established as a coenzyme: B = bacteria; P = plant; A = animal.

<sup>c</sup> Extensive efforts<sup>9, 11, 17</sup> have so far not been able to demonstrate the involvement of pyridoxal phosphate.

lating the hydroxyphenylalanines or hydroxyphenylserines may be of significance in the formation of arterenol and epinephrin and may bear a relation to hypertension.\textsuperscript{4, 56}

C. TRANSAMINASES

These enzymes\textsuperscript{62, 63} catalyze the exchange of amino groups between \(\alpha\)-ketoglutarate and a variety of amino acids yielding glutamate and the corresponding keto acid. The reactions between glutamate and pyruvate to yield alanine and that from glutamate to oxaloacetate to yield aspartate are reversible. These reactions have recently been extended to a variety of other amino acids (listed below),\textsuperscript{64, 65} but these have not yet been shown to be strictly reversible. It would appear, however, that transamination may serve as a general mechanism for the synthesis of amino acids from their keto anlogs. Tissues from pyridoxine-deficient animals are low in trans-

\begin{itemize}
\item O. Schales and S. S. Schales, \textit{Arch. Biochem.} \textbf{11}, 155 (1946).
\item E. Roberts and S. Frankel, \textit{J. Biol. Chem.} \textbf{190}, 505 (1951).
\item S. R. Mardasheu and V. N. Gladkoua, \textit{Biokhimiya} \textbf{13}, 315 (1948); \textit{C. A.} \textbf{42}, 8859 (1948); \textbf{43}, 1073 (1949).
\item P. Holtz, R. Heise, and K. Ludtke, \textit{Arch. Exptl. Pathol. Pharmakol.} \textbf{191}, 87 (1938).
\item P. Holtz, \textit{Naturwissenschaften} \textbf{27}, 725 (1939).
\item O. Schales and S. S. Schales, \textit{Arch. Biochem.} \textbf{24}, 83 (1949).
\item E. W. Page, \textit{Arch. Biochem.} \textbf{8}, 145 (1945).
\end{itemize}
IV. BIOCHEMICAL SYSTEMS

aminase, and pyridoxal phosphate proved to be the coenzyme.

1. Glutamic to pyruvate
2. Glutamic to oxalacetic
3. α-Ketoglutarate to

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Coenzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>Citrulline</td>
</tr>
<tr>
<td>Serine</td>
<td>Ornithine</td>
</tr>
<tr>
<td>Threonine</td>
<td>Arginine</td>
</tr>
<tr>
<td>Cystine</td>
<td>Lysine</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Leucine</td>
</tr>
<tr>
<td>Methionine</td>
<td>Isoleucine</td>
</tr>
<tr>
<td>Valine</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>Proline</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>Histidine</td>
<td>Tryptophan</td>
</tr>
</tbody>
</table>

Chemical transaminations between pyridoxal and most amino acids have been projected to explain the mechanism of enzymatic transamination. It has now been shown that animal transaminase is activated by crystalline pyridoxamine phosphate. This compound requires a long incubation period with the apoenzyme but does not appear to be converted into pyridoxal phosphate either during this period or during active transamination itself, which seems to be a necessary prerequisite for the "shuttle" theories proposed and which fact, therefore, renders them quite unlikely. There seems to be little question but that pyridoxal phosphate is concerned in most reactions of amino acids, but its mechanism of action in these processes is not yet clear. Pyridoxal phosphate has been shown to be the coenzyme of some further transaminases, the enzyme synthesizing cystathionine, that cleaving it to cysteine and α-ketobutyrate, the serine and

70 A. E. Braunstein and M. G. Kritzmann, Nature 158, 102 (1946).
PYRIDOXINE AND RELATED COMPOUNDS

threonine deaminases of *Neurospora*,\(^{83}\) and a D-serine deaminase from bacteria.\(^{84}\)

There apparently also exists a transaminase from glutamine to a variety of keto acids\(^{85}\) in which the α-amino group of the glutamine is removed before that of the amide. This reaction is also catalyzed by pyridoxal phosphate.

**D. RACEMASES**

Pyridoxal phosphate is the coenzyme of an enzyme catalyzing the formation of an equilibrium mixture of DL-alanine from either D- or L-alanine.\(^{86}\) There are indications that a variety of such racemases may exist.

**E. TRYPTOPHAN**

In addition to decarboxylation and transamination, tryptophan is metabolized by pyridoxal phosphate enzymes as follows:

1. Tryptophanase.\(^{87}\) This enzyme catalyses the breakdown of tryptophan to indole, pyruvate, and ammonia.

\[
\text{Indole} \xrightarrow{\text{Tryptophanase}} \text{Tryptophan} \xrightarrow{\text{Indole + pyruvate + ammonia (bacteria)}}
\]

\[
\text{Kynurenine} \xrightarrow{\text{Kynurenine deaminase}} \text{Kynurenic acid}
\]

\[
\text{3-Hydroxykynurenine} \xrightarrow{\text{Xanthurenic acid}} \text{Xanthurenic acid}
\]

\[
\text{Anthranilic acid + alanine} \xrightarrow{\text{3-Hydroxyanthranilic acid}} \text{Quinolinic acid}
\]

\[
\text{Tryptophan Metabolism}
\]

\[
\text{3-Hydroxyanthranilic acid} \xrightarrow{\text{Nicotinic acid}}
\]

2. In the animal, tryptophan is metabolized through kynurenine according to the accompanying scheme. Pyridoxal phosphate is the coenzyme of the reaction converting kynurenine to anthranilic acid and alanine\(^{88},^{89}\) and

---


the step of converting 3-hydroxykynurenine to 3-hydroxyanthranilic acid.

3. Pyridoxal phosphate is concerned with the formation of tryptophan by the condensation of indole plus serine.

F. SULFUR-CONTAINING AMINO ACIDS AND PEPTIDES

Pyridoxal phosphate is the coenzyme of the cystathionine cleavage to homocysteine and serine. It is involved, as well, in the cysteine deaminase by a process which may be identical with the cystathionine cleavage.

V. Specificity of Action

W. W. UMBREIT

A. COENZYME FORM

Gunsalus and Bellamy discovered that the rate of tyrosine decarboxylation of *Streptococcus faecalis* cells was dependent upon the supply of "pseudo-pyridoxine" in the growth medium. Cells grown in media deficient in members of the vitamin B group did not decarboxylate tyrosine, but the resting cells could be activated with respect to this reaction by the addition of pyridoxal. When enzyme preparations from such cells were employed, adenosinetriphosphate (ATP) was necessary in addition to pyridoxal for activation. It was possible to obtain active preparations of the coenzyme in the absence of ATP by treatments designed to phosphorylate the pyridoxal. By purification of yeast fractions showing codecarboxylase activity, a material was obtained which appeared to contain pyridoxal phosphate. The biological properties of the compound were characterized by its activation of some enzyme systems listed previously, and in those cases studied the natural material from yeast and the synthetic preparations were interchangeable.

The purified coenzyme from synthetic sources contained one phosphorus per mole of pyridoxal. In contrast to pyridoxal, there was no maximum absorption at 300 μ in alkaline solution, which would eliminate the possibility that the phosphate was at position 3. Similar conclusions were reached in a direct chemical study. Nevertheless, strong claims including the preparation of a variety of derivatives has now been given. From the data now available, pyridoxal phosphate is unquestionably the monophosphoric acid ester of the 5-hydroxymethyl group of pyridoxal. A 4-phosphate has been postulated to account for some aspects of spectrum, but this possibility has been eliminated.

### B. FORMATION OF THE COENZYME FORM

As mentioned, the only form of vitamin B₆ which is active enzymatically is pyridoxal-5-phosphate. The other members of the vitamin B₆ groups owe their activity to systems capable of converting them into pyridoxal-5-phosphate. The interrelationships are illustrated below. The conversion of pyridoxine, pyridoxal, and pyridoxamine into pyridoxal phosphate during growth of organisms using these materials as sources of vitamin B₆ has been demonstrated biologically. The conversion of pyridoxal to its phosphate by an enzymatic reaction involving ATP has been studied. The conversion of pyridoxamine phosphate to pyridoxal phosphate by way of

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V. SPECIFICITY OF ACTION

an apparently specific type of transamination has also been observed.\textsuperscript{26, 27} Not much is known about the enzymes carrying out the series of reactions illustrated, but there is adequate evidence that they exist.

C. SPECIFICITY AND MECHANISM OF ACTION

In all cases so far known, the enzymatically active form of vitamin B\textsubscript{6} is pyridoxal-5-phosphate and the activity of pyridoxamine phosphate observed occasionally\textsuperscript{26-29} is due to the presence of auxiliary enzyme systems converting it to pyridoxal phosphate. It appears that the principal storage product of the phosphorylated vitamin is pyridoxamine phosphate but that it is itself inactive. The fact that only the pyridoxal-5-phosphate is active in a variety of enzyme systems listed previously makes any general postulate of a mechanism of action somewhat premature. Such mechanisms as have been proposed for either decarboxylation or transamination have postulated the formation of a Schiff's base between the \(\text{4-aldehyde group of pyridoxal phosphate}^{26} \) and the \(\alpha\)-amino group of the amino acid.\textsuperscript{26, 30-39}

\begin{itemize}
\item \textbf{Pyridoxamine}
\item \textbf{Pyridoxal}
\item \textbf{Pyridoxine}
\item \textbf{Pyridoxal-5-phosphate (coenzyme form)}
\item \textbf{Pyridoxamine phosphate}
\item \textbf{Pyridoxine phosphate}
\end{itemize}

Interconversion of Members of the Vitamin B\textsubscript{6} Group

\textsuperscript{26} W. W. Umbreit, D. J. O’Kane, and I. C. Gunsalus, \textit{J. Biol. Chem.} \textbf{176}, 629 (1948).
\textsuperscript{30} H. Blaschko, \textit{Advances in Enzymol.} \textbf{5}, 67 (1945).
Although such a postulate is a possibility, there seems to be no positive evidence for it and in the case of the transaminases there is some evidence against it.\textsuperscript{26}

D. ANTAGONISTS

Only one of the vitamin B\textsubscript{6} antagonists, desoxypyridoxine, has been studied by enzymatic techniques.\textsuperscript{25} This material acts by being itself phosphorylated and competing, in the form of desoxypyridoxine phosphate, with pyridoxal phosphate for the surface of the apoenzyme.

VI. Biogenesis

W. W. UMBREIT

The origin of the vitamin B\textsubscript{6} molecule is not known. The transformations among the various members of the vitamin B\textsubscript{6} group are outlined on p. 241. The original hypothesis of Snell\textsuperscript{1} that, since D-alanine replaced the growth requirement of certain bacteria for vitamin B\textsubscript{6}, condensation between d-alanine and a 4-carbon dicarboxy acid might serve to form pyridoxal has not been borne out by subsequent data, since organisms grown on D-alanine do not contain vitamin B\textsubscript{6}. There appears to be a requirement for D-alanine\textsuperscript{2} which in the presence of vitamin B\textsubscript{6} is met from L-alanine and the pyridoxal phosphate containing racemase.\textsuperscript{3}

VII. Estimation

HENRY SHERMAN

Since many of the compounds which possess vitamin B\textsubscript{6} activity occur in nature as conjugates which are, in large part, firmly bound to tissue, pre-

\textsuperscript{31} E. F. Gale, \textit{Advances in Enzymol.} \textbf{6}, 1 (1946).
\textsuperscript{33} O. Schales, \textit{Advances in Enzymol.} \textbf{7}, 513 (1947).
\textsuperscript{35} E. Werle, \textit{Z. Vitamin- Hormon u. Fermentforsch.} \textbf{1}, 504 (1947).
\textsuperscript{38} F. Schlenk and A. Fisher, \textit{Arch. Biochem.} \textbf{8}, 337 (1945).
\textsuperscript{39} E. Werle and W. Koch, \textit{Biochem. Z.} \textbf{319}, 305 (1949).
\textsuperscript{3} W. A. Wood and I. C. Gunsalus, \textit{J. Biol. Chem.} \textbf{190}, 403 (1951).
liminary tissue digestion is usually required for the quantitative extraction of the active material prior to physical, chemical, and microbiological assay.\textsuperscript{1} Melnick and coworkers\textsuperscript{2} have tested various methods for liberating bound vitamin B\textsubscript{6} and have found the following hydrolytic procedures to be satisfactory: (1) autoclaving the materials at 15 lb. pressure in 0.055 \textit{N} H\textsubscript{2}SO\textsubscript{4} for 90 minutes; (2) autoclaving at 15 lb. pressure in 2 \textit{N} H\textsubscript{2}SO\textsubscript{4} for 30 minutes; (3) suspending the materials in 1 \textit{N} HCl at 100° for 60 minutes. There are modifications of this type of hydrolytic procedure which have been successfully used; they all employ an acid hydrolysis, since it has been demonstrated that the three members of the vitamin B\textsubscript{6} complex are stable in acid solution\textsuperscript{3} and unstable in neutral or alkaline solution. Pepsin and papain digestions in acid media have also been utilized to liberate bound vitamin B\textsubscript{6}.

A. PHYSICAL METHODS

HENRY SHERMAN

1. SPECTROPHOTOMETRIC

Pyridoxine exhibits a typical ultraviolet absorption spectrum which changes markedly with variations in hydrogen ion concentration.\textsuperscript{4, 5} Melnick et al.\textsuperscript{2} have shown that pyridoxal and pyridoxamine have similar absorption curves and behave similarly with changes in pH. They have used these facts as the basis of their spectrophotometric method for the assay of vitamin B\textsubscript{6}. "The ultraviolet absorption curves show no maxima common to all three compounds. However, if readings are taken at 325 \textmu m of solutions at pH 6.75, close estimates of the total amount of the compounds present may be obtained, despite variations in their relative concentrations. At that wave length there is an absorption maximum common to pyridoxine and pyridoxamine, and while pyridoxal absorbs approximately 20 per cent more light at its 315 \textmu m maximum, at 325 \textmu m all three compounds absorb to the same extent. The \textit{E}\textsubscript{1\textmu m} values at 325 \textmu m in pure solutions at pH 6.75 all approximate 410 \pm 1.5 percent expressed in terms of the free bases. Spectrophotometric analyses, of course, cannot be applied unless the test solutions are free from irrelevant light-absorbing materials, or unless blank solutions containing none of these vitamin B\textsubscript{6} factors are available for evaluating the interference." This spectrophoto-


\textsuperscript{3} E. Cunningham and E. E. Snell, \textit{J. Biol. Chem.} \textbf{158}, 491 (1945).


metric method has been adapted to pure solutions, but as yet has not been used successfully with biological materials.

Vacher and Faucquembergue\(^6\) utilized the change in the shape of the ultraviolet absorption curve of pyridoxine with pH as the basis for their assay. They analyzed for pyridoxine in the presence of other substances by obtaining ultraviolet absorption curves at two or more levels of pH.

2. Polarographic

Pyridoxine produces a double wave when it is reduced at the dropping mercury electrode, indicating a reduction in two 2-electron steps.\(^7\) Although reduction was difficult, satisfactory results were obtained when tetramethylammonium bromide was the supporting electrolyte. This method, however, has not yet been used for the estimation of pyridoxine in foods.

B. CHEMICAL METHODS

HENRY SHERMAN

Most of the colorimetric tests that have been used for the assay of the vitamin B\(_6\) group were originally devised when pyridoxine was believed to be the only member of this biologically active complex. Since pyridoxal and pyridoxamine are now considered important members of the vitamin B\(_6\) complex and since they are present in natural materials along with other biologically inactive phenolic compounds which often react similarly, many of the original concepts concerning the various colorimetric tests have had to be revised. It seems unlikely that any simple colorimetric test can be applied toward the assay of all forms of the vitamin B\(_6\) complex. At present, three separate and distinct compounds possess vitamin B\(_6\) activity, and there is some evidence that even these three compounds do not account for all the biological activity.\(^2\) The colorimetric procedures which are now available are most accurate when applied to analytical samples, such as pharmaceutical preparations, which contain only pyridoxine. However, if these tests are employed to assay natural products in which pyridoxine does not constitute the major portion of the vitamin B\(_6\) content, considerable caution must be exercised in interpreting the results.

One class of color reactions includes the formation of cyanine dyestuffs and phthaleins.

1. Cyanine Dye Test\(^8\)

The cyanine dye test is specific for \(\alpha\)-picoline compounds of which the vitamin B\(_6\) group is the only naturally occurring member. It has been ap-


plied to the determination of pyridoxine. The test depends upon the reactivity of the α-methyl groups. The vitamin must first be converted into the methyl ether by diazomethane and then into a quaternary pyridine compound by methyl iodide or dimethylsulfate. The resulting salt, upon heat treatment with a solution of sodium in ethyl alcohol and then with chloroform, produces a violet color, which has absorption maxima at 599 and 555 μ. By this color test, 0.1 mg. of pyridoxine can be detected. It has not been used extensively in food analysis because of the difficulty encountered in the preparation of the phenolic ether in quantitative yields.

2. Phthaleins

Stiller and his associates oxidized pyridoxine to the 4,5-dicarboxylic acid and then fused the resulting acid with resorcinol to obtain the corresponding phthalein, which had a greenish-yellow fluorescence. However, this method is not readily adaptable to assay procedure because of its lack of specificity. It can be used, however, to measure pyridoxine in pure solutions.

The most widely used color reactions depend upon the phenolic nature of the vitamin B₆ group. One series of tests involves the active participation of the phenolic hydroxyl group. Another series of tests depends upon the coupling reaction which is common to most phenolic compounds. Each of the three active forms of the vitamin B₆ group has a phenolic group in the 3 position and an unsubstituted position para to this phenolic group. These two factors favor the condensation of the vitamin with chromogenic reagents to form dyes which can be measured colorimetrically; this fundamental reaction is the basis of the color tests that have been applied most successfully to the chemical assay of the vitamin B₆ complex.

3. Ferric Chloride Method

When pyridoxine is reacted with a 10% aqueous solution of FeCl₃, a red-brown color is formed, indicating the presence of the phenolic hydroxyl group. This reaction has been used to estimate pyridoxine in more highly refined concentrates, by comparing the color developed in the unknown sample with that of standards. Pharmaceutical preparations containing only pyridoxine of the vitamin B₆ group are especially amenable to such a test; other members of the B vitamin group do not interfere. No literature is available on the reactions between pyridoxal or pyridoxamine with FeCl₃.

4. Phenol Test with Folin-Denis Reagent

Pyridoxine, like most phenols, reacts with the Folin-Denis reagent (a mixture of phosphotungstic and phosphomolybdic acids) to form a blue

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5. O. Folin and W. Denis, J. Biol. Chem. 22, 305 (1915).
color which can be measured. This method did not gain wide acceptance due to its lack of specificity.

5. Diazotized Sulfanilic Acid Test

Swaminathan\textsuperscript{12} used the color reaction between pyridoxine and diazotized sulfanilic acid as the basis for its estimation. This reaction is not specific for pyridoxine; complete removal of interfering substances prior to the coupling reaction is essential. The procedure employed to free extracts of interfering substances is long and difficult to perform. Protein and protein derivatives resulting from a pepsin digestion are removed by phosphotungstic acid, and purines, pyrimidines, and imidazole bases are removed by a silver nitrate and barium hydroxide treatment. The pyridoxine is then adsorbed on Clarite from a filtrate adjusted to pH 1 to 2, eluted with hot barium hydroxide, adjusted to pH 6, and then coupled with diazotized sulfanilic acid. This method has been applied to many foods and to urine samples with excellent results.\textsuperscript{13, 14}

Bina et al.\textsuperscript{15} improved the chemical procedure with diazotized sulfanilic acid. They adsorbed pyridoxine on Superfiltrol at pH 3 and then used alkaline ethyl alcohol as the selective eluant; this not only aided and simplified the purification process but also served to stabilize the color which, in the Swaminathan procedure, started to fade after 5 minutes.

Ormsby et al.\textsuperscript{16} observed that the three members of the vitamin B\textsubscript{6} complex gave different colors when treated with diazotized sulfanilic acid. Pyridoxine gives an orange color, pyridoxamine an orange to pink color, and pyridoxal a bright yellow color. Although the stability of the colors was limited, absorption spectra could be obtained. It is evident, therefore, that foodstuffs containing more than one member of the B\textsubscript{6} group can produce a color with diazotized sulfanilic acid which may be difficult to match with standards prepared from pyridoxine. This suggests why individual colorimetric tests are not very accurate when applied to natural materials.

6. Diazotized p-Aminoacetophenone Test

Brown et al.\textsuperscript{17} introduced this new diazo reaction for pyridoxine; it had first been used for the determination of thiamine.\textsuperscript{18} Although the color produced is not as stable as that developed with diazotized sulfanilic acid, its sensitivity and specificity are much better. This increased sensitivity is

\textsuperscript{12} M. Swaminathan, Nature 145, 780 (1940).
\textsuperscript{17} E. B. Brown, A. F. Bina, and J. M. Thomas, J. Biol. Chem. 158, 455 (1945).
\textsuperscript{18} H. J. Prebluda and E. V. McCollum, J. Biol. Chem. 127, 495 (1939).
probably due to the fact that the color reaction is carried out at a pH of 7.0 to 7.3 instead of at the higher pH of 10 to 11, which is required for the coupling reaction with diazotized sulfanilic acid. Solutions containing 0.5 to 2.0 γ of pyridoxine per milliliter can be measured by this procedure. The use of diazotized p-aminoacetophenone instead of sulfanilic acid in the color development permits a simpler and more efficient purification process. A synthetic resin, Amberlite No. 1R-1, is used instead of phosphotungstic acid in the purification process prior to the color development. This resin adsorbs soluble interfering substances, thus preventing their simultaneous adsorption on Superfiltrol with pyridoxine; the vitamin is eluted from the Superfiltrol with alkaline ethyl alcohol.

7. Diazotized p-Nitroaniline Test

Swaminathan\textsuperscript{13} used this phenol reagent to assay for pyridoxine in food-stuffs, but it has not been used by many other investigators. Nassi\textsuperscript{19} modified the method somewhat by introducing a blank determination. He observed that pyridoxine, irradiated with ultraviolet light, lost the capacity to produce an orange color with diazotized p-nitroaniline. He suggested, therefore, that colorimetric tests be conducted on non-irradiated and irradiated aliquots of the sample; the difference between the two values would more closely approximate the true pyridoxine content of the sample. However, considerable caution in interpretation must be exercised in view of the presence of pyridoxal and pyridoxamine in natural materials.

8. Indophenol Test

Gibbs\textsuperscript{20} observed many years ago that phenols which had unsubstituted para positions formed, under the proper conditions, blue indophenol salts. Pyridoxine gives a positive Gibbs test when reacted with 2,6-dichloroquinonechlorimide.\textsuperscript{5} Scudi and his coworkers\textsuperscript{21-24} developed an analytical procedure for the estimation of pyridoxine based upon this indophenol reaction, which has become the most widely used and accepted of all colorimetric tests. As little as 0.5 γ pyridoxine in 1 ml. of solution can be analyzed by this method. However, even this chlorimide reagent, which is more specific than other reagents, reacts with compounds not possessing B₈ activity. Most of the chemical procedures that have utilized this indophenol reac-

\textsuperscript{13} L. Nassi, \textit{Boll. soc. ital. biol. sper.} \textbf{16}, 690 (1941).
\textsuperscript{24} J. V. Scudi, \textit{J. Biol. Chem.} \textbf{139}, 707 (1941).
tion depend for their accuracy upon the complete removal of interfering substances prior to the formation of the color complex.

In this procedure, Scudi\textsuperscript{24} used a two-phase water-butanol solution to aid in the removal of interfering substances at pH 6.8 to 7.2. However, the neutralized solution was then weakly buffered with veronal prior to color development. No provision was made to control interfering compounds like salts and bases. A borate buffer blank was introduced to correct for compounds which would react with the chlorimide reagent, but the results were not very quantitative. Pyridoxine forms a complex with boric acid, which does not give the indophenol salt. It was originally thought that the difference in values obtained in veronal and borate buffers would represent vitamin B\textsubscript{6}-active materials, but with the discovery of pyridoxal and pyridoxamine the borate buffer blank lost its original purpose. Pyridoxal and pyridoxamine react with the chlorimide reagent to form a blue pigment even in the presence of borate. Therefore, when all three compounds are present in natural materials, the total blue color formed with the chlorimide reagent is equal to the sum of the members of the vitamin B\textsubscript{6} group. The difference, in the presence and absence of borate, represents the color contribution of pyridoxine alone, and not the vitamin B\textsubscript{6} group.

Bird \textit{et al.}\textsuperscript{25} modified the Scudi colorimetric method so that it was simpler and more rapid. Pyridoxine was adsorbed on Superfiltrol at pH 3.0, washed, and then eluted by adding a butanol solution of 2,6-dichloroquinonechlorimide to the Superfiltrol. Veronal buffer was then added, so that the pH was raised to 7.8 to 8.0 and the characteristic blue color then developed. Elution and color development were thus carried out simultaneously.

Hochberg \textit{et al.}\textsuperscript{26} developed a method which was based on the coupling of pyridoxine and the chlorimide reagent in isopropanol, a one-phase system. The use of a strong NH\textsubscript{4}OH-NH\textsubscript{4}Cl buffer of high basicity and salinity eliminated the interference caused by different kinds and amounts of bases and salts in the test solution. The color development reached a maximum in 60 seconds and was three times as sensitive as other tests; similar tests required 20 to 40 minutes for complete color development. The use of an internal standard eliminated the influence of other compounds upon the rate, extent, and stability of color formation. A borate blank made the reaction fairly specific for pyridoxine. When this method was applied to biological materials, the pyridoxine was first adsorbed on Lloyd's reagent, eluted, and then submitted to the coupling reaction.\textsuperscript{27} Bottomley\textsuperscript{28} has suggested that products which are high in fat content be subjected to an ether extraction prior to isolation and coupling. Melnick and coworkers,\textsuperscript{2} using

\textsuperscript{28} A. C. Bottomley, \textit{Biochem. J.} \textbf{38}, V (1944).
this colorimetric procedure, observed that pyridoxamine and pyridoxal were about 31% and 15% as reactive, respectively, as pyridoxine on an equimolar basis. They state, therefore, that this method can be used with precision only when pyridoxine constitutes a major portion of the vitamin B₆ content of the material tested. When applied to metabolically active tissues, like yeast, which have large amounts of pyridoxal and pyridoxamine, this method can give only approximate values. Undoubtedly, similar conclusions may be drawn with respect to other chemical assay procedures.

Canbäck and Lindholm have substituted 2,6-dibromoquinonechlorimide for the chloro compound and have reported good results.

9. Cyanogen Bromide Test

Sweeney and Hall have recently developed a chemical assay procedure which is based on the König reaction, often used in the chemical assay for niacin. The pyridine ring is opened by cyanogen bromide and the resulting product coupled with an aromatic amine to give a colored complex. Although pyridoxine is a pyridine derivative, it reacts very little, if at all, with the König reagent, for it has an α-methyl group on the pyridine nucleus. However, this methyl group can be removed by first converting it to the carboxyl group and then heating the resulting α-carboxylic acid at 120 to 150°. The loss of CO₂ results in the formation of a compound which then reacts with the König reagent.

In this method, pyridoxine is removed from niacin and other interfering substances by adsorption on zeolite at pH 7.6 and subsequent elution with hot normal ammonium hydroxide. The eluate is evaporated to dryness and oxidized, while being heated, with sulfuric acid and selenium. The oxidized mixture is neutralized, treated with cyanogen bromide, and then coupled with sulfanilic acid. The resulting color is read at 480 mµ. Excellent recovery experiments (90 to 103%) have been reported.

Pyridoxal gives the same test as pyridoxine, but pyridoxamine gives only a faint color with this procedure. However, pyridoxamine can be converted to pyridoxine with nitrous acid in an additional step. In this way, the procedure affords a means not only for determining the total vitamin B₆ complex but also for differentiating pyridoxamine from the other two members of the group.

C. BIOLOGICAL METHODS

HENRY SHERMAN

The vitamin B₆ activity of natural materials is measured most reliably by animal assay. No preliminary hydrolytic, extractive, and concentrating procedures are required to liberate the many bound forms of the vitamin

so that the resulting total activity may be in a measurable state; the material is fed directly to the assay animals. Errors which are inherent in these chemical manipulations are thus avoided. Moreover, animal assay measures available, not "total," vitamin B₆.

There are two general methods which have been employed for the bioassay of vitamin B₆; one is based upon the cure or prevention of the acrodynia that is produced on a vitamin B₆-deficient diet, the other upon the increase in weight when graded doses of vitamin B₆ are added to the deficient diet.²⁹ Often, a combination of these two procedures is used. The rat is the experimental animal most widely used.

1. Acrodynia Rat Test

The first biological assays of vitamin B₆ were based entirely, or for the most part, upon the cure of rat acrodynia produced by the vitamin B₆-deficient diet.³¹-³⁷ However, the curative procedure was not generally adopted because of its many inherent weaknesses: (1) factors other than vitamin B₆ are involved in the production and cure of rat dermatitis,³⁸, ³⁹ (2) severity of dermatitis varies with season;⁴⁰ (3) interpretations of resulting cures are dependent largely upon subjective influences;⁴¹ and (4) the acrodynia cannot be produced consistently in the hands of some investigators.⁴² Undoubtedly, much of the earlier work was unsatisfactory because the diets were not completely free from traces of vitamin B₆; a complete synthetic diet was not available, thus necessitating the inclusion of natural products, such as "eluate" and "filtrate" factors, in the diet. Supplee et al.,⁴³ on the other hand, have used the onset of acrodynia as the basis of an assay method. They devised a basal diet which permitted the development of acrodynia in practically 100% of their animals in six to eight weeks.

³⁸ T. W. Birch, J. Biol. Chem. 124, 775 (1938).
³⁹ H. Sherman, Vitamins and Hormones 8, 55 (1951).
⁴² W. L. Dann, J. Biol. Chem. 128, XVIII (1939).
2. Rat Growth Test

Assay procedures which have utilized the growth response have been more successful. In spite of its greater lack of specificity, it is preferred today for the biological assay of vitamin B$_6$.

In the first biological assay procedures by the rat growth method, the water-soluble vitamins, other than those that were available in crystalline form, were supplied in the diet by liver extracts, rice polishing concentrates, or other natural products. Clarke and Lechycka replaced the natural products in the diets with pure vitamins, except for biotin, and developed a basal ration which could be used successfully to assay for vitamin B$_6$. The vitamins were fed in solution as separate supplements and given three times each week during both the depletion and assay periods.

Sarma et al. have modified and improved the method of Conger and Elvehjem; theirs is now the biological procedure that is being used most extensively for the bioassay of vitamin B$_6$. They sought to develop a basal ration which would permit the minimum growth in the absence of vitamin B$_6$ and the maximum growth with optimal amounts of vitamin B$_6$. Several carbohydrates (sucrose, dextrin, glucose) and proteins (casein, blood fibrin, egg albumin) were tried in various combinations to meet this criterion. The diet finally selected for the bioassay of vitamin B$_6$ had the following composition: sucrose 75 g., blood fibrin 18 g., salts IV 4 g., corn oil 3 g., thiamine 0.2 mg., riboflavin 0.3 mg., nicotinic acid 2.5 mg., calcium pantothenate 2 mg., 2-methyl-1,4-naphthoquinone 1 mg., inositol 10 mg.; choline chloride 100 mg., and biotin 0.01 mg., per 100 g. of diet. Halibut liver oil, diluted 1:2 with corn oil, was fed at a level of 2 drops per week, with α-tocopherol included at 0.5 mg. per drop.

Male rats were placed on this depletion diet for two weeks after which they were divided evenly with respect to weight into groups of three and placed on diets containing different amounts of pyridoxine, ranging from 0 to 150 γ per 100 g. of ration in increments of 25 γ. They were continued on their respective diets for four weeks. The weight gain per week was approximately linear up to 75 γ of pyridoxine per 100 g. of diet. Twice this amount was required for maximum weight gains of over 30 g. per week.

The biological activity of pyridoxine, pyridoxal, and pyridoxamine was determined for three methods of administration. The components of the vitamin B$_6$ complex were equally active when fed by medicine dropper as

daily supplements to the ration or when injected interperitoneally. However, pyridoxal and pyridoxamine were approximately 25% less active than pyridoxine when the three compounds were mixed with the ration; this may be due to the increased destruction or utilization of pyridoxal and pyridoxamine by intestinal bacteria.

Biological materials to be assayed are mixed in the diet at two levels so that they supply between 25 and 75 γ of vitamin B₆ per 100 g. of ration. Weight gain is recorded weekly; after four weeks, the average weight gain per week is calculated. This value is then compared to a standard curve. In most cases, higher results were obtained at the lower level of assay. The values obtained by this method are somewhat lower than values obtained by the yeast growth method, presumably because of the decreased activity of pyridoxal and pyridoxamine when mixed in the diet.

3. Chick Growth Test

Prophylactic and curative techniques have been employed. In the prophylactic test, day-old white Leghorn chicks were fed the following vitamin B₆-deficient diet: Cerelose 61 g., blood fibrin 18 g., gelatin 10 g., salts IV 5 g., CaHPO₄.2H₂O 1 g., L-cystine 0.3 g., thiamine hydrochloride 0.3 mg., riboflavin 0.6 mg., calcium pantothenate 2.0 mg., choline chloride 150 mg., nicotinic acid 5.0 mg., biotin 0.02 mg., inositol 100.0 mg., folic acid 0.125 mg., vitamin D₃ 0.004 mg., α-tocopherol 0.03 mg., 2-methyl-1,4-naphthoquinone 0.05 mg., and vitamin A 1700 I.U. Chicks on this diet died in two weeks without gaining any weight. Those chicks receiving vitamin B₆, either as a standard dose or a test food, were allowed to grow on their respective diets for three weeks. The response was recorded in terms of body weight. There is no depletion period in this procedure; the test dose of vitamin B₆ is given at the beginning of the assay. Pyridoxal and pyridoxamine exhibited lower activity than pyridoxine when fed in the ration, just as in the rat growth test. Growth response was equal for all three forms of the B₆ complex when the vitamins were fed by dropper or injected interperitoneally.

In the curative test chicks are placed on the basal vitamin B₆-deficient ration for a six-day depletion period. They are then divided into groups of seven, of equal average weight, and continued on the depletion diet for an additional five days. The test substance or standard is given in a single oral dose to each chick and repeated on the following three alternate days. Basal diet and water are given ad libitum. The chicks are weighed before each dose and on the second day after the last dose is given. The basal vitamin B₆-deficient diet used in this test has the following composition: Cerelose 46.5 g., casein (vitamin free) 25.0 g., gelatin 10.0 g., salts IV 5.0 g.,

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calcium gluconate 5.0 g., KH₂PO₄ 1.0 g., liver extract "L" (Wilson and Co.) 2.0 g., wheat germ oil 4.5 g., 400 D fish liver oil 0.5 g., l-cystine 0.2 g., choline 0.2 g., inositol 0.1 g., PABA 30.0 mg., thiamine 2.0 mg., riboflavin 2.0 mg., calcium pantothenate 4.0 mg., nicotinic acid 10.0 mg., biotin 0.01 mg., and 2-methyl-1,1-naphthoquinone 0.4 mg.

4. Rice Moth Larva Test

Sarma⁵¹, ⁵² observed that the rice moth larva (Corcyra cephalonica St.) required vitamin B₆ for growth, and that growth was proportional to the amount of vitamin B₆ in the diet. An assay procedure based on these observations was developed. The results obtained with this procedure agreed well with chemical tests. Pseudopyridoxine (pyridoxal and pyridoxamine) was equivalent to pyridoxine in activity.

D. MICROBIOLOGICAL METHODS

ESMOND E. SNELL

Vitamin B₆ occurs naturally in three unconjugated and several conjugated or "bound" forms. The former are pyridoxal, pyridoxamine, and pyridoxine. The latter include pyridoxal phosphate and pyridoxamine phosphate and their combinations with protein, an unidentified conjugate of pyridoxine present in rice bran concentrate, and possibly additional compounds. The bound forms show activity in animal assay, but not for most microorganisms. Hydrolysis to the unconjugated forms must therefore be carried out preparatory to microbiological assay.⁵³

Pyridoxal and pyridoxamine phosphates are hydrolyzed more rapidly between pH 1.5 and 2.0 than at lower or higher pH values. Where these compounds are among the most important bound forms of the vitamin, lower concentrations of acid are superior to higher concentrations for the liberation of vitamin B₆.⁵⁴, ⁵⁵ This situation exists with many, but not all, natural materials. The procedure of choice for most materials is to heat the finely divided sample with an excess of 0.055 N H₂SO₄ or HCl at 20 lb. pressure in the autoclave for 5 hours. For some materials, higher acid concentrations are more effective. Preliminary to extensive study of a single material, therefore, the most effective hydrolytic procedure should be determined and used.⁵³

Pyridoxal, pyridoxamine, and pyridoxine are equal in activity for animals

under many conditions\textsuperscript{56, 57}; under others, pyridoxal and pyridoxamine may show slightly less activity than pyridoxine. The three forms show very different activities, however, for many microorganisms.\textsuperscript{56} All lactic acid bacteria, for example, are unable to utilize pyridoxine for growth; most use pyridoxal and pyridoxamine, some only pyridoxal.\textsuperscript{56} These organisms, therefore, are not useful for determining the total vitamin B\textsubscript{6} content of natural materials. For many yeasts and molds, however, all three forms show equal activity;\textsuperscript{56} consequently, these organisms are most useful for assay purposes.

Among these latter organisms, assay methods utilizing \textit{Neurospora sitotropa} pyridoxineless\textsuperscript{53, 58, 59} and \textit{Saccharomyces carlsbergensis} \textsuperscript{4228}\textsuperscript{53, 60-62} have been studied most thoroughly. Both yield excellent results; the latter procedure, however, is simpler, faster, and more convenient. Growth of the test organism in the vitamin B\textsubscript{6}-free medium increases with the vitamin B\textsubscript{6} concentration in the range from 0 to about 0.01 γ per 10 ml. of medium. Pure vitamin B\textsubscript{6} (any of the forms may be used as standard) and samples to supply the vitamin at several levels within this range are added to individual 1 in. by 8 in. tubes containing 4 ml. of medium. Each tube is then diluted to 9 ml., plugged or otherwise covered, and sterilized by heating in flowing steam at 100° for 10 minutes. After cooling, 1 ml. of inoculum suspension (0.3 mg. of moist yeast) is added, and the tubes are shaken mechanically for 16 to 18 hours. Yeast growth is then determined turbidimetrically. The responses to various levels of the pure vitamin are plotted to yield a standard curve, from which the vitamin B\textsubscript{6} content of the sample aliquots can be determined by interpolation. Details of the procedure have been treated elsewhere.\textsuperscript{51, 60}

If it is desired to determine pyridoxal plus pyridoxamine, but not pyridoxine, \textit{Streptococcus faecalis} may be used as the assay organism.\textsuperscript{53, 62, 63} Similarly, use of \textit{Lactobacillus casei} as the assay organism permits the specific estimation of pyridoxal.\textsuperscript{53, 62, 64} Differential assays of this type reveal that most of the vitamin B\textsubscript{6} present in yeast, meats, glandular organs, etc., is present as pyridoxal and pyridoxamine, with only traces (if any) of

IX. OCCURRENCE IN FOODS

pyridoxine. In plant tissues, however, all three forms of the vitamin occur in similar amounts. The significance of this difference in distribution is not yet known.

VIII. Standardization of Activity
HENRY SHERMAN

When vitamin B₆ was first discovered it was defined as "that part of the vitamin B complex that is responsible for the cure of the specific dermatitis developed by young rats on a vitamin B-free diet supplemented with purified thiamine and lactoflavin." The standardization of materials possessing vitamin B₆ activity was based upon the curative effect they possessed against this specific dermatitis or rat acrodynia. György defined the provisional unit for the estimation of vitamin B₆ or the "rat-day dose" as "the minimum quantity of the substance that would cause healing of the specific dermatitis." The earlier concentrates possessing vitamin B₆ activity were adsorbates or eluates prepared from yeast or bran extracts. One milliliter of a so-called Peters' eluate possessed one "rat-day dose" of vitamin B₆.

When pyridoxine was isolated as the active principle and was synthesized, it became the reference standard; the pyridoxine content of natural materials, as measured by biological assay, was expressed in equivalent amounts of the compound. One "rat-day dose" of vitamin B₆ became equivalent to approximately 10 μg of pyridoxine. Pyridoxine is available commercially as the hydrochloride; it is a white crystalline powder which is remarkably stable. A U.S.P. reference standard of pyridoxine hydrochloride is available for all assay procedures, chemical, physical, microbiological, and biological.

IX. Occurrence in Foods
ESMOND E. SNELL and CHARLES S. KEEVL, JR.

Most analytical figures for the vitamin B₆ content of foods are not satisfactory, primarily because (1) several forms of the vitamin occur naturally

1 P. György, Nature 133, 498 (1934).
that have different activities for different test organisms, and occur in different ratios in different foodstuffs; (2) the bound forms of these vitamins are inactive for many microorganisms and show unusual hydrolytic behavior, in that they are more rapidly hydrolyzed by low acid concentrations than by high; (3) these facts were not known in earlier studies dealing with chemical and biological estimation of vitamin B₆, and have been insufficiently considered in many later studies.

*Saccharomyces carlsbergensis* and *Neurospora sitophila* respond equally to each of the three forms of vitamin B₆ and, when used with a satisfactory hydrolytic procedure, should give reliable estimates for the vitamin B₆ content of a sample. Values obtained with these organisms and by rat assay are listed as “preferred values” in the tables below. The hydrolytic procedure used for liberating the vitamin is indicated by a letter (a–m) in parentheses; of these procedures, a and h appear most satisfactory at present.

Figures obtained by rat assay, although listed with the “preferred values,” are nonetheless subject to considerable error, since on some but not all rations pyridoxal and pyridoxamine are somewhat less active than pyridoxine in supporting growth.

The various hydrolytic procedures used for liberating vitamin B₆ are listed in summary form below:

<table>
<thead>
<tr>
<th>Extractant</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) 0.055 N H₂SO₄, 180 ml./g. finely divided sample</td>
<td>Autoclave at 15 lb. (120°) for 1 hr.</td>
</tr>
<tr>
<td>(b) 1 N HCl, 40 ml./1-5 g.</td>
<td>Autoclave at 15 lb. for 1 hr.</td>
</tr>
<tr>
<td>(c) 2 N H₂SO₄, 1:200 dilution</td>
<td>Autoclave at 15 lb. for 30 min.</td>
</tr>
<tr>
<td>(d) 1 N H₂SO₄, 50 ml./g.</td>
<td>Autoclave at 15 lb. for 30 min.</td>
</tr>
<tr>
<td>(e) 20 mg. of papain and of takadiastase per gram of sample</td>
<td>Incubate at 37° for 24 hr.</td>
</tr>
<tr>
<td>(f) 2 N HCl, 10 ml./g.</td>
<td>Autoclave at 20 lb. for 5 hr.</td>
</tr>
<tr>
<td>(g) NaOH pretreatment of sample followed by HCl hydrolysis as in (b)</td>
<td>Autoclave at 20 lb. for 5 hr.</td>
</tr>
<tr>
<td>(h) 0.055 N HCl, 180 ml./g.</td>
<td>Autoclave at 20 lb. for 5 hr.</td>
</tr>
<tr>
<td>(i) 0.1 N H₂SO₄ at 80° for 30 min.; pepsin digestion at 38° for 24 hr.</td>
<td>Autoclave at 20 lb. for 5 hr.</td>
</tr>
<tr>
<td>(j) No hydrolysis; unknown finely divided, shaken with adsorbent prior to chemical assay</td>
<td>Autoclave at 20 lb. for 5 hr.</td>
</tr>
<tr>
<td>(k) 0.04 N H₂SO₄, Autoclave at 15 lb. for 1 hr., then 70 ml. papain-takadiastase as in (e)</td>
<td>100° for 2 hr.</td>
</tr>
<tr>
<td>(l) 4 N HCl</td>
<td>100° for 2 min.</td>
</tr>
<tr>
<td>(m) 2% CH₃COOH</td>
<td>100° for 2 min.</td>
</tr>
</tbody>
</table>

Table II gives values for the vitamin B₆ content of several foodstuffs that have been assayed repeatedly by different procedures; an idea of the magnitude of the variation observed (due to variations both in procedure and in the samples themselves) may be gained from it.

In Table III, the vitamin B₆ content of a variety of foods, together with the assay organism and extraction procedure, is summarized. In all cases,
<table>
<thead>
<tr>
<th>Product</th>
<th>Chemical assay</th>
<th>Saccharomyces carlsbergensis</th>
<th>Neurospora sitophila</th>
<th>Other organism</th>
<th>Rat assay, γ/g.</th>
<th>Preferred values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley, ground seed</td>
<td></td>
<td>3.2 (a)</td>
<td>1.6 (b), 2 5.6 (h)</td>
<td>0.2 (a)</td>
<td>3.2-5.6</td>
<td>3.2-5.6</td>
</tr>
<tr>
<td>Beef heart, fresh</td>
<td></td>
<td>2.9 (a)</td>
<td>1.2 (a)</td>
<td>2.07</td>
<td>2.0-2.9</td>
<td>2.0-2.9</td>
</tr>
<tr>
<td>Beef liver, fresh</td>
<td></td>
<td>7.1 (a), 7.1 (a)</td>
<td>1.7 (a)</td>
<td>6.0</td>
<td>6.0-7.1</td>
<td>6.0-7.1</td>
</tr>
<tr>
<td>Beef liver, dry</td>
<td>80.0 (k), 86.1 (k)</td>
<td></td>
<td>1.2 (e), 1.7 (e)</td>
<td>20.5</td>
<td>20.5</td>
<td>20.5</td>
</tr>
<tr>
<td>Beef muscle, fresh</td>
<td></td>
<td>2.3 (a), 3.7 (a)</td>
<td>0.77 (e)</td>
<td>3.21</td>
<td>2.3-3.2</td>
<td>2.3-3.2</td>
</tr>
<tr>
<td>Corn, whole, yellow</td>
<td>7.9 (i)</td>
<td>7.4 (a), 4.8 (h)</td>
<td>0.7 (e)</td>
<td>3.6, 3.9, 4.3</td>
<td>3.6-5.7</td>
<td>3.6-5.7</td>
</tr>
<tr>
<td>Lamb, muscle, fresh</td>
<td></td>
<td>2.9 (h)</td>
<td>0.61 (e)</td>
<td>2.5, 3.7</td>
<td>2.5-3.7</td>
<td>2.5-3.7</td>
</tr>
<tr>
<td>Lamb, muscle, dry</td>
<td></td>
<td>9.3 (a)</td>
<td>2.7 (e)</td>
<td>9.3-9.7</td>
<td>9.3-9.7</td>
<td>9.3-9.7</td>
</tr>
<tr>
<td>Milk, whole</td>
<td>1.8 (i)</td>
<td>0.54 γ/e.c. (a)</td>
<td>0.58 (b), 0.67 γ/e.c. (b)</td>
<td>0.51 (e)</td>
<td>0.54-1.1</td>
<td>0.54-1.1</td>
</tr>
<tr>
<td>Milk whole, dried</td>
<td></td>
<td>5.5 (a), 3.3 (a)</td>
<td>8.2 (b), 7.0 (g)</td>
<td>-</td>
<td>3.8</td>
<td>3.3-8.2</td>
</tr>
<tr>
<td>Pork ham</td>
<td></td>
<td>3.3 (a)</td>
<td>1.7 (e)</td>
<td>1.8, 4.8</td>
<td>3.3-4.8</td>
<td>3.3-4.8</td>
</tr>
<tr>
<td>Pork liver, fresh</td>
<td></td>
<td>5.9 (a)</td>
<td>4.3 (b)</td>
<td>2.9</td>
<td>2.9-5.9</td>
<td>2.9-5.9</td>
</tr>
<tr>
<td>Pork muscle, fresh</td>
<td></td>
<td>6.8 (a)</td>
<td>3.3 (b)</td>
<td>4.1</td>
<td>3.3-6.8</td>
<td>3.3-6.8</td>
</tr>
<tr>
<td>Soybeans</td>
<td>12.0 (k)</td>
<td>8.0 (h)</td>
<td>6.4 (c), 11.8 (c), 20.</td>
<td>7.1</td>
<td>7.1-12.0</td>
<td>7.1-12.0</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>15.0 (l)</td>
<td>13.8 (b)</td>
<td>15.7 (c)</td>
<td>13.8</td>
<td>13.8</td>
<td>13.8</td>
</tr>
<tr>
<td>Wheat germ</td>
<td></td>
<td>14.8 (b), 16.0 (b)</td>
<td>15.7 (c)</td>
<td>10.5, 22, 9.6</td>
<td>8.5-16.0</td>
<td>8.5-16.0</td>
</tr>
<tr>
<td>Wheat meal</td>
<td></td>
<td>3.3 (i)</td>
<td>3.1 (b)</td>
<td>13.0, 8.5</td>
<td>3.3, 12</td>
<td>1.7-3.4</td>
</tr>
<tr>
<td>White flour</td>
<td></td>
<td>1.7 (a)</td>
<td>3.1 (b)</td>
<td>2.0</td>
<td>1.2-2.6</td>
<td>1.2-2.6</td>
</tr>
<tr>
<td>Whole wheat flour</td>
<td>10.1, 11, 8.1 (i)</td>
<td>4.7 (a), 6.0 (f)</td>
<td>2.6 (e)</td>
<td>4.1, 4.6</td>
<td>3.8-6.0</td>
<td>3.8-6.0</td>
</tr>
<tr>
<td>Yeast, brewers, fresh</td>
<td>7.2 (d)</td>
<td>4.1 (h)</td>
<td>-</td>
<td>4.0</td>
<td>5.7-24.2</td>
<td>5.7-24.2</td>
</tr>
<tr>
<td>Yeast, brewers, dry</td>
<td>68.0, 70.0, 52.0 (k), 10</td>
<td>5.7 (a), 24.2 (a)</td>
<td>1.0 (e)</td>
<td>20.0</td>
<td>40.0-57.0</td>
<td>40.0-57.0</td>
</tr>
</tbody>
</table>

*a Hydrolytic procedures used are keyed to the list in the text by the letter in parentheses; references are indicated by superscript numbers.
such figures represent the sum of the pyridoxine, pyridoxal, and pyridoxamine contents.

In Table IV, results of the only study so far made to determine the distribution of the individual forms of the vitamin are recorded. The values for pyridoxine, which usually occurs in the smallest amount, are subject to by far the largest error. The fallacy in referring to the "pyridoxine" content of foodstuffs, rather than to their vitamin B₆ content, is evident from this table.

These data show that vitamin B₆ is widely distributed, and much more uniformly so than most of the other vitamins. Muscle meats, liver, vegetables, whole grain cereals, and especially the bran from cereal grains are among the best sources; few materials can be classed as really poor sources. This wide distribution undoubtedly is a reflection of the important and multiple roles played by the vitamin in anabolic and catabolic reactions of the amino acids and proteins and probably explains the fact that naturally occurring deficiency diseases due to lack of this vitamin have not so far been found.

4 P. F. Sharp, J. B. Shields, and A. P. Stewart, Jr., Inst. Food Technol. 6, 54 (1945).
19 P. R. Burkholder, Science 98, 188 (1943).
20 P. R. Burkholder and I. McVeigh, Plant Physiol. 20, 301 (1945).
22 A. M. Copping, Biochem. J. 37, 12, (1943).
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#### TABLE III

**The Vitamin B<sub>6</sub> Content of Various Foodstuffs**

<table>
<thead>
<tr>
<th>Product</th>
<th>Vitamin B&lt;sub&gt;6&lt;/sub&gt; content, γ/g.*</th>
<th>Range of preferred values, γ/g.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dairy Products</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cheese</td>
<td>0.98 (S. cer.) (c)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Egg, hens, dried</td>
<td>2.3 (S. cer.) (e),&lt;sup&gt;12&lt;/sup&gt; 0.85 (S. cer.) (e)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Egg, hens, fresh</td>
<td>0.48 (S. cer.) (e),&lt;sup&gt;12&lt;/sup&gt; 0.22 (S. cer.) (e)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Milk, cow’s, evaporated</td>
<td>0.62 (S. carls.) (a)&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.62</td>
</tr>
<tr>
<td>Milk, cow’s, dry skim</td>
<td>5.5 (S. carls.) (a)&lt;sup&gt;5&lt;/sup&gt;</td>
<td>5.5</td>
</tr>
<tr>
<td>Milk, cow’s, dry whole</td>
<td>See Table II</td>
<td>3.3–8.2</td>
</tr>
<tr>
<td>Milk, cow’s, whole</td>
<td>See Table II</td>
<td>0.51–1.1</td>
</tr>
<tr>
<td>Milk, goat’s</td>
<td>0.13 γ/ml. (S. cer.) (e)&lt;sup&gt;27&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Milk, mare’s</td>
<td>0.01 γ/ml. (S. cer.) (e)&lt;sup&gt;27&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Milk, human</td>
<td>0.08 γ/ml. (S. cer.) (e)&lt;sup&gt;27&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td><strong>Fish Products</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atlantic mackerel, canned, wet solids</td>
<td>2.1 (S. carls.) (a)&lt;sup&gt;28&lt;/sup&gt;</td>
<td>2.1</td>
</tr>
<tr>
<td>Atlantic sardines, canned, wet solids</td>
<td>1.6 (S. carls.) (a)&lt;sup&gt;28&lt;/sup&gt;</td>
<td>1.6</td>
</tr>
<tr>
<td>Cod, fresh</td>
<td>3.4 (rat)&lt;sup&gt;7&lt;/sup&gt;</td>
<td>3.4</td>
</tr>
<tr>
<td>Flounder, fresh</td>
<td>1.0 (S. cer.) (e)&lt;sup&gt;12&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Halibut, fresh</td>
<td>1.1 (S. cer.) (e)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Pacific mackerel, canned, wet solids</td>
<td>2.7 (S. carls.) (a)&lt;sup&gt;28&lt;/sup&gt;</td>
<td>2.7</td>
</tr>
<tr>
<td>Pacific sardines, canned, wet solids</td>
<td>2.8 (S. carls.) (a)&lt;sup&gt;28&lt;/sup&gt;</td>
<td>2.8</td>
</tr>
<tr>
<td>Salmon, canned, wet solids</td>
<td>4.5 (S. carls.) (a)&lt;sup&gt;28&lt;/sup&gt;</td>
<td>4.5</td>
</tr>
<tr>
<td>Salmon, fresh</td>
<td>5.9 (rat),&lt;sup&gt;7&lt;/sup&gt; 0.33 (S. cer.) (e)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>5.9</td>
</tr>
<tr>
<td>Tuna, canned, wet solids</td>
<td>4.4 (S. carls.) (a)&lt;sup&gt;28&lt;/sup&gt;</td>
<td>4.4</td>
</tr>
<tr>
<td><strong>Fruit and Fruit Products</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apple, fresh</td>
<td>0.26 (S. cer.) (e)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Banana, fresh</td>
<td>3.2 (S. cer.) (e)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Cantaloupe, fresh</td>
<td>0.36 (S. cer.) (e)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Grapefruit juice, canned</td>
<td>0.08–0.18 γ/ml. (S. carls.) (a)&lt;sup&gt;29&lt;/sup&gt; 0.14 (S. carls.) (a)&lt;sup&gt;30&lt;/sup&gt;</td>
<td>0.08–0.18 γ/ml.</td>
</tr>
<tr>
<td>Grapefruit juice, fresh</td>
<td>0.08–0.18 γ/ml. (S. carls.) (a)&lt;sup&gt;29&lt;/sup&gt;</td>
<td>0.08–0.18 γ/ml.</td>
</tr>
<tr>
<td>Grapefruit sections</td>
<td>0.17–0.24 (S. carls.) (a),&lt;sup&gt;29&lt;/sup&gt; 0.09 (S. cer.) (e)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.17–0.24</td>
</tr>
<tr>
<td>Lemon juice, fresh</td>
<td>0.35 γ/ml. (S. carls.) (a)&lt;sup&gt;8&lt;/sup&gt;</td>
<td>0.35 γ/ml.</td>
</tr>
<tr>
<td>Orange juice, canned</td>
<td>0.16–0.31 γ/ml. (S. carls.) (a)&lt;sup&gt;29&lt;/sup&gt;</td>
<td>0.16–0.31 γ/ml.</td>
</tr>
<tr>
<td>Orange juice, fresh</td>
<td>0.18–0.32 γ/ml. (S. carls.) (a)&lt;sup&gt;29&lt;/sup&gt;</td>
<td>0.18–0.56 γ/ml.</td>
</tr>
<tr>
<td>Orange sections, Valencia</td>
<td>0.31 (S. carls.) (a)&lt;sup&gt;29&lt;/sup&gt;</td>
<td>0.31</td>
</tr>
<tr>
<td>Peaches, canned, wet solids</td>
<td>0.16 (S. carls.) (a)&lt;sup&gt;30&lt;/sup&gt;</td>
<td>0.16</td>
</tr>
<tr>
<td>Peaches, fresh</td>
<td>0.16 (S. cer.) (e)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Raisins</td>
<td>0.94 (S. cer.) (e)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Product</td>
<td>Vitamin B&lt;sub&gt;4&lt;/sub&gt; content, γ/g.*</td>
<td>Range of preferred values, γ/g.</td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>------------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>Strawberries, fresh</td>
<td>0.44 (S. cer.) (e)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Tangerine juice, canned</td>
<td>0.33 γ/ml. (S. carls.) (a)&lt;sup&gt;29&lt;/sup&gt;</td>
<td>0.33 γ/ml.</td>
</tr>
<tr>
<td>Tangerine juice, fresh</td>
<td>0.23 γ/ml. (S. carls.) (a)&lt;sup&gt;29&lt;/sup&gt;</td>
<td>0.23 γ/ml.</td>
</tr>
<tr>
<td>Watermelon, fresh</td>
<td>0.33 (S. cer.) (e)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>—</td>
</tr>
</tbody>
</table>

**Grain Products**

<table>
<thead>
<tr>
<th>Product</th>
<th>Vitamin B&lt;sub&gt;4&lt;/sub&gt; content, γ/g.*</th>
<th>Range of preferred values, γ/g.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn, canned, wet solids</td>
<td>0.68 (S. carls.) (a)&lt;sup&gt;30&lt;/sup&gt;</td>
<td>0.68</td>
</tr>
<tr>
<td>Corn, golden bantam, dry</td>
<td>7.0 (S. cer.) (e)&lt;sup&gt;31&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Corn grits</td>
<td>2.0 (rat)&lt;sup&gt;9&lt;/sup&gt;</td>
<td>2.0</td>
</tr>
<tr>
<td>Corn meal, white</td>
<td>0.67 (S. cer.) (e)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Corn meal, yellow&lt;sup&gt;7&lt;/sup&gt;</td>
<td>3.8 (S. cer.) (e)&lt;sup&gt;12&lt;/sup&gt; 5.6 (N. sito.) (g)&lt;sup&gt;18&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Corn steep liquor</td>
<td>9.8 (N. sito.) (b)</td>
<td>9.8</td>
</tr>
<tr>
<td>Corn, whole yellow</td>
<td>See Table II</td>
<td>3.6–5.7</td>
</tr>
</tbody>
</table>

**Rice**

<table>
<thead>
<tr>
<th>Product</th>
<th>Vitamin B&lt;sub&gt;4&lt;/sub&gt; content, γ/g.*</th>
<th>Range of preferred values, γ/g.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice, finished</td>
<td>3.4 (N. sito.) (b)&lt;sup&gt;32&lt;/sup&gt; 4.5 (chem.) (m)&lt;sup&gt;33&lt;/sup&gt; 3.4</td>
<td>—</td>
</tr>
<tr>
<td>Rice paddy</td>
<td>8.1 (N. sito.) (b)&lt;sup&gt;22&lt;/sup&gt;</td>
<td>8.1</td>
</tr>
<tr>
<td>Rice, raw, husked</td>
<td>6.9 (chem.) (i)&lt;sup&gt;13&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Rice, raw, milled</td>
<td>3.3 (chem.) (i)&lt;sup&gt;13&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Rice, whole</td>
<td>10.3 (chem.) (m)&lt;sup&gt;38&lt;/sup&gt;</td>
<td>—</td>
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</tbody>
</table>

**Wheat**

<table>
<thead>
<tr>
<th>Product</th>
<th>Vitamin B&lt;sub&gt;4&lt;/sub&gt; content, γ/g.*</th>
<th>Range of preferred values, γ/g.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat bran</td>
<td>See Table II</td>
<td>13.8–15.7</td>
</tr>
<tr>
<td>Wheat flour, patent</td>
<td>0.9 (S. cer.) (e)&lt;sup&gt;12&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Wheat germ</td>
<td>See Table II</td>
<td>8.5–16.0</td>
</tr>
<tr>
<td>Wheat germ extract</td>
<td>49.0 (chem.) (j)&lt;sup&gt;34&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Wheat, ground seed</td>
<td>2.1 (S. cer.) (e)&lt;sup&gt;6&lt;/sup&gt; 3.7 (N. sito.) (b)&lt;sup&gt;2&lt;/sup&gt; 3.7</td>
<td>—</td>
</tr>
<tr>
<td>Wheat meal</td>
<td>See Table II</td>
<td>1.7–3.4</td>
</tr>
<tr>
<td>White bread</td>
<td>1.0 (S. carls.) (a)&lt;sup&gt;8&lt;/sup&gt; 0.06 (S. cer.) (e)&lt;sup&gt;6&lt;/sup&gt; 1.0</td>
<td>—</td>
</tr>
<tr>
<td>White flour</td>
<td>See Table II</td>
<td>3.8–6.0</td>
</tr>
<tr>
<td>Whole wheat bread</td>
<td>4.2 (S. carls.) (a)&lt;sup&gt;8&lt;/sup&gt; 0.58 (S. cer.) (e)&lt;sup&gt;6&lt;/sup&gt; 4.2</td>
<td>—</td>
</tr>
</tbody>
</table>

**Miscellaneous**

<table>
<thead>
<tr>
<th>Product</th>
<th>Vitamin B&lt;sub&gt;4&lt;/sub&gt; content, γ/g.*</th>
<th>Range of preferred values, γ/g.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley, malt</td>
<td>11.9 (chem.) (k)&lt;sup&gt;11&lt;/sup&gt; 12.8 (chem.) (k)&lt;sup&gt;11&lt;/sup&gt; —</td>
<td>—</td>
</tr>
<tr>
<td>Barley, ground seed</td>
<td>See Table II</td>
<td>3.2–5.6</td>
</tr>
<tr>
<td>Buckwheat, ground seed</td>
<td>3.5 (N. sito.) (b)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>3.5</td>
</tr>
<tr>
<td>Cottonseed meal</td>
<td>13.1 (S. cer.) (e)&lt;sup&gt;12&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Millet</td>
<td>3.3 (N. sito.) (b)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>3.3</td>
</tr>
<tr>
<td>Oats, hulled</td>
<td>0.92 (S. carls.) (a)&lt;sup&gt;35&lt;/sup&gt; 0.3 (S. ovi-formis) (e)&lt;sup&gt;4&lt;/sup&gt; 0.92</td>
<td>—</td>
</tr>
<tr>
<td>Oats, rolled</td>
<td>0.93 (S. carls.) (a)&lt;sup&gt;35&lt;/sup&gt; 1.5 (S. carls.) (h)&lt;sup&gt;9&lt;/sup&gt; 2.5 (rat)&lt;sup&gt;9&lt;/sup&gt; 0.93–1.5</td>
<td>—</td>
</tr>
<tr>
<td>Rye, ground seed</td>
<td>3.0 (S. carls.) (h)&lt;sup&gt;9&lt;/sup&gt; 3.7 (N. sito.) (b)&lt;sup&gt;2&lt;/sup&gt; 3.0–3.7</td>
<td>—</td>
</tr>
<tr>
<td>Sorghum</td>
<td>2.1–8.6 (S. carls.) (a)&lt;sup&gt;36&lt;/sup&gt; 2.1–8.6</td>
<td>—</td>
</tr>
</tbody>
</table>

**Meat Products**

<table>
<thead>
<tr>
<th>Product</th>
<th>Vitamin B&lt;sub&gt;4&lt;/sub&gt; content, γ/g.*</th>
<th>Range of preferred values, γ/g.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef brain, fresh</td>
<td>1.6 (S. carls.) (a)&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1.6</td>
</tr>
<tr>
<td>Beef brain, dry</td>
<td>4.3 (rat)&lt;sup&gt;7&lt;/sup&gt;</td>
<td>4.3</td>
</tr>
<tr>
<td>Beef heart, fresh</td>
<td>See Table II</td>
<td>2.0–2.9</td>
</tr>
<tr>
<td>Beef heart, dry</td>
<td>5.1 (S. cer.) (e)&lt;sup&gt;6&lt;/sup&gt; 9.2 (rat)&lt;sup&gt;7&lt;/sup&gt; 9.2</td>
<td>—</td>
</tr>
</tbody>
</table>
TABLE III—Continued

<table>
<thead>
<tr>
<th>Product</th>
<th>Vitamin B&lt;sub&gt;6&lt;/sub&gt; content, γ/g.*</th>
<th>Range of preferred values, γ/g.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef kidney, fresh</td>
<td>3.9 (S. carls.) (a),&lt;sup&gt;8&lt;/sup&gt; 8.4 (S. carls.) (a),&lt;sup&gt;9&lt;/sup&gt; 9.9 (S. carls.) (b),&lt;sup&gt;10&lt;/sup&gt; 3.5 (rat)&lt;sup&gt;7&lt;/sup&gt;</td>
<td>3.5-9.9</td>
</tr>
<tr>
<td>Beef kidney, dry</td>
<td>16.5 (rat)&lt;sup&gt;7&lt;/sup&gt;</td>
<td>16.5</td>
</tr>
<tr>
<td>Beef liver, fresh</td>
<td>See Table II</td>
<td>6.0-7.1</td>
</tr>
<tr>
<td>Beef liver, dry</td>
<td>See Table II</td>
<td>20.5</td>
</tr>
<tr>
<td>Beef muscle, fresh</td>
<td>See Table II</td>
<td>2.3-3.2</td>
</tr>
<tr>
<td>Beef muscle, dry</td>
<td>See Table II</td>
<td>12.5</td>
</tr>
<tr>
<td>Beef tongue, fresh</td>
<td>1.3 (S. carls.) (a),&lt;sup&gt;5&lt;/sup&gt; 1.0 (rat)&lt;sup&gt;7&lt;/sup&gt;</td>
<td>1.0-1.3</td>
</tr>
<tr>
<td>Beef tongue, dry</td>
<td>3.5 (rat)&lt;sup&gt;7&lt;/sup&gt;</td>
<td>3.5</td>
</tr>
<tr>
<td>Chicken, dark meat, fresh</td>
<td>&lt;2.0 (rat)&lt;sup&gt;7&lt;/sup&gt;</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>Chicken, breast, fresh</td>
<td>1.3 (S. cer.) (c)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Horse muscle, dry</td>
<td>9.8-10.4 (N. sito.) (b)&lt;sup&gt;37&lt;/sup&gt;</td>
<td>9.8-10.4</td>
</tr>
<tr>
<td>Frankfurters</td>
<td>1.3 (S. carls.) (a)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.3</td>
</tr>
<tr>
<td>Lamb liver, fresh</td>
<td>3.0 (rat)&lt;sup&gt;7&lt;/sup&gt;</td>
<td>3.0</td>
</tr>
<tr>
<td>Lamb liver, dry</td>
<td>10.3 (rat)&lt;sup&gt;7&lt;/sup&gt;</td>
<td>10.3</td>
</tr>
<tr>
<td>Lamb muscle, fresh</td>
<td>See Table II</td>
<td>2.5-3.7</td>
</tr>
<tr>
<td>Lamb muscle, dry</td>
<td>See Table II</td>
<td>9.3-9.7</td>
</tr>
<tr>
<td>Lamb stew meat, fresh</td>
<td>2.3 (S. carls.) (a)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2.3</td>
</tr>
<tr>
<td>Lamb stew meat, dry</td>
<td>6.4 (S. carls.) (a)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>6.4</td>
</tr>
<tr>
<td>Pork, bacon</td>
<td>2.0 (S. cer.) (c)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Pork, heart, fresh</td>
<td>2.9 (rat)&lt;sup&gt;7&lt;/sup&gt;</td>
<td>2.9</td>
</tr>
<tr>
<td>Pork, heart, dry</td>
<td>12.8 (rat)&lt;sup&gt;7&lt;/sup&gt;</td>
<td>12.8</td>
</tr>
<tr>
<td>Pork, ham, fresh</td>
<td>See Table II</td>
<td>3.3-4.8</td>
</tr>
<tr>
<td>Pork, ham, dry</td>
<td>19.0 (rat)&lt;sup&gt;7&lt;/sup&gt; 5.1 (S. cer.) (c)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>19.0</td>
</tr>
<tr>
<td>Pork, kidney, fresh</td>
<td>3.3 (rat)&lt;sup&gt;7&lt;/sup&gt;</td>
<td>3.3</td>
</tr>
<tr>
<td>Pork, kidney, dry</td>
<td>14.8 (rat)&lt;sup&gt;7&lt;/sup&gt;</td>
<td>14.8</td>
</tr>
<tr>
<td>Pork, liver, fresh</td>
<td>See Table II</td>
<td>2.9-5.9</td>
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<tr>
<td>Pork, liver, dry</td>
<td>8.2 (rat)&lt;sup&gt;7&lt;/sup&gt;</td>
<td>8.2</td>
</tr>
<tr>
<td>Pork, muscle, fresh</td>
<td>See Table II</td>
<td>3.3-6.8</td>
</tr>
<tr>
<td>Pork, muscle, dry</td>
<td>13.0 (rat)&lt;sup&gt;7&lt;/sup&gt; 5.0 (S. cer.) (c)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>13.0</td>
</tr>
<tr>
<td>Sheep heart, fresh</td>
<td>1.6 (rice moth larva) (i)&lt;sup&gt;26&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Sheep kidney, fresh</td>
<td>1.7 (rice moth larva) (i)&lt;sup&gt;38&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Sheep liver, fresh</td>
<td>6.1 (rice moth larva) (i),&lt;sup&gt;38&lt;/sup&gt; 13.8 (chem.) (i)&lt;sup&gt;13&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Sheep muscle, fresh</td>
<td>4.6 (chem.) (i),&lt;sup&gt;13&lt;/sup&gt; 2.4 (rice moth larva) (i),&lt;sup&gt;38&lt;/sup&gt; 0.43 (S. cer.) (c)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Veal leg, fresh</td>
<td>3.7 (S. carls.) (a)&lt;sup&gt;5&lt;/sup&gt;</td>
<td>3.7</td>
</tr>
<tr>
<td>Veal leg, dry</td>
<td>11.0 (S. carls.) (a)&lt;sup&gt;5&lt;/sup&gt;</td>
<td>14.0</td>
</tr>
<tr>
<td>Veal liver, fresh</td>
<td>3.0 (rat)&lt;sup&gt;7&lt;/sup&gt;</td>
<td>3.0</td>
</tr>
<tr>
<td>Veal liver, dry</td>
<td>8.4 (rat)&lt;sup&gt;7&lt;/sup&gt;</td>
<td>8.4</td>
</tr>
<tr>
<td>Veal muscle, fresh</td>
<td>2.8 (N. sito.) (b),&lt;sup&gt;2&lt;/sup&gt; 2.9 (rat)&lt;sup&gt;7&lt;/sup&gt;</td>
<td>2.8, 2.9</td>
</tr>
<tr>
<td>Veal muscle, dry</td>
<td>11.5 (rat)&lt;sup&gt;7&lt;/sup&gt;</td>
<td>11.5</td>
</tr>
<tr>
<td>Veal shoulder, fresh</td>
<td>3.0 (S. carls.) (a)&lt;sup&gt;5&lt;/sup&gt;</td>
<td>3.0</td>
</tr>
<tr>
<td>Veal shoulder, dry</td>
<td>11.0 (S. carls.) (a)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>11.0</td>
</tr>
<tr>
<td>Veal shoulder, fresh</td>
<td>3.5 (S. carls.) (a),&lt;sup&gt;6&lt;/sup&gt; 1.3 (S. cer.) (c)&lt;sup&gt;6&lt;/sup&gt; 3.5</td>
<td>—</td>
</tr>
<tr>
<td>Veal shoulder chop, dry</td>
<td>12.0 (S. carls.) (a)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>12.0</td>
</tr>
<tr>
<td>Veal sirloin chop, fresh</td>
<td>4.1 (S. carls.) (a)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>4.1</td>
</tr>
<tr>
<td>Veal sirloin chop, dry</td>
<td>11.0 (S. carls.) (a)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>11.0</td>
</tr>
<tr>
<td>Veal stew meat, fresh</td>
<td>3.3 (S. carls.) (a)&lt;sup&gt;5&lt;/sup&gt;</td>
<td>3.3</td>
</tr>
<tr>
<td>Veal stew meat, dry</td>
<td>12.0 (S. carls.) (a)&lt;sup&gt;5&lt;/sup&gt;</td>
<td>12.0</td>
</tr>
<tr>
<td>Product</td>
<td>Vitamin Bs content, γ/g.*</td>
<td>Range of preferred values, γ/g.</td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>---------------------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td><strong>Vegetables</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asparagus, green, canned, wet solids</td>
<td>0.30 (S. carls.) (a)⁴⁰</td>
<td>0.30</td>
</tr>
<tr>
<td>Beans, French, fresh</td>
<td>0.96 (rice moth larva) (i)⁴⁰</td>
<td>—</td>
</tr>
<tr>
<td>Beans, green, canned, wet solids</td>
<td>0.32 (S. carls.) (a)⁴⁰</td>
<td>0.32</td>
</tr>
<tr>
<td>Beans, lima, dry</td>
<td>6.0 (S. cer.) (c)⁶</td>
<td>—</td>
</tr>
<tr>
<td>Beets, fresh</td>
<td>0.13 (S. cer.) (c)⁶</td>
<td>—</td>
</tr>
<tr>
<td>Beets greens, fresh</td>
<td>0.37 (S. cer.) (c)⁶</td>
<td>—</td>
</tr>
<tr>
<td>Beet root, fresh</td>
<td>1.1 (chem.) (i)¹³</td>
<td>—</td>
</tr>
<tr>
<td>Cabbage, fresh</td>
<td>2.9 (chem.) (i),¹¹ 1.2 (S. cer.) (c),⁶</td>
<td>2.5 (rice moth larva) (i)³⁴</td>
</tr>
<tr>
<td>Cauliflower, fresh</td>
<td>0.20 (S. cer.) (c)⁶</td>
<td>—</td>
</tr>
<tr>
<td>Carrots, canned, wet solids</td>
<td>0.22 (S. carls.) (a)⁴⁰</td>
<td>0.22</td>
</tr>
<tr>
<td>Carrots, raw</td>
<td>1.9 (chem.) (i), 2.2 (rice moth larva) (i),¹⁸ 1.2 (S. cer.) (c)⁶</td>
<td>—</td>
</tr>
<tr>
<td>Lettuce, fresh</td>
<td>0.71 (S. cer.) (c)⁶</td>
<td>—</td>
</tr>
<tr>
<td>Mushrooms fresh</td>
<td>0.45 (S. cer.) (c)⁶</td>
<td>—</td>
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<tr>
<td>Okra, fresh</td>
<td>0.75 (S. cer.) (c)⁶</td>
<td>—</td>
</tr>
<tr>
<td>Onions, fresh</td>
<td>0.63 (S. cer.) (c)⁶</td>
<td>—</td>
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<tr>
<td>Peas, blackeyed, fresh</td>
<td>1.9 (S. cer.) (c)⁶</td>
<td>—</td>
</tr>
<tr>
<td>Peas, canned, wet solids</td>
<td>0.46 (S. carls.) (a)⁴⁰</td>
<td>0.46</td>
</tr>
<tr>
<td>Peas, split, dry</td>
<td>1.6 (S. carls.) (a), 3.3 (rat)⁹</td>
<td>1.6-3.3</td>
</tr>
<tr>
<td>Peanuts</td>
<td>3.0 (S. cer.) (c)⁶</td>
<td>—</td>
</tr>
<tr>
<td>Potato, fresh</td>
<td>1.6 (chem.) (i), 2.5 (rice moth larva) (i),¹⁸ 2.2 (S. cer.) (c)⁶</td>
<td>—</td>
</tr>
<tr>
<td>Spinach, canned, wet solids</td>
<td>0.6 (S. carls.) (a)⁴⁰</td>
<td>0.60</td>
</tr>
<tr>
<td>Soybeans</td>
<td>See Table II</td>
<td>7.1-12</td>
</tr>
<tr>
<td>Tomato, canned, wet solids</td>
<td>0.71 (S. carls.) (a)⁴⁰</td>
<td>0.71</td>
</tr>
<tr>
<td>Turnips, fresh</td>
<td>1.1 (S. cer.) (c)⁶</td>
<td>—</td>
</tr>
<tr>
<td>Yams, fresh</td>
<td>3.2 (S. cer.) (c)⁶</td>
<td>—</td>
</tr>
<tr>
<td><strong>Yeast Products</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast, baker’s, fresh</td>
<td>7.0 (S. carls.) (a), 6.2 (S. carls) (a)¹⁴</td>
<td>6.2-7.0</td>
</tr>
<tr>
<td>Yeast, brewer’s, dry</td>
<td>See Table II</td>
<td>40.0-57.0</td>
</tr>
<tr>
<td><strong>Miscellaneous</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ale</td>
<td>0.5 γ/ml. (S. carls.) (c)¹⁴</td>
<td>0.5 γ/ml.</td>
</tr>
<tr>
<td>Beer</td>
<td>0.5 γ/ml. (N. sito.) (b),²⁹ 0.60-γ/ml. (S. carls.) (c)¹⁴</td>
<td>0.5-0.6 γ/ml.</td>
</tr>
<tr>
<td>Chocolate</td>
<td>0.23 (S. cer.) (c)⁶</td>
<td>—</td>
</tr>
<tr>
<td>Grass, dried (Cerophyllum)</td>
<td>9.2 (S. carls.) (a),⁹ 5.3 (rat),⁹ 12.3 5.3-10.3</td>
<td>(S. carls.) (F)³¹</td>
</tr>
<tr>
<td>Honey</td>
<td>0.04-0.27 (S. carls.) (a),¹⁰ 2.4-4.8 0.04-0.27 (chem.)⁴¹</td>
<td>—</td>
</tr>
<tr>
<td>Malt extract</td>
<td>5.4 (N. sito.) (b)²</td>
<td>5.4</td>
</tr>
<tr>
<td>Molasses, blackstrap</td>
<td>20.0 (chem.) (l),²¹ 24.9 (S. cer.) (c)¹²</td>
<td>—</td>
</tr>
<tr>
<td>Molasses, Brown</td>
<td>2.7 (S. cer.) (c)⁶</td>
<td>—</td>
</tr>
<tr>
<td>Sugar, brown</td>
<td>0.7 (S. cer.) (c)¹²</td>
<td>—</td>
</tr>
<tr>
<td>Stout (English)</td>
<td>0.87 (S. carls.) (c)¹</td>
<td>0.87</td>
</tr>
<tr>
<td>Tapioca</td>
<td>3.2 (rice moth larva) (i)³⁸</td>
<td>—</td>
</tr>
<tr>
<td>Wine, burger</td>
<td>0.66-2.0 (rat)⁴²</td>
<td>0.66-2.0</td>
</tr>
<tr>
<td>Wine, Tokay</td>
<td>0.72-2.14 (rat)⁴²</td>
<td>0.70-2.1</td>
</tr>
</tbody>
</table>

* Abbreviations to the assay procedures are as follow: S. cer., Saccharomyces cerevisiae; S. carls., Saccharomyces carlsbergensis; N. sito., Neurospora sitophila pyridoxines; chem., chemical. Hydrolytic procedures are keyed to the list in the text by the letters in parentheses; references are indicated by superscript numbers.
### IX. OCCURRENCE IN FOODS

#### TABLE IV

<table>
<thead>
<tr>
<th>Product</th>
<th>Pyridoxal</th>
<th>Pyridoxamine</th>
<th>Pyridoxine†</th>
<th>Total B₆</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef liver, fresh</td>
<td>5.8 ± 0.58</td>
<td>21.6 ± 2.8</td>
<td>-2.5 ± 3.3</td>
<td>25.0</td>
</tr>
<tr>
<td>Celery, fresh</td>
<td>6.4 ± 0.7</td>
<td>1.7 ± 0.7</td>
<td>7.4 ± 1.6</td>
<td>15.4</td>
</tr>
<tr>
<td>Carrot, fresh</td>
<td>1.7 ± 0.2</td>
<td>0.2 ± 0.2</td>
<td>5.8 ± 0.8</td>
<td>7.7</td>
</tr>
<tr>
<td>Chicken heart, fresh</td>
<td>6.4 ± 0.7</td>
<td>8.4 ± 1.4</td>
<td>-1 ± 2</td>
<td>13.8</td>
</tr>
<tr>
<td>Chicken liver, fresh</td>
<td>31.3 ± 3.3</td>
<td>32 ± 5.6</td>
<td>0 ± 8</td>
<td>63.3</td>
</tr>
<tr>
<td>Corn meal, yellow</td>
<td>1.2 ± 0.8</td>
<td>0.7 ± 0.2</td>
<td>0 ± 0.3</td>
<td>1.9</td>
</tr>
<tr>
<td>Egg white</td>
<td>0.16 ± 0.02</td>
<td>0.09 ± 0.02</td>
<td>0.3 ± 0.07</td>
<td>0.55</td>
</tr>
<tr>
<td>Egg, whole</td>
<td>4.6 ± 0.5</td>
<td>0.84 ± 0.5</td>
<td>0 ± 0.9</td>
<td>5.4</td>
</tr>
<tr>
<td>Egg yolk</td>
<td>9.0 ± 0.8</td>
<td>2.8 ± 1.4</td>
<td>0 ± 2</td>
<td>11.8</td>
</tr>
<tr>
<td>Fish, frozen</td>
<td>8.2 ± 0.8</td>
<td>13.3 ± 2.1</td>
<td>-1 ± 3</td>
<td>22.5</td>
</tr>
<tr>
<td>Lemon, whole</td>
<td>2.5 ± 0.25</td>
<td>0.5 ± 0.3</td>
<td>8.3 ± 1.6</td>
<td>11.3</td>
</tr>
<tr>
<td>Lettuce, leaf</td>
<td>10.0 ± 0.8</td>
<td>-1 ± 2</td>
<td>7.1 ± 1.6</td>
<td>16.4</td>
</tr>
<tr>
<td>Liver powder, Wilson 1:20</td>
<td>5.0 ± 0.5</td>
<td>21.6 ± 2.8</td>
<td>12.0 ± 4.1</td>
<td>38.6</td>
</tr>
<tr>
<td>Milk, whole, fresh, γ/ml.</td>
<td>0.26 ± 0.02</td>
<td>0.06 ± 0.06</td>
<td>-0.02 ± 0.05</td>
<td>0.30</td>
</tr>
<tr>
<td>Peas, split</td>
<td>0.40 ± 0.04</td>
<td>0.3 ± 0.01</td>
<td>1.1 ± 0.2</td>
<td>1.8</td>
</tr>
<tr>
<td>Pepper, green</td>
<td>6.8 ± 0.70</td>
<td>37.0 ± 4.2</td>
<td>16 ± 6.6</td>
<td>60.0</td>
</tr>
<tr>
<td>Rat kidney</td>
<td>27.2 ± 2.5</td>
<td>9.1 ± 3.5</td>
<td>8.3 ± 4.9</td>
<td>44.6</td>
</tr>
<tr>
<td>Rat liver</td>
<td>24.0 ± 2.5</td>
<td>6.3 ± 2.8</td>
<td>7.4 ± 4.1</td>
<td>37.7</td>
</tr>
<tr>
<td>Wheat germ</td>
<td>0.72 ± 0.08</td>
<td>0.5 ± 0.01</td>
<td>2.5 ± 0.6</td>
<td>3.7</td>
</tr>
<tr>
<td>Wheat, whole</td>
<td>1.6 ± 0.16</td>
<td>2.6 ± 0.3</td>
<td>7.4 ± 1.6</td>
<td>11.6</td>
</tr>
<tr>
<td>Yeast, baker's, fresh</td>
<td>6.2 ± 0.7</td>
<td>7.7 ± 1.4</td>
<td>0 ± 2</td>
<td>13.9</td>
</tr>
<tr>
<td>Yeast, brewer's, dry</td>
<td>4.0 ± 0.4</td>
<td>25 ± 2.8</td>
<td>-1 ± 4</td>
<td>28.0</td>
</tr>
</tbody>
</table>

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*From Rabinowizt and Snell.*

†Calculated [total B₆-(pyridoxal + pyridoxamine)]. These values are very inexact, since they bear the cumulative errors of the three values from which they are calculated.

37 L. Perlman and A. F. Morgan, *Food Research* 10, 334 (1915).
X. Effects of Deficiency

A. IN PLANTS

HENRY SHERMAN

The intact green plant is generally able to synthesize adequate amounts of the B vitamins so that it is independent of any external source for these vitamins. Most species of the higher plants can grow in the light in media containing only inorganic salts. At times, however, an external source of the B vitamins, especially thiamine, niacin, and pyridoxine, may accelerate growth.

Individual parts of the plant, however, differ widely in their ability to synthesize these vitamins; those plant tissues which do not synthesize the vitamins at an adequate rate usually obtain their supply from those parts that do. Thiamine, niacin, and pyridoxine function as hormones in plants; they are synthesized in one part of the plant—the leaf—and are transported, usually in the phloem, to those sections where they are needed. Isolated roots of higher plants require thiamine, niacin, and pyridoxine for growth. Tomato root tips have been maintained on a solution of mineral salts, cane sugar, thiamine, and pyridoxine through twenty-five successive passages. They have also been transferred from liquid media to agar cultures but only in the presence of pyridoxine. Isolated roots from carrot, sunflower, acacia, and Jimson weed also require pyridoxine for growth.

In the intact plant these essential vitamins are synthesized in the leaves and translocated to the roots. When isolated roots are cultured in media which are deficient in the above B vitamins, there is evidence of reduced growth in length. Pyridoxine and thiamine deficiency also result in lowered cell division activity in the root meristem.

There is also evidence that thiamine and pyridoxine are also involved in the process of root growth in cuttings.

Fries investigated the role of pyridoxine, thiamine, and biotin in promoting the growth of certain ascomycetes. Pyridoxine was the only vitamin that was required by all the species investigated. In fact, one species, Ophiostoma multianulatum, was used with some success in determining the pyridoxine content of various malt extracts.

1 J. Bonner and H. Bonner, Vitamins and Hormones 6, 225 (1948).
3 F. W. Went, Am. Scientist 31, 189 (1943).
5 D. Day, Science 94, 468 (1941).
6 N. Fries, Symbolae Botan. Upsaliensis 7, 73 (1943).
B. IN ANIMALS
HENRY SHERMAN

It is comparatively simple to induce vitamin B₆ deficiency symptoms in animals; deficient synthetic diets and/or pyridoxine antagonists, such as desoxypyridoxine, have been employed for this purpose. A description of the deficiency varies with the species of animal, but there are a few general symptoms which appear to be common to most animals. The differences among the animals appear to be in degree rather than in kind. In general, vitamin B₆ deficiency in animals induces retarded growth, several types of anemia, epileptiform fits, and characteristic lesions of the skin. The vitamin complex appears, therefore, to play an important role in blood, nerve, and skin metabolism.

1. Rats

Poor weight gain is one of the earliest signs of a vitamin B₆ deficiency; this is often apparent within five days. Acccompanying this failure in growth are usually a decrease in appetite and a marked interference in efficiency of food utilization. With this poor growth there is a reduction in the size of the accessory organs of reproduction and decreased sexual behavior. Simple omission of the vitamin has only a slightly adverse effect on reproduction. However, the administration of desoxypyridoxine in the deficiency diet induces a high incidence of resorptions. The growth of suckling young born from vitamin B₆-deficient mothers is retarded early in the lactation period, and survival to weaning is very rare.

Vitamin B₆ avitaminosis induces a symmetrical dermatitis or acrodynia in the peripheral areas of the body such as the tail, paws, nose, mouth, and ears. Very often, this acrodynia is accompanied by an edema of the corium and a scaliness of the extremities. This pathological condition was, in fact, used as the basis of the first biological assay procedure. There have been reports, however, that the acrodynia cannot be produced consistently and that the vitamin only modifies some of the dermal lesions.

15 W. L. Damm, J. Biol. Chem. 128, XVIII (1939).
essential fatty acids, linoleic and arachidonic acids, are closely interrelated with vitamin B₆ in the etiology of these skin lesions.

The kidneys and adrenal glands are also adversely affected by a vitamin B₆ deficiency. Very early in the deficiency, the rat develops slight lesions in the glomerulus, which eventually result in scarring and pitting.¹⁵a The adrenal glands enlarge during the deficiency, especially the zona fasciculata; this leads to an impairment of function which is generally characterized by a decreased rate of water excretion.¹⁵b, ¹⁵c

Although anemia is not a regular symptom of vitamin B₆ deficiency in the rat, there are other indications that vitamin B₆ is involved in maintaining the integrity of the blood system. The regeneration of red blood cells after hemorrhage is delayed and inadequate in vitamin B₆-deficient rats.¹⁶, ¹⁶a, ¹⁶b Deficient rats show a microcytosis, an increase in red blood cell count, reduction in mean corpuscular diameter, granulocytosis, lymphopenia, and many normoblasts.¹⁷ There is a marked reduction in the ability of the deficient rat to form circulating antibodies to foreign erythrocytes;¹⁷-¹⁹ this may be due to atrophy of the thymus in these deficient rats.²⁰, ²¹ Total body iron and copper are significantly increased in vitamin B₆ deficiency, suggesting an increase in their absorption.²²

Vitamin B₆ deficiency invariably leads to nervous disorders. Chick et al.²³ described the appearance of spontaneous convulsive seizures in rats maintained for long periods of time on diets deficient in vitamin B₆. These seizures were characterized by hyperexcitability, circular running, tonic-clonic convulsions, and a comatose recovery period. These observations were confirmed by Lepkovsky et al.²⁴ Daniel et al.²⁵ described similar seizures in young rats being nursed by mothers maintained on a vitamin B₆-deficient diet. Patton et al.²⁶ observed that spontaneous convulsive seizures developed in young rats being suckled by mothers on a deficiency

¹⁵b R. B. Stebbins, Am. J. Physiol. 166, 538 (1951).
¹⁶b W. W. Hawkins and B. Lechow, Rec. can. biol. 11, 65 (1952).
X. EFFECTS OF DEFICIENCY

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diet toward the end of the lactation period. These symptoms could be alleviated by the administration of 10 \( \gamma \) of pyridoxine per day, but even 50 \( \gamma \) of pyridoxine per day could not protect healthy rats against similar convulsive seizures induced by sound. The inclusion of 25 to 150 \( \gamma \) of pyridoxine per day in the diets of rats protected them against spontaneous seizures but not against sound-induced seizures; with higher levels of pyridoxine the seizures were both delayed and less severe. Davenport and Davenport\(^{27}\) demonstrated that the pyridoxine-deficient rat showed increased brain excitability measured by the decrease in electroshock threshold. Administration of pyridoxine and/or glutamic acid increased the threshold, whereas, tryptophan, which intensifies the B\(_6\) deficiency, reduced it. These observations suggest that maintenance of the transaminase system is essential for normal brain function.

Vitamin B\(_6\)-deficient rats often exhibit muscular dystrophy, especially in the cardiac region.\(^{28,\,29}\)

Since vitamin B\(_6\) is intimately involved in the decarboxylase\(^{30}\) and transaminase\(^{31}\) enzyme systems, it is not uncommon to observe disturbances in protein metabolism in vitamin B\(_6\) deficiency. Acrodynia is more severe in pyridoxine-deficient rats fed a high protein diet than in rats fed a low protein diet.\(^{32}\) Peretti\(^{33,\,34}\) observed that B\(_6\) deficiency had no effect on nitrogen balance or nitrogen metabolism, but Hawkins et al.\(^{35}\) could increase the fasting blood levels of urea and non-protein nitrogen by feeding pyridoxine-deficient rats on a high protein diet. Beaton et al.\(^{35a}\) also demonstrated a significant increase in the fasting level of blood urea, which, they claimed, was the result of a true metabolic disturbance and not renal failure. The avitaminotic rats also exhibited an increased urinary nitrogen excretion, suggesting an impairment in ability to utilize dietary nitrogen. Terroine\(^{36}\) depleted young male rats of vitamin B\(_6\) on a high protein diet; when they were then placed on a protein-free diet, the normal rats lost weight more rapidly than did the deficient animals. Total urinary urea, ammonia, and amino nitrogen were higher in deficient than in normal rats.

\(^{30}\) E. F. Gale, Advances in Enzymol. 6, 1 (1946).
\(^{32}\) L. R. Cerecedo and J. R. Foy, Arch. Biochem. 5, 207 (1944).
\(^{33}\) G. Peretti, Boll. soc. ital. biol. sper. 16, 306 (1941).
\(^{34}\) G. Peretti, Boll. soc. ital. biol. sper. 17, 321 (1942).
\(^{36}\) T. Terroine, Arch. sci. physiol. 4, 91 (1950).
The excretion of creatinine was higher in deficient rats than in control rats. Martin\textsuperscript{37} showed that the toxicity of L-tyrosine was less in pyridoxine-deficient animals than in control animals.

A derangement in tryptophan metabolism is very common in vitamin B\textsubscript{6} deficiency. Vitamin B\textsubscript{6}-deficient rats, or rats receiving desoxypyridoxine, excrete more xanthurenic acid in the urine than do normal animals, when they are fed tryptophan.\textsuperscript{38} Such a derangement in tryptophan metabolism is one of the earliest symptoms of vitamin B\textsubscript{6} deficiency in higher animals and man.\textsuperscript{39} This increased excretion of urinary xanthurenic acid by the deficient rat is diminished by niacin supplementation.\textsuperscript{40}

Sheppard and McHenry\textsuperscript{41} found that in vitamin B\textsubscript{6}-deficient rats the pyridoxine content of liver, kidney, and leg muscle was independent of protein intake. With a constant protein intake, the pyridoxine concentration of liver is proportional to dietary pyridoxine concentration up to 25 \(\gamma\) per rat per day; the content of pyrodixine in the liver can also be increased by keeping the dietary pyridoxine content constant and increasing the protein level. Irradiation does not affect the concentration of vitamin B\textsubscript{6} in the liver of normal or deficient rats.\textsuperscript{42}

Many enzyme systems which are intimately involved in the catabolism of proteins are affected by a vitamin B\textsubscript{6} deficiency. The livers of vitamin B\textsubscript{6}-deficient rats have only one-third the kynureninase activity of livers of normal rats.\textsuperscript{43, 43a} The heart and kidney-cortex tissues of avitaminotic rats had only 40\% transaminase activity of similar tissues from control animals; however, the succinic acid oxidase activity of these deficient tissues was 80 to 90\% the activity of normal tissues.\textsuperscript{44} The glutamic acid decarboxylase activity of the brain of the vitamin B\textsubscript{6}-deficient rat is 50\% less active than that of a normal brain.\textsuperscript{44a} The \textit{in vitro} transfer of sulfur from homocysteine to serine is retarded with liver extracts of vitamin B\textsubscript{6}-deficient rats; the addition of phosphopyridoxal restores this transsulfurization enzyme sys-
tem to normal. These livers show a lower rate of cystathionine cleavage than normal specimens. The cysteine desulfhydrolase content of liver extracts of pyridoxine-deficient rats fed a high protein diet is lower than that obtained from a normal rat. This decrease in desulfhydrolase concentration takes place a few days before many of the other signs of B6 vitaminosis occur. Homogenized kidney from vitamin B6-deficient rats had only one-third the p-amino acid oxidase activity of normal kidney. A supplement of p-amino acids decreased nitrogen utilization by vitamin B6-deficient animals; l-amino acids had no effect. Pyridoxine-deficient rats gained less water and protein than did rats receiving a complete diet.

A vitamin B6 deficiency causes abnormal fat metabolism. The carcasses of pyridoxine-deficient rats contain less fat, and of a higher degree of unsaturation, than the carcasses of normal rats; they also have a higher percentage of arachidonic acid. The low linoleic acid content of carcass fat of pyridoxine-deficient rats is not a specific characteristic of the deficiency, for caloric and thiamine deficiencies produce similar changes. It has been demonstrated that vitamin B6 is essential for the conversion of protein to fat. The rate of absorption of lipids from the intestine does not appear to be affected by the deficiency. The livers of pyridoxine-deficient rats, however, have more fat than those of normal rats. There is a decrease in the rate of oxidation of short-chained fatty acids by liver slices from pyridoxine-deficient rats.

Severe vitamin B6 deficiency depressed the basal metabolic rate and increased the respiratory quotient of rats. Riboflavin deficiency had no effect on the basal metabolic rate. Vitamin B6 deficiency increases the

50 H. Sherman, Vitamins and Hormones 8, 55 (1951).
51 G. Medes, M. V. Mann, and J. B. Hunter, Arch. Biochem. and Biophys. 32, 70 (1951).
specific dynamic action of amino acids such as glutamic acid.\textsuperscript{58, 59} It is believed that vitamin B\textsubscript{6} deficiency delays the deamination of amino acids.\textsuperscript{60} The adult avitaminotic rat cannot adjust to cold very efficiently.\textsuperscript{60a}

Vitamin B\textsubscript{6} deficiency decreased the concentration of pantothenic acid in the liver by 78\%, in the kidney by 28\%, and in muscle by 18\%.\textsuperscript{61} Liver and kidney niacin concentration decreased 15\% and liver riboflavin content decreased 22\% in vitamin B\textsubscript{6}-deficient rats.

2. Mice

In young mice, a vitamin B\textsubscript{6} deficiency induces poor growth and paralysis of the hind legs but no characteristic dermatitis. In adult mice, the deficiency results in failure to maintain body weight and death within two months. Acute stages of the deficiency in adult mice are often accompanied by pathological skin lesions and necrotic tails.\textsuperscript{62} De Renzo and Cerecedo\textsuperscript{63} produced an acrodynia in the mouse by feeding a diet deficient in vitamin B\textsubscript{6} and desoxypyrudoxine. A diet high in casein or an equivalent amount of methionine aggravated the skin lesions and shortened survival time. Boutwell et al.\textsuperscript{63a} have also been able to produce acrodynia in mice with low vitamin B\textsubscript{6} containing diets.

There is the same close relationship between vitamin B\textsubscript{6} and protein metabolism in mice as there is in rats. Deficient mice excrete xanthurenic acid, especially when the diet is high in tryptophan.\textsuperscript{64} Pyridoxine-deficient mice fed diets containing 60\% casein lived only one-third as long as those fed 10\% casein. The tissue reserves of pyridoxine in vitamin B\textsubscript{6}-deficient mice diminished more rapidly when the diet contained 50\% casein than when it contained only 10\%.\textsuperscript{65} The mice on the high protein diet lost more weight and had a higher mortality rate than those on the low protein diet. This effect was not due to variation in caloric intake, or to unequal urinary excretion of pyridoxine, or to the tryptophan content of the diets, according to these authors. At low levels of vitamin B\textsubscript{6} intake, less vitamin was stored on the high protein diet, but with high levels of vitamin B\textsubscript{6} intake, a high

\textsuperscript{61} T. Terroin and J. Adrian, \textit{Arch. sci. physiol.} \textbf{4}, 435 (1950).
\textsuperscript{64} E. C. Miller and C. A. Baumann, \textit{J. Biol. Chem.} \textbf{167}, 551 (1945).
protein diet favored the storage of the vitamin. Mice on deficient diets often develop fatty livers. This condition appeared more rapidly on a high casein diet than on a low one. The addition of tryptophan to the deficient diet hastened fatty liver formation.

Silberberg and Levy demonstrated that, in young mice deprived of pyridoxine, cartilage growth and bone formation were inhibited; a high protein diet accentuated this effect. Interdental bone growth ceased after 2 weeks, and recession changes progressed in all periodontal structures. The mandibular condyle showed slight narrowing of the cartilage cap after 1 week.

Young Swiss mice which had been fed a special vitamin B₆-deficient diet and given an injection of desoxypyridoxine developed granulocytosis and lymphopenia. In leukemic mice of the Ak strain, the deficiency augmented the leukemic granulocytosis and shortened the survival time.

There was a marked regression of lymphosarcoma implants in deficient mice. Tumor implants failed to develop in mice deprived of vitamin B₆ prior to the inoculation.

### 3. Hamsters

Routh and Houchin reported that on a vitamin B₆-deficient diet the Syrian hamster developed an "acrodynia-like" dermatitis around the mouth, lost weight, and died within 24 days.

Schwartzman and Strauss have produced a vitamin B₆ deficiency in the male Syrian hamster. It was characterized by arrest of growth, diminished food and water intake, progressive malnutrition, muscular weakness, and changes of the fur. Increased amounts of xanthurenic acid were excreted in the urine. Deficient animals died in 12 to 13 weeks. There were no cutaneous changes comparable to the classical "rat acrodynia." Epileptiform seizures did not occur in the pyridoxine-deficient hamster, but there were other symptoms of nervous disorder, such as an ataxic gait, or paresis of one extremity, and priapism. Autopsy revealed a loss of fat tissue and atrophy of lymphoid tissues, especially the thymus. The transamination rate of heart muscle of pyridoxine-deficient hamsters was 30 to 40% lower than that of control animals fed ad libitum or restricted amounts of food; succinoxidase activity was comparable to that of control animals.

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4. Dogs

Fouts et al., first observed a nutritional microcytic hypochromic anemia in young dogs receiving a diet that was low in vitamin B₆. Adult dogs also developed this condition when placed on a similar diet. The blood plasma iron concentration progressively increased as the anemia became more severe. It dropped to a low-normal value with pyridoxine therapy. Total blood copper values were at a low-normal level during the anemia and increased to normal during pyridoxine administration. The pyridoxine-deficient dog also exhibited an increased urinary output of urea, ammonia, uric acid, and creatinine; xanthurenic acid was excreted in the urine of these deficient dogs. When desoxypyridoxine was fed to dogs, they exhibited atrophy of spleen, thymus, and lymph nodes; the ratio of spleen to body weight decreased. They developed epileptiform convulsions. The lipid content of the zona fasciculata and zona reticularis of the adrenal glands decreased, and the glands became larger than normal. The "rat acrodynia" symptoms did not appear in the adult dog. McKibbin et al. have shown that young puppies developed a different deficiency picture. No deficiency symptoms other than anemia, loss of weight, anorexia, and death were observed in puppies. Adult dogs kept for 300 days or more on a diet free of vitamin B₆ developed symptoms of cardiac failure including dyspnea, tachycardia, dilatation and hypertrophy of the right ventricle and the right auricle, accumulation of serous fluid in the thorax and chronic passive congestion of the liver; degenerative changes were found in the myelin sheaths of the peripheral nerves and of the spinal cord.

Vitamin B₆-deficient dogs show a decreased ability to convert protein into carbohydrate, as indicated by the decrease in the urinary dextrose:nitrogen ratio in phlorizinized animals on a high protein diet; no such impairment occurs in thiamine and riboflavin deficiencies. Dogs receiving a vitamin B₆-deficient diet and desoxypyridoxine show a sharp fall in the volume of gastric juice.

5. Pigs

Hughes and Squibb\textsuperscript{52} showed that pigs failed to grow on a vitamin B\textsubscript{6}-deficient diet, had rough coats, and had poor appetites. They later developed epileptiform fits which became more and more frequent. A microcytic hypochromic anemia was produced.\textsuperscript{52, 53} Blood hemoglobin and red blood cell counts were low; the size of the red blood cell was also reduced. The omission of pyridoxine and pantothenic acid from the diet of pigs led to the development of abnormal gait and degenerative changes in the peripheral nerves, the posterior root ganglia, the posterior roots, and the posterior funiculi of the spinal cord.\textsuperscript{54}

The pyridoxine-deficient animal continues to absorb iron; tissue iron is abundant, but its utilization is diminished.\textsuperscript{55} The rate of hemolysis does not increase. The hemosiderosis of the liver, the spleen, and the bone marrow of vitamin B\textsubscript{6}-deficient pigs can be prevented by restricting the dietary intake of iron. The urinary excretion of iron in pyridoxine deficiency is insignificant and is not altered from the normal. Serum iron is increased; it is in the ferric state.

Follis and Wintrobe\textsuperscript{56} investigated the nature of the changes in the nervous system produced by the deficiency. Ataxia was first manifested as a slightly high lift of the hind legs, together with a swaying of the hind quarters in walking. The hind legs often twisted in one direction or another. After 9 or 10 weeks of the deficiency, histologic changes were visible. Demyelization of the peripheral nerves occurred (brachial and sciatic); the nerve fibers with the larger diameters were affected most of all. The degeneration was characterized by the appearance of small droplets of neutral fat and by the loss of the fine reticular structure of the myelin sheath. Later, axis cylinder changes became evident. Myelin degeneration was found only in the peripheral nerves in the early stages, but in the later stages necrotic cells were found in the dorsal root ganglia.

Lehrer et al.\textsuperscript{57} produced a vitamin B\textsubscript{6} deficiency in 2-day-old baby pigs. Poor appetite was the earliest sign of the deficiency. Within 23 days the piglets showed muscular incoordination, spastic gait, epileptiform fits, rough hair coats, a brown exudate around the eyes and impairment of

vision. The administration of pyridoxine cured all but the impairment of eyesight.

Tryptophan metabolism is abnormal; xanthurenic acid is excreted in the urine.\(^8\) Fatty livers are often observed in these deficient animals.\(^9\)

### 6. Monkeys

Young rhesus monkeys (Macaca mulatta), weighing 1.5 to 2.0 kg., failed to grow normally when placed on a vitamin B\(_6\)-free diet.\(^8\) They maintained their initial weight for 9 months; then a marked weight loss occurred. During the depletion period, a hypochromic microcytic anemia developed; nucleated red cells appeared in the blood.\(^8\) Hemoglobin values dropped. There was an alteration in ratio of neutrophiles to lymphocytes and a marked polychromatophia developed. The animals were inactive, weak, and very irritable. They lost their appetite; they showed thinning of the fur and some graying of the fur on legs, arms, and back. With pyridoxine administration, growth was resumed and the blood picture improved. In most respects, the deficiency symptoms resembled those observed in swine and dogs. Mushett \textit{et al.}\(^7\) observed a similar picture when monkeys were given desoxypyradoxine.

Greenberg and Rinehart also investigated the symptoms of vitamin B\(_6\) deficiency in the monkey. They showed that the deficient monkey excreted xanthurenic acid in the urine.\(^8\) The blood vitamin B\(_6\) level of fourteen rhesus monkeys fell during the first 2 weeks of the deficiency and remained for one year at a low concentration of 2 to 3 γ per 100 ml. of whole blood.\(^9\) Control animals receiving 1 mg. of pyridoxine per day had corresponding blood levels of 5 to 20 γ. Rinehart and Greenberg\(^9\) maintained five immature monkeys on a synthetic pyridoxine-deficient diet for 5½ to 16 months. In the early part of the experiment, there was decreased food consumption, gradual weight loss, and diminished vigor. After 5 or 6 months, the animals became unkempt, sluggish, and hyperirritable when disturbed. No convulsions occurred. The hair became thinner and lighter; hair growth later ceased. Some animals showed edema of the eyelids, and most developed "some fissuring of the epithelium of the palms of the hands and feet." All developed a moderate leukopenia and anemia. Sclerotic lesions developed in the arteries of all deficient animals; the condition was

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more severe in those monkeys which had been longer on the deficient diet. The lesions were prominent in the coronary arteries, as well as in those of the kidneys and the pancreas. Advanced changes were found in the arteries of the testicle of one animal. The authors stated that "it was somewhat surprising to see an advanced arteriosclerosis in the vessels of an immature testis." The sclerotic lesions produced in the vitamin B_6-deficient monkeys closely resembled those of man.

The arteriosclerotic lesions appear to be a specific characteristic of a pyridoxine deficiency, for no such lesions were observed in rhesus monkeys that were thiamine deficient.\(^{93}\)

The subcutaneous injection of desoxypyridoxine induced a microcytic anemia, leucopenia, and lymphopenia.\(^{77}\) The blood-forming constituents and the fat of the femoral marrow were reduced; the adrenal glands were also affected, as in dogs.

7. Birds

Jukes\(^{94}\) reported that deficient chicks showed nervous symptoms, characterized by convulsive movements. Lepkovsky and Kratzer\(^{95}\) have described three stages of the pyridoxine deficiency syndrome. In stage 1, there was an abnormal excitability; in stage 2, the chick exhibited jerky convulsive movements; in stage 3, convulsions occurred. These convulsions appeared after about 12 days on the deficient diet; rats require 4 to 6 months for similar symptoms to appear. Growth is retarded and is accompanied by anorexia and extreme weakness. It is significant that the apyridoxic chick does not develop any dermatitis. Other symptoms of the deficiency include hyperthrombinemia, decreased clotting time,\(^{96}\) hypoplasia of the spleen, lymphoid atrophy,\(^{77}\) and lowered egg production and hatchability.\(^{97}\)

Turkeys develop nervous disorders on a vitamin B_6-deficient diet, characterized by convulsive seizures, but they do not develop any anemia.\(^{98}\)

Severe pyridoxine deficiency in young white Pekin ducklings is characterized by a very rapid cessation of growth and severe anemia.\(^{99}\) A chronic vitamin B_6 deficiency in older ducklings caused lack of growth, poor feathering, paralysis, convulsions, and severe microcytic anemia (low hemoglobin,


\(^{95}\) S. Lepkovsky and F. H. Kratzer, *J. Nutrition* 24, 315 (1942).


reduced red blood cell count, low hematocrit, and many young red blood cells).

8. Other Animals

The vitamin $B_6$ deficiency symptoms in fox pups are similar to those in dogs.\(^{106}\) There is a cessation of growth, anorexia, and reduced hemoglobin values.

Young rainbow trout (Salmo gairdnerii irideus) develop nervous disorders when vitamin $B_6$ is omitted from the diet; they also lose their ability to judge distances.\(^{101}\)

With the development of synthetic milk diets for young calves, it has been possible to limit the synthesis of vitamins by microorganisms in young ruminants. Young calves do not grow on a vitamin $B_6$-deficient diet. The excretion of vitamin $B_6$ and its metabolites is lowered.\(^{102}\) The deficiency is further characterized by lack of appetite, anorexia, sluggishness, listlessness, poor hair coat (dull and falling out) and, in some cases, epileptiform fits and death; there was no blood pathology.

C. IN MICROORGANISMS

ESMOND E. SNELL

Pyridoxine, the first member of the vitamin $B_6$ group to be recognized, was isolated by virtue of its vitamin activity for animals in 1938, and was synthesized in 1939. Its growth-promoting activity for certain yeasts\(^{103}, 104\) and lactic acid bacteria\(^{105}, 106\) was noted very shortly following its isolation. It is now known to be required for growth of a wide variety of bacteria, yeasts, and molds;\(^{107}, 108\) those not requiring the vitamin appear to synthesize it.

Microbiological assay of natural materials with lactic acid bacteria yielded extremely high values for their apparent "pyridoxine" content, as compared with values obtained by other methods; the existence of additional compounds with high vitamin $B_6$ activity was therefore postulated.\(^{109}\) These were eventually identified as pyridoxal and pyridoxamine.\(^{110}\)

\(^{103}\) R. E. Eakin and R. J. Williams, J. Am. Chem. Soc. 61, 1932 (1939).
\(^{107}\) B. C. J. G. Knight, Vitamins and Hormones 3, 105 (1943).
\(^{109}\) E. E. Snell, B. M. Guirard, and R. J. Williams, J. Biol. Chem. 143, 519 (1942).
became apparent after extensive assays that the lactic acid bacteria were essentially unable to utilize pyridoxine to satisfy their vitamin B₆ requirements, the growth-promoting activities previously observed for this compound being due to the chemical transformation of it, in minute yield, to pyridoxal or pyridoxamine (“pseudopyridoxine”) by interaction with other ingredients of the medium during autoclaving. ⁴¹⁴, ⁴¹² Because of this, many of these organisms grow suboptimally for lack of vitamin B₆ (pyridoxal or pyridoxamine) in media that contain an excess of pyridoxine. ⁴⁹, ⁴³ When such organisms are grown with small amounts of pyridoxine, therefore, they may show metabolic incapacities due to vitamin B₆ deficiency which are not apparent when they are grown with equally small amounts of pyridoxal or pyridoxamine. Examples of this behavior will appear later in the discussion. Different lactic acid bacteria also vary in the ease with which they use pyridoxamine and pyridoxal; the former is almost inactive for Lactobacillus casei, the latter is highly active. ⁴¹² Several lactic acid bacteria are also known for which only the phosphorylated compounds, pyridoxamine phosphate and pyridoxal phosphate, have high activity. ⁴¹⁴, ⁴¹⁵

Various clostridia so far examined, ⁴¹⁵ as well as the protozoan organism Tetrahymena geleii, ⁴¹⁶ resemble lactic acid bacteria in utilizing pyridoxal or pyridoxamine far more effectively than pyridoxine. For most yeasts and molds, on the other hand, all three compounds have very similar activities, ⁴¹² as they do in animals.

A deficiency of vitamin B₆ may produce a variety of effects in microorganisms in addition to those upon growth. These effects often reflect themselves in an altered quantitative requirement for the vitamin for growth under different environmental conditions. Several examples follow. It was early shown that Streptococcus faecalis, which requires vitamin B₆ for growth in a medium based on an acid-hydrolyzed casein supplemented with tryptophan, could grow without the vitamin if D-alanine were added. ⁴¹⁷ When grown under such conditions, little or no vitamin B₆ was synthesized; ⁴¹⁸ however, when cells were grown with vitamin B₆ and without D-alanine, the latter amino acid was synthesized and laid down in the cells. ⁴¹⁹ It was suggested that vitamin B₆ (pyridoxal phosphate) served as

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⁴¹⁷ G. W. Kidder and V. C. Dewey, Arch. Biochem. 20, 133 (1949); 21, 58 (1949).
a coenzyme for the synthesis of d- from L-alanine;\textsuperscript{119} this suggestion was confirmed by the demonstration\textsuperscript{120} that cells of this organism (and many other microorganisms) contain an enzyme capable of racemizing L-alanine, and that pyridoxal phosphate is its coenzyme. Thus in the presence of vitamin B\textsubscript{6} such cells synthesize d-alanine; in the absence of vitamin B\textsubscript{6} the deficient cells will grow only if this essential d-amino acid is supplied to them. Deficient cells grown under the latter conditions contain the aporacemase but cannot racemize L-alanine for lack of pyridoxal phosphate.\textsuperscript{120}

Cells of \textit{Streptococcus faecalis} grown either with DL-alanine under the above conditions or with low levels of vitamin B\textsubscript{6} have no detectable tyrosine decarboxylase activity, whereas when high levels of vitamin B\textsubscript{6} are supplied the activity of this enzyme is high.\textsuperscript{121} It was this finding with vitamin B\textsubscript{6}-deficient bacteria which led to the identification of pyridoxal phosphate as (1) the coenzymatic form of vitamin B\textsubscript{6}\textsuperscript{122} and (2) the coenzyme for the amino acid decarboxylases of both bacterial and mammalian cells.\textsuperscript{123, 124}

When cells of \textit{S. faecalis} are grown with DL-alanine replacing vitamin B\textsubscript{6}, they are unable to carry out transamination between aspartic and keto-glutaric acids, but they do so rapidly on addition of pyridoxal or pyridoxal phosphate.\textsuperscript{125} This unequivocal demonstration of the role of pyridoxal phosphate as a coenzyme for the glutamic-aspartic transaminase not only confirmed a role previously indicated for the vitamin\textsuperscript{126, 127} but showed that greatly differing amounts of vitamin B\textsubscript{6} were required to activate different vitamin B\textsubscript{6}-dependent reactions in the same cells. It had been observed previously that cells of this organism grown with high levels of vitamin B\textsubscript{6} carried out transamination and decarboxylation of tyrosine, whereas cells grown with small amounts of the vitamin carried out transamination, but not decarboxylation.\textsuperscript{128}

Several reports\textsuperscript{129-131} have pointed out that lactic acid bacteria grown with pyridoxine in the medium require certain amino acids for growth that are no longer essential if the pyridoxine is replaced by pyridoxamine.

\textsuperscript{120} W. A. Wood and I. C. Gunsalus, \textit{J. Biol. Chem.} \textbf{190}, 403 (1951).
\textsuperscript{123} E. F. Gale, \textit{Advances in Enzymol.} \textbf{6}, 1 (1946).
\textsuperscript{124} E. Werle, \textit{Angew. Chem.} \textbf{63}, 550 (1951).
\textsuperscript{130} J. L. Stokes and M. Guiness, \textit{Science} \textbf{101}, 43 (1945).
or pyridoxal. It will be apparent from the previous discussion that the pyridoxine-grown cells were somewhat deficient in vitamin $B_6$; the requirement for otherwise non-essential amino acids under these conditions is excellent evidence that vitamin $B_6$ is required for their synthesis. Such amino acids include cystine for $L$. casei, $L$. arabinosus, and $S$. faecalis; lysine, alanine, and threonine for $L$. delbrueckii, $L$. arabinosus, and $L$. casei; and threonine, lysine, alanine, serine, histidine, aspartic acid, phenylalanine, and tyrosine for $L$. arabinosus. In the presence but not in the absence of ample vitamin $B_6$, $L$. arabinosus also synthesizes tryptophan from indole, and $S$. faecalis synthesizes histidine from imidazole pyruvic acid. Indeed, each of the amino acids of casein must be present together with $\alpha$-alanine to permit growth of $S$. faecalis in the absence of vitamin $B_6$. In the presence of ample vitamin $B_6$, $\alpha$-alanine and many of the $L$. amino acids are synthesized by this organism. It thus appears that vitamin $B_6$ is intimately involved in synthesis of each of the "non-essential" amino acids in lactic acid bacteria; in their presence, the magnitude of the vitamin $B_6$ requirement falls either to zero or to such low levels that these organisms can synthesize the small remaining amount required.

In the presence of adequate amounts of vitamin $B_6$, many of the essential amino acids for lactic acid bacteria can be replaced by the corresponding keto or hydroxy acids; in the absence of the vitamin the keto and hydroxy acids are without growth-promoting activity. It was shown that the keto acids were transformed to amino acids by transamination; each of the transaminases involved was vitamin $B_6$-dependent and hence could not function in the absence of supplies of this vitamin.

Neurospora sitophila pyridoxineless requires approximately ten times as much vitamin $B_6$ for growth in the absence of thiamine as is required when thiamine is added. Vitamin $B_6$-deficient cultures of this organism thus require thiamine, which is synthesized when the supplies of vitamin $B_6$ are more liberal. Although not so interpreted by the investigators, the observation may indicate an important role for vitamin $B_6$ in the synthesis of thiamine. A different type of relationship between these two vitamins occurs in Saccharomyces carlsbergensis 4228 and in several similar yeasts in which thiamine suppresses growth. This toxic effect is effectively coun-

teracted by vitamin B₆. The mechanism by which excess thiamine causes a condition of vitamin B₆ deficiency in such organisms is not known.

The requirement for vitamin B₆ in the synthesis of nicotinic acid, noted in animals, becomes apparent in a widely used assay method for vitamin B₆ which employs Saccharomyces carlsbergensis 4228. This organism grows very well in the absence of nicotinic acid when vitamin B₆ is present in excess, and consequently nicotinic acid was not included in the initially proposed assay medium. With suboptimal amounts of vitamin B₆, however, nicotinic acid gives an "extra" growth response and must therefore be added to the medium when this is used for the determination of vitamin B₆ in natural materials.

D. IN MAN

P. GYÖRGY

Vitamin B₆ is required in the nutrition of all species of animals studied. Its role in metabolic processes is multiple and involves a large number of biochemical reactions. Deficiency of vitamin B₆ in animals manifests itself first in retardation of growth, which in itself is no indication for lack of a specific essential nutrient. This is followed by a variety of more characteristic manifestations of vitamin B₆ deficiency, such as cutaneous changes, microcytic anemia, convulsions, etc.

In general, it is expected that vitamins required by a large number of vertebrates are also necessary dietary constituents for man. Thus, it appeared to be reasonable to predict that there must be a human dietary requirement for vitamin B₆. However, in contrast to the great majority of vitamins no pathological or clinical conditions in human beings were known or identified up to the most recent past which could have been related to deficiency of vitamin B₆. As a matter of fact, the very widespread occurrence of vitamin B₆ in food products made it a priori improbable that a major deficiency of vitamin B₆ could develop in man. In the absence of such clear-cut deficiency conditions for vitamin B₆ there were three possibilities open to investigate the need for vitamin B₆ by man: (1) through the experimental production of vitamin B₆ deficiency; (2) through biochemical procedures which should detect even the initial stage of vitamin B₆ deficiency or a metabolic disarrangement indicating an increased requirement for vitamin B₆, and (3) through therapeutic tests in specific clinical conditions.

Furthermore, even these mental supplementation. The basal diet consisted of a mixture of sucrose, corn oil, vitamin-free casein, salt mixture, cod liver oil concentrate, and all known water-soluble vitamins, except pyridoxine. The unpalatability of the mixture made difficult the ingestion of a sufficient amount to supply the caloric needs of a moderately active man, with the net result of a definite weight loss. At the level taken the diet furnished 82.5 g. of protein and 50 mg. of iron daily. It is no wonder that on such an experimental diet the subject toward the end of the experimental period was complaining of "an unusual degree of depression and mental confusion." It may be assumed that the noted beneficial effect of a supplement of vitamin B₆ at this stage of the experiment was due more to a psychological than nutritional-metabolic effect. The authors themselves concluded that the experiment revealed no changes which could unequivocally be considered as resulting from a lack of vitamin B₆. Furthermore, even on the animal experiments it may be definitely stated that the experimental period of 55 days is too short to bring about deficiency of vitamin B₆ in adult animals or man.

More conclusive are the observations reported by Snyderman et al. These authors administered a vitamin B₆-deficient diet for therapeutic reasons to two mentally defective infants for 76 and 130 days, respectively. The first changes noted were of biochemical nature. Pyridoxic acid disappeared from the urine, and the total urinary pyridoxine was reduced to extremely low values ranging from 0.2 to 2 μg per day. "Subsequently, both infants lost the ability to convert tryptophan to nicotinic acid, an effect which was desired in order to block a metabolic path for tryptophan that might be competing with normal tissue synthesis. A plateauing of the weight curve occurred 33 and 73 days after the institution of the regime. On the 76th day one subject developed a series of convulsions which were promptly relieved by the administration of pyridoxine. The other subject developed a hypochromic anemia at approximately the 130th day. This responded dramatically to pyridoxine; a rise of reticulocytes was noted after 72 hours reaching a peak in 4 days after which red cell count and hemoglobin rose to normal. Both subjects gained weight normally after supplementation. In marked contrast to the excellent and prompt clinical response to the administration of pyridoxine was a delay in the reappearance in the ability to convert tryptophan to nicotinic acid."

The development of pyridoxine deficiency may be accelerated in adults with the use of a pyridoxine analog. Mueller and Vilter described symptoms of pyridoxine deficiency after prolonged administration of desoxypyridoxine.\textsuperscript{144, 144a} The pyridoxine analog was given to eight patients suffering from various chronic illnesses in doses of 60 to 150 mg, intramuscularly daily for periods of 18 to 55 days, while the subjects were maintained on a diet low in the vitamins of the B complex.

Seborrhea-like skin lesions developed about the eyes, nose, and mouth within 2 to 3 weeks in seven of the eight patients. In three of the seven the lesions were quite severe. Half of the patients developed erosions in and around the mouth resembling cheilosis of riboflavin deficiency and glossitis resembling morphologically that seen in niacin deficiency. One patient developed severe systemic symptoms: nausea, vomiting, weakness, and dizziness. The subjects with the most severe manifestations of deficiency were two patients with rheumatoid arthritis.

There was no anemia due to desoxypyridoxine. The total white count remained unchanged, but in seven of the eight patients there was a mild absolute lymphocytopenia.

The skin, mucous membrane, and systemic manifestations remained unchanged when a mixture containing thiamine, riboflavin, and nicotinamide was given, but they disappeared 48 to 72 hours after pyridoxine was administered.

During the period of the administration of desoxypyridoxine there was no increased excretion of xanthurenic acid in the urine. No tryptophan load test was performed.

The inhibitory ratio of antimetabolite to metabolite in human beings was not accurately measured but is at least 1:1.

The pathologic changes and clinical symptomatology observed under the influence of desoxypyridoxine as well as those seen in infants fed for a prolonged period of time a vitamin B\textsubscript{6}-deficient diet indicate not only the existing need of man for vitamin B\textsubscript{6} but also give an experimental foundation for the clinical and pathologic vitamin B\textsubscript{6} deficiency in man.

Even before the above-mentioned experimental clinical studies, claims were put forward regarding the usefulness of pyridoxine in various pathologic conditions in man, with the more or less silent assumption that primary or secondary lack of vitamin B\textsubscript{6} might play a part in the pathogenesis of these diseases. In other instances pyridoxine was tried therapeutically in the clinic on the basis of a possible analogy of the clinical conditions in question to similar manifestations in experimental vitamin B\textsubscript{6}.


deficiency in animals. The logic of such and similar attempts is certainly exceedingly thin, and it is no wonder that the observations reported were very contradictory and apparently often marred by subjective factors, emanating either from the authors or from the patients.

One group of such reports dealt with conditions involving the neuromuscular system, such as myasthenia, parkinsonism, chorea, epilepsy, muscular dystrophy, and related diseases. Beneficial results, claimed after administration of pyridoxine in patients with muscular weakness, in some cases of parkinsonism, even in poliomyelitis, in lightning pains accompanying tabes dorsalis and in multiple sclerosis, when given intraspinally, or in chorea, and in hypertrophic muscular dystrophy may be contrasted with negative and, in general, better-controlled observations in parkinsonism, in muscular dystrophy, in amyotrophic lateral sclerosis, and in epilepsy.

The similarity of the cutaneous lesions in experimental pyridoxine deficiency to desquamative and seborrheic or seborrhoid lesions in man stimulated the use of pyridoxine in the therapy of this widely represented clinical group of pathologic skin conditions. Negative results are again to some extent matched with beneficial results in seborrheic or seborrhoid conditions after treatment with pyridoxine.

118 N. Jolliffe, Minnesota Med. 23, 512 (1940).
130 A. M. Keith, J. Pediat. 20, 200 (1942).
133 W. Pehl, Z. Kinderheilk. 61, 613 (1940).
134 P. Gyorgy, unpublished observations.
The designation “acrodynia” for the syndrome of experimental pyridoxine deficiency in rats has been originally proposed without prejudice whether the condition in rats is analogous in its etiology with the identically named condition in children. The name was chosen only on the basis of the outward similarity of cutaneous manifestations and their distribution in rats with severe vitamin B₆ deficiency and in human acrodynia. As a matter of fact, the author found pyridoxine without benefit in human acrodynia. However, in contrast to these negative results, beneficial effects from medication with pyridoxine were claimed in human acrodynia by Frontali and Bose. The unpredictable and variable course of human acrodynia makes the so-called positive results less convincing than the lack of response in other cases on the same medication. Against Frontali’s further claim of lowered B₆ values in blood in acrodynia and their rise after specific medication, it deserves to be pointed out that the assay methods for B₆, especially for blood, are notoriously far from being satisfactory.

In cases of pellagra, pernicious anemia, and Mediterranean anemia, a slight increase in the granulocytic series of the white blood cells was observed. On the basis of this analogy Cantor and Scott have introduced the treatment of agranulocytosis with pyridoxine and claimed striking success in three cases. These observations were confirmed in agranulocytosis after treatment with thiouracil and nitrogen-mustard. Inconsistent response was noticed by Taylor in a case of agranulocytosis after treatment with thiouracil. During more recent years, after the successful introduction of intensive antibiotic treatment of agranulocytosis, it became difficult to assess the value of any other possible therapeutic supplements. One gains, however, the impression that the original claims regarding the value of vitamin B₆ in the treatment of agranulocytosis are shared at the present time by few if any hematologists.

Experimental vitamin B₆ deficiency may depress the lymphatic tissue and may also lead to relative lymphocytopenia, especially marked when lack of vitamin B₆ in the diet is combined with the administration of desoxy-pyridoxine as metabolic antagonist of pyridoxine. Gellhorn and Jones

168 Quoted by Bose in ref. 169.
172 M. M. Cantor and J. W. Scott, Science 100, 545 (1944).
175 H. Fleischhacker, Le Sang 21, 368 (1950).
178 A. Gellhorn and L. O. Jones, Blood 4, 60 (1949).
placed three patients with disseminated lymphosarcoma and three cases of leukemia on a vitamin B₆-deficient semi-synthetic, chiefly casein diet, together with desoxypyridoxine for short periods of up to 14 days. Although there was evidence of malnutrition in the form of weight loss and weakness, no specific signs of vitamin B₆ deficiency developed. Two patients had acute toxic manifestations after the administration of large doses of desoxypyridoxine. These were characterized by transient epileptiform convulsions. There were no sequelae and no recurrence of the symptoms when the dose of the drug was reduced. There was no unequivocal evidence of depression of hemopoiesis, no significant atrophy of lymphoid tissue, and no signs of demyelination of nerves. Tested without a supplementary load of tryptophan, the excretion of xanthurenic acid was not increased. These observations seem to indicate that the restriction of vitamin B₆ in the diet together with desoxypyridoxine for periods up to 2 weeks had no therapeutic effect in lymphosarcoma and leukemia in human patients.

A large number of investigators studied the effect of pyridoxine medication in nausea and vomiting of pregnancy. In thirty-seven cases Willis and his associates have observed very satisfactory results from administration of pyridoxine (50 mg.). Similar beneficial results were observed by other authors. On the other hand Hesseltine, on the basis of a controlled study of sixteen cases, concluded that the use of pyridoxine for hyperemesis gravidarum is valueless and without justification.

It is difficult to assess the results for any medical treatment in a condition such as nausea and vomiting of pregnancy, greatly influenced by purely emotional factors. Such scepticism applies equally well to positive as well as to negative findings.

The same reserved attitude may be observed for the similar syndrome of radiation sickness. Bergman claimed beneficial effect with pyridoxine (in doses of 100 mg. intramuscularly before and after the surgical procedure) in the prevention of nausea and especially that of vomiting following general anesthesia. However, these results were not confirmed by Kernis and Stodsky. Greater unanimity is found regarding the treatment of radi-

179 L. Kernis and B. Stodsky, Anesthesiology 11, 212 (1950).
tion sickness with pyridoxine. Various doses were used, from 25 to 200 mg. of pyridoxine. All reports recorded beneficial effects, in some instances well controlled with placebos.

The greatest difficulty in accepting the beneficial effect of pyridoxine in conditions such as nausea and vomiting of early pregnancy or following general anesthesia as well as in radiation sickness is the lack of any direct indication for a deficiency of vitamin B₆ in these conditions. Further, there is no available proof for a direct pharmacological action of pyridoxine to explain any clinical beneficial effect after administration of pyridoxine.

As in any deficiency of a vitamin, it would be of great diagnostic value if biochemical reactions could be found for the assessment of a specific metabolic disturbance connected with the vitamin in question, in this case with vitamin B₆. In this connection the urea level in the blood may furnish some indication for a specific metabolic aberration. It is known that fasting blood urea is significantly less in normal pregnant than in non-pregnant subjects. The urea level is definitely lower in cases of hyperemesis gravidarum than in normal pregnancy, but it may be re-tored to typical normal values after the administration of pyridoxine. Although the low level of fasting blood urea is opposite to the condition found in pyridoxine-deficient rats, the response to a test load of alanine is entirely similar. Blood urea is increased to a maximum 6 hours after oral administration of 30 mg. of dl-alanine, returning to original values within 12 hours in the non-pregnant and normal pregnant woman. In women with hyperemesis gravidarum, blood urea level's rise but fail to decrease significantly between the sixth and the twelfth hours, thus exhibiting a flat curve. Supportive therapy alone for 72 hours does not alter the response to dl-alanine, but supportive therapy supplemented with 120 mg. of pyridoxine produces normal pregnancy response to the test.

More specific, at least for a relative vitamin B₆ deficiency, is the increased urinary excretion of xanthurenic acid after a load test of tryptophan. Without this extra load of tryptophan, the excretion of xanthurenic acid,

190 A. Oppenheim and B. Lih, Radiology 47, 381 (1946).
even in fully developed vitamin B₆ deficiency, may stay within normal limits. Greenberg and co-workers studied the same phenomenon in three human subjects on a vitamin B₆-deficient diet for 1 to 3 weeks (chiefly casein-sucrose-oil and vitamins), followed by an additional week during which they received daily supplements of pyridoxine (10 or 15 mg.). The results summarized in the following table indicate that human subjects may develop early on a vitamin B₆-deficient diet a derangement of tryptophan metabolism, manifested by the excretion of xanthurenic acid after an extra load of tryptophan (Table V).

The xanthurenic acid index may be used as a biochemical sign of a latent vitamin B₆ deficiency or at least of an increased requirement for vitamin B₆. The xanthurenic acid index is expressed as the percentage of the dose of tryptophan, the latter given in an amount of 0.1 g. per kilogram of body weight. In five non-pregnant women Vandelli found that the index ranged from 0.25 to 1.50. In six pregnant women it ranged from 2.13 to 12.4, the higher values tending to occur later in pregnancy. In three preg-

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### TABLE V

<table>
<thead>
<tr>
<th>Subject</th>
<th>Dietary status</th>
<th>Pyridoxine supplement</th>
<th>Excretion, mg./24 hr.</th>
<th>Before</th>
<th>After</th>
</tr>
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<td></td>
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<td>tryptophan</td>
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<td>D. B.</td>
<td>Synthetic diet for 21 days</td>
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<td>Synthetic diet for 28 days</td>
<td>10 mg. per day for last 7 days</td>
<td>6.2</td>
<td>28.4</td>
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<td>Natural diet (control period)</td>
<td>5 mg. every other day for 14 days</td>
<td>10.1</td>
<td>33.2</td>
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<td>Synthetic diet for 28 days</td>
<td>15 mg. per day for 7 days</td>
<td>5.0</td>
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<td></td>
<td>Natural diet for 38 days</td>
<td>15 mg. per day for 45 days</td>
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<td>38.9</td>
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</table>
nant women who had been treated for nausea and vomiting with pyridoxine, the index was from 0 to 0.58.

This tryptophan load test should be repeated on a larger number of cases of pregnancy and extended to other conditions, such as radiation sickness, in which clinical observations seem to support the therapeutic efficacy of pyridoxine. Until such more exact biochemical metabolic data become available, no definite conclusions may be drawn as to the therapeutic value of pyridoxine in these and similar clinical conditions.

All the above described more or less circumstantial or at least artificially produced evidence in favor of clinical vitamin B6 deficiency and, therefore, in support of vitamin B6 requirement for man, has received most recently striking and unequivocal support in direct clinical observations.

Hunt et al.290 observed a young infant in a very serious convulsive state lasting for several weeks after birth. This infant responded dramatically to injection of pyridoxine or to larger amounts of pyridoxine-HCl given daily by mouth (1 to 2 mg.). The basic, puzzling, and unexplained metabolic defect in this infant has been B6 dependency on a higher level of dietary requirement.

Even more convincing for the essential nature of vitamin B6 in human dietary was the widespread occurrence of nervous irritability and convulsive seizures in young infants in the age period of 6 weeks to 6 months receiving a proprietary liquid, canned (autoclaved) milk formula. This formula represents an approximately one-third diluted cow's milk with added lactose and fat. Thus, the original vitamin B6 content of this formula is also about one-third of that of undiluted cow's milk. During autoclaving, the content of the thermolabile, natural vitamin B6 in this preparation was further reduced by two-thirds of the original amount and reached a level of about 60 μg per liter, far below the level of vitamin B6 in human milk (100 to 120 μg). In contrast, the same, but spray-dried, powdered, and not autoclaved, formula showed no appreciable destruction of vitamin B6, and the vitamin B6 level remained around 180 μg per liter. No cases of nervous irritability or convulsions were seen in infants receiving the spray-dried formula with its higher vitamin B6 content.

The relation of vitamin B6 to this nutritionally induced convulsive disorder is best illustrated by the observation of Coursin201 in a 2½-month-old infant. This infant received the proprietary liquid formula from birth without supplements.

the infant was hospitalized. . . On the 5th day of hospitalization, the infant went into status epilepticus. The eyes remained fixed and staring, pupils contracted with no reaction to light. The body was held rigidly with the head thrown back. Generalized convulsive movements recurred repeatedly with occasional outbursts of an abnormally high-pitched monotonous cry. There was some evidence of cyanosis about the lips. There was no response to painful stimuli. Pulse was rapid and regular at 180. Respirations were gasping at 40. The patient was placed in oxygen with some improvement of color but no effect on the seizures. He was removed to the EEG laboratory and connected by means of needle electrodes to the EEG apparatus. Continuous recording was undertaken with great technical difficulty due to the child's muscular activity. However, successful tracing was obtained with evidence of markedly increased voltage of 200 µv and slowing to 2-3 waves per sec. These appeared in bursts with recurrently accompanying spikes and lasted for 4-6 sec. . . . This pattern of

Fig. 2. Electroencephalogram during convulsion in an infant on a vitamin B₆ deficient milk formula.

Fig. 3. Electroencephalogram taken from the same infant (Fig. 2) 3 minutes after intramuscular injection of 100 mg. of pyridoxine-HCl.
activity (Fig. 2) with accompanying clinical seizures was well established when 100 mg. of pyridoxine-HCl was given intramuscularly. For about 1 minute following injection, there was little change in the EEG. However, by 3 minutes, activity had definitely diminished and by 4–5 minutes after injection, the EEG became a normal sleep record (Fig. 3). Clinically, the infant was of good color without added oxygen, sleeping peacefully and showing no evidence of tremor or rigidity. In 48 hours, although kept on the original formula, there was no evidence of previous difficulties."

It is of interest that, as in animals, young infants appear to be more susceptible to convulsive seizures as manifestations of vitamin B₆ deficiency than older children or adults. The critical level of vitamin B₆ intake lies between 50 and 80 γ per day.

The biochemical defect characterizing the convulsive disorder in vitamin B₆ deficiency has not yet been elucidated.

Isoniazid may also produce grand mal convulsions in man which are counteracted by pyridoxine, although perhaps not as completely as the convulsive seizures occurring in simple, direct vitamin B₆ deficiency.²⁰²

XI. Pharmacology

KLAUS R. UNNA

Pyridoxine has an extremely low toxicity. Doses up to 1 g. per kilogram are tolerated without ill effects by rats, rabbits, and dogs.¹ The metabolism, the circulatory and respiratory systems, and isolated smooth muscle organs of normal animals are not influenced by the vitamin.¹ Pyridoxine has been reported to increase the contraction height and work output of perfused frog muscles.² This effect was obtained only with concentrations between 0.00005 and 0.005 millimole per liter; a similar effect was observed by the same authors with thiamine and pantothenic acid in comparable concentrations.

Little is known about the toxic effects of pyridoxal and pyridoxamine. On injection of graded amounts of the different forms of the vitamin into eggs, 5 mg. of pyridoxal reduced the hatchability more markedly than 5 mg. of pyridoxine, whereas pyridoxamine was without effect in doses of 10 mg.³ Pyridoxamine in doses of 320 mg. per kilogram injected intraperitoneally in mice failed to cause toxic effects.⁴

Pyridoxamine has been found to prevent fatal convulsions caused by thiosemicarbazide in mice; intraperitoneal injections of 320 mg. per kilogram preceding a threefold lethal dose of thiosemicarbazide prevented all toxic manifestations.\(^4\) Pyridoxine and pyridoxal were said to be less effective antidotes. Toxic effects of semicarbazide were less readily antagonized by pyridoxamine. In mice and rats, pyridoxine in doses of 6 mg. to 800 mg. per kilogram raised the convulsive doses of semicarbazide significantly; intravenous injections of 100 to 400 mg. prevented semicarbazide seizures in man.\(^5\) On the other hand, pyridoxine failed to exert an anticonvulsant effect against seizures induced by metrazol, strychnine, ammonium acetate, or by electroshock in mice\(^6\) and by electroshock in rats.\(^6\)

Pyridoxine, in conjunction with other B vitamins, may also counteract the anorexia and anemia caused by promin in rats.\(^7\)

Pyridoxine has been stated to protect mice from death by x-ray radiation, although the leucopenia was not influenced; a similar protection was obtained with folic acid.\(^8\) On the other hand, a protective effect of pyridoxine against injurious effects of P\(^{32}\) could be demonstrated only in pyridoxine-deficient mice but not in normal mice.\(^9\)

Toxic effects of desoxy-pyridoxine in chicks,\(^10\) in rats,\(^11,12\) and in man\(^13\) are readily explained by its conversion into desoxy-pyrididine phosphate and subsequent competition with pyridoxal phosphate for the apoenzyme.\(^14\) The toxic effects of desoxy-pyridoxine resembling the manifestations of pyridoxine deficiency were readily obtained when the diet was restricted in pyridoxine. On an adequate intake of pyridoxine, toxic effects occurred in rats only after the diet was supplemented with 50 mg. % of desoxy-pyridoxine;\(^12\) intramuscular injection of 50 mg. caused no ill effects in normal man.\(^13\)

Pyridoxine is readily absorbed from the gastrointestinal tract and rapidly metabolized. Only small fractions of ingested or injected pyridoxine are excreted as such in the urine. In normal persons on adequate diets a pyridoxine activity corresponding to less than 1 \(\mu\)g. per milliliter was found in the urine.\(^1\) After intravenous administration of 50 to 100 mg., only 5 to 10 %

is detected within 1 hour by colorimetric tests as pyridoxine in the urine;\textsuperscript{15-17} recovery of pyridoxine in the urine after ingestion of 8 to 100 mg. ranged from 4 to 8%.\textsuperscript{15, 18, 19} Negligible amounts of pyridoxine appear in the sweat.\textsuperscript{19} Microbiological assays which differentiate between pyridoxine, pyridoxal, and pyridoxamine have shown that normally only minute amounts of any of the three forms of vitamin are found in urine.\textsuperscript{20} 4-Pyridoxic acid,\textsuperscript{21} the main metabolic product of pyridoxine and also of pyridoxal and pyridoxamine, accounts for 70 to 90% of the excretion products measured after ingestion of either form of vitamin B\textsubscript{6} in man.\textsuperscript{19, 20, 22} It has also been found in the urine of rats, but not of dogs.\textsuperscript{23} Ingestion of large amounts of pyridoxine leads to increased excretion of pyridoxal and also of pyridoxamine. On the other hand, no evidence could be obtained showing the conversion of pyridoxal or pyridoxamine to pyridoxine.\textsuperscript{20}

Median lethal doses of pyridoxine hydrochloride have been determined in animals as follows: on intravenous injection in mice (545 mg. per kilogram) and in rats (657 mg. per kilogram),\textsuperscript{24} on subcutaneous injection in rats (3.7 g. per kilogram); and on oral administration in rats (5.5 g. per kilogram).\textsuperscript{1} Tonic convulsions precede death in mice and rats. Doses larger than 1 g. per kilogram administered to rats, rabbits, and dogs caused in all three species characteristic manifestations which began with marked impairment of coordination and of righting reflexes within two or three days, progressing to severe tonic convulsions and death in the stage of paralysis.\textsuperscript{1} Autopsies showed enlargement of the adrenals with occasional hemorrhages into the cortex. Neurohistologic examination of dogs and rats treated with 2 to 6 g. of pyridoxine per kilogram revealed degeneration of the posterior columns of the spinal cord and in some cases also a well-marked degeneration of the posterior roots, posterior ganglia, and peripheral nerves.\textsuperscript{25}

Prolonged daily administration of pyridoxine to rats (25 mg. per kilogram), dogs (20 mg. per kilogram), and monkeys (10 mg. per kilogram) failed to cause any toxic manifestations or pathologic changes in the tissues.\textsuperscript{1} Rats receiving 2.5 mg. per kilogram daily were raised through three generations. Mice tolerated repeated intravenous injections of 100 mg. per kilo-

\textsuperscript{17} J. Flexner and M. R. Chassin, \textit{J. Clin. Invest.} \textbf{20}, 313 (1941).
gram without ill effects. Prolonged administration of 1 mg. of pyridoxine to rats subsisting on diets restricted in either thiamine, riboflavin, or pantothenic acid failed to aggravate the manifestations of the deficiency state.

Since pyridoxine deficiency in experimental animals causes such specific and striking effects on the skin, on hematopoiesis, and on the central nervous system, pyridoxine has been given in very large amounts to man for therapeutic trials in a large number of conditions. No toxic effects have been encountered with daily administration over periods of months of 50 to 200 mg. of the vitamin by either the oral, intramuscular, intravenous, or intrathecal route.

No pharmacodynamic effects of large doses of pyridoxine are established in man. Administration of large doses in attempts to prevent nausea and vomiting in radiation sickness and during pregnancy rests, in the absence of both an experimental basis and carefully controlled studies, on empiricism. It may be remembered that equally large doses of thiamine and of niacinamide are also recommended for this purpose. Recent observations on tryptophan metabolism in pregnant women have shown that pyridoxine prevents the excretion of abnormally large amounts of xanthurenic acid. These findings may be analogous to the observations on tryptophan metabolism in pyridoxine-deficient animals and, hence, imply an alteration during pregnancy of metabolic functions in which pyridoxine is known to play an essential role. Also, the low fasting blood level of urea of pregnant women can be restored to normal values by pyridoxine.

XII. Requirements and Factors Influencing Them

A. OF ANIMALS

HENRY SHERMAN

It is difficult to record the precise absolute requirement of animals for vitamin B₆, for there are many factors, external and internal, which influence this requirement. No single standard set of assay conditions or criteria has received universal acceptance. Since vitamin B₆ has at least a

30 S. Stone, J. Nervous Mental Disease 100, 185 (1941).
threefold function in the body, it is conceivable that the requirement for one function may not be the same as for the other functions. Many times, growth is used as the criterion of response, but, since this is not specific, it may not be the appropriate criterion to employ; in a deficiency, biochemical systems which require vitamin B₆ may cease to function long before growth is affected. The synthesis of vitamin B₆ by intestinal bacteria also complicates this entire requirement picture.

1. Rats

The majority of investigators have advocated a pyridoxine requirement of 10 γ per day or 100 γ per 100 g. of diet for normal growth.¹ This value also applies to the requirement of the cotton rat (Sigmodon hispidus hispidus).² During lactation, however, this amount is increased to 120 to 200 γ per 100 g. of ration.³ The vitamin B₆ requirement can also be increased by inducing an experimental hyperthyroidism⁴ and by overloading the diet with thiamine.⁵ The vitamin B₆ requirement does not appear to be increased in aging rats; thiamine requirement, however, is increased.⁶

The three active forms of the vitamin B₆ complex are equally effective in promoting growth and in curing acrodermatitis when fed by medicine dropper or injected interperitoneally;⁷ however, when added to the diet, pyridoxine seems to be the most active. It has been suggested that intestinal microorganisms utilize or destroy pyridoxal and pyridoxamine preferentially. Linkswiler et al.⁸ have shown that aureomycin increased the growth of rats fed limiting levels of pyridoxine, pyridoxal, or pyridoxamine. These three forms, however, were equally active in promoting growth when fed in the diet with aureomycin. The authors postulate that the antibiotic may prevent utilization or destruction of the vitamin B₆ group by intestinal microorganisms, thus increasing the amount available to the rat.

The vitamin B₆ requirement also appears to be influenced by the qualitative and quantitative amino acid composition of the diet. The feeding of large amounts of DL-serine⁹ or glycine¹⁰ to rats increases their vitamin B₆ requirement. The addition of cystine or methionine to rats fed a vitamin

² B. S. Schweigert, Vitamins and Hormones 6, 55 (1948).
¹¹ E. Pagé and R. Gingras, Rev. can. biol. 6, 372 (1947).
B_{6}-deficient diet containing 15% casein accelerated the deficiency and decreased survival time.\textsuperscript{12} When levels of methionine slightly greater than that required for normal growth were fed to rats on a limiting amount of vitamin B_{6}, their growth rate was depressed; other amino acids had no effect.\textsuperscript{12a} Cerecedo and Foy\textsuperscript{13} have shown that weanling rats on a 60% casein diet that was deficient in vitamin B_{6} developed more severe skin lesions earlier than did animals on a lower protein diet. These findings suggest an inverse relationship between vitamin B_{6} and protein intake. The presence in the diet of unsaturated fat appears to spare pyridoxine.\textsuperscript{14} Suboptimal amounts of pyridoxine become optimal when supplemented with linoleic acid-containing fats.

Beaton \textit{et al.}\textsuperscript{14a} have demonstrated the importance of other factors in determining the severity of acrodynia in pyridoxine-deficient rats. Male rats appeared to develop the deficiency with more rapidity and severity than female rats, indicating a higher requirement for the former. When the deficient diet contained an incomplete protein as the only source of nitrogen, no acrodynia developed, suggesting that the skin lesions develop under those dietary conditions which allow an increase in body weight when the deficient diet is supplemented with vitamin B_{6}. On the other hand, the injection of growth hormone into pyridoxine-deficient animals aggravated the deficiency symptoms. Adrenalectomy prevented the appearance of acrodynia in vitamin B_{6} deficiency.

When rats are maintained at a temperature of 91°F, they require twice the amount of pyridoxine necessary at 68°F.\textsuperscript{15}

2. Mice

Young weanling mice on a pyridoxine-deficient diet containing 18 to 20% casein showed a good growth response when the diet was supplemented by 1 γ of pyridoxine per gram of diet.\textsuperscript{16,17} When the deficient diet was supplemented by only 0.5 γ of pyridoxine per gram of food, the animals grew at one-half the normal rate. An increase in the protein level of the diet elevated the requirement for vitamin B_{6}. Deficient mice on a 60% casein level lived one-third as long as mice on a 10% casein diet; the mouse requires three times as much pyridoxine on a diet of 60% casein than when 20% was fed. L-Tryptophan administration decreased the survival time of

\textsuperscript{12} L. R. Cerecedo, J. R. Foy, and E. C. De Renzo, \textit{Arch. Biochem.} 17, 397 (1948).
\textsuperscript{13} L. R. Cerecedo and J. R. Foy, \textit{Arch. Biochem.} 5, 207 (1944).
\textsuperscript{14} H. Sherman, \textit{Vitamins and Hormones} 8, 55 (1951).
\textsuperscript{15} C. A. Mills, \textit{Arch. Biochem.} 1, 73 (1942).
\textsuperscript{17} H. P. Morris, \textit{Vitamins and Hormones} 5, 175 (1947).
mice deficient in vitamin B₆, but not as much as casein of equivalent tryptophan content; amino acids other than tryptophan also appear to be involved.

The growth rate and survival time of weanling mice receiving 2 γ or more of pyridoxine per gram of food are unaffected by variations of 10 to 60% in dietary casein. Pyridoxamine and pyridoxal are less active for mice than pyridoxine, especially on high casein diets. ¹⁸

When adult white mice are given a medium dose of x-rays (350 r. in a single exposure), they succumb within a short time. The survival time can be prolonged by the daily injection of 50 γ of pyridoxine for 7 days preceding and 13 days after irradiation. ¹⁹

3. Hamsters

The vitamin B₆ requirement of the hamster has not been adequately established. Schwartzman and Strauss²⁰ have shown that the daily subcutaneous injection of 50 γ of pyridoxine was sufficient for good growth. As in rats, the inclusion of corn oil in the diet of vitamin B₆-deficient hamsters delayed the onset of the deficiency symptoms; fat appeared to have a sparing action.

Unpublished data by Fisk²¹ suggest that the male hamster requires 800 γ of pyridoxine per 100 g. of diet, whereas the female hamster requires 400 γ per 100 g. of diet.

The hamster's requirement for vitamin B₆ appears to be the highest of all rodents studied thus far.

4. Dogs

No reliable data are available for the vitamin B₆ requirement of the dog.

5. Pigs

Although the precise requirement is not known, 200 γ of pyridoxine per kilogram of body weight permits the pig to grow normally and prevents the development of anemia.²² Corn oil does not spare pyridoxine in curing anemia.

6. Monkeys

No quantitative data are available for the monkey's vitamin B₆ requirement. However, it has been observed that monkeys receiving 1 mg. of pyri-

doxine daily grow normally and do not exhibit any deficiency symptoms. In all probability, the daily requirement is less than 1 mg.

7. Chicks

It has been fairly well established that chicks require 200 to 300 γ of vitamin B₆ per 100 g. of ration for normal growth, egg production, and hatchability. When linseed oil meal is fed to chicks at a 30% level, the vitamin B₆ requirement is increased. Treatment of the linseed oil meal with water removes the factor which is responsible for the increased requirement.

The Red Rock breed has a higher requirement for vitamin B₆. When Red Rock cross chicks were fed diets containing 500 γ of vitamin B₆ per 100 g. of ration, vitamin B₆ deficiency symptoms appeared. These symptoms disappeared when the pyridoxine content of the diet was increased. This variation in requirement may be due to genetic factors.

There is no difference in the chick's dietary requirement for vitamin B₆ in cool temperatures and in tropical heat.

![Fig. 4. Relationship of size of a species to its pyridoxine requirement.](image)

The pyridoxine requirement of young ducklings is similar to that of chicks, approximately 250 \( \gamma \) per 100 g. of ration.\(^1\)

8. Other Animals

Fox pups require less than 200 \( \gamma \) per 100 g. of ration.\(^2\) Rainbow trout need 100 to 1000 \( \gamma \) of vitamin \( B_6 \) per 100 g. of diet.

Ruminants do not require an external source of vitamin \( B_6 \), for the vitamin is synthesized in adequate amounts in the rumen and reticulum of the sheep and in the rumen of the cow.\(^3\) Milk from a cow fed a vitamin \( B \)-complex deficient diet has as much pyridoxine as milk from cows fed a normal ration.

It has been fairly well established that with the \( B \) vitamins the smaller species have a higher relative \( B \)-vitamin requirement than the larger ones.\(^4\) Figure 4 illustrates this fact with respect to pyridoxine.\(^5\)

B. OF MAN

P. GYÖRGY

As stated in the section dealing with the effects of vitamin \( B_6 \) deficiency, there cannot be any doubt as to man's need for vitamin \( B_6 \). The human dietary requirement for vitamin \( B_6 \) has not yet been definitely established. It has been estimated to be in the neighborhood of 1.5 mg. per day for adults,\(^6\) or about 0.03 mg. per kilogram per day.\(^7\) According to recent observations on convulsive seizures in young infants receiving a milk formula low in vitamin \( B_6 \), the critical level of minimal requirement seems to lie between 0.06 and 0.1 mg. per day for infants up to 4 to 6 months of age—thus about 0.01 to 0.02 mg. per kilogram per day.

Factors influencing the requirement for pyridoxine in man have not yet been studied. No natural antagonists of pyridoxine are known. It appears from clinical observation that pregnancy and x-ray irradiation might increase the need for pyridoxine. More direct or indirect evidence is needed before such a conclusion could be considered as firmly established.


# Chapter 15

**RIBOFLAVIN**

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I. Nomenclature

ROBERT S. HARRIS

Accepted names: Riboflavin (U.S. Pharmacopeia)
Riboflavine (British Pharmacopoeia)

Obsolete names: Vitamin B₂    Ovoflavin
               Vitamin G    Lyochrome
               Lactoflavin  Uroflavin
               Hepatoflavin

Empirical formula: C₁₇H₂₀N₄O₆
Chemical name: 6,7-Dimethyl-9-(d-1'-ribityl)isoalloxazine
Lactoflavin is the original name of riboflavin, still used in Europe. Ovo-
flavin, hepatoflavin, uroflavin, etc., are historical names, indicating the
origin of the preparations, which are chemically identical with lactoflavin.
Riboflavin, the American designation, indicates that the naturally occur-
ring flavin is a derivative of (d)-ribose. This name was adopted in 1952 by
the International Commission for the Reform of Biochemical Nomenclature.

Riboflavin is identical with vitamin B₂. In former times, in the United
States, the term vitamin G was also used for this nutritional factor.

After vitamin B₁ had been obtained in pure form, the isolation of crys-
tallized riboflavin was one of the most fascinating chapters in the chemistry
of the vitamins. For the sake of its historical interest, the story of the dis-
cover of riboflavin will be told here briefly.¹

In 1927, Paul György, at that time at the pediatric clinic of the University of
Heidelberg, began investigations on the curative factor for egg white injury, which
he called vitamin II.² Beginning in 1931, he and Edgar Lederer, who worked with
Richard Kuhn at the Kaiser Wilhelm Institute for Medical Research, Heidelberg,
attempted to isolate this vitamin. Vitamin II deficiency in rats is characterized by
a dermatitis. Since pellagra is another avitaminosis connected with skin symptoms,
it seemed useful and interesting to make a comparative study of the nutrition factor

¹ Literature references are given mainly for the last ten years. For earlier and more
references, consult textbooks and reviews, for instance: H. R. Rosenberg, Chem-
York, 1945; R. J. Williams, R. E. Eakin, E. Beerstecher, Jr., and W. Shive, The
1950; F. A. Robinson, The Vitamin B Complex, pp. 132–210. John Wiley and Sons,
New York, 1951; G. A. Emerson and K. Folkers, Ann. Rev. Biochem. 20, 584 (1951);
H. Vogel and H. Knobloch, Chemie und Technik der Vitamine, 3rd ed., Vol. 2,

² See also P. György, Nutrition Revs. 12, 97 (1954).

³ In 1940 P. György, V. du Vigneaud, and D. Melville identified this vitamin with
the yeast growth-promoting factor biotin, which had been isolated some years
before by F. Kögl and B. Tönnis.
connected with this disease. A lack of vitamin B₂, the heat-stable companion of the heat-labile vitamin B₁, at that time was considered to be the cause of pellagra. In the beginning of 1932, the author of this article started the isolation of so-called vitamin B₂ at Kuhn's institute. György performed the biological tests on rats, according to the method of Sherman and Bourquin; later on, when our preparations became purer, the diet of the animals had to be modified somewhat, since it was lacking not only in vitamin B₂ but also in another member of the B-vitamin complex.

The literature contained very little and vague data on the concentration of vitamin B₂ from yeast and liver, which turned out to be of little value for the procedure of isolation. For the adsorption of the vitamin, fuller's earth in acid solution had been recommended. Another valuable adsorbent soon was found, which adsorbed vitamin B₂ already from neutral solution; this was "Frankonit KL" (a bleaching earth produced by the Pfirsehnger Mineralwerke, Kitzingen/Main, Franconia), which since that time has been used frequently in biochemical work. Before the vitamin B₂ investigation, the existence of our sample of this adsorbent had been forgotten in one of the laboratory closets, after I had tried it with little success for the polymerization of isoprene.

None of the known methods was fit for elution of the vitamin from the adsorbent in yields worth mentioning. But a wrong hypothesis about the chemical nature of vitamin B₂ helped me to find the right trail. In one paper of the literature it had been assumed that vitamin B₂ might contain iron. With regard to the biological properties of hemin derivatives, one would have been inclined to guess that iron porphyrin complexes were involved. Since pyridine is a solvent for compounds of this type, this substance, diluted with water and alcohol, was tried out for the elution of vitamin B₂ adsorbates. This attempt was a full success. The later progress of our investigation made it clear that iron has nothing to do with vitamin B₂, but the wrong hypothesis had proved useful.

The successful elution drove the isolation procedure one essential step forward. It soon became evident that all eluates, which were active in the animal experiments, had a greenish-yellow color and showed a yellowish-green fluorescence in the light of a quartz lamp. Therefore, the idea came up that vitamin B₂ itself might be colored, and the investigation was continued with attention to this assumption.

It still was difficult to obtain purified preparations of the vitamin from extracts of yeast, liver, heart, or kidney, because of the presence of large amounts of accompanying substances. An 80% methanol extract of egg white turned out to be a much better starting material. The concentrated, greenish-yellow eluates on precipitation with AgNO₃ gave a brownish-red silver salt of the vitamin. In later experiments, a precipitation with Tl₂SO₄ was inserted for further purification. This salt had been chosen with regard to the chemical relationship of certain thallous and silver salts. The first few milligrams of crystallized "ovoflavin" became available for analysis shortly before Christmas, 1932. The animal tests proved without any doubt the growth-promoting nature of the substance.

At this stage of the investigation, we received knowledge that the pharmacologist Ph. Ellinger in Düsseldorf, working on the fluorescence of animal organs, had prepared a colored concentrate from skimmed milk, which obviously was similar to ovoflavin. Our methods of purification proved to be particularly applicable to whey. We therefore changed over to this starting material. It was, however, not possible to handle the large amounts of liquid in the laboratory. Therefore, the first step of the concentration, the adsorption, was carried through in a large cheese dairy in Bavaria. With this procedure we soon were able to obtain 1 g. of crystallized "lactoflavin" from 5400 l. of whey, thus opening a way for the elucidation of the chemical structure of vitamin B₂.
Other investigators had obtained impure preparations of flavin. As early as 1879, A. W. Blyth isolated from whey a resinous preparation of a red-orange color which he called "lactochrom." In 1925, B. Bleyer and O. Kallmann attempted the purification of the yellow pigment of whey. In 1932, I. Banga and A. Szent-Györgyi obtained a golden-yellow pigment from heart muscle, whose colored component they called "cytoflav." In 1933, Ellinger and Kosebara described impure, crystalline preparations of flavin ("lyochrome"), at the same time that the isolation of pure, crystallized lactoflavin was published under the authorship of György, Kuhn, and Wagner-Jauregg. The vitamin nature of the pigment was unknown before the investigations of the latter authors. Shortly after, in 1933, L. E. Booher in the United States described a concentrate from whey powder with the chemical and biological properties of riboflavin. Soon other investigators followed with the isolation of riboflavin from various natural sources.

For the understanding of the biochemical function of riboflavin, the discovery of the "yellow enzyme" by Warburg and Christian in 1932 was of extraordinary importance. The same authors described lumiflavin, a photochemical degradation product of riboflavin, which proved of great value for the elucidation of the chemical structure of riboflavin (Kuhn, Rudy, Wagner Jauregg, and coworkers, 1933-34). The synthesis of riboflavin by Kuhn and Weygand in Heidelberg and by Karrer and his coworkers in Zürich in 1934 finally confirmed the structural formula.

A. ISOLATION

Three factors mainly govern the possibility of isolating riboflavin from natural sources in a pure state and in good yield: (1) the concentration in which the pigment occurs; (2) the amount and kind of accompanying substances; (3) the form (free or bound) in which the vitamin is present.

1. The concentrations of riboflavin in different natural materials are listed in a later section. In cases of low concentration, one will usually have to dispense with the isolation and to confine oneself to the quantitative determination according to either the vitamin test or one of the fluorometric methods.\(^2\)

2. Considerable difficulties in the isolation of riboflavin can be caused by the presence of accompanying substances. As an example the liver might be mentioned. Although this organ has a very high content of riboflavin, the isolation of the crystallized substance was rather difficult and was performed only one year after the preparation of riboflavin from other sources had been described.\(^3\)

3. Riboflavin occurs in its free, dialyzable form only, in the retina of the eye, in whey, and in urine. In organs, tissues, and other living cells, riboflavin is present, as riboflavin monophosphoric acid, and as riboflavin adenine dinucleotide. The two phosphates account for practically all the riboflavin present in rat kidneys, and 70 to 90% of the total riboflavin in

\(^2\) For recent developments of the fluorometric methods to determine vitamin B\(_2\) in plants, see H. Roth, Biochem. Z. 320, 355 (1950).

\(^3\) R. Kuhn and T. Wagner-Jauregg, Ber. 67, 1770 (1934).

all tissues is present in the form of the dinucleotide.\(^4\) Spleen contains an enzyme that rapidly degrades the phosphate-bound forms of riboflavin to the free vitamin.\(^5\)

It has been shown that riboflavin phosphoric acid is able to form loose, non-dialyzable complexes, for instance with a solution of pseudoglobulin or albumin from horse serum. The separation of the flavin component and the protein in this case can be achieved by precipitation of the latter with ammonium sulfate.\(^6\)

In order to liberate riboflavin from its natural protein-bound forms, it is necessary to treat the mashed tissues with suitable solvents at room temperature or at the boiling point of the solvent. Methanol, ethanol, acetone, undiluted or diluted with water, and aqueous acid solutions have been used for extraction of the vitamin. For instance, riboflavin from fresh or dried plants has been extracted in good yields by boiling the material with 70% methanol for 45 minutes.\(^2\)

For the isolation of riboflavin from the extracts, it sometimes is useful first to remove lipids by extraction with ether, in which the vitamin is insoluble. Salts and glycogen in some cases can be eliminated from riboflavin concentrates by fractionate precipitation with alcohol or acetone. Impurities from fermentation liquors may be precipitated by means of acetone, and crude riboflavin can be recovered from the concentrated filtrate by the addition of more acetone.\(^7\) The vitamin can be extracted with butanol and then precipitated from the extract by addition of petroleum ether.\(^8\)

In the isolation of riboflavin from whey, the accompanying creatinine has been removed by picric acid precipitation.

Precipitation of riboflavin occurs with lead acetate and with silver nitrate in neutral solution, or with phosphotungstic acid in \(N \, \text{H}_2\text{SO}_4\); from the latter precipitate the phosphotungstic acid can be extracted with amyl alcohol. Silver nitrate or mercuric sulfate in acid solution leaves the vitamin in solution but precipitates some accompanying substances.

Good adsorbents for riboflavin are fuller’s earth in acid solution, Florisil, Floridin XXF, and Frankonit in neutral solution. One of the best eluents is pyridine, diluted with aqueous methanol or ethanol;\(^8a\) ammonia, triethanolamine, 0.1 \(\text{N} \, \text{NaOH}\) in 60% ethanol, boiling 60% ethanol, 80%\

\(^8a\) P. György, R. Kuhn, and T. Wagner-Jauregg (to I. G. Farbenindustrie A. G.), German Pat. 607,512 (Nov. 22, 1932).
acetone, and polyhydric alcohols have been used for elution. Vitamin B₂ is adsorbed by charcoal very strongly; however, elution is difficult from this adsorbate. Adsorption occurs furthermore with lead sulfide, when this is precipitated in a riboflavin solution; the vitamin can be extracted with hot water from the precipitate. Riboflavin is not adsorbed by kieselguhr, kaolin, tale, aluminum oxide, or calcium carbonate.

A combination of precipitation and adsorption methods mostly will be necessary to isolate pure riboflavin. As examples might be mentioned the isolation of riboflavin from egg white, egg yolk, whey, and urine. A general method for the preparation of pure D-riboflavin from natural sources has been described, which is based on adsorption on fuller’s earth, fractionation with immiscible solvents and acetone, and crystallization from an aqueous acetone-petroleum ether mixture; aqueous alcohol solutions have been used for elution of the adsorbates.

B. CHEMICAL AND PHYSICAL PROPERTIES

C₁₇H₂₆N₄O₆: molecular weight 376.4; C 54.25 %, H 5.36 %, N 14.89 %.

Riboflavin crystallizes from 2 N acetic acid, alcohol, water, or pyridine in fine orange-yellow needles. The decomposition point is 278 to 282° (darkening at about 210°). Values for the decomposition point between 271 and 293° can be found in the literature. The vitamin is odorless and has a bitter taste.

Riboflavin is soluble in water only to an extent of 10 to 13 mg. in 100 ml. at 25 to 27.5°, 19 mg. in 100 ml. at 40°, and 230 mg. in 100 ml. at 100°. The vitamin dissolves in ethanol to 4.5 mg. % and is slightly soluble in amyl alcohol, cyclohexanol, benzyl alcohol, and phenol or amyl acetate. The impure material has a much higher solubility than the pure substance. Alkali dissolves the vitamin well, but these solutions are unstable. There is no solubility in ether, acetone, chloroform, or benzene. Formic acid dissolves more than 1 % of riboflavin.

For intravenous administration, sterile, supersaturated solutions of riboflavin in normal saline might be employed. By heating to the boiling

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9 S. H. Rubin and E. De Ritter, J. Biol. Chem. 158, 639 (1945); Commercial Solvents Corp., U. S. Pat. 2,343,254 (March 7, 1944) [C.A. 38, 3093 (1944)].
13 W. Koschara, Ber. 67, 761 (1934).
point, a temporary concentration of 1 mg. per milliliter is said to be attained. The supersaturated solution of riboflavin is fairly stable; it requires days to crystallize.

In order to obtain more concentrated solutions, riboflavin has been dissolved together with other compounds which are capable of increasing its solubility. N-Methylacetamide, L-tyrosine amide,\textsuperscript{16} tryptophan,\textsuperscript{17} sodium acetyltryptophan, urea, nicotinamide, aliphatic amidines, sodium desoxycholate, veratryl alcohol, salts of boric,\textsuperscript{15, 17a} mono- and dihydroxybenzoic, gallic, aminobenzoic, adenyllic, and other acids,\textsuperscript{18} kynurenic and 2-phenylquinoline-4-carboxylic acid,\textsuperscript{18a} and propylene glycol with or without the addition of a monohydroxymonoalkoxybenzaldehyde\textsuperscript{19} have been used. Solutions containing a concentration of riboflavin up to about 0.6% can be prepared in this way. The solubility of riboflavin in nicotinamide solutions at pH 5 increases from about 0.1% to about 2.5%, when the nicotinamide concentration is increased from 5 to 50%.\textsuperscript{20} When riboflavin is fused with an amide such as urea, urethan, or nicotinamide, products are obtained which yield aqueous solutions containing up to 6% of the flavin.\textsuperscript{20a} Recently a method of solubilizing riboflavin with sodium-3-hydroxy-2-naphthoate has been developed; the solubility of riboflavin is as high as 7.9% in a 10% solution of the solubilizer.\textsuperscript{21} Borax and alkali are said to give a complex with riboflavin of the formula C\textsubscript{17}H\textsubscript{19}O\textsubscript{6}N\textsubscript{4}Na\cdot Na\textsubscript{2}B\textsubscript{4}O\textsubscript{7} \cdot 10H\textsubscript{2}O.\textsuperscript{22}

Water-soluble derivatives of riboflavin\textsuperscript{18} include esters with phosphoric, sulfuric,\textsuperscript{22a} gallic, aminoacetic, phthalic, succinamic,\textsuperscript{22} citric, malic, tartaric, and levulinic acids, and methylol and acetal derivatives.\textsuperscript{23} In the synthesis of methylol derivative, preparations with as high as 55% microbiological activity can be obtained in short reaction time, when only 1 mole of formaldehyde is combined with 1 mole of riboflavin. Upon addition of 2 or more

\textsuperscript{16} Wyeth Inc., U. S. Pat. 2,445,208 (Sept. 13, 1948) [C.A. 42, 6496 (1948)].


\textsuperscript{17a} D. V. Frost, J. Biol. Chem. 145, 693 (1942).

\textsuperscript{18} For literature references, see K. Schoen and S. M. Gordon, Archives Biochem. 22, 149 (1949).

\textsuperscript{18a} C. S. Runti, Farm. sci. e terc. (Pavia) 7, 344 (1952).


\textsuperscript{20} D. V. Frost, J. Am. Chem. Soc. 69, 1064 (1947); Abbott Laboratories U. S. Pat. 2,407,412 (Sept. 10, 1946) [C.A. 41, 254 (1947)].


\textsuperscript{22a} G. B. Stone, Science 111, 283 (1950).

mole of formaldehyde, the activity falls off rapidly. As in the case of tri- and tetrasuccinates, the sulfate is microbiologically active only after previous hydrolysis. Riboflavin mono- and disuccinates have vitamin B₂ activities for the rat which are 100% and 65%, respectively, of riboflavin's activity. Both the mono- and diaceton derivatives of riboflavin are active in the nutrition of rats. Riboflavin-5'-phosphate is fully as active in the rat as riboflavin (oral and parenteral administration), as well as in the microbiological test. The same is true for flavin adenine dinucleotide.

Neutral solutions of riboflavin have a greenish-yellow color. The absorption spectrum shows characteristic absorption maxima at 475, 445, 359-372, 268, and 223 mg.\(^{12a,b}\) The absorption in the visible part of the spectrum has been used for quantitative determination of riboflavin.

Neutral aqueous solutions of riboflavin display intense yellowish-green fluorescence, with a maximum at 565 mg which can be used for quantitative determination of the vitamin. The fluorescence vanishes on the addition of acids or alkalis; optimal fluorescence occurs at pH 4 to 8.\(^{24}\) A recent study of the fluorescence of riboflavin and flavin adenine dinucleotide (FAD) includes the effect of different quenchers. The relatively weak fluorescence of FAD may be caused by internal quenching by interaction of the alloxazine and adenine portions of the molecule.\(^{25}\)

Riboflavin has an amphoteric character. Its dissociation constants are \(K_a = 6.3 \times 10^{-12}\) and \(K_b = 0.5 \times 10^{-5}\); the isoelectric point corresponds to a pH of 6.0. The pH of the saturated aqueous solution is approximately 6.\(^{24}\)

The optical activity of riboflavin in neutral and acid solutions is exceedingly small. In an alkaline medium, the optical rotation is strongly dependent upon the concentration; \([\alpha]^{21°}_D = -70° (c = 0.06%\); 0.1 N NaOH); \([\alpha]^{21°}_D = -117° (c = 0.5%; 0.1 N NaOH).\(^{26}\) Borate-containing solutions are strongly dextrorotatory: \([\alpha]^{20°}_D = +340° (pH 12)\); in this case the rotation depends only slightly upon the riboflavin concentration.\(^{27}\)

Neutral aqueous solutions of riboflavin are relatively heat stable if protected from light and can be sterilized by autoclaving for a short time; only slight destruction occurs by heating to 120° for 6 hours. At room temperature (27°) decomposition of buffered solutions (pH 5 and 6) takes place at rates of 3 and 1.2% per month. No appreciable destruction of the vitamin can be observed during the cooking of food,\(^{28}\) but when milk in bottles


\(^{26}\) R. Kuhn, H. Rudy, and F. Weygand, *Ber.* 68, 625 (1935). For the rule which governs the rotation of different 3-polyhydroxyalkyl flavins, see F. Weygand, *Ber.* 73, 1278 (1940).


is exposed to sunlight, more than half of the riboflavin is destroyed within 2 hours.\textsuperscript{29, 30} The rate of destruction by light becomes higher with increasing temperature and p\textsubscript{H}.\textsuperscript{38} Alkali decomposes riboflavin rapidly.

Riboflavin is stable against acids, air, and the common oxidizing agents (except chromic acid, KMnO\textsubscript{4}, and potassium persulfate), bromine and nitrous acid. The stability of riboflavin has been used for the purification of the crude synthetic product; in acid solution impurities are oxidized at a temperature below 100\textdegree\ with use of Cl\textsubscript{2}, H\textsubscript{2}O\textsubscript{2}, HNO\textsubscript{3}, or HClO\textsubscript{3}.\textsuperscript{31} But the vitamin is destroyed by hydrogen peroxide in the presence of ferrous ions.

Reducing agents such as sodium dithionite (Na\textsubscript{2}S\textsubscript{2}O\textsubscript{4}), zinc in acid solution, catalytically activated hydrogen, and titanous chloride transform riboflavin in alkaline, neutral, or acetic acid solutions directly into a colorless dihydroflavin, which is reoxidized on shaking with air. The potential of an equimolecular mixture of riboflavin and its leuco compound at pH 7.0 is \(-0.185\) volt \((-0.146\) volt at pH 5.9), pretty much on the negative side. Combination with the enzyme protein has been shown to raise the redox potential from \(-0.19\) volt for D-riboflavin-5′-phosphate to \(-0.06\) volt for the “old yellow enzyme.”\textsuperscript{32}

By the action of zinc, tin, or sodium amalgam in strong HCl (p\textsubscript{H} < 1), a red reduction intermediate forms which is a semiquinone radical.\textsuperscript{33, 34} This behavior of riboflavin might be useful for its detection.

Riboflavin gives a red-violet color with concentrated H\textsubscript{2}SO\textsubscript{4}, which changes to yellow on dilution. When heated with 50\% NaOH solution, riboflavin produces a green color, changing to red on dilution.\textsuperscript{35}

Riboflavin shows a heightened affinity for Fe\textsuperscript{2+}.\textsuperscript{35a} It has been found associated with iron in a protein (conalbumin) which occurs in the avian blood stream and in egg white.\textsuperscript{35b}

Bacteriostatic effects of riboflavins have been observed only in the light. These may be explained possibly by the formation of toxic products and in part by destruction of needed nutrients. It has been demonstrated that

\textsuperscript{31} R. Posternack and E. V. Brown (to Charles Pfizer and Co.), U. S. Pat. 2,324,800 (July 20, 1944) [C.A. \textbf{38}, 221 (1944)].
\textsuperscript{32} R. Kuhn and P. Boulanger, \textit{Ber.} \textbf{69}, 1557 (1936).
\textsuperscript{35a} A. Albert, \textit{Biochem. J.} \textbf{54}, 646 (1953); other metal chelates of riboflavin were described recently by W. O. Faye and W. E. Lange, \textit{J. Am. Chem. Soc.} \textbf{76}, 2199 (1954).
\textsuperscript{35b} J. Bain and H. Deutsch, \textit{J. Biol. Chem.} \textbf{172}, 547 (1948).
in the presence of riboflavin irradiation causes destruction of tryptophan and pyridoxine.\textsuperscript{35e}

Riboflavin is practically non-toxic. The toxicity to mice by intraperitoneal injection amounts to 340 mg. per kilogram.\textsuperscript{36} The $L{D}_{50}$ value for rats, using the same form of application, is 560 mg. per kilogram.\textsuperscript{37} The administration of 10 g. per kilogram orally to rats or 2 g. per kilogram orally to dogs showed no toxic effects.\textsuperscript{37a}

\textbf{C. CONSTITUTION}

\[
\begin{align*}
\text{CH}_2 & \text{---C---C---C---CH}_2\text{OH} \\
\text{H} & \text{H} & \text{H} \\
\text{H}_3\text{C} & \text{N} & \text{N} & \text{C} & \text{O} \\
\text{H}_3\text{C} & \text{N} & \text{NH} & \text{O}
\end{align*}
\]

6,7-Dimethyl-9-(p-l'-ribityl)isoalloxazine

The side chain of riboflavin is characterized by the following reactions: Acetylation with acetic anhydride in pyridine gives a chloroform-soluble tetraacetate, melting at 242 to 243°. It is easily saponified by diluted alkali at room temperature; its vitamin $B_2$ activity for rats is almost the same as that of riboflavin. The formation of a tetraacetate indicates the presence of four hydroxyl groups.

Formation of a diacetone compound indicates that two hydroxyl groups in pairs are adjacent. Oxidation of riboflavin with lead tetraacetate yields 0.8 mole of formaldehyde. That proves the presence of a primary hydroxyl group in the $\alpha$ position to a secondary hydroxyl group.

The oxygen-containing part of the side chain of riboflavin can be removed by irradiation in alkaline solution. The resulting lumiflavin (m.p. 330°), in contrast to riboflavin, is chloroform-soluble.\textsuperscript{12a, b, 38} Irradiation of ribo-


\textsuperscript{37a} V. Demole, Z. \textit{Vitaminforsch.} \textbf{7}, 138 (1938).

\textsuperscript{37b} O. Warburg and W. Christian, \textit{Naturwissenschaften} \textbf{20}, 980 (1932); \textit{Biochem. Z.} \textbf{266}, 377 (1933).
flavin in neutral or acid solution removes the entire side chain, yielding lumichrome. Lumichrome is formed also by stoichiometrical oxidation of riboflavin by \textit{Pseudomonas riboflava} or by mycobacteria. Deuteroflavin is a third irradiation product of riboflavin. The photochemical behavior of vitamin B$_2$ is demonstrated in the following scheme:

\begin{center}
\begin{tikzpicture}
\node [text width=5cm,align=center] {Riboflavin} [above] at (0,0) {light};
\node [text width=5cm,align=center] at (0,-1.5) {acid or neutral solution};
\node [text width=5cm,align=center] at (0,-3) {alkaline solution};
\node [text width=5cm,align=center] at (0,-4.5) {neutral solution, absence of O$_2$};
\node [draw,shape=rectangle,minimum width=3cm,minimum height=3cm] at (-2,-2) {Riboflavin};
\node [draw,shape=rectangle,minimum width=3cm,minimum height=3cm] at (2,-2) {Riboflavin};
\node [draw,shape=rectangle,minimum width=3cm,minimum height=3cm] at (-2,-3.5) {6,7-Dimethylalloxazine};
\node [draw,shape=rectangle,minimum width=3cm,minimum height=3cm] at (2,-3.5) {6,7,9-Trimethylisoalloxazine};
\node [draw,shape=rectangle,minimum width=3cm,minimum height=3cm] at (0,-5) {Deuteroflavin};
\node [draw,shape=rectangle,minimum width=3cm,minimum height=3cm] at (-2,-6) {Alloxazine};
\node [draw,shape=rectangle,minimum width=3cm,minimum height=3cm] at (2,-6) {Isoalloxazine};
\node [draw,shape=rectangle,minimum width=3cm,minimum height=3cm] at (0,-7.5) {Deuteroleucoflavin};
\end{tikzpicture}
\end{center}

Lumichrome is the 6,7-dimethyl derivative of alloxazine (I), whereas riboflavin and lumiflavin are substitution products of the hypothetical isoalloxazine (II).

In the photolysis of 9-\((2'-\text{hydroxyethyl})\)isoalloxazine, alloxazine is formed

40 J. W. Foster, \textit{J. Bacteriol}. 47, 27 (1944); 48, 97 (1944) [\textit{C. A}. 38, 1761, 5526 (1944)].
41 The reaction mechanism of the decomposition of riboflavin by light has been discussed recently by R. Brdicka, \textit{Collection Czechoslov. Chem. Commun.} 14, 130 (1949) [\textit{C. A}. 44, 4337 (1950)]; see also ref. 42.
and the side chain produces acetaldehyde, formaldehyde, and an acid, probably formic acid.\textsuperscript{42}

The alkaline hydrolysis of riboflavin gives urea and 1,2-dihydro-6,7-dimethyl-2-keto-1-d-riboyl-3-quinazoline-carboxylic acid (III). (This acid has recently been shown to have a depressant action on cardiac and visceral muscles when injected intravenously in the dog.\textsuperscript{43}) In the case of lumiflavin, 1,2-dihydro-2-keto-1,6,7-trimethyl-3-quinazoline-carboxylic acid (IV) is obtained along with urea.\textsuperscript{12b, 43, 44}

\begin{equation}
\text{H}_3\text{C} \quad \text{N} \quad \text{CO} \quad \text{NH} \quad \text{H}_3\text{C} + \text{NH}_2\text{CONH}_2
\end{equation}

III, \( R = \text{d-riboyl}; \quad \text{IV}, \( R = \text{CH}_3

The oxocarbonic acid (IV) can be decarboxylated by sublimation with formation of the lactam (V). This, when heated with NaOH, gives 1,2-dimethyl-4-amino-5-methylaminobenzene (VI).

\begin{equation}
\text{H}_3\text{C} \quad \text{N} \quad \text{CO} \quad \text{C-} \quad \text{NH}\text{CH}_3 \quad \text{H}_3\text{C} \quad \text{N} \quad \text{CH} \quad \text{H}_3\text{C} \quad \text{NH}_2
\end{equation}

IV \quad \text{V} \quad \text{VI}

On reduction, riboflavin readily takes up two hydrogen atoms with for-

\textsuperscript{44} R. Kuhn and T. Wagner-Jauregg, \textit{Ber.} \textbf{66}, 1577 (1933); R. Kuhn and H. Rudy, \textit{Ber.} \textbf{67}, 892, (1934); R. Kuhn, K. Reinemund, and F. Weygand, \textit{Ber.} \textbf{67}, 1460 (1934).
formation of a leuco compound. The colorless dihydoriboflavin is reoxidized easily to riboflavin by air. Stronger catalytic hydrogenation of flavins yields octahydroflavins, which are easily oxidized in alkaline solution by air to the corresponding hexahydroflavins.

Three intermediate compounds have been obtained in the crystalline state by stepwise reduction of riboflavin to leucoriboflavin. They consist of molecular compounds of reduced and unreduced and radical-like intermediate molecules. In verdoflavin 1 mole of riboflavin and 1 mole of monohydoriboflavin (with a free valence) are associated; chloroflavin is probably partly free monohydoriboflavin and partly a quinhydrone, formed of riboflavin and leucoriboflavin; rhodoflavin contains the hydrochlorides of leucoriboflavin and monohydoriboflavin:

\[ \text{Flavin (yellow)} \]

---

Consumption of moles oxygen upon oxidation

0.25

Verdoflavin (bronze-green)

0.50

Chloroflavin (grass-green)

0.75

Rhodoflavin (carmoisine red)

1.0

Leucoriboflavin (colorless)
By analysis of the titration curves, Michaelis and Schwarzenbach\(^\text{47}\) showed that in solution, at low concentration including the physiological concentration range, there is an intermediate form of reduction which is entirely represented by a free radical. The maximum ratio of this to the total dye is 0.10 at pH 4.62, and 0.14 at pH 6.92, at 30°. In higher concentrations, a partial dimerization of the radical to a bimolecular compound takes place. No other molecular species on an oxidation level between flavin and dihydroflavin could be detected in solution.

On reduction of the yellow enzyme (riboflavin phosphate attached to a specific protein) at 0° by the reduced triphosphopyridine nucleotide (co-enzyme), under anaerobic conditions, a red intermediate is obtained which possesses the same absorption spectrum as rhodoflavin. Thus the red reduction form of riboflavin can be produced under nearly neutral conditions.

D. SYNTHESIS

1. Chemical Methods

In 1891, O. Kühling synthesized alloxazines by condensation of \(o\)-phenylenediamine hydrochloride with alloxan.

\[
\begin{array}{c}
\text{NH}_2 \\
\text{NH}_2 \\
\end{array}
\begin{array}{c}
\text{OC} \\
\text{CO} \\
\text{OC} \\
\text{CO} \\
\text{NH} \\
\end{array}
\rightarrow
\begin{array}{c}
\text{N} \\
\text{N} \\
\text{NH} \\
\end{array}
\begin{array}{c}
\text{OC} \\
\text{CO} \\
\text{NH} \\
\end{array}
\]

Using the same principle, R. Kuhn and P. Karrer worked out methods for the synthesis of flavins, based on \(o\)-xylene, \(D\)-ribose, and alloxan as

\[
\begin{array}{c}
\text{CH}_2\text{OH} \\
\text{HOCH} \\
\text{HOCH} \\
\text{HOCH} \\
\text{HOCH} \\
\text{CH}_2 \\
\end{array}
\begin{array}{c}
\text{NH} \\
\text{H}_3\text{C} \\
\text{NH}_2 \\
\end{array}
\begin{array}{c}
\text{O} \\
\text{CO} \\
\end{array}
+ \begin{array}{c}
\text{C} \\
\text{C} \\
\text{NH} \\
\end{array}
\rightarrow
\begin{array}{c}
\text{CH}_2\text{OH} \\
\text{HOCH} \\
\text{HOCH} \\
\text{HOCH} \\
\text{CH}_2 \\
\end{array}
\begin{array}{c}
\text{H}_3\text{C} \\
\text{NH} \\
\text{H}_3\text{C} \\
\text{NH}_2 \\
\end{array}
\begin{array}{c}
\text{O} \\
\text{CO} \\
\end{array}
\]

starting materials. Riboflavin (III) could be obtained by condensation of 1,2-dimethyl-4-amino-5-(p-l'-ribitylamine)benzene (I) with alloxan, which reacts in its lactam form (II).

The reaction is carried out in acid solution. Boric acid as a catalyst increases the yield considerably. Other catalysts are H₂S, SnCl₂, or alloxantin in the presence of 1 mole of HCl.

Four representative examples of riboflavin synthesis are given in the following, which differ in the preparation of the intermediate I.

(1) This intermediate can be prepared by condensation of o-nitroxyldidine (IV) with D-ribose and catalytic reduction of the formed riboside (V) to the diamine (I). The yield was 16% riboflavin, calculated on the amount of ribose used.

\[
\begin{align*}
\text{IV} & \quad \text{H}_3C \quad \text{NH}_2 \\
& \quad \text{H}_3C \quad \text{NO}_2 \\
\text{H}_3C \quad \text{NHCHCH(OH)}_2 \text{CH}_2 \quad \text{O} \\
\rightarrow & \quad \text{V} \\
\text{H}_3C \quad \text{NHCHCH(OH)}_2 \text{CH}_2 \quad \text{NO}_2 \\
& \quad + \text{d-ribose} \rightarrow \\
& \quad + \text{H} \rightarrow \text{I}
\end{align*}
\]

Recently, 6,7-diethyl-9-(p-l'-ribityl)isoalloxazine has been prepared by this method.

(2) o-Nitrochlorobenzenes have been reacted with amino sugars or amino alcohols, and the condensation product was hydrogenated to the diamine.

Poor yields are obtained with sugars containing four and five hydroxyl groups, but sugars with shorter chains (n < 4) give satisfactory yields.

The required glycamines can be prepared by hydrogenation of the cor-

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48 R. Kuhn, Angew. Chem. 49, 6 (1936).
50 Hoffmann-La Roche and Co., British Pat. 628,410 (Aug. 29, 1949) [C.A. 44, 4935 (1950)].
51 By condensation of aromatic amines with D-ribose, two isomers are obtained which have been considered to be the corresponding N-arylribofuranosylamines (A) and N-arylribopyranosylamines (B); A is converted to B in the presence of water, [L. Berger and J. Lee, J. Org. Chem. 11, 75, 84, 91 (1946); G. P. Ellis and J. Honeyman, Nature 167, 259 (1951); J. Chem. Soc. 1952, 1490 2053]. The Na₂SO₄ complexes of arylamine-N-β-ribopyranosides can be hydrogenated to the corresponding ribitylamines in excellent yield [Hoffmann-La Roche, Inc., U. S. Pats. 2,384,102, 2,384,105 (Sept. 4, 1945) [C.A. 40, 600, 2854 (1946)].
52 R. Kuhn and R. Ströbele, Ber. 70, 773 (1937).
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responding sugars in liquid ammonia containing 3% of water, over a Raney nickel catalyst at 85° and 200 p.s.i.\(^5\)

9-(β-Hydroxyethyl)isoalloxazine,\(^6\) 6-nitro-9-(β-hydroxyethyl)isoalloxazine, 9-(β-diethylaminoethyl)isoalloxazine, 6-nitro-9-(β-diethylaminoethyl) isoalloxazine, and other basically substituted isoalloxazines\(^6\) have been prepared by this method. 9-(Dialkylaminoalkyl)isoalloxazines, the free bases, differ chemically from riboflavin by their solubility in organic solvents, for instance CHCl\(_3\).

(3) Another method for the synthesis of substituted 2-nitroanilines which are needed for the synthesis of riboflavin is the condensation of substituted o-dinitrobenzene with sugar amines. For instance, o-dinitroxyylene and ribamine are condensed in aqueous alcoholic solution and catalytically reduced to the corresponding diamine. The over-all yield of riboflavin amounted to 4.5% of the ribose used.\(^5\)

3-Methylriboflavin\(^5\) and, recently, 6,7-dichloro-9-(1'-D-sorbityl)isoalloxazine and its analogs have been synthesized by this method; a variant uses substituted o-iodonitro-benzenes as starting materials.\(^8\)

(4) A fourth method of riboflavin synthesis starts with the condensation of 3,4-xylidine with D-ribose by boiling the amine and the sugar in alcoholic

\(^{5}\) R. B. Flint and P. L. Salzberg, U. S. Pat. 2,016,962 (1932); F. W. Holly, E. W. Peel, K. Folkers et al., J. Am. Chem. Soc. 72, 5416 (1950); 73, 332 (1951); 74, 4047 (1952).


\(^{5}\) R. Kuhn and F. Weygand, Ber. 68, 1601 (1935).

\(^{5}\) R. Kuhn, K. Reinemund, F. Weygand, and R. Ströbele, Ber. 68, 1765 (1935).

The 3,4-xylidine-N-D-riboside formed is catalytically reduced without isolation of the reaction product prior to hydrogenation. Karrer and Meerwein have shown that coupling with phenyl diazonium salt gives the corresponding azo dye, with a yield of 92% of the theoretical amount. The reduction to (2-amino-4,5-dimethylphenyl)-D-1'-ribamine can be performed with 85% of the theoretical yield.

\[
\begin{align*}
\text{H}_2\text{C} & \quad \text{NH}_2 \\
\text{H}_2\text{C} & \quad \text{H}_2\text{C} \\
\text{H}_2\text{C} & \quad \text{NH} \quad \text{C} \quad \text{C} \quad \text{C} \quad \text{C} \quad \text{CH}_2 \\
\text{H}_2\text{C} & \quad \text{H}_2\text{C} \\
\text{H}_2\text{C} & \quad \text{NHCH}_2(\text{CHOH})_2\text{CH}_2\text{OH} \\
\text{H}_2\text{C} & \quad \text{NHCH}_2(\text{CHOH})_2\text{CH}_2\text{OH} \\
\text{H}_2\text{C} & \quad \text{N}=\text{N} \quad \text{C}_6\text{H}_5 \\
\text{H}_2\text{C} & \quad \text{N} \quad \text{H}_2 \\
\text{H}_2\text{C} & \quad \text{NHCH}_2(\text{CHOH})_2\text{CH}_2\text{OH}
\end{align*}
\]

This method can be used for industrial preparation of riboflavin. The yield obtained is very high, 38% calculated for ribose. The method is limited to the synthesis of 6,7-substituted flavins, because only \(m,p\)-disubstituted aniline derivatives couple with diazonium salts in the ortho position.

61 R. Kuhn and L. Birkhofer, Ber. 71, 621 (1938).
D-Ribose, needed for the riboflavin synthesis described, can be obtained either from natural sources or by synthetic methods. It has been prepared by hydrolysis of yeast nucleic acid.\(^{65}\) From 2 kg. of yeast, only 1 to 2 g. of pure D-ribose have been obtained via yeast nucleic acid and guanosine.

The synthetic method starts with glucose, which, via calcium gluconate, is converted to D-ribose through the following steps: D-arabinose, diacetyl-arabinal, D-arabinal. The latter, by oxidation with perbenzoic acid, gives a mixture of D-arabinose and D-ribose, with a yield of 10 to 17\(\%\).\(^{66}\) The sirupy ribose prepared by this method can be obtained crystallized by conversion to aniline-N-D-ribofuranoside and subsequent hydrolysis (Berger and Lee\(^{64}\)).

Recently processes have been developed whereby ribose can be prepared directly by electrolytic reduction of ribonolactone. The corresponding acid can be obtained by rearrangement of arabonic acid, which usually is produced by the oxidation of corn sugar in alkaline solution with oxygen or air. By a newer method of the Northern Regional Research Laboratory, calcium arabonate is obtained with 85% yield by electrolytic oxidation of 2-ketogluconate.\(^{67}\)

Since the preparation of D-ribose forms a bottleneck in the synthesis of riboflavin, methods have been developed which avoid the use of ribose.

F. Weygand\(^{68}\) in 1940 showed that it is possible to use D-arabinose for the synthesis of riboflavin. N-D-Arabinoside of xylidine (I) is transformed by a so-called Amadori isomerization into the isoarabinose derivative II, which under alkaline conditions (possibly favoring the keto form) can be hydrogenated to the intermediate III of the riboflavin synthesis. The yield is about 13% of the pentose used.

Later, processes of technical importance were developed which avoid the primary use of pentoses altogether and operate with D-ribose acid or its lactone. This sugar acid can be obtained by pyridine epimerization of D-arabonic acid, which in its turn is prepared from D-glucose.

In the procedure of Pfizer and Co.,\(^{69}\) D-ribonamide is acetylated and the

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\(^{69}\) Pfizer and Co., Inc., British Pats. 515,360 (May 21, 1942), 551,401 (Feb. 25, 1943) [C.A. 38, 5845, 2344 (1944)], 585,212 (Feb. 3, 1947) [C.A. 41, 3815 (1947)].
reaction product is converted into tetraacetylribonic acid by treatment with nitrous acid, which then is reacted with PCl₅ to form the acid chloride. This is reduced to give tetraacetyl-β-ribose, palladium supported on BaSO₄ being used as a catalyst. Hydrogenation of tetraacetyl-β-ribose in the presence of o-4-xyldine, with Raney nickel or platinum as catalyst, yields tetraacetyl-1-α-ribityl-o-4-xyldine, which finally is coupled with a phenyl-diazonium salt. A similar method uses tetrabutyrl-β-ribonamide as a starting material.⁷⁰

A somewhat different method starts with β-ribonolactone, prepared from β-arabonic acid via β-ribonic acid.⁷¹ The lactone is reacted with xyldidine,


and the ribonic xylidide, after acetylation, is chlorinated to the imidochloride, which can be reduced smoothly to the amine and then be deacetylated.

\[
\begin{align*}
\text{H}_3\text{C} & \quad \text{H}_3\text{C} \\
\text{NH}_2 & \quad \text{NH}_2 \\
\xrightarrow{\text{d-ribonolactone}} & \\
\text{RNHCO(CHOH)CH}_2\text{OH} & \rightarrow \\
\text{RNHCO(CHOCOCH}_3\text{CH}_2\text{OCH}_3 & \xrightarrow{\text{PCl}_3} \\
\text{RN} & \xrightarrow{\text{H}_2} \\
\text{Cl} & \\
\text{RNHCH}_2\text{(CHOCOCH}_3\text{CH}_2\text{OCH}_3 & \xrightarrow{\text{OH}^-} \\
\text{RNHCH}_2\text{(CHOH)}\text{CH}_2\text{OH}
\end{align*}
\]

In another procedure 3,4-dimethylaniline and tetraacetyl-d-ribonitrile are subjected to catalytic reductive coupling and the resulting acetylated amine is deacetylated.

Alloxan, which was needed for the earlier synthesis of riboflavin, can be obtained by oxidation of uric acid or barbituric acid.

The newer methods use barbituric acid or its derivative directly. The condensation of an appropriate o-aminoazo compound with barbituric acid can be carried through in the presence of a weak organic acid, such as acetic acid.

\[
\begin{align*}
\text{H}_3\text{C} & \quad \text{H}_3\text{C} \\
\text{NHR} & \quad \text{NH} \\
\text{O} & \quad \text{CO} \\
\text{H}_2\text{C} & \quad \text{H}_2\text{C} \\
\text{N} & \quad \text{N} \\
\text{Ar} & \quad \text{Ar} \\
\text{R} = \text{d-Ribityl;} & \quad \text{Barbituric acid} & \quad \text{Riboflavin}
\end{align*}
\]

L-Lyxoflavin recently was synthesized according to this method. Previously, Bergel et al. used a method to convert N-d-ribityl-o-4-

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xyldine into riboflavin by coupling with diazotized aniline and shaking the resulting azo compound with excess alloxantin or dialuric acid in an atmosphere of nitrogen, finally oxidizing any leucoriboflavin by shaking with air.

A method involving neither ribose nor alloxan is the following: 3,4-dimethylaniline (I) is reductively condensed in the presence of a palladium catalyst with tetraacetyl-D-ribonitrile (II) with loss of NH₃. Ribonitrile can be prepared from ribonic acid via the amide. The formed N-tetraacetyl-D-ribitylamino-3,4-dimethylaniline (III) is coupled with p-nitrophenyldiazonium chloride, and the product is reduced in the presence of a platinum catalyst to 1-N-tetraacetyltritylaminol-2-amino-4,5-dimethylbenzene (IV). This compound is then condensed with 5,5-dichlorobarbituric acid (V) to form tetraacetylriboflavin (VI) which is then hydrolyzed to riboflavin.

\[
\begin{align*}
\text{I} & \quad \text{II} & \quad \text{III} & \quad \text{IV} & \quad \text{V} & \quad \text{VI} \\
\text{H}_3\text{C} & \quad \text{CH}_2\text{OAc} & \quad \text{CN} & \quad \text{CH}_2\text{OAc} & \quad \text{Cl}_2\text{C} & \quad \text{CO} \\
\text{NH}_2 & \quad (\text{HCOAc})_3 & \quad & \quad & \quad & \quad \\
\text{H}_3\text{C} & \quad & \quad \text{CH}_2 & \quad & \quad \text{CO} \\
\text{H}_3\text{C} & \quad & \quad & \quad & \quad & \quad \\
\end{align*}
\]

6,7-Dimethyl-9-benzylisoalloxazine can be formed by heating 5,5-dichlorobarbituric acid in pyridine with 1-benzylamino-2-amino-4,5-dimethylbenzene. Similarly, 5-amino-N-ribityl-o-xyldine and 5,5-dichlorobarbituric acid gives riboflavin in excellent yield.

Synthesis of Riboflavin-5'-phosphate (flavin mononucleotide, FMN). The phosphorylation of riboflavin with phosphoryl chloride in pyridine provides

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a method for small-scale preparation of riboflavin-5'-phosphate. The original method of Kuhn and Rudy\(^{77}\) yields mainly a cyclic phosphate, riboflavin-4',5'-phosphate, as shown by Forrest and Todd.\(^{78}\) Acid hydrolysis of the cyclic ester gives riboflavin-5'-phosphate, which is identical with the natural riboflavin phosphate.

\[
\text{OH OH OH} \\
\text{CH_2-C-C-C-CH_2OP=O} \\
\text{H H H} \\
\text{OH}
\]

D-Riboflavin-5'-phosphate

A more complicated way of synthesis was carried out previously by conversion of riboflavin into the 5-trityl ether, acetylation, removal of the ether group, and phosphorylation of the exposed 5'-hydroxyl group with phosphorous oxychloride; hydrolysis then gave D-riboflavin-5'-phosphate.\(^{79}\)

Dichlorophosphoric acid seems to be a more useful reagent for the phosphorylation of riboflavin than phosphorous oxychloride; FMN has been prepared by this method in quantities greater than milligrams.\(^{80}\)

The most recent method for the synthesis of riboflavin-5'-phosphoric acid uses anhydrous metaphosphoric acid as a phosphorylating agent.\(^{80a}\)

FMN gives a crystallized monodiethanolamine salt with a water solubility of more than 200 times that of riboflavin.

**2. Biochemical Formation**

1,2-Dimethyl-4,5-diaminobenzene (I) seems to be the first biogenetic precursor of vitamin B\(_2\) and vitamin B\(_{12}\), both substances containing I as a structural element.\(^{81}\)


II. CHEMISTRY

2-Amino-4,5-dimethyl-l-ribitylaminnobenzene (II) might be the next intermediate of the biosynthesis of riboflavin since in the presence of alloxan it is a potent stimulator of Lactobacillus casei. This substance is used for the synthesis of riboflavin also by Mycobacterium tuberculosis.

As a higher natural intermediate, 6,7-dimethyl-9-β-riboflavidosflavin (III) also has been considered, yielding the vitamin by reduction of the N-glycoside linkage. According to Weygand, not only N-β-riboflavose, but also an N-β-arabinoside could be involved, being transformed by an Amadori rearrangement to the β-ribose derivative.

\[
\begin{align*}
\text{H}_3\text{C} & \quad \text{NH}_2 & \quad \text{H}_3\text{C} & \quad \text{NHCH}_2(\text{CHOH})_2\text{CH}_2\text{OH} \\
\text{H}_3\text{C} & \quad \text{NH}_2 & \quad \text{H}_3\text{C} & \quad \text{NH}_2
\end{align*}
\]

\[
\begin{align*}
\text{H}_3\text{C} & \quad \text{N} & \quad \text{CO} \\
\text{H}_3\text{C} & \quad \text{N} & \quad \text{CO}
\end{align*}
\]

5′-β-Riboflavin-β-glucopyranoside (riboflavinyl glucoside) has been prepared by incubation of riboflavin with an enzyme obtained from rat liver. No function has so far been ascribed to this new derivative of riboflavin.

Recently light has been thrown on the mechanism of riboflavin biosynthesis in Ashbya gossypii by using isotopic compounds. C\textsuperscript{14}-formate introduced into the culture medium gave rise to riboflavin tagged in the carbon-2 position. The tracer atom from C\textsuperscript{14}-bicarbonate ended up in the carbon-4 position. C\textsuperscript{14}H\textsubscript{3}COOH, CH\textsubscript{3}C\textsuperscript{14}OOH, and totally labeled glucose produced riboflavin containing C\textsuperscript{14} in both the side chain and o-xylene portions of the molecule.

The formation of ribose-5-phosphoric acid and the corresponding ketose,

82 H. P. Sarett, Federation Proc. 4, 101 (1945); J. Biol. Chem. 162, 87 (1946).
3. **Microorganisms as Producers**

Different natural sources have been used for the production of vitamin B<sub>2</sub> by fermenting microorganisms. Whey and other milk by-products have been treated with lactose-fermenting yeasts, especially *Saccharomyces fragilis*, or with *Clostridium butylicum*, several species of *Lactobacillli*, or molds. Molasses or other carbohydrate mashses were fermented with various strains of butanol-producing *Clostridia*, especially *Cl. acetobutylicum*; among the bacteria, this microorganism is one of the best producers of riboflavin. Riboflavin is formed by numerous strains of *Mycobacterium tuberculosis*. In *Mycobacterium smegmatis* up to 3.6 mg. of riboflavin is formed per 100 mg. of the dried cells.<sup>86a</sup>

A majority of the varieties of the yeast species *Candida* produce substantial amounts of riboflavin when glucose is used as the carbon source. *Candida guilliermondia* and *Candida flaveri* were found to produce high yields of riboflavin on a simple synthetic medium of low cost.<sup>86</sup> The use of the *Candida* germs for commercial exploitation is very difficult because of their extremely low tolerance for iron. 2,2'-Dipyridyl has been recommended to control the iron content in fermentation media.<sup>86a</sup>

In 1935 Guilliermond, a French mycologist, observed that *Eremothecium ashbyii* in laboratory cultivation produced a yellow pigment which formed crystals in the thread-like cells. The microorganism originally was isolated as a pathogen for cotton plants in the Belgian Congo. The pigment has since been identified as riboflavin. High yields of this vitamin have been observed in fermentations with *E. ashbyii*, using sugar and nitrogenous compounds in a deep fermentation method, with formation of up to nearly 500 mg. per liter of medium.<sup>85</sup>

*Ashbya gossypii* also was found to be of value in the microbiological production of riboflavin.<sup>87</sup> In shake cultures on a medium containing 4% glucose, 0.5% peptone, and 2.5% corn steep liquor solids, titers of 1000 mg. per liter were obtained. A large variety of factors influencing biosynthesis of riboflavin in submerged aerobic cultivation by *A. gossypii* has been studied.<sup>88</sup>

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Among the molds, the following have been recorded as flavin-producing: *Aspergillus niger, Aspergillus flavus, Penicillium chrysogenum*, and species of *Fusarium*.

For recovery of the vitamin from the fermented liquors adsorption-elution methods have been used. Another possibility is the precipitation of reduced forms of riboflavin from nutrient media by the metabolic reducing action of certain bacteria, particularly a group of generally avirulent streptococci. For instance, up to 90% of the dissolved riboflavin can be obtained as a red-orange, amorphous precipitate with *Streptococcus faecalis*, under anaerobic conditions (cf. p. 314). Instead of bacterial reduction, reducing chemicals have been used, for instance, sodium dithionite (Na₂S₂O₄·2H₂O), stannous, titanous, chromous, and vanadous salts. These methods make use of the fact that the various reduced forms of riboflavin are much less soluble in water than the oxidized form. The solubility in 1 ml. of water is for riboflavin 100 to 130 γ, for verdoflavin 50 γ, for chloroflavin 20 γ, and for leucoriboflavin approximately 8 γ.

## E. SPECIFICITY

Some of the more important relations between chemical constitution and biological activity will be discussed here. Details about the specificity of flavin enzymes can be found in a later section.

### 1. Growth Stimulators

Riboflavin tetraacetate and diacetone riboflavin are active for rats, probably as a result of hydrolysis in the organism; the tetraacetate is, however, inactive on lactic acid bacteria. Riboflavin-5'-phosphate and flavin adenine dinucleotide not only are growth-promoting in rats but both were also as effective as riboflavin for growth and acid production of *L. helveticum*. The flaviny1 glucosides are inactive.

It appears that only flavins which serve as a sole source of growth-promoting flavin (in the absence of suboptimal amounts of riboflavin) for *L. casei* and *B. lactis acidi* are capable of supporting growth in animals. To possess this activity, the flavin must be a ribityl derivative and be substituted in either the 6 or 7 position or both by a methyl group or by an ethyl group in the 6 position, together with a methyl group in the 7 position. 6,7-Diethyl-9-(n-1'-ribityl)isoalloxazine recently has been found capable

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61a S. J. Shimizu, *J. Fermentation Technol. (Japan)* 28, 139 (1950) [C.A. 47, 1755 (1953)].
of serving as the sole source of flavin in the growth of *Lactobacillus casei*; however, this flavin has an antiriboflavin activity in the growing rat. 7-Ethyl-9-(p-1’-ribityl)isoalloxazine is effective only in the presence of suboptimal amounts of riboflavin.

Absence of substituents in both the 6 and 7 positions of riboflavin is connected with high toxicity. For biological activity, the imino group in the 3 position has to be unsubstituted.

Substituents in any other than the 6 and 7 positions on the benzene ring destroy and in certain cases reverse the vitamin activity. For instance, isoriboflavin, an isomer of riboflavin, which has its two methyl groups in the 5 and 6 positions, is an antagonist of vitamin B₂.

From the examples given, it is obvious that only slight alterations of the ring substituent in the riboflavin structure can be made without the loss of vitamin activity.

The following L-araboflavins, \( \text{R--CH}_2\text{--C--C--C--CH}_2\text{OH} \), possess some little stimulating activity for rats and lactic acid bacteria in the presence of suboptimal amounts of riboflavin: 6,7-dimethyl-9-(L-1’-arabityl)isoalloxazine (L-araboflavin), 6,7-trimethylene-9-(L-1’-arabityl)-isoalloxazine, 6,7-tetramethylene-9-(L-1’-arabityl)isoalloxazine, 6-methyl-7-amino-9-(L-1’-arabityl)isoalloxazine; the D-arabityl derivative corresponding to the latter substance is said to be about half as effective as riboflavin in promoting the growth of B₂-avitaminotic rats.

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95 Sümi Nishida, *C.A.*, 45, 7127 (1951); 46, 6715 (1952).
It has been suggested\(^9^6\) that the small vitamin B\(_2\) activity of L-araboflavin might be due to contamination with riboflavin, formed during the synthesis by an Amadori rearrangement (see p. 318). In rats receiving 10 \(\gamma\) of riboflavin per day L-araboflavin in higher doses (200 \(\gamma\) per day) reduces the growth very slightly.\(^9^7\)

Of the other stereoisomers of riboflavin which have been synthesized, only \(\Delta\)-xyloflavin, \(\text{R} - \text{CH}_2 - \text{C} - \text{C} - \text{CH}_2\text{OH}\), is said to produce a slow gain of weight in vitamin B\(_2\)-deficient rats.\(^9^8\) L-Lyxoflavin, \(\text{R} - \text{CH}_2 - \text{C} - \text{C} - \text{CH}_2\text{OH}\), was isolated from human myocardium in 1949 and has been made synthetically.\(^9^9\) It is devoid of riboflavin activity in rats when tested by the standard assay, but in a rat assay for unidentified vitamins in liver and other source materials, as well as for \textit{Lactobacillus lactis}, L-lyxoflavin has shown growth-promoting or vitamin activity.\(^1^0^0\) Lyxoflavin seems to be the first exception from the generally accepted rule that flavins isolated from different natural sources are chemically identical with \(\Delta\)-riboflavin; but its natural existence has not been definitely confirmed.\(^1^0^1\)

Besides the mentioned riboflavin isomers, \(\text{L}-1'\)-ribityl-, \(\text{D}-1'\)-arabityl-, and \(\text{D}-1'\)-lyxitylflavin have been prepared synthetically; none of these substances has vitamin B\(_2\) activity.

2. Antagonists of Riboflavin

Diethylflavin, 6,7-diethyl-9-(\(\text{D}-1'\)-ribityl)isoalloxazine, has already been mentioned as an antagonist of riboflavin in the growing rat.\(^9^2^a\)

Isoriboflavin, 5,6-dimethyl-9-(\(\text{D}-1'\)-ribityl)isoalloxazine, in a daily dose of 2 mg. in rats almost completely inhibits the growth-promoting effect of 10 \(\gamma\) of riboflavin per day. The inhibitory effect can be prevented entirely

\(^1^0^1\) T. S. Gardner, E. Wenis, and J. Lee, \textit{Arch. Biochem. and Biophys.} \textbf{34}, 98 (1951).
by the daily administration of 40 $\gamma$ of riboflavin. Two milligrams of isoriboflavin daily restricts the growth of riboflavin-deficient rats much more than does the deficiency of the vitamin alone.\textsuperscript{102} In L. casei isoriboflavin showed no signs of competitive inhibition of riboflavin.\textsuperscript{40}

$d$-Araboflavin evidently is an antagonist of riboflavin. Two hundred micrograms per day decreases the rate of growth of rats receiving 10 $\gamma$ of riboflavin per day to such an extent that no growth takes place by the third week.\textsuperscript{97}

Another strong antagonist of riboflavin is $d$-galactoflavin, 6,7-dimethyl-
\[
\begin{array}{c}
\text{OH} \\
\text{H} \\
\text{H} \\
\text{OH}
\end{array}
\]
9-(d-1'-dulcytl)isoalloxazine, R—CH$_2$—C—C—C—C—CH$_2$OH. In a dose
\[
\begin{array}{c}
\text{H} \\
\text{OH} \\
\text{OH} \\
\text{OH} \\
\text{H}
\end{array}
\]
of 2.16 mg. per day, it inhibits completely the response of the animals to 10 $\gamma$ of riboflavin daily and markedly inhibits the response to 40 $\gamma$ of vitamin $B_2$ daily. The inhibitory effect is almost, but not completely, prevented by 200 $\gamma$ of riboflavin daily.\textsuperscript{103}

Dichloroflavin, 6,7-dichloro-9-(d-1'-ribityl)isoalloxazine (I), inhibits the growth of Staphylococcus aureus, Streptobacterium plantarum, and Bacillus lactis acidi, but not of yeast. The inhibition is competitively prevented by riboflavin. Dichloroflavin has an oxidation reduction potential of $E_0 = -0.095$ volt (pH 7); for riboflavin it is $E_0 = -0.185$ volt (pH 7). This difference has been regarded as an explanation for the inhibitory behavior of dichloroflavin; perhaps the vitamin analog cannot function like riboflavin in the oxidation-reduction reactions which are catalyzed by the riboflavin coenzymes.\textsuperscript{103a} Twelve other halogen-substituted flavins with various sugar chains in the 9 position have been found to be less effective as riboflavin antagonists than dichloroflavin, tested with Streptobacterium plantarum P 32.\textsuperscript{104}

The growth of Eremothecium ashbyii, which produces riboflavin, is inhibited by dichloroflavin, without reduction of its riboflavin production.\textsuperscript{105} Therefore, the inhibition of this microorganism probably is not to be associated directly with displacement of the vitamin.

It has been shown that certain 9-substituted 6,7-dichloroisalloxazines are inhibitors of $d$-amino acid oxidase.\textsuperscript{106} However, neither dichloroflavin

\textsuperscript{103a} R. Kuhn, F. Weygand, and E. F. Möller, Ber. 76, 1014 (1943).
\textsuperscript{104} F. Weygand, R. Löwenfeld, and E. F. Möller, Ber. 84, 101 (1951).
\textsuperscript{105} W. H. Schopfer, Intern. Z. Vitaminforsch. 20, 116 (1948).
nor its 5'-phosphate, even in a 1000-fold excess over riboflavin, has an
influence on the activity of D-amino acid oxidase or xanthine oxidase.\(^{106a}\)

\[
\begin{align*}
\text{Dichloroflavin} & \\
\text{Dichloro-D-sorboflavin}
\end{align*}
\]

For one substance of this type, 6,7-dichloro-9-(1'-D-sorbityl)isoallox-
azine (II), a special biological effect has been observed.\(^{59}\) It exhibits no
significant inhibition of riboflavin microbiologically and \textit{in vivo} in rats.
However, it is effective in producing regression of established lympho-
sarcoma implants in mice. The D-ribityl, L-arabityl, and D-dulcityl analogs
of compound II show, respectively, slight, questionable, and no carcinolytic
activity. 6-Chloro-9-(1'-D-sorbityl)isoalloxazine appeared to have some ac-
tivity in several tests. Other riboflavin analogs in which the substituents
in the 6 and 9 positions were varied gave questionable or negative results.

Lettré\(^{106b}\) first demonstrated that certain flavins, which are antagonists
of riboflavin, have an antimitotic effect. Later, regression of lymphosarcoma
and decreased growth rate of spontaneous mammary carcinomas in mice

\(^{106b}\) H. Lettré, \textit{Angew. Chem.} \textbf{53}, 363 (1940); H. Lettré and M. E. Fernholz, \textit{Ber.} \textbf{73},
436 (1940).
and of Walker carcinomas in rats deficient in riboflavin were reported.\(^{107}\)
In some cases, the animals were rendered strongly deficient in riboflavin by feeding one of the riboflavin antagonists, isoriboflavin, galactoflavin, or diethylflavin, along with a diet deficient in riboflavin. The activity of dichloro-D-sorboflavin (II) shows that the mechanism of regression of the lymphosarcoma need not necessarily occur through riboflavin inhibition.

Lumiflavin, the photolysis product of riboflavin, is either an inhibitor or a stimulator of the utilization of riboflavin or flavin adenine dinucleotide by \(L.\) \(casei\), depending upon the relative amounts of lumiflavin present.\(^{108}\)

The phenazine analog of riboflavin, 2,4-diamino-7,8-dimethyl-10-(\(d\)-ribityl)-5,10-dihydrophenazine (I),\(^{109}\) antagonizes the action of vitamin \(B_2\) in riboflavin-requiring bacteria. The dinitrophenazine derivative from which I is prepared produces mild riboflavin deficiency in mice. Adequate amounts of riboflavin overcome the effects of the compound.

\[
\begin{align*}
\text{CH}_2(\text{CHOH})_3\text{CH}_2\text{OH} & \quad \text{CH}_3 \\
\text{H}_2\text{C} & \quad \text{H}_2\text{C} \\
\text{NH} & \quad \text{NH}_2 \\
\text{NH}_2 & \quad \text{NHCH}(\text{CH}_2)_2\text{N}((\text{C}_2\text{H}_5)_2\text{HCl}
\end{align*}
\]

**Atabrin (Mepacrine)**

Many substances with antimalarial activity were found to inhibit the growth-promoting effect of riboflavin on microorganisms, for instance mepacrine (atabrin) (II),\(^{110, 111}\) substituted pyrimidines,\(^{112}\) and quinine. However there is no strong parallelism between the antimalarial and the antivitamin function; for instance, proguanil (paludrine) does not antagonize riboflavin. The structural similarity of certain antimalarials to the flavin nucleus is probably not directly correlated to their antimalarial action.\(^{113}\)

**Mode of Action of Riboflavin Antagonists.**\(^{114}\) At least three ways are pos-

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113 L. Hellermann, A. Lindsay, and M. Bovarnick, *J. Biol. Chem.* 163, 553 (1946).
sible in which a riboflavin antagonist might bring about growth inhibition: (a) by competition with the prosthetic group of flavoenzymes; (b) by competitively inhibiting the phosphorylation of riboflavin; and (c) by being enzymatically converted to an analog of riboflavin-5-phosphate or flavin adenine dinucleotide, in which form they might competitively inhibit flavoenzymes.

It has been shown that yeast flavokinase, the enzyme which catalyzes the phosphorylation of riboflavin, also phosphorylates d-araboflavin and dichloroflavin to the corresponding nucleotides. The effects of these phosphorylated riboflavin analogs on flavoenzymes have not yet been studied.

Isoriboflavin, galactoflavin, and sorboflavin neither are phosphorylated by flavokinase nor do they inhibit the enzymatic phosphorylation of riboflavin by flavokinase. This enzymatic process is inhibited by lumiflavin, when present in excess over riboflavin.

3. Nitroflavins and Basically Substituted Isoalloxazines

Two 6-nitroflavins have been synthesized: \(^\text{115}\)

\[
\text{CH}_2\text{CH}_2\text{OH} \\
\begin{array}{c}
\text{I} \\
\text{O}_2\text{N} \\
\text{N} \\
\text{N} \\
\text{CO} \\
\text{CO} \\
\text{NH}
\end{array}
\]

6-Nitro-9-(β-hydroxyethyl)-isoalloxazine

\[
\text{CH}_2\text{CH}_2\text{N(C}_2\text{H}_5)_2 \\
\begin{array}{c}
\text{II} \\
\text{O}_2\text{N} \\
\text{N} \\
\text{N} \\
\text{CO} \\
\text{CO} \\
\text{NH}
\end{array}
\]

6-Nitro-9-(β-diethylaminoethyl)-isoalloxazine

Chemotherapeutic tests with substance I did not show noticeable success.

The structural similarity between riboflavin and atabrin suggested the synthesis of basically substituted isoalloxazine derivatives as possible antimalarials. The following types have been prepared: \(^\text{56, 115-118}\)

\[
\begin{array}{c}
\text{R}'''\text{CH(CH}_2)_n\text{N(C}_2\text{H}_5)_2 \\
\text{III} \\
\begin{array}{c}
\text{R}'' \\
\text{R}'
\end{array}
\end{array}
\]

\[
\begin{array}{c}
\text{CH}_2\text{CHOHCH}_2\text{NR}_2 \\
\text{IV} \\
\begin{array}{c}
\text{R} = \text{H} \\
\text{or C}_2\text{H}_5
\end{array}
\end{array}
\]


They were found to be devoid of antimalarial activity and did not show the tendency to inhibit the metabolic effects of riboflavin. 9-(β-Diethylaminoethyl)isoalloxazine hydrochloride was ineffective in mice infected with *Trypanosoma nagana*.115

Summary. The preceding discussions show that only slight changes in the chemical constitution of riboflavin can be made without loss of vitamin B2 activity. This fact is in agreement with the high chemical specificity of vitamins in general. In replacing the ribityl side chain by other sugar alcohols, or by alteration of substituents in the benzene ring of the isoalloxazine nucleus, derivatives can be obtained which are antagonists of riboflavin.

No derivatives of flavins effective against pathogenic organisms have yet been developed but carcinolytic activity was found among chloro-D-sorboflavins.

### III. Industrial Preparation

THEODOR WAGNER-JAUREGG

Pure crystallized riboflavin for therapeutic purposes is made by chemical synthesis. It is difficult to indicate actual manufacturing processes, since they are held as confidential. The best index of determining the methods used in the industry is the patent literature.1

Concerning the pentose component, methods of riboflavin synthesis without the use of D-ribose are of interest, since none of the known syntheses of D-ribose are simple and economical. Different syntheses of N-(D-ribityl)-3,4-dimethylaniline, the key intermediate in the riboflavin synthesis, have been developed which do not involve D-ribose (p. 318). The introduction of the second amino group can be performed with good yield by the method of Karrer and Meerwein (p. 317).

With regard to the isoalloxazine formation, different patented methods have been described: (1) requisite diamine and alloxan; (2) requisite aminoazo compound and barbituric acid;2 (3) requisite aminoazo compound and dialluric acid in the presence of a hydrogen transfer catalyst (in this method, reduction to the diamine occurs); (4) requisite diamine and dichlorobarbituric acid. Examples have been given in the preceding section (pp. 315ff.).

Vitamin B2 concentrates suited to enrich poultry and livestock feeds can be prepared more cheaply by fermentation processes. During World War

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II, preparation of riboflavin was started from the residues of butanol-acetone fermentation with *Clostridium acetobutylicum*.3

Currently, most of the commercial riboflavin production by aerobic fermentation is probably obtained by biosynthesis with *Eremothecium ashbyii*, in submerged culture with continuous aeration and agitation. Patients covering this process were filed by several firms,4, 5 and conditions for the production of riboflavin by *Ashbyii* have been reported.5 By the use of *E. ashbyii* grown on solid media (germ rice and germ wheat), the vitamin B2 production on an industrial scale is said to have reached a maximum at 20,000 γ per gram (2%).6

A medium containing grain stillage from the ethanol fermentation, cerelose and 1% peptone is excellent for the cultivation of *A. gossypii* to produce yields of riboflavin as high as 15,000 γ per gram.7 Studies of riboflavin production for commercial preparation by fermentation using the yeast *Ashbya gossypii* upon a pilot-plant scale were made recently.8 Corn steep liquor, peptone, and tankage are suitable nitrogen sources with the fermentation of nutrient mashes.9, 10

In 1943, more than 75,000 lb. of crystalline riboflavin were produced from all sources. It is priced at about $100 to 130 per kilogram today, whereas in 1938 the price was $7,945 a pound.11

Besides the pharmaceutical use, small amounts of vitamin B2 are incorporated in most bread flours and breakfast foods and in nearly all poultry and hog feeds.

9 Merek and Co., British Pat. 640,452 (July 19, 1950) [C.A. 44, 9622 (1950)].
IV. Biochemical Systems

M. K. HORWITT

Knowledge of the close relationship between vitamins and biological oxidations may be said to date from 1932, the year in which Warburg and Christian\(^1\) discovered the first flavoprotein. This compound, often referred to as the “old yellow enzyme,” which they obtained from the aqueous extract of bottom yeast was soon separated\(^2\) into a protein and a yellow prosthetic group. Stern and Holiday,\(^3\) using spectroscopic methods, found that the prosthetic group of Warburg’s yellow enzyme was a derivative of alloxazine. This fact, when combined with the observations of Ellinger and Koschara,\(^4\) Booher,\(^5\) and Kuhn \textit{et al.}\(^6\) on the correlations between vitamin B\(_2\) and a water-soluble yellow-green fluorescent pigment, was soon corroborated by the synthesis of riboflavin by the Kuhn\(^7\), \(^8\) and Karrer\(^9\) schools. Theorell’s\(^10\) demonstration that Warburg’s enzyme contained one molecule of phosphate and Kuhn, Rudy, and Weygand’s\(^11\) proof of constitution of riboflavin-5-phosphoric acid were the concluding steps in a fascinating story of the first separation, identification, and synthesis of the prosthetic group of an enzyme.

A. COENZYMES

All flavoproteins can be characterized as specific proteins which contain either flavin mononucleotide or flavin dinucleotide as prosthetic groups, or coenzymes. The flavin mononucleotide, riboflavin phosphate, is not in the strict sense a nucleotide, since the compound is derived from D-ribitol rather than from D-ribose.\(^11\) The location of the phosphoric acid at the 5 position has been definitely established.\(^12\) To date at least three flavoproteins with enzymatic activity have been shown to contain the mononucleotide. These are Warburg’s yellow enzyme, cytochrome c reductase, and L-amino acid oxidase.

Flavin adenine dinucleotide (FAD) is isoalloxazine adenine dinucleotide.

\(^{3}\) K. G. Stern and E. R. Holiday, \textit{Ber.} 67, 1104, 1442 (1934).
\(^{4}\) P. Ellinger and W. Koschara, \textit{Ber.} 66, 315, 808 (1933).
\(^{5}\) L. E. Booher, \textit{J. Biol. Chem.} 102, 39 (1933).
\(^{10}\) H. Theorell, \textit{Biochem. Z.} 272, 155 (1934).
\(^{11}\) R. Kuhn, H. Rudy, and F. Weygand, \textit{Ber.} 69, 2034 (1936).
Its structure has not been proved by synthesis. According to Warburg and Christian,\textsuperscript{13} it is composed of one adenine, one flavin, two pentose, and two phosphoric acid molecules and may be visualized as a combination of riboflavin phosphate and adenylic acid with the elimination of one molecule of water.

The enzymatic phosphorylation of riboflavin by an enzyme in yeast named flavokinase has recently been reported by Kearney and Englund.\textsuperscript{14}

\textsuperscript{13} O. Warburg and W. Christian, Biochem. Z. 298, 150 (1938).

\textsuperscript{14} E. B. Kearney and S. Englund, J. Biol. Chem. 193, 821 (1951).
The reaction catalyzed by this enzyme is:

$$\text{Riboflavin} + \text{ATP} \rightarrow \text{Riboflavin-5-phosphate} + \text{ADP}$$

The mechanism of transformation of riboflavin phosphate to flavin adenine dinucleotide is not known, but it has been shown to occur in human blood cells.\(^{15}\)

1. **General Properties of Flavin Coenzymes**

Riboflavin phosphate and flavin adenine dinucleotide resemble the parent vitamin, riboflavin, in many respects. They exhibit the same characteristic yellow color and yellow-green fluorescence. Reduction with hyposulfite, platinum, or \(H_2\) will reduce riboflavin and its coenzymes to colorless compounds which will reoxidize to their original state when shaken with air. If reduced in strongly acidic solution, a red intermediate is formed which has the properties of a semiquinoid radical.\(^{16}\) In solution they are essentially unstable. This decomposition is influenced by light, heat, and pH, riboflavin being rapidly decomposed in strongly alkaline solutions.

Riboflavin phosphate is considerably more soluble in water than free riboflavin and can be precipitated by various salts. It is hydrolyzed quite slowly in weakly alkaline solutions, quite rapidly in acid solutions, and by phosphatases such as \(\alpha\)-glycerophosphatase. Riboflavin phosphate combines with specific proteins,\(^{17}\) the apoenzymes, by attachment at the phosphoric acid group and at the slightly acidic imino group in the 3 position.

The typical fluorescence of riboflavin is dependent upon the presence of a free 3-imino group, and neither 3-substituted riboflavin nor the enzyme systems will fluoresce.


2. Riboflavin Phosphate

Banga et al. obtained a yellow substance from heart muscle in 1932 which may have been the first preparation of riboflavin phosphate. However, the yellow enzyme which Warburg and Christian obtained from yeast in the same year was more thoroughly investigated. They purified an aqueous extract of autolyzed bottom yeast by treatment with lead subacetate, removed excess lead with phosphate, and precipitated the yellow enzyme in the form of a viscous oil at low temperature with carbon dioxide and acetone. After reprecipitation from acetone and precipitation with methanol at 0°, a dry product was obtained which could be readily dissociated into a colorless protein and a yellow prosthetic group. The latter was later proved to be riboflavin phosphate.

Theorell has shown that the combination of riboflavin phosphate with the apoenzyme could be reversibly dissociated as follows: When a solution of the yellow enzyme was dialyzed against 0.02 N HCl at 0°, the dialyzate was slowly decolorized. The colored group (riboflavin phosphate) passed through the membrane, and the colorless protein remained behind. The protein was changed to a metaprotein by its contact with the dilute acid (i.e., precipitated when brought to pH 7), but when the protein was dialyzed against water to remove all traces of hydrochloric acid, 50 to 70% of the metaprotein was renatured. This renatured protein was now capable of recombination with the coenzyme riboflavin phosphate to produce a complex with all the properties of the original yellow enzyme.

The combination between the coenzyme and the apoenzyme takes place in a stoichiometric manner as shown in Fig. 1.

The reversible dissociation procedure of Theorell, which requires several days for its completion, has been replaced by a simpler method by Warburg and Christian in which a good yield was obtained in about 1 hour. To a solution of yellow enzyme, purified by electrophoresis, an equal volume of saturated ammonium sulfate was added. Sufficient 0.1 N hydrochloric acid was added to this solution at 0° to shift the pH to about 2.8. The colorless precipitate formed contained 78% of the apoenzyme in native form while the coenzyme remained in the supernatant fluid. Resynthesis was accomplished by merely remixing the components.

3. Riboflavin Adenine Dinucleotide

The dinucleotide is widely distributed in animal tissues and in microorganisms. It has been isolated from liver, kidney, muscles, tumor tissue, yeast, and Neurospora. 18

19 H. Theorell, Biochem. Z. 275, 344 (1934).
Since riboflavin is necessary for the growth of many bacteria and is a common component of plant products, it is logical to assume that the dinucleotide is a common constituent of the cells of most living things. Yeast offers the most convenient source for preparation. Warburg and Christian\textsuperscript{13} extracted yeast at 75°; the filtrate was two-thirds saturated with ammonium sulfate and extracted with phenol. The phenol extracts were mixed with ether, and the dinucleotide extracted with water. The ether was removed by evacuation, the aqueous solution acidified with nitric acid to about pH 2, and the dinucleotide precipitated as the silver salt. The precipitate was resuspended in water and decomposed with hydrogen sulfide. The dinucleotide, which was almost completely absorbed on the silver sulfide precipitate, was eluted with dilute barium acetate. The eluates were mixed with ammonium acetate solution and concentrated to dryness in \textit{vacuo}. By taking advantage of the fact that the barium salt of the dinucleotide is twice as soluble at 60° as at room temperature, it was possible to separate the barium salts of the dinucleotide from the barium salts of the contaminating adenine nucleotides.

The similarities of the dinucleotide to riboflavin phosphate in color, fluorescence, and reversible reduction and oxidation have been discussed (p. 336). It is less stable than riboflavin because of its tendency to hydro-

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig1.png}
\caption{Synthesis of the Warburg-Christian flavoprotein. The activity of the enzyme increases as more of the prosthetic group, riboflavin phosphate, is added to form the original catalytically active flavoprotein (Theorell\textsuperscript{20}).}
\end{figure}

lyze to riboflavin phosphate and adenylc acid. The absorption spectra of riboflavin and flavin adenine dinucleotide are given in Fig. 2.13

B. ENZYMES

The classification of flavoproteins based upon their behavior during in vitro experiments may lead to erroneous conclusions about their intracellular function. Relatively slight changes in the method of isolation may produce flavoproteins with different properties and with sluggish activities not normally associated with cellular action. Until more definitive information becomes available, one should not discount the suspicion that an artifact may have been produced in some of the isolation procedures. During the development of our present knowledge of flavoproteins a system of nomenclature has evolved which binds specific proteins to specific substrates. Whether or not such activities are as represented, it is necessary to characterize the individual enzymes in this manner in order to appreciate not only the work which has been done but also that which will be reported.

Table I summarizes the known flavoproteins and some of their characteristics.

1. OLD YELLOW ENZYME

a. Preparation and Properties

The discovery, isolation, and some of the properties of the yellow oxidation enzyme have been discussed in the sections describing the coenzyme riboflavin phosphate (see p. 337).
Since homogeneous preparations contain 0.66% riboflavin phosphate, and since ultracentrifuge measurements of the molecular weight give a figure of about 70,000, there can be only one molecule of prosthetic riboflavin phosphate per molecule of flavoprotein. Elementary analysis of the enzyme by Theorell yields typical figures for a protein: C = 51.5%; H = 7.37%; N = 15.9%; P = 0.043%; S = 1.0%. The specific rotation, $[\alpha]$, is $-30^\circ$.

### TABLE I

**FLAVOPROTEINS**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
<th>Prosthetic group[a]</th>
<th>Reducing system</th>
<th>Oxidizing system</th>
<th>Approx. turnover number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warburg yellow enzyme</td>
<td>Bottom yeast</td>
<td>RP</td>
<td>$H_2$-DPN or $H_2$-TPN</td>
<td>$O_2$, M.B.</td>
<td>50</td>
</tr>
<tr>
<td>Crossed yellow enzyme</td>
<td>Synthetic</td>
<td>FAD</td>
<td>$H_2$-DPN or $H_2$-TPN</td>
<td>$O_2$</td>
<td>35</td>
</tr>
<tr>
<td>Haas enzyme</td>
<td>Bottom yeast</td>
<td>FAD</td>
<td>$H_2$-TPN</td>
<td>M.B.</td>
<td>&gt;50</td>
</tr>
<tr>
<td>Diaphorase, coenzyme factor, straub flavoprotein</td>
<td>Various</td>
<td>FAD</td>
<td>$H_2$-DPN</td>
<td>M.B.</td>
<td>8500</td>
</tr>
<tr>
<td>Cytochrome c reductase of yeast</td>
<td>Brewer's yeast</td>
<td>RP</td>
<td>$H_2$-TPN</td>
<td>Cytochrome c</td>
<td>1300</td>
</tr>
<tr>
<td>Cytochrome c reductase of liver</td>
<td>Pig liver</td>
<td>FAD</td>
<td>$H_2$-TPN</td>
<td>Cytochrome c</td>
<td>1150</td>
</tr>
<tr>
<td>Xanthine, aldehyde, xanthopterin oxidase</td>
<td>Milk</td>
<td>FAD</td>
<td>Hypoxanthine, xanthine; aldehyde; xanthopterin; $H_2$-DPN</td>
<td>M.B., $O_2$</td>
<td>300-550</td>
</tr>
<tr>
<td>Aldehyde oxidase</td>
<td>Liver</td>
<td>FAD</td>
<td>Aldehydes</td>
<td>$O_2$</td>
<td>550</td>
</tr>
<tr>
<td>p-Amino acid oxidase</td>
<td>Kidney, liver</td>
<td>FAD</td>
<td>p-Amino acids</td>
<td>$O_2$</td>
<td>2000</td>
</tr>
<tr>
<td>L-Amino acid, l-hydroxy acid oxidase</td>
<td>Kidney, bacteria</td>
<td>FAD</td>
<td>L-Amino acids, lactic acid, etc.</td>
<td>$O_2$</td>
<td>6</td>
</tr>
<tr>
<td>Glycine oxidase</td>
<td>Kidney, liver</td>
<td>FAD</td>
<td>Glycine</td>
<td>$O_2$</td>
<td>2700</td>
</tr>
<tr>
<td>Fumaric hydrogenase</td>
<td>Yeast</td>
<td>FAD</td>
<td>Reduced dyes (leucomethylene violet)</td>
<td>Fumaric acid</td>
<td>2700</td>
</tr>
<tr>
<td>Glucose oxidase</td>
<td><em>Pententillum notatum</em></td>
<td>FAD</td>
<td>Glucose</td>
<td>$O_2$</td>
<td>2700</td>
</tr>
<tr>
<td>Histaminase, diamino oxidase</td>
<td>Kidney</td>
<td>FAD</td>
<td>Di- and polyamines</td>
<td>$O_2$</td>
<td>2700</td>
</tr>
</tbody>
</table>

---

[a] RP = riboflavin phosphate; FAD = flavin adenosine dinucleotide.


Kuhn and Desnuelle obtained results which were approximately the same except that they found 0.48% sulfur instead of 1.0%. Hydrolysis of the apoenzyme by boiling with dilute sulfuric acid and analyses of the resulting hydrolyzate for amino acids gave the following values: arginine

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8.25%, histidine 2.75%, lysine 13.7%, tyrosine 7.75%, phenylalanine 5.75%, tryptophan 4.86%, cystine 0.18%, glutamic acid 7.17%, and aspartic acid 2%. Since only 20% of the total sulfur could be accounted for as cystine, it is probable that other sulfur-containing amino acids are present in the protein. According to these analyses, which account for about 66% of the total nitrogen, the yellow enzyme contains approximately 33 molecules of arginine, 13 molecules of histidine, 66 molecules of lysine, 40 molecules of proline, 30 molecules of tyrosine, 24 molecules of phenylalanine, 17 molecules of tryptophan, 34 molecules of glutamic acid, 12 molecules of aspartic acid, and only 1 to 2 molecules of cystine.

b. Mechanism of Action of Yellow Enzyme

After Barron and Harrop found that methylene blue could catalytically increase the respiration of erythrocytes, Warburg and Christian repeated the experiments, using hexose monophosphate (Robison ester) as a substrate in an extract of horse erythrocytes. From this extract they were later able to separate a thermostable substance, an enzyme designated as *zwischenferment* (Robison ester dehydrogenase), and a thermostable “zwischenferment-cofactor” (triphosphopyridine nucleotide) to form a complete, iron-free, respiratory chain capable of reacting with molecular oxygen. The isolation of the yellow enzyme from bottom yeast led to the chemical characterization of the thermostable substance in blood cells which became known as the yellow oxidation enzyme.

Warburg has stated that in respiring cells the yellow enzyme cannot successfully compete with hemin enzymes which react with oxygen at a much greater rate. In hemin-free cells, e.g., in facultative anaerobic lactic acid bacteria, the respiration is catalyzed by the yellow enzyme which establishes direct contact with molecular oxygen. However, the “turnover number” of the old yellow enzyme (the number of times that a molecule of enzyme is both oxidized and reduced in a minute) under optimum conditions at 38° in pure oxygen is only 55. At the low oxygen tensions which exist in animal tissues, flavoprotein would hardly be autoxidizable. This would mean that the old yellow enzyme, at best, fulfills a highly specialized, not yet understood role in normal cell respiration, that it may react with other unknown acceptors, or that it is an artifact of preparation.

The classical method of describing the action of the old yellow enzyme

is based upon the fact that a mixture of hexose monophosphate, Robison hexose monophosphoric enzyme (zwischenferment), and coenzyme II (TPN) does not react with molecular oxygen. When the old yellow enzyme is added to this mixture, the following reactions take place:

\[
\begin{align*}
\text{H}_2\text{TPN} + \text{flavoprotein} & \rightarrow \text{TPN} + \text{H}_2\text{-flavoprotein} \\
\text{H}_2\text{-Flavoprotein} + \text{O}_2 & \rightarrow \text{flavoprotein} + \text{H}_2\text{O}_2
\end{align*}
\]

As previously stated, this cycle of oxidation and reduction is relatively slow even when pure oxygen is used.

2. Synthetic, or Crossed, Yellow Enzyme

The property of the flavin coenzymes which makes it possible for them to form complexes with foreign apoenzymes was utilized by Warburg and Christian\(^{21}\) to make the first synthetic flavoprotein. They combined the protein part of the old yellow enzyme with flavin adenine dinucleotide and showed that the resulting holoenzyme could, like the old yellow enzyme, catalyze the oxidation of both di- and triphosphopyridine nucleotides. However, the catalytic activity was even less than the slow rates shown by the old yellow enzyme.
3. Haas Enzyme

In 1938, shortly after Warburg and Christian\textsuperscript{37}, \textsuperscript{23}, \textsuperscript{37} had identified flavin adenine dinucleotide as the prosthetic group of \textit{d}-amino acid oxidase, Haas\textsuperscript{38} isolated a flavoprotein from bottom yeast which contained isoalloxazine adenine dinucleotide as the prosthetic group. The catalytic properties were qualitatively the same as those of the old yellow enzyme. Haas' enzyme was somewhat more rapidly reduced by reduced triphosphopyridine nucleotide, but the rate achieved was still too slow to be of biological importance. At physiological tensions of oxygen this flavoprotein was virtually non-oxidizable, and in reconstructed systems it required an intermediate carrier, like methylene blue, for its reaction with molecular oxygen.

The Haas enzyme contained about 0.7\% flavin adenine dinucleotide. The molecular weight was estimated as 70,000. The specific protein could not form a catalytically active complex with riboflavin phosphate.

4. Diaphorase

The term diaphorase (from the Greek \textit{διαφόρεων} = transfer) is applied to a group of flavoproteins capable of catalyzing the oxidation of reduced pyridine nucleotides. Diaphorase, also called coenzyme factor, was discovered independently by von Euler and Hellström\textsuperscript{39} and by Dewan and Green.\textsuperscript{40, 41} It has since been found in bacteria, yeast,\textsuperscript{42} plants,\textsuperscript{43} blood,\textsuperscript{44} milk,\textsuperscript{45} animal muscle,\textsuperscript{39, 43, 45} brain, kidney, intestine, thyroid, and placenta.

Preparations of diaphorase are essentially alkaline phosphate extracts of ground tissues. These crude preparations are more active\textsuperscript{41} per milligram of dried weight than Warburg's pure old yellow enzyme. Most diaphorases are associated with insoluble particles which complicate the task of purification.

The prosthetic group in diaphorase is isoalloxazine adenine dinucleotide. Heating to 60° does not destroy the enzymatic activity. Solutions of diaphorase are yellow and show a green fluorescence. In this respect they differ from other flavoproteins which do not fluoresce. The addition of sodium dithionate or reduced diphosphopyridine nucleotide decolorizes the enzyme solution.

\textsuperscript{38} E. Haas, \textit{Biochem. Z.} \textbf{298}, 378 (1938).
\textsuperscript{40} J. G. Dewan and D. E. Green, \textit{Nature} \textbf{140}, 1097 (1937).
\textsuperscript{41} J. G. Dewan and D. E. Green, \textit{Biochem. J.} \textbf{32}, 626 (1938).
\textsuperscript{43} H. S. Corran and D. E. Green, \textit{Biochem. J.} \textbf{32}, 2331 (1938).
The general diaphorase reaction for the oxidation of either the di- or triphosphopyridine nucleotide is as follows:

\[ H_2\text{-DPN} + \text{diaphorase} \rightarrow \text{DPN} + \text{reduced diaphorase} \]

Reduced diaphorase + methylene blue

\[ \rightarrow \text{Diaphorase} + \text{leucomethylene blue} \]

Leucomethylene blue + O\(_2\) \[\rightarrow\] Methylene blue + H\(_2\)O\(_2\)

Abraham and Adler\(^{46}\) have indicated that there are two separate diaphorases, one for reduced coenzyme I and one for reduced coenzyme II.

5. STRAUB FLAVOPROTEIN, SOLUBLE DIAPHORASE

The chemical nature of the diaphorases was clarified when Straub\(^{47, 48}\) succeeded in separating a soluble flavoprotein from pig heart. This preparation had all the catalytic properties of diaphorase, and it was concluded that they were the same compound in different physical states. The essential step of the preparation was the heating of the crude diaphorase preparation at pH 4.6 in 2% ammonium sulfate and 3% ethyl alcohol at 43° for 10 to 15 minutes. Some 14% of the enzyme activity went into solution and could be centrifuged from the insoluble particles.

Straub's flavoprotein is much more stable to heat than diaphorases which are attached to insoluble particles. The soluble flavoprotein from the pig heart can be heated to 80° without destroying its catalytic activity. Boiling the solution liberates the prosthetic group, flavin adenine dinucleotide.

According to Corran et al.,\(^{49}\) the oxidation of both reduced coenzyme I and reduced coenzyme II can be catalyzed by this flavoprotein. Abraham and Adler\(^{46}\) showed that the heart flavoprotein had very little activity against reduced coenzyme II, and it is now customary to catalog the oxidation of reduced diphosphopyridine nucleotides as a specific function of soluble diaphorase.

The catalytic effect of heart flavoprotein on oxygen uptake in lactic systems and in malic, tissue phosphoric, and \(\alpha\)-glycerophosphoric enzyme systems has been stressed by Corran et al.\(^{49}\) The turnover number of this enzyme was found to be 8500.

6. CYTOCHROME C REDUCTASE OF YEAST

In the living cell cytochrome c is reversibly reduced and oxidized. Cytochrome oxidase provides a mechanism for the participation of oxygen in


IV. BIOCHEMICAL SYSTEMS

this preparation, but the re-reduction of cytochrome c will not take place in vitro unless an enzyme capable of reducing cytochrome c is added. Several flavoproteins capable of reducing cytochrome c have been isolated. One of these is cytochrome c reductase, which, like Warburg's yellow enzyme, has the "mononucleotide" riboflavin phosphate for a prosthetic group.

This enzyme, which was isolated by Haas et al. in 1940 and which catalyzes the transfer of hydrogen between reduced triphosphopyridine nucleotide and cytochrome c, is not to be confused with either the diaphorases, which can oxidize diphosphopyridine nucleotide, or with Haas' flavoprotein from yeast, which, like the diaphorases, contains riboflavin dinucleotide as the prosthetic group.

a. Preparation and Properties of Cytochrome C Reductase

A specially prepared dried brewer's yeast was extracted in 20° water for 33 hours. After centrifuging, the resulting supernatant solution was 51% saturated with ammonium sulfate at pH 4.5. The resulting precipitate was suspended in 31% ammonium sulfate, in which the enzyme is soluble; again precipitated with 51% ammonium sulfate; freed of salt by dialysis; precipitated with ethanol; adsorbed on aluminum hydroxide gel and eluted with alkaline ammonium sulfate; adsorbed on tricalcium phosphate gel and eluted with pH 6.1 phosphate buffer; and finally adsorbed on aluminum hydroxide gel and eluted with alkaline ammonium sulfate, from which solution it was precipitated with 70% saturated ammonium sulfate. The cytochrome c reductase so obtained in 1940 was considered to be 87% pure. It was estimated that the dried yeast contained 0.6 g. of this flavoprotein per kilogram.

The first preparations of cytochrome c reductase were very unstable in environments not considered harmful to other flavoproteins, which may explain why it was not isolated at an earlier date. The enzyme lost 30% of its activity in 2 days at 0° and would have been completely destroyed after 24 hours in 33% acetone, a condition under which Warburg and Christian prepared their old yellow enzyme. Its molecular weight is about 75,000. Its prosthetic group, riboflavin-5-phosphate, can be interchanged with that of the old yellow enzyme. This fact placed this coenzyme in a more significant position as a biologically important compound, since prior to the isolation of cytochrome c reductase it was suspected that riboflavin-5-phosphate might be an artifact.

In 1942, Haas et al. improved the procedure of isolation and obtained

RIBOFLAVIN

a stable product by lyophilization which was 98% pure, and had eight-fold better yield. This enzyme reacted $10^6$ times faster with cytochrome than with oxygen, so it was concluded that the direct action of the reductase with oxygen was of no physiological importance.

b. Mechanism of Action of Cytochrome C Reductase

Reduced cytochrome c reductase reacts with oxidized cytochrome c (CyFe$^{+++}$) according to the equation.

$$\text{Reduced Cy } c \text{ reductase } + 2\text{CyFe}^{+++} \rightarrow$$

$$\text{Cy } c \text{ reductase } + 2\text{CyFe}^{++} + 2\text{H}^+$$

When hexose monophosphate (Robison ester), Robison ester dehydrogenase (zwischenferment), and coenzyme II (TPN) are added to a solution containing cytochrome c and cytochrome c reductase,

$$\text{Glucose-6-phosphate } + \text{TPN } + \text{H}_2\text{O} \xrightarrow{\text{zwischenferment}} \text{H}_2\text{-TPN } + \text{phosphogluconic acid}$$

$$\text{H}_2\text{-TPN } + \text{cytochrome } c \text{ reductase } \rightarrow$$

$$\text{TPN } + \text{reduced cytochrome } c \text{ reductase}$$

which in turn will reduce cytochrome c.

7. Cytochrome C Reductase of Liver

Proof of the reduction of cytochrome c in animal tissue by reduced coenzyme II (H$_2$-TPN) was not reported until 1949, when Horecker$^{53}$ isolated TPN cytochrome c reductase from pig liver. This flavoprotein has flavin adenine dinucleotide as a prosthetic group but otherwise is quite similar to the cytochrome c reductase obtained from yeast by Haas, Horecker, and Hogness.

a. Preparation

The details of the preparation of acetone liver powder and its subsequent extraction, trypsin digestion, salt precipitations, gel adsorptions, and further purifications are described by Horecker.$^{53}$

b. Properties

The enzyme can easily be split into the protein and flavin adenine dinucleotide prosthetic groups. The protein fraction can be reactivated by either flavin adenine dinucleotide or riboflavin phosphate. Of interest is the fact the apoenzymes of both yeast and liver cytochrome c reductases form

more active compounds with riboflavin phosphate than with flavin adenine dinucleotide.

The reaction of liver cytochrome c reductase with oxygen is less than 2% as fast as the reaction with cytochrome c.

The turnover number of the liver enzyme is 1140 (molecular weight 68,000), as compared to a similar turnover number of 1300 (molecular weight 78,000) for the yeast enzyme.

8. XANTHINE OXIDASE, ALDEHYDE OXIDASE, XANTHOPTERIN OXIDASE

The enzymatic oxidation of hypoxanthine and xanthine to uric acid in the presence of tissue brei and oxygen was recognized by Spitzer in 1899. Burian named this enzyme xanthine oxidase. In 1902, Schardinger observed that if formaldehyde and methylene blue were added to fresh milk in the absence of oxygen the methylene blue was rapidly decolorized. These two enzymatic processes remained unrelated until 1922, when Morgan et al. showed that milk was a rich source of xanthine oxidase. The work of Dixon and Thurlow, Booth, and Ball supported the suggestion that the same enzyme is involved in both reactions. Corran and Green isolated a flavoprotein from cow’s milk which showed no activity as a xanthine-aldehyde oxidase but could catalyze the oxidation of reduced coenzyme I. Then Corran et al. prepared a milk flavoprotein which catalyzed the oxidation of hypoxanthine, aldehydes, and reduced coenzyme I, and showed that although all three activities were associated with the same flavoprotein they could be differentially inactivated. Although the Michaelis constant ($K_m$) and the kinetics of the three substrates are not identical, the addition of aldehyde specifically inhibits the oxidation of purine and vice versa. Either the same active group is concerned in the activation of both purines and the aldehydes, or else there are two active groups in such close juxtaposition in the protein molecule that activity at one interferes with activity at the other.

a. Preparation

Ball has prepared xanthine oxidase from unpasteurized cream, since the enzyme was adsorbed on the fat globules. After the cream was shaken with one volume of 0.2 M Na$_2$HPO$_4$ at 38° for 2 hours, the fat was removed by centrifugation at 0°. The enzyme solution was digested with commercial lipase for 3½ hours at 38°, clarified with 0.5 M calcium chloride, and centrifuged. The solution was 60% saturated with ammonium sulfate, and after standing overnight at 0° the enzyme was centrifuged down, dissolved in one-tenth the original volume of water, and further purified by precipitating with 33% ammonium sulfate.

84 W. Spitzer, Pflügers Arch. ges. Physiol. 76, 192 (1899).
The preparation by Corran et al.\textsuperscript{63} started with whole milk and included precipitation in 13% alcohol and adsorption on alumina as well as precipitation with ammonium sulfate. Although their process was a bit more complicated and gave a lower yield, the product obtained had a catalytic activity which was about 1000 times greater than that of milk.

Xanthine oxidase can also be prepared from pig's liver.\textsuperscript{63}

\textit{b. Properties of Xanthine Oxidase}

The xanthine oxidase prepared by Ball\textsuperscript{61} had an isoelectric point at about pH 6.2. The addition of cyanide alone to xanthine oxidase caused an irreversible inhibition. If cyanide and substrate were added simultaneously, no inhibition occurred. The mode of action of cyanide, first noted by Szent-Györgyi\textsuperscript{65} and later studied by Dixon and Keilin,\textsuperscript{66} remains unexplained. This inhibition was utilized by Corran et al.\textsuperscript{63} to eliminate the xanthine-aldehyde without affecting the diaphorase activity. This diaphorase activity to reduced coenzyme I has been confirmed by Ball and Ramsdell.\textsuperscript{67}

The molecular weight of Ball's preparation was calculated as 74,000. It was golden brown in color and did not fluoresce in ultraviolet light. The flavin adenine dinucleotide prosthetic group could be split off from the apoenzyme by dialyzing against running water for 2 weeks.

c. \textit{Action of Xanthine-Aldehyde Flavoproteins}

Hypoxanthine and xanthine are oxidized to uric acid in the presence of the oxidase:

\begin{center}
\begin{tabular}{c c c c}
\hline
Hydrated hypoxanthine & \rightarrow & O=\text{C} & \rightarrow \\
HN\text{--C}=\text{O} & \rightarrow & \text{C=O} & \rightarrow \\
(H\text{H})\text{HC} & \rightarrow & \text{C\text{--N}} & \rightarrow \\
(N\text{--C\text{--N}} & \rightarrow & \text{CH(OH)} & \rightarrow \\
HN\text{--C=O} & \rightarrow & \text{H} & \rightarrow \\
HN\text{--C\text{--N}} & \rightarrow & \text{O=\text{C}} & \rightarrow \\
HN\text{--C=O} & \rightarrow & \text{C\text{--N}} & \rightarrow \\
HN\text{--C\text{--N}} & \rightarrow & \text{CH} & \rightarrow \\
HN\text{--C\text{--N}} & \rightarrow & \text{C=O} & \rightarrow \\
HN\text{--C=O} & \rightarrow & \text{H} & \rightarrow \\
HN\text{--C\text{--N}} & \rightarrow & \text{O=\text{C}} & \rightarrow \\
HN\text{--C=O} & \rightarrow & \text{C\text{--N}} & \rightarrow \\
HN\text{--C\text{--N}} & \rightarrow & \text{CH(OH)} & \rightarrow \\
HN\text{--C=O} & \rightarrow & \text{H} & \rightarrow \\
HN\text{--C\text{--N}} & \rightarrow & \text{O=\text{C}} & \rightarrow \\
HN\text{--C=O} & \rightarrow & \text{C\text{--N}} & \rightarrow \\
HN\text{--C\text{--N}} & \rightarrow & \text{CH} & \rightarrow \\
HN\text{--C=O} & \rightarrow & \text{C=O} & \rightarrow \\
HN\text{--C\text{--N}} & \rightarrow & \text{H} & \rightarrow \\
\hline
\end{tabular}
\end{center}

Hydrated xanthine & \rightarrow & \text{Uric acid}

\text{Hydrated hypoxanthine} & \rightarrow & \text{Xanthine} & \rightarrow \\
\text{Hydrated xanthine} & \rightarrow & \text{Uric acid}
in the presence of air, \( \text{H}_2\text{O}_2 \) is formed. Dixon\(^65\) has reported on the relative velocity with which nine different purines can be oxidized. Xanthine oxidase can catalyze the anaerobic dismutation of xanthine to hypoxanthine and uric acid.\(^69\)

\[ 2 \text{Xanthine} \rightleftharpoons \text{Hypoxanthine} + \text{uric acid} \]

There is no specificity toward aldehydes.\(^70\) Any non-toxic aldehyde can be oxidized by this flavoprotein.

\[ \text{RCHO} + \text{H}_2\text{O} \rightarrow \text{R} + 2\text{H} \]

\( \text{d. Xanthopterin Oxidase} \)

Wieland and Liebig\(^71\) described the presence in liver and milk of an enzyme which catalyzes the oxidation of xanthopterin (2-amino-4,6-dihydroxypteridine). Kalekar and Klenow\(^72\) found that xanthine and xanthopterin oxidases of cream are inhibited by certain preparations of folic acid. Later, Kalekar et al.\(^73\) showed that this inhibition was due to 2-amino-4-hydroxypteridine-6-aldehyde, a fission product of folic acid formed by irradiation. Studies of the inhibitory effects of this and other compounds by Hofstee,\(^7^4\) Lowry et al.,\(^75\) and Williams and Elvehjem\(^76\) showed that

\( ^{65} \text{R. Burian, } Z. \text{physiol. Chem. } 43, \text{ 497 (1905).} \)
\( ^{66} \text{F. Schardinger, Z. Untersuch. Nahr. u. Genussm. } 5, \text{ 1113 (1902).} \)
\( ^{68} \text{M. Dixon and S. Thurlow, Biochem. J. } 18, \text{ 971, 976, 989 (1924).} \)
\( ^{69} \text{V. H. Booth, Biochem. J. } 32, \text{ 494 (1938).} \)
\( ^{70} \text{E. G. Ball, Science } 88, \text{ 131 (1938).} \)
\( ^{71} \text{E. G. Ball, J. Biol. Chem. } 128, \text{ 51 (1939).} \)
\( ^{72} \text{H. S. Corran and D. E. Green, Biochem. J. } 32, \text{ 2231 (1938).} \)
\( ^{73} \text{H. S. Corran, J. G. Dewan, A. H. Gordon, and D. E. Green, Biochem. J. } 33, \text{ 1694 (1939).} \)
\( ^{74} \text{D. E. Green, Mechanisms of Biological Oxidation, p. 97. University Press, Cambridge, 1940.} \)
\( ^{75} \text{A. Szent-Györgyi, Biochem. Z. } 173, \text{ 275 (1926).} \)
\( ^{77} \text{E. G. Ball and P. A. Ramsdell, J. Biol. Chem. } 131, \text{ 767 (1939).} \)
\( ^{78} \text{M. Dixon, Enzymologia } 5, \text{ 198 (1938).} \)
\( ^{79} \text{D. E. Green, Biochem. J. } 28, \text{ 1550 (1934).} \)
\( ^{80} \text{V. H. Booth, Biochem. J. } 29, \text{ 1732 (1935).} \)
\( ^{81} \text{H. Wieland and R. Liebig, Ann. } 555, \text{ 146 (1944).} \)
\( ^{82} \text{H. M. Kalekar and H. Klenow, J. Biol. Chem. } 172, \text{ 349 (1948).} \)
\( ^{83} \text{H. M. Kalekar, N. O. Kjeldgaard, and H. Klenow, J. Biol. Chem. } 174, \text{ 771 (1948).} \)
\( ^{84} \text{B. H. J. Hofstee, J. Biol. Chem. } 179, \text{ 633 (1949).} \)
xanthine oxidase and the xanthopterin oxidase of cream are identical. 2-Amino-4-hydropteridine is also oxidized by xanthine oxidase.75

9. Liver Aldehyde Oxidase

While attempting to prepare the xanthine oxidase of pig liver, Gordon et al.77 isolated a flavoprotein which specifically catalyzed the oxidation of aldehydes to their corresponding acids. The prosthetic group was flavin adenine dinucleotide. Unlike its counterpart in milk, this aldehydase showed no activity toward either hypoxanthine or reduced coenzyme I.

a. Preparation

The removal of four main colored impurities—hemoglobin, xanthine oxidase flavoprotein, a Fe(OH)$_3$-protein complex, and catalase—served as the working hypothesis upon which Gordon et al.77 based their isolation. Hemoglobin was separated by precipitating the aldehydase with half-saturated ammonium sulfate. Xanthine oxidase was destroyed by heating at 48° for 5 minutes in 25% alcohol. The orange-red Fe(OH)$_3$-protein complex (probably ferritin) is slightly less soluble than the aldehyde oxidase in ammonium sulfate solutions. Repeated fractionations between 20 and 35% saturations of ammoniacal ammonium sulfate solutions removed all the iron complex. The removal of catalase proved most difficult, and, since some catalase accompanies the aldehyde enzyme in the course of many salt fractionations, adsorptions, solvent precipitations, cataphoresis, etc., it was believed that catalase formed a compound with the aldehyde enzyme which could not be resolved by known methods.

b. Properties of Liver Aldehyde Oxidase

Purified preparations of the enzyme are yellowish brown in color. This color is partly bleached on reduction with hydrosulfitc and restored after shaking in air. It contains 0.17% flavin phosphate and has a turnover number of about 550 with acetaldehyde. The flavin adenine dinucleotide prosthetic group can be split off by boiling, acidification to less than pH 4, prolonged dialysis against water, or exposure to fat solvents, etc.

Gordon et al.77 reasoned that their liver flavoprotein was different from the milk xanthine-aldehyde-dihydrocoenzyme I oxidase of Corran et al.49 because the liver enzyme was somewhat less soluble in ammonium sulfate solutions and was rapidly and irreversibly inactivated by dialysis against water at 0°C, whereas the milk enzyme was stable over a period of days. Furthermore, the cyclical reduction and oxidation of the flavin group in

the liver enzyme could be easily demonstrated, whereas there was no clear evidence of such a mechanism in the case of the milk enzyme.

Another difference between liver and milk xanthine oxidase was studied by Richert et al.,78 who showed that antabuse (tetraethylthiuram disulfide) inhibited the rat liver oxidase but did not affect milk xanthine oxidase. Richert and Westerfeld79, 80 and Remy and Westerfeld81 have made an extensive study of the xanthine oxidase activities of a variety of animal tissues.

c. Mechanism of Action of Liver Flavoprotein

The liver aldehyde oxidizes a variety of aldehydes such as acetaldehyde, propionaldehyde, butaldehyde, crotonaldehyde, benzaldehyde, salicylaldehyde, and glycollic aldehydes. Formaldehyde is not oxidized, but this may be due to the denaturing effect of formaldehyde on the enzyme. The action between aldehyde and oxygen is not direct but proceeds as follows:

\[
\text{Crotonaldehyde} + \text{flavoprotein} \rightarrow \\
\text{Crotonic acid} + \text{reduced flavoprotein}
\]

\[
\text{Reduced enzyme} + \text{O}_2 \rightarrow \text{Enzyme} + \text{H}_2\text{O}_2
\]

The presence of catalase in the enzyme preparation causes all the peroxide to be decomposed to water and oxygen.

Oxidation-reduction indicators such as methylene blue, nitrate, and cytochrome c can be reduced by the enzyme, but the catalytic reductions of nitrate and cytochrome c are too slow to be of physiological significance.

10. D-Amino Acid Oxidase

The existence of this enzyme was in dispute for years before Krebs82, 83 extracted it from the kidney and liver of rats. He clearly distinguished it from L-amino acid oxidase in 1935.84 Warburg and Christian13 showed that the prosthetic group of this enzyme is flavin adenine dinucleotide. Negelein and Brömel85 isolated the apoenzyme portion in a high degree of purification and estimated molecular weight at 70,000. There is no absolute agree-

80 W. W. Westerfeld and D. A. Richert, J. Biol. Chem. 184, 163 (1950); 192, 35 (1951).
ment on the rates at which different amino acids are attacked, since techniques and preparations differ, but it is generally acknowledged that methionine shows the highest rate of oxidation and that glycine, lysine, and glutamic acid are not oxidized at all. Amino acids with the amino group in the β position, e.g., β-alanine and β-aminobutyric acid, and dipeptides such as alanyl glycine and leucyl glycine are inactive as substrates.

D-Amino acid oxidase is present in most animal organs, kidney and liver being the best sources. There is an active D-amino oxidase in Neurospora. Kisch has claimed that there are at least three different D-amino acid oxidases, depending on the species of animal from which the kidney extracts are prepared.

The physiological role of this enzyme is not understood, since the amino acids which occur in cells are all of levo configuration. It has been postulated that it is present to destroy D-amino acids formed by the racemization of the natural amino acids.

a. Preparation of D-Amino Acid Oxidase

Since it is not inhibited by drying or by treatment with many organic solvents, it is simple to prepare D-amino oxidase by extracting ground tissue (usually kidney) with acetone and drying in vacuo. The tissue powder can be extracted with water.

b. Mechanism of Action

The oxidation of D-amino acids based upon the determination of oxygen uptake, ammonia, and keto acids was represented as

\[
\begin{align*}
\text{R—C—COOH} & \quad \text{NH}_2 \quad \text{O} \\
& \quad \text{H} \quad \text{NH} \\
& \quad \quad \quad \quad \quad \quad \quad \quad -2\text{H} \quad \quad \quad \quad \quad \quad \quad \text{R—C—COOH} \quad +\text{H}_2\text{O} \quad \quad \quad \quad \quad \quad \quad \text{R—C—COOH} + \text{NH}_3
\end{align*}
\]

However, hydrogen peroxide is formed during the oxidation; so the reaction in highly purified preparations devoid of catalase may be written as follows:

\[
\begin{align*}
\text{(1)} & \quad \text{NH}_2 \quad \text{O} \\
& \quad \text{CH}_3\text{C—COOH} + \text{flavin} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{C—COOH} + \text{H}_2\text{-flavin} \\
\text{(2)} & \quad \text{H}_2\text{-flavin} + \text{O}_2 \rightarrow \text{H}_2\text{O}_2 + \text{flavin} \\
\text{(3)} & \quad \text{CH}_3\text{C—COOH} + \text{H}_2\text{O}_2 \rightarrow \text{CH}_3\text{C—COOH} + \text{CO}_2 + \text{H}_2\text{O}
\end{align*}
\]

The turnover number of D-amino acid oxidase is about 2000.

86 J. R. Klein and P. Handler, J. Biol. Chem. 139, 103 (1941).
87 B. Kisch, Enzymologia 12, 97 (1936).
11. L-Amino Acid Oxidase, L-Hydroxy Acid Oxidase

Although its activity in tissue had been investigated by many workers before and after Krebs\textsuperscript{82-84} classical studies, it was not until 1944 that Stumpf and Green\textsuperscript{88} obtained a cell-free preparation of L-amino acid oxidase from \textit{Proteus vulgaris}. The following year, the first electrophoretically homogeneous L-amino acid oxidase was prepared by Blanchard \textit{et al.}\textsuperscript{89, 90} from rat kidneys. A distinguishing feature of this preparation was that riboflavin monophosphate was the prosthetic group. Later, these workers\textsuperscript{91} showed that this enzyme was able to catalyze the oxidation of L-\(\alpha\)-hydroxy acids. The apparent specificity of the enzyme for the L-antipodes was attested by the fact that L-lactic acid is completely oxidized, whereas DL-lactic acid is only 50\% oxidized.

Another water-soluble L-amino oxidase is found in snake venom. This has been studied by Zeller and Maritz\textsuperscript{92} and Singer and Kearney\textsuperscript{93} and may be a flavoprotein.

\textbf{a. Preparation}

Blanchard \textit{et al.}\textsuperscript{90} started with a cold homogenate of 1.5 kg. of rat kidney which was dewatered and dried with the aid of cold acetone. They used an extensive series of extractions and precipitations from salt solutions, including stepwise fractionations between 30 and 60\% ammonium sulfate saturations.

\textbf{b. Properties}

L-Amino acid oxidase is more difficult to extract from ground tissue than D-amino acid oxidase. It is more rapidly denatured by organic solvents, is inhibited by cyanide, and has only a small fraction as much activity as D-amino acid oxidase in liver or kidney slices.\textsuperscript{82}

The enzyme is electrophoretically homogeneous,\textsuperscript{90} but in the ultracentrifuge two components appeared. The lighter component (molecular weight 138,000) contained 2 molecules of the riboflavin phosphate. The heavier component (molecular weight 555,000), which seems to be an aggregate of 4 molecules of the lighter component, had 8 molecules of flavin. The flavin content was 0.66\%. The enzyme from rat kidney and liver cata-


\textsuperscript{93} T. P. Singer and E. B. Kearney, \textit{Federation Proc.} \textbf{8}, 251 (1949).
lyzed the oxidation of the following thirteen natural amino acids to their keto derivatives: leucine, methionine, proline, norleucine, norvaline, phenylalanine, tryptophan, isoleucine, tyrosine, valine, histidine, cystine, and alanine.

The specificities of the L-hydroxy acid oxidase are similar to those of the L-amino acid oxidase; there is a pronounced relationship between the length of the carbon chain and the rate of oxidation. The turnover number of L-amino oxidase is very low (about 6).

c. Mechanism of Action

In the presence of oxygen as hydrogen acceptor, one molecule of oxygen is taken up for each molecule of amino acid oxidized, and one molecule each of keto acid, NH₃, and H₂O₂ are formed. Methylene blue can replace oxygen as the hydrogen acceptor.

12. Glycine Oxidase

Prior to the isolation of glycine oxidase by Ratner et al., three enzymes were known which could deaminate amino acids oxidatively: D-amino acid oxidase, L-amino acid oxidase, and L-glutamic dehydrogenase. None of these has any activity toward glycine.

a. Preparation

Glycine oxidase, which was found in the liver or kidney of all animals tested, was extracted from an acetone powder of pig kidneys and precipitated with 30% saturated ammonium sulfate. The precipitate was dissolved in water and precipitated twice in 24% monopotassium acid phosphate. The enzyme preparation retained activity for 7 to 10 days at 0.5°. It was rapidly destroyed (80% in 12 hours) by dialysis against water.

b. Properties

Solutions are pale greenish yellow but water-clear. These can tolerate 3 minutes’ exposure to 55° without appreciable loss of activity. Flavin adenine dinucleotide is the prosthetic group, and it can be reversibly separated from the apoenzyme. Glycine oxidase catalyzes the oxidation of glycine and N-monomethylglycine (sarcosine), but not other substituted products or peptides of glycine.

c. Mechanism of Oxidation

The oxidation of glycine (where R is H) or sarcosine (where R is CH₃)

is as follows:

\[ CH_2NHRCOOH + \text{oxidized enzyme} \rightarrow CH==NR\text{COOH} + \text{reduced enzyme} \]

\[ CH==NR\text{COOH} + H_2O \rightarrow CHOCOOH + NH_2R \]

Reduced enzyme + O\text{2} \rightarrow \text{oxidized enzyme} + H_2O_2

Methylene blue can be reduced when the reaction is carried out anaerobically.

**13. Fumaric Hydrogenase**

The only example to date of a flavoprotein enzyme system in which an organic substrate is a hydrogen acceptor was discovered in yeast by Fischer and Eysenbach\textsuperscript{55} and Fischer \textit{et al.}\textsuperscript{56} Fumaric hydrogenase from yeast catalyzes the reduction of fumaric acid to succinic acid by the reduced forms of various oxidation-reduction dyes. Fumaric acid seems to be a specific hydrogen acceptor, but to date no naturally occurring hydrogen donor has been found for this reaction.

The enzyme is present as an impurity in the preparation of Warburg and Christian’s old yellow enzyme, from which it can be separated by cathaphoresis in alkaline solutions.\textsuperscript{66} The prosthetic group is flavin adenine dinucleotide.

The reduction of fumarate was demonstrated as follows:

\[
\text{Leuco-methylene violet} + \begin{array}{c} \text{H—C—COOH} \\ \| \\ \text{HOOC—C—H} \end{array} \rightarrow \begin{array}{c} \text{COOH} \\ \| \\ \text{CH}_2 \end{array} + \begin{array}{c} \text{Methylene violet} \\ \| \\ \text{COOH} \end{array}
\]

Fumaric acid Succinic acid

Fumarate can be replaced in this system\textsuperscript{55} by maleate, crotyl alcohol, phenylcrotyl alcohol, and geraniol. Hyposulfite can replace the reduced dyes as reductants of the enzyme.

The turnover number of this reductase is about 2700.

**14. Glucose Oxidase of Molds**

\textit{Penicillium notatum} produces a flavoprotein which oxidizes glucose to gluconic acid in the presence of oxygen. Because hydrogen peroxide accumulates as a result of its action, it was prematurely classified as an anti-

\textsuperscript{55} F. G. Fischer and H. Eysenbach, \textit{Ann.} 530, 99 (1937).

biotic, notatin. The enzyme apparently occurs in many fungi. This enzyme shows a pronounced specificity for glucose and has very little or no activity for about fifty other sugars tested.

\[
\begin{align*}
\text{H} & \quad \text{OH} \\
\text{C} & \\
\text{HCOH} & \\
\text{HOCH} & \quad \text{O} + \text{O}_2 + \text{H}_2\text{O} \rightarrow \\
\text{HCOH} & \\
\text{HC} & \\
\text{H} & \\
\text{HC} & \quad \text{OH} + \text{H}_2\text{O}_2 \\
\text{d-Glucose} & \\
\text{d-Gluconic acid}
\end{align*}
\]

Methylene blue cannot be used instead of oxygen. If hydrogen sulfide is added to poison the catalase in crude extracts, there is a quantitative formation of hydrogen peroxide.

15. Histaminase, DiAmino Oxidase

The flavoprotein nature of histaminase was first noted by Swedin, but a subsequent report by Leloir and Green did not confirm his findings. Kapeller-Adler has presented evidence to show that this enzyme prepared from hog kidney contains flavin adenine dinucleotide.

This flavoprotein will catalyze the oxidation of di- and polyamines to amino aldehydes. Although it has a much higher affinity for histamine, it will also act on cadaverine, putrescine, and agmatine.

According to Zeller, the action of histaminase is represented as follows:

\[
\text{R}--\text{CH}_2\text{NH}_2 + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{RCHO} + \text{NH}_3 + \text{H}_2\text{O}_2
\]

C. MECHANISM OF ACTION

When the various enzyme systems in which riboflavin is a coenzyme are alternately reduced and oxidized, it is believed that they function by accepting and donating hydrogen atoms one at a time. Several intermediates of the reduced and unreduced molecules\textsuperscript{106} have been postulated, but the existence of such intermediates has not been proved by titration curves.\textsuperscript{107} Since the quinhydrone\textsuperscript{108} is the only form that exists in dilute aqueous solution, the transfer of hydrogen atoms may be indicated as:

\[
\text{RCH}_2-\text{CH}_3\overset{\text{+H}}{\underset{-\text{H}}{\rightleftharpoons}}\text{Stabilized semi-quinoid radical} \overset{\text{+H}}{\underset{-\text{H}}{\rightleftharpoons}}
\]

A recent study by Singer and Kearney\textsuperscript{109} demonstrated that isoalloxazine derivatives, in the absence of added proteins, are capable of catalyzing the reaction between reduced pyridine nucleotides and cytochrome c. They did not imply that this process, which is less efficient than the enzymatic reactions, has a significant biological role, but they hoped that the reactions provided a chemical basis for the function of the isoalloxazine nucleus as a mediator of electron transfer between dihydropyridine nucleotides and cytochrome c or molecular O\textsubscript{2}. The most effective compound in this non-enzymatic reduction isisoriboflavin, followed by riboflavin, riboflavin phosphate, and flavin adenine dinucleotide.

\textsuperscript{106} R. Kuhn and R. Ströbele, \textit{Ber.} 70, 753 (1937).
\textsuperscript{107} L. Michaelis and G. Schwartzzenbach, \textit{J. Biol. Chem.} 123, 527 (1938).
\textsuperscript{108} R. Kuhn and P. Boulanger, \textit{Ber.} 69, 1557 (1936).
V. Specificity of Action

M. K. HORWITT

Riboflavin, riboflavin-5-phosphate,¹ and flavin adenine dinucleotide² are the only naturally occurring flavins which have been found to have vitamin B₂ activity. They are equally effective in promoting the growth of rats and Lactobacillus casei.

At this writing, the new vitamin activity of L-lyxoflavin, the 9-L-1'-lyxityl sterioisomer of riboflavin, is being investigated. This compound has been reported as a constituent of heart muscle,³ but its significance awaits confirmation.⁴

Synthetic derivatives of isoalloxazine which have been found to have riboflavin activities include:

(a) 7-Methyl-9-(D-1'-ribityl)isoalloxazine⁵-⁷
(b) 6-Methyl-9-(D-1'-ribityl)isoalloxazine⁶,⁷,⁸
(c) 6-Ethyl-7-methyl-9-(D-1'-ribityl)isoalloxazine⁶

These three compounds were approximately one-half as active as riboflavin in growth tests on rats. Compound c was almost as active as riboflavin in stimulating the growth of lactic acid bacteria,⁹ but compounds a and b stimulated the bacterial growth only moderately.

Among the synthetic arabityl derivatives which have been studied are:

(d) 6,7-Dimethyl-9-(D-1'-arabityl)isoalloxazine¹⁰
(e) 6,7-Dimethyl-9-(L-1'-arabityl)isoalloxazine¹¹
(f) 6-Methyl-9-(L-1'-arabityl)isoalloxazine⁶
(g) 6,7-Trimethylene-9-(L-1'-arabityl)isoalloxazine⁸
(h) 6,7-Tetramethylene-9-(L-1'-arabityl)isoalloxazine⁸

² H. P. Sarett, J. Biol. Chem. 162, 87 (1946).
⁴ T. S. Gardner, E. Wenis, and J. Lee, Arch. Biochem. 34, 98 (1951).
⁹ E. E. Snell and F. M. Strong, Enzymologia 6, 186 (1933).
d-Araboflavin (compound $d$) inhibits growth and increases the mortality rate$^{12}$ more than the absence of riboflavin alone. The other arabityl derivatives can sustain life in rats at a diminished growth rate if given in relatively large amounts.

Isoriboflavin [$5,6$-dimethyl-$9$-(d-$1'$-ribityl)isoalloxazine] is an isomer of riboflavin which, if given to rats at levels of 2 mg. per day, will counteract the growth-promoting effects of 40 $\gamma$ of riboflavin. It is interesting that isoriboflavin has no inhibitory effect on *Lactobacillus casei*.

Riboflavin tetraacetate$^{13}$ and both the mono- and diacetone derivatives$^{13}$ are fully active in supporting rat growth, probably owing to hydrolysis in the mammalian organism, but they are inactive for lactic acid bacteria. Replacement of the d-ribityl group by a glucosidic group$^{14}$ results in a total loss of biological activity. The monomethylol derivative$^{15}$ prepared by reacting riboflavin with formaldehyde retains about half the original activity. Riboflavin mono-, di-, tri-, and teta-succinates$^{16}$ vary in rat growth activity as 100, 65, 21, and 0%, respectively. Substitution of a methyl group in the 3 position$^{17}$ results in complete loss of vitamin activity.

Generally speaking, the activity of esterified derivatives of riboflavin may vary with the ability of the test organism to effect hydrolysis of the ester; the 3 position must remain unsubstituted, and substitution in the 6 or 7 position is necessary.

When the 6 and 7 positions are not substituted, the compounds are toxic.$^{18}$ A recent review by Woolley$^{19}$ has highlighted the increased interest in the inhibitory analogs of the vitamins.

### VI. Biogenesis

**M. K. HORWITT**

Riboflavin is synthesized by most higher plants, yeasts, and lower fungi, and by some bacteria. The tissues of higher animals are unable to synthesize this vitamin, but the gastrointestinal tract of many of these animals harbor bacteria which may be capable of providing riboflavin for their host.

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The riboflavin content of the milk of cows and goats is many times the amount in the feed, as the result of synthesis by organisms which inhabit the rumen of these animals.

Observations that the fecal contents of rats, fowls, and man may have more riboflavin than the food ingested have stimulated research on the nutritional usefulness of intestinal synthesis. It has been common laboratory knowledge that coprophagia by rats must be avoided if nutritional deficiencies are to be produced. Whether the riboflavin formed by microorganisms is absorbed from the lower intestinal tract in significant quantities is not certain. Najjar et al. have reported that there was a rise in urinary riboflavin after normal subjects were given enemas containing 20 mg. of riboflavin, but Everson et al. found no increase in urinary excretion of riboflavin after administering 2 mg. by retention enema. The concept of low utilization of the riboflavin of intestinal bacteria is supported by studies indicating the relative non-availability of the vitamins in ingested yeast.

Although riboflavin is required as a growth factor for a large number of microorganisms, most of them are able to synthesize more than their requirement. Microbiological production of riboflavin by Clostridium acetobutylicum has been promoted as a commercial source. There have been reports on riboflavin production by the yeast Eremothecium ashbyii and by Candida guilliermondia. Certain molds (Ashbya gossypii) produce and excrete so much riboflavin that yellow crystals are formed about the mycelium.

An investigation of riboflavin synthesis by the bacterial flora of the human intestine has been made by Burkholder and McVeigh. The organisms studied were Escherichia coli, Proteus vulgaris, Bacterium aerogenes, Alcaligenes faecalis, Bacillus mesentericus, and Bacillus vulgaris. E. coli,

8 W. H. Peterson and M. S. Peterson, Bacteriol. Revs. 9, 49 (1945).
13 A. Raffy, Compt. rend. soc. bioi. 126, 875 (1937).
VII. ESTIMATION

Normally a dominant organism in human intestinal flora, produced the most riboflavin. Reviews by Knight and Van Lanen and Tanner on growth factors in microbiology have covered other aspects of riboflavin synthesis by microorganisms.

The synthesis of riboflavin in green plants is of major importance in supplying human riboflavin requirements. The locus of this synthesis is not known, but there is apparently a higher concentration in the leaves than in the remainder of the plant.

It is apparent that the biosynthesis of riboflavin phosphate and flavin adenine dinucleotide from riboflavin is a property possessed by all living organisms which have need of this vitamin.

The work of Plaut has thrown some light on the mechanism of riboflavin biosynthesis by Ashbya gossypii. The addition of C\textsuperscript{14}-formate to flask cultures of yeast gave rise to riboflavin tagged in the carbon-2 position. The tracer atom from C\textsuperscript{14}-bicarbonate ended up in the carbon-4 position. C\textsuperscript{14}H\textsubscript{2}COOH, CH\textsubscript{2}C\textsuperscript{14}OOH, and totally labeled glucose produced riboflavin containing C\textsuperscript{14} in both the side chain and o-xylene portions of the molecule.

VII. Estimation

D. M. Hegsted

The most commonly used methods for the estimation of riboflavin are undoubtedly the various modifications of the fluorometric and microbiological methods. These methods have largely replaced the more laborious, expensive, and generally less exact biological assays using chicks and rats. In fact, the early development of the rapid and fairly satisfactory methods discouraged the development of careful animal assays for riboflavin. Colorimetric and polarigraphic methods have been proposed, but these do not appear to be generally applicable to biological materials.

A. PHYSICAL AND CHEMICAL METHODS

D. M. Hegsted

1. Fluorometric Methods

The properties of riboflavin have been discussed in an earlier section of this chapter. Those which have been utilized for the separation and estima-

\textsuperscript{15} B. C. J. G. Knight, *Vitamins and Hormones* 3, 108 (1945).
\textsuperscript{16} J. M. Van Lanen and F. W. Tanner, Jr., *Vitamins and Hormones* 6, 163 (1948).
\textsuperscript{19} G. W. E. Plaut, *Federation Proc.* 12, 251 (1953).
tion by fluorometry are: stability in acid solution, adsorption on fuller's earth from dilute acid, elution from fuller's earth by dilute pyridine, stability to weak oxidizing agents, reversible reduction to the leuco form by hydrosulfite, destruction by visible light or ultraviolet light, transformation to lumiflavin by irradiation in alkaline solution, insolubility in CHCl₃ whereas lumiflavin is soluble in this solvent, solubility in benzyl alcohol and butanol-pyridine mixtures, and a characteristic fluorescence which is optimum between pH 6 and 7 and completely depressed in strong acid or alkali. The object of the various methods is to effect a sufficient separation from other material so that the fluorescence measured is that of riboflavin alone.

a. Methods Utilizing the Fluorescence of Riboflavin

Practically all the modifications of the fluorometric method in common use have been described in detail by Stiller¹ and Jones.² The completeness of these reports makes any extensive description of the technical details unnecessary at this time and allows less reference to the original literature than would otherwise be necessary. Readers are referred to these presentations for a description of the methods. It appears more useful to direct this discussion toward an evaluation of the methods available in so far as this is possible.

The principal steps of the fluorometric analysis include extraction of the sample, removal of interfering materials, and measurement of the riboflavin in the resultant extract. Reference to standard amounts of riboflavin and correction for the fluorescence of suitable blanks must be included in the latter step.

1. Extraction. The usual procedure is a hot dilute acid extraction, or enzymatic hydrolysis, or both. The acid is ordinarily between 0.04 and 0.25 N. Several acids have been used, such as H₂SO₄, HCl, and H₃PO₄, at either boiling temperature or in the autoclave. The enzymatic treatment may be principally the action of phosphatases or nucleotidases (takadiastase, clarase, mylase, etc.) to free riboflavin from the nucleotides, or it may also include a proteolytic enzyme, usually papain, to liberate riboflavin bound to proteins. Since the fluorescence of flavin adenine dinucleotide is relatively much less than that of free riboflavin³ ⁴ and much of the riboflavin may appear in this form³ ⁴ the desirability of sufficient hydrolysis

³ H. B. Burch, O. A. Bessey, and O. H. Lowry, J. Biol. Chem. 175, 457 (1948).
⁷ K. Yagi, J. Biochem. (Japan) 38, 161 (1951).
appears obvious. If only free riboflavin is present (pharmaceuticals), no special extraction should be necessary. The method of Scott et al.\(^8\) utilizes acid-acetone as the extraction medium. Acid methanol has also been used.\(^9\)

Many workers find an acid extraction alone sufficient,\(^1, 10\) yet Watts et al.\(^11\) believe that some of the apparent losses in meats on storage may represent simply insufficient extraction, and the use of acid extraction followed by enzymatic hydrolysis is common practice.

(2) Purification. The procedure in almost universal use is a brief permanganate oxidation introduced originally by Koschara\(^12\) to remove interfering pigments. Excess permanganate is removed by \(\text{H}_2\text{O}_2\), usually within 2 minutes. However, Klatzien et al.\(^13\) recently reported the production of a non-specific blue fluorescence by this oxidation of certain materials. Leviton\(^14\) found that in the presence of appreciable amounts of ferrous iron, riboflavin was readily destroyed by \(\text{H}_2\text{O}_2\) oxidation. Hence, even the use of this common procedure is subject to certain limitations.

Several methods employ the adsorption of riboflavin on fuller’s earth,\(^15\) such as Florisil used by Conner and Straub,\(^16\) followed by elution with pyridine-acetic acid solution. Hoffer et al.\(^17\) indicated some possible limitations to this procedure. Often the values for the same material decrease as the size of the sample is increased. It was thought that this represented losses (incomplete adsorption or elution) on the column or possibly adsorption of riboflavin on residues. These authors also reported that the native riboflavin in wheat flour extracts is less readily adsorbed than pure riboflavin.

In general, the methods which utilize the Florisil adsorption also utilize permanganate oxidation either before or after the adsorption and elution. Whereas permanganate oxidation after the elution is much more effective in decreasing the pigmentation, the non-specific pigment said to be produced by permanganate oxidation in some products\(^18\) may be separated from riboflavin by adsorption on Florisil.

The original procedure of Hodson and Norris\(^19\) and the more recent modification by Rubin et al.\(^19\) includes a reduction of riboflavin and other pig-

\(^9\) A. P. Jansen, Rec. trav. chim. 69, 1275 (1950).
\(^13\) C. Klatzien, F. W. Norris, and F. Wokes, J. Pharm. and Pharmacol. 1, 915 (1949).
\(^17\) A. Hoffer, A. W. Alcoek, and W. F. Geddes, Cereal Chem. 21, 513 (1944).
ments by hydrosulfite and stannous chloride. The riboflavin is then reoxidized to the fluorescent form by shaking with air. Many other pigments are not reoxidized by this procedure. Slater and Morell\textsuperscript{20} have modified the method of Najjar\textsuperscript{21} and applied it to a variety of products. In this procedure (described in detail by Jones\textsuperscript{2}), the riboflavin is extracted into butanol-pyridine mixture after the permanganate oxidation. Anhydrous Na\textsubscript{2}SO\textsubscript{4} is used to effect the transfer and separation. This method, which has not had general application, appears to have advantages and to deserve broader trial.

Pigments and fluorescent materials which are not separated from riboflavin by purification treatment must be taken into account by appropriate blanks. The two parts of the method are therefore inseparable and cannot be easily discussed alone. The criticisms of the hydrosulfite reduction, which is usually used later in the analysis to obtain the blank reading, are discussed below.

(3) Fluorometric Measurement. Although visual comparison of samples with appropriate standards under ultraviolet light has been used,\textsuperscript{1} it is obviously much less satisfactory than the use of a photofluorometer and may be impossible in the presence of interfering materials. A number of fluorometers are available, equipped with appropriate light source, filters, etc. Optical parts must be of low fluorescent glass. (See Loofbourow\textsuperscript{22} for a short discussion of riboflavin fluorometry.) Since riboflavin is sensitive to light, a secondary standard of fluorescein is used to adjust the sensitivity of the instrument, and readings are performed rapidly. A "standard curve," such as is commonly used in colorimetry, is usually not applicable; therefore the instrument must yield an essentially straight-line relationship between riboflavin concentration and reading over the range used. This range is limited, since self-quenching occurs at high concentrations, probably above 0.13 \(\gamma\) per milliliter.\textsuperscript{22} Perhaps the instrument developed by Lowry\textsuperscript{23} deserves special mention because of its stability and adaptability over wide ranges of riboflavin concentration.

(4) Standards. Probably the preferred method of relating fluorometer reading to the amount of fluorescence in the unknown is the inclusion of a "recovery sample" with each unknown. The increment in reading caused by the added riboflavin is used to calculate the riboflavin in the sample. This method automatically allows for losses in preparation (assuming that the riboflavin in the sample behaves as that added; see Hoffier \textit{et al.}\textsuperscript{17}), adsorption of either incident or fluorescent light by colored samples, and

\textsuperscript{21} V. A. Najjar, \textit{J. Biol. Chem.} \textbf{141}, 335 (1941).
\textsuperscript{22} J. R. Loofbourow, \textit{Vitamins and Hormones} \textbf{1}, 143 (1943).
"quenching" of the fluorescence of riboflavin by various materials in the extract. The disadvantage of the method is that the time and work are doubled and errors in the recovery sample are recorded in the unknown also. As indicated above, the recovery from the Florisil column may be critically related to the amount of riboflavin in the sample. Many workers compromise (see Stiller) and effect considerably saving in time by reading the sample, adding a known amount of riboflavin, and taking another reading. After dilution is accounted for, the increment in fluorometer reading is used as a measure of the fluorescence of riboflavin in that extract and the content in the sample is calculated from it.

Essentially all workers have come to use one or the other of the internal standards mentioned above, since the fluorometer reading is influenced not only by the amount of riboflavin but by the other materials in the extract. The increment in reading caused by a constant amount of riboflavin will generally fall in about the same range, however, and samples which show widely divergent values should be viewed with suspicion.

(5) Blank Readings. Since most biological materials do not yield extracts which contain only riboflavin as the fluorescent material, blank determinations for each sample are ordinarily required and simple reagent blanks do not suffice. Reagents must be sufficiently purified to yield low fluorescent extracts, of course. The procedure which is common to most methods is the reduction of riboflavin to the leuco form with sodium hydrosulfite, assuming that only riboflavin is reduced and that no fluorescent materials are produced in the reduction. Others recommend strong alkali or acid to suppress the riboflavin fluorescence, although these have not been widely used as yet. Another, and probably more specific, method is the destruction of riboflavin by irradiation with artificial light or sunlight. After an extensive comparative study of seven methods for the determination of riboflavin in urine, Morell and Slater find serious fault with the hydrosulfite blank. They conclude: "Methods using the hydrosulfite blanks, even those involving adsorption on Florisil, give high answers because they include 'apparent riboflavin,' i.e., compounds other than riboflavin which fluoresce in the same spectral region and the fluorescence of which is destroyed by hydrosulfite.

"Urine contains not only 'apparent riboflavin' but also precursors of 'apparent riboflavin' which are changed to 'apparent riboflavin' by Na2S2O4-SnCl2 reduction, KMnO4 oxidation, or during adsorption on Florisil." The authors feel that their method utilizing a blank obtained by sunlight irradiation.

tion is much superior. De Ritter et al. \(^28\) also compared several methods for the determination of riboflavin in urine and concluded that the method of Slater and Morell gave results most comparable to the microbiological assay. Pearson and Schweigert \(^29\) found the fluorometric method to give very large errors when applied to the urine of sheep or goats given large doses of riboflavin when compared to the microbiological assay, but rat and human urine gave comparable results by either method.

(6) Comment. It may probably be taken as a truism that, when many methods are in use for the determination of the same substance, none is really satisfactory. This may be a little severe in the present case, since very few authors have attempted careful examination of several methods. The work of Morell and Slater \(^26, 27\) and that of De Ritter et al. \(^28\) are commendable exceptions. Most studies compare "their" method with another, usually the microbiological. It is certainly safe to conclude that no "best method" for all materials can be selected at this time. Several of the methods undoubtedly give reasonably accurate and comparative values when applied to materials of a generally similar origin, meats for example, and which contain reasonable amounts of riboflavin. On the other hand, many materials of plant origin are very low in riboflavin and yield highly colored extracts containing numerous and varied pigments. These present numerous problems, and, indeed, each extract to some degree may present a special problem. Not only is the problem difficult but it has received relatively less study, since such materials are not usually important riboflavin sources in the diet and the knowledge that they are low may often be a sufficient answer. On the other hand, as Morell and Slater point out, the principal interest in urinalysis is usually in the samples which are very low and where methods fail or become undependable. Their work appears promising here.

b. Methods Utilizing the Fluorescence of Lumiflavin

The transformation of riboflavin into the chloroform-soluble derivative, lumiflavin, by irradiation in alkaline solution would appear to offer promise as a specific method of determination, especially if combined with some of the procedures now in common use. It is said \(^1\) that the transformation is not quantitative and difficult to standardize, but very few workers in the United States appear to have investigated its possibilities. Foreign workers continue to improve and use the method. Roth, \(^31\) Jansen, \(^9\) and


\(^{31}\) H. Roth, Biochem. Z. 320, 355 (1950).
others have described methods in recent years. In general the procedure includes an acid extraction, preliminary CHCl₃ extraction, photolysis in alkaline solution (pH 13 to 14 or 0.5 N NaOH), reacidification to pH 4.5, and extraction of the lumiflavin into CHCl₃. Illumination with a 200-watt bulb appears as satisfactory as ultraviolet light, and destruction does not occur with over-illumination. Permanganate oxidation after photolysis may be desirable. There is disagreement as to whether one simple extraction is sufficient or whether continuous extraction must be used.

Although some of the technical operations appear cumbersome for routine use, this is probably only a reflection of the lack of effort to improve and simplify the procedures. More intensive study and comparison with other methods is needed.

c. Microfluorometric Methods

Burch et al. developed methods for serum, red blood cells, and whole blood which require only 25 cubic mm. of material. Riboflavin in the white cells may be obtained with only 0.1 ml. of blood. The amounts of free riboflavin, riboflavin phosphate, and flavin adenine dinucleotide (FAD) may also be determined. Under the conditions used, the relative fluorescence of FAD is only 14% of riboflavin. By determinations before and after hydrolysis (5% trichloroacetic acid at 38° for 20 hours) the amount of FAD is obtained. Riboflavin mononucleotide is practically absent from serum and may be ignored. Hydrosulfite reduction is used to obtain blank readings.

This method has proved extremely useful, and as yet no serious criticisms or improvements have been recorded. The small volumes required make it especially useful for field surveys in nutrition. Serum riboflavin values have been shown to be related to riboflavin intake in experimental animals, especially the FAD content, which fluctuates less and falls later than the free riboflavin level. Whole blood levels are less useful, since the riboflavin content is greatly influenced by the number of red cells. The diagnostic value in human beings remains to be proved but appears promising.

d. Estimation of Riboflavin Nucleotides

Bessey et al. developed a method for the fluorometric determination of riboflavin, riboflavin mononucleotide, and flavin adenine dinucleotide in tissues. The differential fluorescence of FAD as compared to riboflavin and

33 K. Yagi, Igaku to Seibutsugaku 18, 264 (1951) [C.A. 45, 9591 (1951)].
the mononucleotide, which fluoresces equally, is utilized for the estimation of FAD. Benzyl alcohol extraction\textsuperscript{37, 38} is used to separate riboflavin from the nucleotides. From 70 to 90% of the riboflavin in tissues was found to be present as FAD, and essentially all the remainder was the mononucleotide. Fujita and Matsuura\textsuperscript{32} used benzyl alcohol separation to distinguish between riboflavin and esterified riboflavin and examined a large number of plant and animal tissues.

Crammer\textsuperscript{39} described the separation of the three forms by paper chromatography using "collidine" or butanol-acetic acid as the developing solvent. He detected only FAD in heart, brain, kidney, and liver, whereas only free riboflavin was found in the spleen. These results are not consistent with those of Bessey et al. The latter authors report the distribution of riboflavin compounds in one sample of spleen to be similar to that found in other tissues mentioned above. Yagi\textsuperscript{7} has extended the qualitatively chromatographic method of Crammer to a quantitative estimation of the three constituents. After separation, the position on the paper was determined under ultraviolet light, and the spots extracted and analyzed by the lumiflavin procedure. It would appear that his results are comparable to those reported by Bessey et al.

Since the only known functions of riboflavin involve the nucleotides rather than free riboflavin, these methods should be of great use and interest. Critical evaluation of the various procedures must, of course, await further comparative work.

2. Colorimetric and Polarigraphic Methods

Since riboflavin is a colored compound, it can obviously be determined by colorimetry.\textsuperscript{1, 40} A few authors apparently use this procedure,\textsuperscript{41} but it is about 100 times\textsuperscript{22} less sensitive than the fluorometric method and therefore not applicable to most biological materials. Its usefulness in the assay of pharmaceuticals should apparently depend upon the equipment of the laboratory and the specificity of the separation from other yellow materials as compared to separation from fluorescent materials. Recently\textsuperscript{42} a colorimetric method based on the production of a red color in the presence of silver nitrate or Deniges reagent has been described. Sensitivity is much below that of fluorometric methods.

\textsuperscript{39} J. C. Crammer, \textit{Nature} \textbf{161}, 349 (1948).
\textsuperscript{40} H. R. Rosenberg, Chemistry and Physiology of the Vitamins, Inter science Publishers, New York, 1945.
A polarigraphic method was described by Lingane and David. This method also appears less sensitive than those in common use and has had very little application.

3. Enzymatic Methods

In their paper on the isolation of the prosthetic group of \( \text{d-amino acid oxidase} \), flavin adenine dinucleotide, Warburg and Christian describe an assay method for this riboflavin derivative. This consists in the measurement of oxygen uptake when the material to be assayed is added to an appropriate buffered solution containing the protein moiety of the enzyme and \( \text{DL-alanine} \) as the substrate. The method has been used by various groups and was modified to measure the apoenzyme instead of the prosthetic group by Negelein and Bromel. Recently Comline and Whatley have used the method to study the destruction of FAD by an enzyme in animal tissues, particularly in spleen.

The method used by Kuhn and Rudy for the estimation of the prosthetic group of the yellow enzyme, riboflavin mononucleotide, appears to have had little use. This also utilizes oxygen uptake but with hexose phosphate as the substrate, the specific protein of the yellow enzyme, and appropriate coenzymes to complete the oxidizing system. Oxygen uptake is a function of the amount of riboflavin phosphate added.

B. BIOLOGICAL METHODS

D. M. HEGSTED

The Bourquin and Sherman rat assay was the first method for the estimation of what was then called vitamin \( B_2 \) or \( G \) to receive rather wide acceptance. When pure riboflavin became available, it was shown that this method gave quantitative responses to graded doses of riboflavin within certain limits of growth. Although biological assays have since been improved, the early development of the microbiological assay apparently satisfied most of the requirements for a biological method, and the riboflavin assay has never been subjected to the study and refinement which some of the other vitamin assays received. The perfect assay diet would be one in which all the nutrients required by the assay organism, with the exception

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of riboflavin, are present in adequate amounts. The numerous diets which have been used since the original Bourquin-Sherman diet have been tabulated by Day and Darby, and the details of the biological assay are discussed by these authors. The diets have been made more complete with the passage of time, the availability of more crystalline vitamins, and more knowledge of the rat's nutritional requirements. Undoubtedly further improvements could now be made.

The usual assay is made with weanling rats which are fed the riboflavin-low diet for 2 or 3 weeks until growth has practically ceased in the majority of the animals. They are then carefully divided according to weight, sex, etc., and groups are fed various levels of the test material. Other groups receive standard amounts of riboflavin. At the end of the test period the growth of the standard groups is used to construct a dose-response curve from which the riboflavin content of the unknown is determined. Bliss and György and Day and Darby have discussed these assays and the calculation of results in detail. Assays utilizing young chicks have also been used in which the general procedure is similar, although the diet must be devised with the nutritional requirements of chicks in mind.

It is commonly assumed that the bioassay which utilizes larger animals has peculiar advantages over other methods, since it measures the response of the vitamin in an organism similar to man or at least more similar than is L. casei. It may be well to point out that this is not necessarily so. There is ample evidence that the response of the rat to known amounts of riboflavin is markedly influenced by the composition of the diet with which it is fed. Specifically, the amount and kind of carbohydrate and fat are known to be important, and other unsuspected relationships may exist. The level of these may not be controllable when low-potency materials are assayed. Thus one reaches the same position found in all assays to date, namely that accurate figures for low-potency materials may probably not be obtained. Furthermore, it would appear logical that these and other dietary inter-relationships will be true of the human species as well. If so, the riboflavin potency of a given material will vary with the diet with which it is fed as well as with the composition of the material itself. This rather unsatisfactory state of affairs must be accepted as the nature of the thing, and the bioassay, even if accurately done, appears to offer no advantages except for research studies on the physiology of riboflavin. The more rapid and accurate methods discussed elsewhere should, and undoubtedly will, be favored.

Summary

The author has attempted to point to some of the faults of existing methods for riboflavin assay as a stimulus for further development in the field rather than a condemnation of the present procedures. Many of the present methods are adequate to obtain the kind of knowledge most generally sought, which is a more or less quantitative idea of the riboflavin content and with special interest in high-potency materials. Methods designed for a special tissue, serum for example, may be quite satisfactory. It is not to be assumed, however, that success with one material, or even several, guarantees the method for general use. The following report may well become a classic example of what may befall the unsuspecting worker who makes a simple analysis. Baude et al.\textsuperscript{55} reported that the riboflavin content of sow's milk as determined by a fluorometric method designed for cow's milk\textsuperscript{56} was quite low, approximately 0.5 $\gamma$ per milliliter, and more stable to light than the riboflavin in cow's milk.\textsuperscript{57} Microbiological studies in another laboratory failed to confirm this figure, and this discrepancy led to a collaborative effort\textsuperscript{58} to uncover the difficulty. The same sample of milk was analyzed by several methods with the following results: original fluorometric assay, 0.4 $\gamma$ per milliliter; microbiological assay,\textsuperscript{59} 1.2 to 1.5 $\gamma$ per milliliter; chick assay, 1.2 to 1.8 $\gamma$ per milliliter; and rat assay, 1.6 to 2.0 $\gamma$ per milliliter. It was provisionally decided that the fluorometric method did not yield complete extraction, and the sample was then hydrolyzed with $\text{H}_2\text{SO}_4$ at pH 1 for an hour, incubated with takadiastase, and the assay completed by the method of Slater and Morell. This also yielded low results, but it was found that if the final extraction into butanol-pyridine was omitted higher values were obtained. Thus it appeared that the riboflavin did not behave normally and did not completely enter this solvent. Finally, an assay by the method of Bessey, Lowry, and Love was said to yield results comparable to those of the microbiological assay. Unfortunately the story is not complete at the present time, but it appears that at least part of the riboflavin of sow's milk is present in a form which does not behave as does previously known compounds. It should be borne in mind that this example is with a material which does not yield highly colored extracts and that cow's milk has been analyzed successfully for riboflavin many times by fluorometric methods.


Riboflavin was found to be a growth factor for certain lactic acid bacteria\footnote{S. Orla-Jensen, N. C. Otte, and A. Snog-Kjaer, \textit{Zentr. Bakteriol. Parasitenk.}, \textit{Abt. II}, 94, 434 (1936).} \footnote{E. E. Snell, F. M. Strong, and W. H. Peterson, \textit{Biochem. J.} 31, 1789 (1937).} shortly after its isolation as a vitamin for rats.\footnote{R. Kuhn, P. György, and T. Wagner-Jauregg, \textit{Ber.} 66B, 317, 576, 1034, 1577, 1950 (1933).} Relatively few microorganisms that require this vitamin are known; most of these are lactic acid bacteria or closely related organisms.

The use of a strain of \textit{Lactobacillus casei} for the quantitative determination of riboflavin was suggested by Snell and Strong\footnote{E. E. Snell and F. M. Strong, \textit{Ind. Eng. Chem. Anal. Ed.} 11, 346 (1939).} in 1939. The procedure recommended has been widely and successfully used since that time, both in its original form and in several slightly modified forms. Indeed, the subsequent widespread use of microorganisms for quantitative assay work was greatly stimulated by the success of this method. The original assay medium is identical with that later recommended by the Association of Official Agricultural Chemists.\footnote{A. R. Kemmerer, \textit{J. Assoc. Offic. Agr. Chemists}, 27, 541 (1944).} The medium adopted by the U. S. Pharmacopeia\footnote{U. S. Pharmacopeia 14th Revision, p. 752, 1950.} differs only in the concentration of glucose, which has been increased from 1.0 to 3.0\%. Procedural details, precision, reliability, and specificity of the method are given in numerous treatises.\footnote{E. E. Snell, \textit{in Vitamin Methods}, Vol. I, p. 327. Academic Press, New York, 1950.} \footnote{E. E. Snell, \textit{in Biological Symposium}, Vol. XII, Estimation of the Vitamins, p. 143. Jaques Cattell Press, Lancaster, Pa., 1947.}

A medium based on peptone, glucose, yeast extract, acetate, and inorganic salts and freed from riboflavin by treatment of the crude ingredients with light, alkali, or lead acetate followed by hydrogen sulfide is used. Growth of \textit{L. casei} in this medium increases with the riboflavin concentration in the range from 0 to about 0.20 \(\gamma\) per 10 ml. of medium. Pure riboflavin and samples to supply riboflavin at several levels within this range are added to individual tubes containing 5 ml. of the double-strength medium. Each tube is then diluted to 10 ml., capped, autoclaved, cooled, and inoculated. Response of the test organism is customarily determined by acid titration after 72 hours incubation at 37°. Turbidimetric estimations of growth can be made as early as 24 hours. Interpolation of the response obtained to several different known amounts of sample onto the curve obtained by plotting the responses to increasing amounts of pure riboflavin
gives figures from which the average riboflavin content of the sample is readily calculated.

The extraction of riboflavin offers little difficulty because of the looseness with which it is bound, its stability to heat and acid, and the fact that the known bound forms, riboflavin phosphate and flavin adenine dinucleotide, have the same activity as riboflavin for the test organism. Autoclaving the finely divided sample with a tenfold excess of 0.1 N HCl at 15 lb. pressure for 15 to 20 minutes has proved very effective. The cooled mixture is diluted and filtered through paper to remove traces of fatty acids, which interfere with the response to riboflavin. Properly diluted aliquots of the filtrate, adjusted to pH 6.8 to 7.0, are used for assay. All operations, during both extraction and assay, should be carried out in darkness or subdued light, since riboflavin is readily destroyed by light.

Accuracy of the procedure is attested by agreement of the results obtained for a great variety of materials with those obtained by chemical and rat growth procedures. A micro modification of the procedure that permits determination of riboflavin in amounts from 0 to 0.004 \( \gamma \) has been described.

Most other microbiological procedures for riboflavin also utilize \( L. \) casei as the test organism in a slightly modified medium. An exception is the procedure of Kornberg et al., which employs a strain of \( L. \) mesenteroides said to be some fifty times more sensitive to riboflavin than is \( L. \) casei. These methods may prove useful for special applications. They have not, however, been tested nearly as widely as the Snell-Strong procedure recommended above.

VIII. Standardization

M. K. HORBWITT

With the elucidation of the chemical nature of riboflavin, the need for a standard of activity for vitamin \( B_2 \) or \( G \) became less urgent. The present U.S. P. Reference Standard is a reconstituted sample of riboflavin obtainable from the U.S. P. Reference Standards Committee. Comparisons of purified riboflavin with the older units of activity showed that one Bourquin-Sherman rat growth unit, the daily addition of which will produce an average gain of 3 g. per rat per week, was equal to about 2.5 \( \gamma \) of riboflavin.

\(^{69}\) O. H. Lowry and O. A. Bessey, \( J. \) Biol. Chem. 155, 71 (1944).

\(^{70}\) H. A. Kornberg, R. S. Langdon, and V. H. Cheldelin, \( Anal. \) Chem. 20, 81 (1948).

\(^{1}\) A. Bourquin and H. C. Sherman, \( J. \) Am. Chem. Soc. 53, 3501 (1931).

\(^{2}\) O. A. Bessey, \( J. \) Nutrition 15, 11 (1938).
Von Euler had proposed a unit which consisted of 5 γ of riboflavin, the amount which produced an increase in weight of 0.8 to 1.0 g. per day in young rats. A Cornell unit was defined as the growth effect on chicks equivalent to that produced by 1 γ of riboflavin.

The need for standards of biological activity continues to exist, especially in the study of derivatives of riboflavin. As an example, consider the assay of a very water-soluble riboflavin derivative prepared by Stone. Fluorometric assay of the material yielded a value of 57.2% riboflavin; microbiological assay by the U.S.P. XIII revision method yielded a value of 33% riboflavin. The biological assay by the standard rat growth method indicated the riboflavin potency was almost nil.

IX. Occurrence in Food

M. K. HORWITT

The best sources of riboflavin are milk, egg white, liver, heart, kidney, and growing leafy vegetables. Beef muscle, veal, apricot, tomato, and poultry muscle are good sources. Fish muscle, unenriched grains, and legumes, although relatively poor sources, supply important minimal amounts to the average regimen. Yeast, the richest natural source of riboflavin, is not normally a major component of non-therapeutic diets.

The primary factors to be evaluated in a consideration of the stability of riboflavin in food products are the effects of heat, light, elution, and the intracellular reactions which take place during storage.

The relative heat stability of riboflavin is a fortunate property which favors its preservation by ordinary cooking procedures. Even the addition of bicarbonate to a pH of 8.8 does not appreciably increase the loss of riboflavin during short cooking procedures. The major losses which occur during home cooking or commercial canning operations are probably attributable to the extraction of the vitamin by the water used in the cooking or

\[ 6 \text{ G. B. Stone, Science 111, 283 (1950).} \]
\[ 1 \text{ H. Levine and R. E. Remington, J. Nutrition 13, 525 (1937).} \]
## IX. OCCURRENCE IN FOOD

### TABLE II

<table>
<thead>
<tr>
<th><strong>Representative Foods</strong></th>
<th><strong>Approximate measure</strong></th>
<th><strong>Riboflavin, mg.</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cereal products:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Refined</td>
<td>1 slice bread (30 g.)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>½ cup cereal (20 g.)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 soda crackers (20 g.)</td>
<td></td>
</tr>
<tr>
<td>Whole grain and enriched</td>
<td>1 slice bread (30 g.), ½ cup cereal (20 g.), 2 graham crackers (20 g.)</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>Dairy products:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cheese, Cheddar</td>
<td>1 cu. in.</td>
<td>0.13</td>
</tr>
<tr>
<td>Cheese, cottage</td>
<td>½ cup</td>
<td>0.31</td>
</tr>
<tr>
<td>Cream, light</td>
<td>½ cup</td>
<td>0.04</td>
</tr>
<tr>
<td>Custard</td>
<td>½ cup</td>
<td>0.26</td>
</tr>
<tr>
<td>Egg</td>
<td>1 medium</td>
<td>0.14</td>
</tr>
<tr>
<td>Ice cream</td>
<td>½ cup</td>
<td>0.15</td>
</tr>
<tr>
<td>Milk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buttermilk, skim</td>
<td>1 cup</td>
<td>0.43</td>
</tr>
<tr>
<td>Whole</td>
<td>1 cup</td>
<td>0.41</td>
</tr>
<tr>
<td><strong>Desserts:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cake, plain, chocolate</td>
<td>1 piece (75 g.)</td>
<td>0.06</td>
</tr>
<tr>
<td>Cookies, plain</td>
<td>2 medium</td>
<td>0.02</td>
</tr>
<tr>
<td>Pie crust</td>
<td>½ shell, single crust</td>
<td>0.04</td>
</tr>
<tr>
<td>Puddings</td>
<td>½ cup</td>
<td>0.22</td>
</tr>
<tr>
<td><strong>Fish:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cod, haddock, cooked</td>
<td>1 medium serving</td>
<td>0.06</td>
</tr>
<tr>
<td>Halibut, herring, whitefish</td>
<td>1 medium serving (75 g.)</td>
<td>0.06</td>
</tr>
<tr>
<td>Salmon, canned</td>
<td>1 medium serving</td>
<td>0.12</td>
</tr>
<tr>
<td><strong>Fruits:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Banana</td>
<td>1 small</td>
<td>0.05</td>
</tr>
<tr>
<td>Cantaloupe</td>
<td>½ melon, 4½-in. diameter</td>
<td>0.06</td>
</tr>
<tr>
<td>Citrus</td>
<td>½ grapefruit, 1 medium orange</td>
<td>0.02</td>
</tr>
<tr>
<td>Yellow</td>
<td>Fresh (100 g.)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dried (30 g.)</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Meat:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef, lamb, veal, cooked</td>
<td>1 medium serving</td>
<td>0.17</td>
</tr>
<tr>
<td>Fowl, cooked</td>
<td>1 medium serving</td>
<td>0.08</td>
</tr>
<tr>
<td>Liver, cooked</td>
<td>1 small serving</td>
<td>2.38</td>
</tr>
<tr>
<td>Luncheon meats, cooked</td>
<td>2 slices sausage, minced ham, dried beef, ½ frankfurter (30 g.)</td>
<td>0.08</td>
</tr>
<tr>
<td>Pork, ham</td>
<td>1 medium serving, cooked</td>
<td>0.17</td>
</tr>
</tbody>
</table>
### TABLE II—Concluded

<table>
<thead>
<tr>
<th>Food</th>
<th>Approximate measure</th>
<th>Riboflavin, mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sweets:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candy bar</td>
<td>1–2 oz. chocolate-coated bar</td>
<td>0.17</td>
</tr>
<tr>
<td>Molasses; sorghum</td>
<td>1 tablespoon</td>
<td>0.03</td>
</tr>
<tr>
<td>Vegetables:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cabbage, cooked</td>
<td>¼ cup</td>
<td>0.05</td>
</tr>
<tr>
<td>Cauliflower, cooked</td>
<td>¼ cup (70 g.)</td>
<td>0.04</td>
</tr>
<tr>
<td>Corn, parsnips, cooked</td>
<td>½ cup corn; 1 large parsnip</td>
<td>0.09</td>
</tr>
<tr>
<td>Green and yellow:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asparagus, cooked</td>
<td>¼ cup</td>
<td>0.17</td>
</tr>
<tr>
<td>Broccoli, cooked</td>
<td>¼ cup</td>
<td>0.15</td>
</tr>
<tr>
<td>Carrots, cooked</td>
<td>¼ cup</td>
<td>0.05</td>
</tr>
<tr>
<td>Green beans, cooked</td>
<td>¼ cup</td>
<td>0.10</td>
</tr>
<tr>
<td>Leafy greens, cooked</td>
<td>½ cup spinach, turnip, kale, other greens</td>
<td>0.21 a</td>
</tr>
<tr>
<td>Peas, fresh, cooked, canned</td>
<td>½ cup</td>
<td>0.14</td>
</tr>
<tr>
<td>Sweet potato, cooked</td>
<td>½ large</td>
<td>0.06</td>
</tr>
<tr>
<td>Potato, cooked</td>
<td>1 small (100 g.)</td>
<td>0.03</td>
</tr>
<tr>
<td>Tomato, fresh, canned or juice</td>
<td>½ cup; 1 small tomato</td>
<td>0.03</td>
</tr>
<tr>
<td>Other, commonly served raw</td>
<td>2 pieces celery; 8 slices cucumber; ½ head lettuce</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* For canned, reduce by one-half.

blanching operations. These losses are usually less than 20%, and they can be further minimized if the cooking fluids are consumed.

Losses of riboflavin due to exposure to light during cooking may prove to be an important economic loss. Cheldelin et al. have shown large losses, up to 48%, incurred in the cooking of eggs, milk, and pork chops in uncovered dishes under conditions where there was no loss of riboflavin when cooking dishes were covered. The loss in milk, which is allowed to stand in glass containers on the consumer's doorstep may be as high as 85% after 2 hours exposure to bright sunlight. This may be compared with prac-

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ally no loss of riboflavin during pasteurization procedures,\textsuperscript{12} where light effects are minimal.

The losses of riboflavin during storage either by quick freezing\textsuperscript{13, 14} or in sterilized containers\textsuperscript{15-17} are relatively small.

In the absence of liver and yeast, which contain from 2 to 4 mg. of riboflavin per 100 g., the riboflavin content of the average diet is usually related to the amount of animal protein consumed. Unenriched cereal products are poor sources. Green and yellow vegetables, although relatively high in riboflavin, are not usually eaten in sufficient amounts to supply a major portion of the daily requirement. The preparation of experimental diets low in riboflavin has been discussed by Horwitt et al.\textsuperscript{18}

Table II contains representative amounts of riboflavin in foodstuffs. This table is adapted from the Leichsenring and Wilson\textsuperscript{19} short method of dietary analysis. It is based upon their compilation of data published by Watt and Merrill\textsuperscript{20} and Bowes and Church.\textsuperscript{21} The figures presented are averages, often of a wide range of analytical results, and should be regarded as working estimates which will vary with the geography, season, and method of preparation.

X. Effects of Deficiency

A. IN MICROORGANISMS

E. E. SNELL

Shortly after the isolation of riboflavin as a vitamin for animals, Orla-Jensen and coworkers\textsuperscript{1} showed that the compound also was an essential growth factor for many lactic acid bacteria. This finding was rapidly con-

firmed, and the effect of several variations in the riboflavin structure on the bacterial response was determined. The response proved very specific, and on these grounds a microbiological assay for the vitamin was developed and proposed. Both growth and acid production of lactic acid bacteria are proportional to riboflavin in the suboptimal range of concentrations. Riboflavin is required as a growth factor by fewer microorganisms than are most of the other vitamins; besides the lactic acid bacteria, however, many of the hemolytic streptococci, some propionic acid bacteria, some clostridia, and some luminescent bacteria require it. Few if any naturally occurring yeasts or other fungi have been found that require riboflavin; mutants of Neurospora crassa that require it have, however, been obtained, and all organisms so far examined that do not require preformed supplies of this vitamin synthesize it. Indeed, the synthesis of riboflavin by certain fungi, e.g., by Ashbya gossypii and related organisms, constitutes a commercial method for the production of this vitamin.

Aside from decreased growth in the absence of sufficient supplies, few other effects of riboflavin deficiency in microorganisms have been described. From the role of this vitamin as a hydrogen carrier, it might be expected that, as in higher animals, the level of certain oxidative enzyme systems would be depressed during growth on suboptimal supplies. That this is true is shown by investigations of Doudoroff with the luminescent organism, Photobacterium phosphorescens. Cultures grown on a yeast autolyzate agar frequently produced, as variants, dull or dark colonies; these luminesced more brightly when riboflavin was added. The same “dark” colonies were stimulated in growth on a riboflavin-deficient medium by additions of this vitamin. Apparently the dull variants had lost the ability to synthesize sufficient riboflavin for their needs; this was not true of the original bright colonies. In this case, amounts of riboflavin sufficient for growth of the “dull” variants were insufficient to permit maximum luminescence. That riboflavin-enzymes are among those concerned in light production by such luminescent organisms has been proved by Eyring and Johnson.

At low concentrations, many fatty materials show a “sparing effect” on the requirement of lactic acid bacteria for riboflavin; this effect, which is sometimes troublesome in microbiological assays, may possibly indicate

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3 E. E. Snell and F. M. Strong, Enzymologia 6, 186 (1939).
6 B. C. J. G. Knight, Vitamins and Hormones 3, 105 (1915).
9 M. Doudoroff, Enzymologia 5, 239 (1938).
that riboflavin participates in fat synthesis by these organisms. If the fatty materials required were supplied preformed, the requirement for riboflavin might well be decreased thereby.

B. IN PLANTS

M. K. HORWITT

Riboflavin is apparently synthesized by higher plant life,\textsuperscript{11, 12} as evidenced by its distribution in our vegetable foods. There is no recorded evidence of riboflavin deficiency in plants.

According to Galston,\textsuperscript{13} riboflavin determines the photooxidation of indoleacetic acid and may be regarded as a photoreceptor in light-growth reactions. Ferri\textsuperscript{14} has emphasized the fact that the induction of the photoinactivation of indoleacetic acid is a property common to many fluorescent substances. It has been known that the photooxidation of indoleacetic acid could be determined by eosin\textsuperscript{15} and that eosin-treated roots yielded less auxin than untreated ones.\textsuperscript{16} It will be interesting to observe the development of this subject.

C. IN INSECTS

M. K. HORWITT

It has been apparent for some time\textsuperscript{17} that riboflavin is essential for many insects. Among those whose requirements have been studied are the larvae of the flesh fly, Sarcophagia sp.,\textsuperscript{18} the cockroach, Blattella germanica,\textsuperscript{19} the larvae of the confused flour beetle, Tribolium confusum,\textsuperscript{20, 21} Drosophila larvae,\textsuperscript{22} and the yellow fever mosquito, Aedes aegypti.\textsuperscript{23}

Fraenkel and Blewett,\textsuperscript{24} who have made extensive studies of the nutritional requirements of beetles, have shown that riboflavin is required by Tribolium and Ptinus, but that Lasiodermia, Sitodrepa, and Silvanus do not need riboflavin in their diet because of the presence of intracellular symbiotic microorganisms which synthesize the vitamin.

\textsuperscript{11} P. R. Burkholder, \textit{Science} 97, 562 (1943).
\textsuperscript{12} J. Bonner, \textit{Botan. Gaz.} 103, 581 (1942).
\textsuperscript{13} A. W. Galston, \textit{Science} 111, 619 (1950).
\textsuperscript{14} M. G. Ferri, \textit{Arch. Biochem.} 31, 127 (1951).
\textsuperscript{15} F. Skoog, \textit{J. Cellular Comp. Physiol.} 7, 227 (1935).
\textsuperscript{17} R. Craig and W. M. Hoskins, \textit{Ann. Rev. Biochem.} 9, 617 (1940).
\textsuperscript{18} G. Di Maria, \textit{Arch. zool. Ital.} 25, 469 (1938).
\textsuperscript{19} C. M. McCay, \textit{Physiol. Zool.} 11, 89 (1938).
\textsuperscript{21} K. Offhaus, \textit{Z. vergleich. Physiol.} 27, 384 (1939).
\textsuperscript{24} G. Fraenkel and M. Blewett, \textit{J. Exptl. Biol.} 20, 28 (1943).
The American cockroach, *Periplaneta americana*, and *Tineola bisselliella* accumulate much more riboflavin in the malpighian tubes than can be accounted for by the diet.

**D. IN ANIMALS**

**M. K. HORWITT**

Riboflavin is essential for growth and normal health for all animals. Its restriction has been studied in nearly all species which are related to human economics. The primary effect of riboflavin restriction is the cessation of growth. Because it is a fundamental constituent of animal tissue, new tissue cannot be formed unless a minimum amount of riboflavin is available. It is therefore necessary not only for growth but also for tissue repair. The amounts of riboflavin needed for normal growth have been discussed in the section on animal requirements. When less than these requirements is provided, a variety of pathological trends become evident. Wolbach and Bessey have published an excellent review of the tissue changes in vitamin deficiencies, in which they summarized most of the studies reported prior to 1942. The large majority of the older papers on the B₂ vitamin dealt with mixed deficiencies and often described syndromes which are not seen in modern work with a "pure" deficiency of riboflavin. At present, most of our interpretations of ariboflavinosis in animals are based upon experiments which deal with the removal of all, or nearly all, of the riboflavin from the diet. The application to man of such work is limited by the fact that one rarely, if ever, observes a human deficiency syndrome due to the complete absence of dietary riboflavin. More work on the effect of long-standing suboptimal intakes of riboflavin is indicated.

**1. RATS**

Goldberger and Lilly were apparently the first to describe symptoms of riboflavin deficiency in the rat, as a result of a study in which they attempted to produce rat pellagra. They reported a severe ophthalmitis and a bilateral, symmetrical alopecia, which almost completely denuded the head, neck, and trunk. The dermatitis which these authors also observed has since been shown to have been complicated by a pyridoxine deficiency. Whereas the lesions of pyridoxine deficiency are characterized by a florid

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dermatitis of the extremities and a swelling of the ears, the lesions of ariboflavinosis are less specific and slower to develop. An eczematous condition of the skin especially affects the nostrils and eyes. The eyelids become denuded of hair and may be stuck together with a serous exudate.

Conjunctivitis, blepharitis, corneal opacities, and vascularization of the cornea are common manifestations of rat ariboflavinosis. The question of specificity of cataract formation, first reported in 1931 by Day et al. in rats deficient in what was then known as vitamin G, has not yet been resolved. There is an apparent inverse relationship between cataract formation and the amount of riboflavin in the diet. There also seems to be a relationship between corneal opacities and amino acid deficiencies. In a study of the growth of the eye during riboflavin and tryptophan deficiencies, Pirie noted that the eye continued to grow at a normal rate, so that the deficient animals had relatively large eyes in undersized bodies.

Before the pathology of choline deficiency was recognized, there were frequent reports of hepatic injury as a consequence of riboflavin deficiency. It is now assumed that the fatty livers associated with deficiencies of B complex vitamins are not directly related to riboflavin depletion.

A variety of neuropathological changes have been reported, and it is likely that the proportions of fat, carbohydrate, and protein in the diet, as well as the severity of the riboflavin depletion, may play an important part in determining the exact nature of the pathology. Partial paralysis of the legs of the rat are produced more easily on a high fat ration. In its severe form this paralysis is characterized by degeneration of the myelin

sheaths of the sciatic nerves, axis cylinder swelling, and fragmentation. Myelin degeneration and gliosis in the spinal cord have also been observed.

Histological changes in the skin have been described by Wolbach and Bessey\textsuperscript{28, 49, 50} as follows: "We find that the initial responses are in the epidermis and its appendages. The vascular engorgement, so characteristic of pyridoxine deficiency, does not occur. The epidermis as a whole shows little change other than a moderate hyperkeratosis. In some locations there is slight hyperplasia of the epidermis, particularly of the snout and sides of the head, possibly related to scratching. Sebaceous glands, including the Meibomian glands of the eyelids, become somewhat atrophic. There is an increased rate of shedding of hair which we believe to be the result of separation of the cornified anchoring cells from the epithelial sheaths. The outstanding and thus far, to us, distinctive feature of the deficiency is the effect upon regeneration of hair follicles and hair formation. In the late stage of the deficiency, regeneration of the hair follicles does not occur or is incomplete. Follicles engaged in hair formation during the establishment of the deficiency undergo atrophy and for a time continue to form imperfect hair. The atrophy is apparent in all parts of the hair follicle but is more evident in the matrix. The cuticular cells continue longest but undergo atypical cornification. Thus various degrees of retardation of hair production are found in a given area of skin; complete suppression, hair roots represented by loosely packed columns of cornified fusiform cells and hair roots consisting of medulla with imperfectly formed cortical substance. Sharply flexed or buckled hair follicles are common, presumably occasioned by the lack of support normally afforded by the forming hair shaft or root. In cross section, the hair roots are often oval or flat in outline. The microscopic appearances account satisfactorily for the gross appearances of the sparsely distributed hair. The gross impression of thickening of the skin may be accounted for by the persistence of many atrophic regenerated follicles because these may and often do extend to the depth of normal active follicles (i.e., to the muscle panniculus), and owing to their number, should affect the texture of the skin. In 48 hours after riboflavin therapy, there is marked restoration of normal appearances of the follicles and in 72 hours the epithelium of the follicle has assumed normal appearances. The matrix cells respond first."

According to Kornberg \textit{et al.}\textsuperscript{51} rats fed a diet deficient in riboflavin developed granulocytopenia in about 5.0\% of the cases and, less frequently, anemia. The granulocytopenia responded to folic acid more frequently than to riboflavin. However, the anemia observed was alleviated in more rats

\textsuperscript{50} S. B. Wolbach and O. A. Bessey, \textit{Science} \textbf{91}, 559 (1940).
by riboflavin than by folic acid. There was a hyperplasia of bone marrow in the riboflavin-deficient rats which was indistinguishable from that seen in rats with folic acid deficiency. There was also an atrophy of the lymphoid tissue and no evidence of blood formation in the spleen. Further study of these observations has led to the assumption that riboflavin deficiency can cause anemia in the rat.

The importance of riboflavin in the reproductive cycle has been quite apparent to animal breeders. Its absence from the diet of rats may result in anestrus, and if riboflavin is not restored within 10 weeks, the damage becomes irreparable. Female rats bred on a deficient diet by Warkany gave birth to litters one-third of which had congenital skeletal malformations including shortening of the mandible, tibia, fibula, radius, and ulna, fusion of the ribs, sternal centers of ossification, fingers, and toes, and cleft palate. There were no abnormal young when riboflavin was added to the diet. Nelson et al. did not observe skeletal abnormalities at birth in the litters of their riboflavin-deficient rats. The principal changes which appeared in their deficient animals were retarded development of the epiphyses, progressive decrease in the width of the epiphyseal cartilage, increased hyalinization of its matrix, and calcification and separation of the epiphyseal cartilage from the marrow cavity by a thin layer of bone. Hematopoietic tissue was replaced by fat in all the rats after they had been on the deficient diet for 144 days.

Riboflavin has been found to be protective to the rat against the rickettsiae of murine typhus. Chronic riboflavin deficiency is often accompanied by a type of pediculosis against which riboflavin seems to have a specific effect.

It has been shown that there is no appreciable loss of appetite during riboflavin deficiency. On the contrary, there is a relatively increased food intake during the final stages of riboflavin deficiency. Pair-fed control rats grew much faster than the riboflavin-depleted rats. Consequently, riboflavin has been associated with an increased economy of food utilization.

57 J. Warkany, Vitamins and Hormones 3, 73 (1945).
60 H. Pinkerton and O. A. Bessey, Science 89, 368 (1939).
2. Dogs and Foxes

Sebrell\textsuperscript{63, 64} was probably the first to critically evaluate the pathological state in dogs now known to be due to a deficiency of riboflavin. Among the signs noted was the characteristic "yellow liver" due to fatty infiltration. Later, during studies of canine black tongue, it was shown that riboflavin often prevents death in animals on black tongue-producing diets.\textsuperscript{65-67} With inadequate riboflavin, collapse, coma, and death occurred in less than 102 days. The onset was sudden and characterized by ataxia, weakness, and loss of deep reflexes, so that the dog was unable to stand. The animal appeared fully conscious and without pain prior to final collapse, as though death were due to a cellular asphyxia brought on by a failing chemical mechanism.\textsuperscript{23} This collapse syndrome was also noted by Street and Cowgill\textsuperscript{68} in dogs on diets which contained not more than traces of riboflavin.

Street \textit{et al.},\textsuperscript{85} while investigating neurological manifestations of riboflavin deficiency, were not able to confirm the finding of fatty degeneration of the liver in their dogs. They noted myelin degeneration in peripheral nerves and in the posterior columns of the spinal cord, becoming more extensive with the length of the period on the deficient diet. Opacities of the cornea were also noted. The suggestion of these authors that inanition was the cause of previously published reports of fatty liver cannot be reconciled with the observations of Potter \textit{et al.},\textsuperscript{69} who noted no fatty liver in their inanition-control dogs, whereas their riboflavin-deficient, choline-supplemented diets produced typical friable, fatty, yellow livers. Also noted was a dry, flaky dermatitis, usually accompanied by a marked erythema on the hind legs, chest, and abdomen, and a purulent discharge from the eye, which was associated with a conjunctivitis. This was followed in a few days by vascularization of the cornea, which in several dogs went on to corneal opacification.

Anemias have been noted in riboflavin-deficient dogs,\textsuperscript{59, 69, 70} but it remains a question whether this is a specific part of the deficiency syndrome.

The deficiency symptoms which develop in the fox closely resemble those

observed in the dog. Loss of weight, muscular weakness, coma, opacity of the lens, and fatty infiltration of the liver have been reported.\textsuperscript{71}

3. Pigs

The similarity of B\textsubscript{2} deficiency in the pig to human pellagra was noted by Hughes\textsuperscript{72} in 1938, and subsequent reports have highlighted the economic importance of adequate riboflavin in the diet of swine.\textsuperscript{73, 74} Patek \textit{et al.}\textsuperscript{75} characterized riboflavin deficiency in the pig as a syndrome including retarded growth, corneal opacities, dermatitis, changes in the hair and hoofs, and terminal collapse associated with hypoglycemia. These pigs showed changes in the corneal epithelium, lenticular cataracts, hemorrhages of the adrenals, and lipid degeneration of the proximal convoluted tubules of the kidneys. Mitchell \textit{et al.}\textsuperscript{76} did not find any cataracts or corneal changes but did note anorexia and vomiting in their riboflavin-deficient pigs. They considered the absolute and relative neutrophilic granulocyte concentrations in the blood as the most sensitive indices of riboflavin deficiency.

4. Young Ruminants

It is generally agreed that ruminants can meet most of their requirement of B-complex vitamins by intestinal synthesis. However, during the first days after birth the rumen of the young animal has not yet reached functional capacity, and unless riboflavin is supplied to the feed of young dairy calves, definite signs of riboflavin deficiency develop. Synthetic milk diets have been devised which do not favor normal rumen function.\textsuperscript{77} On such diets the dairy calf develops hyperemia of the buccal mucosa, lesions in the corners of the mouth, along the edges of the lips, and around the navel, loss of appetite, scours, excessive salivation and lacrimation, and loss of hair.\textsuperscript{77-79} Pounden and Hibbs\textsuperscript{80} have observed that the type of ration fed to calves was a controlling factor in the development of riboflavin-producing flora and fauna\textsuperscript{81} in the rumen.

\textsuperscript{72} E. H. Hughes, \textit{Hilgardia} \textbf{11}, 595 (1938).
\textsuperscript{73} E. H. Hughes, \textit{J. Nutrition} \textbf{20}, 233 (1940).
\textsuperscript{74} M. M. Wintrobe, \textit{Am. J. Physiol.} \textbf{126}, 375 (1939).
\textsuperscript{75} A. J. Patek, Jr., J. Post, and J. Victor, \textit{Am. J. Physiol.} \textbf{133}, 47 (1941).
Riboflavin deficiency has also been observed in young lambs reared on artificial diets.82

5. Other Mammals

Mice show effects83, 84 quite similar to those described for rats. A histological basis for the inhibition of lengthwise growth in riboflavin-deficient animals has been suggested in studies of endochondral ossification in mice.85

Rhesus monkeys86 develop a freckled type of dermatitis on face, hands, legs, and groin, and a hypochromic, normocytic anemia, both of which are improved by riboflavin administration. Fatty livers which cannot be related to inanition have also been demonstrated in these monkeys.

The similarity between periodic ophthalmia in horses during the course of which corneal vascularization and cataracts frequently occur, and riboflavin deficiency in experimental animals, has suggested a possible common etiology.87 It has been reported88 that riboflavin is effective in preventing the appearance of equine periodic ophthalmia but that it does not influence the course of the disease in established cases. Studies of horses on diets low in riboflavin89 have shown a correlation between their urinary excretion and their dietary intake.

6. Birds

The economic importance of poultry raising has stimulated much excellent research on the vitamin requirements of birds. The needs of chicks and fowls for growth and egg laying have been assayed by many investigators.90-98 During these studies peculiar pathological syndromes have been

91 R. M. Bethke and P. R. Record, Poultry Sci. 21, 147 (1942).
observed which not only have advanced our understanding of riboflavin deficiency but also have been of major importance in the discovery of the more recently described vitamins of the B complex.

Phillips and Engel have observed in chicks specific pathology in the main peripheral nerve trunks, characterized by degenerative changes in the myelin sheaths of the nerve fibers, which was quite similar to that seen during riboflavin deficiency in rats on high-fat diets. A prolonged, mild deficiency produced a characteristic "curled-toe paralysis" in chickens.

Riboflavin deficiency in turkeys produced a severe dermatitis. Hegsted and Perry did not observe any characteristic gross signs of riboflavin deficiency in the duckling; the animals failed to grow and died within a week.

E. IN MAN

M. K. HORWITT

A syndrome resembling pellagra (pellagra sine pellagra) has been known for centuries, but its relationship to the diet was first recognized by Stannus in 1911. His findings were generally confirmed and augmented by Bahr (1912 to 1914) in Ceylon, Scott (1918) in Jamaica, Moore (1930) in West Africa, Landor and Pallister (1935) in Singapore, and Ackroyd and Krishnan (1936) in South India. Yeast products were first used therapeutically by Goldberger and Tanner in their classical studies on induced pellagra, and by Fitzgerald (1932), who reported an outbreak in an Assam prison of ulcerations at the angles of the mouth which were benefited by 1 oz. of yeast daily.

The first suggestion that two separate dietary factors might be concerned in the production of clinical pellagra came from Goldberger et al. in 1918, and what appears to have been riboflavin deficiency was produced on a

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casein diet by Goldberger and Tanner.\textsuperscript{130} A clear-cut separation between these two deficiency states was not made until 1938, at which time both nicotinic acid and riboflavin were available. Sebrell and Butler\textsuperscript{113, 114} studied a group of patients on a diet low in riboflavin and nicotinic acid and showed that the manifestations of \textit{pellagra sine pellagra} were due to riboflavin deficiency.

1. Oral and Facial Lesions of Aribioflavinosis

The changes observed by Sebrell and Butler\textsuperscript{113-115} "consisted of lesions on the lips, which began with a pallor of the mucosa in the angles of the mouth. This pallor was soon followed by maceration; and within a few days superficial linear fissures, usually bilateral, appeared exactly in the corner of the mouth. These fissures showed very little inflammatory reaction, remained moist, and became covered with a superficial yellow crust, which could be scraped off without bleeding. In some instances these linear fissures showed a tendency to extend onto the skin of the face but did not extend into the mouth."

In addition, there was a "scaly, slightly greasy, desquamative lesion on a mildly erythematous base in the nasolabial folds, on the alae nasi, in the vestibule of the nose and occasionally on the ears and around the eyelids, especially at the inner and outer canthi."

In the years subsequent to the above presentation there have been many "confirmatory" reports stemming from clinical observations of "aribioflavinosis."\textsuperscript{116-125} On the other hand, the failure of several groups to duplicate Sebrell and Butler's results and the frequency of angular stomatitis refractory to riboflavin therapy led to some skepticism regarding the sym-

\textsuperscript{117} P. Manson-Bahr, \textit{Lancet II}, 317, 356 (1940).
drome. It is now apparent that the cause of failure in those studies in which the typical picture was not produced was either the brevity of the experimental period or too high a level of riboflavin in the diet. Williams et al. fed a diet containing between 0.8 and 0.9 mg. per day for over 9 months, and Keys et al. gave 1.0 mg. for 5 months without producing any clinical changes. Horwitt et al. fed a diet containing between 0.8 and 0.9 mg. of riboflavin daily for over 2 years, and only one of twenty-two subjects showed any signs (angular stomatitis) which might be attributed to a lack of riboflavin. However, when these workers reduced the riboflavin intake to 0.55 mg. per day, a level only slightly higher than that used by Sebrell and Butler, incontrovertible signs of arboflavinosis appeared in less than 6 months. Subsequent studies have confirmed these observations and have indicated that the course of the development and healing of the lesions was not altered by low dietary levels of nicotinic acid (6 mg.) and tryptophan (250 mg.).

The oral lesions which are generally accepted to be part of the clinical picture of riboflavinosis may be summarized as follows: angular stomatitis, fissures in the angles of the mouth which resemble perleche, cheilosis, involvement of the vermilion border of the lips including vertical fissuring, and crusting and desquamation of the mucous membrane. Glossitis, including the magenta tongue, may be seen, but "pure" riboflavin deficiencies have been produced without such defects.

The characteristic facial lesions include seborrheic accumulations in the folds of the skin, especially in the nasolabial folds. Mild infections of the upper respiratory tract may initiate an inflammation of the nostrils and spread as a weeping, crusty lesion over the skin of the septum. Fissures may appear in the nasolabial folds.

2. Lesions of Scrotum and Vulva

Stannus was the first to record that scrotal involvement may be the initial sign of deficiency (nineteen of one hundred cases of "pellagra").

131 E. A. Zeller, Advances in Enzymol. 2, 93 (1942).
134 M. K. Horwitt, Personal communication (1952).
Sydenstricker\textsuperscript{135, 136} noted an itching dermatitis of the scrotum or vulva in patients with pellagra. Purcell\textsuperscript{137} described a scrotal dermatitis that improved with riboflavin treatment. Mitra\textsuperscript{138} reported a urogenital lesion among Indians which responded to riboflavin. Goldberger and Wheeler\textsuperscript{139} showed that six of their eleven patients exhibited scrotal dermatitis before any other lesions of pellagra appeared. In a recent study\textsuperscript{132} scrotal dermatitis was the most frequently observed symptom of riboflavin deficiency; twelve of fifteen subjects had it, either mildly or severely. Typically, this began as a patchy redness associated with scaling and desquamation of the superficial epithelium of the anterior surface of the scrotum. The median commissure was uninvolved in most of the patients. The more prolonged and severe cases showed a lichenification of the involved areas. The far-advanced lesion became quite raw and extended up the shaft of the penis or to the inner aspects of the thigh. The response to treatment with 6 mg. of riboflavin per day was prompt, and in two cases with severe inflammation it was dramatic. This recent study emphasizes the question of the role of ariboflavinosis in the development of those scrotal and vulval lesions which have been considered characteristic of pellagra.

3. Ocular Manifestations

In experimental animals vascularization of the cornea and involvement of the lids are early and constant findings.\textsuperscript{36, 38, 140} In man, ocular pathology is not constant, but it may occur in a high percentage of cases. Conjunctivitis, lacrimation, and burning of the eyes have been observed as manifestations which have been cured by riboflavin by sufficient investigators to be non-controversial; corneal vascularization in human riboflavin deficiencies has not been noted so often under controlled conditions.

Spies and his associates\textsuperscript{120, 121} were among the first to note that the ocular lesions were cured by riboflavin administration. Sydenstricker and co-workers\textsuperscript{116} reported that photophobia was associated with conjunctivitis. Reduced visual acuity, itching, a sensation of roughness of the eyelids, keratitis, and mydriasis have also been reported.\textsuperscript{116, 124, 141-143}

Rubeosis iridis has been suggested as a manifestation of deficiency which

\textsuperscript{135} V. P. Sydenstricker, Am. J. Public Health 31, 344 (1941).
\textsuperscript{136} V. P. Sydenstricker, Ann. Internal Med. 14, 1499 (1941).
\textsuperscript{140} R. E. Eckardt and L. V. Johnson, Arch. Ophthalmol. 21, 315 (1939).
\textsuperscript{141} H. C. Hou, Chinese Med. J. 59, 314 (1941).
\textsuperscript{143} P. B. Wilkinson, Lancet II, 655 (1944).
can be cured by riboflavin.\textsuperscript{144} Vascular networks of the iris were markedly improved after only 2 days of riboflavin supplementation.

Kruse and colleagues\textsuperscript{123, 124} reported corneal vascularization in forty-seven patients with riboflavin deficiency. Proliferation and engorgement of the bulbar conjunctival capillaries of the limbar plexus were considered by them to be the earliest and most common sign of hypervascularization. As a consequence of many controversial reports,\textsuperscript{145-151} the significance of these observations is not clear. No evidence of corneal vascularization was noted by the Elgin group\textsuperscript{132} despite frequent slit-lamp examinations of subjects before, during, and after experimental riboflavin deficiency.

XI. Pharmacology

M. K. HORWITT

The low solubility of riboflavin may be responsible for its relative innocuousness. Unna and Greslin\textsuperscript{1} found that oral administration of 10 g. per kilogram to rats and 2 g. per kilogram to dogs produced no toxic effects. Giving 340 mg. per kilogram to mice intraperitoneally, which is 5000 times the therapeutic dose, or the equivalent of 20 g. per day for a man, had no apparent effect.\textsuperscript{2-4} The rat LD\textsubscript{50} for riboflavin following intraperitoneal administration was 560 mg. kilogram.\textsuperscript{1} Death, which was due to kidney concretions, occurred in 2 to 5 days. Similar results were obtained by Antopol\textsuperscript{5} after intraperitoneal administration of 125 to 500 mg. per kilogram of the sodium salt. In addition, cytological changes were noted in the heart, pancreas, and pituitary gland, and the adrenals were markedly congested.

Since crystalline concretions of riboflavin were readily detectable in the ureter and bladder within a few hours after a saturated solution of riboflavin

\textsuperscript{145} H. R. Sandstead, Public Health Repts. (U.S.) 57, 1821 (1942).
\textsuperscript{150} J. G. Scott, J. Roy. Army Med. Corps 82, 133 (1941).
\textsuperscript{3} R. Kuhn, Klin. Wochschr. 17, 222 (1938).
\textsuperscript{4} V. Demole, Z. Vitaminforsch. 7, 138 (1938).
was given intravenously, Selye\(^6\) studied bilaterally nephrectomized rats to learn more about the role of the gastrointestinal tract in the absorption and excretion of riboflavin. He noted that excess riboflavin was rapidly excreted into the small intestine, especially the duodenum. Destruction of riboflavin proceeded slowly, if at all, in an isolated loop of duodenum, but quickly in an isolated large intestine. The bile does not function in the elimination of this vitamin. If the intestinal canal and kidneys are removed, the tissues of the rat cannot destroy or eliminate any significant percentage of large doses of intravenously administered riboflavin.

The riboflavin lost in sweat under tropical conditions\(^7\) has been considered of nutritional importance. However, the amounts which can be proved present in sweat are too small to be significant.\(^8\), \(^9\)

Riboflavin is excreted predominantly in the feces, which contain not only the part contributed by the intestinal walls but also that which is synthesized by intestinal bacteria.\(^10\)

Urine contains riboflavin,\(^11\) riboflavin phosphate,\(^12\) and a compound called uroflavin,\(^13\) a derivative which has been reported to be more soluble and to contain more oxygen than riboflavin. The methods of analysis ordinarily used for the estimation of riboflavin in urine do not distinguish between these compounds, since they have similar fluorimetric and microbiological activities.

The amount of riboflavin in the urine will vary with the recent dietary intake and with tissue storage. The correlation between urinary excretions and dietary intake is discussed on p. 401. Urinary excretion of riboflavin will also be affected by marked alterations in nitrogen balance.\(^14\)-\(^16\) Less is excreted in the urine, on a given intake, when tissue growth is rapid, as during convalescence after severe trauma,\(^17\) during lactation,\(^18\) or after administration of testosterone propionate;\(^19\) more is excreted after severe

8 O. Mickelsen and A. Keys, J. Biol. Chem. 149, 479 (1943).
burns or surgical procedures where protein losses indicate cellular decomposition.\textsuperscript{20}

The riboflavin content of the blood is relatively constant\textsuperscript{21-23} (approximately 40 $\gamma$ per 100 ml.) when measured by microbiological techniques. However, since the ingestion of riboflavin can cause a 30\% increase in the flavin adenine dinucleotide content of the red blood cell,\textsuperscript{24} it is likely that the use of improved methods\textsuperscript{25} will show a correlation between dietary and erythrocyte content.

Although there is no appreciable storage capacity of riboflavin in animal tissues, it is apparent that the amount can vary, since the organs of animals will lose as much as two-thirds of their original content when the animals are fed riboflavin-deficient diets.\textsuperscript{26-28} A combined protein and riboflavin deficiency is especially effective in decreasing the riboflavin content of the tissues of the growing rat.\textsuperscript{29} Diets on which signs of clinical arboflavinosis are observed are usually low in protein.

The concept of a rational pharmacology based upon antivitamins, which was pioneered by Woolley,\textsuperscript{30} has greatly stimulated the search for riboflavin antimetabolites. Wright and Sabine\textsuperscript{31} have shown that flavin adenine dinucleotide lowered the atabrin inhibition of tissue respiration and of $\alpha$-amino acid oxidase. This, and a similar observation by Haas\textsuperscript{32} with respect to cytochrome reductase, led Hellerman, et al.\textsuperscript{33} to quantitize the metabolic antagonisms of antimalarials like atabrin and quinine.\textsuperscript{34, 35} The phenazine analog of riboflavin,\textsuperscript{36} as well as isoriboflavin,\textsuperscript{37} when fed to mice and rats produced arboflavinosis.

\textsuperscript{22} F. M. Strong, R. S. Feeney, B. Moore, and H. T. Parsons, J. Biol. Chem. 137, 363 (1941).
\textsuperscript{24} J. R. Klein and H. I. Kon, J. Biol. Chem. 136, 177 (1940).
\textsuperscript{25} H. B. Burch, O. A. Bessey, and O. H. Lowry, J. Biol. Chem. 175, 457 (1948).
\textsuperscript{27} F. Vivanco, Naturwissenschaften 23, 306 (1935).
\textsuperscript{29} J. W. Czaczkes and K. Guggenheim, J. Biol. Chem. 162, 267 (1946).
\textsuperscript{30} D. W. Woolley, Science 100, 579 (1945).
\textsuperscript{31} C. I. Wright and J. C. Sabine, J. Biol. Chem. 155, 315 (1944).
\textsuperscript{32} E. Haas, J. Biol. Chem. 155, 321 (1944).
\textsuperscript{33} J. Hellerman, A. Lindsay, and M. R. Bovariuc, J. Biol. Chem. 163, 553 (1946).
\textsuperscript{34} J. Madinaveitia, Biochem. J. 40, 373 (1946).
\textsuperscript{36} D. W. Woolley, J. Biol. Chem. 154, 31 (1944).
The effects of riboflavin derivatives on the growth of neoplasms have been receiving increasing attention. Antopol and Unna\textsuperscript{28} and Miller and Miller\textsuperscript{39} have shown that large amounts of riboflavin retarded the occurrence of pathological changes in liver produced by \textit{p}-dimethylaminoazobenzene. Pollack \textit{et al.}\textsuperscript{40} noted that the concentration of riboflavin was lower in tumor than in normal tissue. Severe riboflavin deficiency decreased the growth rate of carcinomas\textsuperscript{41, 42} in mice. This could be accomplished either by riboflavin deprivation or by administering\textsuperscript{42} isoriboflavin or galactoflavin. Diethyl-riboflavin\textsuperscript{45} and \textit{6,7}-dichloro-9-(1'-D-sorbityl)isoalloxazine\textsuperscript{44} were effective in reducing the growth of tumors in rats.

\section*{XII. Requirements and Factors Influencing Them}

\textbf{M. K. HORWITT}

Any statement regarding the amount of riboflavin required must be a compromise depending upon variations in the heredity, growth, environment, age, activity, and health of the organism. The synthesis of riboflavin by the host and the differences in the availability of the vitamin from diverse sources must also be considered. With all these variables it is unlikely that there will ever be complete agreement among the workers in this field. Why, for example, only three out of fourteen men on identical diets low in riboflavin,\textsuperscript{1} living in the same environment for over a year, should show relatively severe signs of ariboflavinosis, and why three others in the same group should show no signs of deficiency at all, are questions which will confound the investigator for years to come. The concept of \textquoteleft individual susceptibility\textquoteright is an excuse which covers our ignorance and confounds those responsible for statements of recommended allowances.

\section*{A. OF ANIMALS}

Table III presents representative published requirements of some mammals, birds, and fish. It is apparent from the data listed that there is not

\textsuperscript{28} W. Antopol and K. Unna, \textit{Cancer Research} \textbf{2}, 694 (1942).

\textsuperscript{29} E. C. Miller and J. A. Miller, \textit{Cancer Research} \textbf{7}, 468 (1947).


### XII. REQUIREMENTS AND FACTORS INFLUENCING THEM

#### TABLE III

<table>
<thead>
<tr>
<th>Species</th>
<th>Riboflavin Requirement</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Mice</strong></td>
<td>1.5 γ/g. food</td>
<td>2, 3</td>
</tr>
<tr>
<td></td>
<td>4 γ/day</td>
<td>4</td>
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<tr>
<td></td>
<td>0.4-0.6 mg./100 g. body wt.</td>
<td>5</td>
</tr>
<tr>
<td><strong>Rat</strong></td>
<td>2-3 γ/g. food</td>
<td>6, 7</td>
</tr>
<tr>
<td></td>
<td>7.5 γ/day</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>10 γ/day</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>18 γ/day</td>
<td>10</td>
</tr>
<tr>
<td><strong>Dog</strong></td>
<td>60-100 γ/kg. body wt./day</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>100-200 γ/kg. body wt./day</td>
<td>12</td>
</tr>
<tr>
<td><strong>Swine</strong></td>
<td>20-66 γ/kg. body wt./day</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>1.7 mg./kg. feed</td>
<td>14</td>
</tr>
<tr>
<td><strong>Fox</strong></td>
<td>1.2-4.0 γ/g. diet</td>
<td>15</td>
</tr>
<tr>
<td><strong>Horse</strong></td>
<td>44 γ/kg. body wt./day</td>
<td>16</td>
</tr>
<tr>
<td><strong>Holstein calf</strong></td>
<td>1.0 mg./kg. feed</td>
<td>17</td>
</tr>
<tr>
<td><strong>Monkey</strong></td>
<td>25-30 γ/kg. body wt./day</td>
<td>18</td>
</tr>
<tr>
<td><strong>Chick</strong></td>
<td>2.75-3.25 γ/g. food</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>2.9-3.6 γ/g. food</td>
<td>20, 21</td>
</tr>
<tr>
<td><strong>Poulт</strong></td>
<td>3.25-3.75 γ/g. food</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>3-4 γ/g. food</td>
<td>22, 23</td>
</tr>
<tr>
<td><strong>Duck</strong></td>
<td>3 γ/g. food</td>
<td>24, 25</td>
</tr>
<tr>
<td><strong>Trout</strong></td>
<td>5-15 γ/g. food</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>6-9 γ/g. food</td>
<td>27</td>
</tr>
</tbody>
</table>

---

any unanimity of opinion. However, if one considers the ratio of riboflavin to food intake, it can be noted that 2 to 3 mg. of riboflavin per kilogram of diet seems to satisfy most of the suggested requirements. Such a figure is only of approximate value, since the protein, fat, and carbohydrate proportions of the diet will vary widely.

1. Effect of Dietary Constituents

It has been shown\textsuperscript{19, 28, 29} that the fat content of the diet has a marked effect on the riboflavin requirement of the rat. Thus, the replacement of the dextrin in a rat diet with isocaloric amounts of fat increased the amount of riboflavin needed for growth and enabled the production of more severe deficiency symptoms. Riboflavin appeared to play no part in the synthesis of fat, carbohydrate, and protein.\textsuperscript{30, 31} Extra fat gained by rats on high-riboflavin diets may result from the sparing of dietary fat through more efficient utilization of dietary energy.\textsuperscript{32} Reiser and Pearson\textsuperscript{33} found that cottonseed oil in the diet of chicks increased the requirement of riboflavin. Rats fed a diet containing only 2% of calories as fat required half as much riboflavin as rats fed the standard 20% fat diet, and rats on the latter diet, in turn, required half as much riboflavin as those fed 40% of their calories as fat.\textsuperscript{8}

It is not possible to vary the amount of fat in a diet without changing the relative concentration of the other constituents. The effects of high-fat, low-fat, high-protein, and low-protein diets on riboflavin requirements of the rat were studied simultaneously by Czaiczkes and Guggenheim.\textsuperscript{8} They noted that rats on low-protein diets needed at least twice as much riboflavin as animals kept on a "normal" diet. These authors believe that the

different requirements for riboflavin are due to differences in the amounts of riboflavin which are synthesized in an available form.

Recent studies by Everson et al.\textsuperscript{34} have stressed the importance of complete digestion in evaluating the availability of riboflavin in various foods. Working with young women, they observed that a larger proportion of riboflavin was available from ice cream than from legumes or almonds.

Large doses of ascorbic acid or aureomycin can prevent or delay signs of riboflavin deficiency in rats. Daft and Schwarz\textsuperscript{35} have reported that riboflavin-deficient rats died as expected, but that on identical diets plus 2% ascorbic acid or 20 mg.% aureomycin litter mates survived.

2. Effect of Environment

Working with rats at environmental temperatures of 90 and 68° F., Mills\textsuperscript{7} concluded that the dietary concentration of riboflavin needed for maximum growth was not altered by temperature. Mitchell et al.\textsuperscript{36} using pigs as their experimental animals, have claimed that the riboflavin requirement is greater (2.3 p.p.m. at 42° F.) at low temperatures than at high temperatures (1.2 p.p.m. at 85° F.).

3. Reproduction

Barrett and Everson\textsuperscript{37} indicated that the need for B vitamins increased rapidly as pregnancy\textsuperscript{38} progressed in the rat. Hogan and Anderson\textsuperscript{39} showed that a synthetic diet slightly inadequate for growth was seriously inadequate for lactation. It is reasonable to expect lactation to increase the requirement, since logically the need for mother and offspring is greater than that of the mother alone.

4. Inherent Individual Variations

Those who have worked with animals in nutritional studies are acutely aware of the individual variations which will occur, even in closely inbred litter mates. Fenton and Cowgill\textsuperscript{5} have highlighted this problem by studying the riboflavin requirements of two inbred strains of mice. Mice of the C\textsubscript{57} strain showed maximum growth when the diet contained 0.4 mg. of riboflavin per 100 g., whereas those of the A strain required a dietary level of 0.6 mg. At a 0.2-mg. level the C\textsubscript{57} mice had lowered red cell counts and


\textsuperscript{38} G. Everson, E. Williams, E. Wheeler, P. Swanson, M. Spivey, and M. Eppright, \textit{J. Nutrition} \textbf{36}, 463 (1948).

less riboflavin in their muscle and liver than those of the A strain on the same diet.

5. Other Factors

There have been suggestions that the growth requirements of male and female rats are different,\(^2\) the over-all effects of riboflavin deficiency being more prominent in the male. Unlike thiamine deficiency, the lack of riboflavin is not associated with severe anorexia; thus, appetite is not so important a factor in riboflavin depletion studies. The interrelationships between riboflavin and other vitamins of the B group have been studied.\(^4\)

B. OF MAN

In the absence of experimental data on human subjects, the estimation of riboflavin requirements is based upon average dietary consumptions or upon extrapolations of data from animal experiments. Calculation of average consumption is not a satisfying procedure, since different locales will show great variations depending upon dietary habits and the availability of riboflavin-rich foods. Attempts to calculate man's needs from data on rat growth tend to give figures which are too high to be practical. It is therefore necessary to test vitamin requirements on man, himself.

It is logical to expect that the minimum requirements of human beings would be much more variable than for the inbred laboratory animal. That this is the case was illustrated by Horwitt et al.,\(^1\) who studied fifteen men on a diet providing 0.55 mg. of riboflavin per day. Three of the men developed relatively severe dermatological lesions, nine men showed mild symptoms of ariboflavinosis, and three others had no symptoms at all.

Excretion studies which compare the amount of riboflavin intake with the amount excreted in the urine have been used for many years as a means of estimating human requirements. Much confusion has resulted from this approach because there is, as yet, no agreement upon how much riboflavin should be excreted before the intake is considered adequate.

In some of the older studies on riboflavin excretion\(^2\)-\(^4\) the diet was not considered adequate if it contained less than 2 mg. of riboflavin per day. Consequently, urinary excretions of less than 500 mcg. per day were designated as deficient. More recent comparative studies on the amounts of riboflavin excreted in the urine on different levels of intake have shown that

\(^4\) F. M. Strong, R. S. Feeney, B. Moore, and H. T. Parsons, *J. Biol. Chem.* 137, 363 (1941).
a reserve of riboflavin cannot be maintained by men at levels of intake below 1.1 mg. per day by a diet containing approximately 2200 cal. Brewer et al. calculated the requirement of women to be 1.3 to 1.5 mg. per day on a diet providing 2100 to 2300 cal. per day.

The recommended daily allowances of the Food and Nutrition Board of the National Research Council state that 1.8 mg. of riboflavin is adequate for a 70-kg. adult man, and 1.5 mg. for a 56-kg. adult woman. The assumption was made that increased work and greater than average caloric consumption do not increase the need for riboflavin. The allowances during the latter half of pregnancy and during lactation were increased to 2.5 and 3.0 mg., respectively. There is as yet no proof that more than the normal daily allowance is required during pregnancy. This problem was reviewed by Oldham et al. If one estimates the total amount of riboflavin stored during the gestation period, it seems likely that an additional 0.2 mg. per day should satisfy the needs for growth during pregnancy. The increased allowance for lactation makes ample provision for the amount in human milk, which contains about 0.5 mg. riboflavin per liter.

The recommended allowances for children are graduated in accordance with the growth rate at different ages. It has been recommended that children from 1 to 3 years be allowed 0.6 mg. per day, and that children 10 to 12 years be allowed 1.8 mg. per day. During the rapid period of growth from 13 to 15 years, it has been recommended that both girls and boys receive 2.0 mg. of riboflavin per day. It is apparent that, since these allowances are adjusted for growth requirements, the actual need of an individual will vary with his or her own pattern of growth.

One of the major goals of all the research described in this chapter is to determine how much riboflavin is required by man for optimum nutritional health. The techniques used may be classified under four headings: (1) observations of the repair of pathology by riboflavin administration; (2) survey studies of the nutritional status of population groups; (3) experimental production of riboflavin deficiency; and (4) evaluation of urinary excretion of riboflavin in health and disease.

Riboflavin deficiency states ordinarily noted by the clinician, whose primary obligation is to facilitate the repair of apparent pathology, do not often present adequate opportunities to assay the individual's need for riboflavin. The important contributions of these observations are in the classifications of conditions which can be healed by riboflavin, usually given

in amounts which are far in excess of the daily requirement. Without these classifications the researcher in nutrition would not know what to look for.

Excellent examples of the population survey type of study have been described by Goldsmith\(^4^8\) and by Wilkins and Sebrell.\(^4^9\) Wilder\(^5^0\) has summarized the extensive surveys of malnutrition in Newfoundland\(^5^1-5^3\) to show how the enrichment of flour benefited the population. But, although these surveys were of great value to our understanding of the nutrition of population groups, they are difficult to interpret in terms of the requirement of the individual.

The experimental production of riboflavin deficiency has been successful only in those studies in which levels of 0.55 mg. of riboflavin per day or less have been fed. Those investigators who provided 0.8 mg. per day to their subjects could not produce signs of ariboflavinosis. Nevertheless, no nutritional authority has yet suggested that 0.8 mg. is adequate for optimal health. Rather, one recommends amounts which provide somewhat more than the minimal daily need as fortification against unknown contingencies. The concept that riboflavin cannot be stored may not be entirely correct, since even at dietary levels of 0.5 mg. per day about 6 months must elapse before signs of ariboflavinosis appear.

One of the more important advantages of depletion studies is the opportunity provided for simultaneous investigation of urinary excretion. Since the urinary excretion is a reflection of the dietary intake and the dietary intake is, in the last analysis, the cause of riboflavin deficiency, it is understandable why so much effort has been devoted to the study of riboflavin in urine.

Load tests, in which a known amount of riboflavin is administered and the percentage excreted is determined, are useful means of estimating the degree of saturation of the tissues. Although the usual procedure is to administer riboflavin in the post-absorptive state and to analyze the riboflavin excreted during the following 4 hours, a 24-hour collection may be considered a load test if the dietary intake during that period is known. Goldsmith\(^5^4\) has reviewed the literature on the use of urinary excretion tests in

the evaluation of riboflavin nutrition. Oldham et al.\textsuperscript{55} and Pollack and Bookman\textsuperscript{56} have shown that increased amounts of riboflavin are excreted by subjects in marked negative nitrogen balance. Therefore, in those special cases where tests for riboflavin excretion are made on patients who are catabolizing abnormal amounts of their own tissues, the excretion data may

\textbf{TABLE IV}\textsuperscript{45}

\textbf{Comparison of Urinary Excretion with Daily Intake of Riboflavin. Except for Those on the 0.55-Mg.-Diet, the Data Listed Represent Plateau Levels Which Were Reached in 10 Weeks or Less on the Intake Designated}\textsuperscript{a}

<table>
<thead>
<tr>
<th>Number of subjects on each intake</th>
<th>Riboflavin intake, mg.</th>
<th>Duration of diet at time of analyses, weeks\textsuperscript{b}</th>
<th>Riboflavin excretion, 24 hr. Amount, $\gamma$</th>
<th>Ingested riboflavin, %</th>
<th>Excretion during 4 hr. following subcutaneous injection of 1 mg. of riboflavin, $\gamma$</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0.55</td>
<td>15</td>
<td>$51 \pm 11$</td>
<td>9.3</td>
<td>$23 \pm 15$ \textsuperscript{c}</td>
</tr>
<tr>
<td>11</td>
<td>0.75</td>
<td>12</td>
<td>$73 \pm 5$</td>
<td>9.7</td>
<td>$56 \pm 34$</td>
</tr>
<tr>
<td>12</td>
<td>0.85</td>
<td>15</td>
<td>$76 \pm 38$</td>
<td>8.9</td>
<td>$58 \pm 22$</td>
</tr>
<tr>
<td>28</td>
<td>1.1</td>
<td>13</td>
<td>$97 \pm 62$</td>
<td>8.8</td>
<td>$70 \pm 35$</td>
</tr>
<tr>
<td>39</td>
<td>1.6</td>
<td>100</td>
<td>$434 \pm 185$</td>
<td>26.5</td>
<td>$227 \pm 146$</td>
</tr>
<tr>
<td>12</td>
<td>2.15</td>
<td>10-78</td>
<td>$714 \pm 203$</td>
<td>33.2</td>
<td>$297 \pm 124$</td>
</tr>
<tr>
<td>13</td>
<td>2.55</td>
<td>2-44</td>
<td>$849 \pm 258$</td>
<td>33.3</td>
<td>$298 \pm 172$</td>
</tr>
<tr>
<td>13</td>
<td>3.55</td>
<td>1.5</td>
<td>$1714 \pm 300$\textsuperscript{d}</td>
<td>48.3\textsuperscript{d}</td>
<td>$373 \pm 90$\textsuperscript{d}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} At an 0.85-mg. intake or higher the figures obtained at 15 weeks remain at the same level for at least 2 years more. At a 0.55-mg. intake most of the subjects remained at the level designated for 6 months, at which time they were supplemented. A few individuals who remained longer on this diet showed no further change, but whether or not their excretions would have decreased further with additional time is not known.

\textsuperscript{b} The time of analyses for diets providing 0.75, 0.85, and 1.1 mg. of riboflavin represents the first time plateau levels were obtained which were repeated during subsequent months. The results recorded for the diets providing 1.6, 2.15, and 2.55 mg. are averages of voluminous data obtained during the period of time indicated.

\textsuperscript{c} After 7 weeks.

\textsuperscript{d} These results were obtained from the same individuals who received the 2.55-mg. intake. After 62 weeks on this intake, their supplementation was further increased to provide a 3.55-mg. intake. The values for the 24- and 4-hr. excretion periods were obtained 11/2 and 3 weeks, respectively, after the change in supplementation.

be higher than normally expected. There is a high degree of agreement in the results of excretion studies by different laboratories of subjects on low levels of riboflavin intake,\textsuperscript{45, 57-62} but the variations are greater when the


test dose or daily intake is more than 1 mg.\textsuperscript{44}. \textsuperscript{63-66} Table IV presents a summary of the urinary excretions obtained by the Elgin group\textsuperscript{45} at different levels of dietary intake.


# Chapter 16

## THIAMINE

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I. Nomenclature

ROBERT S. HARRIS

Accepted name: Thiamine (U. S. A.)
Aneurin (Brit. Pharm.)

Obsolete names: Vitamin B₁
Oryzamin
Torulin
Polyneuramin
Vitamin F
Antineuritic vitamin
Antiberiberi vitamin

Empirical formula: $C_{12}H_{17}N_4OSCl \cdot HCl$
Chemical name: 3-(4-Amino-2-methylpyrimidyl-5-methyl)-4-methyl-5-$\beta$-hydroethylthiazolium chloride hydrochloride

Structure:

II. Chemistry

B. C. P. JANSEN

A. ISOLATION

After Eijkman’s discovery that polished rice was the cause of polynieritis in birds and of beriberi in men, Grijns,¹ his successor in Batavia, was

¹ G. Grijns, Geneesk. Tijdschr. Ned. Indië 41, 3 (1901). (Dutch; afterwards trans-
the first to state that these diseases were the result of a "partial hunger," a deficiency of some unknown substance that is present in very small quantities in the outer layers (the silverskin) of the rice. Grijns called this substance "the protective substance"; Grijns also made the first attempts to isolate this substance from an extract from the silverskin of the rice. He succeeded in getting highly active fractions, but did not obtain a pure substance. About ten years later Casimir Funk, working in the Lister Institute in London, obtained a crystalline substance from rice polishings. He coined the word "vitamine"—an amine essential for life—for it. It was proved afterwards however, that this product had no antineuritic activity, and consequently it was not the desired substance. As it was shown subsequently that several "vitamines" were not amines at all, Drummond proposed that the final "e" of the name "vitamine" be dropped.

In 1926 Jansen and Donath, working in the same laboratory where Eijkman and Grijns had made their researches, obtained the crystalline substance having a great antineuritic activity. They sent 40 mg. of it to Eijkman, who was at that time in the Netherlands. Eijkman was able to confirm the prophylactic and curative activities of this substance against avian polyneuritis. Eijkman confessed that, before he received these crystals, he had doubted if the vitamin would be a normal chemical single substance. Jansen and Donath succeeded in the isolation because they used the finding of Seidell that the antineuritic substance is adsorbed by fuller's earth, and furthermore they found a fairly quick method (i.e., 10 days) for testing the antineuritic activity of the different fractions by the use of small rice birds (Munia maja).

About ten years later several workers in the United States, Germany, and England improved the method of isolation and thereby obtained sufficient quantities to establish its structural formula and to find methods for its synthesis (Williams and Cline, Grewe, Andersag and Westphal, and Todd and Bergel.)

1 C. Funk, J. Physiol. (London) 43, 395 (1911); Die Vitamine. J. F. Bergmann, München and Wiesbaden, 1922.
2 J. C. Drummond, Biochem. J. 14, 660 (1920).
8 H. Andersag and K. Westphal, Ber. 70, 2035 (1937).
B. CHEMICAL AND PHYSICAL PROPERTIES

Thiamine hydrochloride crystallizes into colorless, monoclinic needles, which have a melting point of about 250°, a characteristic smell, and a slightly bitter taste. These crystals are stable to the atmospheric oxygen. They are very soluble in water, much less so in alcohol, and insoluble in ether, and other fat solvents. Thiamine chloride hydrochloride crystallizes from alcoholic aqueous solutions as the hemihydrate, \( C_{12}H_{17}ON_4SCl \cdot HCl \cdot \frac{1}{2}H_2O \). Thiamine hydrochloride in water forms a strongly acid solution pH of a 5% solution about 3.5; in solution with a pH less than 5 it is fairly stable to heat and oxidation; this solution shows two absorption bands in the ultraviolet at 235 and 267 μm.

At a pH of 5 or higher, it is destroyed by autoclaving, and at a pH of 7 or more by boiling or merely storing at room temperature.

By treatment with sulfite it is readily split into the pyrimidine and thiazole parts.

In a highly alkaline solution thiamine is oxidized by ferricyanide to thiochrome (cf. Section VII).

C. CONSTITUTION

The work of the above-mentioned investigators has shown that the thiamine molecule consists of a pyrimidine compound and a thiazole compound, connected by a CH₂ bridge. The structural formula of the thiamine hydrochloride is:

\[
\begin{array}{c}
\text{H} \\
\text{C} \\
\text{N} \\
\text{CH₂—C—CH₂—N₃} \\
\text{N} \\
\text{CH₂—C—CNH₂—HCl} \\
\end{array}
\]

The elucidation of the constitution was greatly relieved by the discovery of Williams et al.,\(^{11}\) that thiamine is quantitatively split by sulfite in faintly acid solutions into the pyrimidine and the thiazole halves:

\[
C_{12}H_{18}ON_4SCl_2 + Na_2SO_3 = C_6H_5N_3SO_3 + C_6H_4NSO + 2NaCl
\]

D. SYNTHESIS

The synthesis of thiamine has been performed in different ways. It is possible to synthesize the pyrimidine nucleus and the thiazole nucleus sepa-

---

rately and afterwards to connect both parts. It is also possible to synthesize one of the nuclei with an extra side branch and afterwards to build up the other ring from this side branch.

For synthesizing the pyrimidine part, ethyl formate and $\beta$-ethoxyethylpropionate may be condensed with Na.

$$H\cdot COOC_2H_5 + CH_2OC_2H_5\cdot CH_2\cdot COOC_2H_5 + Na \rightarrow NaOC(H \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \q
chloride by treating with AgCl or by precipitating the practically insoluble thiamine picrate and dissolving it in hydrochloric acid.

E. SPECIFICITY

The activity of thiamine seems to be very specific. Even small alterations in the molecule give inactive substances or diminish the activity 100 or 1000 times or actually produce antagonistic effects. Most instructive in this respect is the work of Emerson and Southwick. They replaced the methyl group in position 2 in the pyrimidine ring of thiamine by other alkyl groups. Replacement by ethyl does not change the activity, as measured by rat experiments; replacements by propyl gives a definite reduction of the activity in pigeon tests. When the methyl is changed into an n-butyl group, the activity is reversed. However, Schopfer and Schultz, some years before the work of Emerson and Southwick, had established that a thiamine having in the second position of the pyrimidine nucleus an ethyl in place of a methyl group has a greater activity than normal thiamine on Phycomyces and on animals. Their relative activity is expressed by the ratio of ethylthiamine to methylthiamine having the same physiological activity. This ratio for Phycomyces was found to be 0.83:1.0 (Schopfer). The ratio for the pigeon is 0.85:1.0 (Schultz). The discrepancies with the results of Emerson and Southwick may be due to the inaccuracy of the animal experiments.

Barton and Rogers, in the book of R. J. Williams et al., give a huge

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number of examples of the influence of modifications in the pyrimidine or in
the thiazole moiety of thiamine on the biological activity of these thiamine
analogs. They arrive at the following conclusion: "as a result of these tests
it is evident that the thiamine molecule can undergo very little modific-
tation without extensive loss of vitamin B\textsubscript{1} activity."

Williams and Cline\textsuperscript{18} were able to establish that the synthetic thiamine
hydrochloride was identical with the natural in composition, ultraviolet ab-
sorption and antineuritic potency. Eckler and Chen\textsuperscript{19} in elaborate pharma-
cological studies compared the curative doses and the minimum lethal
doses of natural and synthetic thiamine. This work confirmed the identity
of both substances.

In several microorganisms the thiamine may be replaced by one or both
of its pyrimidine and thiazole moieties.\textsuperscript{20} Abderhalden\textsuperscript{21} has shown that
higher animals too could sustain on the thiazole + pyrimidine moieties
instead of thiamine itself. One would think that this might be brought about
by the phenomenon of "reflection," i.e., the synthesis of thiamine by the
microorganisms in the gut, as was found by Fridericia et al.\textsuperscript{22} But E.
Abderhalden and R. Abderhalden\textsuperscript{23} stated that tissue extracts are capable
of synthesizing thiamine from the pyrimidine and thiazole parts—only to a
very small extent, however, up to about 1% of the theoretical amount.

III. Industrial Preparation

H. M. WUEST

A. FROM NATURAL SOURCES

1. Low Concentrates from Rice Bran or Polishings

Effective thiamine preparations go back as far as 1910, when Robert R.
Williams under the direction of E. B. Vedder prepared the first crude ex-
tracts from rice bran in Manila which were used in the treatment of beriberi
in the Philippine Islands.\textsuperscript{1} The preparation, called tikitiki extract (or just
tikitiki, the Tagalog word for bran) is still used in the Philippines, and
similar preparations are sold in Japan.

In this country preparations of this kind were first made about 1930 by

\textsuperscript{22} L. S. Fridericia, P. Freundenthal, S. Gudjonsson, G. Johansen, and N. Schoubye,
\textit{J. Hug.} \textbf{27}, 70 (1928).
the Vitab Corporation in Emeryville, California, with an activity up to 50 units per milliliter. Colman\(^2\) and Arnold and Schreffler\(^3\) improved the extraction and the taste of the preparation. It is still used today: U. S. Pharmacopeia XIV (1950, p. 513) describes the extract with 0.06 mg. of B\(_1\) per milliliter.

2. Low Concentrates from Yeast

By plasmolysis of yeast, coagulation of the protein material and concentration of the aqueous solution Light and Frey\(^4\) developed a method of preparing yeast concentrates with a thiamine content up to 300 units per gram.

3. High Concentrates from Rice Germs

A highly improved method for isolating crystalline thiamine from rice polish was published in May 1934 by Williams \textit{et al.}\(^5\) By using a richer source than rice bran or polish, namely rice germs, F. Hoffmann-La Roche Ltd. in Basle, Switzerland (F. Elger, A. J. Frey, and H. M. Wuest) developed a method yielding concentrates with 25,000 to 30,000 units per gram. This product was used in 1935–1937 for the multivitamin preparation Nestrovit (vitamins A, B\(_1\), C, and D in a basis of dried skimmed milk, sugar, and cocoa butter); 1200 to 1500 kg. of rice germs were worked up daily.

4. Isolation of Crystalline Thiamine from Natural Sources

The isolation of crystalline thiamine by Jansen and Donath\(^6\), by Windaus \textit{et al.}\(^7\) and by Williams \textit{et al.}\(^8\) are today only of historical interest. Williams\(^8\) gives a good characterization of the efforts in his book: “It is doubtful whether the isolation and identification of any other substance in the history of biochemistry have cost as much labor as have these operations as applied to thiamin. The first gram of the pure vitamin must have cost an aggregate of several hundred thousand dollars” (written in 1938).

In spite of the improvements of the methods\(^9\)–\(^13\) pure natural thiamine

\(^2\) H. B. Coleman, U.S. Pat. 2,369,775 (February 20, 1945).
\(^3\) A. Arnold and C. B. Schreffler, U.S. Pat. 2,390,679 (December 11, 1945).
\(^4\) R. F. Light and C. N. Frey, U.S. Pat. 2,184,748 (December 26, 1939); British Pat. 428,044 (April 30, 1935).
could not compete with the synthetic product; its isolation on an industrial scale never started.

B. BIOSYNTHESIS OF THIAMINE IN YEAST

Using selected trains of Saccharomyces cerevisiae Schultz et al.14 could show that thiamine has a stimulating effect on the growth of these yeasts. The two moieties of the thiamine, thiazole and pyrimidine, had a similar stimulating effect, especially when used together.15 The authors developed their observation to a technical process for the production of yeast with high vitamin content.16 Such yeasts (with 200 units per gram, wet basis) were sold by Standard Brands under the trade name Hi-Yeast for making enriched bread (1938–1943). In dry form the preparation is still used for pharmaceutical purposes.

C. SYNTHESIS

If the isolation of thiamine from natural sources is interesting, the history of the industrial synthesis is dramatic, as from 1935 three groups were in the final race: in this country Williams et al.; in Germany Andersag and Westphal in the laboratories of the I. G. Farbenindustrie in Elberfeld; and Todd and Bergel in England. The scientific priority without any doubt falls to Williams with his first publication of the complete synthesis in August 1936.17 The claims of priority for Andersag and Westphal made by H. Hörlein18 were based on their earlier patent applications, which were published much later. Todd and Bergel19 published their synthesis about eight months after Williams, only to find out that Andersag and Westphal20 had already filed a German patent application for the same process more than a year previously.

11 L. R. Cerecedo and D. J. Hennessy, J. Am. Chem. Soc. 59, 1617 (1937); L. R. Cerecedo and F. J. Kaszuba, ibid. 69, 1619 (1937); L. R. Cerecedo and J. J. Thornton ibid. 59, 1621 (1937); L. R. Cerecedo, U.S. Pat. 2,114,775 (April 19, 1938).
16 Standard Brands, British Pats. 532,013 (January 15, 1941); 529,825 (September 25, 1941); A. S. Schultz, L. Atkins, and C. N. Frey, U.S. Pats. 2,262,735 (November 11, 1941); 2,233,556 (November 9, 1943); 2,354,281 (July 25, 1944).
20 H. Andersag and K. Westphal, German Pat. 685,032 ex. 3 (filed January 29, 1936, issued Nov. 16, 1939).
Williams' process grew in the hands of the chemists of Merck and Co., Rahway, New Jersey; the ring closure of Todd and Bergel was adopted industrially by Hoffmann-La Roche in their three plants in Basle, Nutley, New Jersey, and Welwyn, Herts. Andersag and Westphal's method has not yet reached the production in tons.

The established structure of thiamine, opens three possibilities for the synthesis:
1. Building up the pyrimidine ring and the thiazole ring separately, then connecting both by quaternization to the thiazolium ring.
2. Building up the pyrimidine ring with the group $\text{CH}_2\text{NH}_2$ in the 5 position, elongating this side chain, and forming the second ring by ring closure to the thiazolium compound.
3. Building up the thiazole ring, quaternization to thiazolium by elongation with a suitable side chain, and ring closure of the pyrimidine ring.

All three ways are described in literature. The first method is used by Williams et al. and by Andersag and Westphal: as the two ring systems are built up separately, the best conditions for the ring closure can be chosen, namely, alkaline condensation for the pyrimidine and acid medium for the thiazole nucleus. The quaternization goes easily and gives a nearly theoretical yield, an important factor for the success of the method.

Todd and Bergel follow the second possibility: the elongation of the side chain is done with the simple and elegant step of thioformylation of the amine, the ring closure to the thiazolium being performed in an acid medium, both reactions giving excellent yields.

The third possibility seems to have only slight chances, as pyrimidine rings are best built up in an alkaline medium, a condition highly unfavorable for the thiazolium ring. This way is described for a thiamine homolog in a French patent, but no experimental data are given. There is no evidence that the pyrimidine ring closure was performed.

The following discussion of the industrial synthesis is based on the technical point of view, with special emphasis on the reported yields.

For practical reasons the molecule is divided into four groups.

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A and B serve to build up the pyrimidine ring, C and D for the ring closure to the thiazole or thiazolium nucleus. All three industrial syntheses use these four groups as stepping stones to the end product; in Todd and Bergel's synthesis the nitrogen for the thiazolium ring switches to group B which is then elongated to group D.

1. **Group A, Two Carbon and Two Nitrogen Atoms**

The natural starting material for the left half of the pyrimidine ring is

\[
\text{NH}_2\text{CH}_3-\text{C} = \text{NH}
\]

which is easily available from acetonitrile in two steps: the nitrile is converted to acetimino ether by ethanolic hydrogen chloride in the absence of water, the ether being converted to acetamidine hydrochloride with ethanolic ammonia. Yield is 80 to 91% in small batches. Under optimal conditions, the yield may be raised to 95%. Instead of acetamidine the acetimino ethyl ether can be used if amino methylene malonitrile serves as group B. The use of thioacetamide and amino methylene malonitrile does not offer special advantages. Acetonitrile, the starting material, can be obtained from dimethylsulfate and sodium cyanide. In this country it is available as a commercial item.

2. **Group B, Four Carbon Atoms and One or Two Amino Groups**

\[
(4)\text{C} \\
(5)\text{C} - \text{C} \\
(6)\text{C} \\
(7)
\]

The carbon skeleton with four carbon atoms permits quite a number of variations. The chain of the carbon atoms 4, 5, and 7 can be provided by the esters of ethoxypropionic acid, succinic acid, malonic acid, and malonitrile; carbon atoms 6 is added in most of the cases by a formyl group, either as such or as the methylene ether. The substitution in 7 may lead to a halogen or to an amino group (synthesis of Todd and Bergel).

a. **Indirect Introduction of the Amino Group in the 4 Position**

Cline et al. start from ethoxypropionic ester (obtained from ethyl acrylate by addition of ethanol with sodium ethylate) and convert it with

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23 O. Hromatka, Merek & Co., U.S. Pat. 2,235,638 (March 18, 1941); Merek & Co., German Pat. 667,990 (November 24, 1938); F. Hoffmann-La Roche and Co., A. G., British Pat. 186,441 (June 2, 1938).
sodium and ethyl formate to sodioformylethoxypropionate, using the crude
\[ \text{CH}_2=\text{CH}-\text{COOEt} \rightarrow \text{EtO}-\text{CH} = \text{CH}-\text{COOEt} + \text{HCOOEt} + \text{Na} \rightarrow \text{EtO}-\text{C}-\text{C}:(\text{CHO} \text{Na})\text{COOEt} \]
sodium salt for the ring closure. In the form given by these authors the method is not satisfactory, as formylation and ring closure (see under c) give very poor yields.

Todd and Bergel\(^9\) formylate cyanoacetic ester in acetic anhydride with ethyl orthoformate to ethoxymethylene cyanoacetic ester.\(^{26}\) No yield is given, but from the abundant work of Claisen it can be assumed that the reaction goes well.

An alternative route of the same authors is slightly longer but offers certain advantages at the ring closure. Here ethyl malonate is condensed with ethyl orthoformate in the presence of zinc chloride with a yield of about 60\%, as shown by Claisen.\(^{27}\)

\[ \text{EtOOC}-\text{C}:(\text{CHOEt})\text{COOEt} \]

In the method of Andersag and Westphal\(^{28}\) one more carbon atom is added by starting from ethyl succinate to formyl ethyl succinate. The yield of this formylation (60 to 70\%) is satisfactory (Wislicenus \textit{et al.}\(^{29}\)).

\[ \text{EtOOC} = \text{CH}_2 = \text{CH}:(\text{CHO})\text{COOEt} \]

\textit{b. Direct Introduction of the Amino Group in the 4 Position}

The more reactive the methylene group in this series, the better the yield at the formylation. Grewe\(^{30}\) formylated malonitrile with orthoformic ester in acetic anhydride to ethoxymethylene malonitrile

\[ \text{HC(OEt)}_3 + \text{CN} = \text{CH}_2 - \text{CN} = \text{CN} - \text{C}:(\text{CHOEt})\text{CN} + 2\text{EtOH} \]

As Diels \textit{et al.}\(^{31}\) have shown, a yield of 75\% of ethoxymethylene malonitrile can easily be obtained.

Starting material for malonitrile is chloroacetic acid which is converted to ethyl cyanoacetate with 77 to 80\%.\(^{32}\) The next step, cyanoacetamide, gives a yield of 86 to 88\%,\(^{33}\) and the conversion of the amide to the nitrile


\(^{27}\) L. Claisen, \textit{Ann.} \textbf{297}, 76 (1897).


\(^{29}\) W. Wislicenus, E. Böcklen, and F. Reuthe, \textit{Ann.} \textbf{363}, 347 (1908).


is performed with phosphorus pentachloride at 90°; the evolution of large amounts of hydrogen chloride starts, and the malonitrile is distilled in vacuo as soon as it is formed; yield 70% (Hesse\textsuperscript{24}). Ethyl cyanooacetate is commercially available in this country.

3. Ring Closure to the Pyrimidine, \( A + B \)

The ease with which the pyrimidine nucleus is formed varies considerably and therefore also the yields. In the most favorable cases no condensing agent is necessary, as the alkalinity of the free acetamidine is sufficient to close the ring. In other cases sodium ethylate or sodium hydroxide is used for the ring closure, and in one special case it even happens in neutral or acid solution. Normally the formyl and cyano (or carbethoxy) groups react at the same time, the ring closure taking place in one step. Todd and Bergel\textsuperscript{19} have isolated as an intermediate an acrylo compound which could be used for the ring closure in two different ways.

\textit{a. Indirect Introduction of the Amino Group in the 4 Position}

Williams and Cline\textsuperscript{17} reacted ethyl sodioformyl-\( \beta \)-ethoxypropionate with acetamidine hydrochloride and sodium ethylate in ethanol, forming 2-methyl-4-hydroxy-5-ethoxymethylpyrimidine. The yield, as quoted by these authors, is extremely poor, only 3.5%. If the process has been used for technical purposes, it must have been radically improved.

By treatment with phosphorus oxychloride the hydroxyl group in the 4 position is replaced by chlorine (yield 70%), which is exchanged with ethanolic ammonia under pressure by the amino groups (yield 70%) finally the ethoxy group in the 7 position is replaced by bromine (hydrogen bromide in glacial acetic acid, yield 90%). Starting from acetonitrile and ethyl acrylate, this synthesis of the pyrimidine moiety requires eight steps. With the exception of formylation and ring closure the yields are good and the reactions show no special difficulty.

When Todd and Bergel\textsuperscript{19} reacted free acetamidine with ethyl ethoxy-methylene cyanoacetate, they obtained colorless needles of ethyl \( \alpha \)-cyano-\( \beta \)-acetamidinoacrylate (yield 46%), and all efforts to close the ring directly at various temperatures and various amounts of sodium ethylate gave no satisfactory results. Treatment of the intermediate by boiling with aqueous 2.5% sodium hydroxide closed the ring (yield 37%), giving a total yield for the ring closure of only 17%.

By replacement of the hydroxyl group with chlorine (60 to 70% yield) and the amino group, 2-methyl-4-amino-5-cyanopyrimidine was obtained with a yield of 40%. Finally the cyano compound was catalytically re-

\textsuperscript{24} B. C. Hesse, \textit{Am. J. Chem.} \textbf{18}, 723 (1896).
duced to 2-methyl-4-amino-5-aminomethylpyrimidine, the last step in this group for their synthesis.

Not satisfied with the results, Todd and Bergel found a much better ring closure with ethyl ethoxymethylene malonate and acetamidine in presence of 1 mole of sodium ethylate; the ring is closed with a yield of 60%.

\[
\begin{align*}
\text{NH}_2 & \quad \text{COOEt} & \quad \text{N}=\text{C}=\text{OH} \\
\text{CH}_3=\text{C} & + \quad \text{C}=\text{COOEt} \rightarrow \quad \text{CH}_3=\text{C} & \quad \text{C}=\text{COOEt} \\
\text{NH} & \quad \text{CHOEt} & \quad \text{N}=\text{CH}
\end{align*}
\]

After the replacement of the hydroxyl group by chlorine (yield 65%), the 4-chloro compound is treated with concentrated aqueous ammonia at room temperature.

The yield of amination is 65%, and the following dehydration of the amide to the nitrile yields 50%. The hydrogenation to the diamine is done as above.

Starting from acetonitrile, ethyl cyanoacetate, or malonate and ethyl orthoformate, both methods of Todd and Bergel have eight steps. The yields of the second method were promising, but both methods were abandoned when still better ways became available.

Andersag and Westphal's procedure is interesting in that they could perform a Hofmann degradation in the pyrimidine series, a reaction which the American and British workers had tried without success. The condensation of acetamidine with ethyl formyl succinate in hot alcoholic solution gives ethyl 2-methyl-4-hydroxypyriramidyl[5]acetic ester, which is converted via the 4-chloro compound to 2-methyl-4-aminopyrimidyl[5]acetamide.

This amide is treated with an ice-cold aqueous solution of bromine in 2.5 \(N\) potassium hydroxide and heated, yielding 2-methyl-4-amino-5-aminomethylpyrimidine in a nearly quantitative yield. The diamide dihydrochloride is then treated with a hot solution of sodium nitrite to remove the amino group in the 7 position, and the resulting 2-methyl-4-amino-5-hydroxymethylpyrimidine is converted with hydrogen bromide in acetic acid to 2-methyl-4-amino-5-bromomethylpyrimidine dihydrobromide. As with the exception of the Hofmann degradation no yields are given for the nine different steps, it is not possible to evaluate the method. It seems that the I. G. Farbenindustrie has not used it technically.

b. Direct Introduction of the Amino Group in the 4 Position

If ethyl \(\alpha\)-cyano-\(\beta\)-acetamidinoacrylate (Todd and Bergel) is not treated with alkali but heated in water (with or without a slight amount of acid), the ring closes in a different way, the carbon atom of the cyano group entering the ring and leaving the carbothoxy group untouched.
2-Methyl-4-aminopyrimidyl[5]carbonic acid is formed\textsuperscript{35} with a yield of 65 to 75\%. By transformation of the carbethoxy group to the amide and cyano group the way to the diamine is opened. It is remarkable that the neutral or acid ring closure goes so well. The drawback of the method is the low yield in the formation of the acrylic acid (46\%, see above).

The best manner of the ring closure with direct introduction of the amino group was first described by Grewe.\textsuperscript{30} When the alcoholic solutions of free acetamidino and ethoxymethylene malonitrile are united, the mixture solidifies at once under formation of the crystalline cyano compound.

\[
\begin{align*}
&\text{NH}_2 & \text{CN} & N=\text{C}-\text{NH}_2 \\
&\text{CH}_3-\text{C} & + & \text{C-CN} \rightarrow & \text{CH}_3-\text{C} & \text{C-CN} \\
&\text{NH} & \text{CHOEt} & N-\text{CH}
\end{align*}
\]

Grewe himself gives no yield; in the laboratories of Elberfeld\textsuperscript{36} a yield of 69\% was obtained.

Ethoxymethylene malonitrile can be quantitatively converted to aminomethylene malonitrile;\textsuperscript{31} the condensation with acetamino ethyl ether gives the best yield for the pyrimidine ring closure reported in literature,\textsuperscript{23}

\[
\begin{align*}
&\text{NH} & \text{CN} & N=\text{C}-\text{NH}_2 \\
&\text{CH}_3-\text{C} & + & \text{C-CN} \rightarrow & \text{CH}_3-\text{C} & \text{C-CN} + \text{EtOH} \\
&\text{OEt} & \text{H}_2\text{N}-\text{CH} & N-\text{CH}
\end{align*}
\]

namely 88\%. The resulting 2-methyl-4-amino-5-cyanopyrimidine was catalytically hydrogenated by Grewe\textsuperscript{36} with palladium charcoal as a catalyst and glacial acetic acid with dry hydrogen chloride as a solvent. No yield is given by Grewe, but it can be assumed from similar hydrogenations of nitriles that it is very good.

Glacial acetic acid in the presence of hydrogen chloride is not a very pleasant medium for high-pressure hydrogenation as it corrodes even normally acid-resistant metals considerably. The difficulty can be overcome by specially constructed autoclaves.

4. Group C, Five Carbon Atoms for the Thiazole Ring

The possibilities for the synthesis of the thiazole ring are nearly as numerous as those of the pyrimidine ring, and the different groups in the three countries together with industrial laboratories have developed more than half a dozen ways for the five carbon atoms.

\textsuperscript{35} Chinoin, Budapest, British Pat. 538,713 (August 14, 1941).
The normal way of building up a thiazole ring starts with α-halogenocarbonyl compounds, and all practical syntheses for the 4-methyl-5-β-hydroxyethyl thiazole use the pentanone chain, where X is a halogen, and R

\[
\text{CH}_3-\text{CO}-\text{CHX}-\text{CH}_3-\text{CH}_2\text{R}
\]
is a hydroxyl, ethoxyl, O-acyl, or halogen. As the final thiazole derivative requires a free β-hydroxyethyl group and the exchange of an ethoxyl by hydroxyl is not too easy, it is obvious that compounds with the free hydroxyl or O-acyl are superior; the use of γ-aceto-γ-chloropropylethyl ether as described by Clarke and Gurin\(^{37}\) as early as 1935 is therefore out of the question for technical purposes.

If γ-acetopropylacetate is brominated in ether, bromine enters mainly in the γ-position and γ-aceto-γ-bromopropylethyl acetate is obtained (Andersag and Westphal\(^{38}\)). The bromination can be done under very mild conditions,

\[
\text{CH}_3-\text{CO}-\text{CHBr}-\text{CH}_2-\text{CH}_2\text{OCOCH}_3
\]
i.e., in glacial acetic acid with a mixture of bromine and pyridine hydrochloride.\(^{39}\) Whatever the conditions of direct halogenation with chlorine or bromine, the possibility of substitution on other carbon atoms of the chain is given and any purification of these mixtures of halogenated ketones is difficult.

If a second carbonyl is present at carbon atom 3, however, the chlorination can be performed with sulfuryl chloride and only substitution in 3 position takes place. Buchman\(^{39}\) has shown that α-acetobutyrolactone can be chlorinated with sulfuryl chloride to α-chloro-α-acetobutyrolactone with a very good yield (83%). When this lactone was decarboxylated by boiling with concentrated hydrochloric acid, Buchman expected γ-chloro-γ-acetopropyl alcohol and reported a yield of 73%. As Stevens and Stein\(^{40}\) have shown, only a small part of the reaction product is the free alcohol; by far the larger part forms an ether from 2 moles of the expected alcohol by the elimination of 1 mole of water. The yield was about 13% alcohol and 62% 

\[
\text{OCH}_2-\text{CH}_2-\text{CHCl}-\text{CO}-\text{CH}_3
\]

\[
\text{CH}_3-\text{C}-\text{CHCl}-\text{CH}_2-\text{CH}_2-\text{O}
\]

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\(^{38}\) Roche Products, F. Bergel, and A. Cohen, British Pat. 554,428 (July 2, 1943); Roche Products and F. Bergel, British Pat. 550,197 (December 29, 1942).


ether, totaling 75%. Both compounds can be used for the synthesis of the thiazole, but the chlorine in the free alcohol is much more reactive than the chlorine in the ether. With the bromo compound, the alcohol is even less stable in its free form and converts spontaneously to its ether. The same authors found that a boiling solution of 1% HCl is more satisfactory than the concentrated acid.

If the saponification and decarboxylation are done with hydrochloric acid in glacial acetic acid with just the calculated amount of water and acetic anhydride is added after the decarboxylation, excellent yields of γ-chloro-γ-acetopropylacetate are obtained (93 to 95%, Low and Smith). The use of the acetate was already proposed by Todd *et al.*, but the low yield in the first step of their process (sodium ethyl acetoacetate and β-bromoethyl acetate, 25%) was not encouraging.

The saponification of α-chloro-α-acetobutyrolactone with sulfuric acid in alcohol at 40 to 50° leads to the formation of a ring ketal, namely 2-methyl-2-ethoxy-3-chlorotetrahydrofuran in a yield of 70% (Klingenfuss). These ketals do not react with ketone reagents (semicarbazide), but their chlorine is reactive and they can be used for the synthesis of the thiazole ring.

The starting material for Buchman’s method, α-aceto-γ-butyrolactone, is easily obtainable from sodium ethyl acetoacetate in alcohol and ethylene oxide, with a yield of 60%. Both raw materials, ethyl acetoacetate and ethylene oxide, are commercial items. Starting from them, only three steps are required to the necessary 3-halogenopentanolone; the yields of these steps are good to excellent.

5. GROUP D, NITROGEN, CARBON ATOM 2, AND SULFUR

a. For Closing the Thiazole Ring

Thioformamide, $\text{NH}_2$—$\text{CH}==\text{S}$, is the easiest source for the remaining three atoms of the thiazole ring. Willstätter and Wirth reacted formamide with $\text{CH}_2$—$\text{CHCl}$:

\[
\begin{align*}
\text{CH}_2 & \quad \text{CHCl} \\
\text{CH}_2 & \quad \text{C}==\text{CH}_3 \\
& \quad \text{O} \quad \text{OEt}
\end{align*}
\]

These ketals do not react with ketone reagents (semicarbazide), but their chlorine is reactive and they can be used for the synthesis of the thiazole ring.

- **41** Roche Products, J. A. Low, and R. J. Smith, British Pat. 606,026 (August 5, 1948).
- **43** M. Klingenfuss, U.S. Pat. 2,123,653 (July 12, 1938); British Pat. 496,801 (December 6, 1938).
in ethereal solution under cooling with phosphorus pentasulfide and got the crude product with a yield of 40 to 45%. As thioformamide is very unstable in crystalline state, only a concentrate is prepared for technical purposes. Gabriel improved the method a little by using larger amounts of phosphorus pentasulfide, but even then the crude product contained only 36% of sulfur (theory 52.5%). Most of the workers in the thiamine field have use the Willstätter-Gabriel method.

Todd et al. claim a superior way by reacting free dithioformic acid in ether with aqueous ammonia. A yield as high as 50% is claimed in a United States patent.

\[
\text{CHSSH + 2NH}_3 = \text{CHSNH}_2 + \text{NH}_4\text{SH}
\]

Dithioformic acid is technically available, but it is not known if the method is used for the production of thioformamide.

b. For Closing the Thiazolium Ring

If, instead of thioformamide, compounds with a substituted amino group are used, thiazolium salts are obtained instead of thiazole. The compound necessary for this kind of thiamine synthesis, 2-methyl-4-amino-5-thioformylaminomethylpyrimidine, can be obtained via formylation of 2-methyl-4-amino-5-aminomethylpyrimidine; as has been known for a long time, the 4-amino group remains untouched and only the 5-formylamino-methyl compound is formed. This in turn can be transformed to the thioformylaminomethyl by means of phosphorus pentasulfide, but the yields of this step are extremely poor.

It was one of the greatest improvements in the synthesis of thiamine when Todd et al. found a simple method for the thioformylation of amines.

Dithioformic acid is obtained as potassium salt when potassium sulfide (from potassium hydroxide and hydrogen sulfide) in alcoholic solution is reacted with chloroform. The yield in the hands of the British workers was about 45% in the form of recrystallized salt.

46 S. Gabriel, Ber. 49, 1115 (1916).
48 M. Hoffer, U.S. Pat. 2,220,243, ex. 4 (November 5, 1940).
50 S. Gabriel and J. Colman, Ber. 34, 1246 (1901); C. O. Johns, Am. Chem. J. 41, 58 (1908).
Amines of any kind react with aqueous or alcoholic solutions of dithio-
\[ \text{RNH}_2 + \text{HCSK} = \text{RNH} \cdot \text{CHS} + \text{KSH} \]
formates under very mild conditions. In the case of aniline or aminoquinoline the yield is quantitative; no yields are given for 2-methyl-4-amino-5-aminomethylpyrimidine, but by analogy it must be assumed that the yield is good. As sodium dithioformate is technically easily available with good yields, this process offers a minimum of inconvenience for part 3 of the synthesis of thiamine. The bad odor of unwanted sulfur compounds in the reaction can be overcome by suitable engineering (closed reaction vessels, centrifuges, and good ventilation of the working rooms).

6. C + D, Ring Closure to the Thiazole Ring

The ring closure between this formamide and the halogenated ketones takes place easily (e.g., mixing the two components in the presence of a small amount of alcohol and keeping the mixture for three days at room temperature), but the yields were not too good at the beginning. Buchman got 50\% 4-methyl-5-β-hydroxyethyl thiazole,\(^{39}\) about the same yield as Clarke and Gurin\(^{37}\) obtained for the 5-β-ethoxyethyl compounds. Using the pure "ether" instead of the alcohol, Stevens and Stein\(^{51}\) could increase the yield to 70\%. It can be assumed that the condensation of γ-chloro-γ-acetophenylacetate with thiophenamidate gives still better results.

There was no lack of experiments to replace the thiophenamidate by raw materials of easier accessibility. Ammonium dithiocarbamate (from ammonia and carbon disulfide) condenses with the halogenated ketone to 2-mercaptop-4-methyl-5-β-acetoxyethyl thiazole (excellent yields are claimed, but no figures are given); Spiegelberg\(^{52}\) has shown that the mercaptol group can be replaced by hydrogen when it is oxidized with 30\% hydrogen peroxide in acid solution to the sulfuric acid. Sulfur dioxide is split off and oxidized by more hydrogen peroxide to sulfuric acid. The yield is more than 75\%; in the case of 2-mercaptop-4-methyl-5-β-chloroethyl thiazole it is 87\%. Even with the relatively high price of the 30\% hydrogen peroxide, the process seems promising for technical use.

7. Linking the Two Ring Systems, Quaternization of the Thiazole to Thiazolium

Linking the two ring systems together (the final step of the thiamine synthesis) offers little difficulty, especially when the pyrimidine compound with bromine in the 7 position is used.

\(^{52}\) H. Spiegelberg, British Pat. 492,637 (September 23, 1938); U. S. Pat. 2,179,984 (November 14, 1939).
Williams et al.\textsuperscript{25} did the first reported experiment with 150 mg. of 2-methyl-5-bromomethyl-6-aminopyrimidine hydrobromide and got a yield of 45\% when they heated the bromo compound with 4-methyl-5-\(\beta\)-hydroxyethyl thiazole in the presence of 0.2 ml. of butanol for 15 minutes at 120°. Stein et al.\textsuperscript{55} got 67\% for the 2-ethyl homolog of thiamine. If light petrolatum is used as the medium and the heating is done under optimal conditions, a nearly quantitative yield may be obtained. The petrolatum is removed after the reaction with ether, and the crystalline product is ready for the conversion to the chloride hydrochloride (thiamine). Andersag and Westphal\textsuperscript{28} follow the same procedure (no solvent, 30 minutes at 120 to 130°), but no yield is given in their paper.

To replace the bromine ions by chlorine ions, Williams et al. use an aqueous suspension of silver chloride and shake the bromide solution with it for a half-hour. The ion exchange is quantitative, and no losses of thiamine occur at this step. After filtration of the silver bromide, the aqueous solution of thiamine is concentrated to a small volume and alcohol is added; thiamine crystallizes in the form of needles. After one or two crystallizations from 90\% ethanol the crystals are dried and ready for delivery. From the silver bromide the silver may be recovered by reduction with zinc dust in aqueous suspension and the excess zinc removed by diluted acetic acid. The carefully washed silver is then ready for conversion into silver nitrate and in turn to silver chloride. The bromide hydrobromide can also be precipitated with an aqueous solution of picric acid and the crystalline thiamine picrate decomposed with 10\% hydrochloric acid.\textsuperscript{54} The picric acid is removed by filtration and the small amount in solution by extraction with ether. The process does not offer any advantages which would justify the hazard of picric acid and an ether extraction.

The conversion can be avoided if 2-methyl-5-chloromethylpyrimidine hydrochloride is reacted with the thiazole. The laboratories of the I. G.


\textsuperscript{54} F. Hoffmann-La Roche and Co., Ltd., Basle, Swiss Pat. 197,717 (August 1, 1938).
Farbenindustrie\textsuperscript{36} used this process in 1943 (methyl acetamide as a solvent heating 1 hour at 115\textdegree), but the yield of pure thiamine in batches of 3 kg. was only 45\%. The use of the bromo compound for the quaternization followed by the ion exchange to the chloride hydrochloride seems to be much superior.

S. Thiазolium Ring Closure to Thiamine

The model experiments of Todd \textit{et al.}\textsuperscript{37} for the thiазolium ring closure e.g., N-phenylthioacetamide and chloroacetone (without a solvent at 80\textdegree), gave quantitative yields. But when they heated \(\gamma\)-chloro-\(\gamma\)-acetopropanol with 2-methyl-5-thioformylamine pyrimidine in dioxane (b.p. 101\textdegree), no thiamine could be obtained; heating at 140\textdegree without a solvent gave considerable resinification, darkening, and only a small yield. The results were much better when \(\gamma\)-chloro-\(\gamma\)-acetopropylacetate was used; after heating at 115 to 120\textdegree for a few minutes, the ring closed in a smooth reaction.

\[
\begin{align*}
\text{N}=\text{C} & \text{—NH}_2 & \text{CH}_3 \text{CH}_2 & \text{—CH}_2 \text{OOCOCH}_3 \\
\text{CH}_3 & \text{—C} & \text{C—CH}_2 & \text{—NHCS} & \text{+ CO—CHCl} & \rightarrow \\
\text{N—CH} &
\end{align*}
\]

thiamine monochloride + CH\textsubscript{3}COOH

At this temperature the acetyl group was saponified and thiamine (as chloride) was obtained directly. No indication regarding the yield is given. By using \(\gamma\)-bromo-\(\gamma\)-acetopropylbenzoate, Andersag and Westphal\textsuperscript{26} could lower the temperature for the ring closure to 100\textdegree, but here again figures for the yield are lacking. Roche Products (Welwyn Garden City, Herts) uses \(\gamma\)-bromo-\(\gamma\)-acetopropylacetate for their actual manufacturing process.\textsuperscript{55} The reaction between 2-methyl-2-ethoxy-3-chlorotetrahydrofurane and the thio compound, as described by Klingenhuss,\textsuperscript{56} used even milder conditions: 95 \% formic acid or 80 \% acetic acid as a solvent and reaction temperatures of 40 to 50\textdegree (10 to 20 hours). If calcium bromide was added to the reaction mixture, a yield of 54 \% of thiamine was obtained.

A similar yield (57 \%) is obtained when the thio compound is reacted with a mixture of 2-methyl-2,3-dichlorotetrahydrofurane in formic acid with pyridine; the pyridine splits off hydrogen chloride, and the intermediate reacts under ring closure. It is obvious that this process\textsuperscript{57} was developed to circumvent existing patents, but it does not offer any advantages.

\textsuperscript{36} A Factory for Vitamin B\textsubscript{1} Production, \textit{Ind. Chemist}, June 1947, 359-68.
\textsuperscript{37} M. Klingenhuss, U.S. Pat. 2,127,446 (August 16, 1938).
\textsuperscript{55} Chinoin, Budapest, \textit{British Pat.} 609,803 (October 7, 1948).
A Japanese circumvention process is even more clumsy: it starts from 2-methyl-4-amino-5-formylaminomethylpyrimidine and introduces a mercapto group into the pentanolone chain (γ,γ-diaceto-γ-mercaptopropanol).

\[(\text{CH}_3—\text{CO})_2\text{C(SH)}\text{CH}_2—\text{CH}_2\text{OH}\]

For the ring closure to thiamine (at 100 to 110° in formic acid containing hydrogen chloride) a yield of 40% is claimed.\(^{58}\)

**D. PATENT SITUATION**

As the patent laws vary from country to country, the patent situation for every new important compound shows variations in different countries. In the case of thiamine these variations are especially pronounced, as the three groups mentioned above filed their basic patent applications within a short time. Additional applications originated from the laboratories of Roche in Basle and Welwyn, the Merck laboratories at Rahway and Darmstadt, and some outsiders.

In this country the Williams-Buchman group (assignors to Research Corporation, New York) predommates and has a strong position on the basis of composition of matter claims, protecting not only the important intermediates of the pyrimidine and thiazole groups but also the halogenated pentanolone chain for the thiazole ring closure. It is interesting that the product claims for 2-methyl-4-amino-5-aminomethylpyrimidine and 4-methyl-5-β-ethoxythiazole of Williams are based not on their synthesis but on the degradation of thiamine, a procedure which has no value for building up the end product but which was essential for the knowledge of the structure and formed the basis of the subsequent synthesis of these intermediates. In Germany the I. G. Farbenindustrie has the strongest position on the basis of the patents of Andersag and Westphal which try to claim all possible ways, including such remote possibilities as the fluoro compounds as halogenated intermediates. These inventors were also successful in getting patent protection for the Todd and Bergel process in Germany, Great Britain, and the United States, as their patent application for this process was filed in Germany as early as January 29, 1936. The strong patent position of Hoffmann-La Roche is based on their claims for the thioformylation of amines and the ring closure to the thiazolium ring.

In the United States the whole field from the first patented intermediates to the end product is covered by about 35 patents. The first application for the end product of Williams and Cline was filed June 15, 1936, whereas Andersag and Westphal had filed their first German application for the same step on January 29 of the same year (but with the correct position of the alkyl groups only as of March 24, 1936 as shown by the Brit. Pat.

\(^{58}\) T. Matukawa and M. Ohta, U.S. Pat. 2,184,720 (December 26, 1939).
The priority of the German inventors under the patent convention is not contested.

The first United States patent granted for the synthesis of thiamine fell to Klingenfuss (August 16, 1938), whereas the basic patent of Williams and Cline was issued only five years later (September 7, 1943).

The circumvention patents of the outsiders did not lead to the industrial use; no thiamine produced on the basis of such processes has appeared on the market (1953).

E. COMMERCIAL FORMS AND PURITY

Thiamine is sold commercially in the form of two salts, the hydrochloride and the mononitrate. The hydrochloride is official in most of the countries; U.S. Pharmacopoeia XIV (1950, p. 621) and British Pharmacopoeia (1953, p. 42) describe the product in details and the requirements of purity. An especially pure product is sold for the preparation of ampuled solutions. The mononitrate is not hygroscopic and therefore is preferred in the food industry. Its preparation is described by a Canadian patent; for the salt itself see U.S.P. XIV, third supplement, p. 11. Its pharmaceutical behavior was investigated by Macek et al.

F. PRODUCTION AND PRICES

The United States, England, and Switzerland are the big producers of thiamine. The following figures show the importance of this vitamin in the U. S.

<table>
<thead>
<tr>
<th></th>
<th>production</th>
<th>sales</th>
<th>value</th>
<th>price per kg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1951</td>
<td>268,000</td>
<td>183,000</td>
<td>12,040,000</td>
<td>145.00</td>
</tr>
<tr>
<td>1952</td>
<td>224,000</td>
<td>145,000</td>
<td>9,574,000</td>
<td>146.00</td>
</tr>
</tbody>
</table>

The price for hydrochloride and mononitrate is the same; there is no higher price for the special product for ampules. Since December 1952, the price has remained stable at $135.00 per kg.

IV. Biochemical Systems

B. C. P. JANSEN

A. ENZYMES

Everyone who experiments on animals on thiamine-deficient diets is struck by the fact that these animals lose weight every day, while their

food intake is reduced to a low level. After they receive a small fraction of a milligram of thiamine, however, their appetite is restored at once and their weight increases enormously. This led to the supposition that the vitamin has something to do with cell metabolism. Several workers investigated the influence of thiamine on the metabolic rate of tissues.

Westenbrink\textsuperscript{1} has reviewed all the investigations published between 1920 and 1932 and has analyzed the data from fifteen publications on this subject. About half the workers give a positive, and the other half a negative, answer. However, after statistically evaluating the work, Westenbrink found that in only two of the fifteen investigations the metabolism of vitamin-rich tissues was significantly higher than in vitamin-poor ones. He himself could find no difference.\textsuperscript{2} The definite answer to this question came from the brilliant work of Peters and his school in Oxford. Peters realized that the development of opisthotonus, which is a sign of the last stages of polyneuritis in pigeons and fowls, proved that the disorder in thiamine-deficient animals affects both the peripheral and the central nervous system. In 1929 Peters and Kinnersley investigated the metabolism of polyneuritic pigeons; the only abnormality they could find was a slight increase in the amount of lactic acid in the brain of pigeons in opisthotonus. Peters also studied the oxygen uptake by minced brain from pigeons in opisthotonus in comparison with that of normal birds. The great difference from many of his predecessors was that he added lactic acid (Kinnersley and Peters\textsuperscript{3}) or in later experiments sugar or pyruvic acid to the brain (Peters and Sinclair,\textsuperscript{4} Peters and Thompson,\textsuperscript{5} Thompson,\textsuperscript{6} and Peters\textsuperscript{7}). In a long series of experiments Peters demonstrated that thiamine plays a decisive part in carbohydrate, and especially in pyruvic acid, metabolism. Peters definitely established the fact that the metabolism of brain from polyneuritic pigeons, as measured by oxygen uptake in Barcroft-Warburg tubes, was less than that of the brain of normal pigeons. Furthermore, Peters demonstrated that the addition of a solution of thiamine to minced brain from polyneuritic pigeons suspended in pyruvic acid solution increased the oxygen uptake of this mixture (catatorulin effect of thiamine). This was the first instance of a chemical substance catalyzing an organ tissue preparation (Passmore et al.\textsuperscript{8}). The work of the Oxford school was confirmed by Sherman and Elvehjem.\textsuperscript{9} Further research led to the concept that the catalysis affecting

\textsuperscript{1} H. G. K. Westenbrink, Arch. neerl. physiol. 17, 239 (1932).
\textsuperscript{2} H. G. K. Westenbrink, Arch. neerl. physiol. 17, 549 (1932).
\textsuperscript{3} H. W. Kinnersley and R. A. Peters, Biochem. J. 24, 711 (1930).
\textsuperscript{5} R. A. Peters and R. H. S. Thompson, Biochem. J. 28, 916 (1934).
\textsuperscript{6} R. H. S. Thompson, Biochem. J. 28, 909 (1934).
\textsuperscript{9} W. C. Sherman and C. A. Elvehjem, Am. J. Physiol. 117, 142 (1936).
the oxygen uptake was due not to thiamine itself but to a compound that was synthesized from the free vitamin (Peters et al.\textsuperscript{10} and Westenbrink and Polak\textsuperscript{11}). The nature of this compound became clear from another series of investigations. Neuberg and his collaborators (Neuberg and Karczag\textsuperscript{12} and Neuberg and Rosenthal\textsuperscript{13}) found an enzyme in yeast which decarboxylates pyruvic acid to acetaldehyde. They called it carboxylase. Simola\textsuperscript{14} showed that the tissues of rats on a thiamine-deficient diet had a greatly reduced content of carboxylase.

All the known thiamine-containing enzymes (holoenzymes) consist of a protein part that is usually called the apoenzyme and a coenzyme of lower molecular weight, the thiamine pyrophosphate (or sometimes thiamine tri- or polyphosphates). All these enzymes catalyze either a pure decarboxylation process or an oxidative decarboxylation reaction.

Both take part in the metabolism of pyruvic acid or, more generally speaking, in the metabolism of \(\alpha\)-oxoacarboxylic acids.

We distinguish between pure carboxylases and oxidative carboxylases, which are usually called pyruvic dehydrogenases. The carboxylases are present in yeast and other microorganism, whereas animal tissues contain pyruvodehydrogenases.

Whether the carboxylase and the pyruvodehydrogenase are identical or are different enzymes will be discussed below.

Auhagen\textsuperscript{15, 16} demonstrated that carboxylase could be rendered inactive by washing with a phosphate buffer solution at pH 7 to 8. The activity was restored by adding an extract of boiled yeast. So he assumed that yeast contains a coenzyme, which he called cocarboxylase. Lohmann and Schuster\textsuperscript{17} succeeded in isolating this cocarboxylase in a pure, crystalline state, and they proved that it is the pyrophosphoric ester of thiamine. This coenzyme + alkali-washed yeast + magnesium ions decomposes pyruvic acid according to the reaction:

\[
\text{CH}_3\text{COOCOOH} \xrightarrow{\text{carboxylase}} \text{CH}_3\text{CHO} + \text{CO}_2
\]

Peters and his pupils demonstrated that the cocarboxylase is the active form of thiamine in tissue oxidation, also (Banga et al.\textsuperscript{18, 19}).

The bond between the apo- and the cocarboxylase at neutral or slightly

\textsuperscript{12} C. Neuberg and L. Karczag, Biochem. Z. 36, 68 (1911).
\textsuperscript{13} C. Neuberg and P. Rosenthal, Biochem. Z. 51, 128 (1913).
\textsuperscript{14} P. E. Simola, Biochem. Z. 254, 229 (1932).
\textsuperscript{15} E. Auhagen, Z. physiol. Chem. 204, 149 (1931).
\textsuperscript{16} E. Auhagen, Biochem. Z. 258, 330 (1933).
\textsuperscript{17} K. Lohmann and P. Schuster, Biochem. Z. 294, 188 (1937).
acid pH is very firm. Washing with water and dialysis does not remove a trace of thiamine pyrophosphate. At pH about 8, the dissociation becomes total, so that by washing with an alkaline phosphate buffer the thiamine pyrophosphate can readily be removed. Also at pH 4.6 the thiamine pyrophosphate may be split off (Stumpf et al.\(^{20}\)). One exception, however, has been found in the enzyme that brings about the condensation of acetaldehyde (Green et al.\(^{21}\)), for the coenzyme is completely dissociated even in neutral solutions (Stumpf\(^{22}\)).

Stumpf assumes that the thiamine pyrophosphate enzymes can be classified into two general groups, depending on the relative degrees of dissociation. The first group, to which the majority of the known carboxylases belong, is not dissociated between pH 4.6 and 7.8. In the second group the enzyme is dissociated even in neutral solutions.

Ochoa and Peters (Ochoa\(^{23}\)) found that the addition of thiamine to apo-carboxylase in the form of alkali-washed yeast greatly enhanced the capacity of this apoenzyme for the production of carbon dioxide after addition of the coenzyme thiamine pyrophosphate and magnesium. The activation was maximal with doses of thiamine twenty times larger than the thiamine pyrophosphate. Instead of thiamine itself also the pyrimidine part of the molecule 2-methyl-4-aminopyrimidyl-5-methylaminodihydrochloride could be used (Ochoa and Peters\(^{24}\)). This proved that the activation is not caused by synthesis of the added thiamine to thiamine pyrophosphatase. This stimulation by thiamine of the resynthesis of thiamine pyrophosphate was much greater in baker's than in brewer's yeast (Lipton and Elvehjem\(^{25}\)). Westenbrink and his collaborators succeeded in giving an explanation for this activation (Westenbrink and van Dorp\(^{26}\) and Westenbrink et al.\(^{27}\)). They found a powerful phosphatase in yeast; this phosphatase is capable of hydrolyzing the phosphoric esters of thiamine in a very short time. The thiamine does not actually stimulate the carboxylase system but only inhibits the phosphatase that destroys the carboxylase.

When the coenzyme once is bound to the protein, thiamine pyrophosphate is resistant to this phosphatase (Westenbrink et al.\(^{28}\)). This is in ac-

\(^{22}\) P. K. Stumpf, J. Biol. Chem. 159, 529 (1945).
\(^{23}\) S. Ochoa, Nature 141, 831 (1938).
\(^{27}\) H. G. K. Westenbrink, D. A. van Dorp, M. Gruber, and H. Veldman, Enzymologia 9, 73 (1940).
cordinary with the observation of Lipmann,29 confirmed by Lipton and Elvehjem30 and by Westenbrink, that thiamine is without any effect when it is added to the yeast suspension some minutes after the addition of cocarboxylase, for then the cocarboxylase is destroyed by the phosphatase. Westenbrink et al.31 demonstrated the high enzymic activity of this phosphatase: in 10 seconds it dephosphorylated a large part of the cocarboxylase. The objection of Lipton and Elvehjem30 that phosphate, the other product of the splitting of thiamine pyrophosphate, did not inhibit the phosphatase action on cocarboxylase could be answered by the assumption that in this case there is no shift of the equilibrium according to the law of mass action, but a competitive inhibition. It appeared later that animal phosphatases are not inhibited by thiamine but by phosphate ions (Westenbrink et al.32).

The synthesis of carboxylase by bringing together alkali-washed yeast, magnesium, and thiamine pyrophosphate was thoroughly studied by Parvé.33 Even when a large amount of thiamine is added, a great deal of the thiamine pyrophosphate is hydrolyzed before it is bound to the protein and becomes resistant to the phosphatase. Therefore, only part of the thiamine pyrophosphate is resynthesized to carboxylase; at most about 25%.

In all respects, the effect of washing at pH 6.2 or 5.6, the resynthesis at pH 6.8, the dissociation at pH 7.8, and the activity per microgram of thiamine pyrophosphate left the resynthesized carboxylase equally as stable as the original holoferment. Only one difference was found. The maximum activity, measured by the production of carbon dioxide from pyruvate, is about pH 5.7 for the native carboxylase and pH 6.2 for a mixture of alkali-washed yeast + magnesium ions + thiamine pyrophosphate. This difference is easily explained, for the optimum pH for the resynthesis is 6.8. In the mixture of alkali-washed yeast + coferment, two reactions are going on simultaneously; the synthesis of the holoenzyme and the action of this enzyme on pyruvate. So pH 6.2 is a compromise between pH 5.7 and 6.8.

Lipton and Elvehjem gave another explanation for the stimulation of the formation of carboxylase by thiamine; they postulated that a substance in yeast, probably a protein other than the enzyme protein, adsorbs the cocarboxylase and in this way inhibits the formation of the holoenzyme carboxylase. The addition of excess thiamine saturates this material and thus permits the adsorption of the thiamine pyrophosphate on the apoenzyme.

Most of their experiments could be interpreted just as well by the supposi-

29 F. Lipmann, Enzymologia 7, 142 (1939).
31 H. G. K. Westenbrink, D. A. van Dorp, and M. Gruber, Rec. trav. chim. 60, 185 (1941).
### Table I
Reactions Catalyzed by Thiamine Pyrophosphate Enzymes

<table>
<thead>
<tr>
<th>Reactions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvic acid → Acetaldehyde + CO₂</td>
<td>Lohmann and Schuster,17 Kenstler et al.,35 Cajori36</td>
</tr>
<tr>
<td>Pyruvic acid + oxygen → CO₂ + H₂O</td>
<td>Peters and his school, see Peters,37 Stumpf et al.20</td>
</tr>
<tr>
<td>Pyruvic acid + oxygen → Acetic acid + CO₂</td>
<td>Lipmann,38 Still,39 Stumpf,42 Stumpf et al.20</td>
</tr>
<tr>
<td>Pyruvic acid + phosphate + oxygen → Acetylphosphate + CO₂ + H₂O</td>
<td>Lipmann40</td>
</tr>
<tr>
<td>Pyruvic acid + phosphate + H₂O → Acetylphosphate + H₂ + CO₂</td>
<td>Utter and Werkman,41 Kalnitzky and Werkman42</td>
</tr>
<tr>
<td>Pyruvic acid + phosphate → Acetylphosphate + HCOOH</td>
<td>Utter and Werkman,41 Kalnitzky and Werkman42</td>
</tr>
<tr>
<td>Pyruvic acid + CO₂ → Oxalacetic acid</td>
<td>Krebs and Eggleston,43 Smyth44</td>
</tr>
<tr>
<td>Pyruvic acid + oxygen → Citric acid</td>
<td>Sober et al.,45 Barron et al.46</td>
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<td>Pyruvic acid + oxygen → Acetolactic acid</td>
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<tr>
<td>Pyruvic acid + (O₂) → Carbohydrate</td>
<td>Mann and Quastel,48 Minz49</td>
</tr>
<tr>
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38 F. Lipmann, Enzymologia 4, 65 (1937).
tion of Westenbrink and van Dorp as by that of Lipton and Elvehjem. There was one experiment that was explained more easily by the Wisconsin than by the Amsterdam investigators: Lipton and Elvehjem demonstrated that by incubating the apoenzyme with cocarboxylase at 30° the recovery of carboxylase gradually increased as the incubation period increased. However, Westenbrink and his collaborators could not confirm these results with the kind of yeast they had at their disposal.

Buchman et al. found a competitive inhibition of thiamine pyrophosphate by the thiazole pyrophosphate portion of the cocarboxylase molecule. Therefore they assumed that the thiamine pyrophosphate is bound to the apoenzyme through the pyrophosphate group which is common to both the cocarboxylase and the inhibitor.

We know a great many reactions that are catalyzed by thiamine-containing enzymes; they are listed in Table I. Little is known as yet of the question whether all these reactions are catalyzed by different enzymes (containing different apoenzymes).

These enzymes have not yet been obtained in a pure, crystalline state. All are protein-thiamine pyrophosphate-magnesium compounds. Fairly pure preparations have been made from yeast. The composition of both preparations did not differ very much; per mole of thiamine pyrophosphate they contained 1 g. of atom of magnesium and about 75,000 g. (1 mole?) of protein.

As we have not at our disposition pure, crystalline enzyme preparations, the activity has to be related to one mole of the coenzyme, the thiamine pyrophosphate. Peters and his collaborators stated that the activity of the carboxylase in yeast and that of the pyruvodehydrogenase in brain dispersions were of the same order. The pyruvodehydrogenase at 30° catalyzes the uptake of 1500 moles of O₂ per minute, computed per mole of thiamine pyrophosphate.

Some years later, Green et al.,\(^\text{51}\) and almost simultaneously Kubowitz and Lüttgens,\(^\text{52}\) using a fairly purified enzyme preparation from yeast, found that this carboxylase preparation catalyzes the decarboxylation of 900 and 700 moles of pyruvic acid at 30° and 20°, respectively, per minute per mole of thiamine pyrophosphate.

On the whole, the purity of the pyruvodehydrogenases from animal tissues is less. It is difficult to obtain a solution free from cell particles. Therefore we do not know whether the oxidative carboxylations are brought about by enzymes that contain only thiamine pyrophosphate + magnesium as the coenzyme part, or whether these enzymes contain also another oxidative coenzyme.

Green et al.\(^\text{53}\) and Stumpf et al.\(^\text{22}\) obtained pyruvodehydrogenase preparations that were free from flavin adenine dinucleotide and from cytochrome c.

Stumpf et al. could demonstrate that pyruvic and α-ketoglutaric oxidases from pigeon breast muscle were different enzymes. The most cogent evidence came from summation experiments: by adding α-ketoglutaric acid to a pyruvodehydrogenase preparation that was saturated with pyruvic acid, an extra carbon dioxide production was obtained.

Table I indicates that many different reactions are catalyzed by thiamine pyrophosphate enzymes and that most of them attack pyruvic acid or other α-keto fatty acids.

It may be remarked that pyruvic acid stands at the crossroads of the carbohydrate metabolism; it is the end point of the anaerobic processes, and the starting point of the decarboxylation and oxidation reactions and of the reversal of the anaerobic chain of reactions. This attack on pyruvic acid may be a simple decarboxylation, an oxidative decarboxylation, a carbon dioxide fixation, a dismutation, or a condensation. We may assume that the exact type of reaction depends upon the protein, the apoenzyme, with which the thiamine pyrophosphate is combined.

In the experiments with tissues or crude extracts, the enzyme system contained several other enzymes and catalytic substances, such as the C₄ dicarboxylic acids, besides the carboxylase. In their initial work Long and Peters\(^\text{56}\) concluded that there is a distinct difference between the systems in yeast and in the animal tissue. The yeast system decarboxylates only the pyruvic acid, whereas in the tissue oxidation accompanies decarboxyla-
tion. Animal tissues contain a pyruvic acid dehydrogenase. From these results they inferred that the apoenzymes are different in yeast and in animal tissues.

Thus, thiamine pyrophosphate can act in yeast as the coferment of pure carboxylase, whereas in animal tissues and in several bacteria it acts as the coferment of a pyruvic dehydrogenase. Watt and Krampitz,\(^59\) using C\(^{30}\)O\(_2\) with isotopic C\(^{33}\) as a tracer, were able to demonstrate that the transformation of pyruvic acid into \(\alpha\)-acetolactic acid + CO\(_2\) is a reversible reaction.

1. The Unitarian View

Coenzymes commonly catalyze different reactions, depending upon the nature of the apoenzyme. However, the difficulty with thiamine pyrophosphate is that the two kinds of reactions are so widely different from a chemical point of view—one is oxidative and the other is non-oxidative. Also, none of the mechanisms proposed for the oxidative action of thiamine pyrophosphate has proved to be satisfactory (see below). Krebs drew attention to this difficulty, and he was the first who advanced an attractive unitarian theory, bringing all the different activities of thiamine under one single denominator. Evans,\(^60\) working in Krebs' laboratory, had shown that minced pigeon liver was able to oxidize pyruvate to \(\alpha\)-ketoglutarate. From experiments to elucidate the mechanism of this reaction, Krebs and Eggleston\(^43\) were led to the assumption that the primary step is an assimilation of carbon dioxide to pyruvic acid, a Wood and Werkman reaction.\(^61\), \(^62\)

\[
\text{CO}_2 + \text{CH}_3\text{COO}^- \rightarrow \text{COOHCH}_2\text{COO}^-
\]

The oxalacetate formed in this way is converted into citrate and \(\alpha\)-ketoglutarate according to Krebs' citric acid cycle. Krebs and Eggleston assumed that the first of this cycle of reactions, the formation of oxalacetate, (the reverse of "decarboxylation") is catalyzed by thiamine pyrophosphate. In this way, the vitamin is not directly concerned with the oxidation of pyruvate but with a reaction preliminary to the oxidation; the oxalacetate acts as a hydrogen carrier. However, the experimental evidence for this theory was not very convincing. Their chief argument was based on the demonstration with suspensions of pigeon liver that pyruvate utilization was greatly increased by suspending it in bicarbonate solution instead of in phosphate buffer and by adding carbon dioxide to the gas phase, whereas carbon dioxide and bicarbonate have no general effect on processes in liver suspensions.

\(^60\) E. A. Evans, Jr., *Biochem. J.* 34, 829 (1940).
Krebs also demonstrated that acetoacetate formation is somewhat reduced in livers of thiamine-deficient pigeons and that addition of the vitamin restored this formation.

Furthermore Smyth, as a collaborator of Krebs, prepared cultures of *Staphylococcus aureus* or *S. albus* that were deficient in thiamine. The pyruvate metabolism of these microorganisms was much lower than that of vitamin-saturated cells. Added thiamine greatly enhanced this pyruvate metabolism. In this respect thiamine could be replaced by oxalacetate. This effect could not be obtained in *Staphylococci* grown in vitamin-sufficient media. So these experiments are in accordance with the assumptions that thiamine catalyzes the formation of oxalacetate and that this substance acts as a hydrogen carrier in the dismutation of pyruvate. At the end of his article Krebs drew attention to the work of Ruben and Kamen, who were able to demonstrate, with the aid of radioactive carbon, that animal tissues were able to assimilate carbon dioxide. In the following years several investigators firmly established the fact of the assimilation of carbon dioxide by animal tissues (e.g., Evans and Slotin, Solomon et al., and Utter and Wood). The role of thiamine pyrophosphate in this process is not definitely proved, however, and there are several experimental data which cannot be brought into line with this unitarian view of Krebs. Barron et al., in contradiction to the results of Smyth with bacteria, stated that the condensation reactions of pyruvate in tissues of avitaminotic animals were accelerated by thiamine but not by oxalacetate. The results of Green with the purified carboxylase preparations are not easily reconciled with the Krebs' theory.

2. COUPLING OF PYRUVIC ACID OXIDATION WITH PHOSPHORYLATION

The coupling of oxidations with phosphorylations with high-energy and low-energy phosphate bonds cuts the flow of energy of the oxidation into fractions (Lipmann). In this way it might be thought that the difference between oxidizing and non-oxidizing enzymes becomes less important. Lipmann and studied the oxidation of pyruvate by an extract from *Bacillus delbrückii* (*Bact. acidificans longissimum*). The pyruvic acid is oxidized to acetic acid and carbon dioxide.

\[
\text{CH}_3\text{COCOOH} + \frac{1}{2}\text{O}_2 \rightarrow \text{CH}_3\text{COOH} + \text{CO}_2
\]

The reaction requires inorganic phosphate and adenylic acid. The inorganic

---

phosphate disappears during the process, and an equivalent amount of adenosine polyphosphate is formed. Thus the oxidation generates an energy-rich phosphate bond. The primary oxidation product of the pyruvic acid behaves like acetyl phosphate. Synthetic acetyl phosphate is able to transfer its high-energy phosphate bond to adenylic acid. From these facts Lipmann formulated the oxidation process.

\[ \text{CH}_3\text{COCOOH} + \text{H}_2\text{PO}_4 + \text{O}_2 \rightarrow \text{CH}_3\text{COOPO}_2\text{H}_2 + \text{CO}_2 + \text{H}_2\text{O}_2 \]

Afterwards Lipmann\(^{46}\) calculated the bond energy of acetyl phosphate. As it is some 3 kcal. higher than the average energy of 12 kcal. of the energy-rich bond, it will amount to around 15 kcal. The need of inorganic phosphate for pyruvate oxidation in brain also was demonstrated (Banga et al.\(^{18,19}\)). However, Ochoa et al.\(^{68}\) could find no indication that acetyl phosphate is an intermediate in the oxidation of pyruvic acid by brain or by muscle. Thus there are many carboxylases that require inorganic phosphate for their action. However, there are exceptions to this rule: Stumpf\(^{29}\) studied the pyruvic acid oxidation by *Proteus vulgaris*, which contains an enzyme that, in the presence of thiamine pyrophosphate and magnesium, specifically catalyzes the oxidation decarboxylation of pyruvic acid to acetic acid and carbon dioxide with liberation of considerable free energy:

\[ \text{CH}_3\text{COCOOH} + \frac{1}{2}\text{O}_2 \rightarrow \text{CH}_3\text{COOH} + \text{CO}_2 \]

No disappearance of pyruvic acid could be observed under anaerobic conditions and no carbon dioxide was produced. This enzyme from *Proteus vulgaris* is equally active in the absence, as in the presence, of inorganic phosphate. In several ways Stumpf could demonstrate that the assumption that trace amounts of phosphate should react with pyruvic acid to form acetyl phosphate, which could then act as a catalyst, was not in accordance with the facts. Similarly the pyruvic oxidase of *Escherichia coli* (Still\(^{39}\)) does not require the presence of inorganic phosphate. Ochoa\(^{69}\) thoroughly studied the coupling of phosphorylation with oxidation of pyruvic acid in the brain and the oxidation of the \(\alpha\)-ketoglutaric acid\(^{70}\) by a preparation from the heart of the cat. He confirms the observation that inorganic phosphate and adenylic acid or adenine triphosphate are required for the oxidation. The rate of oxygen uptake by the dehydrogenase from cat heart depended upon the concentration of inorganic phosphate. However, it is not certain that this preparation contained a thiamine pyrophosphate enzyme.

Kalnitzky and Werkman,\(^{42}\) working with extracts of *Escherichia coli*, found that inorganic phosphate was essential for the activity of its pyruvic

---


acid hydrogenase. Utter and Werkman\(^4\) showed that the apparently "hydroelastic" action of this extract

\[
\ce{CH_3COCOOH + H_2O -> CH_3COOH + HCOOH}
\]

was really a phosphoroclastic" split. Utter et al.\(^7\) with the aid of C\(^{13}\) as a tracer demonstrated that this reaction

\[
\ce{CH_3COCOOH + H_3PO_4 <=> CH_3COO(PO_3H_2) + HCOOH}
\]

is reversible. This reversibility shows that a thiamine pyrophosphate enzyme can catalyze a synthesis, as well as a breakdown, of pyruvate.

Acetic acid is formed by the dephosphorylation of acetyl phosphate. The acetyl phosphate that is formed by the oxidative decarboxylation of pyruvic acid by the action of thiamine pyrophosphate is a higher reactive compound. Lipmann was able to prepare acetyl phosphate synthetically. However this product was inactive. So the enzymatic intermediate must have a somewhat different structure.

Most of the thiamine-enzyme preparations from animal sources are easily sedimented, e.g., by centrifuging 30 minutes at 15,000 or 20,000 r.p.m. (Ochoa\(^7\) and Green et al.\(^8\)). Some of the enzyme preparations from bacteria seem to be more resistant to centrifugal force. Thus Silverman and Werkman\(^5\) state that the carboxylase from *Aerobacter aerogenes* remained in the supernatant solution after centrifuging for 1 hour at 250,000 to 300,000 r.p.m. With a preparation from *Proteus vulgaris*, Stumpf\(^2\) found that centrifugation for 1 hour in a gravitational field of 9700g sedimented only a small part of enzyme. However, centrifugation for 1 hour in a gravitational field of 100,000g sedimented all active material. There seems to be a difference between the different enzymes.

3. Inhibitors and Activators

Most of the preparations show no inhibition by 3.3 to 10 \(\times\) 10\(^{-3}\) \(M\) iodoacetate, fluoroacetate, fluoride, or malonate (Green et al.\(^5\)\(^1\) Green et al.\(^7\)\(^1\) Stumpf\(^2\) and Stumpf et al.\(^8\)\(^1\)). Some preparations are more or less inhibited by iodoacetate and fluoride. Thus the enzyme from *Aerobacter aerogenes* is inhibited 85% by 7 \(\times\) 10\(^{-3}\) \(M\) iodoacetate, and 36% by 5 \(\times\) 10\(^{-3}\) \(M\) fluoride (Silverman and Werkman\(^5\)\(^9\)), and the diacetyl mutase from pigeon breast muscle was inhibited 39% by 3.3 \(\times\) 10\(^{-3}\) \(M\) iodoacetate (Green et al.\(^8\)\(^1\)).

Salts of heavy metals were found to be strong inhibitors for the carboxylase from yeast (Green et al.\(^5\)\(^1\)). A \(M/10,000\) concentration of AgNO\(_3\),

CuSO₄, or Hg(NO₃) gave complete inhibition, but M/10,000 ZnSO₄ showed no inhibition at all.

Several other inhibitors are described for carboxylases. Cajori found inhibition for carboxylase from yeast by hemin; the enzyme was activated by cysteine. Kensler et al. showed inhibition of yeast carboxylase by the split products of N,N-dimethylaminobenzene; they confirmed the stimulation by cysteine.

Stumpf, working with a pyruvic oxidase from Proteus vulgaris, found that inorganic pyrophosphate in 3.4 × 10⁻³ M concentration inhibits the enzyme activity; this inhibition is reversed by 0.7 × 10⁻³ M thiamine pyrophosphate. He ascribed this inhibition by inorganic pyrophosphate to its reversible union with the magnesium-protein complex, thus blocking a similar union with thiamine pyrophosphate. Carboxylase activators were demonstrated by Greenberg and Rinehardt. They found that cysteine, NaHSO₄, and phenylhydrazine were able to activate the thiamine pyrophosphate enzymes. Tauber found that sodium cyanide is a good activator. All these compounds combine with aldehyde and ketone groups.

B. COENZYMES

Until recently only one thiamine-containing coenzyme was known, i.e., thiamine pyrophosphate. Thiamine itself and thiamine monophosphate are without any coenzyme activity.

Several years ago, some French authors prepared thiamine triphosphate and thiamine polyphosphates.

In thiamine pyrophosphate the pyrophosphoric acid is attached to the thiazole group of the thiamine molecule. Thus the formula of pyrophosphate is

\[
\text{N—CH—Cl—CH₃} \\
\text{CH₃—C—CH₂—N—C} \\
\text{N=C} \\
\text{II₂N—HC—S} \\
\text{O—O—O} \\
\text{OH—OH}
\]

Roux et al. assumed that in thiamine triphosphate the third phosphate group is attached to the NH₂ of the pyrimidine. Thus it has the following formula.

In the polyphosphates still more phosphate groups are attached to the pyrimidine nucleus. The name cocarboxylase has been reserved for the thiamine pyrophosphate.

Cocarboxylase can be synthesized from thiamine chemically or enzymically. However, because carboxylase is not dissociated (see below), the enzymic synthesis stops when the apoenzyme is saturated. No preparative use can therefore be made of this reaction.

1. Chemical Procedure

Lohmann and Schuster\(^{17}\) described a method for preparing the cocarboxylase. Weyland and Tauber\(^{74}\) gave an extensive description for the preparation of thiamine pyrophosphate which substance Weil-Malherbe\(^{75}\) synthesized by treating the thiamine bromide with silver pyrophosphate. Karrer and Viscontini\(^{76}\) improved the method of Weyland and Tauber. The strong hydrochloric acid that is present during the procedure of Weyland and Tauber\(^{74}\) tended to hydrolyze a large part of the thiamine pyrophosphate to monophosphothiamine. Therefore Karrer and Viscontini\(^{76}\) improved the method by preparing the phosphate of the cocarboxylase. In this way they reduced the acidity of the solution and so obtained a yield that was 55% of the calculated one.

2. Enzymic Synthesis

Yeast, bacteria, and animal tissues contain systems that are able to phosphorylate thiamine. The first reports (von Euler and Vestin\(^{77}\); and Lohmann and Schuster\(^{17}\)) needed confirmation because thiamine strongly stimulates cocarboxylase activity (see Section IV A, p. 425). Ochoa and Peters\(^{74}\) and Ochoa et al.\(^{68}\) studied the phosphorylation process in animal tissues. They found that liver shows a particularly good phosphorylating capacity. Slices, brei, and "dispersions" were equally active. Brain and muscle were much less active; preparations from duodenal mucosa (pig)

showed no activity at all. Ochoa thinks that the reaction

\[
\text{Thiamine + 2 phosphate } \rightarrow \text{ Thiamine pyrophosphate}
\]

is reversible. Oxygen is essential for a reaction to the right. The optimum pH is about 8.5. Goodhart and Sinclair,\(^75\) working with blood from avitaminous pigeons, demonstrated that the nucleated blood cells can phosphorylate thiamine.

D. Siliprandi and N. Siliprandi\(^78a\) demonstrated that in rats and also in human beings\(^78b\) insulin is essential in the conversion of thiamine to cocarboxylase.

A cell-free protein preparation containing the apocarboxylase has been made from yeast (Weil-Malherbe\(^79\)\(^9\)). He was able to demonstrate that phosphorylation of thiamine occurs only in the presence of adenylpyrophosphoric acid or such reactions as entail its intermediate formation.

Leuthardt and Nielsen\(^79a\) succeeded in preparing a purified apoenzyme for the transformation of thiamine into cocarboxylase. This apoenzyme needs the addition of adenosinetriphosphate and magnesium ions. The optimal pH of the purified enzyme was found to be between pH 6.8 and pH 6.9.

Phosphopyruvic acid can act as a phosphate donor for the synthesis of cocarboxylase in the presence of catalytic amounts of adenylic acid or adenylpyrophosphoric acid. Thus a direct transfer of phosphate from adenyl-pyrophosphoric acid is the mechanism for phosphorylating thiamine. These results were confirmed by Lipton and Elvehjem\(^80\) and by Quastel and Webley.\(^55\) The synthesis does not take place via the monophosphate (Weil-Malherbe\(^79\)\(^9\)), for the production of thiamine pyrophosphate goes faster from thiamine itself than from its monophosphate. The synthesis of cocarboxylase is almost completely inhibited by 0.005 \(M\) iodoacetic acid, and only slightly inhibited by 0.04 \(M\) sodium fluoride (Lipton and Elvehjem\(^81\)).

Westenbrink and his collaborators studied the synthesis of cocarboxylase by living yeast cells (Westenbrink \textit{et al.}\(^82\)). By adding a large excess of thiamine to the yeast, much more thiamine pyrophosphate is synthesized than can be bound to the apocarboxylase present in the yeast; it is probably


bound to some other yeast protein. Contrary to the supposition of Sperber,\textsuperscript{83} Westenbrink \textit{et al.}\textsuperscript{82} proved that the synthesis of carboxylase by living yeast cells is not a simple reversion of the action of the phosphatase, the enzyme that decomposes the coenzyme.

As we take into account the tremendous activity of the newly discovered cobalamin (vitamin $B_12$), we must not exclude the possibility that the carboxylase preparations of animal cells contain a small quantity of a powerful oxidizing coenzyme other than thiamine pyrophosphate.

At the International Biochemical Congress in Paris in July, 1952, there was held a symposium on the tricarboxylic acid cycle (the citric acid cycle).\textsuperscript{84} Most of the participants in this meeting considered it established that the oxidation of pyruvic acid is performed by a thiamine pyrophosphate-containing protein with other coenzyme(s) as the oxidizing agent(s). The oxidizing agent might be the protogen\textsuperscript{85} or $\alpha$-lipoic acid that was isolated by Reed \textit{et al.}\textsuperscript{86} in crystalline form. Green\textsuperscript{87} states that the pyruvic oxidase and other $\alpha$-keto fatty acid oxidases require at least four prosthetic groups to catalyze the oxidative decarboxylation of their substrates: (1) thiamine pyrophosphate, (2) magnesium ions, (3) pyridine nucleotide, and (4) coenzyme A.

So the decarboxylation and the oxidation of pyruvic acid are separate steps in the oxidation of pyruvate. Only the first step, the decarboxylation, is catalyzed by the thiamine pyrophosphate as a coenzyme. The product of decarboxylation seems to be not free acetaldehyde but an enzyme- or coenzyme-bound acetaldehyde derivative which, upon transfer of the acetaldehyde-group to coenzyme A (see pantothenic acid page) undergoes oxidation as an acetaldehyde-CoA compound. (cf. Ochoa, p. 81 in ref. 84).

Langenbeck\textsuperscript{88} made model experiments on enzyme reactions. Referring to these experiments, Weil-Malherbe\textsuperscript{89} thinks that a Schiff base is first formed and that this base undergoes an intramolecular oxidation-reduction process. No experimental evidence was given for this view. On the contrary Stern and Melnick\textsuperscript{90} presented experimental data against the Langenbeck cycle.

Lipmann\textsuperscript{91} reduced thiamine with platinum black and $H_2$ or by $Na_2S_2O_4$. He assumed that a dehydro derivative might be formed which forms a redox

\textsuperscript{83} E. Sperber, \textit{Naturwissenschaften} \textbf{29}, 765 (1941).
\textsuperscript{84} Symposium on the tricarboxylic acid cycle held at the Second International Congress of Biochemistry, Paris, 1952.
\textsuperscript{86} L. J. Reed, \textit{Science} \textbf{114}, 93 (1951).
\textsuperscript{87} D. E. Green, \textit{Science} \textbf{115}, 661 (1952).
\textsuperscript{88} W. Langenbeck, \textit{Ergeb. Enzymforsch.} \textbf{2}, 314 (1933).
\textsuperscript{91} P. Lipmann, \textit{Nature} \textbf{138}, 1097 (1936).
system with the vitamin. In 1938, however, Lipmann and Perlman\textsuperscript{92} showed that the activity of thiamine was lost after dehydrogenation. Stern and Melnick\textsuperscript{90} pointed out that the sulfite, formed in the reaction of thiamine with sodium dithionite, must split the vitamin molecule (Williams \textit{et al.}\textsuperscript{93}). This was proved by Karrer \textit{et al.}\textsuperscript{94} and Karrer and Viscontini.\textsuperscript{76} Furthermore, Stern and Melnick\textsuperscript{90} showed that reduction of thiamine with activated hydrogen gave a product that was not autoxidizable and was biologically inactive when tested on polyneuritic pigeons. Cautious reduction of thiamine pyrophosphate with activated hydrogen, however, gave a dihydro-cocarboxylase that also was not autoxidizable but that was active in curing polyneuritic pigeons.

On the other hand Zima and Williams\textsuperscript{95} and Zima \textit{et al.}\textsuperscript{96} oxidized the sodium salt of thiamine with iodine and obtained a product which they assumed was formed from two molecules of thiamine, in which the S—H of the thiazole half of the molecule is transformed to S—S. Thus, it appeared that the thiamine/thiamine disulfide should form a reduction-oxidation system just as does cysteine/cystine or glutathione and its disulfide.

Karrer and Viscontini,\textsuperscript{76, 97} however, chemically synthesized the thiamine disulfide pyrophosphate. This was combined with the apoenzyme, the alkali-washed yeast. The product was inactive with pyruvic acid. Therefore the disulfide form of cocarboxylase is not the active coenzyme. Nevertheless, the disulfide may be active in the animal organism, but only by preliminary reduction to thiamine itself. Similarly, Peters\textsuperscript{98} found that the disulfide is active only after reduction. But the fact that \textit{in vitro} tissue preparations are able to reduce the disulfide appears, in Peters' opinion, to leave room for the suggestion of Williams and Zima that the disulfide may play a part in the dehydrogenation.

Barron and Lyman\textsuperscript{16} showed that the thiamine pyrophosphate was more resistant to oxidation and reduction than thiamine itself. They concluded that the action of thiamine pyrophosphate as a component of enzyme systems does not involve reversible oxidation and reduction. They were led to postulate that the phosphorylated thiamine acts as the integral part of the activating protein. After summarizing all the work that has been performed until now, we must conclude that much more experimental work

\textsuperscript{95} O. Zima, and R. R. Williams, \textit{Ber.} \textbf{73}, 941 (1940).
is necessary, preferably with pure pyruvodehydrogenase preparations, to establish the mechanism for the oxidative activity of thiamine pyrophosphate.

V. Specificity of Action

B. C. P. Jansen

A. THE ESSENTIAL METALS

Most of the carboxylases are thiamine pyrophosphate-magnesium-protein compounds (Ochoa\(^1\)). Magnesium may be replaced by manganese. Green et al.\(^2\) using a preparation from top brewer’s yeast, made an elaborate study of the replacement of magnesium by other metals. All the divalent metals tested could replace magnesium. The trivalent form of iron and the trivalent aluminum were practically inactive (Table II).

Stumpf\(^3\) in his pyruvic oxidase from Proteus vulgaris replaced the Mg by Mn\(^{2+}\), Fe\(^{2+}\), Ni\(^{2+}\), Zn\(^{2+}\) or Co\(^{2+}\), but Ca\(^{2+}\), Ba\(^{2+}\), Cd\(^{2+}\) and the trivalent Fe\(^{3+}\) and Al\(^{3+}\) were ineffective. Green et al.\(^4\) stated that their preparation of diacetyl mutase from pigeon breast muscle did not require magnesium or any other divalent metal.

B. THIAMINE PYROPHOSPHATE

In pure preparations not containing a phosphorylating system, thiamine pyrophosphate cannot be replaced by thiamine, monophosphothiamine, pyrithiamine (Stumpf\(^3\)), diphosphopyridine nucleotide, triphosphopyridine nucleotide, flavin dinucleotide, adenosinetriphosphate, or pyridoxal phosphate (Green et al.\(^4\)).

Thiamine triphosphate and thiamine polyphosphates have an activity somewhat similar to that of thiamine pyrophosphate, but they quantitatively are much less active; their cocarboxylase activity amounts to only 30% of that of thiamine pyrophosphate.\(^5\) \(^6\)

Velluz \etal.\(^7\) tried to restore the carboxylatic activity of apocarboxylase, obtained by washing yeast with an alkaline phosphate solution. From four to five times more thiamine triphosphoric acid than thiamine pyrophosphate was required to saturate the washed yeast. The resynthesized enzymatic system developed 80% of the activity of the one rebuilt with thiamine pyrophosphate.

Plotka \etal.\(^8\) studied the action of thiamine triphosphoric acid on the heart. On the excised frog's heart thiamine triphosphoric acid exerts a slight positive inotropic action on the normal organ and restores the regularity of the fatigued heart. On the rabbit's heart \textit{in situ}, thiamine triphosphoric acid protects the organ against fibrillation induced by faradization. Thiamine pyrophosphate also exhibits some antifibrillatory properties but much less than thiamine triphosphoric acid. Plotka \etal. in the same article discussed the problem of the existence of thiamine triphosphoric acid in organisms, and they think it is justifiable to consider that thiamine triphosphoric acid plays a role in the special metabolism related to nerve impulse transmission.

C. SUBSTRATE SPECIFICITY

Green \etal.\(^9\) studied the activity of a purified carboxylase from yeast on different substrates (Table III).

Thus the \(\alpha\)-ketonic acids, in addition to pyruvic acid, are decarboxylated also, but the higher homologs are attacked at a lower rate.

The specificity of carboxylases from animal tissues was somewhat different (Green \etal.). These preparations had no action on oxalacetic acid, mesoxalic acid, \(\alpha\)-ketocaproic acid, or phenylpyruvic acid. They decarboxylated \(\alpha\)-ketobutyric acid under formation of propioin, according to the equation

\[
2\text{CH}_3\cdot\text{CH}_2\cdot\text{CO}\cdot\text{COOH} \rightarrow \text{CH}_3\cdot\text{CH}_2\cdot\text{CHOH}\cdot\text{CO}\cdot\text{CH}_2\text{CH}_3 + 2\text{CO}_2
\]

and \(\alpha\)-ketoglutaric acid to succinic semialdehyde and \(\text{CO}_2\):

\[
\text{COOH}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CO}\cdot\text{COOH} \rightarrow \text{COOH}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CHO} + \text{CO}_2
\]

The decarboxylation of \( \alpha \)-ketoglutaric acid took place with the same velocity as that of pyruvic acid. Green et al. made it probable that the pyruvo(a apo)carboxylase and the \( \alpha \)-ketoglutaric(a apo)carboxylase were different enzymes. For if thiamine pyrophosphate was split from the carboxylase by washing at pH 4.6, the maximum rate for carbon dioxide development with pyruvate as a substrate was found by the addition of 2 \( \gamma \) of thiamine pyrophosphate per milliliter of Green's preparation. With \( \alpha \)-ketoglutarate as a substrate, the maximum reaction velocity was attained only when 300 \( \gamma \) of thiamine pyrophosphate per milliliter was added. A third enzyme seemed to be present in Green's preparation, an enzyme for the condensation of acetaldehyde to acetoin. For maximum activity it required 30 \( \gamma \) of thiamine pyrophosphate per milliliter. Each enzyme perhaps has a very high specificity for different substrates and may be catalyzed by different apoenzymes plus the same coenzyme.

The pyruvic oxidase, prepared by Stumpf\(^3\) from *Proteus vulgaris* proved to be very specific. It oxidizes only pyruvic acid; \( \alpha \)-ketobutyric, \( \alpha \)-ketocaproic, \( \alpha \)-keto glutaric, acetoacetic, glyoxylic, and lactic acids are not attacked. The enzyme preparation oxidized formic acid and phenylpyruvic acids, even in the absence of thiamine pyrophosphate. Therefore, the oxidation of these two acids cannot be attributed to the thiamine pyrophosphate-pyruvic acid oxidase. The diacetyl mutase preparation of Green et al.\(^3\) from animal tissues was found to be highly specific for diacetyl. Glyoxal, methylglyoxal, dipropionyl, benzil, cyclohexamedione-1,2, alloxan, and cyclohexanhexone were not attacked by the enzyme. However, the same preparation catalyzes the oxidative decarboxylation of pyruvic acid and of \( \alpha \)-keto glutaric acid (Stumpf et al.\(^6\)). Here again, the \( \alpha \)-keto glutaric oxidase, after splitting off the thiamine pyrophosphate, required a larger

amount of thiamine pyrophosphate than the pyruvic oxidase. Here they found still more cogent evidence bearing on the non-identity of the pyruvic acid and the α-ketoglutaric oxidase from summation experiments. For example, with concentrations of substrates that were adequate to saturate the respective enzymes, α-ketoglutaric acid was oxidized at the rate of 59 mm. of O₂ per 30 minutes, whereas the rate for pyruvic acid was 37 mm. in the same period. The rate with both substrates together was 92 mm., which represents a virtual summation of the two rates. Were a single enzyme involved, the rate in the presence of both substrates would be expected to be in between the two rates, but never greater than the rate of either.

VI. Biogenesis

B. C. P. JANSEN

The biogenesis of the active part of the thiamine enzymes comprises three stages:

1. The synthesis of thiamine itself.
2. The synthesis of the pyrophosphate of thiamine, the cocarboxylase.
3. The synthesis of the whole enzyme, the carboxylase.

For the second and third stages see Section IV A. In this section we shall discuss only the first stage.

Thiamine is synthesized by plants and also by a number of microorganisms. Usually it is assumed that no thiamine is synthesized by animal tissues. However, E. Abderhalden and R. Abderhalden¹ demonstrated that animal tissues were capable of synthesizing thiamine from a solution containing both the pyrimidine and the thiazole moieties. But this was true only to a slight extent for about 1% of the theoretical amount was formed (see page 409). R. Abderhalden² protected rats and pigeons on a thiamine-free diet from deficiency symptoms by giving the animals large quantities of the pyrimidine and the thiazole moieties. Therefore it seems that animal tissues have a trifling capacity to couple the pyrimidine and thiazole parts into thiamine.

In plants the thiamine generally is abundant in seeds and often in green leaves and in roots.

As there is a close connection between microorganisms, particularly mycorhizal fungi, and many plants (and often microorganisms are essential for plants), it is difficult to state which organisms are the most important.

² R. Abderhalden, Pflügers Arch. ges. Physiol. 243, 762 (1940).
for thiamine synthesis. In any event the growth of some plant roots, e.g.,
tomato roots, or pea roots, is greatly stimulated by extremely small amounts
of thiamine \((10^{-10} \text{ g. or less per milliliter})\). Organic manure and fertile soils
contain appreciable amounts of thiamine. Experiments under sterile con-
ditions have demonstrated that plants are able to synthesize thiamine.
Most of it is produced in the young green parts of the plant. In experiments
with plants, however, we must take into account the thiamine that is stored
in the plants. Bonner\(^3\) was able to grow freshly isolated pea roots in a pure
synthetic medium containing inorganic salts and sucrose. If such roots were
subcultured by the removal of 10-mM. tips into fresh medium and particu-
larly if this procedure were repeated several times, thiamine was found to
be essential for growth; 0.2 \(\gamma\) of thiamine per milliliter of the culture medium
gave optimum growth, and even 0.002 \(\gamma\) per milliliter had a marked stimu-
lating effect upon the growth of these roots.

The microorganisms vary considerably in their ability to synthesize thia-
mine. Schopfer\(^4\) established the following groups:

1. Organisms autotrophic for thiamine: \(Absidia repens\).
2. Organisms requiring only pyrimidine: \(Rhodotorula rubra\) type.
3. Organisms requiring only thiazole: \(Mucor Ramannianus\) type.
4. Organisms requiring both pyrimidine and thiazole: \(Phycomyces\) type.
5. Organisms requiring the entire molecule of thiamine: \(Glaucoma-Phyto-
phthora\) type.
6. A separate group of organisms which are partially inhibited by excess
thiamine: \(Rhizopus nigricans\) and other \(Rhizopus\) species.

Williams \(et\) \(al.\)\(^5\) give a list of those macro- and microorganisms that re-
quire either the pyrimidine or the thiazole moiety, those that need both
components, and those that do not require any component at all.

By an (artificial) symbiosis of two microorganisms it is possible to per-
form a synthesis of the thiamine molecule, if each organism is able to syn-
thesize a different nucleus.

Schopfer\(^4\) gives the examples of \(Mucor ramannianus\) and \(Rhodotorula
rubra\). When each of these is inoculated separately on a thiamine-free syn-
thetic medium, neither is able to develop. But when they are inoculated
together, they grow luxuriantly. This proves that \(Rhodotorula\) synthesizes
thiazole which it supplies to \(Mucor ramannianus\) and, conversely that
\(Mucor ramannianus\) synthesizes pyrimidine which it furnishes to \(Rhodo-
torula\).

\(^3\) J. Bonner, \(Science\) \(85\), 163 (1937).
\(^4\) W. H. Schopfer, \(Plants\) and \( Vitamins\), pp. 110, 114. Chronica Botanica Co., Wal-
than, Mass., 1949.
\(^5\) R. J. Williams, R. E. Eakin, E. Beerstecher, and W. Shive, \(The Biochemistry of
From our knowledge of the chemical steps that are used in the thiamine synthesis, we may assume that an organism completely autotrophic for thiamine must be able to effect the synthesis of the pyrimidine and thiazole moieties and to condense pyrimidine and thiazole into thiamine.

About the pyrimidine synthesis in vivo we are not well informed. There are indications that the thiazole part may be synthesized by the condensation of methionine, acetaldehyde and ammonia.\(^3\), \(^4\) In this way \(\alpha\)-amino-\(\beta\)-(4-methylthiazole-5)-propionic acid is formed.

It is known that certain microorganisms can perform transformations analogous to the conversion of this acid to the thiaole part of thiamine.\(^6\)

Bonner and Buchman\(^7\) stated that pea roots synthesize thiazole from the precursors thioformamide and chloroacetopropyl alcohol that are used for the in vitro synthesis. When these two precursors of thiazole are given along with the pyrimidine moiety of thiamine to pea roots, growth is as good as when thiamine is supplied. In the in vitro synthesis chloroacetopropyl alcohol is essential; the pea roots are able to use also acetopropyl alcohol.

\[
\text{HCSNH}_2 + \text{CH}_3\text{COCHClCH}_2\text{CH}_2\text{OH} \xrightleftharpoons[\text{in vivo}]{\text{in vitro}} \text{S--C--CH}_2\text{--CH}_2\text{OH} \\
\text{H--C} \quad \text{C--CH}_3 \\
\text{N}
\]

Thioformamide chloroacetopropylalcohol

Thiazole part of thiamine

\[
\text{HCSNH}_2 + \text{CH}_3\text{COCH}_2\text{CH}_2\text{CH}_2\text{OH} \xrightleftharpoons[\text{in vivo}]{\text{in vitro}} \text{Thiazole part of thiamine} \\
\text{Acetopropylalcohol}
\]

Wild Neurospora strains are able to synthesize thiamine. Tatum in his extensive experiments on mutants of Neurospora obtained four strains that had lost the ability to synthesize thiamine. Accumulation of a pyrimidine component by mutant 18558 and of both pyrimidine and thiazole components by 9185 was shown in bioassays. So Tatum and Bell\(^8\) showed that the evidence obtained with strains 9185 and 18558 is consistent with the view that the synthesis in Neurospora takes place through the production of the thiazole and pyrimidine moieties, with their subsequent coupling.

Woolley and White\(^3\) studied the inhibition of the growth of a number of microorganisms by the antithiamine, pyrithiamine. They found that the inhibition of the growth of microorganisms by pyrithiamine is related to their requirements for thiamine or its components. They found furthermore,

among the bacteria, yeast, and fungi investigated, that the growth of those requiring intact thiamine is inhibited by a much lower concentration of pyrithiamine than those which respond to one or both components, whereas those with no thiamine requirements were not appreciably inhibited. In *Neurospora* there seemed to be no definite correlation between capacity for synthesis of thiamine and its components and the degree of inhibition of pyrithiamine.

Several authors have demonstrated a favorable influence of light on the biosynthesis of thiamine. Bonner and Greene\(^{10}\) found, for example, that the thiamine content of pea plants kept in the dark did not increase, whereas in the light this content rose rapidly.

**VII. Estimation**

**B. C. P. JANSEN**

**A. PHYSICAL**

Thiamine may be estimated by its absorption in the ultraviolet at 265 mp. This method has been used in the laboratory of von Muralt\(^{1}\) in Bern by Lüthy\(^{2}\) for measuring the thiamine content of nerve fibers.

To distinguish the thiamine from other substances (nuclein components) absorbing at 265 mp, the absorption may be measured before and after destroying the thiamine by ferricyanide.

**B. CHEMICAL**

Barger *et al.*\(^{3}\) showed that thiamine on oxidation with ferricyanide in alkaline solution is transformed into thiochrome, see facing page.

This thiochrome is characterized by a strong blue fluorescence. Jansen found that in a very strong alkaline solution and with a suitable quantity of ferricyanide the transformation is practically quantitative. The thiochrome is extracted from the aqueous solution by isobutanol. Thus the fluorescence of the thiochrome, which is accurately measured in a fluorometer, may be used as a yardstick for the thiamine.\(^{4}\)

It could be expected that this method is not only sensitive but also specific for use in biological fluids and extracts. Bouman\(^{5}\) found, however, that

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blighted potatoes (infected with *Phytophlera*) contain a substance with a blue fluorescence which is soluble in isobutanol. Also urine and other biological fluids contain interfering substances. In such cases a preceding adsorption of thiamine to Decalso, fuller’s earth, or other suitable adsorbents, is necessary.

More elaborate descriptions of this method are available. By the use of a suitable fluorometer 0.01 \( \gamma \) of thiamine or less may be determined by the thiochrome method. In biological fluids and extracts a large part of the thiamine is present in the form of pyrophosphate. By oxidizing this it is transformed to the pyrophosphate of thiochrome. This is insoluble in isobutanol. Therefore the pyrophosphate must first be hydrolyzed. This hydrolysis can be performed by takadiastase or by a phosphatase, e.g., from yeast.

Burch *et al.* elaborated a micromethod to determine thiamine and thiamine phosphates in very small quantities of blood and blood cells.

Prebluda and McCollum devised a method using the color production of a diazotized aromatic amine (e.g., \( p \)-aminoacetophenone) with thiamine. The method has been further developed by Melnick and Field. This

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azocoloric method is less sensitive and less specific than the thiochrome method, but it is somewhat simpler and quicker and may be useful in special instances, e.g., for clinical determination in urine.\textsuperscript{11}

C. BIOLOGICAL

Obviously the earliest methods that were used for thiamine estimation were biological ones. In the beginning when polished rice, an easily obtainable thiamine-free food, was taken as a basal diet, hens and cocks were used as experimental animals. Soon smaller birds, especially pigeons, were taken, and Jansen worked with very small birds, nonnetjes (\textit{Munia maja}). When mammals (rats) were the experimental animals, polished rice could no longer be used as a basal diet because of its deficiencies in other nutrients. Therefore a complex basal diet was essential. The experiments may be prophylactic or curative. Both are apt to give not very accurate results, and both require large amounts of thiamine-rich extracts. These are serious handicaps, and therefore when the chemical and microbiological methods were brought about, the biological methods were not applied very much any longer. The advantages of the biological methods are that they do not require cumbersome extractions and that they give specific results. For details see, e.g., Coward\textsuperscript{12} and György.\textsuperscript{13}

D. FERMENTATION

Schultz \textit{et al.}\textsuperscript{14} found that yeast fermentation is enhanced by the presence of free thiamine. They utilized this fact for a quantitative method for the estimation of thiamine. Used with a Warburg apparatus, the method is suitable for a microestimation.\textsuperscript{15}

Nearly all the thiamine in blood and in animal tissues is present in the form of thiamine pyrophosphate (cocarboxylase). This cocarboxylase can be measured by its activity as a coenzyme. Ochoa and Peters\textsuperscript{16} were the first to use this method for the quantitative determination of thiamine and cocarboxylase in boiled tissue extracts. Goodhart and Sinclair\textsuperscript{17} applied this method to the determination of cocarboxylase in blood. All the blood cocarboxylase was found within the blood cells, particularly in the nucleated cells.

\textsuperscript{17} R. S. Goodhart and H. M. Sinclair, \textit{Biochem. J.} \textbf{33}, 1009 (1939).
Westenbrink\textsuperscript{18} worked out a micromethod which permits the determination of about 0.00005 $\gamma$ of cocarboxylase and about 0.0005 $\gamma$ of thiamine separately in a mixture of both compounds.

Westenbrink et al.\textsuperscript{19} used alkali-washed yeast as an adsorbant and at the same time as the apoenzyme for the cocarboxylase preparation from blood. In this way, using the "cartesian diver" technique, it was possible to determine the cocarboxylase content of 0.01 ml. of blood (Parv\textsuperscript{20}).

E. MICROBIOLOGICAL

In 1935 the Swiss investigator Schopfer found that the growth of the mould \textit{Phycomyces blakesleeanus} required the presence of thiamine.\textsuperscript{21} He used this fact to develop a method for the estimation of thiamine, by measuring its influence on the growth of \textit{Phycomyces blakesleeanus}.\textsuperscript{22}

This method is very sensitive and especially suitable for series analysis of foodstuffs, etc. A drawback is the fact that it takes 8 to 10 days before growth is complete. The split products of thiamine, the pyrimidine plus the thiazole part, are active also. (For further details, see ref. 7, p. 485.)

In his search for the components of Wildiers' "bios," Williams and Roehm\textsuperscript{23} found that thiamine greatly stimulates the growth of yeast (\textit{Saccharomyces cerevisiae}). So thiamine may be estimated by its influence on the growth of yeast. Here again the pyrimidine and the thiazole parts are both active on yeast growth.\textsuperscript{24}

In 1944 Sarett and Cheldelin introduced the use of \textit{Lactobacillus fermenti}. This microorganism is extremely sensitive to traces of thiamine.\textsuperscript{25, 26}

In this way it is possible to determine quantities of 5 to 50 m$\gamma$ of thiamine. Cocarboxylase is about 30\% more active than an equimolecular quantity of thiamine. The pyrimidine and thiazole moieties are inactive if the incubation is not prolonged beyond the usual time of 16 to 18 hours at 37\(^\circ\). (For details and for the use of other lactic acid bacteria, see ref. 7, p. 372.)

Often chemical or microbiological methods are used for thiamine estimation in the urine of men or animals to establish the state of thiamine nutrition. Mickelsen et al.,\textsuperscript{27} in their extensive research on the thiamine excre-

\textsuperscript{18} H. G. K. Westenbrink, \textit{Enzymologia} \textbf{8}, 97 (1940).
\textsuperscript{24} R. J. Williams, J. R. McMahan, and R. E. Eakin, \textit{Univ. Texas Publ.} \textbf{4157}, 31 (1941).
tions of normal young men on controlled intakes of thiamine from 0 up to 16 mg. per day, found that at intake levels between 0.7 and 1 mg. of thiamine per day the thiamine excretion may be used as an indicator of the

**TABLE IV**

**Collaborative Results by the Recommended Thiochrome Method**

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Malt extract</th>
<th>Wheat germ</th>
<th>Wheat flour, %</th>
<th>Yeast extract</th>
<th>Dried yeast</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sample 1</td>
</tr>
<tr>
<td>A</td>
<td>2.7</td>
<td>22</td>
<td>55</td>
<td>29</td>
<td>17</td>
</tr>
<tr>
<td>D</td>
<td>4.0</td>
<td>21</td>
<td>3.8</td>
<td>54</td>
<td>20</td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
<td>24</td>
<td>14</td>
<td>54</td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>G</td>
<td>3.9</td>
<td>21</td>
<td>52</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td></td>
<td></td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>J</td>
<td>3.7</td>
<td>21</td>
<td>3.8</td>
<td>61</td>
<td>28</td>
</tr>
<tr>
<td>K</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>L</td>
<td>3.9</td>
<td>20</td>
<td>47</td>
<td>27</td>
<td>19</td>
</tr>
<tr>
<td>M</td>
<td>3.5</td>
<td>23</td>
<td>3.8</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td></td>
<td>3.6</td>
<td>37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>3.62</td>
<td>21.3</td>
<td>3.75</td>
<td>51.3</td>
<td>29.1</td>
</tr>
</tbody>
</table>

**Table V**

**Results by Biological and Microbiological Methods**

<table>
<thead>
<tr>
<th>Laboratory Method used</th>
<th>Thiamine found, γ/g.</th>
<th>Dried yeast</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Malt extract</td>
<td>Wheat germ</td>
</tr>
<tr>
<td>K Rat growth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N Bradycardia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P Rat growth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F Yeast fermentation</td>
<td>3.9</td>
<td>21</td>
</tr>
<tr>
<td>J <em>Lactobacillus fermentum</em></td>
<td>4.1</td>
<td>23</td>
</tr>
</tbody>
</table>

Mean value of the collaborative fluorimetric results (from Table IV)

intake. From 1 mg. up to about 5 mg., however, it is better to use the pyramine excretion. Pyramine is the pyrimidine-like component of the thiamine molecule which is excreted in the urine. The pyramine content of the urine was determined by a modification of the yeast fermentation method of Schultz et al. The standard used in these determinations was 2-methyl-4-amino-5-ethoxymethylpyrimidine hydrochloride.
The different methods of estimation vary widely in principle and even the same method (chemical, biological, etc.) may be carried out in different ways. Thus, for obtaining a reliable extract, different proteinases may be used, combined with extraction with diluted hydrochloric acid, sulfuric acid, or sodium acetate solution. The extracts may be purified for removal of interfering substances in different ways. Several different methods are used for the oxidation of thiamine to thiochrome. Therefore it is possible that the results of the thiamine estimation of one laboratory do not agree very well with those of another institute.

Therefore in many countries the results of different laboratories have been compared. The most recent investigation in this respect probably is that of the Aneurine Panel of the Subcommittee on Vitamin Estimations of the British Analytical Methods Committee of the Society of Public Analysts and Other Analytical Chemists.28 The panel prepared large homogeneous amounts of malt extract, wheat germ, wheat flour (85% extraction), yeast extract, and three different samples of dried yeast. Representative samples of these thiamine-containing foodstuffs were sent to the collaborating laboratories in England and in the Netherlands. A detailed description of the recommended thiochrome method was given.28 For the results, see Tables IV and V.

From these results we see that the various results in the different laboratories are not in perfect agreement. Particularly the biological methods gave divergent results. On the other hand the agreement of the results of the thiochrome and the microbiological methods was not too bad.

VIII. Standardization of Activity

B. C. P. JANSEN

Before the large-scale production of pure, crystalline thiamine hydrochloride, it was decided at the Vitamin Standards Conference of the Commission on Biological Standardization of the Health Organization of the League of Nations held at London in 1931 that a certain quantity (10 mg.) of an acid clay activated by an extract from rice polishings should be used as an international unit of antineuritic vitamin B1 (at that time the name of thiamine).1 This activated acid clay was prepared in the Medical Laboratory at Batavia (now Djacarta) and had already been shown to be a very stable material. In experiments with pigeons and rats it appeared that 10 mg. of this activated acid contains about 3 γ of thiamine hydrochloride.

Thus 1 international unit of thiamine is equivalent to $3 \gamma$ of thiamine hydrochloride.

Afterwards Keresztesy and Sampson$^2$ found that this standard preparation was not ideal; they demonstrated that the preparation contained twice as much thiamine as was indicated in the animal experiments, the animal organism being able to elute only one-half of the adsorbed thiamine.

Now that the crystalline synthetic thiamine hydrochloride is abundantly available, the pure synthetic preparation itself is taken as a standard, and the quantity of thiamine usually is expressed not in international units but in milligrams of thiamine.

Before the isolation of the thiamine some authors had recommended units based on rice polish or yeast. Of course these are now obsolete, and a comparison with the pure vitamin is only approximately possible. With this restriction we can say that 1 $\gamma$ of crystalline thiamine hydrochloride is equivalent to 0.166 Smith curative unit or to 0.666 Chase-Sherman unit or to 0.333 Roscoe unit.

IX. Occurrence in Food

B. C. P. JANSEN

A. OCCURRENCE OF THIAMINE AND ITS PHOSPHATE COMPOUNDS

In contrast to other vitamins, e.g., the fat-soluble vitamins and ascorbic acid, thiamine has a widespread distribution in food. It is entirely absent only from oils and fats, cassava, and refined sugar. On the other hand we do not know of any foods that are as rich in thiamine as certain fish liver oils are in vitamins A and D. In animal experiments brewer's yeast is used as a rich source of thiamine (and other B vitamins).

In connection with this lack of abundantly thiamine-containing foods it is important, as stated by Friedemann et al.,$^3$ that the intestinal absorption of thiamine hydrochloride by normal human subjects is extremely limited. The maximum which could be orally taken without an increase of fecal thiamine is about 5 mg. per day.

A large part of thiamine in vegetable products is in the form of thiamine itself; in animal tissues it is present largely in the form of its phosphate or pyrophosphate derivative (as cocarboxylase). With regard to the nutritive value of the food, it does not matter in which form thiamine is present,

because in the animal organism the thiamine is easily phosphorylated (by an adenosinetriphosphate-containing enzyme) and dephosphorylated by a powerful phosphatase.

Thiamine is present as such in blood plasma, but as pyrophosphate in the corpuscles. In human blood only about 10% of the thiamine is present as such in the plasma; the rest is in the corpuscles. Normal blood of a well-fed person contains about 6 to 12 γ of thiamine per 100 milliliters. The mean values of the thiamine pyrophosphate content of well-nourished men were: 1.5 γ per 10^{11} red cells; 290 γ per 10^{11} leucocytes; for women: 1.3 γ per 10^{11} red cells; 270 γ per 10^{11} leucocytes.

As thiamine is fairly resistant to high temperature and O_2 in a weakly acid milieu, it is clear that most processed foods contain thiamine. However, in alkaline and even in the neutral state the resistance to heat is not great. Therefore, if processing of neutral foodstuffs includes treatment at or above 100°C, a substantial loss of thiamine takes place, dependant upon the duration of the heating. Furthermore, in the cereal grains thiamine is found especially in the outer grain layers, and therefore machine-milled rice is very poor in thiamine. Thus it is obvious that beriberi is a disease of rice and cassava (manioc)-eating countries, as here a great deal of the population depends on rice or cassava as their main source of calories.

For the thiamine content of the different foods we may refer to tables as published elsewhere. Special consideration will here be given to those foods that are either particularly rich in thiamine or that forming the main food item for many people.

a. Cereals

(1) Wheat. The thiamine content of wheat depends especially on the variety and to a lesser extent on the conditions under which it is grown, i.e., the quality of the soil, the use of fertilizers, etc. Usually the content is between 500 and 800 γ of thiamine per 100 g. of whole wheat.

Thiamine is very unevenly distributed in the kernel. The content of the inner layer, the endosperm, is the lowest, about 30 γ per 100 g. The aleurone layer and the germ are much richer in thiamine; by far the richest part is the scutellum, the small layer between the germ and the rest of the kernel. This small layer alone, consisting approximately of 1.5% of the whole kernel, contains 60% of its thiamine (Hinton). The scutellum of rye, corn, and barley has a similar high content of thiamine (Hinton).

4 A. Dubois and G. Corin, Bull. soc. pathol. exotique 7, 492 (1914).
In the modern milling machines (high milling) the process can be regulated in such a way that several fractions are obtained corresponding to the different layers of the kernel. For the preparation of white bread usually a fraction is taken that contains all the endosperm plus so much of the other parts that about 70% of the kernel is present in this fraction. We call this a 70% extraction flour. The thiamine content of this flour is about 60 γ per 100 g. In wartime in England, Holland, and in several other countries a flour of about 85% extraction was used for bread making. The thiamine content of this fraction was about 240 γ per 100 g.

In the process of bread-making yeast is added, so that the thiamine content increases somewhat, but because of the high baking temperature part of the thiamine, especially in the crust, is destroyed. White bread contains approximately 40 γ of thiamine per 100 g., whole meal bread about 180 γ, and the 85% extraction war bread about 120 γ. To the “enriched” bread so much synthetic thiamine has been added that the total thiamine amounts to 110 to 180 γ per 100 g.

(2) Rye. Rye bread usually is made of the whole kernel. Sometimes flour is used. The thiamine content of rye bread is somewhat lower than that of the corresponding wheat bread.

(3) Rice. The thiamine content of rice is lower than that of wheat, namely about 300 γ per 100 g. In home-pounded rice, most of the thiamine remains. In machine-milled rice, only about 30 γ per 100 g. is left. Rice is always thoroughly washed before cooking. This reduces the thiamine content still further.

In the native process of parboiling, the rice is first soaked in water, then boiled and dried. This product is milled. The resulting parboiled rice contains nearly all the thiamine of the whole rice kernel. About the same process is used in industry for the preparation of “converted” rice. This also contains nearly all the thiamine of the original rice. “Enriched” rice contains about 500 γ of thiamine per 100 g.

(4) Corn. The thiamine content of corn is not very high. Its chief deficiency is that of niacin and tryptophan. Therefore, pellagra is the disease of the corn-eating countries. However, in some cases of pellagra clinical signs of a thiamine deficiency may be found. For the prevention and therapy of pellagra an extra addition of thiamine, besides niacin, is necessary.

b. Legumes

Peas and beans are among the foods richest in thiamine. They contain about 500 to 600 γ of thiamine per 100 g. After the discovery that beriberi was caused by the use of polished rice, “katjang idjo,” a kind of green peas, was employed in the prevention of beriberi. It Soybeans are just as good a source of thiamine as the common pulses.
In Southeast Asia soybeans are often fermented by the mould *Aspergillus oryzae*. The product is digestible and palatable. However, it has lost a considerable part of its thiamine. So the fermentation process does not mean an improvement in regard to thiamine (Jansen\(^5\)).

c. Nuts

Nuts are pretty rich in thiamine (300 to 500 \(\gamma\) per 100 g.). Coconuts contain only traces of thiamine.

d. Potatoes

In countries where potatoes are much in use, as in Western Europe, potatoes may fulfill a large part of the thiamine need of the population because of their high thiamine content. This content does not appear to be very high, i.e., 80 to 100 \(\gamma\) of thiamine per 100 g., but potatoes contain about 75 to 80\% of water. Thus, in terms of dry matter, the content is four to five times as large.

Boiling potatoes reduces the thiamine content very little. Boiling peeled potatoes in a large quantity of water produces a slight reduction, but almost all the thiamine will remain in the potatoes and only traces are found in the water, provided that the boiling ceases when the potatoes are done. If however, the boiling has been prolonged until the potatoes fall apart, then the thiamine is extracted by the water.

These factors are of consequence for such European countries as the Netherlands where potatoes form a large part of the diet and thus constitute an important source of thiamine.

On account of the fact that the potato was one of the chief food items in wartime in the Netherlands, an investigation was made into the thiamine content of different varieties of potatoes, raised under different conditions of soil, manure, etc. More than 100 varieties were analyzed.\(^9\) It appeared that the thiamine content of different samples varied from 40 \(\gamma\) to 160 \(\gamma\) per 100 g. of fresh potato. However, in the varieties that were in common use the content amounted to 85 to 100 \(\gamma\) per 100 g.

e. Meat and Fish

The vitamin content of the organs, particularly liver and kidney, is higher than that of the muscles. This is also the case with thiamine. Beef contains about 120 \(\gamma\) of thiamine per 100 g. Since thiamine plays such an important part in muscle activity, however, it is obvious that the thiamine content is highest in those muscles which perform most of the work, as, e.g., the breast muscles in flying birds.

It is a remarkable fact that the meat of pigs is very rich in thiamine. Pork is one of the richest sources of thiamine. Lean pork contains nearly 1 mg. of thiamine per 100 g. Fish on the whole contains somewhat less thiamine than beef, but the difference is not very large. In eggs, most of the thiamine is in the yolk; the thiamine content of fresh yolk is about 300 γ per 100 g.

f. Milk

Taking into account the fact that the amount of dry matter in fresh cow’s milk is only 13%, the thiamine content, about 45 γ per 100 g., is fairly good. Human milk contains only one-third as much thiamine as cow’s milk. Part of the thiamine in human milk is in the form of pyrophosphate and of orthophosphate.\(^\text{10}\)

Kon (in Reading, England) estimated the influence of pasteurization and sterilization on cow’s milk that is used for feeding infants. Pasteurization of the milk reduces the thiamine content about 10%, and the more drastic sterilization procedures may destroy 30% or more.\(^\text{11}\) If we compare the content of this sterilized milk with the recommendation of the Food and Nutrition Board of the American National Research Council for infants, we see that the margin of safety for thiamine in the sterilized milk used as an infant food is small. On the other hand, in breast milk the thiamine content is still lower. Holt et al.,\(^\text{12}\) who themselves estimated the thiamine requirement of infants, draw attention to this fact. They suppose that perhaps in infants fed with breast milk part of the required thiamine is synthesized by the microflora in the gut.

Fortunately most infant foods are acidified (by churning or by the addition of an acid), and in these preparations the thiamine is much more stable.

B. OCCURRENCE OF NATURAL ANTITHIAMINES AND THIAMINASES IN FOODS

Not so much is known about natural antithiamines in food. There is quite a literature, however, on thiaminases. The first indications of a harmful influence from the consumption of fresh-water fish were found on a silver fox farm of a Mr. Chastek. These foxes developed a paralysis that was afterward called the Chastek paralysis. Green et al.\(^\text{13}, \text{14}\) demonstrated that this disease arises in foxes on a diet containing 10% or more of fresh

\(^{10}\) S. de Jong, *Enzymologia* 10, 253 (1942).


IX. OCCURRENCE IN FOOD

uncooked fish and that it could be prevented or cured by giving thiamine. Woolley\textsuperscript{15} and Spitzer et al.\textsuperscript{16} found that a heat-labile principle, probably an enzyme from an extract of carp viscera, was able to inactivate a thiamine solution \textit{in vitro}. Krampitz and Woolley\textsuperscript{17} showed the enzyme activity of the fish principle by isolating the split products from thiamine: 4-methyl-5-hydroxyethylthiazole and 2-methyl-4-amino-5-hydroxymethylpyrimidine.

Sealock and Livermore\textsuperscript{18} realized that this reaction gave rise to hydrogen ions.

\[
\begin{align*}
\text{CH}_3 & \quad \text{N—CH} \\
\text{CH}_3 & \quad \text{C—CH}_2—\text{N} \\
\text{N—C—NH}_2\cdot\text{HCl} & \quad \text{H—C—S} \\
\end{align*}
\]

By using a bicarbonate buffer in a Warburg apparatus Sealock and Livermore\textsuperscript{18} were able to study the kinetics of the reaction by measuring the carbon dioxide that was set free by the hydrogen ions. Probably this same reaction might be used to estimate the thiaminase content of foods. This enzyme, the thiaminase, is present in several species of fishes, mostly in fresh-water fishes (Deutsch et al.\textsuperscript{19}), but also in the Atlantic herring and in aquatic animals like the clam (Melnick et al.\textsuperscript{20}). Jacobsohn and Azevedo\textsuperscript{21} found that the tissues of shrimps and some mussels are rich in thiaminase, but crabs do not contain it. Redi and Giri\textsuperscript{22} could demonstrate the presence of two different thiaminases in fresh-water mussel. Thus the substances

\textsuperscript{17} L. O. Krampitz and D. W. Woolley, \textit{J. Biol. Chem.} \textbf{152}, 9 (1944).
\textsuperscript{21} K. P. Jacobsohn and M. D. Azevedo, \textit{Arch. of Biochem.} \textbf{14}, 83 (1947).
\textsuperscript{22} K. K. Redi and K. V. Giri, \textit{Enzymologia} \textbf{13}, 281 (1949).
exercising an antithiamine activity in fish and other sea food are thiaminases. Some reports have been published on plants that show an antithiamine activity, but it has not yet been established whether these plants contain antithiamines or thiaminases.

Weswig et al. found that ferns (Pteris aquilina) have an antagonistic activity against thiamine. Horses fed a fodder containing large amounts of ferns show signs of polyneuritis and can be cured by thiamine.

Bhagvat and Devi found antithiamine activity in extracts from rice polishings, beans (Phaseolus radiatus), mustard seed, etc.

Somogyi, in a comprehensive investigation of antithiamine factors, not only confirmed their existence in carp viscera but also demonstrated that extracts of organs of warm-blooded animals (rabbits or chicks) have a considerable antithiamine activity. The greatest activity was observed with extracts of heart and spleen. Liver extract showed a moderate activity, whereas the extract of intestines of warm-blooded animals had only a slight action in contrast to that of carp extracts. The active principles obtained from warm-blooded animals are thermolabile and do not dialyze, so probably they contain thiaminases.

X. Effects of Deficiency

B. C. P. JANSEN

A. IN MICROORGANISMS

Of the effects of thiamine deficiency on microorganisms, not much is known. The microorganisms that cannot synthesize thiamine can be used for the microbiological estimation of thiamine.

B. IN ANIMALS

As far back as 1892 Eykman published his results about the signs of nerve degeneration in fowls that were fed with thiamine-poor polished rice. From this work started the whole vitamin research. Thus the degeneration of the peripheral nerves was the first pathological symptom noted. Eykman stained the nerves with Marchi solution, and he thought that in the polyneuritic animals the axis of the nerves was degenerated. Half a century afterward in an extensive examination of the peripheral nerve fibers in

thiamine deficiency Swank and Prados\(^1\) made the observation that the first neuronal histologic change in thiamine-deficient pigeons is degeneration of the distal part of the axon, and changes in myelin are secondary to this, and further that opisthotonus (the characteristic manifestation of acute thiamine deficiency in pigeons) may not be attended by any definite neurologic lesions.

About thirty years after the work of Eykman, Peters in Oxford, England, was able to demonstrate that not only the peripheral nerves, but also the central nervous system was affected by a thiamine deficiency. From that work of Peters resulted a large part of our knowledge of the role thiamine plays in carbohydrate, especially in pyruvate, metabolism. It is probable that most of the pathology of thiamine deficiency is due to a disturbance in the carbohydrate metabolism. The comprehensive work of Peters and his school on the details of the biochemical action of substances causing pathological effects and in particular in trying to understand the initial changes from thiamine deficiency has led him to call these initial changes "biochemical lesions."\(^2\) Peters studied the episthotonus signs induced in the rice-fed pigeon by thiamine deficiency. The episthotonus signs clear up very quickly when thiamine is given, and there is no detectable histological damage at this stage. It is in this sense an example of a pure "biochemical lesion."

The clinical symptoms of thiamine deficiency are connected with the metabolic disturbances. How close the connection between both is, is not precisely known. These symptoms are nearly the same in different animals. Usually there are signs of lameness, of convulsions, accompanied in pigeons with head retraction and in rats with walking in a circle, and of "biochemical lesions." Other signs are anorexia, reduction of growth or decline in weight, and emaciation. As Drummond has emphasized, many of these signs are not independent from each other. Thus the anorexia may be the cause of the decline in weight. In rats the heart rate is reduced;\(^3\) in normal rats the rate is 500 beats per second; in severe deficiency it is not more than 250 to 300. This fact was used by Birch and Harris\(^3\) as an indication of the severity of thiamine deficiency in rats. (In human beings just the opposite takes place; thiamine deficiency leads to tachycardia For an extensive investigation on the pathology of thiamine deficiency in monkeys, see, e.g., Rinehart \textit{et al.}\(^4\)

As thiamine in the form of thiamine pyrophosphate is necessary for the metabolism of pyruvate, one would expect that the amount of pyruvate in blood, and perhaps also in urine, may be increased in thiamine deficiency. Platt and Platt and Lu indeed found a large increase of bisulfite-binding substances, consisting mainly of pyruvic acid, in the blood of beriberi patients. Some hours after the administration of thiamine to the patients the amount of the bisulfite binding substances dropped to normal. In animal experiments Thompson and Johnson found that pigeons and rats with symptoms of acute thiamine deficiency had a high blood pyruvate level. One would be inclined to think that these symptoms of acute thiamine deficiency might be caused by a pyruvate poisoning of the animal. The work of de Jong, however, makes this supposition highly improbable. De Jong devised a micromethod for determining the pyruvate level of a small drop of blood; he thus was able to execute several determinations in the course of the development of polynieritis in the animals. In this way he proved that the symptoms of acute polynieritis in pigeons developed before the rise of pyruvate, and the disappearance of the symptoms after the thiamine administration preceded the return of the pyruvate level to normal.

The results of this work of de Jong seem to demonstrate that the polyneuritis signs are independent of the pyruvate metabolism and, for that reason, of the catalytic action of thiamine pyrophosphate.

From the work of Loewi and of Dale we know that a chemical substance, the acetylcholine, plays a role in the transmission of nerve stimuli.

Binet and Minz showed that the acetylcholine not only plays a role in the transmission of the stimulus from the end of the nerve to the effective organ but that it works also in the nerves themselves. The acetylcholine content of the nerves was increased after electric stimulation. However the addition of an extract of the non-stimulated nerve to that of the stimulated nerve intensified the action of this extract, or of a solution of pure acetylcholine. This points to another substance present in the extract of the stimulated nerve. The authors, considering that thiamine deficiency leads to polynieritis, assumed that the active substance of the stimulated nerve might be this vitamin. So they compared the activity of the substance of the stimulated nerve with that of thiamine. They found that their activities in this respect were the same. They further showed that thiamine also stimulates the activity of acetylcholine in the isolated intestine of the rat.

8 S. de Jong, Arch. néerl. physiol. 21, 465 (1936).
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and in the circulatory organ of the cat, in the absence of the eserine that inhibits the enzyme cholinesterase.

The liberation of thiamine (or a thiamine derivative) by nerve action was for the first time demonstrated by Minz.\textsuperscript{10} Lwoff\textsuperscript{11} had devised a highly sensitive microbiological method for the estimation of very small quantities of thiamine, using Flagellatae. With this method Minz compared the liberation of thiamine from excised resting ox nerves with that of electrically stimulated nerves. He was able to prove that the stimulated nerves delivered much more thiamine than the resting nerves (four to eight times as much). Shortly afterward these results were confirmed and extended by von Muralt. This Swiss investigator has given a review of all the work, mostly from his own laboratory, on thiamine and peripheral neurophysiology.\textsuperscript{12} Von Muralt and his collaborators quickly froze exited nerves or resting nerves in liquid air. The frozen nerves were ground in a mortar and extracted for only 10 minutes with Ringer's solution. The thiamine content of the extracts was estimated by several different methods. The extracts of the stimulated nerves were richer in thiamine than the extracts of the resting nerves. This means that thiamine in the exited nerves is in such a state that more can be extracted by Ringer's solution in 10 minutes than can be obtained from a corresponding sample of unexcited nerves. The different methods for estimating the liberated thiamine yielded on the whole practically the same results; however, the yeast ferment method of Atkin, Schultz, and Frey showed much lower values for the thiamine content of both excited and resting nerves; and also the difference between excited and unexcited nerves had disappeared. It was not yet possible to give an explanation for this discrepancy. Furthermore it was difficult to draw conclusions from these experiments because A. Wyss and F. Wyss\textsuperscript{13} in the laboratory of von Muralt found that by poisoning the nerves by monioacetic acid more thiamine is obtained in the extract of resting nerves than in the extract of excited nerves. In most experiments thiamine exerts an inhibiting effect on the vagus or acetylcholine action on the heart. By replacing certain groups in the thiamine molecule by other groups, the effect is mostly reduced but not abolished. It is amazing, however, that the thiamine pyrophosphate has no action at all!

From all these facts it may be assumed that thiamine or a thiamine compound plays a role in neurophysiological activity.

However, there is as yet no convincing evidence that the neurological active substance is thiamine itself or one or more derivative(s) of thiamine.

\textsuperscript{10} B. Minz, Compt. rend. soc. biol. 127, 1251 (1938).
\textsuperscript{11} M. Lwoff, Compt. rend. soc. biol. 128, 241 (1938).
\textsuperscript{12} A. von Muralt, Vitamins and Hormones 5, 93 (1947).
\textsuperscript{13} A. Wyss and F. Wyss, Experientia 1, 160 (1945).
Von Muralt proposes that as long as this uncertainty exists this substance be called "the second Vagusstoff," because Loewi, before he understood the exact nature of the chemical mediator in the heart, called it "Vagusstoff."

Thus we have two well-established facts concerning the pathology of thiamine in animals. The first is the activity of thiamine pyrophosphate as a coenzyme in the carbohydrate metabolism; the second is the role of thiamine or a derivative of thiamine (the "second Vagusstoff") on the neurophysiological activity. We do not yet know whether these facts are closely connected, or whether they are quite independent from each other. From the fact that the thiamine pyrophosphate lacks the neurophysiological action of thiamine it is probable that the neurophysiological activity is different from the activity on the carbohydrate metabolism.

On the other hand we know that thiamine pyrophosphate is active in the production of acetic acid which is essential to restore the active acetylcholine from the inactive choline that is formed from acetylcholine by the action of the cholinesterase.

C. IN MAN

In the beginning of vitamin research it was easy to compose a diet for the study of thiamine deficiency. With fowls or pigeons as experimental animals, polished rice, after being washed in running water to remove the last traces of thiamine, was a suitable diet. Polished rice, however, not only lacks thiamine, but it also shows a shortage of many other nutrients. Therefore when other experimental animals, e.g., rats, were used, it appeared necessary to add these other nutrients (proteins, mineral salts, nearly all other vitamins) or to compose a complete, synthetic diet.

Williams et al.,14 in their experiments to investigate the signs of a pure thiamine deficiency in volunteers on a thiamine-poor diet, found no signs of edema in their experimental persons, whereas the natives in rice-consuming countries recognize beriberi because of the signs of edema in these patients.

Therefore, to study the effects of a pure thiamine deficiency it is necessary to provide a diet that contains all nutrients in physiological amounts, except thiamine. Since we do not yet know all the essential nutrients, it is very difficult to compose a suitable synthetic diet, not considering the cost of some nutrients! So it is better to choose a good natural diet in which only the thiamine is destroyed. In these diets the thiamine is often destroyed by autoclaving for several hours at pH 5. But by this procedure other nutrients are damaged too. A more specific way to destroy the thiamine is to

treat the diet, or the thiamine-containing parts of the diet, with sulfite.\textsuperscript{15} 16 Presumably the most specific method of destroying the thiamine would be treatment with thiaminase. Smith and Proutt\textsuperscript{17} stated that cats fed a diet consisting exclusively of thiaminase-rich raw carp developed all the signs of the thiamine deficiency characteristic for this animal. To counteract specifically the activity of thiamine, the antithiamines, e.g., pyrithiamine or oxythiamine, can be used. Woolley\textsuperscript{18} was able to demonstrate that at least one of the activities of pyrithiamine consists in the antagonizing of the synthesis of cocarboxylase—the active form of thiamine in carbohydrate metabolism. Woolley and White\textsuperscript{19} state that, whereas mice fed a ration free of thiamine develop no characteristic symptoms of thiamine deficiency, the same animals, on administration of pyrithiamine, do show many of these symptoms. Therefore the best way to study the effects of uncomplicated thiamine deficiency presumably is the use of antithiamines and thiaminases, added to an otherwise optimal diet. A great difficulty is the detection of the first signs of a deficiency. This is important because beriberi is not found in Western countries, but it is possible that even here many persons suffer from a mild thiamine deficiency. Usually the excretion of thiamine into the urine per 24 hours is determined, or during 3 hours after giving a measured dose of thiamine. Also the blood thiamine level is used as a yardstick. However, this gives only a vague indication.\textsuperscript{20} Swank and Jasper\textsuperscript{21} compared encephalograms of normal pigeons with those of thiamine-deficient birds. An increase in the brain potentials occurred slowly in the thiamine-deficient pigeons and preceded the development of clinical signs. Shortly before the appearance of preopisthotonus the amplitude of the brain potentials became three times as high as during the control period. The administration of thiamine to pigeons with preopisthotonus caused return of the brain waves to normal.

Horwitt and Kreisler,\textsuperscript{22} from their work on patients on diets with different thiamine levels, tried to devise an index of carbohydrate metabolism, correlating the levels of glucose, lactic acid and pyruvic acid in the blood of the patient after a measured exercise and the carbohydrate metabolism which is influenced by the thiamine intake. This carbohydrate index was

\begin{itemize}
  \item J. E. Kirk and M. Chieffi, \textit{J. Gerontol.} 5, 236 (1950).
\end{itemize}
thought to be an indication of the state of thiamine nutrition before any clinical signs of thiamine deficiency occurred.

Mouriquand and Coisnard\(^23\) observed that pigeons on a thiamine-poor diet demonstrated a fall in the chronaxy of the nerves and that this fall begins before the clinical signs appear.

### XI. Pharmacology

**KLAUS R. UNNA**

Thiamine has been shown to produce a variety of pharmacologic effects. It should be borne in mind that these effects have been obtained in experimental animals maintained on adequate diets only on parenteral administration of thiamine in doses several thousand times larger than those required for optimum nutrition. These pharmacologic effects in animals have no counterpart in the therapeutic use of the vitamin in man.

Death after intravenous injection of thiamine in animals is due to depression of the respiratory center.\(^1\)–\(^4\) The heart is still beating at the time of cessation of the respiration. Artificial respiration enables the animals to survive otherwise lethal doses;\(^3\) doses of thiamine resulting in concentrations of 7 to 10 mg. % in the blood were fatal to dogs (under ether anesthesia), whereas blood levels of 36 mg. % were tolerated when artificial respiration was provided.

Rapid intravenous injections of 5 to 50 mg. per kilogram cause a transient fall in blood pressure in cats and dogs which increases with increasing dosage of thiamine. The fall in blood pressure is not influenced by atropine or antihistamines; it may be accentuated after adrenergic blockade with dibenamine.\(^5\) \(^6\) There is evidence that the fall in blood pressure is due to thiamine acting at several sites: on the vascular smooth muscle itself, on the vasomotor center, and on the heart. Perfusion experiments on the rabbit's ear and on various arterial areas in dogs have shown that part of the vasodilator effect obtained was due to the acidity of the highly concentrated thiamine solution. Experiments on decapitated cats in which the


\(^1\) H. Molitor and W. L. Sampson, *E. Merck's Jahresber.* 51, 3 (1936).


hypotensive effect of thiamine was markedly diminished indicate that the vasodilatation may be of central origin. A moderate decrease in heart rate following the injection of large doses of thiamine may contribute to a minor extent to the fall in blood pressure.

Thiamine has little, if any, effect on the isolated heart of the frog,\(^1\), \(^2\), \(^7\) \(^9\) or of the turtle.\(^3\) Whether the bradycardia observed in dogs\(^3\), \(^5\), \(^10\) is caused by an action of thiamine on the cardiac vagus or on the medullary centers remains undecided. Studies on the dog heart lung preparation\(^3\) have failed to show any change in heart action with concentrations of thiamine far exceeding those which produced hypotension in the intact dog.

Thiamine is without effect on the isolated intestine of rats, rabbits, and guinea pigs and on the guinea pig uterus.\(^1\), \(^5\), \(^11\) The claim that acetylthiamine has an acetylcholine-like effect on the gut\(^12\) has not been confirmed.\(^11\) In large concentrations thiamine inhibits the action of nicotine on the isolated intestine of rabbits and guinea pigs without interfering with responses to acetylcholine or epinephrine; the thiazole moiety of the vitamin, 4-methyl-5-hydroxyethylthiazole, has similar effects.\(^13\) Thiamine also prevents the rise in blood pressure induced by nicotine.\(^14\) It blocks transmission of nerve impulses through the superior cervical ganglion.\(^15\)

Besides its ganglionic depressant action at large doses, thiamine, in still larger doses, depresses the transmission of impulses to the skeletal muscle at the neuromuscular junction.\(^13\), \(^16\), \(^17\) In accord with this curare-like action, it has been found to depress the response of the skeletal muscle to acetylcholine.\(^18\), \(^19\) Curarizing effects could be demonstrated in intact mammals only under artificial respiration following excessive, otherwise lethal doses of thiamine. Curare-like paralysis of the respiratory muscle is not the cause of death by intravenous injection of thiamine, since the diaphragm responds to direct and indirect electrical stimulation at the time respiration has ceased.\(^4\) 4-Methyl-5-hydroxyethylthiazole was found to have curare-like action similar to thiamine.\(^20\)

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\(^2\) V. Ersperger, *Arch. intern. pharmacodyna mie* 63, 261 (1939).
\(^9\) H. Mazella and N. Ferrero, *Arch. intern. pharmacodyna mie* 82, 220 (1950).
Excessive doses of thiamine may produce bronchoconstriction in dogs.\textsuperscript{21} Application of a 2 to 10\% solution of thiamine directly to the motor cortex of dogs caused generalized convulsions; this effect was not obtained with either of the two moieties of the thiamine molecule.\textsuperscript{22}

Since thiamine or a thiamine-like substance has been reported to be released together with acetylcholine on electrical stimulation of cholinergic nerves,\textsuperscript{23, 24} numerous studies have been carried out with the object of studying a possible interdependence of the effects of thiamine and acetylcholine. Thiamine was found to potentiate the effects of acetylcholine on the leech muscle\textsuperscript{25, 26} and on other preparations (for references, see Minz\textsuperscript{27}). The required concentrations of thiamine were large and far beyond those found in normal tissues. Other studies on isolated organs (intestine, uterus, leech muscle, frog heart, frog rectus muscle), however, have failed to demonstrate any sensitizing effect of thiamine on the action of acetylcholine; at concentrations of 1 to 10 mg. \% in the nutrient solution, thiamine depressed the effects of acetylcholine.\textsuperscript{7, 8, 11} Thus, the influence which thiamine may exert on the reactivity of the tissues to acetylcholine appears not to be sufficiently substantiated to allow general conclusions. Thiamine in large concentrations inhibits cholinesterase.\textsuperscript{28, 29} To what extent this action may be involved in some of the pharmacodynamic effects of thiamine is difficult to assess. Lacking data on the actual acetylcholine levels in the tissues of thiamine-treated animals, there is little reason to compare thiamine to such a potent cholinesterase inhibitor as eserine. Elucidation of the interdependence between acetylcholine and thiamine has already been initiated at the biochemical level; an interdependence with regard to the pharmacological effects of the vitamin has yet to receive unequivocal substantiation.

The lethal doses of thiamine by various routes of administration have been determined in a number of species.\textsuperscript{1} On intravenous injection the lethal doses in mice were 125 mg. per kilogram; in rats, 250 mg. per kilogram; in rabbits, 300 mg. per kilogram; and in dogs, 350 mg. per kilogram. The ratios of the lethal doses on intravenous injection to those on subcu-

\textsuperscript{21} M. Post and J. A. Smith, \textit{Am. J. Physiol.} \textbf{163}, 742 (1950).

\textsuperscript{22} M. V. Dias, \textit{Science} \textbf{105}, 211 (1947).


\textsuperscript{24} A. von Muralt, \textit{Nature} \textbf{152}, 188 (1943).


\textsuperscript{26} F. von Bruecke and H. Sarkander, \textit{Arch. expptl. Pathol. u. Pharmakol.} \textbf{195}, 218 (1940).


taneous and oral administration were found to be 1:6:10. These data on lethal doses in mice and rabbits have been confirmed. In monkeys, intravenous administration of 200 mg. per kilogram failed to elicit any symptoms, and only 600 mg. per kilogram caused the first toxic symptoms. It is interesting to note that dogs and particularly monkeys are less sensitive than rodents. Lethal doses of thiamine mononitrate as determined in mice and rabbits were not significantly different from those of thiamine hydrochloride.

On intravenous injection of 50 mg. per kilogram daily for a period of 4 weeks, rabbits failed to show loss in weight, or other toxic manifestation. No pathologic tissue changes were found on autopsy. Rats have been maintained for three generations on a daily intake of 0.08 to 1.0 mg. of thiamine, i.e., doses up to one hundred-fold of the daily requirement for the vitamin, without any untoward effects. Other observations that daily subcutaneous injections of 0.1 mg. of thiamine, though not affecting growth, caused impairment of lactation and cannibalism and decreased fertility in the second generation can hardly be taken as evidence of thiamine toxicity, since these experiments were inadequately controlled, and the same manifestations were obtained in rats without thiamine injections on adding manganese chloride to that particular diet. In the light of subsequent discovery of other nutritional factors essential for the rat, these effects were more likely due to inadequacy of the diet than to the injections of thiamine. Prolonged daily administration of 1 mg. of thiamine to weanling rats maintained on a diet deficient in another B vitamin (riboflavin, pyridoxine, or pantothenic acid) failed to cause significant effects on the weight of these animals or on the manifestations of their deficiency state.

The data on acute toxicity and the absence of evidence of cumulative toxicity give evidence for the very large therapeutic margin of thiamine. The ratio between the daily requirement for thiamine and its lethal dose has been variously estimated at from 600 to 70,000 (depending on species and route of administration).

No toxic effects of thiamine administered by mouth have been reported in man. Parenterally, doses of 100 to 500 mg., in single and repeated injections, have been given to patients. Toxic or other effects have not been

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noted on many thousands of injections by either the subcutaneous, intramuscular, intraspinal, or intravenous route in doses which in many cases were from one hundred to two hundred times larger than the daily maintenance dose. These excessive amounts have been well tolerated apparently without any noticeable or measurable effects on circulation, respiration, or other organ systems. A nut-like taste has been reported on injection of large amounts of thiamine; this taste sensation has been used as criterion for the measurement of circulation time by intravenous injection of 300 mg. of thiamine.\textsuperscript{37}

In relatively rare instances, thiamine has caused reactions resembling anaphylactic shock in man. Such reactions have been recorded in over two hundred cases in the world literature (for extensive case references, see Jaros et al.\textsuperscript{3}). All reactions have occurred exclusively on parenteral administration. They consist in their milder form of a feeling of burning and warmth, urticaria, weakness, restlessness, sweating, nausea, tightness of the throat and chest, dyspnea, hypotension, and tachycardia. In more severe cases the symptoms may rapidly progress to angioneurotic edema, cyanosis, pulmonary edema, hemorrhage into the gastrointestinal tract, and collapse. Five cases of sudden death following intravenous or intramuscular injection of thiamine have been reported.\textsuperscript{38-41} The signs and symptoms of these reactions\textsuperscript{42} are those of anaphylactic shock. Their onset follows the injection within minutes. The patient may, in milder cases, recover quickly. Treatment directed against the symptoms generally consists in injection of epinephrine, artificial respiration, administration of oxygen, and analeptics such as caffeine. The occurrence of these reactions and their severity is not related to the dose of thiamine injected, which has varied between 5 and 100 mg. The great majority of patients in which these reactions have been observed had previously tolerated parenteral injection of equal amounts of thiamine without any untoward effects. Thus, they apparently developed a hypersensitivity to thiamine. Only in rare instances\textsuperscript{43, 44} have such reactions been observed on the first known injection of thiamine. Most of the reactions have been reported after four to ten or more preceding injections.

These reactions are caused by thiamine and not by other solutes, solvents, or preservatives in the solutions which were injected, since they

\textsuperscript{40} Fornara, cited by F. Dotti, \textit{Minerva med.} 1, 720 (1949).
\textsuperscript{42} C. G. Weigand, \textit{Geriatrics} 5, 274 (1950).
\textsuperscript{43} M. M. Mitrami, \textit{J. Allergy} 15, 150 (1944).
\textsuperscript{44} J. Seusing, \textit{Klin. Wochschr.} 29, 394 (1951).
have occurred alike with preparations from different manufacturers and also with aqueous solutions of crystalline thiamine hydrochloride.

Since the symptomatology of these reactions is consonant with most, if not all, aspects of anaphylactic shock, the most likely explanation for the mechanism of the reactions seems to be an anaphylactic one. In many of the cases the observers have obtained immediate whealing on intradermal injection of thiamine, and, in some, positive transference of the sensitivity has been accomplished. Since the manifestations of the thiamine reactions are those known to occur with certain immunologic alterations and since these patients have been shown to have such immunologic alterations, it would appear reasonable to associate the two. It is conceivable that a combination of thiamine with protein develops which is antigenic to the host. The evidence is, at present, not conclusive, and the anaphylactogenic properties of thiamine require further investigation. If thiamine is an obligatory whealing agent, positive intradermal tests may not be valid proof of individual sensitivity. Attempts to sensitize rabbits by massive and repeated injections of thiamine have failed.

Positive patch tests obtained in individuals with these reactions do not present immunologic evidence for the immediate anaphylactic type of reaction. They rather indicate the existence of the delayed eczematous type of hypersensitivity which would be the immunologic substrate for the contact dermatitis type of reaction. In persons handling pharmaceutical preparations of thiamine, occurrence of contact dermatitis on the hands and forearms has been observed.

Recently, on the basis of the similarity of hypotensive effects of thiamine, acetylcholine, and histamine on intravenous injection in dogs, the suggestion has been made that overdoses of thiamine may cause an accumulation of acetylcholine in excessive quantities in tissues which in turn may be responsible for the occurrence of the untoward reactions observed in man. Such an explanation lacks experimental evidence: the sudden onset of these reactions, lack of correlation to dose administered, limitation to parenteral injection, the manifestations comprising the entire spectrum of anaphylactic signs and symptoms, and other facts militate against such an assumption.

XII. Requirements and Factors Influencing Them

A. OF ANIMALS

B. C. P. JANSEN

The animal body is unable to store thiamine to any large extent. An adult human body does not contain more than about 30 mg. of thiamine. As the body continually loses thiamine in the urine, feces, and perspiration, it needs a constant supply.

It is difficult to find an exact criterion for measuring the requirement of an animal. The growth curve of young animals is most frequently used as a criterion. However, a drawback is that the curve indicating the influence of the thiamine content of the diet on the growth of the animal is an asymptotic one. Thus it is difficult to fix the maximum (or "normal") growth. Other methods used are the influence of the diet on the thiamine content of the blood or on normal or abnormal metabolism, i.e., on the pyruvic acid content of the blood.

As a thiamine deficiency produces anorexia, Cowgill\(^1\) used the "normal appetite" as a criterion.

Furthermore it is assumed that a certain (minimum) amount of thiamine is essential to keep an animal alive, to promote normal growth, and to protect it from polynecritis. On the other hand, the work of Rasmussen \(et\ al.\)\(^2\) and of Foster \(et\ al.\)\(^3\) clearly demonstrated that mice are more resistant—or, as Schneider\(^4\) puts it, less susceptible—to a certain strain of poliomyelitis virus if the thiamine content of the diet is reduced to an amount below the content that is required in other respects. Therefore it seems that a diet which may be considered thiamine deficient gives these animals better protection against poliomyelitis.

It is obvious that a great many factors exert influence on the requirement of thiamine in animals. The factors studied in the greatest detail are:

1. Size of the animal.
2. Composition of the diet.
3. Physical state of the animal (hyperthyroidism, pregnancy, lactation, fever, age, etc.).
4. Climate (temperature).
5. Intestinal microflora.
7. Performance of muscular work.

\(^4\) H. A. Schneider, \textit{Vitamins and Hormones} 4, 35 (1946).
(1) *Size of the Animal.* We know that the rate of the metabolism of an animal depends upon its body surface. Thus we expect that the requirement of thiamine, an agent in carbohydrate metabolism, will also depend on the surface area of the body. Cowgill, in studying the thiamine requirements of mice, rats, pigeons, dogs, and human beings, found that their requirement is proportional to their weight.0.06

As the metabolism is connected with the amount of calories an animal consumes per day, it is probably better not to indicate the amount of thiamine an animal needs per day but to record the thiamine content of the food (or, still better, the relation between thiamine and carbohydrate intake: see below).

For the thiamine requirements of different kinds of animals, see: for the growing rat, Brown and Sturtevant;5 for the guinea pig, Mannering;6 for the mouse, Morris;7 for chicks, Bird;8 and for pigeons, Bird.9

(2) *Composition of the Diet.* Thiamine plays a role in carbohydrate metabolism. Thus, in the first place, the thiamine requirement depends on the carbohydrate content of the diet. More than two decades ago Evans and Lepkovsky10 found the "thiamine-sparing" action of fats. Several other authors confirmed this action.

Proteins11 and alcohol12,13 also have a thiamine-sparing action. These components of the diet may depress the thiamine requirement practically to zero. The most probable deduction from this fact is that thiamine is probably not involved in the enzyme system necessary for the metabolism of fats, etc. This was confirmed by the work of de Caro and Rindi.14 These authors produced a state of athiaminosis in rats by feeding them a thiamine-deficient diet, demonstrated by a rise in the pyruvic acid level of their blood. Addition of fat to the diet reduced the pyruvic acid level to normal.

Part of the thiamine-sparing action probably is caused not only by the reduction in carbohydrates in the diet, but also by microbial syntheses of thiamine in the gut (see Section XII A (5)).

We may mention here also the presence of antithiamines or of thiaminase, each of which increases the requirement for thiamine.

(3) Physical State of the Animal. As thyroxine regulates the (basal) metabolism, it is to be expected that hyperthyroidism or the feeding of extra doses of thyroxine will increase the requirements of thiamine. The work of several investigators has confirmed this supposition (e.g., Himwich et al.,\textsuperscript{15} Cowgill and Palmieri,\textsuperscript{16} Drill and Sherwood,\textsuperscript{17} and Peters and Rossiter.\textsuperscript{18} A review of this work is given by Drill.\textsuperscript{19}

It is obvious also that pregnancy, in particular during the latter half, and lactation increase the requirements of thiamine. There are reports that the thiamine requirement of a rat successfully nursing a litter is five times as large as normal (Evans and Burr,\textsuperscript{20} Sure,\textsuperscript{21} and Sure and Walker\textsuperscript{22}).

Mills et al.\textsuperscript{23} have demonstrated that the thiamine requirement of rats per gram of diet increases greatly with old age. The most probable explanation for this fact is the supposition that the efficiency of thiamine utilization is diminished.

Gerrits\textsuperscript{24} observed that 38 infants, 0 to 2\textsuperscript{1/2} months of age, never excrete thiamine in the urine, independent of their nutrition. Hamil et al.,\textsuperscript{25} working with an improved method for the determination of thiamine, also found low values for the thiamine in the urine during the first days of life. In this respect it is interesting that the thiamine pyrophosphate content of the blood of newborn infants is much higher than the content of the blood of adults.\textsuperscript{26a}

(4) The Climate (Temperature). Kline et al.\textsuperscript{26} stated that by raising the environmental temperature from 78°F. to 90°F. the thiamine requirement of the rat is decreased.

Hegsted and McPhee\textsuperscript{27} later found that on lowering of the environmental temperature the thiamine requirement of rats increased considerably. At 78°F. the requirement of adult rats amounted to 164 to 168 \( \gamma \) of thiamine per 1000 non-fat calories; at 55°F. the figures were 191 to 203 \( \gamma \).

\textsuperscript{17} V. A. Drill and C. R. Sherwood, \textit{Am. J. Physiol.} \textbf{124}, 683 (1938).
\textsuperscript{20} H. M. Evans and G. O. Burr, \textit{J. Biol. Chem.} \textbf{76}, 263 (1928).
\textsuperscript{21} B. Sure, \textit{J. Biol. Chem.} \textbf{76}, 685 (1928).
\textsuperscript{22} B. Sure and D. J. Walker, \textit{J. Biol. Chem.} \textbf{91}, 69 (1930).
\textsuperscript{23} C. A. Mills, E. Cottingham, and E. Taylor, \textit{Arch. Biochem.} \textbf{9}, 221 (1916).
\textsuperscript{24} W. B. J. Gerrits, Thesis, Amsterdam Noord-Hollandsche Uitgever Maatschappij, 1940.
This is in agreement with the results of the work of Ershoff, who found that rats could survive on a thiamine-deficient diet for an average of 64.7 days at 23° (about 74°F.), whereas on the same diet the average surviving time was only 27.6 days at 2° (approximately 36°F.).

Furthermore Sarett and Perlzweig demonstrated that with a thiamine-rich diet the tissues laid down by rats at 91°F., were twice as rich in thiamine as the tissues from rats given the same diet at 75°F.

On the other hand Mills found that rats require twice as much thiamine at 91°F. as at 65°F. He explains this by the heavy perspiration at the higher temperature. In experiments on chicks, Mills et al. were able to establish the fact that the thiamine content of the diet required for protecting the animals from polyneuritis was three times as high at 90°F. as at 70°F. Mills points out that 2 to 3 weeks are required for metabolic adaptation to heat, and he believes that the neglect of this fact may explain the different results of Kline et al.

Edison et al. from their experiments came to the conclusion that the thiamine requirements for the growth of rats in a tropical environment (90°F. and 70% relative humidity) were not greater and may be less than in temperate conditions (72°F. and 50% relative humidity).

Considering these conflicting results, it is obvious that other factors also change at different temperatures, so that it is not a simple problem to find the sole influence of the temperature. Kline et al. tried to eliminate the influence of different levels of food intake at different temperatures by giving the thiamine-free diet and the additional thiamine separately.

Not so much from the sum total of all these results but more from a priori reasoning an optimal temperature for a minimum thiamine requirement probably will be found; above and below this temperature the requirement will be higher. However, it is to be expected that this optimum temperature will not be a fixed one but will also depend on other factors, e.g., on humidity. At all events there seems to be a great difference in thiamine requirement at varying temperatures.

(5) Intestinal Microflora. Several of the B vitamins are synthesized by the microorganisms in the gut, some of them to such an extent that this synthesis may replace the intake by food.

In some cases this is also true for thiamine. Thus as far back as 1915

30 C. A. Mills, Am. J. Physiol. 133, 515 (1941).
33 C. A. Mills, Nutrition Revs. 4, 95 (1946).
Theiler et al.\textsuperscript{35} demonstrated that ruminants may be sustained on a thiamine-deficient food: the thiamine is produced by the flora of the rumen.

Fridericia \textit{et al.}\textsuperscript{36} found that rats that normally need the thiamine from their food can produce sufficient thiamine in their intestines, if the diet contains a large amount of fresh potato starch. They called this phenomenon "refection."

Under normal circumstances, however, all non-ruminant higher animals depend on their diets for their supply of thiamine. Apparently no one has yet undertaken the experiments to feed animals on a carbohydrate-free diet to see whether those animals and also the next generation can normally live a whole lifetime without thiamine. However, Dann\textsuperscript{37} was able to maintain rats for more than a year (about half the lifetime of a rat) on a thiamine-free, carbohydrate-free synthetic diet. It is possible that the refection—the production of thiamine by the microorganisms in the gut—in this case is sufficient to produce enough thiamine for protein and fat metabolism.

(6) \textit{Individual Genetic Factors}. Practically all initial research on nutrient requirements has been performed with large groups of animals or human beings.

Already Ancel Keys, in his carefully conducted experiments with healthy volunteers who were maintained for several months under strictly controlled conditions, observed that one "normal" person may excrete two or even three times as much thiamine as another "normal" person on exactly the same diet (Mickelson \textit{et al.}\textsuperscript{38}). These volunteers were all "normal" young men with no history, signs, or symptoms of nutritional, digestive, or metabolic peculiarities. Just recently, Williams\textsuperscript{39} has stressed the fact that individual requirements may differ widely. Thus the need for thiamine in man may vary from 0.5 to 1.5 mg. daily. Therefore it is possible that the quantity contained in a certain nutrient, which is sufficient for the average person or animal, may be too low for some individuals, depending on their genetic makeup. Williams coined the term "genetotropic diseases" for diseases that are caused by a genetically larger requirement of a nutrient in a certain individual.\textsuperscript{39} Everyone experimenting with animals knows that even in largely inbred rats individual requirements are widely different. Therefore it is important to work with groups of at least eight to ten, but preferably with even larger groups of animals, to obtain reliable average


\textsuperscript{36} L. S. Fridericia, P. Freudenthal, S. Gudjonsson, G. Johansen, and N. Schoubye, \textit{J. Hyg.} 27, 70 (1928).

\textsuperscript{37} W. J. Dann, \textit{Federation Proc.} 4, 153 (1945).


\textsuperscript{39} R. J. Williams, L. J. Berryand, and E. Beersteether, Jr., \textit{Arch. Biochem.} 23, 275 (1949).
results for the requirements of animals. Light and Cracas\textsuperscript{40} determined the thiamine requirements of different strains of white rats; one strain needed twice the amount of thiamine as another strain to obtain the same growth rate.

(7) Performance of Muscular Work. We know that thiamine is essential for carbohydrate metabolism. Therefore animals doing heavy muscular work should require more thiamine than those at rest. Of course this holds true only if carbohydrates are metabolized. Otherwise thiamine requirements are not increased during heavy work. This has been demonstrated recently by a series of experiments by Gruber and Ruys.\textsuperscript{41} They compared the thiamine pyrophosphate contents in breast muscle, heart, and liver of carrier pigeons which had performed an uninterrupted flight of about 140 miles with the corresponding contents of resting pigeons. Considering the work expenditure and the available carbohydrates in the bodies of the pigeons, Gruber and Ruys calculated that at least 80\% of the calories for the flight must have come from fat metabolism, and only a very small percentage was supplied by carbohydrates. In accordance with these facts, the thiamine content of the organs proved not to have been decreased by the heavy work expenditure during the flight.

Taking into account all these factors that influence the thiamine requirement (and there are several others on which research has been scanty or nil), it is clear that it is impossible to state precisely the daily required amount for a certain animal. Even so it is possible to indicate a certain quantity for the requirement per 100 g. of diet containing at least 60\% of carbohydrates. This content is about 100 to 150 $\gamma$. There is a surprising agreement between different investigators for different kinds of animals: for pigeons,\textsuperscript{42} for rats,\textsuperscript{43} for chicks,\textsuperscript{44, 45} and even for man.\textsuperscript{46}

As with other nutrients, life is possible at different levels of thiamine intake. To find out the optimal intake, Byerrum and Flokstra\textsuperscript{47} determined the thiamine and the thiamine pyrophosphate content of liver, muscle, and brain of rats fed on different levels of thiamine. As the level of thiamine was increased up to 200 $\gamma$ per 100 g. of food, the thiamine pyrophosphate increased; beyond that level no further increase was found. Normal growth took place even on 100 $\gamma$ of thiamine per 100 g. of food. Therefore for

\textsuperscript{40} R. F. Light and L. J. Cracas, \textit{Science} \textbf{87}, 90 (1938).
\textsuperscript{44} A. Arnold and C. A. Elvehjem, \textit{J. Nutrition} \textbf{15}, 463 (1938).
maximal cocarboxylase content of these tissues twice the amount is required as is needed for normal growth. We cannot tell whether this maximal intake of thiamine has any advantage for the animal or not.

B. OF MAN

W. H. SEBRELL, JR.

The principal factors influencing thiamine requirements are the carbohydrate and the calorie intake. The requirement for thiamine is reduced when fat forms a large part of the diet, but for practical purposes the thiamine need may be based on the total calorie intake.

An early appraisal of the thiamine requirements of man was made by Cowgill in 1934. This appraisal was based on an analysis of dietary data in the literature in relation to the occurrence of beriberi. On the basis of this data the minimum intake of thiamine necessary to prevent beriberi is not less than 0.28 mg. per 1000 cal. (60-kg. man on 2500 cals.) or a total per day of 0.7 mg.

Elsom et al. in a study on women volunteers concluded that 0.65 mg. per day was the minimum intake necessary to maintain health.

Melnick, using saturation tests, reported that adults required 0.35 mg. per 1000 cal. or 0.875 mg. per day on a 2500-cal. diet.

Williams et al. found that an intake of 0.22 mg. of thiamine per 1000 cal. caused a slow depletion of tissue reserves and with an intake of 0.45 mg. per 1000 cal. there was a slight depletion of cocarboxylase.

Keys et al. studied the performance of normal young men on controlled thiamine intakes and found that for a period of 10 to 12 weeks no benefit of any kind was observed with intakes of more than 0.23 mg. of thiamine per 1000 cal. (intake 3050 ± 200 cal. per day).

In 1944 Holt critically reviewed the studies of experimental thiamine deficiency by Williams, Elsom, Keys, and Najjar and their collaborators and reinterpreted the data to conclude that the minimum thiamine requirement of an adult man on a diet of natural foods lies between 0.17 and 0.23 mg. per 1000 cal. He concludes that a range of intake between 0.24 mg. and 0.44 mg. per 1000 cal. appears to protect against thiamine deficiency.

Oldham et al. found no change in blood thiamine levels with intakes above 0.2 mg. per 1000 cal.

Foltz et al., using four medical students under observation in a hospital, found that a daily intake of 0.2 mg. per 1000 cal. resulted in deficiency symptoms within 8 weeks. It is their opinion that the minimum daily requirement of thiamine for young adult men is from 0.33 to 0.45 mg. per 1000 cal.

Keys et al. in further studies on men with restricted intake of the B vitamins found that an intake of 0.185 mg. of thiamine per 1000 cal. (daily intake 3300 cal.) was slightly less than entirely adequate.

Glickman et al. found a daily intake of 0.4 mg. per 1000 cal. entirely adequate.

In an attempt to resolve some of the differences of opinion concerning the minimum human requirement for thiamine, a study was conducted by Horwitt et al. under the auspices of the National Research Council. These investigations showed that 0.4 mg. of thiamine (0.18 mg. per 1000 cal.) was below the minimal requirement of relatively inactive men on 2200 cal. daily.

On the basis of all the evidence available, therefore, the opinion of the National Research Council that the minimal thiamine requirement for adults is 0.23 mg. or more per 1000 cal. is well founded. On this basis and in order to allow a suitable factor of safety for individual variation, differences in type of diet, and variations in body stores, which are never large and easily depleted by various stresses, an intake of 0.5 mg. of thiamine per 1000 cal. is recommended by the National Research Council as a safe allowance for adults at ordinary low levels of calorie intake.

The thiamine requirement of the infant on a calorie basis is similar to that of the adult. Knott et al. concluded that young infants have a minimum thiamine requirement of approximately 0.2 mg. daily, which can just be met if its mother’s milk contains 20 γ or more of thiamine per 100 ml. They suggest that 0.4 mg. of thiamine per kilogram may be a practical standard for the ordinary needs of the young infant. The average thiamine content of human milk was found to be about 0.15 mg. per liter. A more critical analysis showed 0.2 mg. per liter in a group of women whose infants were receiving no other milk in contrast to an average of 0.09 mg. per liter in the milk of women whose infants required supplementary feeding.

54 E. E. Foltz, C. J. Barborka, and A. C. Ivy, Gastroenterology 2, 323 (1944).
Holt et al.\textsuperscript{60} found the thiamine requirement of seven infants to vary between 0.14 mg. and 0.20 mg. per day on the basis of a urinary excretion test. With an average thiamine content of cow’s milk of 0.35 to 0.4 mg. per liter, an infant weighing 7 kg. is calculated to receive at least 0.3 mg. of thiamine a day, but this makes no allowance for destruction by heat in pasteurization or sterilization. The margin of safety is, therefore, regarded as small by Holt and coworkers in the case of either sterilized milk or breast milk, since the latter contains roughly only half as much thiamine as cow’s milk.

The meager data available on the thiamine requirement in pregnancy and lactation\textsuperscript{61-63} indicate that in relation to calories the requirement may be considered to be in the same proportion as for infants and adult men.


# Chapter 17

**THE TOCOPHEROLS**

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I. Nomenclature

ROBERT S. HARRIS

Accepted names: Vitamin E
Tocopherol

Obsolete names: Factor “X”
Antisterility vitamin

Empirical formulas: \( \alpha \)-Tocopherol: \( \text{C}_{29}\text{H}_{50}\text{O}_{2} \)
\( \beta \)-Tocopherol: \( \text{C}_{28}\text{H}_{48}\text{O}_{2} \)
\( \gamma \)-Tocopherol: \( \text{C}_{28}\text{H}_{48}\text{O}_{2} \)
\( \delta \)-Tocopherol: \( \text{C}_{27}\text{H}_{46}\text{O}_{2} \)

Chemical names: \( \alpha \)-Tocopherol: \( 2,5,7,8 \)-tetramethyl-2-(4',8',12'-trimethyldecyl)-6-chromanol, or 5,7,8-trimethyltocol
\( \beta \)-Tocopherol: \( 2,5,8 \)-trimethyl-2-(4',8',12'-trimethyldecyl)-6-chromanol, or 5,8-dimethyltocol
\( \gamma \)-Tocopherol: \( 2,7,8 \)-trimethyl-2-(4',8',12'-trimethyldecyl)-6-chromanol or 7,8-dimethyltocol
\( \delta \)-Tocopherol: \( 2,8 \)-dimethyl-2-(4',8',12'-trimethyldecyl)-6-chromanol, or 8-methyltocol

Structures:

\[ \begin{align*}
\text{H}_3\text{C} & \quad \text{CH}_3 & \quad \text{CH}_3 & \quad \text{CH}_3 & \quad \text{CH}_3 \\
7 & \quad 8 & \quad 1 & \quad 2 & \quad 3 \\
\text{O} & \quad \text{(CH}_3)_2\text{CH(CH}_2)_2\text{CH(CH}_2)_2\text{CHCH}_3 \\
\text{HO} & \quad \text{CH}_3
\end{align*} \]

\( \alpha \)-Tocopherol
II. Chemistry

HENRY A. MATTILL

Vitamin E was first recognized by at least three groups of investigators\(^1\text{-}^3\) as a fat-soluble substance necessary for reproduction in the rat. In place of the original "X factor,"\(^4\) the designation of "E" was suggested by Sure,\(^2\) since this was the next letter in sequence following the identification of the antiricketic vitamin as a separate entity. After the isolation of vitamin E in purity,\(^4\) the name tocopherol was proposed (from the Greek, tokos (offspring), pherein (to bear), and ol, to signify an alcohol). Because of the multiple nature of vitamin E, the new name is used in the plural in the generic sense, and it is supplied with Greek letter prefixes, \(\alpha, \beta,\) etc., for

\(^1\) H. M. Evans and K. S. Bishop, *J. Metabolic Research* 3, 233 (1923); *J. Am. Med. Assoc.* 81, 889 (1923).
\(^2\) B. Sure, *J. Biol. Chem.* 58, 693 (1924); 62, 371 (1924); 63, 211 (1925).
designating the individual specific types. Without these prefixes the term tocopherol is considered synonymous with tocopherols and vitamin E.

Unique in the history of vitamins was the organization of two international symposia dealing with vitamin E. The first, in London in 1939, was sponsored by the Nutrition Panel of the Society of Chemical Industry; the second, in New York ten years later (lacking one week), under the auspices of the New York Academy of Sciences and marking about the twenty-fifth birthday of vitamin E. The volume of the proceedings of the first$^5$ (edited by Bacharach and Drummond) contains less than one hundred pages (fifteen papers and discussions). That of the second$^6$ (edited by Mason) is almost five times this size. These publications not only illustrate the advantages of such conferences for crystallizing opinions and guiding the course of further investigation, but they are also enduring records of progress. They contain valuable bibliographies, as do the many reviews to which reference will be made. Especially useful are the annotated bibliographies prepared by Merck and Company, Inc.$^7$ (1925–1941) and Distillation Products Industries$^8$ (1940–1950), which also contain references to suggested clinical applications and to many of the patents which have been granted for the synthesis of tocopherols, their starting materials and derivatives, and their use, alone and with synergists, in the stabilization of oils, fats, and other autoxidizable substances.

Had the physiological role of vitamin E not been extended far beyond the confines of reproduction, the manner of its action would still be an intriguing problem, unsolved after a quarter of a century. So many connections have been established with other biological processes that even a brief survey of them is little short of bewildering. Attempts at coordinating the activities of vitamin E under some fundamental and unifying concept have not yet been rewarded. An understanding of its chemical nature and properties, although this has progressed much farther, is not complete.

A. CHEMISTRY OF VITAMIN E

The first work on its chemical nature$^9$ indicated that, like vitamins A and D, vitamin E was to be found in the unsaponifiable portion of certain fats, that it was somewhat vulnerable to saponification, and that it was destroyed by bromination and acetylation but not by hydrogenation. Vacuum distillation caused considerable decomposition, but solvent partition as between pentane and 92% methanol effected some concentration.

$^5$ Vitamin E, a Symposium. Heffer and Sons, Cambridge, 1940.
$^7$ Alpha Tocopherol, Merck and Co., Inc., May and December, 1941.
Another approach to the understanding of its chemistry was opened by the observations that diets ordinarily adequate for reproduction gave sterile animals if the diets contained certain fats which readily become rancid or if the usual mixed diets were treated with ethereal ferric chloride.\(^{10-14}\) From the unsaponifiable portion of several vegetable oils and plant extracts concentrates were prepared\(^{15}\) which contained antioxidants (called inhibitols\(^ {16}\) because of the presence of \(\alpha\)- or \(\beta\)-hydroxyl groups) and also vitamin E,\(^ {17,18}\) as judged by bioassays.

From these observations, either of two conclusions could be drawn: (1) that vitamin E was readily oxidized and was protected by the accompanying antioxidants, or (2) that the vitamin and the antioxidant were the same substance. The latter view was favored by the fact that the two could apparently not be separated, but it was not acceptable because the biological and antioxygenic activities were not parallel from one concentrate to another. The uncertainty and confusion were resolved by the demonstration\(^ {19}\) that there were several substances with unequal vitamin E activity and whose antioxygenic action also varied but in the opposite direction.

The several kinds of vitamin E have been shown to be the principal antioxygenic components of natural fats, and their possible function as "biological antioxidants" has been the subject of many discussions and publications.\(^ {20}\)

The parallel observations on the vitamin E and the antioxidant content of concentrates\(^ {21}\) confirmed the earlier findings of Evans and Burr\(^ {9}\) except for the biological inactivation of vitamin E by acetylation; the presence of a hydroxylic group was demonstrated, and the benzoic acid ester as well as the acetylated compound was shown to be biologically active. A previously observed band in the ultraviolet spectrum with maximum at 2940 \(\lambda\) was believed not to be related to vitamin E,\(^ {22}\) since concentrates from other sources, such as palm oil, showed the same band but were biologically inactive.


Drummond and his colleagues\(^{23}\) prepared very potent concentrates from wheat germ oil, being the first to use selective adsorption on aluminum oxide. They confirmed many of the previous observations including this maximum adsorption at 2940 A. with a minimum at 2670 A. Whether it was characteristic of the vitamin or of an associated substance, the band "persisted" in proportion to the vitamin activity when this was destroyed by ultraviolet radiation.

Pure vitamin E was first isolated by Evans and the Emersons\(^{4}\) from the unsaponifiable matter of wheat germ oil. By the use of cyanic acid on the unsaponifiable fraction, three solid amorphous aliphonates were obtained. The alcohol regenerated from one of these, when given in a single dose of 3 mg., always enabled vitamin E-deficient rats to bear young. It was named \(\alpha\)-tocopherol. Of the alcohols obtained from the other two aliphonates, one had less biological potency (later called \(\beta\)), the other none at all. \(\alpha\)-Tocopherol was a light-yellow viscous oil which could not be crystallized, but its conversion to another crystalline derivative (with \(p\)-nitrophenyl isocyanate), followed by reconversion to the aliphonate and regeneration of the alcohol, left its biological activity unimpaired. The peak of its absorption band was at 2980 A., and microanalysis indicated a provisional formula, \(C_{29}H_{50}O_2\). Soon thereafter the same \(\alpha\)-tocopherol was isolated from cotton seed oil,\(^{24}\) and later\(^{25}\) still another active substance, \(\gamma\)-tocopherol, was obtained from the same source. Further investigation revealed that only \(\alpha\)-tocopherol is present in lettuce and that palm oil is qualitatively similar to cottonseed oil and contains no \(\beta\)-tocopherol.

In several other laboratories, tocopherols were isolated from wheat germ oil, the \(\beta\) by Todd and his coworkers\(^{26}\) in London, neotocopherol by Karrer and his colleagues\(^{27},^{28}\) in Zurich, and cumotocopherol by John\(^{29}\) in Göttingen, the name indicating that pseudocumohydroquinone (trimethylhydroquinone) was produced from it by thermal decomposition. Both products were probably identical with \(\beta\)-tocopherol.\(^{30}\) The preponderance of \(\beta\)-tocopherol in European wheat germ oil and of the \(\alpha\) form in that of California has not been explained.

The successful chemical identification of α-tocopherol by Fernholz\textsuperscript{31} began with the isolation of duroquinone (tetramethylhydroquinone) from the products of its thermal decomposition. Dehydrogenation by selenium\textsuperscript{32} also produced it, and the British workers\textsuperscript{30} obtained traces of it from concentrates along with pseudocumoquinone from pure β-tocopherol.

Fernholz’s suggestion\textsuperscript{31} that α-tocopherol might be a mono ether of duroquinone was shown to be untenable by them\textsuperscript{30} and by John,\textsuperscript{33,34} Karrer,\textsuperscript{35} Drummond,\textsuperscript{36} and their coworkers, most of whom proposed a coumaran or chroman nucleus with a side chain (Karrer) of isoprene residues as in phytol (the alcoholic portion of chlorophyll). Fernholz\textsuperscript{37} also concluded from the dissimilarities of the absorption spectra of ethers of duroquinone and of tocopherol that this did not have a simple ether structure. Thermal decomposition of tocopherol produced not only durohydroquinone but also an unsaturated aliphatic hydrocarbon. Mild oxidation of this substance by chromic acid produced a lactone, C\textsubscript{21}H\textsubscript{40}O\textsubscript{2}, whose free hydroxy acid was so readily relactonized that a tertiary hydroxyl had to be postulated. More vigorous oxidation yielded dimethylmaleic anhydride, an acid C\textsubscript{16}H\textsubscript{32}O\textsubscript{2}, along with a ketone C\textsubscript{18}H\textsubscript{36}O, diacetyl, and acetone. Particularly the formation of a 16-carbon acid limited the number of possible structures for the lactone. After close and skillful organic chemical reasoning, the structure he proposed for α-tocopherol was that of a substituted 6-hydroxychroman, with a long aliphatic side chain attached to the pyran ring.

Intensive work in other laboratories confirmed the presence of a chroman nucleus; degradation studies by John and his coworkers\textsuperscript{38} demonstrated this nucleus with two substituents on carbon 2 (adjacent to the chroman oxygen), thus providing a tertiary ether ring and optical activity, and Smith and his coworkers\textsuperscript{39} came to the same conclusion by still different organic chemical procedures and reasoning.

Meantime, still favoring the coumaran structure, but undecided, Karrer and his coworkers\textsuperscript{40} accomplished an almost quantitative condensation of

\textsuperscript{33} W. John, E. Dietzel, and P. Günther, Z. physiol. Chem. 252, 208 (1938).
\textsuperscript{34} W. John, Z. physiol. Chem. 252, 222 (1938).
\textsuperscript{37} E. Fernholz, J. Am. Chem. Soc. 60, 700 (1938).
\textsuperscript{38} W. John, E. Dietzel, P. Günther, and W. Finte, Naturwissenschaften 26, 366 (1938).
trimethylhydroquinone with phytyl bromide, zinc chloride being the catalyst. Except for lacking optical activity the product had physical and chemical properties identical with those of naturally occurring vitamin E. Later\(^1\)\(^2\) this synthetic product was shown also to have practically the same physiological properties as vitamin E, and thus a unique event happened in organic-biochemical history—the synthesis of a complex substance before its actual constitution was known!

A span of approximately fifteen years had elapsed between the first recognition of the existence of vitamin E and the synthesis of physiologically active tocopherol. The last two years of this period witnessed as rapid a succession of significant events as has ever been seen in any field of biochemical endeavor.

The Karrer synthesis with phytyl bromide was duplicated by Smith and his colleagues without a catalyst\(^3\)\(^4\) and by the use of dienes (phytadiene)\(^5\)\(^6\) and of allyl and crotyl compounds and butadiene.\(^7\) For the construction of the phytol chain, John and Pini\(^8\) used (tetra)farnesol, Smith and Sprung\(^9\) used citral.

Further proof for the existence of a chroman nucleus was provided by permanganate oxidation,\(^10\) which produced the same decisive lactone, and later by another method of synthesis, via carbinals,\(^11\) and by the quantitative conversion of tocopherol (from purified tocopherylquinone via a tertiary halide) into the diacetate.\(^12\)

For \(\beta\)-tocopherol, John\(^13\) and also Karrer et al.\(^14\) had proposed the same general structure as that of the \(\alpha\)-tocopherol, less one of the methyl groups, and John\(^15\) had concluded from the products of hydriodic acid treatment of \(\beta\)-tocopherol that the methyl groups were in the 5,8 positions. Emerson\(^16\) confirmed the general structure by obtaining the same lactone from both \(\beta\)- and \(\gamma\)-tocopherol. Both these dimethyl compounds had been synthesized from dimethylhydroquinones and phytyl bromide,\(^17\)\(^18\) the \(\beta\) from the \(\alpha\)-

and the γ from the ortha, and the products had biological activity comparable with that of the respective tocopherols. By degradation studies the structure of γ-tocopherol was confirmed. The suggestion that the unsubstituted tocopherol be called tocol was generally adopted; α-, β-, and γ-tocopherol are 5,7,8-trimethyl-, 5,8-dimethyl-, and 7,8-dimethylocotol, respectively. A fourth tocopherol, 8-methyl-(δ-)tocol, is the principal tocopherol in soybean oil and occurs, normally, in small amounts, in rendered hog fat.

Increased yields from natural sources were obtained by preliminary chromatographic adsorption of unsaponified wheat germ oil and also of the subsequent unsaponifiable portion, as well as by fractional crystallization. Natural α- and γ-tocopherol were both obtained in crystalline form from 2.5% methyl alcohol solution at −35°, and their extinction coefficients were not changed significantly by crystallization. β-Tocopherol could not be crystallized, but crystals of its azobenzene-4-carboxylate were obtained as orange plates. The practicability of molecular distillation was demonstrated; the process will be discussed elsewhere.

B. DERIVATIVES AND THEIR PHYSIOLOGICAL ACTION

The always engaging question as to the relation of organic structure to physiological action prompted the preparation of many closely related derivatives of the tocopherols. Various esters were made, some of them beautifully crystalline, including those with fatty acids and with benzoic, succinic, and phosphoric acids. Lower homologs and higher homologs

59 W. S. Singleton and A. E. Bailey, Oil & Soap 21, 224 (1944).
containing one or two ethyl groups in place of the methyl\textsuperscript{70} (or of H in 5,7-tocotol) and allyl and crotol derivatives\textsuperscript{71} were prepared, as well as compounds containing synthetic phytol or fewer isoprene units in the side chain.\textsuperscript{72} With pseudocumol as starting material and by the use of Grignard reactions, a wide variety of derivatives can be made, containing a side chain other than the phytol group; with cetyl the product was called iso-\(\alpha\)-tocopherol.\textsuperscript{73}

Most of these compounds were tested for their physiological action by the method of rat assay (see below). Even with the earlier and less precise procedures, certain general conclusions were readily apparent and are still valid. If the activity of natural \(\alpha\)-tocopherol is set at 100, that of natural \(\beta\)-tocopherol is 40, and that of \(\gamma\)-tocopherol is 8 (or less).\textsuperscript{74,75} Figures for the synthetic products were reported\textsuperscript{76} as 100:25:19. \(\delta\)-Tocopherol has only 1.\% of the biological activity of the \(\alpha\).\textsuperscript{56} By contrast, nuclear dealkylation increases antioxygenic action\textsuperscript{19,77} and resistance to atmospheric oxidation,\textsuperscript{56} but if the distinction between antioxygenic activity, as usually measured in accelerated tests at 70° to 100°, and antioxidant potency measured at body temperature is valid, the latter and the biological activity run parallel.\textsuperscript{78} Optical activity (residing in carbon 2) is very slight, but the potencies of the synthetic \(dl\) compounds (\(\alpha\) and \(\beta\)) were found to be two-thirds and one-half, respectively, of those of the natural.\textsuperscript{79} For the cure of nutritional muscular dystrophy in rabbits, the ratios of potency for the \(\alpha\), \(\beta\), and \(\gamma\) varieties were reported as 100:30:20, and the synthetic \(\alpha\) and \(\gamma\) about 90 and 30\% of the natural.\textsuperscript{80}

The biopotency of the esters is equal to that of the free alcohols, or even slightly greater, because the esters are not autoxidizable. The use of the crystalline succinate was suggested as a vitamin E standard\textsuperscript{74} in place of


\textsuperscript{73} W. John, P. Gunther, and F. H. Rathmann, \textit{Z. physiol. Chem.} 268, 104 (1941).

\textsuperscript{74} M. Joffe and P. L. Harris, \textit{J. Am. Chem. Soc.} 65, 925 (1943).


\textsuperscript{80} E. L. Hove and P. L. Harris, \textit{J. Nutrition} 33, 95 (1947).
the oily liquid \(dl\)-acetate originally proposed,\(^{81}\) 1 mg. of which was one international unit (I.U.). In terms of this unit, the biological equivalents of \(dl\)-\(\alpha\)-tocopherol, \(d\)-\(\alpha\)-tocopherol, and \(d\)-\(\alpha\)-tocopheryl acetate are 0.68, 0.92, and 1.36 I.U. per milligram, respectively.\(^{82}\)

Substitution of ethyl for one or two methyl groups lessened the activity slightly, but compounds obtained by replacement of the two \(o\)-methyl groups (7, 8) in \(\alpha\)-tocopherol by tri- or tetramethylene rings were inactive in 50-mg. doses.\(^{83}\) When the 2-methyl group was replaced by an ethyl or a propyl group, biological activity was slightly reduced.\(^{84}\) Of several methoxy-tocols, only the 5,7-dimethyl-8-methoxytocols had any biological activity (60 mg. was completely protective), and this was less than that of 5,7-dimethyltocol.\(^{85}\)

The character of the side chain is highly specific; reduction of the number of isoprene units, even by one, is inactivating, but the four units may be of natural or synthetic origin; the asymmetry of the phytol residue is unimportant.\(^{86}\)

An aminotocopherol (NH\(_2\) in place of OH) was biologically equivalent to the hydroxyl-containing compound,\(^{87}\) whether as such or after biological replacement is not known. Methyl and ethyl ethers and the allophanates are inactive.\(^{21}\)

A naphthotocopherol obtained as a by-product in the synthesis of vitamin K has the biological properties of both the vitamins, the adequate rat dose exceeding that of \(\beta\)-tocopherol.\(^{88},^{89}\)

Many of the compounds described in the comprehensive review of vitamin E (up to 1940)\(^{90}\) were demonstrated to have biological activity in massive doses, 50 to 100 mg.\(^{91}\) Not only are such amounts unphysiological, but the results may be suspect, because the control of the bioassay was inadequate according to present practice. Recently some of these compounds were shown to be inactive biologically.\(^{92}\)

\(^{82}\) P. L. Harris and M. I. Ludwig, *J. Biol. Chem.* 179, 1111 (1949); 180, 611 (1949).
C. PHYSICAL PROPERTIES

The absorption maxima, in the ultraviolet, of \( \alpha \)-, \( \beta \)-, and \( \gamma \)-tocopherol are given as 292, 297, and 298 \( \text{m} \mu \), respectively;\(^{25} \) those of the synthetic compounds are the same except for the \( \gamma \), which was at 300 \( \text{m} \mu \); the extinction coefficients\(^{25, 58, 93} \) are 70 to 73.7, 86 to 87, and 90 to 93.

Infrared spectra of some natural and synthetic tocopherols, their esters, tocopherylquinone, and hydroquinone were obtained by Rosenkrantz;\(^{94} \) bands near 8.6 \( \mu \) and 10.9 \( \mu \) are characteristic of tocopherols. Much may be expected from the application of spectrophotometric methods in the elucidation of structure of the tocopherols, their oxidation products, and derivatives.

The specific rotations of the \( \alpha \), \( \beta \), and \( \gamma \) compounds in ethyl alcohol are \(+0.32\), \(+2.9\), and \(+2.2\), respectively; in benzene those of the \( \alpha \) and \( \gamma \) are \(-3.0\) and \(-2.4\).\(^{93} \)

D. PRODUCTS OF OXIDATION

Tocopherylquinone, the first stable oxidation product of tocopherol, is a yellow compound and is obtained by the action of ferric chloride or silver nitrate. It was identified by John\(^{94} \) and is quite without activity for the rat.\(^{94} \) The same quinone was produced by Karrer et al. with various agents;\(^{100} \) with gold chloride a potentiometric titration was made the basis of a quantitative determination.\(^{101} \) This quinone can be reduced to tocopherylhydroquinone by the use of palladium in alcohol or of zinc in glacial acetic acid and is unstable unless acetylated. In strong acid solution with

\[ \text{CH}_3 \quad \text{OH} \]
\[ \text{H}_3\text{C} \quad \text{O} \quad \text{CH}_3 \]
\[ \text{O} \quad \text{C}_14\text{H}_{33} \]
\[ \alpha\text{-Tocopherylquinone} \]

\(^{97} \) C. Golumbic and H. A. Mattill, \textit{J. Biol. Chem.} \textbf{134}, 535 (1940).
\(^{98} \) M. D. Wright and J. C. Drummond, \textit{Biochem. J.} \textbf{34}, 32 (1940).
a reducing agent, the original tocopherol can be regenerated by reduction and cyclization.\textsuperscript{102}

The biological inactivity of tocopherylquinone is an indication that this reaction does not occur \textit{in vivo}, at least not as regards its effect on reproduction.

Tocopherylhydroquinone is also inactive in the rat,\textsuperscript{103} but when it was given intravenously every 5 days in a water emulsion\textsuperscript{104} to dystrophic rabbits it was equivalent to \(\alpha\)-tocopherol itself, the quinone being less effective.\textsuperscript{105} The conclusion was drawn that the hydroquinone was the antidystrophic agent, and, being unstable, it was oxidized before it could be converted to tocopherol for storage purposes.

With more vigorous oxidation of tocopherol, as with nitric acid, a red color develops. Since its depth is proportional to the amount of tocopherol, it was proposed as the basis of a quantitative method.\textsuperscript{106} This “red” quinone, which is biologically inactive\textsuperscript{107} and has resisted all attempts at crystallization, was first assigned a \(p\)-quinone structure.\textsuperscript{108, 109} It remained for Smith and his colleagues\textsuperscript{110} to show that by the action of nitric acid any substituent on carbon 5 is replaced by oxygen and that the substance was a chroman-5,6-quinone.

\begin{center}
\includegraphics[width=0.5\textwidth]{tocopherol.png}
\end{center}

\begin{center}
\textit{a-Tocopherylhydroquinone}
\end{center}

\begin{center}
\includegraphics[width=0.5\textwidth]{quinone.png}
\end{center}

\begin{center}
\textit{Chroman 5,6-quinone}
\end{center}

\textsuperscript{103} A. Issidorides and H. A. Mattill, \textit{J. Biol. Chem.} 188, 313 (1951).
The course of oxidation of the tocopherols in autoxidizing fats depends on the nature of the fat. In animal fats (containing added \( \alpha \)-tocopherol) and in vegetable fats, where tocopherols occur naturally, tocoquinones were the immediate products, but the chroman-5,6-quinone appeared only in vegetable fats. The red quinone appears to have its origin in 7,8-dimethyltoctol (\( \gamma \)), in which position 5 is occupied by \( \mathrm{H} \). This is more readily oxidized than a methyl group, but when pure \( \alpha \)-tocoquinone is gently heated in methanol containing traces of synergistic acids, such as phosphoric, sulfuric, tartaric, and others, chroman-5,6-quinone is formed along with other unidentified oxidation products of the nature of quinones.

At the same time some \( \alpha \)-tocopherylquinone is converted to \( \alpha \)-tocopherol, as demonstrated by antioxygenic and biological activity and by absorption spectrum. This is a kind of dismutation, as if the acids catalyzed the displacement of the 5-methyl group. This behavior is consistent with the more rapid production of the red quinone from \( \gamma \)-tocopherol by the action of silver nitrate and may ultimately contribute to an understanding of the unsolved kinetic problem of synergism of ascorbic, phosphoric, and other polyhydroxy acids with tocopherols. Further oxidation products have been and doubtless remain to be discovered and identified, along with dimers, but it is doubtful that any of them beyond tocopherylquinone will prove to play a role in biological processes.

By analogy with simpler quinols, the transitory existence of a semiquinone or free radical could be assumed. Proof of this was provided by the ingenious technique used by Michaelis and Wollman. A solution of tocopherol in suitable organic solvents and brought to the temperature of liquid air assumed the consistency of glass. When this was irradiated with ultraviolet light, it developed an orange-red color and absorption bands not characteristic of the quinone. Both the color and the absorption bands disappeared when the temperature was slightly elevated. Recently, fresh evidence for the existence of the free radical of tocopherol has been obtained in the author's laboratory. Univalent oxidation does not involve the opening of the ring, and if the formation of this semiquinone is not concerned in the biological action of tocopherol, it certainly finds its place in any explanation of the antioxygenic action of tocopherols in vitro.

111 C. Golumbic, *Oil & Soap* 20, 105 (1943).
115 C. Golumbic, *Oil & Soap* 19, 181 (1942); 23, 184 (1946).
119 G. E. Inglett, Unpublished data.
III. Industrial Preparation

PHILIP L. HARRIS

A. SOURCE MATERIALS

1. Natural Tocopherols

Corn, cottonseed, soybean, and wheat germ oils with tocopherol contents ranging from about 0.1 % to 0.3 % constitute the starting material for practically all natural tocopherol preparations. These oils represent a tremendous potential raw material source but one which challenges the manufacturer in his efforts to increase the tocopherol concentration a hundredfold, to at least 30 % and sometimes to 100 %, for use in pharmaceutical products.

The tocopherols in corn oil are largely \( \gamma \)-tocopherol, those in cottonseed oil are \( \alpha \)- and \( \gamma \)-tocopherols in about equal amounts, those in soybean oil

Still another partial oxidation product, \( \alpha \)-tocopheroxide, was obtained by Boyer and his associates\(^{120, 121}\) by oxidizing \( \alpha \)-tocopherol with two equivalents of ferric iron in the presence of \( \alpha, \alpha \)-dipyridyl. If treated immediately by ascorbic acid, the product could be reduced to \( \alpha \)-tocopherol, as shown by the absorption spectrum, but after some days it was irreversibly converted to tocopherylquinone; in the ultraviolet region its peak is at 2370 A.; in the infrared it lacks the characteristic absorption of the OH groups at 30 A. Its chemical properties are those of an epoxy compound, the epoxy group probably being in the 8,9 position. Given intraperitoneally in the rat test, it had about one-ninth the activity of \( \alpha \)-tocopherol, by mouth only one-thirtieth, perhaps related to its rapid oxidation to the inactive tocopherylquinone. Tocopheroxide may be the second step in the oxidation of tocopherol to its quinone.

\[ \text{2,5,7,8-Tetramethyl-2(4,8,12-trimethyltridecyl)-9,10-epoxy-6(5H)-chromanone} \]


are \( \alpha, \gamma, \) and \( \delta \) forms, and those in wheat germ oil are \( \alpha \)- and \( \beta \)-tocopherols. Consequently, concentrates made from any of these oils represent a preparation of the particular predominant tocopherol or tocopherols of the original oil. The tocopherols in the commercial concentrates of mixed tocopherols are standardized to contain 50% \( \alpha \)-tocopherol and the remainder \( \gamma \) plus \( \delta \)-, with only negligible amounts of \( \beta \)-tocopherol.

The preparation of concentrates of the \( \alpha \) form of tocopherol is of prime interest, since this form possesses the highest physiological activity. However, concentrates of the individual \( \beta, \gamma, \) and \( \delta \)-tocopherols can be prepared.

2. Synthetic Tocopherols

Trimethylhydroquinone and phytol or phytanyl chloride are most often mentioned as source chemicals for the preparation of synthetic, racemic \( \alpha \)-tocopherol. However, the synthesis can be carried out with a variety of other starting materials, but apparently with considerably more difficulty and less efficiency.

Synthetic \( \beta \) - and \( \gamma \)-tocopherols, with two methyl groups, and synthetic \( \delta \)-tocopherol, with only one methyl group, on the chroman ring, can be prepared from the properly constituted dimethyl- and monomethylhydroquinones, respectively, and phytol.

B. PROCEDURES

Details of commercial operations in the vitamin E field have not been published. However, the following references cover procedures which should be usable.

1. Natural Tocopherols

Saponification, with subsequent extraction and concentration of tocopherols in the non-saponifiable fraction, is a feasible method for preparing low potency preparations. It is used, for example, to make about an eight-fold concentration of the tocopherols of wheat germ oil (Table I). Similarly, direct, hot ethanol extraction of wheat germ, followed by low-temperature

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2 M. L. Quaife, J. Biol. Chem. 175, 605 (1948).
treatment of the extract, which freezes out much of the extracted lipid material, and by removal of solvent, yields a tocopherol-enriched oil.10

The details of procedure and equipment used for the solvent extraction of vegetable oils with liquid propane or other hydrocarbons have been described but without much information concerning the potency of the concentrates obtained.11-13 Also, fractional crystallization of cottonseed oil from solvents yields a tocopherol-containing fraction.14

Adsorption chromatography is used to prepare relatively pure individual

| TABLE I |
| Description of Some Typical Commercially Available Tocopherol Preparations |

<table>
<thead>
<tr>
<th>Product</th>
<th>Tocopherol Content, mg./g.</th>
<th>α-Tocopherol or α-tocopherol acetate</th>
<th>Color and form</th>
<th>Refractive index, 20°</th>
<th>Specific gravity, 20°</th>
<th>Absorption spectrum ε, %/1 cm.</th>
<th>Bio-potency, I.U./g. (rat sterility test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Wheat germ oil concentrate (1 to 8)</td>
<td>16</td>
<td>10</td>
<td>Dark-brown viscous oil</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>10</td>
</tr>
<tr>
<td>Natural mixed tocopherol concentrates (Type 4-50)</td>
<td>500</td>
<td>250</td>
<td>Red viscous oil</td>
<td>1.4972</td>
<td>0.9320-0.9550</td>
<td>39 at 294</td>
<td>250</td>
</tr>
<tr>
<td>d-α-Tocopherol acetate concentrates (Type 6-35)</td>
<td>500</td>
<td>350</td>
<td>Brownish-yellow viscous oil</td>
<td>1.4848</td>
<td>0.9320-0.9550</td>
<td>22.4 at</td>
<td>475</td>
</tr>
<tr>
<td>d-α-Tocopheryl acetate (Type 6-100)</td>
<td>1000</td>
<td>1000</td>
<td>Light-yellow viscous oil</td>
<td>1.4950</td>
<td>0.9500-0.9640</td>
<td>40-41 at</td>
<td>1360</td>
</tr>
<tr>
<td>Synthetic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dl-α-Tocopherol (racemic)</td>
<td>1000</td>
<td>1000</td>
<td>Clear viscous oil which darkens readily</td>
<td>1.5030</td>
<td>0.9470</td>
<td>71-76 at</td>
<td>680</td>
</tr>
<tr>
<td>dl-α-Tocopheryl acetate (racemic)</td>
<td>1000</td>
<td>1000</td>
<td>Clear yellow viscous oil</td>
<td>1.4955</td>
<td>0.9545</td>
<td>42.5 ± 1 at 283.5</td>
<td>1000</td>
</tr>
<tr>
<td>Approx.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dl-α-Tocopheryl acetate (racemic)</td>
<td>1000</td>
<td>1000</td>
<td>Clear yellow viscous oil</td>
<td>1.4972</td>
<td>0.9665</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Molecular distillation of tocopherol-containing oils has proved to be an efficient and practical procedure for preparing potent concentrates of natu-
ral tocopherols. In this process, heat-labile tocopherols are distilled without destruction at relatively low temperature and pressure, and under conditions of very short thermal exposure. Concentrates of 14 to 18% tocopherols are readily obtained from soybean oil and also from leaf meal extracts. Further processing permits even greater concentration of tocopherols.

$$\text{CH}_3$$
$$\text{HO-C}^\text{C-H}$$
$$\text{CH}_1-C=C-C-OH$$
$$\text{CH}_3$$

Trimethylhydroquinone

$$\text{H}_2$$
$$\text{HO-}$$
$$\text{C}$$
$$\text{CH}_1-C=\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{C}^\text{H}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2$$
$$\text{CH}_3$$

Phytol

$$\text{HO-C}^\text{C-H}$$
$$\text{CH}_3$$
$$\text{CH}_1-C=C-C-OH$$
$$\text{CH}_3$$

Racemic α-Tocopherol

Fig. 1. Synthesis of α-tocopherol. The asterisks indicate points of asymmetry giving rise to a mixture of isomers. The natural occurring form is d-α-tocopherol.

2. SYNTHETIC TOCOPHEROLS

Condensation of trimethyl hydroquinone and phytol under the conditions shown in Fig. 1 is conveniently carried out with almost quantitative yields of racemic α-tocopherol. This and similar reactions used commercially are modifications of Karrer’s and of Smith’s classic original synthesis in 1938.

23 W. S. Singleton and A. E. Bailey, Oil & Soap 21, 157 (1944).
C. PROPERTIES AND POTENCY OF AVAILABLE PREPARATIONS

1. Natural Tocopherols

Wheat germ oil concentrates and unsaponifiable fractions are primarily of historical interest, since, in the early days of tocopherol use, these preparations alone were available. However, there is even yet some manufacture of wheat germ oil concentrates, and, as shown in Table I, they are brown oil preparations relatively susceptible to oxidative destruction and possessing only about 10 I.U. of vitamin E activity per gram.

The second listed tocopherol preparation in Table I is typical of a type of mixed tocopherol concentrate prepared from vegetable oils in which the total tocopherol content may be 300 mg. per gram to 600 mg. per gram and the α form of tocopherol constitutes at least 50% of the total. Consequently, in the example listed, Type 4–50 concentrate contains 250 mg. of α-tocopherol per gram. Since this is d-α-tocopherol in the free alcohol form, 250 mg. furnishes a biopotency of (250 × 0.92) 230 I.U. per gram. Furthermore, the antioxidant potency of these concentrates is very high because of the free tocopherol content.

d-α-Tocopheryl acetate concentrate, Type 6-35, is representative of a series of preparations in which the tocopherol is present in the acetic acid ester form, has practically no antioxidant activity, but does have exceptional stability and high biopotency. The example in Table I supplies 475 I.U. of vitamin E per gram concentrate as the result of its content of 350 mg. of d-α-tocopheryl acetate per gram (350 × 1.36).

The last natural-type product in Table I is pure d-α-tocopheryl acetate, a light-yellow viscous oil with the highest biopotency of any vitamin E product, 1360 I.U. per gram.

2. Synthetic Tocopherols

The two synthetic preparations available are clear viscous oils with a biological potency of 680 I.U. per gram for the α-tocopherol and 1000 I.U. per gram (by definition) for the α-tocopheryl acetate. Synthetic α-tocopherol and α-tocopheryl acetate are racemic mixtures of from two to eight isomers, depending upon whether natural or synthetic phytol was used in the synthesis (Fig. 1). The separation and isolation of the natural d isomer of α-tocopherol can be effected by resolution of the bromocamphorsulfonates.

Esters of natural or synthetic α-tocopherol, other than the acetates, and tocopheramine are occasionally prepared. For example, succinates, acid

succinates, fatty acid esters, nitrobenzoates, and phosphates of \( \alpha \)-tocopherol are described\(^{34-40} \) but have remained laboratory products.

D. PRODUCTION CONTROL TESTS

Considerable experience with various physical and chemical techniques permits the generalization that the Emmerie-Engel reaction\(^{41} \) is the method of choice for production control in which total tocopherol values suffice.\(^{42-44} \) However, procedures employing eric sulfate titration of tocopherols, modifications of Kofler’s method,\(^{45} \) serve a useful purpose in control analyses on pure tocopherols. The quantity of \( \alpha \)-tocopherol in the preparation, however, is the important question and is obtained by determining the non-\( \alpha \)-tocopherol moiety by the nitroso method and subtracting this from the amount of total tocopherols determined separately.\(^{46} \) Direct determination of \( \alpha \)-tocopherol, after removal of non-\( \alpha \)-tocopherols by careful, arbitrary washing with sulfuric acid, is used by a regulatory agency in inspection control assays.\(^{47} \)

Products containing tocopherol esters must be carefully saponified before analyzing for total and individual tocopherols. Quantitative hydrolysis is possible if proper precautions to prevent tocopherol loss and destruction are observed. For example, exclusion of air during reaction and neutralization and use of an antioxidant are necessary.\(^{48-52} \)

Bioassays of final products and particularly of new types of preparations are desirable, since the chemical methods do not distinguish the various unnatural isomers of \( \alpha \)-tocopherol from the natural \( d \) isomer. The physio-

\(^{34} \) F. Hoffmann-La Roche & Co., Swiss Pat. 208,851 (May 16, 1940).
\(^{35} \) F. Hoffmann-La Roche & Co., German Pat. 711,243 (Aug. 28, 1941).
\(^{36} \) F. Hoffmann-La Roche & Co., German Pat. 712,743 (Oct. 2, 1941).
\(^{37} \) F. Hoffmann-La Roche & Co., Swiss Pat. 216,825 (Sept. 15, 1941).
\(^{38} \) P. Karrer, U.S. Pat. 2,231,125 (Feb. 11, 1941).
\(^{39} \) L. I. Smith, W. B. Renfrow, Jr., and J. W. Opie, J. Am. Chem. Soc. 64, 1084 (1942).
\(^{40} \) U. V. Solmsen and J. Lee, U.S. Pat. 2,457,932 (Jan. 4, 1949).
\(^{41} \) A. Emmerie and C. Engel, Rev. trav. chim. 58, 265 (1939).
\(^{43} \) H. W. Rawlings, Oil & Soap 21, 257 (1944).
\(^{46} \) M. L. Quaife, J. Biol. Chem. 175, 605 (1948).
\(^{48} \) K. Ritsert, E. Merck’s Jahresber. 55, 13 (1943).
\(^{49} \) T. Cauböck and B. Wallenberg, Svensk Farm. Tidskr. 50, 477 (1946).
\(^{50} \) T. Moore and J. Tosic, Biochem. J. 37, xiv (1943).
\(^{52} \) E. Eden and V. H. Booth, Food Manufact. 25, 279 (1950).
logical response of carefully controlled test animals to administration of tocopherol preparations according to standard procedures is the ultimate measure of potency and a desirable quality control test to be conducted on composite production samples.

IV. Biochemical Systems

HENRY A. MATTILL

A. ABSORPTION AND METABOLISM

The absorption of tocopherols, like that of other fat-soluble substances is greater from oil solution than from vegetable tissues. There is no evidence that esterification is a feature of the absorption of vitamin E as it is of vitamin A; indeed, when the ester is fed to rats, the serum contains the free alcohol, suggesting rather rapid hydrolysis; but only about half as much tocopherol is found in the serum as when free tocopherol is given. The rise in human serum was reported to be about the same after the ingestion of either form. The presence of bile salts is essential; in rats and in dogs with bile fistula the amount of tocopherol absorbed from a good diet was sufficiently reduced to cause demonstrable deficiency.

On a normal intake, absorption is fairly complete. After a daily dose of 3.5 mg. in a rat, 3 to 15% of it appeared in the feces; after a large dose, as much as 25%. Its presence in the urine could be demonstrated only spectrophotometrically under those conditions but not chemically; also, no tocoquinone was demonstrable.

Methylation of any of the lower homologs to the trimethyl α variety is unlikely in view of the appearance of γ-tocopherol in the eggs following the feeding of it to laying hens. The deposition in eggs after feeding the three tocopherols was 10:2.5:1 for α:β:γ. The feeding of α-tocopherol to milk

7 L. R. Hines and H. A. Mattill, J. Biol. Chem. 149, 549 (1943).
cows (10 g, daily) raised the tocopherol content of the milk to 0.068 mg. per gram of fat (from 0.025), whereas a mixture of γ- and δ-tocopherols changed it to 0.034 mg. per gram of fat (from 0.022). Studies on normal adults confirmed this idea; α-tocopherol increased the level of serum tocopherols more quickly and for a longer time than γ. There appears to be a selective deposition of α-tocopherol in animal tissues and fluids.

The catabolic pathway is not known, but presumably it is via tocopher-erylquinone, the first stable oxidation product; more of this than of tocopherol was reported in dog plasma.¹⁰ It has recently been found in the stools,¹¹ but it was not demonstrable in rat liver, muscle, or urine.⁷

The presence of some tissue tocopherol in a bound form has been inferred from the additional amounts variably obtained by acid alcohol extraction, after the usual treatment with alcohol-petroleum ether (Skel-lysolve B) mixture.⁷,¹² Furthermore, the normal plasma content (0.9 to 1.2 mg. %) is only slowly extracted by ether or hydrocarbon solvents, whereas prior or simultaneous use of ethyl alcohol rapidly extracts it;¹³ measurements of intrinsic viscosity and other evidence favor the view that conjugates are formed with native proteins, most successfully with lipid-free bovin plasma albumin, such that a concentration of 2 to 3 mg. of toco-pherol per milliliter of a 2 % protein solution can be readily achieved as a stable slightly opalescent solution. The advantages of this procedure over the use of the unphysiological phosphate or succinate are obvious.

**B. STORAGE**

The capacity of various rat tissues to accumulate tocopherol was first demonstrated by Mason¹⁴ in extensive trials with the bioassay method. When the diet contained minimal amounts of vitamin E the liver stored one-half to one-fourth as much as the skeletal muscles, body fat, and visceral organs. With a high intake, the liver stored 14 times as much as at the lower intake, the other tissues only 3 to 4.5 times. The liver is thus the chief re-pository of vitamin E when its intake is high, and the amount found there may be the best index of previous intake.

**C. ANTIOXYGENIC ACTION**

1. In Vivo

Because of the increased stability of fats containing vitamin E, the possibility of enriching the fat stores by adding tocopherols to the diet has

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been explored. It was first demonstrated experimentally in rats\(^\text{15-17}\) that the induction period of rendered abdominal fats could be prolonged in this manner, the maximum deposition of the tocopherols being achieved 7 to 10 days after single doses. \(\alpha\)-Tocopherol was slightly more effective than \(\gamma\)-tocopherol (the opposite is true \textit{in vitro}), \(\beta\)-tocopherol being intermediate. The quantities of the three forms in the fat, as found by chemical determination, varied in the same direction, suggesting that the rate of absorption might be the dominant factor.

Similar protection was imparted to the fat of rabbits on a purified diet (but not on a natural ration) by feeding or injecting tocopherols at high levels;\(^\text{18}\) pork fat was also protected in this manner when tocopherols were fed to baby pigs in sufficient amounts over a 12-week period.\(^\text{19}\) In stored turkeys the peroxide value of the fats and the development of unpleasant flavors were inversely related to the tocopherol content of the tissues, which was increased by extra feedings.\(^\text{20}\)

2. \textit{In Vitro}

The prevention of oxidized flavors in milk has been correlated with increased tocopherol content of the milk produced by feeding.\(^\text{21}\) The factors responsible for the production of these unacceptable qualities are many and complex, and much study has been given to the usefulness of various stabilizers, including tocopherol by itself and in various combinations,\(^\text{22}\) to prevent deterioration in the quality of stored milk, butter, and other fat-containing foods and food products. Tocopherol with ascorbic, citric, and phosphoric acids (or their acid salts) are the principal naturally occurring synergists\(^\text{23-25}\) in milk, but they may not be effective in aqueous systems. Vitamin A and carotene in small concentrations,\(^\text{26}\) cephalin,\(^\text{27}\) methionine,\(^\text{28}\)

sulphhydryl groups, and crude sources of vitamin B complex are known to be effective in other systems. Complications are increased by the factor of actual and relative concentrations; in more than minimal amounts, carotene becomes a pro-oxidant, and there is an optimum concentration of tocopherol for most effective stabilization.

The deterioration of fats and oils in natural and prepared foods and its prevention by tocopherols and synergists are, chemically, still in the empirical stage. In general, the most effective fat antioxidants have a normal oxidation potential between 848 and 484 millivolts. A difference in potential must exist between antioxidant and synergist, and the oxidation of the synergist by the oxidized form of the antioxidant must be faster than the oxidation of the antioxidant by the fat peroxides, which, in turn, must be faster than the oxidation of the synergist by the fat peroxides.

D. SPARING ACTION ON VITAMIN A

Closely allied to the antioxygenic action of the tocopherols is their long-discussed sparing action on vitamin A. The first indications of this physiological property were the observations of Moore and of Bacharach to the effect that the vitamin A reserves in the livers of rats kept for a long time on E-deficient diets were much lower than those of rats receiving supplements of vitamin E. Tocopherol concentrates prevented the autoxidation of carotene in vivo and increased the biological response of vitamin A-deficient rats to minimal quantities of carotene. The vitamin A requirement is thus partly dependent on the adequacy of dietary vitamin E.

Studies by Hickman and his colleagues showed that the growth-promoting action of vitamin A and of carotene in rats was equally enhanced by the simultaneous oral administration of any of the tocopherols; in human subjects, fecal excretion of added dietary carotene and of other reducing materials was increased when tocopherols or tocoquinones were also

35. C. Golumbic, Oil & Soap 20, 105 (1943).
37. C. Golumbic, Oil & Soap 23, 184 (1946).
fed. As mentioned earlier, vitamin E increased the utilization of vitamin A in chicks, and the administration of 1 g. of tocopherol per day to New Zealand cows raised the levels of carotene and vitamin A in their milk fat. This sparing effect must be taken into account in any assessment of the vitamin A value of a diet.

These facts and the ineffectiveness of the acetate ester, previously observed and later confirmed, suggested an antioxygenic action of tocopherols in and near the alimentary tract rather than in the liver. Such limitation on the site of action of tocopherols does not explain the effectiveness of otherwise suboptimal quantities of essential fatty acids in preventing the fatty acid deficiency syndrome in rats when they receive tocopherol, which has not been confirmed or the increased stability of tissue fats.

This sparing action of tocopherols is definite, and the conflicting results are doubtless due to uncontrolled variables, including the form and amounts of vitamins A and E administered, the manner in which they are given, and the presence of inositol or of stabilizers such as cephalin or xanthophyll.

The biological implications of this point of view were recently emphasized by Dam and his colleagues, who found that methylene blue, thiodiphenylamine, and Antabuse (tetraethylthiuram disulfide), when added to vitamin E-deficient diets containing cod liver oil, caused an increased deposition of vitamin A in the livers of chicks. The first two substances did the same in rats. When cod liver oil was replaced by lard, or when the diet contained no fat, methylene blue had no effect on the vitamin A storage.

42. H. Patrick and C. L. Morgan, Poultry Sci. 22, 397 (1943); 23, 525 (1944).
53. R. M. Johnson and C. A. Baumann, J. Biol. Chem. 175, 811 (1948).
The protective effect is believed to be in the tissues, since the vitamin A of the unsupplemented cod liver oil diet did not diminish during storage of the food or under conditions resembling those in the alimentary tract. Of particular interest was the observation that methylene blue could partially replace tocopherol in preventing the sterility of female rats on vitamin E-deficient diets. Vitamin E is thus a biological antioxidant, or it can maintain the integrity of certain enzymatic redox systems in which methylene blue can replace the vitamin.

V. Estimation
HENRY A. MATTILL

A. QUANTITATIVE DETERMINATION

Quantitative methods for the determination of tocopherols include chemical, physical, and biological procedures. All are described and critically evaluated in two recent volumes.¹

1. CHEMICAL ASSAY

The chemical methods are based largely on the assay of oxidation products. The most complete oxidation is that obtained in the Furter-Meyer method with nitric acid, the color of the chroman-5,6-quinone being determined spectrophotometrically or colorimetrically. Volumetric titration methods have been developed with eric sulfate² and lead tetraacetate.³ By means of the latter, almost pure tocopherylquinone can be prepared.

A procedure was also devised for the simultaneous determination of tocopherol, tocopherylquinone, and vitamin K,⁴ ⁵ involving oxidation to quinone, reduction to the respective hydroquinones, and the use of 2,6-dichloroindophenol. Immediate diminution of the blue color is a measure of vitamin K; subsequent slower reduction is due to tocopherylhydroquinone.

The most versatile and commonly used method is based on the oxidation of tocopherol to tocopherylquinone by ferric chloride in the presence of α,α-dipyridyl, the resultant ferrous chloride being measured by the red color produced.⁶ The blue color produced on the addition of potassium fer-

³ A. Issidorides, J. Am. Chem. Soc. 73, 5146 (1951).
rycyanide can also be used.\(^7\) Interfering substances such as vitamin A and other materials readily oxidized under the conditions must be removed by various means,\(^8\) such as selective adsorption on Floridin XSS\(^9\) or clay,\(^10\) treatment with concentrated sulfuric acid,\(^11\) hydrogenation,\(^12\) or high-vacuum\(^13\) or molecular distillation\(^14\) of the tocopherols from extracts. Or the determination can be made before and after acetylation, the difference being a measure of tocopherol.\(^15\)

With minor modifications, this method has had wide use; typical examples are cited of its application to oils,\(^16,\)\(^17\) foodstuffs,\(^18\) plant\(^19\) and animal\(^20-24\) tissues and serum.\(^25-31\)

In the presence of fats, the amount of color produced is greatly depressed, for reasons as yet unknown;\(^32\) the effect varies with the nature and concentration of the fat, and the interference is, of course, avoidable by preliminary separation of the unsaponifiable portion, with due care to prevent loss due to the high temperature of saponification.

The less sensitive potentiometric method by which tocopherol is oxidized to tocopherylquinone with gold chloride\(^33\) is subject to the same interfer-

\(^7\) P. Meunier and A. Vinet, Compt. rend. 211, 611 (1940); Ann. chim. anal. et chim. appl. 23, 145 (1941).
\(^12\) M. L. Quaife and R. Beihler, J. Biol. Chem. 159, 663 (1945).
\(^15\) A. Emmerie and C. Engel, Z. Vitaminforsch. 13, 259 (1943).
\(^16\) A. Emmerie, Rec. trav. chim. 59, 246 (1940); 60, 104 (1941).
\(^22\) L. R. Hines and H. A. Mattill, J. Biol. Chem. 149, 549 (1943).
\(^23\) H. Kaunitz and J. J. Beaver, J. Biol. Chem. 166, 205 (1946).
\(^26\) A. Emmerie, Rec. trav. chim. 61, 305 (1942).
\(^29\) M. L. Quaife and P. L. Harris, J. Biol. Chem. 166, 499 (1944).
\(^32\) H. Kaunitz and J. J. Beaver, J. Biol. Chem. 166, 653, 661 (1944).
ence by other reducing substances. At the dropping mercury electrode, the $\beta$- and $\gamma$-tocopherols are oxidized at more positive potentials than is the $\alpha$, and the latter can be determined, polarigraphically, within 5% in the presence of oils or of traces of cholesterol. The apparent oxidation-reduction potential of $\alpha$-tocopherol seems to be between the normal oxidation potentials of mono- and dimethylhydroquinones, approximately +200 millivolts. The potentials of the four tocopherols ($\alpha$ to $\delta$) are reported as $+273$, $+343$, $+348$, and $+405$ millivolts, respectively.

None of these methods distinguishes between all the different tocopherols, and, since their biological activity is unequal, various chemical methods were sought by which to confirm or replace the tedious bioassay. Chromatographic separation of the pure tocopherols from mixtures of them is incomplete. In one of the differentiating chemical methods, advantage was taken of the fact that at 15° $\beta$- and $\gamma$-tocopherols produce only about half the color (Emmerie and Engel) which they would produce at 35°, whereas temperature has no effect on color production by $\alpha$-tocopherol.

A differential method for $\delta$-tocopherol was developed, depending on the fact that with the ferric chloride-dipyridyl reagent $\delta$-tocopherol gives a slow steady increase in color intensity (in 10 min.) after the initial rapid oxidation is completed (2½ min.). This probably indicates that $\delta$-tocopherol is oxidized beyond the $p$-quinone stage. The absorption spectrum of AgNO$_3$-oxidized $\delta$-tocopherol has a broad maximum, suggesting a mixture of reaction products. With nitric acid oxidation the product has an absorption maximum at 373 m$\mu$ which might be made the basis for spectrophotometric differentiation from the other tocopherols whose maximum is at 460 to 480 m$\mu$.

An actual separation is partially secured by utilizing the fact that $\beta$-tocopherol does not couple with diazotized o-dianisidine. A differential method for the determination of mixtures of $\alpha$-, $\gamma$-, and $\delta$-tocopherols depends on the relative difference in color intensity of the coupled $\gamma$- and $\delta$-tocopherols in alkaline solutions, measured at two wavelengths.

All the tocopherols except the $\alpha$ react with nitrous acid to form nitroso...
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derivatives. The $\beta$, $\gamma$, and $\delta$-nitrosotocopherols can be separated by simple chromatography, estimated separately, and the difference between their sum and the total as determined by the Emmerie and Engel method is $\alpha$-tocopherol. Recently\textsuperscript{46} countercurrent distribution has been employed for the separation of tocopherol from tocopherylquinone in stools. The $\alpha$, $\beta$, and $\delta$ compounds have been cleanly separated by reversed-phase paper chromatography, but the $\beta$ and $\gamma$ isomers could not be.\textsuperscript{47}

Fluorometry has also been applied, preferably to the unsaponifiable matter of fats after chromatographic separation; the nitric acid oxidation product is treated with $\alpha$-phenylenediamine to form the fluorescent phenazine.\textsuperscript{48}

If the purely physical methods for estimation of the tocopherols are limited to their absorption spectra in the ultraviolet region, the first and apparently only attempt to apply the technique quantitatively to natural products\textsuperscript{49} showed that unidentified substances seriously interfere both before and after saponification.

2. BIOASSAY

Tocopherol, the "fertility" vitamin, owes its name to the first demonstration of vitamin E deficiency, the failure of laboratory rats to procreate. This was a chance observation, in Pasteur's sense, and the existence and indispensability of vitamin E might have been discovered in connection with several other unnatural and diseased conditions now known to be produced when it is lacking. Indeed, deprivation of vitamin E is followed by a more baffling array of physiological abnormalities in different species than has ever been encountered with a single vitamin. None of these disorders is as suitable for purposes of bioassay as the restoration of fertility in a vitamin E-deficient female rat. In one or another of its modifications, this circumstantial, tedious, and demanding procedure has been the guide and milestone in the exploratory work on the distribution and chemical nature of vitamin E.

To be valid, such a bioassay must meet several criteria.\textsuperscript{50}

1. Fertilization by a normal male must be established by the finding of sperm in the vaginal contents or of a vaginal plug.

45 M. L. Quafie, J. Biol. Chem. 175, 605 (1948).
2. The 4- to 5-day estrous cycles, having been regular before fertilization, as determined by vaginal smears, should cease.

3. Implantation should be demonstrated by the placental sign or vaginal blood leak about the twelfth day of pregnancy.

4. On the twentieth or twenty-first day, the animal casts a litter if vitamin E supplies have been adequate, or her weight declines steeply but not abruptly, indicating resorption of the young. If there is any question of the fertility of the animal as such, an adequate dose of vitamin E should be given after another positive mating, and it is also essential to recognize a pseudopregnancy.

Because of the possibility of initial or first litter fertility due to vitamin E stores, a resorption gestation was once considered a necessary step before any animal was used for test. It was demonstrated, however, that the animal was a less sensitive indicator after a resorption gestation, and the practice first recommended by Mason and Bryan51 is now generally followed: the rats to be used for assay purposes are reared on a vitamin E-deficient diet beginning in their nursling period. Animals whose vitamin E storage is limited to that obtained through placental and mammary transfer, on such a regime, never show first litter fertility. In any case, placental transfer is very limited, whereas mammary transfer, especially after administration of large doses of tocopherol, has been demonstrated in lactating rats and in other species52, 53 to be considerable, particularly in colostrum.54, 55 Tocopherols in early human milk are reported as 0.13 to 3.6 mg. per 100 ml., with an average of 0.14 mg. in later milk.56 A later study57 confirmed the mammary transfer of tocopherol; premature infants on formulas low in vitamin E demonstrated a rapid decline in serum tocopherol levels. During the first six days after birth, the figures for breast-fed infants increased much more rapidly than those for bottle-fed.

A further improvement in the direction of standardization58 was the examination of the uterus at the sixteenth day by laparotomy, a positive response being the presence of two or more viable fetuses with at least four implantation sites. The presence of placental scars or of resorbing fetuses (or of both) testifies to the positive character of the mating and the in-

57 S. W. Wright, L. J. Filer, Jr., and K. E. Mason, Pediatrics 7, 386 (1951).
adequacy of the test dose. The assay period is thereby shortened, and there is no temptation to use the animal for further and possibly unreliable assays.

There is some question as to the time and manner of administering the test dose, whether on the fourth, fifth, and sixth days, or on each of the first ten days, after conception; the latter should be advantageous if more efficient use can be made of a smaller dose. The test substance is ordinarily administered by mixing it with a small portion of the diet under conditions that assure its consumption or by giving it, dissolved in a suitable oil (olive), with a medicine dropper.

Enough animals must be used (perhaps ten) on each of several levels of ingestion (three or four) to permit statistical treatment of the results which relate dosage to litter efficiency either on a curve or more simply by the method of probits. Litter efficiency is suitably expressed in terms of mean fertility dose, which is the least amount of tested substance on which one-half of the animals give a positive response as defined above. A series of animals on pure tocopherol or an ester of it should accompany the several series of the unknown substance under test.

The results of bioassays made by this standardized procedure are more reliable than earlier results, many of which were disappointing in their wide divergence; thus, the mean fertility dose for synthetic racemic dl-α-tocopherol acetate was 0.56 to 1.71 mg. The MFD of natural α-tocopherol (and its succinic acid ester) given by mouth is now 0.75 mg. The "unit" for vitamin E as originally proposed was 1 mg. of tocopherol acetate in olive oil, an amount greater than the presently accepted MFD for this substance.

Other criteria for the basis of a bioassay have included increase in maternal body weight during gestation, the number of offspring delivered at term, whether living or dead, or the percentage of placental implants resulting in birth of living offspring. The disappearance of symptoms of exudative diathesis in chicks, the reduction of creatinuria in rabbits suffering from muscular dystrophy, the prevention of hemolysis due to an

alloxan-like substance (considered later), and the prevention of testicular atrophy in the male rat\textsuperscript{69} have also been suggested as possible methods of bioassay of vitamin E. The interesting suggestion to use the transparent crustacean \textit{Daphnia magna} for rapid detection and evaluation of vitamin E\textsuperscript{70} seems never to have been further developed.

VI. Occurrence in Food

HENRY A. MATTILL

The distribution of the tocopherols is probably wider than that of any other vitamin, certainly more extensive than that of the other fat-soluble types. They occur as free alcohols, not as esters, and except for their consequent rapid oxidation in experimental rations containing certain unsaturated fats their discovery might have been long delayed. Before the development of trustworthy chemical methods for the separate estimation of each of the tocopherols, the application of the arduous bioassay had provided much information on the occurrence of vitamin E in various plants and in animal tissues. Subsequent chemical determinations have added many items,\textsuperscript{1,2} most of which can be found in the usual food tables. The richest dietary sources are the vegetable oils and cereal products containing them, butter and margarine, eggs and liver, legumes and greens.

In plant tissue the tocopherols usually accompany carotene or highly unsaturated fatty acids (in seed oils),\textsuperscript{3} substances readily oxidized in air. There appears to be a significant correlation between the amounts of linoleic acid and tocopherol found in fats and oils.\textsuperscript{4} As was pointed out,\textsuperscript{5} this association of tocopherol and unsaturated fatty acids in vegetable tissues and the presence of relatively impermeable cellulose walls afford considerable protection against autoxidation, whereas in animal tissue there are no barriers to the diffusion of oxygen. If there is likelihood of peroxidation of the fats in animal tissue and if the tocopherols act as stabilizers, one would expect to find a higher concentration of them in tissues of high fat content. In the rat,\textsuperscript{1} except for the pituitary and suprarenals, this appears to be true, but none of the many rat tissues examined is completely devoid

\textsuperscript{70} A. Viehsoever and I. Cohen, \textit{Am. J. Pharm.} \textbf{110}, 297 (1938).
\textsuperscript{1} M. L. Quaife, \textit{Ann. N. Y. Acad. Sci.} \textbf{52}, 300 (1949).
\textsuperscript{5} K. C. D. Hiekman and P. L. Harris, \textit{Advances in Enzymol.} \textbf{6}, 469 (1946).
of tocopherols when the intake is abundant.\(^6\) Lard\(^7\) and fish oils\(^8\) may contain small amounts. Bacterial synthesis of tocopherol in the intestine\(^9\) is unlikely;\(^10\) indeed, the feeding of sulfaguanidine in a diet high in cod liver oil protected rats against peroxide-produced yellow-brown coloration of adipose tissue,\(^11\) perhaps due to an \textit{in vivo} antioxygenic effect of the sulfa drug.

The amount found in cereal products depends primarily upon the extent of milling, since the tocopherols are mostly in and near the embryo;\(^12\) in durum wheat the non-germ tocopherol is fairly uniformly distributed throughout the endosperm. In animal products the content increases with the rising level of vitamin E in the diet, especially in the liver and body fats, as recently found in swine;\(^13\) more tocopherol was found in the blood cells than in the plasma. The severity of processing and length of storage to which the foodstuffs have been subjected are major factors, whatever the source. In dairy cows the level of plasma tocopherols seems to be directly related to the intake, whether this varies as the result of natural seasonal supply or of supplementary feedings.\(^14,15\) The tocopherol content of human serum seems to vary similarly.\(^16\)

As mentioned in connection with mammary transmission,\(^17\) the amount of vitamin E in milk is closely dependent on the amount in the diet and is greater in colostrum than in later milk. Recently, in a series of human milk samples representing various donors and stages of lactation, the figures were 0.10 to 0.48 mg. per 100 ml.;\(^18\) the mean was 0.24 mg., or about 80 \(\gamma\) per gram of fat, and throughout the period of lactation (3rd through 37th


\(^12\) H. Granados, E. Aaes-Jorgensen, and H. Dam, \textit{Experientia} \textbf{6}, 150 (1950).


week), the values were correlated with the fat content. Pasteurization caused no loss. Evaporated cow's milk and whole milk powder contained 23 to 40 \( \gamma \) of tocopherol per gram of fat; proprietary infant foods, 29 to 171 \( \gamma \).

VII. Effects of Deficiency

KARL E. MASON

A. IN ANIMALS

1. INTRODUCTION

a. General Considerations

More than a quarter century has passed since the existence of vitamin E (\( \alpha \)-tocopherol) was definitely established. Its first recognized function as an antisterility factor for the laboratory rat has been overshadowed by its demonstrated need for maintenance of structural and functional integrity of skeletal muscle, cardiac muscle, smooth muscle, and, in some animals, the peripheral vascular system. Tocopherols play an important role as intracellular antioxidants, related especially to the stabilization of ingested fats and possibly of products arising in the metabolic synthesis and degradation of lipids, and they may also function in a detoxifying capacity. Morphologic alterations arising in the course of vitamin E deficiency may well represent localized reactions of particularly susceptible tissues to loss of these vital antioxidants or, secondarily, to dysfunction of enzyme systems in which tocopherols actively participate.

The histopathologic lesions of vitamin E deficiency are remarkably varied, they represent morphologic alterations in a number of unrelated tissues, they seem not to be related to dysfunction of any specific type of cell or tissue, and they are of such a nature that restoration of normal morphology rarely occurs after tocopherol therapy, even though the physiological or biochemical disturbances are corrected. Most lesions are dependent upon fat in the diet, and their onset and intensity are accentuated in proportion to the amount and degree of unsaturation of the fat used. There are the possibilities that unsaturated fats destroy dietary traces of the vitamin in the diet or the gut, that they or their oxidation products produce a direct cell injury which is superimposed upon that due to lack of vitamin E, and that excess utilization of tocopherols to stabilized unsaturated fats being incorporated into cell lipids hastens depletion of tissue tocopherols and the precipitation of deficiency manifestations (which may or may not be related to lipid metabolism of the cells involved). Conceivably,
the true picture may represent a combination of these postulated interactions. With these possible interactions in mind, the symptomatology and histopathology of experimental vitamin E deficiency may be more comprehensible.

### TABLE II

**Listing of References Describing Histopathologic Changes after Vitamin E Deficiency in Various Species of Animals**

(The numbers refer to references cited throughout the chapter.)

<table>
<thead>
<tr>
<th>Animal species</th>
<th>Reproductive system</th>
<th>Musculature</th>
<th>Nervous system</th>
<th>Vascular system</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Testis degeneration</td>
<td>Fetal resorption</td>
<td>Skeletal</td>
<td>Cardiac</td>
</tr>
<tr>
<td>Monkey</td>
<td>21</td>
<td></td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>Dog</td>
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<td>14, 32, 33</td>
<td>24, 61a</td>
<td>1, 2, 4, 5, 21</td>
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<tr>
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<td></td>
<td>1, 4, 50, 51, 53-67, 70-74, 50, 90, 92, 101a</td>
<td>4, 60-92</td>
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<tr>
<td>Hamster</td>
<td>21</td>
<td>21</td>
<td>57a, 76</td>
<td>21</td>
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<td>Cotton rat</td>
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<td>21</td>
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<tr>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>23</td>
<td>38, 39</td>
<td>63a, 66, 82a, 67-69a</td>
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<td>58, 89</td>
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<tr>
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<td></td>
<td>63a</td>
<td>64a, b, c</td>
</tr>
<tr>
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<td>Pig</td>
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<td>30, 30a</td>
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<tr>
<td>Pheasant</td>
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<td></td>
<td>57, 100</td>
<td></td>
</tr>
<tr>
<td>Guppy fish</td>
<td>31</td>
<td></td>
<td>31</td>
<td></td>
</tr>
</tbody>
</table>

### b. Histopathologic Lesions in Different Species

Those structural and functional derangements which appear to be most characteristic of avitaminosis E, whether experimentally induced or occurring naturally, are listed in Table II, and under each are cited references to pertinent literature dealing with the types of tissue dysfunction observed in various animal species. It will be noted that lesions of skeletal muscles constitute the most universal finding, and that recorded alterations of other organs and tissues are rather spotty. The absence of recorded lesions in tissues or organs of certain species does not necessarily mean that a need for vitamin E on the part of these structures does not exist; for the most part, it indicates either that the muscular lesions are so pronounced
that the duration and degree of depletion necessary to bring about other tissue dysfunctions have not been attained (as in the rabbit and in cattle), or that only a deficiency state during early life of the species has been studied (as in the mink, sheep, pig, horse, duck, turkey) and that little or nothing is known of the effects of prolonged, chronic deficiency during adult phases of life.

c. Pigment

Since frequent reference will be made to an acid-fast pigment often associated with lesions of vitamin E deficiency, it seems appropriate at this point to discuss briefly its nature and possible origin. Martin and Moore first called attention to the occurrence of this pigment in uterine smooth muscle, skeletal muscle, sex glands, and other organs and tissues of rats maintained for prolonged periods on low E diets, and commented on its insolubility, inert and iron-free nature, and the brownish discoloration of the affected tissues. Its brownish-yellow fluorescence was also recognized. Although its major site of formation appears to be in the musculature, it eventually comes to be acquired by macrophages of the adjacent connective tissues and distributed rather widely throughout the reticulo-endothelial system. Chemical analysis of the pigment in rat uteri suggests the presence of oxidation products of protein. Histochemical studies suggest a lipofuchsin type of pigment derived through peroxidation and polymerization of unsaturated fats; except for certain differences in its oxidation potential, it is undistinguishable from a yellowish-brown, waxy pigment commonly observed in association with nutritional cirrhosis of rats on low protein diets and first characterized by Lillie et al., who proposed the term "ceroid" for it. In paraffin sections of tissues exposed to various fixatives and fat solvents, both pigments have similar acid-fast, sudanophilic and other staining reactions. Recent histochemical studies further support the theory that ceroid arises through the autoxidation of unsaturated lipids pathologically accumulated in cells having an insufficiency of biological antioxidants. Victor and Pappenheimer have pointed out that failure to

provide sources of vitamin E, combined with the presence of cod liver oil,
in cirrhosis-producing diets is the primary cause of ceroid production, and
that tocopherol supplements suppress or prevent its formation. Pigment
accumulation in adipose tissue in low-E rats\textsuperscript{10} (see p. 538), which provides
the closest morphologic counterpart to ceroid formation in the fatty in-
filtrated liver, is dependent upon the presence in the diet of fatty acids
having chain lengths of at least 18 carbon atoms and at least two unsatu-
rated bonds and is accentuated as chain length and unsaturation are in-
creased.\textsuperscript{11} The presence of peroxides in the adipose tissue\textsuperscript{12} undoubtedly
plays a role in pigment formation. The evidence thus indicates that the
pigment of vitamin E deficiency and ceroid of nutritional cirrhosis are very
similar, if not identical. It should also be emphasized that this pigment
accumulates at the same sites and has much the same characteristics as
the so-called “wear and tear” pigment normally found to a limited extent
in the adrenal cortex, sex glands, and elsewhere in the body\textsuperscript{3, 4} For these
reasons care must be exercised in relating the occurrence of acid-fast pig-
ment to a state of avitaminosis E.

2. Male Reproductive System

a. Rats

In male rats depleted of vitamin E from early life the seminiferous epito-
thelium shows no injury until active spermatogenesis begins, during the
third month of life, when a progressive and relatively rapid degeneration of
the epithelium occurs. Although there is close agreement among investiga-
tors regarding the distinctive character and irreparable nature of the histo-
pathologic changes\textsuperscript{13-17} there has been no satisfactory elucidation of the
underlying metabolic disturbances. The latter are so profound that vitamin
E therapy must be given 10 to 15 days prior to first appearance of histo-
logic injury in order to give full protection; therapy begun at intermediate
periods results in protection of certain seminiferous tubules but progressive
degeneration in others, or degeneration in all tubules, depending upon the

\textsuperscript{10} K. E. Mason, H. Dam, and H. Granados, \textit{Anat. Record} \textbf{94}, 265 (1946).
\textsuperscript{11} L. J. Filer, Jr., R. E. Rumery, and K. E. Mason, \textit{Trans. 1st Conf. on Biol. Antioxi-
\textsuperscript{15} A. Juhász-Shäffer, \textit{Virchow's Arch. path. Anat. u. Physiol.} \textbf{281}, 3 (1931); \textbf{286}, 834 (1932).
\textsuperscript{16} A. Ringsted, Dissertation, Undersøgelser over testis' histopathologi ved E-avi-
taminose; en eksperimentel-morfologisk studie. Nyt Nordisk Forlag, Copen-
hagen, 1936.
\textsuperscript{17} C. Engel and L. H. Britschneider, \textit{Intern. Z. Vitaminforsch.} \textbf{13}, 58 (1943).
interval. Degeneration is delayed 30 to 40 days by a single dose of 0.5 to 1.0 mg. of tocopherol fed on the 15th day of life; yet daily doses of between 0.5 and 0.75 mg. are necessary to protect the testis in rats reared 16 to 17 months on E-low diets. Prolonged deficiency has no effect on the weight of accessory sex glands, indicating that hormonal functions of the testis are not impaired.

The degenerative process, which reaches completion in 2 to 3 weeks, affects seminiferous tubules unequally as it progresses. It is characterized by the following sequence of events: (1) inhibition of spermatogenesis, associated with abnormal swelling and fusion of mature sperm; (2) marked diminution in sperm, and nuclear chromolysis in spermatids and secondary spermatocytes; (3) extensive sloughing of germ cells, and fusion of many into large multinucleate cells; (4) nuclear fragmentation and hydropic degeneration of remaining germ cells; (5) eventually the shrunken tubules are lined by a vacuolated, fibrous Sertoli syncytium. During the degenerative process many germ cells are sloughed and transported to the epididymis; others undergo dissolution in situ. Moderate amounts of acid-fast pigment are usually demonstrable in the Sertoli syncytium and in macrophages of the interstitial connective tissue. Leydig cells are normal. The testes are grossly atrophied, brownish, flabby, and watery when cut.

b. Other Species

In the hamster there occurs a much more gradual degeneration of the germinal epithelium, accompanied by accumulation of much acid-fast pigment in the germ cells and macrophages of the interstitial tissue, but, unless injury reaches an advanced stage, vitamin E therapy results in relatively successful restoration of the germinal epithelium. Testicular degeneration, associated with some pigment, occurs also in the guinea pig if a chronic deficiency is maintained such that symptoms of muscular dystrophy are kept minimal until after sexual maturity, but therapeutic response has not been studied. Testis damage resembling early phases of injury in the rat has been observed in the rabbit, the dog, and the monkey. There is also a suggestion that the germinal epithelium of young pigs is altered

18 K. E. Mason, Am. J. Physiol. 131, 268 (1940).
21 K. E. Mason, Unpublished studies.
by vitamin E deficiency. The testis of the mouse, on the other hand, is remarkably resistant; most investigators have observed no injury after deficiency periods up to 14 months; however, Menschik et al. report marked atrophy of the germinal epithelium after about 18 months. There is no testis injury in Florida cotton rats depleted to the point of showing marked muscular dystrophy. In lower forms, testis degeneration has been reported only in cockerels and in the guppy fish. The varied response of the testis of different animals to vitamin E deficiency represents an interesting but little-understood phenomenon.

3. Female Reproductive System

a. Fetal Resorption

Intrauterine death and resorption of the fetus in well-nourished rats represents the phenomenon primarily responsible for the discovery of vitamin E and the basis for its subsequent bioassay and identification. The histopathology is presented in the classic monograph of Evans and Burr and the later studies of Urner. All reproductive events are normal up to the time of implantation, which occurs at about the 7th day after insemination in the rat. Several days later there is retardation of fetal development, diminished hemopoietic activity in yolk sac and liver, and rarefaction of the allantois and mesenchymal tissues of the embryo proper. Either the allantoic placenta fails to properly differentiate and invade the maternal decidua, or else the latter offers unusual resistance to this invasion. In either case, impaired vascular relationships between fetal and maternal components of the placenta appear to be responsible for asphyxia, starvation and death of the fetus. This is followed by rapid necrosis and resorption of the fetus, more or less persistent but not severe uterine bleeding, and gradual regression of the placenta until little more than a blood clot remains

27 A. M. Pappenheimer, Am. J. Pathol. 18, 169 (1942).
32 J. A. Urner, Anat. Record 50, 175 (1931).
at term. There is also the interesting observation that offspring of rats dosed at critical periods after the 8th day of gestation sometimes show rather extensive umbilical and skeletal defects.23a

Fetal resorption can be prevented by administration of adequate vitamin E at any time during the first week of pregnancy. If the dosage is critical there may be delivery of dead as well as viable fetuses, the latter rarely surviving more than a few days. Less adequate dosage delays fetal death and resorption for varying periods. Under the latter conditions, fetuses at about the 16th day of pregnancy frequently show pronounced changes in the vascular system (stasis, distention, thrombosis, local hemorrhages) and generalized ischemia prior to death, but no obvious lack of hemopoiesis.33

With prolonged depletion of vitamin E beyond the first few months of reproductive life, there is a progressive increase in vitamin E requirements for the completion of established pregnancies, and also a progressive interference with implantation of the ovum (i.e., decreased fecundity rate) as age progresses.34, 35 Evidence points to a uterine and not an ovarian dysfunction.36

Fetal death and resorption quite comparable to that in the rat, although not studied in great detail, occur in the mouse25, 26 and in the hamster.21 That in the mouse has been attributed to impaired production of histiotrophe.37 In the guinea pig there may occur necrosis of the placenta and fetal death,38 or abortion due to premature separation of the placenta.39 Defective development of the chick embryo deprived of vitamin E will be discussed later (p. 534).

b. Ovary

In rats reared for a year or more on E-low diets, there are no alterations in behavioral estrus, ovulation, fertilization of the ovum, or its early development and tubal transport.36 Histologically, macrophages of the ovarian stroma progressively accumulate pigment arising at sites of follicular atresia and luteal regression,3 a phenomenon which is much more marked in the hamster.21 In resorbing rats there is premature regression of corpora lutea,

but this is secondary to early termination of pregnancy.\textsuperscript{40} Otherwise, most investigators have failed to find any morphologic changes in the rat ovary, except in animals rather advanced in age where senility changes tend to confuse the picture. On the other hand, well-controlled observations on mice\textsuperscript{41} indicate that lack of vitamin E results in fewer primordial ova, less interfollicular tissue, smaller but more numerous corpora lutea, and absence of neutral fat but marked increase in insoluble lipid complexes. Effects on the ovary of other species have not been reported. Histologic changes occurring in the uterus in vitamin E deficiency are discussed in a later section (p. 531).

c. Other Endocrines

There is no convincing evidence that vitamin E deficiency exerts any direct effect upon the function or structure of the anterior pituitary. There exists a rather extensive and controversial literature, much of which has been reviewed elsewhere.\textsuperscript{30a, 42, 43} Alterations observed in the basophiles of the anterior pituitary of male rats, resembling those following castration, are regarded as secondary to the testicular degeneration;\textsuperscript{44-46} they are absent in deficient female rats.\textsuperscript{47} The pituitary of male chicks, showing testicular injury of vitamin E deficiency, shows changes similar to those of the male rat.\textsuperscript{30a} Thyroid hypoplasia reported in low-E rats, and attributed to disturbed anterior pituitary functions,\textsuperscript{45} seem explicable on the basis of a relative inadequacy of dietary iodine.\textsuperscript{45}

The adrenal cortex undergoes no significant change as a result of vitamin E deficiency other than an accumulation of acid-fast pigment which closely resembles the "wear and tear" pigment normally present to a limited extent in the zona reticularis and may reflect diminished ability of the low-E animal to effectively stabilize cortical lipids of certain types. The accumulation of acid-fast pigment is especially marked in the mouse\textsuperscript{29, 49} and occurs to a limited extent in the rat and hamster.\textsuperscript{21}

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\bibitem{mason} K. E. Mason, \textit{in} \textit{Sex and Internal Secretions}, Chapter 22. Williams & Wilkins Co., Baltimore, 1939.
\bibitem{mason2} K. E. Mason, \textit{Vitamins and Hormones} \textbf{2}, 107 (1944).
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\bibitem{tibirica} P. A. T. Tibirica, J. Dutra de Olivera, and A. Aguiar, \textit{Hospital O. (Rio de Janeiro)} \textbf{26}, 585 (1944).
\bibitem{barrie} M. M. O. Barrie, \textit{Lancet} \textbf{233}, 251 (1937).
\bibitem{tobin} C. E. Tobin and J. P. Birnbaum, \textit{Arch. Pathol.} \textbf{44}, 269 (1947).
\end{thebibliography}
4. Muscular System

a. Skeletal Muscle

Nutritional muscular dystrophy constitutes the most universal manifestation of vitamin E deficiency (Table II) and signifies a vitally important, but as yet unknown, function of tocopherol in the metabolism of skeletal muscle. Although the histopathologic changes vary considerably from species to species, and even at different age periods in the same species, there is still a fundamental pattern of change which is expressed as an acute type of reaction in young animals and as a chronic type in adult animals. The former type, of which the "late lactation" paralysis of rats is typical, has received most attention because of ease of production experimentally and occasional spontaneous occurrence in domestic animals. Since combined placental and mammary transfer of tocopherol is often barely sufficient to meet the daily needs of the young offspring of mammals, inadequacy of dietary tocopherol during the lactation and early post-lactation periods, when there is unusually rapid growth and maturation of muscle fibers, can be expected to have a particularly devastating effect. The dystrophic changes occurring in adult animals bear a close resemblance to the lesions of human muscular dystrophy and to the hyaline necrosis (Zenker's degeneration) of muscle which follows prolonged febrile states, such as typhoid fever in man. It should also be kept in mind that at any given stage of vitamin E depletion there are certain differences in the extent to which the dystrophic process affects different muscles in the same animal, or even different regions of any one muscle.

(1) Late-Lactation Paralysis. When vitamin E reserves of lactating rats are critically low, the suckling young frequently exhibit a generalized paralysis, usually between the 18th and 25th days of life. This often appears rather suddenly. There is clenching of the forepaws, weakness and dragging of the extremities, inability to recover posture when placed on their backs, diminution of respiration and body temperature, listlessness and death. Spontaneous recovery may occur when symptoms are mild. Vitamin E therapy prevents the symptoms if given as late as the 15th day of lactation, but it has little or no beneficial effect once symptoms have appeared. Spontaneous recovery with retention of residual paralysis has been observed, but it is of rare occurrence. Lesions of the brain and spinal cord have been described but not confirmed. Most investigators favor a purely myogenic origin of the dystrophic process. Immobilization of a muscle by section of its nerve or its tendon prior to the 18th day markedly

51 H. M. Evans, J. Mt. Sinai Hosp. N. Y. 6, 233 (1940).
52 M. D. Lipshutz, Rev. Neurol. 65, 221 (1936).
52a W. de Gutierrez-Mahoney, Southern Med. J. 34, 389 (1941).
VII. EFFECTS OF DEFICIENCY

Fig. 2. Skeletal muscle from Vitamin E-deficient rat 22 days old, showing "late-lactation" paralysis. Note the strands of fragmented muscle fibers separated by areas of edema and leucocytic infiltration. A few normal fibers can be seen at the periphery of the muscle (left). \( \times 100 \). (From Mason.\textsuperscript{57a})

Fig. 3. Skeletal muscle from same rat, at higher magnification, showing an area where a group of fibers have undergone necrosis. Portions of two fibers, still possessing cross striations, can be seen at left (S), and several basophilic, spindle-shaped, multinucleate strands representing early stages in regeneration of new fibers at (R). Elsewhere there is an intermingling of macrophages (M), fibroblasts (F), muscle nuclei with investments of myoplasm (X), endothelial cells and faint outlines of capillaries. A fibroblast in mitosis is seen at lower right. \( \times 440 \). (From Mason,\textsuperscript{57a})
protects against dystrophy.\textsuperscript{53} Death is usually ascribed to serious impairment of the respiratory musculature, but other metabolic dysfunctions may be involved. The following description is based upon the reports of Olcott,\textsuperscript{54} Telford \textit{et al.},\textsuperscript{55} Pappenheimer,\textsuperscript{53, 56, 57} and Mason.\textsuperscript{58}

Grossly, the skeletal muscles are pale, ischemic, moist, and sometimes grayish and gritty owing to calcium deposition. Microscopically, there is widespread interstitial edema, leucocytic infiltration and segmental fragmentation of muscle fibers (Fig. 2). The affected segments show loss of cross striations, amitotic proliferation of sarcolemma nuclei, hyaline necrosis of fibrillar substance, nuclear fragmentation, rupture of sarcoplemma sheaths, and removal of debris by invading macrophages. In the densely cellular areas marking the point of breakdown of fiber segments, there appear many basophilic, fusiform, or band-like strands with centrally located nuclei; these represent young, regenerating fibers presumably arising from plasmoidal masses and, perhaps, from intact muscle nuclei with investing sarcoplasm released in the degenerative breakdown of the fibers (Fig. 3). Edema and leucocytic infiltration diminish in a few days, and regenerating fibers become increasingly numerous. The frequent presence of a layer of normal fibers at the periphery of the muscle suggests that proximity to good vascular supply may retard the dystrophic process.\textsuperscript{53, 56}

Weanling rats low in E but exhibiting no symptoms of paralysis, and rats showing spontaneous recovery from paralysis, usually show considerable muscle damage histologically.\textsuperscript{53, 55} Similar muscle changes, unassociated with external symptoms, have been observed in newborn rabbits,\textsuperscript{57} in prepubertal mice,\textsuperscript{27} and in young pigs.\textsuperscript{21}\textsuperscript{a} The muscular dystrophy observed in “stiff-lamb” disease,\textsuperscript{58, 59, 59a} in “white muscle disease” of young calves,\textsuperscript{60} and in a similar syndrome in the foal,\textsuperscript{61} all of which occur under

\textsuperscript{53} A. M. Pappenheimer, \textit{J. Mt. Sinai Hosp. N. Y.} 7, 65 (1940); \textit{Physiol. Revs.} 23, 37 (1943).

\textsuperscript{54} H. S. Olcott, \textit{J. Nutrition} 15, 221 (1938).


\textsuperscript{56} A. M. Pappenheimer, \textit{Am. J. Pathol.} 15, 179 (1939).


farm conditions and seem undoubtedly due to inadequacy of vitamin E in early life, closely resembles the early paralysis of laboratory mammals; the same is true of muscle changes observed in puppies,\textsuperscript{61a} mink,\textsuperscript{62} ducks,\textsuperscript{63} goats,\textsuperscript{63a} and chicks\textsuperscript{64} (where definite lesions have been observed only in the pectoral muscles), and guppy fish.\textsuperscript{61} Of particular interest are the classic studies of Blaxter and coworkers,\textsuperscript{64a-6} who describe in detail the symptomatology, gross pathology, biochemical alterations, and histopathology of a muscular dystrophy produced in Ayrshire calves reared on a low-E diet. The syndrome is indistinguishable from the long recognized "white muscle" disease of calves. Dietary cod liver oil accentuated the muscle lesions and also nullified otherwise protective doses of α-tocopherol, as is also true of other herbivorous animals (guinea pig, rabbit, goat). They describe swelling of muscle fibers, fragmentation, evidence of increased breakdown or impaired synthesis of muscle globulins, and massive sarcolemmic proliferation which they think reflects an attempt to maintain constant the volume of the dystrophic muscle; it is their opinion that in different species the latter is accomplished by varying degrees of sarcolemmic proliferation and fatty infiltration, thus accounting for much of the species variation in general character of the muscle lesions observed.

For a more detailed review and discussion of vitamin E as it applies to the nutrition of farm and laboratory animals, the reader is referred to the recent review by Blaxter and Brown.\textsuperscript{64f}

(2) \textit{Dystrophy in Rabbit and Guinea Pig}. Herbivorous animals as a whole appear to be particularly susceptible to withdrawal of vitamin E, and also to the presence of unsaturated fats in the diet.\textsuperscript{63a, 65} It was in the guinea pig and rabbit that nutritional muscular dystrophy was first experimentally produced by Goettsch and Pappenheimer,\textsuperscript{66} although its relationship to lack

\textsuperscript{64a} L. L. Madsen, \textit{J. Nutrition} \textbf{11}, 471 (1936).
of vitamin E was not clearly established until a later date.\textsuperscript{67, 68} Experimental work with this group of animals has centered largely around the rabbit, because of the ease with which symptoms can be produced and made to disappear by vitamin E therapy and the sequence repeated as often as desired.\textsuperscript{69} In respect to this striking responsiveness to tocopherol therapy, which involves rapid biochemical and morphologic repair, the dystrophic process in the rabbit differs from that in the rat. On the whole, the muscle lesions described in rabbits and guinea pigs more closely resemble the acute or explosive type exemplified by late-lactation paralysis than the more chronic type described below. However, it seems probable that the basic alterations occurring within the muscle fibers are fundamentally similar in all instances. Recent studies\textsuperscript{69a} indicate that the initial disturbance, which precedes loss of cross striations or other microscopic change, involves loss of actomyosin or alterations of the submicroscopic pattern in which actomyosin is organized.

(3) \textit{Adult, or Chronic, Dystrophy}. In young rats which spontaneously recover from late-lactation paralysis there is, within a week or so, a dramatic diminution in the intensity and extent of the muscle lesions. At one month of life only occasional fibers are dystrophic; the remaining musculature is normal and shows little or no connective tissue replacement of degenerated fibers. With continued deficiency there is progressive involvement of more and more fibers, usually in groups such that a patchy distribution of lesions results, which may be extensive enough to cause locomotor disabilities by the fifth or sixth month of life. In rats whose vitamin E reserves prevent the occurrence of early lesions, those of the later type may not be evident microscopically until the fourth or fifth month, and gross evidence of dystrophy not apparent until the eight to tenth month of life.

Gross and microscopic details of adult dystrophy in rats have been given by Ringsted,\textsuperscript{70} Einarson and Ringsted,\textsuperscript{71} Evans \textit{et al.},\textsuperscript{72} Knowlton et al.,\textsuperscript{73}


\textsuperscript{71} L. Einarson and A. Ringsted, Effect of Chronic Vitamin E Deficiency on the Nervous System and the Skeletal Musculature in Adult Rats. Levin and Munksgaard, Copenhagen, 1938.


Mackenzie et al., Pappenheimer, Martin and Moore, Monnier, and Mason and Emmel. The first evidence of paresis is a waddling and slightly incoordinated gait; later there is pronounced straddling of the hind legs, hyperflexion of digits, flabbiness and weakness of the musculature involving the adductor thigh muscles particularly, dragging of the hind legs, inability to walk or stand, distinct ataxia, pronounced muscle atrophy, deformity of feet, and general and localized sensory disturbances. The animals are hump-backed, unkempt in appearance; ulcerations of the skin are common. The skeletal muscles are atrophic, somewhat dry or gritty, and somewhat brownish in color. Once the adult paresis is well-established, prolonged vitamin E therapy does no more than arrest the process and improve the growth and well-being of the rat; that is, there results a permanent paralysis of about the degree present at the beginning of therapy. This is quite different from the rabbit, where paresis can be made to disappear and reappear repeatedly by careful regulation of the vitamin E intake, and the late-lactation paralysis in rats where E therapy is of little or no avail once symptoms appear.

The histopathologic picture (Fig. 4) differs from that of late-lactation paralysis chiefly in the relatively smaller number and widely scattered location of affected muscle fibers. Edema, fragmentation, and leukocytic infiltration are less conspicuous features. Necrosis tends to involve longer fiber segments, but the degenerative and regenerative processes, although proceeding perhaps at a somewhat slower rate, are quite similar to those characterizing late-lactation paralysis. Sarcolemma nuclei appear to undergo amitotic proliferation and become irregularly distributed in the fiber, followed by the appearance of coarse interfibrillar granules and vacuoles, loss of cross striations, breakdown of fibrillar substance, and removal of debris by invading macrophages. The latter are often marked by accumulations of acid-fast pigment which commonly appears in the degenerating fibers. Usually the point of segmental degeneration is marked by an area of high cellular concentration which, as in the case of early dystrophy, contains considerable numbers of free muscle nuclei with their investment of sarcoplasm and strongly basophilic multinucleate strands which represent early phases of a regenerative reaction. The basophilic strands give rise to slender muscle fibers possessing rows of centrally placed nuclei, arising presumably through rapid amitotic divisions; such fibers are quite common, and degenerating fibers absent, in muscles from animals given vitamin E therapy


74 M. Monnier, Compt. rend. soc. phys. et hist. nat. (Genève) 57, 252 (1940); Intern. Z. Vitaminforsch. 11, 235 (1941).
after periods of dystrophy. Changes of fundamentally the same type occur in the mouse, the hamster and cotton rat, the dog, and the monkey. Certain differences observed in the histopathologic picture in various laboratory animals may be attributable to species differences in the extent and rate of the degenerative and regenerative processes in dystrophic muscle.

(4) Interrelationships with the Nervous System. Except for conflicting opin-

Fig. 4. Skeletal muscle of hamster after chronic Vitamin E deficiency (200 days), showing areas of necrosis of muscle fibers (X) and other fibers in various stages of regeneration. The latter vary from elongated, basophilic, multinucleate strands (R) to fibers which are normal, except for an irregular distribution of nuclei in chain-like rows located more or less centrally in the fiber (X). In some areas, degenerated fibers have been replaced by fatty tissue (F). X 125. (From Mason.)

ions concerning the rat, the dystrophic muscle lesions of vitamin E-deficient animals are considered to be purely myogenic. The early onset of the lesions, their biochemical nature and response to E-therapy, frequent occurrence of similar alterations in cardiac muscle are in accord with this assumption. It should be mentioned, however, that in most instances the nervous system has not been carefully studied. On the other hand, much attention has been given to the nervous system of the rat. In their pioneer studies on the paralysis of adult rats reared for many months on low-E diets, Einarson and Ringsted described lesions in the dorsal

76 O. B. Houchin and H. A. Mattill, J. Biol. Chem. 146, 301, 309, 313 (1942).
columns, dorsal nerve roots, and ventral horn cells of the spinal cord and stated (p. 134 in ref. 71) that "... the muscular changes in many of the animals, especially in the early stage of the affection, show several points of decided resemblance to the picture of muscular dystrophy, i.e., a myogenous muscular atrophy. Gradually, as the disease in these animals is progressing, however, the muscular changes increasingly assume the typical neurogenous appearance so that finally the muscular features alone would be evidence enough of the presence of an amyotrophic lateral sclerosis. ... On the other hand, we have not been able to exclude the existence of a mixed picture, i.e., the possibility that the muscular changes may in part be dystrophic and partly be due to the spinal changes." Similar conclusions were reached by Monnier,74 Although, in a study of comparable material, some investigators77 have found no lesions of the nervous system, others78, 79 have observed demyelinization and gliosis in the posterior columns and dorsal nerve roots, but not the alterations in the ventral horn cells and pyramidal tracts described by Einarson and Ringsted. In a recent and detailed "criticizing review" of the literature on this subject, Einarson80 admits that in their earlier report73 there was overemphasis of "the myopathogenetic significance of the spinal cord changes," and he expresses the opinion that "vitamin E on the whole acts simultaneously on the musculature and the nervous system, due to some physico-chemical effects it exerts in metabolism." Until there is better agreement regarding lesions of ventral horn cells (see p. 533), it is impossible to say with certainty that an atrophy of spinal origin is superimposed upon a true myopathy.

Peripheral nerves and motor end plates are reported as normal in nutritional myodegeneration of ducklings,81 guinea pigs,82, 82a and young56 and old79 rats; but Telford84 finds that loss of end plates, secondary to degeneration of muscle fibers, occurs in young rats. Einarson and Ringsted83 observed some atrophic muscle spindles in dystrophic muscle of adult rats which they imply may be related to alterations noted by them in ventral root fibers.

An important recent development is the demonstration that \( \alpha \)-tocopherol hydroquinone, which is a potent antidystrophy compound, has no antisterility activity\(^{33a}\) and undergoes little or no conversion to \( \alpha \)-tocopherol \textit{in vivo}.\(^{33b}\) Another oxidation product of tocopherol, \( \alpha \)-tocopherolquinone, is also antidystrophic but appears to have no antisterility activity. Thus \( \alpha \)-tocopherol, the antisterility vitamin, may represent a provitamin for other compounds functioning as antidystrophy vitamins. An excellent discussion of these and other aspects of experimental muscular dystrophy has been presented by Mackenzie.\(^{33c}\)

\[ b. \text{Cardiac Muscle} \]

Hyaline necrosis and replacement fibrosis of cardiac muscle, closely resembling the changes occurring in skeletal muscle, have been observed in the vitamin E-deficient rabbit,\(^{63a, 84, 85}\) guinea pig,\(^{63a, 56}\) young calf,\(^{64a, b, c}\) cow,\(^{87, 88}\) sheep,\(^{59, 89}\) goat,\(^{63a}\) rat,\(^{1}\) mouse,\(^{28}\) hamster,\(^{21}\) and cotton rat.\(^{21}\) The lesions are rapid in onset in herbivorous animals (rabbit, guinea pig, sheep, and cattle), not associated with acid-fast pigment, and frequently the cause of sudden death through myocardial failure. This is in striking contrast to other laboratory animals (rat, hamster, and cotton rat) in which extensive focal necrosis and scarring of the myocardium, with accumulation of pigment in muscle fibers and macrophages, may exist for many months without serious effects. Electrocardiographic changes of varying degrees, indicative of myocardial damage without involvement of the conducting system (Purkinje fibers), have been found in the rat, guinea pig, rabbit, cattle, and monkey. The most dramatic picture is seen in sudden collapse of cattle in cardiac failure, usually with little or no symptomatology prior to exitus.\(^{33}\) The phenomenon in the rabbit has received the most thorough study, largely through the careful studies of Gatz and Houchin,\(^{4}\) who provide an excellent description of the electrocardiographic and histopathologic changes. They believe that a phase of increased muscle metabolism, as reflected in increased \( O_2 \) consumption similar to that in


\(^{87}\) T. W. Gullickson and C. E. Calverley, \textit{Science} \textbf{104}, 312 (1946).


\(^{90}\) S. Americano Freire, \textit{Brasil-med.} \textbf{55}, 308 (1941).


skeletal muscle, precedes the onset of morphologic changes. The latter are characterized by interstitial edema, infiltration of neutrophiles and mononuclear cells, hyaline necrosis of muscle fibers in association with coagulative necrosis and vacuolation of the sarcoplasm, gradual loss of myofibrillae, and appearance of wide constriction bands. Cardiac capillaries and larger vessels are prominently distended with blood; and sometimes small hemorrhages occur. Usually the necrosis is patchy in its distribution. Neither Purkinje fibers, neurons, nor fibers of the autonomic plexi show any changes. When severe, the lesions are grossly visible as circumscribed grayish areas. Lesions are most extensive in the peripheral myocardium of the ventricles but occur also in the papillary muscles, the interventricular septum, and the atrial musculature.

c. Smooth Muscle

Rats deprived of vitamin E for several months exhibit a yellowish discoloration of the uteri which gradually increases to a chestnut brown color as deficiency progresses, due to the accumulation of brownish, fluorescent, acid-fast pigment granules in the smooth muscle cells and macrophages of the myometrium.\(^1\), \(^2\), \(^3\), \(^4\), \(^5\), \(^92\)-\(^95\) A similar but somewhat less pronounced change occurs in the smooth musculature of the fallopian tube, cervix, vagina, seminal vesicle, prostate, vas deferens, ureter, trabeculae and capsule of the spleen, small intestine, bronchi, and uterine and pulmonary veins.\(^1\), \(^4\), \(^92\)-\(^94\)

The uterine changes constitute a prototype for those observed in smooth muscle elsewhere. Pigment granules appearing first at each pole of the nucleus gradually push the myofibrillae peripherally, eventually distending and even distorting the muscle cells such that they are difficult to distinguish from intervening pigment-laden macrophages. It is more presumed than established that much of the pigment in macrophages is derived from muscle cells undergoing necrosis; some of it may be released to macrophages without breakdown of the cells. Macrophages containing large globules of pigment, produced perhaps by alteration and concentration of smaller pigment granules, become numerous and conspicuous in the intermuscular connective tissue and in the outer zone of the endometrium. Endometrial fibrosis seems not to be accentuated.\(^96\)

Uterine pigmentation does not occur in rats ovariectomized before puberty, but does appear if such rats are given estrogen treatment;\(^97\) once

\(^{93}\) W. Hessler, \textit{Intern. Z. Vitaminforsch.} 11, 9 (1941).

\(^{94}\) V. Demole, \textit{Intern. Z. Vitaminforsch.} 8, 338 (1939); \textit{Schweiz. med. Wochschr.} 71, 1251 (1941).


\(^{96}\) J. Lopes de Faria, \textit{Ann. N. Y. Acad. Sci.} 52, 121 (1949); \textit{Hospital O (Rio de Janeiro)} 29, 533 (1946).

established, however, it is but little influenced by prolonged tocopherol therapy, pregnancy, castration, or hormone treatment. Although uterine pigmentation does not appreciable modify the response of the uterus to drugs which act either upon the autonomic nerve supply or as direct muscle stimulants, or interfere with the course of pregnancy if adequate vitamin E is provided after mating, it may be to some degree responsible for the lowered incidence of fertile matings, due to impaired implantation, and for the increased requirements of successful gestation in long-term E-deficient rats.  

There is a remarkable species variation with respect to the response of smooth muscle to vitamin E depletion. In the monkey the pigment changes are especially marked in vascular smooth muscle but occur also in the small intestine, gall bladder, urinary bladder, and bronchi. Although not extensively studied in other species, the lesions appear to be limited largely to the urinary bladder, blood vessels, and small intestine in hamsters, and to the small intestine in the dog, but are absent in the cotton rat. In the mouse only brownish discoloration of the uterus has been reported. Pigmentation of smooth muscle has not been observed in association with other lesions of vitamin E deficiency in herbivorous animals or in birds. These findings suggest that from a metabolic standpoint, at least, smooth muscle may differ widely from species to species, and even in different organs of the same species. In avian species, turkey poults show a patch hyaline necrosis of the smooth musculature of the gizzard, with inflammatory reactions and replacement fibrosis, as the only recognized manifestation of vitamin E deficiency; similar changes of a milder type have been reported in the chick.

5. Nervous System

Studies on the effects of vitamin E deficiency upon the nervous system, based chiefly upon the rat, have given rather controversial results (see review by Einarson). In rats showing late-lactation paralysis, Lipshutz reported extensive cerebrospinal lesions involving especially the vestibular and tectospinal pathways and dorsal sensory columns, and de Guiterrez-Mahoney has described rather widespread cellular hyperchromasia and loss of Nissl pattern; other investigators, however, report no neuropathologic changes in such animals.

Demyelination and gliosis in dorsal nerve roots and dorsal sensory columns (cuneatus and gracilis) of the spinal cord of adult rats subjected to

prolonged vitamin E deficiency, as first reported by Einarson and Ringsted\textsuperscript{13} and Monnier\textsuperscript{74} and considered related to tabes-like symptoms in such animals, although questioned by some investigators,\textsuperscript{77} have been well substantiated by others.\textsuperscript{78, 79}

Einarson and Ringsted\textsuperscript{13} also described extensive degeneration of ventral horn cells and, in a few cases, demyelination of the pyramidal tracts, leading to a picture suggestive of a combination of tabes dorsalis combined with spinal muscular atrophy or amyotrophic lateral sclerosis. Einarson\textsuperscript{80} now considers the pyramidal tract lesions a misinterpretation, due to faulty comparison with control material. The ventral horn changes described also by Monnier\textsuperscript{74} but not observed by other investigators\textsuperscript{77–79} who have searched for them, are characterized by alterations in Nissl substance leading to lipodystrophy and irreparable atrophy of motor horn cells, those located more centrally and at the venous end of the capillary bed where hypoxia is greatest being more affected than the more peripheral cells. Similar changes in dystrophic guinea pigs have been reported.\textsuperscript{100a, 100b} Einarson\textsuperscript{80} thinks that when vitamin E is inadequate adenylic acid, necessary for formation of high-energy phosphate bonds, is not properly liberated from cytoplasmic nucleotides and that accumulation and decomposition of the latter lead to the degenerative cell changes observed. Failure to use, or to properly apply, the gallocyanin-chromalum stain, having a selective affinity for nucleic acids, may explain the failures of others to confirm these findings.\textsuperscript{80} Possibly the widespread cellular hyperchromasia described by de Gutierrez-Mahoney\textsuperscript{52a} in ventral horn cells and elsewhere in the nervous system, and the accumulation of acid-fast pigment observed by Pappenheimer and Victor\textsuperscript{101} and Einarson\textsuperscript{101a} in motor cells of the cord and medulla of old vitamin E-deficient rats, represent changes of a similar type but revealed by other staining methods.

Mackenzie \textit{et al.}\textsuperscript{73a} report marked tremors in adult paralyzed rats and, following a shrill note, an outburst of activity terminating in collapse without convulsions or loss of consciousness. Hamsters on low-E diets have been reported\textsuperscript{101b} to show a sudden onset of incoordination, hyperexcitability, and violent reactions to external stimuli such as touch or noise, culminating in collapse, unconsciousness, stupor, and death; tocopherol orally effected spectacular recovery in some instances. In the writer’s experience, and that of others who have studied E-deficiency in the hamster, this phenomenon has never been observed.

Lesions of the cerebellum and cerebrum resulting from vitamin E deficiency in birds appear to be secondary to vascular changes and, for that reason, are discussed in the following section.

6. Vascular System

a. Chicks

Most of the lesions observed in vitamin E-deficient chicks appear to be the result of alterations in the peripheral vascular system, especially the capillary bed. Like the skeletal muscle lesions of the duck, the chick, and the pheasant, 57, 63, 64, 100 and gizzard necrosis of the turkey and the chick, 81, 99, 109 referred to previously, these manifestations are rarely seen in chicks after the second month of life. Since they occur spontaneously in the field with variable frequency, 81, 100, 102 they are of economic as well as academic importance.

(1) Embryonic Mortality. According to Adamstone, 102 inadequate vitamin E in the chick egg results in embryonic death at about the fourth day of incubation, due to disintegration of blood vessels of the blastoderm, hemorrhage into the coelom and exocoelom, and cellular proliferations in the blastoderm which interrupt the vitelline circulation. If this critical period is passed, there may be spontaneous rupture of vascular channels at various sites within the embryo, usually associated with clusters of pycnotic histiocyte-like cells at the points of extravasation. Whether these cells are responsible for the vascular rupture or represent a protective reaction at the site of injury has not been satisfactorily established.

(2) Exudative Diathesis. Chicks reared from hatching on low-E diets usually exhibit a state of exudative diathesis or of nutritional encephalomalacia, or sometimes both, during the first two months of life. The two manifestations appear to be secondary to dysfunctions of the capillary bed, sometimes regress spontaneously, and are influenced considerably by variations in fats and other dietary components.

Exudative diathesis, as described by Dam and Glavind, 103 is characterized grossly by the appearance of large patches of subcutaneous edema on the breast and abdomen, and less frequently on the neck, legs, or wings. These represent local subcutaneous and interfascial accumulations of a plasma-like fluid frequently tinged greenish by decomposed hemoglobin. The affected tissues show edema, hyperemia, and increased permeability of the capillaries as indicated by increased absorption of intravenously injected Trypan blue. The subcutaneous tissue at the site of old lesions retains a buff color.

102 F. B. Adamstone, J. Morphol. 52, 47 (1931); Arch. Pathol. 31, 622 (1941).
for some time. Bird and Culton describe more severe manifestations of a similar disorder in which edema of brain and lungs, marked distention of heart and pericardium, generalized ascites, and coronary and intestinal hyperemia usually terminate in stupor and death of the chicks. No histopathologic study of the localized lesions of exudative diathesis has been reported.

(3) *Nutritional Encephalomalacia.* This disorder of the nervous system, as described by Pappenheimer et al. and by Adamstone, is characterized by motor incoordination, ataxia, head retraction, coarse tremors, opisthotonos, prostration with legs spastic and claws strongly flexed, somnolence, stupor, and death. At necropsy there is gross swelling, flattening, irregular distortion, and greenish-brown discoloration of the cerebral convolutions. Similar lesions frequently occur in the cerebrum, midbrain, and medulla. They vary from small focal areas to large confluent patches of ischemic necrosis. The cerebellar lesions, microscopically, show edema and disruption of cellular and fibrillar elements of the gray matter, degenerative necrosis of Purkinje cells and the small cells of the granular layer, capillary thrombi which are especially abundant where the blood vessels make a pronounced right-angle turn at the Purkinje cell level, and small hemorrhages in the cortical white matter. Although capillary thrombosis seems to be the primary cause of the ischemic necrosis, it is possible that some of the symptomatology may be secondary to prolonged vasoconstriction or vasomotor paralysis of larger blood vessels. During spontaneous or induced recovery there is active ingrowth of new blood vessels, gliosis and reparative reorganization in the softened tissues, and appearance of phagocytes with brownish pigment. The symptoms and lesions described are identical to those of "crazy chick disease," long recognized by poultrymen in brooder-stage chicks.

Jungherr, who has given particular attention to the seasonal occurrence and histopathology of this spontaneous disease, which is often a more chronic disorder than that produced experimentally, reports that in association with, and sometimes independent of, this ischemic necrosis there occurs extensive fibrosis of the cerebellum. Furthermore, the ischemic and fibrotic lesions are often associated with large areas of increased vascularity in the medulla, the midbrain, and the thalamus, accentuated by adventitial cell proliferation and intervascular gliosis, which he regards as a "new morphologic expression of subacute E-avitaminosis in chickens."

108 F. B. Adamstone, *Arch. Pathol.* 31, 603 (1941); 43, 301 (1947).
(4) Influence of Variations in Diet. According to Dam,104 exudative diathesis is rare and encephalomalacia never occurs in chicks fed purified E-deficient diets containing no added fats; furthermore, dietary unsaturated fats accentuate both manifestations, and by varying the type and proportion of fats and other components of the diet it is possible to produce these two manifestations separately or concomitantly. Essentially the same diet may give a very different incidence of the two symptoms in the hands of different investigators.109 The presence of substances such as ascorbic acid,109 xanthophyll,110 or redox substances of various types,111 by virtue of their capacity to replace tocopherol as an antioxidant in the gastrointestinal tract or elsewhere, may confer considerable protection against the symptoms. Thus it appears that in addition to lack of vitamin E there is a delicate balance of dietary factors which operate to determine the onset and severity of the vascular dysfunctions and the anatomic site at which they occur. In this connection reference may be made to the lymphoblastoma-like growths observed by Adamstone112 in the liver, the intestine, and other visceral organs of the chick, sometimes leading to hemorrhage by invasion of blood vessels, when cod liver oil or sardine oil, but not halibut liver oil, was added to diets previously treated with ferric chloride to destroy traces of vitamin E. On the other hand, additions of halibut liver oil led to an anemia-like condition presumably resulting from extensive erythropagocytosis in the liver.113 These findings have not been confirmed or satisfactorily explained.

b. Mammals

(1) Hemorrhage. The vascular stasis and hemorrhage occurring in the rat fetus, as described in an earlier section (p. 520), bears a certain resemblance to the vascular dysfunctions in chicks. So also do the interfascial, subcutaneous, and thymic hemorrhages observed during the second month of life in rats reared from birth on low-E diets high in total unsaturated fatty acids of cod liver oil,114 and also the hemorrhages into cranial and visceral cavities, lungs, and intestine observed by Elvehjem et al.115 in puppies born of mothers fed mineralized milk diets low in vitamin E. As in the chick studies just discussed, there are again the three factors to be

112 F. B. Adamstone, Am. J. Cancer 28, 540 (1936); Arch. Pathol. 31, 717 (1941).
113 F. B. Adamstone, Arch. Pathol. 31, 613 (1941).
considered—inadequate vitamin E, early age of the animal, and some type of metabolic stress or local insult of tissues. Whether the latter relates to fat peroxides or to other metabolites as well is not known. There is also the question of whether in the young organism the vascular bed is peculiarly susceptible to injury by such metabolic factors, or whether the metabolic factors differ qualitatively or quantitatively as biochemical processes mature.

Of particular interest in this connection are the studies of Holman\(^\text{116}\) demonstrating that a necrotizing arteritis in dogs, produced by induction of renal insufficiency after prior feeding of a high fat diet, can be retarded or prevented by tocopherol. Here vitamin E seems to protect against chemical injury resulting from the presence of abnormal lipids deposited in the vascular wall. Difference of opinion exists as to whether \(\alpha\)-tocopherol does\(^\text{117}\) or does not\(^\text{118}\) protect dogs against stilbestrol-induced thrombocytopenic purpura. In rats tocopherols prevent increased capillary fragility due to \(\alpha\)-irradiation.\(^\text{119}\)

(2) Hemolysis. György and Rose have shown that hemoglobinuria, intravascular hemolysis, and death occurring in alloxan-treated rats are readily prevented by dietary tocopherols, although the diabetic phenomena are not affected;\(^\text{120}\) and that dialuric acid, a decomposition product of alloxan, and several related compounds produce hemolysis of erythrocytes.\(^\text{121, 122}\) \(\alpha\)-Tocopherol, through its function as an antioxidant, protects the red cell against this chemical injury, perhaps by counteracting a free radical or peroxide formed as an intermediate of the oxidation-reduction system of dialuric acid and alloxan. On the basis of these reactions they have developed an hemolysis test which can be applied either in vivo or in vitro as a measure of the biologic activity of tocopherols and of vitamin E depletion in the rat.\(^\text{122, 122a}\) Modifications of this test have demonstrated increased tendency to hemolysis in red cells of vitamin E-deficient monkeys (unpublished observations in the writer's laboratory) and in those of premature infants,\(^\text{123}\) full-term infants, and newborn rats.\(^\text{124}\) Although hemolysis

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can be produced by many other compounds and can be prevented by other antioxidants, \( \alpha \)-tocopherol is unquestionably a most effective natural intracellular antioxidant.

It is generally accepted that vitamin E deficiency does not influence the peripheral blood picture or hematopoietic functions. However, Dinning\textsuperscript{125} has recently reported a pronounced leucocytosis in low-E rabbits, responding to tocopherol therapy, which he concedes may reflect leucocytic infiltration of dystrophic muscle.

7. Other Manifestations

\( a. \) Adipose Tissue

Prolonged vitamin E deficiency in rats results in considerable diminution in body fat, accounting in part for the plateau in body weight occurring during early adulthood and for the rather emaciated appearance of rats showing advanced stages of paresis. Menschik \textit{et al.}\textsuperscript{29, 126} have studied this phenomenon in mice, reporting that adipose tissue develops normally during early life but that after about 9 months of deficiency little or no body fat is evident—except for the brown glandular fat of the interscapular region which is often increased in amount and of deeper brown color than in control mice. They were led to conclude that vitamin E deficiency interferes with the ability to deposit dietary fats as neutral fats, related perhaps to the fact that both ingested and stored fat undergo abnormal or abortive metabolic changes.

A strikingly different reaction occurs in the adipose tissues of the rat when low-E diets contain high levels (about 20\%) of cod liver oil\textsuperscript{10} or highly unsaturated fractions of this oil,\textsuperscript{127} or methyl esters of linseed, corn, or soybean oils.\textsuperscript{11} Under such circumstances there is a brownish discoloration of the subcutaneous and intraperitoneal adipose tissue. Peroxides are usually demonstrable either by chemical\textsuperscript{12} or histochemical\textsuperscript{128-130} methods. Microscopically,\textsuperscript{10, 127} the adipose tissue first appears studded with yellowish-brown islets. The latter represent clusters of fat cells in various stages of development in which small fat globules and the peripheral portions of larger fat vacuoles are composed of acid-fast pigment; pigment-laden macrophages are also present in increased numbers. At later stages these cells, and possibly other connective elements, participate in complex foreign-

\textsuperscript{129} J. Glavind, H. Granados, S. Hartmann, and H. Dam, \textit{Experientia} \textbf{5}, 84 (1949).
\textsuperscript{130} H. Dam, \textit{Ann. N. Y. Acad. Sci.} \textbf{52}, 194 (1949).
body type of reactions leading to the formation of large, pigment-laden giant cells which eventually dominate the picture. Tocopherol therapy arrests the process but in most locations brings about no more than a limited reduction in pigment and very little change in other cellular reactions.

Similar alterations of adipose tissue have been observed in the mouse, pig, and mink; it is of interest that the naturally occurring "yellow fat disease" or "steatitis" of mink, frequently causing serious losses of kits prior to the pelting season, fits into this picture. In all instances a dietary intake of highly unsaturated fats and inadequacy of vitamin E have been involved. The most satisfactory explanation for the histopathologic reactions described above is that unsaturated dietary fats incorporated into adipose tissue cells which lack sufficient tocopherol as an antioxidant to stabilize them, or to counteract peroxides which accumulate in the tissues, undergo polymerization or combine with cell proteins, or both, to form acid-fast pigment which provokes giant cell reactions and perhaps a certain amount of cell necrosis.

b. Liver

Certain histopathologic changes in the liver have been observed in a few species, but, in most instances, lack of vitamin E is merely one of several factors involved. In chicks there has been reported a phenomenon of "erythropagocytosis," in which the liver shows brownish discoloration and, microscopically, enlargement of hepatic cells, widening of sinusoids, and much hemosiderin in hepatic and Kupffer cells; there is also hyperplasia of myeloid tissue. Its occurrence only when ferric chloride-treated diets are supplemented with halibut liver oil raises questions as to its specificity.

After prolonged vitamin E depletion in mice (14 months or more) Menschik et al. have noted progressive accumulation of coarse "lipoproteic" globules, swelling, and nuclear pycnosis in hepatic cells; they also describe sinusoidal dilation, extravasation of erythrocytes or obvious hemorrhage, hemosiderin in Kupffer cells, and disorganization of the parenchyma. Histochemically, the lipoproteic globules are composed of a mixture of unsaturated fatty acids, phospholipids, and cholesterol, probably combined with protein, resembling the "ceroid" of nutritional cirrhosis referred to before and suggesting abnormal metabolic changes in liver fat.

In nutritional cirrhosis (diffuse hepatic fibrosis) of rats fed diets low in lipotropic factors (methionene, choline) there is an extensive fatty infiltration of the liver followed by a progressive deposition of fibrous tissue in the

form of irregular trabeculae with extensive disorganization of the parenchyma. If such diets are low in vitamin E, as was usually the case in the early studies, the livers become grossly brownish-yellow in color and on histologic examination show accumulations of ceroid pigment in cells of the parenchyma and fibrotic areas, especially in the latter. As mentioned previously (p. 516) this ceroid is generally indistinguishable from the acid-fast pigment of vitamin E deficiency. Tocopherols have no influence on the fatty or fibrotic changes but retard or prevent ceroid formation. The cellular reactions which occur in areas of fatty infiltration of hepatic cells are essentially the same as those described above for adipose tissue and indicate the need for tocopherols to prevent undesired oxidative changes in the infiltrating lipids. Dietary unsaturated fats such as cod liver oil accentuate ceroid formation. Except for dissemination of much of this pigment to the lungs, the histopathologic changes in the musculature and other tissues are typical of those seen in rats fed low-E diets containing adequate protein.

A different type of liver injury, known as acute or massive hemorrhagic necrosis, occurs in rats reared on diets low in vitamin E and deficient in sulfur-containing amino acids (alkali-treated casein or low casein diets) or containing as their protein component certain yeasts (high yeast diets) which lack an unidentified protective substance (factor 3 of Schwarz) present in most American yeasts. There is a vast and confusing literature on this subject, which now warrants the statement of Schwarz that "a simultaneous lack of 3 factors—cystine, vit. E and factor 3—is a prerequisite for the development of dietary necrotic liver degeneration, and each one of them alone can protect." It is not yet clear how each of these substances acts in protecting the rat against the rather sudden onset of massive necrosis and hemorrhage in the liver or massive lung hemorrhages, which cause death. In addition to widespread centrolobular necrosis of the liver, there have been noted dystrophic changes in skeletal muscles, ulcers of the forestomach, kidney lesions, and hemorrhage in lymph nodes and intestine.

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c. Kidney

A progressive nephrosis of the kidney occurs in low-E rats, involving isolated convoluted tubules of the outer cortex after 3 to 4 months of deficiency and becoming quite generalized by the tenth month. The glomeruli show little change, but the tubular epithelium becomes coarsely granular, irregular in contour, and separated from the basement membrane to form an amorphous pale-staining layer. In advanced stages, the loops of Henle and even the collecting tubules are involved. It is surprising that the rats can survive with such widespread nephron damage. Acid-fast pigment has been observed in the tubular epithelium of the rat, monkey, and mink in the absence of the nephrotic changes just described.

d. Tooth Depigmentation

Depigmentation of the maxillary incisors of the rat is recognized as a common manifestation of vitamin E deficiency; mandibular incisors are also involved if dietary protein is low. This iron-containing, non-fluorescent pigment is continuously formed and deposited by the enamel organ as the incisor is worn away by attrition. The depigmentation is secondary to atrophic changes in the enamel organ. According to Pindborg, there is edema and disorganization of the papillary layer, probably caused by capillary damage in this region, followed by epithelial derangement and cyst formation in the ameloblast layer. There is also a progressive deposition of acid-fast pigment in macrophages of the highly vascular periodontal connective tissue. After vitamin E therapy, function of the enamel organ is restored and newly deposited enamel acquires its normal color. Depigmentation of maxillary incisors occurs also in the hamster but no histopathologic studies have been reported.

B. IN MAN

1. General Considerations

A state of avitaminosis E has not been shown to occur in man. There are some similarities between certain manifestations of vitamin E deficiency in experimental animals (such as fetal resorption, dystrophic changes in skeletal muscle) and certain clinical disorders in man (habitual abortion, progressive muscular dystrophy); yet, it has not been established that the latter are either due to lack of vitamin E or benefited by therapeutic use of the vitamin. On the other hand, there has arisen an extensive but decidedly

147 H. Granados, K. E. Mason, and H. Dam, J. Dental Research 24, 197 (1945); 25, 179 (1946).
148 J. J. Pindborg, J. Dental Research 29, 212 (1930); 31, 805 (1952).
controversial literature regarding the therapeutic efficacy of vitamin E (α-tocopherol) in a variety of clinical disorders which often have little in common with the deficiency syndrome in lower animals and which are not associated with any known inadequacy of vitamin E in the diet or in body tissues. If tocopherol actually does exert a beneficial effect in such disorders, the high dosage levels reported as necessary suggest a true pharmacologic action on particular tissues or local regions of the body, perhaps through some influence upon deranged metabolic processes at the affected site.

The distribution of vitamin E in various tissues and organs of man, at a concentration quite similar to that in lower mammals, implies that it serves some useful purpose. Depletion of body stores of vitamin E in lower animals produces a variety of symptoms and histopathologic changes which suggest that vitamin E functions as an important intracellular antioxidant; whether it has other metabolic functions remains to be established. Regardless of how it participates in the metabolic economy of the animal organism, it is a logical assumption that it functions in like manner in metabolic processes of man, and that certain of the symptoms and lesions characterizing the deficiency state in animals would also occur in man, provided that comparable dietary conditions prevailed for a sufficiently long period of time. On the basis of animal experimentation, where we have some notion of the relations between previous tissue storage and rate of metabolic utilization (or depletion) of tocopherol as a function of time, the possibility that adult man ever reaches a state of uncomplicated avitaminosis E seems rather remote. However, during early infancy, before tissue storage becomes an important factor, there is real possibility of tocopherol inadequacy, as will be discussed later.

Deficiency states are of two general types: natural deficiency, due to inadequate intake of a nutrient over prolonged periods of time which, in the case of fat-soluble vitamins, are measured in terms of years, if previous intake and body storage have been reasonably normal; and "conditioned" deficiency, arising through factors which chronically diminish the absorption or storage or increase the rate of metabolic utilization of the vitamin. Deficiency of a fat-soluble vitamin rarely appears as a true clinical syndrome in adult man; however, states of suboptimal nutrition with respect to such vitamins are recognized, although they are usually complicated by other dietary inadequacies. To establish whether suboptimal or marked deficiency of vitamin E occurs in man there must be acquisition of many data on the vitamin E status of normal individuals and of others whose status might be considered suboptimal on the basis of dietary habits or organic disease. Such data must relate primarily to four major aspects of vitamin E nutrure: (1) the dietary intake over a period of years, (2) the extent of intestinal absorption and excretion, (3) capacity for tissue storage
and (4) rate of metabolic utilization. In such considerations, it is customary to express vitamin E in terms of d-α-tocopherol, which constitutes about 90% of the total tocopherol in human tissues and seems to be absorbed more efficiently than, or preferentially to, the non-α forms which represent about one-half the usual dietary supply.149

a. Dietary Intake

Chemical and biological analyses indicate that tocopherols are present in essentially every article of diet; yet, the richest sources such as vegetable oils, unmilled cereals, and eggs constitute a much smaller proportion of the usual dietary than animal products, vegetables, and fruits, which represent relatively poor sources of α-tocopherol. It has been estimated that the average American dietary provides approximately 14 to 19 mg. of d-α-tocopherol, daily,150 and that the better diets probably do not provide more than 25 mg.149 Furthermore, the recommended diet of the National Research Council (1945) and certain therapeutic diets (low sodium, diabetic) would convey only about one-half this amount.149 The same is true of the average diet in Holland.151 The estimates do not take into consideration the losses of tocopherol which may occur during storage, commercial handling, and cooking, concerning which very little information is yet available.

b. Intestinal Absorption and Excretion

The question of how efficiently the gastrointestinal tract absorbs ingested tocopherol has received scant attention. It has been recognized that little or none is excreted in the urine151, 152 and that appreciable amounts are lost in the feces.152-154 The tocopherol content of bile is of about the same magnitude as that of the blood, and undoubtedly some absorbed tocopherol is re-excreted into the intestinal tract via the biliary tract.152, 153 On the basis of bioassay tests, Hickman et al.154 concluded that in normal human subjects the ratio between ingested and excreted tocopherol is of the order of 4 to 1.2. On the other hand, recent studies of Klatskin and Molander,153 based on chemical analyses for fecal tocopherol and a larger number of subjects, indicate that normal individuals excrete approximately two-thirds of ingested tocopherol in the feces; they are also of the opinion that there is little or no destruction in the gastrointestinal tract, and concur with others that intestinal synthesis of tocopherol is quite unlikely. On the as-

sumption that approximately 50% of ingested tocopherol is absorbed, it appears that a normal adult on an average American diet would have a net absorption of about 7 to 10 mg. of $d$-$\alpha$-tocopherol, daily.

c. Tissue Storage

Most tissues and organs of man contain tocopherol, chiefly in the form of $\alpha$-tocopherol, as first reported by Abderhalden who carried out a series of analyses on organs from human fetuses, newborn infants, and adults.\cite{155} These findings have been generally verified and extended by other studies\cite{156-159} which begin to give a fairly satisfactory picture of tocopherol distribution and concentration in man. Unlike vitamin A, which is stored largely in the liver, tocopherol is stored chiefly in the adipose tissue. On the basis of tocopherol content of a wide variety of tissues from two healthy adults, both cases of accidental death, total body storage has been estimated to be 3.4 g. for a 20-year-old male and 8 g. for a 40-year-old female (Table III). Accepting 5 g. as the average body storage in well-nourished adults, an individual of 70 kg. body weight would possess ap-

<table>
<thead>
<tr>
<th>Table III</th>
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<tr>
<td>Estimated Content of Total Tocopherols in Human Subjects</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Woman, mg.</th>
<th>Man, mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td>6180</td>
<td>1885</td>
</tr>
<tr>
<td>Muscle</td>
<td>269</td>
<td>285</td>
</tr>
<tr>
<td>Blood</td>
<td>45</td>
<td>64</td>
</tr>
<tr>
<td>Liver</td>
<td>33</td>
<td>45</td>
</tr>
<tr>
<td>Pancreas</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Spleen</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Heart</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Kidney</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Uterus</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>Testis</td>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>

Total

<table>
<thead>
<tr>
<th>Woman, mg.</th>
<th>Man, mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>6560</td>
<td>2309</td>
</tr>
<tr>
<td>6120</td>
<td>3440</td>
</tr>
</tbody>
</table>

a From Quaife and Dju.\cite{156}

\cite{158} M. Y. Dju, K. E. Mason, and L. J. Filer, Jr., *Études Néo-natales* 1, 49 (1952).
proximately 70 mg. of tocopherol per kilogram of body mass. For comparison there are reported levels of 5.6 mg. per kilogram in a newborn infant analyzed in toto, and of 3.1 mg. per kilogram in fetuses of 2 to 6 months gestation age (average of 20 fetuses). Analyses of separate tissues and organs of man, as discussed later (p. 555) suggest that the tocopherol content of adipose tissue provides a much more reliable index of vitamin E nutriture than that of other tissues.

d. Metabolic Utilization

The metabolic utilization of α-tocopherol, which can be regarded as the difference between net absorption and net storage over any given period of time, has at present a rather intangible value. It is possible to control and measure net absorption with a certain degree of accuracy, but total storage or changes in storage over periods of time cannot be measured or estimated in a living individual. Of greater importance is an understanding of those metabolic and other factors which influence the rate of utilization of tocopherol in man, both as it applies to the body as a whole and to isolated organs and tissue. At the present time we have essentially no information on this question and are only able to draw certain inferences from experimental studies in which factors such as increasing age, diets high in unsaturated fats, and other types of metabolic stress exert a noteworthy effect on the total body economy of vitamin E.

2. Vitamin E in Early Life

During recent years considerable attention has been given to the vitamin E status of man during prenatal and early postnatal life. Tocopherol concentration is low in tissues of the fetus and newborn infant but somewhat higher in the placenta which, as in lower mammals, appears to have but a limited capacity to transfer tocopherol to the fetus. Tocopherol levels are also low in cord blood and in blood of the newborn, generally ranging from one-third to one-fifth of that of the mother; yet, maternal blood levels show a natural increment (see Table IV,

163 W. Neunweiler, Intern. Z. Vitaminforsch. 21, 83 (1949).
165 P. Cattaneo and A. Mariani, Rend. ist. super. sanità 13, 124 (1950).
168 S. W. Wright, L. J. Filer, Jr., and K. E. Mason, Pediatrics 7, 386 (1951).
p. 552) during the latter part of gestation.\textsuperscript{164, 165, 169-171} The reason for this is not clear; it may help to overcome the restricted placental transfer of tocopherol, or it may reflect a physiologic preparation for more effective transfer of tocopherol to the milk, better to prepare the infant for the exigencies of early extraterine existence. Human breast milk has a considerably higher content of tocopherol than does cow’s milk;\textsuperscript{165, 171-174} furthermore, in both species colostrum is much richer in tocopherol than is later milk.

There is thus unquestionable evidence that the newborn infant has a rather small endowment of tocopherol at birth. Analysis of one full-term infant in toto\textsuperscript{157, 158} has indicated a total content of about 25 mg., or approximately the daily intake of an adult on a high-quality diet. It also appears that tocopherol concentration in tissues shows no significant increase during the first three years of postnatal life.\textsuperscript{159} It is pertinent at this point to call attention to the fact that certain manifestations of vitamin E deficiency have been produced only during relatively early phases of life of the species, and that in producing states of experimental vitamin E deficiency it has always been a practice to initiate the deficient diet early in life, before any appreciable tissue storage has occurred, because of the recognized difficulty in depleting tissue reserves of the vitamin. The question then arises as to whether the seemingly precarious tocopherol status of the newborn infant provides any basis for a natural, or a conditioned, deficiency state during infancy or early childhood.

\textit{a. Susceptibility of Erythrocytes to Hemolysis}

György and his associates\textsuperscript{175, 176} have demonstrated that erythrocytes of low-E adult rats, and of newborn rats from mothers on stock diets, are readily hemolyzed \textit{in vitro} or \textit{in vitro} by small amounts of dialuric acid, alloxan (both reduction products of alloxan), or hydrogen peroxide, and that small amounts of $\alpha$-tocopherol protect the cells against these effects. In the writer’s laboratory, it has also been shown that erythrocytes of vitamin E-deficient monkeys hemolyze when exposed to small amounts of

\begin{table}
\begin{tabular}{|c|c|}
\hline
Reference & Description \\
\hline
\end{tabular}
\end{table}
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alloxantin having no effect upon the erythrocytes of controls fed the same diet supplemented with tocopherol.

Furthermore, György et al. report that the red blood cells of full-term infants at birth show mild hemolysis when exposed to small amounts of hydrogen peroxide, that incubation of washed erythrocytes with α-tocopherol makes them resistant to this effect, and that tocopherol fed to the infant (but not when given to the pregnant mother) accelerates the disappearance of this fragility which normally occurs during the first week or so of postnatal life. They speculate that "the 'physiologic' vitamin E deficiency of the fetus and the newborn may have practical clinical implica-

![Fig. 5. Effect of α-tocopherol administration, beginning at time indicated by arrow, upon the susceptibility of erythrocytes to hemolysis (H₂O₂) in four premature infants (from Gordon and de Metry).](image)

Of additional significance is the recent report of Gordon and de Metry describing hemolysis of red cells, by the hydrogen peroxide test, in premature infants bottle-fed for periods up to 30 days after birth, and disappearance of this fragility within 2 to 5 days after tocopherol administration to the infant (Fig. 5). They emphasize the low tocopherol content of most artificial formulas for infants, compared to that of breast milk, as have Wright et al., who showed that in bottle-fed full-term infants the postnatal increase in serum tocopherol is much more gradual than in breast-fed infants (Fig. 6), and that in bottle-fed prematures during the first month or so of post-

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natal life there is a gradual decline in serum tocopherols until they reach levels comparable to those which in the experimental animal are associated with manifestations of vitamin E deficiency (Fig. 7). It seems, therefore, that the bottle-fed premature infant, denied the benefit of placental transfer of vitamin E during the latter phases of gestation, physiologically handicapped from the standpoint of suckling and other postnatal adaptations, and usually reared on a low-fat formula (because of poor tolerance for fats) rarely providing more than one-fourth to one-fifth the tocopherol present in breast milk, represents the nearest approach to a natural avitaminosis E in man. It still remains to be determined whether the low-E status of the premature or full-term newborn is etiologically related to any

specific disorders or dysadaptations of the young infant, and also whether the maintenance of abnormally low levels of serum tocopherol in prematures for a considerable period of time before the institution of a mixed diet has any harmful influence upon the subsequent health of the infant. A few clinical states which have been explored from this general standpoint justify comment, even though the results are equivocal.

b. Retrolental Fibroplasia

In view of what has been said regarding the tocopherol status of the fetus and the newborn infant, it is natural that considerable attention has been given to the possible implication of an inadequacy of vitamin E in the etiology of retrolental fibroplasia.\textsuperscript{178-181} This disorder is characterized by

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unusual proliferative activity in retinal capillaries, followed by edema and small hemorrhages which rupture into the vitreous, separation of the retina, and, with further proliferation, the formation of a disorganized membrane-like mass in the vitreous. Its etiology is unknown, but it is generally regarded as related to some metabolic disorder of prenatal or early postnatal life.

There are many observed facts which provide quite logical reasons for suspecting that inadequacy of vitamin E might be involved, as outlined by Owens and Owens. Although the latter investigators found no significant difference in serum tocopherol levels between infants with normal eyes and those who developed retrolental fibroplasia, prophylactic tocopherol therapy given to alternate premature infants with birth weights of 3 pounds or less gave results which were at least encouraging; so also did the use of \( \alpha \)-tocopherol in infants showing early stages of the disease. Unfortunately, however, their subsequent experience, and that of other investigators, has raised doubt as to whether tocopherol has therapeutic value in preventing or ameliorating the lesions. Capillary changes due to physiologic immaturity and electrolyte imbalance and failure to maintain proper oxygenation during early postnatal life are currently under consideration.

c. Other Implications

There are suggested alterations in the peripheral vascular system during early phases of life in connection with certain manifestations of experimental vitamin E deficiency; for instance, exudative diathesis and nutritional encephalomalacia in young chicks, vascular stasis and hemorrhage in the fetus of low-E rats, the reported effect of tocopherol on vascular resistance in guinea pigs, and stilbestrol-induced purpura in dogs. To these may be added the reported, but as yet unestablished, effects of tocopherol in thrombocytopenic purpura and in thromboembolic phenomena in adult man as discussed in a later section. On the basis of these reported findings, clinicians have been led to test the efficacy of α-tocopherol in certain disorders of infancy with results which are somewhat questionable.

Impressed by the frequency with which fetal death in prematures, especially in those with birth weights under 1.5 kg., can be related to cerebro-meningeal hemorrhages due to multiple rupture of capillary vessels, Minkowski\(^{184, 185}\) has attempted to measure the vascular resistance of such infants (on the basis of petechiae produced by vacuum cup applied to skin of the back) and its response to vitamin P-like substances and to α-tocopherol. When mothers were given large doses (600 to 900 mg.) of α-tocopherol several hours prior to premature delivery, there was a definite increase in vascular resistance of the premature infant as compared to that observed in infants of comparable weight from untreated mothers: tocopherol also lessened the visibility of the capillary network of the skin, as visualized by the capillaroscope.\(^{185}\) There were four instances of intracranial hemorrhages in the treated group, and twelve in the untreated group, each represented by 105 infants; Minkowski considers these results suggestive of beneficial effects but recognizes the need for additional data before more conclusive statements can be made.

It has been reported\(^{186, 187}\) that α-tocopherol given to pregnant, Rh-negative mothers protects the infant against erythroblastosis, possibly through reduced permeability of the placenta to the Rh antigen, as indicated by marked reduction of antibody titer in the maternal plasma.

Gerloczy, who was for a period convinced that α-tocopherol, largely through a diuretic effect, greatly benefited premature infants with scleredema, has since reported\(^{188}\) that subsequent experience has led him to doubt the efficacy of tocopherol therapy. Tocopherol is said to be effective in the treatment of erythredema (Pink disease)\(^{189}\) and in effecting increased ap-

\(^{184}\) A. Minkowski, Arch. franç. pediat. 6, 276 (1949); Ann. Paediat. 174, 80 (1950).
\(^{185}\) A. Minkowski, Le Song 22, 701 (1951).
\(^{188}\) F. Gerloczy, Paediat. danub. 6, 83 (1949).
\(^{189}\) G. Forsyth, Med. J. Australia 1, 78 (1941).
petite and improved body growth in infants failing to respond to other measures.\textsuperscript{190, 191} These observations, based on a small number of subjects, require confirmation.

3. Vitamin E in Later Life

As pointed out earlier, it seems rather unlikely that a natural deficiency of vitamin E ever occurs in man during adolescence or adulthood. On the other hand, it is conceivable that a "conditioned" deficiency might arise as a result of (1) prolonged and severe impairment of fat absorption, (2) an inherited defect of metabolism affecting the capacity of certain tissues or organs properly to utilize the vitamin, or (3) certain types of metabolic stress which greatly increase requirements for the vitamin or its rate of loss from tissue stores.

There is no known symptomatology of avitaminosis E in man. Therefore, any evaluation of the tocopherol status of man depends largely upon information pertaining to (1) dietary history, (2) plasma tocopherol levels, (3) tocopherol concentration in tissues, and (4) the demonstration of histopathologic changes which are comparable to those characterizing the experimental deficiency state in other primates and lower mammals. Dietary histories are not particularly informative in the case of vitamins which are stored rather tenaciously by tissues. Chemical and histopathologic analyses of tissues and organs are limited to postmortem material or to small samples obtained through biopsy. Blood levels represent the interplay of many factors and are not necessarily indicative of the tocopherol status of the individual as a whole; however, they constitute the most widely used criterion for evaluating vitamin E nutriture in man.

a. Plasma (or Serum) Tocopherols

(1) Normal Levels. Most of our information on plasma tocopherols is based upon blood samples analyzed by the method of Quaife and Harris,\textsuperscript{192, 193} utilizing the Emmerie and Engel color reaction. A micromethod later developed by Quaife et al.\textsuperscript{194} and requiring minute samples of blood has been of particular value in studies with infants\textsuperscript{165, 195} and will undoubtedly come into much wider use. The values given by these methods are generally higher and more consistent in the hands of different investigators than are those reported by European workers employing other chemical procedures.

\textsuperscript{191} G. W. Schmidt, \textit{Arch. Kinderheilk.} \textbf{138}, 178 (1950).
Plasma tocopherol levels in newborn infants are approximately one-third to one-fourth those found in healthy adults. Average values of 0.23 mg. % (S.D. ± 0.13), 167 0.37 mg. % (S.D. ± 0.15), 168 and 0.43 mg. % (S.D. ± 0.12) 164 have been reported. For young and adolescent children, values of about 1 mg. % are considered normal. 196 Plasma tocopherol levels for healthy adults usually fall within the range of 1.0 to 1.2 mg. % (see Table IV). During pregnancy there is a rather pronounced increment in plasma tocopherols, 164, 165, 169-171 which declines only gradually during lac-

<table>
<thead>
<tr>
<th>No.</th>
<th>Type</th>
<th>Age range</th>
<th>Plasma tocopherols, a mean and S.D. mg. %</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>Normal</td>
<td>17-48</td>
<td>1.20 ± 0.22</td>
<td>192</td>
</tr>
<tr>
<td>70</td>
<td>Normal</td>
<td></td>
<td>1.04 ± 0.32</td>
<td>198</td>
</tr>
<tr>
<td>350</td>
<td>Clinic patients</td>
<td></td>
<td>0.78 ± 0.26</td>
<td>198</td>
</tr>
<tr>
<td>21</td>
<td>Normal</td>
<td></td>
<td>1.09 ± 0.17</td>
<td>199</td>
</tr>
<tr>
<td>42</td>
<td>Patients, unselected</td>
<td></td>
<td>0.92 ± 0.29</td>
<td>199</td>
</tr>
<tr>
<td>62</td>
<td>Patients, cardiacs</td>
<td></td>
<td>0.94 ± 0.35</td>
<td>199</td>
</tr>
<tr>
<td>23</td>
<td>Normal</td>
<td>20-40</td>
<td>1.23 ± 0.31</td>
<td>200</td>
</tr>
<tr>
<td>57</td>
<td>Patients, convalescent</td>
<td></td>
<td>1.02 ± 0.37</td>
<td>200</td>
</tr>
<tr>
<td>43</td>
<td>Patients, liver disorder</td>
<td></td>
<td>0.95 ± 0.32</td>
<td>200</td>
</tr>
<tr>
<td>26</td>
<td>Normal</td>
<td>16-39</td>
<td>0.90 ± 0.19</td>
<td>197</td>
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<td>162</td>
<td>Patients, infirmary</td>
<td>40-101</td>
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<td>197</td>
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<tr>
<td>20</td>
<td>Normal</td>
<td>22-55</td>
<td>1.26</td>
<td>202</td>
</tr>
<tr>
<td>11</td>
<td>Pregnancy, early</td>
<td></td>
<td>1.17 ± 0.19</td>
<td>164</td>
</tr>
<tr>
<td>12</td>
<td>Pregnancy, late</td>
<td></td>
<td>1.62 ± 0.31</td>
<td>164</td>
</tr>
<tr>
<td>54</td>
<td>Pregnancy, delivery</td>
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<td>1.70 ± 0.30</td>
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<tr>
<td>17</td>
<td>Pregnancy, 0-8 wk.</td>
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<td>Pregnancy, 9-16 wk.</td>
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<td>1.02 ± 0.25</td>
<td>169</td>
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<tr>
<td>33</td>
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<tr>
<td>75</td>
<td>Pregnancy, 25-32 wk.</td>
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</tr>
<tr>
<td></td>
<td>Pregnancy, 33-40 wk.</td>
<td></td>
<td>1.51 ± 0.40</td>
<td>169</td>
</tr>
</tbody>
</table>

a Method of Quaife and coworkers, 195, 196

A tendency for values to increase with age has been reported. 170, 171

(2) Effect of Disease. Compared to normal healthy adults, clinic patients 198 and convalescent hospital patients randomly selected 197, 199, 200

167 M. Chieffi and J. E. Kirk, J. Gerontol. 6, 17 (1951).
have somewhat lower levels of plasma tocopherol (Table IV). Patients with liver disease also tend to show low values, but they are not significantly different from those of convalescent patients with no evidence of liver disease; the same is true of cardiac patients. Lower-than-usual values are also commonly observed in diseases where intestinal absorption is defective, as in sprue, celiac disease, fibrocystic disease of the pancreas, biliary obstruction, and diarrhea associated with achlorhydria. This is in accord with experimental evidence that surgical production of a biliary fistula leads to a state of vitamin E deficiency in the rat and dog. On the other hand, higher-than-usual values are frequently observed in diseases associated with hypercholesteremia and in cardiovascular disease and pregnancy, both of which are often associated with increased blood lipids (Fig. 8). These deviations from normal might be explained, accord-

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*Fig. 8. Range of plasma tocopherols in man in relation to good health, pregnancy, and certain broadly classified diseases (from Darby et al.)*

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ing to Darby et al., on the basis of differences in intestinal absorption or differences in lipid-carrying power of the blood.

The effectiveness of intestinal absorption of vitamin E has been measured by the tocopherol "absorption curve" (also termed "tolerance curve," "tolerance test," "blood persistence curve"). Subjects are given a single, large, oral dose of tocopherol (usually 5 to 20 mg. of α-tocopherol per kilogram of body weight) and blood samples are taken at 0, 3, 6, 9, 12, and 24 hours. The curve obtained by plotting the tocopherol values, or the planimeter measurement of the area under the curve, provides a basis for comparing individual responses. Low response curves have been reported in some, but not all, cases of liver disease,\(^2\) in primary fibrosis,\(^3\) and in sprue.\(^4\) Low responses have been observed in cases of fibrocystic disease of the pancreas, diarrhea and cirrhosis in infants, and in children with celiac syndrome and lupus erythematosus, and rather high responses in metabolic disorders with associated hypercholesteremia.\(^5\)

Low response curves may not necessarily reflect impaired intestinal absorption. The fate of tocopherol, once it has passed the intestinal barrier, may be influenced by inadequacy of a lipid or protein "carrier" to which it may be bound either for purposes of transport or for functional activity or by other biochemical alterations of the blood or tissues. Klatskin and Molander,\(^6\) who have studied the combined picture of absorption and fecal excretion in normal, convalescent, and cirrhotic individuals, feel that the low plasma tocopherol level in cirrhosis is "more closely related to tissue depletion than to an impairment of absorption or utilization." Their observation that cirrhotics excrete a considerably smaller fraction of administered tocopherol than do healthy adults suggests also the possibility that in patients with impaired hepatic functions much less tocopherol is re-excreted via the biliary tract.\(^7\) Although bile from patients with operative biliary fistulas has about the same tocopherol content as blood,\(^8\) it is not known whether this represents a true excretion product or a phase in an enterohepatic circulation of tocopherol.

From what has been said, it appears that tendencies for plasma tocopherol to reach different levels in a variety of diseases may reflect previous dietary intake, effectiveness of absorption and excretion, state of tissue depletion, or biochemical changes in the blood which influence its carrying capacity for tocopherol. Low levels observed in diseases where a significant and prolonged impairment of fat absorption is indicated may sometimes reflect a low vitamin E status of the body as a whole, although they may rarely represent degrees of depletion commensurate with typical biochemical or histopathologic manifestations of avitaminosis E.

\(^3\) K. C. D. Hickman and P. L. Harris, Advances in Enzymol. 6, 469 (1946).
b. Tissue Tocopherols

Tissue levels of tocopherols, especially those of the major storage depots, are undoubtedly the most reliable index of vitamin E nutriment. The usual method for analysis of tocopherols in foods and tissues is laborious and time-consuming. Tissues obtainable are limited to postmortem material, except where generous biopsy samples can be secured. Although \( \delta \)-\( \alpha \)-tocopherol is the predominant type found in tissues, variable but usually not very large amounts of \( \gamma \) - and \( \delta \)-tocopherols are also present; however, results are generally expressed in terms of total tocopherols.

Information concerning tocopherols in human tissues is limited to analyses carried out by Abderhalden on a variety of tissues from fetuses, newborn, and adults, those of Quaife and Dju on two cases of accidental death, and a more extensive series of analyses covering the period from early fetal life to old age, carried out in the writer's laboratory. The results of these studies indicate that tocopherols are widely distributed in human tissues from early fetal life to advanced old age. Tocopherol levels, expressed as milligrams per 100 g. of fresh tissue, are low in fetuses of 2 to 6 months' gestation age, and only slightly higher in premature and full term infants at birth. The data obtained are in accord with other evidence that during the early postnatal period of life states of suboptimal vitamin E nutriment may occur. During early postnatal life, tissue levels tend to increase slowly unless suppressed by disease of various types. During childhood and adolescence they reach levels comparable to those of adults which, for muscle, heart, liver, and certain other visceral organs, are approximately twice those at birth, and for adipose tissue are considerably higher than in other tissues, with the exception of the adrenal. Both pituitary and testis contain about four times as much tocopherol per unit of fresh tissue as do other visceral organs, but only about one-third as much as the adrenal. During the latter few decades of life there appears to be a tendency for tocopherols to diminish somewhat in liver and in adipose tissue. It is also of interest that total tocopherols (expressed as milligrams per 100 g. of fresh tissue) in skeletal muscle, heart, liver, and other visceral organs, except the endocrine glands mentioned, are of about the same order as observed in the circulating blood.

On the other hand, when tocopherols are expressed as milligrams per gram of extractible fat the values for adipose tissue are considerably lower than for most of the tissues just mentioned, whereas the values for pituitary, testis, and adrenal appear to be considerably higher. Although tocopherols in adipose tissue appear to increase during early life on the basis of tocopherols per unit of fresh tissue, and to diminish in terms of tocopherols per unit of extractible fat, from a quantitative standpoint the adipose tissue of the

body appears to represent the major site of tocopherol deposition and may therefore best reflect the tocopherol status of the body as a whole.

c. Histopathology

The histopathologic lesions of experimentally induced deficiencies often have a close counterpart in the corresponding deficiency state in man. This is true of vitamin C and of vitamins A, D, and K, insofar as the period of infancy and early childhood is concerned; however, differences in responsiveness of the adult organism and the tenacity with which its tissues retain traces of the fat-soluble vitamins seldom permit outspoken symptomatology or histopathologic changes. It is notably difficult to induce manifestations of vitamin E deficiency in mature animals that have built up considerable stores of tocopherol. Furthermore, the fact that tocopherol storage is chiefly in the adipose tissue, and thereby much less subject to interference by disease processes such as influence storage of vitamin A in the liver, may explain why there is so little evidence of vitamin E inadequacy in man other than during early infancy, as discussed previously.

Histopathologic approaches to the question of vitamin E deficiency in man have been generally twofold. One is based upon the occurrence of an acid-fast pigment commonly associated with the lesions of experimental vitamin E deficiency; the other relates to striking similarities between the muscle lesions of progressive muscular dystrophy in man and those common to the various species of animal in which vitamin E deficiency has been produced.

(1) Acid-Fast Pigment (Ceroid). An acid-fast pigment, sometimes referred to as ceroid, occurs in the smooth and striated musculature and becomes disseminated throughout the reticulo-endothelial system, after prolonged vitamin E deficiency in certain animals; in organs such as the sex glands and the adrenal, where small amounts of this pigment occur normally, there may be a conspicuous increase. Excess of intracellular, unsaturated lipids or fat peroxides and inadequacy of tocopherols as antioxidants are considered primary factors in the genesis of this pigment. A comparable pigment has been described by Wolf and Pappenheimer in various parts of the central nervous system, and by Pappenheimer and Victor in a variety of other tissues and organs from routine autopsies. They report that, in general, the occurrence of acid-fast pigment in human tissues tends to be associated with hepatic cirrhosis and hemochromatosis, celiac disease, pancreatic fibrosis, and non-tropical sprue, except for its occasional location about focal degenerative lesions such as atheromatous plaques and areas of follicular atresia. It is of interest that acid-fast pig-

ment, some of which may be derived from red blood cells, is commonly associated with atheromatous lesions of the aorta and other vessels, and that atheromatous aortas are said to contain peroxides such as are associated with sites of ceroid formation in vitamin E-deficient animals. A ceroid-like pigment in the human ovary has also been described by Brenner and by Reagan, usually in association with follicular atresia, and regarded as an oxidation product of vitamin A (or carotene) and unsaturated fats, catalyzed perhaps by lipoxidase of the ovarian tissues.

It should be made clear that in the studies describing ceroid in human tissues no claim is made that an avitaminosis E is involved. Ceroid may arise in tissues where, in association with unsaturated fats, there are local oxidative disturbances similar to those occurring in vitamin E deficiency but due to other causes; there is also the possibility of a localized destruction of intracellular tocopherol due to metabolic stress or chemical insult even though the tocopherol status of the body in general is normal.

There are several reports which present more definite suggestions that human beings may approach a state of conditioned avitaminosis E. Pappenheimer and Victor have presented in considerable detail the postmortem findings on four individuals exhibiting chronic nutritional disorders (idiopathic hypoproteinemia, gastrocolic fistula subsequent to gastroenterostomy, non-tropical sprue, and chronic jejunitis with cirrhosis) in which there was found an abundance of acid-fast pigment having much the same localization as that seen in vitamin E-deficient animals. In all cases, there was pronounced pigmentation of the muscular coats of the esophagus, stomach, and small intestine, such as seen in the E-deficient monkey and in dogs with biliary fistulae. Acid-fast pigment was also noted in liver cells, Kupffer cells, uterine muscle, phagocytes of ovarian stroma, cardiac and skeletal muscle, media of small arteries, and the Sertoli syncytium and interstitial cells of the degenerate testes of the one male of the series. Tverdy et al. present a detailed clinical history and postmortem findings in a case of non-tropical sprue, in which they noted acid-fast pigment with essentially the same distribution in intestinal smooth muscle, liver, degenerate testes, and macrophages of various organs and tissues. Histopathologic changes were also noted in the central nervous system, including

214a L. van Bogaert and G. Tverdy, Monatsschr. Psychiat. Neurol. 120, 301 (1950).
the presence of much acid-fast pigment, especially in relation to blood vessels and the choroid plexus epithelium. They consider these changes to be strongly indicative of an avitaminosis E associated with sprue.

Reference has been made (p. 544) to the low serum tocopherol levels and flat type of absorption curve observed in sprue, celiac disease, and similar disorders. Also of interest is the comment of Frazer that "Dietary inadequacy is certainly not a common cause of vitamin deficiency in the sprue syndrome. It may account for the occasional case of vitamin E deficiency, especially since oxidative rancidity of fats may be one of the precipitating causes in tropical sprue."

It is unfortunate that in the five cases referred to above no data could be obtained on serum tocopherol levels prior to death or on tissue levels at postmortem. With lack of evidence of this type, the findings reported can be considered only highly indicative that a conditioned state of avitaminosis E may sometimes occur in man as a result of chronic diseases which seriously interfere with the absorption of fats and fat-soluble vitamins.

(2) Progressive Muscular Dystrophy. Many investigators have been impressed by the striking similarity between the skeletal muscle lesions of progressive muscular dystrophy and dermatomyositis in man, and those of nutritional myodegeneration which represent the most characteristic manifestation of vitamin E deficiency in experimental animals (p. 552). This applies also to myocardial lesions, which constitute a rather characteristic finding in progressive muscular dystrophy as they do in many animal species deficient in vitamin E.

There is also evidence of a common biochemical defect, in the form of urinary excretion of ribose-phosphorus-containing complexes which appear to be rather specific for human muscular dystrophy and which occur also in dystrophic vitamin E-deficient rabbits. The suggestion that these complexes may reflect disturbances in nucleotide metabolism is in keeping with other evidence of disturbed nucleic acid metabolism in the vitamin E-deficient rabbit and monkey. Of particular interest is the observation of Minot et al. that "pentose-containing complexes were detected in the urine of an apparently normal 3 year old brother of one of our patients

with muscular dystrophy. There was a familial history of the disease, as one older brother of our patient and a maternal uncle had already died after running a typical course. Within the next 2 years this younger brother developed the typical clinical picture of rapidly progressing muscular dystrophy. It is possible that disintegration of muscle cells was already in progress before the appearance of detectable dysfunction. It is also conceivable that some inborn anomaly of vitally important nucleotide metabolism is responsible for the deterioration seen in clinical dystrophy.\(^{222}\) A generalized aminoaciduric observed in muscular dystrophy\(^{222}\) may reflect muscle breakdown and not a primary metabolic disturbance.

It is beyond the scope of this chapter to review the numerous reports affirming or denying the therapeutic usefulness of wheat germ products or tocopherol in human muscular dystrophy. Over a period of more than 15 years the results have been predominantly negative. This is not surprising in view of the normal levels of tocopherol found in the blood\(^{196,223}\) and in various tissues and organs.\(^{159,223a}\) It is possible, however, that in individuals with muscular dystrophy tocopherols cannot exert their normal functions in skeletal muscles, either because of a metabolic block such as referred to by Minot or because of an inability to effectively convert tocopherols to their hydroquinones or to other compounds which exert the antidystrophy effects of vitamin E. It is this general approach which has been followed by Milhorat \textit{et al.},\(^{221,221a,221b}\) who report that \textit{dl-}α-tocopherylhydroquinone and related compounds are effective in reducing creatinuria in dystrophic patients and also in curing dystrophy of vitamin E deficiency in rabbits. Unquestionably, this constitutes an important area for continued investigation.

Other investigators\(^{225-227}\) are of the opinion that wheat germ oil and tocopherol are effective in treating those diseases of muscle which they classify as collagen diseases; namely, dermatomyositis and menopausal dystrophy. The latter term has been proposed by Shy and McEachern\(^{227}\) for


a type of myopathy occurring predominantly in women, but occasionally in men, at the age of about 40 years or thereafter, and thought to be a close counterpart of the dystrophy in vitamin E-deficient animals; others classify this condition as myositis.\textsuperscript{228} Both diseases are said to respond also to cortisone therapy.\textsuperscript{227} The observations\textsuperscript{227} regarding dermatomyositis are in accord with the favorable response to wheat germ oil reported previously by Milhorat et al.\textsuperscript{229} There is still a question whether wheat germ products possess effective substances apart from tocopherols. It has been suggested\textsuperscript{230} that one such substance, inositol, can interact with tocopherol in the gastrointestinal tract to form a condensation product which diminishes the creatinuria of progressive muscular dystrophy; conceivably, a metabolic mechanism of this type could be defective or absent in patients with dystrophy.

On the basis of different patterns of inheritance, Tyler and Stevens\textsuperscript{231, 232} are of the opinion that most cases of progressive muscular dystrophy can be grouped under two major headings: the childhood type, occurring only in males and inherited as a sex-linked recessive, and the fascioscapulohumeral type, occurring in both sexes and inherited as a Mendelian dominant. It is as yet impossible to say whether the same or different metabolic defects of metabolism are involved, or the extent to which the metabolic defect in each case may eventually be related to oxidation or other conversion products of tocopherol.

\textit{d. Disorders of Reproduction}

The discovery of vitamin E and the long-established method for its bioassay are based upon the phenomenon of fetal resorption in the rat. It is not surprising that over the past twenty-five years there have appeared numerous clinical reports on the therapeutic use of vitamin E in habitual abortion, threatened abortion, threatened miscarriage, premature labor, and eclamptic states. The results are conflicting and confused by differences in definition, vitamin E dosage, and extent to which other therapeutic measures are employed and often not recorded. As is so often the case with unestablished therapeutic agents, favorable reports considerably exceed those which relay negative findings.

(1) \textit{Habitual Abortion}. An habitual aborter is usually defined as one who has spontaneously aborted before the 16th week during three successive pregnancies. It is estimated that 4\% of all spontaneous abortions are ha-

\textsuperscript{232} F. E. Stevens and F. H. Tyler, \textit{Am. J. Human Genetics} \textbf{3}, 111 (1951).
bital, and that about 10% of all pregnancies terminate in abortion, amounting to 240,000 yearly in the United States.223 Bacharach,234 in a statistical analysis of reported cases of habitual abortion treated with vitamin E up to 1940, felt that the chance of a successful pregnancy was definitely increased by this therapeutic measure. Hertig and Livingstone235 later state: “Vitamin E, judging from the literature, has an important effect on the favorable outcome of pregnancy in cases of habitual abortion —this in spite of the fact that the average human dietary cannot be shown to be deficient in vitamin E.” Only about 16% of habitual aborters show plasma tocopherol levels below the average normal range, and these are effectively raised to normal by as little as 25 mg. of α-tocopherol, daily.236 An 80% salvage in 211 patients with from three to eleven previous abortions, by correction of contributory conditions of varied type, has been reported by Javert et al.237 The present status of the problem is well summarized in their statement: “There is such a maze of literature that proper cognizance cannot be taken of all the pertinent articles. As the reader reviews them in order to develop his own philosophy, let him be reminded of three important matters: the high percentage of success irrespective of which vitamin, hormone or method is employed; the lack of specific information as to the pathogenesis of human spontaneous abortion...”

(2) Threatened Abortion and Miscarriage. Evan Shute, although dubious about the merits of vitamin E in habitual abortion, has reported its therapeutic usefulness in threatened abortion and threatened miscarriage,238, 239 premature labor,240 abruptio placentae,241 and non-eeclampsic late-toxemias of pregnancy,242 and is of the opinion that tocopherol may in some way counteract the effects of high blood estrogen rather than compensate for a trueavitamnosis E. Other clinicians have reported similar success with tocopherol, frequently combined with progesterone therapy; the reason that so much doubt still exists concerning these claims is due not so much to other reports in the negative as it is to failure to, or inability to, satisfactorily validate these clinical experiences by control data or by basic information regarding the tocopherol status of the patients. The most

228 E. Shute, Urol. and Cutaneous Rev. 47, 239 (1943).
thorough study in the latter sphere\textsuperscript{169} reports no significant changes in plasma tocopherol in cases of threatened abortion, prematurity, pre-eclampsia, or essential hypertension complicating pregnancy, except for patients with abortion during the 17th to 24th weeks of pregnancy; the interpretation which may be placed upon this latter finding is questionable until more data are available. Whether data on the tocopherol content of aborted fetuses and placentas would contribute to a better understanding of this perplexing problem remains to be determined.

(3) *Sterility in the Male.* The other classic manifestation of experimental vitamin E deficiency, namely, testicular degeneration in the rat, has no known counterpart in man. Although Shute\textsuperscript{245} holds the opinion that vitamin E therapy causes an increase in sperm count and enhances the possibilities that infertility in males can frequently be overcome by vitamin E therapy, largely through improvement in the number and quality of the sperm, Williams\textsuperscript{244} and Farris\textsuperscript{245} report that vitamin E concentrates have no significant effect on sperm concentration, motility, or cytologic aberrations in infertile men. Three other studies, yielding somewhat contradictory results, are summarized by Swyer.\textsuperscript{216}

\textbf{VIII. Pharmacology}

\textbf{KARL E. MASON}

As so aptly stated by Mattill,\textsuperscript{1} "None of the vitamins has been associated with as wide a variety of biological processes as vitamin E, or has been functionally related to so many basically different physiologic and chemical reactions. . . . The search for some unifying principle or correlating idea as to the manner of action has not been rewarding."\textsuperscript{17} Largely through the pioneer researches of Mattill and his associates, the tocopherols have come to be recognized as widely distributed and important biological antioxidants, both \textit{in vivo} and \textit{in vitro}. Compared to other tocopherols (\(\beta, \gamma, \delta\)), \(\alpha\)-tocopherol possesses the greatest biological activity \textit{in vivo} and is regarded as the prototype of vitamin E. It represents 90\% or more of the tocopherols in animal tissues. Whether ingested as the natural \(d\) or the synthetic \(dl\) form, or esters of the same, it is absorbed as \(d\)-\(\alpha\)-tocopherol; the latter is probably bound to lipids or proteins during transport in the blood stream and deposited as such within the cell.

\textsuperscript{244} E. Shute, \textit{Urol. and Cutaneous Rev.} \textbf{48}, 423 (1944).
\textsuperscript{1} H. A. Mattill, \textit{Nutrition Revs.} \textbf{10}, 225 (1952).
α-Tocopherol, like ascorbic acid, is one of the few antioxidants capable of passing the intestinal barrier, reaching intracellular sites, and exerting regulatory control over cell oxidations. Despite hopes and a certain amount of evidence that tocopherol might prove to function in some oxidation-reduction system, or participate in some specific manner in certain enzyme systems, no clear-cut claims can yet be made; nor has there been produced indisputable evidence that α-tocopherol exerts biological effects unrelated to its well-recognized function as an intracellular antioxidant.

A. HYPERVITAMINOSIS E

No state or syndrome of hypervitaminosis E has been described, nor is there evidence that tocopherols per se exert any deleterious effect in animals or man. Demole has shown that mice will tolerate oral doses of 50 g. per kilogram, and rats doses of 4 g. per kilogram daily for 2 months. Adult humans have tolerated oral doses of 1 g. per day for months, or larger doses for shorter periods, with no undesirable effects. Clinical literature contains references to complaints of gastric distress and other symptoms in patients on much smaller dosage levels; these are probably related to fatty substances present in tocopherol concentrates or, in some instances, to psychic factors.

B. MODE OF ADMINISTRATION

The natural and synthetic forms of α-tocopherol and their acetate esters are viscous oils. Intramuscular injections, frequently used in clinical practice, have sometimes led to painful reactions locally and to oleogranulomas at a later date; “solubilized” preparations may be less reactive in these respects. Tocopherol ointments have been used to only a limited extent. In animals, implanted pellets of the crystalline esters (palmitate, succinate, and phosphate) produce marked local tissue reactions; so also does injection of the slightly water-soluble phosphate ester. Neither in animals nor in man are there reliable data concerning the relative effectiveness of absorption and utilization of tocopherols administered in these various ways, as compared to oral dosage which appears to be the most effective mode of administration.

C. METABOLIC STRESS IN ANIMALS

There is a considerable body of evidence that α-tocopherol has a remarkable capacity to protect experimental animals against a variety of metabolic stresses, including those induced by anoxia, high intake of unsaturated fats, low protein intake, restricted intake of other vitamins (A,  

2 V. Demole, Intern. Z. Vitaminforsch. 8, 338 (1930).
of such substances as alloxan, silver nitrate, \( o \)-resyl phosphate and carbon tetrachloride. In many instances, additional tocopherol means the difference between death of the animal or continued survival in good health. Presumably these beneficial effects relate to the antioxidant functions of tocopherol, operating sometimes in the intestinal tract and at other times at sites where local tissue injury would otherwise occur. Furthermore, tocopherol can enhance the curative action of critical amounts of vitamin A and essentially fatty acids, possibly by protecting them up to the point of intestinal absorption.

D. THERAPEUTIC USE

There exists an extensive but rather controversial literature dealing with the therapeutic efficacy of tocopherol in a wide variety of disease states, many of which have little or nothing in common with experimentally induced manifestations of vitamin E deficiency and are not associated with any evidence of a significant inadequacy of tocopherol in the patient. Furthermore, the effects reported are usually obtained only with relatively high doses over a period of many weeks or months. As expressed by Hickman\(^5\) in an interesting review of this subject, “The discrepancy between the few milligrams a day that suffice to maintain the majority of people in health and the hundreds of milligrams being used clinically gives cause for serious thought. . . . Only time and continued study can resolve the dosage paradox with this vitamin . . . .”

Those who report benefit in certain clinical disorders usually stress the importance of sustained, high daily dosage, generally amounting to between 200 and 600 mg. of \( \alpha \)-tocopherol; this represents about twelve to thirty-six times the average daily intake from diet. There is also the common observation that only in a certain proportion of patients suffering from a specified disorder is there a significant remission of symptoms, the remainder showing no benefit other than perhaps an improved sense of well-being and physical vigor. Observations such as these suggest that in certain instances high tocopherol dosage may, through its capacity to regulate and enhance intracellular oxidations, greatly improve states of lowered or otherwise altered metabolism at various localized sites in tissues and organs of the body. The location of the latter, and their relation to the etiology and sequelae of the disease entity under consideration would, of course, vary widely from patient to patient. In other words, as in its protective effect in enabling the experimental animal to resist and overcome the effects of a variety of metabolic stresses, the clinical value of tocopherol may lie in large part in its ability to counteract localized metabolic and toxic stresses.

VIII. PHARMACOLOGY

which may be either primary or secondary to, or quite independent of, a primary disease process.

1. HEART DISEASE

Vogelsang, Shute, and Shute\textsuperscript{6-10} report that intensive tocopherol therapy usually ameliorates or abolishes the clinical signs and symptoms (anginal pain, exertional dyspnoea, fatiguability, lowered exercise tolerance) of coronary, rheumatic, and hypertensive heart disease, and also hastens the resolution of coronary thrombi and the recovery of damaged cardiac tissue. These effects are attributed to a pharmacologic action of \( \alpha \)-tocopherol in decreasing the oxygen requirement of cardiac muscle, in helping to resolve and also in preventing intravascular thrombi, and in improving the functional state of the capillary bed. Although, as they point out, there is considerable experimental evidence to support such postulations, there are also certain etiologic and other differences between cardiac disease in man and the experimentally induced lesions of vitamin E deficiency. Clinical reports\textsuperscript{11-16} which might be considered as supporting these claims record a total of 109 cases of heart disease, of which 40 were regarded as being benefited to a variable degree by tocopherol therapy. On the other hand, another series of clinical reports,\textsuperscript{17-27} representing observations on a total of 252


\textsuperscript{8} W. E. Shute, Summary \textbf{1}, 13 (1949); \textbf{3}, 19 (1951).

\textsuperscript{9} W. E. Shute and E. V. Shute, Summary \textbf{2}, 3 (1950).

\textsuperscript{10} A. L. Pascoe and W. E. Shute, Summary \textbf{1}, 50 (1949).


\textsuperscript{12} L. Pin, Contribution to the Study of the Physiological and Therapeutic Properties of Vitamin E. Maurice Lavergne, Paris, 1947.

\textsuperscript{13} K. P. Ball, Lancet, I, 116 (1948).


\textsuperscript{15} N. Agadjanian, J. Méd. Paris \textbf{68}, 29 (1948); Summary \textbf{3}, 50 (1951).


patients, record no recognizable benefit resulting from this therapeutic procedure. Many variables, some controllable and others not, enter into such clinical studies and markedly influence the final conclusions, which often fall far short of a critical evaluation of patient response in terms of what the result might have been in the absence of tocopherol therapy. Until many of these variables are resolved and more reliable criteria of responses established, the question of the merits of tocopherol therapy in heart diseases must be considered an unsettled one.

2. Peripheral Vascular Disease

Rather remarkable effects of high dosage of α-tocopherol in the management of vascular disorders such as indolent ulcers, early granulone of the extremities, thromboangiitis obliterans, thrombophlebitis, phlebothrombosis and cerebral thrombosis, and also burns, have been reported by Shute et al.; 26, 28-34 these effects are attributed to antithrombic, thrombocytolytic, and capillary-vasodilative functions of tocopherol. Others report beneficial effects in cases of thromboangiitis obliterans, 35, 36 leg ulcers, 37-39 and phlebitis; 40 but contrary views are also expressed. 41-44 Increased peroxide content of subcutaneous tissues in cases of vascular disease of the extremities, suggestive of a local deficiency of vitamin E, 45 and the reported effects of tocopherol on experimentally induced femoral thrombi 46 and in prevention of arterial lesions 47 in dogs, are of interest in this connection.

31 W. E. Shute and E. V. Shute, Seminar 1, 47 (1949).
34 W. R. Cameron, Seminar 3, 9 (1951).
42 L. L. Pummock and A. M. Minno, Angiology 1, 337 (1950).
44 H. I. Lipsmann, in discussion of paper by E. V. Shute, ref. 30.
A practical application of the observation that α-tocopheryl phosphate has antithrombic activity\(^48\) has not been established. Kay,\(^49\) in reviewing an extensive experience, states: "It is conceivable, though not yet proven statistically, that the administration of alpha tocopherol and calcium will reduce the incidence of postoperative phlebothrombosis and pulmonary embolism." This question has been carefully reviewed elsewhere,\(^50-52\) with essentially the same conclusions. The reported effect of tocopherol in countering stilbestrol-induced purpura in dogs\(^53\) has not been confirmed;\(^54\) its reputed antipurpuric action in thrombocytopenic purpura in man\(^55,56\) warrants further study.

3. Menopausal Syndrome

α-Tocopherol, at dosage levels considerably lower than those employed in other clinical disorders, is reported to relieve symptoms of the menopause and to be especially useful where estrogens are contraindicated.\(^57-67\) Perloff\(^64\) reports that results with tocopherol compare very favorably with those obtained with hormone therapy, which seems contradictory to the postulation\(^63\) that vitamin E is antiestrogenic. On the other hand, the more recent report of Blatt et al.,\(^69\) based on a careful comparison of the effects of estrogens, phenobarbital, tocopherol, and placebos on 748 climacteric patients, concludes that α-tocopherol is no more effective than placebo therapy in controlling menopausal symptoms.

\(^{50}\) Nutrition Revs. 10, 16 (1952).
\(^{51}\) I. S. Wright, Circulation 5, 161 (1952).
\(^{54}\) E. V. Shute, Urol. and Cutaneous Rev. 50, 732 (1946).
\(^{60}\) B. B. Rubenstein, Federation Proc. 7, 106 (1948).
\(^{66}\) H. A. Gazan, N. Y. State J. Med. 52, 1280 (1952).
\(^{68}\) M. G. H. Blatt, H. Wiesbader, and H. S. Kupperman, Arch. Internal Med. 91, 792 (1953).
4. PRIMARY FIBROSITIS AND RELATED DISORDERS

Under this heading are included conditions generally regarded as metabolic disorders of the connective tissues and represented by Dupuytren's contracture, Peyronie's disease, and generalized involvement of muscles such as seen in myositis, fibromyositis, muscular rheumatism, lumbago, and bursitis. The reported beneficial effects of tocopherol therapy have been ascribed to effects upon the vascular bed or upon altered connective tissues, perhaps through correction of some localized metabolic disturbances in the involved tissues.

Steinberg's observations have extended by him and confirmed by certain investigators but not by others. It is noteworthy that in the two most recent studies, where tocopherol was the sole form of treatment and plaster casts were used to measure changes in flexion deformity, definite though moderate improvement was observed in 23 of 26 affected hands in one study and no improvement noted in 58 hands in the other.

Peyronie's disease (fibrous infiltration of the intercavernous septum of the penis), which is not uncommonly associated with Dupuytren's contracture, is said to respond favorably to tocopherol therapy, although negative or negligible responses are not infrequent. Similar results have been reported in the treatment of urethral stricture and interstitial cystitis. No contrary findings have as yet been reported.

There is also the interesting observation of Edgerton et al. that tocopherol frequently relieves the pain associated with keloids when other measures have failed but does not prevent the development of these areas of dense scar tissue. This is attributed to softening of the scar tissue, perhaps through correction of underlying metabolic or vascular disturbances.

general hypothesis has also been offered as an explanation of the favorable response observed in other types of primary fibrositis.

Myositis and other disorders usually included in the category of generalized primary fibrositis have been reported to be benefited by tocopherols whether administered orally or in the form of an ointment. If other clinicians have had a different experience, they appear not to have reported it.

Burgess and Pritchard are of the opinion that tocopherol, through some unexplained action upon the connective tissues, exerts beneficial effects in a variety of collagenoses such as chronic discoid lupus erythematosus, scleroderma, and granulomatous ulcers. Although others have reported favorable results in granuloma inguinale and in lupus erythematosus (when combined with pantothenic acid therapy), still others have recorded negative or questionable results. Only further research can clarify this controversial question.

5. DIABETES MELLITUS

α-Tocopherol administration is said to considerably reduce the insulin requirements of diabetics, the effect being attributed to improved glycogen storage in muscle cells rather than to potentiation of insulin action. On the other hand, other investigators, who have studied this question with particular care, have reported only negative results.

84 M. Ant, N. Y. State J. Med. 45, 1861 (1945); Industrial Med. 15, 399 (1946); Rheumatism 6, 114 (1950).
89 R. D. Sweet, Lancet II, 310 (1948).
6. General Comments

Without reference to numerous other clinical reports, many of them unsubstantiated or unchallenged, dealing with beneficial effects of tocopherol in a variety of other human ills, it will be apparent that the status of \( \alpha \)-tocopherol as a chemotherapeutic agent rests on an extensive but decidedly controversial literature. Out of the array of conflicting evidence, there seem to emerge indications that in certain disease processes there may occur more or less local metabolic disturbances which may lead to increased needs for, or even to local destruction of, tissue tocopherol, especially involving the connective tissues and the related capillary bed; conceivably, such local disturbances which may not be compensated for by normal body stores of tocopherol can be overcome by high and sustained tocopherol therapy. Whether this represents a glimmer of a pharmacologic action of tocopherol, and whether functions of \( \alpha \)-tocopherol other than its generally accepted role as an intracellular antioxidant would be necessary to explain such effects, remain for future research to decide.

IX. Requirements and Factors Influencing Them

HENRY A. MATTILL

A. OF ANIMALS

From what has been said, it is obvious that no categorical statements can be made as to the requirements of different species or for the prevention of a particular nutritional impairment in any of them. Even with a highly purified basal diet, the results are subject to considerable biological variation, and with natural foodstuffs the minute variations implied in the experimental work described in the preceding pages can produce profound effects, depending on the nature and history of each component. A diet high in fat, particularly unsaturated fat, appears to increase the need for vitamin E.\(^1\)\(^-\)\(^4\) Any statement regarding the MFD (mean fertility dose) (0.75 mg. of \( \alpha \)-tocopherol) for the rat holds only for the specific conditions of the test. The daily needs of the two sexes are about equal, despite the fact that the male requirement begins early (40th to 50th day of life), whereas that of the female arises only after conception.\(^5\) The minimal daily

---

requirement is \( \frac{1}{15} \) to \( \frac{1}{20} \) of the MFD for adult female rats. The minimum amounts for prevention of muscle dystrophy in nursling rats from E-low mothers depends on the time when supplementation begins; the longer this is delayed, as between the 10th and 17th day, the more is required.\(^6\) There are no essential differences between the antisterility and dystrophy-preventing potencies.\(^7\) As the daily dosage is reduced below 0.75 mg., the length of the reproductive period is reduced.\(^8\) The gradual decline in productivity of rat colonies may often be traced to the inadequacy of the vitamin E supplies in commercial feeds. Such premature sterility appears to have no effect on total span of life.\(^9\) For the rabbit the daily amount of \( \alpha \)-tocopherol to prevent and cure dystrophy has been given as 0.2 to 0.4 mg. per kilogram;\(^10\) the minimum need of the laying hen is 1.2 mg. per day\(^11\) and of the guinea pig 3 mg.\(^12\)

Like many other functions, that of vitamin E might be quantitatively related to the 0.7 power of the body weight.\(^13\) On this basis the daily requirement of an adult person of 70 kg. would be perhaps 30 mg. According to surveys in Holland\(^14\) and in the United States\(^15\) the average individual intake of \( \alpha \)-tocopherol is 6 mg. per 1000 cal. or less,\(^16\) but the tocopherols of vegetables are much less available than those in oils,\(^17\) and they may not all be the \( \alpha \) type.

Few, if any, sufficiently controlled experiments have been made to establish the requirements of domestic animals that are known to need vitamin E. Their natural food ordinarily supplies it in abundance, and the preparation of a ration devoid of it, as by treatment with ferric chloride, introduces other variables and may also fail of its purpose.

The moot question has been whether domestic animals on their natural feeds require supplements of vegetable oils or of tocopherol in order to yield the best returns in rate of growth and in reproduction. Sterility in cows was reported to have been successfully treated with wheat germ oil,\(^18\) the

assumption being that for some unknown reason a deficiency may have existed, notwithstanding dietary abundance. There are numerous and more recent statements both in support and in denial of this contention as applied to all farm animals. The problem is complicated by the fact that their need of vitamin E for reproduction has not been clearly demonstrated;\(^{19}\) the young, through lack of enough E in their milk, suffer degeneration of voluntary or cardiac muscle, from which they may or may not recover if untreated. Tocopherol given to the young effects a cure. The dietary inadequacy in the dams can be due to the lack of green feed, the use of restricted rations, degenerated cereals, or sophisticated concentrates. There is increasing evidence that the responses of animals to tocopherol differ, on the farm as well as in the laboratory.\(^{20, 21}\)

Many more experiments, carefully controlled and with guarded conclusions, like those reported on swine,\(^{22, 23}\) must be performed before the requirements of domestic animals are known.

**B. OF MAN**

**KARL E. MASON**

Requirements of tocopherol for man have not been satisfactorily established and can be inferred in only an approximate manner on the basis of what is known concerning dietary intake, effectiveness of absorption, rate of utilization, extent of excretion, and tissue storage. An interesting discussion of the various factors operating between nature's deposition of tocopherol in plant foods and the subsequent transfer of tocopherol across the intestinal barrier in man has been presented by Hickman and Harris.\(^{24}\) It is reported that part of the dietary tocopherol is oxidized to quinones in the intestine.\(^{25}\) Others,\(^{26}\) who feel that there is very little destruction in the intestine, indicate that perhaps more than half of ingested tocopherol may be excreted in the feces. The measurement of fecal tocopherols is a difficult procedure because of the presence of many interfering substances. Progress has been made,\(^{24, 25-26}\) and it is hoped that improved methods will make possible more informative tocopherol-balance studies.


As previously stated (p. 543), the average daily intake for adult man appears to be about 14 to 19 mg. of \( \alpha \)-tocopherol. On the basis of tocopherol analyses of foods it has been estimated\(^{27}\) that on diets considered low, average, and high in vitamin E there would be provided, respectively, 1, 4, and 10 mg. of \( \alpha \)-tocopherol for infants; 4, 10, and 20 mg. for children; 5, 15, and 35 mg. for adults; and 3, 10, and 15 mg. for aged persons. When consideration is given to losses due to storage, cooking, inactivation in the gut, and fecal excretion, the latter representing by far the major loss, the total available for metabolic utilization and storage might be reduced by one-third more; this would give a net absorption of approximately 5 to 7 mg. of \( \alpha \)-tocopherol daily for an average adult whose daily intake amounts to about 20 mg. per day. The extent to which requirements vary with age is not known. One might expect that, in terms of milligrams of tocopherol per kilogram of body weight, the requirements during infancy and childhood would be greater than during adult life. This would be in keeping with the evidence of slow acquisition of body storage stores during childhood and adolescence.\(^{28, 29}\) It is not clear whether lowered tissue storage observed in old age\(^{28, 29}\) represents diminished dietary intake or increased needs to compensate for the generally lowered oxidative state of body tissues. On the experimental side, there is evidence of increased requirements for reproductive function as age progresses.

The requirements for vitamins are influenced by much the same conditions which necessitate increased intake of vitamin A; namely, conditions which chronically impair the absorption of fats from the gastrointestinal tract. It seems reasonable to assume also that those factors which have been shown to accentuate deficiency manifestations in animals, most of which can be considered to represent types of metabolic stress (excess intake of unsaturated fats, low protein intake, ingestion of chemical substances such as \( \alpha \)- cresyl phosphate or silver nitrate), can be regarded as potentially capable of increasing human requirements as well.

Chapter 18

NEW AND UNIDENTIFIED GROWTH FACTORS

VERNON H. CHELDELIN

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The body of information that has formed the basis for the preceding chapters has enabled us to obtain a clear outline of the chemistry and function of several of the vitamins that have been discussed. Although the pioneer work on vitamins was done only half a century ago, the intensity of research, particularly during the past two decades, has so overcome the time disadvantage that several vitamin systems are now as well or better understood than many of the more classical compounds of biochemical importance.

The purpose of the present discussion is mainly to provide the reader with an up-to-date account of the newly suspected and recognized members of the biocatalyst family—those which have been reported but have not yet been sufficiently developed to merit separate consideration. However, in view of the speed with which problems in this field are being carried forward, the members of the "new factors" group must obviously be transient ones. Compounds listed in the present article may within a few years be expected either to emerge as fully characterized vitamins or to be dropped altogether as duplications are discovered and the occasional artifact or combined effect of known factors is recognized and eliminated. New members are meanwhile being added at a rapid rate.

I. Lipoic Acid (Thioctic Acid, Pyruvate Oxidation Factor, Protogen, Acetate Replacement Factor)

This compound is selected for initial consideration because its chemistry and some of its biological functions have become well outlined and because its development typifies both the intensity of study and the diversity of systems that are being employed in the search for new growth factors. Two
bacterial and one protozoal growth systems and one bacterial oxidative system were under study in the four laboratories that independently reported the existence of this compound.

Priority for the discovery of lipoic acid is assigned to Guirard, Snell, and Williams¹ and to Kidder and Dewey,² ³ who at an earlier date noted a combined growth effect that was later shown⁴ to be due to lipoic acid, pyridoxal, and copper ions. Guirard et al. observed⁵ that the stimulatory effect of acetate upon the growth of Lactobacillus casei could be replaced by small quantities of natural extracts such as liver, yeast, or dried grass juice. They concentrated the active material 44-fold from yeast. The following year, O'Kane and Gunsalus⁶ found that an unidentified factor from yeast (later called pyruvate oxidation factor⁷) was necessary in resting cells of Streptococcus faecalis, for oxidation of pyruvate, or its anaerobic dismutation to lactate, acetate, and carbon dioxide. Preparations were obtained that were approximately 200 times as potent as the starting material (yeast extract). Independently of these investigations, Stokstad et al.⁸ fractionated the natural supplements described by Kidder and Dewey² ³ for the nutrition of Tetrahymena geleii and found that the necessary unknowns could be reduced to two. Both of these unknowns produced the same biological response, and it was concluded that they were two forms of the same compound. They named the substance "protogen" because of its effect upon the protozoon. Later,⁹ these workers found that a strain of Corynebacterium also required protogen for growth. Finally, Kline and Barker⁸ described what appears to have been lipoic acid as a growth factor for Butyribacterium rettgeri.⁹ They concentrated the factor approximately 200-fold from yeast and demonstrated the existence of at least three forms.

The probable identity of protogen, the acetate replacing factor, and the pyruvate oxidation factor was reported by Snell and Broquist,¹⁰ who showed a high degree of correlation to exist among the relative potencies of different concentrates for acetate replacement, pyruvate oxidation, and Tetrahymena growth. The compound had been found to be fat-soluble, and because of this and its adsorption characteristics it was also thought to be similar to

¹ B. M. Guirard, E. E. Snell, and R. J. Williams, Arch. Biochem. 9, 381 (1946).
³ G. W. Kidder and V. C. Dewey, Arch. Biochem. 8, 293 (1945).
vitamin B\textsubscript{12} \textsuperscript{8,11,12} however, the latter identity has not been confirmed experimentally.\textsuperscript{12a}

The vitamin was first isolated in pure form by Reed, Gunsalus, \textit{et al.},\textsuperscript{13} the term “lipoic acid” was also introduced at this point to describe the (fat-soluble) compound as it was obtained from liver after hydrolysis, possibly from ester or amide combination. The isolation procedure from yeast\textsuperscript{14} disclosed the existence of five chromatographically distinct forms of the factor. These have been characterized by Gunsalus \textit{et al.}\textsuperscript{9} as: (1) a bound form, (2) a water-soluble, fat-solvent-insoluble form, referred to as “water-soluble complex,” and three fat-soluble varieties referred to as (3) a weak acid, (4) a strong acid, and (5) a neutral substance. The first form was found very widely distributed; the second was observed principally after enzyme digestion. The three fat-soluble compounds remained after hydrolysis with acid or alkali. However, Reed \textit{et al.}\textsuperscript{14,15} reported only two forms after hydrolysis. This may possibly have been due to production of some forms during operational treatments, or to conversion of natural conjugates (e.g., esters or amides) to the two principal compounds remaining. The two forms, when bioautographed on paper with water-saturated butanol, exhibited \(R_f\) values of 0.90 and 0.60.\textsuperscript{14} The \(R_f\) 0.60 form was largely converted to the \(R_f\) 0.90 variety (a more polar substance). A similar interconversion was reported by the Lederle group in their isolation of protogen;\textsuperscript{16} they observed a marked increase in water solubility and designated the process protogen A \(\rightarrow\) protogen B.

The potency of pure lipoic acid (the \(R_f\) 0.60 form, called \(\alpha\)-lipoic acid)\textsuperscript{13} was found to be 250,000 times that of yeast, based on the stimulation provided in the \textit{S. faecalis} test for pyruvate oxidation. As a replacing agent for acetate in \textit{L. casei}, lipoic acid was 15,000,000 times as potent; half-maximum growth was provided by \(1.7 \times 10^{-6}\) \(\gamma\) per milliliter of culture medium. This compound thus ranks with biotin, vitamin B\textsubscript{12}, and the folic acid group as an extremely active biocatalyst.

Studies on the structure of the lipoic acids have been both intensive and numerous. The picture of the interrelationships among the various forms has rapidly emerged, although the important details are not yet all worked

\textsuperscript{12a} Since this manuscript was prepared, a report has been issued indicating a relationship between vitamin B\textsubscript{13} and orotic acid [L. Manna and S. M. Hauge, \textit{J. Biol. Chem.} 202, 91 (1953)].
\textsuperscript{13} L. J. Reed, B. G. De Busk, I. C. Gunsalus, and C. S. Hornberger, Jr., \textit{Science} 114, 93 (1951).
\textsuperscript{14} L. J. Reed, B. G. De Busk, P. M. Johnston, and M. E. Getzendaner, \textit{J. Biol. Chem.} 192, 851 (1951).
\textsuperscript{15} L. J. Reed, M. E. Getzendaner, B. G. De Busk, and P. M. Johnston, \textit{J. Biol. Chem.} 192, 850 (1951).
out. Reports during the past year have been issued from the Lederle Laboratories and jointly from the universities of Texas and Illinois and the Eli Lilly Laboratories. These all point to the lipoic acids as a family of di-thioöctanoic acids and their derivatives. The first synthesis of a pure compound with lipoic acid activity was carried out by the Lederle group, who prepared the cyclic 6,8-dithiooctanoic acid, which they called 6-thioctic acid. This was accomplished by condensing ethyl adipyl chloride with ethylene to yield $\Delta^2$,6-ketooctenoate, and converting the latter in consecutive steps to 8-thiol-6-hydroxyoctanoic acid (with thiaacetic acid), 6,8-dithioloctanoic acid (thioureia in HI), and finally the cyclic disulfide (I$_2$ in KI). The product was confirmed as being identical with protogen A, after earlier work had suggested the 5,8 ring structure for the factor. Synthetic DL-6-thioctic acid appeared from the tabular data presented to be roughly half as active in pyruvate oxidation as the isolated lipoic acid of Reed et al.

Protogen B (8-lipoic acid) may be presumed to be the corresponding (mono) sulfoxide. This structure appears consistent with the observed transformations (protogen A $\rightarrow$ protogen B) during isolation, and with the properties of the two compounds.

The need for lipoic acid for the oxidation or dismutation of pyruvate places this catalyst in the same particular area of metabolism as diphosphothiamine (DPT) and coenzyme A (CoA). The precise enzymatic role of lipoic acid has not yet been determined, although important indications were provided by the findings that "lipothiamide" (LT) and "lipothiam..."
mide pyrophosphate” (LTPP) were active in the oxidative decarboxylation of pyruvate. These materials were obtained in a reaction mixture of α-lipoic acid or its acid chloride with thiamine and thiamine pyrophosphate, respectively. The biologically active products possessed \( R_F \) values identical with those of two of the forms present in natural materials. Both gave negative thiochrome tests and positive azo tests, and the authors suggested that the carboxyl group of lipoic acid was conjugated with the pyrimidine amino group of thiamine. The test system used was a mutant strain of *Escherichia coli* that would not respond to either vitamin, alone or mixed together, but which grew luxuriantly on LT or LTPP. Soluble enzyme preparations from the mutant catalyzed the anaerobic dismutation of pyruvate or the oxidation of α-ketoglutarate only when LTPP was supplied (LT was inactive in the latter system). These reactions thus reveal a definite enzymatic role for lipoic acid in oxidation of α-keto acids, as a molecular conjugate with thiamine, and point to LTPP as the actual coenzyme. The authors proposed, partly on the basis of previous knowledge of the mechanism of pyruvate oxidation in *E. coli*,\(^{30, 31}\) that LTPP participates according to reaction 1:

\[
\text{Pyruvate (α-Ketoglutarate)} + \text{LTPP} + \text{DPN}^+ \rightarrow \\
\text{Acetyl LTPP (succinyl LTPP)} + \text{CO}_2 + \text{DPNH} + \text{H}^+ \quad (1)
\]

In the presence of coenzyme A, the acyl group could then be transferred as follows:

\[
\text{Acetyl LTPP (succinyl LTPP)} + \text{CoA} \rightarrow \\
\text{Acetyl CoA (succinyl CoA)} + \text{LTPP} \quad (2)
\]

This scheme thus provided a plausible order of participation for LTPP and CoA, although it did not *per se* clarify the question of whether LTPP is needed for decarboxylation or dehydrogenation. Evidence bearing on this is provided in other studies on *E. coli* and *S. faecalis*,\(^{32-36}\) as follows:

1. The complete system for the pyruvate → acetate + CO\(_2\) conversion in *E. coli* requires DPT, diphosphopyridinenucleotide (DPN), CoA, Mg\(^{++}\), and lipoic acid (presumably as LTPP).


2. When ferricyanide replaces oxygen as the final electron acceptor, CoA and DPN may be by-passed, although the requirement for DPT and lipoic acid remains.

3. When no oxidation occurs, as in acyloin formation, CoA, DPN, and lipoic acid may all be omitted; however, DPT is still required.

From these observations it appears that in these organisms LTPP may be involved in dehydrogenation at a very early stage in the breakdown of pyruvate. This is in line with the report by Sanadi et al.\textsuperscript{37} that pig heart \( \alpha \)-ketoglutaric oxidase contains up to 6 moles of lipoic acid and about 1 mole of cocarboxylase per mole of enzyme, but no DPN or CoA. Schemes suggesting possible sequences of reactions have been proposed,\textsuperscript{35, 38-40} which may be summarized approximately as follows:

\[
\text{CH}_3\text{COCOO}^- \xrightarrow{\text{DPT}^+, \text{Mg}} \left[ \begin{array}{c} \text{O} \\ \text{CH}_3-\text{C}^- \end{array} \right] \xrightarrow{\text{DPT}^+} \text{DPT}^+ + \text{CO}_2
\]

\text{(acetaldehyde - DPT complex)}

This complex may then be oxidized by lipoic acid, presumably in the disulfide form. Whether this step involves the lipoic moiety for the first time, or whether LTPP is the actual acceptor in reaction 1, is speculative. In either case the cyclic disulfide may react to produce an acyl lipoate:

\[
\text{[CH}_3-\text{C}^-] \xrightarrow{\text{H}_2} \text{CH}_3-\text{C}^-\text{S-CH}_2
\]

\text{(2)}

\[
\text{CH}_3-\text{C}^-\text{S-CH}_2 \xrightarrow{\text{CoA-SH}} \text{CH}_3-\text{C}^-\text{S-CoA} + \text{H}_3\text{C}-\text{CHR} \]

\text{(3)}

According to this scheme, the acetyl CoA produced in reaction 3 is removed.

\textsuperscript{40} L. J. Reed, Symposium on Metabolic Significance of Vitamins, American Institute of Nutrition, \textit{Federation Proc.} \textbf{12}, 558 (1953).
as "acetate" via such reactions as 4, 5, or 6. CoA-SH is simultaneously regenerated for use in reaction 3, whereas the oxidized functional form of lipoate reappears in reaction 7 for use in reaction 2. Through the use of flavoproteins and oxygen, or lactic dehydrogenase, DPN+ is made available for re-use in reaction 7. When ferricyanide is the electron acceptor, an aldehyde-LTPP complex may be supposed to react according to equation 8:

$$\left[ \begin{array}{c} \ce{O} \\ \ce{CH_2-C=} \end{array} \right]^{-} + \text{LTPP} \xrightarrow{2\text{Fe(CN})_6^-} \left[ \begin{array}{c} \ce{O} \\ \ce{CH_2-C} \end{array} \right]^+ + \text{LTPP}^+ + 2\text{Fe(CN})_6^- \ \ \ (8)$$

Alternately, an aldehyde-DPT complex may combine with a second molecule of aldehyde to produce acetoin.

The foregoing reactions explain fairly well most of the observed requirements for lipoic acid, DPT, CoA, and DPN in the oxidation of pyruvate (but not free acetaldehyde or ethanol\(^{41}\)) by \textit{E. coli} and \textit{S. faecalis}. However, the need for LTPP when oxidation proceeds, in contrast to DPT when acylloins are formed, is not clearly explained. It is possible that the same apoenzyme might bind either co-factor. This has indeed been suggested,\(^{40}\) and an experimental verification of this concept would permit a simpler presentation of reactions 1 and 2 above.

These studies thus suggest that lipoic acid possesses at least three important functions in \textit{E. coli}: first, as a primary (co)dehydrogenase in the oxidation of \(\alpha\)-keto acids; second, as an acyl transferase; and third, in the transfer of energy through the formation of a "high-energy" S-acyl bond, which can be transferred to CoA and utilized in various biosynthetic reactions. This full complement of activities may be restricted to certain organisms, however, since it has been reported\(^{42, 43}\) that in \textit{Tetrahymena} pyruvic dehydrogenase activity was unaffected by the removal of lipoic acid from the system; only the acylation process was impaired by this treatment. It would appear that lipoic acid is not the primary electron acceptor from pyruvate in this organism. A role, as yet undescribed, for lipoic acid in animal systems is suggested by its presence in \(\alpha\)-keto glutaric\(^{37}\) and pyruvic oxidases,\(^{44}\) although dietary requirements for the factor have not been established.\(^{45}\) An active function in animal pyruvic dehydrogenase systems is further hinted through the work of Peters \textit{et al.},\(^{46}\) Stocken and Thomp-

son,47 and Gunsalus.35 The two former groups observed that the vesicant arsenical Lewisite (CHCl—CH—AsCl₂) was particularly effective against the pyruvic oxidase system in pigeon brain, and that the attack was primarily upon —SH groups in this system. Reversal of Lewisite oxide poisoning by BAL (2,3-dimercaptopropanol) was shared by other dithiols, but not by monothiols. It was then shown35 that lipoic acid, like BAL (but unlike glutathione), was highly active in overcoming arsenate and arsenite inhibition of apopyruvate dehydrogenase activity in S. faecalis. Whether the lipoic acid functioned in the latter study as catalyst or substrate is not clear; however, the high reversing power of lipoic acid is noteworthy (0.04 \( \gamma \) was somewhat less active than 100 \( \gamma \) of BAL). It is quite possible that lipoic acid is one of the first compounds in the pyruvic oxidase system to be inactivated by Lewisite.47a

On the basis of the belief that lipoic acid possesses codehydrogenase functions, Calvin and his associates38, 39, 48 have suggested the intriguing possibility that this coenzyme may participate in the primary quantum conversion act of photosynthesis. It was observed49 that the incorporation of \( \text{C}^{14}\text{O}_2 \) into members of the citric acid cycle in green algae was strongly inhibited by light. This was at first interpreted as being due to the maintenance (in light) of too low a concentration of a key intermediate required for entry into the cycle, and, more recently, as a possible depletion of the supply of oxidized lipoic acid available to oxidize pyruvate. The key reaction is envisaged48 as

\[
\text{Chlorophyll (activated)} + \text{S} \rightarrow \text{Chlorophyll (ground state)} + \text{S}
\]

whereby lipoic acid is maintained as thyl-free radicals due to light-induced

47a The postulated activity of lipoic acid in the dithiol form as an anti-arsenical raises some questions as to the correctness of the proposed structures of LT and LTTP,26 if the latter are assumed to be the active forms of the compound. Thus, with a lipoic acid sulfur-protein link ruled out because of the low reversing power of monothiols, only the pyrophosphate group remains for ready binding to the apoenzyme. Such a linkage, however, would be expected to be weaker than that known to exist between lipoic acid and protein.3 Similar reservations apply to the manner of attachment of LTTP to the apoenzyme during transport of the C₃ unit to CoA. Other possibilities are: (a) rupture of the thiazole ring and attachment to protein or thiamine through the S atom; (b) function of lipoic acid without thiamine in the arsenate reversing system; (c) an alternate structure of LT which would permit attachment through the carboxyl or amino groups. Further experiments are needed to determine the exact relationships of the various moieties in lipoic acid systems.
electronic excitation. In the absence of light, reoxidation of the free radicals would occur, through DPN, flavoproteins, and other oxidants, including finally CO$_2$.

Such a proposal implies that the "reduction" of lipoic acid (disulfide) by electronic excitation proceeds more rapidly than its reduction by pyruvate. Since the $E_v$ of the operating lipoic acid coenzyme has not yet been reported, a comparison cannot be made; however spectrophotometric evidence is presented$^{39, 48}$ to show that the energy needed to rupture the S—S bond in 6,8-thioctic acid (30,000 to 40,000 cal. per mole) is approximately equal to the energy available for photosynthesis when one quantum of light is absorbed. By contrast, 5,8-thioctic acid and other disulfides required 55,000 to 70,000 cal. per mole for similar cleavage. The peculiar instability of 6,8-thioctic acid was thought to be due to strain within the ring. This is further suggested by the yellow color of the latter compound; the other disulfides examined were colorless. Further indirect evidence supporting this concept was provided by model systems, whereby zinc porphyrin or tetralin could be used to reduce the disulfide photochemically. Finally, when algae were treated with 6,8-thioctic acid, oxygen production was accelerated, using quinone as the oxidizing agent. 5,8-Thioctic acid was ineffective. These suggestions, if verifiable by direct experiments, will constitute a major step toward a full understanding of the photosynthetic process.

The interest that the discovery of lipoic acid has engendered will doubtless continue for some time; in addition to further studies on the chemistry of the vitamin, the enzymic aspects of the problem deserve major attention. The finding in lipoic acid of a catalytically active thiol has emphasized again the importance of this class of compounds to biochemical systems. The realization that CoA and other thiols possess acetylating power in $in$ vitro systems and that the thiol bond is probably of the "high-energy" variety$^{50-55}$ focuses interest upon the lipoic acid system as a possible participant in energy conservation and transport from pyruvate to ATP and other eventual acceptors.

II. Carnitine (Vitamin B$_7$)

In 1947 Fraenkel and Blewett$^{54}$ observed that yeast extracts contained two apparently unfamiliar growth factors which were required by the mealworm Tenebrio molitor. One of the factors, adsorbable on Norit, was found to be folic acid; the other, a "filtrate factor," was regarded as a new

$^{50}$ F. Lynen and E. Reichert, Angew. Chem. 63, 47 (1951).
$^{53}$ T. Wieland and E. Bakelmann, Angew. Chem. 64, 59 (1952).
$^{54}$ G. Fraenkel and M. Blewett, Biochem. J. 41, 469 (1947).
growth principle. It was designated vitamin \( B_T \) because of its ready water solubility and its importance to the insect.

Assays of vitamin \( B_T \) were carried out by placing 4-week-old \( T. \) molitor larvae on a purified casein-salts-glucose-cholesterol-vitamins diet, and noting the growth response to sources of the vitamin over a 4- to 6-week period. Using this assay, the vitamin was found in yeast, whey, and many animal tissues. Vegetables, with the exception of wheat germ, were poor sources. Isolation of the vitamin was effected from liver and whey\(^{55, 56} \) by adsorption on and elution from fullers' earth, extraction into phenol, chromatography on alumina columns, and countercurrent extraction with phenol-dilute HCl. The purified material was found upon characterization, degradation, and synthesis studies to be identical with carnitine \( [(\text{CH}_3)_3\text{N}^+–\text{CH}_2\text{CH(OH)CH}_2\text{COO}^-] \), the trimethyl betaine of \( \beta \)-hydroxy-\( \gamma \)-aminobutyric acid.

The purified growth factor was active at levels of 0.37 to 0.75 \( \gamma \) per gram of diet. This places the requirement at the catalyst level of activity, despite the fact that in several animal tissues the carnitine content approaches 0.1% (it represents up to 3% of the total water-soluble "extractives" of skeletal muscle). The high activity for growth, in spite of the high content in tissues, is reminiscent of choline. Moreover, the similarity in structure to choline and the thetins suggests the possibility that carnitine may serve as a methylating agent \textit{in vivo}. Carter \textit{et al.},\(^{56} \) acting on this supposition, have tested crotonobetaine (a dehydration product of carnitine) and \( \beta \)-hydroxy-\( \gamma \)-aminobutyric acid as possible replacements for carnitine in \textit{Tenebrio}. Only the latter compound was active, at levels of 12 to 24 \( \gamma \) per gram of diet. The authors therefore suggested that carnitine may participate in transmethylation reactions in animal tissues. A similar postulate was made earlier for carnitine in the human being\(^{57, 58} \) on the basis of an increased excretion of methylated pyridinium compounds (described as trigonelline) after administration of carnitine. Thus, even though only a few insect species closely related to \textit{Tenebrio} (e.g., \textit{Palorus ratzeburgi}\(^{59} \)) appear to require an exogenous supply of carnitine,\(^{54} \) the interesting possibility that this compound may take a place among the group of important transmethylating agents in higher animals has been raised by this work with lower forms. It is not unreasonable to hope that future researches into the growth requirements of other phyla may bring to light additional

\(^{55} \) G. Fraenkel, \textit{Arch. Biochem. and Biophys.} 34, 468 (1951).

\(^{56} \) H. E. Carter, P. K. Bhattacharyya, K. R. Weidman, and G. Fraenkel, \textit{Arch. Biochem. and Biophys.} 38, 405 (1952).


\(^{58} \) W. Ciusa and G. Nebbia, \textit{Acta Vitaminol.} 2, 49 (1948).

\(^{59} \) G. Fraenkel, \textit{Arch. Biochem. and Biophys.} 34, 457 (1951).
III. Peptides

The question of nutritional equivalence of amino acids and intact proteins has been raised often since the classical review of the subject by Rose.60 He and his colleagues had shown that, although rats could grow on a purified diet containing known amino acids, much better performance could be elicited with complete proteins. During the decade that followed, improvements in supplemental rations and in the availability of many of the common amino acids made possible continuing improvements in purified diets. The greater growth that resulted materially reduced the margin of superiority of intact proteins over amino acid mixtures.

Strepogenin.61-66 Against this background, considerable interest was created by the discovery by Woolley and Sprince67, 68 of a peptide-like fraction of natural materials that was necessary for growth of a strain of hemolytic streptococci. The material, which was called strepogenin, was also shown to be needed for early growth of L. casei and to promote growth in mice.69 However, its structure has remained unknown, and to the writer's knowledge no pure samples of strepogenin have been prepared. It is probably a mixture, perhaps of structurally closely related peptides. The best information has been derived indirectly from degradation studies of insulin,70, 71 which was thought to have a strepogenin-like pattern in a portion of its structure, together with inhibition studies on lycomarasmin, the tomato-wilting agent of Fusarium lycopersici SACC.

Lycomarasmin activity in tomatoes could be duplicated by tripeptides containing serine, glycine, and aspartic acid.72 These peptides were also antagonistic to strepogenin for L. casei. Serylglycylglutamic acid was then synthesized and found to possess strepogenin activity, about one-fortieth that of strepogenin concentrates. It was concluded that the latter peptide may be a fragment or a relative of strepogenin.

60 W. C. Rose, Physiol. Revs. 18, 109 (1938).
61 Nutrition Revs. 4, 273 (1946).
63 Nutrition Revs. 6, 223, 277 (1948).
Support for the concept of an intrinsic growth-promoting property associated with the serine-glycine-glutamic acid structure is provided by the experiments of Chattaway and coworkers.\(^{73,74}\) They found that extracts of liver and yeast contained growth-promoting agents for \textit{C. diphtheriae gravis}, \textit{S. faccalis} R., and \textit{L. casei}, which upon concentration proved to be of peptide nature. Two peptides, labeled \(P_1\) and \(P_2\), contained the bulk of the activity; \(P_2\) upon hydrolysis was found to yield serine, glycine, and glutamic acid. Finally, the experiments of Krehl and Fruton\(^{75}\) have confirmed the activity of L-serylglycyl-L-glutamic acid for \textit{L. casei} and have shown that the closely related L-seryl-L-allyl-L-glutamic acid was inactive.\(^{75a}\) Also inactive were several related peptides of glutamic acid, glycine, and tyrosine.

Peptides of other amino acids have also been shown to be more active than their constituent moieties in supporting microbial growth. Malin \textit{et al.}\(^{76}\) reported that certain peptides of glycine were utilized more readily by several lactobacilli than was glycine itself. Simmonds and Fruton observed\(^{79,80}\) that a prolineless mutant of \textit{E. coli} was more responsive to any of several proline peptides tested than it was to proline, and that an isolated species of \textit{Alcaligenes}, termed "SF",\(^{81}\) required leucyl peptides for growth; with these in the medium, no other nitrogen or carbon source was needed. Other peptide requirements have been demonstrated by Snell \textit{et al.};\(^{82-84}\) in a medium in which \(\alpha\)-alanine satisfied the vitamin \(B_6\) requirement, \textit{L. casei} could be shown also to depend upon a peptide factor for its nutrition. Fractionation of the factor from partly hydrolyzed casein produced a mixture

\(^{75a}\) Although recent reports on the structure of insulin\(^{76,77}\) fail to reveal a serine-glycine-glutamic acid sequence, the closest relatives are a cysteine-glycine-glutamic acid, and a cysteine-glycine-serine series. Both of these are found in the "phenylalanine" fraction of insulin rather than in the "glycine" fraction where strepogenin activity was first reported.\(^{79}\) However, the similarity of the first sequence listed here to the strepogenin-active compound (serine replaced by cysteine) may warrant the testing of additional peptides.
\(^{78}\) R. B. Malin, M. N. Camien, and M. S. Dunn, \textit{Arch. Biochem. and Biophys.} \textbf{32}, 106 (1951).
of dipeptides, thought to be the alanyl and tyrosyl peptides of valine, leucine, and isoleucine. Also, Sloane and McKee have shown that the Staphylococcus albus factor of Hughes is replaceable by an intact plasma protein fraction, the activity of which may be due to special peptide structures, especially those of cysteine.

Several influences appear to be operative in determining the response of organisms to peptides; the most obvious is the need for a particular unit per se either because of a more rapid transfer into the cell or because of a paucity of appropriate conjugating enzymes to bring about its biosynthesis from the amino acids. Apart from this, it has been shown that in L. casei, when d-alanine was present in the medium it inhibited the utilization of the L isomer; however, d-alanine had no effect on L-alanine peptides. A similar effect was then suggested for other systems, i.e., an antagonism among certain related amino acids that may not be experienced when peptides are employed instead. The destructive action of tyrosine decarboxylase upon free tyrosine, but not on its peptides, was also noted and offered as an explanation of the greater response of S. faecalis to tyrosine peptides. Finally, it should be pointed out that many peptides have been shown to be less active than their constituents. These may simply become digested, assimilated, and resynthesized into protein patterns in which the peptide sequences in question may not appear at all. Some peptides (in addition to the antibiotic polypeptides) actually delay or inhibit bacterial growth, perhaps by interference with the synthesis of peptides and proteins within the cells.

The role of strepogenin and other peptides in animal nutrition is doubtful. Although Womack and Rose were able to produce more rapid weight gains in rats fed intact protein (casein) than in those maintained on nineteen amino acids, these differences have been eliminated by employing acid-hydrolyzed casein supplemented with tryptophan and cystine (Ramasarma et al.). The casein hydrolyzate was devoid of strepogenin activity. Other workers have also shown that properly balanced amino acid mixtures supported good growth of mice and that these mixtures were not improved

56 T. P. Hughes, J. Bacteriol. 23, 437 (1932).
59 S. Simmonds, J. I. Harris, and J. S. Fruton, J. Biol. Chem. 188, 251 (1951).
by the addition of strepogenin-rich proteins. It is possible that lower taste acceptability may have been chiefly responsible for the poorer performance on amino acid mixtures. The need for special peptides such as strepogenin thus seems to be best established for microbial species. Even with these, the proportion of peptides that is utilized per se is probably very small; in L. casei, strepogenin activity has been claimed for glutamine (although this is not in agreement with the findings of others). In spite of the technical difficulties involved, a major demand in this area of investigation continues for purified fractions, so that the nutritional and biochemical roles of these compounds may be further realized.

IV. Lyxoflavin

In 1947, Pellares et al. isolated a pentose from human heart, which they identified as lyxoflavin. Later, these workers reported the isolation of lyxoflavin from the same source, although this was challenged by subsequent work.

This close relative of riboflavin (and of the corresponding moiety of vitamin B₁₂) was viewed by Emerson and Folkers as a possible new member of the B complex, although they recognized that the experimental evidence for the reported existence of lyxoflavin was not as rigorous as might be desired. They therefore devised a ration based on soybean meal as the major constituent, to which had been added 0.5% desiccated thyroid, and observed the rate of growth of rats on this diet. The growth-depressing effect of large doses of thyroid was overcome by extracts of liver and by fish meal, or alternatively by synthetic lyxoflavin. The lyxoflavin effect was shown not to be due to conversion to riboflavin. The ability of lyxoflavin to completely replace the effect of liver or fish meal gave support for its classification as a vitamin for the rat, and its general importance was further suggested by the findings that it also stimulated swine and chick growth. It seemed possible from this work that lyxoflavin might be

*60 E. S. Pellares and H. M. Garza, Arch. Biochem. 22, 63 (1949).
*61 T. S. Gardner, E. Wenis, and J. Lee, Arch. Biochem. and Biophys. 34, 98 (1951).
related to or identical with the "stress factor" in liver observed by Ershoff,\textsuperscript{106} which was capable of counteracting the growth-depressing effect of thyroid in rats fed a casein diet.

However, Ershoff has since shown\textsuperscript{107} that lyxoflavin was ineffective as an antithyrototoxic factor when this diet was used. Since the diets employed in the two laboratories differed considerably in composition (soybean-dextrose versus casein-sucrose), the possibility has been raised (Cooperman \textit{et al.}\textsuperscript{108}) that lyxoflavin may act as a stimulant in liver factor synthesis by the intestinal flora when the soybean-dextrose diet is employed. Finally, the latter authors were able to demonstrate a slight replacement of riboflavin in rat diets and \textit{L. casei} growth media, and they concluded that the existing evidence did not warrant the classification of lyxoflavin as a new vitamin. Microbiological evidence is inconclusive, for lyxoflavin is stimulatory both in its own right\textsuperscript{109} and as an adjuvant for riboflavin,\textsuperscript{108, 109} or is inhibitory.\textsuperscript{109} Thus, the question whether lyxoflavin may be a member of the B complex remains unsettled. Of greater importance, however, is the question whether lyxoflavin can function in metabolism in any unique fashion. Further experiments are in order to definitely establish the natural occurrence of lyxoflavin, its possible presence in flavoproteins, and its effect upon growth and metabolism.

\textbf{V. Coenzyme III}

\textit{Proteus vulgaris} was shown by Singer and Kearney\textsuperscript{110, 111} to require a previously undescribed cofactor for oxidation of cysteine-sulfinic acid to cysteic acid. Isolation of the factor from bakers' yeast produced a nucleotide which was thought by the authors to be identical with nictotinamide-ribose-(5)-pyrophosphate. Because of the similarity to the other nictotinamide coenzymes, the name coenzyme III was tentatively assigned. Coenzyme III was found in high concentration in yeast and in liver and kidney mitochondria.\textsuperscript{112} In each of these systems, sulfinic dehydrogenase activity was demonstrated, and a fairly general requirement for this cofactor in cysteine metabolism appears possible.

\textbf{VI. Factors Required in Unheated Growth Media}

In 1933 Orla-Jensen\textsuperscript{113} observed that many lactic acid bacteria would not grow properly upon carbohydrates that had been sterilized in distilled


\textsuperscript{110} E. B. Kearney and T. P. Singer, \textit{Biochim. et Biophys. Acta} 8, 698 (1952).

\textsuperscript{111} T. P. Singer and E. B. Kearney, \textit{Biochim. et Biophys. Acta} 8, 700 (1952).

\textsuperscript{112} T. P. Singer and E. B. Kearney, \textit{Federation Proc.} 12, 269 (1953).

water and added aseptically to a sterile yeast-casein medium. Heating of glucose with the medium resulted in normal growth, as did heating of small amounts of methylglyoxal, furfural, or pentoses with the yeast-casein mixture. These observations have been repeated and extended in later years, so that it appears that at least two types of transformation occur during heating: one is the expulsion of oxygen and/or the formation of reducing substances, whereas the other involves interaction of carbohydrate with phosphate and/or the nitrogenous components of the medium. The compounds so produced were presumed to act as the stimulatory agents.

Studies in this laboratory have revealed that products formed by heating glucose with inorganic phosphate and amino acids will greatly stimulate the growth of Lactobacillus gayoni 8289 (strain 45; cf. ref. 123 for description of organism) during a 12-hour incubation period. In a series of experiments, glycine was found to be consistently superior to other amino acids as a precursor of active material, whereas alanine produced substances that were strongly inhibitory. N-Glucosylglycine was then synthesized as the ethyl ester and found to be as active as equal weights of yeast extract in a filter-sterilized medium, at levels up to 1 mg. per 10 ml. of culture. Higher levels of yeast produced greater stimulation, whereas amounts of glucosylglycine above 5 mg. became inhibitory. However, when glucosylglycine was heated separately and added aseptically to the basal medium, additional growth stimulation was provided which approached, although it did not equal, the growth on yeast extract. Of approximately twenty species of lactic acid bacteria tested, three others were stimulated by unheated glucosylglycine: Streptococcus zymogenes 10100, Lactobacillus acidophilus (O.S.C. strain), and Leuconostoc mesenteroides P63. L. gayoni F20 responded only after the compound had been heated. Other organisms, such as L. gayoni 8289, strain 49, L. casei, and Saccharomyces cerevisiae

119 V. Kocher, Intern. Z. Vitaminforsh. 20, 369 (1949).
LM did not respond at all to glucosylglycine under these conditions, although they were stimulated by yeast extract.125

The relative superiority of glycine over other amino acids in producing a biologically active material with glucose, and the strong inhibition produced with alanine, appear to confer a measure of specificity upon the heat activation reaction and to raise the possibility that one or at most a few factors may be involved. Presumably these represent conversion products from glucosylglycine, since the latter compound possesses relatively low activity. The role of phosphate is not yet clear. Further research should disclose the nature of these factors, as well as their relation to the products of the Mail- lard "browning reaction."126 The latter appears to be a more general reaction between carbohydrates and amino acids, resulting in a net loss of nutritional value of the amino acids toward higher animals.127

VII. Guinea Pig Antistiffness Factor

A syndrome in guinea pigs was described several years ago, whereby these animals developed characteristic joint stiffness on diets high in milk (Wulzen and Bahrs128,129). When adequate greens were given, the animals maintained normal health. Intermediate degrees of stiffness at the wrist joints were detected in the animals on the milk diets, and an assay method was developed which attempted to place the stiffening (produced at least in part by the deposition of calcium phosphate in the joints) on a quantitative basis. Constituents of the diet were sought which might protect the animals against the onset of stiffness, and it was believed (van Wagtendonk and Wulzen130) that such a protective factor could be obtained in pure form, either from raw cream or from sugar cane juice. On the basis of this approach, the existence of a nutritional principle, the "antistiffness factor," was claimed, and methods were given for its isolation from cream130 and from cane juice.131

The existence of the syndrome has been confirmed by other experimenters132,133 and has been described in detail in a review of the subject by

125 D. Rogers, T. E. King, and V. H. Cheldelin, unpublished.
126 L. C. Maillard, Compt. rend. 154, 66 (1912).
the principal authors. The condition is accompanied by extensive calcification in the joints, body wall and cavity, and upper skeleton; profound changes have been observed in the skull and teeth. Hearing is impaired in the affected animals. Numerous changes have also been recorded for calcium, phosphorus and protein levels in the blood.

Beyond this, it is difficult, if not impossible, to make further positive statements regarding the "antistiffness factor," owing in the main to the fact that the assay method was developed and used without adequate controls. Later, a critical examination of the assay by Christensen et al. and an examination of the data of Oleson et al. revealed that the assay method could not distinguish between concentrations of active materials that differed by five- or even tenfold. These discoveries necessarily vitiated the claims based on the wrist stiffness assay, whether for isolation of an active principle or for correlation of stiffness with peculiarities in metabolism. The fact that pure compounds have been isolated from cane juice thus simply reflects the success of chemical separations of materials (chiefly steroid) in the ether-extractable fractions of sugar cane, with no connection between the chemical separations and any physiological index.

In spite of the unsatisfactory character of many of the studies, it appears possible that a variety of steroids may possess some antistiffness potency. Thus, positive results have been claimed for stigmasterol and various esters of ergostanol and ergostanol (although this is denied by the work of Smith et al.). All these tests suffer from the inadequacies of the assay described above. It seems, however, that the assay is capable of detecting advanced stages of the condition; addition of these sterols to the "deficient" guinea pig diet over a period of several months might serve qualitatively to establish whether or not the compounds in question can serve as antistiffness agents. Finally, attention should be given to the question whether this is a nutritional or (perhaps more likely) a pharmacological principle, i.e., a condition brought about by high levels and imbalances of calcium and phosphorus in the diet. When cotton rats were maintained on diets high in calcium and phosphorus but low in several other minerals, especially magnesium, a similar "calcinosis" was observed, which led to extremely high (23 to 36 %) ash contents in the heart tissue. The condi-

tion, as in guinea pigs, was alleviated by feeding oatmeal but was aggravated by increasing the phosphate content of the diet. Vitamin E was ineffective. Microscopic appearance of the tissue lesions\textsuperscript{111} was reported to be similar to those in guinea pigs.\textsuperscript{112}

The gross similarities in guinea pig stiffness and human arthritis confer a continuing interest upon this unusual disease. It is hoped that studies will be resumed which will aim at the development of sound analytical procedures and the establishment of the nutritional and metabolic relationships that may exist.

VIII. Miscellaneous Factors

The recent literature contains many depositions describing factors for growth, reproduction, and intermediary metabolism. The evidence is favorable that one or more of these will prove to be of major importance. This is particularly true in the field of poultry nutrition,\textsuperscript{103, 104} where at least ten reports of new factors have been issued. However, caution is urged in accepting the entire group as bona fide factors, for most of them have not yet been purified or sufficiently cross-checked against other factors to determine what aliases may exist. In addition to probable duplications, the newer members of the B\textsubscript{12} group (see Chapter 3) as well as antibiotics may be responsible for some of these effects. Artifacts may be obtained owing to interplay of known factors, e.g., amino acid and amino acid-vitamin imbalances; such derived foods as processed soybean meals, which are used in many poultry diets, may vary considerably in their composition. Finally, the possibility exists that uncontrolled pathogenic organisms in the intestinal tracts of animals may influence performance in a non-uniform manner, particularly where mobility is restricted, as in poultry batteries.

A list of several of these and other newly described factors is given in Table I. Where possible, pertinent information is given regarding the suspected identities which reflects the opinions of either the listed authors or the present writer. Finally, for convenience in summarizing some of the older literature, several earlier "vitamins" and related materials are listed in Table II. These have either been discarded altogether or have been shown to be replaceable by one or more known compounds. It is unlikely that these terms will ever be used again, for the growth factors of the future will probably be given names in keeping with their chemical structure or biological function.

Certain trends in future research on new growth factors may be adumbrated by an analysis of the current list. Thus, factors for rat nutrition are


\textsuperscript{112} P. N. Harris and R. Wulzen, \textit{Am. J. Pathol.} \textbf{26}, 595 (1950).


<table>
<thead>
<tr>
<th>Factor</th>
<th>References</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth factors for chicks</td>
<td>Cravens et al., 145</td>
<td>Probably numerous duplications (see text)</td>
</tr>
<tr>
<td>From bran</td>
<td>Cravens et al., 145</td>
<td>Related to orotic acid?</td>
</tr>
<tr>
<td>From yeast</td>
<td>Savage et al., 146</td>
<td>Thought to be identical with grass juice factor for guinea pigs and rats</td>
</tr>
<tr>
<td>From fish meal, yeast</td>
<td>Combs et al., 147 Menge et al., 148, 149</td>
<td>Effect may be produced by antibiotics</td>
</tr>
<tr>
<td>From liver</td>
<td>Menge et al., 148, 149 Kohler and Graham 150, 151</td>
<td></td>
</tr>
<tr>
<td>From whey</td>
<td>Stokstad et al., 152</td>
<td></td>
</tr>
<tr>
<td>From grass juice</td>
<td>Young et al., 153</td>
<td></td>
</tr>
<tr>
<td>From fermentation products</td>
<td>Couch et al., 154</td>
<td></td>
</tr>
<tr>
<td>From peanut meal</td>
<td>Scott, 157</td>
<td></td>
</tr>
<tr>
<td>Factors for egg production and hatchability</td>
<td>Atkinson and Couch, 158 McGinnis et al., 159 Briggs, 160 Menge and Combs 161</td>
<td>Two factors claimed</td>
</tr>
<tr>
<td>From liver, whey</td>
<td></td>
<td>Effects most pronounced when supplemented with antibiotics</td>
</tr>
<tr>
<td>Growth and antiperosis agents for turkeys</td>
<td></td>
<td></td>
</tr>
<tr>
<td>From yeast, whey</td>
<td></td>
<td></td>
</tr>
<tr>
<td>From liver, fish meal, whey</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antipancreatic fibrosis factor for ducks</td>
<td>Miller 161</td>
<td></td>
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<tr>
<td>From cornstarch</td>
<td>Baxter, 162 Schwarz 163 (&quot;factor 3&quot;)</td>
<td></td>
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<tr>
<td>Antiliver, kidney necrosis factors for rats</td>
<td>King and Hauge 164</td>
<td></td>
</tr>
<tr>
<td>From cornstarch</td>
<td>Ershoff, 165</td>
<td></td>
</tr>
<tr>
<td>From yeast</td>
<td>Bosshardt and Huff 167</td>
<td></td>
</tr>
<tr>
<td>Growth factor for rats</td>
<td>Schaefer et al., 168 Tove et al., 169 Cooperman et al., 171</td>
<td></td>
</tr>
<tr>
<td>From fish solubles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;Stress factors&quot; for rats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>From liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;Stress factors&quot; for mice</td>
<td></td>
<td></td>
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<tr>
<td>From liver, cottonseed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth factors for mink</td>
<td></td>
<td></td>
</tr>
<tr>
<td>From liver, whey</td>
<td>Schaefer et al., 168</td>
<td></td>
</tr>
<tr>
<td>Monkey antianemia factor</td>
<td>Cooperman et al., 171</td>
<td></td>
</tr>
<tr>
<td>Growth factor for foxes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>From liver, whey</td>
<td>Schaefer, 173</td>
<td></td>
</tr>
<tr>
<td>Growth factor for corn borer</td>
<td>Beck et al., 174, 175</td>
<td></td>
</tr>
<tr>
<td>Potato eelworm hatching factor</td>
<td>Calam et al., 176</td>
<td></td>
</tr>
<tr>
<td>Growth factor for Treponemata</td>
<td>Eagle and Steinman, 180, 181</td>
<td>Replaceable by serum albumin, which functions as a carrier for cholic or other lipids</td>
</tr>
<tr>
<td>From enzymatic protein digests</td>
<td></td>
<td></td>
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</table>
### TABLE I—Concluded

<table>
<thead>
<tr>
<th>Factor</th>
<th>References</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth factors for <em>Trypanosoma cruzi</em> From blood</td>
<td>McRay <em>et al.</em></td>
<td>Reportedly a complex derivative of hemoglobin^1^</td>
</tr>
<tr>
<td>Growth factor for <em>Trichomonas vaginalis</em> From pancreas</td>
<td>Sprince <em>et al.</em></td>
<td>Iron porphyrins slightly active;^1^ &quot;coprogen&quot; isolated,^1^ probably related to &quot;ferriochrome&quot; an organo-iron pigment^19^</td>
</tr>
<tr>
<td>Growth factors for <em>P. looslii</em></td>
<td>Hesseltine <em>et al.</em>, Page^1^</td>
<td>May be peptide mixture,^1^ possibly strepogemin-like^1^</td>
</tr>
<tr>
<td>Growth factors for <em>Clostridia</em></td>
<td>Knight and Filde,^1^ Jones and Clifton</td>
<td>Low molecular weight basic protein^1^</td>
</tr>
<tr>
<td>Growth factor for <em>Microbacterium florum</em></td>
<td>Bishop <em>et al.</em></td>
<td>Glycosprotein with special S-containing peptide structure^1^ (see text)</td>
</tr>
<tr>
<td>Growth factor for pleurospomon-like organisms (&quot;PPLO&quot;)</td>
<td>Tang <em>et al.</em></td>
<td>Mixtures, probably peptides</td>
</tr>
<tr>
<td><em>Staphylococcus albus</em> factor (SSF)</td>
<td>Hughes^6^</td>
<td>May be related to Bi, although not identical</td>
</tr>
<tr>
<td>Growth factors for lactobacilli</td>
<td>Kitay and Snell^2^</td>
<td>May be related to above factors from various sources; may be oligosaccharides</td>
</tr>
<tr>
<td>Alkal-stable factors for lactobacilli</td>
<td>Robinson <em>et al.</em>, Östling and Nyberg^1^</td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus leichmanii</em> factor</td>
<td>Peeler and Norris^3^</td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus bifidus</em> factors</td>
<td>Gylenberg <em>et al.</em>, Shorb and Veil^1^</td>
<td></td>
</tr>
<tr>
<td>Growth factor for * Fusobacteria*</td>
<td>György <em>et al.</em>,^3^</td>
<td></td>
</tr>
<tr>
<td>Growth factor for <em>Pythogen</em></td>
<td>Omata^4^</td>
<td></td>
</tr>
<tr>
<td>Coenzyme of alcoholic fermentation</td>
<td>Perlman^5^</td>
<td></td>
</tr>
<tr>
<td>Citric acid oxidation factor</td>
<td>Oldmeyer,^6^ Tria and Barma-</td>
<td></td>
</tr>
<tr>
<td>Formic hydrogenlyase cofactor</td>
<td>Perl^6^</td>
<td></td>
</tr>
<tr>
<td>Bacterial deaminase cofactor</td>
<td>Foulkes^7^</td>
<td></td>
</tr>
<tr>
<td>Coconut milk factor</td>
<td>Lichstein and Boyd^8^</td>
<td>Probably a nucleotide^8^</td>
</tr>
<tr>
<td></td>
<td>Williams and Christman^9^</td>
<td>Probably a nucleotide^9^</td>
</tr>
<tr>
<td></td>
<td>Van Overbeek <em>et al.</em>, Duhamet and Gautheret,^10^ Steward and Caplin^11^</td>
<td>May be derived from fatty acids^10^ Produced by H2SO4 degradation of carbohydrates; functionally related to biotin, adenyllic acid;^10^ may be similar to heat-produced growth factors^11^ Stimulates plant root growth; indoleacetic,^11^ naphthaleneacetic acids^11^ partly active, but not identical with factor^11^</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Factor</th>
<th>References</th>
<th>Remarks</th>
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<tbody>
<tr>
<td>B&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Williams and Waterman&lt;sup&gt;232&lt;/sup&gt;</td>
<td>Probably pantothenic acid&lt;sup&gt;232&lt;/sup&gt;</td>
</tr>
<tr>
<td>B&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Reader&lt;sup&gt;224&lt;/sup&gt;</td>
<td>Replaceable by mixtures of arginine, glycine, and cystine&lt;sup&gt;213&lt;/sup&gt;</td>
</tr>
<tr>
<td>B&lt;sub&gt;5&lt;/sub&gt;</td>
<td>Carter et al.,&lt;sup&gt;234&lt;/sup&gt;</td>
<td>Presumed identical with B&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;232&lt;/sup&gt; or nicotinic acid&lt;sup&gt;237&lt;/sup&gt;</td>
</tr>
<tr>
<td>B&lt;sub&gt;7&lt;/sub&gt;</td>
<td>Centanni&lt;sup&gt;258&lt;/sup&gt;</td>
<td>Prevented digestive disturbances in pigeons; probably a mixture&lt;sup&gt;218&lt;/sup&gt;</td>
</tr>
<tr>
<td>B&lt;sub&gt;8&lt;/sub&gt;</td>
<td>von Euler et al.&lt;sup&gt;219&lt;/sup&gt;</td>
<td>Adenylc acid&lt;sup&gt;213&lt;/sup&gt;</td>
</tr>
<tr>
<td>B&lt;sub&gt;8&lt;/sub&gt; and B&lt;sub&gt;11&lt;/sub&gt;</td>
<td>Briggs et al.,&lt;sup&gt;259&lt;/sup&gt;</td>
<td>Probably a mixture of B&lt;sub&gt;11&lt;/sub&gt; and the folie acid group</td>
</tr>
<tr>
<td>B&lt;sub&gt;14&lt;/sub&gt;</td>
<td>Norris and Majnarich&lt;sup&gt;221&lt;/sup&gt;</td>
<td>Announced as metabolite of xanthopterin, but later work failed to confirm earlier results&lt;sup&gt;222&lt;/sup&gt;</td>
</tr>
<tr>
<td>B&lt;sub&gt;p&lt;/sub&gt;</td>
<td>Hogan et al.,&lt;sup&gt;233&lt;/sup&gt;</td>
<td>Anti-derosis factor in chicks, replaceable by manganese&lt;sup&gt;234&lt;/sup&gt; and choline&lt;sup&gt;223&lt;/sup&gt;</td>
</tr>
<tr>
<td>B&lt;sub&gt;W&lt;/sub&gt;; factor W</td>
<td>Lunde and Kringstad,&lt;sup&gt;226&lt;/sup&gt; Elvehjem et al.&lt;sup&gt;237&lt;/sup&gt;</td>
<td>Regarded as identical with biotin&lt;sup&gt;238&lt;/sup&gt;, although properties also resemble pantothenic acid phosphates, coenzyme A</td>
</tr>
<tr>
<td>B&lt;sub&gt;S&lt;/sub&gt;</td>
<td>Lunde and Kringstad&lt;sup&gt;228&lt;/sup&gt;</td>
<td>Anti-gray hair factor for rats; properties resemble bound forms of pantothenic acid</td>
</tr>
<tr>
<td>L&lt;sub&gt;1&lt;/sub&gt;, L&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Nakahara et al.&lt;sup&gt;209&lt;/sup&gt;</td>
<td>Factors from yeast, liver reportedly related to anthranilic acid, adenosine, necessary for normal lactation&lt;sup&gt;230, 231&lt;/sup&gt;</td>
</tr>
<tr>
<td>Factors R, S (for chick growth)</td>
<td>Schumacher et al.&lt;sup&gt;232&lt;/sup&gt;</td>
<td>R = probably related to folic acid&lt;sup&gt;238&lt;/sup&gt; S = probably identical with strepogenin&lt;sup&gt;234&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin T (torutiline)</td>
<td>Goetsch&lt;sup&gt;235&lt;/sup&gt;</td>
<td>From insects; mixture of folic acid group, B&lt;sub&gt;12&lt;/sub&gt;, desoxyribosides&lt;sup&gt;236&lt;/sup&gt;</td>
</tr>
<tr>
<td>T factor, for Lactobacillus fermenti</td>
<td>Metcalf et al.&lt;sup&gt;237&lt;/sup&gt;</td>
<td>From tomato juice; may be identical with thiamine&lt;sup&gt;238&lt;/sup&gt;</td>
</tr>
<tr>
<td>Factor U</td>
<td>Stokstad and Manning&lt;sup&gt;239&lt;/sup&gt;</td>
<td>Probably a mixture containing B&lt;sub&gt;12&lt;/sub&gt; and folic acid activity&lt;sup&gt;233&lt;/sup&gt;</td>
</tr>
<tr>
<td>Factor V, for Henophilus</td>
<td>Davies&lt;sup&gt;234&lt;/sup&gt;</td>
<td>Diphosphopyridine-nucleotide&lt;sup&gt;232&lt;/sup&gt; Equivalent to biotin&lt;sup&gt;238&lt;/sup&gt;</td>
</tr>
<tr>
<td>Factor X</td>
<td>Boss&lt;sup&gt;236&lt;/sup&gt;</td>
<td>Equivalent to pyridoxine&lt;sup&gt;238&lt;/sup&gt;</td>
</tr>
<tr>
<td>Factor Y</td>
<td>Chick et al.,&lt;sup&gt;214&lt;/sup&gt;</td>
<td>Identical with folic acid&lt;sup&gt;235&lt;/sup&gt;</td>
</tr>
<tr>
<td>Growth factors for guinea pigs: GPF-1</td>
<td>Woolley and Springer&lt;sup&gt;245&lt;/sup&gt;</td>
<td>Replaceable by cellulose plus amino acids&lt;sup&gt;245&lt;/sup&gt;</td>
</tr>
<tr>
<td>GPF-2</td>
<td></td>
<td>Similar to strepogenin&lt;sup&gt;235&lt;/sup&gt;</td>
</tr>
<tr>
<td>GPF-3</td>
<td></td>
<td>Replaceable by B&lt;sub&gt;12&lt;/sub&gt;&lt;sup&gt;218&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gizzard erosion factor</td>
<td></td>
<td>Chloroform-soluble fractions from liver; may be related to folic acid group, also leipoic acid</td>
</tr>
<tr>
<td>Factors for L. helveticus and S. lactis: H&lt;sub&gt;1&lt;/sub&gt;, 1, 2, 3, and 4</td>
<td>Bird et al.,&lt;sup&gt;247&lt;/sup&gt; Barton-Wright et al.,&lt;sup&gt;240&lt;/sup&gt; Colio and Bah&lt;sup&gt;250&lt;/sup&gt;</td>
<td>Partly replaced by nucleotides&lt;sup&gt;232&lt;/sup&gt; plus high levels of folic acid&lt;sup&gt;232&lt;/sup&gt; or asparagine&lt;sup&gt;234&lt;/sup&gt; glutamine or asparagine&lt;sup&gt;234&lt;/sup&gt; glucosylglycine active (see text 127)</td>
</tr>
<tr>
<td>L. gayonii factor</td>
<td>Chehelafin and Riggs&lt;sup&gt;251&lt;/sup&gt;</td>
<td>Replaceable by inorganic molybdate&lt;sup&gt;234&lt;/sup&gt;</td>
</tr>
<tr>
<td>Xanthine oxidase factor</td>
<td>Westerfeld and Richert&lt;sup&gt;235&lt;/sup&gt;</td>
<td></td>
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very few; with the discovery of vitamin B₁₂ and its inclusion in rat diets, dramatic deficiencies could no longer be produced with purified rations fed

in the usual manner. Even without $B_{12}$, the deficiency state often became pronounced only when an abnormal stress was placed on the animal, e.g.,

206 E. Tria and O. Barnabei, *Boll. soc. ital. biol. sper.* 27, 133 (1951) [C. A. 47, 1196 (1953)].
226 E. R. Norris, personal communication.
by feeding thyroid extract. In the future, metabolites essential for the rat and certain other species may be best revealed through the application of external stresses to induce deficiencies. Similar stress devices have been employed successfully upon microbial populations, through the use of anti-metabolites, and the creation of mutants that are incapable of carrying out normal metabolic reactions. Pteroyl glutamic acid (Chapter 13) and p-hydroxybenzoic acid (Chapter 12) were discovered by these means. Since the limit to the number of derived systems that may be produced through these approaches is remote, they offer many possibilities for discovering pathways of metabolism that are at present obscured because of the absence of suitable test systems. Turning again to "normal" strains of organisms, the fruitful researches on Tenebrio and other insects may signal a shift of interest among investigators toward the lesser known phyla of the

animal kingdom, with the probability that many important new metabolites may be exposed to view, and the assurance that our knowledge of comparative biochemistry will be greatly enriched. Though many of these metabolites may come to fall outside the biocatalyst class, it is nevertheless the continued unveiling of metabolic machinery to which the investigators in this field are dedicated, and their progress will be followed keenly by all who would gain an insight into the economy of the cell.
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