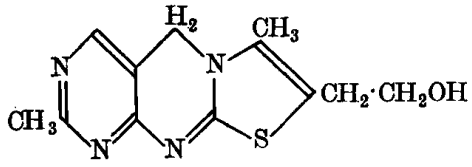
is also formed by the oxidation of thiamine with alkaline potassium ferricyanide (Todd *et al.*, 1935); it has also been synthesised by Todd *et al.* (1936).



thiochrome

§6. Vitamin B_2, riboflavin (lactoflavin), $C_{17}H_{20}O_6N_4$. Riboflavin is a water-soluble, thermostable vitamin which occurs in the vitamin B complex. It is necessary for growth and health, and occurs widely distributed in nature, *e.g.*, in yeast, green vegetables, milk, meat, etc. Chemically, vitamin B_2 is closely related to the yellow water-soluble pigments known as the *flavins* (*iso*alloxazines), and since it was first isolated from milk, vitamin B_2 is also known as *lactoflavin*.

Riboflavin is a bright yellow powder, m.p. 292°, showing a green fluorescence; it is soluble in water and in ethanol, but is insoluble in chloroform and other organic solvents.

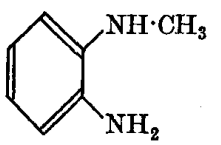
When exposed to light, lactoflavin in sodium hydroxide solution forms mainly lumi-lactoflavin, $C_{13}H_{12}O_2N_4$ (this is soluble in chloroform). Lumi-lactoflavin, on boiling with barium hydroxide solution, is hydrolysed to one molecule of urea and one molecule of the barium salt of a β-ketocarboxylic acid, I, $C_{12}H_{12}O_3N_2$ (Kuhn *et al.*, 1933, 1934). The nature of this acid is shown by the fact that, on acidification of the barium salt, the free acid immediately eliminates carbon dioxide to form the compound, II, $C_{11}H_{12}ON_2$. This compound showed the properties of a lactam, and on vigorous hydrolysis by boiling with sodium hydroxide solution, it forms one molecule of glyoxylic acid and one molecule of the compound $C_9H_{14}N_2$ (III).

$$\underset{\text{lactoflavin}}{C_{17}H_{20}O_6N_4} \xrightarrow[\text{light}]{\text{NaOH}} \underset{\text{lumi-lactoflavin}}{C_{13}H_{12}O_2N_4} \xrightarrow{Ba(OH)_2}$$

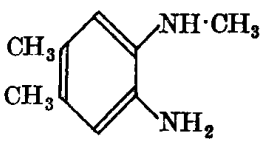
$$CO(NH_2)_2 + \underset{\text{I}}{[C_{12}H_{12}O_3N_2]} \xrightarrow[-CO_2]{\text{acid}}$$

$$\underset{\text{II}}{C_{11}H_{12}ON_2} \xrightarrow{\text{NaOH}} CHO{\cdot}CO_2H + \underset{\text{III}}{C_9H_{14}N_2}$$

The structure of III was elucidated as follows (Kuhn *et al.*, 1934). Preliminary tests showed that III was an aromatic diamino compound. Then it was found that it gave a blue precipitate with ferric chloride, and since this reaction is characteristic of monomethyl-*o*-phenylenediamine, it suggests that III contains the following nucleus, IV. The molecular formula of IV is $C_7H_{10}N_2$, and since III is $C_9H_{14}N_2$, two carbon and four hydrogen atoms



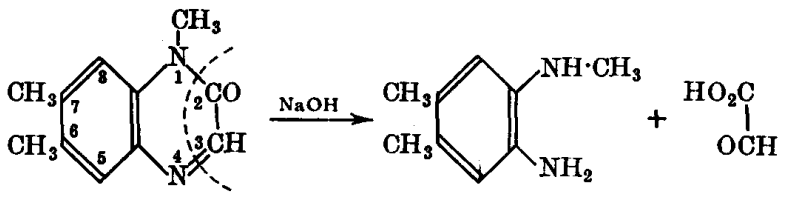
IV



III

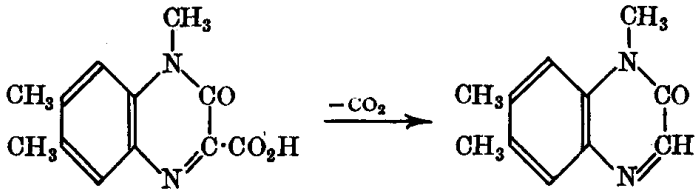
must be accounted for. This can be done by assuming the presence of an ethyl group or of two methyl groups in the benzene ring. Kuhn *et al.*

carried out a series of synthetic experiments and showed that III has the structure given, *N*-methyl-4 : 5-diamino-*o*-xylene. Kuhn then proposed II as the structure of the precursor of III, since this would produce the required products of hydrolysis.



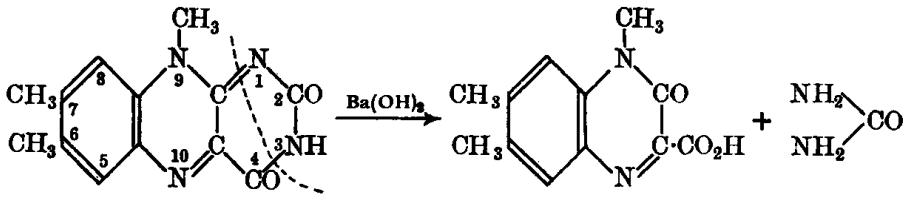
II III

II could therefore have been produced from the β-ketocarboxylic acid I



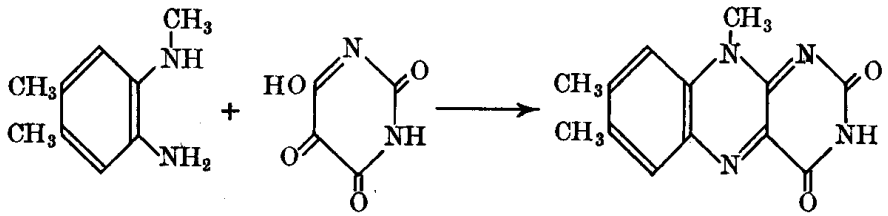
I II

Since I and a molecule of urea are obtained from lumi-lactoflavin, the latter could be 6 : 7 : 9-trimethyl*iso*alloxazine (6 : 7 : 9-trimethylflavin).



lumi-lactoflavin I

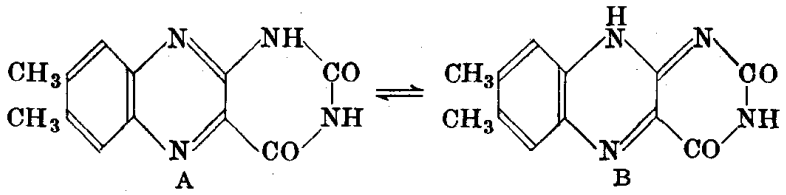
This structure for lumi-lactoflavin has been confirmed by synthesis (Kuhn *et al.*, 1934). *N*-Methyl-4 : 5-diamino-*o*-xylene is condensed with alloxan hydrate (§2. XVI) in aqueous solution at 50–60°.



Methylation (methyl sulphate) of this synthetic product gives a tetramethyl compound identical with the product obtained by the methylation of the natural lumi-lactoflavin.

Side-chain of lactoflavin

Exposure of a *neutral* solution of lactoflavin to light produces *lumichrome*, $C_{12}H_{10}O_2N_4$ (Karrer *et al.*, 1934). Analytical work similar to that described for lumi-lactoflavin showed that the structure of lumichrome is 6 : 7-dimethylalloxazine (A).

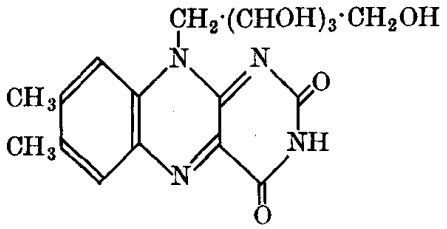


A B

lumichrome

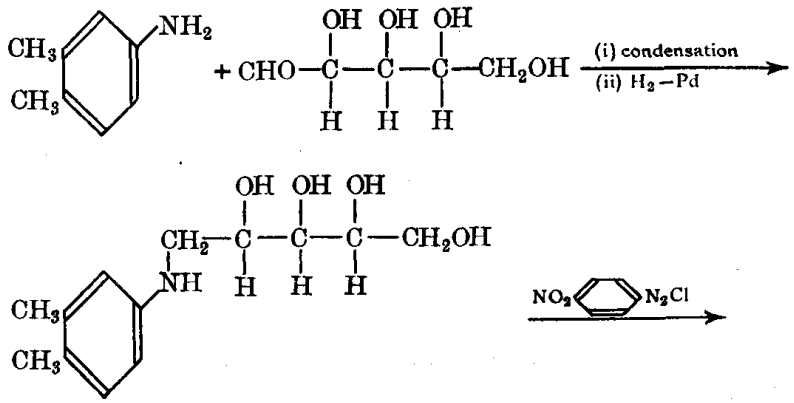
The *iso*alloxazine (structure B) is a tautomer of the alloxazine (structure A); B does not exist as such, but this structure is fixed when there is a substituent at position 9 (see also §25. XII). Stern *et al.* (1934) have shown that the absorption spectra of compounds containing a 9-substituent are different from those in which the mobile 9-hydrogen atom is present. Also, in the latter case, the alloxazine structure (A) predominates.

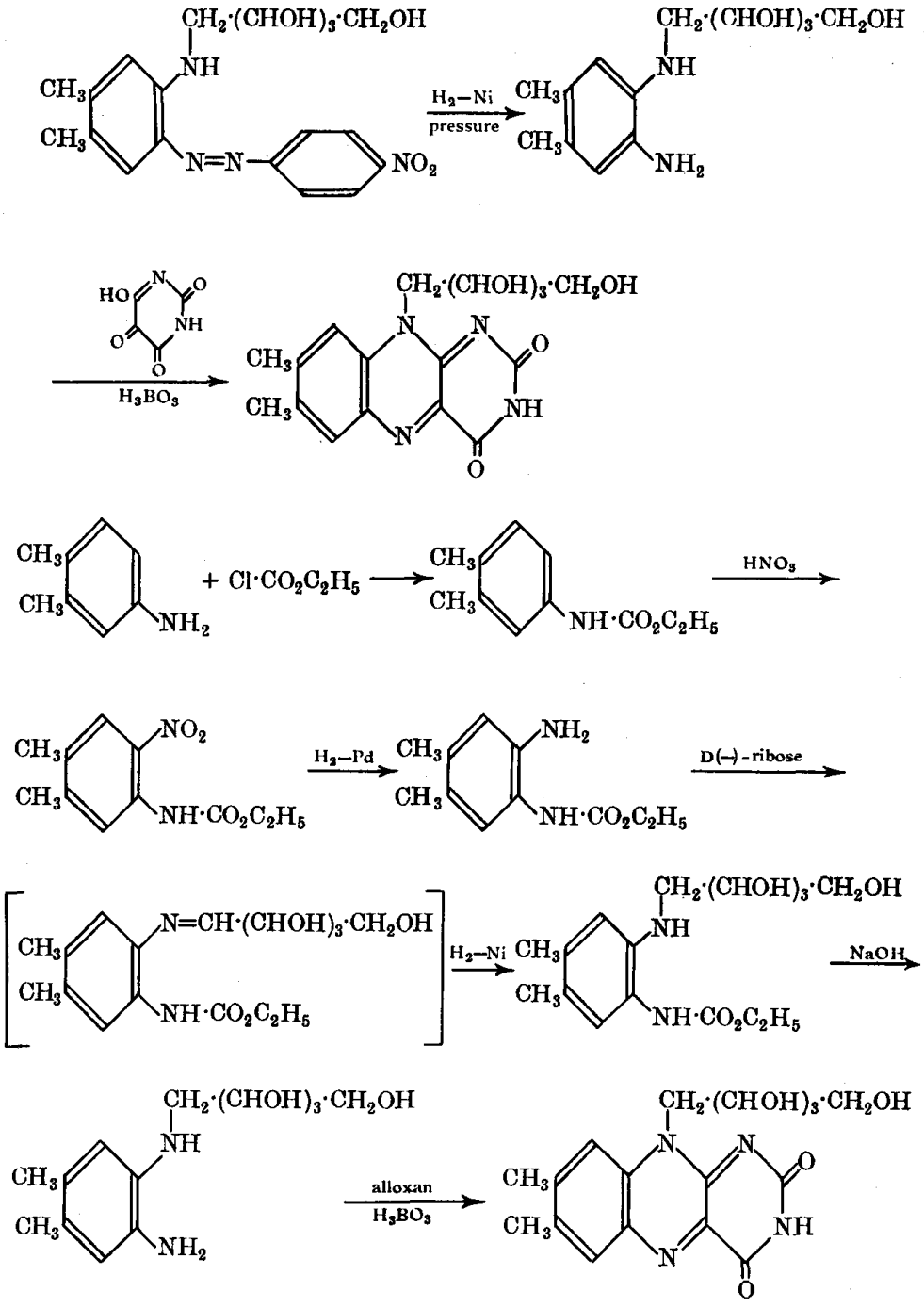
Thus lumichrome is lumi-lactoflavin with a hydrogen atom instead of a methyl group at position 9. This suggests that lactoflavin contains a side-chain (of five carbon atoms) attached to N_9. The Zerewitinoff procedure shows that lactoflavin contains five active hydrogen atoms; thus the molecule contains four hydroxyl groups (one active hydrogen atom is the hydrogen of the NH group at position 3). The presence of these four hydroxyl groups is supported by the fact that the silver salt of lactoflavin (the silver atom replaces the hydrogen of the NH group) forms a tetra-acetate. Thus the side-chain is a tetra-hydroxy derivative, and so a possible structure for lactoflavin is:



lactoflavin

This side-chain contains three asymmetric carbon atoms, and so there are eight optically active forms possible. Which configuration is actually present was solved by synthesising a number of pentose derivatives, and it was finally shown by Karrer *et al.* (1935) that the configuration is that of D(−)-ribose. The following syntheses are due to Karrer *et al.* (1935).





Thus lactoflavin is 6 : 7-dimethyl-9-[D-1′-ribityl]-*iso*alloxazine. Of all the pentoses (and hexoses) used, only the compound from D-ribose shows growth-promoting properties. For this reason vitamin B_2 (lactoflavin) is also known as riboflavin. More recently, however, it has been found that L-lyxoflavin occurs naturally; it has been synthesised and shows some vitamin activity (Folkers *et al.*, 1951).

Biosynthetic experiments have established that riboflavin is formed from purine precursors (McNutt, 1954, 1956; Goodwin *et al.*, 1954; Plaut *et al.*, 1959). It has also been shown that the dimethylbenzene system is derived from acetate

units (Plaut, 1954; Birch *et al.*, 1957; Goodwin *et al.*, 1958). Cresswell and Wood (1960) have synthesised riboflavin by a method which has possible implications in the biosynthesis of this vitamin.

§7. **Pantothenic acid,** $C_9H_{17}O_5N$, is a chick antidermatitis factor, and is also capable of promoting the growth of yeast and of bacteria; it has been isolated from many sources, *e.g.*, liver, kidney, yeast, etc.

Pantothenic acid shows the reactions of a monocarboxylic acid, *e.g.*, it can be esterified to form monoesters (R. J. Williams *et al.*, 1939). The application of the method for determining active hydrogen atoms shows that pantothenic acid contains two hydroxyl groups, and since the acid condenses with benzaldehyde (to form a benzylidene derivative) and with acetone (to form an *iso*propylidene derivative), this suggests that the two hydroxy groups are in either the 1 : 2- or 1 : 3-position (*cf.* §§8, 9. VII). When warmed with dilute hydrochloric acid, pantothenic acid is hydrolysed into compounds I and II. Investigation of I showed that it was β-alanine

$$C_9H_{17}O_5N \xrightarrow{HCl} \underset{\text{I}}{C_3H_7O_2N} + \underset{\text{II}}{C_6H_{10}O_3}$$

(actually present as the hydrochloride, $\overset{-}{Cl}\{H_3\overset{+}{N}\cdot CH_2\cdot CH_2\cdot CO_2H\}$). On the other hand, when hydrolysed with alkali, pantothenic acid forms β-alanine (I) and the salt of an acid which, on acidification, spontaneously forms the lactone II. Thus the free acid of II is probably a γ- or δ-hydroxycarboxylic acid; also, since the rate of lactonisation is fast, II is more likely a γ-lactone than a δ-lactone (*cf.* §7c. VII). As pointed out above, pantothenic acid contains two hydroxyl groups. One of these has now been accounted for, and so the problem is to find the position of the second one. This was shown to be α- by the fact that the sodium salt of the acid of the lactone II gives a canary-yellow colour with ferric chloride (a test characteristic of α-hydroxyacids), and also by the fact that II, on warming with concentrated sulphuric acid, liberates carbon monoxide (a test also characteristic of α-hydroxyacids). Thus II is most probably the γ-lactone of an α-hydroxyacid (R. J. Williams *et al.*, 1940).

II was shown to contain one active hydrogen atom, and the application of the Kuhn–Roth methyl side-chain determination (§3. IX) showed the presence of a *gem*-dimethyl group (Stiller *et al.*, 1940); the presence of this group is confirmed by the formation of acetone when the lactone II is oxidised with barium permanganate. Thus a possible structure for II is α-hydroxy-β : β-dimethyl-γ-butyrolactone:

$$\underbrace{CH_2{-}C(CH_3)_2{-}CHOH{-}CO}_{O} \equiv C_6H_{10}O_3$$

II

This has been confirmed as follows. Treatment of the lactone with methylmagnesium iodide, followed by hydrolysis, gives a trihydric alcohol which, on oxidation with lead tetra-acetate, gives acetone and an aldehyde. This aldehyde, on oxidation with silver oxide, gave a compound III, which was shown to be β-hydroxy-α : α-dimethylpropionic acid. The foregoing reactions may be formulated as follows:

$$\underbrace{CH_2\cdot C(CH_3)_2\cdot CHOH\cdot CO}_{O} \xrightarrow[\text{(ii) } H_2O]{\text{(i) } CH_3MgI}$$

II

$$CH_2OH{\cdot}C(CH_3)_2{\cdot}CHOH{\cdot}C(OH)(CH_3)_2 \xrightarrow{(CH_3{\cdot}CO_2)_4Pb} CH_3{\cdot}CO{\cdot}CH_3 + CH_2OH{\cdot}C(CH_3)_2{\cdot}CHO \xrightarrow{Ag_2O} CH_2OH{\cdot}C(CH_3)_2{\cdot}CO_2H$$

III

Examination of II shows that it contains one asymmetric carbon atom. The lactone, **pantolactone** (the acid is known as **pantoic acid**), obtained from pantothenic acid is lævorotatory, and the structure assigned to it has been confirmed by synthesis (Stiller *et al.*, 1940).

$$(CH_3)_2CH{\cdot}CHO + CH_2O \xrightarrow{K_2CO_3} (CH_3)_2C(CH_2OH)(CHO) \xrightarrow[(ii)\,KCN]{(i)\,NaHSO_3}$$

*iso*butyraldehyde formalin

$$(CH_3)_2C(CH_2OH)(CHOH{\cdot}CN) \xrightarrow[boil]{HCl} (CH_3)_2C(CH_2{-}O{-}CO)(CHOH)$$

(±)-lactone

The (±)-lactone (as the sodium salt of the acid) was resolved with quinine hydrochloride, and the (—)-form was identical with the lactone obtained from pantothenic acid.

In pantothenic acid, the nitrogen atom is not basic. Also, since hydrolysis of pantothenic acid produces a free amino-group (in β-alanine), this suggests that the group —CO·NH— is present, *i.e.*, pantothenic acid is an amide. Thus the hydrolysis may be formulated:

$$CH_2OH{\cdot}C(CH_3)_2{\cdot}CHOH{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}CO_2H$$

pantothenic acid

$$\downarrow HCl$$

$$[CH_2OH{\cdot}C(CH_3)_2{\cdot}CHOH{\cdot}CO_2H] + NH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CO_2H$$

$$\downarrow \text{lactonises}$$

$$CH_2{\cdot}C(CH_3)_2{\cdot}CHOH{\cdot}CO \quad (O\text{ bridge})$$

This interpretation of the results has been proved by the synthesis of pantothenic acid. Stiller *et al.* (1940) warmed pantolactone (synthesised as described above) with the ethyl ester of β-alanine, and removed the ester group by hydrolysis with a cold solution of barium hydroxide.

$$CH_2{\cdot}C(CH_3)_2{\cdot}CHOH{\cdot}CO + NH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CO_2C_2H_5$$

$$\downarrow \text{warm}$$

$$CH_2OH{\cdot}C(CH_3)_2{\cdot}CHOH{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}CO_2C_2H_5$$

$$\downarrow Ba(OH)_2$$

$$CH_2OH{\cdot}C(CH_3)_2{\cdot}CHOH{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}CO_2H$$

A better yield of pantothenic acid is obtained by warming the dry lactone with the dry sodium salt of β-alanine (R. J. Williams *et al.*, 1940).

§8. Folic acid complex. A number of micro-organisms need certain concentrates (prepared from natural sources) for growth; several active principles have been shown to be necessary. In addition to the above property, some of these active principles also exhibit other effects, *e.g.*, the prevention of certain types of anæmia in chicks. The following compounds have been described as constituents of the folic acid complex.

(i) Folic acid. It has been suggested that folic acid from animal sources is different from that from vegetable sources.

(ii) *Lactobacillus casei* factor (three forms).

(iii) *S. lactis* R factor.

(iv) Vitamin B_c factor (this now identified as liver *L. casei* factor).

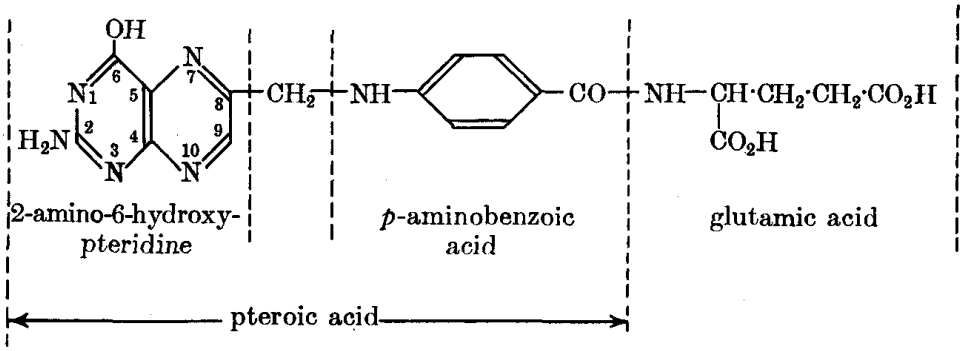
(v) Vitamin B_c conjugate.

(vi) Vitamins B_{10}, B_{11} and factors R, S and U.

(vii) Vitamin M.

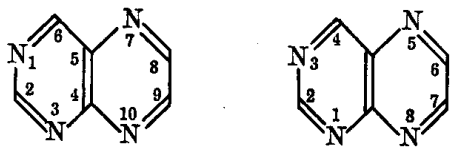
It is possible that some of these are identical; names have been given by different workers to the active substances that they have isolated (see, *e.g.*, iv).

Angier *et al.* (1946) have shown that liver *L. casei* factor (also called vitamin B_c) is:



Fermentation *L. casei* factor contains three glutamic acid residues; yeast vitamin B_c conjugate contains seven glutamic acid residues.

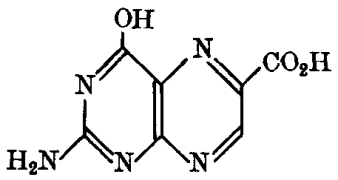
§8a. Structure of *L. casei* factors (Angier *et al.*, 1946). The alkaline hydrolysis of the fermentation *L. casei* factor, in the absence of oxygen, formed two molecules of D-glutamic acid and the DL-form of liver *L. casei* factor. On the other hand, the alkaline hydrolysis of the fermentation *L. casei* factor, in the presence of air, gave two substances, I and II. I was



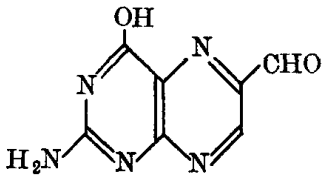
A pteridine B

shown to be a monocarboxylic acid, and the examination of its ultraviolet absorption spectrum led to the conclusion that it was a pteridine derivative (A is the system of numbering used here; B is an alternative system of

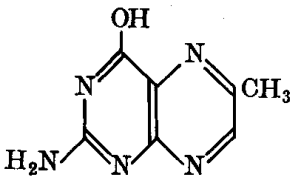
numbering frequently used in American publications). A further examination of compound I showed that it also contained one hydroxyl and one amino-group. Oxidation of I with chlorine water, followed by hydrolysis with hydrochloric acid, produced guanidine, $NH{=}C(NH_2)_2$, as one of the products. The formation of this compound suggests that the amino-group is at position 2. Finally, I was shown to be 2-amino-6-hydroxypteridine-8-carboxylic acid by synthesis.



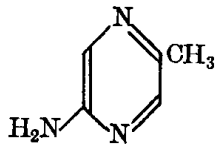
I



IV



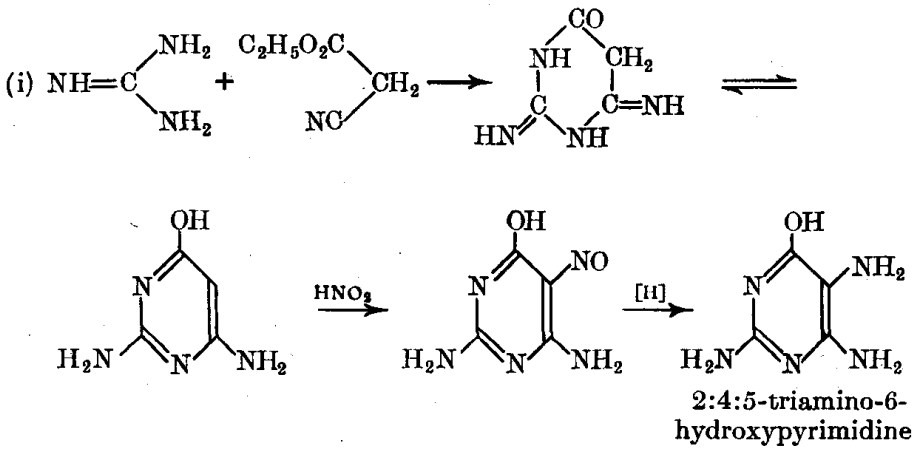
V



VI

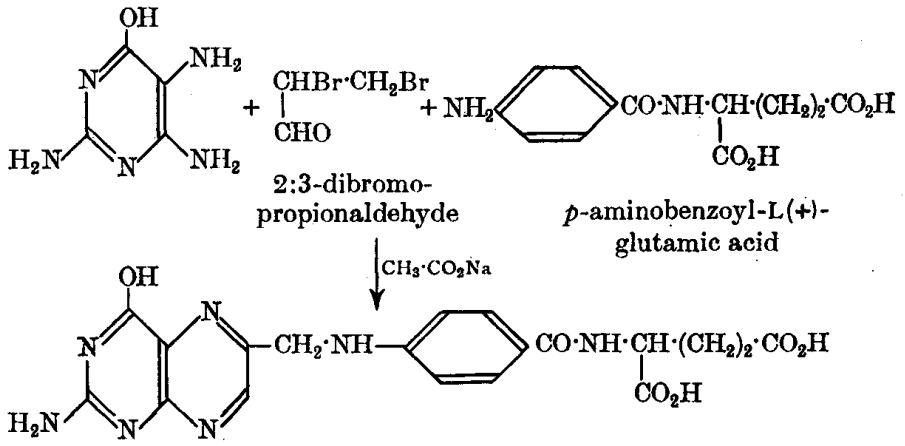
The reactions of compound II showed that it was a primary aromatic amine, and on hydrolysis it gave one molecule of *p*-aminobenzoic acid and three molecules of glutamic acid.

Hydrolysis of the fermentation *L. casei* factor with sulphurous acid gave an aromatic amine, III, and an aldehyde, IV. III, on hydrolysis, gave one molecule of *p*-aminobenzoic acid and three molecules of glutamic acid, *i.e.*, II and III are identical. When the aldehyde IV was allowed to stand in dilute sodium hydroxide solution in the absence of air, compound I and another compound, V, were produced. V, on vigorous hydrolysis, gave 2-amino-5-methylpyrazine, VI. From this it was concluded that V is 2-amino-6-hydroxy-8-methylpteridine, and IV is 2-amino-6-hydroxypteridine-8-aldehyde. Consideration of this evidence led to the suggestion that the liver *L. casei* factor has the structure given in §8; this has been confirmed by synthesis, *e.g.*, that of Angier *et al.* (1946).



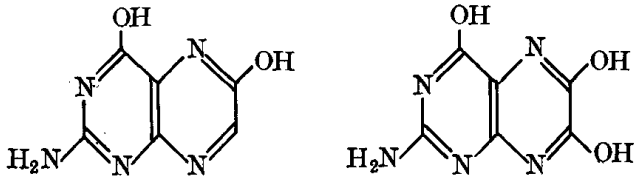
2:4:5-triamino-6-hydroxypyrimidine

(ii)



liver *L. casei* factor

It might be noted, in passing, that the **pterins** are pigments of butterfly wings, wasps, etc.; they were first isolated from butterfly wings.



xanthopterin leucopterin

§9. **Biotins** (vitamin H). Bios, an extract of yeast, was shown to be necessary for the growth of yeast (Wildiers, 1901). It was then found that bios consisted of at least two substances (Fulmer *et al.*, 1922), and two years later, Miller showed that three substances were present in bios. The first of these was named Bios I, and was shown to be *meso*inositol (Eastcott, 1928; see also §13). The second constituent, named Bios IIA, was then shown to be β-alanine (Miller, 1936) or pantothenic acid (Rainbow *et al.*, 1939). The third substance, named Bios IIB, was found to be identical with *biotin*, a substance that had been isolated by Kögl *et al.* (1936) as the methyl ester from egg-yolk. Subsequently other factors present in bios have been isolated, *e.g.*, pyridoxin (see §10) and nicotinic acid (§11).

Biotin is a vitamin, being necessary for the growth of animals. In 1940, du Vigneaud *et al.* isolated from liver a substance which had the same biological properties as biotin. Kögl *et al.* (1943) named their extract from egg-yolk α-biotin, and that from liver β-biotin. Both compounds have the same molecular formula $C_{10}H_{16}O_3N_2S$.

β-Biotin (Bios IIB or biotin), m.p. 230–232°, behaves as a saturated compound (the usual tests showed the absence of an ethylenic double bond). β-Biotin forms a monomethyl ester $C_{11}H_{18}O_3N_2S$ which, on hydrolysis, gives an acid the titration curve of which corresponds to a monocarboxylic acid; thus the formula of β-biotin may be written $C_9H_{15}ON_2S{\cdot}CO_2H$. When heated with barium hydroxide solution at 140°, β-biotin is hydrolysed to carbon dioxide and diaminocarboxylic acid $C_9H_{18}O_2N_2S$ which, by the action of carbonyl chloride, is reconverted into β-biotin (du Vigneaud *et al.*, 1941). These reactions suggest that β-biotin contains a cyclic ureide structure. Furthermore, since the diaminocarboxylic acid condenses with phenanthraquinone to form a quinoxaline derivative, it follows that the two amino-