# BIOCHEMISTRY.

It has again been found necessary to make a somewhat arbitrary selection from the many progressive branches of Biochemistry, and in this the aim has been to choose topics which have arrived at a fairly clear-cut stage of development since they were last considered here. They include aspects of metabolism, hormones, nutrition, and chemotherapy.

## I. Phosphorylation Mechanisms.

### *Phosphate Bond Energy.*

The inter-relationships of phosphorylations in the transport and storage of metabolic energy [1] have been further clarified and their range and interpretation have been extended.[2, 3, 4] It is doubtful if under physiological conditions appreciable synthesis of phosphoric esters could occur by reversal of their hydrolytic (esterase) cleavage, and the primary introduction of phosphoric groups falls into two main classes, though doubtless other routes [5, 6] also exist.

Phosphorolysis of the glucosidic type of linkage in poly- or di-saccharides occurs reversibly, as is described below, and thus provides for the introduction of phosphate into organic metabolites without the need for external energy sources. The second type of phosphorylation is associated with a marked increase of free energy, which is commonly derived from enzymic dehydrogenation accompanying the addition of phosphate to a double bond.

The differences in energy level in these two types of compound are naturally reflected in their widely different properties. The esters of phosphoric acid with alcohols such as hexoses (including the "glucosidic" type represented by glucose 1-phosphate), pentoses, trioses, glycerol, choline, serine, or the 2- or 3-position in glyceric acid, are stable compounds; their hydrolysis whether by acids or enzymes is reversible, and is accompanied by relatively small energy change ($\Delta F$ about $-3000$ cals.). But the cleavage of the second type of phosphoric compound is strongly exothermic ($\Delta F$ about $-11{,}000$ cals.). Lipmann [3] has therefore introduced for the latter the symbol $\sim$ P, denoting the "high energy phosphate bond" by means of which the high potential energy of the phosphorus linkage may be indicated: the phosphate group he writes $\sim$ ph. From the biochemical standpoint this is justified by the emphasis which it places on the ability of such high potential groups to promote synthetic reactions. Examples of this type of compound include anhydrides formed from phosphoric acid, with an organic

[1] See *Ann. Reports*, 1941, **38**, 241; 1940, **37**, 386, 417.
[2] H. M. Kalckar, *Chem. Rev.*, 1941, **28**, 71; *Biol. Rev.*, 1941, **17**, 28.
[3] F. Lipmann, "Advances in Enzymology," Interscience Press, N.Y., 1941, **1**, 99.
[4] A. A. Green and S. P. Colowick, *Ann. Rev. Biochem.*, 1944, **13**, 155.
[5] H. M. Kalckar, *Biochem. J.*, 1939, **33**, 631.
[6] S. P. Colowick, M. S. Welch, and C. F. Cori, *J. Biol. Chem.*, 1940, **133**, 359; 1941, **137**, 343.

phosphate as in the two terminal groups of adenosine triphosphate, or with a carboxyl as in 1 : 3-diphosphoglyceric acid or acetyl phosphate, or with an acidic enol group as in phospho*enol*pyruvic acid. The amidophosphate bond is another type of high energy linkage, present in creatine phosphate and arginine phosphate, the energy reservoirs of muscle and nerve.

Following the primary introduction of inorganic phosphate into metabolites, other enzymic reactions are known to transport these groups, either inter- or intra-molecularly, thus forming the wide variety of phosphorylated intermediates. According to the energy changes involved, such reactions may be reversible or irreversible. Thus reversible interchange of phosphate occurs, on the higher energy level, between the adenosine triphosphate–adenylic acid system and the creatine–creatine phosphate or phosphoglycerate–diphosphoglycerate systems. On the lower potential level it may be between glucose 1-phosphate and glucose 6-phosphate (enzyme, phosphoglucomutase), or between 3- and 2-phosphoglyceric acids (phosphoglyceromutase). The phosphorylation of glucose to glucose 6-phosphate by adenosine triphosphate (hexokinase), *i.e.*, a change from the high to low level, is irreversible. The concept of phosphate bond energy, and in a wider sense of group potential, has many applications to biological syntheses, including acetylations, methylations, and aminations (cf. 3). It plays an important part in animal phosphorylations, as will be briefly considered here.

*Primary Introduction of Phosphate Groups : Phosphorylase.*

The preparation from skeletal muscle of this enzyme, which esterifies a glucose unit of a polysaccharide, or conversely synthesises polysaccharide from glucose 1-phosphate, is fully described by Cori and co-workers.[7, 8, 9, 10] It has been obtained in a crystalline (*a*) and an amorphous (*b*) form. The former is a euglobulin of M.W. 340,000—400,000 and has 60—70% of its maximum activity without addition of adenylic acid. The more soluble *b* form is inactive without addition of adenylic acid, but both *a* and *b* are equally active in its presence. The optimum rate of conversion is $4 \times 10^4$ mols. of glucose 1-phosphate to glycogen/mol. enzyme/min. at 30°. Glucose competitively inhibits the activity, while cysteine increases both activity and solubility. Extracts of muscle and spleen contain an enzyme (" PR ") which, like trypsin, removes the prosthetic group from *a*, converting it into the amorphous *b* form. Simultaneously, pentose (0·3 μg./mg. of protein) is lost, but the substance split off is not adenylic acid. Added adenylic acid does not render the *b* form crystallisable, nor is it firmly bound as is that present in *a*. Possibly this non-dissociable union of the prosthetic group is a protection *in vivo*.

As with vegetable phosphorylase [11] the equilibrium position varies with

[7] A. A. Green and G. T. Cori, *J. Biol. Chem.*, 1943, **151**, 21.
[8] G. T. Cori and A. A. Green, *ibid.*, p. 31.
[9] C. F. Cori, G. T. Cori, and A. A. Green, *ibid.*, p. 39.
[10] G. T. Cori and C. F. Cori, *ibid.*, p. 57.
[11] C. S. Hanes and E. J. Marshall, *Biochem. J.*, 1942, **36**, 76.

pH; polysaccharide is formed from Cori ester only when a little " priming " polysaccharide is added. The reaction is considered to be [9]: glucose 1-phosphate + terminal glucose unit $\rightleftharpoons$ maltosidic chain unit + inorganic phosphate. The terminal glucose units are supplied by the end groups of the highly branched glycogen or amylopectin molecule (starch amylose does not activate animal phosphorylase [10]), polysaccharide synthesis consisting of a lengthening of the side chains by addition of glucose units in 1 : 4-glucosidic linkages [9, 12, 13] to form long unbranched chains of glucopyranose units.

When a supplementary enzyme from heart or liver, obtained free from phosphorylase, accompanies crystalline phosphorylase, a branched-chain type of polysaccharide resembling glycogen results. Presumably branched-chain polysaccharides such as glycogen and amylopectin arise from the joint action of phosphorylase and another enzyme [10] or factor.[12] It is uncertain whether the supplementary enzyme in Cori's experiments is another type of phosphorylase, able to establish 1 : 6-glucosidic linkages, or else some kind of diastase not identical with that of blood serum.[10]

The yield of phosphorylase from rabbit skeletal muscle (40—80 mg./100 g.) is not altered by previous stimulation of the muscle, but the proportion crystallisable is diminished.[8, 14] Phosphorylase occurs in a variety of tissues, and in embryonic tissues is related to the activity of their glycogen metabolism.[14, 15] It is contained in adipose tissue,[15] which utilises glycogen,[15] and in cartilage the enzyme may produce phosphoric esters, yielding phosphate needed for calcification.[16]

Adenylic acid is not a component of potato phosphorylase [17] or of disaccharide phosphorylases,[18] which require no coenzyme. It is noteworthy that in muscle phosphorylase adenylic acid acts as coenzyme without any evidence of its phosphorylation.[19] Adenosine di- or tri-phosphates have no, and inosic acid only feeble, coenzyme activity.[9, 20]

### *Adenosine Triphosphatase.*

The energy liberated by hydrolysis of the final [21] phosphoric group of adenosine triphosphate (ATP) is believed to be directly utilised for muscular contraction.[22] Engelhardt's important discovery in 1939 [22] that myosin

[12] W. N. Haworth, S. Peat, and E. J. Bourne, *Nature*, 1944, **154**, 236.

[13] W. N. Haworth, R. L. Heath, and S. Peat, *J.*, 1942, 55; W. Z. Hassid, G. T. Cori, and R. M. McCready, *J. Biol. Chem.*, 1943, **148**, 89; K. H. Meyer, "Advances in Enzymology," 1943, **3**, 109; W. Z. Hassid, *Ann. Rev. Biochem.*, 1944, **13**, 59.

[14] A. Mirski and E. Wertheimer, *Biochem. J.*, 1942, **36**, 221.

[15] B. Shapiro and E. Wertheimer, *Biochem. J.*, 1943, **37**, 397; A. Mirski, *ibid.*, 1942, **36**, 232; E. Wertheimer, *Nature*, 1943, **152**, 565.

[16] A. B. Gutman and E. B. Gutman, *Proc. Soc. Exp. Biol. Med.*, 1941, **48**, 687; A. B. Gutman, F. B. Warrick, and E. B. Gutman, *Science*, 1942, **95**, 461.

[17] D. E. Green and P. K. Stumpf, *J. Biol. Chem.*, 1942, **142**, 355.

[18] M. Doudoroff, *ibid.*, 1943, **151**, 351; P. H. Hidy and H. G. Day, *ibid.*, 1944, **152**, 477.

[19] Cf. ref. (3), p. 124.

[20] C. F. Cori, *Cold Spring Harbor Symposia on Quantitative Biology*, 1939, **7**, 260.

[21] Cf. refs. (32), (33), and (28). [22] See *Ann. Reports*, 1941, **38**, 241.

" which is the contractile constituent of muscle is at the same time the catalytic agent which promotes the chemical reaction which provides the direct source of energy of muscular activity " [23] has been widely accepted as probable after several careful studies,[22, 24, 25, 26] and so far no more active fraction has been isolated from this globulin. Nevertheless, cataphoresis [25] and sedimentation [26] analyses suggest that myosin may not be quite homogeneous, though nearly so.[27] The most serious challenge to the view that myosin itself is the enzyme is the recent demonstration by Kalckar [28] that an adenosine polyphosphatase, present in the soluble albumin fraction from potato and 50—100 times more active than myosin, is strongly and apparently somewhat specifically adsorbed by myosin. But even if the activity of myosin should eventually prove to be due to adsorbed adenosine triphosphatase, the latter might still be linked to contraction of myosin. This potato enzyme, like that from liver, hydrolyses ATP directly to adenylic acid without intervention of myokinase. It thus differs from the muscle enzyme, which is considered specific for the triphosphate inasmuch as it does not act upon adenosine diphosphate (ADP) except through myokinase;[28] but it attacks inosine triphosphate even faster than ATP.[29]

Although iodoacetic acid does not inactivate adenosine triphosphatase,[30] oxidation does so, and SH compounds reverse the inactivation.[31] Apparently the establishment of the single thioether linkages by the former reagent is to be distinguished from the cross-linked S–S bonds believed to be here produced by oxidants.

Radioactive phosphorus has been used to show in muscle the coupling of oxidation with the phosphorylation of adenylic acid and creatine [32] and the rapidity of resynthesis of ATP after its breakdown.[33, 34]

### *Phosphokinases (Phospherases).*

*Myokinase.*—Although Lipmann's terminology does not differentiate them, the terminal phosphate group of ATP is more reactive than that of the diphosphate (ADP), the latter being unable to transfer phosphate directly (*e.g.*, to glucose in presence of hexokinase), but requiring the presence of a water-soluble enzyme, myokinase, obtained from muscle and other tissues.[35, 36] This enzyme, which is stable to heat and to acid, catalyses the reversible

[23] W. A. Engelhardt, *Yale J. Biol. Med.*, 1942, **15**, 21 (Engl. trans. from Russian orig.).
[24] D. M. Needham, *Biochem. J.*, 1942, **36**, 113.
[25] K. Bailey, *ibid.*, p. 129.
[26] G. Schramm and H. H. Weber, *Kolloid-Z.*, 1942, **100**, 242; Brit. Chem. Physiol. Abs., 1943, III, 348.
[27] M. Ziff and D. H. Morre, *J. Biol. Chem.*, 1944, **153**, 653.
[28] H. M. Kalckar, *ibid.*, p. 355. [29] A. Kleinzeller, *Biochem. J.*, 1942, **36**, 729.
[30] D. M. Needham, *ibid.*, p. 113. [31] M. Ziff, *J. Biol. Chem.*, 1944, **153**, 25.
[32] R. F. Furchgott and E. Shorr, *ibid.*, 1943, **151**, 65.
[33] E. V. Flock and J. L. Bollman, *ibid.*, 1944, **152**, 371.
[34] H. M. Kalckar, J. Dehlinger, and A. Mehler, *ibid.*, 1944, **154**, 275.
[35] S. P. Colowick and H. M. Kalckar, *ibid.*, 1943, **148**, 117.
[36] H. M. Kalckar, *ibid.*, p. 127.

reaction: 2ADP $\rightleftharpoons$ ATP + adenylic acid. Inosin diphosphate is not affected.[29] The enzyme is inactivated by oxidants and activated by SH compounds, and it is capable of transferring 4 times its own weight of phosphorus per min. at 30°.

*Hexokinase.*—The occurrence of the hexokinase reaction: hexose + ATP $\longrightarrow$ hexose 6-phosphate + ADP, is probable in various cells which metabolise glucose, and from several of these this water-soluble enzyme has been extracted.[35, 37, 38] With yeast hexokinase direct phosphorylation of the 6-position of the hexose occurs with glucose or fructose,[39] but it is possible that in aerobic liver suspensions fructose may be directly phosphorylated in position 1, or alternatively, as has been suggested for galactose 1-phosphate,[40] there may be an equilibrium between these 1-phosphates and Cori ester.[39]

Hexokinase is of special importance in the synthesis of glycogen from glucose, the glucose 6-phosphate being reversibly converted *via* the 1-phosphate into glycogen, by means of the enzymes phosphoglucomutase and phosphorylase (cf. 37).

*Phosphorylation of Fructose 6-Phosphate.*—This reaction proceeds by way of an enzyme not yet isolated, sometimes called Neuberg ester phospherase. It catalyses the reaction: fructose 6-phosphate + ATP $\longrightarrow$ fructose 1 : 6-diphosphate + ADP. It is stated to be inhibited by oxidising agents and even by O/R indicators of $E_0 > 0{\cdot}05$ v., and this sensitivity has been held to be the mechanism of the Pasteur effect, by which the fermentation is checked aerobically [41]; but this is perhaps an over-simplification.[42]

*Other Enzymes concerned in Reactions of Phosphorylated Intermediates.*

Recent outstanding advances include the purification of phosphoglucomutase,[37] the isolation of aldolase (or zymohexase) of muscle,[43] now crystallised and its distribution studied,[44] and of enolase (crystalline mercury salt).[45] It is stated that the addition of phosphate to 3-phosphoglyceraldehyde is non-enzymic, and that the unknown intermediate in the dehydrogenation of this triose phosphate (previously considered to be 1 : 3-diphosphoglyceraldehyde[22]) has the nature of a "loose physical addition product," analogies for which are suggested.[46]

*Oxidative Phosphorylations.*

*Preceding the formation of pyruvate.* The reaction just mentioned was the first in which the oxidative formation of high energy phosphate bonds

[37] S. P. Colowick and E. W. Sutherland, *J. Biol. Chem.*, 1942, **144,** 423.
[38] I. Huzák, *Biochem. Z.*, 1942, **312,** 315.
[39] C. F. Cori, *Biological Symposia*, 1941, **5,** 131.
[40] H. W. Kosterlitz, *Biochem. J.*, 1943, **37,** 318, 321, 322.
[41] W. A. Engelhardt and N. E. Sakov, *Biochimia*, 1943, **8,** 9.
[42] Cf. E. S. G. Barron, "Advances in Enzymology," 1943, **3,** 149 (p. 183).
[43] D. Herbert, H. Gordon, V. Subrahmanyan, and D. E. Green, *Biochem. J.*, 1940, **34,** 1108.
[44] O. Warburg and W. Christian, *Biochem. Z.*, 1943, **314,** 149, 399.
[45] *Idem, ibid.*, 1941—2, **310,** 384.
[46] O. Meyerhof and R. Junowicz-Kocholaty, *J. Biol. Chem.*, 1943, **149,** 71.

was clearly demonstrated, inorganic phosphate being incorporated into the carboxyl of the final product, 1 : 3-diphosphoglyceric acid. This is the more common mechanism by which such bonds arise. An alternative is seen in the action of enolase, which merely by the removal of a molecule of water from 2-phosphoglyceric acid reversibly produces the high energy enolic bond in the resulting phospho*enol*pyruvic acid : in this remarkable reaction the considerable energy of dehydration is conserved within the molecule (cf. 3).

Thus in the passage from a glucose unit of glycogen to pyruvate, through the well-known series of phosphorylated intermediates, one externally introduced ~ ph (from ATP) is required in the formation of fructose 1 : 6-diphosphate, and, since two mols. of pyruvate are formed, 2 ~ ph arise at each of the stages resulting in 1 : 3-diphosphoglyceric and phospho*enol*pyruvic acids. Thus the removal of 4 hydrogen atoms should yield a balance of 3 ~ ph per mol. of hexose metabolised. Starting from glucose, instead of glycogen, the primary phosphorylation by ATP and hexokinase consumes a further ~ ph, and at the pyruvate stage only 2 ~ ph remain on balance. Possibly in intact cells, as distinct from extracts, economies are effected by unknown mechanisms (*e.g.*, the formation of fructose 1 : 6-diphosphate could theoretically occur by intermolecular transfer of the 1-phosphate from Cori ester to fructose 6-phosphate).[3] The synthesis in intact cells of ATP from inorganic phosphate during glucose fermentation has been demonstrated.[47]

*During pyruvate metabolism.* The simpler conditions prevailing in bacterial extracts enabled Lipmann [48, 49, 50] to demonstrate the production of high energy phosphate bonds during the bacterial oxidation of pyruvate to acetate and carbon dioxide, and the formation of acetyl phosphate, now isolated as the pure silver salt.[50] The latter has been synthesised from monosilver phosphate and acetyl chloride and the properties and determination of monoacetyl phosphate are described.[51] It is assumed [3, 52] that in the enzymatic synthesis [50] the addition of phosphoric acid to the carbonyl group of pyruvic acid is followed by the dehydrogenation of the resulting (unknown) compound; the analogy with bisulphite and cyanohydrin compounds is suggested. This mechanism finds some support in purely chemical studies of E. Baer.[53, 54] The reaction is represented as a dehydrogenative decarboxylation : $CH_3{\cdot}CO{\cdot}CO_2H + HO{\cdot}ph \rightleftharpoons CH_3{\cdot}C(OH)(O{\cdot}ph){\cdot}CO_2H \xrightarrow{-2H} CH_3{\cdot}CO{\cdot}O \sim ph + CO_2$. The closely similar reaction occurring in cell-free extracts of *Esch. coli* : pyruvic acid + $H_3PO_4$ ⇌ acetyl phosphate + formic

[47] D. J. O'Kane and W. W. Umbreit, *J. Biol. Chem.*, 1942, **142,** 25.
[48] Cf. *Ann. Reports*, 1940, **37,** 417.
[49] F. Lipmann, *J. Biol. Chem.*, 1940, **134,** 463; Symposium on Respiratory Enzymes, Univ. of Wisconsin Press, 1941, 145; *Federation Proc.*, 1942, **1,** 122.
[50] F. Lipmann, *J. Biol. Chem.*, 1944, **155,** 55.
[51] F. Lipmann and L. C. Tuttle, *ibid.*, 1944, **153,** 571.
[52] F. Lipmann, *Ann. Rev. Biochem.*, 1943, **12,** 1.
[53] *J. Amer. Chem. Soc.*, 1940, **62,** 1597.
[54] *J. Biol. Chem.*, 1942, **146,** 391.

acid,[55] has now been shown to be reversible,[56, 57] $^{13}C$ of radioactive formic acid appearing in the carboxyl group of the keto-acid. Since in the bacteria, though not in these cell-free extracts, carbon dioxide is normally in equilibrium with formic acid, the mechanism of a new method of carbon dioxide fixation into the carboxyl of pyruvic acid is revealed by these experiments. The synthetic reaction resulting in carbon dioxide fixation is able to proceed only because the dehydrogenation product is acetyl phosphate, and not the free acid: the formation of acetic acid from pyruvate would result in an energy loss of some 15,000 cals.[57]

As yet the evidence of the formation of acetyl phosphate or homologous compounds in animal tissues is indirect, based on reactions such as acetylations *in vivo*,[58] in isolated tissues [59] or in tissue extracts.[60] However, the oxidative formation of energy-rich phosphate bonds in such material has been repeatedly proved by the phosphorylation of adenylic acid, creatine, or glucose 6-phosphate, for example.[5, 32, 33, 61]

The efficiency of this process is unexpectedly high. The oxidation by a cytochrome system of α-ketoglutarate to succinate [62] causes esterification of 3 atoms of phosphorus for each oxygen atom consumed: in this 4-C dicarboxylic acids did not function as hydrogen carriers. Obviously an oxidative decarboxylation as formulated above could give only a 1 : 1 ratio. Even the highly speculative assumption of a diphosphate formation leaves a deficiency of one phosphorus atom esterified. Perhaps even more remarkable is the observation [63] that the whole pyruvic molecule is oxidised in heart extract with precisely the same efficiency; P/O ratio = 3. This indicates that no less than 15 high-energy phosphate bonds are established by the oxidation of 1 mol. of pyruvate, which at the level of 11,000 cals./bond shows the efficiency of conversion of oxidation into phosphate bond energy to be nearly 60%. If the course of pyruvate oxidation through the "tricarboxylic acid cycle" [64] be accepted, those of the five dehydrogenation reactions involved which have been shown to be accompanied by phosphorylation are as follows: α-Keto-acid oxidation (occurring twice) could generate $2 \times 3 \sim$ ph [62]; succinate $\longrightarrow$ fumarate not more than $1 \sim$ ph [61, 63]; malate to oxalo-

[55] M. Silverman and C. H. Werkman, *Proc. Soc. Exp. Biol. Med.*, 1940, **43**, 777; M. F. Utter and C. H. Werkman, *Arch. Biochem.*, 1943, **2**, 491.

[56] M. F. Utter, C. H. Werkman, and F. Lipmann, *J. Biol. Chem.*, 1944, **154**, 723.

[57] F. Lipmann and L. C. Tuttle, *ibid.*, p. 725.

[58] E. A. Doisy, jun., and W. W. Westerfield, *ibid.*, 1943, **149**, 229; G. J. Martin and E. H. Rennenbaum, *ibid.*, 1943, **151**, 417; K. Block and D. Rittenberg, *ibid.*, 1944, **155**, 243.

[59] P. J. G. Mann, M. Tennenbaum, and J. H. Quastel, *Biochem. J.*, 1939, **33**, 1506.

[60] D. Nachmansohn and A. L. Machado, *J. Neurophysiol.*, 1943, **6**, 397; D. Nachmansohn, H. M. John, and H. Waelsch, *J. Biol. Chem.*, 1943, **150**, 485.

[61] V. A. Belitzer and E. F. Tsibakowa, *Biochimia*, 1939, **4**, 516 (cf. footnote, p. 493, ref. 63); S. Ochoa, *J. Biol. Chem.*, 1941, **138**, 751; S. P. Colowick, H. M. Kalckar, and C. F. Cori, *ibid.*, 1941, **137**, 343.

[62] S. Ochoa, *ibid.*, 1943, **149**, 577; 1944, **155**, 87.

[63] *Idem*, *ibid.*, 1943, **151**, 493.

[64] A. H. Krebs, "Advances in Enzymology," 1943, **3**, 191.

acetate oxidation generates phosphate bonds (phosphopyruvic acid) to an unknown extent.[65] The remaining dehydrogenation, of *iso*citrate to α-ketoglutarate, has not been studied in this respect. Hence, as yet only about half of the 15 ester bonds established in the oxidation of 1 mol. of pyruvate can be accounted for experimentally, and 5 such bonds are as many as could be reasonably expected on the basis of known mechanisms. It follows that there must be yet unexplored mechanisms which enable the enzyme equipment of the cell to tap the large energy range (up to 1·2 v.) between the potential levels of oxygen and of the metabolites, and turn as much as 60% of it into phosphate bond energy. F. D.

## 2. The Intermediary Metabolism of Tryptophan.

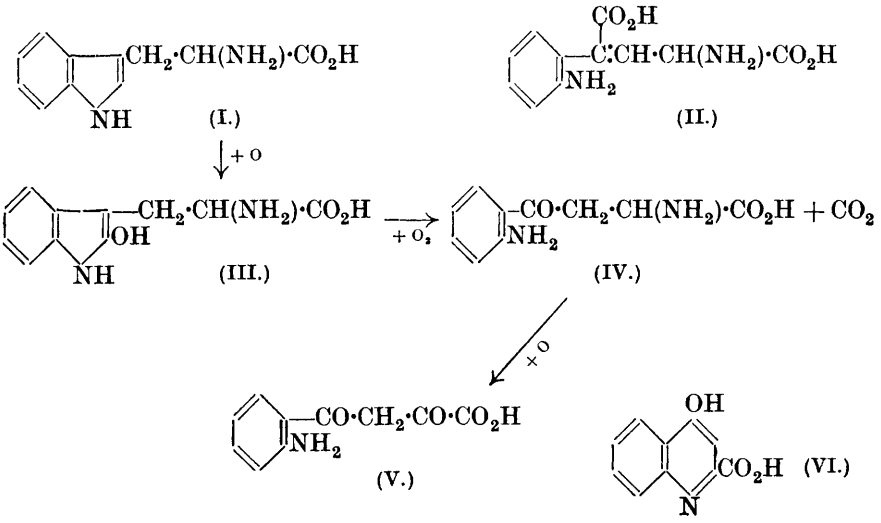
The metabolic importance of tryptophan (I) was realised soon after its discovery by F. G. Hopkins and S. W. Cole [1] in 1901. It cannot be synthesised in the mammal and has to be supplied in the diet. The rat can utilise *d*(+)-tryptophan instead of the natural *l*(−)-isomer for growth; [2,3] this, however, is not the case in the chick.[4] The intermediary metabolism of the two isomerides in many species, including the rat, appears to be different and it can be deduced that an optical inversion does not take place to a great extent under normal dietary conditions. In man ingestion of *d*(+), but not of *l*(−), -tryptophan leads to the excretion of a substance, possibly indole-3-acetic acid, which can be oxidised to a red pigment.[5] Deficiency of (I) in the diet of the rat leads to a decrease of serum proteins and a slight hypochromic anæmia.[6] Apart from these unspecific changes, which are probably common to all deficiencies of essential amino-acids, cataract of the eye and corneal lesions have been observed.[7,8]

The intermediary metabolism of (I) has yielded a number of interesting compounds. Kynurenic acid (VI), which was discovered in 1853 by Liebig,[9] has been isolated from the urine of dogs [9] (as the name implies), rabbits [10] and many other species.[11,12] It is formed from *l*(−)-tryptophan, and from indolepyruvic acid, but not from *d*(+)-tryptophan.[13]

Another substance was isolated from the urine of rabbits fed on polished

[65] H. M. Kalckar, *J. Biol. Chem.*, 1943, **148**, 127.
[1] *J. Physiol.*, 1901, **27**, 418.
[2] C. P. Berg, *J. Biol. Chem.*, 1934, **104**, 373.
[3] V. du Vigneaud, R. R. Sealock, and L. van Etten, *ibid.*, 1932, **98**, 565.
[4] G. R. Grau and H. J. Almquist, *J. Nutrit.*, 1944, **28**, 263.
[5] A. A. Albanese and J. E. Frankston, *J. Biol. Chem.*, 1944, **155**, 101.
[6] *Idem, ibid.*, 1943, **148**, 299.
[7] J. R. Totter and P. L. Day, *J. Nutrit.*, 1942, **24**, 159.
[8] A. A. Albanese and W. H. Buschke, *Science*, 1942, **95**, 584.
[9] *Annalen*, **86**, 125.
[10] A. Ellinger, *Z. physiol. Chem.*, 1904, **43**, 325.
[11] W. G. Gordon, R. E. Kaufmann, and R. W. Jackson, *J. Biol. Chem.*, 1936, **113**, 125.
[12] R. W. Jackson, *ibid.*, 1939, **131**, 469.
[13] R. Borchers, C. P. Berg, and N. E. Whitman, *ibid.*, 1942, **145**, 657.

rice and supplied with excess of (I).[14] It was assigned by its Japanese discoverers the name kynurenine and the structure (II). It was shown recently that structure (II) is incorrect and that kynurenine is represented by (IV).[15] (IV) was synthesised, though in poor yield, by condensation of *o*-nitrophenacyl bromide with ethyl sodiophthalimidomalonate, followed by acid hydrolysis and reduction. The synthetic material had chemical and optical properties identical with those of the natural product; identification is, however, not quite complete, since the synthetic material has not yet been resolved. The chain of reactions leading from tryptophan (I) to kynurenic acid (VI) now becomes clear. (I) is presumably oxidised to α-hydroxytryptophan (III), a substance so far only found in phalloidin, a toxic peptide, obtained from *Aminata phalloides*;[16] (III) is then further oxidised to (IV) with loss of carbon dioxide. Under normal dietary conditions this amino-acid is further broken down, probably through the αγ-diketo-acid (V), which rearranges itself to the quinoline derivative (VI).




Still another substance derived from (I) has been isolated from the urine of rats fed on fibrin.[17] The new substance, which was called xanthurenic acid because of its yellow colour, was shown to be 4 : 8-dihydroxyquinoline-2-carboxylic acid (IX). Being an 8-hydroxyquinoline derivative, it forms complexes with metals and the intense green colour given with ferrous salts is used for its estimation. (IX) is excreted by rats,[17] rabbits,[17] and swine,[18]

[14] Y. Kotake and J. Iwao, *Z. physiol. Chem.*, 1931, **195**, 139.

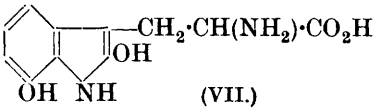
[15] A. Butenandt, W. Weidel, and W. von Derjugin, *Naturwiss.*, 1942, **30**, 51; *Z. physiol. Chem.*, 1943, **279**, 27.

[16] H. Wieland and B. Witkop, *Annalen*, 1940, **543**, 171.

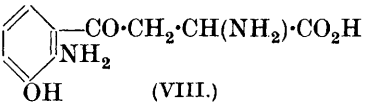
[17] L. Musajo, *Atti R. Accad. Lincei*, 1935, **21**, 368; *Gazzetta*, 1937, **67**, 165, 171, 182.

[18] G. E. Cartwright, M. M. Wintrobe, P. Jones, M. Lauritsen, and S. Humphreys, *Bull. Johns Hopkins Hosp.*, 1944, **75**, 35.

but not by dogs.[17, 19] *d*(+)-Tryptophan, indolepyruvic acid and kynurenic acid do not give rise to excretion of (IX). L. Musajo and M. Minchilli [20] claim that kynurenine does not form (IX), but Reid *et al.*[19] state that it does. The immediate precursor of (IX) may therefore be either a dihydroxytryptophan (VII) or the hydroxykynurenine (VIII).

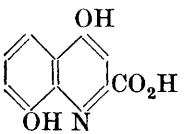



(VII.)




(VIII.)

Xanthurenic acid and kynurenine are not excreted by animals reared on normal diets, but the exact dietary deficiency necessary to produce excretion of these substances was not known until Lepkovsky *et al.*[19] showed that the green pigment found in the urines of pyridoxine deficient rats was the iron complex of (IX). Later work [18, 19] established the following facts: Xanthurenic acid is only found in pyridoxine-deficient animals fed on diets containing *l*(−)-tryptophan and the amount excreted is proportional to the tryptophan intake. Addition of pyridoxine to or omission of tryptophan from the diet leads to the disappearance of (IX) from the urine. Similarly, kynurenine excretion both in rats and in swine [18, 19] depends on pyridoxine deficiency. It seems fairly certain that kynurenine at least is a normal intermediary product of tryptophan metabolism and it appears that in pyridoxine deficiency its further breakdown is impossible. It is likely that pyridoxine is the prosthetic group of an enzyme responsible for the further oxidation of kynurenine and (IV) is excreted unchanged in its absence. (IX) may possibly be a pathological product. The fact, however, that animals on normal diets can metabolise (IX) may indicate that the formation of this 8-hydroxyquinoline derivative is an additional normal pathway of tryptophan metabolism, at least in certain species.




(IX.)

Kynurenine has recently acquired considerable interest in another direction. E. L. Tatum [21] had found that a hormone which stimulates the formation of a brown pigment in the eyes of *Drosophila* and other insects was related to tryptophan. The formation of this hormone is controlled by a specific gene and can be replaced by a substance formed by bacteria from tryptophan. This hormone was isolated by Butenandt and co-workers [15] and identified as kynurenine. E. L. Tatum and A. J. Haagen-Smit [22] showed in 1941 that their crystalline product was a complex of sucrose and kynurenine. The gene is apparently responsible for the ability of the organism to convert tryptophan into kynurenine.

[19] S. Lepkovsky, E. Roboz, and A. J. Haagen-Smit, *J. Biol. Chem.*, 1943, **149**, 195; D. F. Reid, S. Lepkovsky, D. Bonner, and E. L. Tatum, *ibid.*, 1944, **155**, 299.

[20] *Gazzetta*, 1940, **70**, 307.

[21] *Proc. Nat. Acad. Sci.*, 1939, **25**, 486; E. L. Tatum and G. W. Beadle, *Science*, 1940, **91**, 458.

[22] *J. Biol. Chem.*, 1941, **140**, 575.

Another interesting observation has recently been reported by E. L. Tatum and D. Bonner.[23] These authors found that by *X*-ray treatment a mutant of *Neurospora* can be produced which requires tryptophan for growth. This amino-acid can be replaced by a combination of indole and *l*(−)-serine, but not by any other possible intermediates. The growth of this deficient strain in the presence of indole is proportional to the serine added. The formation of tryptophan was actually demonstrated by isolation. It is assumed that a direct combination of these two compounds takes place. It is also suggested that the decomposition of tryptophan brought about by *E. coli*[24] which leads directly to indole may be a reversal of the reaction found in *Neurospora*. A. N.

## 3. Hormones.

### *The Thyroid Gland.*

*Thyroid Gland and Iodine Metabolism.*—The application of radioactive isotopes of iodine to the study of the production of thyroxine by the thyroid gland has been very fruitful.

The investigations may be divided into two main classes: (*a*) those involving the administration of radio-iodine to the intact animal, followed by removal of the thyroid gland and other tissues for examination some hours or days later; and (*b*) those in which the uptake of radio-iodine by respiring slices of isolated thyroid tissue is examined.

(a) *Studies on the intact animal.* That iodine is preferentially retained by thyroid tissue has been amply confirmed in experiments in which radio-iodine, administered orally or parenterally, has been found in greater concentration in the thyroid gland than in any other tissue of the body within a few hours of administration.[1,2,3,4] I. Perlman, I. L. Chaikoff, and M. E. Morton [3] distinguish between "tracer" doses of radio-iodine, which contain too little iodine for detection by ordinary chemical means, and what may be termed "physiological" doses of radio-iodine, measured in mg., in which a minute amount of radio-active material is mixed with ordinary potassium iodide as a carrier. When a relatively large dose (*e.g.*, 2·5 mg./kg. of body weight) of potassium iodide containing some radio-iodine is administered to a rat, 50—60% may be excreted in the urine and fæces within the next 24 hours, and only about 1% may be found in the thyroid gland. Nevertheless the thyroid gland collects, per g. of tissue, over a hundred times as much iodine as other tissues in the body and retains it longer, one-half still being present at the end of 24 hours.[3] When such large doses are administered, the thyroid tissue may become saturated with iodine and then lose its capacity to fix this element selectively, though this power may be regained within a

[23] *Proc. Nat. Acad. Sci.*, 1944, **30**, 30. [24] D. D. Woods, *Biochem. J.*, 1935, **29**, 640.

[1] S. Hertz, A. Roberts, and R. D. Evans, *Proc. Soc. Exp. Biol. Med.*, 1938, **38**, 510.

[2] (*a*) J. G. Hamilton and H. M. Soley, *Amer. J. Physiol.*, 1939, **127**, 557; (*b*) *idem*, *ibid.*, 1940, **131**, 135.

[3] *J. Biol. Chem.*, 1941, **139**, 433.

[4] C. P. Leblond and P. Süe, *Amer. J. Physiol.*, 1941, **134**, 549

few days.[4] Radio-iodine in a non-ionic form (iodate or di-iodotyrosine) is not fixed by the thyroid tissue.[4]

The administration of a tracer dose of iodine to an animal labels its circulating iodine without significantly increasing the total amount in the blood and tissues. Such tracer doses are rapidly taken up by rat thyroid tissue, 15—20% being retained therein within 2 hours, and a maximum of 65% 30—40 hours after administration.[3] Thereafter the amount in the thyroid slowly diminishes.[3] The fact that a tissue constituting only about 0·01% of the body weight and containing approximately 20% of all the iodine in the body [5] takes up as much as 65% of the tracer dose of this element in a relatively short time suggests that the turnover of iodine by the thyroid gland is rapid, and that circulating iodine can be removed by thyroid tissue more rapidly than it can come into equilibrium with tissues other than that of the thyroid.[3] The specific activity of the thyroid gland in this respect is strikingly emphasised.

When tracer doses of iodine are given to a sheep, 2—13% is found in the thyroid gland 4 hours later. Of this about 9% is in the inorganic form, 85% as 3 : 5-di-iodotyrosine, and 6% as thyroxine. Forty-eight hours after administration 30—40% of the tracer dose is found in the gland, about 13% of this being in the inorganic form, 78% as di-iodotyrosine and 9% as thyroxine. Thus at the end of 48 hours 3—4% of the dose of administered iodine is found to be in the form of thyroxine.[6] These findings, which have been adequately confirmed, are compatible with a rapid formation of di-iodotyrosine, followed by a slower conversion of the latter into thyroxine.[6, 7] Hypophysectomy depresses the thyroid's ability to collect radio-iodine and to form di-iodotyrosine and thyroxine, but the administration of pituitary thyrotropin or exposure to cold both enhance these effects.[4, 8] In children with a myxœdema not associated with goitre the thyroid collects less administered radio-iodine than does the normal gland, whereas the thyroid of a child with a goitrous myxœdema collects more than normal.[9] In Graves' disease the hyperactive thyroid gland fixes as much as 80% of a relatively large (2 mg.) dose of administered radio-iodine,[10] and, according to C. P. Leblond,[11] the hyperplastic thyroid of iodine deficiency is also able to collect administered radio-iodine more rapidly than can the normal gland. It seems probable that increased efficiency in the collection of iodine is associated with pituitary

[5] W. T. Salter, *Physiol. Rev.*, 1940, **20**, 345.

[6] I. Perlman, M. E. Morton, and I. L. Chaikoff, *J. Biol. Chem.*, 1941, **139**, 449.

[7] (*a*) W. Mann, C. P. Leblond, and S. L. Warren, *ibid.*, 1942, **142**, 905; (*b*) A. Lein, *Endocrinology*, 1943, **32**, 429.

[8] (*a*) M. E. Morton, I. Perlman, E. Anderson, and I. L. Chaikoff, *ibid.*, 1942, **30**, 495; (*b*) M. E. Morton, I. Perlman, and I. L. Chaikoff, *J. Biol. Chem.*, 1941, **140**, 603; (*c*) C. P. Leblond, *Anat. Rec.*, 1944, **88**, 285; (*d*) C. P. Leblond, J. Gross, W. Peacock and R. D. Evans, *Amer. J. Physiol.*, 1944, **140**, 671.

[9] J. G. Hamilton, M. H. Soley, and K. B. Eichorn, *Amer. J. Dis. Child.*, 1943, **66**, 495.

[10] S. Hertz, A. Roberts, and W. T. Salter, *J. Clin. Invest.*, 1942, **21**, 25.

[11] *Rev. Canadian Biol.*, 1942, **1**, 402.

stimulation of thyroid activity, a phenomenon not observed in myxœdema resulting from pituitary deficiency.

The therapeutic value of radio-iodine retained by the thyroid in Graves' disease [12] and by metastases of thyroid carcinoma [13] is apparently disappointing, but radio-iodine is of proven value for the assessment of the completeness of thyroidectomy [14] and in examination of the functional activity of the developing thyroid gland.[15] In experiments of this type functional thyroid tissue can be detected radioautographcially, *i.e.*, by its power to record its presence on a suitable photographic plate some hours after the administration to the animal of a tracer dose of radio-iodine.

(b) *Studies on slices of thyroid tissue.* When small amounts of radio-iodine in the form of potassium iodide are added to bicarbonate–Ringer solution in which are suspended surviving slices of thyroid gland, 70% of the added radio-iodine is present as di-iodotyrosine 3 hours later, and 12% as thyroxine,[16] though the addition of excess of inorganic iodide (non-radioactive) to the medium inhibits the formation of both di-iodotyrosine and thyroxine from added radio-iodine.[17] Thyroid gland which has been minced is much less effective, and a smooth suspension of finely divided tissue is almost completely inactive.[16] These results show clearly that the process of conversion of the added radio-iodine into the organic form in which it is found depends on the integrity of cell function, and is not merely the result of a chemical interchange of radio-iodine. The process is inhibited by the exclusion of air, and by addition to the medium of small amounts of cyanide, sulphide, azide and carbon monoxide, all of which inhibit the cytochrome–cytochrome oxidase system.[18] But $10^{-3}$M-azide completely inhibits the formation of di-iodotyrosine and thyroxine by the slice while permitting the collection and retention in the inorganic form of 60% of the radio-iodine of the medium.[18] This and other similar evidence suggests that the thyroid mechanism for the collection of inorganic iodine can be differentiated from that responsible for the conversion of inorganic iodine into the organic form.

*Non-thyroidal Production of Thyroid-active Substances.*—The belief that thyroxine-like substances may be formed from administered iodine in the tissue of an animal lacking a thyroid gland [19] has been confirmed by the demonstration that both di-iodotyrosine and thyroxine are produced from

[12] (*a*) S. Hertz and A. Roberts, *J. Clin. Invest.*, 1942, **21**, 624; (*b*) J. G. Hamilton and J. H. Lawrence, *ibid.*, p. 624.

[13] A. S. Keston, R. P. Ball, V. K. Franz, and W. W. Palmer, *Science*, 1942, **95**, 362.

[14] W. O. Reinhardt, *Proc. Soc. Exp. Biol. Med.*, 1942, **50**, 81.

[15] (*a*) A. Gorbman and H. M. Evans, *ibid.*, 1941, **47**, 103; (*b*) *idem*, *Endocrinology*, 1943, **32**, 113.

[16] (*a*) M. E. Morton and I. L. Chaikoff, *J. Biol. Chem.*, 1942, **144**, 565; (*b*) *idem*, *ibid.*, 1943, **147**, 1.

[17] M. E. Morton, I. L. Chaikoff, and S. Rosenfeld, *ibid.*, 1944, **154**, 381.

[18] (*a*) H. Schachner, A. L. Franklin, and I. L. Chaikoff, *ibid.*, 1943, **151**, 191; (*b*) *idem*, *Endocrinology*, 1944, **34**, 159.

[19] (*a*) A. Chapman, *ibid.*, 1941, **29**, 686; (*b*) A. Chapman, G. M. Higgins, and F. C. Mann, *J. Endocrinol.*, 1944, **3**, 392; (*c*) I. Perlman, M. E. Morton, and I. L. Chaikoff, *Endocrinology*, 1942, **30**, 487.

radio-iodine by the fully thyroidectomised rat.[20] The minute amounts of these substances containing radio-active iodine were identified by their consistent behaviour when relatively large amounts of non-radioactive authentic substances were added as carriers during the processes of fractionation.[20] These results are of particular interest in view of the discovery, by W. Ludwig and P. von Mutzenbecher (1939),[21] that preparations of iodinated casein containing 6—8% of organically bound iodine, together with certain other iodinated proteins, possess the physiological activity of thyroid protein and yield, on alkaline hydrolysis, mono-iodotyrosine (cf. [22]), di-iodotyrosine, and pure thyroxine (100—200 mg./100 g. of iodocasein).[21] The correctness of these findings has been completely confirmed.[23] The physiologically active iodinated proteins were prepared by the addition of a limited amount of iodine to a solution of the protein in dilute sodium bicarbonate, followed by incubation at 37° for some hours. For maximal thyroid activity two atoms of iodine should be taken up for each molecule of tyrosine in the protein (Turner *et al.*[23]). P. von Mutzenbecher (1939) also showed that incubation at 37° of 3 : 5-di-iodotyrosine in alkaline solution (pH 8—9) for 1—2 weeks resulted in the formation of thyroxine in gross yield of about 0·25%,[24] and this finding, too, was amply confirmed.[25, 26, 27, 28] Von Mutzenbecher also observed that casein which had been iodinated in the cold in ammoniacal solution exhibited little or no biological activity, but that the development of biological activity resulted from incubation of this iodinated protein in alkaline solution for some days.[24] Finding that the formation of thyroxine from di-iodotyrosine by alkaline incubation was accompanied by a fall of pH (*e.g.*, from 8·8 to 8·4) and the formation of iodide, and further that the addition of sodium sulphite inhibited the formation of thyroxine whereas the addition of sodium thiosulphate did not, von Mutzenbecher suggested that the oxidation of the di-iodotyrosine to thyroxine might be associated with the splitting of iodine from di-iodotyrosine in the form of hypoiodite,[24] a suggestion that received some independent support.[26] On the other hand, the reaction, which is inhibited by the presence of potassium ferricyanide and of 3 : 5-di-iodo-4-hydroxybenzoic acid, requires the presence of air,[27] and C. R. Harington and R. V. Pitt Rivers [28b] find that it is inhibited

[20] M. E. Morton, I. L. Chaikoff, W. O. Reinhardt, and E. Anderson, *J. Biol. Chem.*, 1943, **147**, 757.

[21] *Z. physiol. Chem.*, 1939, **258**, 195.

[22] C. R. Harington and R. V. Pitt Rivers, *Biochem. J.*, 1944, **38**, 320.

[23] (*a*) *Idem*, *Nature*, 1939, **144**, 205; (*b*) E. P. Reineke, *J. Dairy Sci.*, 1942, **25**, 702; (*c*) E. P. Reineke and C. W. Turner, *Univ. Missouri Agric. Exp. Stat.*, 1942, *Res. Bull. 355*, 88 pp.; (*d*) E. P. Reineke, M. B. Williamson, and C. W. Turner, *J. Biol. Chem.*, 1942, **143**, 285; (*e*) E. P. Reineke and C. W. Turner, *J. Clin. Endocrinol.*, 1943, **3**, 1; (*f*) E. P. Reineke, M. B. Williamson, and C. W. Turner, *J. Biol. Chem.*, 1943, **147**, 115.

[24] *Z. physiol. Chem.*, 1939, **261**, 253.

[25] P. Block, jun., *J. Biol. Chem.*, 1940, **135**, 51.

[26] T. B. Johnson and L. B. Tewkesbury, jun., *Proc. Nat. Acad. Sci.*, 1942, **28**, 73.

[27] A. E. Barkdoll and W. F. Ross, *J. Amer. Chem. Soc.*, 1944, **66**, 898.

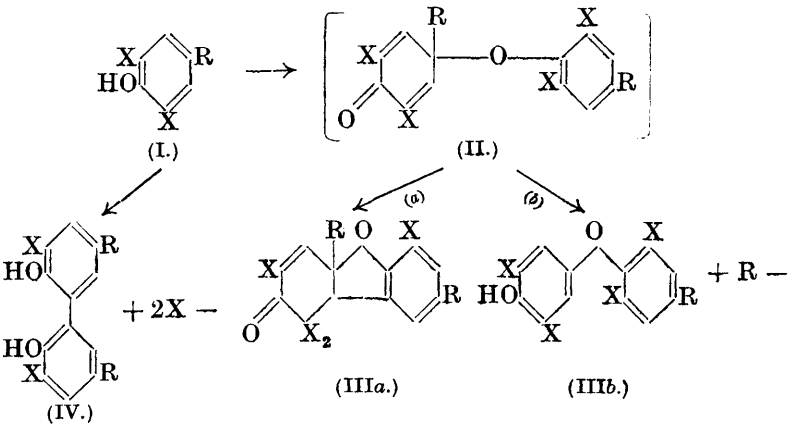
[28] (*a*) C. R. Harington, *J.*, 1944, 193; (*b*) C. R. Harington and R. V. Pitt Rivers, *Biochem. J.*, 1944, **38**, *Proc.* xxxiv.

in conditions under which the formation of hypoiodite would occur most readily. The simplest explanation might be that free iodine, which converts tyrosine into di-iodotyrosine, also brings about the oxidation of the latter to thyroxine, but von Mutzenbecher's observation that the oxidation occurs in the presence of thiosulphate is not in easy agreement with this hypothesis.

C. R. Harington [28a] has recently obtained a net yield of 3·4% of thyroxine by directly oxidising di-iodotyrosine in alkaline solution (pH 9—10) at 100° with hydrogen peroxide, the thyroxine formed being continually shaken out with butyl alcohol (a solvent into which di-iodotyrosine passes to only a slight extent from alkaline solution) in order to protect it against decomposition under the somewhat drastic conditions employed. These experiments unequivocally demonstrate that thyroxine can be formed from di-iodotyrosine by direct oxidation.

The fact that di-iodotyrosine can be so easily converted into thyroxine *in vitro* suggests the possibility that such a conversion may also easily take place in the body, but pure di-iodotyrosine possesses little or no thyroxine-like activity when administered to animals. On the other hand, J. Lerman and W. T. Salter [29] claim that the physiological activity of dried thyroid gland, which contains di-iodotyrosine, is proportional to its total iodine content and not to its variable proportion of thyroxine iodine. It seems possible, therefore, that administered peptide-linked di-iodotyrosine may be convertible into thyroxine in the body.

*Mechanism of Production of Thyroxine* in vitro *and* in vivo.—Harington and Barger (1927) pointed out that thyroxine might be formed *in vivo* by the coupling of two molecules of di-iodotyrosine, and this idea has recently been developed by Johnson and Tewkesbury (1942) [26] to explain the formation of thyroxine by the prolonged incubation of di-iodotyrosine in alkaline medium. These investigators recall that Pummerer *et al.*[30] oxidised *p*-cresol

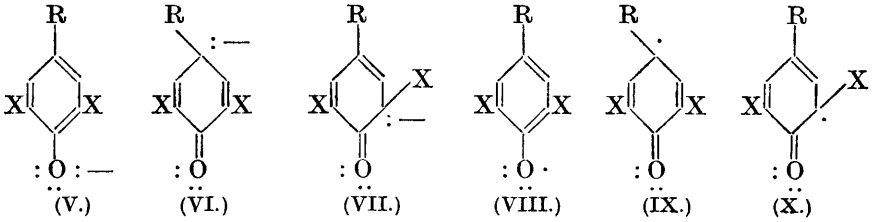



[29] *J. Pharm. Exp. Ther.*, 1934, **1**, 298.

[30] (*a*) R. Pummerer, D. Melamed, and H. Puttfarchen, *Ber.*, 1922, **55**, 3116; (*b*) R. Pummerer, H. Puttfarchen, and P. Schopflocher, *ibid.*, 1925, **58**, 1808.

with potassium ferricyanide in alkaline solution and obtained two main products: a ketotetrahydrodibenzofuran derivative (III*a*; R = Me X = H) and 2 : 2′-dihydroxy-5 : 5′-dimethyldiphenyl (IV; R = Me, X = H). Pummerer explained the formation of (III*a*) as resulting from the rearrangement of the unstable quinonoid compound (II; R = Me, X = H), which, he suggested, was formed intermediately. Johnson and Tewkesbury pointed out that, if an analogous reaction is assumed for di-iodotyrosine, rearrangement of the intermediate quinonoid compound [II; R = $CH_2$·$CH(NH_2)$·$CO_2H$ (alanyl), X = I] to a stable tetrahydrodibenzofuran derivative cannot take place, being prevented by the presence of iodine atoms *ortho* to the phenolic hydroxyl group. They suggest that the molecule may therefore stabilise itself by splitting off the alanyl side chain attached to the carbon carrying the ether oxygen, with the formation of thyroxine (III*b*; R = alanyl, X = I), and claimed to be able to identify pyruvic acid and ammonia among the products of the alkaline incubation of di-iodotyrosine, these presumably having been formed by the decomposition of the discarded alanyl side chain. Subsequently W. W. Westerfeld and C. Lowe [31] showed that the two compounds found by Pummerer *et al.* to be formed by the oxidation of *p*-cresol with ferricyanide were also obtained by oxidation of this substance with horseradish peroxidase and hydrogen peroxide.

In an interesting theoretical discussion of the mechanism of the reactions postulated by Pummerer *et al.*, Johnson and Tewkesbury, and Westerfeld and Lowe, Harington [28a] considers the implications of the assumption that *p*-cresol and di-iodotyrosine are oxidised in the form of the phenoxide ion and that the oxidation consists in the removal of an electron from the ion, followed by reaction of the free radical so formed. Harington points out that the phenoxide ion would be expected to resonate among at least three structures (V), (VI), and (VII), and that the oxidation of a *p*-substituted phenoxide ion might be assumed to consist in the removal of one electron from the oxygen atom of form (V), giving (VIII), and from the carbon atoms *para* and *ortho*, respectively, to the carbon carrying the oxygen atom of forms (VI) and (VII), giving the corresponding free radicals (IX) and (X). With *p*-cresol the interaction of (VII) and (IX) would give (II; R = Me,




X = H), which would rearrange to give (III*a*). Interaction of two molecules of (X) would give (IV). With di-iodotyrosine (R = alanyl, X = I), compound (II), formed by the interaction of (VIII) and (IX), could not stabilise as (II*a*) owing to the presence of the iodine atoms *ortho* to the phenolic

[31] *J. Biol. Chem.*, 1942, **145**, 463.

hydroxyl group, and would therefore give (III*b*) (thyroxine). For similar reasons (IV), formed from *p*-cresol, would probably not arise from di-iodotyrosine.

Chaikoff [18] suggests that the formation of both di-iodotyrosine and thyroxine by the thyroid gland is linked with aerobic oxidations in which the cytochrome–cytochrome oxidase system is involved, but E. W. Dempsey [32] believes that peroxidase is present in the thyroid follicular cells and that this enzyme may catalyse the conversion of di-iodotyrosine into thyroxine. It is possible that hydrogen peroxide is formed in living cells by the action of flavoprotein systems, and any peroxidase present might catalyse the oxidation of iodide ions to free iodine and then assist the oxidation of the di-iodotyrosine, thus formed, to thyroxine. With milk, which contains the flavoprotein system xanthine oxidase, peroxidase, and the readily iodisable protein casein, A. S. Keston [33] finds that the addition of xanthine as substrate for the xanthine oxidase system, together with a small amount of radio-iodine in the form of iodide ion, results in the rapid formation of organically bound radio-iodine. This may provide a model for further investigation of the mechanism for the organic incorporation of iodine in animal cells.

C. R. Harington [34] supports the simplest view, namely, that " the essential biochemical reaction leading to the synthesis of thyroxine may be the liberation of iodine from iodide by an oxidising enzyme system; if this were to occur conditions would be set up, namely, the presence of iodine in a faintly alkaline medium, which would not only be suitable for the iodination of tyrosine but would be analogous with those which . . . will effect the formation of thyroxine from di-iodotyrosine *in vitro*." [34] Certainly the ease with which thyroxine can be formed from tyrosine *in vitro* in the absence of enzymes but under physiological conditions not only emphasises the possibility that the formation of thyroxine from tyrosine and iodine, in the thyroid gland and elsewhere, may be a non-enzymic process but also allows consideration of the simplest hypothesis concerning the rôle of the thyroid, namely, that the primary function of this gland is the collection of circulating iodine.

*Goitrogenic Substances*.—For many years the existence has been realised of substances, both naturally occurring and artificial, which are capable of inducing enlargement of the thyroid gland on experimental administration to animals, and until recently it has been accepted that the goitrogenic action of such substances is neutralised by the addition of iodine to the diet. In 1941 J. B. MacKenzie, C. G. MacKenzie, and E. V. McCollum [35] reported that sulphaguanidine, employed to combat intestinal infection, produced a remarkable enlargement of the thyroid gland in the rat, and in the following year J. B. MacKenzie and C. G. MacKenzie showed that this goitrogenic activity was shared by a series of sulphonamides and thioureas.[36] The thyroid hypertrophy, which was accompanied by a fall in basal metabolic

[32] *Endocrinology*, 1944, **34**, 27.
[33] *J. Biol. Chem.*, 1944, **153**, 335.
[34] *Proc. Roy. Soc.*, 1944, *B*, **132**, 223.
[35] *Science*, 1941, **94**, 518.
[36] (*a*) *Federation Proc.*, 1942, **1**, 122; (*b*) *Endocrinology*, 1943, **32**, 185; (*c*) *Johns Hopkins Hosp. Bull.*, 1944, **74**, 85.

rate, was not prevented by the administration of iodine but was inhibited by the injection of thyroxine.[36] These findings were quickly confirmed,[37] and analogous results with substituted thioureas [37,38,39] and natural goitrogens [40] reported. The thyroid hyperplasia induced by these goitrogens was accompanied by signs of increased activity of the anterior pituitary gland, and was lacking in hypophysectomised animals.[36,37,40] The suggestion was then made that these substances depressed thyroid hormone production, and that the thyroid hyperplasia was secondary to increased pituitary activity evoked by the depression.[36,37] About the same time it was observed that the prolonged administration of potassium thiocyanate to human beings could induce the appearance of thyroid goitres, associated with a fall in basal metabolic rate,[41] though the development of this type of goitre could be prevented by the administration of dietary iodine.

Acting on the assumption that thiourea interferes with the production of thyroid hormone E. B. Astwood [42] successfully treated clinical hyperthyroidism by the daily administration of thiourea and showed that 2-thiouracil also was effective. The therapeutic efficacy of this new treatment of hyperthyroidism quickly received widespread confirmation,[43] and it was shown also that the administration of thiourea or thiouracil to experimental animals duplicated the effects of thyroidectomy with respect to growth,[44] metabolism of isolated tissues,[45] organ morphology,[46] thyrotropin-induced

[37] (*a*) E. B. Astwood, J. Sullivan, A. Bissell, and R. Tyslowitz, *Endocrinology*. 1943, **32,** 210; (*b*) E. B. Astwood, *J. Pharm. Exp. Ther.*, 1943, **78,** 79; (*c*) E. W, Dempsey and E. B. Astwood, *Endocrinology*, 1944, **32,** 509.

[38] C. P. Richter and K. H. Clisby, *Arch. Path.*, 1942, **33,** 46.

[39] T. H. Kennedy, *Nature*, 1942, **150,** 233.

[40] (*a*) W. E. Greisbach and H. D. Purves, *Brit. J. Exp. Path.*, 1943, **24,** 171; (*b*) V. I. E. Whitehead, *ibid.*, p. 192.

[41] (*a*) R. W. Rawson, S. Hertz, and J. H. Means, *J. Clin. Invest.*, 1942, **21,** 624; (*b*) J. L. Kobacker, *Ohio Sta. Med. J.*, 1942, **38,** 541; (*c*) M. P. H. Foulger and E. Rose, *J. Amer. Med. Assoc.*, 1943, **122,** 1072; (*d*) R. W. Rawson, S. Hertz, and J. H. Means, *Ann. Int. Med.*, 1943, **19,** 829.

[42] *J. Amer. Med. Assoc.*, 1943, **122,** 78.

[43] (*a*) R. H. Williams and G. W. Bissell, *New England J. Med.*, 1943, **229,** 97; (*b*) H. P. Himsworth, *Lancet*, 1943, ii, 465; (*c*) R. W. Rawson, R. D. Evans, J. H. Means, W. C. Peacock, J. Lerman, and R. E. Cortell, *J. Clin. Endocrinol.*, 1944, **4,** 1; (*d*) P. B. Newcombe and E. W. Deane, *Lancet*, 1944, i, 179; (*e*) J. L. Gabrilove and M. J. Kert, *J. Amer. Med. Assoc.*, 1944, **124,** 504; (*f*) E. C. Bartels, *ibid.*, 1944, **125,** 24; (*g*) K. E. Paschkis, A. Cantarow, A. E. Rakoff, A. A. Walking, and W. J. Tourish, *J. Clin. Endocrinol.*, 1944, **4,** 179; (*h*) R. H. Williams and H. M. Chute, *New England J. Med.*, 1944, **230,** 657; (*i*) E. B. Astwood, *J. Clin. Endocrinol.*, 1944, **4,** 229; (*j*) T. H. McGavick, A. J. Gerl, M. Vogel, and D. Schwimmer, *ibid.*, p. 249; (*k*) F. L. Ritchie and B. L. Geddes, *Med. J. Aust.*, 1944, **1,** 381; (*l*) M. H. Sloan and E. Shorr, *Endocrinology*, 1944, **35,** 200; (*m*) E. B. Astwood, *ibid.*, p. 200; (*n*) H. P. Himsworth, C. A. Joll, H. Evans, G. Melton, and S. L. Simpson, *Proc. Roy. Soc. Med.*, 1944, **37,** 693; (*o*) E. M. Martin, *Canadian Med. Assoc. J.*, 1944, **51,** 39; (*p*) J. K. McGregor, *ibid.*, p. 37; (*q*) E. M. Watson and L. D. Wilcox, *ibid.*, p. 29.

[44] (*a*) A. M. Hughes, *Endocrinology*, 1944, **34,** 69; (*b*) R. H. Williams, A. R. Weinglass, G. W. Bissell, and J. B. Peters, *ibid.*, p. 317.

[45] B. J. Jandorf and R. E. Williams, *Amer. J. Physiol.*, 1944, **141,** 91.

[46] C. P. Leblond and H. E. Hoff, *Endocrinology*, 1944, **35,** 229.

metamorphosis of tadpoles,[47] development of fish,[48] pigmentation of bird plumage,[49] and insulin sensitivity.[50] Thiouracil has also been successfully employed in an evaluation of the amount of thyroxine secreted by the thyroid gland under different conditions.[37] These results all support the view that thioureas and thiouracil inhibit the formation of its hormones by the thyroid gland, but do not interfere with the action of the hormone once it has been liberated into the blood stream.

*Mechanism of the Action of Thiouracil and of Other Goitrogens on the Production of Thyroid Hormone.*—The daily administration of thiouracil to young rats for 8 days reduces the iodine content of the thyroid gland almost to zero, though the weight of the gland may be increased nearly threefold.[51] If the daily administration of thyroxine is now begun, with continuation of thiouracil treatment, the iodine content of the gland remains low but the follicles fill with densely staining colloid material.[32, 51] Similar results follow the removal of the pituitary gland during thiouracil administration.[51] It seems that under these conditions the secreted colloid material contains little or no thyroxine,[32, 51] and that the incorporation of iodine into this material has been inhibited by the thiouracil.

When radio-iodine is injected into rats previously made goitrous by the administration of thiouracil, the power of the thyroid gland to collect the administered iodine may be only 10—20% of normal,[52, 53, 54] and the formation of di-iodotyrosine and of thyroxine is also inhibited.[53] The capacity of thiocyanate-induced goitres to collect administered radio-iodine may be supernormal, however,[52] a finding which is significant in view of the fact that thiocyanate is an iodine-inhibited goitrogen.

A. L. Franklin, I. L. Chaikoff, and S. R. Lerner [55] found that the addition, to the medium in which surviving slices of thyroid tissue were maintained, of $10^{-3}$M-thiouracil, or of a like concentration of thiourea or of potassium thiocyanate, depressed the ability of the tissue to convert added radio-iodine into di-iodotyrosine and thyroxine. This concentration of thiourea and of thiouracil had little effect on the capacity of the slices to collect iodine from the medium, although potassium thiocyanate in a similar amount significantly diminished the collection of added radio-iodine. The latter results are at variance with the data from intact animals cited above. Sulphanilamide also depresses the formation of di-iodotyrosine and thyroxine in slices

[47] A. M. Hughes and E. B. Astwood, *Endocrinology*, 1944, **34**, 138.

[48] E. D. Goldsmith, R. F. Nigrell, A. S. Gordon, H. A. Charipper, and M. Gordon, *ibid.*, 1944, **35**, 132.

[49] M. Juhn, *ibid.*, p. 278.

[50] G. J. Martin, *Arch. Biochem.*, 1943, **3**, 61.

[51] E. B. Astwood and A. Bissell, *Endocrinology*, 1944, **34**, 282.

[52] (*a*) R. W. Rawson, J. F. Tannheimer, and W. Peacock, *ibid.*, p. 245; (*b*) R. Larson, F. R. Keating, jun., R. W. Rawson, and W. Peacock, *ibid.*, 1944, **35**, 200; (*c*) R. W. Rawson, R. E. Cortell, W. Peacock, and J. H. Means, *ibid.*, p. 201.

[53] A. L. Franklin, S. R. Lerner, and I. L. Chaikoff, *ibid.*, 1944, **34**, 265.

[54] (*a*) E. J. Baumann, N. Metzger, and D. Marine, *ibid.*, p. 44; (*b*) A. S. Keston, E. D. Goldsmith, A. S. Gordon, and H. A. Charipper, *J. Biol. Chem.*, 1944, **152**, 241.

[55] *Ibid.*, 1944, **153**, 151.

of thyroid tissue, without depressing the capacity of the slices to collect iodide from the medium.[18, 56]

As was suggested above (p. 241), the simplest hypothesis regarding the specific function of the thyroid is that this gland possesses special ability to collect iodine from the circulation. Since free iodine is presumably the iodinating agent in the formation of di-iodotyrosine from tyrosine, and since iodide ions constitute the form in which this element is collected from the blood stream, it seems probable that the first process which the collected iodide ions undergo is enzymic oxidation to free iodine. Inhibition of this process might not only inhibit the formation of di-iodotyrosine and therefore that of thyroxine but also depress the power of the gland to collect more iodide. D. Campbell, F. W. Landgrebe, and T. N. Morgan [57] recall E. A. Werner's observation [58] that free iodine can oxidise thiourea to formamidine disulphide, $NH{:}C(NH_2)\cdot S\cdot S\cdot C(NH_2){:}NH$, being itself reduced to iodide ions in the process, and suggest that this may be a mechanism whereby thiourea might interfere with the synthesis of the thyroid hormone. Another possibility is that thiourea and other similar goitrogens inhibit the action of an enzyme which catalyses the formation of iodine from iodide in the thyroid gland. Thiouracil does not poison cytochrome oxidase,[32] though cytochrome oxidase inhibitors do prevent the formation of thyroxine from inorganic iodide in surviving slices of thyroid tissue.[18] Thiouracil poisons peroxidase [32, 59] and polyphenol oxidases [60] and protects *p*-cresol against enzymic oxidation when present molecule for molecule of substrate.[60] Dempsey believes that, although peroxidase may be concerned in the formation of iodine from iodide in the thyroid gland, this enzyme also catalyses the conversion of di-iodotyrosine into thyroxine.[32] This belief is based on the observation by Dempsey and Astwood [37] that di-iodotyrosine, unlike thyroxine, does not prevent the goitrogenic action of thiouracil, the assumption being made that thiouracil must therefore inhibit the conversion of di-iodotyrosine into thyroxine. Since, however, the thyroid gland appears to be unable to utilise administered di-iodotyrosine, for the formation of thyroxine, in the absence of goitrogenic agents,[4, 43] the assumption would appear on the available evidence to be of doubtful validity.

It may be concluded that thiourea and thiouracil interfere with the formation of iodine from iodide ions, either by reducing any iodine formed back to iodide ions, or by poisoning the enzyme system catalysing the oxidation of iodide ions to iodine. Whether or not these goitrogens interfere in any other way with the formation of thyroxine in the body is as yet uncertain.

*Nature of the Thyroid Hormone.*—Canzanelli *et al.*[61] found that the addition of thyroglobulin, but not of thyroxine, to tissues respiring *in vitro*

[56] A. L. Franklin and I. L. Chaikoff, *J. Biol. Chem.*, 1943, **148**, 719; 1944, **152**, 295.
[57] *Lancet*, 1944, *i*, 630. [58] *J.*, 1912, **101**, 2166.
[59] J. B. Sumner and G. F. Somers, "Chemistry and Methods of Enzymes," Academic Press Inc., New York, 1943.
[60] F. Chodat and G. Duparc, *Helv. Chim. Acta*, 1944, **27**, 334.
[61] (*a*) A. Canzanelli and D. Rapport, *Endocrinology*, 1937, **21**, 779; (*b*) A. Canzanelli, R. Guild, and D. Rapport, *ibid*, 1939, **25**, 707.

increases the rate at which oxygen is taken up, and a stimulating action on tissue respiration *in vitro* has also been observed with plasma from patients with hyperthyroidism.[62] These observations suggest that thyroglobulin might be the circulating thyroid hormone, but immunological tests fail to reveal the presence of this protein in the blood stream under a variety of conditions,[63, 64] including that of hyperthyroidism.[63] Only under such an abnormal condition as thyroid trauma was thyroglobulin detected in the blood stream [63] and it seems probable that thyroglobulin as such does not normally leave the thyroid follicles. Proteolytic enzymes are present in the thyroid gland, and their activity varies under physiological conditions [65] and the hydrolysis of thyroglobulin to a less complicated thyroxine-containing molecule is probably a preliminary step in the secretion of the thyroid hormone. Harington, whose earlier results suggested that the secretion of the thyroid gland might be a thyroxine-containing peptide rather than thyroxine itself, has recently reviewed the evidence on this point [34] and concludes that there is no satisfactory reason to abandon the simplest hypothesis, namely, that thyroxine itself is the circulating hormone. As Harington and his colleagues had earlier shown,[66] the administration to rats of antisera raised against thyroxyl derivatives of horse-serum albumin and globulin confers resistance against the usual metabolism-increasing activity of administered thyroxine or thyroglobulin. That the administration of these antisera was without effect on the metabolic rate of the treated rats, though such treatment prevented the normal action of administered thyroxine and thyroglobulin, was explicable on the basis of the great power of the normal thyroid gland to respond to a call for increased secretory activity.[66] Harington [34] suggests that the simplest explanation of the facts is that the circulating antibodies of the passivity immunised animal, possessing serological combining sites adapted to thyroxine, interfere with the access of the latter to its normal sites of action in the tissues, so that it is most probable that the injected thyroxine is present as such in the circulation. He points out that this simple interpretation can be avoided only by the assumption that injected thyroxine follows the devious route of synthesis into thyroglobulin, followed by release as such (which seems on other grounds to be unlikely) or as a peptide, which is the real hormone, and such a complicated process is at least unnecessary to account for the immunological phenomena observed.[34] Harington concludes that thyroxine is "the true thyroid hormone as it circulates in the body." [34] J. H. Means [67] in another recent review concludes that "the thyroid hormone travels from the thyroid to its end-organs in a form lower than the protein level, and that it acts upon its end-organ in a form of higher level than that of the amino-acids. It may

[62] W. T. Salter and F. W. Craige, *J. Clin. Invest.*, 1938, **27**, 502.
[63] J. Lerman, *ibid.*, 1940, **19**, 555.
[64] L. I. Stellar and H. G. Olken, *Endocrinology*, 1940, **27**, 614.
[65] A. J. Dziemian, *J. Cell. Comp. Physiol.*, 1943, **21**, 339.
[66] R. F. Clutton, C. R. Harington, and M. E. Yuill, *Biochem. J.*, 1938, **32**, 1119
[67] *Ann. Int. Med.*, 1943, **19**, 567.

both travel and act in the form of a polypeptide or peptone,"[67] a conclusion also compatible with the immunological evidence provided by Harington. If one accepts as significant the observation that thyroxine fails to stimulate tissue respiration *in vitro* whereas thyroglobulin and plasma from patients with hyperthyroidism are effective under these conditions,[61, 62] the simplest explanation of all the available evidence, including the results of the immunological investigations, appears to be that thyroxine stimulates tissue respiration only when it is combined in peptide form, and that it is transported from the thyroid tissues to the gland in this form.

In the sea, the liberation of iodine from iodine ions might occur on a minute scale wherever the oxidative catalysts of respiring cells of unicellular organisms were active. Thus the tissue proteins of a primitive protozoon might, as the result of the oxidative capacity of its enzyme systems, come to contain organically bound iodine, in the form of thyroxine, with the aid of the mechanisms reviewed above. With Means [67] we may conclude that the elaboration of the thyroid hormone preceded that of the thyroid gland in the process of evolution, and that the gland developed as an organ specialised for the production and subsequent distribution of a substance which originally was produced in the tissues in general, and which, even in higher animals, can still be made in tissues other than that of the thyroid. Thyroxine, in a combined form, may therefore be a general constituent of living protoplasm, essential for the maintenance of respiration at the high level which is characteristic of the cells of the highly developed metazoon. That being so, we might regard thyroxine not as a specific internal secretion of one ductless gland, but as an essential amino-acid.

In some respects the position with respect to choline also is analogous. Choline is an essential constituent of the normal body and the body is apparently able to manufacture all but one portion of the molecule of this important substance, namely, the methyl groups. Provided that a source of exogenous methyl groups is available to the body, *e.g.*, from methionine, choline can be manufactured in sufficient amount for its particular requirements, though otherwise this substance becomes an essential food factor and qualifies for the description of vitamin. Similarly, the only portion of the thyroxine molecule that the body cannot provide from its own resources—tyrosine is not an essential amino-acid—is iodine, and once free iodine is available the manufacture of thyroxine can proceed. In higher animals the presence of the specialised thyroid gland is essential if the rate of collection of iodine and thyroxine production are to keep pace with the demand for this amino-acid, but in lower animals the tissues in general can probably produce *in situ* all they need, provided that the essential constituent is to hand. As Means points out,[67] man could live happily without a thyroid gland if his food proteins were properly iodinated, and it is true to say that to the higher animals from which the thyroid gland has been removed thyroxine, or a thyroxine-containing iodinated protein, has become an essential constituent of the diet, and might therefore be regarded as a vitamin for such an animal.

Thyroxine, or a compound containing it in peptide linkage, can be regarded as a hormone. But such a description does not preclude the possibility of regarding it, from some points of view, as an essential amino-acid, or as a vitamin or coenzyme. Once more the overriding of boundaries which were once thought to divide different departments of scientific activity may be regarded as the natural concomitant of progress and development.

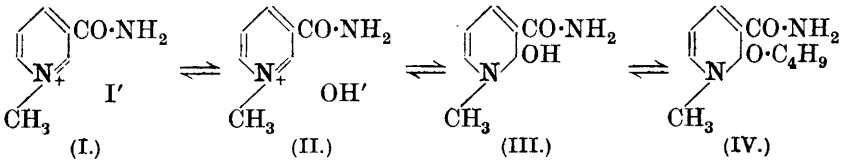
F. G. Y.

## 4. Nutrition.

The excretion of methylated derivatives of nicotinic acid, the relation of pyridoxine to hæmatopoiesis and iron metabolism, and the nutritional value of "folic acid" and vitamin $B_c$ are reviewed in this section.

### *The Excretion of Nicotinic Acid.*

In 1940 V. A. Najjar and R. W. Wood [1] described the presence in urine of a substance with a characteristic blue fluorescence, which they called $F_2$. Its excretion was related to the availability of nicotinic acid and increased in proportion to the nicotinic acid intake. In view of the uncertainty of the form taken by the substance in urine, the symbol $F_2$ is a convenient means of denoting it. $F_2$ is absent from the urine of pellagrins [2] and it slowly disappears from the urine of dogs fed upon a nicotinic acid-deficient diet,[3] whereas the administration of nicotinic acid or its derivatives increases its excretion.[4, 5] The chemical nature of $F_2$ has been elucidated and the substance has been isolated as waxy, hygroscopic, needle-shaped crystals, which in neutral or weakly alkaline conditions have a greenish-blue fluorescence, and in acidic solution a blue.[6, 7, 8] A substance identical in physical and chemical properties can be obtained by the treatment of nicotinamide methiodide with baryta. It is well known that alkalinisation of such a compound (I) will lead to the formation of a quaternary base (II), which subsequently may suffer transformation into a carbinol (III) by attachment of the hydroxyl group to one of the α-positions of the pyridine ring :




[1] *Proc. Soc. Exp. Biol. Med.*, 1940, **44**, 386.
[2] V. A. Najjar and L. E. Holt, *Science*, 1941, **93**, 20.
[3] V. A. Najjar, H. J. Stein, L. E. Holt, and C. V. Kabler, *J. Clin. Invest.*, 1942, **21**, 263.
[4] V. A. Najjar and L. E. Holt, *Proc. Soc. Exp. Biol. Med.*, 1941, **48**, 413.
[5] P. Ellinger and R. A. Coulson, *Biochem. J.*, 1944, **38**, 265.
[6] J. W. Huff and W. A. Perlzweig, *Science*, 1943, **97**, 538; *J. Biol. Chem.*, 1943, **150**, 395.
[7] P. Ellinger and R. A. Coulson, *Nature*, 1943, **152**, 583; *Biochem. J.*, 1943, **37**, Proc. xvii.
[8] V. A. Najjar, V. White, and D. B. N. Scott, *Bull. Johns Hopkins Hosp.*, 1944, **74**, 378.

It would appear that the substance actually isolated from urine is the carbinol. But it is uncertain [6, 7, 8] whether the fluorogenic substance in urine is the quaternary base, the $\psi$-base, a pyridinium salt, or a mixture of these dependent upon the conditions. The fluorescence observed *in vitro* is considered [7] to be due to a mixture of 6-hydroxy-1-methyl-1 : 6-dihydropyridine-3-carboxyamide and another carbinol with an *o*-quinonoid structure. Atmospheric oxygen and ferricyanide oxidise an alkaline solution of $F_2$.[8] This treatment, which leads to a deep violet fluorescence, might be expected to cause the formation of a pyridone from the base. An alternative possibility [8] is that the fluorescence is due to the formation of a carbinol ether (IV) from the $\psi$-base and the *iso*butanol used to extract $F_2$. Either suggestion would explain the slow increase in fluorescent intensity of *iso*butanol extract of $F_2$ from alkaline solutions.

Upon the fluorescence of the derivative of the pyridinium salt have been based methods for its estimation in urine.[9, 10, 11] The essential feature of some of them is a base exchange between the substance and permutit. The use of these methods has shown that there is a distinct individual variation and a fluctuation throughout the day in the excretion of $F_2$.[5] The proportionality between the amount eliminated and the intake of nicotinamide has led naturally to the development of load tests for gauging nutritional status as regards nicotinamide. Investigation of this kind of test has diminished the value of trigonelline excretion as a nutritional index, since its determination may include the pyridinium salt, which on acid or alkaline hydrolysis is converted into trigonelline.[6, 12] Nevertheless as a nutritional index the excretion of $F_2$ will need to be used with careful discrimination. Its dependence on the body reserves of methyl donators whose level depends upon the diet, has been indicated.[12] This view is well attested by experiments upon rats, guinea-pigs, and rabbits.[13, 14, 15] The feeding of unusual amounts of nicotinamide to rats adversely affected their growth and their livers—effects which could be remedied by choline or methionine. In guinea-pigs and rabbits no ill effects arise from the ingestion of large amounts of nicotinamide. There is a clear difference in the response of these animals to nicotinamide. The rat excretes the *N*-methylnicotinamide in the urine; the rabbit and the guinea-pig do not. Methylation of nicotinamide has been demonstrated *in vitro* with rat liver slices.[16] A consequence of a high level of nicotinamide in the diet of the rat is a depletion of its store of methyl donators, the sequelæ of which are retarded growth and fatty livers. The reverse of this, diminished stores of methionine and choline from faulty diet creating low excretion of $F_2$, is thus conceivable.

[9] V. A. Najjar, *Bull. Johns Hopkins Hosp.*, 1944, **74**, 392.
[10] R. A. Coulson, P. Ellinger, and M. Holden, *Biochem. J.*, 1944, **38**, 150.
[11] J. W. Huff and W. A. Perlzweig, *J. Biol. Chem.*, 1943, **150**, 483.
[12] H. P. Sarett, *ibid.*, p. 395.
[13] P. Handler and W. J. Dann, *ibid.*, 1942, **146**, 357.
[14] P. Handler and F. Bernheimer, *J. Biol. Chem.*, 1943, **148**, 649.
[15] P. Handler, *ibid.*, 1944, **154**, 203.
[16] W. A. Perlzweig, M. L. C. Bernheim, and F. Bernheim, *ibid.*, 1943, **150** 401.

*The Relation of Pyridoxine to Anæmia.*

In the last decade accumulative evidence has indicated that deficiency of the B vitamins, particularly pyridoxine and nicotinic acid, may interrupt normal erythropoiesis. Mention in this review is confined to work of recent times, which attempts to define clearly the type of anæmia from blood and bone marrow investigations. The most careful work upon the effect of pyridoxine deficiency has been done on the pig and the dog.

In dogs a hypochromic anæmia would seem to be a feature of deprivation of vitamin $B_6$.[17,18,19,20] The anæmia does not respond to iron or copper; yet prompt improvement follows oral or intravenous administration of crystalline pyridoxine. The fact that the initial improvement is not always maintained has led to the suggestion that other factors may be involved.[19,21,22] With progressing severity of the anæmia the plasma iron rises, and rapidly falls with the administration of pyridoxine and with the initial blood regeneration.[22]

In 1938 Chick and her collaborators [23] showed that omission of the eluate fraction of a liver concentrate, a source of pyridoxine, from a synthetic diet led to a microcytic anæmia and epileptic fits in young pigs. In pigs lacking the filtrate fraction a normocytic anæmia developed. Wintrobe and his co-workers [24,25,26] found that pigs fed on a diet supplemented with vitamins A and D and all the known crystalline B vitamins except pyridoxine develop a severe microcytic anæmia which is most clearly hypochromic at its height. As the anæmia progresses, anisocytosis becomes more marked; large polychromatophilic corpuscles and cells containing blue-staining granules make an appearance. An irregular reticulocytosis may also occur. The anæmia is associated with hyperplasia of the bone marrow and an irregular reticulocytosis. The anæmia is not hæmolytic in type; no significant changes occur in the serum bilirubin or in the excretion of urobilinogen or urinary porphyrin. Fatty infiltration of the central portion of the hepatic lobules also occurs. Epileptiform convulsions are seen in the majority of $B_6$-deficient pigs. An outstanding feature was hæmosiderosis of the spleen, liver, and bone marrow and an increase in the serum iron, which is apparently chiefly in the ferric

[17] P. J. Fouts, O. M. Helmer, S. Lepkovsky, and J. H. Jukes, *J. Nutrition*, 1938, **16**, 197.

[18] *Idem*, *Amer. J. Med. Sci.*, 1943, **199**, 163.

[19] H. J. Borson and S. R. Mettier, *Proc. Soc. Exp. Biol. Med.*, 1940, **43**, 429.

[20] H. R. Street, G. R. Cowgill, and H. M. Zimmerman, *J. Nutrition*, 1941, **51**, 275.

[21] *Idem*, *ibid.*, p. 275.

[22] J. M. McKibbin, A. E. Schaeffer, D. V. Frost, and C. A. Elvehjem, *J. Biol. Chem.*, 1942, **142**, 77.

[23] H. Chick, J. F. Macrae, A. J. P. Martin, and C. P. Martin, *Biochem. J.*, 1938, **32**, 2207.

[24] M. M. Wintrobe, M. Samter, and H. Lisco, *Bull. Johns Hopkins Hosp.*, 1939, **64**, 399.

[25] M. M. Wintrobe, R. H. Follis, M. H. Miller, H. J. Stein, R. Alcayago, S. Hymphreys, A. Suksta, and G. E. Cartwright, *ibid.*, 1943, **72**, 1.

[26] G. E. Cartwright, M. M. Wintrobe, and S. Hymphreys, *J. Biol. Chem.*, 1944, **153**, 171.

state. Administration of pyridoxine produced a rapid regeneration of blood with a return of the red cells to normal size. This response was accompanied by a mobilisation of iron, which was indicated by the disappearance of the hæmosiderosis and a fall in the serum iron. These interesting results clearly imply a rôle for pyridoxine in iron metabolism. From the fact that in combined pyridoxine and iron deficiency no hæmosiderosis or elevated serum iron occurs despite the development of anæmia, it would appear that the disturbances in iron metabolism are due to increased absorption or decreased excretion. This is an interesting possibility, since it is contrary to the idea that the animal absorption of iron is dependent upon its needs. In many respects—the ferræmia, hæmosiderosis, hyperplastic bone marrow and neurological disturbances—the pyridoxine anæmia is similar to pernicious anæmia, although it differs in being characterised by a microcytosis and lack of response to liver extract. Nevertheless the study of the mechanism of $B_6$ anæmia may provide some help towards the solution of pernicious anæmia.

The possible relationship of the anæmia and kindred symptoms of vitamin $B_6$-deficiency with tryptophan metabolism has been referred to in Dr. Neuberger's Report (p. 237).

### *Folic Acid and Vitamin $B_c$.*

During the last four years it has become evident that certain micro-organisms need for their growth one or more factors distinct from any of the known vitamins. It has also become apparent that these factors have a rôle in animal nutrition which consists, in the main, in promoting growth, countering the effect of sulphonamides, and in stimulating the formation of the cells of the blood.

In 1940 E. E. Snell and W. H. Peterson [27] described a factor of acidic nature needed by *Lactobacillus casei E*; to it they gave the name norit eluate factor. From spinach concentrates another acidic factor, named folic acid, was prepared,[28] defined as the material necessary for the growth of *Streptococcus lactis R* on a given medium. This nutrilite is abundant in green leaves and occurs in animal tissues and yeast.

Williams and his co-workers [29,30,31] have now obtained folic acid in amorphous form from spinach. It is a substance of M.W. about 400, not easily soluble in organic compounds and extremely labile. Esterification, acylation and methylation destroy its biological activity. It is also sensitive to oxidation and reduction, and is none too stable in acid or alkaline solution. From analysis it has an approximate empirical formula of $C_{15}H_{15}O_8N_5$ and absorption spectra indicate that it may contain a structural unit similar to xanthopterin.[32]

[27] *J. Bact.*, 1940, **39**, 273.
[28] H. K. Mitchell, E. E. Snell, and R. J. Williams, *J. Amer. Chem. Soc.*, 1941, **63**, 2284.
[29] *Idem, ibid.*, 1944, **66**, 267.
[30] E. H. Frieden, H. K. Mitchell, and R. J. Williams, *ibid.*, p. 269.
[31] H. K. Mitchell, and R. J. Williams, *ibid.*, p. 271.
[32] H. K. Mitchell, *ibid.*, p. 275.

From most of the work on concentrates it would appear that the norit eluate factor and folic acid are either the same substance or closely similar compounds. Concentrates of folic acid are active in stimulating the growth of yeast and other organisms, including *Lactobacillus casei*, and B. L. Hutchings, N. Bohonos, and W. H. Peterson have concluded [33] that the eluate factor was similarly of general nutritional significance for the lactic acid bacteria and the growth of *Streptococcus lactis*. From descriptions [31,34] of concentrates of folic acid it would appear that, together with folic acid, other substances of biological importance are present; these include *p*-aminobenzoic acid and xanthopterin, which are capable of counteracting the inhibitory effect of sulphonamides upon the growth of bacteria and rats. The fact that concentrates prepared from different sources stimulate the growth of *Lactobacillus casei* and *Streptococcus lactis* probably led to the interchangeable use of the terms folic acid and eluate factor. The term "folic acid" may therefore be used to indicate this group of growth stimulants.

On animals, concentrates of the eluate factor and folic acid exert effects which may be attributable to similar groups of substances. In the chick,[35] eluate factor has been found to promote growth; in the rat, folic acid.[28] Concentrates of both factors share with *p*-aminobenzoic acid the property of antagonising the noxious effects of sulphonamides, sulphaguanidine and sulphathiazole, which are poorly absorbed from the intestine. Besides producing a reduction in growth,[36] sulphonamides may cause agranulocytopenia, leucopenia, and often anæmia and other pathological conditions when they are incorporated in synthetic diets adequately supplied with vitamins.[37] Their action may be partly due to an interference with enzyme systems of the body or to suppression within the intestine of bacterial synthesis of essential factors; folic acid [38] and biotin are synthesised by intestinal bacteria. Both biotin and concentrates of the eluate factor and folic acid counteract the growth inhibition which is produced by sulphonamides.[39] Biotin and folic acid also appear to influence the utilisation of pantothenic acid by the rat. On diets abundantly supplemented with pantothenate and containing succinyl sulphathiazole, rats developed the characteristic symptoms associated with deficiency of this vitamin.[40] This change was corrected by the administration of folic acid and crystalline biotin. Although agranulocytopenia and leucopenia, produced in rats by feeding sulphonamides, respond to crystalline folic acid from different sources,[41] the effect of concentrates upon growth and blood formation may not be due solely to

[33] *J. Biol. Chem.*, 1941, **141**, 521. [34] H. K. Mitchell, *Science*, 1943, **97**, 442.
[35] B. L. Hutchings, N. Bohonos, D. M. Hegsted, C. A. Elvehjem, and W. H. Peterson, *J. Biol. Chem.*, 1940, **140**, 647.
[36] S. Black, R. S. Overman, C. A. Elvehjem, and K. P. Link, *ibid.*, 1942, **145**, 137.
[37] F. S. Daft, S. S. Ashburn, and H. H. Sebrell, *Science*, 1942, **96**, 322.
[38] H. K. Mitchell and E. R. Isbell, *Univ. Texas Pub.* No. 4327, 1942, 125.
[39] E. Nielsen and C. A. Elvehjem, *J. Biol. Chem.*, 1942, **145**, 713.
[40] L. D. Wright and A. D. Welch, *Science*, 1943, **97**, 423.
[41] F. S. Daft and W. H. Sebrell, *Pub. Health Reps. U.S.A.*, 1943, **58**, 1542.

their folic acid content. In concentrates obtained from liver, Elvehjem and his co-workers [42] claimed to have identified a growth factor, vitamin $B_{11}$, and a factor necessary for good feathering in chicks, vitamin $B_{10}$, in addition to folic acid. In several respects the properties of vitamins $B_{10}$ and $B_{11}$ are akin to those of folic acid.

In 1940 A. G. Hogan and E. M. Parrott [43] observed that on simplified rations chicks developed a macrocytic hypochromic anæmia which was attributed to the lack of a dietary factor, vitamin $B_c$, present in aqueous extracts of liver. A greater incidence of anæmia in chicks is produced by feeding sulphaguanidine.[44] Vitamin $B_c$ is insoluble in organic solvents, more stable in alkali than in acids, adsorbable on fuller's earth and super-filtrol, and precipitable with metallic salts and phosphotungstic acid [44]—properties, in fact, similar to those of folic acid and the eluate factor. Its antianæmic action could not be reproduced by xanthopterin or by the anti-pernicious anæmia factor. Vitamin $B_c$ has now been obtained in the crystalline form both as the free acid and as the methyl ester.[45] Incorporated in a synthetic diet amply supplemented with all the known vitamins, the crystalline substance prevented retardation in growth (both body weight and feathering) and the development of anæmia and leucopenia.[46] Given parenterally, it produced the same effects.[46] This observation has been taken to indicate that vitamin $B_c$ produces those effects which have been claimed for folic acid and vitamins $B_{10}$ and $B_{11}$. Furthermore vitamin $B_c$ was highly active as a growth stimulant for *Lactobacillus casei E*. This led to the suggestion that vitamin $B_c$, the norit eluate factor and folic acid are the same substance.

The isolation of other crystalline substances has complicated rather than clarified the relationship among the microbial and the animal factors. Two crystalline compounds have been obtained; one from yeast and the other from liver.[47] Both are acids with similar absorption spectra and highly active towards *Lactobacillus casei*. There is a striking difference in their activities; towards *Lactobacillus casei* they are equally active, towards *Streptococcus lactus R* the yeast product is half as active as the liver one. Contrary to the behaviour of these crystalline acids, certain concentrates show activities greater towards *Streptococcus* than *Lactobacillus*. These facts can be harmonised by assuming the existence either of two or more substances or of different forms of one substance. In milk and in yeast folic acid may be present in a combined form, inactive to the two micro-organisms. Whole milk is more effective in inhibiting the harmful action of sulphon-

[42] G. M. Briggs, T. D. Luckey, C. A. Elvehjem, and E. B. Hart, *J. Biol. Chem.*, 1943, **148**, 163; 1944, **153**, 423.

[43] *Ibid.*, 1940, **132**, 507.

[44] B. L. O'Dell and A. G. Hogan, *ibid.*, 1943, **149**, 323.

[45] J. J. Pfiffner, S. B. Binkley, E. S. Bloom, R. A. Brown, O. D. Bird, A. D. Emmett, A. G. Hogan, and B. L. O'Dell, *Science*, 1940, **97**, 404.

[46] C. J. Campbell, R. A. Brown, and A. D. Emmett, *J. Biol. Chem.*, 1944, **152**, 483; **154**, 721.

[47] E. L. R. Stokstad, *ibid.*, 1943, **149**, 573.

amides upon rats than would be expected from its low folic acid content.[48] Yeast extracts have a high vitamin $B_c$ activity and low microbiological activity. When submitted to enzymatic hydrolysis, they stimulate the growth of the micro-organisms. From such extracts by the same method as that used in the isolation of the antianæmic factor, a crystalline compound has been obtained which contains the same percentage amount of carbon, hydrogen, and nitrogen as vitamin $B_c$.[49] The individualisation of combined forms of this vitamin and the bacterial growth factors will be an important step towards an explanation of the discrepancies which have been observed in the microbial activities of different materials. It may also elucidate the relation of folic acid and vitamin $B_c$ to vitamins $B_{10}$ and $B_{11}$.

It is too early to say how important the pterins may be in animal nutrition and lack of space prohibits their inclusion here. J. R. P. O'B.

## 5. The Assay of Vitamins B, with Special Reference to Microbiological Methods.

The necessity of establishing nutritional requirements and levels for vitamins of group B has stimulated investigations of assay methods, and great strides have been made in recent years. These methods are of three main types: biological, microbiological, and chemical; and each has its difficulties and objections. Ideally, the three methods should be so developed that each furnishes an accurate check on the others. The development of assay methods provides an interesting example of modern collaborative work; a number of teams in this country and the United States are engaged in this manner, and some examples are quoted later.

*Aneurin.*—The stimulatory effect of aneurin on fermentation by living yeast has been shown to be highly specific and formed the basis of one of the first microbiological methods for the assay of the vitamin as described by A. S. Schultz, L. Atkin, and C. N. Frey.[1] These authors [2] have found that by sulphite treatment the fermentation activity of aneurin is completely (99%) destroyed, whilst interfering substances are unaffected. The authors describe a differential method, employing a new fermentometer, in which the measurement of fermentation activity is determined before and after sulphite treatment.

H. H. Bunzell [3] employs the same principle, but a different type of apparatus. Results are obtained more rapidly and are accurate for amounts of the vitamin as small as 0·01 μg. A modified Warburg technique is described by E. S. Josephson and R. S. Harris.[4]

There is in general good agreement between results by the microbiological and the chemical methods, but sometimes differences are observed when biological assays are compared. J. C. Moyer and D. K. Tressler [5] reported

[48] A. D. Welch and L. D. Wright, *Science*, 1944, **100**, 153.

[49] S. B. Binkley, O. D. Bird, E. S. Bloom, R. A. Brown, D. G. Calkins, C. J. Campbell, A. D. Emmett, and J. J. Pfiffner, *ibid.*, p. 36.

[1] *J. Amer. Chem. Soc.*, 1937, **59**, 948, 2547. [2] *Ind. Eng. Chem. Anal.*, 1942, **14**, 35.

[3] *Ibid.*, p. 279. [4] *Ibid.*, p. 755. [5] *Ibid.*, p. 788.

assays on a number of frozen vegetables in which they used fermentation and thiochrome methods successfully. R. H. Hopkins and S. Wiener [6] in a study of aneurin in the materials and process of brewing found good agreement between results by the fermentation method and the thiochrome method as described by R. G. Booth.[7] Similar satisfactory comparisons were made in a collaborative study by the Accessory Food Factors Committee of the Medical Research Council [8] in which flours and bread were assayed by several biological methods, the fermentation method, the thiochrome method and an azo method elaborated by B. S. Platt and G. E. Glock.[9]

A number of workers have reported assays using other organisms, including *Phycomyces*,[10, 11] *Phycomyces blakesleeanus*,[12–14] and *Staphylococcus aureus*.[15] These methods, however, have been criticised by C. F. Niven and K. L. Smiley [16] on various grounds. The authors claim that *Streptococcus salivarius* (Strain S20B) is more suitable. The growth response is determined turbidimetrically, and owing to the extreme sensitivity of the organism no difficulty is experienced due to incidental turbidity of added food extracts. Co-carboxylase is some 40% more active than aneurin, a fact which has not yet found explanation, and which renders enzymatic hydrolysis necessary for precise determinations in some foods.

The stability of aneurin to heat has been studied by B. W. Beadle, D. A. Greenwood, and H. R. Kraybill.[17] Stability is a function not only of the hydrogen-ion concentration of the solution, but also of the particular electrolyte system employed. Results were obtained by chemical and spectrographic examination and indicated that for a heating period of one hour at pH 5·4, there was 100% destruction at the boiling temperature in the presence of borates, as compared with 57% in unbuffered solution, 10% in the presence of acetates, and 3% where phosphates were used. R. G. Booth [18] confirms many of these findings and has extended his observations to co-carboxylase, which he finds very much less stable than aneurin at the same pH. Destruction of aneurin is not primarily an oxidation effect, although, as copper can catalyse destruction, oxidation may be involved. An everyday application of work of this type concerns the losses of the vitamin which occur in cooking. Booth found that his estimate of loss agreed reasonably well with published figures.

*Riboflavin.*—Although both the microbiological and the fluorimetric methods for assay of riboflavin have yielded results in reasonable accord

[6] *J. Inst. Brew.*, 1944, **41**, 124.
[7] *J. Soc. Chem. Ind.*, 1940, **59**, 181.
[8] *Biochem. J.*, 1943, **37**, 433.
[9] *Ibid.*, p. 439.
[10] M. Malm and H. Lundeen, *Svensk Kem. Tid.*, 1941, **53**, 246.
[11] J. Lehmann and H. E. Nielsen, *Acta Med. Skand.*, Suppl., 1941, **123**, 374.
[12] W. H. Schopfer and A. Jung, *Compt. rend.*, 1937, **204**, 1500.
[13] J. Bonner and J. Erickson, *Amer. J. Bot.*, 1938, **25**, 685.
[14] J. Meiklejohn, *Biochem. J.*, 1943, **37**, 349.
[15] P. M. West and P. W. Wilson, *Science*, 1938, **88**, 334.
[16] *J. Biol. Chem.*, 1943, **150**, 1.
[17] *Ibid.*, 1943, **149**, 339, 349.
[18] *Biochem. J.*, 1943, **37**, 518.

with those obtained by biological methods, much evidence has accumulated that interfering substances may be present in natural products. It is necessary that each type of product should be treated in relation to its own peculiarities and the problems arising therefrom. In the fluorimetric method, originally developed by A. Z. Hodson and L. C. Norris,[19] later modified by V. A. Najjar,[20] pigments and non-flavin fluorescent substances must either be removed or allowed for.

The original microbiological assay method of E. E. Snell and F. M. Strong[21] used *Lactobacillus casei*-ε as test organism. Henceforth this organism will be denoted by its more convenient synonym, *Lactobacillus helveticus*.

In a study of assay methods for cereals, J. S. Andrews, H. M. Boyd, and D. E. Terry[22] found that the method of extraction is of great importance if satisfactory results are to be obtained. Extraction with taka-diastase was necessary in order to eliminate the effects of undesirable impurities. In this manner agreement was obtained between results by the microbiological method and the fluorimetric method in the case of patent and whole wheat flours, but there were discrepancies in the case of other cereal products. On the other hand, M. I. Wegner, A. R. Kemmerer, and G. S. Fraps[23] found taka-diastase (and also papain) treatment unsatisfactory in microbiological work on similar products, nor could the difficulty be obviated by adding photolysed extracts to the basal medium.

J. C. Bauernfeind, A. L. Sotier, and C. S. Boruff[24] found that the effect of additional growth substances in some foodstuffs was observable in assays using *L. helveticus*, especially when the amounts of riboflavin were below the optimum. The authors described methods for countering these effects, and suggested that the interfering substances were of the nature of fatty acids. This suggestion was followed up in an important paper by F. M. Strong and L. E. Carpenter,[25] who examined the effects of added fatty acids, to which the organism was sensitive, and showed that the difficulty did in fact arise from their presence. If they are removed by suitable preliminary treatment, reliable values for riboflavin may be obtained.

Satisfactory concordance in results by the microbiological method, which was modified by E. C. Barton-Wright and R. G. Booth,[26] and the fluorimetric method, as adapted by V. A. Najjar,[20] has been achieved by these authors in the assay of many cereals and cereal products. D. W. Kent-Jones and M. Meiklejohn[27] also have obtained satisfactory results by these methods.

R. H. Hopkins and S. Wiener[6] give figures for riboflavin in brewing materials by the microbiological method, but indicate that additional investigation of the fluorimetric method is necessary owing to disturbing factors in such materials as hops.

[19] *J. Biol. Chem.*, 1939, **131**, 621.
[20] *Ibid.*, 1941, **141**, 355.
[21] *Ind. Eng. Chem. Anal.*, 1939, **11**, 346.
[22] *Ibid.*, 1942, **14**, 271.
[23] *J. Biol. Chem.*, 1942, **144**, 731.
[24] *Ind. Eng. Chem. Anal.*, 1942, **14**, 666.
[25] *Ibid.*, p. 909.
[26] *Biochem. J.*, 1943, **37**, 25.
[27] *Analyst*, 1944, **69**, 330.

Finally, a collaborative study of the riboflavin content of meals served in R.A.F. messes may be mentioned. In this instance good agreement was obtained between the biological and the microbiological methods and it is concluded by T. F. Macrae, E. C. Barton-Wright, and A. M. Copping [28] that the adult riboflavin requirement does not exceed 2 mg. per day.

*Nicotinic Acid.*—An excellent review on nicotinic acid is contributed by C. A. Elvehjem and L. J. Tepley.[29]

There are a large number of chemical methods and their modifications for the estimation of nicotinic acid. All depend on the reaction with cyanogen bromide, followed by colour production with an amine.[30] Probably the most extensive study has been made by E. Kodicek,[31] who later modified the procedure in collaboration with Y. L. Wang.[32] The colour-producing base employed in both methods is *p*-aminoacetophenone; other bases proposed include orthoform (orthocaine),[33] *p*-phenylenediamine dihydrochloride,[34] and procaine.[35] The last gave good results with animal products such as meat extract and meat juice; but in general it may be said that the chemical methods are unreliable for plant products.

The method of E. E. Snell and L. D. Wright [36] was modified by W. A. Krehl, F. M. Strong, and C. A. Elvehjem,[37] who employed *Lactobacillus arabinosus* 17/5 and a synthetic medium.

In a study of methods of extraction V. H. Cheldelin and R. R. Williams [38] find that many materials yield their nicotinic acid completely under digestion with taka-diastase and papain, and that similar values in the case of meats and milk are obtained whether hydrolysis is enzymatic or by acid or alkali. On the other hand, acid or alkaline extracts of cereals give higher values than those prepared by enzyme action.

Comparison of results by microbiological and chemical methods of assay has shown that higher results by microbiological assays are obtained when plant products, particularly cereals, are treated in the preliminary stage with acid. R. D. Greene, A. Black, and F. O. Howland [39] employed a method similar to that of Snell and Wright [36] for microbiological assays, and a modified cyanogen bromide method due to W. S. Jones.[40] With some products, good agreement was found between the two types of method, although the authors prefer the microbiological method where small quantities of nicotinic acid are present. J. A. Andrews, H. M. Boyd, and W. A. Gortner [41] have studied the nicotinic acid content of cereals and cereal

[28] *Biochem. J.*, 1944, **38**, 132.
[29] *Chem. Reviews*, 1943, **33**, 185.
[30] W. König, *J. pr. Chem.*, 1904, **69**, 105.
[31] *Biochem. J.*, 1940, **34**, 724.
[32] *Ibid.*, 1943, **37**, 530.
[33] R. G. Martinek, E. R. Kirch, and G. L. Webster, *J. Biol. Chem.*, 1943, **149**, 245.
[34] A. E. Teeri and S. R. Shimer, *ibid.*, 1944, **153**, 307.
[35] E. C. Barton-Wright and R. G. Booth, *Lancet*, 1944, 565.
[36] *J. Biol. Chem.*, 1941, **139**, 675.
[37] *Ind. Eng. Chem. Anal.*, 1943, **15**, 471.
[38] *Ibid.*, 1942, **14**, 671.
[39] *Ibid.*, 1943, **15**, 77.
[40] *J. Amer. Pharm. Assoc.*, Sci. Ed., 1941, **30**, 272.
[41] *Ind. Eng. Chem. Anal.*, 1942, **14**, 663.

products, and also conclude that the microbiological assay is influenced by the type of hydrolysis procedure employed.

Nevertheless, the method of Krehl, Strong, and Elvehjem [37] is proving most valuable, and has recently reached a high level of accuracy as modified by E. C. Barton-Wright,[42] who has applied it to a wide range of materials, which are extracted under pressure with N-hydrochloric acid. Fats and fatty acids do not appear to have any effect on the organism. D. W. Kent-Jones and M. Meiklejohn [27] have applied the method with success.

*Pyridoxine.*—Colorimetric methods for assay of pyridoxine have been proposed by M. Swaninathan [43] and by J. V. Scudi.[44] Modifications of the latter method have been suggested by O. D. Bird, J. M. Vanderbelt, and A. D. Emmett,[45] and by A. F. Bina, J. M. Thomas, and E. B. Brown.[46] The most recent reference to such methods is probably that by A. C. Bottomley.[47]

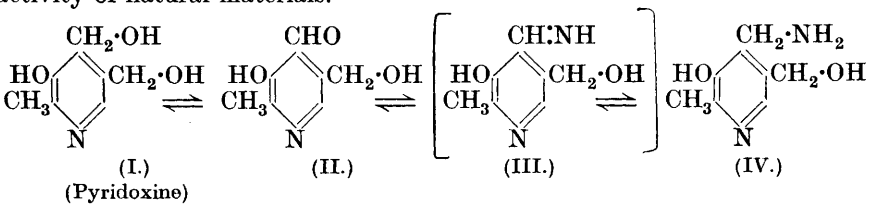
A yeast growth method originally presented by L. Atkin, A S. Schultz, and C. N. Frey [48] has been modified by these authors together with W. L. Williams.[49] The organism used is a yeast strain (No. 4228) which is characterised by a specific response to pyridoxine. Extracts of the materials for assay are prepared by acid treatment, and yeast growth is estimated turbidimetrically. Satisfactory assays on a large number of substances are reported. Bound pyridoxine is liberated also by acid treatment under pressure by L. Siegel, D. Melnick, and B. L. Osler.[50] Their results for a number of natural materials agreed well with those obtained by biological methods.

It was shown by E. E. Snell, B. M. Guirard, and R. J. Williams [51] that *Streptococcus lactis* R would grow on a medium if in addition to the usual constituents pyridoxine were present. Growth on such a medium, however, was many times as great as could be accounted for on the basis of actual content of pyridoxine. The indications were that pyridoxine is converted into a more highly active metabolite, called $\psi$-pyridoxine for the present, prior to utilisation by the organism, and that $\psi$-pyridoxine exists in natural products. The original presence or derivation of pyridoxine renders microbiological assays for pyridoxine invalid, and the case is complicated by the fact that the effect varies with different organisms; *e.g.*, very high values are obtained as indicated with *Streptococcus lactis* R, but low values are obtained with *Saccharomyces cerevisiæ* as test organism.

In a later communication, E. E. Snell [52] advances suggestions as to the nature of $\psi$-pyridoxine, and shows that mixtures having enhanced growth-promoting properties for *Lactobacilli* may be formed from pyridoxine by processes involving (*a*) possible amination and (*b*) partial oxidation. The latter change had also been noted by L. E. Carpenter and F. M. Strong.[53]

[42] *Biochem. J.*, 1944, **38**, 314.
[43] *Indian J. Med. Res.*, 1941, **29**, 561.
[44] *J. Biol. Chem.*, 1941, **139**, 707.
[45] *Ibid.*, 1942, **142**, 317.
[46] *Ibid.*, 1943, **148**, 111.
[47] *Biochem. J.*, 1945 (in the press).
[48] *J. Amer. Chem. Soc.*, 1939, **61**, 1931
[49] *Ind. Eng. Chem. Anal.*, 1943, **15**, 141.
[50] *J. Biol. Chem.*, 1943, **149**, 361.
[51] *Ibid.*, 1942, **143**, 519.
[52] *Ibid.*, 1944, **154**, 313.
[53] *Arch. Biochem.*, 1944, **3**, 375.

An amine (IV) and an aldehyde (II), " pyridoxamine " and " pyridoxal " respectively, have been synthesised,[54] and there is much evidence that these compounds or their higher combinations are responsible for the $\psi$-pyridoxine activity of natural materials.




The use of biochemical mutants in the mould *Neurospora* induced by means of ultra-violet and *X*-rays is an interesting development in microbiological methods of assay of vitamins of the B group. The production of these mutants has been described by G. W. Beadle and E. L. Tatum,[55] and they are characterised by an inability to carry out specific syntheses which can be effected by the normal unmutated strain.

An *X*-ray-induced mutant of *Neurospora sitophila*, produced by Beadle and Tatum is utilised as test organism by J. L. Stokes, A. Larsen, C. R. Woodward, and J. W. Foster [56] in a microbiological method for pyridoxine. Growth response is determined by actual dry weight of the mould, and the method is thus free from some objections which arise in turbidimetric assays. Under the conditions employed, the organism exhibits a specific response to pyridoxine, but none to $\psi$-pyridoxine. The results obtained are in good agreement with those obtained by biological assay.

*Biotin.*—The elucidation of the structure of biotin has been discussed in detail.[57] The importance of this needs no stressing, since, apart from scientific interest in the substance itself, it is a valuable tool in much modern microbiological work.

It may be some time before a chemical test for biotin of the required delicacy and specificity is forthcoming. In the meantime, the microbiological methods are being intensively studied, one of the more important problems centring on the question of free and bound biotin. Earlier methods of extraction included treatment merely with hot water,[58] but it was later found that much larger amounts of biotin were yielded by autolysis of tissues such as liver.[59] Later still,[60, 61] a combination of autolysis and acid hydrolysis was resorted to, and in 1941, after a series of tests of all types of treatment, R. C. Thompson, R. E. Eakin, and R. J. Williams [62] came to the conclusion that the best method for many types of material consists in drastic

[54] S. A. Harris, D. Heyl, K. Folkers, and E. E. Snell, *J. Biol. Chem.*, 1944, **154**, 315.
[55] *Proc. Nat. Acad. Sci.*, 1941, **27**, 499; 1942, **28**, 234.
[56] *J. Biol. Chem.*, 1943, **150**, 17.
[57] *Ann. Reports*, 1943, **40**, 172.
[58] F. Kögl and W. van Hasselt, *Z. physiol. Chem.*, 1936, **243**, 189.
[59] E. E. Snell, R. E. Eakin, and R. J. Williams, *J. Amer. Chem. Soc.*, 1940, **62**, 175.
[60] R. E. Eakin, W. A. McKinley, and R. J. Williams, *Science*, 1940, **92**, 224.
[61] *Univ. Texas Publication*, 1941, No. 4137.
[62] *Science*, 1941, **94**, 589.

acid treatment. Some destruction of the biotin occurs, but it is remarkably stable in acid solution. The problem is complicated by the fact that biotin appears to exist in different combinations which are broken down with varying degrees of ease, each type of product requiring individual treatment.

In earlier methods for the assay of biotin, the growth of yeast was usually measured turbidimetrically,[63, 64] a procedure which involved serious difficulty with solutions which were already cloudy or highly coloured. Similarly, P. M. West and P. W. Wilson [65] used *Rhizobium trifolii* as test organism. In order to overcome inherent difficulties in these methods, G. M. Shull, B. L. Hutchings, and W. H. Peterson [66] proposed the use of *Lactobacillus helveticus* as test organism, and measured the effect of added biotin by the increase in titratable acidity. An added advantage of this method lies in the fact that the same organism may be used for assay of pantothenic acid and riboflavin, thus obviating additional cultures. G. M. Shull and W. H. Peterson [67] later suggested two modifications in the assay. The eluate factor level in the yeast supplement in the basal medium is increased so that optimal growth of the organism is obtained. A procedure whereby the inoculum is independent of drop size is described.

The chemistry and biochemistry of biotin is reviewed by K. Hofmann.[68] A detailed account of methods and results of microbiological assay of all vitamins in the B group is provided by R. J. Williams and his collaborators.[69]

*Pantothenic Acid.*—No satisfactory chemical method of assay of pantothenic acid has as yet been devised. The earlier microbiological methods based on stimulation of yeast growth have largely given place to methods in which *L. helveticus* is used as test organism.[70–75]

The method of Pennington *et al.* employed autoclaving with or without previous autolysis under benzene in order to free pantothenic acid from test materials. Various enzymatic methods have been employed.[74, 76–78]

In more recent studies on the microbiological assay, A. L. Neal and F. M. Strong [79] have endeavoured to overcome some of the difficulties previously

[63] F. Kögl and B. Tönnis, *Z. physiol. Chem.*, 1936, **242**, 43.
[64] E. E. Snell, R. E. Eakin, and R. J. Williams, *J. Amer. Chem. Soc.*, 1940, **62**, 175.
[65] *Enzymologia*, 1940, **8**, 152. [66] *J. Biol. Chem.*, 1942, **142**, 913.
[67] *Ibid.*, 1943, **151**, 201.
[68] "Advances in Enzymology," 1943, **3**, 289. Interscience Publishers, New York.
[69] *Univ. Texas Publications*, 1941, No. 4137; 1942, No. 4237.
[70] E. E. Snell, F. M. Strong, and W. H. Peterson, *Biochem. J.*, 1937, **31**, 1789.
[71] *Idem, J. Amer. Chem. Soc.*, 1938, **60**, 2825.
[72] *Idem, J. Bact.*, 1939, **38**, 293.
[73] D. Pennington, E. E. Snell, and R. J. Williams, *J. Biol. Chem.*, 1940, **135**, 213.
[74] F. M. Strong, R. E. Feeney, and A. Earle, *Ind. Eng. Chem. Anal.*, 1941, **13**, 566.
[75] D. Pennington, E. E. Snell, H. K. Mitchell, J. R. McMahan, and R. J. Williams, *Univ. Texas Publication*, 1941, No. 4137, 14.
[76] H. A. Waisman, L. M. Henderson, J. M. McIntire, and C. A. Elvehjem, *J. Nutrition*, 1942, **23**, 239.
[77] A. H. Buskirk and R. A. Delor, *J. Biol. Chem.*, 1942, **145**, 707.
[78] E. Willerton and W. H. Cromwell, *Ind. Eng. Chem. Anal.*, 1942, **14**, 603.
[79] *Ibid.*, 1943, **15**, 654.

encountered by modifying the medium employed and improving the method of growing the inoculum. Enzymatic methods of liberating "bound" pantothenic acid were studied until satisfactory results were obtained and steps were taken to eliminate interfering fat-soluble substances.[24,25] The effect of water-soluble substances, present particularly in brans, was minimised by modifications in the basal medium. The authors claim that the modified method gives concordant results at increasing levels of dosage, and that very small amounts of the vitamin may be estimated with accuracy.

There appears to be an additional growth factor or factors for *L. helveticus* in the concentrate of rice polishings according to M. F. Clarke, M. Lechycka, and A. E. Light.[80] Notable increases in acid production were observed over and above those normally experienced with pure calcium pantothenate. The high values obtained by these workers may not, however, necessarily be due to a supplementary growth stimulator. J. L. Stokes and B. B. Martin [81] report that high acid production may be obtained merely by increasing the amounts of glucose and sodium acetate in the medium. With a view to increasing acid production and hence the titration range, A. E. Light and M. F. Clarke [82] propose a modification in the medium.

Other test organisms have been employed, among which *Streptococcus lactis*,[83] *Streptobacterium plantarum*,[84] *Proteus morganii*,[84a] and *L. arabinosus* [84b] may be mentioned.

A useful review of pantothenic acid is contributed by R. J. Williams.[85]

p-*Aminobenzoic Acid.*—Chemical methods are not greatly in evidence as yet, but are being developed. Colorimetric methods are described by E. R. Kirch and O. Bergeim [86] and by H. W. Eckert.[87]

*Acetobacter suboxydans* is recommended as test organism for *p*-aminobenzoic acid by M. Landy and D. M. Dicken,[88] who describe a suitable basal medium. Related or derived compounds of *p*-aminobenzoic acid have little or no biological activity, and the method has high specificity.

A mutant strain of *Neurospora crassa* of G. W. Beadle and E. L. Tatum [55] is used by R. C. Thompson, E. R. Isbell, and H. K. Mitchell.[89] Additions of graded amounts of *p*-aminobenzoic acid to a synthetic medium stimulate a specific growth response in the mould which is determined by measurement of the growth produced. The extraction of *p*-aminobenzoic acid by water and by acid hydrolysis is compared. The latter treatment involves a certain loss of the vitamin, but this loss is not significant in comparison with the enhanced yield of "bound" *p*-aminobenzoic acid. The same authors [90] have later shown that complete extraction is effected only by acid hydrolysis

[80] *J. Biol. Chem.*, 1942, **142**, 957. [81] *Ibid.*, 1943, **147**, 483. [82] *Ibid.*, p. 739.
[83] H. K. Mitchell, H. H. Weinstock, E. E. Snell, S. R. Stanbury, and R. J. Williams, *J. Amer. Chem. Soc.*, 1940, **62**, 1776.
[84] R. Kuhn and T. Wieland, *Ber.*, 1940, **73**, 962.
[84a] M. J. Pelczar and J. R. Porter, *J. Biol. Chem.*, 1941, **139**, 675.
[84b] H. R. Skeggs and L. D. Wright, *ibid.*, 1944, **156**, 21.
[85] "Advances in Enzymology," 1943, **3**, 253. Interscience Publishers, New York.
[86] *J. Biol. Chem.*, 1943, **148**, 445. [87] *Ibid.*, p. 197.
[88] *Ibid.*, 1942, **146**, 109. [89] *Ibid.*, 1943, **148**, 281. [90] *Ibid.*, 1943, **147**, 485.

under pressure. They suggest that the method of Landy and Dicken [88] responds to only a fraction of the total yielded by acid hydrolysis.

Quantitative response to *p*-aminobenzoic acid is evinced by *Clostridium acetobutylicum* Strain S9, which attains maximal growth in 24 hours on a suitable medium proposed by J. O. Lampen and W. H. Peterson.[91] These authors claim that the vitamin is rapidly destroyed by acid hydrolysis, and prefer to hydrolyse with alkali under pressure. This method of extraction is also favoured by J. C. Lewis,[92] who uses *L. arabinosus* as test organism.

Reference should not be omitted to the synthetic medium of M. Landy and D. M. Dicken [93] for use with *L. helveticus* and applicable to assay of each member of the group. Whilst this ideal has not perhaps been realised, the medium or modifications of it have proved useful to many workers.

The family of B vitamins is ever-increasing and it is too early to discuss assay methods for new members. It may be mentioned, however, that methods for " folic acid " are available.[69] F. W. N.

## 6. Actions of Chemotherapeutic Agents and Related Compounds.

Chemotherapy concerns interactions of drug, parasite and host, but the majority of investigations of chemotherapeutic agents during the period reviewed have been of their effects upon bacteria. The present account is mainly limited to such effects and is arranged according to their type. Factors involved in the comparison of *in vivo* and *in vitro* actions of drugs have been examined,[1] and their relations to other interactions in the complete chemotherapeutic system have been reviewed elsewhere.[2]

### I. *Biological Effects.*

(a) *Morphological.*—Abnormal size or shape in bacterial cells is induced by many agents;[3, 4] sometimes but not always [5] by sulphanilamide, and frequently by compounds without known chemotherapeutic action.[5] Their occurrence in response to changes in media has been ascribed to independent effects of the change upon chemical factors conditioning cell elongation and division.[5, 6]

(b) *Upon Growth.*—Sulphonamides increase the mean generation time during the logarithmic phase, and the length of the lag phase, of *Bact. lactis ærogenes*; [7] pantoyltaurine, in concentrations active *in vivo* against *Strepto-*

[91] *J. Biol. Chem.*, 1944, **153**, 193. [92] *Ibid.*, 1942, **146**, 441.
[93] *J. Lab. Clin. Med.*, 1942, **27**, 1086.
[1] Symposia, *Trans. Faraday Soc.*, 1943, **39**, 319; *Ann. N.Y. Acad. Sci.*, 1943, **44**, 445.
[2] H. McIlwain, *Biol. Rev.*, 1944, **19**, 135.
[3] *E.g.*, J. W. Foster and H. B. Woodruff, *Arch. Biochem.*, 1943, **3**, 241.
[4] G. H. Spray and R. M. Lodge, *Trans. Faraday Soc.*, 1943, **39**, 424.
[5] C. N. Hinshelwood and R. M. Lodge, *Proc. Roy. Soc.*, 1944, *B*, **132**, 47.
[6] R. M. Lodge and C. N. Hinshelwood, *Trans. Faraday Soc.*, 1943, **39**, 420.
[7] D. S. Davies and C. N. Hinshelwood, *ibid.*, p. 431.

*coccus hæmolyticus*, has effects upon that organism which are similar and which, like the *in vivo* activity, are annulled by pantothenate.[8] The effects of many other metabolite-analogues upon overall growth have been reported. Pyrithiamine, in which a pyridine ring replaces the thiazolé ring of aneurin, inhibits several bacteria [9, 10] and has greatest effects on those most exacting in their requirements for aneurin,[9] which antagonises its action. Inhibition by benziminazole is counteracted by some aminopurines.[11] Dethiobiotin,[12] biotin sulphone,[13] and an analogous imidazolidone derivative [14] competitively inhibit certain organisms but to others may be indifferent or in some cases act as source of biotin; or they may make available to bacteria, biotin which is inactivated by avidin. 6 : 7-Dichloro-9-ribityl*iso*alloxazine [15] and phenazine analogues of riboflavine [16] inhibit bacterial growth and this may be restored by riboflavine. New analogues of *p*-aminobenzoate [17] and pantothenate,[18] some of which are antibacterial, have been reported. Inhibitory substances designed in this way can act upon strains of organisms resistant to the agent used as model,[19] though cross-resistance can be developed to agents apparently different in type.[20] Orthanilamide does not inhibit an organism to which anthranilic acid is a growth-factor.[21]

Considering existing chemotherapeuticals, the inhibition of growth of *Escherichia coli* caused by atebrin [22] and of a lactobacillus and streptococcus caused by diamidines [23] are antagonised by spermidine and polyamines. The interaction of *p*-aminobenzoate and sulphonamides has been investigated under various conditions of aeration [24] and temperature.[25] The latter factor influences also the mutual interaction of *p*-aminobenzoate, sulphonamides and urea; [26] joint action of the last two can be additive, as urea is sometimes bacteriostatic.[27] Effects of sulphonamides on certain micro-

[8] H. McIlwain, *Biochem. J.*, 1944, **38**, 97.
[9] D. W. Woolley and A. G. C. White, *J. Exp. Med.*, 1943, **78**, 489.
[10] O. Wyss, *J. Bact.*, 1943, **46**, 483.
[11] D. W. Woolley, *J. Biol. Chem.*, 1944, **152**, 225.
[12] K. Dittmer, D. B. Melville, and V. du Vigneaud, *Science*, 1944, **99**, 203; V. G. Lilly and L. H. Leonian, *ibid.*, p. 205.
[13] K. Dittmer, V. du Vigneaud, P. Gyorgi, and C. S. Rose, *Arch. Biochem.*, 1944, **4**, 229.
[14] K. Dittmer and V. du Vigneaud, *Science*, 1944, **100**, 129.
[15] R. Kuhn, F. Weygand, and E. F. Möller, *Ber.*, 1943, **76**, 1044.
[16] D. W. Woolley, *J. Biol. Chem.*, 1944, **154**, 31.
[17] O. H. Johnson, D. E. Green, and R. Pauli, *ibid.*, 1944, **153**, 37.
[18] J. Barnett, *J.*, 1944, 5; J. Barnett, D. J. Dupré, B. J. Holloway, and F. A. Robinson, *ibid.*, p. 94.
[19] H. McIlwain, *Brit. J. Exp. Path.*, 1943, **24**, 203.
[20] J. McIntosh and F. R. Selbie, *ibid.*, p. 246.
[21] E. E. Snell, *Arch. Biochem.*, 1943, **2**, 389.
[22] M. Silverman and E. A. Evans, jun., *J. Biol. Chem.*, 1943, **150**, 265; 1944, **154**, 521.
[23] E. E. Snell, *ibid.*, 1943, **152**, 475.
[24] J. W. McLeod, A. Mayr-Harting, and N. Walker, *J. Path. Bact.*, 1944, **56**, 377.
[25] S. W. Lee and E. J. Foley, *Proc. Soc. Exp. Biol. Med.*, 1943, **53**, 243.
[26] S. W. Lee, J. A. Epstein, and E. J. Foley, *ibid.*, p. 245.
[27] W. M. M. Kirby, *ibid.*, p. 109.

organisms differ from the compounds' normal antibacterial effects in not being antagonised by *p*-aminobenzoate.[28] Lack of such antagonism is a valuable feature in the homosulphonamides, of which new members are active *in vivo*.[29]

(c) *Upon Viability.*—An outstanding finding of the period under review is of the unusual action of penicillin. At concentrations approximating to those attained during therapy, penicillin has little effect upon the viability of staphylococci,[30] hæmolytic streptococci [31] and meningococci [32] under conditions which do not permit growth of the organisms; *e.g.*, in salt solutions or in very dilute broth, in rich media in the cold or in rich media when growth is inhibited by sulphonamides [33] or by boric acid.[30] Under conditions otherwise permitting growth, an extremely small concentration of penicillin is bacteriostatic, 0·0009 unit/ml. (*c.* 0·0005 μg./ml.) having an effect comparable with that of 100 μg./ml. of sulphadiazine; concentrations comparable with those used therapeutically (*e.g.*, of 1/24 unit/ml.) are, however, bactericidal. Factors which normally increase the rate of growth of streptococci, in the presence of penicillin increase their rate of death. A proportion of organisms in staphylococcal cultures is not susceptible to being killed by penicillin; such " persistent " organisms are considered to be in a particular cultural phase. The proportions of organisms of a culture which are persistent can be altered by manipulation of the culture; [30] they increase on chilling. Recommendations in the clinical use of penicillin have been made on the basis of the new findings.[30] Varying susceptibility of bacteria at different phases of the culture-cycle has frequently been observed [34] and a further example has appeared recently in the greater sensitivity to acriflavine of *B. salmonicida* while it is in its logarithmic phase.[35]

Of agents already known to be bactericidal, the relations between concentration and action [36] and time of exposure and action [37] of phenol have been further studied. The significance of rates of death has been discussed.[38] Surface-active cations such as benzylalkylammonium chlorides are bactericidal, but their toxic action upon bacteria can be prevented, and when in progress halted, by anions of large molecular weight such as sodium dodecyl sulphate.[39] This shows two phases in the action of the cation: a pre-

[28] J. T. Tamura, *J. Bact.*, 1944, **47**, 529; F. Hawking, *Brit. J. Exp. Path.*, 1944, **25**, 63.

[29] D. M. Hamre, H. A. Walker, W. B. Dunham, H. B. van Dyke, and G. Rake, *Proc. Soc. Exp. Biol., N.Y.*, 1944, **55**, 170; D. G. Evans, A. T. Fuller, and J. Walker, *Lancet*, 1944, **247**, 523.

[30] J. W. Bigger, *ibid.*, p. 497.

[31] G. L. Hobby and M. H. Dawson, *Proc. Soc. Exp. Biol. N.Y.*, 1944, **56**, 178.

[32] C. P. Miller and A. Z. Foster, *ibid.*, p. 205.

[33] G. L. Hobby and M. H. Dawson, *ibid.*, p. 181.

[34] C.-E. A. Winslow and H. H. Walker, *Bact. Rev.*, 1938, **3**, 147.

[35] W. W. Smith. *Proc. Soc. Exp. Biol. N.Y.*, 1944, **56**, 238.

[36] D. P. Evans and A. G. Fishburn, *Quart. J. Pharm.*, 1943, **16**, 201.

[37] R. C. Jordan and S. E. Jacobs, *J. Hygiene*, 1944, **43**, 275.

[38] O. Rahn, *Biodynamica*, 1943, **4**, 81.

[39] E. I. Valko and A. S. DuBois, *J. Bact.*, 1944, **47**, 15.

liminary reversible one and later irreversible changes associated with death. The reversible one is considered to be the attachment of the agent to the cell and was shown to have some of the characters of ionic exchange; the action was reduced by the additional presence of less toxic cations. Such antagonism was effective against only limited concentrations of toxic cation. Similar phases in the action of other bactericides have been proposed;[36] here also the second phase was considered to be fundamentally different and to consist in denaturation or precipitation of the bacterial protein. The activities of antiseptics at different pH have been related to the concentrations of ionised and undissociated molecules; undissociated and not ionised benzoic, salicylic, and sulphurous acids were found antiseptic.[40] Œstrogens and related compounds are bactericidal,[41 42] but optimal antibacterial activity is not shown by members of greatest œstrogenic activity.[43] Propamidine is bactericidal as well as bacteriostatic to staphylococci [44] and to *Escherichia coli* [45] and both effects are antagonised by lecithin.[45]

## II. *Biochemical Effects.*

(a) *Upon Energy-yielding Processes.*—Evidence has been collected [46] suggesting a correlation of the inhibitions of bacterial respiration or anaerobic carbon dioxide production, with inhibition of growth, by sulphonamides. The respiratory inhibition is only partial (and by some investigators has been reported absent) at concentrations of sulphonamides which are completely bacteriostatic. To affect glycolysis or respiration of streptococci in the presence of glucose and a few other substrates, pantoyltaurine is required in much greater preponderance over pantothenate than is required for it to inhibit growth; [8] these metabolic inhibitions also are relatively small or may be absent. Oxidation of amino-acids by *Escherichia coli* is inhibited by low concentrations of propamidine and is more sensitive to the compound than is oxidation of glucose.[47] The inhibitions are markedly increased by adding the inhibitor before the substrate, and by increase in pH.[48] The activity of antimalarials in inhibiting oxygen uptake of malarial parasites is correlated with their therapeutic efficacy.[49]

(b) *Upon Metabolism of Vitamin-like Compounds.*—The system at which sulphonamides and *p*-aminobenzoate are believed to interact has not yet been specified biochemically, but further interpretations of actions of sulphonamides in terms of their competing with *p*-aminobenzoate for enzymes

[40] O. Rahn and J. E. Conn, *Ind. Eng. Chem.*, 1944, **36**, 185.
[41] G. H. Faulkner, *Lancet*, 1943, **245**, 38.
[42] B. Heinemann, *J. Lab. Clin. Med.*, 1944, **29**, 254.
[43] G. Brownlee, F. C. Copp, W. M. Duffin, and I. M. Tonkin, *Biochem. J.*, 1943, **37**, 572.
[44] W. R. Thrower and F. C. O. Valentine, *Lancet*, 1943, **244**, 133.
[45] W. O. Elson, *J. Biol. Chem.*, 1944, **154**, 717.
[46] R. J. Henry, *Bact. Rev.*, 1943, **7**, 175.
[47] F. Bernheim, *Science*, 1943, **98**, 223.
[48] F. Bernheim, *J. Pharm. Exp. Ther.*, 1944, **80**, 199.
[49] S. R. Christophers, *Trans. Faraday Soc.*, 1943, **39**, 333.

have been given.[50, 51] Increased synthesis of *p*-aminobenzoate has been found to be associated with development of sulphonamide-resistance in staphylococci.[52] By training certain strains of *Corynebacterium diphtheriæ* to synthesise pantothenate, strains resistant to pantoyltaurine were produced in the absence of that compound and of any other inhibitor; [53] but not all drug resistance is by synthesis of specific antagonists.[19] The system through which pantoyltaurine inhibits streptococcal growth has to some extent been characterised [54] and its functioning is associated with pantothenate-inactivation. In the preparations studied, the pantothenate metabolism required a concurrent energy-yielding process such as glycolysis. The pantothenate metabolism, but not glycolysis, was inhibited by concentrations of pantoyltaurine even lower than those affecting growth and the activities of a series of pantothenate analogues in inhibiting growth were correlated with their activities in inhibiting pantothenate-inactivation. A bacterial degradation of riboflavine is inhibited by structurally related compounds but occurs independently of a process such as glycolysis and its inhibition does not affect growth.[55]

### III. *Chemical or Physical Effects.*

Analyses of sulphonamide action, the effect of pH upon it, and its antagonism, base these processes upon reversible combination of the drug, antagonist, or their ions with enzymes in accordance with the law of mass action.[50, 51, 56] The bulk of the *p*-aminobenzoate of preformed organisms is not, however, displaced by bacteriostatic concentrations of sulphanilamide;[57] the equilibria may obtain during or before *p*-aminobenzoate assimilation. Similar lack of displacement of pantothenate by pantoyltaurine has been observed.[57] Correlation of the action of drugs with properties which they exhibit apart from biological systems has been reported [36, 50, 58] and reviewed.[59, 60]

### IV. *Chemotherapeutic Mechanisms.*

The year's findings have shown the multiplicity of types of antibacterial action exhibited by chemotherapeuticals. The connection of these with chemotherapeutic activity *in vivo* is established only in certain cases and in others would not be expected to be very close. Discussion of such connections (chemotherapeutic "mechanisms") is beyond the scope of the present account, but it will be seen that evidence for such connections is

[50] W. D. Kumler and T. C. Daniells, *J. Amer. Chem. Soc.*, 1943, **65**, 2190.
[51] I. M. Klotz, *ibid.*, 1944, **66**, 459.
[52] M. Landy, N. W. Larkum, E. J. Oswald, and F. Streightoff, *Science*, 1943, **97**, 295.
[53] H. McIlwain, *Brit. J. Exp. Path.*, 1943, **24**, 212.
[54] H. McIlwain and D. E. Hughes, *Biochem. J.*, 1944, **38**, 187.
[55] J. W. Foster, *J. Bact.*, 1944, **48**, 97.
[56] F. H. Johnson, H. Eyring, and W. Kearns, *Arch. Biochem.*, 1943, **3**, 1.
[57] H. McIlwain, *Proc. Biochem. Soc.*, 1944, **38**, viii.
[58] A. Albert and R. Goldacre, *J.*, 1943, 454.
[59] A. Albert, *Australian J. Sci.*, 1944, **6**, 137.
[60] W. S. Gledhill, *ibid.*, p. 170.

most fully provided in the cases of pantoyltaurine and the sulphonamides, by observations *in vivo* and of categories I (*b*), II (*a*), II (*b*), and III. Other compounds are of radically different mode of action and one compound may act in more than one way.[61] As the normal life of organisms involves a working together of processes which include all the above categories, many other means can be envisaged for their disturbance. H. McI.

F. DICKENS.
H. MCILWAIN.
A. NEUBERGER.
F. W. NORRIS.
J. R. P. O'BRIEN.
F. G. YOUNG.

[61] C. E. Hoffmann and O. Rahn, *J. Bact.*, 1944, **47**, 177.