BIOLOGICAL METHYLATION

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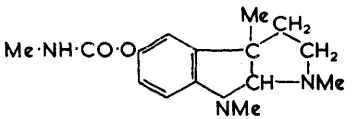
IN the strictest sense the term " biological methylation " implies either (1) the transfer under biological conditions of an intact methyl group from a compound (A) to a second compound (B), or (2) the fission under biological conditions of some compound (C), not necessarily containing a methyl group, so as to eliminate a molecule such as formaldehyde or formic acid, a " one-carbon fragment " ; this is thereupon captured by a compound (D) and afterwards the resulting group, *e.g.*, $-CH_2 \cdot OH$ or $H-\overset{|}{C}=O$, is reduced to $-CH_3$.

In (1), compound (A) is known as a methyl donor, and the process is a true transmethylation ; in (2), compound (C) is called a methyl source and the term transmethylation is, perhaps, better not employed to designate the process. This review presents a discussion of such reactions, as observed in animals, in higher and lower plants, and in micro-organisms.[1-5] Much work has been carried out in order to determine the mechanism of these processes and, although much remains to be learned about the intermediate stages, the use of isotopic indicators has thrown considerable light on the main lines along which biological methylation proceeds.

The CMe *group.* The angular methyl groups attached to a quaternary carbon atom in the sterols or in an alkaloid such as physostigmine (I), and the methyl of the :CH·Me group of corydaline (I*a*) are of particular interest. Robinson considers that formaldehyde or some closely related compound which can behave in an analogous manner (the formaldehyde equivalent) is concerned in their formation in alkaloids and that the " heteroenoid " system —N—C=C— is involved.[6-10] A valuable discussion of the



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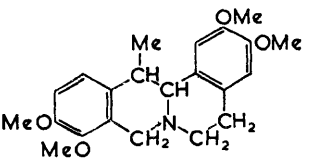


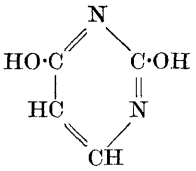
(I*a*)

[1] Challenger, *Chem. and Ind.*, 1942, **61**, 399, 413, 456. [2] *Chem. Rev.*, 1945, **36**, 315. [3] *Ann. Reports*, 1946, **43**, 262. [4] *Adv. Enzymology*, 1951, **12**, 429. [5] du Vigneaud, " A Trail of Research ", Cornell Univ. Press, Ithaca, New York, 1952.

[6] Robinson, *J.*, 1917, **111**, 877 ; 1918, **113**, 868 ; 1940, 509. [7] Robinson and Shah, *J.*, 1934, 1491. [8] Cornforth, Cornforth, and Robinson, *J.*, 1942, 682. [9] Robinson, *J. Roy. Soc. Arts*, 1948, **96**, 796. [10] Robinson, " The Structural Relations of Natural Products " (Weizmann Memorial Lectures, 1953), Oxford Univ. Press, 1955.

biosynthesis of cholesterol and the origins of its methyl groups has recently appeared.[10a]

The recent proof that the methyl group of thymine (II) is furnished by glycine $NH_2{\cdot}CH_2{\cdot}CO_2H$ or serine $HO{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$, through a "one-carbon fragment", probably formaldehyde or formate,[11] affords an excellent biological example of Robinson's generalisation, as $C_{(5)}$ of thymine and of its precursor uracil (*Ib*) forms the third member of a system —N—C=C— (see above). Uracil, thymine, cytosine (III), and 5-methylcytosine (IV) are four pyrimidines formed by hydrolysis of nucleic acids. Probably the formation of 5-methylcytosine in Nature also proceeds by methylation of the —N—C=C— system present in cytosine (see p. 286).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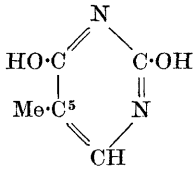
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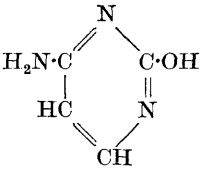
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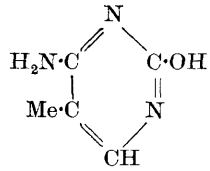
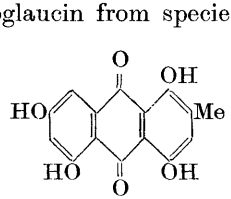

(IV)

The *C*-methyl groups of the anthraquinones isolated by Raistrick and his colleagues from the mycelia or culture media of various moulds should also be mentioned (see p. 286). A few examples of such mould metabolites are: catenarin (V) from *Helminthosporum catenarium*, and physcion (VI) and erythroglaucin from species in the *Aspergillus glaucus* series.[12]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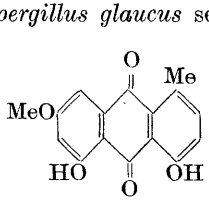

(VI)

The $CH_3{\cdot}N$ *group.* The frequent occurrence of this grouping in natural bases is most simply explained by again invoking Robinson's formaldehyde equivalent, probably arising from glycine or serine (see p. 255), thus: $—NH_2 + CH_2O \rightarrow —NH{\cdot}CH_2{\cdot}OH \rightarrow —NHMe$.

A few typical examples of compounds containing the $CH_3{\cdot}N$ group may be cited: tetramethylammonium hydroxide and methylpyridinium hydroxide occur in the sea-anemone *Actinia equina*,[13] betaine $^+NMe_3{\cdot}CH_2{\cdot}CO_2^-$ in sugar beet and many plants and in crustacea and the muscle tissues of animals. Choline $HO{\cdot}CH_2{\cdot}CH_2{\cdot}NMe_3^+\}OH^-$ is found in

[10a] Popják, "Chemistry, Biochemistry and Isotopic Tracer Technique", Lectures, Monographs, and Reports of the Royal Institute of Chemistry, 1955, No. 2, p. 23.

[11] Elwyn and Sprinson, *J. Amer. Chem. Soc.*, 1950, **72**, 3317; *J. Biol. Chem.*, 1950, **184**, 465.

[12] Raistrick, *Ann. Rev. Biochem.*, 1940, **9**, 571; *Ann. Reports*, 1939, **36**, 386; 1941, **38**, 261; Birkinshaw and Chaplen, *Biochem. J.*, 1955, **60**, 255.

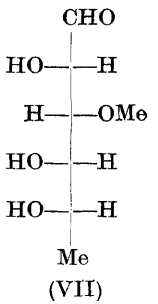
[13] Ackermann, Holtz, and Reinwein, *Z. Biol.*, 1923, **79**, 113; 1924, **81**, 61.

almost all living cells. Hordenine (p-HO·C_6H_4·CH_2·CH_2·NMe_2) occurs in barley seedlings, and adrenaline in the suprarenal gland. Other alkaloids containing CH_3·N groups are cocaine, nicotine (see p. 276), and pilocarpine. Betaine is completely methylated glycine (see p. 260), and a large number of other amino-acids have their counterpart in natural betaines, *e.g.*, hypaphorine from tryptophan, ergothioneine from mercaptohistidine, and γ-butyrobetaine $^+NMe_3$·CH_2·CH_2·CH_2·CO_2^- in *Actinia equina*.[13]

The MeO *and the* CH_2O_2 *groups.* These are obviously to be regarded as being formed directly or indirectly by interaction of a " formaldehyde equivalent " with one or two hydroxyl groups. The occurrence of these linkings in natural products is well known, *e.g.*, in vanillin, anethole, safrole, and piperine. Cotarnine, an oxidation product of the opium alkaloid narcotine, contains a methoxyl, a methylenedioxy, and a *N*-methyl group. Cryptopine, another opium alkaloid, contains one NMe, two OMe, and one CH_2O_2 group. In the closely related alkaloid protopine (see p. 277), a second methylenedioxy-group replaces the two methoxy-groups and engages the same two carbon atoms, suggesting that a similar mechanism is responsible for the formation of both these oxygenated linkages.

Methylated carbohydrates are extremely rare in Nature. One example is the partially methylated L-thevetose (VII) which is formed along with its aglucone, digitoxigenin, on hydrolysis of the glycosides nerifolin and thevetin occurring in *Nerium oleander* and *Thevetia nerifolia*. Digitalose is a stereoisomer of thevetose and is obtained by hydrolysis of the digitalis glycosides.

The cardiac glycosides,[14] cymarin, diginin, oleandrin, and sarmentocymarin, furnish on hydrolysis an aglucone (of the sterol type, as is also digitoxigenin) and the stereoisomeric 3-methyl-2 : 6-dideoxyhexoses, D-cymarose, D-diginose (VIII), L-oleandrose, and D-sarmentose respectively.



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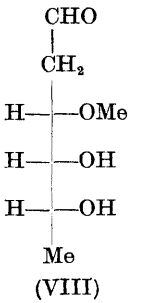

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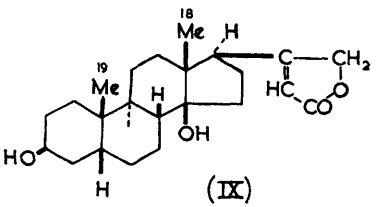


(IX)

The constant presence of the methoxy-group on $C_{(3)}$ of these compounds is most striking. The 2-deoxy-group is not essential for the presence of a 3-methoxy-group, nor is any particular stereochemical configuration. It may be noted that, in every case but one, the aglycone contains the two angular methyl groups (see p. 255) characteristic of the sterols. Thus digitoxigenin which is esterified with thevetose in the glycoside nerifolin

[14] Shoppee and Shoppee, " The Chemistry of Carbon Compounds ", Ed. E. H. Rodd, Elsevier Pub. Co., 1953, Vol. IIB, p. 983.

is (IX). In the case of cymarose its aglucone (cymarigenin or strophanthidin) contains only the 18-methyl group, the 19-group being oxidised to —CHO.

Another 3-methylhexose, 3-*O*-methyl-D-galactose, has been found among the products of hydrolysis of an acidic polysaccharide in the mucilage of *Ulmus fulva* (slippery elm).[15] On the other hand the hemicelluloses of aspen wood and beech wood, and the oligosaccharides of mesquite gum, myrrh gum, and lemon gum, contain 4-*O*-methyl-D-glucuronic acid units. A few key references may be cited.[16]

Methyl esters $R{\cdot}CO_2Me$. The pectins are methyl esters of polygalacturonic acids; cocaine is the methyl ester of benzoylecgonine; methyl salicylate occurs in oil of wintergreen, and methyl anthranilate in oil of jasmin. The *N*-methyl derivative of methyl anthranilate is found in oil of mandarin, and its 3-methoxy-*N*-methyl compound damascenin in certain of the *Ranunculaceæ*.

Here again it is difficult to avoid the impression that a similar mechanism is involved in the formation of these different methyl linkages.

The MeS *group*. The biological importance of this group is becoming more and more apparent. Methionine $MeS{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$, its methylsulphonium salt,[17] cheirolin[18] $Me{\cdot}SO_2{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}NCS$, sulphoraphene[19] $Me{\cdot}SO{\cdot}CH{:}CH{\cdot}CH_2{\cdot}CH_2{\cdot}NCS$, and the corresponding nitrile,[19] dimethyl-β-propiothetin[20] $^+SMe_2{\cdot}CH_2{\cdot}CH_2{\cdot}CO_2^-$, the dimethyl sulphide[21] to which it gives rise, and dimethyl sulphone[22] are a few examples.

In many cases a reaction analogous to that mentioned in connection with the $CH_3{\cdot}N$ group may be involved—the interaction of formaldehyde and a thiol group,[23] followed by reduction: $RSH + CH_2O \rightarrow RS{\cdot}CH_2{\cdot}OH \rightarrow R{\cdot}S{\cdot}CH_3$.

Formic acid may possibly also react with a thiol, to give $RS{\cdot}CHO$, analogous to the *S*-acetyl derivative[24] formed by co-enzyme A. Reduction of this might produce $R{\cdot}S{\cdot}CH_3$.

The djenkolic acid[25] of the djenkol bean of Java contains the CH_2S_2 group, analogous to the methylenedioxy-group. It has the structure $CH_2[S{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H]_2$, was isolated both from the bean and from the urine of natives who consumed it, and was synthesised[26] from cysteine

[15] Hirst, Hough, and Jones, *J.*, 1951, 323.

[16] Hough, Jones, and Wadman, *J.*, 1952, 798; Jones and Wise, *J.*, 1952, 2750, 3389; Aspinall, Hirst, and Mahomed, *J.*, 1954, 1734; Gorrod and Jones, *J.*, 1954, 2522.

[17] McRorie, Sutherland, Lewis, Burton, Glazener, and Shive, *J. Amer. Chem. Soc.*, 1954, **76**, 115; Challenger and (Miss) Hayward, *Chem. and Ind.*, 1954, **73**, 729.

[18] Armstrong and Armstrong, "The Glycosides", Longmans, London, 1931, p. 66.

[19] Schmid and Karrer, *Helv. Chim. Acta*, 1948, **31**, 1017, 1087, 1497.

[20] Challenger and (Miss) Simpson, *J.*, 1948, 1591.

[21] Haas, *Biochem. J.*, 1935, **29**, 298.

[22] Karrer and Eugster, *Helv. Chim. Acta*, 1949, **32**, 957, 2397.

[23] Levi, *Gazzetta*, 1932, **62**, II, 775.

[24] Lynen, Harvey Lectures, Williams and Wilkins, Baltimore, 1952—53, Vol. 38, p. 211.

[25] Van Veen and Hyman, *Rec. Trav. chim.*, 1935, **54**, 493.

[26] Armstrong and du Vigneaud, *J. Biol. Chem.*, 1947, **168**, 373.

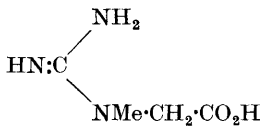
and methylene chloride in liquid ammonia. Its structure suggests that it may be formed in the plant from cysteine and formaldehyde or its " equivalent ".

The development of the conception of biological methylation (cf. refs. 1—4)

His in 1887 showed that pyridine acetate given to dogs is excreted as methylpyridinium acetate. The same reaction occurs in turtles and an analogous methylation of quinoline in dogs. As early as 1824, however, Gmelin mentioned the exhalation of a strong garlic odour on administration of potassium tellurite to animals. In 1853 Hansen described the same effect in man and stated that the odour resembled that of diethyl telluride $TeEt_2$. In 1876 Brownen described the phenomenon of " bismuth breath ", formerly well known to patients undergoing treatment with bismuth carbonate, and showed that it was due to traces of tellurium compounds. So far, however, the intervention of a methyl group in these phenomena had not been discussed.

In 1894 Hofmeister, aware of the work of His, regarded the " tellurium gas " as dimethyl telluride, though without proof. In that paper we note the first, rather vague, conception of the possibility of methyl transfer. Hofmeister considered that " the methyl group is already present in the tissues which possess the capacity for methylation. In presence of pyridine and tellurium these are methylated, whereas under normal conditions methyl derivatives such as choline and creatine (X) are produced." Hofmeister did not mention any particular compound as the source of the methyl group.

Jaffé [27] suggested in 1906 that the increased urinary excretion of creatinine (XI) by rabbits receiving guanidinoacetic acid (glycocyamine) might be due to methylation by the organism.



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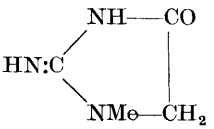

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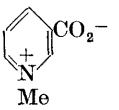


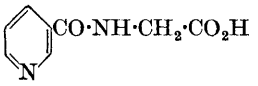
(XII)

A distinct advance was made in 1913 by Riesser [28] who concluded that the methyl groups of the (assumed) dimethyl telluride synthesised by the animal body from potassium tellurite, and the methyl group of creatine (X) were probably furnished by choline or betaine. He also found that injection of choline or betaine into rabbits led to an increased creatine content of the muscle. The Reviewer extended Riesser's experiments.[29]

Further progress was recorded in 1912 when Ackermann [30] showed that in dogs nicotinic acid is converted into trigonelline (XII) and into nicotinuric acid (XIII). Later work has shown that a similar process occurs in other

[27] Jaffé, *Z. physiol. Chem.*, 1906, **48**, 430.
[28] Riesser, *ibid.*, 1913, **86**, 440.
[29] Challenger, Taylor, and Taylor, *J.*, 1942, 48.
[30] Ackermann, *Z. Biol.*, 1912, **59**, 17.

animals and that in most cases, at any rate, N^1-methylnicotinamide (XIV) and not trigonelline is produced.[4]



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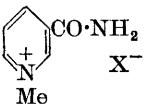


(XIV)

Glycine is obviously concerned in the formation of the nicotinuric acid. Similar " detoxications " are summarised by R. T. Williams.[31] Challenger [32] suggested in 1935 that glycine might also be responsible for the methylation of the heterocyclic nitrogen atom of nicotinic acid, possibly after oxidative deamination to formaldehyde and, in another connection, that glycine might in a similar manner methylate itself to betaine (see p. 271). Fifteen years later the work on one-carbon fragments (see p. 271) indicated that this suggestion may be somewhere near the truth.

In 1917 Thomson,[33] when discussing the increased creatinine formation in animals receiving arginine and methyl citrate, associated this with a methyl transfer from the citrate, or possibly from acetone arising from the citric acid residue. The mechanism of creatine formation has been discussed by Bach [34] in two monographs.

Methylation by moulds

Arsenic (refs. 1—4).—About 1815 several cases of arsenical poisoning occurred in Germany owing to the use of domestic wall-papers, the pigments on which contained copper hydrogen arsenite. Gmelin in 1839 noticed a garlic odour in " arsenical " rooms. He ascribed the poisoning to a volatile arsenic compound liberated from the damp and mouldy wall-paper. Selmi suggested in 1874 that the moulds might play a definite part in the volatilisation of the arsenic and that they produced hydrogen from the paper and paste which then gave rise to arsine, AsH_3.

The work of Gosio and Biginelli. In 1891 Gosio exposed a potato-mash containing arsenious oxide to air; it became infected with moulds and bacteria and evolved a garlic odour. Some organisms were isolated in pure culture and their effect on various media containing arsenious oxide or arsenical pigments studied. The bacteria produced no volatile arsenic compounds, but some moulds were intensely active, especially *Penicillium brevicaule* (*Scopulariopsis brevicaulis*).

Biginelli aspirated " Gosio gas " evolved from the mould cultures through mercuric chloride in dilute hydrochloric acid. The resulting precipitate was assigned the composition $Et_2AsH,2HgCl_2$. He therefore stated that the gas was diethylarsine.

Meanwhile Cevey had observed that a garlic odour is also evolved when

[31] Williams, " Detoxication Mechanisms ", Chapman & Hall, London, 1947.
[32] Challenger, *Chem. and Ind.*, 1936, 900.
[33] Thompson, *Biochem. J.*, 1917, **11**, 307; *J. Physiol.*, 1917, **51**, 347.
[34] Bach, *Biol. Rev.*, 1945, **20**, 158; " The Metabolism of Protein Constituents in the Mammalian Body ", Oxford Univ. Press, 1952.

the inorganic arsenic of the cultures is replaced by sodium cacodylate, $Me_2AsO{\cdot}ONa$.

The work of the Leeds School. Further work was commenced by Challenger *et al.* in 1931. Sterile bread crumbs were inoculated with *S. brevicaulis* and incubated. Sterilised aqueous solutions of various arsenic compounds were added and the cultures arranged in series. Sterile air was then passed through them, volatile arsenic compounds being absorbed in Biginelli's solution. When arsenious oxide (0·2—0·25% in the bread) was used two different deposits were obtained according to the concentration of the mercuric chloride, consisting of the dimercurichloride and the monomercurichloride of trimethylarsine $Me_3As,2HgCl_2$ and $Me_3As,HgCl_2$. Gosio gas is therefore trimethylarsine, Me_3As. This conclusion was confirmed by absorption in nitric acid and in benzyl chloride, hydroxytrimethylarsonium nitrate and benzyltrimethylarsonium chloride (characterised as picrates) being respectively obtained.[35] With sodium methylarsonate $Me{\cdot}AsO_3Na_2$ (1—1·5% in the bread) or sodium cacodylate (0·1—0·3%) (free from inorganic arsenic), the evolved gas gave the same mercurichloride.

It seemed possible that, with sodium methylarsonate and cacodylate, the mould might have broken the As–C link, giving inorganic arsenic. With sodium ethylarsonate in bread cultures of the mould, ethyldimethylarsine $AsMe_2Et$ was evolved and identified as the mercurichloride, thus eliminating this possibility. Absorption in benzyl chloride yielded benzylethyldimethylarsonium chloride, and in nitric acid ethylhydroxydimethylarsonium nitrate (characterised as picrates). Addition of other alkylarsonic acids to the mould in concentrations varying from 0·2% to 0·5% gave mixed methylated arsines. The relations, $R{\cdot}AsO_3H_2 \longrightarrow As{\cdot}RMe_2$ and $RR'AsO_2H \longrightarrow As{\cdot}RR'Me$, summarise these results.

Inorganic Compounds of Selenium and Tellurium (cf. refs. 1—4).— Rosenheim showed that unpleasant odours were evolved when *S. brevicaulis* was grown upon sterile bread containing inorganic compounds of selenium and tellurium. The substances responsible were not identified.

The gas evolved from the selenium cultures was identified by Challenger and North. The volatile products from cultures of *S. brevicaulis* on bread containing sodium selenate or selenite were characterised as dimethyl selenide mercurichloride and mercuribromide $SeMe_2,HgX_2$, hydroxydimethylselenonium nitrate $HO{\cdot}Me_2Se^+\}NO_3^-$, bis(dimethyl selenide)dichloroplatinum, $PtCl_2,2Me_2Se$, and benzyldimethylselenonium chloride (isolated as the derived picrate). *Aspergillus niger* also converts sodium selenate into dimethyl selenide (see p. 272).

(Miss) Bird and Challenger aspirated the gases evolved from cultures of *S. brevicaulis* on bread containing potassium tellurite through various reagents. Dimethyl telluride mercurichloride was obtained and converted into the dibromide. Absorption in alcoholic iodine gave the di-iodide. The mould gas is therefore dimethyl telluride.

No proof exists that the odour exhaled by men and animals in receipt of tellurite is actually dimethyl telluride; however, the well-established

[35] Challenger, (Miss) Higginbottom, and Ellis, *J.*, 1933, 95.

instances of biological methylation by animals being borne in mind, no reasonable doubt can remain that both animals and moulds methylate tellurium and selenium.

Sulphur.—*Fission of the disulphide link by* S. brevicaulis *and methylation of the* C_nH_{2n+1}·S *group.* Unsuccessful attempts were made to produce dimethyl sulphide by addition of sulphur or some of its compounds to bread cultures of two different strains of *S. brevicaulis.* This was surprising because Pohl [36] noticed a leek-like odour in the breath of animals receiving injections of thiourea. The odorous product was not an alkanethiol. It was absorbed by sulphuric acid and gave a precipitate with mercuric chloride. Pohl therefore concluded that it was an alkyl sulphide.

It seemed possible that compounds containing thiol or disulphide linkages might be more susceptible to the methylating action of the mould. Dialkyl disulphides (methyl to *n*-pentyl) were added in dilute aqueous suspension to bread cultures of *S. brevicaulis* and volatile products aspirated first through mercuric cyanide and then through mercuric chloride.[37] The products consisted of the alkanethiol RSH [absorbed in mercuric cyanide, giving $(RS)_2Hg$], the unchanged disulphide RS·SR, and the alkyl methyl sulphide RSMe. The precipitates obtained with mercuric chloride were mixtures of the mercuric chloride addition product of the alkyl methyl sulphide with varying amounts of RS·HgCl,$HgCl_2$ arising from fission of RS·SR. On treatment of these mixtures with sodium hydroxide pure alkyl methyl sulphide was evolved and was then converted into the mercurichloride, the alkylbenzylmethylsulphonium picrate, or the double compound with platinous chloride. The fission and methylation of the disulphide link by *S. brevicaulis* appears therefore to be a general reaction of the simple aliphatic disulphides. It is not clear whether the fission is reductive or hydrolytic. Birkinshaw, Findlay, and Webb [38] showed that the wood-destroying fungus *Schizophyllum commune* Fr. converts inorganic sulphate into methanethiol, MeSH. Dimethyl disulphide and traces of hydrogen sulphide and dimethyl sulphide are also formed. This is the only established instance of the biological methylation of inorganic sulphur.

Fission of the monosulphide link by moulds and in the animal body

The behaviour of DL-methionine in bread cultures of *S. brevicaulis* was examined by Challenger and Charlton ; [39] both methanethiol and dimethyl sulphide were evolved and detected as bismethylthiomercury $(MeS)_2Hg$ and the mercurichloride of dimethyl sulphide, $2Me_2S,3HgCl_2$, respectively. Under identical conditions *S*-methyl-, *S*-ethyl-, and *S*-*n*-propyl-L-cysteine, $RS·CH_2·CH(NH_2)·CO_2H$, gave the corresponding alkanethiol RSH and alkyl methyl sulphide RSMe.

These fissions are closely related to that of cystathionine

[36] Pohl, *Arch. exp. Pathol. Pharmacol.*, 1904, **51**, 341.

[37] Challenger and Rawlings, *J.*, 1937, 868 ; Blackburn and Challenger, *J.*, 1938, 1872.

[38] Birkinshaw, Findlay, and Webb, *Biochem. J.*, 1942, **36**, 526.

[39] Challenger and Charlton, *J.*, 1947, 424.

$CO_2H{\cdot}CH(NH_2){\cdot}CH_2{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$ in the presence of rat liver or its saline extracts or kidney slices, giving cysteine.

Binkley [40] has obtained results with the enzyme thionase which bear closely on this work. Preparations of purified thionase, activated with glutathione, convert *S*-alkylcysteines (alkyl = methyl, ethyl, propyl, and butyl) into the corresponding thiols. Challenger and Walshe [41] have recently investigated a case of severe necrosis of the liver, in which a strong odour was present in the breath (foetor hepaticus) and in the urine. Aspiration of nitrogen through the urine into mercuric cyanide gave bismethylthiomercury $Hg(SMe)_2$, identified by m.p. and mixed m.p. and by the evolution of dimethyl sulphide at its m.p. Chromatographic examination of the plasma showed a marked increase in methionine content, suggesting that the methanethiol (which gives rise to the mercury derivative) arises from methionine by a fission analogous to those observed by Challenger and Charlton and by Binkley.

Normally, by transmethylation and remethylation, often occurring in the liver, the methionine level in the body is kept constant. In severe liver damage transmethylation may be prevented ; consequently methionine may accumulate, as in this and other cases of severe liver disease. In such circumstances the organism resorts to C–S fission in order to reduce the concentration of methionine, thus producing methanethiol. Canellakis and Tarver [41a] discuss their somewhat analogous observation that when D- or L-[*Me*-^{14}C]- or L-[^{35}S]-methionine or the corresponding D-[^{14}C]keto-acid is incubated with rat-liver mitochondria the —SMe group is taken up by the protein and liberated therefrom as methanethiol by 2-mercaptoethanol $HS{\cdot}CH_2{\cdot}CH_2{\cdot}OH$. Further work is needed before a clear picture of these results can be obtained. The authors have also shown that methanethiol is readily oxidised to sulphate and carbon dioxide in rats and can furnish the methyl groups of methionine, choline, and creatine and the β-carbon atom of serine (cf. pp. 269, 271).

Natural sulphonium compounds related to dialkyl sulphides

A Thetin Derivative in Marine Algæ.—In 1935 Haas [21] showed that the red alga *P. fastigiata* evolves a strong odour in air. When air is passed over the weed and then through (*a*) bromine in carbon tetrachloride, (*b*) mercuric chloride, and (*c*) potassium chloroplatinite, the resulting derivatives were shown to be identical with those of dimethyl sulphide. *P. nigrescens* also evolved this sulphide. Miss Margaret Simpson and the Reviewer [20] showed that, when the volatile products from the alga, removed from its host *Ascophyllum nodosum*, were aspirated through (*a*) mercuric cyanide and (*b*) mercuric chloride, no precipitate was formed in (*a*), indicating the absence of methanethiol. The deposit in (*b*) consisted solely of the mercurichloride of dimethyl sulphide. The sulphur compound was, therefore,

[40] Binkley, *J. Biol. Chem.*, 1950, **186**, 287.
[41] Challenger and Walshe, *Biochem. J.*, 1955, **59**, 372.
[41a] Canellakis and Tarver, *Arch. Biochem. Biophys.*, 1953, **42**, 387, 446.

homogeneous. The evolution of the sulphide by the weed was shown to be an enzymic process.

The alga was extracted with ethanol and the extract, when concentrated, solidified. After successive conversion into the reineckate, sulphate, chloride, and chloroplatinate, and again into the chloride, a picrate was obtained; this gave a pure chloride, $C_5H_{11}O_2ClS$ which was optically inactive and evolved dimethyl sulphide on treatment with cold aqueous sodium hydroxide. It appeared probable that the chloride was either an α- or a β-thetin derivative (XV) or (XVI). The authentic bromides were

$Cl^-\{^+SMe_2\cdot CHMe\cdot CO_2H$ (XV) $Cl^-\{^+SMe_2\cdot CH_2\cdot CH_2\cdot CO_2H$ (XVI)

converted into the chlorides, picrates, styphnates, and chloroplatinates, comparison [20] of which with corresponding derivatives from the alga left no doubt that the algal product is the chloride of the β-propiothetin derivative, 2-carboxyethyldimethylsulphonium chloride (XVI). This sulphonium compound was then isolated in the Reviewer's laboratory from two green algæ, *Enteromorpha intestinalis* and *Spongomorpha arcta*, as the picrate and chloroplatinate respectively. Several other marine (and two freshwater) algæ evolved dimethyl sulphide with alkali and presumably contain the same or a related sulphonium salt.

The Sulphur Compounds of Asparagus and Horsetail.—Nencki stated in 1891 that the strong odour which is readily detectable in the urine after ingestion of asparagus is due to methanethiol, of which he analysed the lead salt. The first serious investigation of the sulphur compounds of asparagus was made by Jensen [42] who isolated $\beta\beta'$-dimercapto*iso*butyric acid $HS\cdot CH_2\cdot CH(CO_2H)\cdot CH_2\cdot SH$ as the disulphide. This acid on ingestion did not give rise to excretion of methanethiol.

Dr. Margaret Whitaker (Simpson) [43] found that a concentrated acetone extract of asparagus evolved dimethyl sulphide when boiled with alkali. [The seaweed thetin (see above) evolves the sulphide in the cold.] By the use of two ion-exchange resins which retained (*a*) the basic diamino-acids and a sulphonium compound and (*b*) the monoamino-acids, an eluate was obtained which gave a chloroplatinate and thence a picrate. Challenger and (Miss) Hayward [17] identified the sulphonium compound which is the source of the dimethyl sulphide as a methylmethioninesulphonium salt $X^-\{^+SMe_2\cdot CH_2\cdot CH_2\cdot CH(NH_2)\cdot CO_2H$. Paper chromatography of the chloride obtained from the picrate, and of the methionine and homoserine from the alkaline decomposition of this chloride, confirmed the identity.

About 6 months previously this sulphonium compound had been isolated as the bromide from cabbage and detected in other vegetables by Shive and his co-workers.[17] *Equisetum telmateia* (the giant horsetail) evolves small quantities of dimethyl sulphide, characterised as the mercurichloride, on long boiling with alkali.[43] Larger quantities were obtained from the

[42] Jensen, *J. Biol. Chem.*, 1948, **176**, 657.

[43] Challenger, Leaver, and Whitaker, *Biochem. J.*, 1953, **56**, ii; Leaver, Thesis, Leeds, 1953.

spore cones of another species of *Equisetum* and also from the fresh green fronds and leaves of another cryptogam, the common bracken (*Pteris equilina*). It seems, therefore, that the precursor of the dimethyl sulphide obtained from these plants by alkali treatment is also a sulphonium compound. The work of Challenger and Simpson on *P. fastigiata* in 1947 furnished the first example of the occurrence of a sulphonium compound in plants and it is clear that further instances are likely to be discovered.

Natural occurrence of dimethyl sulphone. This sulphone has been isolated from dried ox blood, from the adrenal cortex of oxen, and also from *Equisetum palustre, E. arvense, E. hiemale,* and (probably) *E. telmateia* (*E. maximum*) by Karrer and his school.[22] The sulphone may be formed by [44] methylation and oxidation of hydrogen sulphide which can be produced from cystine in liver slices. It might also arise by fission of a dimethylsulphonium compound, giving dimethyl sulphide (as happens in certain mould cultures [4]), followed by oxidation, or by oxidation of methionine to methylsulphonylacetic acid and decarboxylation: $MeSO_2 \cdot CH_2 \cdot CO_2H \longrightarrow Me_2SO_2 + CO_2$.

The Sulphonium Compounds of Dogs' Urine and their Decomposition Products.—In 1895 Abel concluded on slender evidence that diethyl sulphide is evolved when dogs' urine is boiled with alkali and in 1905 Neuberg and Grosser claimed, without experimental details, to have obtained the phosphotungstate and bismuthi-iodide from diethylmethylsulphonium hydroxide in the urine, and stated that the quantity was increased on administration of diethyl sulphide to the dogs, the sulphide being methylated by the organism.

A further study of the subject was begun in Leeds and Dr. Margaret Whitaker and D. Leaver [43] decomposed several litres of dogs' urine with alkali and absorbed the volatile sulphur compounds in (*a*) mercuric cyanide and (*b*) mercuric chloride. Very little thiol was detected in (*a*) but the sulphide mercurichloride in (*b*) was converted into the mercuribromide and into the benzylsulphonium picrate and styphnate $X^-\{^+SRR' \cdot CH_2Ph$. The m.p.s differed from those of the corresponding diethyl sulphide derivatives, approximating to those of the analogous derivatives of methyl *n*-propyl sulphide, but were unsharp. The sulphilimine $RR'S{\rightarrow}N \cdot SO_2 \cdot C_6H_4Me$, however, was purified and had m.p. 103—104° (and 104—105° in admixture with methyl *n*-propyl sulphilimine, m.p. 105°). Presumably a small quantity of another sulphide is contained in the unpurified natural sulphide. This was confirmed by two methods.

(1) The crude natural sulphide was converted into the methylsulphonium base. Paper chromatography of this presumed mixture of bases and comparison of the pattern with those given by known mixtures suggested the presence of dimethyl-*n*-propylsulphonium hydroxide and a small quantity of *n*-butyldimethylsulphonium hydroxide.

(2) The crude sulphilimine was chromatographed on special paper and developed with potassium iodide in hydrochloric acid which hydrolyses the sulphilimine to sulphoxide. This then liberates iodine. Undeveloped bands corresponding to the spots were separately boiled with sodium hydrogen

[44] Smythe, *J. Biol. Chem.*, 1942, **142**, 387.

sulphite solution, and the sulphides thus liberated by reduction of the sulphilimines were converted into mercurichlorides. That from the main band was shown by m.p. and mixed m.p. to be $2SMePr^n,5HgCl_2$. That from the smaller band was indistinguishable in m.p. from the mercurichloride of *n*-butyl methyl sulphide.

The sulphide from dogs' urine is therefore mainly methyl *n*-propyl sulphide with most probably some *n*-butyl methyl sulphide. In spite of the extended use of ion-exchange resins, the sulphonium compounds in the urine which are the precursors of these two sulphides have not yet been identified. *n*-Butylmethyl-*n*-propylsulphonium hydroxide is probably not the source of both sulphides. No butyl propyl sulphide was detected in the natural sulphides. If there are two sulphonium bases in the urine the third group in each may be acidic or completely decomposed by alkali, as it does not appear in the volatile sulphides from the urine.

Mechanism of biological methylation by moulds

The Acetic Acid Hypothesis.—At first it seemed possible that, if the mould is assumed to have acetic acid at its disposal, Gosio gas (trimethylarsine) might be produced thus:

$$As(OH)_3 + 3Me{\cdot}CO_2H = AsMe_3 + 3CO_2 + 3H_2O$$

In spite of much work designed to test this hypothesis no evidence in its favour was obtained.[1–4]

The Formaldehyde Hypothesis.—In its application to the production of trimethylarsine from arsenious acid this postulates the formation of hydroxymethylarsonic acid $HO{\cdot}CH_2{\cdot}AsO_3H_2$ from formaldehyde and $AsHO_3H_2$ as the first stage, followed by reduction to methylarsonic acid, $Me{\cdot}AsO_3H_2$. After further reduction to $Me{\cdot}As(OH)_2$ the isomeric form $Me{\cdot}AsHO{\cdot}OH$ could react again with formaldehyde, yielding cacodylic acid, $Me_2As{\cdot}O_2H$ and finally trimethylarsine.[1–4] Hydroxymethylarsonic acid could not be synthesised and its homologue $HO{\cdot}CH_2{\cdot}CH_2{\cdot}AsO_3H_2$ in bread cultures of the mould gave no volatile product. Had reduction of the 2-hydroxyl group occurred the formation of ethyldimethylarsine would have been expected.

If selenious and tellurous acids can react as $SeHO_2{\cdot}OH$ and $TeHO_2{\cdot}OH$ the formaldehyde hypothesis could explain their methylation in mould cultures.

As applied to the fission of disulphides and methylation of the resulting thiol, the formaldehyde hypothesis demands the formation of $RS{\cdot}CH_2{\cdot}OH$.[1–4, 37] Such compounds have been described but are easily hydrolysed. The compound $EtS{\cdot}CH_2{\cdot}OH$ could not be freed from traces of ethanethiol and its reduction to ethyl methyl sulphide in mould cultures could not be tested. This reaction of thiols and formaldehyde has again been invoked by Berg [44a] and by Greenberg [44b] to explain the conversion of homocysteine into methionine in animal enzyme systems.

[44a] Berg, *J. Biol. Chem.*, 1953, **205**, 145.

[44b] Greenberg, *ibid.*, 1951, **190**, 611; *Fed. Proc.*, 1954, **13**, 221; *J. Amer. Chem. Soc.*, 1952, **74**, 6307.

The choline and betaine of higher plants, fungi, and animals have usually been regarded as arising by methylation of a precursor by formaldehyde or glyoxylic acid resulting (in the case of animals) from the deamination of glycine.

An enzyme (glycine oxidase) which converts glycine into glyoxylic acid and ammonia has been detected in the liver and kidneys of many animals.[45] Moreover, formate and formaldehyde are produced from $C_{(\beta)}$ of serine and $C_{(\alpha)}$ of glycine in liver slices. The formaldehyde hypothesis, merged in the wider conception of the one-carbon fragment (see p. 271), now affords a satisfactory explanation of some, though not all, aspects of biological methylation. Thus if sodium formate labelled with ^{14}C is added to certain mould cultures containing arsenious oxide or sodium selenate the trimethylarsine and dimethyl selenide are radioactive, but see p. 273.

The Transfer of a Methyl Group.—Shortly after the formaldehyde hypothesis was advanced to explain mycological methylation, Challenger and (Miss) Higginbottom [46] suggested an alternative mechanism and stated in 1935 that " it is not impossible that some ingredient of the cell substance containing a methylated nitrogen atom may, under the special conditions obtaining in the cell, lose a methyl group *which if it be eliminated with a positive charge could be easily co-ordinated by the unshared electrons of tervalent arsenic or quadrivalent selenium and tellurium* ". The italics indicate the degree to which this suggestion extends those of Hofmeister and Riesser.

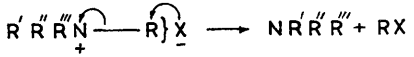
This conception was developed by the Reviewer in 1942.[1] " Almost all the compounds which undergo methylation by moulds or in animals can furnish negative ions so that co-ordination of a positive methyl group by the unshared electrons of the ion would yield a neutral molecule which could then undergo *reduction and ionisation* followed by further co-ordination of a positive methyl radical." This positive methyl ion " may be derived from betaine, choline, or methionine ". By 1942 the work of du Vigneaud and his school (see p. 269) on transmethylation in animals had rendered these speculations particularly attractive.

The application of this suggested process to arsenic and selenium has been set out elsewhere in full.[1-4] It will therefore be sufficient here to indicate the sequence of the various postulated intermediate compounds : arsenious acid, methylarsonic acid $Me{\cdot}AsO_3H_2$, cacodylic acid Me_2AsO_2H, trimethylarsine oxide (see p. 261) ; selenious acid, methaneselenonic acid, methylseleninic acid, dimethyl selenone, dimethyl selenoxide.

The postulated intermediate selenium compounds have not been detected in the media, but Bird and Challenger [47] showed that *S. brevicaulis* and certain *Penicillia* convert methyl-, ethyl-, and propyl-seleninic acid $R{\cdot}SeO_2H$ into dimethyl, ethyl methyl and methyl *n*-propyl selenides RSeMe, as required by the suggested mechanism. The nitrate $NO_3^-\{^+SeMe_2{\cdot}OH$ also gives dimethyl selenide.

[45] Ratner, Nocito, and Green, *J. Biol. Chem.*, 1944, **152**, 119.
[46] Challenger and (Miss) Higginbottom, *Biochem. J.*, 1935, **29**, 1757.
[47] (Miss) Bird and Challenger, *J.*, 1942, 574.

The alternative to methylation by a positive methyl ion (reaction of S_N1 type) is a bimolecular reaction of the S_N2 type, thus:



Here X represents the arsenite, tellurite, etc., ion.[1]

This differs from the S_N1 reaction in its kinetics, but not in its products. If, as seems probable, this bimolecular mechanism is preferable, then the co-ordination of Me^+ by the arsenite or other negative ion should be replaced by a scheme in which the transfer of methyl occurs without actual separation as an ion. This, however, also involves the attachment of methyl to the unshared electrons of the metalloid, so that the earlier formulations [1–4] are still convenient for representing the intermediate stages in the methylation process. Further work using tracers containing $^{14}CH_3$ (see p. 272) points strongly to methionine rather than a quaternary ammonium salt as the methyl donor.

Cantoni has shown [48] (see p. 279) that in certain liver- or kidney-enzyme systems methionine is converted into a sulphonium compound, the *S*-adenosinylmethionine ion or "active methionine". This has the formula $C_5H_4N_5{\cdot}C_4H_6O_3{\cdot}CH_2{\cdot}S^+Me{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$ (see p. 280 for full structure).

If it is formulated as $RR'MeS^+$ the bimolecular S_N2 reaction with, *e.g.*, arsenite, could be represented thus, on the assumption that methionine is similarly "activated" in moulds:

$$RR'MeS^+ + :As(OH)_3 \longrightarrow [RR'S\leftarrow{}^+Me{:}As(OH)_3] \longrightarrow$$
$$RR'S + [Me{\cdot}As^+(OH)_3] \longrightarrow Me{\cdot}AsO(OH)_2 + H^+$$

The attraction of the positive sulphur pole for the electrons of the S–Me link might allow nucleophilic attack on the methyl group by the arsenic atom with its unshared electrons. The resulting transition state would lead to a neutral sulphide RR'S [*S*-adenosinylhomocysteine $C_5H_4N_5{\cdot}C_4H_5O_3{\cdot}CH_2{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$] which has recently been detected in the enzymic methylation of guanidinoacetic acid,[48a] methylarsonic acid, and a proton, without formation of a free positive methyl ion at any stage. The dominant position occupied by methionine in all aspects of biological methylation, and Cantoni's discovery that a sulphonium ion is an intermediate, renders it unlikely that methyl is transferred as a neutral radical.

Similar considerations would explain the methylation of pyridine and quinoline [1–4] in the dog and the formation of N^1-methylnicotinamide from nicotinic acid in animals.

The alkyl methyl sulphides formed from dialkyl disulphides by *S. brevicaulis* may arise from alkanethiol, by co-ordination of Me, or this may occur before fission.

[48] Cantoni, *J. Amer. Chem. Soc.*, 1952, **74**, 2942.
[48a] Cantoni and Scarano, *ibid.*, 1954, **76**, 4744.

Transmethylation. Du Vigneaud's experiments with isotopic indicators

Transmethylation from methionine and choline. The conclusion that biological methylation in animals or moulds might be effected by methyl groups detached from choline or betaine was established by the work of du Vigneaud *et al.*[49] who showed that homocystine can replace methionine in the diet of the white rat only in presence of choline or betaine. It was suggested that a methyl group is transferred from the nitrogen of choline or betaine to the sulphur of homocysteine (transmethylation ; but see pp. 272, 281) to give methionine, and that the reaction might be reversible, methionine acting as a methyl donor to a choline precursor. This hypothesis was tested by feeding deuteromethionine containing (*a*) 83·6 and (*b*) 87·5 atom % of deuterium in the methyl group, to rats kept on a methionine- and choline-free diet. [The deuterium content of urinary creatinine (XI) closely follows that of the creatine (X) and choline of the tissues.] The experiment with specimen (*a*) was, therefore, continued for 94 days until the methyl group of the creatinine contained 72·4 atom % of deuterium. The animal was then killed and the choline isolated from the tissues as the chloroplatinate. The content of deuterium in the methyl groups of this choline was 74·2, and in the tissue creatine 73 atom %. These figures represent in each case approximately 83% of the theoretically possible amount of deuterium, on the assumption that all the methyl groups had come from deuteromethionine. This figure is the " deuterium ratio ", *i.e.*, 100 × atom % of deuterium in the methyl group of the isolated compound ÷ atom % of deuterium in the methyl group of the deuteriomethionine administered. On oxidation of the choline to trimethylamine all the deuterium was found in the methyl groups.

These reactions are true transmethylations (the methyl group being transferred as a whole) and do not involve the elimination of dideuterioformaldehyde CD_2O. This, if produced, would react with the amino-group of the choline precursor, presumably ethanolamine, to give —NH·CD_2·OH which on reduction in the organism would give —NH·CD_2H and not —NH·CD_3. Consequently the deuterium content of each methyl group of the choline could not exceed two-thirds of that in the methyl group of the methionine administered, *i.e.*, the " deuterium ratio " would be at most 66·6%. Similar arguments hold for the deuterocreatine. This conclusion was completely established when deuteromethionine and methionine containing ^{14}C in the methyl group were fed to a rat. The ratio of D to ^{14}C in the isolated choline and creatine was the same as in the original mixture. Du Vigneaud's school has recently discussed the scope of this method.[49a]

Du Vigneaud then administered trideuterocholine chloride $Cl^-\{^+N(CD)_3\cdot CH_2\cdot CH_2\cdot OH$ to rats, on a methionine- and choline-free diet containing homocystine, for 23 and 56 days respectively. The deuterium content of the creatine was 24% and 29% of the theoretical maximum

[49] Du Vigneaud, Cohn, Chandler, Schenk, and Simmonds, *J. Biol. Chem.*, 1941, **140**, 625 ; Simmonds, Cohn, Chandler, and du Vigneaud, *ibid.*, 1943, **149**, 519.

[49a] Rachelle, Kuchinskas, Knoll, and Eidinoff, *J. Amer. Chem. Soc.*, 1954, **76**, 4342.

and the deuteromethyl group was also detected in tissue methionine. The methyl groups of choline can therefore take part in methylation, but this experiment does not prove that they were transferred intact. It appears that homocysteine is formed from methionine by the animal, and that methionine is continuously re-formed through the methyl group or a one-carbon fragment (see pp. 271, 278) supplied by choline. When deutero-methionine and an adequate supply of ordinary choline were administered, formation of deuterocholine still occurred.

Methionine can also provide a methyl group in the rabbit [50] and in man.[51]

Biological Importance of the Thetins.—When du Vigneaud discovered the biological mobility of the methyl group in choline, methionine, and betaine, he tested many other methyl derivatives but of these only the sulphonium compound dimethylacetothetin chloride, $Cl^-\{^+SMe_2{\cdot}CH_2{\cdot}CO_2H$ (which has not as yet been detected in Nature), exhibited methyl mobility.[52] Toennies, and Toennies and Kolb,[53] had already suggested that sulphonium derivatives of methionine might play a part in biological phenomena. (Du Vigneaud remarked that methionine is rather resistant to purely chemical demethylating reagents, but that formation of a sulphonium compound might loosen the methyl group.)

After the isolation of 2-carboxyethyldimethylsulphonium chloride $Cl^-\{^+SMe_2{\cdot}CH_2{\cdot}CH_2{\cdot}CO_2H$ from seaweed,[20] du Vigneaud and his colleagues confirmed the finding that dimethylacetothetin chloride can replace methionine in the diet of the white rat, the resulting growth being comparable with that obtained with choline or betaine *plus* homocystine. Maw and du Vigneaud [54] also showed that the seaweed thetin chloride has a mobile methyl group and will support the growth of rats on a basal methionine-free diet containing homocystine. They pointed out that the chlorides of choline, ethyl-2-hydroxyethyldimethylammonium hydroxide (" monoethyl-choline "), betaine, dimethylacetothetin, ethylmethylacetothetin, and dimethyl-β-propiothetin—all of which can, directly or indirectly, supply a methyl group to homocystine *in vivo*—contain a methyl group or groups directly linked to an 'onium pole.

Dubnoff and Borsook [55] showed that the two thetin chlorides can methylate homocysteine to methionine in liver or kidney preparations of rats. The enzyme " dimethylthetin transmethylase " was partly purified by fractional precipitation with ethanol. It is separable from the accompanying enzyme " betaine transmethylase " as the latter is destroyed at pH 4·5. The transmethylation is independent of oxygen (see p. 278) and is not inhibited by oxidative poisons such as azide, cyanide, arsenate,

[50] Schenk, Simmonds, Cohn, Stevens, and Du Vigneaud, *J. Biol. Chem.*, 1943, **149,** 355.

[51] Simmonds and Du Vigneaud, *ibid.*, 1942, **146,** 685.

[52] Du Vigneaud, Harvey Lectures, Williams and Wilkins, Baltimore, 1942—43, Vol. 38, p. 39.

[53] Toennies, *J. Biol. Chem.*, 1940, **132,** 455; Toennies and Kolb, *J. Amer. Chem. Soc.*, 1945, **67,** 849.

[54] Maw and Du Vigneaud, *J. Biol. Chem.*, 1948, **174,** 381, 477; **176,** 1029, 1037.

[55] Dubnoff and Borsook, *ibid.*, p. 789.

or arsenite. Only one methyl group is transferred from dimethylthetin when homocysteine is in excess ; (methylthio)acetic acid $MeS{\cdot}CH_2{\cdot}CO_2H$ is inactive, as was also found by Maw and du Vigneaud in their experiments *in vivo*. Neither dimethylacetothetin nor dimethyl-β-propiothetin has yet been detected in animal tissues.

Synthesis of labile methyl in the body

Until about 6 years ago it was believed that the animal organism is incapable of synthesising methyl groups and that methyl sources such as methionine and choline must be present in the diet. Du Vigneaud [5] and also Bennett,[56] however, occasionally found animals capable of showing some growth on a homocystine diet without added choline.

Reactions involving One-carbon Fragments.—Du Vigneaud, Ressler, and Rachele [57] showed that germ-free rats maintained under completely sterile conditions with D_2O in their drinking water can synthesise choline containing deuterium in the methyl groups to the extent of 3·3 and 6·4% of that in the body water, after 10 and 23 days respectively. Since intestinal bacteria were absent this synthesis must have been achieved by the tissues of the rats.

In some fundamental work carried out by Sakami [58] shortly afterwards, rats received glycine containing $^{13}CO_2H$ and [^{14}C]formate. They were then killed and serine was isolated from the liver. The serine contained ^{13}C, located almost exclusively in the carboxyl group, and ^{14}C, mainly in the β-position. The reaction may be represented thus :

$$H{\cdot}{}^{14}CO_2H + NH_2{\cdot}CH_2{\cdot}{}^{13}CO_2H \longrightarrow HO{\cdot}{}^{14}CH_2{\cdot}CH(NH_2){\cdot}{}^{13}CO_2H$$

Whether the formate reacts as such with the CH_2 of glycine, giving $H{\cdot}{}^{14}CO{\cdot}CH(NH_2){\cdot}{}^{13}CO_2H$ as an intermediate, or undergoes reduction to formaldehyde, giving serine, was not decided. Formation of $HO_2{}^{14}C{\cdot}CH(NH_2){\cdot}{}^{13}CO_2H$ by fixation of $^{14}CO_2$, arising from $H{\cdot}{}^{14}CO_2H$, or by dehydrogenation of a molecule of formate and one of glycine, appears to be excluded since such a compound would be expected to yield serine containing at least 50% of $HO{\cdot}{}^{13}CH_2{\cdot}CH(NH_2){\cdot}{}^{14}CO_2H$ on reduction.

Sakami also showed that, when glycine $NH_2{\cdot}{}^{14}CH_2{\cdot}CO_2H$ was fed to rats, the liver serine contains ^{14}C at the α- and the β-position to an almost equal extent. Glycine is, under these conditions, a major source of formate which then reacts with unchanged glycine.

Siekevitz, Winnick, and Greenberg [59] observed the reverse change with serine. Formate and formaldehyde are produced in liver slices from $C_{(\beta)}$ of serine and $C_{(\alpha)}$ of glycine. Further, Ratner *et al.*[45] described an oxidase, present in the liver and kidneys of all animals examined, which converts glycine into glyoxylic acid and ammonia. Sakami [58] also found that when [*Me*-^{14}C]choline was administered to rats the tracer element appeared in the β-position in serine. This suggests that formate or some closely related

[56] Bennett, for references see Sakami and Welch, *J. Biol. Chem.*, 1950, **187**, 379.
[57] Du Vigneaud, Ressler, and Rachele, *Science*, 1950, **112**, 267.
[58] Sakami, *J. Biol. Chem.*, 1948, **176**, 995 ; 1949, **179**, 495 ; *Fed. Proc.*, 1950, **9**, 222.
[59] Siekevitz, Winnick, and Greenberg, *J. Biol. Chem.*, 1949, **180**, 845.

derivative is an intermediate in the oxidation of the choline-methyl group. Moreover this reaction might be reversible and the methyl groups of choline might arise, in some circumstances and to some extent, from compounds such as methanol or sodium formate. If so, some light might be thrown on those cases reported by du Vigneaud and by Bennett in which rats appeared to be capable of synthesising methyl groups on a methyl-free diet.

Consequently Arnstein [60] fed isotopically (^{14}C) labelled formate, methyl alcohol, and various potential sources of these compounds such as DL-[β-^{14}C]serine, L-[β-^{14}C]serine, [α-^{14}C]glycine, [CO_2H-^{14}C]glycine, and D-[β-^{14}C]serine to rats on a normal diet. After 1—5 days the rats were killed, the choline was isolated as the reineckate, and converted into the chloroplatinate, and thence into trimethylamine chloroplatinate. The first five compounds gave rise to [*Me*-^{14}C]choline.

Carbon dioxide is known to arise by oxidation of D-[β-^{14}C]serine and [CO_2H-^{14}C]glycine *in vivo*. These compounds were not converted into [*Me*-^{14}C]choline, from which it follows that the intact rat is unable to reduce carbon dioxide to methyl to an appreciable extent. This agrees with the results of du Vigneaud, Verly, and Wilson [61] who found that after isotopic sodium hydrogen carbonate $NaH^{14}CO_3$ had been fed to rats the choline of the tissues was free from the isotope.

Arnstein pointed out that the methyl precursor was unknown; serine and glycine both yield formaldehyde and formate as degradation products but these compounds may be involved as derivatives.

Three important points now emerge: (1) the attachment of a methyl group to a choline or creatine precursor can take place by some process which in its early stages, at any rate, is not identical with the "transfer of methyl as a whole", which we associate with the "transmethylation" so thoroughly established by du Vigneaud; (2) carbon dioxide does not appear to be the source of the methyl group—this is important in view of the significance of carbon dioxide fixation in other fields of biochemistry; and (3) the body is not entirely dependent on exogenous methyl groups. Verly and du Vigneaud showed that methyl alcohol can make appreciable amounts of methyl groups available for creatine and choline.

Use of radioactive carbon ^{14}C in the investigation of mycological methylation

Workers at Leeds have recently studied the effect of various potential sources of the methyl group, labelled with ^{14}C, on the production of trimethylarsine and dimethyl selenide in bread cultures of *S. brevicaulis*, and of dimethyl selenide in cultures of *Aspergillus niger* on liquid media or on bread.[62] The sources were choline chloride, betaine, DL-methionine and sodium formate. Only one methyl group in choline and betaine

[60] Arnstein, *Biochem. J.*, 1951, **48**, 27; cf. Arnstein and Neuberger, *ibid.*, 1953, **55**, 259.

[61] Du Vigneaud, Verly, and Wilson, *J. Amer. Chem. Soc.*, 1950, **72**, 2819.

[62] Challenger, Lisle, and Dransfield, *J.*, 1954, 1760; Dransfield and Challenger, *J.*, 1955, 1153.

was labelled. The products were collected as the mercurichlorides $AsMe_3,2HgCl_2$ and $SeMe_2,HgCl_2$, and their radioactivity was measured. The " methylation percentage " was calculated from the ratio

$$\frac{100}{nf} \times \frac{\text{Radioactivity of methylated product per mole}}{\text{Radioactivity of methyl source per mole}}$$

where n is the number of methyl groups produced by methylation per molecule of the product and f is the fraction of the total of labelled methyl groups or carbon atoms per molecule which are theoretically labile.

Thus, $n = 1$ when the product is $SMeEt,2HgCl_2$ and $n = 2$ for the product $SMe_2,HgCl_2$. The value f is of importance since in choline, betaine, dimethylacetothetin $^+SMe_2{\cdot}CH_2{\cdot}CO_2^-$, and dimethyl-$\beta$-propiothetin $^+SMe_2{\cdot}CH_2{\cdot}CH_2{\cdot}CO_2^-$ only one methyl group is labile in animals or in tissue preparations.

The methylation percentage was small with choline chloride, betaine, and sodium formate, varying from 1 to 5, but was very considerable in all experiments with methionine, ranging from 25 to 95. Moreover, with methionine a very much smaller proportion of the total ^{14}C is converted into $^{14}CO_2$ than with the other sources. In bread cultures of *A. niger* containing selenate and methionine, the methylation percentage was only 45 whereas on a synthetic medium containing sucrose, glycine and inorganic salts the figure rose to over 90. This is probably because the protein of bread is an excellent source of natural methionine which considerably dilutes the radioactive amino-acid.

It therefore appears probable that the $^{14}CH_3$ of methionine is transferred intact to arsenic and selenium and that in mycological methylation the part played by this amino-acid is as dominating as in the analogous animal processes. The demonstration that the methylation percentage for the systems [*Me*-^{14}C]betaine–selenate and [^{14}C]formate–selenate in bread cultures of *A. niger* under comparable conditions is 2—5 times greater in presence of homocystine than in its absence. This agrees well with Borsook's observation that homocystine or homocysteine is necessary before the methyl-carbon atom of choline can be transferred to guanidinoacetic acid, yielding creatine.

These results suggest that methionine is produced from homocysteine by acceptance of a methyl group or other one-carbon fragment, detached from betaine, choline, or formate. *S*-Adenosinylhomocysteine (see p. 281) is formed when active methionine [63] transfers its methyl group to guanidinoacetic acid, giving creatine.[48a] Homocysteine or some derivative of it is probably concerned with the maintenance of the correct level of methionine in metabolism.

The Leeds authors conclude that carbon dioxide is probably not an intermediate in the production of methionine, for reasons which are discussed in the original paper.

The methylation of sulphur in bread cultures of *S. brevicaulis* containing diethyl disulphide or *S*-ethylcysteine has been studied in presence of

[63] Cantoni, *J. Biol. Chem.*, 1951, **189**, 208, 211 ; 1953, **204**, 403.

[*Me*-^{14}C]choline and [^{14}C]formate. Fission of the S–S and the S–C linkages produced inactive ethanethiol and radioactive ethyl methyl sulphide.

Methylsulphonium Compounds as Methyl Sources.—The work of du Vigneaud with intact animals and of Borsook and Dubnoff with animal tissues (see p. 278) has shown that dimethylacetothetin and dimethyl-β-propiothetin salts can act as methyl sources. Handler and Bernheim [64] found that this is also true of another thetin, methylmethioninesulphonium iodide $I^-\{^+SMe_2\cdot CH_2\cdot CH_2\cdot CH(NH_2)\cdot CO_2H$ which can replace methionine in the diet of young rats. It was of interest to determine whether these three compounds are comparable with methionine in mould methylations, *i.e.*, are methyl donors or are methyl sources and comparable with choline or betaine. This was tested by competition experiments in which the non-radioactive thetins were added to *A. niger* cultures on the usual sucrose–glycine–salts medium in presence of [*Me*-^{14}C]methionine and selenate. With the first two thetins and with betaine the usual high values for the methylation percentage (80—100) were obtained, indicating that no appreciable dilution of the radioactive methyl groups had occurred and that, consequently, these compounds are not direct methyl donors.

It remained to be seen whether the thetins are methyl sources in mould cultures. When these compounds labelled with ^{14}C in one methyl group were added to bread cultures of *S. brevicaulis* containing selenate the methylation percentage was only 1—1·6, whereas betaine hydrochloride under similar conditions gave a value of 4·0. Clearly the thetins are also very poor methyl sources. Hence it would seem that the " thetin transmethylase " reported by Borsook and Dubnoff to occur in rat-liver preparations is not present in the moulds *A. niger* and *S. brevicaulis* under the experimental conditions.

The position of methylmethioninesulphonium iodide is more complex. When this was unlabelled and in competition with labelled methionine, in liquid cultures of *A. niger* containing selenate, the methylation percentage was depressed to 60 ; when it was labelled and used in similar cultures the value was 35.[62] As there was strong evidence of the formation of methionine in this second experiment, it is possible that the sulphonium iodide is not itself a methyl donor or a source, but that the effective agent is the methionine to which it gives rise. As only one methyl group in the sulphonium iodide was labelled, 50% of the methionine produced would be non-radioactive which would explain the low methylation percentage.

Availability of the Optical Isomers of Methionine as Methyl Donors.—Cantoni [63] found L-methionine to be twice as active as the DL-isomer in the methylation of nicotinamide to N^1-methylnicotinamide by the enzyme nicotinamide methyl kinase in presence of adenosine triphosphate and phosphoglycerate $H_2O_3P\cdot O\cdot CH_2\cdot CH(OH)\cdot CO_2H$. This system involves the formation of *S*-adenosinylmethionine. Cantoni concluded that D-methionine was inactive. Betaine and dimethylacetothetin were also inactive as methyl donors, but, when they were used in conjunction with DL-homocysteine, methylation of nicotinamide occurred readily, presumably owing

[64] Handler and Bernheim, *J. Biol. Chem.*, 1943, **150**, 335.

to formation of methionine. Cantoni found that choline was inactive in absence or presence of homocysteine, probably owing to the absence of choline oxidase (see p. 278). His result with dimethylacetothetin agrees with those obtained in mould cultures (see above).

Handler and Bernheim [64] found that L-methionine was twice as effective as the D-isomer in the methylation of guanidinoacetic acid to creatine by rat-liver slices. However, the α-keto-acid $MeS{\cdot}CH_2{\cdot}CH_2{\cdot}CO{\cdot}CO_2H$ which would arise by enzymic oxidative deamination of D(or L)-methionine was as effective as L-methionine itself. Possibly, therefore, D-methionine is first converted into the keto-acid which is then re-aminated asymmetrically to the L-amino-acid before methylation. This view is supported by the observation that transmethylation from D-methionine did not take place in the presence of benzoic acid, which is a D-amino-acid oxidase inhibitor.

It was therefore decided to study the relative utilisation of D- and L-methionine in the methylation of sodium selenate by *A. niger* giving dimethyl selenide. The results showed that D-methionine is quite as effective as L- or DL-methionine.[62]

Notable recent developments in the study of methylation in plants

In 1942 Barrenscheen and Válgy-Nagy [64a] reported that, in extracts of wheat seedlings, the methylation of guanidinoacetic acid to creatine was greatly stimulated by methionine and by betaine. Eight years later, work in Canada and the United States threw light, not only on the origin of the NMe group, but also on that of the methoxy- and the methylenedioxy-group.

Origins of the Methyl Groups of Hordenine.—Hordenine, *N*-dimethyltyramine $p\text{-}HO{\cdot}C_6H_4{\cdot}CH_2{\cdot}CH_2{\cdot}NMe_2$, was isolated from barley by Léger in 1906. Kirkwood and Marion [65] showed that certain strains of the plant produce instead the corresponding *N*-monomethyl derivative. Moreover, the plant *Trichocereus candicans* contains, not only hordenine, but also its betaine candicine, so that all stages of the methylation of tyramine are found in Nature.

Kirkwood and Marion then showed that when sodium [^{14}C]formate is fed to sprouting barley the activity is recovered almost entirely in the NMe groups of hordenine and choline, as shown by degradation to trimethylamine. Matchett, Marion, and Kirkwood [65] observed a similar result on administration of L-[*Me*-^{14}C]methionine, but [*Me*-^{14}C]choline contributed no labelled methyl to hordenine ; and as choline produces much labelled carbon dioxide under these conditions it appears that this is not a source of the methyl group. Possibly choline oxidase (see p. 278) is absent.

Origin of the Methyl Group of Nicotine.—On the other hand, Brown

[64a] Barrenscheen and Válgi-Nagy, *Z. physiol. Chem.*, 1942, **277**, 97.

[65] Kirkwood and Marion, *Canad. J. Chem.*, 1951, **29**, 30 ; cf. Matchett, Marion, and Kirkwood, *ibid.*, 1953, **31**, 488 ; Dubeck and Kirkwood, *J. Biol. Chem.*, 1952, **199**, 307 ; Brown and Byerrum, *J. Amer. Chem. Soc.*, 1952, **74**, 1523 ; Byerrum, Dewey, and Ball, *ibid.*, 1954, **76**, 3997 ; Byerrum and Wing, *J. Biol. Chem.*, 1953, **205**, 637 ; Byerrum, Hamill, and Ball, *ibid.*, 1954, **210**, 645 ; Sribney and Kirkwood, *Nature*, 1953, **171**, 931.

and Byerrum,[65] and Byerrum and Wing [65] found that the methyl-carbon atom of methionine and choline, and the carbon atom of formate, furnished the NMe group of nicotine in *Nicotiana rustica*. Formate is probably the precursor of methionine. "The differences in the age of the plants and conditions of growth (light or darkness) could account for differences in the availability of the methyl groups of choline." In view of the demonstration of a true transmethylation from methionine in the formation of the methoxy-groups of barley lignin (see below) and of the similar rate at which choline and methionine furnish the methyl group of nicotine, the authors consider it reasonable to assume that some of the methyl groups of choline were also transferred intact. This, however, has not been proved. The nicotine was isolated as the dipicrate and then demethylated, the resulting methyl iodide was absorbed in triethylamine, and the quaternary iodide was assayed.

Origin of the NMe and OMe Groups of Ricinine.—This was investigated by Dubeck and Kirkwood [65] by feeding the germinating seeds of *Ricinus communis* (the castor oil plant) with [*Me*-^{14}C]methionine and choline and [^{14}C]formate. Only with methionine was the ricinine (3-cyano-1 : 2-dihydro-4-methoxy-1-methyl-2-oxopyridine) appreciably labelled. The methyl of the methoxy-group was removed by hydriodic acid and that of the methylimino-group by more vigorous treatment of the residue. The methyl iodide was assayed as tetramethylammonium reineckate. The radioactivity was found wholly in the methylimino- and the methoxy-group, which had almost the same activity. A similar study on damascenin, which in addition to NMe and OMe, also contains the group CO_2Me (see p. 258), would be interesting.

Origin of the OMe Groups of Lignin.—The work now to be described and that of Byerrum, Dewey, and Hall[65] afford the first proof of the origin of the methoxy-groups of plants. It was shown by the use of ^{14}C that the methyl group of methionine provides the methoxy-groups of the lignin of barley and tobacco. The lignin was demethylated as before and the methyl iodide assayed as methyltriethylammonium iodide. Further, by employing the device introduced by du Vigneaud [48a] (see p. 269) and using a mixture of 10% of [*Me*-^{14}C]methionine and 90% of deuteromethionine Byerrum *et al.* showed that the methyl group of methionine is transferred intact. The ^{2}H : ^{14}C ratios in the methyltriethylammonium iodide were 94 and 95% of the ratio in the methionine fed to barley and tobacco respectively.

If all of the methyl group of the administered methionine had been oxidised to the state of formaldehyde and then reduced to give the methoxy-group of lignin, the ^{2}H : ^{14}C ratio in the methyl group of the quaternary salt would be 67% of that in the original methionine (cf. p. 269).

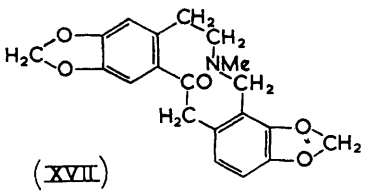
This is only the third completely authenticated case of true transmethylation and the authors are to be congratulated.

Formate is also a precursor of lignin-methoxyl, but when it was administered along with an equimolecular quantity of methionine about 26 methoxy-groups were formed from the amino-acid for each one arising from

formate. This agrees with the conclusion that methionine is very close to the final stage in the methylation process.

Origin of the Methylenedioxy-group in Protopine.—The Canadian school has announced that the carbon atoms of the methylimino- and the methylenedioxy-group of the alkaloid protopine (XVII) are derived from methionine. This second group appears to be peculiar to plants and has not been detected in animal products.

Sribney and Kirkwood [65] grew *Dicentra* hybrids in liquid media containing L-[*Me*-^{14}C]methionine, [*Me*-^{14}C]choline, and sodium [^{14}C]formate. On demethylation of the resulting radioactive protopine the NMe group finally yielded tetramethylammonium iodide and reineckate. The methylenedioxy-groups were assayed by hydrolysis of a separate portion of the alkaloid with 20% sulphuric acid, followed by isolation and counting of the liberated formaldehyde as its dimedone derivative. With methionine, the activity of the methylimino- and the methylenedioxy-groups was very similar. Formate contributed ^{14}C to the molecule, but to a much smaller degree. Apparently the methylenedioxy-groups arise from the oxidation of a methoxyl to a hydroxymethoxy-group, followed by ring closure with an adjacent hydroxyl group, rather than by a formylation followed by reduction and ring closure. Otherwise the alkaloid from the formate should be at least as active as that from methionine. (The activity of the formate was 4 times that in the methionine experiment.)



(XVII)

Choline did not contribute any activity to protopine, which recalls earlier negative results obtained by the Canadian school with this base. The authors suggest that the activity not accounted for by the methylimino- and methylenedioxy-groups may be found in the central carbon atom of protopine (XVII) which could originate from methionine by way of the " formate pool " of the plant. Byerrum, Hamill, and Ball [65] have recently shown that in tobacco plants the α-carbon atom of glycine is incorporated into the methylimino-group of nicotine, which was isolated and assayed as the dipicrate and, after degradation, as triethylmethylammonium iodide. This occurred at least as rapidly as the incorporation of the methyl group of methionine or choline and ten times faster than with formate. Consequently they considered that formate was not the direct product of the breakdown of glycine and suggested that a one-carbon unit at the oxidation stage of formaldehyde, reduced to give the methyl group of nicotine, is produced (cf. Robinson's " formaldehyde equivalent "). This suggestion is in line with earlier work (see p. 255).

Moreover formaldehyde has been shown to furnish the methyl group

of choline in rats, but by the use of pigeon-liver extracts Berg [44a] found that it was not oxidised to formate before incorporation into the methyl group of methionine. Consequently, Byerrum *et al.* remark " the proposal of Kisliuk and Sakami [66] that tetrahydro-5-hydroxymethylfolic acid is ' active formaldehyde ' in animal metabolism suggests that it might also be involved in the glycine to nicotine reaction " (see p. 283).

Methylation in enzyme systems

Dubnoff [67] has shown that in animal enzyme preparations the methyl groups of choline contribute to methionine formation from homocystine only if the animal can oxidise choline to betaine. Methionine is rapidly formed from betaine, dimethylacetothetin, and dimethyl-β-propiothetin in suitable organs of all animals tested, but choline is effective only if the animal possesses choline oxidase. Liver and kidney homogenates of rabbit, guinea-pig, and chick, which have none, do not form methionine aerobically or anaerobically. At pH 6·7 where choline oxidation proceeds only to the aldehyde stage there is no significant aerobic formation of methionine from choline.

The work by Muntz [68] supports these conclusions. He allowed [^{15}N]choline chloride to react anaerobically with homocysteine in a rat-liver homogenate. 2-Dimethylaminoethanol and dimethylglycine were added as carriers ; [^{15}N]dimethylaminoethanol, the product to be expected from the demethylation of choline, was not detected. [^{15}N]Dimethylglycine, however, which should arise from betaine, was obtained and shown not to be formed from the dimethylaminoethanol. Apparently, in these systems, choline does not lose a methyl group directly, but must first be converted into betaine. Cromwell and Rennie [69] have studied the precursors of choline in wheat seedlings.

Influence of Conditions on Methylation in Enzyme Systems.—Borsook and Dubnoff [70] in 1947 distinguished between two types of methylation in preparations of rat or guinea-pig liver. One is dependent on oxygen and is prevented by oxidation inhibitors. Catalytic activity is lost by homogenisation of the tissue and is not restored by simple addition of methionine, although in intact slices of rat liver methionine accelerates the reaction. The activity is restored to the homogenate, however, when adenosine triphosphate (XX) is added in addition to methionine. In this, category I, is the methylation by methionine of guanidinoacetic acid to creatine and of nicotinamide to N^1-methylnicotinamide. Characteristic of category II is independence of oxygen, non-susceptibility to oxidation inhibitors, and persistence of catalytic activity after cell structure is destroyed. Examples are the methylation of homocystine or homocysteine

[66] Kisliuk and Sakami, *J. Amer. Chem. Soc.*, 1954, **76**, 1456.
[67] Dubnoff, *Arch. Biochem.*, 1949, **22**, 474 ; **24**, 251.
[68] Muntz, *J. Biol. Chem.*, 1950, **182**, 489.
[69] Cromwell and Rennie, *Biochem. J.*, 1953, **55**, 189 ; 1954, **58**, 318, 322.
[70] Borsook and Dubnoff, *J. Biol. Chem.*, 1947, **169**, 247.

to methionine by choline (see, however, p. 278), betaine, dimethylacetothetin, and dimethyl-β-propiothetin.

In category I adenosine triphosphate is ineffective without methionine. Borsook and Dubnoff [70] considered that the chief function of oxidation in methylation by methionine in liver slices or in homogenates is the continuous production of the necessary adenosine triphosphate. Biochemical oxidation is frequently associated with vigorous phosphorylation.

Formation of " Active Methionine " in Enzyme Systems.—An entirely new light was thrown on these observations by Cantoni.[48, 63, 71, 72] He showed that the effect of adenosine triphosphate did not depend on the formation of a phosphorylated derivative of methionine. By addition of methionine, adenosine triphosphate, magnesium ions, and glutathione to an enzyme preparation obtained from rat, pig, ox, or rabbit liver, he finally obtained a product which he named " active methionine ". He showed that orthophosphoric acid was also formed.

In presence of an enzyme from pig liver which he called nicotinamide methylpherase (a transmethylation enzyme) this " active methionine " was able to methylate nicotinamide to N^1-methylnicotinamide in absence of adenosine triphosphate or any other source of high-energy phosphate links (see p. 272). Similarly the quite different enzyme guanidinoacetic acid methylpherase (from rabbit liver) in presence of " active methionine " and absence of adenosine triphosphate converted guanidinoacetic acid into creatine.

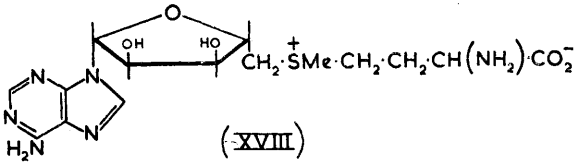
Not only was adenosine triphosphate unnecessary for these methylations (after its initial participation in the conversion of methionine into the " active methionine "), but treatment of an impure preparation of this substance with barium acetate precipitated phosphate, leaving a filtrate which was free from phosphate and contained the " active methionine " which was readily separated. It was shown not to contain phosphorus, and analysis and a study of its breakdown products disclosed that it was *S*-5′-deoxyadenosinyl-5′-methionine (XVIII).

Acid hydrolysis at 100° gave adenine (6-aminopurine), homoserine $HO{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$, and a sulphur compound, probably a thioribose or methylthioribose. More gentle hydrolysis at 100° in an acetate buffer at pH 7·0 gave some adenine, but mainly a crystalline compound which was conclusively shown to be identical with the adenine methylthiopentoside isolated many years earlier from yeast and shown by synthesis to be 5′-deoxy-5′-methylthioadenosine (XIX). Its unusual structure had always interested a few chemists but its biological origin was not clear and no natural compounds of analogous constitution were known. Its formation by micro-organisms requires an ample supply of methionine in the culture media. It may arise from " active methionine " which has

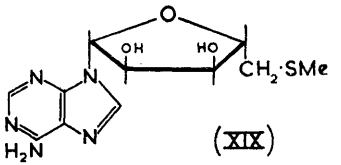
[71] Baddiley, Cantoni, and Jamieson, *J.*, 1953, 2662 ; Baddiley and Jamieson, *J.*, 1954, 4280.

[72] Woolley, *Nature*, 1953, **171**, 323 ; Cantoni, " Phosphorus Metabolism ", Johns Hopkins Press, Baltimore, 1951, Vol. I, p. 641 ; 1952, Vol. II, p. 129 ; Sealock and Davis, *J. Biol. Chem.*, 1949, **177**, 987 ; Alivasatos and Woolley, *Fed. Proc.*, 1955, **14**, 172.

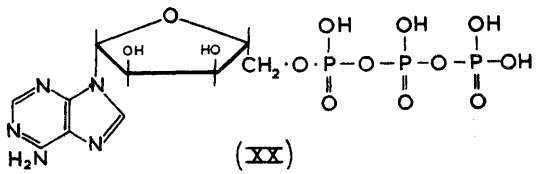
not yet, however, been detected in plants or micro-organisms. "Active methionine" has been synthesised by Baddiley and Jamieson [71] from the hydrobromide of α-amino-γ-bromobutyric acid and 5′-deoxy-5′-methylthioadenosine (XIX). The product was separated by paper chromatography and was indistinguishable from the natural substance in its behaviour on paper chromatography and electrophoresis (it moves to the cathode).



(XVIII)



(XIX)



(XX)

Boiling water regenerates the adenine methylthiopentoside. Enzyme tests show that the synthetic DL-material has 40—50% of the activity of the natural substance in the methylation of nicotinamide and guanidinoacetic acid. It appears that methionine must be converted into a sulphonium compound before it can transfer its methyl group and in view of the results of du Vigneaud and those of Byerrum *et al.* (see pp. 269, 276) it may be assumed that the methyl group of "active methionine" is transferred intact. This is not to say that methionine cannot also by oxidation furnish a one-carbon fragment which can finally appear as a methyl group. The ready oxidation of methionine-methyl in animals is quite in keeping with such an alternative mode of methylation.

It was suggested by Toennies [53] in 1945 that methionine might undergo conversion into a sulphonium compound before release of its methyl group. This possibility was put forward before any sulphonium compound had been satisfactorily detected in Nature. The actual reaction suggested by Toennies was unsupported by experiment but the essential idea was most stimulating and was borne in mind by many chemists. The 'onium structure of the methyl sources choline, betaine, and dimethylacetothetin had already been emphasised. Then followed the isolation of dimethyl-β-pro-

piothetin from seaweed and its recognition as a methyl source and possibly a methyl donor.

The following facts may now be summarised: (1) Methionine can transfer its methyl group intact. (2) Methylation by methionine in enzyme systems *in vitro* requires *either* air and an intact tissue slice *or* adenosine triphosphate, in which case the tissue can be homogenised. (3) Methionine and adenosine triphosphate with the appropriate enzyme system give the sulphonium compound *S*-5′-deoxyadenosinyl-5′-methionine. (4) Methylation *in vitro* by the use of 'onium compounds, such as betaine, choline (which is first converted into betaine), and the two thetins, does not require oxygen or adenosine triphosphate and is not inhibited when the tissue is homogenised. This is presumably because these compounds are already of 'onium type. The conclusion follows, but has not been experimentally established, that betaine and the thetins may also be able to transfer their methyl groups intact, though methylation by an oxidised one-carbon fragment may also be possible. It is desirable that this suggestion, which is not new but receives support from Cantoni's work, should be tested, *e.g.*, by the use of a compound containing a $^{14}CD_3$ group (see p. 269). (5) Betaine, dimethylaceto- and dimethyl-β-propio-thetin, " active methionine ", and methylmethioninesulphonium iodide (the last two are also thetins) are all either methyl donors or sources. These all contain the twin-ion structure $^{+}X{\cdot}[CH_2]_n{\cdot}CO_2^{-}$ which is absent in choline. Possibly not simply an 'onium but also a betaine or thetin type of structure is necessary for the transfer of an intact methyl group.

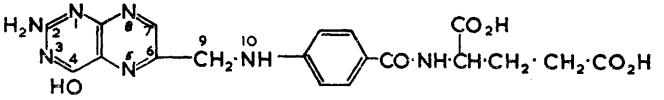
Transmethylation and Derivatives of Thiamine.—Woolley [72] has discussed some important transmethylation reactions of thiamine (aneurin, vitamin B_1) and some structural analogues in which a substituted methyl group CH_2R (where R is the pyrimidine residue of thiamine, or a pteridine ring) is transferred from the quaternary nitrogen atom of the thiazolium ring of the thiamine derivative to a tertiary nitrogen atom of an acceptor molecule. This occurs in presence of an enzyme thiaminase present in carp, clams, and certain fresh water fish, in bracken, and in some micro-organisms. Woolley regards these enzymic reactions as exactly similar to Cantoni's methylation of guanidinoacetic acid to creatine by means of active methionine. The other compound which should be formed in this last-named reaction, *S*-adenosinylhomocysteine (see p. 273) has now been detected by Cantoni and Scarano [48a] and synthesised by Baddiley and Jamieson.[72a]

During the last 15 years much attention has been directed to compounds containing the so-called high-energy linkages of which adenosine triphosphate, acetyl phosphate [72] and acetyl co-enzyme A [24] are examples. The extreme mobility of a phosphate group in adenosine triphosphate and of the acetyl groups in the other two compounds is attributed to this type of link. Cantoni considers that the sulphonium ions of active methionine and of the other thetins and the quaternary ammonium ion of betaine and aneurin contain this link, which is responsible for the mobility of the methyl or substituted methyl group.

[72a] Baddiley and Jamieson, *J.*, 1955, 1085.

Possible mechanism of methylation by one-carbon fragments

A study of the anti-anæmic principles of liver has revealed the importance of two compounds, pteroylglutamic acid (XXI) and its 5-formyl-5 : 6 : 7 : 8-tetrahydro-derivative, folinic acid. Both have been isolated from liver and the former is probably identical with the folic acid first obtained in 1941 from spinach.



(XXI)

Sauberlich and Baumann [73] showed that *Leuconostoc citrovorum* did not grow on a synthetic medium that was satisfactory for other micro-organisms used in vitamin assay. It was found that crude extracts of liver, yeast, or peptone could support growth when added in traces to the medium. The active material was named citrovorum factor or leucovorin. During a study of the inhibition of *Lactobacillus casei*, which requires folic acid (pteroylglutamic acid) for growth, by the antimetabolite methylfolic acid, it was found that liver extracts were 15 times more effective in reversing the inhibition than could be accounted for by their content of pteroylglutamic acid. This led ultimately to the isolation and synthesis [74, 75, 76, 77] of folinic acid. In a mixture of acetic and formic acid pteroylglutamic acid is *N*-formylated, probably at position 10. Catalytic reduction, followed by autoclaving and chromatotraphy, yields 5-formyl-5 : 6 : 7 : 8-tetrahydrofolic acid. The migration of the formyl group to $C_{(5)}$ probably occurs through an intermediate cyclic compound. Folinic acid has also been synthesised biologically.[78] When folic acid is incubated with liver slices from folic acid-deficient rats, folinic acid is produced in amount equal to that found in normal livers. This suggests that a one-carbon fragment has been supplied through the agency of an enzyme.

We now come to some observations which link folic and folinic acid with biological methylation. In 1950 Sakami and Welch [79] showed that, in the system just mentioned, addition of folic acid increased the synthesis of methyl groups (probably as methionine) from [^{14}C]formate. Moreover a similar effect was noticed in intact rats. Other workers [80] found that rats, fed with folic acid and [^{14}C]formate, incorporated almost ten times as much ^{14}C into body-protein and three times as much into viscera-protein

[73] Sauberlich and Baumann, *J. Biol. Chem.*, 1948, **176**, 165.
[74] Flynn, Bond, Bardos, and Shive, *J. Amer. Chem. Soc.*, 1951, **73**, 1979, 3067.
[75] Keresztesy and Silvermann, *ibid.*, p. 5510.
[76] Pohland, Flynn, Jones, and Shive, *ibid.*, p. 3247.
[77] Welch and Nichol, *Ann. Rev. Biochem.*, 1952, **21**, 633.
[78] Nichol and Welch, *Proc. Soc. Exper. Biol. and Med.*, 1950, **74**, 52.
[79] Sakami and Welch, *J. Biol. Chem.*, 1950, **187**, 379.
[80] Plant, Betheil, and Lardy, *ibid.*, 1950, **184**, 795.

as did rats deficient in folic acid. Much ^{14}C was found in the β-position of serine. Similar results were obtained on administration of [^{14}C]methanol to (*a*) rats deficient in, and (*b*) rats in receipt of, folic acid. Less labelled choline was found in (*a*) than in (*b*). More recent work by Kisliuk and Sakami [66] emphasises the relation of tetrahydrofolic acid to systems involving one-carbon fragments and has enabled a suggestion as to mechanism to be made.

They studied the system glycine–serine in pigeon-liver extracts :

$$HO\cdot{}^{14}CH_2\cdot CH(NH_2)\cdot CO_2H \rightleftarrows H_2N\cdot{}^{14}CH_2\cdot CO_2H + \text{“ C ”}$$

Formate and formaldehyde were employed as one-carbon sources. This reaction is prevented by filtering the liver extracts through Dowex-1 chloride (an ion-exchange resin) and suitable dialysis, thus giving a “ treated extract ”. Such extracts can be re-activated by a single addition of tetrahydrofolic acid, giving rise to an incorporation of the ^{14}C *of glycine* into serine equal to 40 times that observed with the “ treated ” extract and equal to that of the untreated extract *plus* tetrahydrofolic acid. Folic acid and leucovorin are not effective as substitutes for tetrahydrofolic acid.

The treated extracts *plus* [^{14}C]*formaldehyde* introduced very little ^{14}C into serine in presence of glycine. Addition of tetrahydrofolic acid raised the amount of incorporated isotope to the figure obtained with untreated extract, which was 36 times greater than that observed with the treated extract. It was also shown that, in this enzyme system, *the β-carbon of* L-*serine* provides a one-carbon fragment for conversion of labelled glycine into serine.

The authors state : “ These results, in which a rapid interconversion of serine and glycine and utilisation of formaldehyde for serine β-carbon formation has been stimulated in inactivated pigeon liver extracts by a single addition of tetrahydrofolic acid, are consistent with a co-factor role of this substance in certain biosyntheses.”

We now turn to the utilisation of *formate*. In “ treated ” pigeon liver extracts utilisation of formate could be restored by addition of tetrahydrofolic acid, adenosine triphosphate, diphosphopyridine nucleotide (DPN), glucose 2-phosphate, and Mn^{++}, but not by tetrahydrofolic acid alone. [^{14}C]*Formate* incorporated into serine under these conditions was 13 times greater than with untreated extracts.

When folic acid was substituted for its tetrahydro-derivative in this complex mixture the incorporation of formate into serine by the treated extracts was one-fifth of that obtained with tetrahydrofolic acid. Presumably, in presence of adenosine triphosphate formate reacts with folic acid and with tetrahydrofolic acid to give the corresponding 5-formyl derivatives which are then reduced by an enzyme system employing diphosphopyridine nucleotide to give tetrahydro-5-hydroxymethylfolic acid. (The reducing action of enzymes utilising this nucleotide is well-known through the study of carbohydrate metabolism.) This tetrahydro-5-hydroxymethylfolic acid is thought by Kisliuk and Sakami [66] to be the carrier of formaldehyde and indirectly of formic acid in one-carbon metabolism (see above.) The compound has, in fact, been designated “ active formaldehyde ”. The authors

suggest that biosynthesis of serine is allied to the Mannich reaction. "The α-carbon atom of glycine, activated by Schiff base formation with pyridoxal phosphate, combines with a condensation product of formaldehyde and tetrahydrofolic acid." The resulting product gives serine by hydrolysis.

The authors' communication is in abstract and only the role of this hydroxymethyl derivative in serine formation is considered. Presumably the conversion of homocysteine into methionine by a one-carbon fragment would involve the formation of an intermediate *S*-hydroxymethylhomocysteine $HO{\cdot}CH_2{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$ and regeneration of tetrahydropteroylglutamic acid.

Blakley [81] describes a comprehensive study of the interconversion of glycine and serine and the formation of serine from glycine and formaldehyde in extracts of pigeon liver, using ^{14}C. His results and conclusions are very similar to those of Kisliuk and Sakami [66] and he also discusses the probable significance of tetrahydro-5-hydroxymethylpteroylglutamic acid as the carrier of formaldehyde in one-carbon metabolism. He also considers the possibility that this might be the cyclic compound (see p. 282) which could arise by the internal condensation of the 5-hydroxymethyl compound at position 10 or by direct reaction of formaldehyde at positions 5 and 10 of tetrahydropteroylglutamic acid as suggested by Neuberger.

A study [82] of the synthesis of serine in cell suspensions of *Streptococcus faecalis* R emphasises the importance of formate and of pteroylglutamic acid (folic acid) which can, moreover, be replaced by its 10-formyl derivative and its 5-formyltetrahydro-compound (leucovorin, see p. 282). It is considered that folic acid is converted into a derivative (similar to, but probably not identical with, leucovorin) before it is active in serine synthesis by this organism. This may be Kisliuk and Sakami's hydroxymethyl derivative.

The capacity of glycine and serine to furnish the methyl group of thymine (II) has already been mentioned (see pp. 255, 286). Evidence which suggests that cell suspensions of *Bacillus subtilis* can convert uracil (I*b*) (p. 256) into thymine has recently been obtained.[83] Addition of glycine to the suspensions increases the yield by 68%. This is one of the very few cases where, under controlled conditions, the methylating capacity of a bacterium has been demonstrated.

Greenberg [44b] has shown that purine synthesis is connected with formate metabolism. It was found (1948—1950) that the CH_2 group of glycine, the β-carbon atom of serine, and formate can furnish $C_{(2)}$ and $C_{(8)}$ of the purine ring.

Berg [44a] has discussed the incorporation of formate into serine, purines, and the methyl group of methionine in extracts of pigeon liver, processes which are stimulated by homocysteine. Formate and formaldehyde might give $HO_2C{\cdot}CH(NH_2){\cdot}CH_2{\cdot}CH_2{\cdot}S{\cdot}CHO$ and the corresponding $—S{\cdot}CH_2{\cdot}OH$ derivative respectively. These could carry the one-carbon fragment and

[81] Blakley, *Biochem. J.*, 1954, **58**, 448.
[82] (Miss) Lascelles and Woods, *ibid.*, p. 486.
[83] Rege and Sreenivasen, *J. Biol. Chem.*, 1954, **208**, 471.

be interconvertible and reducible to methionine. By reaction with glycine the —S·CH_2·OH compound might yield

$$HO_2C{\cdot}CH(NH_2){\cdot}CH_2{\cdot}CH_2{\cdot}S{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$$

which, Berg suggests, might then yield serine and homocysteine, presumably by hydrolysis. Thymine would presumably arise in a similar manner from uracil (see pp. 256, 284).

The formyl derivative of homocysteine recalls the reversible acetylation of co-enzyme A.[24] The possible biological reduction of S·CH_2·OH to SMe was considered by Challenger and Rawlings [37] in 1937. Blakley [81] suggests, however, that the effect of homocysteine observed by Berg may be more simply explained in connection with its reducing action on formate or on leucovorin.

Vitamin B_{12} (Cobalamin) and Biological Methylation.—It was found at the Lankenau Hospital in Philadelphia [84] that B_{12} allows rats to grow on diets deficient in choline and methionine. Arnstein [85] points out that this might be due to facilitation of the synthesis of growth factors (normally derived from methyl groups) by stimulation of an alternative route. Alternatively B_{12} might be directly concerned with actual methyl synthesis. He therefore studied [85] the quantitative utilisation in rats of serine, glycine, and formate labelled with ^{14}C in presence and in absence of vitamin B_{12}. The vitamin caused a marked increase in the radioactivity of the methyl group of methionine. This was independent of the methyl source and presumably due to stimulation of methionine synthesis from formate or formaldehyde and homocystine. The synthesis of choline was also increased by vitamin B_{12}.

That the vitamin does not affect transmethylation is indicated by the observation that when [*Me*-^{14}C]choline was fed to rats on a diet deficient in vitamin B_{12} there was no significant change in the radioactivity ratio choline : methionine. Were the vitamin concerned in transmethylation its absence would be expected to alter this ratio, the activity of the isolated methionine being lowered and that of the choline being raised.

Although the contrary view has been expressed,[86] a conclusion similar to that of Arnstein was reached by American workers.[87, 88] It was found that synthesis of methionine from homocysteine and betaine can proceed in poults and chicks respectively in absence of vitamin B_{12}. This assumes that the betaine–methionine conversion is a true transmethylation (see p. 281).

Arnstein [89] also found that vitamin B_{12} does not affect the conversion of glycine into serine. Since folic acid is concerned with this relation it appears unlikely that vitamin B_{12} is directly involved in the conversion of folic acid into a co-enzyme form, *e.g.*, " active formaldehyde ".

[84] Bennett, *Science*, 1949, **110**, 589; Toennies, *Fed. Proc.*, 1950, **9**, 234.

[85] Biochemical Society, Symposium on the Biochemistry of Vitamin B_{12}, February 19th, 1955; Arnstein and Neuberger, *Biochem. J.*, 1953, **55**, 259.

[86] Goldthwait and Benedich, *J. Biol. Chem.*, 1952, **196**, 841.

[87] Williams *et al.*, *ibid.*, 1953, **202**, 151, 607.

[88] Smith, *Ann. Rev. Biochem.*, 1954, **23**, 258.

[89] Arnstein, *Adv. Protein Chem.*, 1954, **9**, 1.

He suggests [85] that the effect of vitamin B_{12} on methylation may be exerted on either (*a*) the formation of hydroxymethylhomocysteine

$$HO{\cdot}CH_2{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$$

or (*b*) its reduction to methionine. A formyl derivative of homocysteine R·S·CHO, as envisaged by Berg (see p. 284), may also be involved.[89a]

The relation of the hydroxymethyl group of serine to thymine (II) discussed on p. 255 suggests that a hydroxymethyl derivative, *e.g.*, 5-hydroxymethyluracil, might be an intermediate stage in thymine synthesis. Great interest attaches to the recent isolation [90] of 5-hydroxymethylcytosine on hydrolysis of the deoxyribonucleic acids of certain viruses which infect *Escherichia coli* (*B. coli*) and are known as coliphages. Folic acid appears to be implicated in the synthesis of the methyl group of thymine as well as in the glycine–serine relation and it may be concerned with the hydroxymethylation. The question arises whether cytosine (III) gives the hydroxymethyl derivative directly or whether it is first deaminated to uracil which then undergoes hydroxymethylation and re-amination.

It is remarkable that, although 5-methylcytosine (IV) occurs in small amounts in the deoxyribonucleic acids of all higher animals and plants yet examined, it is absent [91] from bacteria, phages, and insect and plant viruses. The coliphages at any rate appear unable to reduce the hydroxymethyl group to methyl.

Asperthecin,[92] the purple-red dye from *Aspergillus quadrilineatus*, appears to be either 3 : 4 : 5 : 6 : 7- or 1 : 4 : 5 : 6 : 7-pentahydroxy-2-hydroxymethylanthraquinone, structures which may be compared with those of the natural methylpolyhydroxyanthraquinones (p. 256). Further, rhodocladonic acid, another polyhydroxyanthraquinone derivative obtained from lichens, contains a hydroxymethyl and a methoxycarbonyl group, which have presumably a similar origin.

These compounds are of interest on account of the increased attention now being given to the hydroxymethyl group in plant biochemistry.

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[89a] Elwyn and Sprinson, *J. Biol. Chem.*, 1954, **207**, 459, 467.

[90] Putnam, *Adv. Protein Chem.*, 1953, **8**, 222, 235, 262; Wyatt and Cohen, *Biochem. J.*, 1953, **55**, 774.

[91] Wyatt, *ibid.*, 1951, **48**, 581, 584.

[92] Howard and Raistrick, *ibid.*, 1955, **59**, 475.