Studies on Biological Methylation. Part XIV. The Formation of Trimethylarsine and Dimethyl Selenide in Mould Cultures from Methyl Sources containing* ^{14}C.

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The mechanism of 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, has been studied by the use of choline chloride, betaine, sodium formate, and DL-methionine as potential sources of the methyl group labelled with ^{14}C. The mercurichlorides of the trimethylarsine and dimethyl selenide were radioactive in each case. The activity was very considerable with methionine, from which the $^{14}CH_3$ is probably transferred intact to arsenic and selenium. Addition of [^{14}C]choline and [^{14}C]formate to cultures of *S. brevicaulis* containing diethyl disulphide or *S*-ethylcysteine gave inactive ethanethiol and radioactive ethyl methyl sulphide.

WORK carried out in Leeds since 1931 has established that in bread cultures of the mould *Scopulariopsis brevicaulis* arsenious oxide or sodium arsenate, sodium selenate or selenite, and potassium tellurate or tellurite are converted into trimethylarsine, dimethyl selenide, and dimethyl telluride respectively. These volatile products were characterised as the mercurichlorides. Numerous other derivatives were also employed for this purpose [Challenger, Higginbottom, and Ellis, *J.*, 1933, 95; Challenger and Higginbottom, *Biochem. J.*, 1935, **29**, 1757; Challenger and North, *J.*, 1934, 68; Bird and Challenger, *J.*, 1939, 163].

Methylation is also observed with the alkylarsonic and dialkylarsinic acids, $R{\cdot}AsO(OH)_2$ and $RR'AsO{\cdot}OH$, giving $AsRMe_2$ and $AsRR'Me$, and with the alkaneseleninic acids $R{\cdot}SeO_2H$ giving SeRMe, where R and/or R′ = Me, Et, or Pr^n [Challenger and Ellis, *J.* 1935, 396; Challenger and Rawlings, *J.*, 1936, 264; Bird and Challenger, *J.*, 1942, 574]. Other moulds (including *Aspergillus niger* and *Penicillium notatum*) which can effect one or more of these methylations are cited by Bird, Challenger, Charlton, and Smith (*Biochem. J.*, 1948, **43**, 78).

The methylating action of *S. brevicaulis* is also exerted on aliphatic disulphides $RS{\cdot}SR$ (R = Me to n-C_5H_{11}) and on *S*-alkylcysteines $RS{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$ (R = Me to Pr^n) giving RSH and SRMe in each case (Challenger and Rawlings, *J.*, 1937, 868; Challenger and Charlton, *J.*, 1947, 424). Methionine $MeS{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$ also yields methanethiol and dimethyl sulphide. The importance of the last result will be considered below.

These results, and experiments designed to test the hypotheses which were put forward to explain them, have been discussed by one of us in various reviews (*Chem. and Ind.*, 1942, 399, 413, 456; *Chem. Reviews*, 1945, **36**, 315; *Ann. Reports*, 1946, **43**, 262; *Adv. Enzymology*, 1951, **12**, 429). It seemed possible that formaldehyde, glyoxylic acid, or some closely related compound (Robinson's " formaldehyde equivalent," *J.*, 1917, **111**, 879; *J. Roy. Soc. Arts*, 1948, **96**, 796; IX Int. Congr. Pure and Appl. Chem., Madrid, 1934), arising

* Part XIII, Challenger and Liu, *Rec. Trav. chim.*, 1950, **69**, 334.

from glycine or possibly from purines, might by reaction with arsenious acid yield $HO{\cdot}CH_2{\cdot}AsO_3H_2$. This on reduction might give $Me{\cdot}AsO_3H_2$. Repetition of the process would finally yield trimethylarsine. Similar arguments could be employed to explain the formation of the methylated sulphur, selenium, and tellurium compounds. Conclusive evidence in favour of this hypothesis was difficult to obtain and attention was gradually focused on a mechanism involving the loss of a methyl group from some labile methyl compound present in the mould cell, followed by its attachment as such to arsenic and other elements. This was discussed by one of us and Higginbottom in 1935, and again in 1942.

The suggestion of a methyl transfer in animal metabolism was first advanced by Hofmeister (*Arch. exp. Path. Pharm.*, 1894, **33**, 209, 213) who, when discussing the formation of methylpyridinium hydroxide and dimethyl telluride in animals stated " (Es) ist anzunehmen dass die CH_3 Gruppe in den Geweben welche das Vermögen der Methylierung besitzen, als solche vorgebildet ist. . . . Bei Anwesenheit von Pyridin und Tellur käme es zur Methylierung dieser, während normalerweise methylhaltige Stoffe anderer Art, z.B. die Körper der Cholin und der Kreatingruppe entständen." Hofmeister did not mention any particular compound as a possible methyl source. Riesser (*Z. physiol. Chem.*, 1913, **86**, 440) described experiments in which the methyl of betaine and choline was transferred at a high temperature to selenium and tellurium. This work was expanded by Challenger and Higginbottom (*loc. cit.*, 1935) and by Challenger, Taylor, and Taylor (*J.*, 1942, 48).

Between 1939 and 1942 du Vigneaud and his school (see du Vigneaud, " A Trail of Research," Cornell Univ. Press, Ithaca, New York, 1952) provided the first experimental proof that methyl transfer (" transmethylation ") can occur in young white rats, the methyl sources being shown to be choline and methionine. The samples administered contained a high proportion of deuteromethyl groups. Much further work by du Vigneaud, using various methyl sources labelled with ^{14}C or with deuterium or with both, has more clearly defined the mechanism and suggested various intermediate stages.

It is convenient, though not always possible, to distinguish between a methyl " donor " and a methyl " source." It has been rigidly demonstrated that the methyl group of methionine can be transferred intact (du Vigneaud, Cohn, Chandler, Schenck, and Simmonds, *J. Biol. Chem.*, 1941, **140**, 625). In the cases of choline (Simmonds, Cohn, Chandler, and du Vigneaud, *ibid.*, 1943, **149**, 519), betaine (du Vigneaud, Simmonds, Chandler, and Cohn, *ibid.*, 1946, **165**, 639), acetothetin chloride $Cl^-\{Me_2S^+{\cdot}CH_2{\cdot}CO_2H$ (du Vigneaud, Moyer, and Chandler, *ibid.*, 1948, **174**, 477), and dimethyl-β-propiothetin chloride $Cl^-\{Me_2S^+{\cdot}CH_2{\cdot}CH_2{\cdot}CO_2H$ [first isolated from the red alga *Polysiphonia fastigiata*, by one of us and Miss M. I. Simpson (*J.*, 1948, 1591)] (Maw and du Vigneaud, *J. Biol. Chem.*, 1948, **174**, 381, 477; **176**, 1029, 1037; Dubnoff and Borsook, *ibid.*, 1948, **176**, 789) the carbon atom of a methyl group is certainly transferred to homocysteine, giving rise to methionine, but the exact mechanism, which may involve many stages, has not been determined. It is possible that the carbon atom of the methyl group is detached as formaldehyde or formic acid which by reaction with a thiol or an amino-group and subsequent reduction may give rise to SMe or NHMe.

It is fairly clear, however, that the effective fragment is not carbon dioxide (du Vigneaud, Verly, and Wilson, *J. Amer. Chem. Soc.*, 1950, **72**, 2819; Arnstein, *Biochem. J.*, 1951, **48**, 27; Sakami, *J. Biol. Chem.*, 1948, **176**, 995; 1949, **179**, 495) although the elimination of carbon dioxide has frequently been observed during the metabolism of methylthio- and methylamino-derivatives in animals (for references see *Adv. Enzymology*, 1951, **12**, 480).

Sakami (*J. Biol. Chem.*, 1948, **176**, 995; 1949, **179**, 495), du Vigneaud, Verly, and Wilson (*loc. cit.*), and Arnstein (*loc. cit.*) developed the conception of the one-carbon fragment which again brought the formaldehyde hypothesis, or some very similar scheme (see p. 1760), into the foreground as an alternative mechanism for biological methylation. An example may be cited. On administration of glycine, serine, or formate labelled with ^{14}C as shown, $H_2N{\cdot}{}^{14}CH_2{\cdot}CO_2H$, $HO{\cdot}{}^{14}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$, and $H{\cdot}{}^{14}CO_2Na$, the labelled carbon atom appeared in the methyl groups of the choline of the tissues of rats. Reference may also be made to two recent publications in this field by Berg (*J. Biol. Chem.*, 1953, **205**, 145) and Greenberg (*Fed. Proc.*, 1953, **12**, 651).

It follows that true transmethylation involving an intact methyl group is not the only

process concerned in biological methylation. This phenomenon may involve (*a*) true transmethylation, (*b*) transfer of a one-carbon fragment followed by reduction, or (*c*) process (*b*) followed by process (*a*) as the last stage.

Recently we have 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. The sources were choline chloride, betaine, sodium formate, and DL-methionine. Only one methyl group in choline and betaine was labelled. The trimethylarsine and dimethyl selenide 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

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

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 $SMeEt,2HgCl_2$; $n = 2$ for $SMe_2,HgCl_2$. The value f is of importance since in choline, betaine, dimethylacetothetin $Me_2S^+\cdot CH_2\cdot CO_2^-$, and dimethyl-β-propiothetin $Me_2S^+\cdot CH_2\cdot CH_2\cdot CO_2^-$ only one methyl group is labile in animals or in tissue preparations (du Vigneaud, Simmonds, Chandler, and Cohn, *J. Biol. Chem.*, 1946, **165**, 639; du Vigneaud, Simmonds, and Cohn, *ibid.*, 1946, **166**, 47; Borsook and Dubnoff, *ibid.*, 1948, **176**, 789; Maw and du Vigneaud, *ibid.*, p. 1037).

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.

We have also shown in separate preliminary experiments in cultures of *A. niger* on medium (G) (see p. 1765) that the D- and L- enantiomorphs of $^{14}CH_3$-methionine can both act as methyl donors to selenium to a considerable extent. These results will be described in a forthcoming publication.

It therefore appears 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 importance of methionine and formate for transmethylation has also been established for the dimethylamino-group of hordenine in barley seedlings (Kirkwood and Marion, *Canad. J. Chem.*, 1951, **29**, 30; Dubeck and Kirkwood, *J. Biol. Chem.*, 1952, **199**, 307) and the NMe group of nicotine (Brown and Byerrum, *J. Amer. Chem. Soc.*, 1952, 74, 1523). The methyl of the >NMe and methoxyl groups of ricinine of castor beans (Dubeck and Kirkwood, *loc. cit.*) arise from methionine, and the methyl of the methoxyl group of barley lignin is transferred intact from this amino-acid (Byerrum and Flokstra, *Fed. Proc.*, 1952, **11**, 193; Byerrum, Dewey, and Ball, *ibid.*, 1953, **12**, 186).

The dominance of methionine in mould methylation is also suggested by our observations that the methylation percentage for the systems [^{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 with the observation of Borsook (*J. Biol. Chem.*, 1945, **160**, 635) that homocystine or homocysteine is necessary before the methyl-carbon atom of choline can be transferred to guanidoacetic acid, yielding creatine.

These results suggest that methylation by *S. brevicaulis* and *A. niger* involves a true transmethylation mechanism from methionine which may, however, be produced from homocysteine by acceptance of some other one-carbon fragment, detached from betaine, choline, or formate.

It seems probable, nevertheless, that carbon dioxide is not an intermediate. This follows from a consideration of the " CO_2 ratio " which is obtained by dividing the specific activity of the carbon dioxide evolved from the cultures by the specific activity of the

methylated product per carbon atom (see Tables 1, 2, and 5). If radioactive carbon dioxide arising from the labelled sources by mycological oxidation is in equilibrium within the cell with the carbon dioxide evolved by the normal metabolism of the mould this ratio must be at least unity if carbon dioxide is an intermediate. Except in the case of *A. niger* and sodium formate this ratio was always fractional, and with methionine as methyl source was extremely low.

There is much evidence from animal metabolism to show that carbon dioxide is not a source of the methyl group (du Vigneaud, *J. Amer. Chem. Soc.*, 1950, **72**, 2819; Arnstein, *loc. cit.*). Ressler, Rachele, and du Vigneaud (*J. Biol. Chem.*, 1952, **197**, 1) showed by the use of [^{14}C,α-D]formic acid, D·$^{14}CO_2H$, that no oxidation of the non-carboxylic hydrogen atom of formate occurs during synthesis of choline-methyl groups in the rat, thus excluding carbon dioxide as a possible precursor of the methyl group. Kirkwood and Marion (*loc. cit.*) reached similar conclusions from their work on hordenine formation in barley seedlings.

Methylation of sulphur by [^{14}C]choline and [^{14}C]formate has also been detected in bread cultures of *S. brevicaulis* containing diethyl disulphide or *S*-ethylcysteine. Fission of the S–S (Challenger and Rawlings, *J.*, 1937, 868; Blackburn and Challenger, *J.*, 1938, 1872) and S–C linkages (Challenger and Charlton, *J.*, 1947, 424) produces ethanethiol [detected as non-radioactive $(EtS)_2Hg$] and radioactive ethyl methyl sulphide (detected and counted as the mercurichloride).

It is seen from Tables 2, 5, and 7 that the percentage methylation of selenium is much greater in cultures of *A. niger* on a liquid medium (G) containing sucrose, inorganic salts, glycine, and DL-methionine than when this organism or *S. brevicaulis* is grown on bread. This we attribute to the dilution effect of the methionine in the protein of the bread. A similar explanation may account for the much lower methylation figure observed when non-radioactive methionine methiodide is added to *A. niger* cultures on medium (G) containing [^{14}C]methionine and selenate (Table 8). Presumably methionine is produced by demethylation of the methiodide and dilutes the radioactive methionine.

Another reaction, however, occurs here—fission of the methiodide to dimethyl sulphide —a decomposition observed by Challenger and Charlton (*loc. cit.*) using bread cultures of *S. brevicaulis* and by Challenger and Liu (*Rec. Trav. chim.*, 1950, **69**, 334) with *Penicillium notatum* on both liquid and bread media. We have shown, however (see p. 1770), that this is not the cause of the low activity of the dimethyl selenide mercurichloride.

In the experiments with sodium selenate the purity of the dimethyl selenide mercurichloride was checked by m. p., often by mixed m. p. (Challenger and North, *J.*, 1934, 68), and in several cases by recrystallisation without alteration in specific activity. In one case crystallisation from aqueous mercuric chloride lowered the activity (owing to partial conversion into a higher mercurichloride, see p. 1770), but on decomposition with sodium hydroxide and re-absorption in mercuric chloride the original activity was observed. It appeared conceivable that part of the radioactivity of the trimethylarsine and dimethyl selenide mercurichlorides might be due to a mercuric chloride compound of an alkylamine or alkylamine salt arising through mycological fission of the carbon–nitrogen link in choline or betaine. This possibility was eliminated when it was shown that the hydrochlorides of methylamine, diethylamine, and trimethylamine gave no precipitate in either acidified or neutral mercuric chloride. No evidence for such a C–N fission was obtained by Challenger and Higginbottom with betaine in cultures of *S. brevicaulis* on a modified Czapek–Dox medium (*Biochem. J.*, 1935, **29**, 1768), or by us in cultures of *A. niger* on bread (see p. 1769).

In the experiments where methionine was used as a methyl donor it seemed possible that complications due to subsidiary reactions might arise. *S. brevicaulis* in bread cultures containing the *S*-alkylcysteines gives alkanethiol and alkyl methyl sulphide (see p. 1760). This action is exerted to a much smaller extent on methionine, especially in liquid media. With *A. niger* Challenger and Charlton (*loc. cit.*) observed only a slight thiol or sulphide odour in bread cultures containing 0·5% of DL-methionine. Nevertheless, when using this amino-acid labelled in the methyl group with ^{14}C, radioactive methanethiol, dimethyl sulphide, and traces of dimethyl disulphide (from oxidation of the thiol) might be evolved. Contamination of the precipitated dimethyl selenide and trimethylarsine mercurichlorides

must therefore be avoided. The methanethiol is not a danger, as it is completely removed by mercuric cyanide. In no case were more than traces of its mercury derivative observed. Dimethyl sulphide, the amount of which is usually roughly equivalent to that of methanethiol, yields a mercurichloride which is rather unstable in Biginelli's solution (mercuric chloride in hydrochloric acid, *J.*, 1933, 99) and most of it would pass on and be absorbed in the third reagent, neutral mercuric chloride, giving $2SMe_2,3HgCl_2$. Any disulphide would give a precipitate of the formula $MeS{\cdot}HgCl,xHgCl_2$ in this reagent (Blackburn and Challenger, *J.*, 1938, 1874) which could therefore deposit a mixture containing traces of these two sulphur compounds as well as dimethyl selenide mercurichloride, $SeMe_2,HgCl_2$. On treatment of the precipitate with sodium hydroxide and aspiration afresh into acid mercuric chloride, however, only the dialkyl selenide and sulphide volatilise and again most of the dimethyl sulphide would pass through the Biginelli's solution. This is indicated by Experiment 3, Table 8, and in Table 9, where the *A. niger* cultures contained, in addition to selenate and [^{14}C]methionine, inactive dimethyl-β-propiothetin bromide which with *S. brevicaulis* on bread yields only about 3% of dimethyl sulphide (Challenger and Liu, *loc. cit.*) but in liquid cultures of *A. niger* produces considerable quantities. Here the methylation percentage and m. p. observed with the first deposit are very low, owing to contamination with inactive dimethyl sulphide mercurichloride. Repeated decomposition with alkali and slow aspiration afresh into acid mercuric chloride raise the m. p. considerably and the methylation percentage then agrees with that obtained in other experiments, where only traces of dimethyl sulphide could have been produced.

This suggests that, in comparison with methionine, dimethyl-β-propiothetin bromide is negligible as a direct methyl-donor, otherwise a dilution effect would have been observed at constant activity. A similar result was obtained with dimethylacetothetin chloride, except that this does not yield dimethyl sulphide in mould cultures (Challenger and Liu, *loc. cit.*). The same conclusion may be reached in the case of betaine where no dilution effect was detected with selenate in *A. niger* cultures (Table 8). It may appear surprising that these 'onium compounds, including the methylmethioninesulphonium ion are so much inferior to methionine as methyl sources, especially as a methylated 'onium structure has frequently been suggested as being particularly suitable as a methyl source.

The recent work of Cantoni and his colleagues (*J. Amer. Chem. Soc.*, 1952, **74**, 2942; Baddiley, Cantoni, and Jamieson, *J.*, 1953, 2662; Cantoni, *J. Biol. Chem.*, 1953, **204**, 403) explains these findings. They have shown that, in an enzyme system obtained from rabbit liver and containing adenosine triphosphate, methionine yields orthophosphoric acid and an " active methionine " which they regard as responsible for transmethylation to guanidinoacetic acid, nicotinamide, and dimethylaminoethanol, giving creatine, *N*-methylnicotinamide, and choline respectively. To this compound they assign the annexed quaternary

$$NH_2{\cdot}C_5H_2N{-}\overbrace{CH{\cdot}CH(OH){\cdot}CH(OH){\cdot}CH}^{O}{\cdot}CH_2{\cdot}\overset{+}{S}Me{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2^-$$

structure. It is possible, therefore, that this complex—the *S*-adenosylmethionine ion—is concerned also in the transfer of the methionine-methyl group to arsenic or selenium in mycological methylation. If so it is obvious that the simple methylsulphonium derivative of methionine cannot enter into such a combination unless it first loses a methyl group to form methionine and that methionine is therefore a more effective methyl source than its own methylsulphonium derivative or, probably, any other simple 'onium compound.

EXPERIMENTAL

The radioactive choline was prepared by interaction (in a vacuum-train cooled by liquid air) of [^{14}C]methyl iodide with dimethylaminoethanol (Ferger and du Vigneaud, *J. Biol. Chem.*, 1950, **185**, 53.) The labelled choline iodide was converted into the chloride which was then diluted with inactive chloride (Found, for the corresponding picrate: C, 42·6; H, 10·5. Calc. for $C_{11}H_{16}O_8N_4$: C, 43·0; H, 10·1%). It showed 637·7 counts per minute (c.p.m.), *i.e.*, 212,000 c.p.m. per mmole. The activity was 308×10^{-6} μc per mg. by comparison with a sample of the picrate standardised by the Atomic Energy Research Establishment, Harwell. The activity calculated as choline chloride was $102{\cdot}2 \times 10^{-3}$ μc per mmole. The choline chloride was oxidised to betaine in 86% yield by potassium permanganate in very dilute, slightly acid solution (Lintzel and Fomin, *Biochem. Z.*, 1931, **238**, 438). The radioactive betaine was converted into the hydro-

chloride, m. p. 238° (Found: C, 39·0; H, 7·6; Cl, 22·9. Calc. for $C_5H_{12}O_2NCl$: C, 39·1; H, 7·9; Cl, 23·1%). Its activity was $102·2 \times 10^{-3}$ μc per mmole. It was converted into the picrate, m. p. 184°, which gave 614·5 c.p.m., *i.e.*, 213,000 c.p.m. per mmole. This shows that the plating method described below gives consistent results.

Radioactive sodium formate was diluted with " AnalaR " material by dissolution in water and evaporation to dryness *in vacuo* over phosphoric oxide. Titration with neutral potassium permanganate gave: equiv., 67·6 (Calc.: 68·0). The specific activity was: $126·5 \times 10^{-3}$ μc per mmole.

Radioactive methionine was prepared from *S*-benzylhomocysteine, sodium, and [^{14}C]methyl iodide in liquid ammonia (Melville, Rachele, and Keller, *J. Biol. Chem.*, 1947, **169**, 419) and finally diluted to a convenient activity. Dissolution of *S*-benzylhomocysteine in ammonia and interaction with sodium were facilitated by stirring with a stream of pure hydrogen. Sodium wire was added until a blue colour persisting for 10 min. was produced. (When carefully dried nitrogen, from a cylinder, was used as a stirrer no permanent blue colour could be obtained regardless of the amount of sodium added. This was attributed to the effect of traces of oxygen in the nitrogen.) The colour was then discharged by careful addition of inactive methyl iodide. The [^{14}C]methyl iodide, warmed to 50°, was then introduced in a stream of hydrogen. Inactive methyl iodide was then added till the ammonia solution no longer gave a positive nitroprusside reaction for the thiol group. Any radioactive methionine remaining in the mother-liquors after crystallisation of the final product was removed by addition of the non-active amino-acid and separated as described by Melville *et al.* (*loc. cit.*). On the second operation the isolated methionine had only a low radioactivity. The united fractions were then crystallised from aqueous alcohol and dried *in vacuo* over phosphoric oxide (radioactive yield 48%). Paper-chromatography with 80% phenol or butanol–acetic acid–water as the moving phase gave two spots with ninhydrin, due to methionine and its sulphoxide. The sulphoxide is known to be produced by oxidation of methionine on the filter-paper during the chromatographic process (Dent, *Biochem. J.*, 1948, **43**, 169). The methionine was analysed (Found: C, 40·3; H, 7·3; N, 9·0; S, 21·1. Calc. for $C_5H_{11}O_2NS$: C, 40·25; H, 7·45; N, 9·4; S, 21·5%); its specific activity was 356×10^{-3} μc per mole. The radioactive sodium formate and methyl iodide were obtained from the Radiochemical Centre, Amersham.

Optically active methionines were prepared by resolution of DL-homocystine obtained from DL-methionine and sulphuric acid (Butz and du Vigneaud, *J. Biol. Chem.*, 1932, **99**, 135). *S*-Benzyl-*N*-formylhomocysteine was resolved by brucine (du Vigneaud and Patterson, *ibid.*, 1935, **109**, 97) and after removal of the formyl and benzyl groups was converted into methionine with [^{14}C]methyl iodide. The radioactive D- and L-methionine had $[\alpha]_D^{20}$ +7·75° and −7·6° in 1% aqueous solution. Du Vigneaud and Patterson (*loc. cit.*) give $[\alpha]_D^{25}$ +8·0° and −7·7°. Windus and Marvel (*J. Amer. Chem. Soc.*, 1930, **52**, 2675) give $[\alpha]_D^{25}$ +8·7° and −8·1°. The purity of both isomers was checked as in the case of DL-methionine by paper chromatography. Two spots were obtained as before. Activities were for D-methionine 725×10^{-3} and for L-methionine 1440×10^{-3} μc per mmole.

Culture Media.—(a) *Solid.* Bread crumbs (150 g.) and distilled water (25 c.c.) were sterilised in 1-l. conical flasks plugged with cotton wool for 25 min. at 15 lb. pressure.

(b) *Liquid.* A modified Czapek–Dox medium was prepared from sodium nitrate (2·0 g.), potassium chloride (0·5 g.), magnesium sulphate (0·5 g.), ferrous sulphate (0·01 g.), and potassium dihydrogen phosphate (1·0 g.) in water (1 l.). The medium (300 c.c.) with 10% of sucrose and 0·25% (10 mmole) of glycine was sterilised as before in 1·5-l. conical flasks and designated Medium G.

Cultivation of Organisms and Addition of Substrates.—Two moulds were used: (1) *Scopulariopsis brevicaulis*, strain Washington 2, N.C. Type Cultures No. 580 (designated Strain D by Challenger, Higginbottom, and Ellis, *J.*, 1933, 98), was used only on bread medium, which was heavily inoculated from a sub-culture on a potato–agar slope. Incubation was usually at 35° for 4—5 days followed by a similar period at room temperature. (2) *Aspergillus niger*, strain No. 17, obtained from Dr. T. K. Walker of the Manchester College of Technology, was sub-cultured on wort–agar slopes. When grown on sterile bread the cultures were incubated for 2 days at 35° and 1—2 days at room temperature, growth and sporing being more rapid than with *S. brevicaulis*. The Medium G after inoculation with *A. niger* was incubated at 35° for 2—3 days and left at room temperature for a similar time, to yield a thin, even, lightly sporing mycelial pad. Too vigorous growth is disadvantageous. The substrates and methyl sources, if stable, were sterilised as before. Radioactive materials were usually sterilised in separate tubes, each containing 15 c.c. of water, which was then added to the cultures. Arsenious oxide

or sodium selenate solutions were sterilised in bulk and added by sterile pipette. Compounds unstable to heat were sterilised in aqueous solution by Seitz filtration and added from the graduated vacuum-receiver.

After addition of the materials to the cultures the cotton-wool plugs were replaced by two-hole rubber bungs fitted with sterilised inlet and outlet tubes loosely plugged with cotton wool. The flasks (usually two or three) were then connected in series, and volatile matter was removed by a slow stream of air which was freed from carbon dioxide and any traces of aliphatic sulphides or selenides which might be present in the laboratory air by previous passage through 2N-sodium hydroxide, 3% mercuric chloride, and sterile cotton wool. On issuing from the cultures the air passed through a series of selective reagents : (*a*) aqueous mercuric cyanide which removed hydrogen sulphide, thiols, and selenothiols—only in special cases were these present; (*b*) acid mercuric chloride which removed trimethylarsine as the dimercurichloride, and most of the dimethyl selenide as the mercurichloride; (*c*) neutral mercuric chloride which absorbed the remainder of the dimethyl selenide, together with dialkyl sulphides and disulphides; (*d*) two flasks of aqueous potassium hydroxide (10—15%), containing phenolphthalein, which absorbed carbon dioxide. When the original colourless solution became pink a fresh flask was employed. The carbon dioxide was then estimated volumetrically.

Purification of End-products for Counting.—Carbon dioxide was precipitated as barium carbonate by addition of barium chloride to the 10—15% potassium hydroxide solution used as absorbent, separated in a centrifuge, washed with water, and dried at 100°. The trimethylarsine and dimethyl selenide, which were known to be pure (*J.*, 1933, 95; 1934, 68; *Biochem. J.*, 1948, **43**, 78), were precipitated as the mercurichlorides. It was, therefore, considered unnecessary to recrystallise these compounds to constant activity in every case, though this was often done when using dimethyl selenide mercurichloride. Contamination with a higher or lower mercurichloride would affect the specific activity and this factor has been investigated for dimethyl selenide (Table 10). The object of the work was to compare the effectiveness of various methyl sources and rather less stress has been laid on obtaining an accurate value for the specific activity of each deposit. The results are, however, comparable and repeatable.

In some experiments (see p. 1769) it seemed possible that traces of dimethyl sulphide might contaminate the dimethyl selenide. In a few cases its presence was definitely established. Consequently the mercurichloride was treated with aqueous sodium hydroxide, and the liberated selenide and sulphide were removed in a stream of purified air. Passage through (*a*) mercuric cyanide and (*b*) acidified and (*c*) neutral mercuric chloride as before gave no precipitate in (*a*), while dimethyl selenide collected as the mercurichloride in (*b*) and any dimethyl sulphide was precipitated in (*c*). When only traces of the sulphide were present the aspiration was usually continued for 14 hr., so as to remove all sulphide from (*b*). With larger quantities (see Table 9) the process was repeated several times with the precipitate in (*b*). The purity of the deposits was checked by m. p. and often by mixed m. p. determinations.

Details of Radioactive Assay.—The active barium carbonate, sodium formate, methionine, and choline and betaine picrates were spread evenly in a circular depression in a " Perspex " disc (2·55 sq. cm.) and compacted with a close-fitting chromium plated metal plunger. The mercurichlorides adhered to the metal and could not be treated in this way. The " Perspex " discs were perforated, fitted with a filter paper, and made the base of a cylindrical funnel. This was half-filled with water, the mercurichloride added as a fine aqueous suspension, and gentle suction applied. The uniform layer so formed was dried in air. The weight of material on the plates was wherever possible greater than 0·07—0·08 g., the minimum weight required to obtain a deposit of " infinite thickness " with the particular discs used. This was determined by recognised procedure, with choline picrate as standard.

Other active materials were also compared with this standard, but normally absolute values in μc per mmole were not required because the methylation percentage (see p. 1762) was usually calculated by direct comparison of the c.p.m. of the product and the methyl source. Specimens were dried by 15 minutes' air-suction, followed by 2—3 hours' storage in a vacuum-desiccator. A thin end-window Geiger–Müller counter, " lead castle," Standard H.T. scaler, and probe units were used.

Counting and calculation of errors were carried out as described by Fisher (" Statistical Methods for Research Workers," Oliver and Boyd, Edinburgh, 1950) and by Kamen (" Radioactive Tracers in Biology," 2nd Ed., Academic Press, New York, 1950). Counting errors are quoted only where they are of approximately the same order of magnitude as the observed figures, as, *e.g.*, in Table 6. The effect of " back-scatter " due to the heavy mercury atoms was neglected and may render all methylation percentages 6—10% high (Dransfield and

Challenger, unpublished work) but this factor will be constant, and since all calculations of methylation percentage are based on mercurichlorides the results will be strictly comparable.

" Biological " errors arising from variations in intensity of inoculation, rate of growth, or rate of metabolism are difficult to assess but the results were reproducible. Controls were set up wherever necessary.

Results.—Results are shown in Tables, to which the following notes are added.

TABLE 1.

Mould : *S. brevicaulis*, Washington 2.
Medium : Bread, 150 g. per flask.
Substrate : Arsenious oxide, 0·25 mmole per flask.
Trimethylarsine collected and counted as $AsMe_3,2HgCl_2$.

Methyl source	Concn. (mmole per flask)	Days of aspiration	Methylation (%)	CO_2 (mmole)	Activity (%) recovd. as CO_2	CO_2 ratio
[*Me*-^{14}C]Choline chloride	1	14	1·9	968	—	—
[*Me*-^{14}C]Betaine	1	21	0·7	2900	—	—
Na [^{14}C]formate	5	14	3·1	2035	71	0·18
DL-[*Me*-^{14}C]Methionine ...	1	5	28·3	450	0·3	0·00008
		15	13·5	488	0·4	

Table 1. The m. p. of the five deposits of trimethylarsine dimercurichloride were respectively 265°, 263—265°, 262—264°, 261—263° (mixed m. p. 261—263°), and 262° (mixed m. p. 261—262°) (all with decomp.). Challenger, Higginbottom, and Ellis (*loc. cit.*) give m. p. 265° (decomp.). The yields of the mercurichloride varied from 9 to 18%. Addition of methionine does not affect the yield (see Tables 3 and 6). Unpublished work by Dr. Marjorie L. Bird showed that, in similar experiments without addition of any methyl source, after more than a year's aspiration the yield of trimethylarsine calculated on the arsenious oxide was only about 85%.

TABLE 2.

Mould : *S. brevicaulis*, Washington 2.
Medium : Bread, 150 g. per flask.
Substrate : Sodium selenate, 2·5 mmole per flask.
Dimethyl selenide collected and counted as $SeMe_2,HgCl_2$.

Methyl source	Concn. (mmole per flask)	Days of aspiration	Methylation (%)	CO_2 (mmole)	Activity (%) recovd. as CO_2	CO_2 ratio
[*Me*-^{14}C]Choline chloride	1·0	14	1·2	1080	—	—
	5·0	7	4·1	1240	47	0·3
		14	3·1			
[*Me*-^{14}C]Betaine	1·0	14	1·1	1260	—	—
	5·0	7	4·0	1230	43	0·3
		14	2·3			
Na [^{14}C]formate	(1) 5·0	14	4·4	1288	100 ± 10	0·2
	(2) 5·0	1·5	—	346	57	
		5 [a]	5·8	441	10	
		7	3·0	422	4·7	
		9	2·7	335	2·5	
		14	0·9	513	1	
DL-[*Me*-^{14}C]Methionine ...	1·0	4 [b]	25·5	351	0·15	0·00005
		15 [c]	12	582	0·8	

Table 2. On three occasions the m. p. of the mercurichloride was taken : (*a*) 152—154°, (*b*) 152—154° and mixed m. p. 153—154°, (*c*) m. p. and mixed m. p. 152—153°. Dimethyl selenide mercurichloride has m. p. 153—154° (Challenger and North, *loc. cit.*). The yield averaged 11% except when methionine was present, the figure then being 6%. Other examples of this effect are mentioned in Tables 3 and 6.

TABLE 3.

Mould : *S. brevicaulis*, Washington 2.
Medium : Bread, 150 g. per flask
Substrate : Diethyl disulphide, 4·1 mmole per flask.
Methyl ethyl sulphide collected and counted as $SMeEt,2HgCl_2$.

Methyl source	Concn. (mmole per flask)	Days of aspiration	Methylation (%)	CO_2 (mmole)	Activity (%) recovd. as CO_2
[*Me*-^{14}C]Choline chloride	3·6	7	2·4	980	24·2
Na [C^{14}]formate	3·6	3	6·5	360	55
		6	4·8	490	13·5
		9	—	350	7
		12	—	380	3

Table 3. The m. p. of the ethyl methyl sulphide mercurichloride was 125—126°. Challenger and Rawlings (*J.*, 1937, 868) give m. p. 127—128°. The yields were 7·5—8·5%, calc. on the disulphide.

TABLE 4.

Substrate : *S*-Ethylcysteine, 1·66 mmole per flask. Other conditions as in Table 3. Yield of mercurichloride, 8%.

Methyl source	Concn. (mmole per flask)	Days of aspiration	Methylation (%)	CO_2 (mmole)	Activity (%) recovd. as CO_2
[*Me*-^{14}C]Choline chloride	3·6	12	0·8	2180	38·7
Na [^{14}C]formate	3·6 *	9	8·1	470	89

* Very slow aspiration.

TABLE 5.

Mould : *A. niger* 17.
Medium : Bread, 150 g. per flask. Here, and in Tables 6—8 the substrate was sodium selenate (2·5 mmole per flask) and the dimethyl selenide was collected and counted as $SeMe_2,HgCl_2$.

Methyl source	Concn. (mmole per flask)	Days of aspiration	Methylation (%)	CO_2 (mmole)	Activity (%) recovd. as CO_2	CO_2 ratio
[*Me*-^{14}C]Choline chloride	5·0	3	0·1	300	1	0·5
		11	1·0	300	5	
[*Me*-^{14}C]Betaine	5·0	5	1·1	340	3·6	—
		10	5·3	470	9·8	0·1
		19	1·8	400	4·0	
Na [^{14}C]formate	5·0	3	2·0	410	37	—
		11	0·9	370	25	0·9
DL-[*Me*-^{14}C]Methionine ...	5·0	7	44	600	0·3	0·0001
		14	—	550	0·4	
		25	40	268	0·1	
		42	33·5	925	1·0	

Table 5. The m. p. of the dimethyl selenide mercurichloride varied from 152—154° to 153—154° (mixed m. p. 153—154°). Yields of $SeMe_2$ produced averaged 24%, except in presence of methionine (yield 7%, see Tables 3 and 7).

TABLE 6. *Effect of adding homocystine.*

Mould : *A. niger* 17. Medium : Bread, 150 g. per flask.

Days of aspirn.	Methylation (%)	CO_2 (mmole)	Activity (%) recovd. as CO_2	Days of aspirn.	Methylation (%)	CO_2 (mmole)	Activity (%) recovd. as CO_2
	Homocystine, 1·25 *mmole.* [*Me*-^{14}C]*Betaine*, 5·0 *mmole.*				[*Me*-^{14}C]*Betaine only.*		
3	5·1 ± 0·3	—	—	3	2·5 ± 0·2	730	26·5
6	7·8 ± 0·2	780	44·6	6	1·6 ± 0·15	—	—
15	7·4 ± 0·2	1450	43·4	15	5·2 ± 0·3	1370	29·7
	Homocystine, 1·25 *mmole.* *Sodium* [^{14}C]*formate*, 5·0 *mmole.*				*Sodium* [^{14}C]*formate only.*		
3	3·2 ± 0·1	550	79·8	3	1·9 ± 0·1	570	71·5
6	1·4 ± 0·07	550	11·5	6	0·45 ± 0·05	1070	16·4
9	0·52 ± 0·05	1700	8·9	9	0·15 ± 0·03	1640	11·8
15	0·25 ± 0·04	—	—	15	0·11 ± 0·02	—	—
	Homocystine, 1·25 *mmole.* [*Me*-^{14}C]*Choline chloride*, 3·0 *mmole.*				[*Me*-^{14}C]*Choline chloride only.*		
3	1·8	1050	30	3	0·8	900	6
6	3·0	—	—	6	4·3	—	—
9	4·5	940	17	9	2·0	960	25
13	3·3	—	—	13	6·7	—	—
19	2·9	830	7	19	6·3	910	7·5

Table 6. M. p.s of $SeMe_2,HgCl_2$ were 152—153°. The average yield of $SeMe_2$ over 14 days was 46% in absence, and 45% in presence, of homocystine.

The results of experiments without homocysteine, where formate or betaine was used as methyl source, compared quite well with those of similar experiments given in Table 5. The marked increase in the percentage methylation when homocystine is also present is discussed on

p. 1762. When choline is used as the methyl source, however, there are marked differences. The results in Table 5 suggest that, unlike *S. brevicaulis*, *A. niger* cannot utilise choline so readily as betaine. The results in Table 6 for choline with or without homocystine show, however, that the percentage methylation, although initially less than that for betaine, is ultimately about the same. The only known difference in conditions between the two sets of experiments is that in the case of those in experiments of Table 6 air was passed over the cultures faster than in those of Table 5. Thus it appears that the utilisation of choline as a methyl source for *A. niger* depends largely on an oxidation process. This is in agreement with animal experiments, where choline is said only to be effective as a methyl source if choline oxidase is present. It is suggested that the choline is first oxidised to betaine before the methyl group is available for methylation (Dubnoff, *Fed. Proc.*, 1949, **8**, 195; *Arch. Biochem.*, 1949, **24**, 251; Muntz, *J. Biol. Chem.*, 1950, **182**, 489; Soloway and Stetten, *ibid.*, 1953, **204**, 207).

Neither trimethylamine nor ammonia is evolved from choline chloride in bread cultures of *A. niger*. When a tube containing dilute hydrochloric acid was inserted before the mercury salts in the absorption train, and the acid finally evaporated, no residue was obtained.

TABLE 7.

Mould : *A. niger* 17. Medium G (300 c.c. per flask).

Methyl source	Concn. (mmole per flask)	Days of aspiration	Methylation (%)	CO_2 (mmole)	Activity (%) recovd. as CO_2
[*Me*-^{14}C]Betaine	2·0	11	11·1	—	—
		22	19·6	948	22·1
		40	22·6		
DL-[*Me*-^{14}C]Methionine ...	* 2·0	16	96	1190	16
		42	55·4		
	2·0	10	95·2		
		26	86·6	1420	16
		41	13·3	170	13·9
		50	3·2		

* Here the liquid medium contained ammonium nitrate (0·5%) instead of the usual glycine (0·25%). These cultures did not spore, but a good even pad of white mycelium was formed in 4 days. They appeared to produce dimethyl selenide only half as rapidly as those grown on medium G.

Table 7. The $SeMe_2,HgCl_2$ had m. p. and mixed m. p. 152—153°. Yields of $SeMe_2$ from selenate were here more difficult to assess owing to loss in the purification. They were of the order of 10% in 28 days, except in the [*Me*-^{14}C]betaine experiment without added methionine, where the yield was of the order of 20%.

Table 8. M.p.s and mixed m. p.s for $SeMe_2,HgCl_2$ were 152—153°.

TABLE 8. *Competition experiments.*

Mould : *A. niger* 17. Culture : Medium G.

Methyl source	Concn. (mmole per flask)	Aspirn. (days)	Methyln. (%)
[*Me*-^{14}C]methionine (2 mmole per flask) plus :			
Betaine	5·0	16	99
		31	79
Dimethylacetothetin chloride	5·0	13	98
		28	80·5
Dimethyl-β-propiothetin bromide *	5·0	13	94·5
		28	82
S-Methyl-DL-methioninesulphonium iodide *	5·0	28	59

* These cultures also produced *ca.* 15 and 20% respectively of inactive dimethyl sulphide. This was separated from active dimethyl selenide as described on p. 1766. The dimethyl sulphide was roughly determined from the weight of mercurichloride $2SMe_2,3HgCl_2$ obtained after separation.

Examination of Radioactive Mercurichlorides.—Radioactive specimens of $SeMe_2,HgCl_2$ collected from several experiments with DL-methionine as methyl donor were diluted with an inactive sample, giving specimen (A), m. p. 152—153°; this was recrystallised from aqueous 3% mercuric chloride, giving (B), m. p. 156—157°, and finally decomposed with sodium hydroxide; the dimethyl selenide was again absorbed in Biginelli's solution, giving specimen (C), m. p. 152—153°. The three samples were assayed. The c.p.m. were 77, 49, and 79 respectively. The experiment provides further evidence that constant activity had been attained for sample (A) of dimethyl selenide mercurichloride. Sample (B) presumably contained some mercurichloride containing a larger proportion of mercury. Further evidence for the formation of this

was furnished in the first experiment in Table 7 where the original deposit (m. p. 154—155°) in neutral mercuric chloride gave 79 c.p.m.; on purification by aspiration as usual the m. p. fell to 152—153° and the c.p.m. rose to 98. See also Table 10.

Slight traces (5—10 mg.) of $(MeS)_2Hg,Hg(CN)_2$, m. p. 138—139° (see p. 1760; cf. Briscoe, unpublished work), are normally produced in experiments with *A. niger* on medium G containing selenate and methionine. A somewhat larger amount (about 25 mg.) was, however, produced in the experiment with selenate + [*Me*-^{14}C]methionine + betaine (Table 8), and, as would be expected, was found to be radioactive. Its specific activity per methyl-C atom was 98% of that of the methionine added to the culture. The experiment with DL-[^{14}C]methionine + *S*-methyl-DL-methioninesulphonium iodide + selenate was of particular interest because here 80 mg. of this compound were produced. Although its m. p. and mixed m. p. were identical with the corresponding figures for the 25-mg. specimen, the specific activity per C atom was only 63% of that of the radioactive methionine added, instead of the expected 98%. It is interesting that here there was also a dilution in the radioactivity of the dimethyl selenide from the usual figure of about 90% to 59%. This dilution could be wholly or partly due to direct methylation by the non-radioactive methylmethioninesulphonium iodide. It seems more likely, however, since non-radioactive methanethiol is certainly produced, that the methylmethioninesulphonium iodide has lost a methyl group, to yield non-radioactive methionine. This then undergoes S–C fission along with the radioactive methionine already present, to yield the " diluted " methanethiol. This non-radioactive methionine so produced would also itself methylate the selenium. It was shown by Challenger and Charlton (*J.*, 1947, 429; *Ann. Reports*, 1946, **43**, 262) and by Challenger and Liu (*loc. cit.*) that methylmethioninesulphonium iodide itself undergoes fission in cultures of *S. brevicaulis* and *Penicillium notatum*, yielding dimethyl suphide but no methanethiol. The conditions, however, differed considerably from those of the experiments summarised in Table 8, in that neither selenate nor added methionine was present.

In view of the importance of *S*-adenosylmethionine (see p. 1764) in biological methylation it is possible that the methylsulphonium iodide of methionine must be converted into methionine before transmethylation to selenium can take place. In that case, the methyl group necessarily removed may be captured by homocysteine, giving rise to more methionine. Oxidation to carbon dioxide would appear to be inconsiderable, judging from the results of an experiment with radioactive methylmethioninesulphonium iodide (unpublished work).

Separation of the Mercurichlorides of Dimethyl Sulphide and Dimethyl Selenide.—Table 9 illustrates the gradual separation, as shown by activity and m. p., in experiment 3 of Table 8.

TABLE 9.

No. of aspiration	Time of aspiration (hr.)	Wt. (mg.) of ppt. in acid $HgCl_2$ soln.	" % Methylation " as $SeMe_2$	M. p.
1	1	240	25	147—148°
2	5	75	65	150—151
3 *	14	40	88	152—153
4	14	30 *	89	152—153
5	14	90 †	94·5	152—153

* These weights were inconveniently low and were corrected to infinite thickness. † Check on infinite thickness correction by dilution with inactive $SeMe_2,HgCl_2$ and aspiration as before.

The original deposit was decomposed with sodium hydroxide, and the dimethyl selenide and sulphide were volatilised in an air-stream through (*a*) acid and (*b*) neutral mercuric chloride. After 1 hr. (aspiration 1) the deposit in (*a*) was 240 mg. This was again made alkaline, and the operation repeated (aspiration 2), and so on.

Further Investigation of the Higher Mercurichloride of Dimethyl Selenide.—Radioactive dimethyl selenide monomercurichloride collected from various absorption trains attached to mould cultures was made alkaline and the liberated dimethyl selenide separately aspirated into 20 c.c. of each of the mercuric chloride solutions shown in column 1 of Table 10, so that approx.

TABLE 10.

Reagent	C.p.m. of $SeMe_2$ derivative	M. p. of $SeMe_2$ derivative	" Fractional activity of maximum "
(1) Biginelli's solution (10% $HgCl_2$ in 2N-HCl)	1450	152—153°	1·00
(2) 3% Aq. $HgCl_2$	1139	154—155	0·78
(3) 5% Aq. $HgCl_2$ (*a*)	1096	155—156	0·75
(*b*) after washing of ppt. with water ...	1384	153—154	0·95
(4) 10% Ethanolic $HgCl_2$	1455	152—153	1·00

0·1 g. of solid collected in each reagent. These deposits were suspended in about 5 c.c. of water, quickly filtered on to the standard discs, and counted. The deposit from (3) was then replaced in the filtration apparatus and repeatedly washed with water (6 × 10 c.c.). The activity and m. p. of this precipitate (3*b*) were then determined.

It is seen that with neutral aqueous mercuric chloride (2) and (3*a*) the activity declined and the m. p. rose, compared with (1) and (4) where m. p. and activity are the same. The precipitate formed in (1) is known to be $SeMe_2,HgCl_2$ (Challenger and North, *J.*, 1934, 70; North, Thesis, Leeds, 1934), and that in (4) must be the same. In precipitates in (2) and (3*a*) extra mercuric chloride must be attached to the dimethyl selenide molecule. The composition $2SeMe_2,3HgCl_2$ is suggested for this complex by the figures in column 4. These ratios represent the c.p.m. of the sample divided by the maximum c.p.m. obtained (*i.e.*, for sample 4). Since the activity of the dimethyl selenide is the same in every case this ratio will be invariably proportional to the ratio of the molecular weights of the mercuric chloride complexes. The " fractional activity of maximum " for $2SeMe_2,3HgCl_2$ would be $381/516{\cdot}5 = 0{\cdot}74$, which is very near to the value obtained for the precipitate in (3). The extra mercuric chloride must be loosely attached because washing with water removes most of it, and the m. p. and c.p.m. then approximate to those of the monomercurichloride. It should be noted that the higher mercurichloride is formed in the aqueous, less concentrated mercuric chloride solutions. In acidic and alcoholic solutions the greater solubility of mercuric chloride presumably favours the formation of the lower derivative. It may be mentioned that dimethyl sulphide mercurichloride has the composition $2SMe_2,3HgCl_2$ (for references see Blackburn and Challenger, *J.*, 1938, 1878) and that ethyl methyl selenide forms a stable dimercurichloride, m. p. 141·5°, and a monomercurichloride, m. p. 100°, which loses ethyl methyl selenide in air with rise in the m. p. This monomercurichloride forms the derivative with more mercury on recrystallisation from 3% aqueous mercuric chloride (Bird and Challenger, *J.*, 1942, 573).

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