BIOLOGICAL METHYLATION

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I. INTRODUCTION

As long ago as 1815, more or less severe cases of arsenical poisoning occurred in Germany and were ascribed to the use of domestic wall-papers the pigments on which were shown to contain arsenic. At that time the use of copper hydrogen arsenite and similar colors (Scheele's green and Schweinfürter green) for the decoration of houses was common. Since then, in spite of rigorous legislation, several similar cases—some fatal—have occurred from time to time.

Summaries of the earlier literature on this subject (with extensive bibliographies containing numerous references to its medical aspects) have been published by Abel and Buttenberg (4), Huss (136), and Maassen (152). The next few pages of this review contain a chronological account of the development of ideas concerning the origin of these toxic effects and the nature of the arsenical compounds producing them. It may be stated at once that the toxic compound has been shown to be trimethylarsine and that moulds growing on the damp wall-paper are responsible for its production. This conclusion gave to the phenomenon a much wider significance, methylation being a well-recognized bi-

ological process which, in some instances, seems to be employed for detoxication purposes.

The earliest and most obvious explanation put forward to explain the absorption of arsenic by persons living in the rooms was the inhalation of particles detached from the paper. The presence of arsenic in the dust of such rooms had been demonstrated (for references see Abel and Buttenberg (4) and Huss (136)), but poisoning had also been observed where the original arsenical paper had been covered by a fresh one containing no arsenic (Fleck (86); see also Huss). Consequently it was necessary to look for some other cause than mechanical disintegration of the pigment. Gmelin (98) in 1839 noticed that a garlic odor was usually present in rooms where the symptoms had developed. He ascribed the poisoning to a volatile arsenic compound liberated from the wall-paper which, in his experience of such cases, was usually found to be damp and mouldy. Further indications in this direction were obtained in 1872 by Fleck (86), who exposed strips of paper coated with Schweinfürter green (copper arsenite containing copper acetate) to moist air in flasks. The strips were attached to the walls with starch paste and after a time became mouldy. Air was passed through the flasks and led into silver nitrate solution, giving a deposit which appeared to be silver. On removing this and adding ammonia, a precipitate of silver arsenite was thrown down. No chemical evidence for the identity of these deposits was given by Fleck.

Two years later, Selmi (189) suggested that the moulds on the wall-paper and in Fleck's experiments might play a definite part in the volatilization of the arsenic by producing hydrogen from the paper and paste which, acting on the arsenical pigment, gave rise to arsine. This hypothesis had at first sight much to recommend it. Hydrogen is now known to be produced from carbohydrate by many microörganisms, though not by moulds, but it would appear probable that Fleck's strips of paper ultimately harbored a very mixed microbiological flora.

A suggestion that the gas was arsine had already been made by Martin (153) in 1847 but without reference to mould action. Later, however, this view had to be abandoned. There is no evidence that arsine is concerned in the phenomenon in any way, but moulds have been shown to play an essential part in the process.

In their very useful summary Abel and Buttenberg refer to a case where an arsenical paint on a wall gave rise to no trouble until it was covered up with arsenic-free paper attached by means of some, presumably organic, adhesive. Vallance (203) quotes a case recorded by Scheringa (187) where an arsenical odor was reported in a room. The outer wall-paper gave a negative test for arsenic, but older paper underneath gave a strong positive test, the arsenic being almost completely soluble in water. It was discovered, however, that fifteen years previously the paperhanger had mixed rat poison with the paste to prevent mice from gnawing the paper. Re-papering from time to time had evidently furnished nutrient material for the fungi which converted the arsenic to volatile compounds.

In the light of more recent work it is interesting to recall that in 1846 Basedow

(15) suggested that the air of the "arsenical rooms" might contain cacodyl oxide, $(CH_3)_2AsOAs(CH_3)_2$, which was originally obtained by Cadet (48) in 1760 by heating a mixture of arsenious oxide and potassium acetate, and was exhaustively studied later on by Bunsen (40–46). Basedow (15) brought forward no evidence in support of his suggestion, which was doubtless made owing to the very great interest aroused by Bunsen's experiments at the time.

The work of Gosio and Biginelli

Up to 1891, therefore, very little reliable information existed as to the manner in which toxic products are evolved from arsenical wall-paper and opinions on their nature were based on little or no experimental evidence. In this year Gosio (101) began a systematic study of the whole question. He exposed a potato-mash containing arsenious oxide to the air and observed that it quickly became infected with various moulds and bacteria and evolved a garlic odor. He isolated some of these organisms in pure cultures and studied their effect on various media containing carbohydrate and arsenious oxide and also certain arsenical pigments. The bacteria produced no volatile odorous compound of arsenic under these conditions (see also page 327), but some of the moulds were intensely active in this respect, especially one which Gosio named *Penicillium brevicaule* and which Saccardo (184) had first isolated from decomposing paper. Gosio also isolated this mould from a carrot. Other organisms which exhibited this phenomenon were *Aspergillus glaucus*, *A. virens*, and *Mucor mucedo*. Thom and Raper (202) have recently extended this list to include *A. fischeri*, *A. sydowi*, and a few soil organisms.

With the aid of pure cultures of *Penicillium brevicaule*[1] Gosio elaborated a biological method (101, 102, 103) for the detection of minute traces of arsenic in materials of the most varied type. The substance to be tested was extracted with water or dilute acid, the solution evaporated, and small quantities of the residue added to a slice of sterile potato previously inoculated with the mould. On maintaining the culture at a temperature of about 25–30°C. the presence of any inorganic compound of arsenic could be detected after a few hours by the production of a more or less intense garlic odor. Smith and Cameron (194) have stated that as little as one-millionth of a gram of arsenious oxide in 1 g. of material can be recognized in this manner; the reaction is qualitatively much more delicate than the Marsh test but is not readily adaptable to quantitative purposes.

Gosio then turned to the chemical examination of the volatile arsenic compound to which the odor is due and which is often known as Gosio gas. He removed the gases from a large number of cultures of *S. brevicaulis* on potato-mash in a stream of air and passed them through a red-hot tube, weighing the carbon dioxide and water produced. He concluded that the gas contained an alkylarsine, probably diethylarsine, $(C_2H_5)_2AsH$, although at the time this substance had not been prepared.

[1] The modern nomenclature is *Scopulariopsis brevicaulis*, which will be used in future references to this organism.

Gosio's work was continued by his assistant Biginelli (24), who aspirated the gas evolved from cultures of *S. brevicaulis* on potato-mash containing arsenious oxide through mercuric chloride dissolved in dilute hydrochloric acid. The resulting precipitate was assigned the composition $(C_2H_5)_2AsH \cdot 2HgCl_2$. Biginelli, therefore, concluded that the gas was diethylarsine. Klason (143), however, from a reconsideration of Biginelli's analyses and some further work, regarded it as diethylarsine oxide. Wigren (216) synthesized both these compounds and showed that their behavior towards acid mercuric chloride (Biginelli's solution) was different from that of Gosio gas, a conclusion confirmed by the later experiments of Ellis (61) in Leeds.

Meanwhile Cevey (51) had made the important observation that a garlic odor is also evolved when the inorganic arsenic of the cultures is replaced by sodium cacodylate, $(CH_3)_2AsOONa$. This was confirmed by Pool (175) with the mould *Monilia sitophila* Saccardo.

The Forest of Dean case

While the identity of Gosio gas was still in doubt, the deaths of two children in the Forest of Dean were reported (71) in December, 1931. The parents and two other children were also affected. The following account of the proceedings at the inquest is taken from *The Analyst* (8): Professor H. A. Scholberg, of University College, Cardiff, said that he had made a microscopical and bacteriological examination of the lungs of the boy. He attributed the death to bronchial pneumonia and blood poisoning. The jury were of the opinion that there was not sufficient evidence to show that the arsenic found had contributed to the death.

At the inquest on the girl, Mr. R. H. Ellis, F.I.C., County Analyst, said that he found arsenic in certain organs of the body (*viz.*, intestines, liver, kidneys, and lungs), the total amount (as arsenious oxide) being 2.65 mg. He had also analyzed samples of the wall-paper and of the plaster. In the paper from a dry part of the wall he had found 8.3 parts per million of arsenious oxide; in samples from a part where the mould was most pronounced there were 2.3 parts per million; and in the plaster there were 91 parts. An unused roll of wall-paper, purchased at the same time, contained 4.4 parts of arsenic per million. He had found definite traces of arsenic being given off in gaseous form from the wall that was affected by mould, and it was significant that the arsenic content of the mouldy wall-paper was only half that in a portion of the new paper, and only a quarter of that in a sample of the same paper taken from a dry part of the wall. In his opinion, the arsenic in the paper was present as an impurity, and he attributed the trouble to the plaster, and not to the paper. The arsenic in the plaster, which was composed of coke-breeze and cement, would dissolve in the moisture coming through the wall from the bank of soil outside, and the mould would then grow on the paper and would liberate the arsenic in the form of a very deadly organic compound. Mr. Ellis added that other tests made by him showed that four of the six members of the family had traces of arsenic in their systems. The jury returned a verdict that death was due to dysentery and to exposure to arsenic, which was generated in the house in a gaseous form.

Note by the Editor of The Analyst:

Mr. Ellis has informed the Publication Committee that consideration was given to the possibility of the quantity of arsenic found being present without any question of poisoning, but the distribution of the quantities found was also taken into account and considered in connection with the pathological condition of the organs. One of the chief factors which led to the conclusion formed was the fact that the amount of arsenic found in the lungs was greater in parts per million than in any other part of the body, except the large intestine, and this agreed with the condition of the lungs.

The problem of proving the presence of arsenic in the air was more difficult, and an attempt to detect it by simple aspiration gave negative results. Experiments were, therefore, made by exposing filter papers, saturated with silver nitrate, on the walls of the house, and these were left for 7 and 9 days, respectively. When these filter papers were destroyed, in the usual way, and the amount of arsenic was determined by the electrolytic Marsh test, small mirrors of arsenic were obtained.

The desirability or otherwise of the use for building purposes of materials which contain arsenic and also the use of arsenical preservatives in building construction has been briefly discussed elsewhere by the author (52; see also references 21, 77, 144, 146, 207, 219). In a recent letter to the author Mr. R. H. Ellis (80) states that he has investigated two other cases of (non-fatal) poisoning in a room in an ecclesiastical building used as an office and has demonstrated the presence of a volatile compound of arsenic in the air of the room. The walls were damp and stained and "there was some evidence of the growth of moulds, though nothing like so much as was present in the Forest of Dean case." As Mr. Ellis may be publishing his results elsewhere, further details can be omitted.

Identification of Gosio gas

Owing to the uncertainty regarding the nature of Gosio gas, a study of the subject was commenced at the University of Leeds (61) in the late autumn of 1931. In May 1932 the gas was identified as trimethylarsine, $(CH_3)_3As$.

Four strains of *Scopulariopsis brevicaulis* (Thom) were employed. Bread crumbs (with or without added water) were used in conical flasks such that after sterilization (25–30 min. at 120°C. or 30 min. at 100°C. on three successive days) a layer 1–1.5 in. deep was obtained. For a 1-liter flask 150–200 g. of fresh crumbs was required. These were inoculated with an aqueous spore suspension of the mould from a potato–agar slope culture, incubated for 3–4 days at 32°C. and then at room temperature for 4–5 days more until spores just tinged with brown were obtained.

Aqueous solutions of various arsenic compounds, sterilized for 25–30 min. at 120°C., or alternatively, at 100°C. as indicated above, were added and the usual cotton-wool plugs replaced by rubber bungs carrying tubes lightly plugged with cotton-wool. These had been sterilized at 120°C. for 25–30 min. The flasks were arranged in series and a continuous stream of sterile air was passed through, volatile arsenic compounds being absorbed in Biginelli's solution (mercuric chloride in dilute hydrochloric acid, see page 318). Sterilized solutions of all arsenic compounds other than arsenious oxide were found to be free from inorganic arsenic. The average concentration of the arsenious oxide was 0.2–0.25 g., of sodium methylarsonate 1–1.5 g., and of the sodium cacodylate 0.1–0.3 g.

per 100 g. of fresh crumbs. The ethylarsonate was used in concentrations of 0.2–0.25 and 0.5 g. of the acid sodium and potassium salts, respectively, per 100 g. of crumbs.

When arsenious oxide was used, the precipitate (B_1) which formed in the acidified mercuric chloride solution had a melting point of 264°C. and was identical with Biginelli's second compound of melting point 270°C. On passage of the gas for some weeks, the melting point of the precipitate fell to about 221°C., recrystallization from hot water giving needles (B_2) of melting point 224–226°C.; these were also obtained when Gosio gas was passed into dilute Biginelli's solution.

A comparison of B_1 and B_2 with the precipitates obtained from Biginelli's solution with arsine, diethylarsine oxide, diethylarsine, and triethylarsine showed them to be entirely different and conclusively proved that Gosio gas could not be identical with any one of these compounds.

The properties of the mould gas are also different from those of monoethylarsine or monomethylarsine (74), which oxidize in air to form red solids.

Analyses of B_1 and B_2 proved that these compounds are the dimercurichloride and monomercurichloride of trimethylarsine, $(CH_3)_3As \cdot 2HgCl_2$ and $(CH_3)_3As \cdot HgCl_2$, and that Gosio gas is therefore trimethylarsine,[2] a volatile liquid of boiling point 53°C. which has long been known. Direct comparison confirmed this conclusion. Trimethylarsine with Biginelli's solution gave a precipitate identical with B_1, as shown by melting point, mixed melting point (265°C.), and all other properties. The mercurichloride precipitated with dilute Biginelli's solution melted at 224°C. and was identical with B_2 similarly obtained from Gosio gas.

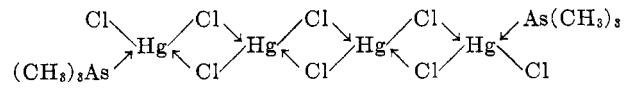
When the arsenious oxide of the bread-crumb cultures was replaced by sterilized solutions of sodium methylarsonate, $CH_3AsO(ONa)_2$, or sodium cacodylate, $CH_3AsOONa$ (free from inorganic arsenic), the evolved gas gave a mercurichloride which was shown by melting point and mixed melting point to be identical with that obtained with arsenious oxide.

The identity of Gosio gas was then confirmed by several further observations:

[2] Trimethylarsine when kept in a tube with limited access of air gave, after 6 weeks, a white solid which on solution in alcohol and precipitation with ether gave cacodylic acid. The filtrate on treatment with picric acid yielded hydroxytrimethylarsonium picrate, indicating the formation of trimethylarsine oxide.

During the preparation of allyldimethylarsine (see page 322) a dilute ethereal solution was allowed to evaporate slowly. From the residual white solid cacodylic acid was isolated as in the case of trimethylarsine, and treatment of the filtrate with picric acid yielded allylhydroxydimethylarsonium picrate, $(CH_3)_2As(CH_2CH{=}CH_2)(OH)OC_6H_2(NO_2)_3$. Triethylarsine with limited access of air yields diethylarsonic acid, $(C_2H_5)_2AsOOH$ (95a).

Evans, Mann, Peiser, and Puride (83) suggest that trimethylarsine dimercurichloride may have double the normal molecular weight and that a probable formulation is



An alternative structure is also discussed by these authors.

(*a*) Compounds B_1 and B_2 with nitric acid gave hydroxytrimethylarsonium nitrate, $(CH_3)_3As(OH)NO_3$, shown by melting point and mixed melting point to be identical with the product obtained from the synthetic arsine and nitric acid or by passing Gosio gas into nitric acid. (*b*) Both these nitrates with sodium picrate gave hydroxytrimethylarsonium picrate, $(CH_3)_3\dot{A}s(OH)\bar{O}C_6H_2(NO_2)_3$. (*c*) Synthetic trimethylarsine and hydrogen peroxide gave trimethylarsine oxide, $(CH_3)_3AsO$, which with picric acid yielded the picrate (melting point and mixed melting point 218–219°C.). (*d*) Passage of Gosio gas through alcoholic benzyl chloride gave a quaternary salt and thence a picrate, shown by melting point and mixed melting point to be benzyltrimethylarsonium picrate, $(CH_3)_3\dot{A}s(CH_2C_6H_5)\bar{O}C_6H_2(NO_2)_3$.

Since many aliphatic arsines are oxidized in air, it was necessary in order fully to establish the identity of Gosio gas (which is obtained in highly aerated cultures) to show that trimethylarsine can be volatilized unchanged in an air stream. On passage of air over or through the amyl ether–xylene solution obtained in the preparation of trimethylarsine (139) and then into Biginelli's solution, a precipitate was formed which had a melting point of 265°C. and gave a mixed melting point of 265°C. with substance B_1 obtained from similar treatment of Gosio gas.[2]

II. ALKYLARSONIC ACIDS AND *S. brevicaulis*

It seemed possible (*i*) that in the case of the formation of trimethylarsine from sodium methylarsonate and sodium cacodylate the mould might cause preliminary fission of the arsenic–carbon link, giving rise to inorganic arsenic. This could not, however, be detected at the close of the experiment, by extracting the bread crumbs and mycelium with hot water, filtering, acidifying, and treating with hydrogen sulfide. No arsenious sulfide was precipitated. There still remained the further possibility (*ii*) that in the methylarsonate and cacodylate experiments the trimethylarsine might have arisen by reduction, followed by dismutation:

$$CH_3AsO(OH)_2 \rightarrow CH_3As(OH)_2 \rightarrow CH_3AsO$$

$$3CH_3AsO = (CH_3)_3As + As_2O_3$$

and

$$(CH_3)_2AsOOH \rightarrow (CH_3)_2AsOH$$

$$3(CH_3)_2AsOH \rightarrow 2As(CH_3)_3 + As(OH)_3$$

Grischkewitsch-Trochimovski's observation (109) that the action of alkali on chlorodiethylarsine gives triethylarsine and other compounds rendered it necessary to bear such a possibility in mind. He states, however, that cacodyl chloride, $(CH_3)_2AsCl$, gives the pure oxide with alkali.

It appeared therefore of importance to study the behavior of sodium ethylarsonate to cultures of the mould on sterile bread crumbs, and this was done. A garlic odor was again evolved and Biginelli's solution gave a solid (B_3) which depressed the melting point of the mercurichloride (B_1) obtained from the

methylarsonate cultures. B_3 was also obtained when synthetic ethyldimethylarsine, $(CH_3)_2AsC_2H_5$, was treated with Biginelli's solution (139).

The gas from the ethylarsonate cultures after passage through benzyl chloride yielded a picrate, identical with synthetic benzylethyldimethylarsonium picrate, $(CH_3)_2(C_2H_5)\dot{A}s(CH_2C_6H_5)\bar{O}C_6H_2(NO_2)_3$, prepared from ethyldimethylarsine and benzyl chloride. Absorption of the mould gas in nitric acid gave a nitrate and thence a picrate, identical with synthetic hydroxyethyldimethylarsonium picrate, $(CH_3)_2(C_2H_5)\dot{A}s(OH)\bar{O}C_6H_2(NO_2)_3$.

These results show that neither removal of an alkyl group according to (*i*) nor dismutation (*ii*) occurs, since in the first case trimethylarsine would have been obtained and in the second a mixture of trimethylarsine and triethylarsine. This reaction, involving both methylation and reduction to a derivative of trivalent arsenic, was then further studied (58, 63). By addition of (*a*) diethylarsonic acid, $(C_2H_5)_2AsOOH$, (*b*) *n*-propylarsonic acid, and (*c*) allylarsonic acid, $CH_2{=}CHCH_2AsO(OH)_2$, to similar cultures of the same strain of the mould in concentrations varying from 0.2 to 0.5 per cent, mixed methylated arsines were produced. These were removed in a sterile air stream, and absorbed in suitable reagents. From (*a*) methyldiethylarsine was obtained and converted to the dimercurichloride and to diethylhydroxymethylarsonium picrate. Similarly (*b*) gave dimethylpropylarsine (58), which was identified as the dimercurichloride, as benzyldimethylpropylarsonium picrate, and as hydroxydimethylpropylarsonium picrate. Dimethylpropylarsine was also obtained (63) upon addition of methylpropylarsonic acid to bread cultures of *S. brevicaulis*. Under similar conditions ethylpropylarsonic acid gave ethylmethylpropylarsine, characterized by formation of the usual derivatives (63).

In an analogous manner (*c*) gave rise to allyldimethylarsine, $(CH_3)_2AsCH_2CH{=}CH_2$, which was characterized as the dimercurichloride and as allylbenzyldimethylarsonium picrate.

It is interesting that in spite of the powerful reducing action[3] exercised by *S. brevicaulis* the double bond of the allyl group in allylarsonic acid is unaffected, the melting points of the dimercurichloride and the benzylarsonium picrate from the resulting arsine being different from those of the corresponding *n*-propyl compounds.

III. *S. brevicaulis* AND INORGANIC COMPOUNDS OF SELENIUM

Attention was then turned to some early experiments of Rosenheim (183) in England in 1902, who showed that when *S. brevicaulis* was grown upon sterilized bread crumbs in the presence of inorganic compounds of selenium and tellurium, gaseous products possessing powerful and unpleasant odors were evolved. In

[3] In bread cultures of *S. brevicaulis* hydroxytrimethylarsonium nitrate (the nitrate of trimethylarsine oxide) and tri-*n*-propylarsine oxide are reduced to trimethylarsine and tri-*n*-propylarsine, respectively (60). The conversion of sodium arsenate and the salts of mono- and di-alkylarsonic acids to methylated tertiary arsines is clearly also a reduction (58, 60, 61, 63). Furthermore diethyl sulfoxide, $(C_2H_5)_2SO$, and hydroxydimethylselenonium nitrate (the nitrate of the selenoxide, $(CH_3)_2Se(OH)NO_3$) are converted by bread cultures of the mould to diethyl sulfide and dimethyl selenide, respectively (62).

the case of tellurium the smell resembled that of Gosio gas, but with selenium a characteristic and quite different odor was produced. The substances responsible for the odors were not identified. Maassen (152), working in Berlin almost simultaneously, obtained analogous results and, as the result of decidedly insufficient experimental work based entirely on odor, stated that the volatile products consisted of diethyl selenide and diethyl telluride. He also superficially examined the expired air of animals which had received injections of soluble inorganic selenites and tellurites and concluded that in these cases the unpleasant odor of the breath was due to dimethyl selenide and dimethyl telluride. A similar conclusion on equally unsatisfactory evidence had been reached as regards animals injected with tellurium compounds by Hofmeister (127) in 1894. Maassen concluded therefore that the animal body deals differently with compounds of selenium and tellurium than the organism of the mould. This statement has been fairly frequently quoted. It is clear that in deciding that the gas from the selenium or tellurium mould cultures was an ethyl derivative, Maassen was somewhat unduly influenced by Biginelli's incorrect identification of Gosio gas as diethylarsine, a report which had been published one year previously (24).

The formation of odorous compounds in the breath of animals treated with inorganic derivatives of tellurium was first observed by Gmelin (99). Hansen (117), on administration on five successive days of potassium tellurite to dogs or men, detected a garlic odor, similar to that of diethyl telluride, in the breath after a few minutes. This lasted for weeks and the persons in question were obliged to forsake the society of their fellows. A similar effect was observed by Japha (137) with inorganic selenium compounds. The odors have also been attributed to hydrogen telluride or selenide. Other references to the same phenomenon in human subjects have been recorded from time to time, but neither in the case of "selenium breath" nor "tellurium breath" was the odorous substance satisfactorily identified.

The gas evolved from the cultures in Rosenheim's early experiments with selenium compounds has now been identified (62). The volatile product arising from several pure cultures of two different strains of *S. brevicaulis* on sterile bread crumbs in the presence of either sodium selenate or sodium selenite was separately aspirated in a stream of sterile air through (*a*) Biginelli's solution, (*b*) mercuric bromide, (*c*) nitric acid, (*d*) potassium platinochloride, and (*e*) benzyl chloride. The products obtained were: in (*a*) dimethyl selenide mercurichloride, $(CH_3)_2Se \cdot HgCl_2$; in (*b*) dimethyl selenide mercuribromide; in (*c*) hydroxydimethylselenonium nitrate, $(CH_3)_2Se(OH)NO_3$; in (*d*) dimethyl selenide α-platinochloride, $PtCl_2 \cdot 2(CH_3)_2Se$ (95b); and in (*e*) benzyldimethylselenonium chloride, $(CH_3)_2Se(CH_2C_6H_5)Cl$, isolated as the picrate. Diethyl selenide mercurichloride was also prepared and found to be different from the mould product. The mould gas is therefore dimethyl selenide.

IV. *S. brevicaulis* AND INORGANIC COMPOUNDS OF TELLURIUM

Reference has already been made to the observations of earlier workers on the production of a garlic odor resembling that of an alkyl telluride when potassium

tellurite is administered orally to men or animals or added to cultures of *S. brevicaulis*. Blyth (33) refers to the case of a student who swallowed "a dose of tellurium" and had to be segregated. He also mentions the phenomenon of "bismuth breath," formerly well known to pharmacists and attributed to the presence of traces of tellurium in medicinal preparations of bismuth. Further details are given by Brownen (36), Letts (147), and Reissert (179). During a recent investigation of inorganic derivatives of tellurium in Leeds the odor could easily be detected in the vicinity of those engaged in the work, although they had never come into contact with organic compounds of tellurium.

On the basis of work which is discussed in an earlier paper (62), Maassen (152) concluded that the animal body elaborated dimethyl selenide and dimethyl telluride, and *Scopulariopsis brevicaulis* the corresponding diethyl derivatives. (Maassen's conclusion is incorrectly quoted in *British Chemical Abstracts* (35) and by Mellor (155), the contrary view being attributed to him.) In the case of the mould and sodium selenite and selenate this was disproved by Challenger and North (62), the product from cultures on bread or glucose-Czapek-Dox medium being shown to be dimethyl selenide. Difficulty was, however, experienced with similar cultures containing potassium tellurite. Aspiration of the volatile products through Biginelli's solution (mercuric chloride in dilute hydrochloric acid) gave traces of precipitate which decomposed without melting. Other absorbents gave equally unsatisfactory results.

Several factors appeared to contribute to this lack of success. Soluble tellurites are readily reduced to black amorphous tellurium by cultures of the mould. Maassen (152) states that this is unavailable for conversion into the volatile product, a conclusion confirmed by Blackburn in Leeds (56). Furthermore, the alkyl tellurides readily undergo atmospheric oxidation, giving complex products (13, 205).

Success was at last achieved (25) by growing *S. brevicaulis* upon bread crumbs in test-tubes and absorbing the volatile product in about 5 cc. of Biginelli's solution or other reagent. In this way contact of the mould gases with large volumes of air was somewhat diminished and dimethyl telluride mercurichloride (50) was obtained.

With sodium hydroxide the mercurichloride gave mercury and soluble dimethyl telluride dihydroxide or oxide or a compound of this with some other methyl derivative of tellurium (compare reference 13). The alkaline solution with hydrobromic acid gave dimethyl telluride dibromide, melting point 94–95°C. (204), thus confirming the identification of the mould gas. Furthermore, by absorption in alcoholic iodine, dimethyl telluride diiodide (melting point 125°C. with decomposition) was obtained. The recorded melting points of the dibromide (24°C.) and diiodide (57°C.) of diethyl telluride (151) differ widely from those of the corresponding dimethyl derivatives.

The mould gas is therefore dimethyl telluride, and Maassen's statement (152) that it consists of the diethyl compound is incorrect. This conclusion was also confirmed by the use of cultures on 2 per cent glucose-Czapek-Dox medium. The behavior of tellurium compounds in cultures of *S. brevicaulis* thus falls into

line with that of inorganic derivatives of arsenic (61) and selenium (62). It is of interest that arsenic resembles selenium and tellurium in its toxicological properties much more than it resembles antimony (70).

In order to discover whether the deposition of tellurium in tellurite cultures of *S. brevicaulis* was due to a reducing action of the bread or of some product elaborated by the mould, bread crumbs moistened with a tellurite solution were left in a corked test-tube. Practically no deposition of tellurium occurred, but after some days a green mould appeared and a strong odor of dimethyl telluride was noticed. A culture of this organism was sent to Dr. Thom of the United States Department of Agriculture, Washington, through the courtesy of Dr. St. John Brooks of the Lister Institute, who stated: "I place the organism near *P. notatum*, not necessarily identical with Westling's strain of *P. notatum*, since biochemical differences between strains are the rule rather than the exception."

Bread cultures of the "green mould" containing tellurite were then examined, and the evolved dimethyl telluride identified as before and as benzyldimethyltelluronium picrate. There was only very slight formation of tellurium, which would appear to be the special advantage of this particular organism. Dimethyl telluride was also produced in cultures on 2 per cent glucose-Czapek-Dox medium.

In view of Dr. Thom's results, pure cultures of *P. chrysogenum* Thom (Washington 26) and *P. notatum* were obtained from the Lister Institute. In bread–tellurite cultures the former gave dimethyl telluride, identified as the mercurichloride and the dibromide, but only a very faint odor could be observed when *P. notatum* was used. Both organisms readily gave dimethyl selenide in bread cultures containing sodium selenite or selenate. This was also produced in bread–selenate cultures by the "green mould." (For the behavior of these three organisms with salts of alkyl seleninic acids, $RSeO_2Na$, see page 343.)

None of these green *Penicillia* give any odor of trimethylarsine in bread cultures containing arsenious acid,[4] but all convert sodium methylarsonate in bread cultures to trimethylarsine, which is also produced in similar cultures of *P. chrysogenum* and *P. notatum* containing sodium cacodylate (26). Here, although dismutation appears improbable (see page 321), it must be conceded that methyl groups are present in the substrate. It is therefore interesting to note that bread cultures of *P. chrysogenum* convert sodium allylarsonate to allyldimethylarsine, $CH_2{=}CHCH_2As(CH_3)_2$ (26).

V. COMPARISON OF THE ACTION OF LIVING ORGANISMS ON COMPOUNDS OF ARSENIC, SELENIUM, AND TELLURIUM

Bearing in mind the methylating powers of the animal body (59, 62) there can be no doubt that men and animals also evolve dimethyl telluride after administra-

[4] Later experiments by Mr. P. T. Charlton (June, 1944) indicate that bread cultures of *P. notatum* containing approximately 0.2, 0.3, and 0.4 per cent of arsenious oxide evolve faint garlic odors, recognizable with difficulty. Lower concentrations were without effect. The other two *Penicillia* again failed to produce any garlic odor with concentrations of arsenious oxide ranging from 0.02 to 0.4 per cent. Further work is in progress.

tion of tellurium. This has already been stated both by Hofmeister (127) and by Maassen (152), but their conclusions, which have been widely quoted, were based on considerations of odor and there exists no proof of the nature of the alkyl telluride evolved by experimental animals.

Dudley (76) refers to the garlic odor of the breath of persons suffering from selenium poisoning and to the presence of a volatile, ether-soluble selenium compound in the urine of a horse after ingestion of sodium selenite. As concluded by Challenger and North from analogous experiments with *S. brevicaulis* (62), the exhaled product is almost certainly dimethyl selenide.

It would therefore appear that arsenic also should be methylated in the animal body and exhaled as trimethylarsine. That no odor comparable in intensity to that produced by tellurite follows administration of medicinal doses of inorganic compounds of arsenic is well known (see also Reissert (179)), but occasional references to the presence of a garlic odor in the perspiration following upon arsenical poisoning occur (34).

Pleschtizer and Preobrajensky (172) passed the breath from patients in receipt of inorganic arsenic through bromine water. Treatment with ammonia followed by evaporation gave a slight residue in which the presence of arsenic was detected by addition to cultures of *S. brevicaulis*, the garlic odor of Gosio gas being obtained. The presence of some volatile compound of arsenic in the breath was thus rendered extremely probable, but the quantity was too small for identification. These authors, at that time unaware of the identification of Gosio gas as trimethylarsine (61), merely quoted Biginelli's statement (24) that it consists of diethylarsine.

Keeser (140), however, while citing this latter work, states without further comment that according to the Russian workers the gas from cultures of *S. brevicaulis* on arsenical media is diethylarsine and not trimethylarsine, thus unintentionally creating a wrong impression.

Carlson (49), Montgomery (158), and Puntoni (176) refer to the production of a garlic odor in the breath after ingestion or injection of cacodylic acid or its sodium salt. Bloemendal (31) passed the exhaled air of a rabbit which had received 20 mg. of sodium cacodylate through alkaline potassium permanganate solution, which then contained arsenic. The odorous product was not identified. Montgomery suggested that it was cacodyl. From the behavior of sodium cacodylate in cultures of *S. brevicaulis* (61), the formation of trimethylarsine would be expected by reduction and further methylation. However, methylation does not occur readily in animals receiving arsenious oxide (see above), and trimethylarsine, if formed from cacodylic acid in the animal body, might conceivably arise by reduction and dismutation.

$$(CH_3)_2AsOOH \xrightarrow{\text{reduction}} (CH_3)_2AsOH$$

$$3(CH_3)_2AsOH = 2As(CH_3)_3 + As(OH)_3$$

(See page 321 and compare the action of chlorodiethylarsine with alkali; 109, see also 12.) Such dismutations are well established in the case of the alkyl

derivatives of tellurium (75). As shown on page 322, however, aliphatic arsonic acids other than the methyl compounds do not undergo dismutation in mould cultures. The mechanism by means of which methylarsonic acid and cacodylic acid yield trimethylarsine in mould cultures cannot be rigidly established, but by analogy with other alkylarsonic acids dismutation appears very improbable.

Puntoni attributed the garlic odor after oral administration of cacodylate to the effect of intestinal organisms, some of which he cultivated on cacodylate media, obtaining a similar odor. Using strains of the same and two other bacteria, Challenger and Higginbottom (60) were unable to detect any odor in media containing arsenious oxide, sodium arsenate, sodium methylarsonate, or sodium cacodylate. Many bacterial species were tested by Abel and Buttenberg (4), Hildebrandt (123), Sanger (186), Huss (136), and Emmerling (82), and also by Simons in the author's laboratory (192), but in no case was a garlic odor obtained in cultures containing arsenious oxide. Gosio (104, 105) studied the behavior of inorganic tellurites and selenites in cultures of numerous bacteria and frequently observed reduction to the free element, but makes no mention of any odor arising from the cultures. Maassen (152), however, states that eighteen species of bacteria, including many pathogenic forms, can give odorous products in the presence of soluble compounds of selenium and tellurium, especially when a good growth is obtained. He recommends the use of an 18–20 hr. old agar culture and the addition of a sterile solution of sodium selenite or potassium tellurite (0.005 g. in 0.2 to 0.5 cc. of water) followed by an equal volume of bouillon. After incubation of the material for 24 hr. at 30–35°C. he observed a strong odor. Some bacteria employed by Maassen were tested by Simons (192) under similar conditions in the presence not only of arsenious acid but also of sodium methylarsonate, sodium cacodylate, and sodium selenite, but no odor was obtained.

The weight of the evidence would indicate that bacteria are unable to produce volatile methyl derivatives of arsenic, selenium, and tellurium, the statements to the contrary being based on observations of odor only.

Negative results were also obtained by Emmerling (82) and Huss (136), using various yeasts. Challenger and Higginbottom (60) cultivated *Saccharomyces cerevisiae*, *S. carlsbergensis*, *S. monacensis*, and "Rasse XII" on beer-wort or 5 per cent glucose mineral salt solution in the presence of arsenious oxide, but no garlic odor was produced. Verona (206) refers to a volatile arsenical product detected in very small quantity in cultures of the yeast *Saccharomyces ellipsoideus* Hansen, containing arsenic. Dr. C. Simons, working in the author's laboratory, was unable to confirm this statement with the particular strain of this yeast available to him.

VI. ARSENIC-TOLERANT MOULDS

Reference may here be made to some observations which, although not concerned with biological methylation, possess certain superficial analogies with the subject. In a paper which has already been cited, Thom and Raper (202) state that fungi which volatilize arsenic and also arsenic-tolerant organisms are

more numerous than was previously supposed. These tolerant organisms survive in arsenical media without decomposing the arsenic compound or producing volatile odorous products.

Further examples of this were recorded by various pharmacists in 1932. About that time the *British Pharmacopoeia* had modified the composition of Liquor Arsenicalis B.P. by omitting the Compound Tincture of Lavender and altering the pH. This change was soon followed by several letters to the *Pharmaceutical Journal* stating that a mould growth had appeared in specimens of the Liquor prepared according to the new formula. The first of these, by Sheard and Tribley, may be quoted:

"We have prepared several batches of Liquor Arsenicalis B.P. 1931 and find that if prepared with ordinary unsterilised distilled water, the solution rapidly develops extensive colonies of moulds of the Mucor type and also smells most foul in less than a week after preparation. If, however, the solution is sterilised by any of the customary methods no moulds, of course, develop. We conclude that Liquor Arsenicalis B.P. 1932 which has a p_H approximating to 7 is much more prone to the growth of moulds than the corresponding acid and alkaline solutions of arsenic of the B.P. 1914, and that it should be recently prepared, sterilised and carefully stored."

These statements were confirmed by Bennett (18, 19), who found that the most satisfactory pH for Liquor Arsenicalis is below 2 or above 9. Hampshire (115) stated that specimens of the new Liquor had been prepared which had kept satisfactorily for 12 months and suggested that "pharmacists should look carefully into their methods of making the Liquor."

Moore (159) identified one of the fungi:

At a recent meeting of the Science Section of the Birmingham Pharmaceutical Association a locally prepared sample of Liquor Arsenicalis B.P. 1932 was exhibited, which showed an abundant grey-black growth. On investigation this was found to be the common mould, *Cladosporium herbarum* (Link.), a member of the Hyphomycetes. This fungus is somewhat variable in form, but always possesses a well-branched mycelium which buds-off conidia, usually unicellular, but sometimes with a median septum; in a moist medium the conidia produce globose spores. All stages of the fungus were found in the sample examined. *Cladosporium herbarum* is a fungus of wide occurrence, growing saprophytically on a variety of substrata. It is known to occur on damp walls, especially of wine cellars, where it also occurs on casks; it has even been known to penetrate the corks of wine bottles in storage. It may be a factor in the blackening of cheese, contamination occurring in the dairy. It has also been reported as causing putrefaction in stored eggs, the conidia passing through the untreated shell and the lining membrane, then germinating to produce a mycelium in the "white." It is common in water supplies: Bewley and Budden, investigating the fungus flora of greenhouse water supplies from wells, tanks, brooks, and ponds, found *Cladosporium* present in thirty-four of forty-one samples. Salacz found *Cladosporium herbarum*, amongst other fungi, to be capable of growth and spore-formation in solutions containing 2 per cent. arsenic, and that growth would continue so long as the percentage did not exceed 4, above which the solution became lethal. Work on the physiology of this fungus and on various points arising from its occurrence in Liq. Arsenicalis is now in progress.

The reference to the odor suggested to the author that possibly the mould or moulds in question might be producing trimethylarsine and that the necessary organic matter might be introduced by accidental contamination.

A bottle of Liq. Arsenicalis B.P. 1932, in which a mould growth had formed at the bottom was obtained through the courtesy of Messrs. Goodall, Backhouse, and Co., Leeds. The solution had an odor which could not be identified, but was not that of trimethylarsine. A specimen of the growth was removed by Miss C. Higginbottom and inoculated on to sterilized slants of (*a*) potato–agar, (*b*) wort–agar, and (*c*) meat extract agar. Growth occurred, and (after a preliminary purification had been made on the same medium) a few spores from a potato–agar plate were inoculated on to sterile bread crumbs, as in the experiments of Challenger, Higginbottom, and Ellis (61). Growth occurred slowly at 28°C., and after 3 days arsenious oxide solution was added in quantity sufficient to give a concentration in the bread of about 0.2 per cent. Incubation was continued, but not the slightest garlic odor was developed by the culture, even after 10 days, the behavior being entirely different from that of the true "arsenical moulds" employed by Challenger and his collaborators. It should be borne in mind, however, that in these preliminary experiments no claim is made that the mould obtained from Liquor Arsenicalis was isolated in pure culture. Furthermore, Challenger inoculated samples of sterile Liq. Arsenicalis B.P. 1932 with pure cultures of *S. brevicaulis*. No germination occurred after several weeks and no odor developed. This work has been summarized by Dyer (78).

Further details were published by Milne and Rattray (156, 157). The growths from several samples of the Liquor were compared with *S. brevicaulis*, no resemblance being noted. The moulds appeared to be species of *Fusarium* and *Torula* and an organism which was not identified.

Samples of the Liquor adjusted to different pH values were inoculated with *Rhizopus nigricans*, *Aspergillus glaucus*, "*Penicillium glaucum*," and *S. brevicaulis* and left at room temperature and at 32°C. No definite increase in growth took place, although in the case of the samples at about pH 7 the moulds appeared to be healthiest, "but growth at best is slight." It may be mentioned that these authors refer to the "musty" odor of three different samples of the infected Liquor but do not mention any odor of garlic.

It is clear, therefore, that the organisms concerned with these observations are not true "arsenic organisms," i.e., they do not volatilize the arsenic, and that the odor is not due to trimethylarsine. The results appear, however, worthy of mention if only to avoid any possible confusion.

VII. FISSION OF THE DISULFIDE LINK IN $C_nH_{2n+1}S—SC_nH_{2n+1}$ BY *S. brevicaulis* AND METHYLATION OF THE $C_nH_{2n+1}S$ GROUP

In view of the successful experiments with selenium and tellurium, attempts were made to obtain dimethyl sulfide by addition of sulfur or certain of its compounds to bread cultures of two different strains of *S. brevicaulis*. Negative

results were obtained with sulfur, sodium sulfite, sodium thiosulfate, sodium tetrathionate, thiourea, thiodiglycolic acid and its sodium salt, sodium formaldehydesulfoxylate (Rongalite), and also with sodium ethanesulfonate and ethanesulfinite, the last-named compound in liquid cultures.

This was somewhat surprising in view of the experiments of Pohl (173), who noticed a leek-like odor in the expired air of animals receiving subcutaneous or intravenous injections of thiourea. The odorous product was non-reactive to sodium hydroxide or mercuric cyanide, and was therefore not an alkylthiol. It was, however, absorbed by sulfuric acid and gave a precipitate with mercuric chloride which, on oxidation, yielded a sulfate. Pohl therefore concluded that the product was an alkyl sulfide. Hofmeister (127) was unable to detect any odor in the expired air of dogs and rabbits fed with powdered sulfur or injected with sodium sulfide or thiosulfate.

Neuberg and Grosser (167) stated that the precursor of the diethyl sulfide which was shown by Abel (3) to be evolved on warming the urine of dogs with alkali is diethylmethylsulfonium hydroxide. They also state that administration of diethyl sulfide to dogs gives rise to this compound, but experimental details have not been published.

Particular interest attaches to the observation of Haas (114) that the seaweeds *Polysiphonia fastigiata* and *P. nigrescens* evolve dimethyl sulfide shortly after being gathered. The occurrence in nature of methylated compounds of sulfur such as cheirolin, $CH_3SO_2CH_2CH_2CH_2N{=}C{=}S$, erysolin, $CH_3SO_2CH_2CH_2$-$CH_2CH_2N{=}C{=}S$ (see 9 for references), and particularly methionine, CH_3-$SCH_2CH_2CH(NH_2)COOH$, demonstrates the possibility of a biological methylation of sulfur. The relation of methionine to cysteine and to cystine suggested that compounds containing the —SH or —S—S— links might be more amenable to the methylating action of the mould.

Neuberg and Schwenk (168) showed that, on addition to a solution of sugar undergoing fermentation by bottom yeast, diethyl disulfide is reduced to ethylthiol.

Dr. H. E. North (unpublished observation) found that on addition of diethyl disulfide to bread cultures of *S. brevicaulis* ethylthiol was evolved; it was identified as the mercaptide, $Hg(SC_2H_5)_2$, by absorption in mercuric cyanide. The gases issuing therefrom reacted with mercuric chloride, giving a precipitate which was clearly a mixture. Diethyl disulfide, boiling point 153°C., is volatile in a stream of air and, as it is inert to mercuric cyanide, any which escaped reaction in the culture flasks would reach the mercuric chloride.

Morin (160) and Otto (169) mention the formation of a heavy white crystalline precipitate from diethyl disulfide and alcoholic mercuric chloride, but give no further details.

The behavior of aliphatic disulfides RSSR (R = C_2H_5 or n-C_3H_7) with excess of saturated aqueous mercuric chloride was therefore examined. In each case a white precipitate of the composition $RSHgCl \cdot HgCl_2$ was formed. Its weight accounted for only about 70 per cent of the disulfide, the remainder having formed

soluble products.[5] The insoluble compounds were shown by analysis, melting points, and mixed melting points with compounds of known composition to be identical with the compounds obtained from the corresponding alkylthiols and excess of aqueous mercuric chloride. Bertram (22) obtained CH_3SHgCl from methylthiol. The product formed from ethylthiol in alcoholic solution is stated by Débus (73) to be C_2H_5SHgCl, but no mention of a double compound with mercuric chloride is made. Sachs (185), however, states that with mercuric chloride in ether C_2H_5SHgCl is converted into $C_2H_5SHgCl \cdot HgCl_2$. The same addition takes place slowly in aqueous solution.

Since the compounds RSHgCl or $RSHgCl \cdot HgCl_2$ would clearly be formed in the mercuric chloride absorption bottles during experiments with the mould and RSSR, their properties were studied. Neither of these compounds liberates any thiol when warmed with sodium hydroxide in an air stream, aspiration through mercuric cyanide giving no precipitate. The mercurichlorides of the alkyl sulfides readily eliminate R_2S under these conditions.

VIII. *S. brevicaulis* AND DIALKYL DISULFIDES

The behavior of disulfides toward mercuric chloride having been established, ethyl and *n*-propyl disulfides were added in dilute aqueous suspension to the bread cultures. In every case the products issuing from the culture flasks consisted of the alkylthiol, RSH, the unchanged disulfide, RSSR, and the methyl alkyl sulfide, $RSCH_3$. In the case of the relatively non-volatile di-*n*-propyl disulfide very little of this came over. The precipitates in the mercuric chloride flasks consisted of mixtures of the mercuric chloride addition product of the methyl alkyl sulfide with varying amounts of $RSHgCl \cdot HgCl_2$. On treatment of these mixtures with sodium hydroxide in a slow stream of air, pure methyl alkyl sulfide was evolved and converted to the mercurichloride. No alkylthiol volatilized under these circumstances (see above). The methyl alkyl sulfides were also characterized as the benzylmethylalkylsulfonium picrates, and (in the case of ethyl methyl sulfide) as the double compound with platinous chloride.

[5] It was suggested by Challenger and Rawlings (64) that, in the case of diethyl disulfide, the soluble product might be ethanesulfinic acid, $C_2H_5SO_2H$, arising from the chlorothiol. C_2H_5SCl, by way of the sulfenic acid, C_2H_5SOH, which then undergoes dismutation:

$$C_2H_5SSC_2H_5 + 2HgCl_2 = C_2H_5SHgCl \cdot HgCl_2 + C_2H_5SCl$$
$$C_2H_5SCl + HOH = C_2H_5SOH + HCl$$
$$2C_2H_5SOH = C_2H_5SH + C_2H_5SO_2H$$

The sulfinic acid was later isolated by Blackburn and Challenger (56) through the sodium salt as ethyl *p*-nitrobenzyl sulfone. The fission of dimethyl disulfide by mercuric chloride is similar and the methanesulfinic acid was identified as methyl *p*-nitrobenzyl sulfone. The intermediate sulfenic acid (RSOH) could, of course, arise directly by incipient hydrolytic fission thus:

$$R_2S_2 + HOH = RSH + RSOH$$

the thiol being removed as the very insoluble $RSHgCl \cdot HgCl_2$. Such fission was demonstrated for diethyl disulfide and water at 170°C. (64).

In view of the reducing properties of cultures of *S. brevicaulis* (58, 59), the formation of thiols from the disulfides was not surprising. Addition of ethylthiol and *n*-propylthiol to bread cultures of the mould under the same conditions as obtained for the disulfides gave very similar results, methylation being observed in each case.

The reaction was also extended by Blackburn and Challenger (56) to di-*n*-butyl and di-*n*-amyl disulfides, which in bread cultures of the mould are converted to *n*-butylthiol and *n*-butyl methyl sulfide and to *m*-amylthiol and *n*-amyl methyl sulfide, respectively. These were removed, separated, and identified as in the case of the ethyl and *n*-propyl derivatives. Here again the amount of methyl and alkyl sulfides is larger than that of the alkylthiols, but the total yield is very low. With diethyl disulfide as substrate much passes over unchanged and, as already stated, the product $C_2H_5SHgCl \cdot HgCl_2$ accompanies the ethyl methyl sulfide mercurichloride, $CH_3SC_2H_5 \cdot 2HgCl_2$. The di-*n*-propyl disulfide is less volatile and very little reaches the mercuric chloride. This is still more obvious with di-*n*-butyl and di-*n*-amyl disulfides, the methyl alkyl sulfide mercurichloride being entirely free from chloromercury alkylthiol derivatives. The fission of the disulfide link by *S. brevicaulis* appears therefore to be a general reaction of the simple aliphatic disulfides.

This biological conversion of *n*-butyl disulfide to the corresponding thiol is of interest in view of the occurrence of these two compounds and of isoamylthiol in the anal secretion of the skunk (6, 17). Traces of methylthiol are also stated to be present.

Earlier workers also considered that the higher homologues of methylthiol were contained, along with basic nitrogenous compounds, in the secretions of various animals allied to the skunk. References are given by Nencki and Sieber (166). Furthermore the secretion of the zorrino, a South American marten, appears to contain a thiol with four atoms of carbon and probably the corresponding disulfide (85).

It was at first uncertain whether di-*n*-butyl and di-*n*-amyl disulfides would volatilize from the cultures and react with the mercuric chloride in the absorption bottles. Their behavior toward this reagent was therefore studied in water for the butyl compound and in alcohol in the case of di-*n*-amyl disulfide. The products were chloromercury *n*-butylthiol, C_4H_9SHgCl, and chloromercury *n*-amylthiol, respectively. Unlike the fission products of the first three dialkyl disulfides (57), these contain no coördinated molecule of mercury chloride.

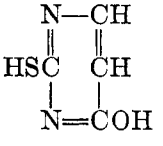
At the commencement of this account of the biological formation of methyl derivatives of sulfur it was stated that numerous inorganic sulfur compounds failed to undergo methylation in cultures of *S. brevicaulis*. It is therefore particularly interesting to refer to the work of Birkinshaw, Findlay, and Webb (27), who have recently shown that the wood-destroying fungus *Schizophyllum commune*, Fr., when grown on an aqueous medium containing glucose, inorganic salts, and a trace of "Marmite," converts inorganic sulfate to methyl mercaptan, CH_3SH. This was absorbed in mercuric cyanide and chloride and characterized as mercury dimethylthiol, $(CH_3S)_2Hg$, and chloromercury methylthiol,

CH_3SHgCl, respectively. Traces of hydrogen sulfide are probably formed, but no dimethyl sulfide. This is the only recorded instance of the biological methylation of inorganic sulfur (compare 64). It will be recalled that *S. brevicaulis* forms dimethyl selenide, but no selenothiol, CH_3SeH, from selenate or selenite.

IX. BEHAVIOR OF THIOUREA AND SIMILAR COMPOUNDS IN THE HUMAN BODY

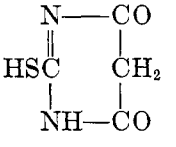
Pohl's experiments on the production of a leek-like odor in the expired air of animals receiving thiourea, which were described at the commencement of Section VII, have lately acquired an enhanced interest. During the last two years thiourea has found application in the treatment of thyrotoxicosis (hyperthyroidism) arising from an excessive secretion of thyroxine by the thyroid gland (10, 11, 125). Good results have been obtained, but a minor disadvantage is the peculiar sweetish odor which is imparted to the breath. The author is much indebted to Dr. C. A. Mawson of the Pathology Department, Royal Berkshire Hospital, Reading, for drawing his attention to this phenomenon. Dr. Mawson and his colleagues compare the odor to that of seaweed (*cf.* 114).

2-Thiouracil (I), which is also employed in the treatment of thyrotoxicosis, does not give rise to this odor. In view of the close relation of this uracil to thiourea, this difference is remarkable. It would appear as if thiourea is not produced, at any rate in quantity, from thiouracil in the organism.

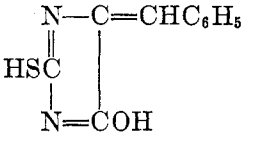


I
2-Thiouracil

II
Thiourea



III
2-Thiobarbituric acid



IV
5-Benzalthiohydantoin

Astwood (10, 11) found that the antithyroid activity of 2-thiouracil (I), 2-thiobarbituric acid (III), *sym*-diethylthiourea, and 5-benzalthiohydantoin (IV) is greater than that of thiourea (II).

In view of the work of Haas, of Rawlings, and of Blackburn (see above) it would appear probable that the odor is due to methylthiol or to dimethyl sulfide, probably the latter. Experiments are at present in progress in collaboration with Dr. Mawson and with Dr. Leese of the Leeds General Infirmary with the object of identifying the odorous compound.

X. THE MECHANISM OF BIOLOGICAL METHYLATION

A. The acetic acid hypothesis

The first of the suggested mechanisms (59) might proceed thus in the case of arsenious acid:

$$As(OH)_3 + 3CH_3COOH = As(CH_3)_3 + 3CO_2 + 3H_2O$$

Such a reaction would be analogous to the well-known cacodyl oxide test. Alternatively, arsonoacetic acid might result by a process of dehydrogenation:

$$HOOCCH_2 \cdot H + H \cdot AsO(OH)_2 = 2H + HOOCCH_2AsO_3H_2$$

This by loss of carbon dioxide might yield methylarsonic acid, $CH_3AsO(OH)_2$, which, on reduction to $CH_3As(OH)_2$, isomerization to $CH_3AsO(OH)H$, and renewed reaction with acetic acid, could finally yield trimethylarsine. The suggested dehydrogenation would be analogous to the formation of succinic acid from calcium acetate by *Mucor stolonifer* (47).

Challenger and Higginbottom (59) were unable to obtain any supporting evidence for this hypothesis; arsonoacetic acid in bread cultures of *S. brevicaulis* gave small quantities of trimethylarsine in some, but not all, experiments; α-arsonopropionic acid gave traces of this arsine, probably owing to the formation of a little arsenious acid which is readily eliminated from compounds of this type. Decarboxylation would have yielded ethylarsonic acid, which in the presence of the mould would have been converted to ethyldimethylarsine; this was not observed. Using α-arsonobutyric acid in bread cultures of *S. brevicaulis*, Challenger and Rawlings (63) observed neither trimethylarsine nor *n*-propyldimethylarsine in two experiments, each lasting 42 days. Moreover, only pure trimethylarsine was evolved from cultures containing arsenious acid with salts of formic, propionic, and butyric acids, whereas on the acetic acid hypothesis some formation of hydrogen arsenide, triethyl- and tri-*n*-propylarsines, or even of mixed methylalkylarsines might conceivably have been expected, e.g.:

$$3H\cdot COOH + As(OH)_3 = 3CO_2 + 3H_2O + AsH_3$$

$$3HOOC\cdot CH(CH_3)H + (HO)_3As = 3CO_2 + 3H_2O + As(CH_2CH_3)_3$$

$$HOOCCH(CH_3)\cdot H + H\cdot AsO(OH)_2 = 2H + HOOCCH(CH_3)AsO(OH)_2$$

$$HOOCCH(CH_3)AsO(OH)_2 \xrightarrow[-CO_2]{} CH_3CH_2AsO(OH)_2 \longrightarrow CH_3CH_2As(CH_3)_2$$

It may be mentioned that thiodiglycolic acid $(HOOCCH_2)_2S$ gave no dimethyl sulfide. These results suggest that decarboxylation of the group $\rangle CHCOOH$ does not readily occur in cultures of *S. brevicaulis*.

B. The formaldehyde hypothesis

The view that methylation in green plants is effected by formaldehyde is generally accepted by chemists. Emde (81) differentiates between "primary" formaldehyde produced by photosynthesis and that arising by "secondary" breakdown processes. In moulds and animals any formaldehyde involved in methylation reactions is presumably of secondary origin and even in plants some may arise by the demethylation of NCH_3 groups (or in other ways, see pages 338, 339) and become again available for methylation (see 110, 122, 182). Numerous methylations can be effected by formaldehyde (see Hess (119) for summary, also Werner (214); Clarke, Gillespie, and Weisshaus (68)). The presence of an oxygen acceptor is, of course, necessary. As will be seen later this may be either formaldehyde itself, formic acid, or a suitable group present in the molecule of the compound undergoing methylation.

It has unfortunately not been possible to apply successfully a crucial test to the formaldehyde hypothesis as regards the methylations effected by moulds. In its application to the production of trimethylarsine, this postulates the formation of hydroxymethylarsonic acid, $HOCH_2AsO(OH)_2$, as the first stage, followed by reduction to methylarsonic acid. After further reduction to $CH_3As(OH)_2$, the isomeric form, $CH_3AsO(OH)H$, might be expected to react again with formaldehyde (59, 61), repetition of the process yielding cacodylic acid, $(CH_3)_2AsOOH$, and finally trimethylarsine. Hydroxymethylarsonic acid could not be synthesized and its homologue, $HOCH_2CH_2AsO(OH)_2$, when purified from traces of inorganic arsenic, is inert in bread cultures of the mould or, at least, gives no volatile product. Had reduction of the β-hydroxyl group occurred in the cultures, the formation of ethyldimethylarsine would have been expected.

Challenger and Higginbottom (59), using cultures of *S. brevicaulis* on glucose-Czapek-Dox medium containing arsenious acid, found that addition of sodium formate or of formaldehyde (free or as various derivatives), with or without formate, had no appreciable influence on the yield of trimethylarsine.

If we assume that selenious and tellurous acids can react in the forms $H \cdot SeO_2OH$ and $H \cdot TeO_2OH$, the formaldehyde hypothesis can similarly be employed to explain their conversion to dimethyl selenide and dimethyl telluride in mould cultures. There is some doubt, however, as to whether selenious acid can react in this form. Strecker and Daniels (198) found that the product from the action of silver selenite on ethyl iodide is identical in boiling point and other physical properties with that obtained from selenium oxychloride ($SeOCl_2$) and sodium ethoxide. They conclude, therefore, that, unlike sulfurous acid, selenious acid or its salts are not capable of tautomerism to the forms $HSeO_2OH$ and $AgSeO_2OAg$. Loevenich, Fromdling, and Fohr have, however, shown that β-naphthylseleninic acid, $C_{10}H_7SeO_2H$, can give rise to the normal ester and also to a selenone (150).

As applied to the fission of disulfides and methylation of the resulting mercaptan, the formaldehyde hypothesis demands the formation of $RSCH_2OH$. Several compounds of this type have been described (148), but they are unstable and easily hydrolyzed. The compound $C_2H_5SCH_2OH$ could not be freed from traces of ethylthiol, and so its capability of reduction to $C_2H_5SCH_3$ in mould cultures could not be determined (64).

Possible origins of formaldehyde

Leaving photosynthesis in green plants out of consideration, we may now consider some possible modes in which formaldehyde might arise and become available as a methylating agent in moulds, animals and, to some extent, in plants.

(*1*) *Deamination of glycine:* Schweitzer (188; cited by Robinson (182)) found that potato tyrosinase can oxidize glycine with formation of formaldehyde, carbon dioxide, and ammonia. Similar decompositions of glycine and other amino acids are summarized by Challenger and Higginbottom (59).

Such compounds as choline and betaine, which occur in higher plants, fungi,

and animals, have usually been regarded as arising through methylation of a precursor by formaldehyde or glyoxylic acid resulting (in the case of animals) from the deamination of glycine. It was, in fact, suggested by the author (53) that glycine might by oxidative deamination methylate itself to betaine. On the other hand, du Vigneaud and his colleagues have recently shown in animal experiments that the methyl group of methionine, $CH_3SCH_2CH_2CH(NH_2)COOH$, is concerned in the formation of choline (see page 349) and that the reverse relationship also occurs.

(*2*) *Oxidative demethylation of* $\rangle NCH_3$ *compounds:* The remarkable experiments of Hess and his coworkers (119–122) established that when formaldehyde reacts with certain primary and secondary alcohols containing a cyclic $\rangle NH$ group (pyrrolidine and piperidine derivatives) this is converted to $\rangle NCH_3$ and the $—CH_2OH$ or $\rangle CHOH$ group is oxidized to —CHO or =C=O. Conversely, the resulting *N*-methylated keto acid on treatment with phenylhydrazine or semicarbazide yields a secondary alcohol, the $\rangle NCH_3$ group giving rise to $\rangle NH$ and the phenylhydrazone or semicarbazone of formaldehyde. The reduction of $\rangle NCH_2OH$ to $\rangle NCH_3$ is regarded by Hess as being effected by the $\rangle CHOH$ group and not by excess formaldehyde. Thus, an external secondary alcohol can act as an oxygen acceptor: isopropyl alcohol with formaldehyde and piperidine or diethylamine gives acetone. On the other hand, the conversion of hexahydronicotinic acid to its $\rangle NCH_3$ derivative, formic acid, and carbon dioxide when heated with 2 moles of formaldehyde shows that this can act as an oxygen acceptor in the absence of other suitable substances. See also in this connection (*a*) the absence of carbon dioxide pressure in the sealed tubes used for the interaction of the alkanolamine and formaldehyde, and (*b*) its production when formic acid is also present.

There is much evidence to show that demethylation of methylated amino acids or amines can be effected by animals or animal tissues.

Some interesting results were obtained by Fuchs (96) in a study of the behavior of choline in the body of the dog. Large subcutaneous injections of choline chloride yielded only traces in the urine, indicating considerable breakdown. That this does not take place by way of trimethylamine (as is occasionally the case with microörganisms (174)) is shown by the absence of any abnormal amount of trimethylamine oxide in the urine; trimethylamine normally gives rise to this oxide in animals.

Monomethyl- and dimethyl-aminoethanols, which are allied to choline, also disappear on injection but do not give rise to appreciable amounts of trimethylamine or its oxide. The same is true of methylamine and dimethylamine.

Fuchs concludes that choline and these related compounds undergo demethylation in the dog.[6] For other work leading to similar conclusions see Guggenheim (111).

In this connection the conversion of dimethylaniline to the glycuronate of *p*-monomethylaminophenol in the rabbit (128) is of interest. Small quantities of monomethylaniline could also be detected in the urine. Demethylation of dimethylaniline is also effected by dogs and *o*-aminophenol is excreted (129). Lewis and Tager (149) state that *N*-methyl- and *N*,*N*-dimethylsulfanilamides are demethylated when administered to men or mice. (For the recent work on the demethylation of *p*-dimethylaminoazobenzene in rats and its curative effect on renal hemorrhage in these animals see page 352.)

Bloch and Schoenheimer (30) fed rats with (*a*) isotopic glycine and (*b*) isotopic sarcosine (*N*-methylglycine). Glycine was then isolated from the tissue protein as the trioxalatochromate, the concentration of isotopic nitrogen being almost identical in each case. It is suggested that sarcosine is demethylated in the tissues without loss of nitrogen. This is in agreement with the work of Gordon and Jackson (100) and of Abbott and Lewis (1) on the capacity of sarcosine to replace glycine as a detoxicating agent when benzoic acid is fed to albino rats. On the other hand, *N*-ethylglycine causes no increase in the rate of excretion of hippuric acid when administered with benzoate to rabbits, suggesting that deëthylation is at any rate a much slower process (2). The oxidative demethylation of sarcosine has now been definitely established in the presence of broken cell preparations of the liver of cats and rabbits, formaldehyde being detected colorimetrically and the resulting glycine determined by van Slyke's method (116). The results with sarcosine are of interest in view of the work of Hess (already cited). The authors point out that other *N*-methylamino acids are not necessarily metabolized in the same way. Thus Keilin and Hartree (141) found that *N*-methylalanine gives pyruvic acid and methylamine with amino acid oxidase.

In consequence of the behavior of sarcosine it was to be expected that attempts would be made to discover whether it could act as a methylating agent. Work by du Vigneaud and his colleagues has shown that, unlike certain closely related compounds (which do not eliminate a methyl group as formaldehyde), sarcosine

[6] The possibility does not appear to be excluded that in Fuchs's experiments choline and aminoethanol might disappear through direct conversion to lecithin and cephalin, and that demethylation of choline is not involved. The natural phosphatides probably contain no mono- or di-methylaminoethanol (16, 180, 213; see page 340) and such an explanation leaves the disappearance of these two compounds unexplained.

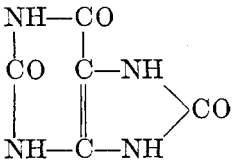
Preliminary methylation of mono- and di-methylaminoethanols to choline is an obvious explanation, but this is considered improbable by Fuchs because he obtained no betaine in the urine after subcutaneous injection of sarcosine (methylgycine) in a dog. A further alternative explanation of Fuchs's results is that choline might be oxidized to the corresponding aldehyde or to betaine. No search for these compounds in the urine appears to have been made, although Fuchs refers to the work of Mann and Quastel (152a), who observed the conversion of choline to the aldehyde by rat-liver slices.

exerts no methylating action in animal experiments. It can, therefore, be stated that, attractive as is the hypothesis that biological methylation in moulds and animals arises through the agency of formaldehyde produced by deamination of glycine or demethylation of *N*-methyl compounds, confirmation on the biological side is lacking. There are, however, no grounds for discarding it as a possible mechanism under certain circumstances, although du Vigneaud, by his work on methylation processes in animals, has shown that it cannot be the only one.

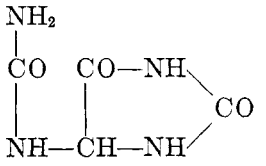
(3) *The breakdown of purines through uric acid to glyoxylic acid*: In animal and vegetable tissues there are present enzyme systems which convert nucleic acids to purines (adenine and guanine) and thence by oxidation and deamination to uric acid. Uricase, an enzyme converting uric acid (I) into allantoin (II), occurs in the liver and kidneys of various animals (215) and was found by Nemec (165) in several leguminous plants and by Fosse, Brunel, and de Graeve (91) in higher fungi

Sumi (199) isolated uric acid from the spores of *Aspergillus oryzae*, and Fosse, de Graeve, and Thomas (92) detected it in numerous plants. Allantoin has long been recognized as a product of purine metabolism, and Fosse *et al.* (93, 94) have shown it to be widely distributed in animals and plants, e.g., in *Phaseolus lunatus* and *Acer pseudoplanus*. Fosse and Brunel (90) showed that these two plants, and also others, contain an enzyme *allantoinase*, which hydrolyzes allantoin to allantoic acid (III), which had already been obtained from the same source by Fosse (87, 88).

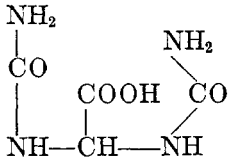
Brunel (37) has shown that material from many animal and vegetable sources contains an enzyme *allantoicase*, which hydrolyzes allantoic acid to glyoxylic acid and urea (for references to the occurrence of urea in plants, see Brunel's thesis (37)). He then showed that uricase, allantoinase, and allantoicase are all present in the mollusc *Mytilus edulis* (39) and in the mycelium of *Aspergillus niger* when grown on certain media (37, page 134). Uricase was only present when the medium contained uric acid; formation of allantoinase required the presence, among other compounds, of allantoin, whereas allantoicase was formed even when ammonium sulfate was the only source of nitrogen.



I
Uric acid



II
Allantoin



III
Allantoic acid

The existence of an enzymic system in animals, plants, and at least one mould, capable of producing glyoxylic acid, which is so closely related to formaldehyde, suggested a search for this system in the mycelium of *S. brevicaulis*, although the very special conditions required for its formation by *A. niger* were not a very hopeful sign. Dr. S. Blackburn (29) failed to detect glyoxylic acid in cultures of *S. brevicaulis* grown on glucose-Czapek-Dox medium by the usual sensitive color

reactions, although Dakin (72) mentions its presence in media on which bacteria and moulds have grown. Challenger, Subramaniam, and Walker (65) showed that small amounts of glyoxylic acid are produced by the growth of *A. niger* on citric acid, malonic acid, and acetates (for similar observations, see reference 20).

When the mycelium of *S. brevicaulis*, grown on glucose-Czapek-Dox solution, was incubated for a few hours with allantoin and a few drops of chloroform, no glyoxylic acid could be detected. The mycelium, therefore, does not contain both allantoinase and allantoicase. On similar incubation with allantoin, and boiling the resulting solution with dilute hydrochloric acid, no glyoxylic acid was formed. Hence allantoinase, which produces allantoic acid, was absent, as this acid if formed would have yielded glyoxylic acid on hydrolysis with acid (89, 94). Finally the mycelium was incubated with potassium allantoate; no glyoxylic acid was detected and therefore allantoicase was also absent. A culture of the strain of *S. brevicaulis* used in this work (*Scopulariopsis brevicaulis* (Sacc.) Bainier, in the Baarn *List of Fungi*, 1932) was sent to Dr. Brunel in Paris, who grew the mycelium and confirmed our findings. (The author and Dr. Blackburn are much indebted to him for this coöperation.) There would, therefore, appear to be no evidence for the suggestion that methylation by *S. brevicaulis* is effected by glyoxylic acid arising by the progressive breakdown of uric acid. There remains the somewhat remote possibility that when grown upon arsenical media the mycelium might contain the necessary enzymes, but this has not been investigated. The methylated xanthines, theophylline, theobromine, and caffeine, have often been regarded as waste products of purine metabolism in certain plants, being in this respect analogous to the alkaloids which are, also, frequently highly methylated. To these must now be added 1,3,7,9-tetramethyluric acid, recently separated from the residues accumulated during the isolation of caffeine from tea by Johnson (138).

It is interesting to speculate on the mechanism of these biological methylations in the purine series. There is, of course, no experimental evidence for regarding it as different from that involved in the formation of other natural methyl derivatives. It is, however, clear that the work of Fosse and his colleagues has demonstrated a hitherto unrecognized source of "secondary" formaldehyde in plant metabolism, and it is not impossible that the methylated purines in plants may actually arise by way of glyoxylic acid or formaldehyde originating from nucleic acids through uric acid.

(*4*) *Assimilation of carbon dioxide:* The possibility that biological methylation may, in some of its aspects, be connected with the utilization of carbon dioxide by animals or moulds would appear worthy of investigation. References are given elsewhere by the author (55).

C. The transfer of a methyl group

The third hypothesis, based on the transfer of a methyl group from some already methylated compound such as choline or betaine, had already been put forward by Riesser (181) to explain the production of creatine in animals and also the formation of alkylated (presumably methylated) derivatives of selenium

and tellurium on administration of compounds of these elements to men and animals (59).

Betaine is of frequent occurrence in plants and has recently been detected in various crustacea (53). It has also been found in yeast, ergot, mushrooms, and in other fungi (112). Betaine is very resistant to attack by many, though not by all, microörganisms and by most animals except ruminants (for references see 118).

Choline, however, is of even more general occurrence in plants and is found combined in almost all organs of men and animals. Its occurrence as acetylcholine is of great importance in animal physiology. Choline has been isolated from the mycelium of certain moulds (e.g., from *A. sydowi*, where it occurs as the betaine-like sulfate, $(CH_3)\overset{+}{N}_3CH_2CH_2OSO_2\bar{O}$) by Wooley and Peterson (218) and from the pathogenic fungus *Blastomyces dermatiditis* by Peck and Hauser (170). Moreover, a demethylated derivative of choline, aminoethyl alcohol or cholamine, is found combined in the phosphatide cephalin, and also in the mycelium of *Blastomyces dermatiditis*, although various workers (16, 180, 213) failed to detect mono- or di-methylaminoethanol in the products of the hydrolysis of phosphatides. Simons (193) was unable to detect any demethylation products of choline when *S. brevicaulis* was grown on a glucose-choline chloride-Czapek-Dox medium with or without arsenious oxide.

Faltis and Holzinger (84) find that dimethylaminoethanol occurs as an ester of cassaic acid in the alkaloid cassaine, obtained from *Erythrophleum guineense*. Blount, Openshaw, and Todd (32) isolated monomethylaminoethanol by hydrolysis of erythrophleine, another alkaloid of the same bean. Here it is combined with erythrophleic acid, which is probably a methoxycassaic acid. Cassaic acid is stated by Faltis and Holzinger to be a hydroxy ketonic acid containing three six-membered rings and one double bond.

Guggenheim (113) discusses two possible origins of aminoethanol in nature: (*1*) the decarboxylation of serine, $CH_2OHCH(NH_2)COOH$; (*2*) the condensation of formaldehyde to glycolaldehyde, followed by reaction with ammonia and reduction. It is probable that aminoethanol and choline are interconvertible by way of the methyl derivatives of the former, which is in agreement with the work of du Vigneaud (195) and of Stetten (212).

The origin of Riesser's suggestion regarding the transfer of a methyl group is perhaps to be found in a communication by Hofmeister (127), who, when referring to the formation of methylpyridinium hydroxide and of dimethyl telluride (the formation of the telluride was assumed and not proved) in the animal body stated: "Nach dem Ausgeführten ist anzunehmen dass die CH_3 Gruppen in den Geweben welche das Vermögen der Methylierung besitzen als solche vorgebildt ist. . . . Bei Anwendung von Pyridin und Tellur käme es zur Methylierung dieser während normalerweise methylhaltige Stoffe anderen Art z.B. die Körper der Cholin und der Kreatingruppe entstehen."

Hofmeister does not mention betaine or choline as sources of the methyl group, but only suggests that choline or creatine are the normal products of the methylation process. In support of his views Riesser stated that on heating betaine hydrochloride or choline chloride and sodium formate with sodium

selenite or tellurite, odors resembling those of dimethyl selenide and telluride were produced, but no chemical identification was carried out.

(1) Transfer of methyl groups from betaine

Challenger and Higginbottom (59) and Challenger, Taylor, and Taylor (66) have shown that (*a*) sodium sulfite, (*b*) organic disulfides, (*c*) sodium selenite, and (*d*) sodium tellurite when heated with betaine (free from hydrochloride, to avoid the formation of methyl chloride) and in the absence of sodium formate yield dimethyl sulfide, methyl alkyl or methyl aryl sulfide, dimethyl selenide, and dimethyl telluride. All these products were identified by the formation of derivatives. The last three reactions (b, c, and d) exhibit a rather close parallel with the behavior of the corresponding compounds in cultures of *S. brevicaulis* (see pages 323, 324, 331). For a reason as yet unexplained the analogous experiment with sodium arsenite gave no trimethylarsine. The suggestion for these experiments was found in the early observation of Willstätter (217) that, on heating, betaine is converted to the methyl ester of dimethylaminoacetic acid, a reaction clearly involving the migration of a methyl group (compare also Straw and Cranfield (197)). It was suggested by Challenger (54) that these pyrogenic reactions proceed somewhat as follows:

$$(1)\quad (CH_3)_3\overset{+}{N}CH_2C\overset{-}{O}O + Na_2SeO_3 = (CH_3)_2NCH_2COONa + CH_3SeO_3Na$$

With selenites and tellurites a quaternary salt is probably first formed. The dimethyl selenide presumably arises by decomposition of the sodium methaneselenonate.

$$(2)\quad (CH_3)_3\overset{+}{N}CH_2C\overset{-}{O}O + RSSR = (CH_3)_2NCH_2COOSR + RSCH_3$$

Under similar conditions primary aromatic amines yield *N*-monomethyl derivatives.

Challenger, Taylor, and Taylor (66) then refer to the two mechanisms discussed by Ingold and his collaborators (97, 132–135), who have shown that the reactions

$$(R\overset{+}{N}R'R''R''')\overset{-}{X} \rightarrow RX + NR'R''R''' \quad \text{and} \quad (R\overset{+}{S}R'R'')\overset{-}{X} = RX + R'SR''$$

may be unimolecular or bimolecular according to the polar character of R and X. The unimolecular reaction proceeds by the separation of an ion R^+, which then unites with X^-, but in the bimolecular reaction no free ion is eliminated. The authors then state that "in the absence of any evidence as to the kinetics of the various betaine decompositions—they occur at high temperatures—it is impossible to say whether a free methyl ion is concerned in the reactions."

Experimental evidence is equally lacking as regards the kinetics of the production of methyl derivatives by living cells. Considering first a unimolecular mechanism as given above (Type S_N1, Hughes and Ingold (133), Gleave, Hughes, and Ingold (97)), it was first pointed out by Challenger (54) that almost all the compounds which have been found to undergo methylation by

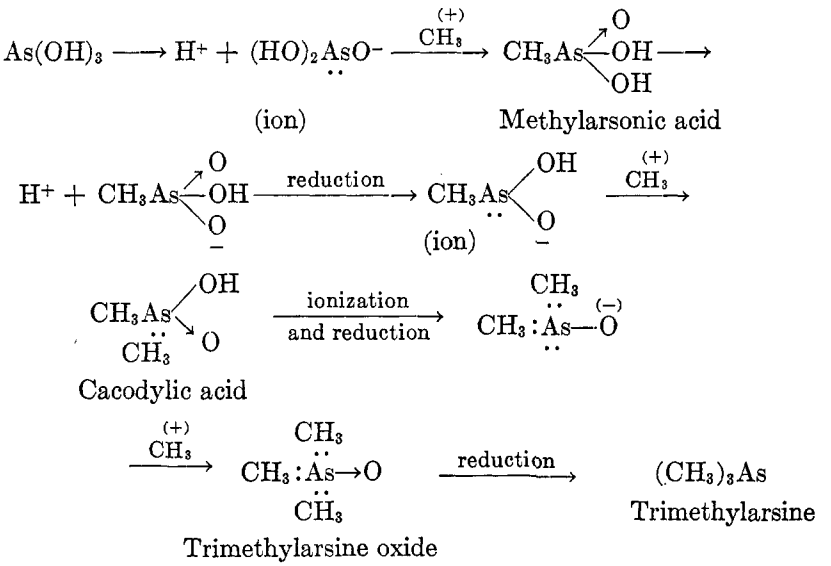
moulds or on introduction into the animal body are capable of furnishing negative ions, e.g., sodium arsenite, selenite, and tellurite, alkylthiols (arising from dialkyl disulfides) and also nicotinic acid, which gives trigonelline (5, 130). Moreover, all these compounds contain unshared electrons, so that coördination of a positive methyl group by the ion would lead to the formation of a neutral molecule which could then undergo reduction and ionization, followed by further coördination of a CH_3^+ radical. The positive methyl group may be assumed to be derived from either betaine, choline, or methionine, leaving in either case a negative ion which would compensate the hydrogen ion of the compound RH undergoing methylation (66):

$$(CH_3)_3\overset{+}{N}CH_2\overset{-}{C}OO + RH = (CH_3)_2NCH_2COOH + RCH_3$$

$$\underbrace{(CH_3)_3\overset{+}{N}CH_2}_{OH^-}CH_2OH + RH = (CH_3)_2NCH_2CH_2OH\cdot + RCH_3 + HOH$$

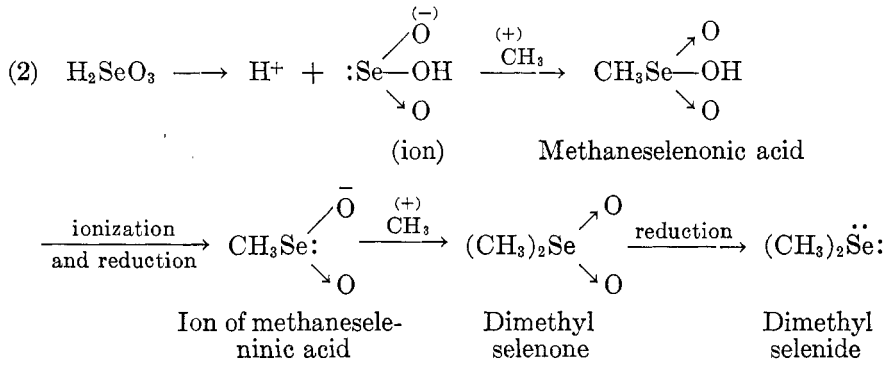
(2) Methylation of arsenic, selenium, and tellurium compounds

The process suggested by the Leeds School may be illustrated in the case of arsenious and selenious acids:



None of the suggested intermediate compounds have been detected in mould cultures, but methylarsonic acid, cacodylic acid, and hydroxytrimethylarsonium nitrate (the nitrate of trimethylarsine oxide) all yield trimethylarsine when present as substrates in bread cultures of *S. brevicaulis* (61). Furthermore, it has been shown earlier in this review that alkyl- and dialkyl-arsonic acids, $RAsO(OH)_2$ and R_2AsOOH (where R may be ethyl, *n*-propyl, or allyl), similarly

yield mixed arsines, $RAs(CH_3)_2$ and R_2AsCH_3. Ethylmethyl-*n*-propylarsine was obtained in this manner from ethyl-*n*-propylarsonic acid (58, 63; see page 322).



A similar series of reactions would explain the formation of dimethyl telluride from salts of tellurous acid.

It will be seen that on this view of the mechanism the postulated intermediate products are also required by the formaldehyde hypothesis, which, however, demands the prior formation of a hydroxymethyl group at each stage of the methylation. None of the three postulated intermediate selenium compounds have been detected in the culture media. Dimethyl selenone has not been prepared. For experimental work designed to test the possibility of this scheme, see Bird and Challenger (25a), who have shown that bread cultures of *S. brevicaulis* and certain *Penicillia* convert methane-, ethane-, and propane-1-seleninic acids, $RSeO_2H$, to dimethyl-, ethyl methyl, and methyl *n*-propyl selenides, $RSeCH_3$, as required by the mechanism outlined above. The authors point out, however, that the simultaneous production of traces of alkylselenothiols, RSeH, or of dialkyl diselenides introduces some ambiguity into the interpretation of these results since, by analogy with the behavior of alkylthiols and disulfides in cultures of *S. brevicaulis* (see below) either type of compound might be converted to a methyl alkyl selenide without passing through the selenone, CH_3SeO_2R. Neglecting this possibility, which the authors regard as somewhat improbable, the formation of methyl alkyl selenide may be represented thus:

$$\overset{-}{RSeO_2} + \overset{+}{CH_3} \rightarrow RSeO_2CH_3 \rightarrow RSeCH_3$$

Bird and Challenger also examined the behavior of the potassium salts of methane-, ethane-, and propane-1-selenonic acids, $RSeO_2OK$, in cultures of the same moulds with a rather surprising result, only dimethyl selenide being formed in each case. This is presumably due to breakdown of the selenonate in the cultures, giving ROH and $KHSeO_3$. This reaction takes place in the test-tube on warming with dilute acid or alkali. This observation is not regarded as vitiating the suggested mechanism, since it is possible that the methaneselenonic acid postulated as the first intermediate product might be sufficiently stable, when formed within the cell, to pass on to the next stage without hydrolysis.

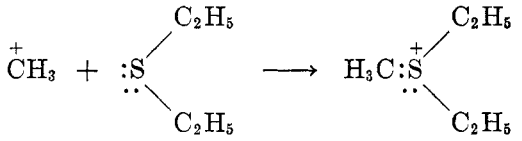
(3) Methylation of sulfur compounds

Work carried out with Blackburn and with Rawlings (56, 63) suggests that the methyl alkyl sulfides obtained by addition of dialkyl disulfides to cultures of *S. brevicaulis* arise by methylation of an alkylthiol first produced. Ionization of this, followed by coördination of CH_3, would explain the observed facts:

$$RSH \rightarrow \overset{-}{R}\overset{}{S} + \overset{+}{H} \qquad (1)$$

$$\overset{-}{R}\overset{}{S} + \overset{+}{C}H_3 \rightarrow RSCH_3 \qquad (2)$$

Alternatively, coördination of the methyl ion by the disulfide may occur prior to fission (66). Neuberg and Grosser (167) state that diethylmethylsulfonium hydroxide is a normal ingredient of a dog's urine, yielding diethyl sulfide on warming with alkali. They also state that diethyl sulfide on administration to dogs is converted to the methylsulfonium base. This can be expressed thus:



and represents a reversal of the reaction

$$NR_3 + Alk\overset{+}{S}R_2 \rightleftharpoons Alk\overset{+}{N}R_3 + R_2S$$

envisaged by Hughes and Ingold (134), who remark that instances of this reaction have not been recorded. Attempts by B. Taylor (201) to detect the formation of this sulfonium base on addition of diethyl sulfide to cultures of *S. brevicaulis* on glucose-Czapek-Dox medium failed.

Addition of aqueous hydrogen sulfide, or of sodium sulfide, thiosulfate, tetrathionate, sulfite, or methanesulfonate to cultures of the mould failed to give any dimethyl sulfide. Sodium ethanesulfinate, $C_2H_5SO_2Na$, gave no ethyl methyl sulfide. The apparent inertness of the last three compounds, both in bread and in liquid cultures, was at first somewhat surprising, in view of the ready reactivity of sodium selenite and sodium alkylseleninate. It appeared possible that this failure might be ascribed to the formation of methanesulfonic acid or of dimethyl sulfone by reactions analogous to those postulated in the case of sodium selenite.

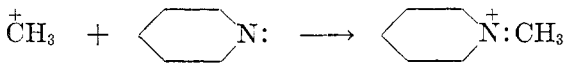
Diethyl sulfone, unlike diethyl sulfoxide (62), is not reduced to diethyl sulfide in bread cultures of *S. brevicaulis*, and sulfones, if formed, would probably accumulate. (This difference may be compared with the difference in the case of the chemical reduction of sulfites and sulfates to hydrogen sulfide.) Careful extraction of the liquid culture media containing sodium sulfite, methanesulfonate, or ethanesulfinate with chloroform gave no dimethyl or ethyl methyl sulfone. Methanesulfonic acid, also containing the stable $\rangle SO_2$ group, might also be expected to resist further reaction, in which case neither sulfone nor

sulfide would be formed. Attempts to detect this acid in liquid cultures containing sodium sulfite failed, although several attempts were made. The detection of small quantities of alkylsulfonic acids is, however, very troublesome. These experiments were carried out by Dr. Bird and Mr. J. W. Fletcher.

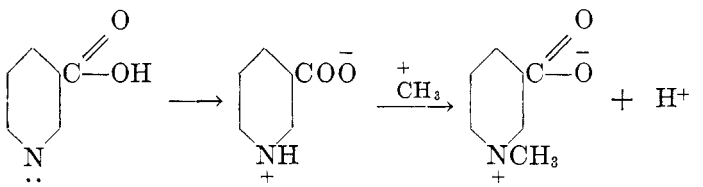
Even on the assumption that sodium methanesulfonate escaped detection in the sulfite cultures, it is difficult to explain the non-formation of ethyl methyl sulfone from sodium ethanesulfinate.

(4) Methylation of nitrogen compounds

Coördination of a positive methyl ion would also explain the well-known conversion of pyridine and quinoline to methylpyridinium and methylquinolinium hydroxides in the body of the dog (126, 145, 200).



The formation of trigonelline or of *N'*-methylnicotinamide (see below) on administration of nicotinic acid to various animals may be explained in the same way.



This mechanism is in agreement with the twin-ion structure for betaines such as nicotinic acid and trigonelline. The hydrogen ion of the nicotinic acid yields RH, as before.

Ackerman (5) showed that this formation of trigonelline in the dog is accompanied by that of nicotinuric acid (see also 130, 131)

and in previous communications by the author (53, 59) this has been cited in support of the view that glycine (with or without previous oxidative deamination to formaldehyde) is concerned in both changes. It should be pointed out that the formation of nicotinuric acid is not incompatible with the view that the methyl group of trigonelline is derived from choline, betaine, or some similar substance. Complete demethylation of the first two compounds would lead to aminoethyl alcohol ($NH_2CH_2CH_2OH$) and to glycine (NH_2CH_2COOH) either of which, assuming preliminary oxidation in the first case, could yield nicotinuric acid.

Further work by Najjar *et al.* (161, 162, 163) and by Huff and Perlzweig (130, 131) indicates that in man and in rats doses of either nicotinic acid or its amide result in the excretion of *N'*-methylnicotinamide as the chief end product

rather than trigonelline. After large doses of nicotinic acid to rats, however, there appears in the urine a considerable fraction of the total methylated product which is not the amide.

Perlzweig, Bernheim, and Bernheim (171) have shown that nicotinamide when incubated with rat liver slices at 37°C. is converted into its *N'*-methyl derivative. The process is strictly aerobic, and requires unbroken cells; minced liver even in the presence of oxygen is inert. Nicotinic acid is not methylated under these conditions, nor is the amide methylated by rat kidney or muscle. The necessity for an intact cell recalls the results of Challenger and Higginbottom (59), who were unable to observe the formation of trimethylarsine from arsenious acid and various sterile preparations obtained from cultures of *S. brevicaulis* e.g., by submitting the mycelium to great pressure, thus obtaining a "press juice," by treating the mycelium with acetone, or by filtering the culture medium through porcelain.

Maassen (152) states that a press juice obtained from the mycelium of *S. brevicaulis* or the mycelium itself, after killing with alcohol, chloroform, or ether, gave no odor with sodium tellurite. Smith and Cameron (194) also failed to obtain an active enzyme preparation.

Hofmeister (127) and Maassen (152) showed that whereas the intact, minced, or crushed tissue of the liver of dogs and especially the lungs and testicles of dogs and the testicles of fishes readily convert inorganic compounds of selenium and tellurium into odorous substances, presumably dimethyl selenide and dimethyl telluride, attempts to obtain an active press juice from the organs failed. Exposure of the tissues to low temperatures had no harmful effect on the activity, but heating at 40–50°C. or treatment with acids, alkalis, concentrated salt solutions, or alcohol destroyed it at once. The methylating process is therefore presumably enzymic, but owing to their failure to obtain active preparations after separation from the tissue both Maassen and Hofmeister concluded that it was definitely bound up with the life of the cell.

In their experiments with an entirely different substance—nicotinamide—Perlzweig *et al.* (171) observed no methylation using minced liver. Further work under strictly comparable conditions will be necessary before the effect of destruction of the cell structure on biological methylation in animals can be satisfactorily assessed.

As already stated (see page 341), the transfer of a methyl group might take place by a bimolecular mechanism of Ingold's S_N2 type. Hughes and Ingold (134), when discussing the kinetics of the reaction between alkyl halides and sodium thiosulfate or ethyl sodioacetoacetate, state, ". . . mechanism S_N1 if present would not normally be observed, the carbon cation produced by a primary ionization would react much more often with the solvent than with the ionic reagent, and the result would be a hydrolysis or alcoholysis." From this it would appear that, as biological processes occur in an aqueous medium, the participation of a positive methyl ion would involve the simultaneous if not preponderating production of methyl alcohol.

Raistrick (177), in an investigation of the carbon balance sheet of three

different strains of *S. brevicaulis* grown on aqueous glucose and inorganic (Czapek-Dox) salts, found no metabolic products other than carbon dioxide. One strain of *Penicillium chrysogenum* and one of *P. notatum* were shown by Bird and Challenger (25) to produce dimethyl selenide and dimethyl telluride in bread cultures containing selenite or tellurite. Raistrick *et al.* (178) record that three species of *P. chrysogenum* and one of *P. notatum* do not form alcohol when grown on glucose-Czapek-Dox solution, except in very small amounts, although they produce other non-volatile metabolic products. The term "alcohol" refers, of course, to ethyl alcohol, but methyl alcohol would not have escaped detection.

It is not possible to state whether the strains of *P. chrysogenum* and *P. notatum* used by Bird and Challenger were identical with any of those cited by Raistrick and it should be mentioned that (178a) some species of *Aspergillus nidulans* produce alcohol, whereas others do not. An examination by Dr. Higginbottom of cultures of *S. brevicaulis* on the same medium (200, 200, and 1000 cc. were separately distilled and the "first runnings" tested with acidified potassium dichromate) failed to reveal the presence of any methyl alcohol. As it seemed possible that the methylation processes of the mould might only function in presence of a poison, one culture (200 cc.) containing arsenious acid was also examined with, however, a negative result.

There is at present, therefore, no evidence for the production of methyl alcohol by *S. brevicaulis*. Further experiments with larger quantities are in progress.

The alternative to methylation by elimination of a positive methyl ion is a bimolecular reaction of the S_N2 type (97, 133), which they express thus:

$$R'R''R'''\overset{\curvearrowleft}{N} - \overset{\curvearrowleft}{R}\}X \rightarrow NR'R''R''' + RX$$

Here X would represent the arsenite, tellurite, etc., ion. This differs from the S_N1 reaction only in its kinetics and not in its products. If, as seems probable for the reasons just stated, this bimolecular mechanism would appear preferable, then the representation of the coördination of CH_3^+ by the arsenite or other negative ion should be replaced by a scheme in which the transfer of methyl takes place without actual separation as an ion. Since, however, this also ultimately involves the attachment of methyl to the unshared electrons of the metalloid, the formulations on pages 342 and 343 may be retained for convenience in representing the suggested intermediate stages in the methylation process.

(5) Transmethylation: du Vigneaud's experiments using isotopic indicators

The suggestion that certain biological methylations in animals might be conditioned by choline or betaine, first outlined by Riesser (181), was amplified by Challenger and Higginbottom (59) and expanded to include the similar reactions exhibited by *S. brevicaulis* and certain other moulds. These authors stated, "... it is not impossible that some ingredient of the cell substance containing a methylated nitrogen atom may, under the special circumstances obtaining in the cell, lose a methyl group which, if it be eliminated with a positive charge, could

be easily coördinated by the unshared electrons of tervalent arsenic or of quadrivalent selenium or tellurium." This suggestion, now further developed in this communication (the sections dealing with the coördination of a methyl ion have been in typescript since June, 1939), receives support from the recent work of du Vigneaud and his colleagues. They have shown (67, 209, 210) that homocystine (IV) (after conversion to homocysteine (V) can replace methionine (VI) in the diet of the white rat only in the presence of choline and certain related substances such as betaine, which, however, produces the effect more slowly than choline. The authors suggested that a methyl group is transferred from the nitrogen of choline or betaine to the sulfur of homocysteine to give methionine and considered that the reaction might be reversible, methionine acting as a donor of methyl groups to a choline precursor.[7]

$(HOOCCH(NH_2)CH_2CH_2S)_2$

IV

Homocystine

$HOOCCH(NH_2)CH_2CH_2SH$

V

Homocysteine

$HOOCCH(NH_2)CH_2CH_2SCH_3$

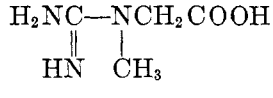
VI

Methionine

$HOOCCH(NH_2)CH_2CH_2SCD_3$

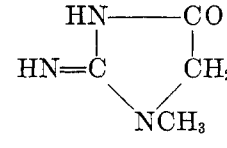
VII

Deuteriomethionine



VIII

Creatine



IX

Creatinine

It will be seen later (page 352) that choline prevents a pathological condition known as "fatty infiltration" of the liver in rats. It appeared possible, though rather improbable, that the growth observed in the dietary experiments just outlined might have been simply due to this particular effect of choline, the liver thus being enabled to remain healthy and to carry out methylation by some other means than a transference of methyl from choline.

This explanation was, however, disproved when the choline was replaced by its ethyl analogue,

$$(C_2H_5)_3\underset{\underset{OH}{\frown}}{N}CH_2CH_2OH$$

which can also prevent fatty infiltration. This compound did not allow of the growth of rats maintained on a choline-methionine-free diet containing homocystine. du Vigneaud points out (208) that had an ethyl group been transferred, ethionine (*S*-ethylhomocysteine, $C_2H_5SCH_2CH_2CH(NH_2)COOH$) would have been formed and this was shown by Dyer (79) to be incapable of replacing

[7] Professor du Vigneaud very kindly informed the authors of these early experiments in April, 1939.

methionine in the diet. du Vigneaud also showed that on feeding ethionine and choline on a methionine-free diet to rats no growth resulted, indicating that homocysteine is not formed from ethionine in the body. This stability of the $—SC_2H_5$ link in ethionine recalls the difficulty experienced in deëthylating ethylglycine in rabbits (see page 348) or certain *N*-ethylphenazine derivatives under purely chemical conditions (154a).

du Vigneaud's "transmethylation" hypothesis, based on his dietary experiments with white rats, was tested by the use of specimens of deuteriomethionine (VII) containing (*a*) 83.6 and (*b*) 87.5 atom per cent of deuterium in the methyl group. These were fed to rats kept on a methionine-choline-free diet (211). Earlier work had shown that the deuterium content of the urinary creatinine (IX) closely follows that of the creatine (VIII) 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 per cent. The animal was then killed and the choline isolated from the tissues as the chloroplatinate. The atom percentage of deuterium in the methyl groups of this choline was found to be 74.2, the corresponding figure for the tissue creatine being 73. These figures represent in all three cases approximately 85 per cent of the theoretically possible amount of deuterium, assuming that all the methyl groups had come from the deuteriomethionine. This figure is the "deuterium ratio," i.e., atom per cent deuterium in methyl group of isolated compound per atom per cent deuterium in methyl group of deuteriomethionine administered $\times 100$. For other results with specimens (*a*) and (*b*) the original paper must be consulted. By oxidation of the choline to trimethylamine with potassium permanganate and analysis of the hydrochloride it was shown that the whole of the deuterium was contained in the methyl groups.

du Vigneaud and his colleagues conclude that these reactions are true transmethylations (the methyl group being transferred as a whole) and that they do not involve the elimination of dideuterioformaldehyde, CD_2O. On the formaldehyde theory of methylation dideuterioformaldehyde, if produced, would react with the amino group of the choline precursor, presumably ethanolamine, $HOCH_2CH_2NH_2$ (see 195), to give $—NHCD_2OH$, which on reduction in the organism would give $—NHCD_2H$ and not $—NHCD_3$. It would then follow that the deuterium content of the methyl groups of the choline could not rise above two-thirds of the concentration of the deuterium in the methyl group of the methionine administered, i.e., the "deuterium ratio" would have a maximum of 66.6 per cent. Similar arguments hold for the deuteriocreatine.

du Vigneaud *et al.* (212) then administered trideuteriocholine

$$(\underbrace{CD_3)_3\overset{+}{N}OH}CH_2CH_2OH$$

to rats maintained on a methionine-choline-free diet containing homocystine for 23 and 56 days, respectively. On isolation of the creatine from the body tissues the deuterium content of the two samples was 24 per cent and 29 per cent

of the theoretical maximum, thus proving that the methyl groups of choline can also take part in transmethylation. This transfer also takes place, though to a lesser extent, when no homocystine is given or when ordinary methionine is given instead of homocystine.

The most interesting result obtained by du Vigneaud in this particular investigation is, however, the demonstration of the transfer of methyl from choline, giving rise to methionine by the detection of the deuteriomethyl group in tissue methionine. Furthermore this transmethylation was shown to occur when deuteriocholine was administered without homocystine in the diet and even when ordinary methionine was given along with deuteriocholine.

The authors consider that homocysteine is formed during the catabolism of methionine by the animal, thus enabling methionine to be re-formed by means of the methyl group supplied by choline. Continuous synthesis of methionine therefore occurs, although more than enough methionine is supplied in the diet. Similarly, experiments in which deuteriomethionine and ordinary choline were fed together show that the formation of choline from methionine proceeds even with an adequate supply of choline.

The determination of the deuteriomethionine was carried out by fission of the $—SCD_3$ group by heating with hydriodic acid. The methyl iodide (CD_3I) thus formed was absorbed in alcoholic trimethylamine cooled with solid carbon dioxide. The resulting tetramethylammonium iodide was converted to the chloride with silver chloride and the percentage of deuterium determined in the corresponding chloroplatinate.

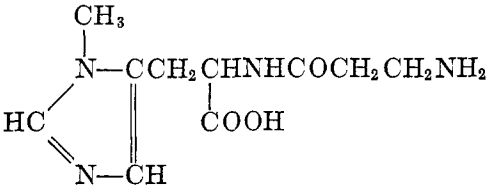
Control experiments showed that some loss, i.e., exchange, of deuterium occurs due to fission of the $C—D_3$ linkage by the hydriodic acid. Thus the tetramethylammonium iodide so obtained contained 66–72 per cent of the calculated amount of deuterium. The values obtained for the isotopic content of tissue methionine are therefore minimal figures.

Deuterium–hydrogen exchange also occurs when deuteriomethionine is heated with aqueous 20 per cent sodium hydroxide for 23 hr. On the other hand, the C—D bond in the $N—CD_3$ group of deuteriocholine is not labilized by boiling normal hydrochloric acid, boiling 5 per cent barium hydroxide, or hot alkaline potassium permanganate solutions.

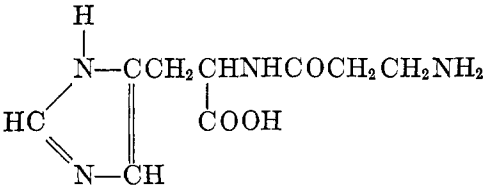
Further experimental work by du Vigneaud and his colleagues (186a) has established the occurrence of transmethylation in another animal, the rabbit. Deuteriomethionine (79 atom per cent D in the methyl group) was administered to the extent of 0.5 per cent of the diet for 8 days and 1.0 per cent for a further 20 days. At the end of that time the creatinine of the urine, the free (water-soluble) and bound (ether-soluble) choline of the tissues, and the anserine (X) of the muscle were analyzed, the deuterium ratios being 21.4, 9.2, 4.9, and 1.9, respectively. The basal diet contained fibrin and hence some ordinary methionine was present. Consequently the above ratios are minimal values. The anserine was separated by alternate formation of the mercury and copper derivatives and analyzed as the copper compound. Choline and creatinine were analyzed as the chloroplatinate and double potassium picrate, respectively.

The rate of transfer of methyl from methionine giving anserine is much slower than the analogous process which yields creatinine.

The authors point out that although rabbit muscle contains both carnosine (XI) and anserine, the latter compound greatly predominates.



X
Anserine



XI
Carnosine

A further advance was marked by the recent announcement of Simmonds and du Vigneaud (191) that, using the isotope technique, they had shown that the methyl group of dietary methionine can be used by man in the synthesis of choline and creatinine. A healthy adult male ingested 6 g. of trideuteriomethionine (73.3 atom per cent D in the methyl group) during 3 days. After 72 hr. the deuterium ratio was 0.54 ± 0.06 in the case of the creatinine zinc chloride compound of the urine and 1.9 ± 0.1 for the choline chloroplatinate obtained from 350 cc. of the blood. These low figures are due, of course, to the short duration of the experiment.

(6) Transmethylation in wheat germs

Barrenscheen and Valyi-Nagy (14a) have recently shown that methionine increases the creatine synthesis from glycocyamine (guanidinoacetic acid) by wheat germs, six to eight fold. The process is obligatory aerobic, the sulfur of the methionine being oxidized to sulfate, corresponding to 25 per cent of the transformed methionine. Plant tissues transform glycine in presence of methionine to betaine. Here again oxidation of the methionine occurs.

XI. LABILE METHYL GROUPS IN RELATION TO OTHER BIOLOGICAL PROCESSES

The work of du Vigneaud and his colleagues has clearly established that choline is concerned with methylation in white rats by virtue of its capacity to transfer its methyl groups.

Two pathological conditions have been extensively studied in rats: namely, fatty livers and hemorrhagic kidneys. These conditions can be produced on a

diet deficient in choline. A valuable summary of work in this field is given by McHenry (154) and Griffith (106; this reference also contains an account by du Vigneaud (208) of much of his recent work on transmethylation).

McHenry states that a marked increase in liver fat can be noted within a day after rats are placed on a choline-free diet and the renal hemorrhages are well marked within 10 days. Both these effects can be cured by administration of choline, methionine, or betaine with the diet; by analogy with du Vigneaud's work this suggests that labile methyl groups may be concerned. The curative action on fatty livers is known as a lipotropic effect.

McHenry summarizes the possible modes of action of choline in the animal body as (*1*) stimulation of the formation of phospholipoids, (*2*) formation of acetylcholine, (*3*) transmethylation. Both in the case of fatty livers and hemorrhagic kidneys he is inclined to ascribe the curative action of choline to the first mode of action rather than to the third, and cites the established case of the lipotropic action of the ethyl analogue of choline

$$(CH_3CH_2)_3\overset{+}{N}CH_2CH_2OH$$
$$\underbrace{\qquad\qquad}_{OH^-}$$

as showing that labile methyl is not an essential requirement for this effect. (This triethylammonium base cannot, however, replace choline in du Vigneaud's experiments on the utilization of homocystine by white rats.)

Griffith (106) states that it seems probable that the choline phospholipoids are involved in the problem of the formation (and spontaneous cure) of the renal lesions, but that the whole picture may be complicated by variations in the dietary or metabolic supply of compounds containing labile methyl groups or of other substances.

It appears impossible at present to decide on the mechanism by which fatty livers and the kidney lesions are cured by choline, betaine, and methionine—and hemorrhagic kidneys by *p*-dimethylaminoazobenzene—but it seems probable that labile methyl groups play a part in many if not all of the effects. There is no doubt that in recent years the methyl group has acquired a greatly enhanced importance in biochemistry.

Griffith and Mulford (107) in quantitative experiments have compared the choline-like activity of methionine and betaine with respect to fatty livers and kidney lesions in rats and find that "the methyl of methionine is efficiently utilised whereas betaine has but one-third the protective action of choline, as if only one of its methyl groups is used in the synthesis of choline."

This recalls the observation of du Vigneaud that as a source of labile methyl for converting homocystine to methionine and so promoting the growth of white rats, betaine is inferior to choline. Aminoethanol and its monomethyl and dimethyl compounds all occur in nature, and the complete transfer of methyl from choline may be possible. It will be recalled that Willstätter and his colleagues (66, 217) showed that betaine is converted by heat to the methyl

ester of dimethylaminoacetic acid, owing to the transfer of one methyl group. Of course, *in vivo*, further demethylation may occur giving finally the methyl ester of glycine—possibly by transmethylation, possibly by oxidative demethylation. Evidence on this point is lacking. It is, however, interesting to speculate as to whether one or three of the methyl groups of betaine are available for transmethylation. Here it should be recalled (see page 337) that sarcosine or methylglycine, CH_3NHCH_2COOH, is stated to lose a methyl group as formaldehyde in the presence of kidney slices (116) but is incapable of participating in transmethylation under the conditions employed by du Vigneaud.

Griffith and Mulford (107) find that nicotinic acid has an opposite effect to that of choline on the incidence and severity of renal lesions or the deposition of liver fat, "possibly because of the diversion of some labile methyl for the formation of trigonelline" (5, 130). It would be useful to determine whether a similar antagonism could be demonstrated between choline and such compounds as pyridine, quinoline, dialkyl disulfides, selenites, tellurites, and glycocyamine, all of which are well-recognized as methyl acceptors in animals or moulds.

Another compound which is antagonistic to choline as regards the lipotropic effect in rats is cystine. The effect is not, however, proportional to the amount of cystine fed and is regarded by Griffith (106) as "not directly related to the metabolism of choline but due to a stimulation of metabolism which is the result of a supplement of cystine in a cystine-deficient diet."

The effect of *S*-methylcysteine,

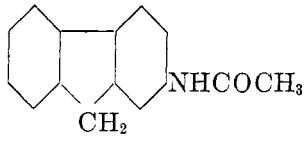
$$CH_3SCH_2CH(NH_2)COOH$$

on the production or cure of fatty livers does not seem to have been investigated. Should this compound prove to have a lipotropic action as has methionine, further examination of the cystine effect might be worth while.

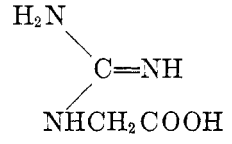
The "diversion of some labile methyl", which Griffith and Mulford regard as possibly accelerating the production of fatty livers and the kidney lesions already discussed, may also play an indirect part in facilitating the formation of liver tumors in rats receiving 2-acetylaminofluorene. Bielschowsky (23) finds that 2-acetylaminofluorene is carcinogenic for white rats; 4 mg. per rat and day added to the standard diet for 20–30 weeks produces malignant tumors in different organs. In female rats the majority of these tumors are adenocarcinomas of the breast evident on the average after 250 days. Addition of 20 mg. of glycocyamine per rat and day to the acetylaminofluorene diet accelerates the appearance of breast tumors and at the same time increases considerably the number of liver tumors in female rats. The results suggest that the reduction of available labile methyl groups by the conversion of glycocyamine into creatine enhances the carcinogenic action of 2-acetylaminofluorene.

The full details of this work have not yet been published, but it appears possible that the reduction of available methyl groups may facilitate the formation of fatty livers and later of cirrhotic livers. There is evidence for believing that these conditions are frequently preliminaries to the formation of liver

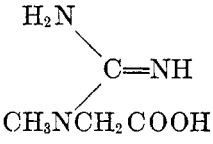
tumors. Here again it would be interesting to study the effect of other methyl acceptors.



2-Acetylaminofluorene



Glycocyamine



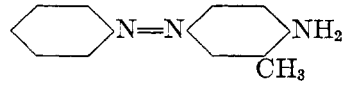
Creatine

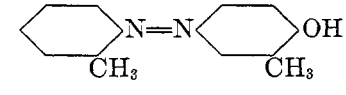
In this connection it may be mentioned that Nelson, Fitzhugh, and Calvery (164) have shown that liver tumors following cirrhosis can be produced in rats by a diet containing selenium at levels of 5, 7, and 10 parts per million. The selenium was administered either as a mixture of potassium ammonium sulfide and selenide, containing 48 g. of selenium per liter, or in the form of seleniferous grain containing sulfur and selenium in organic combination, probably as an amino acid or amino acids of the sulfide type. Tumors were not observed earlier than 18 months but many occurred at 24 months. (Liver damage in rats fed on seleniferous cereals was first observed by Franke (95).)

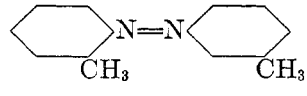
The authors make no reference to a possible "side-tracking" or "diversion" of labile methyl with subsequent liver damage in these selenium experiments, but selenites, selenates, and alkylseleninic acids, $C_nH_{2n+1}SeO_2H$, are methyl acceptors (25a, 62) in mould cultures and the first two classes of compound doubtless in the animal body also (see 25, 62). The effect of inorganic selenides in mould cultures has not been studied, but the negative selenide ion with its unshared electrons should readily give rise to dimethyl selenide. Apart from this probability the partial oxidation of inorganic selenides to selenites or selenates might be expected and this would probably also be true of selenium in amino acid. combination since the sulfur of methionine is converted to sulfate in the body.

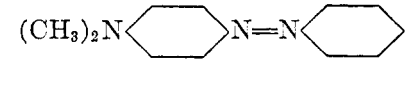
At this early stage in the development of these studies of new carcinogenic agents too much stress must not be laid on analogies, but the fact that on certain diets nicotinic acid is antagonistic to the lipotropic effect of choline and that selenium and glycocyamine are concerned directly or indirectly with tumor formation should certainly be noted, these three agents being methyl acceptors.

This survey may conveniently close with a short account of some aspects of a subject which is at present attracting much attention—the carcinogenic action of derivatives of azobenzene. Four such compounds have been found to produce this effect in mice (146a).









Among these the most important is *p*-dimethylaminoazobenzene, which produces liver tumors when fed to rats, an effect first observed by Kinosita and recently

discussed from the chemical standpoint by Cook (69). Kinosita found that *p*-aminoazobenzene is not carcinogenic (142). Diets high in protein and vitamin B markedly reduce the carcinogenicity of *p*-dimethylaminoazobenzene, and the question arose whether these ingredients of the diet act by causing demethylation (136a).

Stevenson, Dobriner, and Rhoads (196) found that in rats demethylation does occur accompanied by fission and reduction of the azo linkage, and that the urine contains *p*-aminophenol, *N*-acetyl-*p*-aminophenol, *p*-phenylenediamine, and *N*,*N'*-diacetyl-*p*-phenylenediamine. The acetylation is presumably a detoxication and is frequently observed on administration of aromatic amines. This behavior recalls the demethylation of monomethyl- and dimethyl-anilines in the rabbit (128, 129). For the behavior of other *N*-methylated aromatic amines in animals see Hildebrandt (124).

XII. CONCLUSION

When discussing transmethylation, du Vigneaud *et al.* (211) state, "...we do not know whether methionine and choline act directly or whether they are precursors of derivatives from which the methyl groups are released.... The ability of choline to give up methyl groups in the metabolic process is ... puzzling in view of the stability of the bond between methyl ... and nitrogen in choline in ordinary *in vitro* reactions." This stability is contrasted with "the well-known conversion of betaine to dimethylglycine methyl ester" (217). du Vigneaud *et al.* continue, "Because of the existence of this relatively stable N-methyl bond one is tempted to postulate the existence of some derivative of choline in which the methyl groups are similarly chemically labilized by a group more electronegative than the alcoholic hydroxyl." In an earlier communication (210) they had suggested that phosphorylation of choline might induce mobility of a methyl group. In this connection it may be mentioned that choline sulfate, $(CH_3)_3\overset{+}{N}CH_2CH_2OSO_2\bar{O}$, which has a "betaine" structure, occurs in the mycelium of *Aspergillus sydowi*, and that fourteen strains of this organism were found to volatilize arsenic when cultivated on "Czapek's solution agar" containing arsenious oxide (202). The volatile arsenic compound was not identified, but from numerous analogies (see also 26) there is little doubt that it was trimethylarsine. It is at present, however, impossible to assess the significance of these interesting observations. Furthermore, the mobility of the methyl group of betaine observed by Willstätter (217) and recently studied by Challenger, Taylor, and Taylor (66) occurs at 200°C. or higher, and some preliminary work by Dr. C. Simons in the author's laboratory suggests that under similar conditions a methyl group of choline may also be mobile.

The work of the American authors, while establishing the occurrence of transmethylation in animals, does not at this stage exclude formaldehyde (of secondary origin) as an alternative route, nor enable us to decide between the two mechanisms in the case of mould methylations (see page 338). A possible bridge between the animal and the mycological problems might be furnished by a study of the elimination of selenium, doubtless as dimethyl selenide, in the breath

of animals which have received injections or oral doses of selenates or selenites. The production of an unpleasant odor in the breath of such animals has long been known (see 62). A garlic odor in the breath of workmen engaged in the extraction of selenium from electrolytic copper "slimes" has also been observed (76).

Schultz and Lewis (see 54) found after subcutaneous injection of sodium selenite into adult white rats that 17 to 52 per cent of the administered selenium was excreted by the lungs within 8 hr. The product was absorbed in sulfuric acid, but was not identified. The amount was not materially influenced by administration of either methionine or choline chloride. Absorption in a very small quantity of Biginelli's solution (mercuric chloride in dilute hydrochloric acid) as employed by Bird and Challenger (25, 25a) in mould experiments might have led to a rapid decision. Similarly Dudley (76) reported the elimination of selenium as a volatile compound in the urine after administration of sodium selenite to a horse. It was not identified. McConnell (54) found that after single subtoxic injections of selenate containing radioactive selenium into adult white rats, 3 to 10 per cent of the original dose was excreted by the lungs in 23 hr., and absorbed in a hydrobromic acid–bromine mixture. The excretion was chiefly by the kidney in a non-volatile, ether-insoluble form.

Once the volatile product—presumably dimethyl selenide—has been identified, it is to be hoped that the elimination of selenium in the breath after simultaneous administration of trideuteriocholine or trideuteriomethionine will be studied. Absorption in nitric acid and analysis of the resulting hydroxydimethylselenonium nitrate, $(CH_3)_2Se(OH)NO_3$ (62), might bring a decision regarding the mechanism of this process. Similar experiments could be carried out with sodium tellurite, but the identification of the dimethyl telluride which would presumably result might, owing to its ready oxidation, present difficulties (25).

An obvious extension of the work of du Vigneaud and his colleagues would be to add arsenite, selenite, or tellurite and a compound having one or more mobile deuteriomethyl groups to cultures of *S. brevicaulis*. It might then be possible to decide whether transmethylation, established for certain animal methylations, also holds for the mycological process. As already stated, it is at present impossible to decide upon this point, owing to insufficient evidence. In the author's opinion it will, probably, be very profitable to seek experimental confirmation of the transmethylation hypothesis. The mobile methyl group has undoubtedly acquired considerable biochemical significance in the last few years, whereas the formaldehyde theory has not received any further experimental support. As suggested on page 339, however, the possibility that the utilization of carbon dioxide produced by the moulds is concerned with the phenomenon should also be considered.

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