CCVIII. THE PRODUCTION OF TRIMETHYL- ARSINE BY *PENICILLIUM BREVICAULE* (*SCOPULARIOPSIS BREVICAULIS*).

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CHALLENGER *et al.* [1933] showed that the volatile odorous product evolved from bread cultures of *Penicillium brevicaulis* containing arsenious acid, sodium methylarsonate $\text{CH}_3\text{AsO}(\text{ONa})_2$ or sodium cacodylate $(\text{CH}_3)_2\text{AsO} \cdot \text{ONa}$ —the so-called Gosio-gas—is trimethylarsine. It was also found that when the arsenical substrate contains an ethyl or heavier alkyl group attached to arsenic, as in the case of ethylarsonic acid $\text{C}_2\text{H}_5\text{AsO}(\text{OH})_2$, a mixed arsine, *e.g.* dimethylethylarsine, $(\text{CH}_3)_2\text{AsC}_2\text{H}_5$, is obtained. Challenger and Ellis [1935] established also the formation of dimethyl-*n*-propyl-, dimethylallyl- and methyldiethyl-arsines using bread cultures containing *n*-propylarsonic, allylarsonic and diethylarsinic acids respectively.

This remarkable methylating action is not confined to arsenic. In bread or glucose cultures containing sodium selenate or selenite the mould gives rise to dimethyl selenide [Challenger and North, 1934]. The work of Rosenheim [1902] and of Maassen [1902] and experiments of the present authors which are still in progress render it probable that the intense odour evolved from similar cultures containing potassium tellurite is due to dimethyl telluride. The present paper outlines certain theories which have been put forward to account for this type of biological methylation both by earlier workers and during informal discussions with our colleagues. It also describes experimental work which has been designed to test them.

These theories fall under three main heads according to which the methylating agent of the mould may be (a) formaldehyde, (b) acetic acid, (c) a free methyl group detached from, *e.g.*, choline. In (a) it is necessary to consider two possibilities (a_1) that formaldehyde arises from the breakdown of carbohydrate, (a_2) that it is formed by deamination of glycine. There is very little published evidence in favour of (a_1). Fernbach [1910] states that *Tyrophrix tenuis* converts dihydroxyacetone, sucrose or starch into methylglyoxal, acetic acid and formaldehyde. The latter was detected with Schiff's reagent and isolated as its condensation product with phenylhydrazine [Wellington and Tollens, 1885]. No M.P. or analysis of this derivative was recorded.

There seem to be no other well-authenticated instances of the production of formaldehyde by the biological decomposition of carbohydrate and this has been pointed out by Emde and Hornemann [1931]. Emde [1929] remarks however that bio-methylation in plants can only be a question of secondary formaldehyde arising from carbohydrate, primary formaldehyde from photosynthesis being only available in the chloroplasts; all other parts of the cell must employ carbohydrate breakdown products for methylation. Two criticisms may be made

regarding these statements. Firstly, formaldehyde may arise from glycine by deaminative oxidation and secondly the production of formaldehyde itself is not essential—glyoxylic acid $\text{CHO} \cdot \text{COOH}$ could function equally well as a methylating agent. The formation of traces of this acid in cultures of *A. niger* on citric acid, malonic acid and acetates has been demonstrated both by Challenger *et al.* [1927] and by Bernhauer and Scheuer [1932].

In Table III, p. 1770, Exps. 31–35 show that the addition of sodium formate with or without formalin, paraformaldehyde, hexamethylenetetramine or rongalite (sodium formaldehydesulphoxylate) exercises no beneficial effect on the yield of trimethylarsine in liquid cultures.

The results obtained on addition of methyl alcohol are discussed on p. 1771.

As regards (a_2) it has been shown by Clarke *et al.* [1933] that dimethylglycine with silver oxide gives dimethylamine, carbon dioxide and formaldehyde. Glycine itself is similarly converted into ammonia, carbon dioxide and water [Herbst and Clarke, 1934] and alanine yields ammonia, acetaldehyde, acetic acid and carbon dioxide. These results are, of course, in agreement with the generally accepted theory of the deamination of amino-acids as developed by the work of Neubauer and Fromherz [1911] and later by Krebs [1933]. Glycine on exposure to sunlight in aqueous solution was found by Ganassini [1912] to give ammonia, carbon dioxide and formaldehyde. Illumination of glycine in nitric acid solution in presence of ferric nitrate gives carbon dioxide, formaldehyde and glyoxylic acid [Benrath, 1911; 1912].

Assuming the intervention of formaldehyde as the methylating agent its reaction with arsenious acid might proceed by a mechanism which has been previously outlined [Challenger *et al.*, 1933] and may be briefly summarised thus:

There is unfortunately no direct evidence in favour of this mechanism. That arsenious acid can react in the tautomeric form $\text{H} \cdot \text{AsO}(\text{OH})_2$ is well known, sodium arsenite and alkyl halides giving alkylarsonic acids $\text{R} \cdot \text{AsO}(\text{OH})_2$. Again acetaldehyde reacts with phosphorous and hypophosphorous acids giving the compounds $\text{CH}_3\text{CHOH} \cdot \text{PO}(\text{OH})_2$ and $\text{CH}_3\text{CHOH} \cdot \text{PH} \cdot \text{O} \cdot \text{OH}$ as has been shown by Ville [1891] and by Marie [1904]. Moreover formaldehyde reacts with phosphine and hydrochloric acid to give $(\text{CH}_2\text{OH})_4\text{PCl}$ [Hoffman, 1921; 1930]. The experimental section contains results which show clearly that *P. brevicaulis* exercises a reducing action on derivatives of quinquevalent arsenic.

Attempts to synthesise hydroxymethylarsonic acid have failed, so this suggested scheme has not yet been tested. β -Hydroxyethylarsonic acid, $\text{HOCH}_2 \cdot \text{CH}_2\text{AsO}_3\text{H}_2$, which would be expected to yield ethylarsonic acid and then ethyldimethylarsine, is unaffected in bread cultures.

All attempts to detect the formation of methylarsonic or cacodylic acid or trimethylarsine oxide in the various culture media employed in this work have entirely failed, although several litres of medium were examined.

The suggestion (*b*) that the methyl group of the trimethylarsine is derived from acetic acid appears at first sight to have much to recommend it. Cadet's production of dimethylarsine oxide $(\text{CH}_3)_2\text{As} \cdot \text{O} \cdot \text{As}(\text{CH}_3)_2$ (cacodyl oxide) and of cacodyl itself by heating sodium acetate and arsenious oxide is a well-known reaction (for references and a historical account see Morgan [1917]).

If acetic acid be the source of the methyl group the reaction can be formulated in two ways:

Arsonoacetic acid

Methylarsonic acid

Methylarsonic acid readily gives trimethylarsine in mould cultures and a possible mechanism was discussed on p. 1758.

Mechanisms (1) and (2) labour under the objection that no, or at any rate very few, examples of the simple decarboxylation of fatty acids to hydrocarbons by micro-organisms are recorded in the literature. Exceptions are the conversion of cinnamic acid into phenylethylene by *A. niger* [Herzog and Ripke, 1908] and the formation of methane from acetic and other aliphatic acids by various bacteria.

Mechanism (2) is formulated as a dehydrogenation and appears more probable. Several experiments have been carried out to obtain some idea as to the possibility of this reaction.

Arsonoacetic and arsonopropionic acids were prepared according to the method of Mulder [1929]. If the biological production of trimethylarsine from arsenious acid normally proceeds by scheme (2) above one would expect arsonoacetic acid in bread cultures of *P. brevicaule* to be readily converted by way of methylarsonic acid into trimethylarsine. Arsonopropionic acid

might similarly be expected to yield ethylarsonic acid $\text{CH}_3\text{CH}_2\text{AsO}(\text{OH})_2$ and thence dimethylethylarsine [Challenger *et al.*, 1933].

In the latter case the mould was found to produce only minute quantities of arsenical gas which was identified as trimethylarsine. This presumably arose from arsenic acid produced by gradual hydrolysis of the arsonopropionic acid in the cultures. (This readily occurs on heating arsonopropionic acid with water at 110°.) Using sodium arsonoacetate in bread and glucose cultures and the free acid in bread cultures, small quantities of trimethylarsine were detected. The quantities were however so small as to suggest that slight hydrolysis of the arsonoacetic acid had occurred. If decarboxylation and intermediate formation of methylarsonic acid had taken place the yield of arsine might have been expected to be much larger, since of all the arsenic compounds examined during the last four years sodium methylarsonate most readily gives arsine. It may therefore be concluded that these results afford no evidence in favour of the participation of acetic acid according to scheme (2).

The results of these experiments are in agreement with those of Challenger and North [1934] who failed to obtain any dimethyl sulphide from bread cultures containing thiodiglycollic acid $\text{S}(\text{CH}_2\text{COOH})_2$ indicating the difficulty which the mould experiences in decarboxylating the group $-\text{CH}_2\text{COOH}$.

Further, though not conclusive, evidence against the suggestion that reaction (1) or (2) represents the mechanism of methylation is afforded by the fact that addition of sodium formate, propionate or butyrate, or of propionic or butyric acid to bread or glucose cultures containing arsenious oxide gave always pure trimethylarsine, identified as the dimercurichloride. Had reactions analogous to (1) or (2) occurred the trimethylarsine might have been expected to be con-

taminated with arsine, triethylarsine and tripropylarsine respectively, or possibly with dimethylethyl- and dimethylpropyl-arsines, *e.g.*

In this connection it may be mentioned that many of the early workers on the nature of Gosio-gas, *e.g.* Martin [1847], Fleck [1872], Selmi [1874] and Wolf [1887], suggested that it consisted of arsine. In none of our experiments, whether in presence or absence of formate, have we obtained the slightest evidence of the presence of this gas. Even small quantities would be readily detected by the formation of yellowish to brown precipitates in Biginelli's solution, an acid solution of mercuric chloride employed by this worker in his researches on the nature of Gosio-gas [1901].

The interesting suggestion that a methyl group may be detached from a methylated derivative such as choline or some other ammonium base was made by Riesser [1913] in order to explain the assumed formation of dimethyl telluride in the animal body after ingestion of inorganic tellurium compounds [Hofmeister, 1894; Maassen, 1902]. It has however been pointed out by Challenger and North [1934] that the identification of the odorous compound as dimethyl telluride in these animal experiments, though probably correct, is based on entirely insufficient evidence. This does not, however, invalidate the importance of Riesser's suggestion which can be applied equally well to explain the biological methylation of arsenic and selenium by moulds. He states that on heating a mixture of choline chloride or betaine, sodium tellurite and sodium formate (the latter acting as reducing agent) the odour of dimethyl telluride is produced. No further identification of this compound was attempted. The wide occurrence of choline in plants has been emphasised by Schulze and Trier [1912].

Riesser brought forward little or no other evidence in favour of his suggestion, though after injecting rabbits with choline and betaine he observed some increase in the creatine content of the muscle. He suggested that the methyl group of the creatine might be derived from the choline. His attempt to prove the possibility of this reaction by a chemical experiment was not convincing.

It nevertheless appeared desirable to repeat and extend Riesser's experiments on the transfer of methyl groups from choline. When anhydrous betaine was heated with a mixture of sodium tellurite and sodium formate in a stream of carbon dioxide, dimethyl telluride was identified among the volatile products by absorption in benzyl chloride and conversion into dimethylbenzyltelluronium picrate.

In this and some other experiments betaine was used instead of choline chloride in order to exclude the possibility of the evolution of methyl chloride which, acting on sodium tellurite, might produce a compound (*e.g.* $\text{CH}_3\text{TeO}_3\text{Na}$) which could possibly give rise to dimethyl telluride on heating. Using betaine however it appears that the reaction must be due to transfer of a methyl group.

By substituting sodium selenite for sodium tellurite and heating with choline chloride or with betaine in a stream of carbon dioxide, dimethyl selenide was readily detected and characterised as the mercurichloride and the platinochloride [Challenger and North, 1934]. Analogous experiments in which sodium arsenite, sodium formate and choline chloride were similarly heated failed to give any trimethylarsine, although this is very easily recognised [Challenger *et al.*, 1933].

In this connection the work of Willstätter [1902] and of Willstätter and Kahn [1904] should be cited. These authors showed that on heating betaine to 280° it undergoes isomerisation to the methyl ester of dimethylaminoacetic acid and

this reaction has recently been shown by Kuhn and Giral [1935] to hold for the homologous ξ -trimethylpentadeca- and π -trimethylheptadeca-betaine. These latter authors are of the opinion that the reaction is inter- rather than intra-molecular. Further evidence for this is afforded by our observation that mono-methylaniline is produced on boiling betaine (free from hydrochloride) with aniline for several hours.

Experiments in which choline chloride was added to cultures of the mould on glucose or in which betaine was used as sole source of carbon, both in presence of arsenious oxide, showed no beneficial effect on the yield of arsine. Nevertheless it is not impossible that some ingredient of the cell substance containing a methylated nitrogen atom, may under the special conditions obtaining in the cell, lose a methyl group which, if it be eliminated with a positive charge, could be easily coordinated by the unshared electrons of trivalent arsenic or of quadri-valent selenium and tellurium.

Another possible origin of a free methyl group may be sought in the decomposition, possibly enzymic, of a complex ion formed from glycine and arsenious acid:

This suggestion may possibly have some bearing on the observation of Ackerman [1912] who showed that in the body of the dog nicotinic acid is methylated giving rise to trigonelline, the corresponding betaine, the formation of which is accompanied by that of nicotinuric acid. These results have been confirmed by Komori *et al.* [1926].

It appears not unreasonable to assume that glycine may be concerned in the formation of the first as well as the second of these compounds thus:

It was also shown by Cohn [1893] that α -picoline is similarly converted by the dog into α -pyridinuric acid, obviously by way of picolinic acid. One would expect that this change should also be accompanied by the formation of homarin, the corresponding methylbetaine, although this was not detected. Homarin has however recently been isolated by Kutscher and Ackermann [1933] from the muscle of *Arca noae*.

The results quoted above are extensions of the well-known work of His [1887] on the conversion of pyridine into methylpyridinium hydroxide in the body of dogs. Komori *et al.* [1926] have confirmed this for turtles. This base has since been detected in the sea anemone *Actinia equina*.

It was noticed by Hausmann [1904] that the actinia *Aiptasia diaphana* Rapp. when placed in sea-water containing 0.03 mg. of arsenious oxide per 100 ml. evolved a garlic odour which was also noticed when this organism was similarly treated with very dilute sodium tellurite. Sodium selenite produced a mercaptan-like odour. Hausmann regarded this effect as analogous to the results of Gosio [1893; 1897; 1901] and Maassen [1902] with *P. brevicaulis*. He was inclined to attribute it, however, to the brown algae (Zooxanthellen) which lived in symbiosis with the actinia. Under the influence of the arsenious acid most of these became detached from the actinia, and when separated were found still to be capable of producing odours with selenite and tellurite.

The odorous compounds were not identified but it appears almost certain that they consisted of alkyl, and probably methyl, derivatives of arsenic, tellurium and selenium.

In this connection it is of particular interest that a rather closely allied organism, *Actinia equina*, contains large quantities of tetramethylammonium hydroxide which is believed to exercise a paralysing (curare) action on the small fish which serve as its food. This organism also contains other highly methylated compounds, e.g. methylpyridinium hydroxide and γ -butyrobetaine (actinine), [Ackermann *et al.*, 1923; 1924]. The related compound trimethylamine oxide occurs in many marine animals [Hoppe-Seyler, 1933].

It appears possible, assuming the actinia *Aiptasia diaphana* to exercise an alkylating action on arsenic, selenium and tellurium apart from the algae with which it is in symbiosis, that this may be effected by the transfer of a methyl group from one of its nitrogenous bases to the metalloid.

Particular interest attaches to a recent observation of Haas [1935] that the seaweed *Polysiphonia fastigiata* evolves dimethyl sulphide very shortly after being gathered. Whether this arises by the breakdown and methylation of cysteine or from methionine, or on the other hand is produced by methylation of the sulphur of the sulphuric esters present in seaweed [Haas and Russell-Wells, 1923] awaits investigation. This result recalls the observation of Henze [1904] that the sponge *Suberites domuncula* gives off an intense odour reminiscent of mercaptan. The odorous product removed in a stream of air was absorbed in sulphuric acid with loss of odour which reappeared on dilution of the acid with ice. Henze was unable to prove the presence of sulphur in the odorous compound but from its behaviour with sulphuric acid it would appear very probable that he was dealing with an alkyl sulphide. (Compare the analogous experimental procedure of Pohl [1904] and Abel [1895] for the investigation of volatile sulphur compounds produced under biological conditions.)

EXPERIMENTAL.

The ability to produce trimethylarsine when grown on a suitable nutrient medium containing arsenious oxide appears to be confined to moulds, especially *P. brevicaulis*, although other species have been shown to give the garlic odour but to a very much less extent. [Gosio, 1893; Maassen, 1902; Thom and Raper, 1932.]

A large number of bacterial species have been tested by Emmerling [1896], Abel and Buttenberg [1899], Maassen [1902] and Huss [1914], but in no case was a garlic odour detected during growth in the presence of arsenious oxide. Puntoni [1917] however states that *B. mesentericus vulgatus*, *B. mesentericus ruber* and *B. subtilis* gave garlic odours in presence of sodium cacodylate. We have tested one strain of each of these organisms on glucose meat extract at 37° in the presence of 0.2 % arsenious oxide, sodium arsenate, sodium methyl-arsenate or sodium cacodylate and obtained negative results. It should be

mentioned, however, that our strains of these three bacteria were obtained from the Collection of National Type Cultures at the Lister Institute whereas those employed by Puntoni were isolated from the faeces of patients undergoing treatment with sodium cacodylate.

B. coli communis and *B. lactis aerogenes* have also been cultivated in a series of six 1 litre conical flasks containing 200 ml. 2 % glucose, with 1 % peptone and 0.025 % arsenious oxide, and the products aspirated through Biginelli's solution. A similar experiment was made using a stream of nitrogen in place of air. Neither organism under either aerobic or anaerobic conditions gave any trace of trimethylarsine dimercurichloride.

Negative results were also obtained by Emmerling [1896] and Huss [1914] using various yeasts. In this research *Saccharomyces cerevisiae*, *S. carlsbergensis*, *S. monacensis* and "Rasse XII" were grown on beer wort or 5 % glucose-mineral salt solution in the presence of arsenious oxide but no arsenical odour was detected.

Gosio [1901] states that the well-washed mycelium of *P. brevicaule* after growth in the presence of arsenious oxide contains arsenic. No essential difference can be detected in the microscopic appearance of the strain used in this research when grown on 5 % glucose-Czapek-Dox solution with or without 0.2 % arsenious oxide. The arsenic content of the growth from 30-day cultures on 5 % glucose-Czapek-Dox solution has also been determined. The mycelium was well washed with a large volume of water, until the washings were free from inorganic arsenic, and dried at 100°. 6–7 g. of dry mycelium analysed by a slight modification of the method of the Sub-Committee on arsenic determination [1930] contained not more than 0.032 % arsenic.

Experiments illustrating the reducing action of P. brevicaule.

Hydroxytrimethylarsonium nitrate. Two one-litre flasks each containing 150 g. bread crumbs were sterilised, inoculated with a spore suspension of *P. brevicaule*, incubated for 5 days at 30° and treated with 25 ml. of 0.8 % hydroxytrimethylarsonium nitrate, which had been previously sterilised at 120° for 20 minutes, giving a concentration of 0.13 % in the bread. The gases produced from the cultures were aspirated with sterile air through Biginelli's solution for 95 days. The resulting precipitate was frequently removed and fresh Biginelli's solution placed in the absorption bottle. On the 17th day, 0.55 g. of white crystals (M.P. 264° and 264° in admixture with an authentic specimen of trimethylarsine dimercurichloride of M.P. 265°) were removed.

Further deposits of M.P. 263°, 264° and 263–4° were obtained after 32, 48 and 75 days respectively giving a total of 0.87 g. of trimethylarsine dimercurichloride, a yield of 65.4 % calculated on 0.4 g. of the arsonium nitrate.

Two similar, sterilised but uninoculated (control) flasks were similarly treated with the arsonium nitrate and the gases aspirated through Biginelli's solution as before. No precipitate formed during the whole of the 95 days.

Tri-n-propylarsine oxide. Tri-n-propylarsine dibromide (kindly supplied by Prof. W. J. Jones, University College, Cardiff) was converted into the oxide by two successive treatments with water and excess of silver oxide followed by evaporation to dryness. The solid which resulted was free from halogen but very deliquescent. It was therefore not analysed but treated with picric acid giving *hydroxy-tri-n-propylarsonium picrate*. (Found C, 40.3; H, 5.5; N, 9.6 %. $C_{15}H_{24}O_8N_3As$ requires C, 40.1; H, 5.4; N, 9.4 %.)

Four bread flasks were prepared as in the last experiment, two were inoculated as before, and incubated for 4 days at 30° and for 4 days at room temperature.

To each of the four flasks were added 25 ml. of 0.8 % tri-*n*-propylarsine oxide (sterilised in the autoclave). The products from the inoculated and control flasks were separately aspirated by sterile air through Biginelli's solution for 69 days. No mercurichloride was obtained from the control flasks but the mould cultures gave after 9 and 30 days deposits (0.02 and 0.02 g.) both of m.p. 106–7° and 107° (constant) after recrystallisation from alcohol. Yield 4.65 %. During the remaining 30 days no further precipitate was obtained except a trace due to the gradual evolution of ammonia from the cultures.

Tri-*n*-propylarsine monomercurichloride was prepared for comparison. Tri-*n*-propylarsine dichloride m.p. 84.5–85° (Dyke *et al.* [1931] give m.p. 84°) was reduced with arsenic-free zinc and hydrochloric acid and the resulting mixture of hydrogen and tripropylarsine passed into aqueous mercuric chloride giving a product m.p. 106–7° and 107° on recrystallisation as before. (Dyke *et al.* [1931] give m.p. 106° for the monomercurichloride $(C_3H_7)_3As.HgCl_2$.) In admixture with the mould product there was no depression of m.p.

One of the uninoculated control flasks which had given no arsine during 74 days was then inoculated with the mould. Growth occurred at ordinary temperature and aspiration during 24 days gave 0.05 g. of tri-*n*-propylarsine monomercurichloride m.p. 106°. The yield in this case was 11.6 % as compared with 4.65 % from two flasks in the earlier experiment.

Hydroxytrimethylammonium chloride and trimethylamine oxide. The first-named substance $(CH_3)_3N(OH)Cl$ was prepared by the method of Dunstan and Goulding [1899] and melted constantly at 213° (decomp.) the m.p. depending somewhat on the rate of heating.

It gave the picrate of const. m.p. 200° (decomp.) for which these authors give m.p. 196–8°; Hantzsch and Hilland [1898] give m.p. 197–8°. Treatment of the chloride with excess of freshly precipitated silver oxide in ice-water, filtration and evaporation gave the hydrated amine oxide m.p. 79–81° on recrystallisation from alcohol.

The hydroxychloride (0.1 g. in 20 ml. sterile distilled water) was added to a culture of the mould grown for 3 days at 30° on 50 g. bread and a similar quantity to sterile uninoculated bread. No odour of trimethylamine was obtained after 41 and 11 days respectively at room temperature.

The amine oxide (0.1 g.) under similar conditions added to a culture grown for 4 days at 30° and one day at room temperature gave no odour in either culture or uninoculated control during 50 days.

Sodium arsenate. Four cultures on 150 g. bread were prepared as usual and incubated for 10 days at 30° and 2 days at room temperature. 25 ml. of 1.2 % sodium arsenate were added to each. After 19 and 51 days, 0.2 g. and 0.9 g. of mercurichloride m.p. 264° were obtained. On the 58th day two bottles containing 2:4-dinitrophenylhydrazine in 2*N* HCl were placed in the absorption train followed by Biginelli's solution as usual. No dinitrophenylhydrazone separated but a further deposit of 1.25 g. of mercurichloride m.p. 264° was obtained. Recrystallisation of the united deposits gave plates m.p. 265° not depressing the m.p. (265°) of authentic trimethylarsine dimercurichloride.

Salts of β -hydroxyethylarsonic acid. The calcium salt, for which we are indebted to the kindness of Dr H. King, was purified from traces of inorganic arsenic by solution in hot dilute hydrochloric acid and reprecipitation with ammonia, the operation being twice repeated. The sodium salt was prepared in 1.2 % solution by boiling with sodium carbonate. No precipitate was obtained in the acidified sterile solution on rapid passage of hydrogen sulphide for 5 minutes.

Four bread cultures in 1 litre flasks were grown for 2 days at 32° and 4 days at room temperature. 25 ml. of the sterile sodium salt solution were added to each culture and the gases aspirated through Biginelli's solution as usual. No odour was detected and no precipitate obtained even after 44 days. Using similar cultures and comparable concentrations of (a) the original unpurified calcium salt and (b) the sodium salt prepared from it, one or two crystals formed on the delivery tube in (a) and a trace of trimethylarsine dimercurichloride m.p. 262° was deposited in (b) after a few days, but did not increase on continuing the experiment for a total of 76 days. The slight positive result here was clearly due to the trace of inorganic arsenic originally present. Had the mould reduced the β -hydroxyl group to hydrogen the resulting ethylarsonic acid should have given dimethylethylarsine (see p. 1757).

Effect of compounds containing the group $-(CH_2)_nCOOH$ on the formation of trimethylarsine.

In the following experiments the mould was grown on 150 g. sterile bread crumbs in four 1 litre flasks for 4 days at 30° and in (b) for one day more at room temperature and treated with (in addition to the appropriate carboxylic acid or its salt) 25 ml. of 1.2 % aqueous arsenious oxide. The gaseous products were continuously aspirated as usual through Biginelli's solution which was renewed after removal of each deposit.

(a) *Sodium propionate.* To each culture were added 25 ml. of 6 % aqueous sodium propionate (concentration in the bread 1 %). A mercurichloride was deposited by the third day and after 10, 14, 25, 38, 56 and 66 days deposits of 0.015, 0.07, 0.18, 0.21, 0.05 and 0.37 g. were obtained having m.p. 261°, 264°, 265°, 263°, 264–5° and 265° respectively. Yield 11.1 %.

(b) *Propionic acid.* 25 ml. of 6 % propionic acid were added to each of the four flasks and after 17, 21, 34, 47 and 60 days deposits of 0.02, 0.18, 0.21, 0.16 and 0.22 g. having m.p. 263–4°, 265°, 265°, 265° and 265° respectively were obtained; 0.79 g. in all. Yield 9.8 %. Trimethylarsine dimercurichloride melts at 265°, tri-*n*-propylarsine monomercurichloride at 107° (see p. 1764).

(c) *Sodium butyrate.* 25 ml. of the 6 % aqueous solution (sterilised at 120° for 20 minutes) were added to each of the four flasks. The first trace of mercurichloride separated on the third day and after 10, 14, 25, 38, 56 and 66 days deposits of 0.05, 0.15, 0.12, 0.26, 0.05 and 0.45 g. (total 1.10 g. yield 13.7 %) were obtained. The m.p. varied from 264° to 265°. A further quantity of mercurichloride could probably have been obtained but the cultures had become very dry and, as is usual under such circumstances, much ammonia was evolved, so the experiment was discontinued.

Behaviour of P. brevicaule with sodium propionate or butyrate as sole source of carbon.

A small seed culture of the mould was first grown on 1 % sodium butyrate containing the inorganic salts of the Czapek-Dox medium employed by Raistrick [1931]. The growth was poor.

500 ml. of the same medium in each of four 3-litre flasks were sterilised at 120° for 20 minutes and inoculated with a portion of the seed culture. After 8 days at 30° a thin surface growth began to form and the culture was removed to room temperature and to each flask 1 g. of arsenious oxide in 60 ml. water (final concentration 0.2 %) was added. Aspiration into Biginelli's solution for 25 days gave only one or two crystals on the delivery tube and there was no

increase in the mould growth. Addition of 2.8 ml. hydrochloric acid in sterile distilled water to each flask did not assist growth or cause any more deposit to form during a further 24 days.

An analogous experiment using 1 % sodium propionate but incubating for 15 days at 30° gave equally unsatisfactory results after 10 days. Addition of 3.16 ml. hydrochloric acid in 20 ml. water effected no improvement during a further 24 days.

Sodium arsonoacetate.

(a) The sodium salt of arsonoacetic acid was prepared according to the method of Palmer [1925] and twice recrystallised from water. In acidified aqueous solution it gave no precipitate on passage of hydrogen sulphide for 10 minutes.

To four cultures on 150 g. bread in 1-litre conical flasks grown for 4 days at 32° and one day at room temperature were added 25 ml. of sterile 3 % sodium arsonoacetate. Final concentration 0.5 %. No odour of trimethylarsine and no precipitate in Biginelli's solution were obtained on aspiration for 33 days.

(b) 8 cultures on 100 ml. 2 % glucose-Czapek-Dox salt solution were grown for 6 days at 30° and 25 ml. sterile 4 % solution of sodium arsonoacetate added. Final concentration 1 %. Sterile air was passed over the cultures and through Biginelli's solution. No trimethylarsine dimercurichloride was obtained during 29 days.

Arsonoacetic acid.

(a) To four cultures on bread crumbs grown for 3 days at 30° and 2 days at room temperature, were added 25 ml. 6 % arsonoacetic acid. Final concentration 1 %. The acid was dissolved in sterile water and not itself sterilised owing to its tendency to liberate inorganic arsenic on heating with water. Aspiration through Biginelli's solution gave in 31 days 0.04 g. $\text{Me}_3\text{As} \cdot 2\text{HgCl}_2$ m.p. 263–4° and by the 65th day a further 0.02 g. m.p. 260°. Yield 0.27 %.

(b) This experiment was made exactly as in (a) but with a final concentration of arsonoacetic acid of 0.5 %. 0.37 g. $\text{Me}_3\text{As} \cdot 2\text{HgCl}_2$ m.p. 263–4° was obtained in 36 days; 0.27 g. m.p. 264–5° by 51st day and 0.03 g. m.p. 260° by 68th day. Yield 6.2 %.

(c) Using cultures grown for 3 days at 30° and 10 days at room temperature and a final concentration of 0.5 % arsonoacetic acid as in (b), no trimethylarsine was formed in 14 days. Although these cultures were 13 days old, numerous other experiments have shown that the mould is still unimpaired in activity after this time.

Arsonopropionic acid.

Two series of four bread cultures were grown for 3 days at 30° and one day at room temperature. Arsonopropionic acid (solution in sterile water) was added to give a final concentration 0.5 %. In 50 days only a very small amount of precipitate (barely sufficient for the necessary m.p. determinations) was obtained in the Biginelli's solution in each series and this was shown by m.p. and mixed m.p. to be trimethylarsine dimercurichloride and not the dimercurichloride of dimethylethyl arsine.

Action of P. brevicaulis on glycine.

Sterile air was passed over four cultures on 300 ml. of Czapek-Dox medium containing 2 % glycine as sole source of carbon, previously incubated for 6 days at 30°. Volatile products were absorbed in (a) 40 ml. of a mixture of conc. HCl (8 g.) and water (40 ml.) and (b) 30 ml. of Biginelli's solution. In 24 hours traces

of an amorphous finely divided precipitate formed in (b) but did not increase appreciably throughout the experiment. It contained no calomel. Warm, but not cold, NaOH caused blackening but no odour was produced. This might indicate the presence of traces of an alkylphosphine. The amount available was too small for further examination, but from a second set of cultures in which the phosphate content of the Czapek-Dox solution was increased from 0.1 to 0.5 % no similar deposit could be obtained.

After 28 days a strong odour of ammonia was noticed. Evaporation of the acid in (a) gave 0.16 g. of a non-deliquescent solid. This was converted into the platinichloride by evaporation of a portion with aqueous chloroplatinic acid and removing excess of the latter by extraction with alcohol. The residue was dried. (Found Pt, 44.1 %. $(\text{NH}_4)_2\text{PtCl}_6$ requires Pt 43.96 %.)

After 22 days more the fresh acid in (a) yielded another 1.35 g. of ammonium chloride which was identified as before by analysis of the platinichloride. (Found Pt 43.8 %; calc. 43.96 %. $\text{PtCl}_4 \cdot 2\text{CH}_3\text{NH}_2\text{HCl}$ requires Pt 41.31 %.) The volatile products clearly contained no alkylamines.

The culture medium still contained glycine, as shown by the deep blue colour with copper sulphate. It had p_{H} about 9.0 and an ammoniacal odour. 400 ml. were distilled, the distillate acidified and evaporated. Conversion of the residue into chloroplatinate showed absence of alkylamines. (Found Pt 43.8 %; calc. 43.96 %.)

Distillation of 300 ml. of the acidified culture medium indicated the absence of volatile fatty acids. The residue was added to the remainder of the medium, evaporated and a portion distilled with 80 % NaOH and any volatile matter absorbed in hydrochloric acid. Evaporation left only ammonium chloride. (Found, for the platinichloride Pt 43.9 %; calc. 43.96 %.) Had any betaine been formed by methylation of the glycine this would have eliminated trimethylamine under such treatment.

P. brevicaule and glycine in presence of arsenious oxide.

The procedure was the same as in the last experiment except that the cultures were incubated for 6 days at 33° and 50 ml. of aqueous 1.2 % arsenious oxide (final concentration 0.2 %) were added to each culture before aeration. Traces of the amorphous white precipitate were obtained in the Biginelli's solution at the beginning of the experiment but the quantity was insufficient for examination. Evaporation of the hydrochloric acid after 28 days gave a trace of solid insufficient for analysis. Aeration was continued for 22 days longer using fresh absorption solutions (a) and (b) when traces of a mercurichloride m.p. 262° and 262° in admixture with unrecrystallised trimethylarsine dimercurichloride (m.p. 261–2°) were obtained. The hydrochloric acid on evaporation gave 0.13 g. of ammonium chloride. (Found, for the platinichloride Pt 43.6 %; calc. 43.96 %.) After removal of the mycelium the medium had p_{H} about 8.5 and a faint ammoniacal odour. The distillate obtained from 400 ml. of the medium was acidified, evaporated and converted into platinichloride. (Found Pt 43.85 %; calc. 43.96 %.) The two analyses indicate that ammonia but no methylamines are evolved under the conditions employed. The object of the experiment was to determine whether cultures on glycine afforded better yields of trimethylarsine than the usual bread or glucose media. As this was clearly not the case, the test for betaine (distillation with 80 % sodium hydroxide) was omitted.

P. brevicaula and alanine.

Four 1-litre flasks with 200 ml. of sterile Czapek-Dox solution containing 2 % alanine as sole source of carbon were inoculated, incubated for 5 days at 30° and aerated, the volatile products being absorbed in 2 bottles containing dilute hydrochloric acid (1:5) and finally in Biginelli's solution. No precipitate formed in the latter during 54 days. The mould growth was just submerged and there was no aerial mycelium. The dilute acid was evaporated and the non-deliquescent residue converted into the platinichloride. (Found Pt 43.94 %; calc. for $(\text{NH}_4)_2\text{PtCl}_6$ Pt 43.96 %.) Two of the cultures (p_{H} 8.5–9.0) after removal of mycelium were evaporated to dryness, no ammonia being evolved. The solid residue (2.5 g.) was distilled with 80 % NaOH; absorption in hydrochloric acid and treatment as usual gave a platinichloride. (Found Pt 43.98 %; calc. Pt 43.96 %.) Neither alkylamines nor compounds of the betaine type are therefore detectable when the mould is grown on alanine.

Action of P. brevicaula on betaine.

Four 1-litre flasks each containing 200 ml. of Czapek-Dox solution from which the nitrate was omitted and which contained 1 % betaine as sole source of carbon were inoculated from a seed culture on the same medium and incubated for 14 days at 30°, by which time the growth was still poor. The volatile products were aspirated through dilute hydrochloric acid and Biginelli's solution as usual. No deposit formed in the latter even after 97 days. The acid yielded on evaporation 1 g. of a very slightly deliquescent solid which was extracted with cold alcohol, the solution evaporated and the residue extracted with cold chloroform. The latter yielded a trace of solid giving a picrate sintering at 210–215° and decomposing from 265°. This was apparently mostly ammonium picrate with a trace of some other substance. Trimethylamine picrate melts at 220°. It is clear that betaine is not a good nutrient for the mould. This is confirmed by the next experiment.

Action of P. brevicaula on betaine in presence of arsenious oxide.

Four flasks each containing 200 ml. of the modified medium used in the last experiment were inoculated as before and incubated for 14 days at 30° and treated with 25 ml. of 1.6 % arsenious oxide. Aeration and absorbents as before. Deposition of mercurichloride occurred after 11 days but increased only very slowly. After 50 days one flask was removed owing to an accidental contamination. After 53 days the mercurichloride (M.P. 259°) was removed and a further quantity (M.P. 264–5°) on the 76th day. The total amount was very small. No further deposit formed and the experiment was discontinued after 97 days.

The hydrochloric acid gave only 0.25 g. of solid which appeared to be ammonium chloride since it was converted as before into a picrate which had no M.P.

Discussion of tabulated results.

In the experiments described in Table I all cultures of *P. brevicaula* were grown on media containing Czapek-Dox salt solution with glucose as the sole source of carbon. When a moderately good mycelium had begun to form, the arsenic compound was added, the cultures connected in series and a continuous stream of sterile air drawn over. The air stream together with any trimethylarsine was passed through acid mercuric chloride (Biginelli's) solution where the arsine was retained as the insoluble dimercurichloride $\text{Me}_3\text{As} \cdot 2\text{HgCl}_2$. M.P. 265° (decomp.).

TRIMETHYLARSINE PRODUCTION BY *P. BREVICAULE* 1769

Table I.

Exp. No.	Glucose conc. %	C.D. salt solution No. of flasks and vol./flask ml.	Pre-incubation time and temperature	As ₂ O ₃ conc. %	Na ₂ HAsO ₄ , 7H ₂ O conc. %	CH ₃ AsO(ONa) ₂ , 6H ₂ O conc. %	Duration of exp. days	(CH ₃) ₃ As % yield
1	5	5 250	11 days 32° 11 days R.T.	0.2	—	—	52	Trace
2	5	4 300	8 days 32° 4 days R.T.	0.16	—	—	59	0.37
3	2	4 250	3 days 32°	0.2	—	—	99	0.65
4	2	4 150 (Pumice)	3 days 32°	0.2	—	—	71	Trace
5	2	4 100	3 days 30°	0.2	—	—	78	0.65
6	5	8 500	3 days 30° 3 days R.T.	0.2	—	—	30	0.42
7	5	6 800	7 days 30°	0.2	—	—	51	0.51
8	15	4 100	5 days 30°	0.2	—	—	32	0.37
9	2	4 200	3 days 30° 2 days R.T.	0.2	—	—	104	1.35
10	2	6 200	3 days 30° 2 days R.T.	0.2	—	—	90	1.93
11	2	6 200	4 days 30°	0.2	—	—	55 (restricted) 77 (continuous aeration)	0.68
12	2	6 200	4 days 30°	0.2	—	—	55 (restricted) 77 (continuous aeration)	0.62
13	2	4 200	6 days 30° 2 days R.T.	—	0.37	—	58	0.74
14	2	8 100	4 days 30°	—	—	1.0	34	0.88
15	2	7 100	4 days 30°	—	—	1.0 (½ neut.)	65	1.34

Exps. Nos. 1–12 give the yields of trimethylarsine (calculated on added arsenic compound) using arsenious oxide, and Nos. 13–15 those with other sources of arsenic. In No. 15 the sodium methylarsonate was treated with the calculated amount of hydrochloric acid for half-neutralisation of the disodium salt.

In Exp. No. 4 well-washed lumps of pumice stone were introduced into the medium before sterilisation so that only a very shallow layer of liquid remained unabsorbed. By this means a much greater area of growth was exposed to the

Table II.

Exp. No.	C.D. salt solution. No of flasks and vol./flask ml.	Pre-incubation time and temperature	As ₂ O ₃ conc. %	Fructose conc. %	Xylose conc. %	Glycerol conc. %	Ca acetate conc. %	Na acetate conc. %	Duration of experiment days	(CH ₃) ₃ As % yield
16	6 200	6 days 30° 3 days R.T.	0.2	2.0	—	—	—	—	79	0.87
17	6 200	6 days 30° 3 days R.T.	0.2	2.0	—	—	—	—	79	0.72
18	4 200	3 days 30° 1 day R.T.	0.2	—	2.0	—	—	—	56	1.07
19	2 300	16 days 30° 40 days R.T.	0.2	—	—	5.0	—	—	72	Small amount
20	4 200	7 days 30° 39 days R.T.	0.2	—	—	—	—	5.0	82	Small amount
21	4 200	7 days 30° 4 days R.T.	0.2	—	—	—	1.0	—	55	Small amount

Table III.

Exp. No.	Glucose conc. %	C.D. salt solution No. of flasks and vol./flask ml.	Pre-incubation time and temperature	As ₂ O ₃ conc. %	CH ₃ OH conc. %	C ₂ H ₅ OH conc. %	H. COONa conc. %	HCHO as formalin conc. %	HCHO as paraformaldehyde conc. %	HCHO as C ₆ H ₁₂ N ₄ conc. %	HOCH ₂ OSO ₂ Na conc. %	Duration of exp. days	(CH ₃) ₂ As % yield
22	2	4	200	0.2	2	—	—	—	—	—	—	99	1.70
23	2	3	200	0.2	2 (+ excess Na ₂ SO ₃)	—	—	—	—	—	—	86	1.00
24	2	6	200	0.2	1.6 (M/2)	—	—	—	—	—	—	90	1.37
25	2	6	200	0.2	1.6 (M/2)	—	—	—	—	—	—	105	2.12
26	2	6	200	0.2	1.6 (M/2)	—	—	—	—	—	—	105	1.99
27	2	4	200	0.2	—	2	—	—	—	—	—	105	1.20
28	2	6	200	0.2	—	2.3 (M/2)	—	—	—	—	—	105	0.62
29	2	6	200	0.2	—	2.3 (M/2)	—	—	—	—	—	105	0.59
30	2	6	200	0.2	—	2.3 (M/2)	—	—	—	—	—	105	0.93
31	2	4	200	0.2	—	—	1.0	—	—	—	—	65	0.84
32	2	6	200	0.2	—	—	1.0	—	—	0.005	—	70	0.53
33	2	6	200	0.2	—	—	1.0	—	0.005	—	—	70	0.50
34	2	3	200	0.2	—	—	—	—	—	—	0.026 (0.006HCHO)	55	1.40
35	2	4	200	0.2	—	—	—	0.004	—	—	—	61	0.74

air in relation to the quantity of medium used. The growth however was very thin compared with that usually obtained on a liquid surface.

Exps. Nos. 11 and 12 were set up in the usual way, but the system was only aerated for $1\frac{1}{2}$ –3 hours per week and after 55 days a current of nitrogen was drawn through to drive out any accumulation of trimethylarsine in the culture flasks. No arsine was obtained. A continuous stream of air was then passed over the cultures for a further 77 days. Arsine formation began immediately and the yield was comparable with that obtained under normal conditions.

All other experiments described in Tables I to IV received continuous aeration. The duration of the experiments as recorded in all these tables dates from the addition of the source of arsenic to the culture.

Exps. Nos. 16–21, Table II, show the yields of trimethylarsine obtained when arsenious oxide was added to cultures of the mould on Czapek-Dox salt solution with various carbon compounds, other than glucose, as the sole source of carbon.

The influence of various simple carbon compounds on the yield of arsine obtained during growth on glucose is shown in Table III. The cultures were grown in the usual way on glucose-Czapek-Dox salt solution and then the carbon compounds added at the same time as the arsenious oxide.

The sodium formate was sterilised in aqueous solution at 120° for 20 minutes; methyl and ethyl alcohols added directly using sterile pipettes whilst the other compounds were not sterilised but dissolved in sterile water before addition to the cultures. All solutions containing sugars were sterilised in steam for 30 minutes on each of three successive days and in all other cases, unless specifically mentioned, sterilisation at 120° for 20 minutes was employed.

The average yield (Table I) of arsine from arsenious oxide in cultures on glucose-Czapek-Dox salt solution is 0.78 % with limiting values 0.37–1.93 %. It is evident that under the conditions shown in Table III ethyl alcohol, sodium formate and formaldehyde (as various derivatives) with or without formate, have no appreciable influence on the production of arsine.

The average yield in the presence of methyl alcohol was somewhat higher *i.e.* 1.63 % although within the highest value obtained with glucose as the sole source of carbon.

The effect of various nitrogenous compounds with or without glucose on arsine formation from arsenious oxide is shown in Table IV. In Exp. No. 45

Table IV.

Exp. No.	C.D. salt solution No. of flasks and vol./flask ml.	Pre-incubation time and temperature	As ₂ O ₃ conc. %	Glucose conc. %	Witte peptone conc. %	Glycine conc. %	Betaine (base) conc. %	Choline chloride conc. %	Duration of exp. days	(CH ₃) ₃ As % yield
36	4 200	2 days 30° 2 days R.T.	0.2	2.0	1.0	—	—	—	87	3.14
37	5 200	3 days 30°	0.2	2.0	1.0	—	—	—	84	7.55
38	6 200	3 days 30°	0.2	2.0	1.0	—	—	—	77	5.26
39	6 200	3 days 30°	0.2	2.0	1.0	—	—	—	77	3.52
40	4 100	4 days 30°	0.2	—	1.0	—	—	—	48	0.74
41	6 200	2 days 30° 2 days R.T.	0.2	—	1.0	—	—	—	87	0.34
42	4 200	2 days 30° 1 day R.T.	0.2	2.0	—	2.0	—	—	65	0.28
43	4 300	6 days 30°	0.2	—	—	2.0	—	—	50	Trace
44	8 100	11 days 30°	0.2	2.0	—	—	—	2.0	51	0.34
45	4 200 (N.-free)	14 days 30°	0.2	—	—	—	1.0	—	97	Trace

betaine was used as the sole source of both carbon and nitrogen but in all other cases the nitrate was not omitted from the Czapek-Dox salt solution. In all experiments the nitrogenous compound was present before inoculation.

Although peptone alone gave a low yield, glucose and peptone together gave by far the greatest yields of trimethylarsine obtained on a liquid medium, the average being 4.87 %, with limiting values 3.14–7.55 %. This was to a certain extent associated with a more extensive growth of the mould.

*Reaction between choline chloride or betaine and arsenite,
selenite and tellurite.*

IA. *Selenite and choline chloride.* An intimate mixture of anhydrous sodium formate (5 g.), choline chloride (5 g.) and sodium selenite (6 g.) was placed in a 100 ml. round-bottomed flask, which was connected with an absorption train of 3 bottles containing HCl (1:1) and 3 containing saturated aqueous HgCl_2 . The mixture was strongly heated on a sand-bath in a stream of CO_2 and the volatile products passed through the absorption train. The acid retained the alkaline vapours whilst a white precipitate (0.50 g.) m.p. 152° (decomp.) formed in the mercuric chloride. This gave yellow mercuric oxide and an odour of alkyl selenide on treatment with dil. NaOH. Recrystallised from much acetone it formed shining crystals m.p. 155° (decomp.) and mixed m.p. 155° with a freshly recrystallised authentic specimen of dimethylselenide monomercurichloride, $\text{Me}_2\text{Se} \cdot \text{HgCl}_2$. m.p. 155° (decomp.).

A portion of this product was shaken with dilute sodium hydroxide and ether and the ethereal solution treated with aqueous potassium platinochloride K_2PtCl_4 . Removal of solvent gave yellow crystals, m.p. 163° on one recrystallisation from CHCl_3 and mixed m.p. $163\text{--}4^\circ$ with authentic dimethylselenide platinochloride (α -form) m.p. $163\text{--}4^\circ$ [Challenger and North, 1934].

IB. *Selenite and betaine.* This experiment was exactly similar to IA except that betaine (anhydrous base) replaced the choline chloride. 0.54 g. mercurichloride m.p. 150° (decomp.) was obtained. Recrystallisation from acetone gave crystals m.p. and mixed m.p. 155° (decomp.).

IC. *Selenite-betaine without formate.* Equal weights (5 g.) of sodium selenite and betaine in the absence of formate heated in a stream of carbon dioxide gave 2.7 g. white precipitate in the aqueous mercuric chloride. Extraction with much acetone gave about 2.0 g. $\text{Me}_2\text{Se} \cdot \text{HgCl}_2$ m.p. and mixed m.p. 154° . The fraction insoluble in acetone with warm dilute sodium hydroxide gave a black precipitate, soluble in potassium sulphide, and probably consisted of the double compound mentioned below (If). It seems that the methylation proceeds better in the absence of formate.

ID. *Selenite-tetramethylammonium hydroxide.* Equal weights (5 g. each) sodium selenite, tetramethylammonium hydroxide and anhydrous sodium formate treated in the usual way gave 0.85 g. mercurichloride m.p. 150° and 155° on recrystallisation from acetone.

IE. *Selenite and glycine.* Equal weights (5 g.) of glycine, sodium selenite and anhydrous sodium formate were treated as in IA. A very small quantity of a white solid m.p. above 270° was deposited in the mercuric chloride. Extraction with acetone gave no trace of $\text{Me}_2\text{Se} \cdot \text{HgCl}_2$. With sodium hydroxide in the cold there was no odour of alkyl selenide but alkaline vapours were evolved on warming. Evidently no methylation of selenium occurs using glycine.

IF. *Control.* 5 g. each of sodium selenite and anhydrous sodium formate were treated as before but omitting the nitrogenous compound. The white mercurichloride so obtained had no m.p. With dil. NaOH a yellow precipitate formed

which gradually became black and was then completely soluble in aqueous potassium sulphide. The white solid was probably a double compound of mercuric selenide and mercuric chloride. Dimethyl selenide was therefore not produced.

IIA. *Tellurite and choline chloride*. 5 g. each of anhydrous sodium formate and choline chloride with 4 g. potassium tellurite were treated as in the analogous case with selenite.

A trace of mercurichloride m.p. 151° (decomp.) was obtained in the absorption flask. This compound blackened slowly with dilute ammonia and with dilute NaOH gave a yellow precipitate (quickly turning black) and an odour of alkyl telluride. The mercurichloride of dimethyl telluride behaves similarly but the compound is difficult to characterise owing to its insolubility.

IIB. *Tellurite and betaine*. Equal weights (5 g.) of potassium tellurite, anhydrous sodium formate and betaine (anhydrous base) were treated in an atmosphere of carbon dioxide as before but using a modified absorption train i.e. (a) 4 bottles hydrochloric acid (1:1), (b) 2 bottles alcoholic benzyl chloride, (c) 2 bottles Biginelli's solution. This was repeated three times using the same absorption solutions (b) and (c) but changing the acid after each run. A small amount of mercurichloride m.p. $> 270^{\circ}$ giving an odour of Me_2Te with dil. NaOH was formed. The benzyl chloride solution was extracted with water, the aqueous extract washed with ether and then evaporated to dryness. The solid residue with saturated sodium picrate solution gave a picrate m.p. $117-18^{\circ}$ on recrystallisation from absolute alcohol, and mixed m.p. $117-18^{\circ}$ with an authentic specimen of benzyldimethyltelluronium picrate m.p. $117-18^{\circ}$.

III. *Arsenite and choline chloride*. 6 g. NaAsO_2 were heated with 5 g. each choline chloride and anhydrous sodium formate as in I and II but with Biginelli's solution to replace the aqueous mercuric chloride. No $\text{Me}_3\text{As} \cdot 2\text{HgCl}_2$ was obtained.

A similar experiment, using powdered metallic arsenic in place of sodium arsenite, also gave a negative result. Other experiments were carried out in which (a) sodium arsenite (5 g.) and betaine (5 g.) in the absence of formate and (b) equal weights (5 g.) of sodium arsenite, tetramethylammonium hydroxide and sodium formate were treated as before. No formation of trimethylarsine could be detected. This result was decidedly unexpected and the possibility that both biological and chemical methylations of arsenic may proceed by a different mechanism from that of selenium and tellurium must be borne in mind. In view of the ease with which *P. brevicaule* methylates both selenium and arsenic this suggestion may appear somewhat improbable.

It is, however, perhaps significant that, apart from an observation of Puntoni [1917] (who ascribed a garlic odour in the breath of patients taking sodium cacodylate to the action on this substance of intestinal organisms), there appear to be no well-established instances of the production of arsenical breath, whereas tainted breath is a well-recognised phenomenon in the case of tellurium (see p. 1760).

There is a certain amount of evidence for believing that the formaldehyde mechanism suggested on p. 1758 for the biological methylation of arsenic, involving a tautomeric form of arsenious acid, may not hold for selenium. Strecker and Daniels [1928] find that the product from the action of silver selenite, Ag_2SeO_3 , on ethyl iodide is identical in b.p. and optical constants with that obtained from selenium oxychloride, SeOCl_2 , and sodium ethoxide. They conclude therefore that, unlike sulphurous acid, selenious acid or its salts are not capable of tautomerism to the forms $\text{H} \cdot \text{SeO}_2 \cdot \text{OH}$ or $\text{Ag} \cdot \text{SeO}_2 \cdot \text{OAg}$.

Enzyme preparations.

A. *Press juice.* *P. brevicaulis* was grown on Czapek-Dox salt solution containing 2 % glucose and 1 % (Witte) peptone until a firm pellicle had formed, i.e. for 9 days at room temperature. The medium was removed and replaced with sterile distilled water for 16 hours and the growth again treated with water for 5 hours. The mycelium was filtered through muslin, again washed and excess moisture removed in a hand press.

To 269 g. pressed mould were added 80 g. each of sand and kieselguhr and the mixture ground for 5–10 minutes in a large iron mortar. The resulting mass was wrapped in a wet filter-cloth (from which excess moisture had been removed in a hand press) and the juice expressed in two fractions, (a) up to a pressure of 100 kg./cm.² and (b) 100–300 kg./cm.² The juice as it formed was filtered through paper and collected in ice-cooled receivers.

(a) Gave 113 ml. and (b) 55 ml. After standing 3 hours in ice-water the juices were examined separately as follows:

- (a) 1. 100 % juice + 0.4 % As_2O_3 .
2. 100 % juice + 0.4 % As_2O_3 + 2.0 % glucose.
3. 100 % juice + 0.4 % As_2O_3 + 10.0 % glucose.
4. 50 % juice + 50 % phosphate buffer p_{H} 7.0 + 0.2 % As_2O_3 + 2.0 % glucose.
5. 10 % juice + 90 % phosphate buffer p_{H} 7.0 + 0.2 % As_2O_3 + 2.0 % glucose.
- (b) 1. 100 % juice + 0.4 % As_2O_3 + 2 % glucose.
2. 50 % juice + 50 % phosphate buffer p_{H} 7.0 + 0.2 % As_2O_3 + 2.0 % glucose.
3. 10 % juice + 90 % phosphate buffer p_{H} 7.0 + 0.2 % As_2O_3 + 2.0 % glucose.

No odour of trimethylarsine was detected during three days nor was any stain obtained on filter-paper soaked in aqueous HgCl_2 and suspended above the liquid, indicating the absence of hydrogen arsenide.

B. *Acetone-dried mycelium.* *P. brevicaulis* was grown on Czapek-Dox salt solution containing 2 % glucose for 2 days at 30° and 11 days at room temperature. The mycelium was allowed to stand over tap-water for 5 hours, filtered through muslin, washed with water and excess moisture removed in a hand press.

The pressed mass (100 g.) was immediately added to 600 ml. acetone, well stirred for 10 minutes, filtered quickly, again treated with acetone (300 ml.) for 2 minutes, filtered and finally well stirred with 100 ml. ether. After removal of ether by filtration the product was dried at 40° for 24 hours and the resulting mass (25 g.) well ground before use.

The enzyme preparation in concentrations 0.125, 0.25, 0.5, 1.0, 2.0, 5.0 and 10.0 % was added to phosphate buffer solution p_{H} 7.0 together with 0.2 % As_2O_3 and 2 % glucose. Similar tests were made at p_{H} 6.0 and p_{H} 8.0 with concentrations of enzyme preparation 0.125 to 2.0 %; also at p_{H} 7.0 with 2 % enzyme preparation and 5 and 10 % glucose instead of the usual 2 %.

In no case was an odour of trimethylarsine detected during 9 days at room temperature.

A further test was done on a larger scale. To each of four 1-litre conical flasks containing 2 % glucose in 200 ml. phosphate buffer solution p_{H} 7.0, were added 25 ml. 1.6 % As_2O_3 (final conc. 0.2 %) and 2 g. enzyme preparation (final conc.

1 %). The flasks were connected in series and a continuous stream of sterile air was drawn over and through Biginelli's solution. No trace of $\text{Me}_3\text{As} \cdot 2\text{HgCl}_2$ formed during 18 days' aeration at room temperature. This acetone-dried preparation nevertheless contained active enzymes since the tests after 48 hours gave an immediate precipitate with 2:4-dinitrophenylhydrazine. This was not given in absence of the enzyme preparation or by an aqueous extract of the latter.

C. *Berkefeld filtrate*. Two cultures of *P. brevicaula* on 2 % glucose in 200 ml. Czapek-Dox salt solution were grown for 3 days at 30° and 4 days at room temperature. The medium was filtered through a Berkefeld candle, and the filtrate (shown to be sterile) tested immediately as follows:

- (a) 50 ml. filtrate + 0.2 % As_2O_3 .
- (b) 25 ml. filtrate + 25 ml. phosphate buffer solution containing 2 % glucose + 0.2 % As_2O_3 .
- (c) 10 ml. filtrate + 40 ml. phosphate buffer solution containing 2 % glucose + 0.2 % As_2O_3 .
- (d) 5 ml. filtrate + 45 ml. phosphate buffer solution containing 2 % glucose + 0.2 % As_2O_3 .

Tests (b), (c) and (d) were made with buffer solutions p_{H} 6.0, p_{H} 7.0 and p_{H} 8.0. No odour of arsine was detected in any test during 8 days at room temperature.

A further series of experiments was made using a concentrated filtrate prepared from similar cultures grown for 3 days at 30° and 7 days at room temperature. The Berkefeld filtrate was concentrated at 40–45° under reduced pressure, to $\frac{1}{3}$ volume and used immediately as follows:

- (a) 25 ml. conc. filtrate + 0.2 % As_2O_3 .
- (b) 25 ml. conc. filtrate + 25 ml. phosphate buffer solution p_{H} 7.0 containing 2 % glucose + 0.2 % As_2O_3 .
- (c) 10 ml. conc. filtrate + 40 ml. phosphate buffer solution p_{H} 7.0 containing 2 % glucose + 0.2 % As_2O_3 .

No odour of arsine was detected in any experiment during 18 days at room temperature.

CONCLUSIONS.

The experimental results so far obtained do not enable any definite conclusion to be reached regarding the mechanism of biological methylation either by moulds or in the animal body. Bearing in mind the complexity of the problem this is hardly surprising. Of the three mechanisms suggested on p. 1757 namely participation of (a) formaldehyde, (b) acetic acid, (c) a free methyl group detached from a more complex molecule, there appears to be a certain amount of evidence against (b). This is indicated by the experiments with arsonoacetic, arsonopropionic and thiodiglycollic acids and those in which fatty acids or their sodium salts were added to bread cultures in presence of arsenious acid. It should be remembered however that whereas decarboxylation of arsonoacetic acid might be expected (by analogy with malonic acid) to be easy the same does not necessarily follow in the case of arsonopropionic and arsonobutyric acids where the acidic groups are further apart. That the formation of trimethylarsine from arsonoacetic acid by *P. brevicaula* occurs with much difficulty and sometimes not at all is certainly hard to reconcile with mechanism (b). The non-production of dimethylethylarsine from arsonopropionic acid is not necessarily such strong evidence against the acetic acid theory. The suggestion that, whatever its origin, formaldehyde (or glyoxylic acid) is the methylating agent allows the formation of trimethylarsine to be represented by a relatively simple, and from a chemical

point of view unobjectionable, scheme. The difficulty experienced in the synthesis of hydroxymethylarsonic acid $\text{HOCH}_2\cdot\text{AsO}_3\text{H}_2$ has so far prevented the application of a possible crucial test, since this compound in mould cultures should undergo reduction to methylarsonic acid and methylation to trimethylarsine.

An attempt to test the capacity of *P. brevicaulis* to effect the reduction of a $-\text{CH}_2\text{OH}$ group to $-\text{CH}_3$ was made by growing the mould on a 2 % glucose Czapek-Dox solution containing hydroxymethylphosphonic acid or its monosodium salt $\text{HOCH}_2\cdot\text{PO}(\text{OH})_2$ [Page, 1912]. No conversion either into a volatile phosphine or into methylphosphonic acid could be demonstrated, although a method for the separation of this acid from the hydroxymethyl compound was devised.

The work of Ackermann [1912] and of Cohn [1893] on the behaviour of pyridinecarboxylic acids in the dog suggests the participation of glycine and of a methyl group according to the scheme outlined on p. 1761. For the elimination of methyl from a methylated nitrogen compound, either free or forming part of a larger molecule, there is again no conclusive evidence. The widespread occurrence of methylated bases free or combined in animal, and especially fish, tissue and the methylating (see however p. 1760) action exercised by many such tissues on sodium tellurite [Hofmeister, 1894] render this hypothesis (c) distinctly attractive. Chemical evidence shows that this elimination may occur (p. 1760).

The results described in the present communication and those of Maassen [1902] and of Smith and Cameron [1933] indicate that it is either impossible or at any rate difficult to obtain active enzyme preparations from the mould. Hofmeister and Maassen 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 (see p. 1760) 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 $40-50^\circ$ 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.

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Addendum.

During the discussion of a communication by Dr Haas (see p. 1762) at the Oxford meeting of the Biochemical Society in May 1935 it was suggested by Mr G. A. D. Haslewood that the production of dimethyl sulphide from seaweed might possibly depend on the elimination of a methyl group from the quaternary carbon atom in the molecule of a sterol.

Such elimination of alkyl groups from quaternary carbon atoms is known to occur during dehydrogenation of sesquiterpenes [Ruzicka and Stoll, 1922; 1923] and sterol derivatives [Diels and Gädke, 1927] by sulphur or selenium, and if the female hormone folliculin be regarded as arising from cholesterol this elimination must also be effected in the animal body. It is possible however that in nature this occurs by oxidation to carboxyl and loss of carbon dioxide especially since the hexahydrobenzene nucleus to which the methyl group in question is attached in the cholesterol molecule, appears as a benzene ring in folliculin.

The suggestion of Mr Haslewood is however extremely interesting and may give rise to further experimental work since sterols are constituents of mould mycelium.

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