114. *Studies in Biological Methylation. Part IX.* The Action of* Scopulariopsis brevicaulis *and certain* Penicillia *on Salts of Aliphatic Seleninic and Selenonic Acids.*

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Addition of the sodium salts of methane-, ethane-, and propane-1-seleninic acids or their hydrogen nitrates to bread cultures of *Scopulariopsis brevicaulis* and of certain *Penicillia* gives dimethyl, methyl ethyl, and methyl *n*-propyl selenides respectively. Addition of the potassium salts of the corresponding selenonic acids $R{\cdot}SeO_2{\cdot}OK$ to similar cultures gives only dimethyl selenide, owing to hydrolysis of the selenonates to ROH and $KHSeO_3$. The bearing of these results on the hypothesis put forward by Challenger (see below) to explain the phenomenon of methylation, and particularly the conversion of selenites and selenates into dimethyl selenide, by certain moulds, is discussed.

In Part VIII a hypothesis was proposed (Challenger, *Chem. and Ind.*, 1942, in the press) to account for most of the observed cases of biological methylation in animals and in moulds. On this view methaneselenonic acid, $CH_3{\cdot}SeO_2{\cdot}OH$, methaneseleninic acid, $CH_3{\cdot}SeO_2H$, dimethylselenone, $(CH_3)_2SeO_2$, and dimethyl selenoxide, $(CH_3)_2SeO$, may be intermediate stages in the conversion of selenious acid or its salts into dimethyl selenide by *Scopulariopsis brevicaulis*. The process might be initiated by the transfer of a methyl group from some compound such as choline, betaine, or methionine.

The present communication describes experiments designed to test the behaviour of the first two of the postulated intermediates in bread cultures of *S. brevicaulis* and of certain other moulds, all of which have been found by us (J., 1939, 163) to convert tellurites and tellurates into dimethyl telluride. The nitrate of dimethyl selenoxide has already been shown by one of us and North (J., 1934, 68) to give dimethyl selenide with *S. brevicaulis*.

Volatile products from the cultures were aspirated through aqueous mercuric cyanide to remove alkylselenothiols and diselenides and then through Biginelli's solution (mercuric chloride, 10 g.; water, 80 c.c., hydrochloric acid, 20 c.c.) to remove dialkyl selenides.

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* Parts I (J., 1933, 95) to VII (J., 1939, 163) of this series were published under the title "The Formation of Organometalloidal and Similar Compounds by Micro-organisms." The revised title more clearly indicates the scope of the investigation.

The behaviour of potassium methaneselenonate (preceding paper) would at first appear to be in agreement with the hypothesis outlined above, since with *S. brevicaulis*, *Penicillium chrysogenum*, *P. notatum*, and the green Penicillium allied to *P. notatum* (J., **1939**, **163**) dimethyl selenide was produced in small amount. This might have arisen in at least three ways: (*a*) according to the suggested mechanism; (*b*) by a rearrangement of potassium methaneselenonate, thus: $2CH_3{\cdot}SeO_2{\cdot}OK = (CH_3)_2SeO_2 + K_2SeO_4$, when the selenate would be methylated and the (unknown) selenone might be reduced, giving dimethyl selenide in each case. Finally (*c*) hydrolysis with elimination of methyl alcohol would yield potassium hydrogen selenite, which would then give dimethyl selenide (Challenger and North, *loc. cit.*). Addition of potassium ethaneselenonate and propane-1-selenonate to cultures of these moulds showed that (*c*) is the correct explanation, dimethyl selenide being obtained in each case. With these substrates methyl ethyl and methyl *n*-propyl selenides should have been obtained according to (*a*), whereas process (*b*) should have yielded a mixture of dimethyl selenide with diethyl selenide and di-*n*-propyl selenide respectively. The results with the three potassium alkaneselenonates are due to the instability of these compounds to hydrolysing agents (preceding paper) but are strikingly differentiated from the behaviour of the mono- and di-alkylarsonic acids $R{\cdot}AsO(OH)_2$ and $R_2AsO{\cdot}OH$. These compounds are very stable and invariably undergo methylation, giving $RAsMe_2$ and R_2AsMe, respectively, no trace of trimethylarsine being evolved except where $R = CH_3$ (see Challenger *et al.*, J., **1933**, **95**; **1935**, **396**; **1936**, **264**). It is not, however, impossible that methaneselenonic acid may be an intermediate in the conversion of inorganic selenite or selenate into dimethyl selenide, since it might be sufficiently stable to pass on to the next stage without loss of selenite when actually formed in the mould cell.

The salts of the alkaneseleninic acids $R{\cdot}SeO_2H$ (R = Me, Et or *n*-Pr) with most of the moulds mentioned above give the corresponding alkyl methyl selenides $R{\cdot}Se{\cdot}CH_3$. In the case of the methane- and ethane-seleninates the selenide was accompanied by either methyl- and ethyl-selenothiol $R{\cdot}SeH$ or the corresponding diselenide. Fredga (Dissert., Uppsala, **1935**) states that seleninic acids give selenium and diselenides on decomposition, but no details are available. Cyanomercury methylselenothiol $Me{\cdot}Se{\cdot}HgCN$ and (probably) mercury diethylselenothiol $(EtSe)_2Hg$ respectively separated in the mercuric cyanide absorption tubes, and alkyl selenocyanate was produced: $R{\cdot}Se{\cdot}SeR + Hg(CN)_2 = R{\cdot}Se{\cdot}HgCN + R{\cdot}SeCN$ (see preceding paper). Owing to the ready oxidation of RSeH (Tschugaeff, *Ber.*, **1909**, **42**, 49) the reaction in the mercuric cyanide is probably due to diselenide.

The formation of dimethyl and methyl alkyl selenides from salts of the seleninic acids is in agreement with the suggested scheme in which methaneseleninic acid is an intermediate product. Dimethyl selenide might conceivably arise from methaneseleninic acid thus: $2CH_3{\cdot}SeO_2H = (CH_3)_2Se + H_2SeO_4$; this was not observed in control experiments using uninoculated bread, nor does it seem to be recorded in the literature. Such a decomposition of ethane- and propane-1-seleninic acids in the mould cultures would give a mixture of dimethyl selenide with diethyl and di-*n*-propyl selenides respectively. This was never observed, the mercuric chloride of the methyl alkyl selenide separating in a pure condition in the absorption tubes.

The production of diethyl diselenide or ethylselenothiol in cultures containing potassium ethaneseleninate creates some slight ambiguity. By analogy with the behaviour of alkylthiols and disulphides (Challenger and Rawlings, J., **1937**, **868**; Challenger and Blackburn, J., **1938**, **1872**) either of these compounds might yield methyl ethyl selenide without passing through the selenone $(CH_3{\cdot}SeO_2{\cdot}C_2H_5)$ stage. The behaviour of alkylselenothiols in bread cultures was not studied, but the diselenides gave no methyl alkyl selenides in such cultures.

In the conversion of sodium selenite into dimethyl selenide by *S. brevicaulis* red selenium is produced and with numerous moulds and bacteria selenium is deposited within the organisms (Gosio, *Atti R. Accad. Lincei*, **1904**, **13**, I, 642). Sodium selenate readily gives dimethyl selenide with *S. brevicaulis* but no selenium is produced.

According to the hypothesis cited on p. 574, selenate is first reduced to selenite before attachment of the methyl group. If so, it is surprising that the resulting selenite escapes reduction to selenium, unless attachment of methyl occurs so rapidly in the cell as to obviate this.

An alternative mechanism might appear possible. Dimethyl selenate $(MeO)_2SeO_2$ might be produced from the selenate ion and its reduction to dimethyl selenite $(MeO)_2SeO$, followed by isomerisation to the methyl ester of methaneselenonic acid $Me{\cdot}SeO_2{\cdot}OMe$. The methaneselenonic acid resulting on hydrolysis could then react according to the original scheme. Dimethyl selenite (Michaelis and Landmann, *Annalen*, 1887, **241**, 159) is, however, readily decomposed by water, giving selenious acid, and Strecker and Daniel (*ibid.*, 1928, **462**, 189) failed to observe any isomerisation of dialkyl selenites to alkyl esters of the alkaneselenonic acids. Nevertheless the formation of derivatives of methyl methanesulphonate on heating dimethyl sulphite with glycine (Voss and Wulkan, *Ber.*, **1937**, **70**, 390) and with dimethylaniline or pyridine (Voss and Blanke, *Annalen*, 1931, **485**, 258) suggests that isomerisation of dimethyl selenite may not be impossible.

EXPERIMENTAL.

The strain of *Scopulariopsis brevicaulis* (*Penicillium brevicaule*) used is registered in the Baarn List of Fungi 1932 as *S. brevicaulis* (Sacc.) Bainier. In one experiment only the strain *S. brevicaulis* Saccardo (strain Washington 2) was used. Details of these strains, designated for convenience (A) and (D), are recorded by Challenger, Higginbottom, and Ellis (*loc. cit.*, p. 98). The strains of *Penicillium chrysogenum*, *P. notatum* and the green Penicillium (closely allied to *P. notatum*) were those used by us in an earlier investigation (J., 1939, 163). Bread crumbs, slightly moistened with water, were placed in test-tubes of about 40 c.c. capacity so as to fill ⅔ of the space, or in 250 c.c. conical flasks, 40 g.

in each. Sterilisation of the media, apparatus, and stopper with delivery tubes and of the air stream was ensured as before (J., 1933, 98). The seleninic acids were used as their sodium salts or as the sodium salts of their hydrogen nitrates, the selenonic acids as the potassium salts. Owing to their instability these compounds were not sterilised, but solutions in sterile water (0·15—0·2 g. in 2 c.c.) were used. The mould was allowed to form a good mycelium before the substrates were added; the time required was usually 2 days at 32° and 2 at room temperature for *S. brevicaulis*, 3 days at room temperature for *P. chrysogenum* and the " variety " of *P. notatum*, and 5—6 days at room temperature for *P. notatum*. Volatile products produced in the cultures were aspirated by sterile air through aqueous mercuric cyanide (5%, 5 c.c.) and then through Biginelli's solution (5 c.c.) contained in small tubes. Control experiments showed that addition of the sodium or potassium salts of all the seleninic or selenonic acids used in this work to tubes of sterile bread crumbs, followed by aeration for as long as 2 months into mercuric cyanide and Biginelli's solution in series, gave no precipitate. No odour was detected in these control flasks and no selenium was deposited in the media.

S. brevicaulis *(Strain D) and the Sodium Salt of Methaneseleninic Acid Hydrogen Nitrate.*—(Five tubes). After 3 days traces of a yellow precipitate, probably $CH_3\cdot Hg\cdot SeCN$ (m. p. 101° decomp.), formed in the mercuric cyanide and a white precipitate in the Biginelli's solution (0·05 g. after 10 days, when the experiment was discontinued). This had m. p. and mixed m. p. 153° with authentic dimethyl selenide mercurichloride of m. p. 153°—all with decomposition. The mercuric cyanide solution had an odour of methyl selenocyanate. Some orange crystals (0·04 g.) appeared in the Biginelli's solution later in the experiment. These had m. p. and mixed m. p. 123° (decomp.) with the product of the same m. p. obtained on aeration of dimethyl diselenide into Biginelli's solution (preceding paper).

S. brevicaulis (Strain A) in a similar experiment using sodium methaneseleninate (six tubes) gave after 6 weeks 0·07 g. of dimethyl selenide mercurichloride, m. p. and mixed m. p. 153° (decomp.), and 0·1 g. of the yellow crystals, m. p. and mixed m. p. 101°, with the product of the same m. p. obtained from dimethyl diselenide and mercuric cyanide (Bird and Challenger, *loc. cit.*).

S. brevicaulis *and the Sodium Salt of Ethaneseleninic Acid Hydrogen Nitrate.*—(Five tubes). In 2 days small amounts of yellow solid, sintering from 85° and blackening and decomposing at 130°, formed in the mercuric cyanide, which then smelled strongly of an alkyl selenocyanate. In the Biginelli's solution a white solid, m. p. and mixed m. p. 100°, with methyl ethyl selenide mercurichloride, m. p. 100°, was deposited. Three weeks' aeration produced very little more solid.

S. brevicaulis *and the Sodium Salt of Propane-*1*-seleninic Acid Hydrogen Nitrate.*—(Seven tubes). No trace of yellow solid formed in the mercuric cyanide during 6 weeks, but, after 5, 25, and 35 days respectively, deposits of 0·025 g., 0·04 g., and 0·01 g. of white crystals formed in the Biginelli's solution. These all melted at 88° and the first deposit did not depress the m. p. (88°) of an authentic specimen of methyl *n*-propyl selenide mercurichloride. Replacement of the Biginelli's solution by neutral aqueous mercuric chloride (4%) gave a white deposit of m. p. and mixed m. p. 160° (decomp.) with the compound of the same m. p. obtained similarly from methyl *n*-propyl selenide (Bird and Challenger, *loc. cit.*).

Penicillium notatum *and the Seleninic Acids.*—The sodium salts of methaneseleninic hydrogen nitrate and of ethaneseleninic acid being used, no methylation was observed during 3—4 weeks, no trace of deposit being formed in the Biginelli's solution. The characteristic yellow precipitates were obtained in the mercuric cyanide after 14—48 hrs. from the commencement of the experiment, that from the methyl compound (0·02 g.) decomposing at 101° with previous softening at 90°, and that from the ethyl derivative (0·12 g.) having m. p. 85°. This experiment was repeated and the yellow solid had m. p. and mixed m. p. 85° (decomp. 130°) with that obtained directly from authentic diethyl diselenide and mercuric cyanide. Alkyl selenocyanate odour in the mercuric cyanide was very strong in both experiments with the ethyl compounds (see above).

Four flask cultures containing the sodium salt of propane-1-seleninic acid hydrogen nitrate gave no deposit or odour in the mercuric cyanide during 5 weeks. The deposit in Biginelli's solution (0·06 g. in 8 days) had m. p. and mixed m. p. 86—87° with methyl *n*-propyl selenide mercurichloride. A further deposit (0·07 g.) was removed on the 21st day and had m. p. and mixed m. p. 85·5°.

The green Penicillium allied to P. notatum *and the Three Seleninic Acids.*—Three tube cultures of sodium methaneseleninate gave no dimethyl selenide after 19 days, but the usual deposit, m. p. 101°, and an odour of methyl selenocyanate formed in the mercuric cyanide. Undoubted methylation was observed with the sodium salts of the hydrogen nitrates of the ethyl and *n*-propyl derivatives, 3 flask cultures being used in each case. 0·13 G. of methyl ethyl selenide mercurichloride (white needles), m. p. and mixed m. p. 100°, formed in Biginelli's solution in 4 days, and in the same time 0·03 g. and in 3 weeks 0·2 g. of methyl *n*-propyl selenide mercurichloride, m. p. and mixed m. p. 88°, were similarly obtained.

A second experiment was carried out with this mould and the sodium salt of ethaneseleninic acid hydrogen nitrate. The resulting mercurichloride, m. p. and mixed m. p. 100° as before, was recrystallised from 3·5% aqueous mercuric chloride; it then melted at 141·5° (decomp.), a figure which was unchanged by three further crystallisations, indicating its homogeneity. This melted at 140—141° (decomp.) in admixture with an authentic specimen of methyl ethyl selenide dimercurichloride, m. p. 140—141° (decomp.).

P. chrysogenum *and the Seleninic Acids.*—No methylation was observed with the sodium salt of methaneseleninic acid (6 tubes; 4 weeks), but the usual yellow solid, m. p. 101° (decomp.), and odour of methyl selenocyanate formed in the mercuric cyanide. With the sodium salt of ethaneseleninic acid hydrogen nitrate and the corresponding *n*-propyl derivative (3 tubes) methylation occurred and methyl ethyl selenide mercurichloride (0·075 g., m. p. and mixed m. p. 100°) and methyl *n*-propyl selenide mercurichloride (0·15 g., m. p. and mixed m. p. 88°) separated from the Biginelli's solution. As usual, no odour or deposit was produced in the cyanide from the *n*-propyl compound, but 0·015 g. of yellow solid, m. p. 85°, and an odour of alkyl selenocyanate were formed from the ethaneseleninic acid salt.

S. brevicaulis *and Potassium Methaneselenonate.*—(Four flasks). After 9 days a slight deposit, m. p. 153—155° (decomp.), formed in the Biginelli's solution. After 9 weeks a further small amount, sintering at 145° and melting at 155° (decomp.), was removed. This, on recrystallisation from acetone, had m. p. and mixed m. p. 153° (decomp.) with dimethyl selenide mercurichloride. During this period only a trace of yellow deposit, blackening after a week or two, formed in the mercuric cyanide. The experiment was repeated, but after 7 weeks the deposits which formed were too small to remove.

S. brevicaulis *and Potassium Ethaneselenonate.*—(Four flasks). During 8 weeks only traces of a yellow deposit, quickly blackening, formed in the cyanide tubes and some white crystals in Biginelli's solution. Their quantity was just sufficient for a m. p. and mixed m. p. (151—153° decomp.) with dimethyl selenide mercurichloride.

S. brevicaulis *and Potassium Propane-*1*-selenonate.*—(Six tubes). No deposit formed in the cyanide during 3 weeks, but 0·001 g. of white crystals, m. p. and mixed m. p. 150—153° (decomp.) with dimethyl selenide mercurichloride, formed in the Biginelli's solution.

P. chrysogenum *and the Potassium Salts of the Three Selenonic Acids.*—(Five tubes in each case. Duration, 7 weeks). No odour of alkyl selenocyanate or precipitate was produced in the mercuric cyanide during 7 weeks, although in the case of the methyl compound the solution became clear brownish-yellow. Dimethyl selenide mercurichloride separated from the Biginelli's solution in each case, the amount from the methaneselenonate being only sufficient for the determination of m. p. and mixed m. p. 153°. The other two salts gave 0·08 g. and 0·075 g. respectively.

The Green Penicillium allied to P. notatum.—This gave similar results with the three selenonates. Four tube cultures were used for the methyl and ethyl compounds and 3 flask cultures for the *n*-propyl derivative. There was no deposit in the cyanide and after 3 weeks the weights and m. p.'s of the dimethyl selenide mercurichloride were (*a*) 0·007 g., 150—152° (decomp.); (*b*) 0·001 g., 152—153° (decomp.); and (*c*) 0·005 g., 150° (decomp.) and mixed m. p. 153° (decomp.).

P. notatum *and the Potassium Salts of the Three Selenonic Acids.*—(Methyl and ethyl compounds, 3 flask cultures; *n*-propyl compound, 4 tube cultures). In each case only a trace of precipitate was obtained in the Biginelli's solution. A m. p. determination (155° decomp.) was possible with that from the ethyl compound. In the other cases the precipitate gave an odour of dimethyl selenide on treatment with sodium hydroxide. Traces only of yellow precipitate formed in the mercuric cyanide from the methyl and the ethyl derivative and none with the *n*-propyl compound during 2—4 weeks. Odour of selenocyanate was absent.

S. brevicaulis (*Strain A*) *and Sodium Benzeneseleninate.*—Six tube cultures on bread were used and the benzeneseleninate, dissolved in sterile water, was added. In a few days a strong odour closely resembling that of phenyl methyl selenide, prepared for comparison, was observed. Aspiration for 6 weeks into concentrated nitric acid yielded, however, only ammonium nitrate containing, by the flame test, traces of a selenium compound. Repetition, cultures on aqueous 3% glucose–Czapek-Dox medium being used, also gave a strong odour in 3 days. After 4—6 weeks, extraction of the medium and of the mycelium with chloroform yielded respectively a slight residue and diphenyl diselenide, m. p. 60° and mixed m. p. 61° with an authentic specimen of m. p. 61°.

In control experiments on the liquid medium no odour was produced during 2 months.

The decomposition of the seleninic acid into the diselenide in cultures of the mould may prevent the formation of more than traces of phenyl methyl selenide, which, moreover, is difficulty volatile.

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