81. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS

65. (A) A SURVEY OF CHLORINE METABOLISM BY MOULDS

(B) CALDARIOMYCIN, $C_5H_8O_2Cl_2$, A METABOLIC PRODUCT OF *CALDARIOMYCES FUMAGO* WORONICHIN

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THREE mould metabolic products containing chlorine have recently been described: geodin, $C_{17}H_{12}O_7Cl_2$, and erdin, $C_{16}H_{10}O_7Cl_2$, from *Aspergillus terreus* Thom, strain No. 45 [Raistrick & Smith, 1936; Clutterbuck *et al.* 1937; Calam *et al.* 1939] and griseofulvin, $C_{17}H_{17}O_6Cl$, from *Penicillium griseo-fulvum* Dierckx [Oxford *et al.* 1939].

In view of these findings a quantitative survey has now been made of the chlorine metabolism of 139 species or strains of moulds grown on the well-known Czapek-Dox 5% glucose solution containing 0.5 g. KCl per litre as the sole source of chlorine. The organisms studied included 27 species of *Aspergillus*, 96 species of *Penicillium* and species in the following genera: *Absidia* (1); *Alternaria* (2); *Botrytis* (1); *Caldariomyces* (3); *Cephalosporium* (1); *Cladosporium* (1); *Clasterosporium* (1); *Helminthosporium* (1); *Mucor* (1); *Stemphylium* (1); *Stysanus* (1); *Syncephalastrum* (1) and *Trichoderma* (1).

The results obtained show that extensive conversion of inorganic chloride into organic chlorine-containing metabolic products is of rather rare occurrence. Of the 139 species or strains examined, 57 utilized less than 5% of the KCl originally present in the medium; 77 utilized from 5 to 25% of the KCl and certain species in this group might well repay further investigation since it includes *Penicillium griseo-fulvum*, the species giving griseofulvin; only 5 cultures comprising three different species metabolized more than 25% of the KCl. Two of these were different strains of *Aspergillus terreus* Thom including strain No. 45 which gives geodin and erdin, one was *Absidia spinosa* Lendner which is being investigated at present and the remaining two were different strains of *Caldariomyces fumago* Woronichin, an investigation of which is described in Part B of the present communication. An interesting feature of this species is the fact that it also metabolizes considerable amounts of bromine in a medium containing KBr in place of KCl. Although no bromine-containing metabolic products of *Caldariomyces fumago* have at present been obtained in a state of purity, crude mixtures of metabolic products containing considerable amounts of bromine in organic combination have been isolated.

Caldariomyces fumago, which was formerly known as *Fumago vagans* Persoon, is one of the common "sooty" greenhouse moulds. A considerable part of the chloride metabolized by this species appears in the metabolism solution as a hitherto undescribed substance which we propose to name *caldariomycin*.

Caldariomycin, $C_5H_8O_2Cl_2$, forms colourless needles, M.P. 121° , which are dextrorotatory in aqueous solution. The molecular constitution suggested for caldariomycin illustrates the following experimentally established facts.

- (1) It contains no methoxyl or side-chain methyl groups.
- (2) Its two chlorine atoms are particularly labile since they are almost quantitatively liberated as chloride when a cold solution of caldariomycin in $N/10$ NaOH is kept overnight.
- (3) It contains no ketonic or aldehyde groups but, since it has two "active" hydrogen atoms (Zerewitinoff), the probable presence of two actual or potential hydroxyl groups is indicated though no satisfactory derivatives proving the presence of these groups have been obtained.
- (4) It is oxidized by cold aqueous chromic acid with the formation of succinic acid thus proving the presence of the grouping $\begin{array}{c} \diagup C-CH_2-CH_2-C \diagdown \end{array}$.
- (5) It is reduced by hydrogen and a palladium-charcoal catalyst to cyclopentanone, C_5H_8O . Hence one may justly argue that caldariomycin is a derivative of cyclopentane, C_5H_{10} , so that the five carbon atoms in its molecule are accounted for.
- (6) Assuming, as indicated in (3), the presence of two hydroxyl groups then it is clear that these cannot be attached to adjacent carbon atoms as the grouping $\begin{array}{c} \diagup C(OH)-C(OH) \diagdown \end{array}$ since caldariomycin is not attacked by the Malaprade [1928] reagent, i.e. HIO_4 , in acid solution. This belief is strengthened by the fact that if the hydroxyl groups were attached to adjacent carbon atoms caldariomycin would be a derivative of cyclopentane-1:2-diol, but both high- and low-melting forms of cyclopentane-1:2-diol are known and have been prepared and shown to be oxidized by the Malaprade reagent, the low-melting form to the extent of 40% and the high-melting form almost quantitatively.
- (7) It is very stable to heat and does not lose either H_2O or HCl at moderate temperatures. Above 180° , however, it decomposes to give, along with H_2O , HCl and black resinous products, a mixture of two isomeric ketones, each having the empirical formula C_5H_5OCl and each giving a mono-2:4-dinitrophenylhydrazone. One of these ketones is also formed when a solution of caldariomycin in $2N$ H_2SO_4 is boiled.
- (8) It does not contain the grouping $-CH_2.CO-$ since it gives no ketonic reactions. This grouping is however formed on treatment with dilute caustic alkali since such a solution now gives a precipitate with Brady's reagent (2:4-dinitrophenylhydrazine in $2N$ HCl). Further, caldariomycin itself does not immediately give the Zimmermann reaction for an active CH_2 group as modified by Callow *et al.* [1938] though an alkaline solution of caldariomycin, after standing for some time, quickly gives an intense reaction. Finally, although caldariomycin reduces Fehling's solution in the cold this reduction is only apparent after a considerable lag period during which a reducing substance is presumably formed in the strongly alkaline solution.

The above experimental facts indicate clearly that caldariomycin is a dichlorodihydroxycyclopentane. No dichlorodihydroxycyclopentanes are described in the literature but of the many possible structural isomerides only three (I, II and III) will satisfy the following four essential conditions.

- (a) The presence of at least one asymmetric carbon atom.

(b) The presence of the grouping $\diagup\text{C}.\text{CH}_2.\text{CH}_2.\text{C}\diagdown$ to account for the succinic acid formed on oxidation.

(c) The absence of the grouping $\diagup\text{C}(\text{OH}).\text{C}(\text{OH})\diagdown$.

(d) The absence of the grouping

since a compound containing this grouping, even if stable in the solid state, would almost certainly give free HCl at once in aqueous solution.

II and III are essentially hydrated forms of two different dichlorocyclopentanones but cannot be excluded on that account alone since a $\diagup\text{CO}$ group so hydrated might not react with Brady's reagent (cf. chloral hydrate $\text{CCl}_3.\text{CH}(\text{OH})_2$ which does not give the characteristic aldehyde reactions).

The main objection to II and III is that they provide no explanation for the comparatively great stability of caldariomycin towards heat. At least two solid hydrates of polychloro-aldehydes and -ketones are known: (a) chloral hydrate which boils at 97.5° to yield a vapour phase containing both chloral and water molecules; (b) *sym*tetrachloroacetone tetrahydrate, $\text{CHCl}_2.\text{CO}.\text{CHCl}_2.4\text{H}_2\text{O}$, which loses all its water at $110\text{--}115^\circ$ and distils in the anhydrous state without decomposition at 180° [Levy & Jedlicka, 1888]; the hydrate is also completely dehydrated on standing over conc. H_2SO_4 [Zincke & Kegel, 1889]. Since caldariomycin does not begin to lose water on heating until a temperature of 180° is reached and can even be distilled unchanged at $149\text{--}150^\circ$ at 20 mm. it seems improbable that it is a hydrate of a dichlorocyclopentanone. By a process of elimination I is therefore indicated as the most probable structure for caldariomycin, which is thus probably 2:2-dichlorocyclopentane-1:3-diol.

I offers a simple explanation for almost all the experimental facts available including the formation of the two isomeric ketones, $\text{C}_5\text{H}_5\text{OCl}$, referred to above under (7). It does not, however, give such a simple explanation for the formation of cyclopentanone on catalytic reduction as do II and III, i.e. by mere replacement of both Cl atoms by H and subsequent loss of 1 mol. of water. Accepting I, a possible mechanism for this reaction is the following: replacement of one Cl atom by H followed by loss of water to give a chlorohydroxycyclopentene, the double bond in which is reduced to give a chlorohydroxycyclopentane which, by loss of HCl, gives the enolic form of cyclopentanone.

It is worthy of note that in all four chlorine-containing metabolic products so far reported—geodin, erdin, griseofulvin and caldariomycin—chlorine atoms are always found adjacent to hydroxyl or methoxyl groups in a grouping of the form $\diagup\text{C}(\text{Cl})\text{—C}(\text{OR})\diagdown$. This fact suggests that a reaction analogous to that by which ethylene chlorohydrin, $\text{CH}_2\text{Cl}.\text{CH}_2\text{OH}$, is formed by the action of dilute hypochlorous acid on ethylene, $\text{CH}_2=\text{CH}_2$, may possibly serve to account for the formation of chlorine-containing metabolic products by moulds.

EXPERIMENTAL

Part A. A survey of chlorine metabolism by different species of fungi

The medium used throughout this part of the investigation was the well-known Czapek-Dox solution having the following composition: glucose, 50 g.; NaNO_3 , 2.0 g.; KH_2PO_4 , 1.0 g.; KCl, 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g.; distilled water, 1 litre. As the object of the work was to follow quantitatively the chloride remaining in solution at different stages of growth particular care was taken to ensure that each flask of medium contained the same amount of chloride. This was done as follows. All the constituents of the medium except the KCl, and in amount sufficient for 35 litres of medium, were dissolved in 32.5 litres of distilled water and 325 ml. amounts were measured into each of 100 one-litre conical flasks. 25 ml. of a 0.7% aqueous solution of KCl were now accurately measured, by pipette, into each flask. All the flasks were plugged with cotton wool and sterilized. Batches of four flasks were sown with 2.5 ml. of a spore suspension, in 10 ml. of distilled water, of a Czapek-Dox or beer-wort agar culture of the mould under investigation. The flasks were incubated in the dark at 24°.

The glucose content (by polarimeter) and KCl content (by Volhard method) of duplicate unsown flasks from each batch of 100 flasks were estimated. At suitable intervals, so arranged that in the last of the four flasks examined for each species the glucose had been almost completely metabolized, the chloride remaining in solution was estimated as follows. The metabolism solution under investigation was filtered through a Büchner funnel and the mycelium was thoroughly washed with hot distilled water. The filtrate and cooled washings were made up to 500 ml. and duplicate estimations of the chloride present in aliquot portions were carried out by the method described by Raistrick & Smith [1936, p. 1316] which involves the removal of interfering substances with norite charcoal and estimation of chloride in the purified solution by the Volhard method. This technique has been proved to give accurate and reproducible results. Residual glucose (by polarimeter) was also estimated in all metabolism solutions.

The results obtained are summarized below. The species examined are arranged in groups according to the degree of chloride metabolized. Only the *maximum* amount of chloride metabolized by each species is recorded and is expressed as % of the chloride originally present. The figures in brackets following the name of the species examined denote (a) the incubation period in days in which chloride absorption reached its maximum, and (b) the residual glucose on this day.

Group I. *Metabolized 0–1% of the total chloride originally present.* 7 species = 5% of all species examined. *Aspergillus tamarii* Kita (85; 0.0%); *Penicillium brunneo-violaceum* Biourge (19; 0.18%); *P. Charlesii* G. Smith (36; 0.0%); *P. expansum* Link em. Thom (42; 0.05%); *P. puberulum* Bainier (38; 0.0%); *P. schneeggii* Boas (24; 3.94%); *Alternaria* sp. (70; 2.28%).

Group II. *Metabolized 1–2% of the total chloride originally present.* 14 species = 10% of all species examined. *Penicillium aurantio-candidum* Dierckx (68; 3.65%); *P. aurantio-griseum* Dierckx var. *poznanensis* Zaleski (54; 0.0%); *P. aurantio-virens* Biourge (67; 2.10%); *P. brunneo-rubrum* Dierckx (strain P. 9) (64; 0.81%); *P. citreo-viride* Biourge (25; 0.34%); *P. crateriforme* Abbott (33; 2.35%); *P. cyaneo-fulvum* Biourge (strain P. 34) (22; 1.26%); *P. Dierckxii* Biourge (42; 0.08%); *P. jantho-citrinum* Biourge (23; 0.92%); *P. olivino-viride*

Biourge (24; 0.29 %); *P. purpurogenum* Fleroff-Stoll (24; 0.29 %); *P. spiculisporum* Lehman (25; 1.58 %); *P. subcinereum* Westling (strain P. 20) (61; 0.0 %); *P. viridi-dorsum* Biourge (25; 1.31 %).

Group III. Metabolized 2-3% of the total chloride originally present. 21 species = 15 % of all species examined. *Aspergillus repens* (Corda) Saccardo (57; 0.81 %); *A. terreus* Thom (strain Ac. 24) (60; 0.60 %); *Penicillium atramentosum* Thom (67; 0.11 %); *P. Biourgei* Dierckx (20; 1.76 %); *P. Brefeldianum* Dodge (41; 1.33 %); *P. chrysogenum* Thom (strain P. 18) (64; 0.08 %); *P. citreo-sulfuratum* Biourge (strain P. 59) (65; 0.08 %); *P. citrinum* Thom (strain P. 25) (25; 0.29 %); *P. decumbens* Thom (70; 0.0 %); *P. digitatum* Saccardo (42; 1.47 %); *P. fellutanum* Biourge (strain P. 67) (54; 0.0 %); *P. flavido-marginatum* Biourge (20; 0.08 %); *P. frequentans* Westling (strain P. 29) (39; 0.0 %); *P. griseo-fulvum* Dierckx (strain P. 38) (64; 1.1 %); *P. notatum* Westling (23; 0.65 %); *P. patris-mei* Zaleski (22; 0.29 %); *P. roqueforti* Thom (strain P. 54) (25; 2.10 %); *P. subcinereum* Westling (strain P. 21) (65; 0.21 %); *P. terlikowskii* Zaleski (67; 0.18 %); *Cephalosporium* sp. (49; 3.15 %); *Helminthosporium monoceras* Drechsler (70; 4.97 %).

Group IV. Metabolized 3-5% of the total chloride originally present. 15 species = 11 % of all species examined. *Aspergillus candidus* Link (84; 0.0 %); *A. flavus* Link (77; 0.0 %); *A. niger* van Tieghem (strain No. 33) (48; 0.0 %); *A. oryzae* (Ahlburg) Cohn (84; 0.0 %); *A. terreus* Thom (strain Ac. 100) (77; 0.0 %); *A. versicolor* (Vuillemin) Tiraboschi (strain No. 18) (46; 0.05 %); *Penicillium Adametzi* Zaleski (64; 0.08 %); *P. cinerascens* Biourge (71; 1.0 %); *P. granulatum* Bainier (22; 0.37 %); *P. Niklewskii* Zaleski (26; 0.37 %); *P. Raciborskii* Zaleski (22; 0.08 %); unidentified *Penicillium* from soil (33; 1.90 %); *Clasterosporium* sp. (83; 0.0 %); *Mucor pyriformis* Fischer (22; 0.56 %); *Stemphylium* sp. (47; 0.47 %).

Group V. Metabolized 5-6% of the total chloride originally present. 16 species = 12 % of all species examined. *Aspergillus fumigatus* Fresenius (strain No. 47) (47; 0.35 %); *A. nidulans* (Eidam) Winter (46; 0.05 %); *A. ochraceus* Wilhelm (47; 0.24 %); *A. sydowi* (Bainier and Sartory) Thom and Church (46; 0.0 %); *Penicillium biforme* Thom (19; 0.05 %); *P. chrysogenum* Thom (strain P. 57) (25; 0.0 %); *P. citreo-roseum* Dierckx (strain P. 22) (23; 0.45 %); *P. fellutanum* Biourge (strain P. 60) (84; 0.0 %); *P. frequentans* Westling (strain P. 80) (70; 0.0 %); *P. griseo-fulvum* Dierckx (strain P. 68) (70; 0.0 %); *P. Hagemi* Zaleski (68; 0.0 %); *P. ochraceum* (Bainier) Thom (68; 0.0 %); *P. roqueforti* Thom (strain P. 93) (69; 0.26 %); *P. Siemaszki* Zaleski (69; 0.05 %); *P. spinulosum* Thom (25; 0.05 %); *Stysanus stemonites* (Persoon) Corda (48; 0.13 %).

Group VI. Metabolized 6-7% of the total chloride originally present. 14 species = 10 % of all species examined. *Aspergillus fumigatus* Fresenius (strain H.A. 1) (56; 0.46 %); *A. terreus* Thom (strain Ac. 107) (60; 0.0 %); *A. Wentii* Wehmer (46; 0.58 %); *Penicillium baculatum* Westling (62; 0.58 %); *P. baiiolum* Biourge (67; 0.0 %); *P. bialowiezense* Zaleski (53; 0.0 %); *P. caseicolum* Bainier (64; 0.0 %); *P. citreo-roseum* Dierckx (strain P. 23) (25; 0.37 %); *P. citrinum* Thom (strain P. 26) (60; 0.0 %); *P. cyaneo-fulvum* Biourge (strain P. 31) (21; 1.57 %); *P. lividum* Westling (33; 0.01 %); *P. solitum* Westling (39; 0.08 %); *P. subcinereum* Westling (strain P. 98) (69; 0.08 %); *P. sublateralium* Biourge (42; 0.0 %).

Group VII. Metabolized 7-10% of the total chloride originally present. 11 species = 8 % of all species examined. *Aspergillus flavipes* (Bainier and Sartory) Thom and Church (56; 0.02 %); *Penicillium aurantio-brunneum* Dierckx (80; 0.0 %); *P. brunneo-rubrum* Dierckx (strain P. 8) (19; 0.21 %); *P. carminoviolaceum* Dierckx (71; 0.0 %); *P. flavo-cinereum* Biourge (70; 0.08 %); *P.*

frequentans Westling (strain P. 82) (70; 0.05 %); *P. fusco-glaucum* Biourge (21; 0.47 %); *P. Paczoskii* Zaleski (71; 0.08 %); *P. roqueforti* Thom (strain P. 83) (69; 0.21 %); *P. roseo-citreum* Biourge (25; 1.36 %); *Botrytis cinerea* Persoon (28; 1.46 %).

Group VIII. *Metabolized 10–15% of the total chloride originally present.* 23 species = 17 % of all species examined. *Aspergillus terreus* Thom (strain No. 3) (62; 0.0 %); *A. terreus* (strain No. 37) (62; 0.0 %); *A. terreus* (strain Ab. 2) (60; 0.0 %); *Penicillium chlorophaeum* Biourge (22; 0.0 %); *P. chrysogenum* Thom (strain P. 17) (22; 0.0 %); *P. chrysogenum* (strain P. 19) (23; 0.39 %); *P. chrysogenum* (strain H.P. 1) (22; 0.55 %); *P. citreo-sulfuratum* Biourge (strain P. 66) (71; 0.0 %); *P. citrinum* Thom (strain P. 6) (80; 0.0 %); *P. citrinum* (strain P. 27) (21; 0.50 %); *P. Daleae* Zaleski (68; 0.0 %); *P. flavi-dorsum* Biourge (70; 0.0 %); *P. frequentans* Westling (strain P. 28) (78; 0.0 %); *P. griseo-brunneum* Dierckx (22; 0.13 %); *P. meleagrinum* Biourge (23; 3.38 %); *P. minio-luteum* Dierckx (77; 0.13 %); *P. obscurum* Biourge (68; 0.0 %); *P. Oledzkii* Zaleski (69; 0.08 %); *P. phaeo-janthinellum* Biourge (22; 0.05 %); *P. Raistrickii* G. Smith (53; 0.02 %); *P. rugulosum* Thom (22; 0.0 %); *Cladosporium herbarum* (Persoon) Link (69; 0.0 %); *Caldariomyces fumago* Woronichin (strain X. 14) (41; 2.28 %).

Group IX. *Metabolized 15–25% of the total chloride originally present.* 13 species = 9 % of all species examined. *Aspergillus itaconicus* Kinoshita (24; 0.42 %); *A. niger* van Tieghem (strain Ac. 1) (21; 0.34 %); *A. terreus* Thom (strain Am. 1) (77; 0.0 %); *A. terreus* (strain Ab. 1) (18; 0.75 %); *A. versicolor* (Vuillemin) Tiraboschi (strain No. 35) (35; 0.85 %); *Penicillium candido-fulvum* Dierckx (20; 2.66 %); *P. chloro-leucon* Biourge (22; 0.32 %); *P. commune* Thom (21; 0.45 %); *P. griseo-roseum* Dierckx (22; 0.71 %); *P. Westlingi* Zaleski (70; 0.0 %); *Alternaria tenuis* Nees (35; 0.85 %); *Syncephalastrum racemosum* (Cohn) Schröter (74; 2.33 %); *Trichoderma viride* Persoon ex Fries (74; 1.57 %).

Group X. *Metabolized over 25% of the total chloride originally present.* 5 species = less than 4 % of all species examined. *Absidia spinosa* Lendner, 26.6 % of chloride metabolized (74; 0.0 %); *Aspergillus terreus* Thom (strain Am. 2), 33.5 % of chloride metabolized (77; 0.0 %); *A. terreus* (strain No. 45), 96.8 % of chloride metabolized (38; 0.13 %); *Caldariomyces fumago* Woronichin (strain X. 13), 71.5 % of chloride metabolized (33; 1.5 %); *Caldariomyces fumago* (strain Ag. 92), 95.7 % of chloride metabolized (46; 1.47 %).

These results indicate that extensive utilization of chlorides by the lower fungi is of very rare occurrence. Thus of 139 species and strains in a number of different genera by far the greater number utilized less than 10 %, and only five utilized more than a quarter, of the chloride present in the medium.

Cl balance sheet for Aspergillus terreus Thom, strain No. 45

The course of Cl metabolism by *A. terreus*, strain No. 45, has already been examined by Raistrick & Smith [1936] but no complete Cl balance sheet for this organism has been reported previously. In order to prove whether geodin and erdin are the only Cl-containing metabolic products formed by this strain the following experiment was carried out. 100 one-litre flasks each containing 350 ml. of the usual Czapek-Dox medium (see p. 667) were sown with *A. terreus*, strain No. 45, incubated at 24° and examined periodically for residual glucose (by polarimeter) and residual chloride (by the Volhard method). After 26 days, when the residual glucose was 0.48 % the contents of 89 flasks were filtered, the mycelium carefully washed and dried and the washings combined with the filtrate.

In a portion of the measured filtrate and washings there were estimated:

(I) Residual inorganic chloride (see p. 667).

(II) Total Cl in solution. An aliquot was evaporated to dryness in a nickel crucible, fused with KOH (10 g.) and Na_2O_2 (5 g.), and the inorganic chloride was estimated by the Volhard method.

(III) Organic Cl in solution. Obtained by subtracting I from II.

To the main bulk of the solution 2N H_2SO_4 (825 ml.) was added and the flocculent precipitate formed was separated by filtration after standing overnight. Cl estimations were then carried out on

(IV) The dried H_2SO_4 precipitate (44.45 g.). By fusion with KOH and Na_2O_2 as in II.

(V) The filtrate from IV. As in IV.

(VI) The dried mycelium (307 g.). As in IV.

The results obtained are given in Table I.

Table I

Fraction	Cl as KCl g.	Cl as % of KCl originally present
Original metabolism solution	15.575	100.0
(I) Residual inorganic chloride	0.460	2.95
(II) Total Cl in solution	13.809	88.65
(III) Organic Cl in solution	13.349	85.70
(IV) Cl in H_2SO_4 precipitate	9.672	62.10
(V) Total Cl in acid filtrate	4.019	25.80
(VI) Cl in mycelium	1.835	11.78

Sum of (II) and (VI) equals 100.43%.

Ether extraction of the dried acid precipitate (IV) led to the isolation of considerable amounts of geodin and erdin but no other Cl-containing compounds could be detected.

Ether extraction of the acid filtrate (V) removed the whole of the organic Cl in solution very readily and from the evaporated ether solution a little over 3 g. of erdin and a small amount of geodin were obtained but no other Cl-containing substances could be isolated.

The mycelium (VI) containing 11.78% of the Cl originally present in the medium was extracted with light petroleum, B.P. 50–60°, and then with ether but no geodin or erdin was obtained nor could any Cl be detected in any form in either extract. The form in which the Cl is contained in the mycelium must await future investigation.

Part B. Cl metabolism of Caldariomyces fumago Woronichin. Isolation, properties and molecular constitution of caldariomycin, $\text{C}_5\text{H}_8\text{O}_2\text{Cl}_2$

The two cultures used were:

(a) *Caldariomyces fumago*, strain Ag 92. Obtained from Centraal-bureau voor Schimmelcultures, Baarn, Holland, in 1925. Some of the metabolic products of this strain, none of which contained Cl, have already been described by Birkinshaw & Raistrick [1931].

(b) *Caldariomyces fumago*, strain X. 13. Obtained from Baarn in 1937.

Caldariomyces fumago Woronichin was formerly known as *Fumago vagans* Persoon and was given its present name by Woronichin [1926]. Van Beyma the Kingma [1931] states that it is of fairly common occurrence as sooty mould of greenhouse plants in Northern Europe.

Course of Cl metabolism by Caldariomyces fumago, strain Ag 92. The figures given in Table 2 indicate the course of Cl metabolism by *Caldariomyces fumago*, strain Ag 92, when grown at 24° on (a) Czapek-Dox 5% glucose solution (see p. 667) containing the usual amount of KCl, i.e. 0.5 g. per litre (see first five items in Table), and (b) Czapek-Dox 5% glucose solution containing double the usual amount of KCl, i.e. 1.0 g. per litre (see second five items in Table). All weights are expressed as g. per flask containing 350 ml. of medium.

Table 2

Incubation period days	Residual glucose by polarimeter %	Mycelium (dry wt.) g.	Total Cl in solution calc. as KCl g.	Cl in solution as inorganic chloride calc. as KCl g.	Cl in solution in organic combination calc. as KCl g.	Cl in solution in organic combination calc. as % of KCl originally present %
0	5.00	—	0.1750	0.1750	—	—
14	3.43	—	0.1731	0.0992	0.0739	42.2
18	2.47	3.78	0.1675	0.0627	0.1048	59.9
29	1.18	—	0.1498	0.0084	0.1414	80.8
37	1.62	—	0.1091	0.0082	0.1009	57.6
0	5.00	—	0.3500	0.3500	—	—
14	3.31	2.40	0.3215	0.2013	0.1202	34.3
29	1.08	6.10	0.2050	0.0522	0.1528	43.6
35	1.10	6.10	0.2610	0.0615	0.1995	57.0
42	1.28	5.30	0.2330	0.0746	0.1584	45.3

Investigation of the metabolic products of Caldariomyces fumago. One hundred 1-litre conical flasks, each containing 350 ml. of Czapek-Dox solution having preferably double the usual amount of KCl, i.e. 1 g. per litre instead of 0.5 g., were sterilized, sown with a spore suspension of *Caldariomyces fumago*, strain Ag 92, and incubated at 24° for about 35 days. The strongly acidic contents of the flasks (residual glucose, 1.3–1.5%) were filtered and the mycelium was washed and dried. The mycelium washings were added to the main filtrate, evaporated *in vacuo* at 40–45° to about 1 litre, and exhaustively extracted with ether. Estimation of the total Cl and Cl as residual KCl in solution showed that ether extraction removes the whole of the Cl present in organic combination.

The ether solution, dried over anhydrous MgSO_4 , was concentrated to a small volume and was then allowed to evaporate to dryness spontaneously in air. There remained a mixture of a yellowish-brown oil and colourless crystalline material. The pasty mass was pressed on a porous plate and the solid material remaining on the plate was boiled with chloroform (10 ml. per 1 g. of total solid) and filtered. On cooling the chloroform solution practically pure *Caldariomycin* separated as large colourless needles, M.P. 120°.

The solid material insoluble in chloroform proved to be fumaric acid. It was purified by sublimation in a high vacuum at 110–120° and was obtained as a colourless, microcrystalline sublimate, M.P. 290° (in a sealed tube), alone or in admixture with authentic fumaric acid. (Found: C, 41.41; H, 3.62; equiv. by titration, 59.1. $\text{C}_4\text{H}_4\text{O}_4$ requires C, 41.36; H, 3.47%; equiv., as a dibasic acid, 58.0.)

A little more caldariomycin was obtained by extraction with ether of the porous tile containing the yellowish-brown oil and repeating the process described above. An oil remained finally from which nothing crystalline has been separated but which contains considerable amounts of Cl in organic combination.

Since caldariomycin contains Cl the use of chloroform as a solvent is open to an obvious theoretical objection and it became necessary to prove that the Cl in caldariomycin does not arise from the solvent used. This was readily done by both the following methods. (a) In a second preparation a portion of the crude tiled mixture of metabolic products direct from the ether extraction and without treatment with chloroform was sublimed in a high vacuum at 60°. The colourless crystalline sublimate containing 40.7% Cl melted at 120–121° alone or in admixture with caldariomycin prepared by crystallization from chloroform. The residue in the sublimation tube consisted of crude fumaric acid. (b) A second portion was extracted with boiling light petroleum, B.P. 40–50°, 15 ml. per 1 g. Fumaric acid remained undissolved and caldariomycin separated from the filtered hot light petroleum solution on cooling, M.P. and mixed M.P. 121°. Chloroform is however a much better solvent for the crystallization of caldariomycin than is light petroleum.

Addition of 4 vol. of alcohol to the ether-extracted metabolism solution precipitated a crude polysaccharide which was dried by grinding with absolute alcohol and then with ether. There were thus obtained 285 g. of a greyish-brown powder (ash, 13.7%), obviously mainly polysaccharide in nature since it gives large amounts of reducing sugars on acid hydrolysis and is essentially the same polysaccharide as was isolated from this strain of *Caldariomyces fumago* by Birkinshaw & Raistrick [1931].

In three experiments with *Caldariomyces fumago*, strain Ag 92, in which the Czapek-Dox solution contained 0.5 g. KCl per litre in exp. 1 and 1.0 g. KCl per litre in exps. 2 and 3, the following yields of metabolic products were isolated.

	Pure caldariomycin g.	Fumaric acid g.	Uncrystallizable oil g.
Exp. 1	4.3	2.1	11.6
Exp. 2	6.1	2.5	14.4
Exp. 3	6.3	3.5	16.95

Since the uncrystallizable oil has a considerable Cl content it is obvious that a large proportion of the Cl metabolized by this strain is present as metabolic products which have not yet been characterized.

With *Caldariomyces fumago*, strain X. 13, 100 flasks of Czapek-Dox solution containing 0.5 g. of KCl per litre gave only 0.4 g. of pure caldariomycin, traces of fumaric acid and 15.6 g. of uncrystallizable oil.

A Cl balance sheet was prepared for *Caldariomyces fumago*, strain Ag 92, grown on Czapek-Dox solution containing 0.5 g. KCl per litre. In this experiment 73 flasks were incubated at 24° for 37 days. At the end of the incubation period the average residual glucose was 0.53%, the total mycelium weighed 373 g. and the mixed metabolism solution had the analysis given in Table 3. There were isolated from the evaporated mixed metabolism solution 3.10 g. of pure caldariomycin, 2.65 g. of fumaric acid and 7.35 g. of uncrystallizable oil. It will be seen from Table 3 that rather less than one-third of the organic Cl in solution (III) was recovered as pure caldariomycin (V).

It will be seen that the mycelium of *Caldariomyces fumago*, strain Ag 92, contains over 20% of the Cl originally present in the medium as KCl. All attempts to isolate any product from the dried mycelium which would account for this Cl-containing constituent have so far been unsuccessful. Extraction of the dried mycelium successively with light petroleum (B.P. 40–50°), ether and methanol removed about 13% of material which however did not contain any

Table 3

Fraction	Cl as KCl g.	Cl as % of KCl originally present
Original metabolism solution	12.775	100.0
(I) Total Cl in solution	9.7073	75.99
(II) Residual inorganic chloride	0.3087	2.42
(III) Organic Cl in solution	9.3987	73.57
(IV) Total Cl in mycelium	2.9280	22.92
(V) Cl in caldariomycin (3.1 g.)	2.705	21.17

Sum of (I) and (IV) equals 98.91 %.

Cl. Extraction with cold saturated NaHCO_3 solution removed about 35 % and with $N/10$ NaOH about 71 % of the total Cl in the mycelium, while boiling for 15 hr. with 33 % H_2SO_4 removed 85 %.

General properties of caldariomycin. Caldariomycin, $\text{C}_5\text{H}_8\text{O}_3\text{Cl}_2$, forms fine, colourless needles, m.p. 121° , without obvious decomposition. (Found: C, 35.19, 35.00; H, 4.73, 4.62; Cl, 41.2, 41.4 %; CH_3O , nil; N, nil; mol. wt., cryoscopic in dioxane, 158, cryoscopic in nitrobenzene, 197. $\text{C}_5\text{H}_8\text{O}_3\text{Cl}_2$ requires C, 35.09; H, 4.71; Cl, 41.5 %; mol. wt. 171.) Optical rotation $[\alpha]_{5461}^{20} +59.2^\circ$ ($c=0.338$ in water).

A Zerewitinoff determination carried out in anisole at room temperature indicated the presence of 1.94 active H atoms per mol.

A Kuhn-Roth chromic acid oxidation gave only 0.2 mol. of acetic acid per mol. of caldariomycin, thus indicating the absence of CH_3 side-chains.

Caldariomycin is readily soluble in cold water, ethanol, ether and in most other organic solvents with the exception of light petroleum and chloroform, in which however it is still appreciably soluble at 0° . Its aqueous solution is neutral in reaction, does not give any colour with FeCl_3 , or any reaction with Schiff's or Brady's reagents, nor does it decolorize aqueous KMnO_4 . It reduces Fehling's solution in the cold but only after standing for several minutes, owing probably to the formation in the strongly alkaline solution of some degradation product (see below). On warming solid caldariomycin with conc. H_2SO_4 a reddish-brown solution, with an intense green fluorescence, is obtained.

The two Cl atoms in its molecule are particularly labile. Although they are almost unaffected by treatment with cold saturated NaHCO_3 solution they are almost completely liberated as chloride on keeping a cold, dilute solution in $N/10$ NaOH overnight. This alkaline solution, after acidification, now gives a precipitate with Brady's reagent.

No crystalline product was obtained when caldariomycin was treated with phenyl isocyanate under the usual conditions for the formation of a urethane. Similarly all attempts to replace the Cl atoms by other groups failed to yield crystalline products. These included refluxing with a solution of silver acetate or potassium acetate in methanol (no action); treatment with KCN under conditions which convert 2-chlorocyclohexanone into 2-cyanocyclohexanone [Mayer, 1933]; and an attempt to condense caldariomycin with the sodium derivative of ethyl malonate.

Caldariomycin gives a good reddish purple colour with alcoholic *m*-dinitrobenzene and alcoholic KOH under the conditions for the modified Zimmermann reaction for active CH_2 groups laid down by Callow *et al.* [1938]. The following experiment shows however that it is not caldariomycin itself which contains the active CH_2 group but a product formed from it by the action of the KOH . An alcoholic solution of caldariomycin (1 ml. containing a few mg.) was mixed with

18% alcoholic KOH (4 drops) and left for 20 min. at room temperature; 2% alcoholic *m*-dinitrobenzene (1 ml.) was then added and the coloration became perceptible in 10 sec. and very intense in 1 min. In a control experiment, using the same alcoholic solution of caldariomycin (1 ml.) but adding the alcoholic KOH and *m*-dinitrobenzene together, the colour was only just perceptible in 1 min. and not very pronounced after 3 min.

Action of heat on caldariomycin. Caldariomycin (3 g.) was heated in a slow stream of N_2 in a boiling tube connected to two bubblers containing a solution of 2:4-dinitrophenylhydrazine in 2*N* HCl. Ebullition of the melt began at 185° and proceeded vigorously at 195–200° at which temperature the oil bath was maintained for 30 min. when ebullition ceased. Water and streams of HCl gas were evolved and the bulk of the caldariomycin was converted into a black, non-volatile, viscous resin. A small amount of a colourless, mobile liquid passed over into the bubblers and gave an immediate orange-red precipitate in the Brady's solution. This precipitate was collected at once (0.10 g.) and the filtrate, on standing overnight, deposited a further amount of a very similar product (0.20 g.). By fractional crystallization from ethanol two dinitrophenylhydrazones were isolated. The less sparingly soluble one consisted of light orange-red rods, M.P. 238° (decomp.), whilst the more sparingly soluble constituent formed very dark red irregular crystalline aggregates, M.P. 224° (decomp.). (Found: (a) On the dinitrophenylhydrazone, M.P. 238°, C, 44.86; H, 3.22; Cl, 11.1%. (b) On the dinitrophenylhydrazone, M.P. 224°, C, 44.98, 45.07; H, 3.22, 3.27; N, 18.7; Cl, 10.8%. $C_{11}H_9O_4N_4Cl$, i.e. the mono-dinitrophenylhydrazone of C_5H_5OCl , requires C, 44.51; H, 3.06; N, 18.9; Cl, 12.0%.) The two compounds are therefore isomeric and since they both gave a red coloration with alcoholic NaOH they are both mono-dinitrophenylhydrazones.

The mono-dinitrophenylhydrazone, M.P. 224°, was also isolated in small yield from the products of the acid hydrolysis of caldariomycin. A solution of caldariomycin (0.5 g.) in 2*N* H_2SO_4 (50 ml.) was boiled for 2 hr. in an atmosphere of N_2 . At the end of this time 15.3% of the Cl in the caldariomycin had been converted into HCl. There was isolated from the hydrolysis products a small amount of an orange dinitrophenylhydrazone which was purified by crystallization, first from ethanol, then from xylene and finally from a large volume of ethanol. It formed small red needles, M.P. 226° (decomp.), which gave the red colour with alcoholic NaOH typical of a mono-dinitrophenylhydrazone. (Found: C, 44.23, 44.28; H, 2.97, 3.20; N, 19.6; Cl, 10.4%. $C_{11}H_9O_4N_4Cl$ requires C, 44.51; H, 3.06; N, 18.9; Cl, 12.0%.)

Proof that caldariomycin does not contain the grouping $\text{>C} \cdot (\text{OH}) \cdot \text{C} \cdot (\text{OH}) \text{<}$. Malaprade [1928] and Fleury & Lange [1932; 1933] have shown that polyhydric alcohols containing hydroxyl groups attached to adjacent carbon atoms react with periodic acid with the formation of aldehydes. Malaprade [1934] and Clutterbuck & Reuter [1935] have also shown that α -ketols, α -diketones and α -ketonealdehydes are readily oxidized by this reagent. Since caldariomycin is unaffected by HIO_4 while the two forms of cyclopentane-1:2-diol are both oxidized it is clear that caldariomycin does not contain the grouping

(a) A solution of caldariomycin (0.1122 g.) in 0.3% KIO_4 solution (100 ml.) was acidified with 2*N* H_2SO_4 (10 ml.) and kept overnight side by side with a blank experiment. Excess KI was then added to each flask and the liberated iodine was titrated with *N*/10 $Na_2S_2O_3$. The experimental flask required 106.7 ml. and the blank 106.8 ml. thus proving that no oxidation had taken place.

(b) The lower-melting *cyclopentane-1:2-diol* (m.p. 10°; 0.234 g. [Godehot & Taboury, 1912, 1913]) when oxidized as above utilized oxygen equivalent to 18.4 ml. *N*/10 iodine which corresponds to about 40 % of complete oxidation of .CHOH.CHOH. to .CHO + .CHO.

(c) The higher-melting *cyclopentane-1:2-diol* (m.p. 46–48°; 0.117 g. [Meiser, 1899]) when oxidized similarly utilized oxygen equivalent to 22.4 ml. *N*/10 iodine corresponding to 97.7 % complete oxidation.

Oxidation of caldariomycin with chromic acid. Formation of succinic acid. To a solution of caldariomycin (0.344 g.) in 2*N* H₂SO₄ (30 ml.) was added a solution of CrO₃ (0.54 g.) in 2*N* H₂SO₄ (20 ml.). The mixture was kept at room temperature for 7 days and was then extracted with ether. The ether extract was evaporated to dryness, the residue (0.225 g.) titrated with *N*/10 NaOH to phenolphthalein and re-extracted with ether to remove some unchanged caldariomycin. The aqueous layer was then acidified, extracted with ether, the solvent removed and the crystalline residue was purified by sublimation in a high vacuum to give a colourless crystalline sublimate (0.1 g.) of succinic acid, m.p. 186°, alone or in admixture with an authentic specimen of succinic acid. (Found: equiv. by titration, 64.0. C₄H₆O₄, titrating as a dibasic acid requires equiv. 59.0.)

Catalytic reduction of caldariomycin. Formation of cyclopentanone. When caldariomycin is reduced with hydrogen, using a palladium-charcoal catalyst, uptake of H₂ is slow throughout the reduction which appears to indicate the absence of a double bond from caldariomycin. Cl is removed from the molecule and appears as HCl in the solution.

Caldariomycin (1.0 g.) dissolved in water (50 ml.) was shaken for 8 hr. with a catalyst prepared from PdCl₂ (1 g.) and norite (3 g.) in an atmosphere of H₂ until no further absorption of H₂ took place. The volume of H₂ absorbed was about 80 % of that required by the equation,

Excess of Brady's reagent was added to the filtered liquid and the crude Cl-free dinitrophenylhydrazone which was precipitated was collected after several hours, washed and dried (0.25 g., m.p. about 140°). It formed long orange needles from ethanol, m.p. 144°, alone or in admixture with an authentic specimen of *cyclopentanone dinitrophenylhydrazone* (m.p. 144°; lit. 142°) prepared from *cyclopentanone* [Gilman, 1932] and recrystallized similarly. The two specimens had the same crystalline form, and both gave with alcoholic NaOH the same red colour typical of a mono-dinitrophenylhydrazone. (Found: on dinitrophenylhydrazone of reduction product, C, 50.25; H, 4.39; N, 22.4 %. C₁₁H₁₂O₄N₄, i.e. the mono-dinitrophenylhydrazone of C₅H₈O, requires C, 49.98; H, 4.58; N, 21.2 %.)

The yield of *cyclopentanone dinitrophenylhydrazone* obtained obviously accounts for only a fraction of the caldariomycin used. In a second experiment 4 g. of caldariomycin were reduced; the mixture was filtered, neutralized with *N* NaOH and evaporated to dryness under reduced pressure at 40°. 1.12 g. of *cyclopentanone dinitrophenylhydrazone*, m.p. 140–142°, were obtained from the distillate after treatment with excess of Brady's reagent. The residue remaining after evaporation was extracted with methanol. On removal of the solvent there remained a solid mixed with some oily material from which the only pure product that could be isolated was unchanged caldariomycin.

Since neither of the *cyclopentane-1:2-diols* (see above) gives an appreciable precipitate with Brady's reagent, even on long standing, and since neither of

them would distil under the conditions described, it is clear that the primary reduction product of caldariomycin cannot be a *cyclopentane-1:2-diol*. It appears therefore that *cyclopentanone* is itself a primary reduction product of caldariomycin and does not arise by dehydration of some intermediate dihydroxy compound through the action of the HCl in Brady's reagent.

Br metabolism of Caldariomyces fumago, strain Ag 92. When *Caldariomyces fumago*, strain Ag 92, was sown on the ordinary Czapek-Dox glucose solution containing equivalent amounts of KBr or KI instead of the usual 0.5 g. per litre of KCl no growth was obtained on the KI medium though fairly normal growth resulted on the KBr medium with the formation of metabolic products containing bromine in organic combination which, however, have not yet been characterized.

100 flasks each containing 350 ml. of Czapek-Dox 5% glucose solution (see p. 667) with 29 g. of KBr per 35 litres in place of 17.5 g. KCl were inoculated with *Caldariomyces fumago*, strain Ag 92, and incubated at 24°. At intervals single flasks were selected and the following estimations were carried out on each flask. (a) Residual glucose by polarimeter. (b) Total Br in solution. (c) Br in solution as inorganic bromide. (d) Br in solution in organic combination, i.e. (b)–(c). (e) Weight of mycelium. The results obtained are given in Table 4 in which all weights are expressed as g. per flask. Growth was very slow during the first 7 days but after about 10 days a thin mycelial felt was established and growth then became almost normal with the ultimate formation of a thick brownish-black compact felt. The metabolism solution gradually became reddish brown in colour and viscous owing to the formation of a polysaccharide which interfered with the Br estimations. This difficulty was overcome by precipitating the polysaccharide with 4 vol. of alcohol. The Br in solution was then estimated by the usual volumetric and gravimetric methods on the polysaccharide-free solution after removal of the alcohol.

Table 4

Incubation period days	Residual glucose by polarimeter %	Mycelium (dry wt.) g.	Total Br in solution calc. as KBr g.	Br in solution as inorganic bromide calc. as KBr g.	Br in solution in organic combination calc. as KBr g.	Br in solution in organic combination calc. as % of KBr originally present %
0	5.00	—	0.290	0.290	—	—
14	4.83	0.62	0.290	0.244	0.046	15.9
21	4.36	—	0.279	0.200	0.079	27.2
28	3.00	3.75	0.248	0.163	0.085	29.3
35	2.70	4.75	0.279	0.149	0.130	44.8
38	1.02	3.77*	0.244	0.136	0.108	37.2
(average of 96 flasks)						

* The Br content of the mycelium = 0.026 g. KBr per flask.

After 38 days' incubation the metabolism solution from the 96 flasks remaining was filtered, evaporated *in vacuo* at 40° to about 1 litre and then exhaustively extracted with ether. The reddish-brown ethereal extract, on removal of the solvent, gave 11 g. of a dark reddish-brown oil containing a considerable proportion of Br. So far the only pure substance isolated from this oil is fumaric acid. The investigation of this somewhat intractable material is being continued in the hope of isolating from it in a state of purity the Br-containing metabolic product which is obviously present.

SUMMARY

A quantitative survey has been made of the chlorine metabolism of 139 species or strains of moulds grown on Czapek-Dox 5% glucose solution containing 0.5 g. per litre of KCl as the sole source of chlorine. Extensive conversion of inorganic chloride into organic chlorine-containing metabolic products is of rather rare occurrence, though with a number of species this conversion is by no means negligible. The chlorine metabolism of one of the species which is most active in this respect—*Caldariomyces fumago* Woronichin—has been investigated in detail and has resulted in the isolation of a hitherto undescribed mould metabolic product, *caldariomycin*, $C_5H_8O_2Cl_2$, which has been shown to be probably 2:2-dichlorocyclopentane-1:3-diol. The same species also converts KBr, when present in the same medium in place of KCl, into organic bromine-containing metabolic products which have not yet been isolated in a state of purity.

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