Studies in the Biochemistry of Micro-organisms

73. METABOLIC PRODUCTS OF *ASPERGILLUS FUMIGATUS* FRESENIUS

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The mould *Aspergillus fumigatus* Fresenius affords several crystalline metabolic products, no less than three of which possess high antibacterial activity. Anslow & Raistrick (1938*a*) reported the isolation, from cultures of a strain of the organism grown on Raulin-Thom medium at 24°, of fumigatin, which they showed to be 3-hydroxy-4-methoxy-2:5-toluquinone. Another strain of the same organism when grown under the same cultural conditions produced spinulosin, i.e. 6-hydroxy-fumigatin (Anslow & Raistrick, 1938*b*), previously reported (Birkinshaw & Raistrick, 1931) as a metabolic product of *Penicillium spinulosum* Thom. The activity (limiting dilution) of fumigatin against *Staph. aureus* was found by Oxford & Raistrick (1942) to be 33,000–100,000, whereas that of spinulosin was <6000–17,000.

Chain, Florey, Jennings & Williams (1943) described the isolation from culture filtrates of *Aspergillus fumigatus* mut. *helvola* Yuill of an antibiotic substance, helvolic acid, with an activity (limiting dilution, *Staph. aureus*) of 80,000–1,280,000. Waksman, Horning & Spencer (1943) had previously reported the isolation from culture filtrates of *Aspergillus fumigatus* of a new antibiotic agent, fumigacin, which has now been shown by Menzel, Wintersteiner & Hoogerheide (1944) to consist of a mixture of helvolic acid and gliotoxin. Gliotoxin, which had already been isolated from culture filtrates of *Gliocladium fimbriatum* Gilman & Abbott by Weindling & Emerson (1936) and Weindling (1937), has an antibacterial activity (limiting dilution, *Staph. aureus*) of 1,000,000 (Dutcher, 1941).

In a systematic investigation in this laboratory of the production of antibiotics by moulds, a freshly isolated strain of *Aspergillus fumigatus*, which showed considerable antibacterial activity, was examined. Chloroform extraction of the acidified culture filtrates gave a highly active extract from which a crystalline substance was isolated, agreeing in most of its properties with helvolic acid. The only marked deviation was in the optical rotation. The value of $[\alpha]_D^{20}$ for our product was -138° (chloroform solvent for all these rotations), whereas for helvolic acid the value $[\alpha]_D^{20} = -49.4^\circ$ is recorded (Chain *et al.* 1943). However, the value obtained by

Menzel *et al.* (1944) for pure fumigacin, which is regarded as identical with helvolic acid, was $[\alpha]_D^{20} = -132^\circ \pm 2^\circ$, in good agreement with our own figure. Hence our product is identified as helvolic acid. On the other hand, our combustion values support the formula $C_{32}H_{42}(44)O_8$ assigned to helvolic acid by Chain *et al.* (1943), which is based mainly on crystallographic X-ray data (Crowfoot & Low, 1943), and we consider that the C_{30} formula put forward by Menzel *et al.* is less probable.

The greater part of the chloroform extract, although possessing antibacterial activity at least as high as helvolic acid, could not be induced to crystallize. It contained N and S, and in view of the fact that Menzel *et al.* obtained and identified gliotoxin from their culture filtrate, although they were unable to isolate the gliotoxin moiety from Waksman's (impure) fumigacin in crystalline form, it seems probable that our fraction containing N and S consisted in the main of gliotoxin or closely related products of similar antibacterial activity.

From the barium salts obtainable on evaporation of our chloroform-extracted culture filtrate we succeeded in isolating small amounts of oxalic acid and larger amounts of another acid identified as the *laevo* rotatory form of ethyleneoxide- α -dicarboxylic acid. This acid has been previously recorded as a mould product, Sakaguchi, Inoue & Tada (1939) having isolated it from the culture fluids of *Monilia formosa* n.sp. and *Penicillium viniferum* n.sp. Although this product possesses no antibacterial activity it is of considerable interest as an addition to the list of metabolic products of *Aspergillus fumigatus* and may eventually throw some light on the complex metabolic processes of that organism.

EXPERIMENTAL

Culture. The strain of *Aspergillus fumigatus* Fresenius M400 employed was isolated from Beaconsfield soil and was identified in this laboratory.

Cultural conditions. The culture medium used was a Czapek-Dox solution of the following composition: glucose, 50 g.; $NaNO_3$, 2.0 g.; KH_2PO_4 , 1.0 g.; KCl, 0.5 g.; $MgSO_4 \cdot 7H_2O$, 0.5 g.; $FeSO_4 \cdot 7H_2O$, 0.01 g.; distilled water, 1000 ml.; initial pH, 4.3. The medium was distributed in 350 ml. amounts in 1 l. conical flasks plugged with cotton-wool and

sterilized by autoclaving. After sterilization the flasks were sown with a spore suspension of *A. fumigatus* M400 prepared from cultures grown for 12–14 days on Czapek-Dox agar, and were then incubated at 24°.

At 7 days the mycelium was well grown, thin, smooth, and friable with a dark green upper surface, the reverse surface being deep brownish green; the culture fluid was a pale lemon-yellow. The colour of the solution deepens with age, and the surface of the mycelium becomes mottled with patches of white mycelium. The pH of the solution rises during the early stages and falls again with prolonged incubation, the pH values at 7, 14 and 21 days being respectively 6.5, 7.2 and 4.5. Even after 21 days there was still 2% of glucose in the culture solution.

Isolation and purification of products

The cultures were harvested after 17–19 days' incubation, when the sugar content of the solution had fallen to about half its initial value and the solution completely inhibited *Staph. aureus* at a dilution of 1 in 40 to 1 in 80. As the behaviour of all batches was similar the method adopted and the results obtained for one representative batch will be described.

The culture fluid from 50 flasks was filtered from the mycelium and collected after 17 days' incubation. It had the following characteristics: volume 16.15 l., pH 7.2, glucose (by polarimeter) 2.6%, permitted no growth of *Staph. aureus* in 24 hr. at a dilution of 1 in 40, only a trace at 1 in 80 when tested by the dilution method.

The solution was acidified to pH 3.6 by the addition of 26.4 ml. of conc. HCl, and extracted twice with 2800 ml. of chloroform. It was necessary to centrifuge the mixture to effect complete separation since a small amount of dark-coloured solid which separated seemed to promote emulsification. All the antibacterial substance was removed by this extraction since the extracted liquor after neutralization permitted growth of the test organism at 1 in 2. The chloroform extract was evaporated *in vacuo*, affording a yellowish friable solidified gum of weight 3.97 g. with antibacterial activity of 1 in 80,000.

Examination of chloroform extract. Isolation of helvolic acid. The crude chloroform extract dissolved in 400 ml. of chloroform to give a brownish red solution. This was passed through a column of acid-washed Al_2O_3 and washed through with a further 400 ml. of chloroform. Little adsorption occurred; the resulting solution was, however, pure yellow, with no red tinge. On removal of solvent *in vacuo*, 3.04 g. of a yellow friable dried froth were obtained of activity 1 in 160,000. This was dissolved in 100 ml. of ethanol at 30–40° and treated with 100 ml. of water at the same temperature. On cooling, 0.12 g. of colourless crystals (needles) was obtained of m.p. 218°. By two recrystallizations from ethanol the melting-point was raised to 226° (temperature taken up quickly from bath at 210°) or 220° (temperature raised slowly from room temperature). Chain *et al.* (1943) record the melting-point of helvolic acid as 212°; Menzel *et al.* (1944) give 215–220°. The substance had an antibacterial activity (*Staph. aureus*) of 1 in 160,000 and

agreed in properties (except optical rotation) with helvolic acid as described by Chain *et al.* (1943). (Found, after drying at 100° in high vacuum: C, 69.20; H, 7.85%; mol. wt. (Rast) 533. $\text{C}_{32}\text{H}_{44}\text{O}_8$ requires C, 69.02; H, 7.97%; mol. wt. 556.7. $\text{C}_{32}\text{H}_{44}\text{O}_8$ requires C, 69.28; H, 7.63%; mol. wt. 554.7.) $[\alpha]_{5461}^{18} = -167^\circ$ in chloroform ($c = 2.37\%$ w/w). $[\alpha]_{5790}^{18} = -138^\circ$ in chloroform ($c = 2.37\%$ w/w). This value for $[\alpha]$ differs from that recorded by Chain *et al.* (1943), but is in fair agreement with the figure of Menzel *et al.* (1944).

For further confirmation the methyl derivative was prepared by means of diazomethane. The substance dissolved in CHCl_3 was treated with an excess of diazomethane in ether, and after 4 hr. the solvent was removed in vacuum. After recrystallization from methanol the product had m.p. 262°. The melting-point figures recorded by Chain *et al.* (1943) and Menzel *et al.* (1944) are respectively 261 and 260–261°. (Found, after drying at 100° in high vacuum: C, 70.18; H, 8.11; OCH_3 , 6.5%. $\text{C}_{33}\text{H}_{46}\text{O}_8$ requires C, 69.44; H, 8.13; OCH_3 , 5.44%. $\text{C}_{33}\text{H}_{44}\text{O}_8$ requires C, 69.69; H, 7.80; OCH_3 , 5.46%.)

The chloroform-soluble residue, after crystallization of the helvolic acid, consisted of non-crystalline material of high antibacterial titre (1 in 160,000). All attempts to induce crystallization failed, but in view of the fact that it contained N and S and of the findings of Menzel *et al.* (1944), who were able to isolate gliotoxin from short-period cultures of *Aspergillus fumigatus*, it is probable that the material contained gliotoxin or closely related products possessing the activity of gliotoxin.

Treatment of the chloroform-extracted liquor. Isolation of ethylene oxide- $\alpha\beta$ -dicarboxylic acid and oxalic acid. The chloroform-extracted culture filtrate was restored to approximate neutrality by the addition of 40 ml. of 20% NaOH giving a pH of 6.9. The solution was treated with 50 g. of barium acetate dissolved in 200 ml. of water. The slight precipitate obtained on keeping overnight was filtered off (wt. 1.75 g.).

The solution was evaporated *in vacuo*, the precipitate of the barium salts which was produced being removed from time to time. When evaporation was stopped the volume of the concentrate and washings was 650 ml. The barium salts weighed 41.5 g. The filtrate gave no precipitate with barium acetate.

On treating the barium salts with 100 ml. (i.e. an excess) of 2N-HCl, the bulk, but not all, dissolved. The solution with the undissolved solid was now extracted with ether in a continuous extractor. The partly crystalline residue, after removal of the ether, was collected in several fractions. The crystals were filtered off and well washed with ether. The total weight of crystals obtained was about 9 g., the m.p. ranged from 176° for the first fraction to about 140–150° for the latest fraction. The later fractions

and the mother liquor were found to contain oxalic acid. This was removed by dissolving in water, neutralizing, and adding a solution of calcium acetate. The calcium oxalate was filtered off and the oxalic acid isolated and positively identified by melting-point and mixed melting-point determination.

The filtrate from the calcium oxalate was re-acidified and extracted, yielding further crystals. The main crystalline product was recrystallized from ethyl acetate-petrol; melting-point thus raised to 186° (slight decomposition), colourless bipyramidal crystals, yield 4.1 g. (Found on sublimed sample C, 36.10; H, 3.07%. N, S, halogen, absent. Mol. wt. (Rast), 143, 135. Equivalent by titration 67.2. $C_4H_4O_6$ requires C, 36.38; H, 3.05%; mol. wt. 132; equivalent (dibasic), 66.0.) The acid was optically active, $[\alpha]_{5461}^{18} = -119.3^\circ$, $[\alpha]_{5790}^{18} = -105.1^\circ$ in 10% aqueous solution. It formed a sparingly soluble crystalline barium salt and had similar properties to the ethylene oxide- $\alpha\beta$ -dicarboxylic acid isolated by Sakaguchi *et al.* (1939) from the metabolic products of *Monilia formosa* n.sp. and *Penicillium viviferum* n.sp. This acid had $[\alpha]_D^{20} = -100.6^\circ$. Our melting-point is somewhat higher than that re-

corded by Sakaguchi *et al.* (1939) and by Kuhn & Zell (1937), which was 180°, but this may depend on the rate of heating.

For further confirmation our acid was converted into the *l*-rotatory chloromalic acid by means of HCl in ether. After crystallization from ether-light petroleum the product had m.p. 173° but showed definite signs of softening at 166°. Sakaguchi *et al.* and Kuhn & Zell give the melting-point of the chloro acid as 166°. (Found: Cl, 20.91%; $C_4H_5O_5Cl$ requires C, 21.04%.) The optical rotation was determined in ethyl acetate. Found: $[\alpha]_{5461}^{18} = -36.4^\circ$, $[\alpha]_{5790}^{18} = -31.6^\circ$ ($c = 0.92\%$ by wt.). Sakaguchi *et al.* record the rotation as $[\alpha]_D^{25} = -31.5^\circ$ in ethyl acetate.

SUMMARY

1. The metabolic products of an authentic strain of *Aspergillus fumigatus* Fresenius, grown on Czapek-Dox glucose solution, were investigated.

2. The antibiotic, helvolic acid, was isolated, together with considerable amounts of ethylene oxide- $\alpha\beta$ -dicarboxylic acid and small amounts of oxalic acid.

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The Quantitative Estimation in Urine of a Base Closely Allied to *l*-Nicotine, and the Absence of Alteration in Output when the Diet is Changed

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In a previous paper (Lockett, 1944*b*), a base was isolated from human urine, both of smokers and non-smokers, and was identified in final form as *l*-nicotine. Evidence was presented that the original unstable form of the base differed from *l*-nicotine. A similar base was detected in the urine of dogs;

solutions of this base and of *l*-nicotine, both as the hydrochlorides, which gave identical ultra-violet absorption spectra, were found to have identical pressor activity when injected intravenously into a cat under chloralose anaesthesia. No evidence as to the physiological significance of this base has yet