ANTIBIOTICS FROM PENICILLIA.

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MANY penicillia, aspergilli and basidiomycetes have been shown to produce antibacterial substances when grown under suitable conditions. A summary of the information produced by surveys in this field will shortly be published (Florey, Chain, Heatley, Jennings, Sanders, Abraham, Florey, 1949). This present paper records some results of a systematic attempt to fill in gaps in our knowledge of antibiotics known to be produced by fungi, but so far not further investigated. It is interesting in this respect to note that a high proportion of the penicillia examined produced already well known substances.

EXPERIMENTAL.

General conditions of culture.

All the penicillia investigated were grown in glass vessels on 500 ml. of medium 2 cm. in depth. Incubation was carried out at 24° C., the activity developing during culture being followed by the cylinder plate method of assay (Heatley, 1944).

Phoenicin from Penicillium rubrum.

The culture fluid of *P. rubrum* was reported to inhibit the growth of *Staph. aureus* by Miss M. E. Johns.

Conditions of culture.

The organism was grown on glucose Sabouraud medium containing 1 per cent peptone, 2·6 per cent malt extract and 4 per cent glucose for eight days.

Isolation.

The culture fluid at the time of harvesting was a reddish-purple colour. On acidification this colour disappeared, and the active principle was completely extracted from the acid solution into chloroform by shaking successively with one-half, one-quarter and one-eighth volumes of the solvent. The yellow chloroform solution was shaken three times with one-tenth of its volume of sodium bicarbonate solution, when the active principle passed to the aqueous phase, which became deep violet in colour.

The extraction into chloroform and re-extraction with sodium bicarbonate was repeated, using smaller volumes of solvent, in order to effect some purification. Final purification was effected by alumina chromatography. The final chloroform solution was evaporated, and the resulting yellow-brown crystalline material was recrystallized from ethanol.

Chemical properties.

The active substance crystallized in plates, m.p. 229–230° C. Found: C 58·61, H 4·17, CH_3(C), 9·7 per cent, active H 1·03 per cent. $C_{14}H_{10}O_6$ requires: C 60·5, H 4·02, CH_3(C) 10·9 per cent. It was slightly soluble in water, but soluble in chloroform, acetic acid and warm ethanol.

In aqueous solution it liberated iodine from acid potassium iodide, and it was reduced to a colourless compound by sodium hydrosulphite.

The substance appeared to be identical with the diquinone phoenicin, a compound isolated from *P. phoenicium* v. Beyma by Friedheim (1938) and examined by Posternak (1938). Phoenicin was reported to have the molecular formula $C_{14}H_{10}O_6$ and to melt at 230° C.

Antagonistic properties.

The crude culture fluid showed activity against *Staph. aureus*, *C. xerosis* and *Myco. phlei.*

Only a few milligrammes of the antibiotic were available for examination. At a dilution of 1 : 1000 it gave a zone of inhibition 22 mm. in diameter when tested by the cylinder plate method against *Staph. aureus.*

Expansine from Penicillium equinum.

The culture fluid of *P. equinum* was reported to inhibit the growth of *Staph. aureus* and *Bact. coli* by Miss M. E. Johns.

Conditions of culture.

The fungus was grown on modified Czapek-Dox medium containing 0·3 per cent sodium nitrate, 0·1 per cent potassium dihydrogen phosphate, 0·05 per cent potassium chloride, 0·05 per cent magnesium sulphate $MgSO_4 \cdot 7H_2O$, 0·001 per cent of hydrated ferrous sulphate $FeSO_4 \cdot 7H_2O$ and 4 per cent of glucose. Activity was maximal after about 10–15 days.

Isolation.

The culture fluid was shaken with $2\frac{1}{4}$ per cent acid charcoal and the active principle eluted from the latter with acetone. After distilling off most of the acetone the remaining fluid was passed through a short alumina column (pH 5), which removed much of the pigment but none of the activity. The antibiotic was extracted from the percolate with ether, and on evaporation of the ether an impure crystalline mass remained which was recrystallized from hot benzene or chloroform, giving pure colourless crystals.

Chemical properties.

After two recrystallizations the crystals melted at 111° C. On admixture with a sample of expansine (m.p. 111° C.) the m.p. of the mixture was 111° C.

When a solution of the crystalline material was warmed with 0·1 N sodium hydroxide solution a bright yellow colour developed. After neutralizing the alkaline solution the addition of aqueous ferrous chloride gave a deep purple-red coloration. The original aqueous solution gave no colour with ferric chloride solution.

The substance gave a yellow precipitate with 2:4-dinitrophenylhydrazine in aqueous 2-N-hydrochloric acid which after recrystallization from methyl alcohol melted at 216° C. (decomp.). The corresponding product from an authentic sample of expansine melted at 216° C. The substance gave an orange precipitate with phenylhydrazine hydrochloride which after recrystallization from hot water melted at 152° C. The corresponding product from expansine had the same melting point (Raistrick, Birkinshaw, Bracken and Michael, 1943).

Expansine from Penicillium novae zeelandiae.

The culture fluid of *P. novae zeelandiae* was reported to inhibit the growth of *Staph. aureus* and *Bact. coli* by Miss M. E. Johns.

Conditions of culture.

The fungus was grown on glucose Sabouraud medium containing 1 per cent peptone, 2·4 per cent malt extract and 4 per cent glucose. Activity was maximal at about the tenth day.

Isolation.

The active principle was removed from the culture fluid at pH 4 by continuous extraction with ether. On evaporation of the ether impure crystals were obtained. These were purified by recrystallization from hot benzene.

Chemical properties.

The substance melted at 110° C. The mixed melting point with a sample of expansine of m.p. 111° C. was 110°C. On warming a solution of the substance with 0·1 N sodium hydroxide solution a bright yellow colour developed. After neutralizing the alkaline solution addition of aqueous ferric chloride gave a deep purple-red coloration. The original aqueous solution gave no colour with ferric chloride solution. With phenyl-hydrazine hydrochloride the substance gave an orange precipitate, which after crystallization from hot water melted at 152° C. With respect to all these properties the substance was identical with expansine.

Mycophenolic Acid from Penicillium viridicatum.

The culture fluid of *P. viridicatum* was reported by Miss M. E. Johns to inhibit the growth of *Staph. aureus*.

Conditions of culture.

The fungus was grown on the modified Czapek-Dox medium used for *P. equinum* containing 5 per cent neutralized corn steep liquor. Activity was maximal at about the seventh to the tenth day.

Isolation.

The active substance was removed from the acidified culture fluid by continuous extraction with ether. The brown gum which remained on evaporating the ether was dissolved in ethanol, and the potassium salt of the substance was precipitated by the addition of ethanolic potassium hydroxide. After centrifugation the precipitate was washed with ethanol, dissolved in water, and the free acid then precipitated by the addition of 2 N sulphuric acid. The precipitate was recrystallized from boiling water, giving small needle crystals.

Chemical properties.

The compound melted at 141° C. On admixture with an authentic sample of mycophenolic acid no depression in melting point was observed.

The acetyl derivative was formed when 0·25 g. substance was refluxed in an oil-bath at 120° C. with 2 g. acetic anhydride and 1 g. anhydrous sodium acetate. The mixture was poured into water, when the derivative formed an insoluble precipitate which was recrystallized from glacial acetic acid. M.p. 161° C. Mixed melting point with authentic acetyl mycophenolic acid 161° C. The compound gave a blue violet colour with ferric chloride in aqueous solution and a blue colour in alcohol.

Mycophenolic Acid from Penicillium bialowiezense.

The culture fluid of *P. bialowiezense* was reported by Miss M. E. Johns to inhibit the growth of *Staph. aureus*.

Conditions of culture.

The fungus was grown on glucose Sabouraud medium containing 1 per cent peptone, 2·6 per cent malt extract and 4 per cent. glucose. Activity was maximal between the twentieth and twenty-fifth days, when the culture fluid was harvested.

Isolation.

The culture fluid contained a penicillin-like antibiotic which was destroyed by incubation of the culture fluid for two hours at 37° C. with penicillinase. The presence of this substance, presumably a penicillin, was responsible for about a third of the total activity. After incubation with penicillinase the culture fluid was extracted with ether at pH 3, which removed the residual active substance. The ethereal solution was concentrated and washed three times with one twentieth of its volume of saturated sodium bicarbonate solution. The main bulk of the active principle was extracted into 0·5 N sodium hydroxide solution. On addition

of hydrochloric acid to the resultant aqueous solution a white precipitate formed, which crystallized readily on scratching the vessel with a glass rod. The substance was recrystallized from hot benzene, hot water, or preferably from 4 per cent aqueous alcohol, when small colourless needles were obtained.

Chemical properties.

The compound melted at 141° C., and on admixture with an authentic sample of mycophenolic acid no depression of m.p. was observed. Found: C 63·7, 64·0, H 6·04, 6·6, CH_3(C) 7·1 per cent, OCH_3 7·95 per cent. Active H 0·46 per cent, M.W. (Camphor) 354, 359, 360, (Barger) 314. $C_{17}H_{20}O_6$ requires: C 63·7, H 6·3, M.W. 320.

The acetyl derivative was prepared as in the previous example and melted at 161° C. Upon admixture with an authentic sample of acetyl mycophenolic acid no depression in m.p. was observed. Found: C 62·5, H 6·13. $C_{19}H_{23}O_7$ requires: C 62·4, H 6·07. Barer, Cole and Thompson (1949) reported that the infra-red spectrum of the antibiotic isolated was identical with that of the authentic sample of mycophenolic acid.

Penicillic Acid from Penicillium baarnense.

The culture fluid of *P. baarnense* was reported by Miss M. E. Johns to inhibit the growth of *Staph. aureus*.

Conditions of culture.

The fungus was grown on a modified Czapek-Dox medium, as used for *P. equinum*, together with 5 per cent neutralized corn steep liquor. Activity was maximal in 10 to 15 days.

Isolation.

Incubation with penicillinase destroyed about one-third of the total activity of the culture fluid. The activity of the residual material was not appreciably diminished in the presence of catalase.

After incubation with penicillinase, continuous extraction of the culture fluid at pH 2 with ether and evaporation of the ether yielded an oily material from which impure orange-coloured crystals were obtained.

The material was passed through an acid-washed alumina column (pH 5), when an active fraction was obtained which yielded almost colourless crystals.

Chemical properties.

The crystalline material was recrystallized from hot water, yielding crystals melting at 65° C. After recrystallization from petroleum ether the substance melted at 84° C. It was found that specimens of penicillic acid from *P. baarnense*, *A. quercinus* and *A. melleus*, partly melted at 38° C. when crystallized from water, ethanol or ether. The low-melting compound was a hydrate which was dehydrated in a vacuum desiccator over phosphorus pentoxide to give the non-hydrated penicillic acid m.p. 84° C. Found: C 56·4, H 6·4. $C_8H_{10}O_4$ requires: C 56·4 per cent, H 5·89 per cent. With ammonium hydroxide

a marked pink colour characteristic of penicillic acid developed, and upon admixture of the crystals with an authentic sample of penicillic acid no depression in melting point occurred.

With phenylhydrazine the substance gave a yellow crystalline derivative, m.p. 171° C.

The corresponding derivative from penicillic acid has been reported to melt at 171° C. and 175° C. (Alsberg and Black, 1913; Birkinshaw, Oxford and Raistrick, 1936).

Herquein from Penicillium herquei.

The culture fluid of *P. herquei* was reported by Miss M. E. Johns to inhibit the growth of *Staph. aureus*. The antibacterial substance in the fluid has been isolated and named herquein.

Conditions of culture.

The fungus was grown on a modified Czapek-Dox medium, as used for *P. equinum*.

Activity appeared between the 8th and 15th days and disappeared within 24 h urs. Frequent examination of the culture fluid and rapid extraction of the active principle when produced were essential for the successful isolation of the antibiotic.

Isolation.

The culture fluid was acidified to pH 2 when a precipitate formed carrying with it all the activity. The precipitate was centrifuged, suspended in water and extracted with chloroform. The active principle was extracted from the chloroform into aqueous solution at pH 7·5, and was then precipitated as a yellow microcrystalline solid by addition of hydrochloric acid. It was recrystallized from aqueous alcohol or benzene.

Chemical properties.

Herquein forms yellowish-brown crystals, m.p. 129° C. (decomp.). Found: C 59·84, H 5·42, OCH_3 5·33, $CH_3(C)$ 10·5 per cent. $C_{19}H_{20}O_8$ requires: C 60·6, H 5·2. The substance is sparingly soluble in water, giving a yellow solution which exhibits a bottle-green fluorescence on addition of alkali.

It is readily soluble in ethanol and chloroform, moderately soluble in ether and carbon tetrachloride, slightly soluble in benzene and insoluble in petroleum ether. It can be precipitated from aqueous solution by means of lead acetate, and recovered by decomposing the lead with sodium sulphate.

Antibacterial properties.

Dr. J. S. Robertson tested the crude culture fluid against a wide variety of organisms, and reported activity against *Sh. shigae*, *Myco. phlei*, *Str. pyogenes*, *V. cholerae*, *Staph. albus*, *Staph. aureus* and *Ps. pyocyanea*.

Tested by the wheel plate method, a 1/500 solution of herquein inhibited the growth of the El Tor vibrio, *V. cholerae*, *C. xerosis* and *Staph. aureus*.

In serial dilution tests it inhibited the growth of *Staph. aureus* and *V. cholerae* at 1:2500, but not that of the El Tor vibrio.

Penicillin-like Antibiotics from Penicillia.

The culture fluids of *P. euglaucum*, *P. meleagrinum*, *P. divaricatum*, *P. roseo-citreum* Biourge (Baarn), *P. roseo-citreum* Biourge (N.C.T.C.) and *P. roseo-citreum* (P. 94) were all reported to inhibit the growth of *Staph. aureus* by Miss M. E. Johns.

Conditions of culture.

P. euglaucum was grown on a modified Czapek-Dox medium, as used for *P. equinum*, together with 5 per cent neutralized corn steep liquor. Activity was maximal in about 10 to 15 days.

P. meleagrinum was grown on the above medium. Activity was maximal in 5 to 10 days.

P. roseo-citreum (N.C.T.C. strain) and *P. roseo-citreum* (P. 94) were grown on the above medium, whilst *P. roseo-citreum* (Baarn) was grown using a similar medium, but without the addition of corn steep liquor. Activity was maximal in 10 days.

Extraction.

In all these cases the culture fluid was acidified to pH 2 with hydrochloric acid, and extracted with one-seventh volume of either ether or ethyl or amyl acetates. All the activity was extracted from the culture fluid at pH 2 and none at pH 7 or pH 10. The organic solvent layer was extracted with phosphate buffer at pH 7 when the activity passed entirely into the aqueous layer.

Chemical properties.

In each case activity was totally destroyed by incubation of either the crude culture medium or the extract for two hours at 37° C. with penicillinase or by incubation in the presence of copper sulphate.

The activity was also destroyed by heating the extract at 100° C. for 15 minutes at pH 2 and at pH 9, and was partially destroyed by heating for 15 minutes at pH 7.

It is concluded that these penicillia produce penicillin-like substances.

SUMMARY.

Antibacterial substances have been extracted from the culture fluids of 13 penicillia.

It has been found that six of these, *P. meleagrinum* Biourge, *P. euglaucum*, *P. divaricatum* and three strains of *P. roseo-citreum* produce penicillin-like antibiotics alone, and that two others, *P. baarnense* v. Beyma and *P. bialowiezense* Zal., produce a penicillin-like antibiotic, together with other antibiotics. *P. baarnense* produces penicillic acid, *P. bialowiezense* and *P. viridicatum* produce mycophenolic acid, and *P. equinum* v. Beyma and *P. novae zeelandiae* v. Beyma produce expansine. *P. herquei* N.C.T.C. 1721 produces an antibiotic named herquein, which inhibits the growth of *Staph. aureus* and some vibrios at high concentrations. *P. rubrum* produces a compound which has antibacterial properties which has been shown to be phoenicin.

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