CCXIII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXII. THE ISOLATION AND CHARACTERISATION OF ERGOSTEROL FROM *PENICILLIUM PUBERULUM* BAINIER GROWN ON A SYNTHETIC MEDIUM WITH GLUCOSE AS SOLE SOURCE OF CARBON.

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WHILST sterols are generally known to occur in moulds, and the presence of ergosterol in a number of species grown on synthetic media has been demonstrated by the development of antirachitic activity on irradiation [Preuss *et al.*, 1931], ergosterol has not previously been actually characterised after isolation from a mould grown on a synthetic medium in which glucose is the only source of carbon.

In the case of the sterol isolated from *Penicillium puberulum* Bainier, it has been found that the physical properties of the sterol itself and the benzoate prepared from it correspond closely with those of purified ergosterol from yeast and ergosteryl benzoate, respectively, and final confirmation of its identity with ergosterol is afforded by measurements of the absorption spectrum and of the antirachitic activity acquired after irradiation. These observations are significant in yielding experimental evidence of the synthesis of ergosterol from glucose.

EXPERIMENTAL.

The organism employed was the strain of *P. puberulum* Bainier, used by Alsberg and Black [1913] in the preparation of penicillic acid, and was supplied by the courtesy of Dr Charles Thom, of the United States Department of Agriculture. Its history is given in Dr Thom's "Penicillia," p. 272.

The medium used was a Czapek-Dox solution containing glucose as sole source of carbon, and was identical in composition with the basal metabolism solution of Raistrick and Rintoul [1931]:

Glucose	...	...	...	...	50 g.
NaNO ₃	...	...	...	...	2 g.
KH ₂ PO ₄	...	...	...	...	1 g.
KCl	...	...	...	...	0.5 g.
MgSO ₄ , 7H ₂ O	...	...	...	...	0.5 g.
FeSO ₄ , 7H ₂ O	...	...	...	...	0.01 g.
Water	...	...	...	...	1000 cc.

The cultures were grown in 1000 cc. flasks, each containing 350 cc. of medium, and were incubated at 24°, or in trays each containing 5 litres in the incubator-steriliser designed for mould cultures by Birkinshaw, Charles, Lilly and Rais-trick [1931]. In the latter case the temperature was maintained at 16–20°.

In order to obtain spores for sowing, Roux bottles containing Czapek-Dox agar were inoculated from the stock culture. When a good growth of spores was attained, 150 cc. of sterile water were added to each bottle, and the surface of the culture lightly rubbed with a sterile wire. The suspension was poured into a flask, and 2.5 cc. of this suspension were used for sowing each flask, or 100 cc. for each 5 litre tray. After an incubation period averaging 2–3 weeks, corresponding with the disappearance of about 90 % of the glucose present, the mycelium was separated from the metabolism solution by filtration, and after washing with water was squeezed out, and dried by exposing in a thin layer to the air at a temperature of about 25°. When most of the water had been removed in this manner, the mycelium was further dried *in vacuo* over CaCl_2 or H_2SO_4 . When quite brittle it was finely ground in a mortar and extracted with ether in an apparatus of the Soxhlet type. A crystalline substance could be isolated from the extract showing an intense reaction for sterols. Further work was therefore undertaken with the object of identifying the sterol or sterols present.

In a preliminary examination made of the ether-soluble material from one batch of mould the residue left after evaporation of the ether was twice recrystallised from alcohol. The product (0.35 g.) was now not completely soluble in ether. The crystals obtained from the ethereal extract (0.28 g.) had m.p. 123–133°, $[\alpha]_{5461}^{20} - 122^\circ$ in chloroform, gave an intense blue coloration with trichloroacetic acid characteristic of ergosterol [Rosenheim, 1929] and contained 73 % of sterol (calculated as ergosterol) precipitated by digitonin.

The ether-soluble material from another batch of mould was dissolved in a large volume of light petroleum, from which three fractions were obtained. (I) on cooling, white crystals (0.55 g.), (II) after evaporation (0.70 g.), and (III) a pasty residue after complete evaporation (2.25 g.). Fraction I did not redissolve completely in ether. The residue (I a) (0.1 g.) had m.p. 106–121° and contained nitrogen. The ethereal extract (I b) was evaporated to dryness. The following data were obtained for these fractions.

Fraction	M.P.	$[\alpha]_{5461}^{20}$ in CHCl_3	Free sterol precipitated by digitonin %	Reaction with $\text{CCl}_3\text{CO}_2\text{H}$
I b	127–139°	– 139.5°	70	Strongly +
II	65–110°	– 19.4°	7.2	Strongly +
III	Below 75°	– 6.3°	2.9	Weakly +

Fraction I b in 0.0025 % alcoholic solution gave an absorption spectrum similar to, but less intense than, that of ergosterol, with the following extinction coefficients: 9.9 at 290 $m\mu$, 18.7 at 282 $m\mu$, 18.7 at 271 $m\mu$, 8.5 at 247 $m\mu$. The corresponding figures for ergosterol are $\epsilon = 13.5, 25.6, 24.5, 6.6$.

These data appear to indicate that the first fraction contains the bulk of the free ergosterol, whilst fraction II probably contains ergosterol largely as ester, and fraction III consists mostly of other compounds, possibly including other sterols.

0.25 g. of fraction I *b* was boiled with 10 cc. of 3 % alcoholic potassium hydroxide for a few minutes. After the addition of a little water crystals separated on cooling. These were collected and dried over calcium chloride; weight 0.134 g., m.p. 154–158°, $[\alpha]_{5461}^{20} - 162^\circ$ in chloroform. Pure hydrated ergosterol has m.p. 160–163°, $[\alpha]_{5461}^{20} - 167.2^\circ$ [Callow, 1931].

The benzoate was prepared by the addition of a slight excess of benzoyl chloride to 0.11 g. of the material dissolved in 3 cc. of pyridine. The product was isolated by pouring the reaction-mixture into water and recrystallised from ethyl acetate. It separated, in the manner characteristic of ergosteryl benzoate, in leaflets changing to bulky thin needles which had m.p. 161–163°, $[\alpha]_{5461}^{20} - 85.3^\circ$ in chloroform. Pure ergosteryl benzoate has m.p. 169–171.5°, $[\alpha]_{5461}^{20} - 88.3^\circ$.

Finally, the benzoate was hydrolysed, the absorption spectrum of the resultant sterol was measured, and the antirachitic activity which it acquired on irradiation was determined. In 0.002 % alcoholic solution the extinction coefficients were 15.0 at 290 $m\mu$, 28.4 at 282 $m\mu$, 26.9 at 271 $m\mu$, and 6.2 at 247 $m\mu$. The curve showed the maxima characteristic of ergosterol, but with slightly greater intensity. A solution of the sterol in alcohol (0.01 %) stirred with a stream of nitrogen was irradiated in a silica cell by a mercury vapour lamp. A solution of pure ergosterol from yeast was irradiated under the same conditions for the same period and, after dilution in olive oil, the antirachitic potencies of the two products were compared on rats by the radiographic method described by Bourdillon *et al.* [1931]. Three pairs of rats were used, to which graduated doses were given, and three rats of the same litter were used as controls and given doses of a standard solution of vitamin D. The antirachitic potencies of the two products were found to be equal.

An estimate of the yield of ergosterol from the mycelium was made in a typical experiment, using 100 flasks (*i.e.* 35 litres of medium) and incubating at 24° for 20 days. The dry mycelium weighed 391 g. and yielded an ether extract weighing 19.9 g. after removal of solvent *in vacuo*. The solid portion of the extract was collected and dried on a porous plate. It weighed 1.8 g., and in alcoholic solution gave the absorption spectrum characteristic of ergosterol. The intensity of absorption indicated the presence of 28 % of ergosterol, corresponding to 0.13 % in the dried mycelium.

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