CLVIII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXXIII. THE MYCELIAL CONSTITUENTS OF *PENICILLIUM BREVI-COMPACTUM* DIERCKX AND RELATED SPECIES.

PART I. ERGOSTERYL PALMITATE.

BY ALBERT EDWARD OXFORD AND HAROLD RAISTRICK.

*From the Division of Biochemistry, London School of Hygiene
and Tropical Medicine, University of London.*

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IN the course of the studies reported in Part XXIV of this series [Clutterbuck *et al.*, 1932], considerable amounts of mycelia from the growth on synthetic media of 15 species and strains in the *P. brevi-compactum* series were accumulated, and in view of the close biochemical relationship of 14 of these organisms as regards their water-soluble metabolic products (see Part XXIV) it became a matter of some interest to investigate their mycelial constituents in order to discover whether this biochemical relationship is of even wider scope. The mycelium of the type species *P. brevi-compactum* Dierckx (Cat. No. P 75) was first investigated, the method being to extract the dried and powdered mycelium with a succession of solvents, *viz.* light petroleum followed by ether, then by acetone and finally by methyl alcohol. This paper is concerned only with the mycelial fat, *i.e.* the material extracted from the mycelium by hot light petroleum, B.P. 50–60°.

It was noticed that a considerable part of the mycelial fat from this species was not soluble in cold absolute alcohol, the insoluble constituent giving all the colour reactions of ergosterol. This insoluble substance, which was much more sparingly soluble in most organic solvents than ergosterol itself, had m.p. 106–108°; $[\alpha]_{5461}^{20^\circ} - 67^\circ$, $[\alpha]_{5790}^{20^\circ} - 52^\circ$ when pure, and could not be made to yield a benzoyl or an acetyl derivative. Its carbon and hydrogen content and molecular weight corresponded to an empirical formula of the order of $C_{44}H_{74}O_2$ and the substance was evidently an ergosteryl ester. When hydrolysed by alcoholic potash it yielded ergosterol and a fatty acid identified as palmitic acid, and was therefore clearly ergosteryl palmitate, $C_{44}H_{74}O_2$, a substance which had previously been prepared synthetically by Windaus and Rygh [1928] by heating ergosterol and palmitic acid *in vacuo* at 190°. The physical constants quoted by these authors, *viz.* m.p. 107–108°, $[\alpha]_{D(5890)}^{18^\circ} - 51^\circ$ are in good agreement with those of the mould product quoted above.

The same substance, or at any rate a substance melting at about 100° whose melting-point was not depressed on admixture with the product from *P. brevi-compactum* (P 75), was obtained from the mycelium of each of the 14 remaining species and strains of the *P. brevi-compactum* series investigated, with the solitary exception of *P. stoloniferum* Thom (Ad 89). The yield of recrystallised ergosteryl palmitate was usually of the order of 0.02 % of the dry weight of

mycelium but in one solitary instance, viz. *P. aurantio-griseum* Dierckx var. *Poznaniensis* Zaleski, the yield of purified ester was as high as 0.5 % and corresponded to 0.06 % of the glucose metabolised in the experiment during which the mycelium was formed. This ester also yielded ergosterol and palmitic acid on hydrolysis.

It is worthy of note that this appears to be the first recorded instance of the isolation of an ergosteryl ester in a pure state from a natural source, although the results of Smedley-MacLean and Thomas [1920], recorded some 13 years ago, indicated clearly that a large proportion of the ergosterol in yeast-fat was present in the form of esters. King *et al.* [1929] showed that the cholesterol esters extracted from a mummified brain 14 centuries old were undoubtedly admixed with ergosterol esters. Pruess *et al.* [1931] estimated the total sterol content of the mycelia of a number of moulds and showed that while most of the alcohol-soluble sterol was present in the free state in the extract, a definite, and in some cases a considerable, proportion of the total sterol in the mycelium was in a form which was not extracted by alcohol, but which yielded an alcohol-soluble sterol on hydrolysis by alcoholic potash. These authors concluded that this latter sterol is probably present in a conjugated form. Birkinshaw *et al.* [1931] found that a considerable fraction of the crude sterol obtained from the mycelium of *P. puberulum* Bainier grown on a synthetic medium containing glucose as the sole source of carbon could not be precipitated by digitonin, although the reaction with $\text{CCl}_3\text{CO}_2\text{H}$ was strongly positive, and concluded that this fraction contained ergosterol largely as ester.

It appeared unlikely therefore that ergosteryl palmitate would prove to be a mycelial constituent specific for the *P. brevi-compactum* series, and it has, in fact, been isolated in relatively good yield (0.4 %) from the mycelium of *P. italicum* Wehmer grown on liquid Czapek-Dox medium at 30°. The yield of this ester from the mycelium of *P. aurantio-griseum* Dierckx var. *Poznaniensis* Zaleski (Cat. No. P 69) would, however, appear to be exceptionally high (the greater part of the sterol in the mycelium being present as palmitate) and it may be significant that although this organism is morphologically definitely a member of the *P. brevi-compactum* series [see Clutterbuck *et al.*, 1932, p. 1443], yet biochemically it has no affinities with the other members of this group, since, unlike them, it yields no trace of mycophenolic acid or of any of the other phenolic acids, $\text{C}_{10}\text{H}_{10}\text{O}_5$, $\text{C}_{10}\text{H}_{10}\text{O}_6$, $\text{C}_{10}\text{H}_{10}\text{O}_7$ and 3:5-dihydroxyphthalic acid when grown on Raulin-Thom medium [see Clutterbuck *et al.*, 1932, p. 1446].

EXPERIMENTAL.

The sources of the organisms employed are given in Part XXIV [Clutterbuck *et al.*, 1932, p. 1443]. Each was grown on Raulin-Thom medium containing both glucose and tartaric acid as sources of carbon, and ammonium tartrate, phosphate and sulphate as sources of nitrogen. The temperature of incubation was 24° and the period of incubation was 20–30 days, the experiment being concluded when all the sugar in the medium had been utilised. The mycelium was filtered off, washed, pressed and dried in a vacuum-oven. The mycelium was ground to a fine powder in a coffee-mill and extracted.

Isolation of ergosteryl palmitate from the dried mycelium. The usual procedure was to extract 50 g. of powdered mycelium with light petroleum (b.p. 50–60°) for 2 working days (15 hours) in an all-glass Soxhlet apparatus. The extract, filtered from a little mycophenolic acid if necessary, was evaporated to small volume and the remaining solvent removed *in vacuo*. The residual yellow fat

was usually of a fairly hard consistency, but the mycelium of *P. aurantio-griseum* Dierckx var. *Poznaniensis* Zaleski (Cat. No. P 69) yielded a very dark coloured and much more fluid fat. Cold absolute alcohol (20 cc. per g. of crude fat) was added and the flask shaken until no fat particles remained adhering to the walls of the flask. The insoluble residue was filtered off, washed twice with alcohol, dried in air and recrystallised from the minimum volume of boiling acetone, about 40 cc. being required for each 0.1 g. of residue. The results for the various mould mycelia are summarised in Table I, the yields in each experiment

Table I.

Catalogue No.	Species [see Clutterbuck <i>et al.</i> , 1932, p. 1443]	Weight of dried mycelium g.	Ergosteryl palmitate (once crystallised from acetone)		
			Yield in g.	Yield % of mycelium	M.P. and remarks
P 75	<i>P. brevi-compactum</i> Dierckx	276	0.25	0.1	102–104° (a), (b)
M 3 (1)	Unnamed. Isolated in 1931 from mouldy Italian maize	386	Trace	—	100–102° (a), (b)
M 3 (3)	Unnamed. Isolated in 1931 from mouldy Italian maize	285	Trace	—	100–102° (a), (b), (c)
M 3 (4)	Unnamed. Isolated in 1931 from mouldy Italian maize	388	Trace	—	(a)
P 151	<i>P. scabrum</i> Biourge	243 (fat content 0.6 %)	Trace	—	92–96° (a)
A 11	<i>P. brevi-compactum</i> Dierckx. Isolated from cotton	419	Trace	—	94–100° (a), (c)
S 30	Unnamed. Isolated from soil	293	0.06	0.02	100–103° (a), (b), (c)
P 84	<i>P. Hagemi</i> Zaleski	450	0.09	0.02	97–102° (a), (b), (c)
P 99	<i>P. Szaferi</i> Zaleski	446	0.05	0.01	102–104° (a), (b), (c)
D 8	Unnamed. Plate contaminant	432	0.13	0.03	98–103° (a), (b), (c)
P 90	<i>P. patris-mei</i> Zaleski	334	0.17	0.05	102–104° (a), (b), (c)
P 37	<i>P. griseo-brunneum</i> Dierckx	524	0.23	0.04	96–100° (a), (b), (c)
Ad 89	<i>P. stoloniferum</i> Thom	547	None. Fat completely soluble in alcohol		
Ad 87	<i>P. Biourgeanum</i> Zaleski	363	0.06	0.02	98–101° (a), (b), (c)
P 69	<i>P. aurantio-griseum</i> Dierckx, var. <i>Poznaniensis</i> Zaleski	229 (d) (fat content, 8 %)	1.14	0.50	102–105° (a), (b), (c)
Ad 85	<i>P. italicum</i> Wehmer. Isolated from a mouldy orange in 1926	195 (e) (fat content, 5 %)	0.80	0.41	102–104° (a), (b), (c)

(a) Gives positive reaction with Callow-Rosenheim reagent (mercuric acetate in nitric acid).

(b) Gives positive Tortelli-Jaffé reaction (specific reaction for ergosterol and its esters).

(c) Mixed m.p. with ergosteryl palmitate from P 75 showed no depression.

(d) The mycelium contained less than 0.1 % of free sterol precipitated by digitonin.

(e) Grown on Czapek-Dox medium and incubated at 30° for 37 days.

being calculated on the basis of the total mycelium from 90 flasks, *i.e.* from the metabolism of about 1575 g. glucose. The fat content of each mycelium was 2–3 % unless otherwise stated.

Investigation of the ergosteryl ester from the mycelium of P. brevi-compactum Dierckx (Cat. No. P 75).

The crude alcohol-insoluble product from about 2.5 kg. of ground mycelium (2.5 g.; m.p. 90–100°) was crystallised first from light petroleum (b.p. 60–80°; 40 cc.) then from acetone, and the purified product so obtained (0.5 g., m.p. 102–107°) was dissolved in hot benzene (30 cc.) and boiling acetone added until a turbidity appeared. The substance crystallised from the last-named mixture in colourless plates, m.p. 106–108° (0.4 g.). The melt became yellow at 150–160°, but solidified on cooling and remelted at 105–107°. The melting-point was not

altered by a further crystallisation from alcohol. The ester crystallised unchanged from 1 % alcoholic digitonin solution. It was also recovered unchanged when attempts were made to benzoate it with benzoyl chloride in pyridine at 60–80° or to acetylate it by boiling with acetic anhydride.

Micro-analysis (Schoeller): C, 83.11, 83.20; H, 11.71, 11.63 %. $C_{44}H_{74}O_2$ requires C, 83.20; H, 11.76 %.

$[\alpha]_{5461}^{20} - 67^\circ$; $[\alpha]_{5790}^{20} - 52^\circ$. (0.0968 g. in chloroform (25 cc.) at 20° in a 2 dm. tube.) The chloroform solution turned yellow on standing for a day.

Molecular weight by hydrolysis. 0.4606 g. was boiled for 1½ hours with *N*/10 ethyl alcoholic potash (60 cc.) when it was found that a nett amount of 7.18 cc. *N*/10 alkali had been neutralised, corresponding to a molecular weight of 642 ($C_{44}H_{74}O_2$ requires mol. wt., 635). Ergosterol (m.p. 151–155°) was precipitated by adding much water to the strongly alkaline solution.

Isolation of ergosterol and palmitic acid by hydrolysis. 0.31 g. (m.p. 99–102°) was hydrolysed as above and the alcohol removed *in vacuo*. Water (50 cc.) and 2*N* NaOH (10 cc.) were then added, and the solution was extracted 3 times with much chloroform. The chloroform extract was dried over anhydrous sodium sulphate and evaporated, the solid residue being crystallised from alcohol to yield ergosterol (0.05 g.), m.p. 152–156°. The alkaline aqueous layer from which the chloroform had been separated was acidified with dilute sulphuric acid and extracted with light petroleum (b.p. 40–50°). The extract was dried over anhydrous sodium sulphate, evaporated and the residue crystallised from aqueous alcohol to yield a fatty acid (0.07 g.) melting at 53–57°. A further crystallisation from aqueous alcohol raised the m.p. to 55–59°. The mixed m.p. with an authentic specimen of palmitic acid (m.p. 62–63°) was 56–61°.

Micro-analysis (Schoeller): C, 74.83; H, 12.43 %. $C_{16}H_{32}O_2$ requires C, 74.91; H, 12.60 %; stearic acid (m.p. 69°), $C_{18}H_{36}O_2$ requires C, 75.98; H, 12.77 %.

Investigation of the ergosteryl ester from the mycelium of P. aurantio-griseum Dierckx var. Poznaniensis Zaleski (Cat. No. P 69).

The crude alcohol-insoluble product required less purification than the corresponding product from P 75. 1.1 g. was crystallised first from acetone (400 cc.) and then from alcohol (150 cc.). It formed shining plates, m.p. 103–106° (0.7 g.).

Formation of ergosterol and palmitic acid by hydrolysis. The above substance (0.66 g.) was hydrolysed by boiling *N*/10 alcoholic potash in the usual way, the alcohol removed, water and 10 cc. 2*N* NaOH added to the residue, which was then extracted 3 times with much chloroform. The product obtained by evaporation of the dried chloroform extract was crystallised once from alcohol to yield almost pure ergosterol (0.19 g.), m.p. 158–160°; $[\alpha]_{5461}^{24} - 160^\circ$; $[\alpha]_{5790}^{24} - 133^\circ$. Pure hydrated ergosterol has m.p. 160–163°, $[\alpha]_{5461}^{20} - 167.2^\circ$ [Callow, 1931].

The alkaline aqueous layer from which the chloroform layer had been partially separated was centrifuged until almost complete separation into two layers had taken place. The upper aqueous layer was then acidified with dilute sulphuric acid and the precipitated palmitic acid collected, well washed and dried (0.16 g.; m.p. 56.5–59°).

0.0893 g. in 25 cc. alcohol required 13.95 cc. *N*/40 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 256. $C_{16}H_{32}O_2$ (palmitic acid) has an equivalent of 256. The acid recovered from the above titration after crystallisation from aqueous alcohol had m.p. 57–61°. Found: C, 75.08; H, 12.58 %. $C_{16}H_{32}O_2$ requires C, 74.91; H, 12.60 %.

A part of the crude acid (M.P. 56·5–59°, see above) was crystallised twice from light petroleum to yield a minute amount of colourless flakes, M.P. 61–62°. Mixed with authentic palmitic acid (M.P. 62–63°), the M.P. was 61–62°.

SUMMARY.

Ergosteryl palmitate has been isolated from the mycelia of 14 out of 15 species and strains in the *P. brevi-compactum* Dierckx series investigated, and also from the mycelium of *P. italicum* Wehmer, all the moulds having been grown on synthetic media. The mycelium of *P. aurantio-griseum* Dierckx var. *Poznaniensis* Zaleski, grown on Raulin-Thom medium and incubated at 24° for 30 days, contains as much as 0·5 % of this ester.

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