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BIOSYNTHESIS OF STEROL ESTERS IN *PHYCOMYCES BLAKESLEEANUS*

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Key Word Index—*Phycomyces blakesleeanus*; Mucoraceae; biosynthesis; sterol esters; acyl-CoA: sterol acyltransferase; phosphatidylcholine: sterol acyltransferase; phospholipases C and D; sterol ester hydrolase.

Abstract—A cell-free enzyme preparation of *P. blakesleeanus* has been shown to possess phosphatidylcholine: sterol acyltransferase, sterol ester hydrolase and to a lesser extent acyl-CoA: sterol acyltransferase activities. Phospholipase C and D activities were also detected.

INTRODUCTION

WHILST the nature of the sterol esters of *P. blakesleeanus* has been investigated,[1] no information is available as to the mechanism of their formation. Indeed virtually nothing is known about the way in which sterol esters are formed in the plant kingdom. However, the waxes, also highly hydrophobic esters of monohydric alcohols, have been shown to be formed by transfer of acyl groups from both acyl-CoAs and phospholipids to fatty alcohols.[2] In the animal kingdom cholesterol ester formation has been shown to involve phosphatidylcholine:cholesterol acyltransferase activity in several tissues.[3,4]

In this paper we report on the mechanism of sterol ester formation in *P. blakesleeanus* using a mycelial cell-free enzyme preparation.

RESULTS AND DISCUSSION

Incubations with free fatty acids and sterol

Effect of different sterols. The ability of the *P. blakesleeanus* cell-free enzyme system to form sterol esters from palmitic acid-[1-^{14}C] and four sterols, ergosterol, 5-dihydroergosterol (ergosta-7,22-dien-3β-ol), episterol (ergosta-7,24(28)-dien-3β-ol) and lanosterol was tested by incubating 2 μCi (0·4 μmol) fatty acid, 5 μmol ATP, 0·2 μmol CoA, 5 μmol $MgCl_2$, 0·25 μmol α-bromopalmitin, 0·5 mg Tween 80 and 0·4 μmol of each sterol separately with the enzyme system (19·7 mg protein) in 1 ml 0·1 M phosphate buffer, pH 7; equivalent boiled enzyme controls were also carried out. Palmitic acid and the four sterols are known to be components of the sterol ester of *P. blakesleeanus*.[1] α-Bromopalmitin, a known inhibitor of β-oxidation,[5] was included in the incubation mixture to minimize any catabolism of the palmitic acid-[1-^{14}C]. Tween 80 was used to solubilize the water-insoluble components. The incubations were stopped by boiling for 5 min. The lipid was then extracted

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[1] MERCER, E. I. and BARTLETT, K. (1974) *Phytochemistry* **13**, 1099.
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[4] ILLINGWORTH, D. R. and GLOVER, J. (1970) *Biochim. Biophys. Acta* **220**, 610.
[5] BURGES, R. A., BUTT, W. D. and BAGGALEY, A. (1968) *Biochem. J.* **109**, 38P.

and the sterol ester isolated by TLC (system 1; R_f sterol ester, 0·55–0·75; palmitic acid, R_f 0·08) and an aliquot assayed for radioactivity. To check that the sterol ester zone from the TLC was indeed sterol ester an aliquot was saponified; the diluted saponification mixture was extracted with Et_2O to remove sterol and then acidified to pH 1 with HCl and re-extracted with Et_2O to remove the fatty acid. The sterol extract was then compared with the appropriate authentic sterol on TLC (systems 2 and 3) and the fatty acid extract with palmitic acid-[1-^{14}C] on TLC (system 2); in all cases the expected identifications were made. The results of this experiment, seen in Table 1, show that the percentage incorporation of palmitic acid-[1-^{14}C] into sterol ester was very low (0·019–0·042%) and only two to three times that of the boiled control. Of the four sterols, 5-dihydroergosterol appeared to be esterified rather better than the others.

TABLE 1. INCORPORATION OF PALMITIC ACID-[1-^{14}C] INTO STEROL ESTER

Sterol in incubation mixture	% Incorporation of palmitate into sterol ester		Sterol in incubation mixture	% Incorporation of palmitate into sterol ester	
	Test	Control*		Test	Control*
Ergosterol	0·027	0·010	Episterol	0·021	0·010
5-Dihydroergosterol	0·042	0·013	Lanosterol	0·019	0·011

2 μCi (0·4 μmol) palmitic acid-[1-^{14}C], 0·4 μmol sterol, 5 μmol ATP, 0·2 μmol CoA, 5 μmol $MgCl_2$, 0·25 μmol α-bromopalmitin and 0·5 mg Tween 80 were incubated with the cell-free enzyme preparation (19·7 mg protein) in 1 ml 0·1 M phosphate buffer, pH 7, for 2 hr at 24° in the absence of light.

* Boiled enzyme control.

Effect of different solubilizing agents. It was suspected that the poor incorporation of labelled palmitic acid into sterol esters seen above may have been due to the presence of Tween 80 which, although an excellent solubilizing agent, is known to inhibit some enzymes. To test this, the effect of five different solubilizing systems, Tween 80, bovine serum albumin (BSA), 30% propylene glycol, 50% propylene glycol and acetone, on the ability of the *P. blakesleeanus* cell-free enzyme system to catalyse the formation of 5-dihydroergosterol palmitate from 5-dihydroergosterol and palmitic acid-[1-^{14}C] was studied. The quantities of 5-dihydroergosterol, palmitic acid-[1-^{14}C], α-bromopalmitin and cofactors in both test and boiled enzyme control incubations were identical with those given previously. The test and control incubations using Tween 80 as the solubilizing agent were prepared in the manner described above. In the BSA test and control incubations the ATP, CoA and $MgCl_2$, each dissolved in 0·1 ml 0·1 M phosphate buffer, pH 7, were added to 0·2 ml of a 5 mg/ml aq. soln of delipidized BSA; the 5-dihydroergosterol, palmitic acid-[1-^{14}C] and α-bromopalmitin, dissolved in the minimal vol. of acetone were then added, thoroughly mixed and the acetone evaporated by a stream of nitrogen. The resulting clear soln was then mixed with 1 ml of the enzyme preparation. In the test and control incubations using acetone as the solubilizing agent the procedure for the preparation of the incubation mixture was identical with that for the BSA incubations save that 0·2 ml H_2O replaced the 0·2 ml BSA soln. In the propylene glycol test and control incubations 0·2 ml of either 30 or 50% (*v/v*) propylene glycol in water was added to 0·1 ml aliquots of each of the cofactor solns and the lipid components, dissolved in the minimal vol. of acetone, were then added. The resulting clear soln was thoroughly mixed and the acetone evaporated by a stream of nitrogen. The experiment was carried out in duplicate using enzyme preparations (33·7 and 31·0 mg protein/ml) prepared on different occasions from the same

sample of lyophilized mycelium. The results of this experiment, seen in Table 2, again show very low incorporations of palmitic acid-[1-^{14}C] into sterol ester which are only three to four times those of the controls. However, BSA and 50% propylene glycol appear to be the solubilizing systems which support sterol ester formation best.

TABLE 2. EFFECT OF DIFFERENT SOLUBILIZING SYSTEMS ON THE INCORPORATION OF PALMITIC ACID-[1-^{14}C] INTO 5-DIHYDROERGOSTEROL PALMITATE

Solubilizing system	% Incorporation of palmitate into sterol ester			
	Experiment 1		Experiment 2	
	Test	Control*	Test	Control*
Tween 80	0·074	0·023	0·042	0·013
Bovine serum albumin (BSA)	0·144	0·036	0·129	0·042
30% Propylene glycol	0·091	0·030	0·066	0·022
50% Propylene glycol	0·165	0·036	0·129	0·042
Acetone	0·049	0·036	—	—

2 μCi (0·4 μmol) palmitic acid-[1-^{14}C], 0·4 μmol 5-dihydroergosterol, 5 μmol ATP, 0·2 μmol CoA, 5 μmol $MgCl_2$, 0·25 μmol α-bromopalmitin were incubated in the presence of different solubilizing agents, phosphate buffer, pH 7, and the cell-free enzyme preparation (Expt. 1, 33·7 mg protein; Expt. 2, 31·0 mg protein) in a total of 1·5 ml for 2 hr at 24° in the absence of light. The enzyme preparations were made from the same batch of lyophilized mycelium on different occasions.

* Boiled enzyme control.

Effect of guanosine triphosphate. The experiments described above were intended to test the postulate that the *P. blakesleeanus* cell-free preparation contains an acyl-CoA synthetase which catalyses the formation of palmityl-CoA-[1-^{14}C] which then becomes the substrate of an acyl-CoA:sterol acyltransferase. Accordingly CoA, ATP and Mg^{2+} were included in the incubation mixture. However, since GTP-dependent acyl-CoA synthetases are known,[6,7] it was decided to compare the efficacy of GTP and ATP in promoting sterol ester formation from palmitic acid-[1-^{14}C] using 5-dihydroergosterol and BSA as the solubilizing system. Test and boiled-enzyme control incubations using ATP and GTP as the energy sources were set up and carried out using the same quantities of reactants and procedure as described above. The experiment was carried out in duplicate using enzyme preparations (40·7 and 93·8 mg protein/ml) prepared from different batches of mycelium. The results of this experiment, seen in Table 3, show a greater incorporation of palmitic acid-[1-^{14}C] into sterol ester in the presence of GTP. The incorporation of label into sterol ester in the presence of both ATP and GTP in experiment A, although still very low, is rather better than in the earlier experiments; moreover the incorporations in the test incubations are 16–23 times greater than those in the controls.

Effect of different fatty acids. It has been shown[1] that palmitic, oleic and linoleic acids are the major fatty acids to be found in the sterol esters of *P. blakesleeanus.* An experiment was, therefore, carried out to see whether the *P. blakesleeanus* cell-free system would catalyse the incorporation of the unsaturated fatty acids, oleic acid-[1-^{14}C] and linoleic acid-[1-^{14}C], into sterol ester better than palmitic acid-[1-^{14}C] using 5-dihydroergosterol, BSA as the solubilizing system and ATP as the energy source. Test and boiled-enzyme control incubations were set up for each fatty acid using the same quantities of reactants and procedure as before but a different preparation of the enzyme system (32·8 mg protein/ml).

[6] ROSSI, C. R. and GIBSON, D. M. (1964) *J. Biol. Chem.* **239,** 1694.
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The results (Table 4) show that all three fatty acids are incorporated into sterol ester to about the same extent.

Incubations with palmityl-CoA and sterol

Since the incorporation of free fatty acids into sterol esters by the *P. blakesleeanus* cell-free preparation was so poor it was decided to utilize palmityl-CoA and so avoid the necessity of an acyl-CoA synthetase-catalysed step.

TABLE 3. EFFECT OF GTP ON THE INCORPORATION OF PALMITIC ACID-[1-^{14}C] INTO 5-DIHYDROERGOSTEROL PALMITATE

Nucleotide	% Incorporation of palmitate into sterol ester			
	Experiment 1		Experiment 2	
	Test	Control*	Test	Control*
ATP	0·175	0·011	0·088	0·020
GTP	0·367	0·016	0·199	0·088

2 μCi (0·4 μmol) palmitic acid-[1-^{14}C], 0·4 μmol 5-dihydroergosterol 5 μmol nucleotide, 0·2 μmol CoA, 5 μmol of $MgCl_2$, 0·25 μmol α-bromopalmitin and 1 mg BSA were incubated in 1·5 ml phosphate buffer, pH 7, with the cell-free enzyme preparation (Expt. 1, 40·7 mg protein; Expt. 2, 93·8 mg protein) for 2 hr at 24° in the absence of light. The enzyme preparations were from different batches of mycelium.

* Boiled enzyme control.

Incubation of palmityl-CoA with ergosterol-[28-^{14}C]. 0·42 μmol ergosterol-[28-^{14}C] (0·6214 μCi/μmol), 0·42 μmol palmityl-CoA and 0·5 mg Tween 80 were suspended in 0·2 ml H_2O and mixed with 5 ml of *P. blakesleeanus* cell-free preparation (11 mg protein). The ergosterol palmitate isolated from the test incubation contained 3150 dpm representing a 0·554% incorporation of label whilst that from the boiled-enzyme control contained 406 dpm representing a 0·079% incorporation of label. This incorporation, seven times greater than that of the control, is still low although rather better than that experienced with free fatty acids.

Incubation of palmityl-CoA-[1-^{14}C] *with ergosterol.* 0·484 μmol ergosterol, 0·484 μmol palmityl-CoA-[1-^{14}C] (2·088 μCi/μmol) and 0·5 mg Tween 80 were suspended in 1 ml H_2O and incubated with 1 ml of *P. blakesleeanus* cell-free preparation (38 mg protein). The ergosterol palmitate isolated from the test incubation contained 4977 dpm representing a 0·222% incorporation of label whilst that of the boiled-enzyme control contained 448 dpm representing a 0·020% incorporation. This incorporation, 11 times greater than the control, is only about half of that in the experiment with ergosterol-[28-^{14}C]. The very poor incorporation of free fatty acids and the CoA ester of palmitic acid into sterol esters suggested that the acyl-CoA:sterol acyltransferase activity in the *P. blakesleeanus* cell-free preparation was very low, perhaps due to its being rather labile. However two other explanations are possible; firstly the cell-free preparation contains a sterol ester hydrolase which catalyses the hydrolysis of much of the sterol ester formed during the incubation and secondly that the fatty acid moiety of sterol esters is more readily derived from a source other than fatty acyl-CoAs, possibly a phospholipid. It is known that cholesterol esters in plasma[3] and cerebrospinal fluid[4] can arise by enzyme-catalysed transfer of an acyl group from phosphatidylcholine to cholesterol. These two possibilities were therefore tested.

Incubations with phosphatidylcholine-[acyl-^{14}C] and ergosterol. 1·263 μmol ergosterol and 0·5 μmol phosphatidylcholine-[acyl-^{14}C] (2 μCi/μmol) was suspended in 0·5 ml H_2O with the aid of ultrasonication and thoroughly mixed with 2 ml of *P. blakesleeanus* cell-free preparation (34 mg protein/ml). Aliquots of the lipid extracted from the test and boiled-enzyme control incubations were subjected to TLC (systems 4–6) and the zones of phosphatidylcholine (PC), lysophosphatidylcholine (lyso PC), phosphatidic acid (PA), 1,2-diglyceride (DG), fatty acid and ergosterol ester isolated and radio assayed. The only radioactive components of the control lipid were unchanged PC and traces of fatty acid.

TABLE 4. EFFECT OF DIFFERENT FATTY ACIDS ON STEROL ESTER FORMATION

Fatty acid-[1-^{14}C]	% Incorporation of fatty acids into sterol ester	
	Test	Control
Palmitic	0·310	0·020
Oleic	0·289	0·020
Linoleic	0·327	0·014

2 μCi fatty acid-[1-^{14}C], 0·4 μmol 5-dihydroergosterol, 5 μmol ATP, 0·2 μmol CoA, 5 μmol $MgCl_2$, 0·25 μmol α-bromopalmitin and 1 mg BSA were incubated in 1·5 ml phosphate buffer, pH 7, with the cell-free preparation (39·7 mg protein) for 2 hr at 24° in the absence of light.
* Boiled enzyme control.

The percentage incorporation of label into ergosterol ester from the test incubation was 4·35%. This is an order of magnitude greater than was obtained with free fatty acid or its CoA derivative and is clearly due to phosphatidylcholine:sterol acyltransferase activity since it cannot be explained by phospholipase-catalysed hydrolysis of PC followed by esterification of the resulting labelled fatty acids with sterol. The presence of phosphatidylcholine:sterol acyltransferase in the cell-free preparation may explain the differences in incorporation of label in the earlier experiments utilizing labelled ergosterol and unlabelled palmityl-CoA in one case and unlabelled ergosterol and labelled palmityl-CoA in the other. The transfer of unlabelled acyl residues from the small amount of endogenous PC which may have been present in the cell-free preparation to ergosterol would have raised the incorporation of label into sterol ester in the first but not the second experiment. The percentage incorporations of label into DG, PA, lyso PC and free fatty acid were 4·76, 6·07, 2·23 and 76·74 respectively. The formation of DG and PA indicate presence of phospholipases C (E.C. 3.1.4.3) and D (E.C. 3.1.4.4) respectively in the cell-free system. The formation of lyso PC may be indicative of the activity of phospholipase A (E.C. 3.1.1.4) but would also result from acyl transfer from PC to sterol. The fact that only 2·23% of the label resulting from PC catabolism accumulates in lyso PC whilst 4·35% accumulates in sterol ester suggests the presence of a lysophospholipase (E.C. 3.1.1.5). The high level of label in the free fatty acids could have arisen as a result of the combined activity of several enzymes including phospholipases A, C and D, lysophospholipase, acyl lipases acting on DG and sterol ester hydrolase.

Sterol ester hydrolase activity

A sterol ester-[G-^{14}C] mixture was isolated from the mycelium of *P. blakesleeanus* grown from spores on a medium containing acetate-[1-^{14}C] since some or all of the components of this mixture would be the natural substrate of any sterol ester hydrolase present

in the *P. blakesleeanus* cell-free preparation. 1·81 mg of this sterol ester mixture (0·291 μCi) was solubilized in 0·5 ml 0·1 M phosphate buffer, pH 7, containing 2·5 mg BSA, and incubated with 1 ml of cell-free preparation (61·5 mg protein/ml).

After the addition of 1 mg of ergosterol to act as carrier, the lipids extracted from the test and boiled-enzyme control incubations were subjected to TLC (system 2) and the free sterol zone, co-chromatographing with marker ergosterol, extracted and radioassayed. The sterol from the test incubation contained 10630 dpm whilst that from the control contained 1506 dpm.

Assuming that the fatty acid moieties of the sterol ester-[G-^{14}C] mixture are all C_{18}* and knowing which carbon atoms of both sterol and fatty acid moieties will be derived from the ^{14}COOH of acetate it can be deduced that 12/21 of the radioactivity of the sterol ester mixture is in the sterol residue. Thus of the 0·291 μCi sterol ester in each incubation mixture, 0·166 μCi (368 500 dpm) resides in the sterol component. The test incubation released 10630 dpm of this radioactivity representing a 2·89% hydrolysis of the sterol ester mixture; similarly the control incubation released 0·41%. The difference of 2·48% thus represents the degree of hydrolysis affected. The cell-free system therefore appears to possess sterol ester hydrolase activity.

The finding of sterol ester hydrolase activity in the *P. blakesleeanus* cell-free preparation may explain the very low sterol ester formation from fatty acids and palmityl-CoA since the quantity of sterol ester detected in the incubation mixture represents the net result of the sterol ester formation and hydrolysis. This would suggest that the level of acyl-CoA:-sterol acyltransferase activity is rather higher than the results indicate. The same reasoning also applies to the phosphatidylcholine:sterol acyltransferase activity. However, even allowing for hydrolysis of some sterol ester, the results indicate clearly that phosphatidylcholine:sterol acyltransferase activity in the *P. blakesleeanus* cell-free preparation is about 10 times higher than that of the acyl-CoA:sterol acyltransferase.

EXPERIMENTAL

Preparation of Phycomyes blakesleeanus *cell-free enzyme system*—P. blakesleeanus. Burgeff, (−)-strain, was grown in 10-l. batches under the cultural conditions described previously.[8] The mycelium was harvested after 36 hr and lyophilized. It was then rubbed through a fine sieve (200 mesh) to yield a fine, pale yellow powder. Portions (5–10 g) of this powder were then mixed with ice-cold 0·1 M phosphate buffer, pH 7, which was 20 mM with respect to mercaptoethanol (~10 ml), to give a smooth, viscous paste. This was then centrifuged at 10000*g* for 20 min at 0°; the resulting supernatant was used as the cell-free enzyme system.

Protein assay. The protein content of the cell-free preparation was determined by the method of Gornall *et al.*[9]

Incubation conditions. All incubations were carried out for 2 hr at 24° in the absence of light.

Post-incubation procedure. All incubation mixtures were boiled to stop enzyme activity. The lipid was then extracted. In most experiments the boiled incubation mixture was cooled, diluted with 5 ml EtOH and extracted four times with 20 ml Et_2O. The bulked Et_2O extract was washed with H_2O, dried over anhyd. Na_2SO_4 and evaporated to dryness under N_2. However, in the experiment with phosphatidylcholine, the lipid was extracted by the method used by Galliard[10] when assaying potato tuber homogenates for endogenous lipolysis. After extraction of the lipid, the subsequent experimental procedure, except where stated otherwise, was identical with that described in the first section of "Results and Discussion".

Thin layer chromatography. System 1: silica gel G impregnated with Rhodamine 6G[11] (0·25 mm) developed with C_6H_6–petrol. (2:3); system 2: silica gel G impregnated with Rhodamine 6G (0·25 mm) developed with $CHCl_3$; system 3: silica gel G impregnated with $AgNO_3$ (10% w/w) developed with C_6H_6–petrol. (2:3); system

* About 65% of the sterol esters of *P. blakesleeanus* are esters of C_{18} fatty acids.[1]

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4: system used by Skipski *et al.*[12] for the separation of neutral lipids; system 5: system described by Skipski *et al.*[13] for the separation of phospholipids; system 6: system described by Skipski *et al.*[14] for the separation of acidic phospholipids.

Saponification of sterol esters and isolation of the resulting sterols and fatty acids. This was carried out as described previously.[1]

Preparation of Ergosterol-[28-^{14}C]. 200 ml of Klein's medium II[15] containing 25 μCi methionine-[methyl-^{14}C] were incubated with 20 ml of a logarithmic-phase culture of Baker's yeast (*Saccharomyces cerevisiae*) growing on Klein's medium II and cultured aerobically for 24 hr at 18 . The cells (3·50 g, wet wt) were harvested, washed free of methionine-[methyl-^{14}C] with ice-cold 0·1 M phosphate buffer, pH 7, and saponified in 10 ml 90% (v/v) aq. EtOH containing 1 g KOH and 25 mg pyrogallol. The unsaponifiable material (96·5 mg; 2·92 μCi) was extracted with Et_2O in the usual way and then chromatographed on a 10 g column of acid-washed, Brockmann Grade 3 alumina (Woelm) developed in a stepwise manner with increasing percentages of Et_2O in petrol. (E/P). The fractions eluted with 15% E/P (13·2 mg; 1·97 μCi) and 20% E/P (8·0 mg; 0·27 μCi) were bulked and the 4-demethylsterol isolated by TLC (system 2; R_f 0·20). Ergosterol-[28-^{14}C] was isolated from the 4-demethylsterols by argentation TLC (silica gel G impregnated with 10% (w/w) $AgNO_3$ developed with 5% (v/v) Me_3CO in $CHCl_3$). The ergosterol-[28-^{14}C] (1·58 μCi/mg) produced a single peak on GLC (3% OV-1) which cochromatographed with authentic ergosterol; moreover the UV spectrum showed the characteristic absorption maxima of $\Delta^{5,7}$-sterols (272, 282, 293 nm with a shoulder at 262 nm in EtOH).[16]

Preparation of Palmityl-CoA. Unlabelled palmityl-CoA was prepared by the method of Seubert.[17] Palmityl-CoA-[1-^{14}C] was prepared by the method of Al-Arif and Blecher.[18]

Preparation of Phosphatidylcholine-[*acyl*-^{14}C]. This was prepared biosynthetically from acetate-[U-^{14}C] using aged potato tuber slices by the method of Galliard.[19]

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