However the observations made point to the value of continuing effort in solving the problems of triglyceride structures.

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Relation of Triglycerides to Phosphoglycerides in Fungi

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Abstract

Lipids of the fungi *Phycomyces blakesleeanus*, *Lipomyces lipoferus*, *Glomerella cingulata* and *Coprinus comatus* have been analyzed by physical and chemical methods. Triglycerides were the largest fraction of all the lipids in these fungi but significant amounts of phosphoglycerides were also present. The presence of relatively large amounts of triglycerides and phosphoglycerides, and the fatty acid patterns of these glycerides, suggests that formation of the tri- and phosphoglycerides involves participation of key intermediates from a common pathway of synthesis. The triglycerides of *Glomerella cingulata* have been studied in more detail than those of the other species. It has been found, using preparative thin-layer chromatography and analytical gas-liquid chromatograhpy, that *G. cingulata* triglycerides comprise one fraction of saturated and monoenoic fatty acids, another fraction of saturated, mono-, and dienoic acids and two fractions containing varying proportions of saturated, mono-, di-, and trienoic fatty acids.

Introduction

PREVIOUS WORK from this laboratory (2,3) has shown that triglycerides constitute the predominant class of lipids in conidia of the fungus *Glomerella cingulata*. In addition, these studies have shown that phosphoglycerides are the predominant phosphatides in the conidia. Arising from these results is the question, does the mycelium of *G. cingulata* and that of other fungi also contain large amounts of tri- and phosphoglycerides, and, if so, is there any relationship between the composition and structure of fungal triglycerides and the composition and structure of fungal phosphoglycerides?

The work presented in this paper is a preliminary report on studies that were undertaken to investigate the distribution of fatty acids among triglycerides and phosphoglycerides obtained from the fungi *Phycomyces blakesleeanus*, *Lipomyces lipoferus*, *Glomerella cingulata* and *Coprinus comatus*.

Materials and Methods

Growth of Fungi

The fungi used in this study were *Phycomyces blakesleeanus* (ATCC 6200), *Lipomyces lipoferus* (ATCC 10742), *Glomerella cingulata* (originally provided by Dr. Jack Ziffer, Pabst Laboratories, Milwaukee, Wis.) and *Coprinus comatus* (ATCC 12640). These fungi were maintained on potato dextrose agar slants. The composition of the nutrient medium (in grams per liter) for obtaining cells of *L. lipoferus* and *G. cingulata* was as follows: sucrose, 20; ammonium tartrate, 5; NH_4NO_3, 1; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.5; NaCl, 0.1; $CaCl_2$, 0.1; and trace element solution, 1 ml. The composition of the trace element solution (in milligrams per liter) was: $Na_2B_4O_7.10H_2O$, 88; $(NH_4)_6MoO_{24}$, 64; $FeCl_3.6H_2O$, 960; $ZnSO_4.7H_2O$, 8,800; $CuCl_2$, 270; $MnCl_2.4H_2O$, 72; biotin, 5. The composition of the nutrient medium (in grams per liter) for obtaining mycelium of *P. blakesleeanus* was: glucose, 50; asparagine, 2; KH_2PO_4, 1.5; yeast extract, 1; $MgSO_4.7H_2O$, 500; thiamine, 50μg. The composition of the nutrient solution (in grams per liter) for obtaining mycelium of *C. comatus* was: glucose, 100; K_2HPO_4, 3; $(NH_4)_2SO_4$, 1; yeast extract, 1; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.5; $MnSO_4.7H_2O$, 0.1; $FeSO_4.7H_2O$, 0.01; $ZnSO_4.7H_2O$, 0.01. These nutrient solutions are essentially the same as those given in references 1, 6, and 8, respectively. They were sterilized at 15 psig (125C) for 15 min. Conidia of *G. cingulata* were obtained by culturing the fungus on potato dextrose agar as described previously (2). Mycelial pellets of *P. blakesleeanus* and *G. cingulata* were harvested after 6 days' growth, and cells of *L. lipoferus* and pellets of *C. comatus* after 10 days' growth.

Standard Lipid Compounds

Cholesterol stearate, ergosterol, phosphatidylethanolamine and cardiolipin, bis(phosphatidyl)glycerol were obtained from Nutritional Biochemicals Corporation. Phosphatidylserine, phosphatidylcholine and fatty acid methyl esters were obtained from Applied Science Laboratories. Tripalmitin was obtained

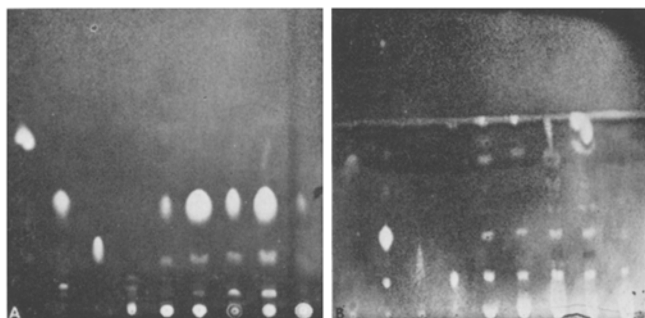

FIG. 1. Thin-layer chromatography of standard compounds and total lipid extracts on silica gel G. *A*, spotted left to right, 2 cm apart: cholesterol stearate; tripalmitin; stearic acid; ergosterol; extracts from *P. blakesleeanus, L. lipoferus, G. cingulata* mycelium, *G. cingulata* conidia, and *C. comatus*. Solvent: hexane-ether-HOAc, 80:20:1 (v/v/v). *B*, spotted left to right, 2 cm apart: bis(phosphatidyl)glycerol, phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, extracts from *P. blakesleeanus, L. lipoferus, G. cingulata* mycelium, *G. cingulata* conidia, and *C. comatus*. Solvent: $CHCl_3$-MeOH-HOH, 65:25:4 (v/v/v). Indicator: 2',7'-dichlorofluorescein.

from Mann Research Laboratories and stearic acid from California Corporation for Biochemical Research.

Solvents

Acetone, benzene (thiophene-free), chloroform, anhydrous diethyl ether, and methanol were Mallinckrodt A.R. grade and were used as supplied. The diethyl ether was stored in a refrigerator after being opened. *n*-Hexane (95%) was obtained from Ace Scientific Laboratories and was redistilled over potassium permanganate dissolved in a small volume of acetone. The fraction distilling between 67.5C and 69.0C was collected.

Extraction of Lipids

Immediately after harvesting, the mycelial pellets were placed on Büchner funnels, washed with water, and the excess water removed by suction. The mycelial pellets were then macerated in $CHCl_3$-MeOH (2:1, v/v) in a Waring Blendor for 2 min, and extracted three times for a total of 8 to 9 hr. The extractions were carried out with continuous shaking on a rotary shaker using 20 ml of $CHCl_3$-MeOH (2:1, v/v) per gram (fresh weight) of tissue. Conidia of *G. cingulata* were not broken prior to extraction.

This extraction procedure has been in use in this laboratory for three years and there has been no evidence that it leads to oxidation of the lipids.

Thin-Layer Chromatography and Column Chromatography

The analysis of total lipid extracts by thin-layer chromatography (TLC) and the separation of triglycerides from the other classes of lipids by column chromatography were carried out as described previously (2). The phospholipids (phosphoglycerides) were obtained after removal of the neutral lipids from silicic acid columns by elution with 300 ml of chloroform (20%) in methanol. In order to identify the principal spots of the commercial standards and to identify the component lipid classes of the total lipid extracts analyzed by TLC, several different qualitative tests were used (2,7). The purity of the triglyceride and phosphoglyceride fractions obtained by column chromatography also was checked by TLC on silica gel G. TLC of triglyceride fractions was carried out on a preparative scale with silver nitrate-impregnated silica gel G. The chromatoplates were prepared and developed by the methods previously described (2,7). The triglyceride samples were applied in bands, and, after development and visualization, blank areas were scraped off the chromatoplates along with the triglyceride bands. Both the blank areas and the triglyceride bands were extracted with diethyl ether. These extracts were retained for analysis of fatty acids by gas-liquid chromatography (GLC).

Gas-liquid Chromatography

GLC was carried out with an Aerograph Hy-Fi Model 600-B chromatograph equipped with a flame ionization detector. The columns were packed with poly(diethyleneglycol succinate), 10% on Gas-Chrom P, and Apiezon L, 7% also on Gas-Chrom P. Column dimensions were 5 ft x 1/8 in. Temperatures of about 175C were used for the polyester and 220C for Apiezon L. Flow rates of carrier gas were 30–36 ml/min. The hydrolysis of lipid samples and methylation of recovered fatty acids were handled as described previously (2,4). Peak areas were calculated by multiplying peak heights by the widths at half the peak heights.

Results and Discussion

It was found, both by TLC and by column chromatography, that the four fungi contained the same major classes of neutral lipids and phospholipids. Glycerides were the predominant classes of lipids in the four fungi. Figure 1 shows thin-layer chromatograms of the neutral lipids and phospholipids, respectively. Triglycerides comprised the major component of the neutral lipids in all cases. The phospholipids contained the glycerides phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine and another phosphatide, tentatively identified as bis-(phosphatidyl) glycerol or a related glyceride. The tentative identification of a phosphatidylglycerol compound is based both on the results of these experiments and on previous results obtained by infrared spectroscopy and mild alkaline hydrolysis followed by paper chromatography (2,3).

Results of GLC of fatty acid methyl esters prepared from the triglycerides and phosphoglycerides of *P. blakesleeanus, L. lipoferus, G. cingulata* and *C. coprinus* are presented in Table I. In all four fungi, the ratios of unsaturated to saturated fatty acids were greater in the phosphoglycerides than in the triglycerides. However, the same major fatty acids were present in the two classes of lipids.

Taken together, the similarities and differences in tri- and phosphoglyceride patterns could be interpreted to mean that these glycerides are formed from a common pool of diglycerides. Thus, in the formation of phosphoglycerides, the 3-positions of the diglycerides would be esterified with phosphoryl compounds to yield phosphoglycerides, while in the formation of triglycerides, the 3-positions of the diglycerides would be acylated with, on the average, a relatively large proportion of saturated fatty acids, yielding the triglycerides. It is probable that formation of diglycerides involves the esterification of L-glycerol-3-phosphate to L-1,2-diglyceride 3-phosphate and the subsequent dephosphorylation of the diglyceride phosphate (5). If the assumption of a common precursor pool is correct, then one of the more obvious deductions that could be made from the data of

TABLE I

Fatty Acid Composition of Triglycerides and Phosphoglycerides as Determined by Gas-Liquid Chromatography (Area %)

Fatty acid	Triglycerides				Phosphoglycerides			
	PB	LL	GC	CC	PB	LL	GC	CC
14:0	Trace	Trace	Trace	4.2	Trace	Trace	Trace	1.2
16:0	28.8	11.9	40.8	28.1	14.8	10.5	28.8	23.0
16:1	Trace	2.8	1.0	1.8	1.9	5.8	1.4	2.9
18:0	15.7	5.6	4.2	17.3	3.8	Trace	1.5	7.1
18:1	29.2	76.7	30.1	23.4	33.4	76.3	14.8	16.0
18:2	15.2	2.8	20.7	25.7	44.4	6.2	48.9	44.2
18:3	Trace	Trace	3.2	Trace	1.3	1.5	5.8	5.4
20:0[a]	10.8	Trace	....	Trace	....	....	....	....

PB = *Phycomyces blakesleeanus*
LL = *Lipomyces lipoferus*
GC = *Glomerella cingulata*
CC = *Coprinus comatus*
[a] Perhaps either or both 20:0 and γ18:3.

Table I is that, since fungal triglyceride fractions (like those from other biological material) seem to consist of several different triglyceride species, the phosphoglycerides also must consist of several phosphoglyceride species. Further, the tri- and phosphoglycerides should be equivalent in terms of the fatty acids located at positions 1 and 2 of their glycerol moieties. In the fungi then, the distribution of fatty acids among the triglycerides might bear a close relationship to the distribution of fatty acids among the phosphoglycerides.

During this investigation, a start was made on determining how the fatty acids are distributed among triglycerides of fungal origin. Table II shows the fatty acids that were present in four bands recovered from the separation of *G. cingulata* conidial triglycerides on silver nitrate-impregnated silica gel G. In Table II, A, B, C and D refer to bands located nearest to the solvent front on the silver nitrate-silica gel G plates, and to the bands second, third, and farthest away, respectively, from the solvent front. The third band, represented by column C, consisted of two triglyceride species. These species, however, were not well resolved in the present experiments. It is evident from Table II that band A contained saturated and monoenoic fatty acids, that band B contained saturated, mono- and dienoic acids and that bands C and D contained varying proportions of saturated, mono-, di-, and trienoic fatty acids. Band D, however, contained a much greater proportion of unsaturated acids than band C.

TABLE II

Distribution of Fatty Acids Among Triglycerides of *G. cingulata* as Determined by Gas-Liquid Chromatography (Area %)

Fatty acid	Band A	Band B	Band C	Band D
14:0	Trace	Trace	Trace	Trace
16:0	61.0	44.0	27.0	15.0
16:1	Trace	0.9	2.3	2.8
18:0	1.8	5.5	2.6	Trace
18:1	37.6	33.0	34.0	21.4
18:2	....	16.5	30.4	39.6
18:3	....	...	4.0	21.2

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