LIPIDS OF *ACANTHAMOEBA CASTELLANII*

Composition and Effects of Phagocytosis on Incorporation of Radioactive Precursors

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ABSTRACT

The lipids of *Acanthamoeba castellanii* (Neff) consist of 52% neutral lipids and 48% polar lipids. Triglycerides account for 75% and free sterols for 17% of the neutral lipids. The major phospholipids are phosphatidylcholine (45%), phosphatidylethanolamine (33%), phosphatidylserine (10%), a phosphoinositide (6%), and diphosphatidylglycerol (4%). The phosphoinositide is unique in that it contains fatty acids, aldehyde, inositol, and phosphate in the ratio of 1.4:0.5:1.1, but it contains no glycerol. Sphingomyelin, cerebrosides, psychosine, and glycoglycerides were not detected, but small amounts of unidentified long chain bases and sugars are present. The rates of uptake of palmitate-1-^{14}C and of its incorporation into glycerides and phospholipids were not affected by the phagocytosis of polystyrene latex beads. Although phagocytosis usually decreased the uptake by amebas of phosphate-^{32}P, serine-U-^{14}C, and inositol-2-^{3}H, their subsequent incroporation into phospholipids was not demonstrably stimulated or inhibited by phagocytosis. Phagocytosis did seem to increase the incorporation into ameba phospholipids of phosphatidylcholine-1,2-^{14}C but not that of phosphatidylethanolamine-1,2-^{14}C. These experiments, in which the incorporation of radioactive precursors into total cell lipids was measured, do not, of course, eliminate the possibility that localized effects may occur.

INTRODUCTION

Weisman and Korn (1, 2) have described some of the initial biochemical and morphological events of phagocytosis in *Acanthamoeba castellanii* (Neff). The amebas will ingest large quantities of latex beads within 15–30 min by a highly selective process. When the ingested beads are of diameter 1 μ or greater, most of the beads are individually contained within a phagocytic vesicle closely surrounded by plasma membrane (2, 3). This allows one to calculate the amount of plasma membrane that has been internalized during the phagocytic process. It is not unusual (1, 3) for the cell to convert to phagocytic vesicles an amount of plasma membrane equal to 50–100% of the surface area of a sphere of the same volume as the ameba. Since no detectable volume change occurs during this process (1), it seemed reasonable to expect (although other explanations are possible [1]) that phagocytosis might stimulate the synthesis or, at least, the turnover of the plasma membrane. Lipids, especially phospholipids, are major constituents of membranes and, therefore, it is of

interest to investigate their metabolism within such a context.

MATERIALS AND METHODS

Materials

Phosphatidylcholine-1,2-^{14}C (5 μc/μmole), phosphatidylethanolamine-1, 2-^{14}C (5 μc/μmole), and phosphate-^{32}P were obtained from Tracerlab, Waltham, Mass. Inositol-2-^{3}H (1.27 mc/μmole), palmitic acid-1-^{14}C (2 μc/μmole), and serine-U-^{14}C (150 μc/μmole) were purchased from New England Nuclear Corp., Boston, Mass. Phospholipid standards and nonradioactive palmitic acid were obtained from either Supelco, Inc., Bellefonte, Pa. or Applied Science Laboratories Inc., State College, Pa. Bovine serum albumin (Fraction V, Armour Pharmaceutical Co., Kankakee, Ill.) was extracted with isooctane and glacial acetic acid according to Goodman (4) to free it of associated fatty acids. Precoated silica gel plates for thin-layer chromatography were obtained from Brinkmann Instruments Inc., Westbury, N. Y., and silicic acid (Unisil, 100–200 mesh) for column chromatography was purchased from Clarkson Chemical Co., Williamsport, Pa. Monodisperse preparations of polystyrene latex beads of diameter 1.099 μ were obtained from The Dow Chemical Co., Midland, Mich.

The fatty acid-albumin complex was prepared with radioactive palmitic acid diluted with nonradioactive palmitic acid to a specific activity of 2.5 $\times$ 10^5 cpm/μmole. The palmitic acid was converted to the sodium salt and was then mixed with a 7% solution of fatty acid-free albumin in the molar ratio of 8:1 (fatty acid:albumin).

Cells

Acanthamoeba castellanii (Neff) was grown on proteose peptone-glucose medium, pH 6.8, in shaking cultures at room temperature for 6–7 days (5). Encysted cells were few (about 1–4%) but increased in number in older cultures.

Lipid Extraction for Determination of Composition

Amebas were harvested by centrifugation at 500 *g* for 5 min, and the cells were extracted with 20 vol of chloroform:methanol, 2:1, for 3 hr at room temperature on a magnetic stirrer. Insoluble material was removed by filtration, and the filtrate was washed with 0.73% NaCl (6) to remove water-soluble components. The chloroform phase was evaporated to dryness under reduced pressure, and the lipids were dissolved in a small volume of heptane.

Column Chromatography

A solution of lipids in heptane was applied to a column of silicic acid (which had previously been activated at 110°C for at least overnight), using 1 g of silicic acid for 20 mg of phospholipid. Sterol esters were eluted from the column with 1% ether in heptane, triglycerides with 8% ether, sterols and diglycerides with 50% ether, and monoglycerides with 100% ether. Phospholipids were then eluted from the same column. Diphosphatidylglycerol and several acidic phosphatides were eluted with 5% methanol in chloroform; 96% of the phosphatidylethanolamine, and 48% of the phosphatidylserine with 15% methanol; 4% of the phosphatidylethanolamine, 44% of the phosphatidylserine, 85% of the phosphoinositide, and 16% of the phosphatidylcholine with 25% methanol; 8% of the phosphatidylserine, 15% of the phosphoinositide, and 82% of the phosphatidylcholine with 50% methanol; and the remaining 2% of phosphatidylcholine and lysophosphatidylcholine with 100% methanol. 20 ml of each solvent were used for each gram of silicic acid.

Thin-Layer Chromatography

The composition of each of the above fractions was determined by analytical thin-layer chromatography, and bulk separation of lipids within each fraction was accomplished by thin-layer chromatography on preparative plates. All plates were activated at 105°C for 30 min immediately before use. The neutral lipids that were eluted from the silicic acid column by 1% ether in heptane were chromatographed with a solvent system of heptane:ether:acetic acid, 70:30:1, which separated sterol esters from triglycerides. All other neutral lipid fractions were chromatographed with a solvent composed of benzene:ether:acetic acid, 45:70:1, which provided good separation of triglycerides, 1,3-diglycerides, 1,2-diglycerides, sterols, and monoglycerides.

The polar lipids that were eluted from the silicic acid column with 5% methanol in chloroform were chromatographed on thin-layer plates in a solvent of chloroform:methanol:water, 100:40:6. The polar lipids in the 15, 25, and 50% methanol eluates were further separated by thin-layer chromatography using either chloroform:methanol:water, 60:60:3, or chloroform:methanol:water:acetic acid, 80:80:8:4. Choice between these two solvents was dictated by differences in the several batches of preparative plates used.[1] Both systems gave good separation of phosphatidylethanolamine, phosphatidylinositol, phos-

[1] Preparative thin-layer plates (2 mm-thick) that were obtained recently from Brinkman Instruments Inc. have not provided satisfactory separation of phospholipids.

phatidylserine, phosphatidylcholine, and lysophosphatidylcholine. Better separation of sphingomyelin from lysophosphatidylcholine and phosphatidylcholine was obtained with chloroform:methanol:water:acetic acid, 100:60:16:8, so that system was used for the 100% methanol eluate from the column. All of the polar lipids were also chromatographed in a basic system of chloroform:methanol:7 N ammonia, 60:35:5.

For all separations, paper-lined chromatographic tanks were allowed to equilibrate with the solvent for 1 hr before use. Full development of the preparative plates required approximately 2.5–3 hr. Lipids were detected by exposing the plates to iodine vapors. The iodine was then allowed to evaporate, and the desired areas of silica gel were scraped off the plate, powdered, and extracted twice with ethanol:chloroform:water:acetic acid, 100:30:20:2 (7). Between 75 and 85% of the phospholipid was recovered by this procedure.

Hydrolysis Procedures

In addition to their chromatographic behavior most of the lipids were identified by chemical analyses. Some of the methods required prior hydrolysis of the phospholipids. Phospholipids were hydrolyzed in 6 N HCl for 18 hr at 122°C in the autoclave. The tubes were covered with marbles and an inverted beaker to prevent dilution of the samples by condensing steam vapors.

Before hydrolysis of the glycolipids and the compounds containing long chain base, the phospholipids were removed by methanolysis at room temperature for 30 min in 0.5 N NaOH in methanol. The mixture was acidified and diluted with water and then extracted with chloroform. The aqueous phase was evaporated to dryness and hydrolyzed in 4 N HCl in methanol for 4 hr at 105°C using screwcap tubes with Teflon-lined caps (8). This hydrolysate contained sugars but no long chain base. The chloroform phase was separated by chromatography on a column of silicic acid that was eluted first with chloroform and then with methanol. The chloroform eluate was discarded, and the methanol eluate was hydrolyzed as above. This hydrolysate contained long chain base but no sugar.

Analytical Methods

Sterols were determined by the Liebermann-Burchard reaction (9) and by gas-liquid chromatography on 3% OV–17 (a 50:50 phenyl methyl siloxane; Applied Science Laboratories Inc., State College, Pa.) at 270°C using a hydrogen flame detector (10). Esters were measured as hydroxamates (11), and plasmalogens were analyzed by the method of Rapport and Alonzo (12). Total phosphate (after ashing the sample by heating with $Mg(NO_2)_2$) and inorganic phosphate were determined by the method of Chen et al. (13). Total nitrogen was measured by the Kjehldahl procedure (14). Serine and ethanolamine were determined in the hydrolysates either by reaction with ninhydrin (15, 16) or with 2,4-dinitrofluorobenzene (17). Sulfate was measured according to Spencer (18), and choline was measured by the method of Collins and Wheeldon (19) but without dilution with ethanol. Glycerol was determined by periodate oxidation to formaldehyde, which was measured with chromotropic acid (20). Inositol was measured by quantitative gas-liquid chromatography of the trimethylsilyl derivative on a column of 17% ethylene glycol succinate at 165°C using a hydrogen-flame detector (21). Total sugars were determined by the method of Walborg and Christensson (22), amino-sugars by the method of Blix (23); and glucose and galactose were estimated enzymatically using the glucostat and galactostat kits obtained from Worthington Biochemical Corporation, Freehold, N.J.

To determine the fatty acid composition of the lipids, samples were saponified in 1 N NaOH in 50% ethanol for 3 hr at 80°C. The solution was acidified and the fatty acids were extracted into heptane which was then evaporated to dryness under a stream of nitrogen. The fatty acids were converted to their methyl esters by heating for 2 min at 80°C in 5% BF_3 in methanol. Samples were analyzed by gas-liquid chromatography on a column of 17% ethylene glycol succinate at 180°C.

Incubations

Amebas were harvested by centrifugation at 500 *g* for 5 min and resuspended in fresh medium at a concentration of 3–4 $\times$ 10^6 cells/ml as determined by measuring the absorbance at 660 mμ (3). The cell suspension was placed on a rotating shaker and allowed to equilibrate for 20 min at room temperature (22–26°C) before addition of latex beads or radio-active precursors. Incubations were terminated by the addition of 2,4-dinitrophenol to a final concentration of 2 $\times$ 10^{-4} M (1). The amebas were collected by centrifugation at 500 *g*, washed twice with cold 0.02 M phosphate buffer, pH 6.8 (to dilute the specific radioactivity of phosphate-^{32}P if present), and once with cold 0.02 M tris(hydroxymethyl)aminomethane, pH 6.8 (to remove most of the inorganic phosphate).

Lipids were extracted with 20 vol of 95% ethanol for 3 hr at room temperature. Chloroform:methanol could not be used because it also dissolved the polystyrene latex, which then interfered with the subsequent chromatographic and analytical procedures. Control experiments proved that all of the lipids that could be extracted with chloroform-methanol were also extracted with ethanol. The ethanolic extract was evaporated to dryness under reduced pressure

and the lipids were dissolved in a small volume of chloroform:methanol, 2:1, which was washed with 0.73% NaCl. The chloroform phase was evaporated to dryness, the lipids were dissolved in heptane and were fractionated in the usual way on columns of silicic acid, and sometimes by thin-layer chromatography. In some experiments fatty acids and glycerides were separated by partitioning the neutral lipid fraction between aqueous, alkaline isopropanol and heptane (5). Glycerides were in the heptane phase, and fatty acids were extracted from the aqueous isopropanol with heptane after acidification.

The uptake of latex beads was measured on other aliquots of cells taken from the incubation flasks, and the cells were washed in the same way as those used for the lipid analyses. Polystyrene was extracted from the washed cells with dioxane and quantitatively measured by the absorbance at 259 mμ (1).

For determination of the specific radioactivity of adenosine triphosphate, additional samples of washed cells from the incubation flask were extracted with trichloroacetic acid and nucleotides were adsorbed onto activated charcoal. Acid-labile phosphate was released by hydrolysis for 10 min in 1 N HCl (24). Aliquots were taken for measurement of inorganic phosphate and radioactivity.

Measurements of Radioactivity.

Lipids were dissolved in toluene containing 0.4% 2,5-diphenyloxazole. Water-soluble compounds were dissolved in a solution of dioxane:ethylene glycol:methanol, 88:2:10, containing 6% naphthalene and 0.4% 2,5-diphenyloxazole. Counting efficiencies in a Liquid Scintillation Counter Model 720, Nuclear-Chicago Corporation, Des Plaines, Ill., were 80% for ^{14}C and 24% for ^{3}H and was assumed to be 100% for ^{32}P. The samples were unquenched, so that no correction was necessary.

RESULTS

Lipid Composition

The total lipid composition of *Acanthamoeba castellanii* (Neff) is given in Table I. The composition of the neutral lipids is not remarkable. The sterols consisted almost entirely of ergosterol and 7-dehydroporiferasterol[2] in the ratio of 2:3 as reported previously by Smith and Korn (10). Minor sterols, probably the 22,23-dihydroderivatives of the major sterols, 22-dihydroergosterol and 7-dehydroclionasterol, were also present.

The identities of the phospholipids in the column eluates were established by thin-layer chromatography and by the analytical data shown in Table II. The composition of the total phospholipid fraction is summarized in Table I. Phosphatidylcholine and phosphatidylethanolamine were the major phospholipids. No other phospholipid accounted for more than 10% of the total. Sphingomyelin was not present in any of the preparations.

The acidic phosphatides included two compounds that were eluted with 5% methanol in chloroform and had R_f's on thin-layer chromatography (0.06 and 0.18) that were much less than that of diphosphatidylglycerol (0.39). Nitrogen was not associated with either of these compounds. A third phosphatide that also contained no nitrogen but had an R_f of 0.6, sometimes occurred in the 15% methanol eluate. These unknown acidic phosphatides are of special interest because of their rapid labeling by radioactive precursors.

The phosphoinositide, which accounted for about 6% of the total phospholipids, is a unique compound in that it contains no glycerol. The absence of glycerol was not due to an inability to detect it. Under identical conditions an authentic sample of phosphatidylinositol was found to contain 2.1 μmoles of glycerol, 1.9 μmoles of inositol, and 1.9 μmoles of phosphorus in good agreement with the theoretical ratio of 1:1:1. No ninhydrin-positive material, long chain base, or sugar was associated with the ameba phosphoinositide. The ester:inositol:phosphorus ratio of one sample was 1.4:0.9:1 (Table II), and another sample contained 15.7 μmoles ester, 6.0 μmoles aldehyde, and 11.3 μmoles phosphorus (Table III) for an ester:aldehyde:phosphorus ratio of 1.4:0.5:1. This was a much higher content of aldehyde than was present in any of the other lipid fractions (Table III). Although detailed structural studies have not yet been done, the phosphoinositide is probably a disubstituted inositol phosphate in which two molecules of fatty acid plus fatty aldehyde (in the ratio of approximately 2:1) are directly linked to the inositol moiety. The R_f of the unknown phosphoinositide was identical with that of phosphatidylinositol in both the acidic and basic systems.

Glycolipids and compounds containing long

[2] This compound was previously identified as 7-dehydrostigmasterol, the C-24 isomer of 7-dehydroporiferasterol. Since 7-dehydroporiferasterol has the same configuration as ergosterol (25), it is more likely that it, and not 7-dehydrostigmasterol, is the major sterol of this ameba. None of the criteria used will distinguish the two isomers, nor will they distinguish ergosterol from its C-24 isomer, 7,22-didehydrocampesterol.

TABLE I

The Lipid Composition of Acanthamoeba castellanii (Neff)

	Molar per cent of total lipids*	Molar per cent of lipid class*
Neutral lipid	52.2 (50.3–55.3; 3)	
Sterol, ester		0.7 (0.6–0.7; 3)
Sterol, free		16.7 (16.5–16.8; 3)
Triglycerides		74.7 (74.0–75.3; 3)
1,2-Diglycerides		1.9 (1.6–2.2; 3)
1,3-Diglycerides		2.4 (1.4–3.1; 3)
Monoglycerides		0.5 (0–1.0; 3)
Unknown ester		3.3 (2.3–3.8; 3)
Phospholipids	43.2 (41.7–45.3; 3)	
Diphosphatidylglycerol		4.2 (4.0–4.4; 6)
Acidic phosphatides		2.3 (2.0–2.7; 4)
Phosphatidylethanolamine		33.2 (32.9–34.6; 7)
Phosphatidylserine		9.5 (9.0–10.0; 7)
Phosphoinositide		5.8 (5.7–5.9; 3)
Phosphatidylcholine		44.5 (43.2–46.7; 6)
Lysophosphatidylcholine		0.5 (0.3–0.6; 4)
Unknown phosphatides		1.8 (1.5–2.6; 4)
Long chain base‡	2.8 (2.7–2.9; 2)	
Glycolipids‡	1.8	

* The mean values are listed and the range of values and number of determinations are given within the parentheses. The absolute amounts of lipid in 10^8 amebas are: neutral lipid 6.16 μmoles, phospholipid 5.11 μmoles, long chain base 0.33 μmoles, and glycolipids 0.22 μmoles.

‡ Distributed among several fractions obtained by silicic acid column chromatography. See Table IV.

chain base were distributed among the methanol-chloroform eluates of the silicic acid column as shown in Table IV. Neither the sugars nor the bases were identified. Galactose, glucose, and amino-sugars were not detected. Compounds such as glycoglycerides, monoglycocerebrosides, or psychosine were not detected by thin-layer chromatography. No evidence was obtained to indicate that the sugars and long chain bases were in the same compounds.

There were no major differences among the fatty acids of the glycerides and phospholipids except for a somewhat higher content of palmitic acid in the neutral lipids (Table V). With two exceptions, oleic acid was the predominant fatty acid. The major fatty acid of phosphatidylserine was 11,14-eicosadienoic acid, and linoleic acid was the major fatty acid of the phosphoinositide. Diphosphatidylglycerol had more arachidonic acid, and the phosphoinositide had less, than did the other lipids. Also of interest, was the presence in the phosphoinositide of an unknown fatty acid with a retention time slightly less than that of methyl eicosadienoate.

Incorporation of Radioactive Precursors into Lipids during Phagocytosis

Weisman and Korn (5) have shown that amebas rapidly take up fatty acids, which are subsequently incorporated into glycerides and phospholipids. The rates of the esterification reactions are slower than the initial rate of uptake of the fatty acid. If phagocytosis were to stimulate membrane synthesis or turnover, the rates of uptake or of esterification of fatty acids might be increased. However, no effect of phagocytosis on the rates of labeling of glycerides or phospholipids was observed in several experiments in which amebas were incubated simultaneously with latex beads and radioactive palmitate, and in experiments in which amebas were preloaded with unesterified radioactive palmitate and then incubated with latex beads in the absence of exogenous fatty acid, in order to separate the esterification reactions from the process of fatty acid uptake.

The incorporation of palmitate-1-^{14}C into individual phospholipids was also measured (Table VI). No significant, reproducible differences in the

TABLE II

Analytical Data for the Major Phospholipids of Acanthamoeba castellanii (Neff)

Compound	Phosphorus*	Ester	Glycerol	Total nitrogen	Ethanolamine	Serine‡	Choline	Inositol
	μmoles	*μmoles*	*μmoles*	μmoles	*μmoles*	*μmoles*	*μmoles*	*μmoles*
Diphosphatidylglycerol	9.5 (1)§	19.2 (2.0)	14.1 (1.5)					
Phosphatidylethanolamine	23.8 (1)	47.1 (2.0)	23.9 (1.1)	25.9 (1.1)				
	13.1 (1)	29.8 (2.3)	15.7 (1.2)		15.2 (1.2)			
Phosphatidylserine	1.6 (1)	3.0 (1.9)	1.3 (0.8)	1.6 (1.0)		1.3 (0.8)		
Phosphoinositide	1.6 (1)	2.3 (1.4)	0.1 (0.06)					1.4 (0.9)
Phosphatidylcholine	7.5 (1)	15.0 (1.1)	7.3 (1.0)				7.1 (0.9)	

* Inorganic phosphorus after hydrolysis. This accounted for 95–100% of the total phosphorus.

‡ Ninhydrin

§ Values in parentheses are normalized to the phosphorus analysis. The theoretical ratios were obtained for standard samples of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, and phosphatidylinositol.

TABLE III

Plasmalogen Content of Purified Lipids and Silicic Acid Column Fractions

Fraction analyzed	Plasmalogen
	% of ester
Triglycerides	5.9
Diphosphatidylglycerol	6.0
Phosphoinositide	37.2
Phosphatidylethanolamine	3.9
Phosphatidylcholine	5.0
5% Methanol fraction	8.0
15% Methanol fraction	4.8
25% Methanol fraction	6.9
50% Methanol fraction	6.4
100% Methanol fraction	(12.4)*

* This value may be too high because of the difficulty in measuring accurately the very small amount of ester present.

TABLE IV

Distribution of Glycolipids and Long Chain Base Among the Fractions from the Silicic Acid Column

Column fraction	Long chain base	Glycolipids
% methanol in chloroform	*μmoles/10³ μmoles phospholipid*	
5	21.5	3.9
15	11.4	2.2
25	3.4	6.5
50	24.1	30.6
100	5.5	—

TABLE V

Fatty Acid Composition of the Major Lipids of Acanthamoeba castellanii (Neff)

	14:0	16:0	16:1	18:0	18:1	18:2	20:1	20:2	20:3	20:4
	%	%	%	%	%	%	%	%	%	%
Diphosphatidyl-glycerol	4.8	1.2	0.8	1.8	50.1	5.9	0.3	6.8	4.7	23.5
Phosphoinositide*	1.0	2.9	2.6	1.3	31.0	42.7	1.8	3.9	5.9	3.0
Phosphatidylserine	0.9	3.2	1.1	2.8	26.5	12.7	2.5	28.8	6.9	14.5
Phosphatidyl-ethanolamine	3.7	5.1	1.4	9.1	47.4	7.8	0.6	8.5	5.0	11.6
Phosphatidyl-choline	0.7	5.4	0.7	9.4	39.3	9.5	1.1	9.2	9.2	15.5
Diglyceride	3.6	10.8	2.3	6.2	39.3	12.1	1.4	8.8	4.4	11.0
Triglyceride	7.2	9.5	2.3	2.9	44.9	8.4	3.1	7.6	4.2	9.9

The fatty acids have been previously identified (26) as follows: 14:0, myristic; 16:0, palmitic; 16:1, palmitoleic; 18:0, stearic; 18:1, oleic; 18:2, linoleic; 20:1, 11-eicosenoic; 20:2, 11,14-eicosadienoic; 20:3, 8,11,14-eicosatrienoic; 20:4, arachidonic.

* An unidentified compound that appears on the chromatogram just before 20:2 accounts for 4.0% of the fatty acids of phosphoinositide. It appears to be present only in this fraction.

TABLE VI

Incorporation of Palmitate-1-^{14}C into the Phospholipids of Acanthamoeba castellanii (Neff) During Phagocytosis

Experiment	Uptake of beads	Uptake of palmitate into total lipids	Specific radioactivity of phospholipids				
			AP	PE	PS	PC	LPC
	mg	*μmoles*	*cpm* × 10^{-3}/*μmole P*				
I	0	2.2	12.9	2.0	0.5	3.3	0.6
	116	1.9	11.3	2.7	0.3	3.3	0.5
II	0	5.4	67	8.1	0.7	11.6	2.8
	72	5.1	50	8.4	—	13.3	1.9

Amebas (1.8 × 10^6/ml) were incubated at 30°C with palmitate-1-^{14}C (0.8 μmoles/ml) as a complex with albumin (8:1) without and with latex beads (1 mg/ml) in a total of 200 ml of medium. Incubations were continued for 30 min at which time the cells were analyzed for the uptake of beads, and the distribution of radioactivity among the phospholipids was determined. The results of two experiments are shown.
AP, acidic phosphatides; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; LPC, lysophosphatidylcholine.

distribution of radioactivity among the phospholipids were induced by phagocytosis. It should be noted that the acidic phosphatides were the most highly labeled phospholipids in these experiments.

Phagocytosis also had no demonstrable effect on the incorporation of phosphate-^{32}P into the phospholipids extracted from the whole cell. The results of a representative experiment are shown in Table VII. In all the experiments the specific activity of ATP was reduced in those cells that were phagocytosing when compared to the control cells. At the same time, the specific activities of all of the phospholipid fractions were reduced by the same factor. No evidence was found for an increase (or decrease) in the rate of conversion of ATP to the phospholipids due to phagocytosis. This was true when the phosphate-^{32}P was added to the incubation flask at the same time as the latex beads or 30 min after the onset of phagocytosis; when cells of different ages were compared to insure that actively growing cultures were used; and when, in an effort to observe a possible recovery period after the termination of phagocytosis, phosphate-^{32}P was added to the amebas at different times after phagocytosis was complete and beads were no longer present.

TABLE VII

Incorporation of Phosphate-^{32}P into the Phospholipids of Acanthamoeba castellanii (Neff) During Phagocytosis

Flask	Time of Addition to flask: Beads	Time of Addition to flask: $^{32}PO_4$	Uptake of beads	ATP	Column fraction: 5%	15%	25%	50%	100%*
	min		*mg*	*cpm* × $10^{-3}/\mu mole$	*cqm* × $10^{-3}/\mu mole$				
1	—	30	—	814	88	17	26	16	89
2	30	30	36.5	459	57	10	14	10	64
3	0	30	68.4	431	63	9	16	12	92

Each flask contained 3.3 × 10^6 amebas/ml in a total volume of 150 ml of medium. Polystyrene latex beads were added to Flask 3 at the beginning of the incubation (0 min) and to Flask 2 at 30 min. No beads were added to Flask 1. At 30 min, 3.6 mc of phosphate-^{32}P were added to all three flasks and the incubation was continued for an additional 30 min. The incubation was stopped by the addition of dinitrophenol, and the cells were washed, and the lipids were extracted as described under Materials and Methods. The phospholipids were separated into fractions that eluted from silicic acid in 5%, 15%, 25%, 50% methanol in chloroform, and 100% methanol. Each fraction was analyzed for phosphorus and for radioactivity. The specific activity of the ATP was determined on a separate sample of cells as described under Materials and Methods. The distribution of phosphorus was 5%, 1.1 μmoles; 15%, 8.2 μmoles; 25%, 4.5 μmoles; 50%, 8.3 μmoles; 100%, 0.5 μmoles.

* The very low amount of phosphorus in this fraction introduces the possibility of error in the calculation of specific activity.

These conclusions were strengthened by determining the specific activity of the individual phospholipids. The 50% methanol fraction is 95% phosphatidylcholine so that even without further purification the specific activity of that fraction can be taken to be the specific activity of phosphatidylcholine. Similarly, the specific activity of the 15% methanol fraction is essentially that of phosphatidylethanolamine, which comprises 90% of that fraction. Diphosphatidylglycerol and the two acidic phosphatides were separated from the 5% methanol fraction in several experiments, two of which are reported in Table VIII. Again, the effect of phagocytosis was a decrease in the specific activity of the phospholipids, which can be entirely explained by the reduction in specific activity of the ATP. It is of interest that, as was found in the experiments with palmitate-1-^{14}C, the two unidentified acidic phosphatides were always the most highly labeled phospholipids.

Experiments with inositol-2-^3H and serine-U-^{14}C confirmed the negative results. Of the total radioactive inositol incorporated into the lipids, 90% was found in the 25% methanol fraction. The specific radioactivity of this fraction was reduced by about 50% in the cells that had phagocytosed beads, indicating that the uptake of inositol was also inhibited by concomitant phagocytosis. Phagocytosis had only a slight inhibitory effect on the incorporation of radioactive serine into phosphatidylserine and phosphatidylethanolamine.

TABLE VIII

Specific Radioactivity of Diphosphatidylglycerol and the Acidic Phosphatides Isolated by Thin-Layer Chromatography

Phospholipid	Specfic radioactivity: I −Beads	I +Beads	II −Beads	II +Beads
	cpm × $10^{-3}/umole\ P$			
Diphosphatidylglycerol	4.7	1.3	3.8	1.6
Acidic phosphatide 1	255.8	108.5	318.5	131.3
Acidic phosphatide 2	223.9	134.4	263.2	161.9

Two experiments have been carried out to determine the effect of phagocytosis on the incorpo-

ration into the amebas of radioactive phospholipids. Both gave similar results and demonstrated little or no effect of phagocytosis on the incorporation of phosphatidylethanolamine into ameba lipids. The incorporation of phosphatidylcholine, however, was stimulated twofold by concomitant phagocytosis. Only insignificant amounts of the radioactive phospholipids were adsorbed onto the latex beads, and more phosphatidylethanolamine was adsorbed than was phosphatidylcholine.

Quantitative and qualitative analyses of the ameba lipids before and after phagocytosis revealed no changes in the total amount of phospholipid nor its per cent composition.

DISCUSSION

Karnovsky and his collaborators (27–30) demonstrated that phagocytosis by polymorphonuclear leukocytes stimulated the incorporation of phosphate-^{32}P and acetate-1-^{14}C into the lipids of the whole cell. Synthesis of new membrane *in toto* did not seem to be involved, however, because the increased incorporation of phosphate-^{32}P was specific, in their first experiments, for phosphatidylserine, phosphatidylinositol, and phosphatidic acid, which are not the major phospholipids of the membrane. Moreover, Sastry and Hokin (31), although able to confirm the results for phosphatidylinositol (into which the incorporation of inositol-2-^{3}H was also increased) and phosphatidic acid, did not observe a stimulation of the turnover of phosphatidylserine. In the meantime, Karnovsky (32) was unable to confirm his initial results for phosphatidic acid. Thus, phosphatidylinositol appeared to be the only phospholipid of polymorphonuclear leukocytes whose metabolism is consistently influenced by phagocytosis. Similar experiments by Oren et al. (29) revealed no effect of phagocytosis on the incorporation of radioactive precursors into any of the lipids of alveolar macrophages.

In marked contrast to these observations, Elsbach (33) has found that phagocytosis stimulates the incorporation of lysophosphatidylcholine and lysophosphatidylethanolamine into the phosphatidylcholine and phosphatidylethanolamine, respectively, of both polymorphonuclear leukocytes and alveolar macrophages. Thus, turnover, of major membrane phospholipids may be stimulated by phagocytosis but be undetected in experiments in which phosphate-^{32}P is used as tracer.

In the experiments described in the present paper, phagocytosis did not stimulate the incorporation of radioactive precursors into the lipids of *Acanthamoeba castellanii* (Neff). This was true despite the fact that in many of these experiments, as in previous ones (1, 3), the amount of plasma membrane that was internalized as phagocytic vesicles approximates the surface area of a sphere of the same volume as the ameba.

Any of several possibilities will explain this apparent paradox. There may be no replacement of plasma membrane other than by the extension of infoldings of the surface, or by stretching of the membrane itself. Surface membrane might be replaced by intracellular membranes, although no morphological evidence for this exists. New membrane may, indeed, be synthesized but from macromolecular precursors that do not equilibrate rapidly with the radioactive substrates that were added. Possibly the apparent stimulation of the incorporation of phosphatidylcholine is an indication of this latter process, although the absence of a similar effect for phosphatidylethanolamine argues against it. Such a process would not be expected to be specific for a particular phospholipid. In view of the recently published observations of Elsbach (33), it is possible that the incorporation of radioactive phospholipids may have been, in part, due to the presence of small amounts of undetected lyso-compounds. If this were so, then the differences between phosphatidylcholine and phosphatidylethanolamine might be the result of differences in the concentration of radioactive lysophosphatidylcholine and lysophosphatidylethanolamine.

A final possibility that must be seriously considered is that there are, in fact, local effects on the turnover of phospholipids that are not reflected in measurements of the turnover of total cell lipids. The cell may be synthesizing phospholipids at a maximal rate, so that stimulation of synthesis or turnover at the plasma membrane is balanced by a decrease elsewhere in the cell. No change would be detected when measurements are made on the lipids of total extracts as in the present experiments. The converse of this hypothesis is, of course, equally possible, i.e. the specific effects observed by others in similar experiments with other cell types might not reflect events occurring at the cell surface. To test for this properly one must isolate the plasma membrane as a discrete entity and measure the incorporation of radioactive pre-

cursors into its lipids and proteins. Experiments are under way in this laboratory to investigate the labeling of the plasma membrane and the labeling of the membrane of the phagocytic vesicles (3) during phagocytosis.

The identity of the unknown phosphoinositide is being investigated further. To our knowledge no compound has hitherto been found that contains inositol and phosphorus but no glycerol or long chain base. The acylinositolmannoside of bacteria (34) does provide an analogy for the direct esterification of long chain fatty acids to inositol, but that compound does not contain phosphorus. Other compounds of interest are the unidentified acidic phosphatides which are much more extensively labeled by radioactive fatty acid and phosphate than is any other phospholipid. These compounds are chromatographically separable from phosphatidic acid whose presence could not be detected in the ameba phospholipids.

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