ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 171, 55–64 (1975)

Phospholipids of *Entamoeba invadens*

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Received April 18, 1975

The major phosphoglycerides present in *Entamoeba invadens* are phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol. Furthermore, three different sphingolipids could be isolated from the amoeba. In addition to sphingomyelin and a phosphonolipid, ceramide phosphonylethanolamine, a previously unknown sphingolipid was present. This sphingolipid contained a long chain base, inositol, and phosphorus in the ratio of 0.97:0.97: 1.0 and could be identified as ceramide phosphorylinositol. The various individual phospholipids showed different rates of turnover. Phosphatidic acid and phosphatidylinositol had, relative to the other phospholipids, a short half-time of about 12 h. Phosphatidylethanolamine and ceramide phosphorylinositol had a half-time of about 24 and 30 h, respectively. The major phospholipid, phosphatidylcholine, and also sphingomyelin and phosphatidylserine showed no turnover. In contrast to the phosphoglycerides, the sphingolipid composition of the amoeba cultivated in different media was rather variable, while the total sphingolipid content remained at 21% of the total amount of phospholipids. The amount of ceramide phosphorylinositol was almost doubled in the cells cultivated on the serum-free medium (T), whereas the amount of sphingomyelin and ceramide phosphonylethanolamine decreased. Evidence is presented that these alterations in the sphingolipid composition of *E. invadens* are related to the amount of unsaturated fatty acids which were present in the culture medium.

INTRODUCTION

The phospholipid composition of a small number of protozoa has been described; among these are *Acanthamoeba castellanii* (1), *Entodinium caudatum* (2), *Tetrahymena pyriformis* (3), *Crithidia* (4), *Trypanosoma vivax* (5) and *Plasmodium knowlesi* (6). In general the phospholipid composition of these protozoa resembles that of mammalian tissue to a certain extent; large amounts phosphatidylcholine and phosphatidylethanolamine and minor amounts of phosphatidylserine, phosphatidylinositol and sphingomyelin were reported to be present (1–3, 4, 6). Besides these common lipids, several protozoa appear to contain one or more unusual phospholipids. In *E. caudatum* and *T. pyriformis* aminoethylphosphonate-containing lipids have been detected in the form of diacylglycerols as well as ceramides and plasmalogens (2, 3). Also a monomethylaminoethylphosphonate has been detected (7). *A. castellanii* was found to have an inositol-containing phospholipid which does not contain glycerol or a long chain base (1). Phosphatidyl-*N*-(2-hydroxyethyl)-alanine has been isolated from *E. caudatum* (8).

The phospholipid composition of *Entamoeba invadens* and *Entamoeba histolytica* has not been studied in detail. Sawyer *et al.* (9) reported that ethanolamine, choline, serine, inositol, sphingosine and amino compounds of unknown structure are present in the water-soluble fraction of the saponified total lipid extract of this

[1] The data presented in this paper have been taken from a thesis submitted in March 1974 by H.H.D.M. van Vliet in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Utrecht. Present address: Gaubius Institute, Health Research Organization T.N.O., Herenstraat 5d, Leiden, The Netherlands.

organism. This present report describes the phospholipid composition of *E. invadens* in detail, emphasizing the structure of a new, inositol-containing phospholipid that has been characterized as ceramide phosphorylinositol.

MATERIALS AND METHODS

Growth of the Amoebae. Entamoeba invadens (PX strain clone IV, a gift of Dr. L. S. Diamond) was cultivated in 4-liter culture medium without stirring at 28°C. For identification of the phospholipids, the amoebae were grown on the serum-containing medium (S) described by Diamond, with the exception that serum prepared from cow blood was used instead of horse serum as originally described (10). For the quantitative determination of the phospholipids, cells were cultivated in medium S as well as in a serum-free medium (T). Medium T was basically the same as medium S, only the serum was replaced by a mixture of cholesterol and oleic acid, solubilized by albumin, and Tween 80 (or Tween 40), as described before (11). Labeling of the phospholipids for autoradiography was obtained by the addition of [^{32}P]phosphate to the medium. The cells were harvested after 8–10 days by centrifugation at 800*g* and washed with a NaCl/phosphate buffer (pH 7.0).

Extraction and isolation of the phospholipids. The cells were extracted with chloroform/methanol (2:1, v/v) and washed as described by Folch (12). The lipid extract was dried *in vacuo* and stored at −20°C in chloroform. The total lipid extract was eluted in four fractions from a TEAE-cellulose[2] column with chloroform/methanol (2:1, v/v); chloroform/methanol (2:1, v/v) containing 1% acetic acid; glacial acetic acid; and 0.1 N K-acetate in chloroform/methanol (4:1, v/v) containing 2.0 ml of concentrated aqueous ammonia per liter (13). The phospholipids from these four fractions were further purified by silicic-acid column chromatography by elution with increasing amounts of methanol in chloroform or by preparative tlc using one of the following solvent systems: (a) chloroform/methanol/acetic acid/H_2O (85:15:10:4, by volume): (b) chloroform/methanol/NH_3/H_2O (70:30:3:2), by volume); (c) chloroform/methanol/H_2O (65:35:8, by volume). Traces of silicic acid and salts were removed by elution over a Sephadex G-25 column (13).

Identification of the phospholipids. Preliminary identification of the phospholipids was obtained by chromatographic comparison of the total lipid extract and the purified phospholipid with reference phospholipid on silica-gel plates using solvent systems (a), (b), (c), and a two-dimensional separating system, as described by Broekhuyse (14). A ninhydrin reagent for the detection of free amino groups, the Dragendorff reagent for the detection of tertiary amino groups, periodate–Schiff reagent for vicinal hydroxyl groups and the molybdate reagent for the detection of phosphorous were used.

Alkaline hydrolysis was performed at 37°C for 20 min by the method of Dawson *et al.* (15). The reaction was stopped by the addition of Dowex 50W-X8 (H^+) to neutrality which was removed by centrifugation before extracting the water-soluble products. Acid hydrolysis was carried out in screw-capped tubes in 6 N HCl at 120°C for at least 2 h and in 1 N HCl at 100°C for 1 h. The water-soluble alkaline and acid hydrolysis products were separated and compared with appropriate reference compounds by paper electrophoresis (50 V/cm for 45 min in pyridine/acetic acid/H_2O (1:10:89, by volume, pH 3.6) and by descending paper chromatography in the solvent systems (d), phenol (H_2O saturated)/acetic acid/methanol (50:5:6, by volume), and (e), propanol/ammonia/H_2O (7:1:2, by volume), and by a two-dimensional combination of paper electrophoresis and chromatography. The amino-group-containing water-soluble products were separated by ascending paper chromatography using KCl-impregnated Whatman III paper in solvent system (f), phenol/butanol/formic acid (80%)/H_2O (50:50:3:5, by volume) saturated with KCl, according to Bremer (16), and by descending paper chromatography in butanol/pyridine/acetic acid/H_2O (60:40:12:48, by volume), system (g).

For periodate oxidation of inositol-containing phospholipids, the lipids were suspended by sonication in H_2O/methanol (1:1, v/v) containing 20 mg of periodic acid per ml. The oxidation was carried out at 37°C for 2 h. When indicated, the products of periodate oxidation were dissolved in absolute ethanol and reduction was carried out by addition of $NaBH_4$ or by exposure to hydrogen in the presence of Pd as catalyst. The products of these procedures were extracted and identified chromatographically (solvent d) after hydrolysis in 1 N HCl at 100°C for 1 h.

Analytical methods. Quantitative determination of phospholipids was carried out according to Rouser *et al.* (17) after two-dimensional tlc (Broekhuyse *et al.* (14)) and visualizing the phospholipids by charring at 180°C after spraying with 20% H_2SO_4. Phosphorous was determined by the method of Fiske and Subbarow (18) or of Rouser *et al.* (17), nitrogen by the method of Lang (18), acyl esters by the hydroxamate method (18), and inositol by periodate oxidation (18). Sphingosine was determined according to Siakatos *et al.* (19).

Turnover experiment. The amoebae were cultivated in 2 liters of medium (T) containing 1 mCi of [^{32}P]phosphate and incubated with 40 ml of preculture. After 10 days of growth, the cells were harvested by low speed centrifugation and washed and

[2] Abbreviations used: CAEP, ceramide phosphonylethanolamine; TEAE, triethylaminoethyl.

resuspended in 200 ml of fresh medium. Incubation at 28°C was continued, and at appropriate time intervals samples were taken for analysis.

The labeled phospholipids were extracted, separated by two-dimensional tlc and visualised by charring, as described under *Analytical methods*. The spots were scraped off the plates into centrifuge tubes and the phospholipids digested with perchloric acid at 180°C. Next, the digest was diluted with H_2O and the silica removed by centrifugation. The supernatant fluid was taken off and ratioactivity was determined by Cerenkov counting (20). On the same sample in the counting vial, phosphorus was determined according to Rouser *et al.* (17).

RESULTS

Identification of the Phospholipids

Entamoeba invadens, cultivated in medium (S) with [^{32}P]-phosphate, contained at least 11 different labeled phospholipids as shown on the autoradiogram (Fig. 1). Preliminary studies on the total lipid extract demonstrated that three of these phospholipids are alkali stable without undergoing a change in their characteristic R_f value (Fig. 2). This characteristic behavior suggests that compounds II, VI and X are sphingolipids. The exact nature of these sphingolipids will be discussed in detail together with the corresponding phosphoglycerides. The total lipid extract was applied to a TEAE-cellulose column, and elution was carried out as described above, resulting in the separation of the lipid extract into four fractions. Fraction A contained three phospholipids I, II and III; fraction B the compounds IV, V, VI and VII; fraction C contained as its major component compound VIII and traces of minor components; and fraction D contained three phospholipids, compounds IX, X and XI (numbers are as indicated in Fig. 1). Further separation of the phospholipids was obtained by silicic-acid column chromatography of each fraction as described

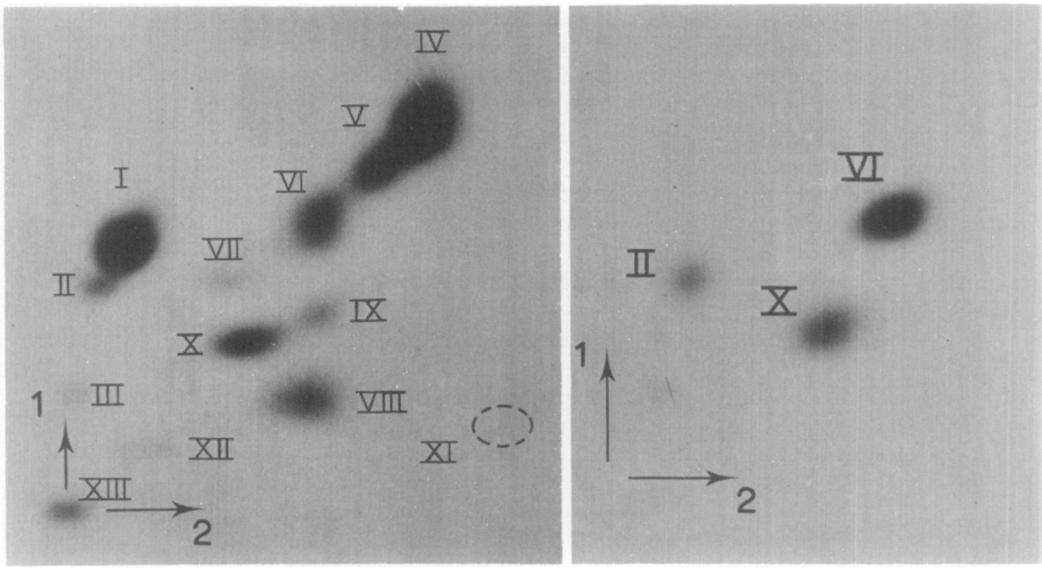

FIG. 1 (left). Autoradiogram of ^{32}P-labeled phospholipids of *Entamoeba invadens*. Total lipid extract was separated by two-dimensional tlc with the solvent system chloroform/methanol/concentrated ammonia/H_2O (90:54:55:55, by volume) in the first direction (1) and the solvent system chloroform/methanol/acetic acid/H_2O (90:40:12:2, by volume) in the second direction (2). The compounds are: (I) phosphatidylcholine, (II) sphingomyelin, (III) lysophosphatidylcholine, (IV) and (V) phosphatidylethanolamine, (VI) ceramide phosphonylethanolamine, (VII) lysophosphatidylethanolamine (VIII) phosphatidylserine, (IX) phosphatidylinositol, (X) ceramide phosphorylinositol, (XI) phosphatidic acid, (XII) unknown, (XIII) origin.

FIG. 2 (right). Autoradiogram of ^{32}P-labeled phospholipids that were not degraded during alkaline hydrolysis of the total lipid extract. Separation of the lipids and explanation of the numbers is given in the legend to Fig. 1.

in Materials and Methods. Final purification was achieved by preparative tlc using different solvent systems.

Phosphatidylcholine, sphingomyelin and lysophosphatidylcholine (Fraction A). Compounds I, II and III (staining positive with the Dragendorff reagent) were eluted from a TEAE-cellulose column with chloroform/methanol (2:1, v/v) and further purified by preparative tlc in solvent systems b and c. Alkaline hydrolysis of the purified compounds I and III both gave glycerolphosphorylcholine, and acid hydrolysis of the intact phospholipids liberated choline and glycerolphosphate as water-soluble products. These observations, together with the quantitative data which are presented in Table I, prove that compound I is identical to phosphatidylcholine and compound III to its lysoderivative. As shown above, compound II was stable to alkaline hydrolysis (Fig. 2). After acid hydrolysis, choline and phosphate could be identified as the only water-soluble products. These data, in combination with other analytical data (Table I), demonstrate that compound II is identical to sphingomyelin (Fig. 3). The nature of the long chain base in this sphingolipid has not been investigated.

Phosphatidylethanolamine, ceramide phosphonylethanolamine (CAEP), lysophosphatidylethanolamine (Fraction B). Elution of the TEAE-cellulose column with chloroform/methanol (2:1, v/v) containing 1% acetic acid gave a mixture of four phospholipids, all of which stained with the ninhydrin reagent indicating the presence of a free amino group. Separation of the compounds and final purification was achieved by preparative tlc in solvent system a. The purified compounds IV, V and VII gave rise to the formation of glycerylphosphorylethanolamine after acid hydrolysis. The results obtained by different hydrolytic procedures and the quantitative determinations strongly indicate that compounds IV and V have identical structure. The separation between the two compounds obtained by chromatography using acidic solvent systems might be due to a difference in fatty acid composition. It could be demonstrated, in agreement with the data of Table I, that the structure of compounds IV and V was phosphatidylethanolamine and that of compound VII the corresponding monoacyl derivative.

Compound VI remained stable to alkaline hydrolysis. After prolonged strong-acid hydrolysis a water-soluble product was isolated which appeared to contain both a free amino group and phosphorus, as determined by chromatographic techniques. This strongly indicates that compound VI contains a carbon–phosphorus bond as is present in phosphonates. By chromatographic comparison with a number of reference compounds, the basic structure of the water-soluble moiety of compound VI appeared to be identical to aminoethylphosphonate. The stability of com-

TABLE I

QUANTITATIVE ANALYSIS OF THE PHOSPHOLIPIDS OF *E. invadens*

Compound	Composition[a]			
	Nitrogen	Acylester	Inositol	Long chain base
I Phosphatidylcholine	1.0	1.7	—	—
II Sphingomyelin	2.0	—	—	0.79
III Lysophosphatidylcholine	1.0	1.1	—	—
IV Phosphatidylethanolamine	0.92	1.9	—	—
V Phosphatidylethanolamine	1.3	1.9	—	—
VI Ceramide phosphonylethanolamine	2.3	—	—	0.89
VII Lysophosphatidylethanolamine	1.1	1.3	—	—
VIII Phosphatidylserine	1.2	1.8	—	—
IX Phosphatidylinositol	—	1.7	0.95	—
X Ceramide phosphorylinositol	1.1	—	0.97	0.97

[a] Data are expressed in μmol/μmol lipid-P.

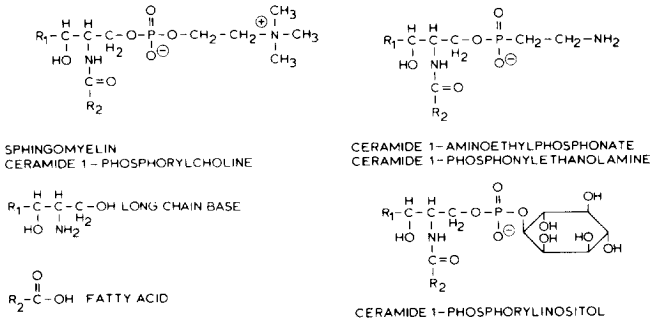

FIG. 3. Proposed structure of sphingolipids of *Entamoeba invadens*.

pound VI under alkaline hydrolysis conditions (Fig. 2) furthermore indicated that the aminoethylphosphonate is covalently linked to a ceramide. The nature of the long chain base has not been determined. Quantitative analysis of the amount of long chain base demonstrated that the compound contains one aminoethylphosphonate per long chain base. These observations demonstrated that compound VI is identical with ceramide phosphonylethanolamine (Fig. 3).

Phosphatidylserine (Fraction C). The chromatographic behavior of compound VIII both on the TEAE-cellulose column (elution with acetic acid) and on silica-gel tlc (solvent a) indicated that this compound is identical to phosphatidylserine. This was confirmed by alkaline hydrolysis and by acid hydrolysis which resulted in the formation of glycerylphosphorylserine and serine, respectively. The quantitative data of Table I agree with this conclusion.

Phosphatidylinositol, ceramide phosphorylinositol and phosphatidic acid (Fraction D). Compounds IX, X and XI were eluted from the cellulose column in Fraction D. Compounds XI behaves in tlc as a reference phosphatidic acid. Alkaline hydrolysis gave glycerolphosphate as the only water-soluble product. Compounds IX and X appeared to contain vicinal hydroxyl groups, as was noticed by staining with the periodate–Schiff reagent. The major alkaline hydrolysis product of compound IX behaved identically, both in paper chromatography (solvent d) and electrophoresis, as glycerylphosphorylinositol. Compound IX was identified as phosphatidylinositol by the release of inositol on acid hydrolysis and by a ratio of inositol to phosphorus of 0.95 (Table I).

Compound X was stable to mild alkaline hydrolysis (Fig. 2), suggesting that this lipid contained a ceramide structure comparable to compounds II and VI (Fig. 3). After strong acid hydrolysis (1 N HCl, 120°C) the only Schiff-positive and phosphorus-containing compounds which could be detected by paper chromatography (solvent d) and paper electrophoresis were inositol and inorganic phosphate, respectively. Glycerol and glycerophosphate appeared to be absent. Mild acid hydrolysis in 1 N HCl for 1 h at 100°C gave rise to the formation of two water-soluble products both containing inositol and phosphorus. The main product appeared to be inositolphosphate as was determined by chromatographic comparison (solvent d) with reference inositol-2-phosphate. The minor hydrolysis product was identical to the cyclic inositolphosphate, which arises by treatment of inositol-2-phosphate under the same conditions as used for the acid hydrolysis of the intact lipid. Periodate oxidation of the intact phospholipid changed the chromatographic properties as was observed by tlc, suggesting that the lipid part of the molecule remained intact but that the polar headgroup had been changed. Periodate oxidation followed by reduction and a mild hydrolysis for 1 h at 100°C resulted in the formation of a phosphorus-containing water soluble product with chromatographic properties different from those of inositolphosphate. The exact structure of this hydrolysis product was not elucidated. However, treatment of phosphatidylinositol in the same manner

gave a water-soluble product which behaved identically with respect to paper chromatography and electrophoresis. The latter data demonstrated that the basic structure of compound X with respect to the polar part is the same as in phosphatidylinositol. The presence of a long chain base was detected, after acid hydrolysis (6 N HCL, 120°C), by tlc (chloroform/methanol, 80:20, v/v) and by a specific assay procedure (29). The nature of the long chain base was not investigated. Quantitative analysis demonstrated a ratio of phosphorus to inositol, long chain base and nitrogen of 0.97, 1.1 and 1.0, respectively (Table I). The quantitative data as well as the results from the different hydrolysis procedures led us to conclude that compound X is identical with ceramide 1-phosphorylinositol (see Fig. 3).

The Phospholipid Composition

The original Diamond medium in which *E. invadens* was cultivated contains a mixture of lipids due to the addition of serum. The variability of the lipid composition of the serum was overcome by the development of a medium in which the serum was replaced by a mixture of cholesterol, oleic acid, and Tween 80 as described before (11). To see whether the lipid composition of the medium influenced the amount and nature of the different phospholipids in *E. invadens*, the amoebae were cultivated on medium S and serum-free medium T. As shown in Table II, the percentile phospholipid composition of *E. invadens* differed when the amoebae were grown on medium S or on medium T. The relative amounts of the ceramide-containing phospholipids especially appeared to be altered. There was a remarkable shift from ceramide phosphonylethanolamine to ceramide phosphorylinositol when medium T was used instead of medium S, whereas the total amount of ceramide-containing lipids remained constant at about 21% of the total amount. The relative amounts of the other phospholipids did not vary much (Table II).

Whether this shift between the relative amounts of the different ceramide phospholipids is due to the change in fatty acid composition of the medium was investigated by varying both the amount and the nature of the fatty acids which were added to the growth medium. The autoradiograms in Fig. 4 demonstrated that an increase in the degree of unsaturation of the fatty acid mixture which is added to the growth medium can be correlated with a decrease in the relative amount of ceramide phosphonylethanolamine.

Turnover of the Phospholipids

The turnover of the various phospholipids was determined by growing the cells in the presence of [^{32}P]phosphate in medium T, followed by a chase of radioactivity by subsequent reincubation of the cells in fresh medium without radioactive phosphate. During the chase experiment the total phospholipid content of the cells increased linearly with time, indicating that the amoebae were still growing during the experiment (Fig. 5). A decrease of the specific activity of the phospholipids could therefore be the result of a net increase in

TABLE II

PHOSPHOLIPID COMPOSITION OF *E. invadens*[a]

Phospholipid	Medium S	Medium T
I Phosphatidylcholine	37.2 (2.2)	33.7 (1.4)
II Sphingomyelin	7.1 (1.3)	4.0 (0.2)
III Lysophosphatidylcholine	2.9 (1.2)	4.1 (0.9)
IV Phosphatidylethanolamine	23.5 (2.7)	25.4 (1.9)
V Phosphatidylethanolamine		
VI Ceramide phosphonylethanolamine	5.3 (0.7)	Not detectable
VII Lysophosphatidylethanolamine	1.2 (0.3)	±
VIII Phosphatidylserine	7.4 (0.8)	7.1 (1.3)
IX Phosphatidylinositol	2.7 (0.5)	3.3 (0.5)
X Ceramide phosphorylinositol	9.0 (1.5)	17.6 (1.2)
XI Phosphatidic acid	2.4 (0.4)	3.6 (0.5)
Residual phospholipid	1.2 (0.4)	1.0 (0.4)

[a] The results are expressed as the mole percentage of total lipid phosphorus, followed in parentheses by the standard deviation. The data are the averages of eight determinations. The compounds are numbered according to Fig. 1.

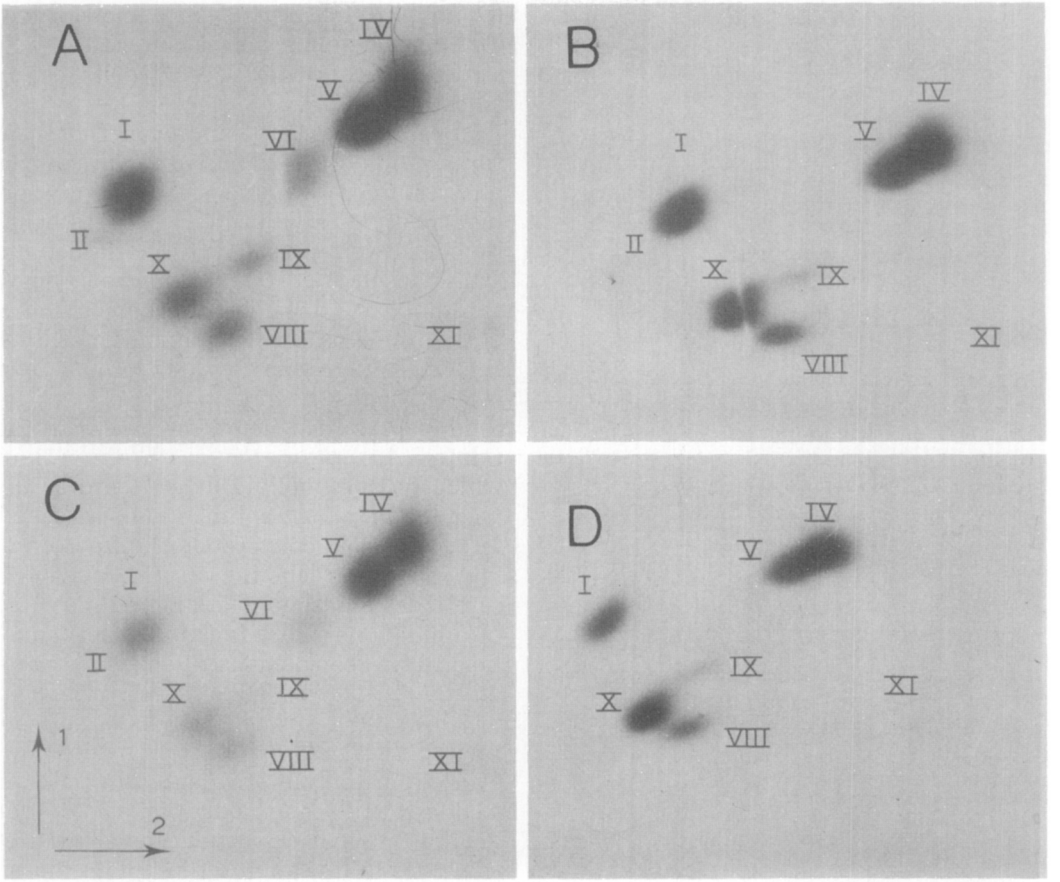

FIG. 4. Autoradiograms of ^{32}P-labeled phospholipids extracted from *Entamoeba invadens* which was grown on medium T to which different fatty acids in various amounts per 600-ml culture medium were added. (A), 60 mg of linoleic acid, 0.6 g of Tween 40; (B), 180 mg of linoleic acid, 0.3 g of Tween 40; (C), 60 mg of linolenic acid, 0.6 g of Tween 40; (D), 180 mg of linolenic acid, 0.3 g of Tween 40. The lipid extracts were separated by two-dimensional tlc, and the phospholipids are numbered as described in the legend to Fig. 1. Tween 40 consists of a mixture of polyoxyethylene sorbitan palmitate (95%), stearate (4%) and myristate (1%).

the amount of phospholipid and/or an active turnover of the phosphate moiety of the phospholipids. However, the decrease in the total radioactivity of the phospholipids indicated that the phospholipids or at least a part of the phospholipids were turning over. In order to investigate which phospholipids contributed to this turnover, the decrease in specific radioactivity of the individual phospholipids was determined. The decreases of the specific activities of the various phospholipids were corrected for the increase of the phospholipid pool size (cell growth) while assuming that the phospholipid composition was not changed during the experiment. The decrease in the corrected specific activities, plotted semilogarithmic against time, demonstrated a different metabolic behavior of the various phospholipids (Fig. 6). Three distinct classes of phospholipids, with respect to their turnover rates, could be distinguished. The first class of phospholipids, including phosphatidic acid and phosphatidylinositol, demonstrated a relatively fast turnover. A half-time of about 12 h for phosphatidic acid and phosphatidylinositol was measured. With respect to the second class of phospholipids, including phosphatidylethanolamine and ceramide phosphorylinositol, a half-time of about 24 and 30 h, respectively, could be

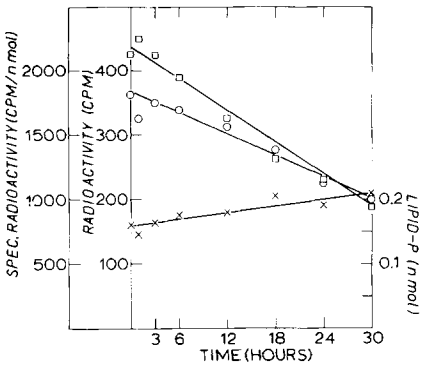

FIG. 5. Turnover of phospholipids of *E. invadens*. Amoebae were grown in [^{32}P]phosphate-containing medium (1 mCi/2 liters) for 10 days, washed and recultured in fresh medium. Samples were taken at given time intervals for determination of phospholipid content (×), absolute radioactivity (○) and specific radioactivity (□) of the total phospholipid extract. For experimental details see Materials and Methods.

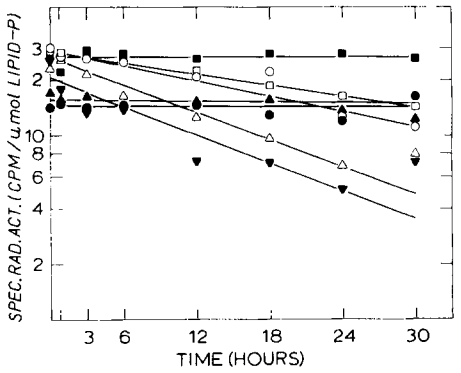

FIG. 6. Semilogarithmic plot of the specific radioactivities of the individual phospholipids. The experiment was carried out as described in the legend to Fig. 5. The results are expressed as specific radioactivities (cpm/nmol of lipid-P) and corrected for cell growth. (●), phosphatidylcholine; (□), ceramide phosphorylinositol; (△), phosphatidylinositol; (○), phosphatidylethanolamine; (▲), sphingomyelin; (■), phosphatidylserine; (▼) phosphatidic acid.

determined. Of the third group of phospholipids, consisting of phosphatidylserine, phosphatidylcholine and sphingomyelin, no decrease in the specific activity was observed which indicated that these phospholipids were not turning over.

DISCUSSION

The occurrence of at least 11 different phospholipids in the total lipid extract of *Entamoeba invadens* was demonstrated. The phospholipids were isolated and their structural characteristics established by chemical methods.

Phosphatidylethanolamine and phosphatidylcholine were found to be the major phospholipids, whereas phosphatidylserine, phosphatidylinositol, and phosphatidic acid were present in minor amounts. This phospholipid composition is strongly similar to the phospholipid composition of other protozoa and of mammalian cells. A typical feature of the *E. invadens* lipid composition is the high amount of sphingolipid, especially when compared with the related *Acanthamoeba castellanii* (1) in which no sphingolipid at all could be detected. Among the sphingolipids three different compounds were identified: Ceramide phosphorylcholine (sphingomyelin), ceramide phosphonylethanolamine and ceramide phosphorylinositol. Phosphonolipids have been demonstrated also in *Entodinium caudatum* (2) and *Tetrahymena pyriformis* (3), but not in the more closely related *A. castellanii* (1). It is obvious however, from the data presented in Table II that growth conditions can affect the amount of CAEP. It should be of interest therefore to investigate the lipid composition of *A. castellanii* after growth under several conditions, especially because it has been suggested that CAEP might serve as a precursor for the lipophosphonoglycans which are present in the membrane of this organism (21).

The ceramide phosphorylinositol from *E. invadens* is probably identical with the inositol-containing sphingolipid from *Saccharomyces cerevisiae* which has been identified by Smith and Lester (22). An inositol-containing phospholipid different from phosphatidylinositol was isolated from *A. castellanii* by Ulsamer *et al.* (1), but long chain bases could not be detected in this compound indicating that the structure of this compound must be different from ceramide phosphorylinositol. The occurrence of ceramide phosphorylinositol together with phosphatidylinositol in *E. invadens* is very intriguing. It is known that the turnover of phosphatidylinositol and phosphatidic acid is stimulated in processes in

which the turnover of membranes is involved, processes like phagocytosis (23), secretion (23) and nerve stimulation (22–24). Evidence for a different metabolic behavior of phosphatidylinositol and ceramide phosphorylinositol were obtained by measuring the turnover of the various phospholipids. As demonstrated in Fig. 6, the various phospholipids exhibited different rates of turnover. Phosphatidylinositol and phosphatidic acid showed a fast turnover. To what extent the rapid turnover of phosphatidic acid and phosphatidylinositol, as observed in *E. invadens*, was also a consequence of phagocytotic stimulation is not known. Ceramide phosphorylinositol and phosphatidylethanolamine turned over at a slower rate. Sphingomyelin, phosphatidylcholine and phosphatidylserine showed no turnover. Why these phospholipids, in contrast to the other phospholipids showed no turnover is not understood. In *Escherichia coli* phosphatidylethanolamine also showed no turnover in contrast to phosphatidylglycerol (25). As in *E. invadens*, the turnover of phosphatidylserine, phosphatidylcholine and sphingomyelin was also shown to be limited in mammalian tissue (26–29) in contrast to the other phospholipids.

It was observed that, after 10 days of growth on a [^{32}P]phosphate-containing medium, both phosphatidylcholine and sphingomyelin had a lower specific activity than the other phospholipids. After such a time interval one would expect that the specific activity for all phospholipids would be the same. This discrepancy can be explained by assuming the presence of a precursor of both phosphatidylcholine and sphingomyelin in the culture medium which could dilute the [^{32}P]phosphate labeling of the precursor pool (CDP-choline or cholinephosphate).

It is noteworthy that the amount of ceramide phosphorylinositol and ceramide phosphonylethanolamine seems to depend strongly on environmental conditions, as demonstrated by the replacement of medium S by medium T. These alterations in the phospholipid composition might be related to the relatively higher content of unsaturated fatty acids in medium T compared to medium S. An increase in the amount of unsaturated fatty acids in the medium relative to the amount of saturated fatty acids, results in a decrease of the amount of CAEP. This observation demonstrates a possibility of changing the phospholipid composition of amoebal membranes. Moreover, it suggests some kind of interrelationship between the fatty acid incorporation and the phospholipid composition of the membranes.

ACKNOWLEDGMENTS

The present investigation was carried out under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.) and with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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