PLASMA MEMBRANE LIPID COMPOSITION OF DICTYOSTELIUM DISCOIDEUM DURING EARLY DEVELOPMENT IN AQUEOUS SUSPENSION

by

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ABSTRACT

Cell-cell contact must be made and maintained for normal development and eventual differentiation of D. discoideum to occur. Certain plasma membrane components are known to alter in activity or abundance during the organism's developmental cycle although no quantitative measurement of plasma membrane neutral lipid and phospholipid content has been reported to date.

Optimal conditions for the extraction, separation and assay of lipid components were derived and tested by quantification of the neutral lipid and phospholipid components of intact cells of strain Ax-2. Development was initiated in D. discoideum populations suspended in aqueous buffer and plasma membrane fractions were purified from both exponentially growing and aggregation-phase cells by a modified procedure which minimized phospholipid degradation during the plasma membrane isolation. Neutral lipid and phospholipid compositions of the plasma membrane fractions PM1 and PM2 from exponentially growing cells and from those in early aggregation phase were determined.

Exponential phase cells' plasma membranes contained large proportions of phosphatidylethanolamine, phosphatidylcholine and lysophosphatidylethanolamine. Lysophosphatidylcholine was absent. A significant quantity of phosphatidylinositol was detected and cardiolipin, phosphatidylglycerol, phosphatidic acid and lysophosphatidic acid were each present in small amounts. The presence of phosphatidylethanolamine plasmalogen was suspected but not proven. No acylglycerol components were detected, the major

neutral lipid fraction being that of free sterol which largely comprised stigmast-22-en-3β-ol; sterol ester was present in extremely small quantities. An unidentified neutral lipid component of plasma membranes was detected by its characteristic absorption and fluorescence upon irradiation at ultraviolet wavelengths. After sixteen hours aggregation the phosphatidylcholine content of the plasma membranes was greatly reduced, a significant proportion of the phosphatidylethanolamine appeared to have been converted to lysophosphatidylethanolamine, and phosphatidylglycerol, phosphatidic acid and lysophosphatidic acid were all in greater abundance than in growing cells' membranes. The free sterol component remained relatively constant but sterol ester had increased dramatically (7 to 10-fold) and the fatty acid composition of the plasma membrane phospholipids was more saturated, primarily because of the accumulation of palmitate and stearate and a reduction of the octadecadienoic fatty acid components.

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INTRODUCTION

Dictyostelium discoideum is a cellular slime mould distributed in the soils and rotting vegetation of temperate forests throughout the world. This simple eucaryotic organism is amoeboid in the vegetative phase of its life cycle during which it ingests bacteria, but on starvation populations undergo a process of synchronous development. This comprises aggregation, grex formation and migration, and fruiting body construction. Within the grex cells differentiate into distinct anterior and posterior types, and ultimately into stalk and spore cells respectively with the formation of the fruiting body. The stalk cells are not viable but the spores germinate into small myxamoebae after dispersal (1,2).

In 1889 MacBride stated of slime moulds: "Their minuteness retires them from ordinary ken; but such is the extreme beauty of their microscopic structure, such the exceeding interest of their life history, that for many years enthusiastic students have found the group one of peculiar fascination, in some respects at least, the most interesting and remarkable that falls beneath our lenses" (3). This description applies to the Acrasiales (cellular slime moulds) as well as to the Myxomycetes (true slime moulds). The two have frequently been confused although major distinctions were noted more than a century ago (3,4). Their differences and similarities have been reviewed recently (5).

D. discoideum has several advantages for the laboratory study of biological development, and its cellular differentiation mechanisms may be of relevance to studies of development in multicellular

organisms (1,2); embryological development, tissue regeneration and cancerous proliferation being examples of the latter. Certain strains of D. discoideum grow quite rapidly in axenic media allowing biochemical manipulation during growth and facilitating the isolation and cloning of mutants. The organism has been the subject of diverse investigations which have provided a comprehensive background of interrelated information (1). In contrast to the majority of developmental systems, that of D. discoideum is not complicated by the effects of growth and division, both of which cease before aggregation begins; the developmental phenomena are thus restricted to morphogenesis and differentiation. Slime mould development has been discussed in general terms by Bonner (6) and elsewhere with particular emphasis on D. discoideum (1,2,5,7).

Cell-cell contacts established during aggregation of D. discoideum are maintained throughout development and are required at all stages for the process to advance. The precise mechanisms of these cellular interactions and their molecular basis have not been determined, although several plasma membrane proteins, glycoproteins and a glycolipid are known to undergo quantitative alterations or activity changes during differentiation (8-17), and univalent antibodies to aggregation-specific surface antigens block cell aggregation and arrest further development (8,18).

Developmentally regulated modification of plasma membrane lipid components may be associated with cell-cell interaction processes. Specific qualitative or quantitative lipid changes might be necessary to allow proteins or glycoproteins to bind to the cell surface or to be incorporated within the membrane. The

activities or specificities of certain developmentally regulated cell surface components might be influenced *in situ* by alterations of lipids in direct association with these components or through more general membrane fluidity effects brought about by changes in lipid composition. In developmental biology there has been no direct observation of such mechanisms, although in certain cultured animal cells the physical state of the plasma membrane alters during cell adhesion (mouse L929 cells) (19), cell fusion (avian myoblasts) (20) and cellular differentiation (neuroblastoma) (21). Moreover, studies outside the developmental field suggest that lipid 'micro-domains' exist in natural membranes and that specific lipid-protein interactions as well as membrane lipid phase transitions are able to affect certain membrane enzyme activities and active transport processes (22-27).

There is circumstantial evidence that lipid composition may be a critical factor of *D. discoideum* development. The antibiotic cerulenin which inhibits fatty acid biosynthesis prevents aggregation and the ensuing differentiation of the organism (28), and the fatty acid composition of the axenic strain of *D. discoideum* can be modified during growth such that its subsequent development is impaired (29). Furthermore both the phospholipid (30) and neutral lipid (31) compositions of the intact organism have been shown to alter during development suggesting that there is either a wide-spread adjustment of these components occurring throughout the cells' various membranes or that there are significant lipid alterations within particular membranes. Although it is directly involved in cell-cell interaction, the *D. discoideum* plasma membrane

had not had its lipid composition investigated until this study was undertaken to determine whether significant lipid changes occur during early stages of the organism's development.

MATERIALS AND METHODS

a. Materials

Standard lipids were of the highest purity available commercially and were purchased from Supelco Inc. and Applied Science Inc.. Solvents were redistilled in glass and stored dry over molecular seives. In order to prevent solvent oxidation and free radical formation 0.005% (w/v) 2,6-di-tert-butyl-4-methylphenol (BHT) twice recrystallized from carbon tetrachloride was added to all ethers: this additive also protects dissolved lipids from autoxidation (32). All chromatography, storage and assay glassware was acid-washed in 5M HCl. Thick glass chromatoplates were also washed in 2M KOH and thoroughly rinsed with distilled, deionized water before being spread with the appropriate thin layer.

b. Organism and Membrane Preparation

The axenic D. discoideum strain Ax-2 was grown in the liquid medium HL-5 at 22°C (33,34). Cells were harvested during exponential growth at a concentration of approximately 5×10^6 cells ml^{-1} by centrifugation for 6 min at 700 xg and 4°C. Three ice-cold distilled water washes and subsequent centrifugations were used to remove all traces of HL-5 medium before immediate lipid extraction of the intact cells.

Cells destined for membrane fractionation were harvested and washed twice with ice-cold 8.6% (w/v) sucrose-5mM tris(hydroxymethyl)

aminomethane hydrochloride (sucrose-Tris), pH 7.4, using 6 min centrifugations of 700 xg as above. Aggregation-phase cells were prepared under standard conditions (36) by resuspension of the washed cell pellets in 17mM phosphate buffer, pH 6.0, at 7 x 10^6 cells ml^{-1} and rotating these cell suspensions at 150 rpm at 22°C for either 8 or 16 hours before reharvesting. Harvested exponential and aggregation-phase cells were immediately resuspended in ice-cold sucrose-Tris saturated with phenylmethylsulphonylfluoride (sucrose-Tris-PMSF), pH 7.4, at 10^8 cells ml^{-1} (35).

In order to minimize lipid degradation the standard plasma membrane purification procedure (35,37) was modified in that the 105,400 xg pellet was not washed but was either used directly as 'crude membrane' material for preliminary experiments or resuspended in 20% (w/v) sucrose-Tris-PMSF, pH 7.4, and immediately layered onto the discontinuous sucrose density gradients. These gradients were centrifuged for 16 hours at 75,800 xg, centrifugation being initiated within 45 minutes of the start of cell fracture. Two distinct bands of membranous material (PM1 and PM2) (35) were recovered as described previously (37) and aliquots were assayed or extracted immediately. The criteria used to assess the purity of the plasma membrane preparations have been discussed by Gilkes and Weeks (37).

c. Lipid Extraction and Isolation

In preliminary experiments lipid extraction was carried out either by the method of Bligh and Dyer (38) according to Kates (39, 40) or by that of Folch *et al* (40,41), the latter being the more efficacious (see Results). The aqueous wash solution for the

Folch procedure comprised 4.5 mM $CaCl_2$ and the resulting phase interface was rinsed at least six times with the Folch 'pure solvents upper phase' of chloroform-methanol-3.6 mM aq. $CaCl_2$ (3:48:47 by vol). A second extraction of each Folch residue was performed overnight at 4°C with 15 times the original sample volume of either chloroform-methanol (7:1 v/v) saturated with 5% (w/v) aq. NH_3, or chloroform-methanol-glacial acetic acid-water (8:4:2:1 by vol.), or chloroform-methanol-concentrated HCl (50:50:0.3 by vol.). The second extract was thoroughly dried under nitrogen and the washed Folch extract was added to it (42). Extracts were concentrated in chloroform-methanol (2:1 v/v) under nitrogen and stored, if necessary, in Teflon sealed vials at -70°C.

Separation of extracts into a phospholipid fraction and individual neutral lipid classes was achieved by biphasic thin-layer chromatography in one dimension on a 500 μm layer of silica gel H (Merck & Co.). Before use the chromatoplates were subjected to an ascending wash with chloroform-methanol (2:1 v/v) for at least 12 hours. Samples were deposited at the origin of activated chromatoplates (43) under a stream of dry nitrogen and the plates were immediately developed in a nitrogen atmosphere using the biphasic system of solvents described by Skipski and Barclay (43). Chromatoplates were stored in a dessicator under aspiration for 20 min after each development to remove all traces of solvent. Routine neutral lipid identification was made by reference to the mobility of standard lipids developed on each plate. Rhodamine 6 G at 0.05% (w/v) in aqueous ethanol (95% v/v) was selectively sprayed onto the standards and the fluorescence of the resulting lipid complexes viewed under ultraviolet radiation.

Techniques for the extraction of lipids from the chromatoplates were derived from methods proposed by Skipski and Barclay (43). Areas of silica gel containing individual lipid fractions were transferred rapidly and quantitatively to 15 ml conical, ground-glass stoppered centrifuge tubes and extracted with three 5 ml aliquots of solvent for 10 min each at room temperature with occasional vortexing. Triacylglycerols, 1,2-diacylglycerols and 1,3-diacylglycerols were extracted with diethyl ether whereas chloroform-methanol (4:1 v/v) was used to extract monoacylglycerols, sterol esters, sterols and hydrocarbons from the silica gel. The extracted neutral lipid fractions were concentrated under nitrogen and stored, if necessary, at -70°C in Teflon-sealed vials. Phospholipids were extracted at 37°C from a band 1.5 cm in width at the chromatoplate origin under conditions similar to those for neutral lipid extraction but using 5 ml aliquots of the following solvent mixtures in sequence: two treatments of chloroform-methanol-glacial acetic acid-distilled, deionized water (25:15:4:2 by vol.), followed by methanol, and then by methanol-glacial acetic acid-distilled, deionized water (94:1:5 by vol.). The extracted phospholipids were concentrated under nitrogen and stored under the same conditions as the neutral lipids.

Phospholipids were separated by biphasic, two-dimensional thin-layer chromatography. A technique was developed that fractionated all fully acylated phospholipids and lysophospholipids known to be present in D. discoideum, although plasmalogen forms were not resolved (30,44). A 97.5 ml volume of 0.5 mM aqueous magnesium acetate was added rapidly to 45 g Camag silica gel H and shaken vigorously for 90 seconds before being spread onto chromatoplates

in a 300 μm wet layer. Phospholipids containing vicinal hydroxyl groups could be selectively retarded during development by incorporating boric acid into the aqueous solution at 0.4 M (45). After drying slowly the thin-layers were washed by ascending development with acetone for at least 12 hours, dried again and activated overnight at (115 ± 5)^{o}C until immediately prior to use. After cooling, each chromatoplate received one phospholipid sample of up to 300 μmol lipid phosphorus (optimum c.175 μmol.) at its point origin. Cooling, spotting and development of the thin-layer were carried out entirely in a dry nitrogen atmosphere using equipment similar to that described by Skipski and Barclay (43). Thin-layer chromatography tanks were lined with Whatman 3MM paper and wetted with the solvent mixture, the vapours of which were allowed to equilibrate with the enclosed atmosphere for an hour before use. Each chromatoplate was developed in the first dimension with chloroform-methanol - 28% (w/v) aqueous ammonia (65:25:8 by vol.) to a height of 18 cm. Residual solvent was immediately evaporated by a powerful stream of cold air followed by aspiration in a dessicator for thirty minutes. The plate was then developed for 18 cm in the second dimension with chloroform-acetone-methanol-glacial acetic acid-distilled, deionized water (35:35:7:10:3 by vol.). Residual solvent was removed as before and individual phospholipids were routinely located by brief immersion of the chromatoplate into an iodine-saturated atmosphere.

The characteristic positions of individual phospholipids on the thin-layer chromatogram were identified by a combination of methods after separating standard phospholipids with acyl chains

of known saturation. These methods included the following: relative speed and reversibility of brown complex formation during immersion in iodine vapour, both factors being dependent on the degree of saturation of the lipids' fatty acid substituents; detection of phosphate esters with the molybdenum-blue reagent of Dittmer and Lester (46); ninhydrin reagent to detect the free amino groups of phosphatidylethanolamine, phosphatidylserine and their lyso derivatives (43); Dragendorff reagent to detect phosphatidylcholine, lysophosphatidylcholine and sphingomyelin (47); the incorporation of boric acid into the thin-layer, as described above, in order to selectively retard phosphatidylglycerol migration (45). Individual phospholipids were either extracted from the silica gel by the method previously described or assayed in the presence of the gel after quantitative transfer to a Pyrex assay tube.

d. Assays

Protein was routinely assayed by the method of Lowry et al (48). The 'biuret technique' (49) was used in measurements of proteolytic activity during membrane purification, the total reagent volume being reduced to 1.0 ml. Total phospholipid was estimated by assaying the extracted lipid-phosphorus by Ames' method (50), although this assay could not be used for the determination of individual phospholipids recovered directly from thin-layer chromatoplates because silica gel interfered with both the digestion step and the colorimetric reaction. The Bartlett lipid-phosphorus assay (51) was modified to increase its sensitivity while remaining

unaffected by iodine pretreatment of lipids or the presence of silica gel. These properties were determined by assaying the phosphorus content of identified phospholipid aliquots after removal of the lipid from an iodine-treated chromatoplate. The modified Bartlett procedure was as follows: 0.50 ml 70% (w/v) aqueous perchloric acid was added to the sample in a small, acid-washed tube and heated for 2.5 hours at 150°C (52). After the tube had cooled, 0.60 ml distilled, deionized water, 0.20 ml 5% (w/v) aqueous ammonium molybdate and 0.02 ml fresh Fiske-SubbaRow reagent (51) were added. Tubes were sealed with Parafilm, the contents mixed and heated for 30 min at 70°C, allowed to cool and centrifuged in swinging buckets for at least 5 min at 1250 xg. Each supernatant was carefully removed with a Pasteur pipette and its absorption measured at 807 nm versus a reagent blank. This absorption maximum differs from the 830 nm maximum of the phospho-molybdate complex in the standard Bartlett assay (51). It is especially important that all glass and quartz apparatus be acid-washed for each of these phosphorus assays.

Neutral lipids of the plasma membranes were measured by gas-liquid chromatography. After thin layer chromatographic separation of the individual neutral lipid fractions internal standards of eicosanoic acid (arachidic acid) and cholest-5en-3β-ol (cholesterol) were added to those fractions containing fatty acid and sterol moieties respectively. The individual neutral lipid fractions were saponified in 1.0 ml aliquots with an equal volume of 15% (w/v) KOH in methanol for 1 hour at 70°C: the methanol was then evaporated under nitrogen and 1.0 ml distilled water was added.

Non-saponifiable lipids were extracted directly with four equal volumes of n-pentane and saponifiable lipids were similarly extracted following acidification of the hydrolysate with 0.25 ml 12 M H_2SO_4, The non-saponifiable fractions were dried separately under nitrogen and acetylated with 0.5 ml acetic anhydride for 30 min at 135°C in Teflon-sealed, screw-capped tubes. Samples were then dried under nitrogen and redissolved in minimal quantities of hexanes in preparation for isothermal gas-liquid chromatography. The non-saponifiable fractions of the sterol ester samples were rechromatographed by thin-layer chromatography in order to separate contaminating hydrocarbons from the derived sterols. These sterols were eluted, acetylated and assayed by gas-liquid chromatography as described above. Sterol esters were also quantified by isothermal gas-liquid chromatography of their fatty acid methyl ester derivatives as were acylglycerols, free fatty acids and certain phospholipid fractions. These derivatives were obtained by drying the individual saponifiable fractions under nitrogen, adding 1.0 ml boron trifluoride in methanol and boiling for 2 min in Teflon-sealed, screw-capped tubes: 1.0 ml of distilled water was then added and the methyl esters were extracted with three equal volumes of n-pentane. The pooled pentane extracts of each sample were dried down under nitrogen, resuspended in a minimal volume of hexanes and analyzed by gas-liquid chromatography. Acetylated sterols were separated using 3% (w/w) methyl silicone (SE-30) phase on 100/120 mesh Gas Chrom Q (Applied Science Inc.) at 245°C in 6ft, 2 mm i.d. sialysed glass tubes with helium as carrier gas. Fatty acid methyl esters were separated on 10% (w/w) diethyleneglycol succinate treated

with orthophosphoric acid (DEGS-PS) on 80/100 mesh Supelcoport (Supelco Inc.) at 140°C in 6 ft, 2 mm i.d. metal columns using helium as carrier gas.

Intact cells provided sufficient neutral lipid for free glycerol and acylglycerol to be measured by the method of Eggstein (53) as modified by Schmidt et al (54), using a 0.001% (w/v) aqueous picric acid reference solution. In order to accomplish full hydrolysis of the individual, separated neutral lipid samples, the KOH saponification step was lengthened to 12 hours from the 20 minutes recommended in the standard, clinical procedure. Assay components were purchased from the Boehringer-Mannheim Corporation.

Lipid measurements were standardized by reference to the protein content of the whole cell, crude membrane or plasma membrane preparation from which the lipids were derived.

RESULTS

a. Comparison of Lipid Extraction Procedures

The techniques of Folch *et al* (41) and of Bligh and Dyer (38) according to Kates (39,40) were compared (Table 1). Incomplete phospholipid extraction from tissues by the Bligh and Dyer method, especially of acidic phospholipids, has been reported by Palmer (18,55). The amounts of phospholipid (Table 1), fatty acid (Table 2) and sterol (Table 3) extracted from *D. discoideum* cells showed that the method of Folch *et al* was superior to that of Bligh and Dyer for this study. In view of the results of the original analysis of lipid extraction by Folch *et al* (41), little of the phosphorus measured in 'Folch fraction II' (Table 1) is likely to have been derived from lipid phosphorus. Similarly, most of the phosphorus in the Bligh and Dyer aqueous methanol phase was probably non-lipid phosphorus. Confirmation of these conclusions was provided by the fatty acid analyses (Table 2). Of the total fatty acid recovered in each procedure only 1.5% was lost to 'Folch fraction II' and 2.8% to the 'Bligh and Dyer fraction II' and it is likely that in both cases this was largely from glycolipid sources. Table 3 indicates that the procedure of Folch *et al* was also more efficient for the extraction of sterols from *D. discoideum* Ax-2. Although Long and Coe estimated that stigmast-22-en-3β-ol (stigmastenol) comprised more than 99% of free and esterified sterol in such cells (31) more recent analyses have measured only 88% of total sterol in the plasma membrane of vegetative cells as stigmastenol (37).

Table 1: Lipid-phosphorus extracted from exponentially growing cells of D. discoideum Ax-2

Extraction Procedure	Fraction No.	Description of Fraction	(1) Phosphorus in Fraction (nmol mg^{-1} total protein)	(2) Phosphorus in Fraction (nmol mg^{-1} total protein)
Folch et al	I	Initial 19.0 vol. C-M[a] (2:1 v/v) extract	118.1 ± 0	135.3 ± 20
	II	Aqueous $CaCl_2$ phase and combined aqueous washings.	12.4 ± 0	9.3 ± 3.0
	III	Second extract of 15.0 vol. C-M[a] (7:1 v/v) saturated with 5% aq. NH_3.	4.0 ± 1.5	4.5 ± 2.5
	IV	Residue	not determined	not determined
Bligh & Dyer	I	1st and 2nd 6.25 vol. aqueous C-M[a] lower phase extracts combined.	88.9 ± 1.5	
	II	Aqueous methanol phase after second C-M[a] extraction.	9.5 ± 0.9	
	III	Residue	not determined	

1. Extraction of duplicate samples by the techniques of Folch et al (41) (incorporating a second, ammoniacal extraction) and of Bligh & Dyer (38). Results are the mean of two extractions.

2. Extraction of multiple samples by the method of Folch et al. Results are the mean of seven independent extractions ± standard deviation.

[a]C-M indicates 'chloroform-methanol'.

Table 2: Fatty acid analysis of hydrolyzed total lipid from Folch et al (41) and Bligh & Dyer (38) extractions of exponentially growing cells of D. discoideum Ax-2.

Fatty Acid	Folch et al Extraction Fractions				Bligh & Dyer Extraction Fractions		
	I	II	III	IV	I	II	III
	(μg fatty acid mg^{-1} total protein)						
14:0	0.5 ± 0.2	trace	trace	trace	2.9 ± 0.4	trace	0
Palmitaldehyde	0.3 ± 0.1	trace	trace	trace	1.5 ± 0.3	trace	0.3 ± 0.1
16:0	5.2 ± 0.7	0.4 ± 0.2	0.5 ± 0.1	0.7 ± 0.1	5.2 ± 0.1	0.5 ± 0.3	1.5 ± 0.1
$16:1^{(\Delta 9)}$	2.1 ± 0.1	trace	trace	trace	1.3 ± 0.7	trace	0.3 ± 0.1
$16:2^{(\Delta 5,9)}$ & $17:0^{a}$	1.6 ± 0.1	trace	trace	trace	1.3 ± 0.7	trace	trace
18:0	1.7 ± 0.1	0	0	0	2.0 ± 0.3	0	0
$18:1^{(\Delta 9)}$ & $18:1^{(\Delta 11)a}$	30.1 ± 0.7	0.4 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	26.5 ± 0.8	0.8 ± 0.3	1.5 ± 0.1
$18:2^{(\Delta 5,9)}$ & $18:2^{(\Delta 5,11)a}$	49.5 ± 1.1	0.5 ± 0.3	0.7 ± 0.1	0.3 ± 0.1	43.6 ± 1.1	0.8 ± 0.4	0.5 ± 0.1
$18:2^{(\Delta 9,12)}$	0.9 ± 0.4	trace	0	trace	trace	0	0
Others[b]	1.5 ± 0.7	trace	0.3 ± 0.2	0.3 ± 0.2	3.2 ± 0.5	0.5 ± 0.3	2.3 ± 0.6
Total fatty acid per fraction (μg mg^{-1} protein)	93.7 ± 4	1.5 ± 1	2.4 ± 1	2.3 ± 1	87.5 ± 5	2.7 ± 2	6.4 ± 1
% Total Recovered	93.8 ± 4	1.5 ± 1	2.4 ± 1	2.3 ± 1	90.6 ± 5	2.8 ± 2	6.6 ± 1

Lipid extraction fractions were as described in Table 1. Results are presented as the mean of three determinations ± standard deviation; 'trace' indicates <0.1 μg fatty acid mg^{-1} total protein.

[a] These fatty acids were not separated under the conditions used but were shown to be present by Davidoff & Korn (56).

[b] Several unidentified minor components.

Table 3: Sterol analysis of total lipid from Folch et al (41) and Bligh & Dyer (38) extractions of exponentially growing cells of D. discoideum Ax-2.

Retention [a]	Folch et al Extraction Fractions				Bligh & Dyer Extraction Fractions	
	I	II	III	IV	I	II + III
	(µg cholesterol equivalent mg^{-1} protein)					
1.17	1.6 ± 0.1	trace	0.1 ± 0.0	trace	0	0
1.30	1.8 ± 0.1	0.2 ± 0.1	trace	trace	2.0 ± 0.4	trace
1.43	17.1 ± 3.5	0.2 ± 0.0	0.2 ± 0.1	trace	13.6 ± 2.1	1.6 ± 1.3
1.59	0.5 ± 0.1	trace	trace	trace	0.2 ± 0.2	0
Others [b]	0.4 ± 0.1	0	trace	trace	0.5 ± 0.1	0.4 ± 0.3
Total sterol per fraction (µg mg^{-1} protein)	21.4 ± 3.9	0.4 ± 0.1	0.3 ± 0.1	trace	16.3 ± 2.8	2.0 ± 1.6
% Total Recovered	96.8 ± 17.6	1.8 ± 0.5	1.4 ± 0.5	trace	89.1 ± 15.3	10.9 ± 8.7

Lipid extraction fractions were as described in Table 1. Results are presented as the mean of three determinations ± standard deviation; 'trace' indicates <0.1 µg cholesterol equivalent mg^{-1} protein. Absolute values vary more than '% total recovered' for the latter are measured within each experiment.

[a] Retention figures are the ratio of (sterol acetate:cholesteryl acetate) retention under the gas-liquid chromatography conditions used.

[b] Several unidentified minor components.

The present study confirmed that other sterols were present in significant proportions (Table 3) and showed that both stigmastenol (standard retention 1.43) and minor sterols remained after extraction of the cells by the Bligh and Dyer technique.

Having determined that for vegetative cells of D. discoideum the Folch lipid extraction procedure was superior to that of Bligh and Dyer, the efficiency of the 'Folch final extraction' (Folch fraction III) was analyzed. Certain residual lipids, notably the polyphosphoinositides, may not have been quantitatively extracted by the ammoniacal solvents (55-57) used in the early experiments and acid conditions have been found necessary to remove such lipids from tissues (30,56,58). Table 4 shows the fatty acid analyses of various final extractions of the 'Folch residue' in which extraction time and volumes were standardized to permit direct comparison of extraction efficiency. The chloroform-methanol-concentrated HCl (50:50:0.3 by vol.) solvent mixture has been used routinely for extracting phosphatidyl-inositol and related compounds from various tissues (T. Buckley, personal communication). Both acid conditions tested were superior to the alkaline solvent mixture and the strongly acidic conditions were the most efficient. The reasons for these differences were not apparent.

As a result of these studies, D. discoideum lipids were extracted in all subsequent experiments by the procedure of Folch et al (41), followed by an extraction with chloroform-methanol-concentrated HCl (50:50:0.3 by vol.).

Table 4: Fatty acid analysis of various final extractions (Fractions III) following initial procedures of Folch et al (41).

Fatty Acid	Fraction I	Fraction III		
		A	B	C
	(μg fatty acid)			
14:0	0.58	0.01	0.03	0.08
Palmitaldehyde	0.85	0.02	0.02	0.03
16:0	3.62	0.06	0.34	0.29
$16:1^{(\Delta 9)}$	1.76	0.01	0.02	0.04
$16:2^{(\Delta 5,9)}$ & 17:0[a]	0.80	0.04	0.03	0.03
18:0	2.59	0.06	0.11	0.10
$18:1^{(\Delta 9)}$ & $18:1^{(\Delta 11)}$[a]	23.75	0.09	0.20	0.57
$18:2^{(\Delta 5,9)}$ & $18:2^{(\Delta 5,11)}$[a]	34.76	0.12	0.16	0.18
$18:2^{(\Delta 9,12)}$	0.45	0.00	0.04	0.05
Others[b]	1.26	0.02	0.11	0.47
Total Fatty Acid	70.42	0.43	1.06	1.84

All extractions were of equivalent samples from one preparation of exponentially growing D. discoideum Ax-2. Fraction I was as described in Table 1. Fraction III was an overnight extraction at 4°C comprising: A, 15 vol (chloroform-methanol (7:1 v/v) saturated with 5% (w/v) aq. NH_3);

B, 15 vol (chloroform-methanol-glacial acetic acid-water (8:4:2:1 by vol));

C, 15 vol (chloroform-methanol-concentrated HCl (50:50:0.3 by vol)).

Values are the mean of three determinations.

[a] These fatty acids were not separated under the conditions used but were shown to be present by Davidoff and Korn (60).

[b] Several unidentified minor components.

b. Isolation and Qualitative Analysis of Lipid Classes

Separation of phospholipid from individual neutral lipid classes by thin-layer chromatography is illustrated in Figure 1. Figure 2(i) shows routine thin-layer chromatographic separation of individual phospholipid classes and Figure 2(ii) an alternative phospholipid separation in the presence of boric acid which retards the migration of phosphatidylglycerol and assisted identification of the characteristic positions of particular phospholipids after their development on both chromatoplates.

Exponentially growing intact cells of D. discoideum Ax-2 were found to contain all major phospholipid groups with the exception of sphingomyelin. While Wilhelms et al have shown glycosphingolipids to be active as surface antigens of D. discoideum (36), most glycolipids would have been removed from the lipid extract by the aqueous wash incorporated in the extraction method of Folch et al (41) used in this investigation. Lysophosphatidylethanolamine, lysophosphatidylcholine and lysophosphatidic acid were detected in the intact cell extracts, in agreement with previous studies (30). Phosphatidylglycerol and phosphatidylserine were shown to be components of D. discoideum grown under standard axenic conditions. Mono-, di- and triacylglycerols, ubiquinones, free sterols and sterol esters were all present, stigmastenol being a major component of both the free sterol and sterol ester fractions.

Qualitatively similar phospholipid results were obtained from plasma membrane samples as from intact cell extracts. The quantities of free sterol and sterol ester in the plasma membranes were measured

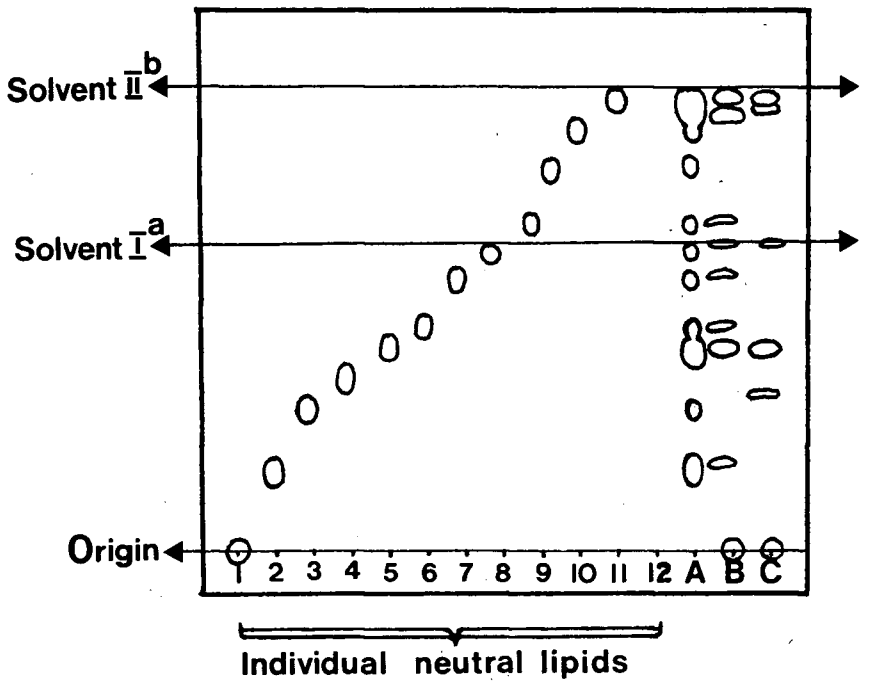

Fig. 1: Separation of neutral lipids by monodirectional, biphasic thin-layer chromatography on silica gel H plates.

Lipids 1 and 3 appear dark under short wavelength ultraviolet irradiation. Lipids 1, 4 and 8 yield blue fluorescence under long wavelength ultraviolet irradiation.

Standard lipids[c]:

1. phospholipid
2. monoacylglycerol
3. ubiquinone
4. unidentified lipidic compound[d]
5. free sterol
6. 1,2-diacylglycerol
7. 1,3-diacylglycerol
8. free fatty acid
9. triacylglycerol
10. fatty acid ester
11. steryl ester
12. hydrocarbon

A. standard neutral lipid mixture
B. D. discoideum Ax-2 whole cell lipid extract
C. D. discoideum Ax-2 plasma membrane (PM1) lipid extract

[a] Solvent I comprised: isopropyl ether-glacial acetic acid (96:4 v/v).

[b] Solvent II comprised: n-hexane-diethyl ether-glacial acetic acid (90:10:1 by vol.).

[c] Each lipid was identified as described under Materials and Methods.

[d] This compound was isolated from D. discoideum membrane preparations as stated under Results.

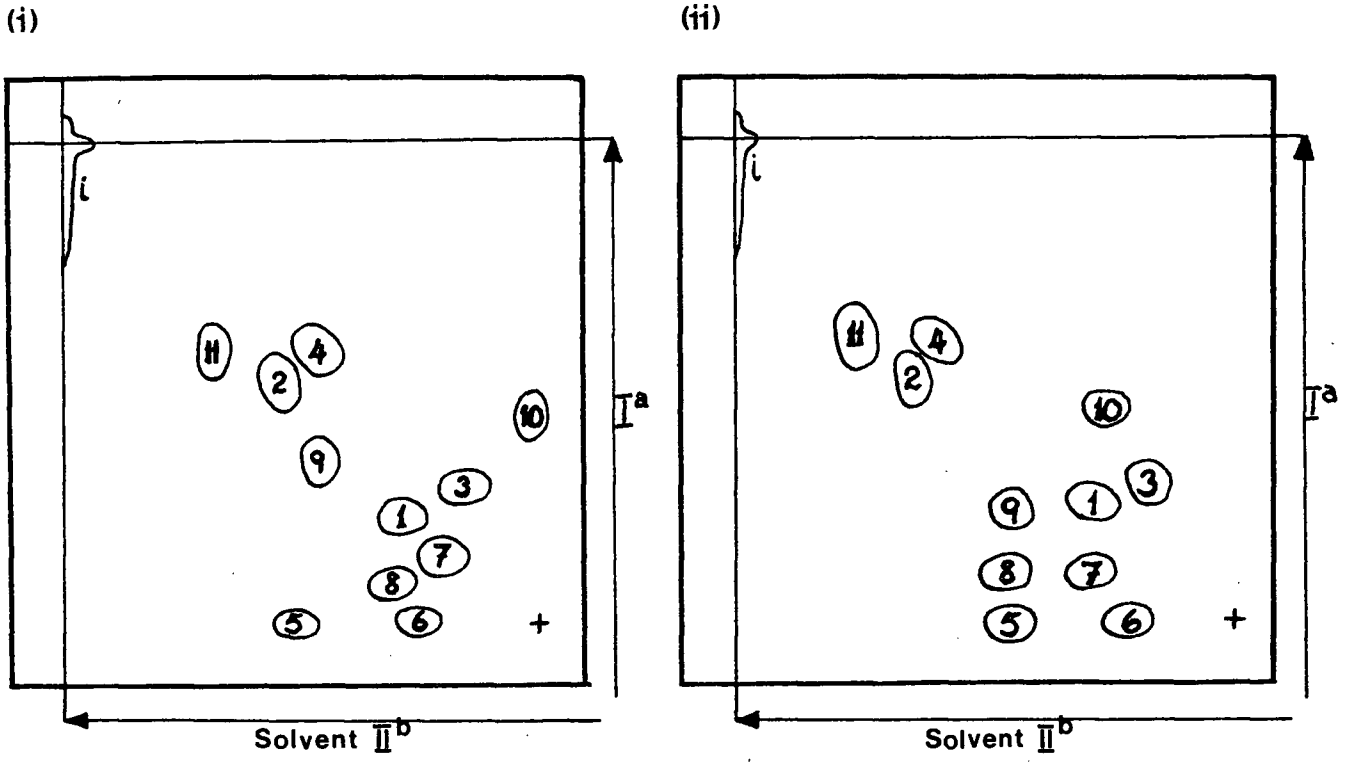

Fig. 2: Two-dimensional, biphasic thin-layer chromatography of a mixture of standard phospholipids on silica gel H plates with additives.

(i) magnesium acetate-silica gel H thin layer of 300 µm thickness[c],

(ii) magnesium acetate-boric acid-silica gel H thin layer of 300 µm thickness[c].

Standard lipids[c]:

1. lysophosphatidylethanolamine
2. phosphatidylethanolamine
3. lysophosphatidylcholine
4. phosphatidylcholine
5. phosphatidic acid
6. lysophosphatidic acid
7. phosphatidylinositol
8. phosphatidylserine
9. phosphatidylglycerol
10. sphingomyelin
11. cardiolipin
i. chromatoplate impurities

[a] Solvent I comprised: chloroform-methanol-28% (w/v) acqueous ammonia (65:25:8 by vol.).

[b] Solvent II comprised: chloroform-acetone-methanol-glacial acetic acid-distilled, deionised water (35:35:7:10:3 by vol.).

[c] Each phospholipid was identified as described under Materials and Methods.

but only trace amounts of acylglycerol were present and quinone derivatives were not detected.

Short wavelength ultraviolet absorption revealed the presence of a currently unidentified neutral lipid compound on chromatoplates following thin layer chromatography of crude and plasma membrane lipid extracts (Fig. 1). Initially this was thought to be one of the organism's less common sterol components migrating with a different mobility than the standard cholesterol, for stigmastenol has a marginally lower R_f than cholesterol in this thin layer chromatographic system. However, the compound's ultraviolet absorption spectrum features radical differences from that of stigmastenol and gas-liquid chromatographic analysis following hydrolytic procedures showed that the unidentified neutral lipid contained a negligable amount of either sterol or fatty acid. The compound emitted blue fluorescence upon irradiation with long wavelength ultraviolet energy. Fluorescence was also observed under both long and short wavelength ultraviolet radiation after treatment of the unknown lipid with rhodamine 6G, from which it was dissociated by solution in chloroform-methanol (4:1 v/v).

c. Quantitative Lipid Determinations of Intact Cell Extracts

To enable comparison of intact cell lipid data with that in the literature (1,30,31,59-62) protein and dry weight measurements were taken from exponentially growing cells and a ratio of 0.59 (protein mass:mass of dry cells) was obtained.

Table 5 displays the individual and total phospholipid content

Table 5: Lipid-phosphorus analysis of intact cells of exponentially growing D. discoideum Ax-2 following phospholipid separation by thin-layer chromatography.

Phospholipid	Current Analysis		Ellingson Analysis
	(nmol mg^{-1} total protein)	(molar percentage)	(molar percentage)
Lysophosphatidylethanolamine	11.4 ± 7.7	8.9 ± 6.0	10.6[b]
Phosphatidylethanolamine	47.1 ± 8.0	36.9 ± 6.3	47.2[b]
Lysophosphatidylcholine	3.1 ± 0.5	2.4 ± 0.4	3.4
Phosphatidylcholine	41.3 ± 7.3	32.4 ± 5.7	16.8
Phosphatidic acid	2.3 ± 0.5	1.7 ± 0.4	3.4[b]
Lysophosphatidic acid	3.5 ± 2.4	2.8 ± 1.9	1.4[b]
Phosphatidylinositol	6.8 ± 1.1	5.3 ± 0.9	8.7
Phosphatidylserine	4.2 ± 3.3	3.3 ± 2.6	-
Phosphatidylglycerol	2.2 ± 0.7	1.7 ± 0.6	0
Sphingomyelin	0	0	-
Cardiolipin	3.6 ± 1.7	2.8 ± 1.5	2.6
Lysocardiolipin	0	0	1.3
Others [a]	3.5 ± 2.5	2.7 ± 1.9	8.3
Total Phospholipid	129.0 ± 16.9		

Data from the current analysis are the mean of five determinations ± standard deviation.

[a] Minor phospholipid components, possibly including lysobisphosphatidic acid (30).

[b] Undetermined amounts of plasmalogen forms also present (30).

of exponentially growing intact cells of D. discoideum Ax-2. In comparison with Ellingson's data for Ax-2 (30), also shown in Table 5, the current analysis yielded major differences. Primarily phosphatidylcholine was recovered in much greater proportion to other phospholipids (32.4 moles %) and phosphatidylethanolamine to a significantly lower extent (36.9 moles %) than they were in the previous study (16.8 moles % and 47.2 moles % respectively), although they were the two most abundant phospholipids in both analyses. However, the earlier report provided percentage values only (30), preventing precise measurement of quantitative differences between these two studies. Ellingson detected no phosphatidylglycerol in Ax-2 and attributed the trace found in cells of strain NC-4 to bacterial sources whereas in the present study phosphatidylglycerol comprised 1.7 moles % of the total phospholipid: the reasons for these discrepancies are not apparent. Ellingson did not record any data for phosphatidylserine (30) although in many similar thin-layer chromatography systems this compound is poorly resolved from phosphatidylinositol. The previously published phosphatidylinositol figure is similar to the total of phosphatidylserine plus phosphatidylinositol detected in this analysis, suggesting that Ellingson did not separate these two components. The levels of lysophosphatidylethanolamine, lysophosphatidylcholine, lysophosphatidic acid, phosphatidic acid and cardiolipin were similar to the previous analysis (30).

Acylglycerol assays of lipid extracts from intact cells were undertaken before and after their separation in the neutral lipid thin-layer chromatography system. Table 6 shows that the total

Table 6: Acylglycerol analysis of intact cells of exponentially growing D. discoideum Ax-2.

Neutral Lipid	Intact Cell Content (nmol mg^{-1} protein)	Intact Cell Content (molar percentage)
Monoacylglycerol	5.9	32
1,2-diacylglycerol	4.2	23
1,3-diacylglycerol	1.1	6
Triacylglycerol	7.4	40
Total Acylglycerol[a] Recovered	18.2 ± 0.4	

[a] Total acylglycerol was measured as an independent parameter.

quantity of acylglycerol in vegetative D. discoideum Ax-2 was small relative to phospholipid (cf. Table 1). Triacylglycerol, a storage lipid in most eucaryotic cells, was the most abundant but significant quantities of the other acylglycerols were also present. Long and Coe quantified triacylglycerol in cells of strain NC-4 growing exponentially on bacteria and their figure of 3.2 mg g^{-1} dry weight (31) (c. 7 nmol mg^{-1} protein) agrees closely with the 7.4 nmol mg^{-1} protein determined here in Ax-2 cells grown under axenic conditions.

The intact cell free sterol analysis shown in Table 7 confirmed that the major free sterol was stigmastenol (31,37), which comprised 85.5 moles % of the total. Two other components of the free sterol fraction were found in significant amounts, their acetates having gas-liquid chromatography retention values relative to cholesteryl acetate of 1.30 (7.5 moles % total free sterol) and 1.59 (4.2 moles % total free sterol). Three other minor constituents could be distinguished. Long and Coe, however, measured in excess of 99% of the free sterol from strain NC-4 as stigmastenol. This was present at approximately 40 nmol mg^{-1} protein (9 mg g^{-1} dry weight) (31) compared to the total of 56.8 nmol mg^{-1} protein reported in this study of Ax-2.

Sterol ester analysis of lipid extracts from intact cells by quantitative gas-liquid chromatography yielded a figure of 1.28 nmol mg^{-1} protein, 2.3 moles % of the free sterol total (Table 7). Previous determinations of sterol ester content of intact cells of strain NC-4 yielded values from 1 to 3 nmol mg^{-1} protein (up to 2 mg g^{-1} dry weight) (31). In contrast to Long and Coe's finding that stigmastenol was the sole sterol present in the sterol ester

Table 7: Sterol analysis of the free sterols and sterol esters extracted from intact cells of exponentially growing D. discoideum Ax-2.

Retention[a]	Free Sterol of Intact Cells, as Stigmastenol[b]		Sterol Ester Sterol of Intact Cells, as Stigmastenol[b]	
	nmol mg^{-1} protein	molar percentage	nmol mg^{-1} protein	molar percentage
1.17	trace	trace	0.40 ± 0.01	31.2 ± 1.0
1.30	4.3 ± 0.8	7.5 ± 1.4	0	0
1.43[c]	48.5 ± 1.0	85.5 ± 1.8	0.71 ± 0.01	55.5 ± 0.8
1.59	2.4 ± 1.0	4.2 ± 1.7	0.17 ± 0.02	13.5 ± 1.7
Others	trace	trace	0	0
Total Sterol Recovered	56.8 ± 2.8		1.28 ± 0.04	

Free sterol values are the mean of five determinations ± standard deviation; 'trace' indicates <0.4 nmol mg^{-1} protein. Sterol ester values are the mean of two determinations.

[a] Retention figures are the ratio of (sterol acetate:cholesteryl acetate) retention under the quantitative gas-liquid chromatography condition used.

[b] The relative amounts of each sterol were determined by reference to an internal cholesterol standard and were converted to molarities using the molecular weight of stigmastenol, the most abundant sterol of D. discoideum.

[c] Stigmastenyl acetate.

fraction of NC-4 (31) the present study shows that it constitutes only 55.5 % of the sterol moieties of Ax-2 sterol esters (Table 7). The second most abundant sterol of the Ax-2 sterol ester fraction was a minor component of the free sterol fraction and had a retention value of 1.17. The second most abundant free sterol (retention value 1.30) was absent from the sterol ester fraction. Thus the sterol profiles of these two lipid classes were very different.

d. Evaluation of Phospholipid Degradation During Plasma Membrane Preparation.

Membrane lipid measurements have been recorded relative to the protein content of their respective membrane fractions. Crude membrane preparations underwent minimal protein loss in the twelve hours following homogenization, indicating that protein degradation was insignificant under the conditions of preparation.

Ferber et al discovered high activities of phospholipase A and lysophospholipase in D. discoideum v12/M1 grown in association with bacteria (63). These two activities would lead to the formation from phosphoglycerides of non-acylated (sn) glycerol-3-phosphatidyl derivatives which would be fully soluble in the aqueous phase of the standard extraction washing procedure (41) and excluded from subsequent lipid-phosphorus analysis. No evidence of phospholipase C activity has been found in cell homogenates (63). Any lipase activity was of great significance to this determination of plasma membrane lipid composition for the membrane preparation procedure required a minimum of 17½ hours.

Figure 3 demonstrates that phospholipid degradation was observed in cell homogenates at 22°C but that this was reduced at 4°C. When lipid-phosphorus is expressed as the logarithm of the percentage remaining at least two distinct rates of loss are revealed (Fig. 3). This suggests that if enzymatic degradation was responsible there was either more than one pool of phospholipid as substrate or that lipases were isolated within particular subfractions. Both the short and the long-term degradation rates increased at the higher temperature. Figure 4 confirms that the early phospholipid loss following homogenization was considerably greater at 22°C than at 4°C. Consequently the entire plasma membrane preparation procedure was carried out at 4°C or on ice and each step performed as rapidly as possible. The PMSF used to inhibit serine-protease activity during plasma membrane preparation had no apparent effect on the rate of loss of lipid-phosphorus from homogenates. Also ineffective was the polyene antibiotic filipin which Ferber *et al* had found to inhibit the phospholipase A and lysophospholipase activities of *D. discoideum* v12/M1 (63).

Crude membranes were prepared rapidly at 4°C and then immediately incubated at 22°C with either 8.6% sucrose-Tris-PMSF buffer, pH 7.4, or with the 105,400 xg supernatant fraction (see Materials and Methods (b)). Figure 5 shows that the rate of lipid-phosphorus loss from the crude membranes was increased approximately four-fold by the presence of the supernatant suggesting that much of the lipase activity observed in the earlier experiments was not membrane bound. Crude membranes were also prepared and incubated in the presence of excess of the following putative *D. discoideum* phospholipase

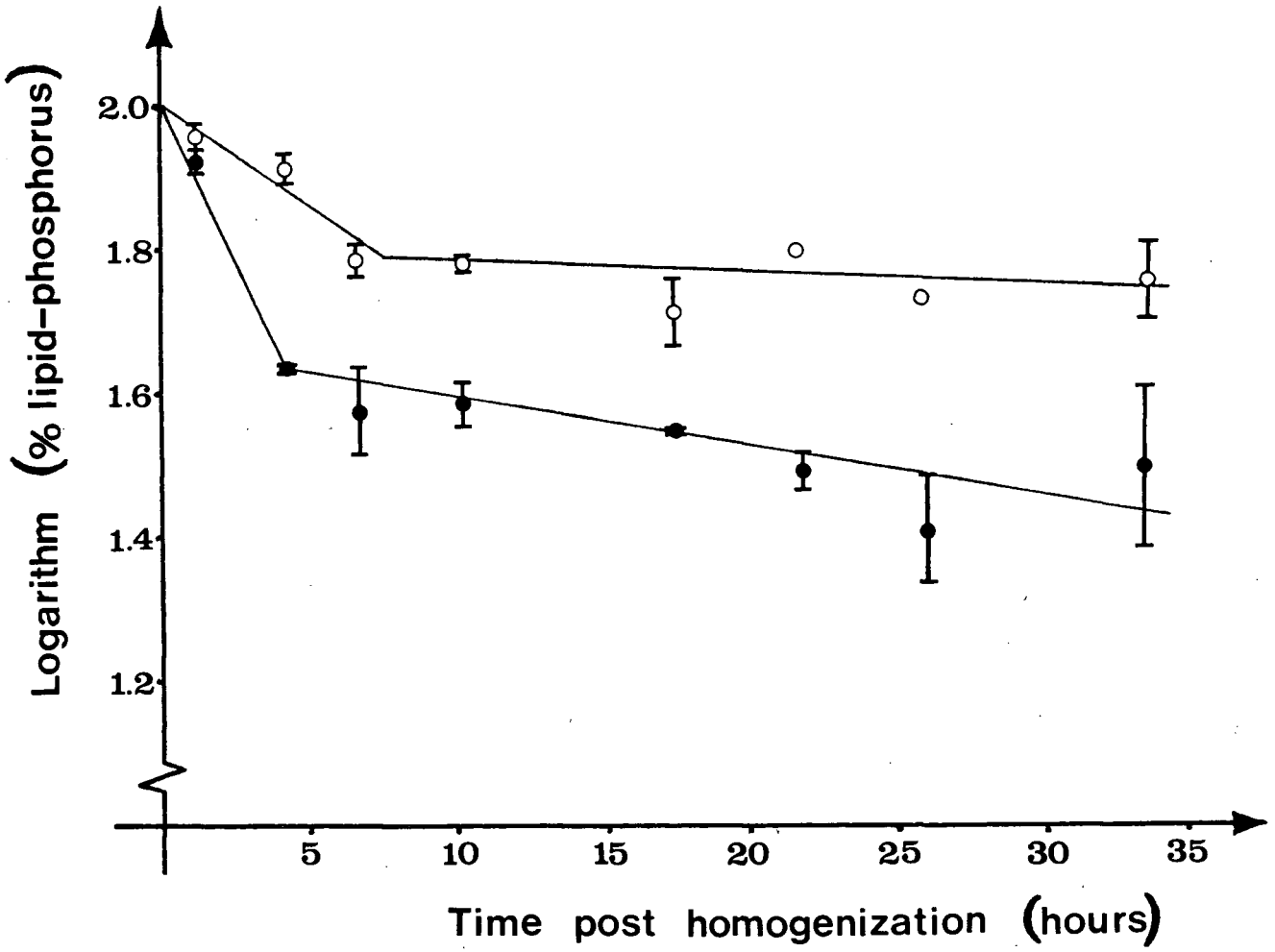

Fig. 3: The effect of temperature on the rates of loss of lipid-phosphorus from D. discoideum Ax-2 homogenates over an extended period.

Intact cells were removed from the homogenate by centrifugation at 700 xg and incubation was carried out at 4°C (○) or at 22°C (●) in 8.6% sucrose-Tris-PMSF buffer, pH 7.4, as described under Materials and Methods.

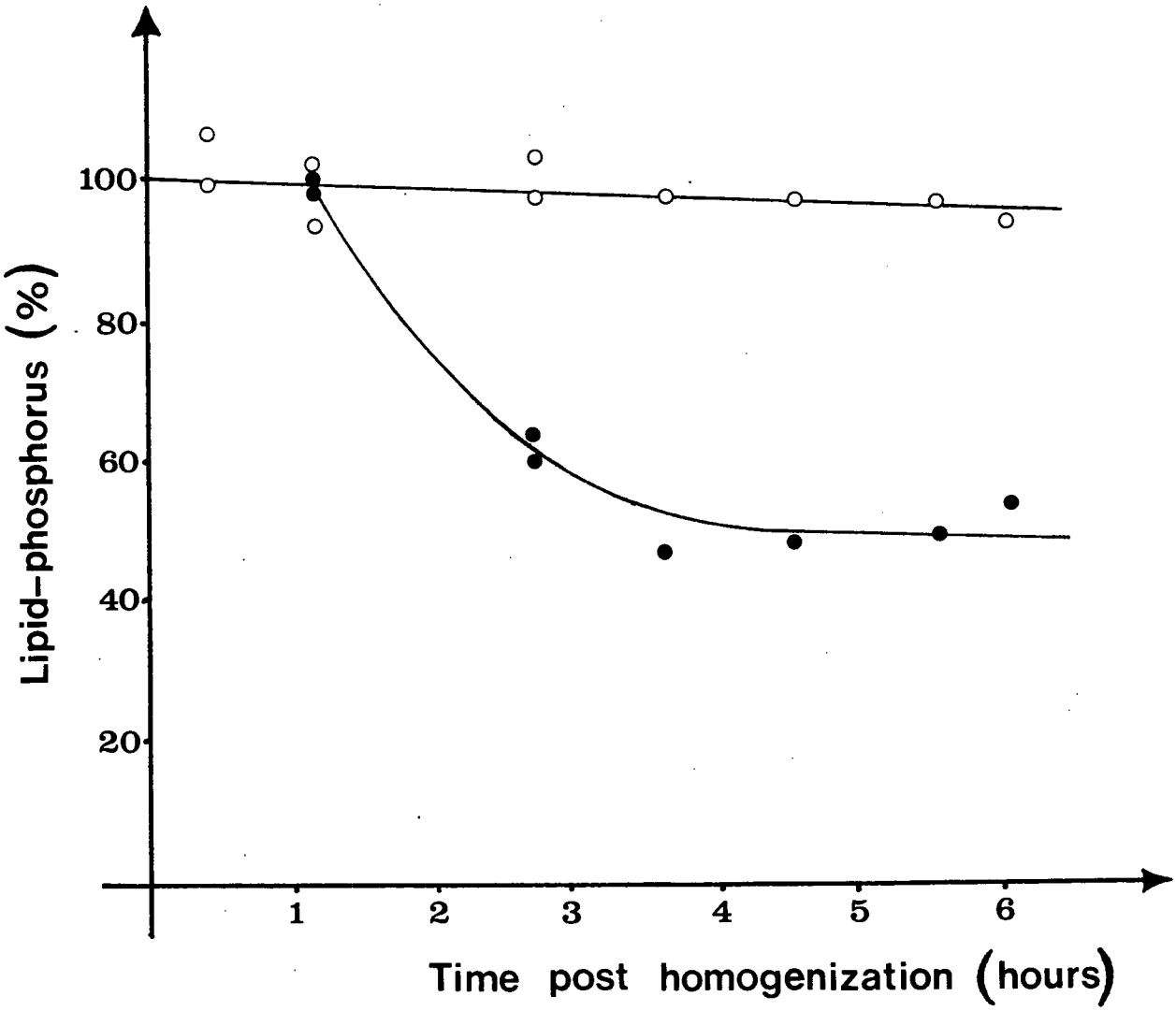

Fig. 4: The effect of temperature on the early loss of lipid-phosphorus from D. discoideum Ax-2 homogenates.

Intact cells were removed from the homogenate by centrifugation at 700 xg and incubation was carried out at 4°C in 8.6% sucrose-Tris-PMSF buffer, pH 7.4, as described under Materials and Methods (○). After 65 minutes half of the preparation was transferred from 4°C to 22°C (●).

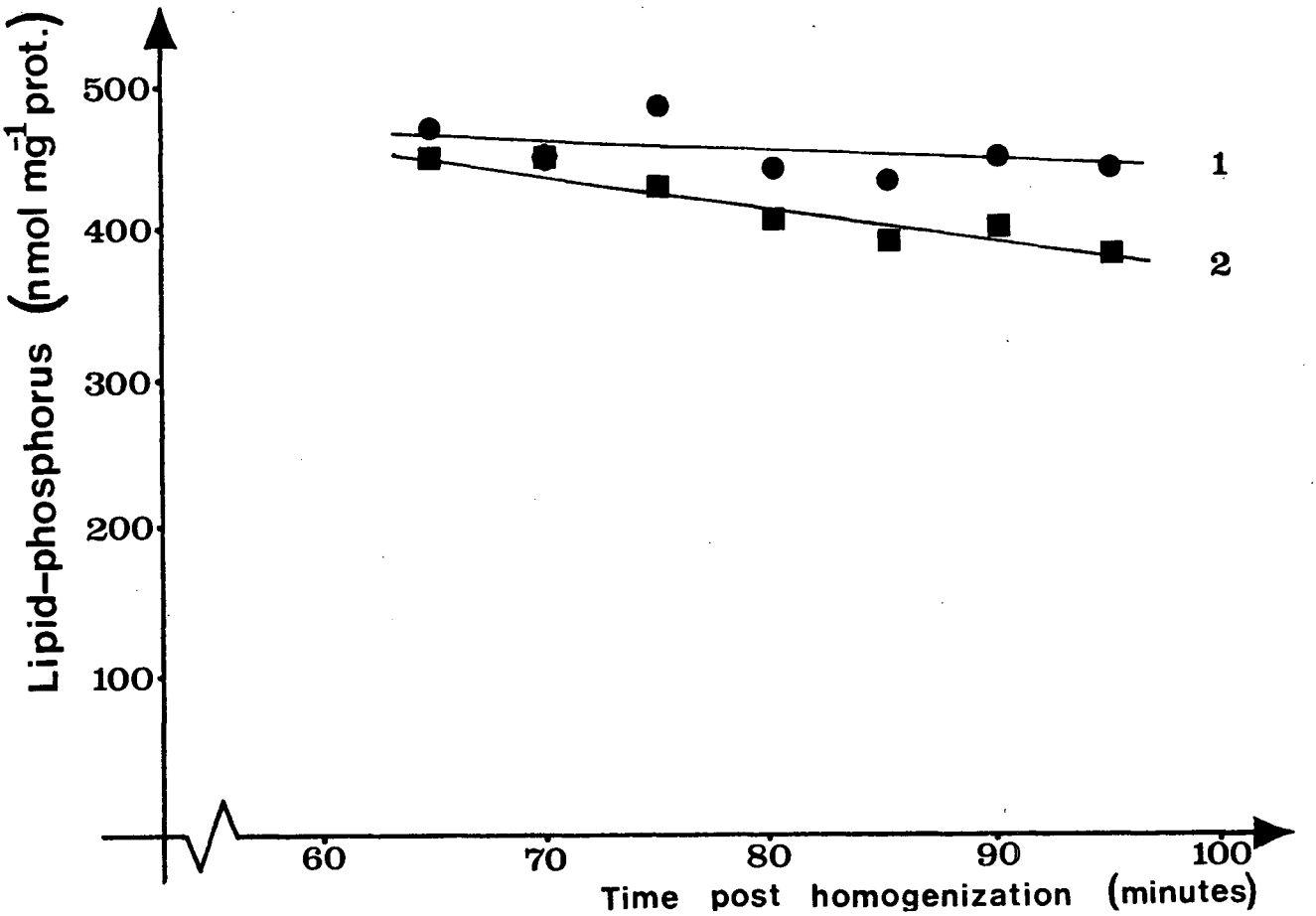

Fig. 5: The effect of the soluble fraction of cell homogenates of D. discoideum Ax-2 on the lipid-phosphorus content of crude membrane preparations incubated at 22°C.

The crude membrane samples were prepared at 4°C as described under Materials and Methods.

(1) Crude membranes resuspended in 8.6% sucrose-Tris-PMSF buffer, pH 7.4, and incubated at 22°C (●).
Rate of lipid-phosphorus loss[a] = 0.6 nmol mg^{-1} protein min^{-1}.

(2) Crude membranes resuspended in the supernatant from the 105,400 xg preparatory centrifugation step (homogenate soluble fraction) and incubated at 22°C (■).
Rate of lipid-phosphorus loss[a] = 2.3 nmol mg^{-1} protein min^{-1}.

[a] Data was subjected to linear regression analysis in order to obtain the average rate of change of lipid-phosphorus over the sampling period.

inhibitors: 4-chloromercuribenzoic acid, sodium 4-hydroxymercuribenzoate, digitonin in both aqueous suspension and methanolic solution, ethylenediaminetetraacetic acid (63). However, none were effective at reducing lipid phosphorus loss from crude membranes. Figure 6 shows that this loss was minimal over a 20 hour sampling period when the incubation temperature was maintained at $4^{o}C$. Plasma membranes were routinely isolated within this time.

In order to determine whether particular phospholipids were modified crude membranes were incubated in 8.6% sucrose-Tris-PMSF buffer, pH 7.4, for a time period equivalent to that of a plasma membrane preparation (Table 8). Phosphatidylcholine was observed to decrease very dramatically from 95.0 to 30.5 nmol mg^{-1} protein in 20 hours at $4^{o}C$ and even further at $22^{o}C$. Lysophosphatidylcholine remained at a low level throughout and phosphatidic acid and lysophosphatidic acid did not increase greatly suggesting that a phospholipase C or a combination of phospholipases were functioning specifically. Phosphatidylethanolamine also decreased significantly, virtually all of the loss occurring in the first 10 hours of the incubation. Lysophosphatidylethanolamine increased to a degree that was almost equivalent to the loss of phosphatidylethanolamine indicating that the disappearance of lipidic choline from the incubation may have been due to a selective activity. Of the minor components phosphatidylinositol and cardiolipin quantities fluctuated to some extent, the amount of phosphatidylserine remained small and phosphatidylglycerol was entirely degraded. The category of 'others' contains an increasing amount of material with time. It includes unidentified new spots which appeared on the chromato-

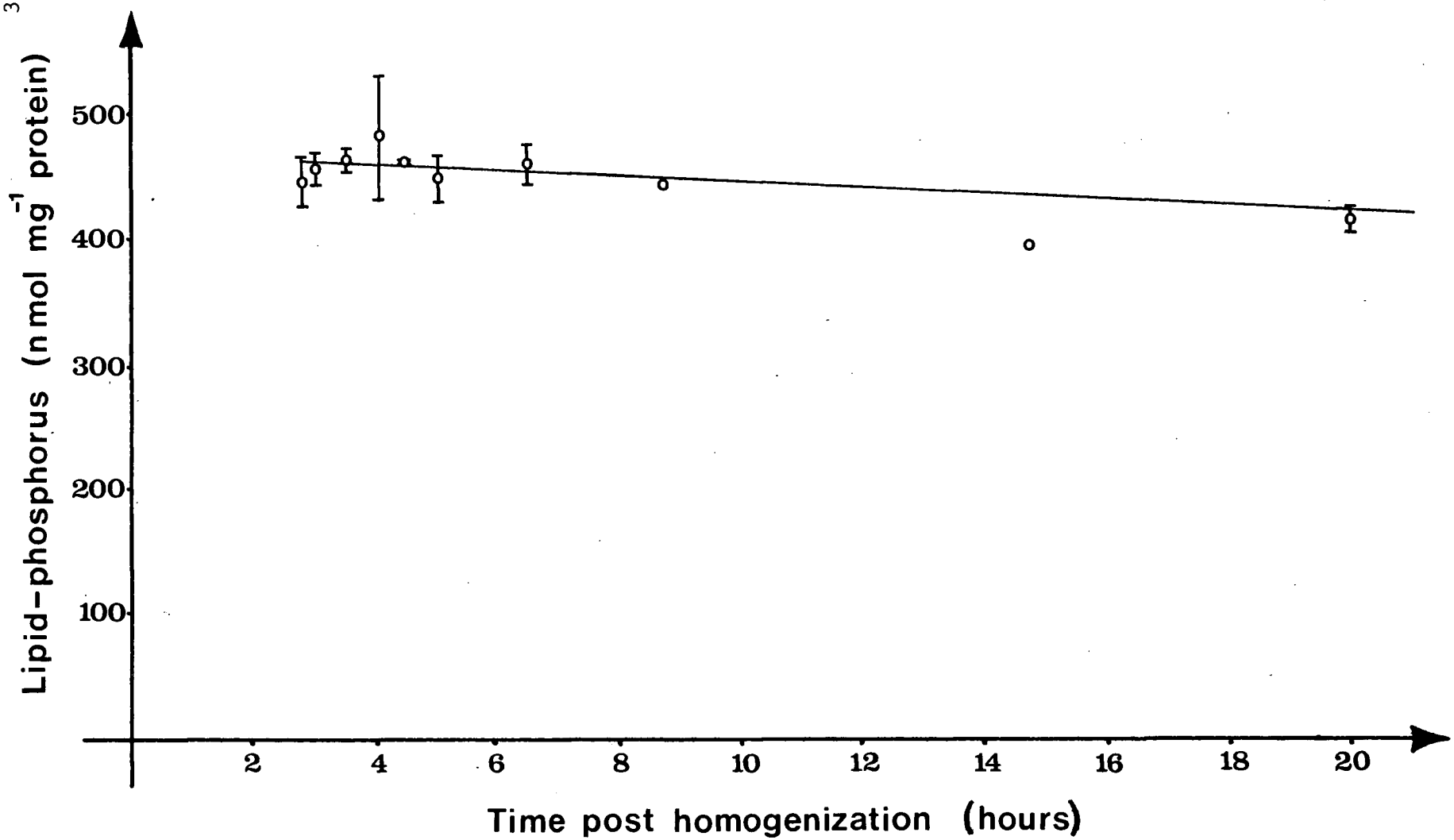

Fig. 6: Lipid-phosphorus content of D. discoideum Ax-2 crude membranes during extended incubation at 4°C.

Data was subjected to linear regression analysis using the least squares procedure. Rate of change of lipid-phosphorus = -2.2 n mol mg^{-1} protein hour^{-1}.

Table 8: Proportion of individual phospholipid components of D. discoideum Ax-2 crude membranes resuspended in 8.6% sucrose-Tris-PMSF buffer, pH 7.4, incubated at either 4°C or 22°C as shown.

Phospholipid	Zero Time	10 hr at 4°C	20 hr at 4°C	20 hr at 22°C
	(nmol mg^{-1} protein)			
Lysophosphatidylethanolamine	128.5 ± 36.0	143.5 ± 23.0	151.0 ± 14.0	164.5 ± 31.0
Phosphatidylethanolamine	120.5 ± 23.5	72.0 ± 9.0	69.0 ± 4.0	71.0 ± 18.5
Lysophosphatidylcholine	11.5 ± 5.0	8.0 ± 3.5	5.0 ± 3.5	1.0 ± 1.0
Phosphatidylcholine	95.0 ± 4.5	44.0 ± 6.0	30.5 ± 8.0	21.5 ± 11.0
Phosphatidic acid	8.0 ± 8.0	13.0 ± 8.5	20.5 ± 12.5	25.5 ± 18.0
Lysophosphatidic acid	22.0 ± 2.5	43.0 ± 2.0	19.0 ± 18.5	19.5 ± 13.0
Phosphatidylinositol	41.5 ± 14.5	38.5 ± 23.0	54.0 ± 9.0	48.5 ± 15.0[b]
Phosphatidylserine	6.5 ± 5.0	2.5 ± 2.5	3.0 ± 3.0	
Phosphatidylglycerol	5.5 ± 4.5	0	0	0
Sphingomyelin	0	0	0	0
Cardiolipin	16.5 ± 6.5	17.5 ± 6.5	34.5 ± 18.5	15.5 ± 10.5
Others[a]	9.0 ± 8.0	55.5 ± 34.5	30.0 ± 14.0	23.0 ± 14.0
Total Phospholipid	464.5	437.5	416.5	389.0

Values of lipid-phosphorus recovered are presented as the mean of dual lipid extractions. The phospholipids were separated by thin-layer chromatography before being assayed.

[a] Unidentified minor components.

[b] These two phospholipids did not separate fully in this analysis.

grams as the incubation proceeded and probably comprised phosphorylated degradation products. Incubation at 22°C generally enhanced the changes observed at 4°C.

Thus the plasma membrane phospholipid results are probably accurate for phosphatidic acid, lysophosphatidic acid, phosphatidylserine, phosphatidylinositol and cardiolipin whereas phosphatidylcholine, lysophosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol are possibly underestimated and lysophosphatidylethanolamine may be overestimated.

e. Plasma Membrane Lipid Composition

The individual phospholipids, the sterols and the phospholipid fatty acid compositions of the two plasma membrane fractions PM1 and PM2 (35) at early stages of the organism's development are shown in Tables 9, 10 and 11. It has been suggested that PM1 may be a purer plasma membrane preparation than PM2 or that the two may represent different areas of surface specialization (37).

The total phospholipid of both PM1 and PM2 decreased substantially in the first eight hours of development (Table 9). The loss was 29% in PM1 (from 690.3 to 491.5 nmol mg^{-1} protein) and 38% in PM2 (from 583.3 to 362.4 nmol mg^{-1} protein). This reduction in phospholipid:protein ratio might reflect the synthesis or incorporation of membrane protein or the degradation or removal of membrane lipid. It is not possible to distinguish between these possibilities from the data in Table 9 although protein content per cell has been shown to decrease over this period (1). Both plasma membrane fractions

Table 9: Plasma membrane lipid-phosphorus analysis at three stages of development of D. discoideum Ax-2 following phospholipid separation by thin-layer chromatography.

Phospholipid	PM1[a]			PM2[a]		
	0 hrs	8 hrs	16 hrs	0 hrs	8 hrs	16 hrs
	(% lipid phosphorus recovered)			(% lipid phosphorus recovered)		
Lysophosphatidylethanolamine	38.5 ± 1.5	17.9	42.8 ± 1.0	27.9 ± 4.4	36.7	37.4 ± 0.3
Phosphatidylethanolamine	19.4 ± 1.0	17.9	13.8 ± 3.5	29.7 ± 11.2	17.7	12.3 ± 3.1
Lysophosphatidylcholine	0	0	0	trace	5.9	1.8 ± 0.3
Phosphatidylcholine	31.1 ± 2.0	43.4	5.3 ± 1.1	25.6 ± 6.0	25.0	4.1 ± 2.0
Phosphatidic acid	0	0	trace	trace	0	16.0 ± 6.0
Lysophosphatidic acid	0	1.9	8.9 ± 5.7	4.6 ± 4.4	0	4.5 ± 0.2
Phosphatidylinositol	8.6 ± 0.5	14.2	8.4 ± 3.7	6.5 ± 2.1	8.8	7.8 ± 0.7
Phosphatidylserine			trace	0	0	0
Phosphatidylglycerol	trace	0	8.7 ± 2.6	trace	0	14.8 ± 0.9
Sphingomyelin	0	0	0	0	0	0
Cardiolipin	trace	trace	0	trace	5.9	0
Others[b]	1.2 ± 0.2	3.8	7.9 ± 1.7	3.4 ± 1.8	0	1.2 ± 1.2
Total Phospholipid (nmol mg^{-1} protein)	690.3 ± 27.2	491.5	487.4 ± 66.5	583.3 ± 41.9	362.4	360.5 ± 12.5

'0 hrs' indicates that cells were growing exponentially and were either harvested for plasma membrane analysis or washed free of nutrients and placed in buffer to initiate development to permit analysis at later time points. 0-, 16- and 8 hr determinations are the mean of four, two and one set of plasma membrane lipid extractions respectively. Errors are recorded as standard deviations where appropriate; 'trace' indicates <1% total phospholipid.

[a]: PM1 and PM2 are the two plasma membrane preparations obtained by the technique described under Materials and Methods.

[b]: Unidentified minor components.

maintained their eight hour phospholipid:protein total after sixteen hours of development, yet numerous membrane-associated protein changes are known to occur during early development (1,9-12).

Compared with the whole cell exponentially grown D. discoideum Ax-2 PM1 fractions (cf. Table 5, column 2; Table 9, column 1) contained a far greater percentage of lysophosphatidylethanolamine (38.5 moles %) and a substantially lower proportion of phosphatidylethanolamine (19.4 moles %). Lysophosphatidylcholine, phosphatidic acid and lysophosphatidic acid were absent from PM1 and cardiolipin was reduced in both plasma membrane fractions (0.9 moles %). The PM2 fraction differed from PM1 in that before development began phosphatidylethanolamine and lysophosphatidylethanolamine were present in equivalent proportions, approximately 29 moles % each (Table 9, column 4). Lysophosphatidic acid and traces of lysophosphatidylcholine and phosphatidic acid were also detected in PM2. Phosphatidylcholine was present in similar proportion (c. 30 moles %, relative to total phospholipid) in the intact cells, PM1 and PM2 fractions alike.

The phosphatidylcholine in PM1 was found to decrease dramatically relative to protein between the eighth and sixteenth hours of Ax-2 development (Table 9, columns 1 to 3). Phosphatidylethanolamine also decreased by a factor of two but this reduction occurred progressively from zero time. The lysophosphatidylethanolamine content of PM1 fluctuated considerably. In vegetative cells and after 16 hours development approximately 57 moles % of the total phospholipid in PM1 contained ethanolamine, although the proportion dropped to 36 moles % at the 8 hour stage. Lysophosphatidylcholine was not detected at any time in PM1, yet phosphatidic acid and

lysophosphatidic acid increased as development progressed. In view of the substantial loss of phosphatidylcholine in the latter half of the observed period these results may indicate that one or more phospholipases acted preferentially on phosphatidylcholine in the plasma membrane during development. Previous data had shown that crude membranes were capable of degrading phosphatidylcholine in the presence of lysophosphatidylethanolamine without an accumulation of lysophosphatidylcholine (Table 8). Phosphatidylglycerol and cardiolipin were present in small quantities in the PMI fraction of exponentially growing cells, although after 16 hours phosphatidylglycerol had increased substantially to 8.7 moles % of total phospholipid. The proportion of unidentified compounds also increased as development progressed (Table 9, 'others'). Phosphatidylinositol and phosphatidylserine remained relatively constant in PMI during the observed period.

Plasma membrane fraction PM2 provided similar phospholipid results to PM1 during early development, the major differences being a greater reduction of phosphatidylethanolamine with time, a smaller amount of lysophosphatidylethanolamine initially and more of the minor components although phosphatidylserine was absent throughout and there was less unidentified phospholipid (Table 9).

Table 10 shows the sterol compositions of the free sterol and sterol ester fractions of PM1 and PM2 from both exponentially growing cells and those which had undergone 16 hours development. The total quantity of free sterol in PM1 dropped slightly during

Table 10: Plasma membrane free sterol and sterol ester sterol analysis at two stages of development of D. discoideum Ax-2.

Retention	Plasma Membrane Fraction PM1[a]				Plasma Membrane Fraction PM2[a]			
	0 hours		16 hours		0 hours		16 hours	
	free sterol	sterol ester	free sterol	sterol ester	free sterol	sterol ester	free sterol	sterol ester
	(nmol mg^{-1} protein)[c]							
0.85	11.4 ± 6.4	0	1.9 ± 0.7	0	8.5 ± 2.8	0	18.1 ± 8.1	0
1.17	16.8 ± 4.1	1.5 ± 0.1	2.8 ± 0.2	4.6 ± 0.8	18.6 ± 11.6	0.9 ± 0.5	17.4 ± 7.2	6.6 ± 2.1
1.30	25.1 ± 0.5	0	22.8 ± 6.1	0	19.6 ± 2.6	0	12.2 ± 2.1	0
1.43[d]	170.1 ± 32.2	3.7 ± 0.3	200.3 ± 7.7	37.1 ± 0.6	189.4 ± 23.5	1.7 ± 0.4	275.9 ± 12.5	22.3 ± 1.5
1.59	17.8 ± 8.4	1.0 ± 0.2	5.6 ± 0.0	2.8 ± 1.2	21.7 ± 8.5	0.6 ± 0.2	5.3 ± 0.0	0.7 ± 0.7
1.83	12.9 ± 6.5	0	1.4 ± 0.4	0	0	0	0	0
Total Sterol	253.8 ± 19.3	6.2 ± 0.9	234.5 ± 14.3	44.4 ± 8.2	257.8 ± 10.6	3.2 ± 1.8	328.5 ± 67.3	29.6 ± 2.5

'0 hour' and '16 hour' data were determined from three and two sets of plasma membrane lipids respectively.

Values are expressed as the mean ± standard deviation.

[a] PM1 and PM2 are the two plasma membrane preparations obtained by the technique described under Materials and Methods.

[b] Retention figures are the ratio of (sterol acetate: cholesteryl acetate), retention under the gas-liquid chromatography conditions used.

[c] Molar equivalents were calculated as described in Table 7 [(b)]. These values were related to membrane protein content.

[d] Stigmastenyl acetate.

this period, whereas that of PM2 increased. The proportion of stigmastenol in the free sterol fraction of vegetative cells' plasma membranes was less than that of intact cell extracts (cf. Tables 7 and 10), being 67 moles % and 74 moles % in PM1 and PM2 respectively. However, after 16 hours development the free sterol composition of both membrane fractions resembled that of exponentially growing whole cells, each containing 84-86 moles % stigmastenol. There was a component with a standard retention of 1.83 which was present in fraction PM1 at zero time, yet became a very minor component after 16 hours development: it was not observed in PM2.

During exponential growth of strain Ax-2 the quantity of sterol ester was virtually twice as large in PM1 as in PM2 (Table 10) and yet, as in the intact cell, the plasma membrane sterol ester content was only 1-3 moles % of the total sterol. The sterol compositions of the plasma membrane sterol ester fractions were also similar to that of whole cell sterol esters (cf. Tables 7 and 10). The 6.2 nmol sterol ester mg^{-1} protein recovered in PM1 at zero time constitutes a five-fold enrichment from the homogenate, suggesting that the sterol ester is largely confined to membranous structures within the cell. After 16 hours development the plasma membranes' sterol ester content had increased dramatically; that of PM1 seven-fold and that of PM2 nine-fold. Only three sterol moieties were detected in the ester fraction: initially stigmastenol comprised 60 moles % of the sterol ester sterol in PM1 and 54 moles % in PM2, these figures rising to 84 and 75 moles % respectively after sixteen hours development. The differences between the sterol compositions of the free sterol and sterol ester fractions of whole cells, and

plasma membranes at both the zero and at the 16 hour time points, suggest that a selective esterification mechanism may be operating (Tables 7 and 10).

The fatty acid compositions of the total phospholipid isolated from the two plasma membrane fractions of D. discoideum at zero and sixteen hours development are displayed in Table 11. In exponentially growing cells the phospholipid acyl chains of both PM1 and PM2 were approximately 14% fully saturated and 86% unsaturated, whereas after sixteen hours development the saturated components had risen to approximately 29% leaving 71% unsaturated. Before development began almost 80% of both membrane fractions' acyl chains comprised 18:1 $^{(\Delta 9 \text{ or } \Delta 11)}$ or 18:2 $^{(\Delta 5,9 \text{ or } \Delta 5,11)}$, these two being represented in similar quantities. After sixteen hours development the total of these fatty acids had dropped to 53% in PM1 and 64% in PM2. Moreover, most of this loss was from the 18:2 pool. Consequently one might expect the membrane fluidity to decrease substantially over the same period.

Table 11: D. discoideum Ax-2 plasma membrane phospholipid fatty acid composition at two stages of the organism's development.

Fatty Acid	Plasma Membrane Fraction PM1[a]		Plasma Membrane Fraction PM2[a]	
	0 hours	16 hours	0 hours	16 hours
	(% recovered)	(% recovered)	(% recovered)	(% recovered)
14:0	0.9	1.7 ± 0.8	0.9	1.2 ± 0.0
Palmitaldehyde	0.4	3.1 ± 1.9	0.6	1.0 ± 0.4
16:0	9.4	14.3 ± 1.2	10.9	12.9 ± 2.9
$16:^{(\Delta 9)}$	3.0	3.5 ± 0.8	2.2	1.6 ± 0.6
$16:2^{(\Delta 5,9)}$ & 17:0[b]	1.7	4.2 ± 0.7	1.8	2.1 ± 0.4
18:0	3.9	13.5 ± 2.0	2.5	12.3 ± 3.5
$18:1^{(\Delta 9)}$ & $18:1^{(\Delta 11)}$[b]	40.9	35.2 ± 2.1	37.4	45.0 ± 1.2
$18:2^{(\Delta 5,9)}$ & $18:2^{(\Delta 5,11)}$[b]	37.5	17.9 ± 2.5	39.9	19.0 ± 0.9
$18:2^{(\Delta 9,12)}$	1.2	2.1 ± 2.1	1.4	2.1 ± 0.5
Others[c]	1.1	4.5 ± 1.4	2.4	2.8 ± 1.2

[a] PM1 and PM2 are the two plasma membrane preparations obtained by the technique described under Materials and Methods.

[b] These fatty acids were not separated under the conditions used but were shown to be present by Davidoff and Korn (61).

[c] Several unidentified minor components.

DISCUSSION

In this analysis D. discoideum Ax-2 lipid quantitation data has been expressed in both molar percentages and in relation to the protein content of the intact cells or the membrane preparation from which the lipids were extracted. Although decreases in both protein and dry weight per cell are among many parameters known to alter during the organism's development (1,59,64), the criteria of reliability and reproducibility (31) favoured the use of the protein standard for the current investigation.

The superiority of the lipid extraction method used in this study, modified from that of Folch et al (41), is apparent from the data in Tables 1, 2, 3 and 4, although a disadvantage of acid extraction conditions is the possibility of plasmalogen hydrolysis (65). Throughout this investigation the acid extraction following each Folch treatment was kept separate from the initial chloroform-methanol extract until the HCl had been removed in a stream of nitrogen. Nevertheless it is possible that sufficient acid remained to degrade plasmalogens to their lyso derivatives when the two extracts were pooled and that previous reports of a high lysophosphatidylethanolamine content of intact D. discoideum cells (30,61) may also be due to hydrolysis of the phosphatidylethanolamine plasmalogen. Ellingson detected plasmalogens in certain phospholipid isolates (Table 5) but did not report the quantities present (30). Thus the higher lysophosphatidylethanolamine content of lipid extracts of crude membranes and plasma membranes compared with those of intact cells (Tables 5, 8 and 9) may indicate that the

phosphatidylethanolamine plasmalogen is enriched in these membranes at the early developmental stages tested. Membranes might be expected to contain plasmalogens rather than lysophospholipids because of the powerful detergent effect of the latter. There is no evidence for the presence of phosphatidylcholine plasmalogen in D. discoideum (30).

The majority of plasma membrane purification methods, including those for obtaining plasma membranes of D. discoideum (35,37), are of extended duration and consequently allow the possibility of considerable phospholipid degradation. Extremely active phospholipases have been detected in the organism by Ferber et al (63) and in the present study substantial phospholipid degradation occurred during incubation of cell-free homogenates in vitro (Figures 3 and 4). There was far less phospholipid degradation during incubation of crude membrane preparations in vitro (Figure 5) indicating that under these conditions most of the phospholipase activity was not membrane bound. The higher lysophosphatidylethanolamine content of both crude and plasma membranes compared with that of intact cells (Tables 5, 8 and 9) may indicate that a certain amount of lysolipid formed after cell breakage and this proposal is reinforced by the apparent conversion of phosphatidylethanolamine to lysophosphatidylethanolamine which was revealed upon prolonged incubation of crude membranes (Table 8). However, the lysolipid may have been produced from plasmalogen during the extraction procedure, a possibility discussed above. Upon incubation of the crude membranes there was also a substantial loss of phospholipid containing choline in contrast to the relatively small loss of lipidic ethanolamine,

which suggests that a mechanism may exist in these membranes for the specific removal or degradation of choline-containing phospholipids.

Phospholipid degradation during plasma membrane purification was minimized by shortening the previously published procedure (37) wherever possible. The method of sucrose gradient centrifugation used to purify plasma membranes from crude membranes may eliminate the residual lipid degradation by physical separation of the plasma membrane fractions from the enzymes responsible. However, any lipase activity specifically associated with the plasma membrane would be simultaneously enriched, which might lead to an exaggeration of lipid changes brought about by developmentally controlled plasma membrane enzyme activities. There are precedents for phospholipase activities in plasma membranes: phosphatidic acid phosphorylase is present in chick embryonic muscle cell plasma membranes (66) and phospholipase A, lysophospholipase, acyl-CoA hydrolase and palmitoyl-CoA synthetase activities have been measured in plasma membranes of Acanthamoeba castellanii (67). Nevertheless plasma membrane phospholipases do not appear to interfere with the analysis of D. discoideum plasma membrane lipid composition for prolonged incubation of this organism's plasma membranes in vitro causes no decrease in the amount of membrane bound phospholipid nor alteration of the membranes' phospholipid composition (G. Weeks, unpublished observations).

A number of D. discoideum plasma membrane lipid changes occurred during early development of the cells in buffered shake suspension. After 16 hours a considerable reduction of the membranes' phospha-

tidylcholine content had taken place (Table 9). In addition there were several smaller alterations of other phospholipids. These changes comprised a decrease in the proportion of phosphatidylethanolamine, which was accompanied by an increase in the proportions of lysophosphatidylethanolamine and phosphatidylglycerol and by the accumulation of lysophosphatidic acid and phosphatidic acid in plasma membrane fractions PM1 and PM2 respectively. Apart from the increase of phosphatidylglycerol these results reflect an overall degradation of phospholipid species.

The most dramatic modification of plasma membrane lipid during early development was the eight-fold increase in the sterol ester content which was accompanied by a slight reduction in the amount of free sterol (Table 10).

There was also a substantial alteration of the plasma membrane phospholipid fatty acid composition after 16 hours development (Table 11). The proportions of palmitate and stearate increased markedly at the expense of the octadecadienoic fatty acids (18:2 $^{\Delta 5,9}$ and 18:2 $^{\Delta 5,11}$). Thus the saturation of the phospholipid acyl chains increased substantially over this period. Whether these changes are caused by specific degradation or by specific synthesis of particular fatty acids is unknown, although the total phospholipid of both PM1 and PM2 plasma membrane fractions decreased substantially relative to protein during the first eight hours of development (Table 9).

Electron spin resonance and fluorescence depolarization analyses of D. discoideum plasma membranes prepared from cells that had developed for 16 hours under the conditions used in the present study revealed a pronounced decrease in membrane fluidity (Herring, F.G. and I. Tatischeff, unpublished observations) which is in

keeping with the changes in lipid composition reported above. In the suspension buffer aggregation was initiated within 8 hours of transfer and at this time the cells exhibit no alteration of plasma membrane fluidity (Herring, F.G. and I. Tatischeff, unpublished observations) although changes in membrane phospholipid were apparent (Table 9). Thus the establishment of cell-cell contacts does not appear to be correlated with overall changes in plasma membrane fluidity even though alterations of phospholipid composition are associated with this stage of development under these conditions. It is possible that the fluidity changes occurring between 8 and 16 hours are unconnected with normal development since plasma membranes prepared from cells developing on a solid surface for the same time period show no alteration of fluidity at any stage. The 16 hour aggregates formed in suspension were of normal size and proceeded to develope and differentiate normally when transferred to a solid surface despite their decreased membrane fluidity. Thus the altered membrane fluidity imposed under the standardised but artificial conditions of suspension culture (36) do not appear to impair normal cell-cell interaction although at present it is unknown whether the membrane fluidity reverts to 'normal' when the differentiation of these aggregates is permitted on a solid surface.

The results presented in this study indicate that substantial lipid changes occur in the plasma membrane of D. discoideum during early development in shake suspension. Most intriguing is the large increase in sterol ester. A possible function for such a molecule in development is not easily visualized at present although stalk differentiation has been induced in cell monolayers of the

organism (68), and the inducing component appears to be a neutral lipid (Stanfield, E. and G Weeks, unpublished observations). Sufficient quantities of this material for the determination of its precise molecular nature are currently unavailable.

The plasma membrane lipid measurements outlined in this study provide a background for further analysis throughout the organism's development. If the phospholipid changes, including those of the fatty acid moieties, which have been observed in the present study are of developmental importance then they should also be observed during development of D. discoideum on a solid surface. It may be especially pertinent to determine the sterol ester content of the plasma membrane during development under these conditions.

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