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ISOLATION OF THE PLASMA MEMBRANE OF A TRYPANOSOMATID FLAGELLATE: GENERAL CHARACTERISATION AND LIPID COMPOSITION

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SUMMARY

A technique is described for the isolation of a fraction that contains the plasma membrane of the trypanosomatid flagellate *Leptomonas collosoma*. This fraction has been investigated by electron microscopy and has been shown to be mostly membranes associated with microtubules, a known plasma membrane marker in this organism. The fraction is enriched in Mg^{2+}-dependent ATPase but has a decreased specific activity of succinate dehydrogenase. Lipid has been extracted from whole cells and the isolated plasma membrane fraction. A fraction of the total lipid that is eluted from a silicic acid column by acetone is found to be concentrated in the plasma membrane. Also enriched in the plasma membrane fraction is a 5,7-diene sterol identified as ergosterol. The major phospholipids of the whole cell and the plasma membrane are phosphatidylethanolamine and phosphatidylcholine. Approximately 60% of the fatty acids of the cell and plasma membrane have a carbon chain length of eighteen, and half of this is in the form of the mono-unsaturated fatty acid.

INTRODUCTION

There have been many attempts to isolate the plasma membranes of eucaryotic organisms. In the main these have utilised multicellular organisms. Reports of attempts to isolate the plasma membranes of protozoans include *Leishmania donovani* [1], *Amoeba proteus* [2], *Acanthamoeba castellanii* [3, 4], *Paramecium aurelia* [5] and *Tetrahymena pyriformis* [6]. Two properties make the plasma membrane of a trypanosomatid flagellate especially interesting. First, these organisms are parasites. Their hosts include insects (in the case of the organism investigated here) or mammals and insects (in the case of those flagellates that are of economic importance as agents of disease). Second, the cell suface may be the site of action of some present or future trypanocidal drugs.

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Accordingly, we have set out to prepare and examine the properties of the plasma membrane of *Leptosomas collosoma*. Detailed analysis of the lipid composition has also been carried out. For comparison, the lipid content of the whole cells has been examined in an effort to identify lipids preferentially occurring in the plasma membrane fraction. In this way a 5,7-diene sterol identified as ergosterol, and a lipid fraction which eluted from silicic acid with acetone have been shown to be enriched in the plasma membrane fraction.

METHODS

The organism

The trypanosomatid flagellate *L. collosoma* was used throughout this work [7]. The original stocks were obtained from the Wellcome Laboratories, Beckenham, Kent, England.

Growth of the organism

Cultures of the organism were grown in 750 ml of medium in 2-l flasks at 25 °C. The medium contained proteose peptone (Difco Laboratories) 20 g/l; dehydrated liver infusion (Difco) 2 g/l; yeast extract (Difco) 2 g/l; glucose 10 g/l; folic acid 0.005 g/l; adenine 0.5 g/l; sodium phosphate 3 g/l, and haemin 0.01 g/l. The haemin was added as a 2.5 mg/ml solution in triethanolamine/water (1 : 3, v/v). The pH of the medium was 8.0.

Analytical procedures

Protein was estimated by the method of Lowry et al. [8]; carbohydrate by the method of Devor [9], and the total nucleic acid by extraction in $HClO_4$ at 95 °C [10]. Lipid was estimated by extraction in chloroform–methanol (2 : 1, v/v), purification on Sephadex G-25 (Pharmacia) [11, 12] and weighing.

In order to determine the sugar composition of the plasma membranes, cellular material was hydrolysed in 0.5 M H_2SO_4 at 125 °C for 1 h. An autoclave with a pressure of 1.05 kg/cm^2 was used. The trimethyl silyl derivatives of the neutral sugars were prepared [13] and analysis was carried out using a Pye Series 104 argon chromatograph with hydrogen flame ionisation detectors. A linear temperature program was used. The initial temperature was 90 °C and rose at 4° per min to 190 °C. The stationary phase was 10% polyethylene glycol adipate on celite.

Enzyme assays

Mg^{2+}-dependent adenosine triphosphatase (EC 3.6.1.4) was assayed according to the procedure of Munoz et al. [14]. In some assays 3 mM $MgCl_2$, 100 mM NaCl, 8 mM KCl, 10 mM $CaCl_2$ and 0.5 mM ouabain were included either alone or together.

5′-Nucleotidase (EC 3.1.3.5) was assayed by the method of Bosmann and Pike [15] or by that of Avruch and Wallach [16].

Succinate dehydrogenase (EC 1.3.99) was assayed as follows. Cell homogenate or purified membranes were preincubated for 1 min with 33.3 mM disodium succinate, 1 mM neutralised KCN, 5 μM 2,6-dichlorophenolindophenol and 0.083 mM Tris–HCl (pH 7.5). The reaction was started by adding phenazine methosulphate

(final concentration, 0.34 mM) and the initial rate of decrease of absorbance at 600 nm was measured.

Cytochrome oxidase (EC 1.9.3.1) was measured by the method of Cooperstein and Lazarow [17].

Acid phosphatase was measured by the release of *p*-nitrophenol from *p*-nitrophenyl phosphate (0.117 mM) in the presence of 0.2 M acetate buffer (pH 4.0).

Electron microscopy

Membrane samples or cells were washed in 0.2 M phosphate buffer (pH 7.6) and were resuspended for 2–24 h in 4% (w/v) redistilled glutaraldehyde buffered with 0.2 M sodium phosphate. They were then washed in the same buffer containing 10% sucrose and resuspended in 4% (w/v) OsO_4 for 3 h. The sample was again washed in buffered sucrose and resuspended in a small volume of noble agar (4%, w/v) at 45 °C. The agar was allowed to solidify and 1-mm cubes were cut and stained in saturated uranyl acetate in acetate–veronal buffer (pH 6.1). After dehydration in a graded series of ethanol concentrations, the samples were treated for two 20-min periods with propylene oxide and for 1 h in a 1 : 1 mixture of propylene oxide and epoxy resin. Thin sections were cut on an LKB ultrotome.

For negative staining the membrane samples were washed in ice-cold sodium phosphate buffer (0.2 M, pH 7.6) and resuspended in buffered glutaraldehyde. The suspension was mixed with 1% aqueous ammonium molybdate and spread on formvar coated grids. An AEI EM 801 electron microscope was used at 80 kV.

Extraction of lipids

All solvents were redistilled before use.

Cell material (1 mg/ml) was heated to 65 °C in methanol for five min. After cooling, 2 vol. of chloroform were added and the material extracted for 3 h.
The suspension was filtered and re-extracted for 12 h in chloroform–methanol (2 : 1, v/v). The combined extracts were dried and redissolved in chloroform–methanol (19 : 1, v/v) saturated with water. This crude lipid fraction was purified by passage down a column of Sephadex G-25 (Pharmacia) [18, 19].

Silicic acid chromatography

Silicic acid in *n*-heptane was poured into a column 10 mm × 100 mm and washed as described by Vorbeck and Marinetti [20]. The purified lipid was dissolved in chloroform and applied to the column. The lipid was eluted sequentially with chloroform, acetone, and chloroform–methanol (1 : 1, v/v). The eluted lipid was dried and weighed.

Thin-layer chromatography

Glass plates were spread with a 0.25-mm layer of silica gel G (Merck) and allowed to dry. They were activated by heating at 105 °C for 1 h before use.

Neutral Lipids. One-dimensional chromatography was carried out using the solvent hexane–diethyl ether–acetic acid (70 : 30 : 1, v/v/v) [21].

Sterols. The solvent systems of Van Lier and Smith [22] Avignan et al. [23] and Dixon et al. [24] were used.

Phospholipids. Two two-dimensional solvent systems were employed. In one sys-

tem the plate was developed in the first dimension with chloroform–methanol–water (65 : 25 : 4, v/v/v) and in the second dimension with chloroform–acetone–methanol–acetic acid–water (50 : 20 : 10 : 10 : 5, by vol.) [19]. In the second solvent system [25] the first dimension was developed with chloroform–methanol–diisobutyl ketone–acetic acid–water (12 : 5 : 23 : 13 : 2, by vol.) and the second dimension with chloroform–methanol–diisobutyl ketone–pyridine–ammonium acetate (0.5 M, pH 10.4) (15 : 9 : 13 : 18 : 3, by vol.).

Lipid eluted from silicic acid by acetone. One-dimensional chromatography was carried out using chloroform–methanol–water (90 : 10 : 1, v/v/v) [26].

Visualisation of lipid classes on thin-layer plates

General lipid visualisation was carried out with chromic acid [27] or iodine [28]. Spots containing amino groups were detected with 0.5% ninhydrin in acetone; those containing choline with Dragendorf's reagent [29]; those containing vicinal–OH groups with Schiff periodate stain [30]; phosphate with acid–molybdate [31]. Glycolipids were stained with diphenylamine and anisaldehyde [30] and sterols with anisaldehyde [30] and vanillin [32].

Estimation of lipid classes after thin-layer chromatography

A semi-quatitative estimate of the relative amounts of neutral lipid classes was obtained by microdensitometry of plates charred with chromic acid.

After visualisation of the phospholipid spots by charring, they were scraped quantitatively into Kjeldahl incineration flasks and refluxed for 20 min with 70% $HClO_4$. The phosphate released was assayed [33].

Paper chromatography of the water-soluble glyceryl phosphoryl derivatives

This was carried out as described by Dawson [34].

Identification of fatty acids

Conversion of the fatty acids to fatty acyl methyl esters was carried out by transmethylation [35] or saponification of the lipid in ethanolic KOH followed by methylation in diazomethane [36].

Gas–liquid chromatographic analysis

Gas–liquid chromatography of the fatty acyl methyl esters was carried out on a Pye Series 104 argon chromatograph equipped with hydrogen flame ionisation detectors. Two stationary phases were used. (1) 10% apiezon L on celite was run isothermally at 200 °C. (2) 10% polyethyleneglycol adipate on celite was run isothermally at 190 °C.

Fatty acids were identified from their retention times which could be predicted by James' plots [37, 38] and cochromatography with authentic standards. Further confirmation of the identity of each fatty acid was obtained by bromination [38, 39] and reduction [40].

Extraction of sterols

Sterols were extracted as described by Breiviic and Owades [41]. The ultraviolet spectrum was determined in ethanol–heptane [41] using a Cary 15 spectrophotometer.

RESULTS

Preparation of plasma membranes

A method of homogenisation was sought that disrupted the cells and released their contents while preserving the plasma membrane as a ghost visible by phase contrast microscopy. The method that fulfilled this criterion best was blending of the cell suspension with glass beads (Ballotini No. 12) in ice-cold 40 mM EDTA buffered with 50 mM Tris–HCl, pH 7.8. The blending was carried out using an MSE microhomogeniser (MSE Ltd. London) at full speed for 5 min.

The homogenate was then centrifuged at $116 \times g$ for 2 min to remove glass fragments, some unruptured cells and some flagellae. The supernatant from this centrifugation was centrifuged at $4000 \times g$ for 5 min and the supernatant from this was further centrifuged at $39\,000 \times g$ for 1 h. The pellets from both the 4000 and $39\,000 \times g$ centrifugations were resuspended in the homogenisation medium.

Linear 40–55% (w/v) sucrose gradients with a cushion of 55% sucrose were prepared. The sucrose was dissolved in the homogenisation medium. The resuspended pellets from the differential centrifugations were layered onto the gradients which were centrifuged at 25 000 rev./min using a SW25.1 rotor in a Beckman L2-65 ultracentrifuge for 4 h. Fig. 1 shows the distribution of material absorbing at 280 nm throughout the gradient. After density gradient centrifugation, the bands of membrane material were collected, diluted with homogenisation medium and centrifuged in a Beckman type 30 rotor at 30 000 rev./min for 30 min. The membranes were washed in the homogenisation medium up to ten times and finally were washed in distilled water or in the buffer in which they were to be assayed.

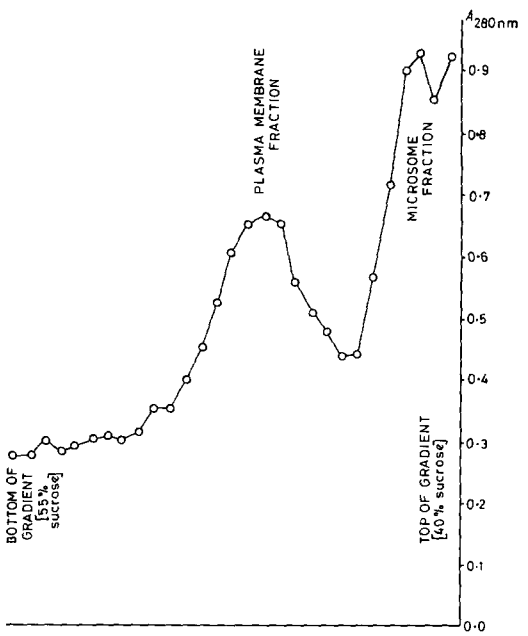


Fig. 1. The distribution of material that absorbs at 280 nm in a 27-ml gradient. The fraction of the cell homogenate that sedimented after centrifugation at $4000 \times g$ for 5 min was layered onto the gradient. The gradient was centrifuged at 25 000 rev./min in an SW 25-1 rotor for 4 h.

Chemical composition

The chemical composition of the lyophilised plasma membrane fraction is given in Table I. Total lipid and carbohydrate are enriched in the plasma membrane fraction compared to the whole cell material.

TABLE I

CHEMICAL COMPOSITION OF THE PLASMA MEMBRANE AND WHOLE CELLS OF *LEPTOMONAS COLLOSOMA*

	Cell homogenate	Plasma membrane
Protein*	71.5±2.9	52.0±4.6
Lipid*	12.2±2.6	29.0±2.8
Total nucleic acid*	6.8±0.4	1.3±0.5
Carbohydrate*	4.8±0.8	8.2±1.1
Neutral sugars**		
Mannose		23.5±2.1
Glucose		43.5±0.3
Galactose		24.5±0.3
Xylose		6±0.4

* Results are expressed as mean percentage of the dry weight of each fraction±S.D. for four separate experiments.

** Results are expressed as the mean percentages of total neutral sugar ±S.D. for three separate experiments.

Enzyme composition

The Mg^{2+}-dependent ATPase was enriched in the plasma membrane 7–8 times over the corresponding specific activity found in the whole cell homogenate (Table II). No stimulation of the enzyme activity was observed in the presence of Na^+ or K^+. Indeed, there appeared to be an inhibition of the enzyme.

A 10-fold increase in the level of acid phosphatase specific activity was found in the membrane fraction compared to the whole cell homogenate.

Both assays for 5'-nucleotidase gave similar results indicating only a 2-fold enrichment of the specific activity of the enzyme in the plasma membrane.

The 10-fold decrease in the specific activity of succinate dehydrogenase in the plasma membrane is indicative of purification of this fraction with respect to mitochondrial contamination. The procedure used to obtain the plasma membrane fraction involves osmotic swelling and violent blending. Since trypanosomatid flagellates probably have only one mitochondrion [42, 43] which may ramify throughout the cell, it is probable that this organelle is extensively damaged. In excess of 90% of the activity of this enzyme is recovered in the supernatant after centrifugation of the homogenate for 1 h at 39 000 × *g*. It is possible that the low activity of succinate dehydrogenase observed in the plasma membrane fractions represents contamination with mitochondrial fragments.

Electron microscopy

Fig. 2 shows cells sectioned to show the microtubules in transverse and longi tudinal section. A striking feature of the surface layers of all trypanosomatid flagel

TABLE II

ENZYME ACTIVITIES OF THE PLASMA MEMBRANE FRACTION AND THE CELL HOMOGENATE

Plasma membranes were isolated as described in the text. 3 mM $MgCl_2$ was included in all assays for ATPase.

Enzyme	Enzyme activity (nmoles substrate consumed per mg protein per min)	
	Cell homogenate	Plasma membrane
Mg^{2+}-ATPase*	1.6±0.2	12.2±2.7
(Na^+-K^+)-ATPase*	1.3±0.4	8.5±3.7
(Na^+-K^+)-ATPase (+ouabain)*	1.3±0.4	7.2±1.8
(Na^+-K^+)-ATPase $(+Ca^{2+})$*	1.7±0.4	12.3±2.4
5′-Nucleotidase**	2.4±0.1	5.0±0.3
Cytochrome oxidase	1.5	0
Succinate dehydrogenase*	33.0±2.5	3.5±2.5
Acid phosphatase	43.0	500

* Results are expressed as mean specific activities ±S.D. for five separate experiments.

** Results expressed as the mean of duplicate determinations of two separate experiments ±S.D.

lates is this skeleton of microtubules running the length of the cell [44–46] (see Fig. 3). This structure, if it were preserved during the isolation of the plasma membrane, would serve as a useful marker. Using 40 mM EDTA buffered with 50 mM Tris–HCl pH 7.8, as the homogenisation medium, the microtubules are preserved, but when 0.2 M phosphate buffer is substituted, extensive degradation of the microtubule system takes place during plasma membrane isolation. Fig. 3 is a negatively stained plasma membrane ghost which has been ruptured to reveal both the inner and outer surfaces of the plasma membrane. Figs 4 and 5 show similar preparations in thin section. The major part of the membrane material in the preparation is associated with microtubules and therefore originates in the plasma membrane.

Lipid composition

Lipid was found to comprise 12% of the dry weight of the cell whilst the plasma membrane fraction contained 29% lipid. Two samples from the whole cell and the plasma membrane were fractionated on silicic acid columns to give neutral lipid, "acetone-eluting" lipid and phospholipid. Table III gives the proportion of each lipid class.

Neutral lipids

The composition of the neutral lipid classes is given in Table IV. The most notable features are the large proportion of free sterol in the plasma membrane and of free fatty acid in the whole cell. The identity of the component that does not move from the origin in this system is not known.

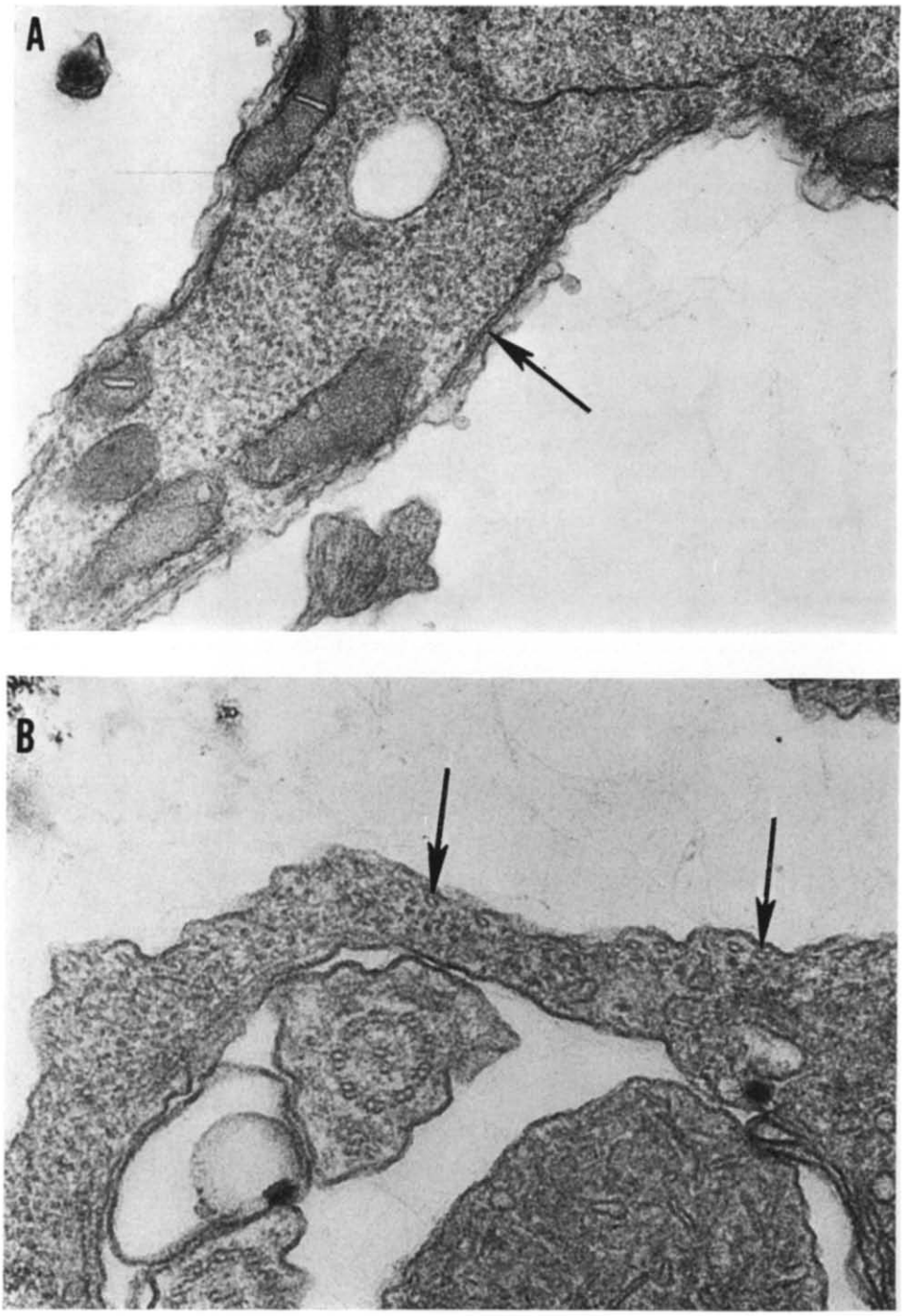


Fig. 2. *L. collosoma*. Thin sections of osmium-fixed and uranyl acetate-stained cells showing the microtubules (arrows) that lie under the plasma membrane in longitudinal (A) and transverse section (B). Magnifications: A × 32 000; B × 7800.

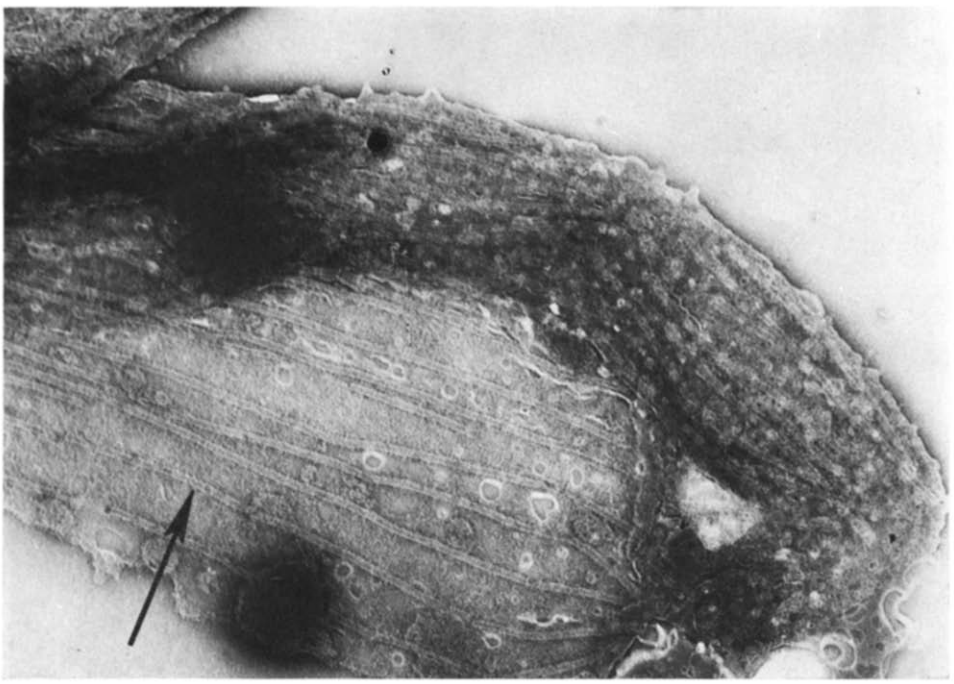


Fig. 3. A plasma membrane 'ghost' after negative staining. The cell has been ruptured to reveal both the inner surface of the plasma membrane. Microtubules (arrow) run longitudinally through the cell on the inner surface of the plasma membrane. Magnification: ×32 600.

TABLE III

THE LIPID COMPOSITION OF WHOLE CELLS AND THE ISOLATED PLASMA MEMBRANES OF *L. COLLOSOMA*

Lipid was extracted from cellular material and dissolved in chloroform. The lipid was eluted sequentially from a silicic acid column with chloroform, acetone and chloroform–methanol (1:1, v/v). Each fraction was dried and weighed. The results are expressed as percent dry weight of total lipid.

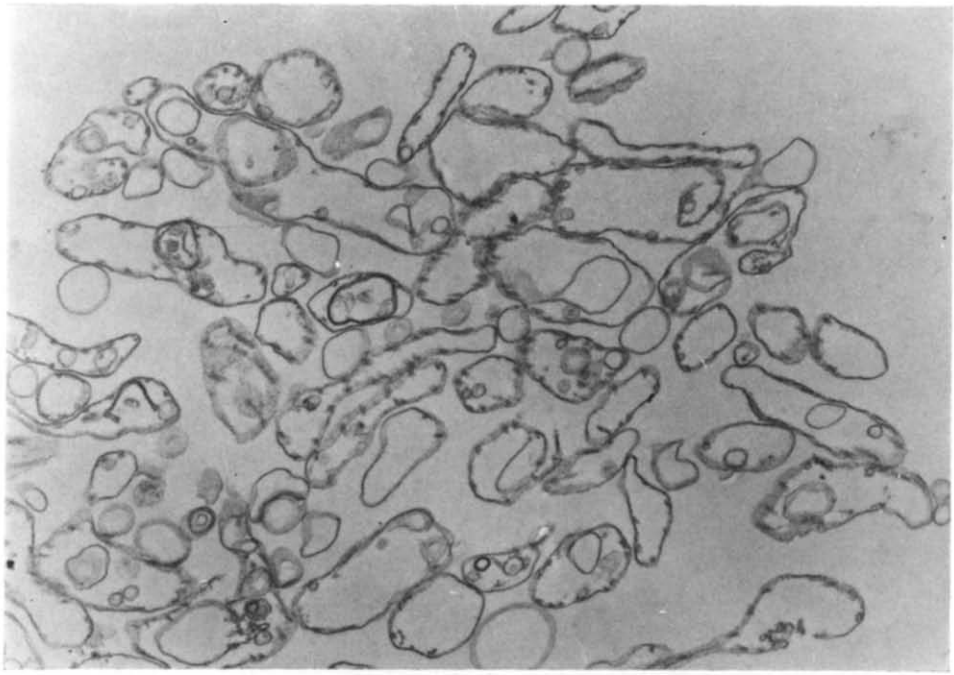
Lipid class	Plasma membrane		Cells	
	Expt 1	Expt 2	Expt 1	Expt 2
Neutral lipid	34	34	38	34
'Acetone-eluting' lipid	32	20	9	10
Phospholipid	34	46	51	56

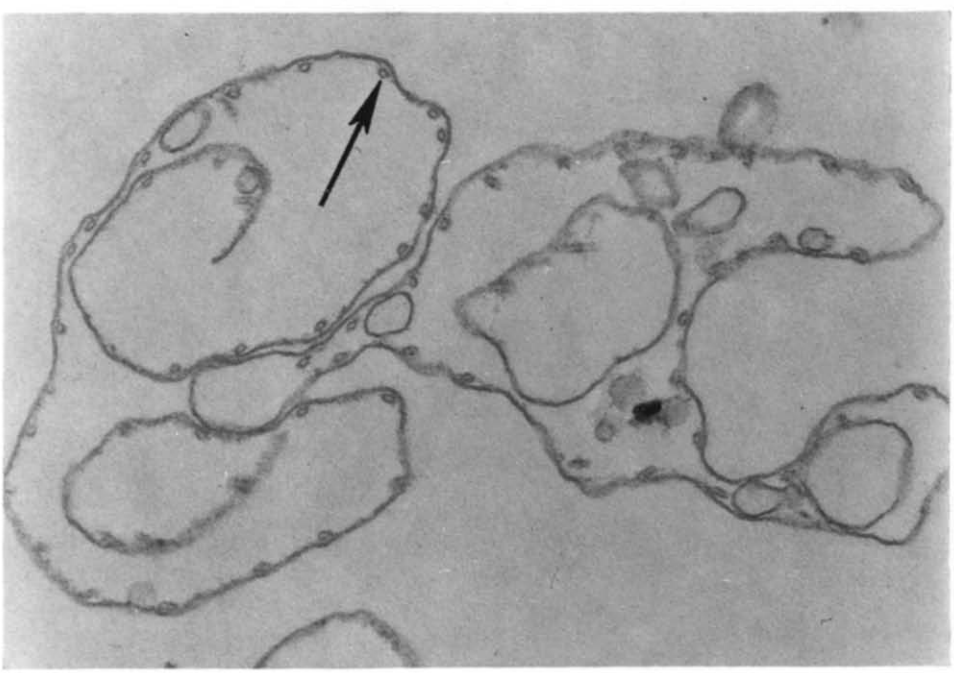
Sterols

In all thin-layer chromatographic systems used, the sterol cochromatographed with ergosterol. Extracted sterol had an ultraviolet spectrum identical with that of ergosterol. It was calculated that 1.5% of the dry weight of the cell is sterol. The plasma membrane contained 5.8% sterol.

Phospholipids

Table V lists the spots identified from thin-layer chromatograms using the





Figs 4 and 5. Purified plasma membrane fraction. The fraction consists mainly of plasma membrane 'ghosts' and associated microtubules (arrow). Magnifications: Fig. 4. ×14 850; Fig. 5. ×32 000.

TABLE IV

THE COMPOSITION OF THE NEUTRAL LIPID FRACTION OF *L. COLLOSOMA*

The neutral lipid fraction, eluted from silicic acid with chloroform, was fractionated by thin-layer chromatography. After development of the thin-layer plate the lipids were charred and the plates scanned with a microdensitometer. The results are expressed as a percentage of the total area under the microdensitometer peaks.

Neutral lipid class	Plasma membrane	Cell
Origin material*	9.7	31.5
Monoglyceride	4.3	16.5
Free sterol	61.0	20.0
Free fatty acids	17.4	22.8
Triglyceride	2.1	5.1
Sterol ester	5.5	4.0

* Material that did not move from the origin on thin-layer chromatography. Its identity is unknown.

TABLE V

PHOSPHOLIPID COMPOSITION OF THE CELLS AND ISOLATED PLASMA MEMBRANE FRACTION OF *L. COLLOSOMA*

The phospholipids were isolated by silicic acid chromatography. Thin-layer chromatography was carried out with the solvent system described by Rouser et al. [19]. The phosphorus in each spot after chromatography was estimated colorimetrically. The figures in brackets are the standard deviations of the means of five experiments. The phospholipids denoted by X have not been identified.

Phospholipid	Phosphorus (weight per cent)	
	Plasma membrane	Cell
Cardiolipin	0.5 (0.4)	7.4 (1.9)
X_1*	N.D.***	3.3 (1.9)
Phosphatidylethanolamine	33.0 (6.9)	26.2 (3.4)
X_2**	4.3 (2.5)	N.D.***
Phosphatidylcholine	29.0 (7.0)	24.6 (7.9)
Phosphatidylserine	12.0 (5.0)	6.0 (0.8)
Phosphatidylinositol	10.0 (1.6)	21.3 (9.5)
Lysophospholipids	11.0 (2.0)	11.0 (2.5)

* Ninhydrin negative.
** Ninhydrin positive.
*** N.D., not detected.

solvent systems of Rouser et al. [19]. Considerable variation in the distribution of phosphorus between the spots was observed in different preparations of whole cells and the plasma membranes isolated from them. This may be due to variations in the stage of the life cycle at which the cells were harvested or variations in the complex medium in which the cells were grown.

Cellular lipid eluted from silicic acid by acetone

It has been shown that glycolipids, sulpholipids and pigments are among the

classes of lipids that are eluted by acetone from a silicic acid column to which extracted lipids have been applied [19]. Acetone elutes a lipid class that constitutes a much larger fraction of the plasma membrane lipid than of the whole cell lipid from *L. collosoma*. The chromatographic system described by Jonah and Erwin [26] was used to separate the components of this fraction and the results obtained parallel those of the latter authors using *Tetrahymena pyriformis*. Nine separate spots were observed in both the purified plasma membrane fraction and in the whole cell material. All of these were positive in the Schiff-periodate reaction but none gave a reaction with ninhydrin, Dragendorf's reagent, acid molybdate, diphenyl amine or anisaldehyde. Thus there appear to be neither phospholipids nor glycolipids in the fraction.

Fatty acid composition

Table VI lists the fatty acids identified in the cells together with the percentage composition based on the area under each peak on the chromatogram. The three preparations show a similar pattern with oleic acid (18 : 1) accounting for about a third of the fatty acids in the cell. About 60% of the fatty acids have a chain length of eighteen carbon atoms. Those that show the greatest variability in amount are 17 : 0 and 17 : 1 and an unsaturated seventeen carbon fatty acid which moves between 17 : 1 and 18 : 0 on polyethyleneglycol adipate columns, precedes 17 : 1 on apiezon L, is reduced to 17 : 0 on hydrogenation and is removed from the mixture on bromination. However, its retention time is not exactly that predicted for 17 : 2.

TABLE VI

FATTY ACID COMPOSITION OF WHOLE CELLS OF *L. COLLOSOMA*

t denotes fatty acid recovered in 'trace' amounts-less than 0.5 % of the total. U denotes an unidentified component of the mixture.

Fatty acid	Weight per cent*	Range
14:0	2.3	t-5.6
14:1	1.0	t-3.1
14:2	0.7	t-2.2
15:0	1.4	t-2.5
15:1	1.5	t-2.5
16:0	4.7	4.4-5.3
16:1	2.0	1.0-3.6
17:0	2.1	t-5.2
17:1	3.6	t-9.8
17:?	5.8	2.3-12.7
18:0	12.2	8.1-15.5
18:1	35.5	34.0-36.8
18:2	4.7	3.2-5.5
18:3/19:1**	16:1	13.8-17.8
20:0	0.9	t-2.7
20:2	0.8	t-2.3
21:0	3.1	2.0-4.6
U	1.4	t-4.4

* Mean of three preparations.

** On polyethyleneglycol adipate the triunsaturated 18-C and monounsaturated 19-C fatty acyl methyl ester cochromatograph. Using apiezon L this peak was shown to be 65 % 18:3 and 35 % 19:1.

Table VII lists the percentage composition of the fatty acids found in the purified plasma membrane fraction. Little difference is seen between the composition of fatty acids in the plasma membranes and in the whole cell.

TABLE VII

FATTY ACID COMPOSITION OF THE ISOLATED PLASMA MEMBRANE OF *L. COLLO SOMA*

Fatty acid	Weight per cent	
	1	2
14:0	t	2.5
15:1	3.1	5.6
16:0	8.3	21.5
16:1	4.5	4.6
17:1	1.1	2.6
17:?	t	3.9
18:0	13.7	16.1
18:1	36.4	37.1
18:2	3.9	6.4
18:3/19:1*	9.8	10.1
20:0	t	1.5
20:2	2.4	2.7
21.1	t	2.1

* Using apiezon L the relative amounts of 18:3 and 19:1 were found to be 56 and 44 %, respectively, of the combined peak observed on chromatography on polyethyleneglycol adipate.

To further identify the fatty acids, catalytic hydrogenation was carried out. This shifted the unsaturated fatty acyl methyl esters to the position of the corresponding saturated fatty acyl methyl esters. Table VIII gives the total fatty acyl

TABLE VIII

THE EFFECT OF REDUCTION BY HYDROGEN ON THE FATTY ACIDS OF *L. COLLOSOMA*

The percentage of material in each peak of the chromatogram after reduction is compared with the percentage of each fatty acid chain length before reduction

After reduction		Before reduction	
Position on chromatogram	%	Total fatty acid of chain length	%
14:0	1.3	14	1.4
15:0	2.0	15	1.8
16:0	5.9	16	5.9
17:0	25.0	17	28.2
17:1	3.0		
18:0	57.8	18	57.7
19:0	1.6	19	4.9
19:1	3.3		

methyl esters of each chain length (saturated plus unsaturated) expressed as a percentage of the total fatty acyl methyl esters. This is compared with the percentage in each peak after reduction. With the exception of the seventeen and nineteen carbon fatty acids, there is a quantitative shift to the saturated positions on the chromatogram. After hydrogenation and gas–liquid chromatography on apiezon L there is however material that still runs at the 19 : 1 and 17 : 1 positions. Of the material that runs at the 17 : 1 position from whole cell lipid, 30% is not reduced to 17 : 0 and of that at the 19 : 1 position, 67% is not reduced to 19 : 0. Bromination also failed to remove the material that ran at these positions.

DISCUSSION

No single criterion can be used to judge the purity of a plasma membrane fraction. Accordingly, we have used a number of criteria, including electron microscopy and enzymic markers to characterise our plasma membrane fraction. For example, the data on chemical composition are similar to those obtained for other plasma membrane fractions. The significance of the nucleic acid that represents 1–2% of the dry weight of the preparation is not clear. It may represent contamination by ribosomes and nuclei or may be an integral part of the plasma membrane as has been suggested by some workers [47–50].

The use of an enzyme marker as a measure of the purity of the plasma membrane fraction requires that the enzyme be located solely or mainly in that fraction. Sodium and potassium ion stimulated ATPase, Mg^{2+}-dependent ATPase and 5′-nucleotidase are among the several enzymes that are thought to be located in the plasma membranes of many mammalian tissues. However, there is no a priori reason for believing that because they are so located in these tissues, they should have the same location in a protozoan. The specific activity of 5′-nucleotidase is increased approximately two times over the level in the cell homogenate. This compares with no purification at all in the plasma membranes of the amoebae of the slime mould *Dictyostelium discoideum* [51], and a purification of 120-fold in the plasma membranes of HeLa cells [52]. The low level of 5′-nucleotidase may be due to a non-specific phosphatase.

In accord with the results for other unicellular organisms [3, 4, 51] there appears to be no significant stimulation of the Mg^{2+}-dependent ATPase by Na^{+} and K^{+}.

Acid phosphatase is an enzyme usually associated with lysosomes. It is not known whether the high specific activity of this enzyme found in the plasma membrane is due to contamination by this organelle or by soluble enzyme released on rupture of the lysosomes in the hypotonic homogenisation medium, or whether it is a true component of the plasma membrane. An indication that the latter might be the case comes from the results of Seed et al. [54] who used a cytological technique to localise acid phosphatase in another trypanosomatid flagellate species. In an electron micrograph not stained with lead citrate a deposition of stain due to acid phosphatase can be seen at the cell surface.

The best evidence that the fraction that has been obtained is largely purified material from the plasma membrane, comes from electron microscopy which shows that a large proportion of the membrane material is associated with microtubules.

The remaining small portion of membrane which is not associated with micro-

tubules, may quite possibly be derived from that region of the plasma membrane that is infolded to form the flagellar pocket. Anderson and Ellis [55] suggest that the paucity of microtubules in this region might be associated with its pinocytotic function.

The percentage of the total dry weight of the cells of *L. collosoma* that is lipid, is in agreement with the results obtained for whole cells of other trypanosomatid flagellates [21, 56–59]. Only one value is available for the lipid content of trypanosomatid flagellate membranes [59] and this is similar to that reported here.

The technique of silicic acid fractionation has been used to fractionate the lipid. Stein and Slawson [60] have noted the action of methanol on silicic acid when esters that dissolve in methanol bleed from the column. This action will invalidate gravimetric determinations of the lipid composition. However, we have shown that no material bled from the column during elution with chloroform–methanol (1 : 1, v/v). No glycolipid was found in the chloroform–methanol extract. Hack, Yaeger and McCaffery [61] found no glycolipids in *Trypanosoma cruzi*, *Leishmania donovani* or *Crithidia fasciculata*. Jonah and Erwin [26] identified the lipid components of *Tetrahymena pyriformis* that were eluted from silicic acid by acetone as "ceramide-like" lipids. Such lipids have been detected in *T. pyriformis* [62] and the trypanosomatid flagellate *C. fasciculata* [63]. The classes of neutral lipids found in *L. collosoma* are similar to those reported for other trypanosomes [21, 64, 65].

Free sterol is the major neutral lipid in the plasma membrane. The sterol of *L. collosoma* is a 5,7-diene sterol, which is identified as ergosterol on the basis of the work to date. Many other trypanosomatid flagellates have been shown to have ergosterol as their main or only sterol. [24, 66–68].

The fatty acid composition of the whole cell material and the isolated plasma membranes show few differences. The identity of those fatty acids that appear monounsaturated by their retention times on gas–liquid chromatography but which are not reduced by hydrogen, has not been elucidated. Meyer and Holz [58] have reported the presence of 9,10-methyleneoctadecanoic acid in a number of insect trypanosomatid flagellates. However, Korn et al. [69] did not report this fatty acid in the protozoa that they studied. The fact that bromination does not remove these fatty acids from *L. collosoma* lipid preparations argues against their being cyclopropane derivatives [70].

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