PLASMA AND PHAGOSOME MEMBRANES OF *ACANTHAMOEBA CASTELLANII*

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ABSTRACT

Plasma membranes were isolated from the ameba *Acanthamoeba castellanii* by low-speed velocity centrifugation followed by equilibrium centrifugation in a sucrose gradient. The isolated membranes had a high ratio of sterol to phospholipid (0.98 moles/mole) and of phospholipid to protein (0.43 mg/mg). The plasma membranes had very low concentrations of DNA, RNA, lipid inositol, and glycerides. Glycolipids and glycoproteins were enriched in the plasma membranes relative to their concentrations in the whole cell. The plasma membranes were also judged to be of high purity by the absence, or very low level, of enzymatic activities considered to be indicative of other cell membranes, and by electron microscope examination. Alkaline phosphatase and 5′-nucleotidase activities were enriched in the plasma membranes 13-fold relative to the whole homogenate and had higher specific activities in the plasma membranes than in any other cell fractions. A Mg^{++} adenosine triphosphatase (ATPase) was enriched sixfold in the plasma membranes relative to the whole homogenate. The phospholipids of the plasma membranes contained more phosphatidylethanolamine and phosphatidylserine and less phosphatidylcholine than did the phospholipids of the whole cells. There were differences in the fatty acid compositions of corresponding phospholipids in the plasma membranes and whole cells but no difference in the ratios of total saturated to unsaturated fatty acids. The membranes of phagosomes isolated from amebae that had ingested polystyrene latex had essentially the same phospholipid, sterol, and enzymatic composition as plasma membranes.

INTRODUCTION

An understanding of membrane structure and membrane function ultimately depends on the complete chemical characterization of the membrane. Pursuant to our studies of pinocytosis and phagocytosis (Weisman and Korn, 1967; Korn and Weisman, 1967; Wetzel and Korn, 1969; Ulsamer et al., 1969) we have undertaken the isolation and characterization of the plasma membrane of the soil ameba, *Acanthamoeba castellanii*. The experimental challenge is the isolation of the plasma membrane of the ameba (Fig. 1) in reasonable yield and uncontaminated by other membranous and soluble constituents of the cell. One major difficulty is establishing adequate criteria for identifying plasma membranes and determining their purity.

When the cells have been ruptured by a gentle procedure, light and electron microscopy can establish the presence of large fragments whose only conceivable origin was the plasma membrane.

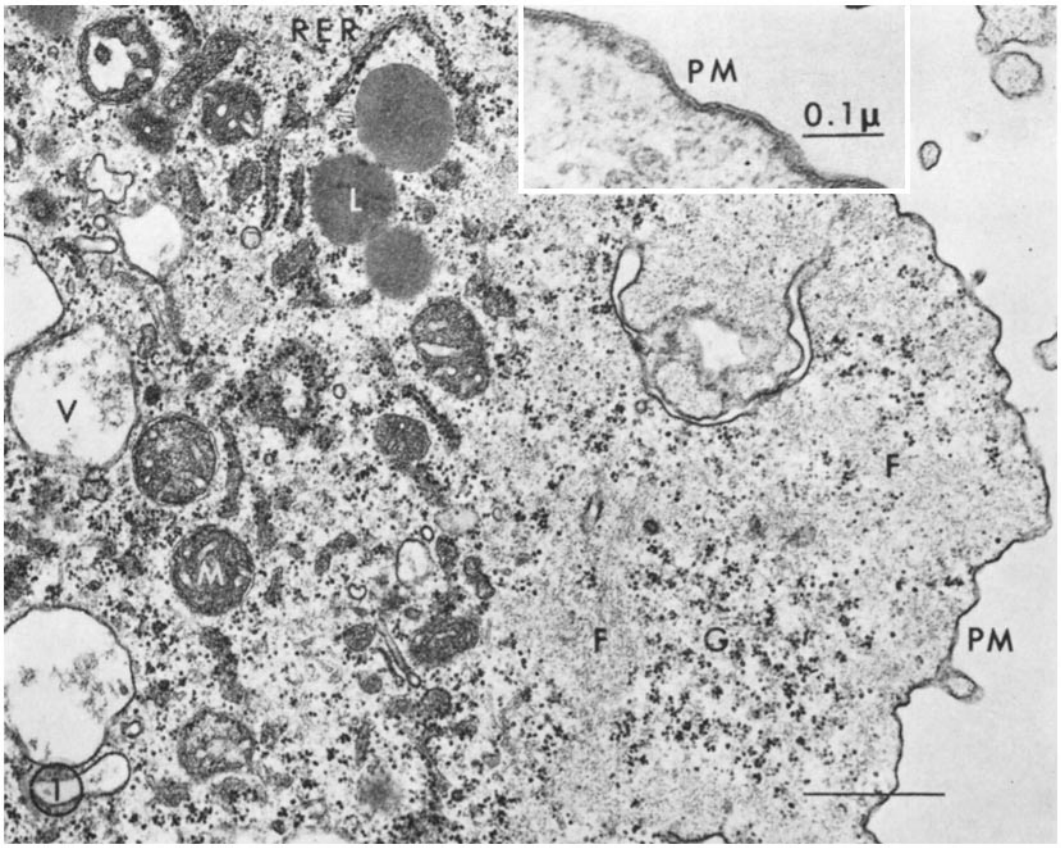

FIGURE 1 A representative segment of a cross-section of *Acanthamoeba castellanii* showing digestive vacuoles (*V*), mitochondria (*M*), rough endoplasmic reticulum (*RER*), fibrils (*F*), glycogen (*G*), lipid droplets (*L*), and plasma membranes (*PM*). × 18,616. The inset shows the plasma membrane at higher magnification. × 108,000. For a detailed description of the ultrastructure of *Acanthamoeba castellanii*, see Bowers and Korn (1968, 1969).

Electron microscopy can also establish the absence from the isolated plasma membrane fraction of distinctive intracellular membranes and organelles such as rough endoplasmic reticulum, mitochondria, and nuclei, but the nature and origin of all of the smooth membranes cannot be unambiguously determined microscopically. On the other hand, assays of succinic dehydrogenase can establish the extent of mitochondrial contamination, and, with less certainty, glucose-6-phosphatase has often been used as indicative of the presence of endoplasmic reticulum. Intact "lysosomes" (pinosomes, phagosomes, and digestive vacuoles in the ameba) can probably be detected by assaying for appropriate acid hydrolases. To calculate from such data the extent of contamination of the plasma membranes by other membranes it is necessary to compare the specific activity of the plasma membrane preparation to that of the purified intracellular membrane thought to be the source of the contamination.

It is more difficult to employ enzyme assays in a positive way. To measure the purification of plasma membranes by the purification of a specific enzyme depends on the enzyme being a true component of the plasma membrane and only of the plasma membrane. For mammalian plasma membranes, 5′-nucleotidase has been thought to satisfy these criteria. Chemical analyses are also of help. It is uniquely characteristic of plasma membranes that the molar ratio of sterol to phospholipid approaches 1:1 (Korn, 1969 *a*, *b*). Analysis of DNA content can give some indication of the extent of contamination by intact nuclei and

analysis of RNA content is useful for assessing the contamination by ribosomes or rough endoplasmic reticulum although residual DNA and RNA might be true components of the plasma membrane. Glycoproteins and glycolipids may also be enriched in plasma membranes.

Until much more is known about the chemical and enzymatic composition of plasma membranes one must proceed with caution and employ all of the available criteria. It should be emphasized that it is easier to detect the presence of specific contaminants than it is to measure the absolute purity of the plasma membranes, and it is yet more difficult to determine if a component of the plasma membranes has been lost during the isolation procedure. We believe that the plasma membrane fraction prepared as described in this paper is at least as pure as any yet obtained. The analytical data reported in this paper define some of the major enzymatic and chemical constituents of the ameba plasma membranes as isolated.

This is not, of course, the first description of the isolation of plasma membranes from animal cells but previous instances are fewer, and the analyses of the isolated membranes less consistent, than one might have thought. Earlier observations relevant to this paper will be discussed later. While this work was in progress Schultz and Thompson (1969) described the isolation of plasma membranes from the same strain of *Acanthamoeba* but they have not yet reported a chemical and enzymatic analysis of their preparation. We have successfully repeated their procedure and the plasma membranes prepared by the two methods are briefly compared in this paper.

METHODS

Amebae were grown in rotating cultures as described previously (Weisman and Korn, 1966) for periods varying between 5 and 7 days. Such cultures contained between 1 and 4% encysted cells. The isolation of plasma membranes from these cultures is described under Results.

Isolation of Mitochondria and Microsomes

For the isolation of mitochondria cells were homogenized and the homogenate was adjusted to 10% sucrose (w/v) in 0.01 M Tris chloride, pH 7.5, as described later for the isolation of plasma membranes. The homogenate was centrifuged at 1800 *g* (max centrifugal force) for 15 min and the supernatant was collected and centrifuged at 9000 *g* (max) for 15 min. The pellet was washed twice by suspension in 10% sucrose (w/v) in 0.01 M Tris and centrifuged at 9000 *g* for 15 min. All these centrifugations were carried out in a Beckman 30 rotor (Beckman Instruments, Inc., Palo Alto, Calif.). The washed pellet was suspended in 30% sucrose (w/v), layered over a discontinuous gradient of 40, 45, and 50% sucrose in 0.01 M Tris, pH 7.5, and centrifuged at 90,000 *g* (max) for 3 hr in a Beckman SW 25 rotor. Mitochondria which were homogeneous by electron microscopy were collected from the 40/45 interface.

A microsomal fraction was prepared from the supernatant obtained by centrifuging the original cell homogenate at 14,000 *g* (max) for 15 min. This suspension was adjusted to 30% sucrose (w/v), layered over 35% sucrose, and centrifuged at 105,000 *g* for 90 min in a Beckman 30 rotor. The pellet was suspended in 35% sucrose, layered over 40, 45, and 50% sucrose and centrifuged at 131,000 *g* (max) for 90 min in a Beckman SW 27 rotor. The material which collected at the 40/45 interface was considered to be representative of the microsomal fraction most likely to contaminate the plasma membranes which were also found to accumulate at that interface (see Results). The microsomal fraction consisted of smooth and rough vesicles and some free ribosomes. The rough vesicles clearly originated from the rough endoplasmic reticulum; the origin of the smooth vesicles was less apparent.

For the isolation of mitochondria and microsomal membranes we have purposely used conditions as similar as possible to those used for the isolation of plasma membranes so that comparison of their enzymatic contents has maximal validity. All procedures were carried out at 0–4°C.

Isolation of Phagosomes

Amebae (3×10^6/ml) were incubated with polystyrene latex beads (1.10 μ diameter; 1 mg/ml) for 60 min and washed with 0.01 M Tris, pH 7.4 until free of excess beads. All subsequent steps were carried out at 0–4°C. The amebae were homogenized in 30% sucrose (w/v) and the homogenate was layered under 25, 20, and 10% sucrose. Upon centrifugation at 105,000 *g* (max) for 90 min the phagosomes accumulated at the interface between 10 and 20% sucrose (Wetzel and Korn, 1969).

Extraction and Fractionation of Lipids

The procedures for extraction and fractionation of neutral lipids and phospholipids have been described previously (Ulsamer et al., 1969). Briefly, cells were extracted at room temperature by stirring for 3 hr with 20 vol of chloroform-methanol (2:1 v/v) (in special instances absolute ethanol was used), and sucrose and other water-soluble materials were removed by washing once with 0.73% NaCl and

twice with clean upper phase (Folch et al., 1957). Neutral lipids and phospholipids were separated by silicic acid column chromatography before quantitation. In some experiments phospholipids were fractionated by thin-layer chromatography (Ulsamer et al., 1969).

Membranes to be analyzed for glycolipid and glycoprotein were first dialyzed in washed cellophane tubing against three changes of 500 vol of 0.01 M Tris, pH 7.5, at 2°C for 12 hr each. The contents of the dialysis bag were lyophilized and then extracted twice for 3 hr with ethanol to obtain glycolipids. The ethanol was evaporated and the residue was heated in 0.5 N KOH in chloroform-methanol (1:1, v/v) at 30°C for 1 hr to remove fatty acids. The solution was neutralized, made 2:1 in chloroform-methanol, and extracted with water. The water-methanol phase contained the sugar derivatives released from hydrolyzable glycolipids. These were hydrolyzed in 2 N HCl at 100°C for 2 hr and analyzed for sugars.

The chloroform-methanol phase contained fatty acids, sterols, long-chain bases and nonhydrolyzable lipids. It was dried, and the residue was dissolved in chloroform and separated by column chromatography on silicic acid into a chloroform eluate and a methanol eluate.

The methanol eluate was dried under pressure and the residue was taken up in 2 N HCl in methanol and heated at 80°C for 18 hr in screw-capped tubes in order to hydrolyze any cerebrosides to long-chain bases and sugars. The long-chain bases were separated and estimated by reaction with methyl orange as described by Lauter and Trams (1962) with the addition of a small amount of ethanol to maintain a single phase in the cuvette.

Chemical Analyses

Proteins were estimated by the method of Lowry et al. (1951) with bovine serum albumin as standard. DNA was determined by the diphenylamine reaction as described by Hatcher and Goldstein (1969). Sucrose did not interfere in the DNA assay but a lipid-soluble component which reacted strongly had to be removed by extracting the membranes with ethanol.

RNA content was determined by a modification of the Schmidt-Thannhauser procedure as described by Munro and Fleck (1966). The unmodified procedure produced erroneously high values for the plasma membrane fraction because of proteins (Lowry-positive material) and lipids (ethanol-soluble material) which were present in the extract and absorbed light at 260 mμ. Membranes were extracted with ethanol to remove lipids, and the RNA in the residue was then hydrolyzed in 10% piperidine at 100°C for 90 min to form 2′- and 3′-nucleotides (Sedat and Hall, 1965). The piperidine was removed under a stream of N_2 and the nucleotides were dissolved in water and separated by high-voltage electrophoresis in 0.05 M ammonium formate, pH 3.5 (Smith, 1967). The nucleotides were visualized under ultraviolet light and the spots and appropriate blank areas of paper were cut out and eluted with 0.10 N HCl. The concentration of nucleotides was measured by the absorbance at 260 mμ.

Total hexoses were determined by the method of Walborg and Christensson (1965) and individual sugars were detected by gas-liquid chromatography of their trimethylsilyl derivatives on 3% OV-17 (Applied Science Laboratories, Inc., State College, Pa.) (Richey et al., 1964). Amino sugars were analyzed by the Elson-Morgan reaction (Blix, 1948). Sialic acid was determined by the thiobarbituric acid assay (Warren, 1959) after hydrolysis of ethanol-extracted proteins in 0.05 N H_2SO_4 for 1 hr at 80°C (Eylar and Jeanloz, 1962). Phosphate was determined as inorganic phosphate by the method of Chen et al. (1956) after combustion by fusion with $Mg(NO_3)_2$ (Ames and Dubin, 1960). Esters were determined as hydroxamates (Rapport and Alonzo, 1955 *a*). Phosphoinositides were hydrolyzed in 6 N HCl at 122°C for 18 hr and inositol was quantitated as its trimethylsilyl derivative by gas-liquid chromatography on 17% ethylene glycol succinate (Well et al., 1965).

Sterols giving a positive Liebermann-Burchard reaction were quantitiated by the method of Moore and Baumann (1952). The ratio of aromatic, Liebermann-Burchard-negative sterols to Liebermann-Burchard-positive sterols (Korn et al., 1969) was determined by gas-liquid chromatography of the sterols on 3% OV-17 and used to calculate the total sterol concentration. The correction factor was in the range of 2–3. Fatty acids were analyzed as methyl esters by gas-liquid chromatography on a column of 17% ethylene glycol succinate, and long-chain bases were chromatographed on 3% SE-30 (Applied Science Laboratories, Inc.)

Plasmalogens were determined by the procedure of Rapport and Alonzo (1955 *b*) and their aldehyde composition was analyzed by gas-liquid chromatography of the dimethylacetal derivatives on 17% ethylene glycol succinate after removal of methyl esters of fatty acids by saponification.

Enzyme Analyses

5′-nucleotidase was assayed according to the procedure of Michell and Hawthorne (1965) with adenosine monophosphate (AMP)[1] as substrate and using Na^+K^+ tartrate to inhibit acid phosphatase. The addition of 0.05% Triton X-100 (Rohmand

[1] *Abbreviations*: AMP, adenosine monophosphate; ATPase, adenosine triphosphatase; EDTA, ethylenediaminetetraacetate; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate.

Haas Co., Philadelphia, Pa.) caused a 35–50% increase in specific activity in both whole cell homogenates and plasma membranes.

Adenosine triphosphatase (ATPase) was assayed according to the method of Fitzpatrick et al. (1969) using Triton X-100 instead of deoxycholate.

Acid phosphatase was analyzed by the procedure of Rosen et al. (1966) with β-glycerophosphate as substrate and fluoride added to inhibit alkaline phosphatase.

Glucose-6-phosphatase was assayed by the method of Hübscher and West (1965) in the presence of both ethylenediaminetetraacetate (EDTA) and Na^+K^+ tartrate to inhibit alkaline and acid phosphatases, respectively.

Alkaline phosphatase was assayed according to the method of Bosmann et al. (1968) with *p*-nitrophenylphosphate as substrate. Triton X-100 was added to a concentration of 0.05% which increased enzymatic specific activities by approximately 50%.

Succinic dehydrogenase was assayed according to Müller et al. (1968). Nicotinamide adenine dinucleotide (NADH)–cytochrome *c* reductase (Mahler, 1955) and nicotinamide adenine dinucleotide phosphate (NADPH)–cytochrome *c* reductase (Williams and Kamin, 1962) were assayed as described in the references cited.

Adenyl cyclase was assayed as described by Vaughan and Murad (1969).

Glucosidase was assayed according to Fleischer and Fleischer (1969) except that glucostat reagent (Worthington Biochemical Corp., Freehold, N.J.) was used to measure free glucose.

Electron Microscopy

Pellets obtained by centrifugation were fixed in 3% glutaraldehyde in 0.1 M phosphate, pH 7.0, containing 1 mM $CaCl_2$, washed in cold buffer, and postfixed in 1% OsO_4 in phosphate buffer. The fixed pellets were washed with the buffer, and dehydrated with a graded series of ethanol solutions. Final dehydration and removal of the ethanol was accomplished by use of propylene oxide. The pellets were embedded in Epon 812 and thin sections were stained in sequence with 1% lead citrate and 1% uranyl acetate and were examined with a Siemens 101 electron microscope. Sections were cut across the pellet from top to bottom in order to have material representative of the entire fraction. Phase contrast microscopy was carried out on material that had been fixed in glutaraldehyde only.

RESULTS

Isolation of Plasma Membranes

Amebae were harvested by centrifugation at room temperature at 500 *g* for 5 min, washed once with ice-cold 0.01 M Tris chloride, pH 7.5, and suspended at a concentration of 2×10^7 cells/ml in cold buffer. All subsequent operations were carried out at 0–4°C and all solutions were buffered with 0.01 M Tris chloride, pH 7.5.

After allowing the cells to swell for 15 min with occasional gentle shaking, they were broken by four gentle strokes with a large, tight-fitting Dounce homogenizer (Kontes Glass Co., Vineland, N. J.) (50 ml capacity). This procedure ruptured approximately 95% of the cells but left encysted cells intact. Relatively large membrane fragments were formed. As each batch of cells was broken sufficient 60% sucrose was added to produce a final concentration of 10% sucrose (w/v). The homogenate was then centrifuged at 500 *g* (max) for 20 min in the SS 34 rotor of a Sorvall model RC 2B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). The pellet was suspended in half of the previous volume of 10% sucrose and again centrifuged at 500 *g* (max) for 20 min. The pellet was suspended once more and centrifuged at 750 *g* (max) for 20 min. This pellet was then suspended in the same volume of 25% sucrose and layered over 30% sucrose in 250-ml glass bottles, and the bottles were centrifuged at 200 *g* (max) for 20 min in an IEC rotor 284 in a PR-2 centrifuge (International Equipment Co., Needham Heights, Mass.). The 25% layer was collected, diluted to 10% with 0.01 M Tris pH 7.5, and centrifuged at 750 *g* (max) for 20 min in the SS 34 rotor. The pellet was suspended in enough 60% sucrose to reach a final density of 50%, and additional 50% sucrose was added to obtain 1 ml of suspension for the crude membranes derived from 10^8 cells. This suspension (16 ml) was placed in the bottom of 3 1/2 in × 1/2 in cellulose nitrate tubes, overlayed with the following sucrose solutions: 45 (8 ml), 40 (7 ml), and 35% (6 ml), and centrifuged at 131,000 *g* (max) for 90 min in a Beckman SW 27 rotor. The entire procedure is outlined in Scheme 1.

Distribution of Membrane Markers during the Isolation Procedure

The initial centrifugation of the cell homogenate at 500 *g* (step 2, Scheme 1) resulted in an enrichment of phospholipid and sterol relative to protein and RNA (Table I). Similar results were obtained by centrifuging at only 200 *g* but the higher centrifugal force was chosen to facilitate packing of the crude membrane pellet. Large membrane

SCHEME 1

Isolation of Plasma Membranes from Acanthamoeba castellanii

Washed cells

Step 1 ↓ 2 x 10^7/ml of 0.01 M Tris, pH 7.4, for 15 min at 0°C. Homogenize with 4 strokes of tight Dounce homogenizer.

Homogenate

Step 2 ↓ Adjust to 10% sucrose and centrifuge at 500 *g* for 20 min.

Pellet

Step 3 ↓ Suspend in 10% sucrose and centrifuge at 500 *g* for 20 min.

Pellet

Step 4 ↓ Suspend in 10% sucrose and centrifuge at 750 *g* fpr 20 min.

Pellet

Step 5 ↓ Suspend in 25% sucrose, layer over 30% sucrose and centrifuge at 200 *g* for 20 min.

25% Layer

Step 6 ↓ Dilute with 0.01 M Tris to 10% sucrose and centrifuge at 750 *g* for 20 min.

Pellet

Step 7 ↓ Suspend in 60% sucrose to make 50%, layer under 45, 40, and 35% sucrose and centrifuge at 131,000 *g* for 90 min.

40/45 Interface = plasma membranes

fragments were not seen in the supernatant that was discarded.

Further enrichment of the sterol and phospholipid relative to protein was obtained by the two washes (steps 3 and 4). The washed pellets had a molar ratio of sterol to phospholipid of 0.88 which was very near that of the purified plasma membranes, and the specific activity of the succinic dehydrogenase of the washed pellet was reduced to only 4% that of the whole homogenate. When the discarded supernatants were centrifuged at the higher force of 1000 *g* a pellet was obtained which had a ratio of sterol to phospholipid similar to that of the washed crude membrane pellet but a specific activity of succinic dehydrogenase equal to that of the whole homogenate, indicating much greater contamination by mitochondria. A pellet that was obtained by centrifugation at 2000 *g* contained a lower sterol to phospholipid ratio of 0.48 and succinic dehydrogenase activity 50% greater than that of the whole homogenate. Acid phosphatase activity was also greater in the pellets obtained at the higher forces. The centrifugal conditions chosen, therefore, were an attempt to maximize the yield of plasma membranes with minimal contamination although this meant that plasma membranes were discarded in the supernatant obtained at each of the above steps.

Step 5 of the isolation procedure served mainly to remove intact cells, cysts, and nuclei and thus lowered still further the RNA and protein content of the crude plasma membranes (Table I). The crude plasma membranes were recovered as a pellet in step 6 with little change in composition.

In the final sucrose gradient centrifugation (step 7) most of the plasma membranes were recovered at the interface between 40 and 45% sucrose as judged by phase contrast microscopy and chemical analysis of all fractions (Table I). Identical results were obtained when this centrifugation was carried out for 1 hr or for 4 hr. When the step gradient was altered by inserting a 42.5% sucrose solution between the 40 and 45% layers the plasma membranes divided approximately equally between the 40/42.5 and 42.5/45 interfaces. The material which accumulated at the latter interface had a slightly lower ratio of total lipid to protein but the ratios of phospholipid to sterol were identical in the two fractions.

The yield of plasma membranes may be calculated from the percentage of total cell sterol recovered. On this basis the yield of plasma membranes at the 40/45 interface would be 9% divided by the fraction of total cell sterol that occurs in the plasma membrane. The mitochondrial and microsomal fractions contained about 40% of the total cell sterol. An unknown amount of sterol must occur in other cell membranes especially those derived from the plasma membrane. As a minimal estimate, however, if it were assumed that about half of the cell sterol is in the plasma membrane, the yield of plasma membranes would be approximately 20%.

Attempts have been made to modify this basic

TABLE I

Distribution of Total Cell Proteins, Ribonucleic Acid, and Lipids in the Isolation of Plasma Membranes

Step number (Scheme 1)	Fraction	Percentage of whole homogenate values			
		Protein	Ribonucleic acid	Phospholipid	Sterol
2	500 *g* pellet	37.0	28.4	47.4	62.1
3	500 *g* washed pellet	10.8	5.0	14.7	30.5
4	750 *g* washed pellet	8.5	3.8	9.8	24.3
5	25% Sucrose layer	2.7	0.9	5.3	19.0
6	750 *g* pellet	1.5	0.6	4.0	16.1
7	Sucrose gradient centrifugation 35/40 Interface	0.09	—	0.28	1.45
	40/45 Interface	0.60	—	2.38	9.20
	45/50 Interface	0.15	—	0.44	1.13
	Pellet	0.22	—	0.40	1.13

These are the results of one experiment which was typical of several.

isolation procedure. When the initial homogenization was more vigorous, smaller membrane fragments were formed which increased the losses in the low-speed centrifugations and made it more difficult to distinguish plasma membranes from smooth intracellular membranes microscopically. When steps 3 and 4 were omitted (and sometimes steps 2 and 6 altered to 750 *g* and 1000 *g*, respecmuch more material accumulated at the 30/35 and 45/50 interfaces but the plasma membranes at the 40/45 interface were chemically and microscopically essentially identical to those obtained by the normal isolation procedure. Plasma membranes prepared by these abbreviated procedures, however, were contaminated by more acid phosphatase.

Electron Microscopy of Membrane Fractions

The material that accumulated at the 40/45 interface was a nearly homogeneous preparation consisting mostly of relatively large open-ended and closed membrane fragments (Fig. 2). At higher magnifications (Fig. 3) the plasma membranes were indistinguishable from plasma membranes of intact cells (Fig. 1) with no distinguishing features, such as coated pits (Bowers and Korn, 1968) for example, on either surface. Examination of many fields at low magnification revealed only rarely structures recognizable as mitochondria or fragments of rough endoplasmic reticulum. Very occasionally, structures were seen that might have been remnants of contractile vacuoles. Recognizable contaminants could account for only a negligible portion of the protein and lipid of the plasma membrane fraction.

Plasma membranes were also present in the material that accumulated at the 35/40 and 45/50 interfaces but these fractions were more heterogeneous (Fig. 4). The 35/40 interface contained mostly plasma membranes contaminated with what might be collapsed contractile vacuoles, dense amorphous aggregates, and unidentified membranous structures. Mitochondria and rough endoplasmic reticulum were less common contaminants. The 40/50 interface contained fewer plasma membranes than did the 35/40 interface (but they were still the major constituent) with mitochondria and what are thought to be remnants of early forms of cyst walls as the major contaminants. It may seem contradictory that mitochondria contaminated the membrane fraction isolated at the 45/50 interface but not at the 40/45 interface since in the isolation of mitochondria described under Methods, mitochondria were isolated at the 40/45 interface. In the latter case mitochondria were top loaded in 30% sucrose whereas in the former the crude plasma membrane fraction was bottom loaded in 50% sucrose. This evidently affected the density of the mitochondria.

Enzymatic Criteria of Plasma Membrane Purity

The purity of the plasma membrane fraction (the 40/45 interface) was assessed by analysis of enzymes generally regarded as markers of other cell membranes (Table II). Succinic dehydro-

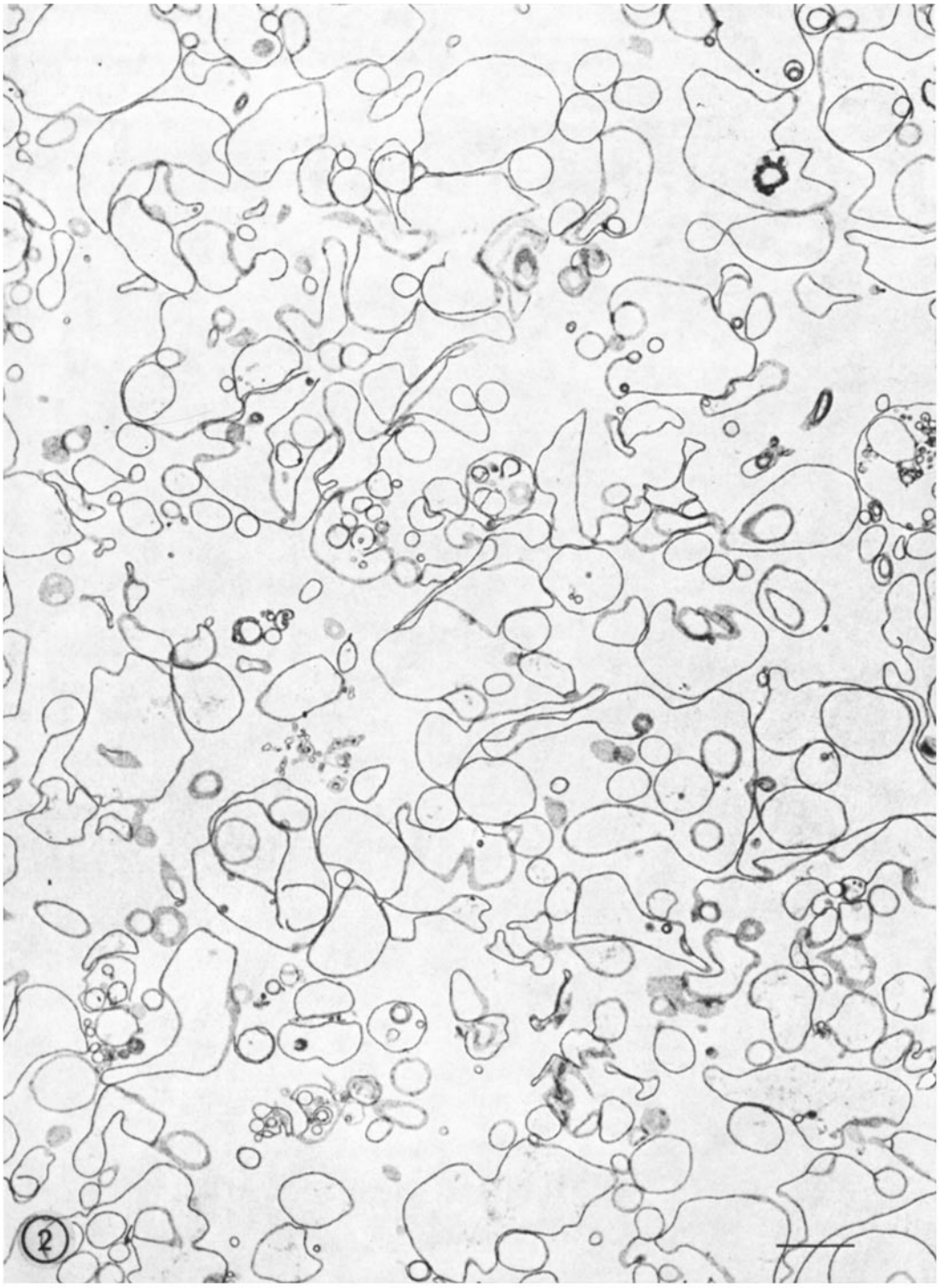

FIGURE 2 Plasma membranes isolated at the interface between 40 and 45% sucrose. This is typical of hundreds of fields of many different preparations. Recognizable contaminants were rarely seen. × 11,200.

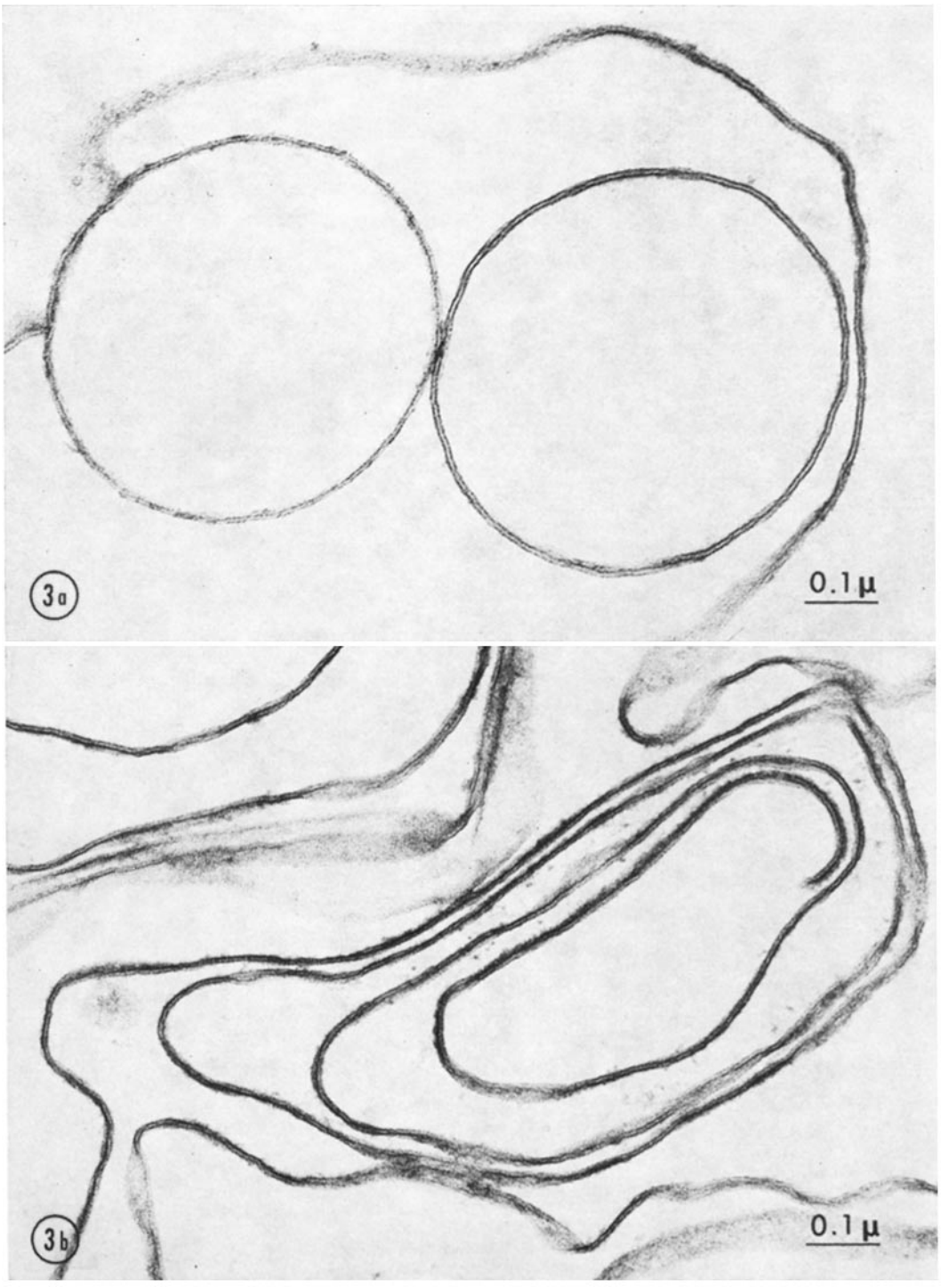

FIGURE 3 Representative areas of a typical plasma membrane fraction at higher magnification. Both closed vesicular membrane profiles and membranes with free ends are common. There are no distinguishing structures on either membrane surface. Magnification is the same as in Fig. 1, inset. × 108,000.

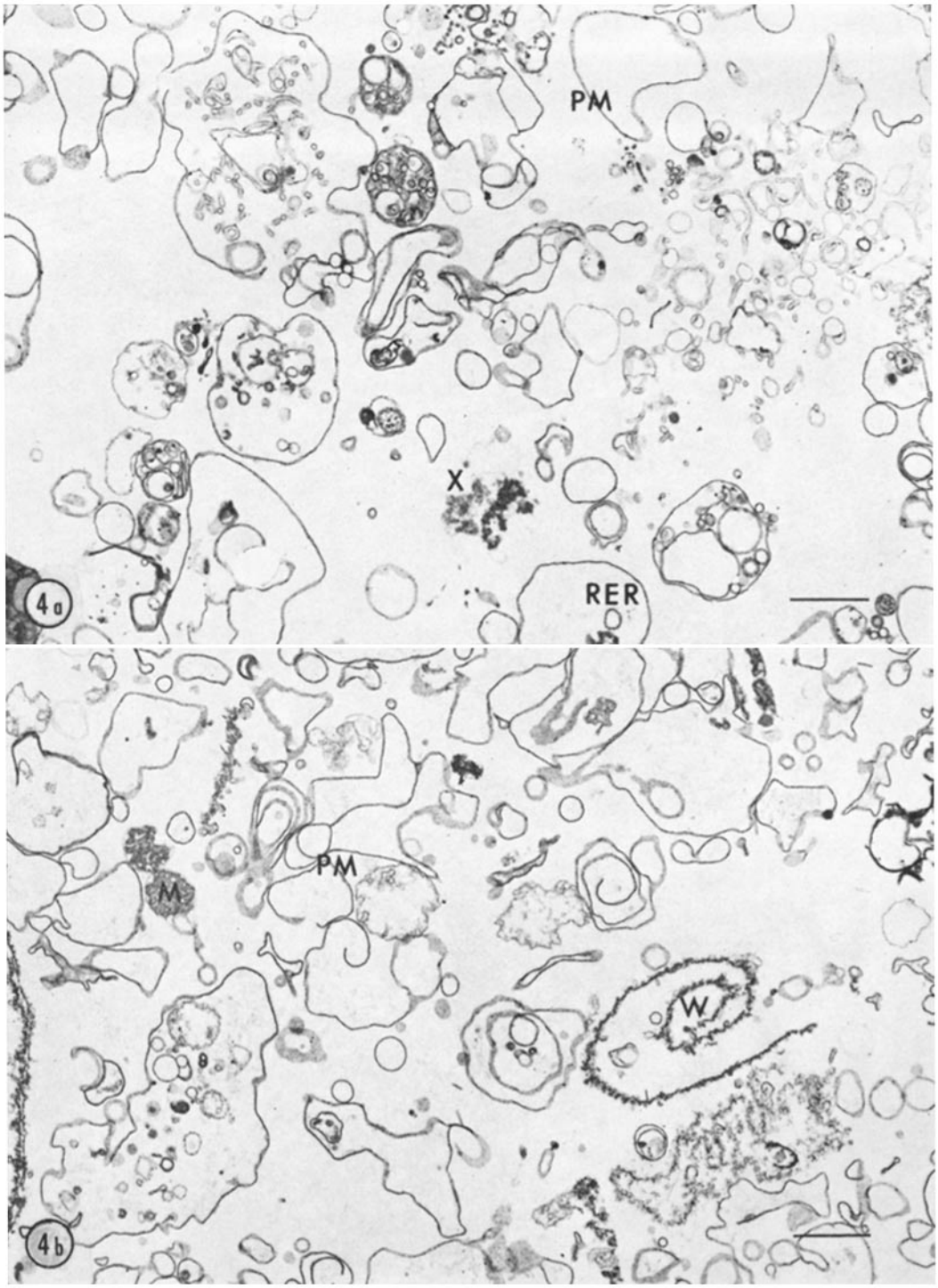

FIGURE 4 Representative portions of the material that collected at the 35/40% sucrose interface (*A*) and the 45/50% sucrose interface (*B*). Both micrographs are at the same magnification as the plasma membranes in Fig. 2. In addition to plasma membranes (*PM*), rough endoplasmic reticulum (*RER*), amorphous aggregates (*X*), and membranes of unestablished origin are present in the 35/40 fraction. Mitochondria (*M*) and what may be remnants of early cyst walls (*W*) are present in the 45/50 fraction. × 11,200.

TABLE II

Enzymatic Criteria of Purity of Isolated Plasma Membranes

Enzyme	Plasma membrane	Homogenate	Mitochondria	Microsomes
		units/mg protein*		
Succinic dehydrogenase	0.00	0.51	2.32	0.35
NADH–cytochrome *c* reductase	0.00	0.21	0.44	1.06
NADPH–cytochrome *c* reductase	0.00	0.47	0.53	0.50
Acid phosphatase	1.32	21.0	13.2	6.6
Glucose-6-phosphatase	0.22	0.96	—	1.01

* One unit of enzyme produces 1 μmole of product in 1 hr.

genase was not detected in the plasma membranes under conditions of assay that would have revealed as little as 2% contamination by mitochondrial protein. Neither NADH–cytochrome *c* reductase nor NADPH–cytochrome *c* reductase was detected in the plasma membranes although these enzymes were easily measured in the whole homogenate and in the mitochondrial and microsomal fractions.

Acid phosphatase was present at a very low level presumably derived from contamination by lysosomal-like vacuoles (pinosomes, phagosomes, digestive vacuoles) which are a major feature of the ameba cytoplasm. It is difficult to quantitate accurately the possible membrane contamination from such sources but the observed specific activity of the acid phosphatase in the plasma membrane fraction was only 0.5–1% of the specific activity we have found for phagosomes isolated from amebae after the ingestion of latex beads (Wetzel and Korn, 1969). Acid phosphatase was a more significant contaminant of the purified mitochondria and microsomal fractions.

Glucose-6-phosphatase is usually assumed to be a microsomal enzyme (Ginsberg and Hers, 1960) but we detected significant activity in the plasma membrane fraction (Table II). In view of the inability to detect other microsomal enzymes in the plasma membrane fraction, and the chemical evidence presented below, it is likely that the glucose-6-phosphatase reflects an enzymatic activity of the plasma membranes. These assays were carried out in the presence of tartrate and EDTA in quantities sufficient to inhibit acid and alkaline phosphatases, respectively. The glucose-6-phosphatases of the plasma membrane and microsomal fractions were not further studied.

It should be pointed out that no attempt was made to purify the mitochondrial and especially the microsomal fractions as extensively as the plasma membranes. They were undoubtedly each contaminated by other cell membranes. The presence of ribosomal protein in the microsomal fraction further reduced the calculated specific activities of the microsomal membrane-associated enzymes. The specific activities reported for these membrane fractions are, therefore, undoubtedly too low which would lead to an overestimate of the extent to which they might contaminate the plasma membrane fraction. Finally, assessments of contamination based on enzymatic specific activities are estimates of contamination by protein. Contamination by lipids would be less since the lipid to protein ratio of plasma membranes is higher than that of other membranes.

Chemical Analysis of Plasma Membranes

The chemical analyses of the plasma membranes (Table III) provide another measure of their purity and also a general description of their composition. The plasma membranes contained little RNA. If all of that RNA were derived from microsomal contaminants similar in analysis to the purified microsomal fraction, then only 2% of the protein of the plasma membrane would be microsomal protein. If the RNA in the plasma membrane fraction were derived from mitochondria, then contamination by mitochondrial protein might be as high as 10%. Contamination of the plasma membrane lipids by mitochondrial and microsomal lipids would be very much less. In fact these are probably overestimates because the values are incompatible with the estimate of contamination calculated from the enzymatic data and from the electron microscope evidence. Furthermore, the RNA of the plasma membrane fraction might, in fact, represent a unique fraction

TABLE III

Chemical Analysis of Plasma Membranes, Whole Cells, Mitochondria, and Microsomes

	Plasma membrane	Whole cells	Mitochondria	Microsomes
RNA (*mg/mg protein*)	0.005	0.09 (0.08–0.11; 7)	0.043 (0.032–0.056; 7)	0.26 (0.21–0.34; 4)
DNA (*mg/mg protein*)	0.0013	0.0049	—	—
Protein-bound sugar (*mg/mg protein*)	0.052 (0.045–0.58; 4)	0.004 (0.001–0.0063; 4)	0.011	—
Total lipid (*mg/mg protein*)	0.78	0.23	—	—
Phospholipid (*mg/mg protein*)	0.43 (0.38–0.47; 7)	0.10 (0.09–0.12; 7)	0.25 (0.21–0.29; 7)	0.22 (0.18–0.24; 4)
Glycerides (*mg/mg protein*)	0.08 (0.07–0.09; 3)	0.10	—	—
Sterol (*mg/mg protein*)	0.212 (0.188–0.236; 7)	0.014 (0.012–0.015; 7)	0.015 (0.012–0.018; 7)	0.026 (0.024–0.029; 4)
Lipid-bound sugar (*mg/mg protein*)	0.024 (0.017–0.027; 4)	0.002 (0.001–0.004; 4)	0.0012	—
Long-chain base (*mole/mole phospholipid*)	0.3 (0.27–0.32; 2)	0.11	0.04	0.06
Lipid inositol (*mole/mole phospholipid*)	0.002 (0.001–0.004; 4)	0.058 (0.057–0.059; 3)	0.115	0.075
Sterol (*mole/mole phospholipid*)	0.98 (0.93–1.07; 7)	0.27 (0.23–0.34; 7)	0.11 (0.08–0.13; 7)	0.22 (0.20–0.24; 4)

* The numbers in the parentheses are the range of values followed by the number of analyses.

TABLE IV

The Lipid Composition of Plasma Membranes, Phagosome Membranes, and Whole Amebae

	Plasma membranes	Phagosome membranes	Whole cells
		*molar per cent of lipid class**	
Neutral lipids			
Sterol ester	0		0.7 (0.6–0.7; 3)
Sterol free	84.3 (83.0–85.6; 2)		16.7 (16.5–16.8; 3)
Triglycerides	7.3 (6.9–7.7; 2)		74.7 (74.0–75.3; 3)
Diglycerides	7.3 (6.5–8.1; 2)		3.3 (3.0–5.3; 3)
Monoglycerides	1.1 (1.0–1.2; 2)		0.5 (0.0–1.0; 3)
Unknown	0		3.3 (2.3–3.8; 3)
Phospholipids			
Acidic phosphatides	5.0 (3.5–8.3; 4)	2.1 (1.4–2.9; 3)	2.3 (2.0–2.7; 4)
Diphosphatidylglycerol	2.6 (2.0–3.0; 4)	1.2 (0.8–1.6; 3)	4.2 (4.0–4.4; 6)
Phosphatidylethanolamine	47.2 (44.1–50.1; 5)	43.1 (41.4–44.5; 3)	33.2 (32.9–34.6; 7)
Phosphatidylserine	26.9 (24.7–29.5; 4)	25.0 (23.5–25.5; 3)	9.5 (9.0–10.0; 7)
Phosphoinositide	0.2 (0.1–0.4; 4)	0.6	5.8 (5.7–5.9; 3)
Phosphatidylcholine	18.6 (16.9–20.1; 4)	23.3 (21.8–24.6; 3)	44.5 (43.2–46.7; 6)
Lysophosphatidylcholine	0	4.5 (3.6–5.1; 3)	0.5 (0.3–0.6; 4)
Unknown phosphatides	0	0.7 (0.5–1.0; 3)	1.8 (1.5–2.6; 4)
Plasmalogen (μmoles/μmoles phospholipid ester)	0.16	—	0.11

* The range of values and the number of analyses are given within the parentheses. The composition of the lipids from the whole cell have been published previously (Ulsamer et al., 1969) and in that earlier paper the compounds were fully characterized. The phosphoinositide contains no glycerol (Ulsamer et al., 1969). The neutral lipids of the phagosome membranes were not analyzed because those membranes were contaminated by varying amounts of triglycerides from cytoplasmic fat droplets (Wetzel and Korn, 1969).

as has been claimed previously for the RNA in the plasma membranes of L cells (Glick and Warren, 1969). The plasma membrane contained very little if any DNA since there is no assurance that the colorimetric assay is specific for DNA.

The protein-bound sugars were enriched in the plasma membrane fraction about 13-fold relative to the whole homogenate. Neither amino sugars nor sialic acid were detected.

The ratio of phospholipid to protein was considerably enriched in the plasma membranes over values for the whole cell; sterols and phospholipids were present in nearly equimolar amounts in the plasma membranes; and there was very little phosphoinositide in the plasma membranes (Table III). If all of the phosphoinositide were derived from microsomes, then a maximum of 2.5% of the phospholipids (5% of the protein) of the plasma membranes would be of microsomal origin. It is at least as probable that this residual phosphoinositide is a true component of the plasma membranes. The enrichments of lipid-bound sugar and

TABLE V

Fatty Acid Compositions of the Major Phospholipids of Plasma Membranes and Whole Cells

Phospholipid	Source	Fatty acids*									
		14:0	16:0	16:1	18:0	18:1	18:2	20:1	20:2	20:3	20:4
		%‡	%	%	%	%	%	%	%	%	%
Phosphatidylethanolamine	Plasma membrane	2.9	4.0	1.3	6.5	32.5	3.7	1.9	30.4	4.7	11.8
	Whole cell	3.7	5.1	1.4	9.1	47.4	7.8	0.6	8.5	5.0	11.6
Phosphatidylserine	Plasma membrane	1.6	12.5	2.5	12.3	27.3	4.1	1.0	23.0	4.3	11.1
	Whole cell	0.9	3.2	1.1	2.8	26.5	12.7	2.5	28.8	6.9	14.5
Phosphatidylcholine	Plasma membrane	1.1	9.8	1.6	16.5	42.2	5.6	1.5	9.1	5.8	7.0
	Whole cell	0.7	5.4	0.7	9.4	39.3	9.5	1.1	9.2	9.2	15.5

* In the designation of fatty acids the first number indicates the chain length and the second number indicates the number of double bonds. The fatty acids have been identified previously (Korn, 1963) as myristic (14:0), palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2), 11,14-eicosadienoic (20:2), 8,11,14-eicosatrienoic (20:3), and arachidonic (20:4).

‡ Per cent for all columns is per cent of total

of long-chain base were about 12-fold relative to the whole cell.

The chemical data, then, are entirely in accordance with the enzymatic and electron microscope evidence that the 40/45 interface is a highly purified preparation of plasma membranes.

The chemical data in Table III were obtained from cells grown for 7 days to a density of about 1–1.5 $\times$ 10^6/ml. Plasma membranes isolated from amebae grown for 5 days (0.4–0.6 $\times$ 10^6/ml) contained more phospholipid (0.56 mg/mg protein) but the same concentration of sterol. Therefore, the molar ratio of sterol to phospholipid was about 0.81 as compared to a ratio of 0.98 for amebae from more densely grown cultures.

Lipid Composition of Plasma Membranes and Phagosome Membranes

The neutral lipids of the plasma membranes contained very much less triglycerides than did the lipids of the whole cell, and sterol esters were not present in detectable quantities in the plasma membranes (Table IV). The sterols consisted of ergosterol and dehydroporiferasterol in the same ratio, 60:40, as in whole cells (Smith and Korn, 1968; Ulsamer et al., 1969). About 70% of the sterols had been enzymatically dehydrogenated to aromatic derivatives (Korn et al., 1969) by the time the plasma membranes were isolated. Relative to the total cell phospholipids, the phospholipids of the plasma membranes contained very much less phosphatidylcholine, much more phosphatidylethanolamine and phosphatidylserine, and very little, if any, phosphoinositide. Very nearly the same composition was found for the phospholipids isolated from plasma membranes of younger cells.

The phospholipid composition of the phagosome membranes (Table IV) was very similar to that of the plasma membranes. It is not known whether the small differences that were observed represent experimental difficulties in analyzing the relatively small amount of phagosome membranes or a real difference between the two membranes which may arise during the phagocytic process. The lysophosphatidylcholine in the lipids of the phagosome membranes may have been formed by hydrolytic reactions during the preparation of the material for analysis but it may be a real difference between the phagosome membranes and the plasma membranes (Lucy, 1970).

The fatty acid compositions of the major phospholipids of the whole cell and plasma membranes (Table V) show several differences. The membrane phosphatidylethanolamine had less stearate, oleate, and linoleate, and more eicosadienoate than the phosphatidylethanolamine from the whole cells. The membrane phosphatidylserine contained more palmitate and stearate and less linoleate than the phosphatidylserine from the whole cell. The membrane phosphatidylcholine contained more palmitate and stearate and less linoleate and arachidonate than the phosphatidylcholine from the whole cell lipids.

TABLE VI

Specific Activities of Alkaline Phosphatase and 5'-Nucleotidase of Plasma Membranes Homogenate, Mitochondria, and Microsomes

	Fraction analyzed			
	Plasma membrane	Homogenate	Mitochondria	Microsomes
Alkaline phosphatase (*units*/mg protein*)	197 (132–321; 7)‡	15 (12–23; 7)	20	18
Relative to homogenate	14 (11–28)	1	1.3	1.2
5'-Nucleotidase§ (*units/mg protein*)	21.4 (15.1–38.2; 6)	1.7 (1.2–2.5; 6)	3.5	1.6
Relative to homogenate	12 (8–32)	1	2	1

* One unit of enzyme produces 1 μmole of product in 1 hr.

‡ The range of values and the number of experiments are shown in parenthesis. These data were obtained from 5-day cultures.

§ It is possible that hydrolysis of AMP is catalyzed by the alkaline phosphatase.

The plasmalogens of both the whole cells and the plasma membranes consisted of 80% stearaldehyde and 20% myristaldehyde. As tentatively identified by their relative retention times the long-chain bases were 65% C_{18}-dehydrophytosphingosine, 6% anhydro-C_{18}-dehydrosphingosine, 8% C_{18}-dehydrophytosphingosine, 9% C_{20}-dihydrosphingosine, and 10% unidentified. Glucose accounted for about 60% of the sugars of the glycolipids of the whole cells and of the plasma membranes. The remainder of the sugars were, unidentified but were not galactose, fructose, or ribose.

Enzymes of the Plasma Membrane

Schultz and Thompson (1969) have made the interesting observation that the specific activity of 5′-nucleotidase of plasma membranes prepared from amebae that were grown to a cell concentration of approximately 4×10^5 cells/ml was approximately three times greater than that of plasma membranes isolated from cells grown to a concentration of about 1×10^6/ml despite the fact that the specific activities of the whole cell homogenates were identical. We have confirmed this observation and have obtained essentially the identical specific activity as that found by Schultz and Thompson for the plasma membranes isolated from the younger cells (Table VI). Similar results have also been obtained by us for alkaline phosphatase which had a specific activity about 10 times greater than that of the 5′-nucleotidase (Table VI). The data do not eliminate the possibility, and might even suggest, that the nucleotidase activity is due to the alkaline phosphatase assayed under suboptimal conditions. For both enzymatic activities purifications of about 13-fold were obtained in the plasma membrane fraction relative to the starting homogenate. In one experiment the purification was 30-fold. Similar purifications and final specific activities were obtained when Mg^{++} or Ca^{++} (Ray, 1970) was present in all the solutions and when 1 mM $NaHCO_3$, pH 7.5 (Schultz and Thompson, 1969) was substituted for 0.01 M Tris. The alkaline phosphatase hydrolyzed *p*-nitrophenylphosphate about three times more rapidly than β-glycerophosphate. Both the alkaline phosphatase and 5′-nucleotidase were about 50% more active in the presence of Triton-X 100.

About 5% of the total cell content of both enzymatic activities was recovered in the purified plasma membrane fraction. This was less than the per cent recovery of sterol in that same fraction and was not due to inactivation of the enzymes since both 5′-nucleotidase and alkaline phosphate activities could be totally accounted for in the sum of the activities of all of the fractions. Furthermore, the specific activities of both enzymes were sometimes higher in the material that accumulated at the 35/40 interface than in the plasma membrane fraction (40/45 interface) (Table VII). This is a curious and unexplained result since the chemical and electron microscope data indicated that plasma membranes were less pure at the 35/40

TABLE VII

Distribution of Alkaline Phosphatase, 5′-Nucleotidase, and Protein in the Discontinous Sucrose Gradient Centrifugation

Experiment	Sucrose interface analyzed		
	35/40	40/45	45/50
(1) Protein (*mg*)	4.9	8.8	13.4
Alkaline phosphatase (*units/mg*)	255	284	102
5′-Nucleotidase (*units/mg*)	19.5	22.3	7.8
(2) Protein (*mg*)	0.6	1.9	1.0
Alkaline phosphatase (*units/mg*)	274	140	41
5′-nucleotidase (*units/mg*)	31.8	17.6	4.0

These values were obtained with cells from 5-day cultures. An abbreviated isolation procedure was used in which steps 3 and 4 (Scheme 1) were omitted. This results in a plasma membrane fraction (40/45) of essentially unchanged composition and more material at the 30/35 and 45/50 interfaces (compare protein values to those of Table I).

interface than at the 40/45 interface. Both enzymatic activities were also present in the material at the 45/50 interface but of lower specific activities as would be expected from the obvious heterogeneity of that fraction. It is possible, therefore, that alkaline phosphatase and 5′-nucleotidase are not uniformly distributed within the plasma membranes and that membrane fragments of different enzymatic content can be separated. If this were so, then the plasma membrane fraction obtained at the 40/45 interface might represent only one portion of the plasma membrane of the ameba. Alternatively, the alkaline phosphatase and 5′-nucleotidase might occur in other membranes closely related to the plasma membrane. This latter possibility might also explain why plasma membranes from "young" cultures were more active than plasma membranes from "old" cultures while the specific activity of the total cell homogenates was the same.

A Mg^{++}-ATPase behaved in a similar fashion (Table VIII) with variable distribution between the 35/40 interface and 40/45 interface. The purifications relative to the whole homogenate and the final specific activities of the ATPase in the plasma membrane fraction were in excellent agreement with those reported by Schultz and Thompson (1969).

The plasma membranes did not have a (Na^+-K^+)ATPase, adenyl cyclase, or glucosidase, each of which has been associated with plasma membranes from mammalian cells.

Comparison with the Procedure of Schultz and Thompson

In the procedure developed by Schultz and Thompson the amebae were homogenized in 1 mM $NaHCO_3$, pH 7.5, filtered through cheesecloth, and the filtrate centrifuged at 1500 *g* for 10 min. The resulting pellet was washed several times by centrifugation at 1220 *g* for 10 min until a fluffy residue was obtained free of a more dense pellet. Plasma membranes were obtained by sucrose gradient density centrifugation at the interface between 43 and 48% sucrose (w/v). The procedure differs from the one described in this paper in that the crude membrane fraction is obtained from a less dense solution using higher centrifugal forces, and the purified plasma membranes are collected at a slightly more dense interface (43/48 compared to 40/45).

TABLE VIII

Mg^{++}-ATPase Activities of Membrane Fractions

Experiment	Fraction analyzed: 35/40 interface	40/45 interface	Whole homogenate
	units/mg protein*		
(1)	38.8	24.0	10.8
(2)	69.5	103.9	13.5
(3)	31.7	28.4	10.8

* One unit of enzyme forms 1 μmole of product in 1 hr.

Insofar as we have compared them, the two procedures gave similar results. For membranes isolated by the procedure of Schultz and Thompson we found a weight ratio of phospholipid to protein of 0.37, which is at the lower end of our range (Table III), and a molar ratio of sterol to phospholipid of 0.96 which is in good agreement with our values. Schultz and Thompson have not published chemical analyses of their plasma membranes. Electron micrographs of plasma membranes as we have prepared them using the procedure of Schultz and Thompson showed more contamination by rough vesicles, what may be contractile vacuoles, and other unidentified material than we found in plasma membranes isolated by our procedure. By enzymatic criteria the two preparations were essentially identical as shown by one experiment in which we prepared plasma membranes from the same culture of amebae by both methods (Table IX). In three other experiments using the procedure of Schultz and Thompson we found the purification of alkaline phosphatase and 5′-nucleotidase (plasma membrane relative to homogenate) to vary between 6- and 26-fold which is in reasonable agreement with the data in Table VI, and with the data of Schultz and Thompson for 5′-nucleotidase (these investigators did not assay for alkaline phosphatase). We obtained higher yields of plasma membranes and less contamination by acid phosphatase using the procedure described in this paper than that described by Schultz and Thompson (Table IX).

Enzymes of the Phagosome Membrane

As shown in Table X, membranes of phagosomes isolated from amebae that were actively ingesting latex beads were very similar in enzymatic com-

TABLE IX

Comparison of Plasma Membranes as Prepared by Methods of Schultz and Thompson and this Paper

	Homogenate	Plasma membrane	
		Schultz-Thompson	This paper
Protein (*mg*)	610	1.2	3.5
Alkaline phosphatase (*units/mg*)	23	325	284
5′-Nucleotidase (*units/mg*)	2	31	23
Mg^{++}-ATPase (*units/mg*)	11	37	28
Succinic dehydrogenase (*units/mg*)	0.4	0	0
Acid phosphatase (*units/mg*)	12	6	2

Amebae cultures were divided into half and plasma membranes were isolated from one portion according to the method described by Schultz and Thompson (1969) and from the other portion by the method described in this paper.

TABLE X

Alkaline Phosphatase and 5′-Nucleotidase Activities of Phagosome Membranes Compared with Plasma Membranes

	Alkaline phosphatase	5′-Nucleotidase	Acid phosphatase
	units/μmole of sterol		
Phagosome membranes	334	48	171
Plasma membranes	365	33	6

Amebae were allowed to phagocytose polystyrene latex beads of diameter 1 μ for 1 hr (Wetzel and Korn, 1969). The culture was divided into half and plasma membrane were isolated from one portion in the usual way and phagosomes were isolated from the other portion according to Wetzel and Korn (1969). Enzymatic activities are expressed as micromoles of product formed per hour per micromole of sterol in order to relate the activities to the membranes since the phagosomes contain soluble proteins. The acid phosphatase is one of the components of these soluble proteins. For the assays of alkaline phosphatase and 5′-nucleotidase, tartrate and fluoride were added which inactivated the acid phosphatase.

position to plasma membranes isolated from cells from the same culture. The enzymatic activities are compared relative to the sterol content of the membranes since the phagosomes contain appreciable nonmembranous proteins (Wetzel and Korn, 1969). Acid phosphatase activities are provided for comparative purposes; it is not a membrane enzyme but is present in the phagosome contents (Wetzel and Korn, 1969).

DISCUSSION

Purity of the Plasma Membranes

The extensive homogeneity of the plasma membranes isolated at the 40/45% sucrose interface indicated by the electron micrographs is supported by the enzymatic analyses. The absence of detectable succinic dehydrogenase, NADH–cytochrome *c* dehydrogenase, and NADPH–cytochrome *c* dehydrogenase indicates very little contamination of the isolated plasma membranes by mitochondria or microsomal membranes. On these bases the purity of the plasma membranes of *Acanthamoeba* isolated by this procedure is at least equivalent to the purity of membranes isolated by the procedure of Schultz and Thompson (1969). Contamination by mitochondria can present a major problem in the isolation of plasma membranes from mammalian cells, and the specific activity of succinic dehydrogenase has frequently been as high as 20% of the specific activity of mitochondria (Coleman et al., 1967). Similarly, the NADH–cytochrome *c* reductase activity in rat liver plasma membranes is commonly as high as 20% of the specific activity of purified microsomes (Emmelot et al., 1964). These problems seem to have been overcome adequately in the isolation of ameba plasma membranes.

The very low activity of acid phosphatase provides reasonable evidence for only minor contamination of the plasma membranes by intact pinosomes, phagosomes, or digestive vacuoles. It is very difficult, however, to eliminate the possibility that membranes of disrupted vacuoles are present in the plasma membrane fraction. Until more detailed evidence is available we would tentatively assume that contamination from such sources is rather low because it has been shown that phagosomes which contain latex beads are not disrupted by more vigorous homogenization than has been used to isolate the plasma membranes (Wetzel and Korn, 1969). In any case, the data in this and the earlier paper indicate that, as would be expected, the membranes of pinosomes and phagosomes are very similar to plasma membranes.

The relatively high purity of the plasma mem-

branes is also indicated by the chemical analyses. One characteristic of plasma membranes is their high molar ratio of sterol to phospholipid (Korn, 1969 *a*, *b*). The ratio of 0.98 found for the *Acanthamoeba* plasma membranes (0.81 for membranes from younger cells) exceeds the highest of a wide range of reported values for liver cell plasma membranes and the values reported for the plasma membranes of the L cell and of platelets, and is similar to values reported for the plasma membranes from intestinal mucosal brush border, HeLa cells, and lymphocytes (Table XI). The observed concentrations of RNA and DNA in the ameba plasma membranes are as low as or lower than most values reported for other preparations of plasma membranes (Table XI). In this regard it should be noted that digestion of the ameba plasma membranes with ribonuclease, a method that failed to detect any RNA in the HeLa cell plasma membrane (Bosmann et al., 1968), did not detect any RNA in the ameba plasma membranes. The very low level of phosphoinositide in the ameba plasma membranes relative to the whole cell homogenate and to other ameba membranes (Table III) provides additional evidence for the purity of the plasma membranes.

The gross chemical composition of the ameba plasma membranes is not very different from the compositions reported for the plasma membranes of L cells, HeLa cells, and lymphocytes (Table XI), except for the uniquely high level of RNA found in the L cell plasma membrane. The plasma membranes of intestinal brush border cells may have significantly less phospholipids and plasma membranes of platelets may have much more phospholipids (Table XI). The compositional data reported for rat liver cell plasma membranes (Table XI) by a number of laboratories vary widely around the values found for the ameba plasma membranes.

The chemical analyses of the *Acanthamoeba* mitochondria and microsomes with which the plasma

TABLE XI

Comparison of Chemical Composition of Plasma Membranes from Animal Cells

	Acanthamoeba*	Rat liver‡	Rat intestinal brush border§	HeLa cell‖	L cell¶	Platelet**	Lymphocyte‡‡
Total lipid (*mg/mg protein*)	0.78	0.39–0.97	0.61	0.67	0.68	—	0.74
Phospholipids (*mg/mg protein*)	0.43	0.26–0.95	0.14	0.31	0.39	1.4, 0.98	0.44
Glycerides (*mg/mg protein*)	0.08	0.01–0.2	0.02	—	0.13		—
Sterol (*mg/mg protein*)	0.21	0.02–0.23	0.08	0.17	0.15	0.35, 0.22	0.22
RNA (*mg/mg protein*)	0.005	0.001–0.07	0.013	0	0.04	0.11, 0.10	0.03
DNA (*mg/mg protein*)	0.001	0.004	0.005	—	—	—	0
Sterol/phospholipid (*mole/mole*)	0.98	0.26–0.82	1.26	1.05	0.74	0.49, 0.45	1.01
Succinic dehydrogenase (relative to whole homogenate)	0	0–0.5	—	0	—	0.07, 0.14	0
NADH–cytochrome *c* reductase (relative to whole homogenate)	0	0.2–2.1	—	—	—	—	—

* This paper.

‡ Emmelot et al. (1964); Skipski et al. (1965); Takeuchi and Terayama (1965); Ashworth and Green (1966); Coleman and Finean (1966); Coleman et al. (1967); Dod and Gray (1968); Pfleger et al. (1968); Stahl and Trams (1968); Stein et al. (1968); Berman et al. (1969); Fleischer and Fleischer (1969); Ray et al. (1969); Evans (1970); Ray (1970). Not all of the analyses have been done by all of the investigators.

§ Forstner et al. (1968 *a*, *b*).

‖ Bosmann et al. (1968).

¶ Warren et al. (1967); Weinstein et al. (1969).

** Barber and Jamieson (1970); two plasma membrane fractions were isolated.

‡‡ Allan and Crumpton (1970).

membranes were compared (Table III) were well within the values usually found for mammalian tissues (Korn, 1969 *a*, *b*), with the possible exception of the rather high sterol to phospholipid ratio of the ameba microsomes. This may have been due to the presence of fragments of plasma membrane or related membranes (phagosomes, etc.) in the microsomal fraction. Since the purpose of these analyses was to characterize cellular membranes likely to contaminate the plasma membrane fraction, and not to define the microsomal fraction, this is not important in the present context.

Phospholipid Composition

The phospholipids of the plasma membranes of *Acanthamoeba* contain much more phosphatidylethanolamine and phosphatidylserine and much less phosphatidylcholine than do the plasma membranes from rat liver and L cells (Table XII). The ameba plasma membranes (and whole cells) contain no sphingomyelin, a compound which is particularly enriched in plasma membranes from mammalian sources. Long-chain bases are present (Table III) but the nature of the lipids which contain them has not been determined. Compounds which contain long-chain bases are eluted in several different fractions from silicic acid. The ameba glycolipids are also unidentified. Although the fatty acid composition of individual phospholipids of the plasma membrane and whole cell differ (Table V) the percentages of saturated and unsaturated fatty acids are similar in the total phospholipids of the plasma membrane and the whole cell. The fatty acids of mammalian cell plasma membranes on the other hand tend to be more saturated than are the total cell fatty acids.

TABLE XII

Phospholipid Compositions of Plasma Membranes of Animal Cells

	Acanthamoeba*	Rat liver‡	L cells§
		mole %	
Acidic phospholipids‖	8	0–9	13
Phosphatidylethanolamine	47	11–19	10
Phosphatidylserine	27	7–9	4
Phosphatidylinositol¶	0.2	7	5
Phosphatidylcholine	19	33–41	32
Sphingomyelin	0	13–33	24

* This paper.

‡ Skipksi et al (1965); Pfleger et al. (1968); Dod and Gray (1968); Ray et al. (1969).

§ Weinstein et al. (1969).

‖ Includes diphosphatidylglycerol, phosphotidylglycerolphosphate, phosphatidic acid and, in the ameba, unidentified compounds.

¶ In *Acanthamoeba* this is a phosphoinositide of unknown structure that contains no glycerol (Ulsamer et al., 1969).

Enzymes of the Plasma Membrane

Alkaline phosphatase and 5′-nucleotidase would seem to be components of the plasma membrane of *Acanthamoeba*. Both are enriched in the isolated plasma membrane fraction 12–14 times relative to the specific activity of the whole homogenate. The specific activity of the 5′-nucleotidase of the ameba plasma membrane is very similar to that found for plasma membranes from rat liver and from HeLa cells (Table XIII). The highest values reported for rat liver membranes are for plasma membranes isolated in the presence of 0.5 mM $CaCl_2$ (Ray, 1970), a modification which had no effect on the specific activity of ameba membranes. The specific activity of alkaline phosphatase was much higher in the ameba plasma membranes than in the plasma membranes of liver, HeLa cells, or platelets and resembled the high specific activity reported for plasma membranes isolated from intestinal brush borders (Table XIII). Since the specific activity of the alkaline phosphatase at pH 10 is about nine times greater than the specific activity of the 5′-nucleotidase at pH 7.5 and since *p*-nitrophenylphosphophosphate is hydrolyzed about three times faster than AMP at pH 7.5, it is quite possible that the 5′-nucleotidase activity is due to the alkaline phosphatase.

We cannot say from these data that either of these two enzymatic activities is specifically a component only of the plasma membranes of amebae. They certainly are present in phagosome membranes (Table X) which are derived from the plasma membranes and there may be yet other intracellular membranes in amebae (pinosomes, digestive vacuoles) which are in equilibrium with the plasma membrane. The fact that the specific activities of both the alkaline phosphatase and 5′-nucleotidase were sometimes higher in fractions that seem to be less rich in plasma membranes than the major plasma membrane fraction suggests that these enzymes may indeed be distributed within more than one membrane. This tentative conclusion is supported by two other observations.

TABLE XIII

Comparison of Enzymes of Plasma Membranes from Animal Cells

	Acanthamoeba*							
	A	*B*	Rat liver‡	Rat intestinal brush border§	HeLa Cell‖	L cell¶	Platelet**	Lymphocyte‡‡
Alkaline phosphatase								
Specific activity (*units/mg*)	197	—	0.9	355, 407	0.67	—	0.13	—
Relative to homogenate	14	—	—	—	21	—	1	—
5′-Nucleotidase								
Specific activity (*units/mg*)	21§§	28§§	7–91***	—	35	—	—	10
Relative to homogenate	12	13	4–28	—	115	—	—	—
Mg^{++}-ATPase								
Specific activity (*units/mg*)	24–100	29	40–200***	6, 25	1.5	2.7	0.6	—
Relative to homogenate	2.5–8	6	6	—	—	1.9	4	—

* A: this paper; B: Schultz and Thompson (1969).

‡ Emmelot et al. (1964); Coleman et al. (1967); Michell et al. (1967); Dod and Gray (1968); Graham et al. (1968); Stein et al. (1968); Berman et al. (1969); Fleischer and Fleischer (1969); Wattiaux-DeConinck and Wattiaux (1969); Ray et al. (1969); Ray (1970); Evans (1970).

§ Forstner et al. (1968 *a*); Eichholtz (1967); Malathi and Crane (1969).

‖ Bosmann et al. (1968); Boone et al. (1969).

¶ Warren et al. (1967).

** Barber and Jamieson (1970).

‡‡ Allan and Crumpton (1970).

§§ The 5′-nucleotidase activity of *Acanthamoeba* plasma membranes may be due to the alkaline phosphatase.

*** Highest value when plasma membrane was isolated in the presence of 0.5 mM Ca^{++}.

The recovery of the enzymes in the plasma membrane fraction (40/45 interface) was generally less than the recovery of sterol in that fraction. Also, Schultz and Thompson observed, and we have confirmed, that although amebae from older cell cultures contain the same amount of enzyme as the cells from younger cultures, the purified plasma membranes have a much lower specific activity. This must mean either that less of the enzymes in the older cultures is in the plasma membranes or that the enzyme is less tightly bound to the plasma membrane and is lost during its isolation. With both young and old cultures we can account for essentially all of the enzymatic activity in the sum of the activities of all of the fractions obtained during the isolation, so there is no significant inactivation of enzyme. It seems possible, then, that alkaline phosphatase and 5′-nucleotidase may be components of cell membranes other than the plasma membrane. Glaumann and Dallner (1970) have recently suggested that 5′-nucleotidase might occur in some subfractions of smooth microsomes from rat liver, and these might be related to some of the fractions which we obtain from the amebae.

Another enzyme, Mg^{++}-ATPase, is probably also a component of the ameba plasma membrane. Its specific activity in the ameba plasma membrane fraction is similar to that reported for liver and intestinal plasma membranes and greater than that found in plasma membranes of HeLa cells (Table XIII). Its purification in the plasma membrane is less than that of alkaline phosphatase and 5′-nucleotidase (but similar to that found for liver cells), undoubtedly because similar activity is present in other cell membranes.

Plasma membranes from mammalian cells are generally thought to contain, among other enzymes, $(Na^{+}$-$K^{+})$ATPase, glucosidase, and adenyl cyclase (Korn, 1969 *a*, *b*). We could not detect these activities in the ameba plasma membranes.

It is of course possible that the plasma membranes contain such activities but that the enzymes were removed or inactivated during the isolation procedure. (Na^+-K^+)ATPase was not detected, however, in whole cell homogenates. Other enzymes could be present that were not assayed.

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