ISOLATION AND CHARACTERIZATION OF SUBCELLULAR MEMBRANES OF *ENTAMOEBA INVADENS*

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ABSTRACT

A method is described for the isolation of subcellular membranes of *Entamoeba invadens*. Plasma membranes were obtained by rate centrifugation followed by isopycnic centrifugation on a sucrose gradient. Intact phagolysosomes floated in a 10% sucrose solution providing a simple technique for isolation. Phagolysosomal membranes were collected by isopycnic centrifugation, after lysis of the phagolysosomes. Microsomes were obtained by differential centrifugation. Membrane fractions were examined by electron microscopy, and the contamination of each fraction was determined with marker enzymes. Mg^{2+}-ATPase is associated with the plasma membrane. Acid phosphatase (β-glycerophosphate) was associated mainly with phagolysosomal membranes. Plasma membranes also contained acid phosphatase activity which hydrolyzes p-nitrophenylphosphate but not β-glycerophosphate. The localization of the two phosphatases was confirmed cytochemically.

Isolated plasma membranes were contaminated with phagolysosomal membranes (15%) and with microsomes (25%). No more than 5% of the phagolysosomal membrane fraction consisted of plasma membranes. Contamination of the microsomes by plasma and phagolysosomal membranes was 10% and 7%, respectively. Plasma membranes and phagolysosomal membranes had a high ratio of cholesterol to phospholipid (0.93 and 1.05 μmol/μmol, respectively). Microsomes were relatively poor in cholesterol (0.39 μmol/μmol).

Microsomes, plasma, and phagolysosomal membranes contained increasing amounts of sphingolipids (12%, 17%, and 28%). Phagolysosomal membranes had a high percentage of phosphatidylserine but little phosphatidylcholine. Microsomes were rich in phosphatidylcholine (45%). Differences in phospholipid composition between plasma and phagolysosomal membranes are discussed in view of the phagocytic process.

Electron micrographs of *Entamoeba* reveal the relatively simple structure of these protozoan cells (Fig. 1). The main organelles are the phagolyso- somes (31, 32) which are scattered throughout the cell. A nucleus is present as well as a Golgi appa- ratus (9, 30) and endoplasmic reticulum (22, 23,
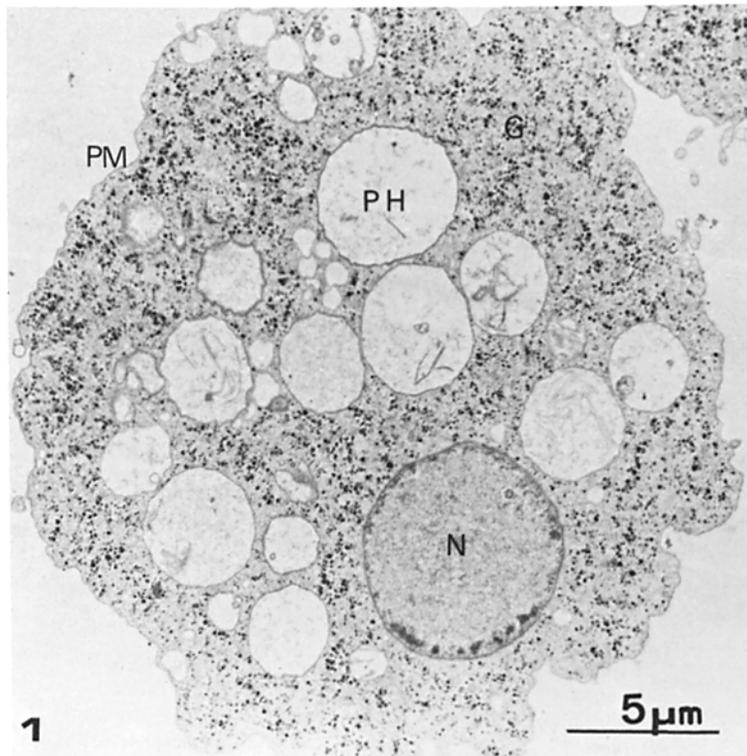
FIGURE 1   Axenically cultivated *Entamoebae invadens* showing phagolysosomes (*PH*), nucleus (*N*), glycogen (*G*), and plasma membrane (*PM*). × 3900.

21) although the latter intracellular membrane structures are scarcely developed. The cell is surrounded by a unit membrane without a typical cell wall (36). Mitochondria are absent in these anaerobic organisms (8). Because of this relative simplicity of the intracellular organization, *Entamoeba* might be particularly suitable for the isolation and characterization of the plasma and lysosomal membranes and, consequently, for investigations on the biogenesis of these membranes. Cell fractionation has been carried out on *Entamoeba invadens* (36), *Tetrahymena pyriformis* (28), and *Acanthamoeba palestinensis* (7); however, apart from electron micrographs, no evidence was presented in these studies as to the purity of the isolated membranes. Plasma and phagolysosomal membranes of the *Acanthamoeba castellanii* (35, 41) have been isolated and identified by means of marker enzymes.

In this study, a procedure is described for the subcellular fractionation of *E. invadens*, designed in particular for the isolation of phagolysosomal and plasma membranes. The membranes were characterized by determination of typical enzymes and by electron microscopy. The possible interrelationship between plasma and phagolysosomal membranes was investigated by analysis of the lipid composition of both membranes.

MATERIALS AND METHODS

Cultivation of Amoebae

The amoebae were cultivated in 4 liters of liquid medium containing cholesterol, Tween 80 and oleic acid, as described before (44). After 8–10 days of growth, the amoebae were harvested and washed twice with NaCl-phosphate buffer by centrifugation at 500 *g* for 5 min. The yield of amoebae was normally about 15–20 g of cells (wet wt).

Isolation Procedure of Subcellular Fractions

The cells were resuspended in 9 vol of a cold sucrose solution (10% sucrose, 1 mM EDTA, 10 mM NaHCO₃ adjusted at pH 7.0) and homogenized by means of 10–15 strokes of a loose-fitting Dounce homogenizer (Kontes Glass Co., Vineland, N. J.). Disruption of the

cells was examined by phase-contrast microscopy. All operations were carried out at 4°C.

The steps in the isolation procedure are summarized in Fig. 2. The homogenate was centrifuged at 500 g for 10 min, and nuclei, cell debris, and whole amoeba cells were collected. After centrifugation of the supernate at 4,500 g for 20 min, a fraction was isolated which floated as a layer on the top of the supernate. The "floating pellet" was carefully taken from the top with a Pasteur pipette and washed twice by resuspension in 10% sucrose and centrifugation at 4,500 g for 10 min. Part of this washed floating pellet containing the phagolysosomes (by criteria to be presented in the Results section) was lysed by suspension in hypotonic buffer (1 mM EDTA, 10 mM NaHCO$_3$, pH 7.0) and a few strokes of a Dounce homogenizer. The "crude" phagolysosomal membranes and the supernate of the phagolysosomes were separated by centrifugation at 15,000 g for 10 min. The crude membranes were resuspended in 10% sucrose and layered on top of a continuous sucrose gradient (25%–40% sucrose) to obtain purified phagolysosomal membranes. The phagolysosomal membranes banded as a single layer at about 33% sucrose during centrifugation for 150 min at 25,000 rpm in a Beckman SW 27 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The band was carefully collected, and the membranes were isolated, after dilution of the sucrose solution with distilled H$_2$O, by centrifugation for 60 min at 30,000 rpm in a Beckman 30 rotor.

After removal of the floating pellet, the 4,500 g supernate, together with the washing solutions, was centrifuged for 10 min at 15,000 g. The pellet was washed twice by resuspending it in 10% sucrose and subsequent centrifugation. This fraction, consisting mainly of the crude plasma membranes, was resuspended in 10% sucrose, layered on a continuous sucrose gradient (30–50% sucrose) and centrifuged for 150 min at 25,000 rpm in a Beckman SW 27 rotor. The major part of the plasma membranes banded at 47% sucrose, whereas minor bands were also observed at 43%, 39%, 37%, and 33% sucrose and, in addition, some membrane-like material floated on the top of the gradient. The purified plasma membranes, banding at 47% sucrose, were collected and further isolated as described above. The small amounts of material at the interfaces of 43%, 39%, 37%, and 33% sucrose were not isolated or analysed further.

Microsomes were obtained by centrifugation of the 15,000 g supernate for 60 min at 30,000 rpm in a Beckman 30 rotor. The refractive indices were measured at 20°C in a Bausch and Lomb Abbe-3L refractometer (Bausch and Lomb, Inc., Scientific Optical Products Div., Rochester, N. Y.). All fractions were, after isolation, immediately resuspended in distilled H$_2$O and stored at −20°C.

Electron Microscopy

Aliquots of the isolated membrane fractions were centrifuged for 5 min at maximal speed in a Beckman microfuge. The pellets were fixed for 1 h in a mixture of 3% glutaraldehyde, 2% formaldehyde, 1% acrolein, and 2.5% dimethyl sulphoxide (DMSO) in 0.08 M phosphate buffer pH 7.0. The fixed material was rinsed in phosphate buffer (four changes over 1 h) and postfixed for 1 h in 1% OsO$_4$ in 0.08 M phosphate buffer pH 7.0. Fixation and washings were carried out at room temperature. The fixed material was dehydrated in graded acetone and embedded in Epon 812. Thin sections were cut on a Reichert OMU 2 microtome (American Optical Corp., Scientific Instrument Div., Buffalo, N. Y.) and stained for 5 min in a saturated uranyl acetate solution which was followed by a 1 min staining in lead citrate solution (50 mg lead citrate in 25 ml 0.01 N NaOH). Electron micrographs were taken with a Siemens Elmiskop I and a Philips EM 200 (37).

Cytochemical Localisation of Acid Phosphatase Activity

The acid phosphatase activities in the membranes of the amoebae were localized by a modified Gomori staining for liberated phosphate, using either p-nitrophenylphosphate or β-glycerophosphate as substrate. The prefixed cells in glutaraldehyde were incubated at room temperature for 30 min in 50 mM Tris-maleate buffer, pH 4.5 containing 2.3 mM lead nitrate and 8.2 mM p-nitrophenylphosphate or 8.2 mM β-glycerophosphate, respectively. After incubation, the cells were prepared for electron microscopy as described above.

Enzyme Assays

Adenosine triphosphatase (Mg^{2+}) activity was determined according to Fitzpatrick et al. (10). Acid phosphatase (II) activity was measured by determination of the release of p-nitrophenol from p-nitrophenylphosphate. Acid phosphatase (I) was measured as described by Wattiaux and de Duve (46), with β-glycerophosphate as substrate; both phosphatases showed an optimum at pH 5. Glucose-6-phosphatase was assayed by the procedure of Kaulen et al. (18) in the presence of tartrate (10 mM) to inhibit acid phosphatase at pH 5.0. 5'-Nucleotidase activity was measured according to Michell and Hawthorne (26) in the presence of 10 mM tartrate.

Phosphate was determined as described by Widnell and Unkeless (49). β-N-acetylglucosaminidase was assayed by the method of Aronson and de Duve (1). L-leucine-aminopeptidase activity was measured according to Goldbarg and Rutenberg (13). Catalase and peroxidase were measured as described by Lück (25). Cholinephosphotransferase activity was assayed according to Sarzala et al. (34). All enzymatic assays were carried out under optimal conditions.

Lipid Analysis

Membrane fractions were extracted with 20 vol of chloroform-methanol (2:1 by vol). The extract was washed according to Folch et al. (11) and stored at

−20°C. Lipid-phosphorus was determined after perchloric digestion as described by Rouser et al. (33). Cholesterol content was estimated after the procedure of Chiamori and Henry (6). The phospholipid composition was determined according to Rouser et al. (33), after separation of the phospholipids by two-dimensional thin-layer chromatography (TLC) as described by Broekhuyse (5).

RESULTS

Distribution of Marker Enzymes in the Subcellular Fractions

The different subcellular fractions were isolated as outlined in Materials and Methods (Fig. 2). The recovery of protein distributed over the various subcellular fractions is shown in Table I.

The purity of the membrane fractions, especially of the plasma and phagolysosomal membranes, was established by the use of marker enzymes. Table I also shows the distribution of the different enzymes which were tested.

Acid phosphatases are commonly used as marker enzymes for (phago)-lysosomes, using both β-glycerophosphate and p-nitrophenylphosphate as substrate. The activity of acid phosphatase (I), assayed with β-glycerophosphate as substrate, was mainly recovered with the phagolysosomes. A 20-fold increase in specific activity of acid phosphatase (I) in the phagolysosomal membranes compared to the whole homogenate suggested that the enzyme is predominantly bound to the phagolysosomal membrane. It could be concluded, from the recovery of acid phosphatase activity (I) in the other membrane fractions, that maximally 25% of the phagolysosomes would be disrupted by the homogenization procedure.

However, acid phosphatase (II) activity, assayed with p-nitrophenylphosphate as substrate, demonstrated a different subcellular distribution. The enzymatic activity was 10-fold enriched in the phagolysosomal membranes and sixfold in the plasma membranes, with a recovery of activity of 40% and 18%, respectively. These observations were confirmed by cytochemical localization of acid phosphatase activity *in situ* using both substrates. When β-glycerophosphate was taken as substrate deposits were exclusively observed on the phagolysosomal membranes, whereas with the aid of p-nitrophenylphosphate the plasma membranes stained also for acid phosphatase activity (Fig. 3).

In addition to acid phosphatase (I), N-acetylglu-cosaminidase activity was also taken as a marker enzyme for phagolysosomes. The activity was shown to be mainly (50%) associated with the phagolysosomes. A sixfold enrichment of enzymic activity in the phagolysosomes is of the same order of magnitude as found for acid phosphatase (I). It follows from the specific activities in phagolysosomal membrane and phagolysosomal supernate that the enzyme is partly present as a soluble enzyme.

Of three different marker enzyme activities that were looked for in plasma membrane preparations, only Mg^{2+}-ATPase was readily demonstrated. L-leucine aminopeptidase activity and 5'-nucleotidase activity could not be detected (45). 70% Of the Mg^{2+}-ATPase activity was associated with the plasma membrane fraction. The specific activity of the enzyme in purified plasma membranes was 80 times greater than that of the whole homogenate (Table I). Glucose-6-phosphatase is commonly taken as a marker enzyme for microsomal membranes. However, the results obtained with this enzyme revealed that the activity was not restricted to any particular fraction. More reliable markers of microsomes are phospholipid-synthesizing enzymes (43). We selected cholinephosphotransferase as marker enzyme. The results shown in Table I indicate that the activity was indeed mainly associated with the microsomal membrane fraction.

The presence of peroxisomes was tested for, using catalase and peroxidase as marker. Both enzyme activities were found to be nonsedimentable (85% was found in the 100,000 g supernate), and therefore, the occurrence of the compartmentalized catalase and peroxidase, and hence the presence of peroxisomes appears unlikely.

With the aid of the various marker enzymes, the degree of purity of the different membrane fractions could be estimated. Judging by acid phosphatase activity, the plasma membrane fraction is contaminated to about 15% by phagolysosomal membranes. Microsomal impurity as estimated by choline phosphotransferase activity is about 25%. The phagolysosomal membranes are contaminated with microsomal protein to about 7%. Impurity of this fraction caused by plasma membrane protein, based on Mg^{2+}-ATPase activity, is less than 3%. Plasma and phagolysosomal membrane protein contribute about 10% and 7%, respectively, to the microsomal fraction, as estimated by the Mg^{2+}-ATPase and acid phosphatase (I) activities of this fraction.
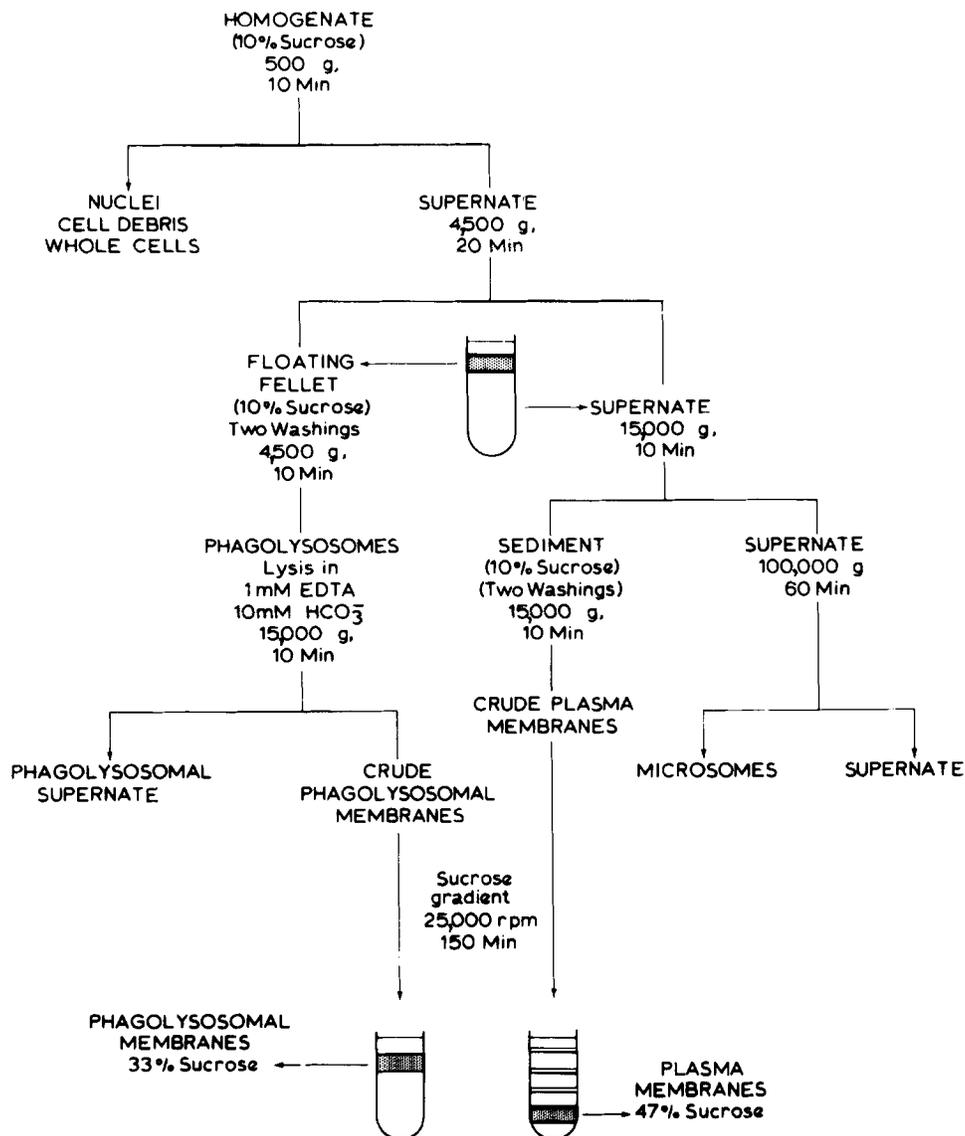
HOMOGENATE
(10% Sucrose)
500 g,
10 Min

NUCLEI
CELL DEBRIS
WHOLE CELLS

SUPERNATE
4500 g,
20 Min

FLOATING
FELLET
(10% Sucrose)
Two Washings
4500 g,
10 Min

SUPERNATE
15,000 g,
10 Min

PHAGOLYSOSOMES
Lysis in
1 mM EDTA
10mM HCO₃
15,000 g,
10 Min

SEDIMENT
(10% Sucrose)
(Two Washings)
15,000 g,
10 Min

SUPERNATE
100,000 g
60 Min

CRUDE PLASMA
MEMBRANES

PHAGOLYSOSOMAL
SUPERNATE

CRUDE
PHAGOLYSOSOMAL
MEMBRANES

MICROSOMES   SUPERNATE

Sucrose
gradient
25,000 rpm
150 Min

PHAGOLYSOSOMAL
MEMBRANES
33% Sucrose

PLASMA
MEMBRANES
47% Sucrose

FIGURE 2   Subcellular fractionation scheme for the isolation of phagolysosomal and plasma membranes.

Morphology of Plasma and Phagolysosomal Membranes

Previous electron microscope studies on intact *E. invadens* had already revealed the absence of mitochondria and the presence of nuclei, ribosomes, and a scarcely developed endoplasmic reticulum and Golgi apparatus (8, 9, 21, 22, 23, 30, 31, 32). The electron micrograph of the plasma membrane fraction isolated in the present study shows the absence of nuclei and ribonucleoprotein and little contamination with glycogen (Fig. 4a).

The presence or absence of smooth endoplasmic reticulum and Golgi apparatus membranes is difficult to establish because these membranes cannot be distinguished from plasma membrane vesicles (cf. Figs. 4a and b). The typical trilaminar organisation of the plasma membrane (Fig. 4a) can be observed. In electron micrographs of the microsomal fraction, very small vesicles probably derived from smooth endoplasmic reticulum and Golgi apparatus could be observed, in addition to glycogen particles (Figs. 4b and 5a).

Electron micrographs of the intact phagolyso-

TABLE I

Assays of Marker Enzymes for Cell Fractions

	Recovery of protein	Acid phosphatase I (β-glycerophosphate)		Acid phosphatase II (p-nitrophenylphosphate)		β-Acetylglucose aminidase		Mg²⁺-ATPase		1.2 Diacylglycerol cholinephosphotransferase	
		sp act	total activity	sp act	total activity	sp act	total activity	sp act	total activity*	sp act‡	total activity
Homogenate	100	0.10 (0.010)	100	0.210(0.015)	100	0.063(0.005)	100	0.001	100	0.19(0.01)	100
Nuclei, whole cells and debris	1.5	0.088(0.038)	0.2	0.340(0.14)	0.2	0.040(0.016)	0.2	0.012(0.007)	9	0.06(0.01)	0.2
Phagolysosomes	7.3	0.66 (0.050)	75.0	0.56 (0.14)	39.9	0.33 (0.06)	48.7	0.006(0.002)	30	0.06(0.01)	2.8
Crude plasma membranes	2.3	0.42 (0.095)	9.9	1.40 (0.14)	18.1	0.089(0.020)	3.2	0.051(0.012)	70	0.52(0.11)	5.9
Microsomes	9.1	0.143(0.053)	6.9	0.54 (0.11)	11.4	0.048(0.013)	3.3	0.008(0.004)	58	1.15(0.13)	44.4
100,000 g supernate	72.8	0.016(0.002)	12.5	0.046(0.002)	15.8	0.022(0.005)	31.6	—	—	—	—
Phagolysosomal membranes	1.0	2.05 (0.042)	—	2.40 (0.22)	—	0.48 (0.17)	—	0.002	—	0.09(0.04)	—
Plasma membranes	6.0	0.30 (0.13)	—	1.20 (0.74)	—	0.078(0.028)	—	0.083(0.021)	—	0.30(0.04)	—
Phagolysosomal supernate	1.2	0.13 (0.031)	—	0.033(0.002)	—	0.26 (0.04)	—	—	—	—	—

Protein recovery data are expressed as percentages of the total amount of protein (24) present in the homogenate; specific activity as μmol/mg protein/min; total activity is calculated as the percentage of the initial activity in the homogenate; and numbers in parentheses represent the SD ($n=3$).

* The apparent recovery in the subcellular fractions of more than 100% is attributed to errors in the estimation of the extreme low activity in the homogenate.

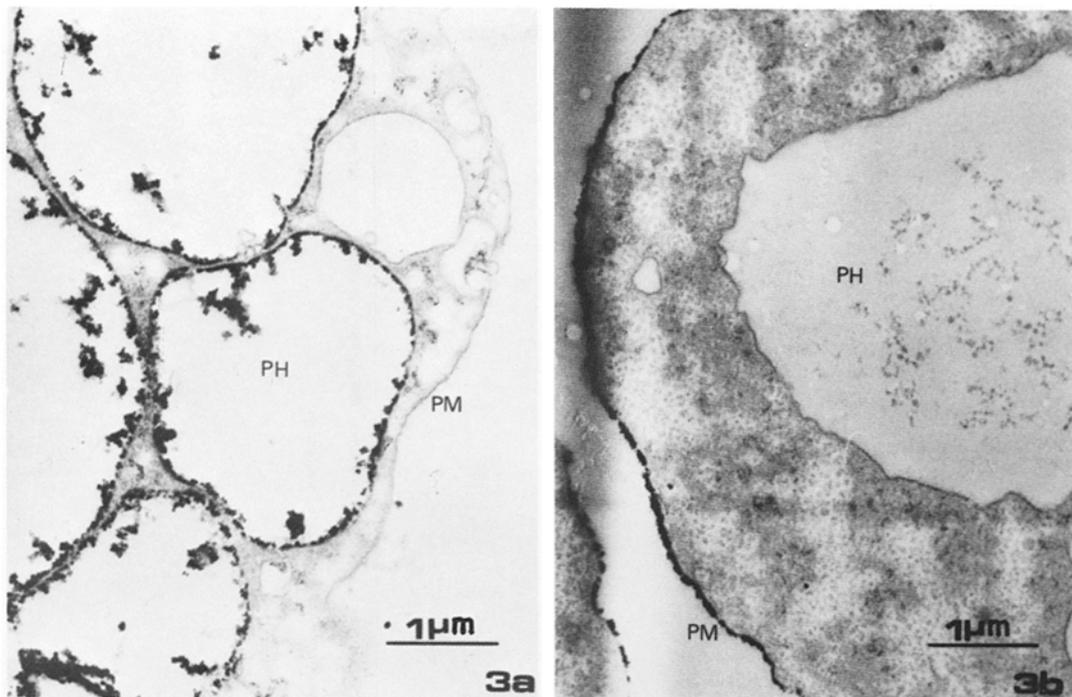‡ Specific activity, nmol/mg protein/min.

FIGURE 3 Histochemical localization of acid phosphatase. Prefixed amoebae were incubated with β-glycerophosphate (a) or p-nitrophenylphosphate (b). The deposits of the reaction products were stained as described in the section Materials and Methods. × 14,930.

somes isolated as a floating pellet showed a heterogeneous population of large vesicles (Fig. 5b–d). Small vesicles inside the phagolysosomes could be observed. The purified phagolysosomal membranes demonstrated a homogeneous picture of vesicles, filled with smaller vesicles or whorls of membranes.

Lipid Analysis of the Membranes

Comparison of the lipid content of the phagolysosomal and plasma membranes showed that the former contained more cholesterol and lipid phosphorus per milligram protein (Table II). In contrast, microsomal fraction contained only one-third of the amount of cholesterol per milligram protein found in the phagolysosomal and plasma membrane. The phospholipid content of the microsomal fraction, however, was about the same as that of the phagolysosomal and plasma membranes. The characteristic ratio of cholesterol to phospholipid for plasma membranes was 1.0. In phagolysosomal membranes this ratio was always somewhat higher than in plasma membranes,

whereas this ratio was 0.39 for the microsomal fraction.

Between the phagolysosomal and plasma membranes, only small differences in cholesterol and phospholipid content were observed; however, more pronounced differences were noticed in the phospholipid composition of these membranes (Table III). The phagolysosomal membranes contained about 1.5 times more sphingolipid than the plasma membranes. This enhancement was accounted for by a rise in the content of both sphingomyelin and ceramide phosphorylinositol.

With respect to the glycerophospholipids, the phosphatidylcholine and phosphatidylethanolamine contents of the membrane fractions were different. The phosphatidylcholine content was lower in the phagolysosomal membranes compared to the plasma membranes, and the reverse was found for phosphatidylethanolamine. Furthermore, the phosphatidylserine content of plasma membranes was twice that of the phagolysosomal membranes. Little lysophosphatidylcholine was present in all membrane fractions including the phagolysosomal membranes.
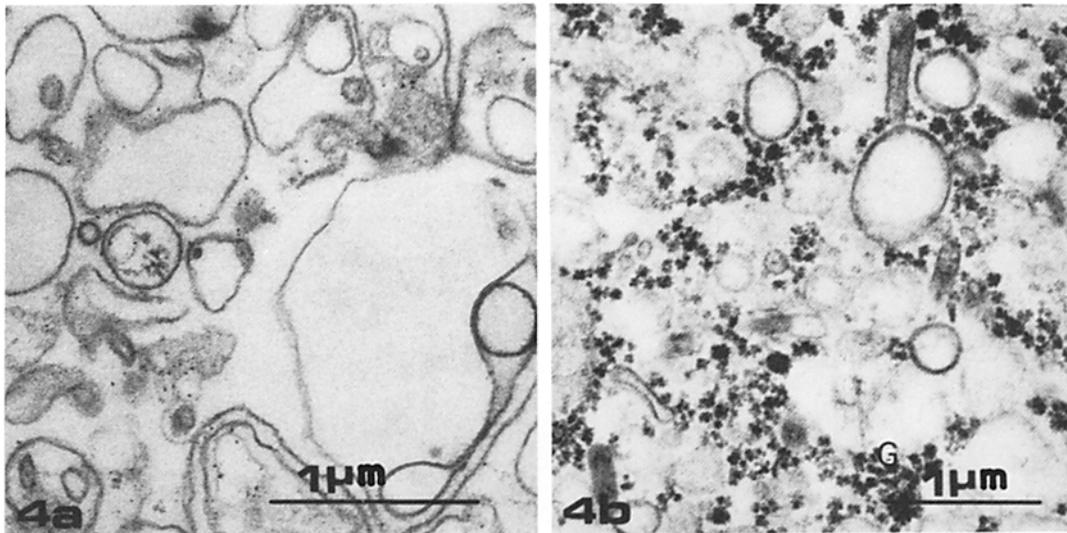
FIGURE 4   (a) Plasma membrane fraction collected at 45% and 47% sucrose. × 30,000. (b) Microsomal fraction: glycogen (G). × 21,390.

DISCUSSION

A procedure is described for the isolation of plasma and phagolysosomal membranes of *E. invadens*. Probably as the result of the uptake of lipid from the culture medium, the intact phagolysosomes could be collected by flotation. Comparable techniques, involving phagocytosis of latex beads (48) or parrafin oil droplets (38, 39), have been used for the isolation of phagolysosomes of both amoebae and mammalian cells.

The phagolysosomal and plasma membranes demonstrated different sedimentation properties in a continuous sucrose gradient, as previously reported for *A. castellanii* (41, 48). The fractions were characterized by the use of marker enzymes. Typical lysosomal enzymes, such as acid phosphatase (I) and *N*-acetylglucosaminidase, were associated with the phagolysosomes. Acid phosphatase (I) activity in contrast to the soluble *N*-acetylglucosaminidase activity was demonstrated to be bound to the phagolysosomal membrane, in agreement with histochemical observations on *Entamoeba histolytica* (9, 32). The small amounts of acid phosphatase (I) activity found in the other cell fractions indicated that only few phagolysosomes are disrupted during isolation.

A second acid phosphatase (II) activity could be identified by the use of *p*-nitrophenylphosphate as substrate; β-glycerophosphate is not a substrate for this enzyme. The specific activity of this en-

zyme was increased in both phagolysosomal and plasma membranes in contrast to the predilection of the acid phosphatases I for the phagolysosomes. Because acid phosphatase (I) hydrolyzes β-glycerophosphate as well as *p*-nitrophenylphosphate, acid phosphatase (II) hydrolyzing *p*-nitrophenylphosphate appears more specifically associated with the plasma membrane. These results were confirmed histochemically. Acid phosphatase (II) activities have also been observed in plasma membranes of different origin (2, 3, 12, 18, 19, 42). An alkaline nitrophenylphosphatase activity associated with plasma and phagolysosomal membranes as described for *A. castellanii* (41), was absent in *E. invadens*.

In agreement with observations on plasma membrane preparations of *A. castellanii* (35, 41), rat liver (45), thymocytes (42), platelets (3), and rat myometrium (19), our results show that Mg^{2+}-ATPase can also serve as a typical marker enzyme for plasma membrane of *E. invadens*.

Peroxisomes, such as those demonstrated in *T. pyriformis* (29), were not present, because both marker enzymes catalase and peroxidase were recovered in the 100,000 g supernate. Identical observations have been reported for *Tritrichomonas foetus* (27). Glucose-6-phosphatase, a marker enzyme for microsomal protein in other tissues, was found not to be associated with any particular membrane fraction in *E. invadens*. This is also the case for *A. castellanii* (41) and *D. discoideum*

(12). As demonstrated for rat liver (43), phospholipid-synthesizing enzymes are bound to microsomal protein and can be taken as marker enzyme for this fraction. Despite low recovery, it could be established that choline phosphotransferase activity was mainly associated with microsomal protein.

Using these results, we could estimate the relative purity of the plasma and phagolysosomal membranes. The plasma membranes seem practically free of phagolysosomal membrane protein, and the main impurity stems from contamination with microsomes. The phagolysosomal membranes were contaminated to a small extent with
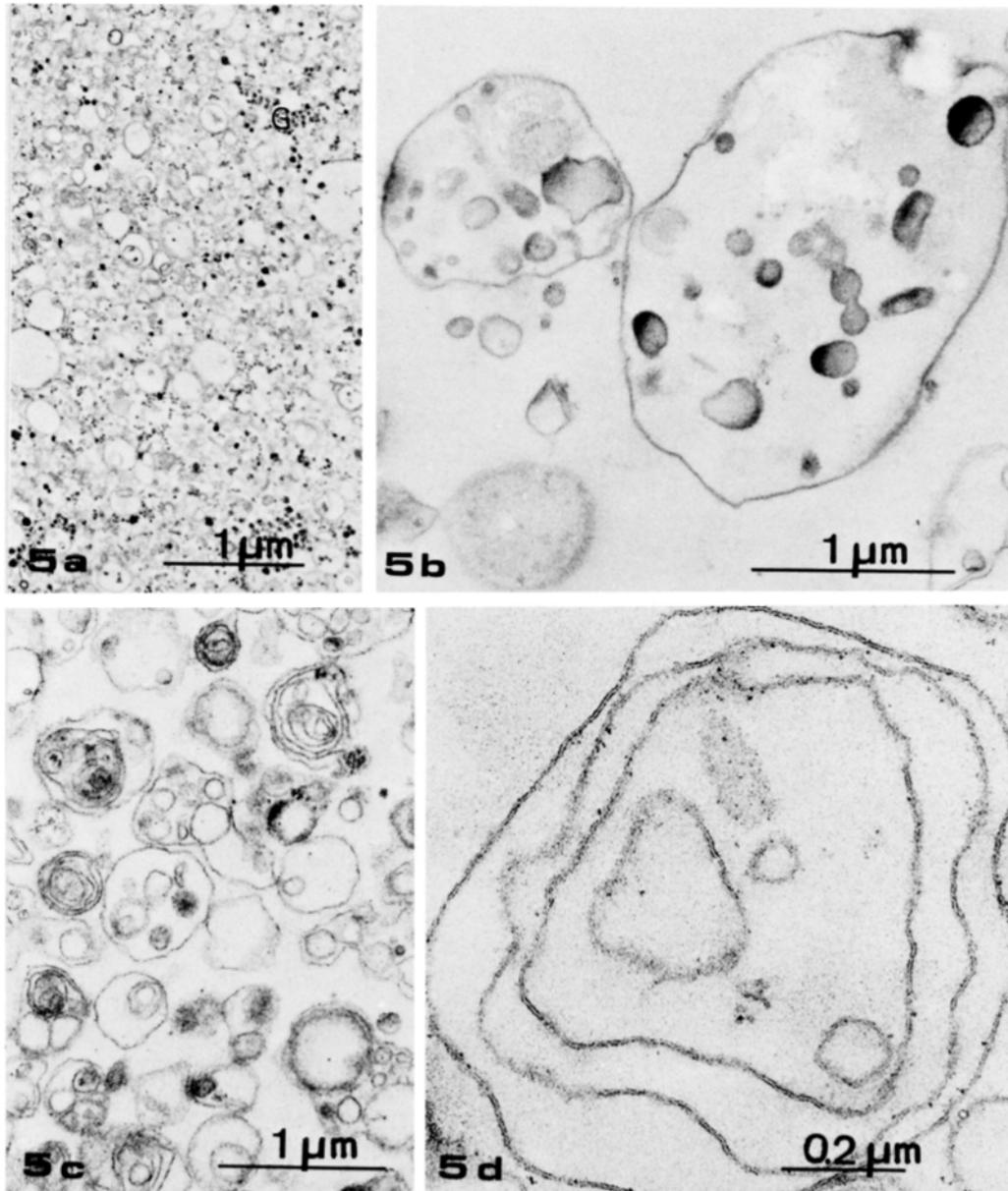

FIGURE 5   (a) Microsomal fraction. × 18,500. (b) Intact phagolysosomes isolated as a "floating pellet." × 27,000. (c) Phagolysosomal membranes collected at 31% and 36% sucrose. × 22,500. (d) Phagolysosomal membranes at higher magnification. × 88,000.

TABLE II

Lipid Composition of the Membrane Fractions

Membrane fractions	Lipid composition		
	μmol cholesterol/mg protein	μmol phospholipid/mg protein	μmol cholesterol/μmol phospholipid
Phagolysosomes	0.46(±0.01)	0.44(±0.03)	1.06(±0.04)
Crude plasma membranes	0.84(±0.35)	0.89(±0.07)	0.92(±0.22)
Microsomes	0.36(±0.04)	0.91(±0.06)	0.39(±0.03)
Phagolysosomal membranes	1.28(±0.25)	1.22(±0.19)	1.05(±0.05)
Plasma membranes	0.90(±0.07)	0.98(±0.05)	0.93(±0.03)

Numbers in parentheses represent the SD (*n*=3).

TABLE III

Phospholipid Composition of Membrane Fractions of E. Invadens

	Whole cells	Plasma membranes	Phagolysosomal membranes	Microsomes
Phosphatidylethanolamine	25.4(1.9)	30.1(0.8)	37.2(3.3)	22.8(1.0)
Phosphatidylcholine	33.7(1.4)	38.3(2.6)	13.9(2.2)	54.4(1.1)
Sphingomyelin	4.0(0.2)	9.3(1.2)	13.7(0.1)	7.1(1.0)
Phosphatidylserine	7.1(1.3)	8.4(0.5)	17.8(2.5)	4.3(0.3)
Ceramidephosphorylinositol	17.3(1.2)	7.6(1.2)	13.9(1.5)	5.0(0.7)
Phosphatidylinositol	3.3(0.5)	3.7(0.2)	1.7(0.5)	4.9(0.3)
Phosphatidic acid	3.6(0.5)	1.4(0.1)	0.7(0.4)	0.8(0.2)
Origin	5.1(1.0)	1.1(0.1)	0.9(0.3)	0.8(0.2)
Total sphingolipid	21.3	16.9	27.6	12.1

Phospholipid composition is expressed in percentage of total lipid-P. Numbers in parentheses represent the SD (*n*=3).

other membranes. It has to be emphasized that this manner of estimating the purity of the different membrane fractions gives maximal values for possible contaminations. The technique of marker enzyme analysis does not rule out the possibility that the "contaminating" activity is part of the content of the fraction. It will be obvious, furthermore, that the isolated membrane fractions denoted as "lysosomal membranes" and "microsomal membranes" are obtained after a number of manipulations which might alter the actual membranes of the subcellular components. Especially the phagolysosomal fraction might be contaminated, by phagocytosis or autophagy, with fractions of other subcellular membranes. Establishment of the purity by electron microscope survey was difficult since the microsomal pellet consisted mainly of smooth-membrane vesicles; however, the micrographs showed rather homogeneous fractions of both plasma and phagolysosomal membranes.

Lipid analysis revealed distinct differences in composition between the different membrane fractions. The measured cholesterol to phospho-

lipid ratio of about 1.0 for the plasma membrane (20) is in good agreement with the value normally found for plasma membranes of *A. castellanii* (41), rat liver (14, 16), and macrophages (47).

Also, with respect to lipid analysis, the data have to be interpreted carefully. The membranes are isolated as vesicles, but the extent of possible inclusions in these vesicles is hard to assess.

With respect to the phospholipid composition, large differences were noticed between plasma, microsomal, and phagolysosomal membranes. Also, in rat liver, these membranes showed distinct differences (14, 18), in contrast to *A. castellanii* in which both plasma and phagolysosomal membranes had about the same phospholipid composition. As reported for *A. castellanii*, the phagolysosomes of *E. invadens* contained a relatively high proportion of phosphatidylserine. Intriguing were the small content of phosphatidylcholine and the elevated sphingo-lipid content in the phagolysosomal membranes of *E. invadens* when compared to plasma membranes. Similar findings have been described for tritosomes of rat liver (14, 18).

During phagocytosis, particles are surrounded by plasma membrane, occluded in a phagosome and transformed into a digestive phagolysosome (17). Because of this interrelationship, the finding of large differences in phospholipid composition of plasma and phagolysosomal membranes was surprising. A possible explanation for this difference in phospholipid composition is that phagocytosis proceeds at specific areas on the plasma membrane, relatively rich in sphingolipid and phosphatidylserine. These areas would be marked by surface-active lysosomes, as observed in *E. histolytica* (31). The results of Tsan and Berlin (40), demonstrating that phagocytosis does not proceed randomly at the plasma membrane, actually supports this notion. Alternatively, the high content of sphingolipid, cholesterol, and phosphatidylserine can be caused by a secondary effect. Phagolysosomes contain different lipolytic enzymes (manuscript in preparation) which might be able to break down the phosphoglycerides in the inner leaflet but not in the outer leaflet of the newly engulfed plasma membrane (phagolysosome). As demonstrated in erythrocyte membrane (4, 15, 50), the phospholipids are distributed asymmetrically over the plasma membrane; this might also be the case in *E. invadens*. Phosphatidylcholine and sphingolipid might be situated in the outer leaflet and almost all of phosphatidylethanolamine and phosphatidylserine in the inner leaflet of the lipid bilayer of the plasma membrane. If, during phagocytosis, the outer leaflet of the plasma membrane becomes the inner leaflet of the phagolysosome, consequently phosphatidylcholine becomes available for degradation by phospholipases, whereas phosphatidylethanolamine and phosphatidylserine would not be susceptible. The percent distribution of these phospholipids, together with the sphingolipids, which will not be degraded by phospholipases, might increase. However, further investigation will be necessary to explain the actual features responsible for the differences in phospholipid composition of the plasma and phagolysosomal membranes.

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