

PLASMA MEMBRANE OF *ENTAMOEBEA HISTOLYTICA**

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Entamoeba histolytica is a common human pathogen that produces extensive cellular destruction in both the intestine and parenchymatous organs (1). It is a relatively simple anaerobic protozoan that lacks mitochondria, contains little endoplasmic reticulum or Golgi apparatus, and has large numbers of ill-defined vesicles filling the cytoplasm (2, 3). A significant portion of the amoebal membrane is localized at the cell surface in the form of an active plasmallemma that is involved in endocytosis and translational motility. Prior studies have suggested that the surface of *E. histolytica* also plays an important role in the contact-dependent lysis of mammalian cells (4, 5). It is therefore surprising that so little information exists concerning the chemical and antigenic composition of the surface membrane. This is, in part, the result of the absence of well-defined subcellular markers and the difficulties in separating the plasma membrane from other cytoplasmic components.

In this article we describe the isolation and selected properties of a highly enriched plasma membrane preparation obtained from axenically propagated *E. histolytica* (HK9:NIH strain). This was accomplished by selectively labeling the externally disposed polypeptides of the plasma membrane with ¹²⁵I by the lactoperoxidase-glucose oxidase technique and isolating concanavalin A-stabilized plasma membrane fragments.

Materials and Methods

Amoeba Cultivation. The *E. histolytica* strain HK9:NIH was obtained from Dr. L. S. Diamond (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md) and cultured axenically in Diamond's TYI-S-33 medium (6) in 20-cm screw-cap tubes or in 200-ml T-flasks. Cells were grown at 37°C to late log phase and harvested by chilling on ice for 5 min and centrifuging at 200 g for 5 min. The yield per flask ranged from 1×10^7 to 2×10^7 amoeba. Cells were washed twice in 19 mM potassium phosphate buffer, pH 7.2, that contained 0.27 M NaCl (PD).¹

Cell Surface Iodination. Amoebal surface proteins were labeled with ¹²⁵I by a modification of the lactoperoxidase-glucose oxidase method of Hubbard and Cohn (7). Briefly, amoeba were harvested, washed twice in PD, and resuspended in 2 ml ice-cold PD that contained 40 mM glucose and 2.5 μM NaI. 1 mCi of carrier-free Na[¹²⁵I] (Amersham Corp., Arlington Heights, Ill) was added to this mixture. Lactoperoxidase (300 o-dianisidine units) (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif) and glucose oxidase (50 o-dianisidine units)

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¹ Abbreviations used in this paper: CAEP, ceramide aminoethyl phosphonate, NAGase, N-acetyl-β-glucosaminidase, PA, phosphatidic acid, PC, phosphatidylcholine, PD, 19 mM potassium phosphate buffer, pH 7.2, that contained 0.27 M NaCl, PE₁, PE₂, two phosphatidylethanolamine species, PI, phosphatidylinositol, PS, phosphatidylserine, SDS, sodium dodecyl sulfate, SDS-PAGE, SDS-polyacrylamide gel electrophoresis, tris buffer, 10 mM tris-HCl buffer, pH 7.5, that contained 2 mM phenylmethyl sulfonyl fluoride.

(Sigma Chemical Co., St. Louis, Mo.) were added to initiate the labeling reaction. Cells were agitated every 2 min. After 15 min, cells were washed with PD, spun through 5 ml of bovine serum, and washed twice with PD, all by centrifugation at 200 *g* for 5 min. Samples of cells from controls (incubated without enzymes) and from the full incubation mixture were saved for determination of cell viability and ^{125}I incorporation. Cell viability, as monitored by trypan blue exclusion, remained >95%. The amount of ^{125}I incorporated into protein was determined by trichloroacetic acid (10%) precipitation. The incorporation of ^{125}I into protein of controls was <1% of the enzyme-mediated incorporation.

Lectin Chromatography A total of 4×10^6 iodinated amoeba were solubilized in PD that contained 1 mM Mg^{++} , 2 mM phenylmethyl sulfonyl fluoride, and 0.5% Zwittergent 3-12 (*N*-dodecyl-*N,N*-dimethyl-3-ammonio-1-propanesulfonate) (Calbiochem-Behring Corp., American Hoechst Corp.). A column of 0.4 ml concanavalin A, immobilized on agarose beads (Sigma Chemical Co.), was washed extensively with PD that contained 1 mM Mg^{++} and 50 mM α -methyl mannoside followed by the same buffer without α -methyl mannoside. The cell lysate was run into the column and flow was stopped for 10 min. The column was then washed with PD that contained Mg^{++} and 0.1% Zwittergent 3-12 until no further radioactivity was detected in the effluent. 50 mM α -methyl mannoside was then added to the buffer and the specifically eluted radiolabel was collected, concentrated by trichloroacetic acid precipitation, and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE).

Isolation of Plasma Membrane. (Fig. 1) 10 flasks of amoebae (10^8 cells) were harvested, washed twice with PD, and pooled with ^{125}I -labeled cells. All subsequent steps were performed on ice or in refrigerated centrifuges. The cell pellet was resuspended to 2×10^7 cells/ml in PD that contained 10 mM MgCl_2 and rapidly mixed with an equal volume of 1 mg/ml concanavalin A in the same buffer. Cell aggregation was apparent within 1 min. After 5 min, cells were gently spun at 50 *g* for 1 min to remove excess concanavalin A. The supernate was discarded, and the cell pellet resuspended in 12 ml of 10 mM tris-HCl buffer, pH 7.5, that contained 2 mM phenylmethyl sulfonyl fluoride (tris buffer) and MgCl_2 added to 1 mM. After swelling for 10 min in the hypotonic buffer, cells were homogenized by 18–20 strokes of a glass Dounce homogenizer with a tight-fitting pestle (Wheaton Scientific Div., Wheaton Industries, Millville, N. J.). Cell lysis and the formation of membrane sheets was verified by phase-contrast microscopy. The homogenate was layered over a two-step gradient consisting of 8 ml of 0.5 M mannitol over 4 ml of 0.58 M sucrose, both in tris buffer, and spun at 250 *g* for 30 min. For analysis, material remaining at the top of the 0.5 M mannitol (supernate I) was centrifuged at 40,000 *g* for 1 h to separate soluble molecules from small membrane fragments and vesicles (supernate II and pellet II). Large plasma membrane fragments and other heavy debris formed a tight pellet at the bottom of the gradient (pellet I). This pellet was resuspended in 1 ml tris buffer that contained 1 M α -methyl mannoside and left on ice for 40 min with occasional mixing. The plasma membranes, now free of the bulk of the concanavalin A, were diluted into three volumes of tris buffer and homogenized by 80 strokes with a glass Dounce homogenizer. This second homogenate was layered on a single-step gradient that consisted of 20% sucrose in

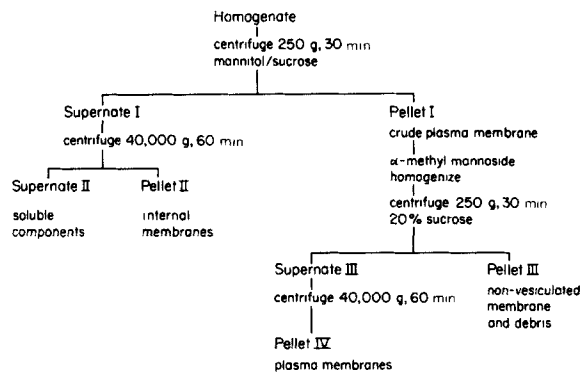


FIG. 1. Isolation of *E. histolytica* plasma membranes

tris buffer and spun for 30 min at 250 g. Vesiculated plasma membranes floating above the initial sucrose layer (supernate III) were collected and were concentrated by centrifugation at 40,000 g for 1 h. The pellet (pellet IV), containing the enriched plasma membranes, was resuspended directly in tris buffer. All samples were either assayed immediately or frozen at -20°C for future use.

Enzyme Assays. Alcohol dehydrogenase activity of *E. histolytica* was assayed as described by Reeves et al. (8). The Ca^{++} -dependent (Mg^{++} -inhibited) ATPase activity was measured as described by McLaughlin and Müller (9). Acid phosphatase activity was assayed using either *p*-nitrophenyl phosphate (10) or β -glycerol phosphate (11) as the substrate. *N*-Acetyl- β -glucosaminidase was assayed using 4-methyl-umbelliferyl-2-acetamido-2-deoxy- β -D-glucopyranoside (Koch-Light Laboratories Ltd., Colindale, Buckinghamshire, England) as substrate (12). Protein was measured by the Coomassie dye-binding assay (13) and compared with a standard curve constructed with bovine serum albumin. Nucleic acid was estimated from OD 280:260 ratios (14). Protein-associated ^{125}I was determined by trichloroacetic acid (10%)-precipitable radiolabel.

Electrophoresis. Proteins were separated by electrophoresis in 5–15% gradient polyacrylamide gels in SDS, as described by Maizel (15). Slab gels were run at constant current with an initial voltage of 40 V. Electrophoresis was continued until the bromphenol blue marker dye was ~1 cm from the lower edge of the gel. Gels were fixed in 30% methanol and 7.5% acetic acid that contained 0.01% Coomassie R-250. Gels were washed with fixing solution until the background staining had cleared, then dehydrated. Iodinated proteins were visualized by autoradiography on Kodak X-OMAT XR-1 film (Eastman Kodak Co., Rochester, N. Y.).

Lipid Analysis. Amoebal lipids were extracted and washed by the method of Folch et al. (16). Phospholipids were separated by two-dimensional thin-layer chromatography on Redi-coat-2D silica gel plates (Supelco, Inc., Bellefonte, Pa.) (17). Separated phospholipids were visualized by exposure to iodine vapors. Dragendorff and ninhydrin reagents were used to detect choline- and amine-containing compounds, respectively (18).

The ashing procedure described by Ames and Dubin (19) was used to estimate total phosphate content. Inorganic phosphate was determined as described by Chen et al. (20). Lipid phosphones were characterized by their resistance to digestion by 70% perchloric acid at 80°C for 36 h, and ceramide lipids by their resistance to digestion by 0.03 N NaOH (21).

For determinations of cholesterol, 5α -cholestane was added to lipid extracts as an internal standard. Samples were saponified in 10% ethanolic-KOH:water (9:1, vol/vol) at 56°C for 30 min and analyzed on a gas-liquid chromatograph equipped with a flame ionization detector. Separations were carried out isothermally at 250°C on 0.25-in \times 6-ft glass columns packed with 3% OV-17 on Chromosorb W HP (Supelco, Inc.) with N_2 as the carrier gas. The cholesterol content of amoebal extracts was corrected for recovery of the internal standard.

Total cell fatty acids were converted to their respective methyl esters by transesterification in 6% methanolic-HCl at 80°C for 16 h. Fatty acid methyl esters were analyzed by gas-liquid chromatography on 0.125-in \times 10-ft stainless steel columns that contained 10% SP-2330 on 100/120 Chromosorb W AW (Supelco, Inc.) at 185°C .

Electron Microscopy. Cells and membrane fractions were fixed with 2.5% glutaraldehyde for 15 min on ice. They were then pelleted in a Beckman microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) and stained with 1% osmium tetroxide for 60 min and with 0.25% uranyl acetate for 30 min, both on ice. Samples were solidified in 1% agarose and dehydrated through a graded series of alcohols. Dehydrated samples were embedded in epon, thin-sectioned, counterstained with lead citrate, and viewed in a Siemens Elmiskop electron microscope (Siemens Corp., Medical/Industrial Groups, Iselin, N. J.) (22).

Results

Surface-labeled Peptides. Lactoperoxidase-glucose oxidase-mediated iodination, when performed at low temperatures, has been shown to selectively label the externally disposed peptides of a variety of cell types (7, 23). When intact trophozoites of *E. histolytica* were labeled in this manner and solubilized in detergent, ~12 major radiolabeled peptides were resolved by SDS-PAGE (Fig. 2A and B). These proteins

ranged from $\sim 12 \times 10^3$ to 200×10^3 mol wt and presented a pattern distinct from that of Coomassie-stained protein of total cell lysates (compare Fig. 2 A and B). When ^{125}I -labeled intact amoebae were lysed and the homogenate spun at $40,000 g$ for 1 h, $>80\%$ of trichloroacetic acid (TCA)-precipitable radioactivity was recovered in the membrane pellet, suggesting that only membrane-associated proteins were iodinated.

To determine which of the peptides were glycoproteins, iodinated intact cells were solubilized and passed through a column of immobilized concanavalin A. The glycoproteins, specifically eluted with α -methyl mannoside, were separated by SDS-PAGE (Fig. 2C). Each of the high molecular weight iodinated peptides was present in this glycoprotein fraction.

These surface-labeled peptides were used as a marker for the cell surface membrane throughout the plasma membrane-isolation procedure.

Plasma Membrane Isolation. Photomicrographs of intact *E. histolytica* demonstrate

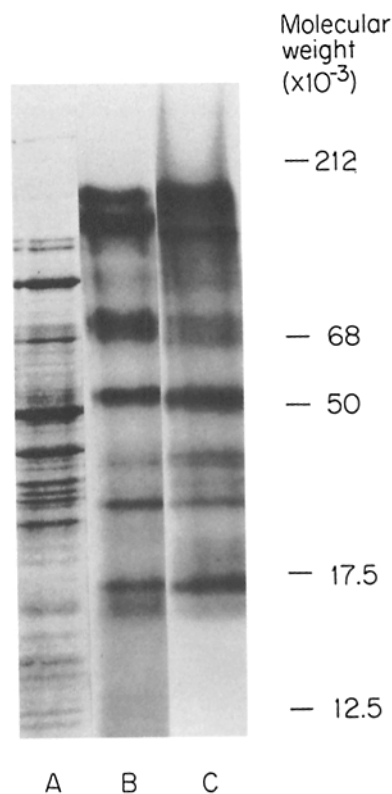


FIG. 2. SDS-PAGE of *E. histolytica* lysates. (A) Total peptide composition of amoebae solubilized in SDS, visualized by Coomassie blue staining. (B) Iodinated surface proteins. Intact amoebae were iodinated by the glucose oxidase-lactoperoxidase procedure and solubilized in SDS. The radiolabeled peptides were then separated by SDS-PAGE and visualized by autoradiography. (C) Iodinated surface glycoproteins. Iodinated intact amoebae were solubilized in Zwittergent 3-12, and the glycoproteins were isolated by affinity chromatography on columns of immobilized concanavalin A. The radiolabeled glycoproteins, specifically eluted with α -methyl mannoside, were visualized by autoradiography after SDS-PAGE. Molecular weight standards included myosin (212,000), bovine serum albumin (68,000), immunoglobulin G (50,000), concanavalin A (17,500), and cytochrome *c* (12,500).

the simple structure of the organism (Fig. 3). The bulk of the internal membrane consists of small vesicles of unknown origin or function. When amoebae were homogenized under hypotonic conditions, the plasma membrane fragmented, and a distinct plasma membrane fraction could not be separated by techniques of differential or isopycnic centrifugation. To overcome this problem, the plasma membrane was stabilized by cross-linking the surface glyco-conjugates of intact cells with concanavalin A. When concanavalin A-treated cells were similarly homogenized, a substantial number of large membrane structures, presumably plasma membrane, were evident in cell lysates (Fig. 4A).

¹²⁵I-labeled amoeba were treated with concanavalin A, homogenized, and spun gently through the mannitol/sucrose gradient. Large membrane scrolls were concentrated in the pellet fraction (Fig. 4C), whereas smaller membrane vesicles were retained above the mannitol phase of the gradient (Fig. 4B). As shown in Table I, the bulk of the radiolabel was recovered in pellet I. Although 22% remained in the supernate (supernate II), this soluble isotope was largely in the form of free iodide. Only 10% of the radiolabel was associated with the membranes of pellet II. These observations suggested that pellet I consisted of crude plasma membrane, whereas the membrane components of supernate I and pellet II consisted largely of internal (i.e., inaccessible to enzymatic label) vesicles.

The crude plasma membrane fraction was treated with α -methyl mannoside to release bound concanavalin A and vesiculated in a second homogenization step. This homogenate was gently spun onto a 20% sucrose cushion. The pellet (pellet III)

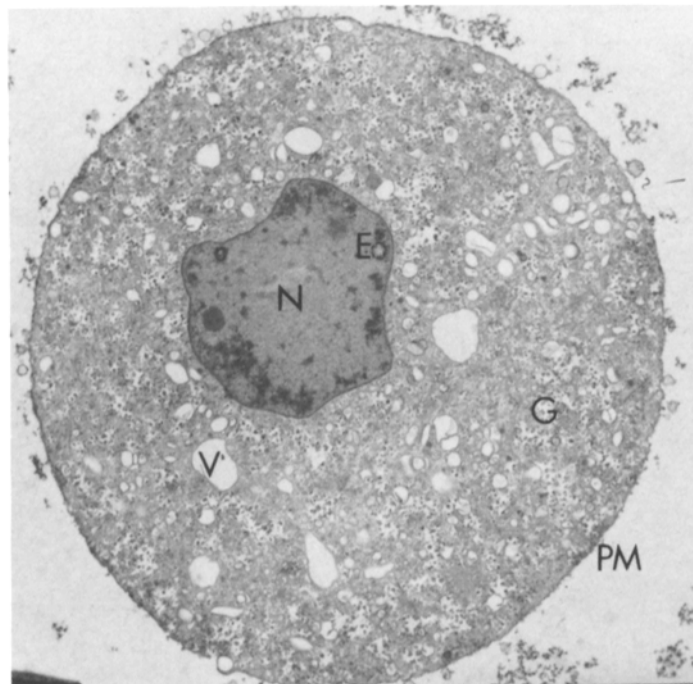


FIG 3 Electron micrograph of *E. histolytica*. PM, plasma membrane, V, endoplasmic vacuole, N, nucleus, E, nuclear endosome, G, glycogen granules $\times 640$

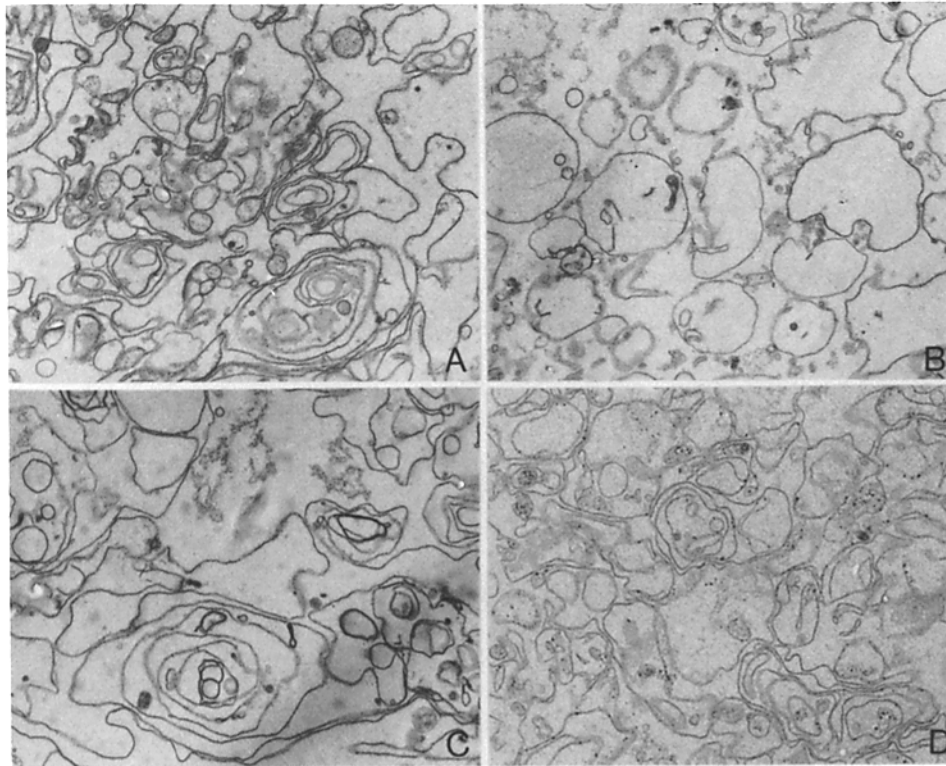


FIG 4 Morphology of subcellular fractions of concanavalin A-treated amoebae as determined by electron microscopy ($\times 4,500$). (A) Cell homogenates. Scrolls and large membrane sheets are visible. (B) Internal membrane vesicles of supernate I (Fig. 1). Few scrolls and membrane sheets are seen. This morphology is also typical of cell homogenates prepared from amoebae not treated with concanavalin A. (C) Crude plasma membranes of pellet I (Fig. 1). Large membrane structures are concentrated in this fraction. (D) Plasma membranes of pellet IV after removal of concanavalin A. Density changes after removal of the lectin are accompanied by fragmentation and some vesiculation of the membranes.

consisted of nonvesiculated plasma membrane and aggregated material. Much of the ^{125}I that was associated with this material was found to pellet through 60% sucrose, away from any apparent membrane structures, and was probably associated with extracellular debris from the original cultures. The plasma membranes retained above the sucrose (supernate III) were shown by electron microscopy to consist of smaller fragments and vesicles (Fig. 4C). This fraction was concentrated by centrifugation at 40,000 g for 1 h (pellet IV, Fig. 4C). Less than 1% of the total protein and $\sim 28\%$ of the total radiolabel were associated with this fraction, for a final enrichment of >30 -fold (Table I). Overall recovery of the ^{125}I marker was 84%.

Distribution of Iodinated Peptides. The polypeptide composition of the plasma membrane was investigated by SDS-PAGE (Fig. 5). Each of the major iodinated peptides of intact cells was present in the final plasma membrane preparation, indicating that these peptides were associated with the membrane and that a representative portion of the plasma membrane had been isolated (compare Fig. 5 with Fig. 2B). The extra radiolabeled peptide at 43,000 mol wt in Fig. 5 was found in variable amounts in

TABLE I
Summary of Plasma Membrane Isolation*

| Fraction | Protein recovery | ¹²⁵ I | | ATPase | | Acid phosphatase | |
|--|------------------|------------------|--------|------------|--------|------------------|--------|
| | | Recovery | sp act | Recovery | sp act | Recovery | sp act |
| | % | % | | % | | % | |
| Homogenate | 100 | 100 | 1 | 100 | 1 | 100 | 1 |
| Soluble proteins (supernate II) | 87.5 ± 1.32 | 22.2 ± 8.1 | 0.3 | 3.8 ± 2.1 | 0.1 | 7.0 ± 1.3 | 0.1 |
| Internal vesicles (pellet II) | 10.2 ± 1.3 | 10.5 ± 1.7 | 1.0 | 59.6 ± 4.1 | 5.8 | 70.0 ± 7.8 | 5.7 |
| Crude plasma membranes (pellet I) | 4.1 ± 0.4 | 67.3 ± 9.6 | 16.4 | 36.7 ± 3.9 | 9.0 | 15.9 ± 7.6 | 3.7 |
| Nonvesiculated membranes and debris (pellet III) | 0.8 ± 0.8 | 23.3 ± 8.0 | 28.4 | 6.2 ± 3.9 | 4.6 | 8.7 ± 5.6 | 11.0 |
| Plasma membranes (pellet IV) | 0.9 ± 0.1 | 27.7 ± 6.8 | 31.8 | 19.4 ± 7.4 | 22.3 | 5.1 ± 5.6 | 2.85 |

* Values represent means ± SE of three experiments. Recoveries were calculated assuming a value of 100% for cell homogenates. Overall recoveries were 97.6, 83.7, 89.0, and 93.8 for protein, ¹²⁵I, ATPase, and acid phosphatase, respectively. Specific activities of cell homogenates were arbitrarily set equal to 1.0 and were calculated as units of activity or counts per minute ¹²⁵I/mg of protein.

different preparations and may be associated with cell debris found in the growth cultures.

The majority of proteins in the cell homogenate (Fig. 2A) were not membrane-associated. The subset of membrane proteins enriched in the plasma membrane preparation presented an SDS-PAGE profile that was distinct from the homogenate and that closely resembled the pattern of surface-labeled peptides (Fig. 5). Each of the major surface-labeled peptides was present in our plasma membrane preparation. The existence of six additional peptides not labeled by surface iodination was not unexpected because this procedure labels only externally disposed tyrosyl residues.

Distribution of Markers. Protein, nucleic acid, and four enzymatic activities were monitored throughout the plasma membrane-fractionation procedure. Alcohol dehydrogenase, a soluble enzyme (8), was found solely in supernate II. No activity was found in any membrane-containing fraction. Likewise, nearly all of the nucleic acid was recovered in the soluble fraction, and no intact nuclei were seen or nucleic acids were detected in the plasma membrane fractions.

In mammalian cells and cell lines, acid phosphatase is a soluble enzyme sequestered within lysosomes. In *E. histolytica*, however, this activity was strongly membrane associated. Greater than 85% of the activity was pelleted by centrifugation of frozen and thawed cells at 40,000 *g* for 1 h. When cell fractions were assayed for acid phosphatase (Table I), 70% of the activity was found associated with the internal membrane vesicles. The final plasma membrane fraction contained only 5% of the total activity yielding a threefold enrichment. We compared the pH optimum and the substrate specificity of the acid phosphatase activities of the internal vesicles and of the purified plasma membrane. The activity from both fractions had an optimal pH of 5.0 and used either *p*-nitrophenyl phosphate or β -glycerol phosphate as substrate equally well. We also assayed subcellular fractions for a second lysosomal hydrolase, *N*-acetyl- β -glucosaminidase (NAGase), which is a soluble intralysosomal enzyme both in mammalian cells and in *E. histolytica*. NAGase levels, corrected for

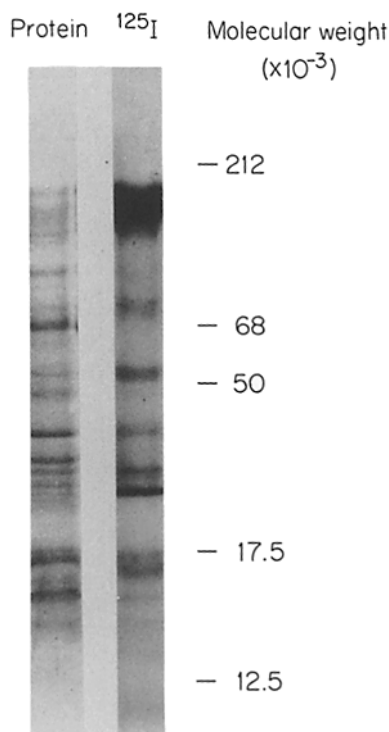


FIG. 5 Peptides of *E. histolytica* plasma membranes. Membranes of pellet IV (Fig. 1) were solubilized in SDS and subjected to SDS-PAGE. ^{125}I Autoradiogram of the plasma membrane radiolabeled peptides prepared from iodinated intact amoebae. Each of the major surface-labeled peptides is isolated in this fraction (compare with Fig. 2B). Protein: Total peptides of the plasma membrane visualized by Coomassie blue staining. Note that the position of each radiolabeled band corresponds to a Coomassie blue-stained peptide. Molecular weight standards are the same as those in Fig. 2.

latency, indicated that the final plasma membrane fraction was contaminated with 6–12% of total lysosomes.

A calcium-dependent ATPase recently described by McLaughlin and Müller (9) was included in this analysis (Table I) as it is one of the few known membrane-associated enzymatic activities in *E. histolytica* that does not have an acid pH optimum. The internal membrane fraction (pellet II) contains 60% of the total ATPase activity, comparable to the percentage for acid phosphatase. However, >19% of the activity was localized in the plasma membrane fraction, with an enrichment of ~20-fold (Table I). This is three times the enrichment of acid phosphatase and comparable to the enrichment of ^{125}I marker in the plasma membrane fraction. We suggest that a major portion of the ATPase activity is associated with the plasma membrane. Neither acid phosphatase nor ATPase activity could be detected in fresh or conditioned culture media.

Sterol Analysis. Cholesterol is required for *in vitro* growth of *E. histolytica* (24) and is the only sterol found in amoebae grown in Diamond's TYI-S-33 media. The cholesterol:phospholipid molar ratio in both cell homogenates and the internal vesicle membranes (pellet II) was found to be 0.30. The purified plasma membranes (pellet

IV), however, had a cholesterol:phospholipid molar ratio of 0.87, an enrichment of almost threefold.

Phospholipid Composition. The phospholipids of *E. histolytica* were identified by thin-layer chromatography (Fig. 6). The major phospholipid in extracts of amoebae was identified as phosphatidylcholine (PC) by comigration with standards and reaction with Dragendorff's reagent. Phosphatidic acid (PA), phosphatidylinositol (PI), and phosphatidylserine (PS) were also identified by comigration with standards. PS was confirmed by its reaction with ninhydrin. Ceramide aminoethyl phosphonate (CAEP), also ninhydrin positive, was resistant to digestion by mild alkali, suggesting either a plasmalogen or a ceramide linkage (21). Phosphate could not be liberated from this phospholipid by digestion with perchloric acid, although phosphorus could be shown to be present either by ashing samples or by ^{32}P incorporation. These properties are consistent with our identification of this lipid as ceramide aminoethyl phosphonate, a phospholipid found as a minor component in several protozoan species (25, 26).

Two phosphatidylethanolamine species (PE_1 and PE_2) were tentatively identified. Both were ninhydrin positive, and both had R_f values appropriate for phosphatidylethanolamine. A similar assignment has been made for *Entamoeba invadens* (27).

Sphingomyelin was identified by comigration with a standard and by its resistance to mild alkaline digestion. Two minor phospholipids (X and Y) were not identified.

The phospholipid composition of whole cells, internal vesicles, and the plasma membrane are compared in Fig. 7. Whole cells and internal vesicles have similar compositions, with PC as the dominant phospholipid, followed by the two classes of

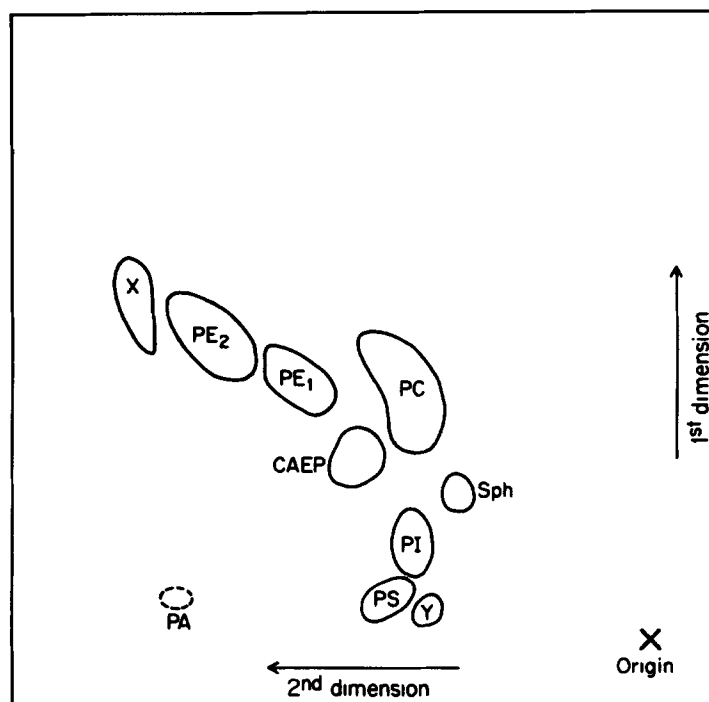


FIG. 6 Separation of *E. histolytica* phospholipids by two-dimensional thin-layer chromatography. Sph, sphingomyelin

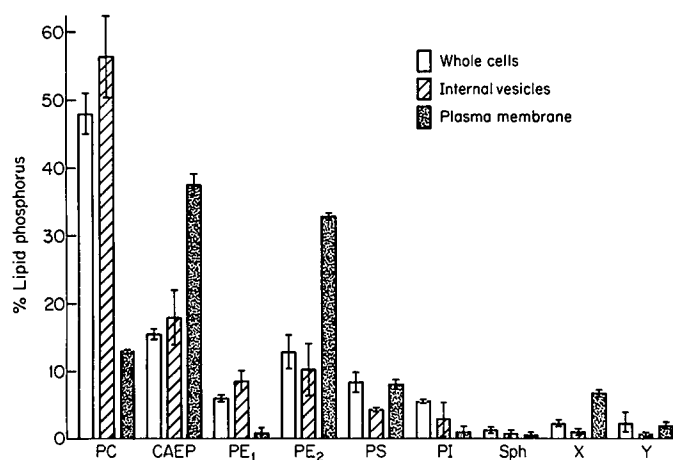


FIG 7 Comparison of the phospholipid composition of whole cells, internal vesicles (pellet II, Fig 1), and plasma membranes (pellet IV, Fig 1). The lipids of whole amoebae and the isolated membrane fractions were extracted and subjected to two-dimensional thin-layer chromatography, and the phosphorus content of individual phospholipids determined. Values are means \pm SE of three experiments.

PE. Plasma membranes, however, were greatly enriched in CAEP and PE₂ and correspondingly deficient in PC and PE₁.

The total fatty acid composition of whole cell extracts and of plasma membranes showed no significant differences.

Discussion

The invasiveness of *E. histolytica* and the contact dependence of in vitro trophozoite cytotoxicity suggests an important role for cell surface molecules in these activities (4, 5). In spite of the apparent importance of the plasma membrane of this organism, little information exists concerning its composition. Classical techniques of differential and isopycnic centrifugation have failed to separate distinct organelle fractions, and analysis of the fractions obtained has been complicated by the lack of defined enzymatic markers for subcellular fractions. We have demonstrated that preincubation with concanavalin A stabilized the plasma membrane and maintained it in large sheets through homogenization. It could be separated from internal vacuoles and vesicles by low-speed centrifugation. Subsequent removal of the lectin with a competitive sugar resulted in changes in membrane size and density and permitted further separation of plasma membrane from aggregates and debris. This technique was first developed for use with the fungus *Neurospora crassa* (28) and has recently been applied to both yeast (29) and astrocytoma cells (30).

To provide an unambiguous marker for the plasma membrane, we labeled cell surface proteins with ¹²⁵I. The relatively mild conditions of this coupled enzyme system did not modify the viability or function of amoeba. Surface iodination of intact amoebae labeled ~12 peptides, ranging from 12 × 10³ to 200 × 10³ mol wt. These labeled peptides were distinct from the major soluble proteins of amoebal lysates, as judged by migration on SDS-PAGE, and were glycoproteins—a common trait among secreted and membrane proteins. These radiolabeled peptides were all

tightly bound to the cell surface and could be distinguished from two of the major serum protein components of the culture medium, bovine serum albumin and immunoglobulin G, on the basis of molecular weight (Figs. 2 and 5). Although we cannot rule out adsorption of minor protein components of the medium to the cell surface; such tightly bound components would still serve as a plasma membrane marker.

The plasma membrane and internal vesicle membranes were identified by the presence and absence, respectively, of TCA-precipitable ^{125}I . Each of the 12 surface-labeled peptides could be found in the plasma membrane preparation, indicating that the entire surface membrane had been isolated. A total of ~ 18 peptides were recovered with the plasma membrane. Of these, 12 comigrated with the radiolabeled peptides demonstrated by autoradiography. Although comigration on SDS-PAGE of peptides from complex mixtures cannot be considered proof of identity, we found this correspondence encouraging. This complex pattern of surface peptides greatly contrasts with that of the free living amoeba, *Acanthamoeba castellanii*. Korn and Wright (31) demonstrated that a single peptide of 15,000 mol wt constituted $>80\%$ of plasma membrane-associated protein. Similarly, the related parasite *E. invadens* is reported to have only 5–9 peptides associated with its plasma membrane (26). The complexity in range and number of peptides of the surface of *E. histolytica* compares favorably with many mammalian cells.

Cholesterol was enriched in the plasma membrane fraction to a molar ratio of 0.87 with phospholipid. This high concentration of nonesterified cholesterol is characteristic of plasma membrane preparations of a variety of cell types, including *A. castellanii* (32) and *E. invadens* (26, 27).

A substantial portion of an alkaline ATPase was found to co-purify with the plasma membrane. This activity could not, however, be used in place of ^{125}I as a surface membrane marker, as 60% of the activity was associated with membranes of the internal vesicles. It is not clear at this time whether this internal activity is associated with membrane distinct from the plasma membrane or with plasma membrane-derived endocytic vesicles. Acid phosphatase activity has been proposed as a surface marker in *E. invadens* (27, 33). In this organism, two membrane-bound acid phosphatase activities have been distinguished on the basis of pH optimum and substrate specificity. One activity, assayed with β -glycerol phosphate, was found only among the internal vesicles. The second activity, assayed with *p*-nitrophenyl phosphate, was found both with the plasma membrane and in phagocytic vesicles. We could detect acid phosphatase activity in our *E. histolytica* plasma membrane preparation, but at a level only slightly above the amount of lysosomal contamination estimated from the remaining latent NAGase activity. Comparison of internal and plasma membrane activity showed that both have an optimal pH of 5.0 and that both can use either substrate equally well. Thus, we cannot demonstrate a distinct plasma membrane acid phosphatase activity in *E. histolytica*.

The phospholipid components of *E. histolytica* were similar to those reported for *E. invadens* and included one unusual phospholipid, CAEP. The phospholipid composition of the plasma membrane differed substantially from the composition of both whole cells and internal vesicles. In particular, PC levels were substantially lower in the plasma membrane, and both CAEP and PE_2 were correspondingly increased. PS may also be increased. Similar differences in composition have been noted in other

organisms, including plasma membrane of *A. castellanu* (32) and the phagolysosomes (27) and plasma membrane (26) of *E. invadens*. The high proportion of phospholipid CAEP in the plasma membrane is more unusual and would have important ramifications in any proposed phospholipase cytotoxic mechanism (25).

The availability of enriched plasma membrane preparations has now permitted us to analyze the antigenic components of the cell surface, to compare the chemical and antigenic composition of virulent and nonvirulent strains, and to assay directly receptors and surface cytotoxins. In addition, we are in a position to further separate and characterize other organelles, including lysosomes and phagolysosomes, of *E. histolytica*. If our results with the plasma membrane are any indication, the apparently simple morphology of *E. histolytica* conceals a complex network of membrane organelles.

Summary

Axenically propagated *Entamoeba histolytica* (HK9:NIH strain) were employed as starting material for the isolation of plasma membrane by a novel procedure. In the absence of known enzymatic markers, the externally disposed polypeptides of intact amoebae were iodinated and the incorporated label used to monitor membrane separation and recovery. 12 major plasma membrane polypeptides (12×10^3 – 200×10^3 mol wt) were labeled and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Each of these was a glycoprotein.

Preincubation of amoebae with concanavalin A stabilized the plasma membranes as large sheets, facilitating its separation by low-speed centrifugation. Dissociation of the lectin with α -methyl mannoside, followed by additional homogenization led to vesiculation and further purification. The isolated plasma membrane was recovered in high yield (28%) and enriched 30-fold in terms of incorporated iodide. All iodinated surface glycoproteins of the intact organism were present in the plasma membrane fraction. A Ca^{++} -dependent ATPase was enriched in the plasma membrane to a similar extent, but over one-half of the total activity was associated with internal, unlabeled membranes, suggesting a dual localization of this activity.

The isolated plasma membrane was enriched in cholesterol and had a cholesterol: phospholipid molar ratio of 0.87. It also contained larger amounts of an unusual phospholipid—ceramide aminoethyl phosphonate—a phospholipase-resistant species.

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References

- 1 Krogstad, D. J., H. C. Spencer, Jr., and G. R. Healy. 1978 Current concepts in Parasitology *Amebiasis. N. Engl. J. Med.* **298**:262
- 2 Lowe, C. Y., and B. C. Maegraith. 1970 Electron microscopy of an axenic strain of *Entamoeba histolytica* *Ann. Trop. Med. Parasitol.* **64**:293
- 3 Proctor, E. M., and M. A. Gregory. 1972 The ultrastructure of axenically cultivated trophozoites of *Entamoeba histolytica* with particular reference to an observed variation in structural pattern. *Ann. Trop. Med. Parasitol.* **66**:335.
- 4 Jarumilinta, R., and F. Kradolfer. 1964. The toxic effect of *Entamoeba histolytica* on leukocytes *Ann. Trop. Med. Parasitol.* **58**:375

- 5 Trissl, D., A. Martinez-Palomo, C. Arguello, M. de la Torre, and R. de la Hoz. 1977. Surface properties related to concanavalin A-induced agglutination. A comparative study of several *Entamoeba* strains. *J. Exp. Med.* **145**:652
- 6 Diamond, L. S., D. R. Harlow, and C. C. Cunnick. 1978. A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. *Trans. R. Soc. Trop. Med. Hyg.* **72**:431.
- 7 Hubbard, A. L., and Z. A. Cohn. 1972. The enzymatic iodination of the red cell membrane. *J. Cell Biol.* **55**:390.
- 8 Reeves, R. E., F. E. Montalvo, and T. S. Lushbaugh. 1971. Nicotinamide-adenine dinucleotide phosphate-dependent alcohol dehydrogenase: the enzyme from *Entamoeba histolytica* and some enzyme inhibitors. *Int. J. Biochem.* **2**:55.
- 9 McLaughlin, J., and M. Müller. 1979. Calcium dependent ATPase in *Entamoeba histolytica*. *J. Protozool.* **26**:10 (Abstr.).
- 10 Müller, M. 1973. Biochemical cytology of *Trichomonad* flagellates. I. Subcellular localization of hydrolases, dehydrogenases, and catalase in *Tritrichomonas foetus*. *J. Cell Biol.* **57**:453.
- 11 Wattiaux, R., and C. de Duve. 1956. Tissue fractionation studies. VII Release of bound hydrolases by means of Triton X-100. *J. Biol. Chem.* **247**:2439.
- 12 Peters, T. J., M. Müller, and C. de Duve. 1972. Lysosomes of the arterial wall. I. Isolation and subcellular fractionation of cells from normal rabbit aorta. *J. Exp. Med.* **136**:1117.
- 13 Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248.
- 14 Layne, E. 1957. Spectrophotometric and turbidometric methods for measuring proteins. *Methods Enzymol.* **3**:447.
- 15 Maizel, J. V. 1971. Polyacrylamide gel electrophoresis of viral proteins. *Methods Virol.* **5**: 179
- 16 Folch, J. M., M. Lees, and G. H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226**:497.
- 17 Rouser, G., S. Fleischer, and A. Yamamoto. 1970. Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids.* **5**:494.
- 18 Skipski, V. P., and M. Barclay. 1969. Thin layer chromatography of lipids. *Methods Enzymol.* **16**:530.
- 19 Ames, B. N., and D. T. Dubin. 1960. The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. *J. Biol. Chem.* **235**:769.
- 20 Chen, P. S., Jr., T. Y. Toribara, and H. Warner. 1956. Microdetermination of phosphorus. *Anal. Chem.* **28**:1756.
- 21 Dawson, R. M. C. 1960. A hydrolytic procedure for the identification and estimation of individual phospholipid in biological samples. *Biochem. J.* **75**:45.
- 22 Fedorko, M. E., and J. G. Hirsch. 1971. Studies on transport of macromolecules and small particles across mesothelial cells of the mouse omentum. *Exp. Cell Res.* **69**:113.
- 23 Hubbard, A., and Z. Cohn. 1975. Externally disposed plasma membrane proteins. I. Enzymatic iodination of mouse L cells. *J. Cell Biol.* **64**:438.
- 24 Latour, N. G., R. E. Reeves, and M. A. Guidry. 1965. Steroid requirement of *Entamoeba histolytica*. *Exp. Parasitol.* **16**:18
- 25 Kittredge, J. S., and E. Roberts. 1969. A carbon-phosphorus bond in nature. *Science. (Wash. D. C.)* **164**:37
- 26 McLaughlin, J., and E. Meerovitch. 1975. The surface and cytoplasmic membranes of *Entamoeba invadens*. II. Polypeptide and phospholipid composition. *Comp. Biochem. Physiol. [B]* **52**:487.
- 27 van Vliet, H. H. D. M., F. Spies, W. A. M. Linnemans, A. Klepke, J. A. F. Op den Kamp, and L. L. M. van Deenen. 1976. Isolation and characterization of subcellular membranes of *Entamoeba invadens*. *J. Cell Biol.* **71**:357.

28. Scarborough, G. A. 1975. Isolation and characterization of *Neurospora crassa* plasma membranes. *J. Biol. Chem.* **250**:1106.
29. Duran, A., B. Bowers, and E. Cabib. 1975. Chitin synthetase zymogen is attached to the yeast plasma membrane. *Proc. Natl. Acad. Sci. U. S. A.* **72**:3952.
30. Lutton, J. K., R. C. Frederich, Jr., and J. P. Perkins. 1979. Isolation of adenylate cyclase-enriched membranes from mammalian cells using concanavalin A. *J. Biol. Chem.* **254**:11181.
31. Korn, E. D., and P. L. Wright. 1973. Macromolecular composition of an amoeba plasma membrane. *J. Biol. Chem.* **248**:439.
32. Ulsamer, A. G., P. L. Wright, M. G. Wetzel, and E. D. Korn. 1971. Plasma and phagosome membranes of *Acanthamoeba castellanii*. *J. Cell Biol.* **51**:193.
33. McLaughlin, J., and E. Meerovitch. 1975. The surface membrane and cytoplasmic membranes of *Entamoeba invadens* I. Gross chemical and enzymatic properties. *Comp. Biochem. Physiol. [B]*. **52**:477.