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Composition and Dynamics of the Plasma Membrane of Entamoeba Histolytica

Stephen Blaine Aley

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Composition and Dynamics
of the
Plasma Membrane
of
Entamoeba histolytica

A thesis submitted to the Faculty of the Rockefeller University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

by

Stephen B. Aley

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The Rockefeller University

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Introduction

INTRODUCTION

The study of the parasitic protozoa presents a multifaceted opportunity for biological research. These organisms are eucaryotic cells with many of the biological systems described in higher eucaryotic cells. However, they are adapted to highly specialized environments, invoking sometimes subtle and often unique variations of these biological systems. More important, an understanding of the biology of the parasite and the differences between the parasite and mammalian cells can be invaluable in the development of pharmacological and immunological methods of control of the parasite.

The subject of this thesis, the parasitic amoeba Entamoeba histolytica, is an ideal subject for such research. While the pathogenic form may be grown in vitro, it is in nature an obligate parasite. It has specifically adapted to growth in the human intestine and has retained or evolved several unique biochemical pathways. Furthermore, E. histolytica displays several interesting phenomena, including immune avoidance and contact dependent cytotoxicity, which have yet to be explained. Finally, amoebic dysentery, the disease caused by infection with E. histolytica, remains a problem both in the tropics and across the globe.

Though sometimes labeled a "tropical disease," amoebic dysentery is more properly a disease of poor sanitation and hygiene. While the highest rates of infection are often associated with the tropics, as high as 50 to 83% in parts of Egypt and Mexico (1), it ranges as far north as man is found. Significant endemic rates are found among the

indigenous peoples of Canada and Alaska, and surveys of persons passing cysts in Leningrad range from 14 to 25% (2). Estimates for the United States range from 1.5 to 33% (2), depending upon regions and populations surveyed. It should be noted that the true infection rate is probably higher than these estimates as a single stool examination can miss as many as two-thirds of E. histolytica infections (1). Treatment of the disease is readily accomplished, with metronidazole as the preferred drug. Reinfection is, however, common in areas of high endemic infections. Left untreated, the disease can result in severe wasting, metastatic infections, and death.

The known life cycle of E. histolytica is deceptively simple. It consists of only two main stages, trophozoite and cyst, and transition forms (fig 1) (3). The amoeboid trophozoite is the form of the parasite responsible for the pathogenesis of the disease. It is an anaerobic organism, capable of survival only within the host. It is readily destroyed by heat and osmotic shock. It is also destroyed by gastric juices and is thus not infective by oral route. The infective stage of the parasite is a dormant cyst formed by the encystation of a single trophozoite. The mature cyst contains four nuclei, the result of two consecutive mitotic divisions, and is surrounded by a polysaccharide wall. It can survive in moist environments for a month or more. It is sensitive to heat but is not killed by most chlorine based water treatment systems (4). Upon ingestion, the cyst passes through the stomach into the intestine where the combination of digestive enzymes and anaerobic conditions cause excystation. Each cyst produces four trophozoites which can then proceed to populate the large intestine of the new host.

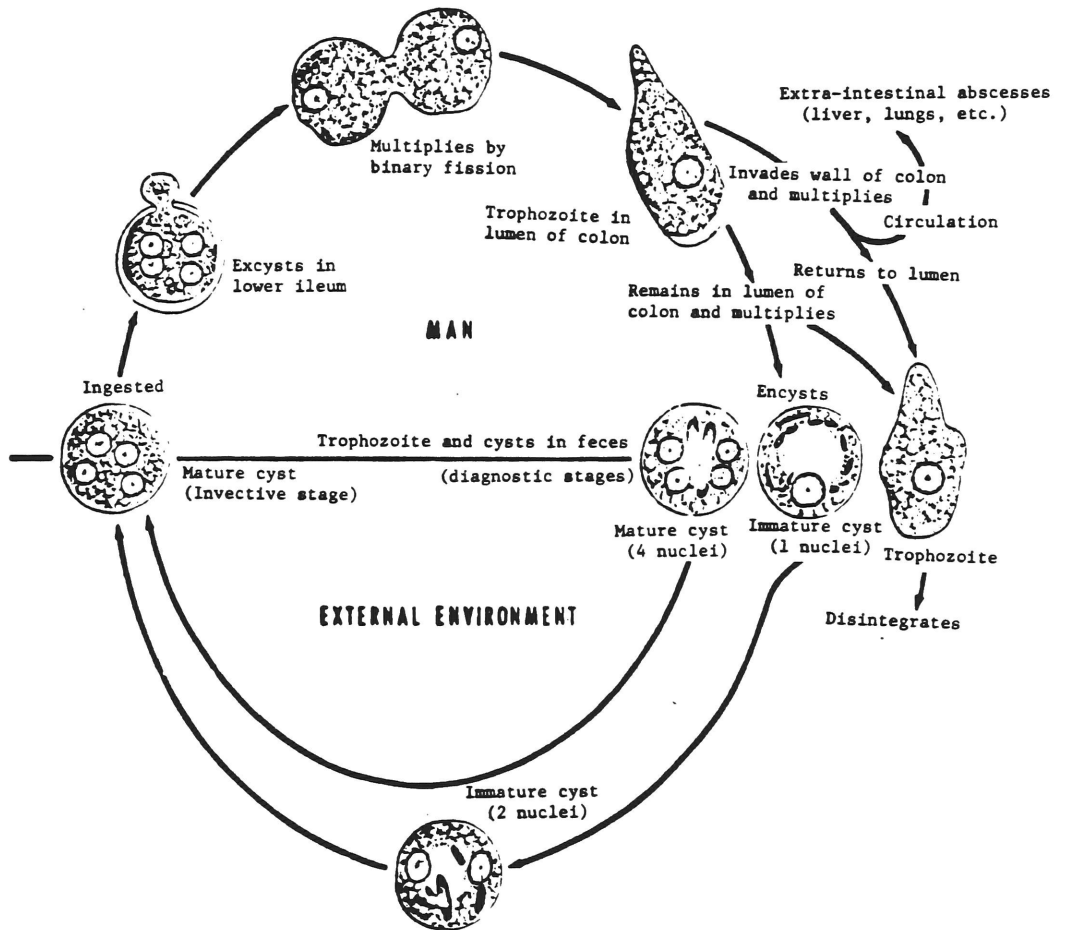


Fig. 1. Life cycle of *E. histolytica*, from Brooke and Melvin (3).

The pathology of the disease results from the invasion of tissue by the trophozoites and the resulting tissue degeneration. The mucosa appears to be largely resistant to the action of trophozoites, and E. histolytica can reside in the intestinal lumen above the mucosa with no apparent ill effects to the host. However, if amoebae should reach the submucosa, rapid and apparently non-inflammatory necrosis of that tissue occurs. The resulting flask shaped ulcers with nearly intact mucosa over degenerated submucosa are characteristic of intestinal amebiasis (1,2,4).

The underlying musculature of the intestine may also be resistant to invasion, as complete penetration of the large bowel, and the resulting peritonitis, is not common (1). However, trophozoites can invade the vasculature of the intestine and be carried through the portal circulation to the liver. Secondary abscesses in the liver are commonly found, although the exact frequency and efficiency of their formation is difficult to estimate. Hepatic abscesses can involve large portions of the liver and can also rupture, causing infection to spread to other areas of the body, most commonly the lungs and brain (1,2,4). All infections outside of the intestine appear to be non-productive in that no cysts are formed in these secondary loci, and bacteria are associated with these loci only in occasional late stages of necrosis. However, these secondary infections, especially of the liver, are also the most detrimental to the host, being the most common cause of death.

The hypothesis that a parasitic amoeba might be responsible for some types of human dysentery was first proposed by Lösch in 1875 (5). He reported the clinical aspects, treatment, and eventual autopsy of a Russian farmer, J. Markow, who had come to the clinic with severe

dysentery. Lösch correlated the severity of symptoms with the appearance of amoebae in the stools of the patient (fig 2). On autopsy he found that the large bowel was severely ulcerated and that amoebae were present in the abscesses. In experiments where he fed fecal and autopsy material to dogs he was able to infect only one of the four animals. He concluded that the amoebae, which he named Amoeba coli, were associated with the severe inflammation of the intestine, but were most likely not the original cause of the inflammation.

Amoebic dysentery was clinically separated from other (bacterial) dysenteries by Councilman and Lefleur in 1891 (6). Of particular interest was their demonstration of extensive hepatic and pulmonary involvement in amoebic dysentery and their detailed description of intestinal, hepatic, and pulmonary ulcers. The cyst form of the parasite was first described in 1893 by Quincke and Roos (7). Schaudinn, in 1903, first clearly separated two Entamoeba species and established the nomenclature in use today (8). The genus name Entamoeba was taken from the nomenclature of Casagrandi and Barbegello (9). The commensal form of the parasite received the original species name of Lösch, coli. To demonstrate the harmless nature of Entamoeba coli, Schaudinn even infected himself with this organism. The more virulent form, which Schaudinn declared was only found associated with "tropical dysentery," was named histolytica "on account of its tissue destroying ability" (8).

The differences of parasitic and non-parasitic strains were further investigated in a dramatic series of human infectivity experiments by Walker and Sellards in 1913 (10). They confirmed the commensal nature of E. coli and introduced the concept of a "carrier" in infections with E. histolytica. Test subjects with no apparent symptoms were found to

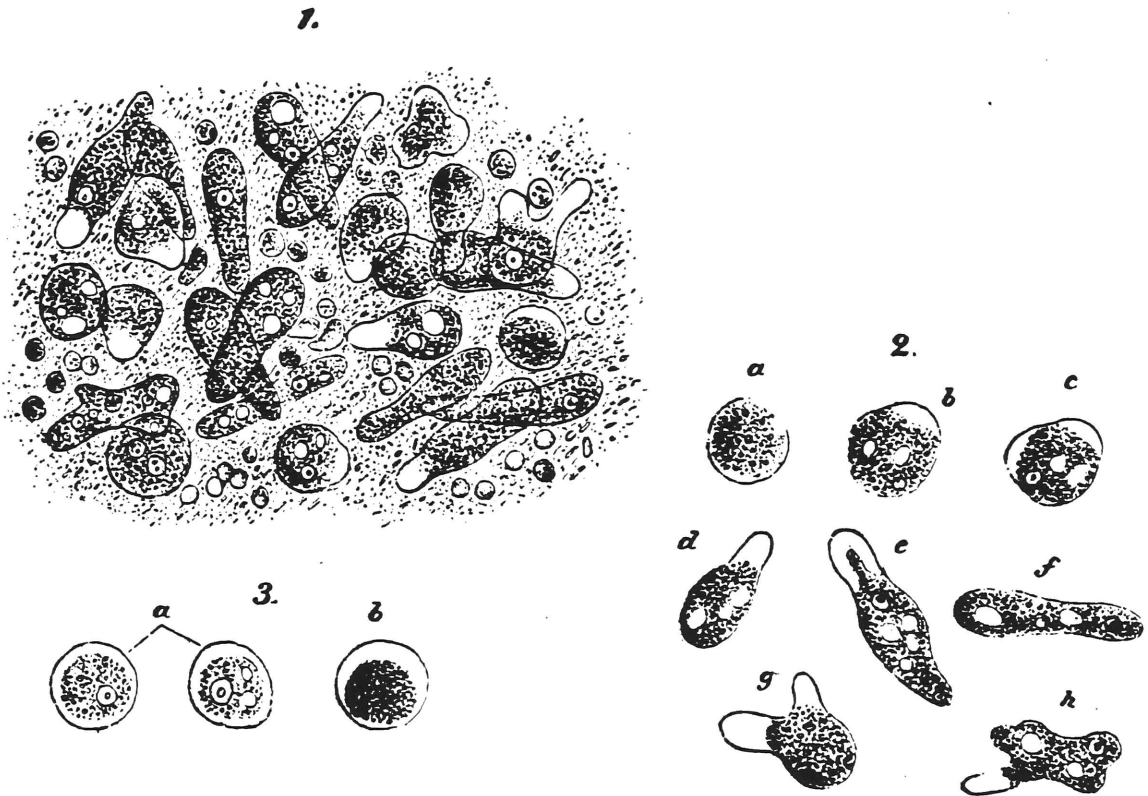


Fig. 2. Trophozoites of Amoeba coli (E. histolytica?) from Lössch (5).

pass amoebic cysts capable of causing severe disease in other subjects. In the same paper they reported that they were able to culture E. coli but not E. histolytica.

The first successful cultivation of E. histolytica did not occur until 1925. Using a combination of Locke-egg-albumin with undefined bacterial associates Boeck and Drbohlav were able to passage serially one strain of amoeba for over 8 months (11). Cyst formation was not seen in those cultures. Modifications of this medium by Dobell in 1928 eventually permitted the full life cycle of E. histolytica to occur in vitro (12). Trophozoites maintained in this or related media were used to investigate the growth requirements of E. histolytica. Of special interest was the discovery by Reeves et al that E. histolytica was capable of using inorganic pyrophosphate as an energy source via a pathway not found in other animal cells (13). With the advent of an axenic culture system developed by Diamond in 1962 (14), more complete analysis of metabolic and biochemical pathways of E. histolytica could be undertaken. Weinbach et al demonstrated that the metabolism of small amounts of oxygen by E. histolytica, first described by Wittner (15), proceeded via a pathway utilizing flavins and non-heme-iron sulfur proteins (16).

Also at this time the question of virulence or disease causing potential of trophozoites of E. histolytica came under increasing scrutiny. This question has been the subject of investigations since the initial work of Lösch. With the advent of reliable culture systems, different strains of E. histolytica were found to vary in their pathogenicity in animals (17). Several hypotheses have been advanced to explain these variations in virulence, including associated bacteria (18), culture conditions (19), and further speciation (20). One

additional species of non-pathogenic amoebae, E. hartmanni, has been defined (21,22), however, it cannot account for the reported variations in virulence. Most of these early studies used cultures of populations of amoebae, leaving open the possibility that much of the supposed variation was due to changes in the composition of the culture populations. Strains of E. histolytica cloned either by micromanipulation (23) or agar dilution (24) are now available, but have not been widely used in virulence studies. Currently the evidence seems to favor the expression of a spectrum of virulence by E. histolytica, controlled by unknown factors and perhaps related to the patient carrier phenomenon, where asymptomatic patients can spread cysts which cause active disease in others. There is some speculation of a third species of non-pathogenic amoebae encompassing the so called Laredo strains (25,26), but true speciation has not yet been demonstrated.

A major consideration in measurement of virulence has been the selection of a model system. Studies on cyst mediated intestinal infections have been performed on dogs (5), kittens (27), and humans (28). The monoxenic and axenic culture systems now in use do not, however, produce cysts. Even using Dobell's medium, cyst production is seen only in selected instances and not by all strains used (29). Attempts to assay virulence of trophozoites, generally by injecting live trophozoites into the caecum, have suffered from a lack of consistency. Injection into the caecum of a number of strains of mice and rats, for example, resulted in at best a 40% infection rate (30,31). For study of the extraintestinal disease, the golden hamster has proven to be the animal of choice. Injection of trophozoites into the liver of the hamster consistently resulted in hepatic abscess formation, but required

large numbers of amoebae (32). Use of newborn hamsters simplified the injection procedure (laparotomy was not required) and permitted the use of much lower numbers of amoebae (33). As few as 10,000 trophozoites from axenic cultures of strain HM1:IMSS results in 100% infection. Cranial injection of trophozoites into newborn mice has also been used as an assay system, with death rather than lesion formation being the endpoint (34). Other animal models in use include injection of trophozoites intracaecally in guinea pigs (35) and intrahepatically in monkeys (36). Currently there is no reliable animal model for secondary infection of the liver after inoculation of the caecum with trophozoites.

Trophozoites have long been known to have a cytotoxic effect on other cells in vitro. Jarumilinta demonstrated a cytotoxic effect of trophozoites against neutrophils and provided evidence that cell killing was contact dependent (37). In 1972, Westphal and Michel reported that the binding of Trypanosoma cruzi to trophozoites was followed by the immediated cessation of movement by the trypanosome (38). The cytotoxic effect has been used to develop several in vitro assays of virulence (39,40). Mattern et al demonstrated that their in vitro assay of virulence, based on destruction of a cell monolayer, correlated well with both the newborn hamster liver assay and newborn mouse cranial assay (40). While the original assay used 3T3 and BHK cells, numerous other cell types, including CHO (41), human neutrophils (42), and mouse macrophages or macrophage-like cell lines (personal observations), can be used. Release of ^{51}Cr from target cells has also been reported as an in vitro assay for virulence (43), but endocytosis of released label by amoebae complicates the analysis (personal observation). More recently Ravdin et al have used both trypan blue staining of and release of ^{121}In

from target cells as in vitro virulence assays (41).

A number of attempts have been made to correlate virulence or invasiveness of strains of E. histolytica with other cell characteristics. Secretion of proteases (44) and hyaluronidase (45) by trophozoites has been suggested and, in each case, ruled out as a factor in virulence. It was suggested by Das et al that cholesterol was important in determining virulence, and that addition of cholesterol to the medium could change an avirulent strain into a virulent one (46,47). This study was strongly contested by Neal in a report using identical strains and procedures (48), such that the relationship of cholesterol to virulence remains, at best, controversial. More recently, trophozoite size (49), ability to ingest erythrocytes (50), and agglutination by the plant lectin concanavalin A (51) have been suggested as hallmarks of virulence. In each case the correlation has eventually broken down.

Other factors of proposed importance in trophozoite virulence are the requirements for a bacterial associate and for encystation of the trophozoite (52,53,54). Both have been inferred from the apparent lessening of virulence in axenic culture-- cultures which are devoid of bacteria and in which the amoebae do not encyst. More direct evidence for the involvement of gut flora also comes from studies of intestinal infection of mice and guinea pigs (52). Germ free animals were infected to a lesser degree than normal animals (55). Host diet is also believed to have some effect on progress of the disease, with a high cholesterol diet reported to augment the severity of the disease (56).

Studies on the biochemical mechanism of pathogenicity have centered on the in vitro models of cytopathic effects. Trophozoites can kill tissue culture cells in vitro. However, conditioned medium from amoeba

cultures has no effect on target cells. Early studies by Jarumilinta et al demonstrated that contact, or at least close proximity of amoebae and target cells was required for cell killing (37). This has been repeatedly confirmed by numerous laboratories (40,41, personal observations). Ultrastructural studies, both in vivo (57) and in vitro (58,59) have demonstrated tight contact, but no recognizable cell junctions or obvious local destruction has been associated with contact. Other ultrastructural investigators have proposed that surface vesicle structures, some associated with a membrane "trigger," might function as surface active lysosomes (60,61), but no biochemical evidence in support of that hypothesis has been reported. It is not surprising that some vesicles are found closely associated with the surface of the trophozoite. As will be shown later, trophozoites contain numerous vesicles and vacuoles that are constantly interacting with the cell surface. Lysosomes, or organelles containing latent acid hydrolases, have been demonstrated in E. histolytica by McLaughlin and Lindmark (62). However, the origin of these organelles in a cell that appears to lack an endoplasmic reticulum and a Golgi apparatus (63,64) is unknown, and the interaction of these organelles with either the endocytic compartments or the surface of the trophozoites has not been reported. Until more direct evidence is obtained, the concept of a specific surface associated lysosome can only be considered an interesting speculation.

In 1972 Lushbaugh et al reported the isolation of a substance from a sonicate of E. histolytica which showed "toxin-like" activity on a guinea pig ileum (65,66). This factor caused the release of target cells from a monolayer in the absence of serum and was designated an amoeba toxin. The serum factor responsible for inhibition was

subsequently identified as fetuin (67). The factor appears to be proteolytic, possibly related to a neutral protease demonstrated by McLaughlin et al (68). However, this factor did not kill target cells and is probably not directly responsible for the cytotoxic effect of E. histolytica trophozoites.

López-Revilla et al have reported the isolation of lipid soluble factors from homogenates of E. histolytica trophozoites that can lyse target cells in vitro (70). Preliminary results indicate that a heat sensitive lipase activity is responsible for the generation of a fatty acid(s) which is then lytic for target cells (personal communication). These results are consistent with an earlier report that the phospholipid inhibitor DL-dimethyl-2,3-distearoyloxypropyl-2-hydroxyethylammonium acetate could reduce the cytotoxic effect of trophozoites (43). However, the generation of fatty acids in homogenates of protozoa is not uncommon, and the phospholipase inhibitor, a phospholipid analogue, may bind to the surface of the cells involved, preventing amoeba - target cell contact. In contrast to the above findings, numerous investigators have been unable to find any cytotoxic effect except in the presence of live trophozoites (41,42, personal observations).

Recently Kobiler and Mirelman have reported on a membrane associated lectin from E. histolytica, hypothesizing that it may be involved in the binding of erythrocytes, and possibly target cells, to the trophozoite (71). However, its apparent ligand, polymers of N-acetylglucose (eg. chitotriose), and lack of activity at neutral pH have raised some doubt as to its function under physiological conditions. Further experiments must be done before definite conclusions may be drawn.

The role of the host immune response in E. histolytica infections is likewise uncertain. As a commensal, E. histolytica elicits no apparent inflammatory or immune response. Serum antibodies against trophozoite proteins are detected in patients with either intestinal or hepatic ulcers (72), but serum titer does not correlate with the severity or duration of symptoms (73), and the antigenic targets have not been well defined. Furthermore, previous infection with E. histolytica does not protect the host from subsequent infection (1). In contrast, spontaneous cures of amoebiasis can occur at any stage of the disease (4), consistent with the development of true immunity. Moreover, recent attempts to vaccinate guinea pigs (74), hamsters (75), and monkeys (76) have yielded promising results.

Our interest in E. histolytica was first aroused by its cytotoxic activity towards cells and tissues. The requirement for trophozoite - target cell contact indicated that the surface of the trophozoite was intimately involved in at least target recognition and in delivery of the cytotoxic agent, and our initial efforts were directed towards investigating the role of that organelle in the cytotoxic process. However, the lack of basic biochemical information about both the trophozoite surface and the cytotoxic process forced a reconsideration of our research goals. We elected to focus our research on three areas of investigation: 1) Structural and biochemical composition of the plasma membrane, 2) Antigenic composition of the trophozoite surface, and 3) Interaction of the trophozoite surface membrane with endocytic and lysosomal compartments of the cell. Our hope is that these studies will aid in the understanding and eventual control of the pathogenic effects of this human parasite.

Materials

and

Methods

CULTIVATION AND HANDLING OF *E. histolytica*

A). *E. histolytica* Strains

All strains of *E. histolytica* used in these experiments were obtained from cultures maintained by Dr. L. Diamond, National Institute of Allergy and Infectious Diseases (NIAID). Strains 200, HK9, and H303 were isolated at NIAID. Strain HM1 was isolated by Dr. B. Sepulveda at the Centro de Estudios sobre Amibiasis in Mexico City. Strain H303 was originally cultured from an aspirate of an hepatic abscess in a human patient with amebiasis. All other strains were originally cultured from the feces of symptomatic human patients. All strains except HK9 had been rederived as clones of single trophozoites by Dr. F. Gillan at NIAID (24).

B). Axenic Cultivation

Amoebae were cultivated after the method of Diamond et al., using trypticase-Yeast-Iron medium (TYI) (77). This medium consisted of 2 gm trypticase broth (Becton, Dickinson and Co., Cockeysville, Md.), 1 gm Yeast Extract (Becton, Dickinson, and Co.), 1 gm glucose, 200 mg NaCl, 100 mg K_2HPO_4 , 60 mg KH_2PO_4 , 100 mg L-cysteine-HCl, 20 mg L-ascorbic acid, and 2.28 mg ferric ammonium citrate (purified brown pearls, Mallinkrodt), dissolved in 87 ml of water. The pH of the medium was adjusted to 6.8 with 5 N NaOH, and the medium was filtered through one layer of Whatman #1 filter paper. 15 ml portions were dispensed into 15 x 150 mm borosilicate glass tubes, and autoclaved at 15 lbs pressure for 20 min. Heat inactivated adult bovine serum (56°C for 30 min) was added

to a final concentration of either 10%, for strains H303, HK9, and 200, or 15% for strain HM1. The vitamin mixture described by Diamond (77) was found not to be essential and was not routinely used. Medium was made freshly each week and was stored at 4°C.

For subculture, tubes containing confluent, motile trophozoites were selected. Cultures were chilled on ice for 5 to 10 min to release adherent amoebae. From 0.05 to 1.0 ml of the suspended cultures were used to inoculate fresh tubes of TYI-S. Cultures were tightly capped and incubated on their sides in a 37°C incubator. All strains were subcultured twice a week, after three and four days of incubation.

For large quantities of trophozoites, cultures were grown in Wheaton T-200 borosilicate glass T-flasks. The protocol was the same as above, except that 170 ml of TYI was distributed to each flask, and flasks were inoculated with 5 to 20 ml of established cultures.

Cells were grown at 37°C to late log phase, harvested by chilling on ice for 5 min to detach amoebae from the sides of the tube or flask and centrifuged at 200 x g for 5 min. The yield per flask ranged from 1 - 2 x 10⁷ amoeba. Cells were washed twice in 19 mM potassium phosphate buffer, pH 7.2, containing 0.27 M NaCl (PD).

C). Assay of in vitro Cytotoxicity

The strains described above were originally selected to represent a range of disease causing ability or virulence. Mattern et al, using both in vivo (33,34) and in vitro (40) assays of virulence, had classified these strains into categories of low (H303), medium (HK9 and 200) and high (HM1) virulence. At various times throughout this work these

rankings were confirmed by us using a modification of the in vitro assay of Mattern et al (40).

J774 cells, a macrophage-like cell line, were suspended to 5×10^5 cells/ml in Eagles minimum essential medium containing 20 mM HEPES (Dulbecco,) with 5.7 mM cysteine and 10 mM ascorbic acid added. One ml of the cell suspension was added to a 1 dram glass vial and incubated at 37°C in an upright position. After the J774 cells had attached to the vial bottom, forming a loose monolayer, 5×10^4 trophozoites were added and allowed to settle onto the monolayer. The medium was covered with mineral oil and the cultures incubated at 37°C . At 1, 2, 3, or 4 hr, cultures were fixed in 1.25% glutaraldehyde in medium. Vials were viewed in a Zeiss inverted microscope and the status of the J774 monolayer was assessed. J774 cells released from the vial bottom were verified as dead by their inability to exclude trypan blue.

D). General Techniques

Viability of trophozoites was established by trypan blue dye exclusion. Amoebae in PD were mixed with an equal volume of 0.4% trypan blue (Grand Island Biologicals), and read within 10 min. Cells excluding dye were always phase lucent and frequently motile. Cells including dye were never phase lucent and showed obvious degeneration when viewed with phase optics.

The number of cells was estimated by counting in a Neubauer counting chamber. Cell counts of $200/\text{mm}^2$ or less were found to be reproducible. Cell densities of over $400/\text{mm}^2$, however, resulted in an underestimate of cell numbers, presumably because of crowding effects.

Old cultures were destroyed by aspiration of fluid and washes into a solution of not less than 2% GERMITOL (Hexcel, Lodi, N.J.), and all glassware was subjected to either disinfectant or autoclaving prior to normal wash procedures. These procedures were maintained primarily as protection against cysts, although cysts have never been reported to develop in axenic culture.

PEPTIDE ANALYSIS

A). Cell Surface Iodination

Amoebal surface proteins were labeled with ^{125}I by a modification of the glucose oxidase-lactoperoxidase method of Hubbard and Cohn (78). Briefly, amoebae were harvested, washed twice in PD, and resuspended to $5 \times 10^6/\text{ml}$ in ice cold PD containing 40 mM glucose and $2.5 \mu\text{M}$ NaI. One mCi/ml of carrier free Na^{125}I (Amersham, Arlington Heights, Il.) was added to this mixture. 150 o-dianisidine mU/ml of lactoperoxidase (Calbiochem, La Jolla, Ca.) and 25 o-dianisidine mU/ml of glucose oxidase (Sigma, St. Louis, Mo.) were added to initiate the labeling reaction. Cells were incubated on ice and agitated every two min. Samples of cells from controls, incubated without enzymes, and of the full incubation mixture were saved for determination of cell viability and ^{125}I incorporation. After 15 min, cells were washed three times with 15 ml of ice cold PD by centrifugation at $200 \times g$ for 5 min. Cell viability, as monitored by trypan blue exclusion, remained greater than 95%. The incorporation of ^{125}I into protein of control cells was always less than 1% of the enzyme-mediated incorporation.

B). Lectin Chromatography

A total of 4×10^6 iodinated amoebae were solubilized in PD containing 1 mM Mg^{++} , 2 mM phenylmethyl sulfonyl fluoride (PMSF), and 0.5% Zwittergent 3-12 (N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, Calbiochem, La Jolla, Ca.). A column of 0.4 ml concanavalin A, immobilized on agarose beads (Sigma, St. Louis, Mo.), was washed extensively with PD containing 1 mM Mg^{++} and 50 mM alpha-methyl mannoside followed by the same buffer without alpha-methyl mannoside. The cell lysate was run into the column and flow was stopped for 10 min. The column was then washed with PD containing Mg^{++} and 0.1% Zwittergent 3-12 until no further radioactivity was detected in the effluent. Alpha-methyl mannoside, 50 mM, was then added to the buffer and the specifically eluted radiolabeled peptides were collected, concentrated by trichloroacetic acid precipitation, and separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE).

C). Tritiated Borohydride Reductive Labeling

Exposed galactose residues on the trophozoite were labeled with tritium after the method of Critchley (79). Trophozoites were optionally pretreated with neuraminidase, a process that exposes surface galactose residues on most mammalian cells. Exposed terminal galactose residues were then enzymatically oxidized by treatment with galactose oxidase and then chemically reduced using tritiated borohydride.

Neuraminidase treatment: 1.5×10^6 cells were resuspended in 1.5 ml PD plus 2 mM PMSF. 75 Calbiochem units of neuraminidase (Calbiochem) were added, and samples were incubated at $37^{\circ}C$ for 30 min. Treated

cells were washed twice with PD.

Galactose oxidase treatment: 1.5×10^6 cells were resuspended in 1.5 ml PD plus 2 mM PMSF. 15 Sigma units of Galactose oxidase (type III, Sigma) were added and mixture was incubated at room temperature for 60 min. Cells were washed twice with PD.

Reductive labeling: Tritiated borohydride (50 mCi/ml 0.01 N NaOH; 5 - 15 Ci / mmole, New England Nuclear, Boston, Ma) was divided into 100 μ l aliquots and frozen at -70°C . For labeling, 1.5×10^6 cells were suspended in 1 ml PD, and 1 mCi freshly thawed tritiated borohydride (20 μ l) was added. Cells were incubated at room temperature for 20 min, and then washed twice with PD.

D). SDS-PAGE Electrophoresis

Proteins were separated by electrophoresis in 5-15% gradient polyacrylamide gels in SDS, as described by Maizel (80). Slab gels were run at constant current with an initial voltage of 40 V. Electrophoresis was continued until the bromphenol blue marker dye was about 1 cm from the lower edge of the gel. Gels were fixed in 30% methanol and 7.5% acetic acid containing 0.01% Coomassie R-250. Gels were washed with fixing solution until the background staining had cleared, then dehydrated. Peptides were visualized by autoradiography at -70°C on Kodak XOMAT XR-1 film. Gels of ^{35}S - or ^3H -radiolabeled peptides were impregnated with Autofluor (National Diagnostics, Somerville, NJ) before drying. Exposure of ^{125}I -radiolabeled peptides was enhanced by using a Cronex Lightning Plus intensifying screen (Dupont, Wilmington, DE).

E. histolytica PLASMA MEMBRANE ISOLATION

The development and justification of the plasma membrane isolation procedure will be presented in the Results section (see fig. 14, p 60). The following is a detailed outline of the procedure.

One flask of HM1:IMSS (10^7 cells) was harvested and washed twice with cold PD. The cells were resuspended in 2 ml PD plus glucose and NaI, radiolabeled, and washed as described. Ten flasks of HM1:IMSS (10^8 cells) were harvested and pooled with the [^{125}I]-radiolabeled cells after washing. All subsequent steps were performed on ice or in refrigerated centrifuges. The cell pellet was resuspended to 2×10^7 cells/ml in PD containing 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 x g for 1 min. The supernate, containing unbound concanavalin A, was discarded, and the cell pellet was resuspended in 12 ml of 10 mM Tris-HCl buffer, pH 7.5, containing 2 mM phenylmethyl sulfonyl fluoride (Tris buffer) with 1 mM MgCl_2 . After swelling for 10 min in the hypotonic buffer, cells were homogenized in a glass Dounce homogenizer fitted with a tight pestle (6 to 8 strokes). 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 x g for 30 min. For analysis, material remaining at the top of the 0.5 M mannitol (Supernate I) was centrifuged at 40,000 x 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 containing 1 M alpha-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 added to 3 ml of Tris buffer and homogenized with 80 strokes in a glass Dounce homogenizer. This second homogenate was layered onto a single step gradient consisting of 4 ml of 20% sucrose in Tris buffer, and spun for 30 min at 250 x g. Vesiculated plasma membranes floating above the initial sucrose layer (Supernate III) were collected and were concentrated by centrifugation at 40,000 x g for 1 h. The pellet (Pellet IV), containing the enriched plasma membranes, was resuspended in 1 ml Tris buffer. All samples were either assayed immediately or frozen at -20°C for future use.

ENZYME AND BIOCHEMICAL ASSAYS

A). Alcohol Dehydrogenase

Alcohol dehydrogenase activity of E. histolytica was assayed as described by Reeves et al. (81). 0.1 ml of appropriately diluted sample was added to 2.9 ml of reaction mixture, consisting of 0.3 M glycylglycine - NaOH buffer, pH 8.8, plus 6 mM NADP. To initiate the reaction, 25 µl of 5 mM 2-propanol was rapidly combined with the mixture. The rate of reaction was determined by measuring the rate of increase in absorbance at 340 nm in a Zeiss PM6 spectrophotometer at 1.0 Abs full scale. Alcohol dehydrogenase activity in E. hystolytica was sensitive to freezing. Consequently only fresh samples could be used for assay.

Low concentrations of Triton X-100 did inhibit the reaction.

B). Alkaline ATPase Assay

The assay of the Ca^{++} dependent, alkaline ATPase activity of E. histolytica was based on the method of McLaughlin and Müller (82). The reaction mixture for the ATPase assay consisted of 75 mM glycylglycine buffer, pH 8.8, 500 μM Ca^{++} acetate, 0.075% Triton X-100, and 500 μM Na-ATP (Boehringer Mannheim, West Germany). ATP was omitted from control mixtures. 0.1 ml of sample was added to 0.9 ml reaction mixture or control and incubated at 30°C for 15 min. The reaction was terminated by the addition of 0.25 ml 8% sulfosalicylic acid and 10 mM CaSO_4 and placed on ice. Turbid samples were cleared by centrifugation at 500 x g for 10 min. Inorganic phosphate in assay supernates was determined following the addition of an equal volume of development buffer, which consisted of 1 part 2.5% $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot\text{H}_2\text{O}$, 3 parts 2% Triton X-100 in 2 N H_2SO_4 , and 1 part freshly mixed 10% ascorbic acid. To facilitate color development, samples were incubated at 30°C for 10 min. The absorbance at 790 nm was measured in a Zeiss PM6 spectrophotometer. H_2PO_4 was used to generate a standard curve. Homogenates of E. histolytica contained approximately 50 mU (1 mU = 1 nmole inorganic phosphate per min at 37°C) of ATPase activity per mg protein.

C). Acid Phosphatase Assay

Acid phosphatase activity was assayed by the procedure described by Müller (83). Reaction mixtures consisted of 0.1 M acetate buffer, pH 5.0, 0.02% Triton X-100, and 15 mM p-nitrophenylphosphate in a final

volume of 1 ml. Assays were initiated by the addition of 0.1 ml of enzyme and were incubated at 37°C for 10 min, at which time the reaction was stopped and the color developed by the addition of 3.0 ml 133 mM glycine, 83 mM NaCO₃, and 67 mM NaCl, pH 10.7. Absorbance at 410 nm was read in a Zeiss PM6 spectrophotometer. Homogenates of E. histolytica contained approximately 215 mU (1 mU = 1 nmole p-nitrophenol/min at 37°C) per mg protein.

D). Coomassie Protein Assay

Coomassie assay for protein was adapted from Bradford et al (84). 100 mg of Coomassie Brilliant Blue G-250 (Sigma) was dissolved in 50 ml of 95% ethanol. After the stain had dissolved, 100 ml of 85% w/v phosphoric acid was added with constant stirring. Water was added to 1 litre, and the reagent filtered through Whatman #1 filter paper into a dispenser bottle. For measurement of protein, 0.1 ml sample, in 0.01 M Tris-HCl, pH 7.2, was added to 3 ml of reagent and vortexed. Absorbance at 595 nm was read after 10 min. Bovine serum albumin, Cohen fraction V, in 10 mM Tris-HCl, pH 7.2, was used to generate a standard curve with each assay. Cuvettes were rinsed immediately after use with acetone to prevent dye buildup.

The Coomassie reagent was stable at room temperature for at least 3 months. 1 M Tris, 1 M sucrose, and 0.1% Triton X-100 had little or no effect on protein determinations. Alkaline conditions are not compatible with this assay.

E). N-acetyl-beta-glucosaminidase

N-acetyl-beta-glucosaminidase was assayed using 4-methyl-umbelliferyl-2-acetamido-2-deoxy-beta-D-glucopyranoside (Koch-Light Laboratories, Colnbrook, Buckinghamshire, England) as substrate (85). A 10 mM stock solution was made in dry methoxy ethanol. The reaction mixture consisted of 0.5 mM substrate in 0.1 M citrate buffer, pH 4.8, containing 0.2% Triton X-100. 0.1 ml of reaction mixture was combined with 0.1 ml of enzyme solution and incubated at 37°C for 10 min. The reaction was stopped by the addition of 2 ml of glycine buffer, pH 10.4, containing 5 mM EDTA. Tubes were transferred to a Perkin-Elmer fluorescence spectrophotometer and the fluorescence $E_{365}F_{460}$ was measured and compared with a standard curve constructed with 4-methylumbelliferone.

F). Peroxidase Assay

Peroxidases were assayed by a modification of the Worthington assay system for horse radish peroxidase (86) using o-dianisidine as a hydrogen donor. The reaction mixture for HRP consisted of 50 mM acetate buffer, pH 5.0, 135 mM NaCl, 0.003% hydrogen peroxide, and 0.01% o-dianisidine. 0.1 ml of enzyme dilution was added to 0.9 ml of reaction mixture and the rate of change in absorbance at 460 nm was measured in a Zeiss PM6 spectrophotometer. Units of activity were calculated by the formula:

$$\frac{\text{Change in A } 460 \text{ nm / min}}{11.3 \times \text{ml enzyme / test}}$$

and corresponded to decomposition of 1 μM H_2O_2 per minute at 25°C. Lac-

toperoxidase was assayed by a similar procedure, using 9.5 mM phosphate buffer, pH 7.2, in place of acetate buffer.

G). Glucose Oxidase assay

Glucose oxidase was assayed by coupling H_2O_2 production to the above peroxidase assay. 0.5 mg / ml of horseradish peroxidase was added to a reaction mixture consisting of the peroxidase reaction mixture minus peroxide and plus 0.5% glucose. 0.1 ml of enzyme solution was added to this mixture and the rate of reaction measured. Units of activity were calculated as above.

H). Miscellaneous

Protein associated radiolabel was determined after precipitation with 5% trichloroacetic acid (TCA). Up to 100 ug of protein was diluted into 1 ml of 10 mg / ml bovine serum albumin in 50 mM NaI, on ice. 1 ml of ice cold 10% TCA was added, and the samples incubated at 4°C for 1 hr. Samples were centrifuged at 500 x g for 15 min. Pellets were washed twice with 10% TCA. ^{125}I was directly determined in a Packard 5220 gamma counter. Pellets of protein samples containing ^{35}S or 3H were first dissolved in 0.4 ml 0.1 N NaOH. An aliquot was then diluted into Hydrofluor (National Diagnostics) and radiolabel was determined in an LKB 1210 Ultrabeta liquid scintillation counter.

A tritiated amino acid mixture (TRK.440) and ^{35}S -methionine were obtained from Amersham. ^{35}S -cystine was obtained from NEN (New England Nuclear, Boston, MA). Nucleic acid was estimated from Abs 280/260 ratios (87).

LIPID ANALYSIS

A). Lipid Extraction

Amoebic lipids were extracted and washed by the method of Folch et al. (88). All steps were carried out at 4°C. Amoebae were resuspended in a small volume of PD. 17 volumes of chloroform : methanol (2:1) were added to the sample and mixed well. This mixture was clarified by filtering through Whatman #1 filter paper, and the volume brought to 20 volumes by the addition of chloroform : methanol (2:1). After 10 min on ice, 4 volumes 0.58% NaCl were added. After mixing, the phases were separated by centrifugation at 500 x g for 5 min. The upper phase was discarded, and the interface washed four times with 4 ml of upper phase solvent, consisting of chloroform : methanol : 0.58% NaCl (3:48:47).

After the final wash, the upper phase was quantitatively removed. 0.1 ml of 0.1 mg butylated hydroxytoluene per ml methanol and 1 gm of Na_2SO_4 : NaCO_3 (4:1) were added to the lower phase. The extracts were flushed with N_2 (oxygen free), tightly capped, and stored at -20°C for at least 1 hr. The extracts were filtered through Whatman #1 filter paper into a round bottom flask and concentrated under reduced pressure. The concentrated extracts were stored under N_2 at -20°C.

B). Total Phospholipid Digestion

Total phospholipid digestion was accomplished by the ashing procedure of Ames and Dubin (89). Phospholipid samples or samples of silica gel containing phospholipid were placed in the bottom of a 13 x 100

mm Pyrex tube. 30 μ l of 10% $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ in ethanol was added, and all solvents were removed by heating. The tube was then shaken over an open flame until brown fumes had evolved and subsided. 0.3 ml of 0.5 N HCl was added, and the covered tubes were placed in boiling water for 15 min.

The amount of liberated inorganic phosphate was determined by the addition of 0.7 ml of a reagent consisting of 6 parts 0.42% $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ in 1 N H_2SO_4 plus 1 part fresh 10% ascorbic acid (90). After incubation for either 20 min at 45°C or 1 hr at 37°C, the absorbance at 820 nm was measured. H_3PO_4 was used to generate a standard curve.

In some experiments phospholipids were metabolically labeled by culturing amoebae in TYI-S medium containing 5 $\mu\text{Ci/ml}$ $^{32}\text{PO}_4$ (Amersham) for three days. For quantitation, phospholipids were extracted and dissolved directly in Hydrofluor for scintillation counting.

C). Separation of Phospholipids

Phospholipids were separated by two dimensional thin layer chromatography (91) on Redicoat-2D silica gel plates (Supelco, Inc., Bellefonte, PA) (TLC plates). TLC plates were activated by heating overnight at 110°C. 100 μ l of amoebic lipid extract was loaded onto a TLC plate. Chromatograms were developed in the first dimension in chloroform : methanol : ammonium hydroxide (65:25:5) until the solvent was 1 cm from the top of the plate and then air dried. When ammonia could no longer be detected, chromatograms were developed in the second dimension in chloroform : acetone : methanol : acetic acid : water

(30:40:10:10:5), again until solvent was 1 cm from the top of the plate.

Phospholipids were visualized by brief exposure of the chromatograms to iodine vapor. Individual phospholipids were characterized by either quantitative or qualitative methods.

D). Qualitative Phospholipid Analysis

Dragendorff reagent was used to identify choline containing phospholipids (92). Reagent A consisted of 1.7 g bismuth subnitrate dissolved in 100 ml of 20% acetic acid. Reagent B consisted of a solution of 40% potassium iodide. Immediately before use, 20 ml of reagent A was mixed with 5 ml of reagent B and 70 ml of H₂O, and the mixture was sprayed onto the silica gel plate. Areas containing choline phospholipids acquired an orange or red color, contrasting with a background color of light orange.

Phospholipids containing primary amines were visualized by reaction with ninhydrin (92). Ninhydrin was dissolved to 0.2% in acetone containing 10% acetic acid. This reagent was lightly sprayed over the developed TLC plate, and the plate was heated to 105°C for 5 min in a ventilated oven. The presence of primary amines caused the reagent to turn red to red violet against a colorless background,

Ceramide phospholipids were detected by their resistance to mild alkaline digestion (93). Up to 550 µg of lipid phosphorous was placed in a small round bottom flask and evaporated to dryness. The sample was redissolved in 0.8 ml carbon tetrachloride, and 7.5 ml ethanol was added. To this mixture 0.65 ml H₂O and 0.25 ml aqueous 1 N NaOH was added. The sample was incubated at 37°C for 20 min, and the pH was

measured (neutral or acid pH implied that too much material in the initial sample and that the procedure must be repeated). The reaction was terminated and the NaOH neutralized by the addition of 0.4 ml ethyl formate and incubation at 37°C for 5 min (sodium hydroxide + ethyl formate --> sodium formate + ethanol). The sample was evaporated to dryness. One vol H₂O was mixed with 2 vol isobutanol : chloroform (1:2), and the phases were allowed to separate. One ml of aqueous (top) and 2 ml of organic (bottom) phase were added to redissolve the sample. Undigested phospholipids, contained in the organic phase, were separated and detected as described above.

Lipid phosphones were detected by their resistance to digestion by 70% perchloric acid at 80°C for 36 hr (94). Individual phospholipids were eluted from the TLC plate with chloroform : methanol (2:1) and either digested by ashing or by perchloric acid. Differences in quantities of inorganic phosphorous liberated were attributed to phosphone linkages.

E). Sterol Analysis

For determinations of cholesterol, 5-alpha-cholestane was added to lipid extracts as an internal standard. Samples were saponified in 10% ethanolic-KOH : water (9:1) at 56°C for 30 min and analyzed by gas-liquid chromatography, using a flame ionization detector. Separations were carried out isothermally at 250°C on 1/4 in x 6 ft glass columns packed with 3% OV-17 on Chromosorb W HP (Supelco) with 50 ml/min of N₂ as the carrier gas. The cholesterol content of lipid extracts was corrected for recovery of the internal standard. For detection of

cholesterol esters, unsaponified samples were separated by gas liquid chromatography as before and compared quantitatively to saponified samples.

F). Fatty Acid Analysis

Total cell fatty acids were converted to their respective methyl esters by transesterification in 6% methanolic-HCl at 80°C for 16 hr. Fatty acid methyl esters were analyzed by gas-liquid chromatography on 1/8 in x 10 ft stainless steel columns containing 10% SP-2330 on 100/120 Chromosorb W AW (Supelco, Inc., Bellefonte, Pa.) with a N₂ flow rate of 30 ml/min at 185°C.

IMMUNOLOGICAL ANALYSIS

A). Preparation of Precipitation Reagent

Formalin fixed Staphylococcus aureus (staph reagent) was prepared by the method of Kessler (95). Staphylococcus aureus (Cowan strain) was grown in a broth consisting of 17.5 mg/ml Penassay broth (Difco), 10 mg/ml Casitone (Difco), 2.5 mg/ml yeast extract (Difco), and 5 mg/ml beta-glycerophosphate (Calbiochem), and supplemented with 4 µg/ml niacin and 2 µg/ml thiamin.

Three litres of bacteria were grown to late log phase and harvested by centrifugation at 5000 x g for 5 min. Pellets were resuspended in 9.5 mM phosphate buffer plus 13.5 mM NaCl and containing 0.05% sodium azide (PDA) and washed twice in that buffer. Bacteria were resuspended to 10% v/v in PDA and formalin was added to 1.5%. This suspension was

stirred at room temperature for 1.5 hr. Fixed cells were washed once in PDA, resuspended to 10% and heated in an 80°C water bath for 5 min. Heated cells were cooled rapidly, washed twice in PDA, adjusted to 10%, and stored at 4°C.

Immediately before use, staph reagent was resuspended in a buffer of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 0.02% NaN₃ (NET), containing 0.5% Nonidet NP-40 (NP-40, Particle Data Laboratories, Elmhurst, IL).

B). Normal and Immune Sera

To raise antibodies against plasma membrane proteins, we prepared a plasma membrane fraction (Pellet IV, fig 14) from 10⁸ trophozoites of strain HM1. The membranes were suspended in 0.5 ml PD, emulsified with an equal volume of Freund's complete adjuvant, and injected subcutaneously into a rabbit. A second plasma membrane preparation (10⁸ trophozoites, HM1) was injected intra-venously one month later. Serum was collected one week after the boost. The serum agglutinated trophozoites of all four strains to a reciprocal titer of 1024, but was not cytotoxic under normal conditions. Serum collected from this same rabbit, but before any immunization, was used as normal rabbit serum.

Human sera from patients with amoebic dysentery were generously provided by Dr. H. Murray and designated HC-1 and HC-2. Normal human serum was obtained from a volunteer with no known history of symptoms of dysentery or exposure to *E. histolytica*. Goat IgG against bovine serum proteins was obtained from Cappel Laboratories (Cochranville, PA).

C). Precipitation of Antigens

Lysates of radiolabeled trophozoites were prepared by resuspending sample in NET plus 0.5% NP-40 and incubating on ice for 40 min. Samples were then spun in a Beckman microfuge for 4 min. The supernate was collected and used for the following precipitations.

For antigen precipitation, a staph reagent-antibody complex was constructed by pelleting staph reagent by centrifugation in a Beckman microfuge and resuspending in 0.2 ml neat antiserum. This mixture was incubated for 20 min at room temperature and washed twice with NET plus 0.5% NP-40. Staph-antibody complexes were resuspended in 100 μ l NET plus 0.5% NP-40. 100 to 500 μ l of lysate were added and the mixtures were incubated for 60 min on ice. In some experiments, 0.5 ml of neat bovine serum was added to the staph - antibody complexes 20 min prior to the addition of lysates. The addition of these unlabeled proteins served to saturate the binding sites of those antibodies against bovine serum proteins. Staph reagent with bound antigen was washed twice in NET plus 0.5% NP-40, then resuspended in 200 μ l of NET plus 4% SDS and incubated at room temperature for 20 min to release the bound antigen. Staph particles were pelleted, and the supernatant was saved for SDS-PAGE analysis.

For gel analysis, 2-mercapto ethanol was added to 2% and samples were boiled for 2 min.

D). Isolation of IgG

Prior to radiolabeling, IgG was isolated from either immune or non-immune serum. One ml of serum was diluted with 0.5 ml PD + 0.5 ml

H₂O and placed on ice. 0.9 ml of cold, saturated ammonium sulfate, adjusted to pH 7.0 with ammonium hydroxide (SAS), was added slowly while stirring. The sample was incubated on ice for 40 min and then centrifuged at 2000 x g for 15 min. The supernate was removed, and 1.1 ml of SAS added. The sample was again incubated on ice for 40 min and centrifuged as above. The pellet was dissolved in 2 ml H₂O and precipitated by addition of 2 ml SAS. The pellet from this third precipitation was dissolved in 1 ml PD. The entire sample was run through a 1.4 x 80 cm column of Sephadex G-200 (Pharmacia, Uppsala, Sweden) in PD + 0.01% sodium azide. The fractions containing the 7S peak were pooled and concentrated under pressure in an Amicon pressure filter using a PM-10 membrane (Amicon Corporation, Lexington, MA).

E). Radiolabeling of Immunoglobulins

Immunoglobulins were labeled by the method of Fraker and Speck (96). 20 µl of 50 µg 1,3,4,6-tetrachloro-3,6-diphenylglycouril (IODO-GEN, Pierce Chemical Co., Rockford, Il.) per ml chloroform was placed in the bottom of a glass tube, and the chloroform removed under a nitrogen stream. These activated tubes were stored anhydrous at room temperature.

To radiolabel immunoglobulin, 50 to 1000 µg of IgG was combined with 0.5 mCi of ¹²⁵I in a total of 200 µl PD. This mixture was placed in the bottom of an activated tube and incubated at room temperature for 3 min. Radiolabeled protein was separated from free ¹²⁵I by applying the entire sample to a 0.2 ml column of Dowex 1 (Sigma) and washing the column with 1 ml of PD.

F). Southern Blot for Proteins

We used an adaptation of the Southern DNA blot to analyze antigens after separation on SDS-PAGE (97,98). Polyacrylamide gels from SDS-PAGE were placed onto a filter paper wick connected to a reservoir of 50 mM Tris-glycine buffer, pH 8.3, containing 0.1% SDS. A moistened nitrocellulose filter was placed over the gel, and 2 in of dry blotting paper was layered on top of the filter. Care was taken to avoid formation of any air pockets in the buffer circuit. Buffer was drawn by osmosis from the paper wick through the gel and nitrocellulose filter to the blotting paper. This flow of buffer elutes separated peptides from the gel and transfers them to the nitrocellulose filter where they are irreversibly bound. The blot was allowed to develop for 24 hr.

Following the blot, the gel was stained as usual, detecting residual protein. The nitrocellulose filter was added to a solution of 10 mM tris buffer, pH 7.5, and 150 mM NaCl (GS), also containing 1 mg/ml gelatin, and incubated at 37°C for 3 hr. The filter was then washed in GS for 30 min, and radiolabeled antibody was added to a level of 2 μ Ci/ml. The filter was incubated with the antibody for 3 hr at 37°C, then washed with three changes of GS containing 0.1% SDS and 0.05% NP-40 for 1 hr each. The filter was dried in air and exposed to x-ray film to visualize bound antibody.

visualize bound antibody.

FLUORESCENCE STUDIES

A). Materials

Fluorescein labeled dextran (FITC-dextran) with an average molecular weight of 70,000 was obtained from Sigma and stored dessicated at 4°C until use. Acridine orange was obtained from Sigma and stored at 4°C as a solution of 100 mg/ml in methanol.

B). Microscopy

Trophozoites containing fluorescent markers were either fixed for 10 min at room temperature with 1% formalin before viewing or viewed directly on a warmed stage. For observation of fluorescence, a Zeiss Photomicroscope III with epi UV illumination and Neofluar objectives was used.

C). Determination of Fluorescence Spectra

All fluorescence spectra were determined on a Perkin-Elmer fluorescence spectrophotometer. FITC-dextran was quantitated by measuring fluorescence emission at 520 nm with an excitation wavelength of 480 nm. Samples in culture medium were measured directly. Cells containing fluorescent markers were first lysed by resuspending in PD plus 0.5% Nonidet NP-40.

The method of Ohkuma and Poole was used to estimate the pH of the

background fluorescence was subtracted the ratio of the fluorescence, from excitations of 450 nm and 495 nm was determined and compared to a standard curve generated by measurement of FITC-dextran fluorescence in solutions of known pH. For measurement on FITC-dextran contained in viable amoebae, trophozoites were plated on glass coverslips and measurement made in a thermostated cuvette. To obtain consistent coverslip orientation, we used a special Teflon holder designed, made, and very generously loaned to us by the late Dr. B. Poole of the Rockefeller University.

ELECTRON MICROSCOPY

A). General Processing

Cells and membrane fractions were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 15 min on ice, then pelleted in a Beckman microfuge. Fixed cells were stained with 1% osmium tetroxide for 60 min, washed with 0.9% NaCl, and stained with 0.25% uranyl acetate for 30 min, all on ice. Samples were washed with 0.9% NaCl, 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 I electron microscope (100).

B). Peroxidase Staining

For cytochemical staining of horseradish peroxidase we used the method of Graham and Karnovsky (101). Cells were fixed with 2.5%

glutaraldehyde for 10 min at room temperature, then washed twice with 0.05 M Tris-HCl, pH 7.5 (TEM). Cells were equilibrated with TEM for at least 2 hours on ice, then pelleted and resuspended in TEM plus 0.5 mg / ml diaminobenzidine. After 10 min at room temperature, H_2O_2 was added to a concentration of 0.03% to initiate the reaction. After an additional 15 minutes at room temperature, cells were washed and processed as above, except that the lead citrate counterstain was omitted.

C). Cytochemical Stain for Acid Phosphatase

Cells used for cytochemical staining of acid phosphatase were fixed 15 min in 1.25% glutaraldehyde in PD, then washed once in 0.9% NaCl and incubated in 0.9% NaCl on ice for 60 min.

Beta-glycerophosphate substrate was mixed by adding 120 mg of lead nitrate in 100 ml 0.05 M acetate buffer, pH 5.0, to 10 ml of 30 mg/ml beta-glycerophosphate. This mixture was incubated at 37°C for 1 hr and then filtered through a 0.45 μ m filter before use.

p-Nitrophenyl phosphate substrate was made by mixing 40 mg/ml p-nitrophenyl phosphate with an equal volume of 1 M $MgCl_2$ and adding HCl to adjust the pH to about 5. 400 μ l of this mixture was combined with 60 mg/ml lead nitrate in 50 ml 0.05 M acetate buffer, pH 5.0. The resulting mixture was incubated at 37°C for 1 hr and then filtered through a 0.45 μ m filter. Controls did not contain beta-glycerol phosphate, p-nitrophenyl phosphate, or $MgCl_2$.

Fixed cells were suspended in prewarmed substrate and incubated at 37°C for 20 min. Cells were washed once in 1% acetic acid, exposed to a 1% dilution of $(NH_4)_2S$ light solution (Fisher Scientific, Fairlawn,

N.J.) for 20 sec, and washed with 0.9% NaCl. Cells were then processed for electron microscopy as above.

D). Cytochemical Stain for Alkaline ATPase

Trophozoites used for ATPase cytochemical stain were fixed and washed as described for acid phosphatase staining. ATP substrate was made by combining 60 mg ATP in 1 ml H₂O, plus NaOH to neutral pH, with 1 ml of 30 mM CaCl₂ and with 0.7 ml 0.2 M Tris, pH 8.8. Two ml of 0.5% lead citrate (pH 10) was then added. This mixture was incubated for 1 hr at 37°C and filtered through a 0.45 µm filter. Controls did not contain ATP or CaCl₂.

Trophozoites were incubated in substrate for 20 min at 37°C and processed as for acid phosphatase stain.

Results:

Part I

Morphology

MORPHOLOGY OF TROPHOZOITES

The E. histolytica strains used in these studies were obtained from Dr. L. Diamond at the NIH and were grown exclusively under axenic conditions. The full life cycle of this organism does not occur in these cultures (29). In particular, only the trophozoite, the vegetative stage of the parasite responsible for the pathology of amebiasis, is present. Trophozoites cultured in axenic conditions display both the morphology and activities of trophozoites obtained from human patients with active amebiasis.

A). Light Microscopy

The clear endoplasm and granular ectoplasm of the trophozoite are clearly visible in phase microscopy (fig 3). The small nucleus, often difficult to resolve in unstained amoebae, is also revealed by phase contrast observation. Blunt pseudopods of clear endoplasm can be seen protruding out of several of the trophozoites pictured. The trophozoites move through the extension of a single, rarely two, blunt pseudopod.

A large number of vesicles are visible throughout the ectoplasm. Endocytosis of large volumes of fluid in the absence of any apparent phagocytic stimulus (food cup formation) could occasionally be observed. The food cup formation resulted in the interiorization of a segment of surface membrane as a large vacuole. After interiorization we were unable to distinguish the newly formed vesicle from other large vacuoles of the cytoplasm. Food cup formation could be a major source of these vacuoles.

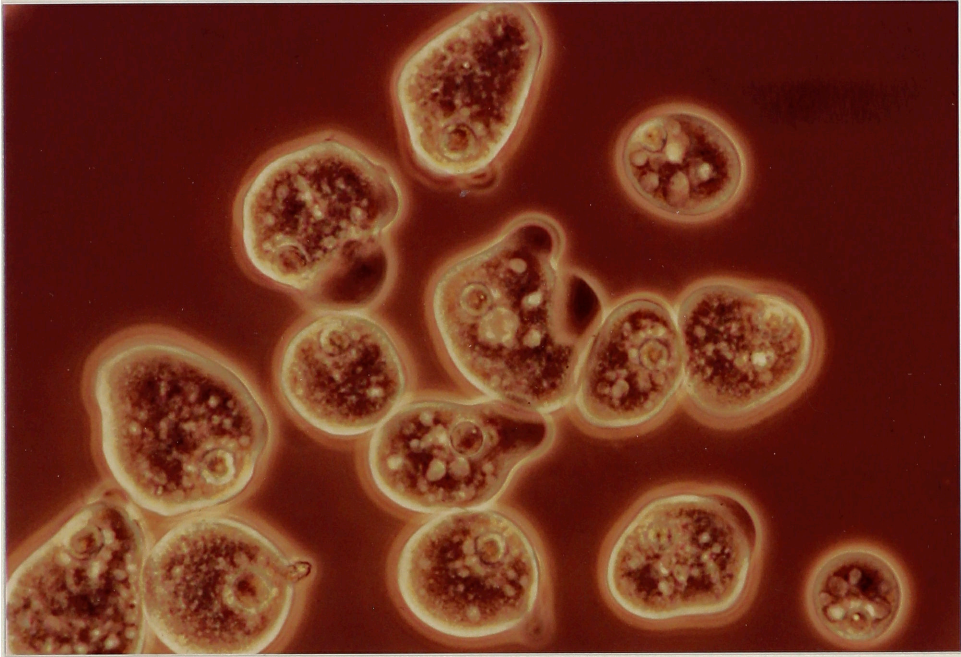


Fig. 3. Phase micrograph of E. histolytica trophozoites, strain H303. (x500).

Addition of human erythrocytes of several blood types, including A+, B+, AB+, AB-, and O+, resulted in immediate and extensive phagocytosis of the erythrocytes by all strains of E. histolytica used in this study. The presence of human erythrocytes in intestinal amoebae is considered diagnostic of E. histolytica (1,2,4). Gluteraldehyde fixation or age of the erythrocytes had no apparent effect on ingestion. In contrast, latex beads of a variety of sizes (1 to 7 μm) were not readily phagocytosed. These properties will be analysed in more detail in a later section.

Trophozoites varied in size among the strains used, with H303 the smallest at 20 - 30 μm and HM1 the largest at 40 - 50 μm . Intestinal trophozoites of E. histolytica can range in size from 20 to 60 μm (1,2,4). HM1 also contained the highest proportion of giant cells.

B). Nuclear Fine Structure

When axenically cultured trophozoites were prepared as for electron microscopy, thick sectioned, and stained with azure A, the nuclear fine structure of E. histolytica was clearly visible (fig 4). Nuclei were ringed with chromatin and typically showed a centrally located karyosome. Connecting filaments could occasionally be seen.

The cytoplasm of these thick sections was fine grained and filled with a number of large and small vesicles.

C). Electron Microscopy

By electron microscopy, the cytoplasm of E. histolytica is devoid of many of the prominent organelles found in mammalian cells (fig 5).

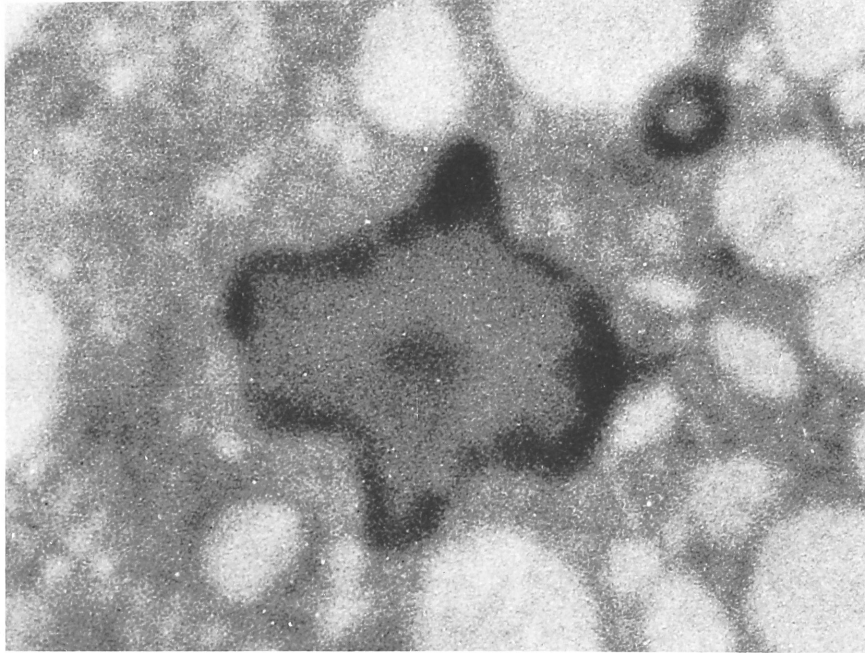


Fig. 4. Thick section of the trophozoite nucleus stained with azure A and visualized by light microscopy. (E. histolytica strain HM1). The nucleus is ringed with dense chromatin and shows a central karyosome. (x2,400).

The lack of mitochondria is expected, as E. histolytica is an anaerobic organism. However, there is also no apparent endoplasmic reticulum or Golgi apparatus (63,64). We can recognize the numerous vacuoles (2-5 μm) visible in light microscopy, as well as many smaller vesicles (<2 μm), distributed throughout the cytoplasm and even within the nucleus. The granules visible throughout the cytoplasm (fig 5 "G") are glycogen stores artifactually condensed by the fixation process. The surface of the trophozoite is bounded by a single unit membrane (fig 5 "PM"). No extensive surface coat is visible. The nucleus is likewise surrounded by a unit membrane which contains well defined pores. Chromatin like material is frequently present about the rim of the nucleus. In addition, nuclei frequently contain a unique organelle, of unknown origin and function, termed a "button body" (fig 5 "Bb").

Scanning electron microscopy was performed by Dr. G. Kaplan (fig 6). The rounded surface with only a limited number of blunt pseudopods is clearly presented. The indentations seen on most cells are presumably the early stages of food cup formation. Also visible are small processes connecting two interacting trophozoites.

D). Cytotoxic Activity

A number of investigators have reported the loss of virulence or infectivity of strains of E. histolytica after prolonged culture (102). To insure that the strains we used retained the ability to cause disease, we assayed their ability to lyse target cells in vitro. Mat-tern et al have demonstrated the relationship of this assay to the in vivo assay of hamster infection (40). Trophozoites were added to mono-

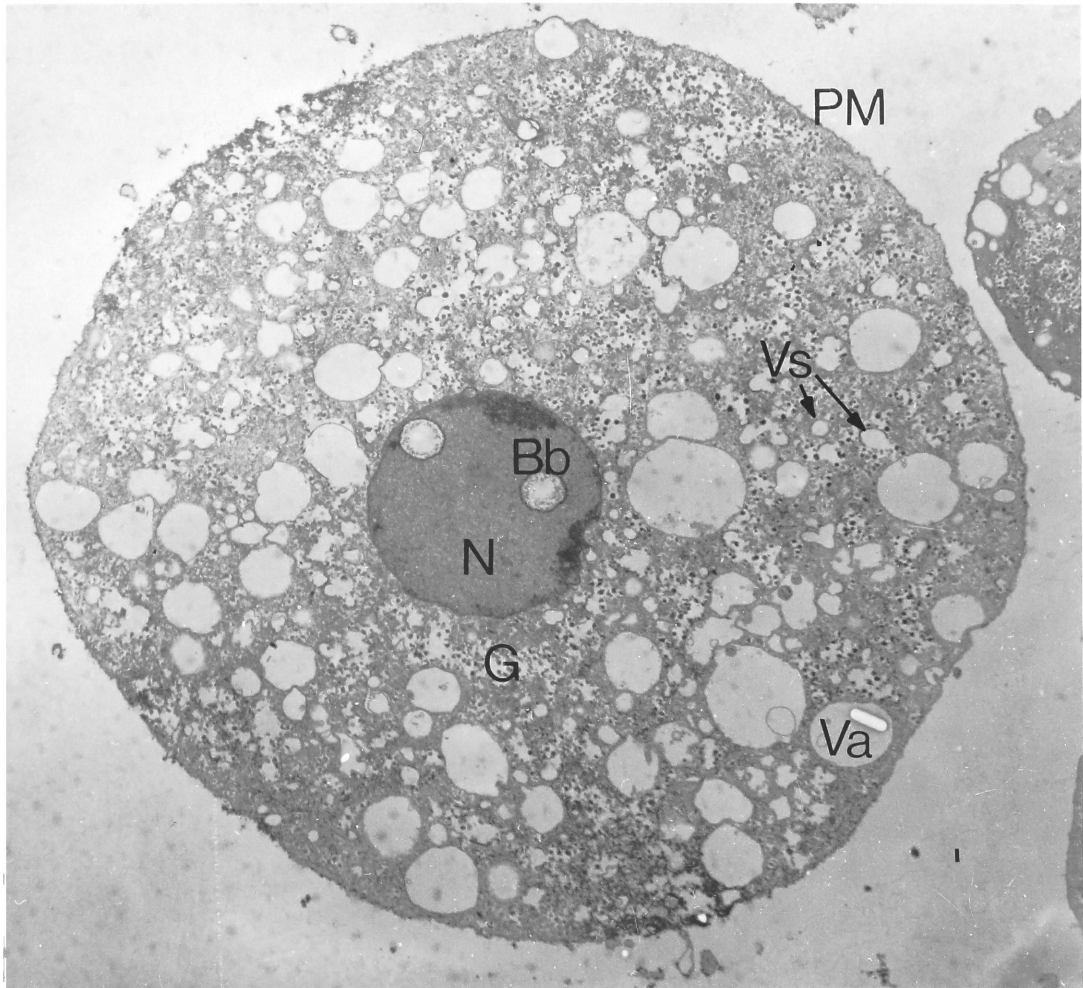


Fig. 5. Electron micrograph of an *E. histolytica* trophozoite, strain HK9. PM, plasma membrane; N, nucleus; Va, vacuole; Vs, vesicle; G, glycogen granules; Bb, button-like body. (x3,800).



Fig. 6. Scanning electron micrograph of E. histolytica trophozoites, strain HK9. Arrows indicate food cup initiation sites. (x1,520).

layers of either J774 cells or primary explants of mouse peritoneal cells, and the destruction of the target cell monolayer was monitored with time. An example of this assay is presented in fig 7. Trophozoites of HK9 were added in a 1 : 10 target ratio with J774 cells and photographed at 0, 1, 2, and 3 hr. By three hours the J774 monolayer was completely destroyed. Trophozoites of strain HM1 were consistently the most active in this assay. Trophozoites of strain H303 were the least active, and those of strains 200 and HK9 were of moderate activity.

E). Cytochemistry

We analysed the cytochemical distribution of two membrane associated enzymes, acid phosphatase and Ca^{++} -dependent alkaline ATPase. Two other potential membrane associated enzymatic activities, alkaline phosphatase and neutral ATPase, were not detected in homogenates of E. histolytica or tested for cytochemically.

Acid phosphatase is frequently used as an enzymatic marker for lysosomes (83). When E. histolytica was stained for acid phosphatase activity, reaction product was found deposited in most, but not all large vesicles of the trophozoites (fig 8). A few small vesicles also contained reaction product. In addition, the surface membrane of some but not all trophozoites was stained. Variable staining of large vacuoles was also seen by Rosenbaum and Wittner (103), although they did not detect surface activity. Van Vliet et al (104), using both subcellular fractionation and cytochemical techniques, demonstrated acid phosphatase activity to be associated with both the internal vesicles and the cell

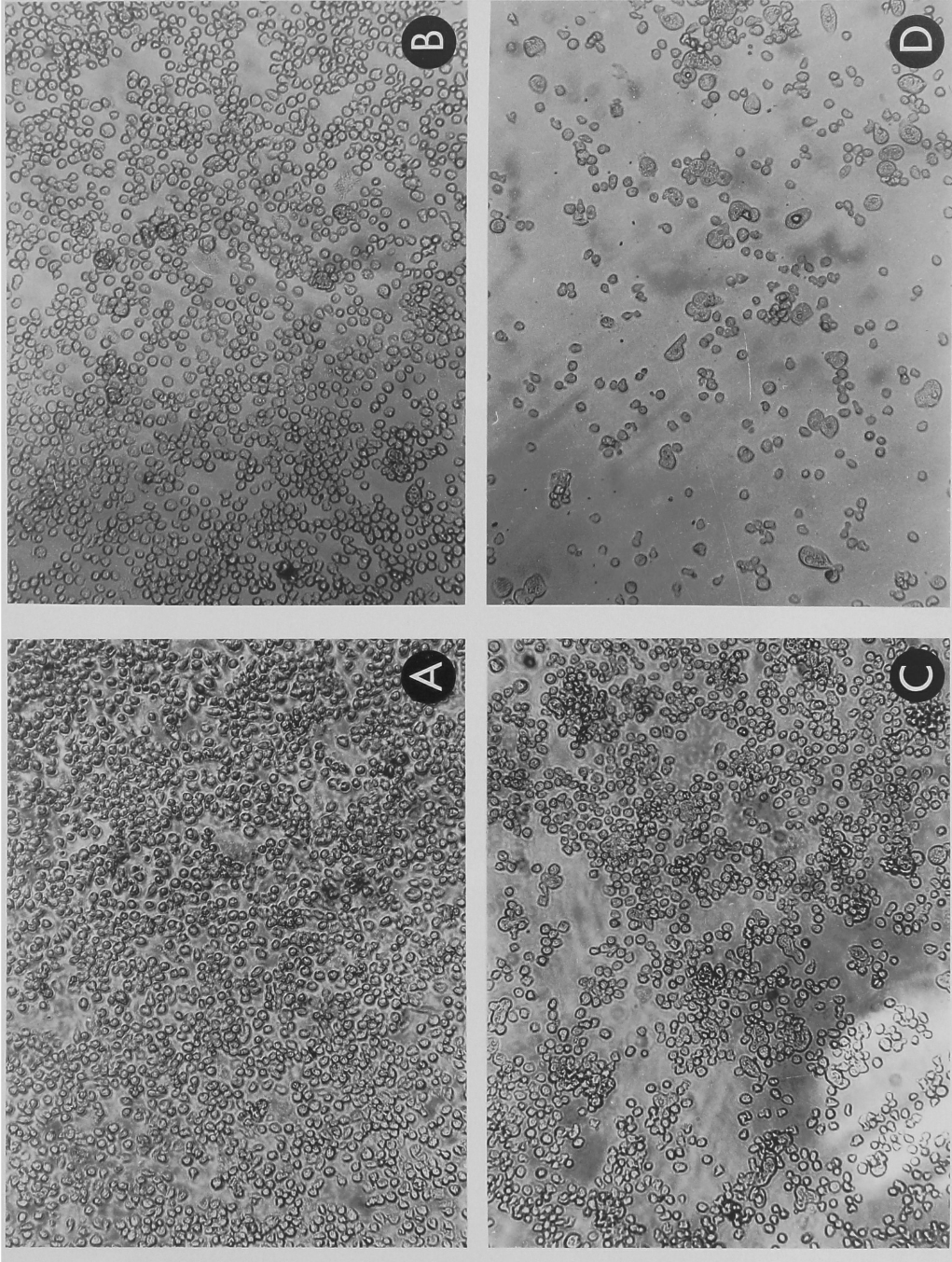


Fig. 7. Destruction of J774 cell monolayer by *E. histolytica* strain HK9. 10^4 trophozoites were added to monolayers consisting of 10^5 cells of the macrophage-like cell line J774 and incubated at 37°C . (A) Time zero. (B) 1 hr. (C) 2 hr. (D) 3 hr. (x100).

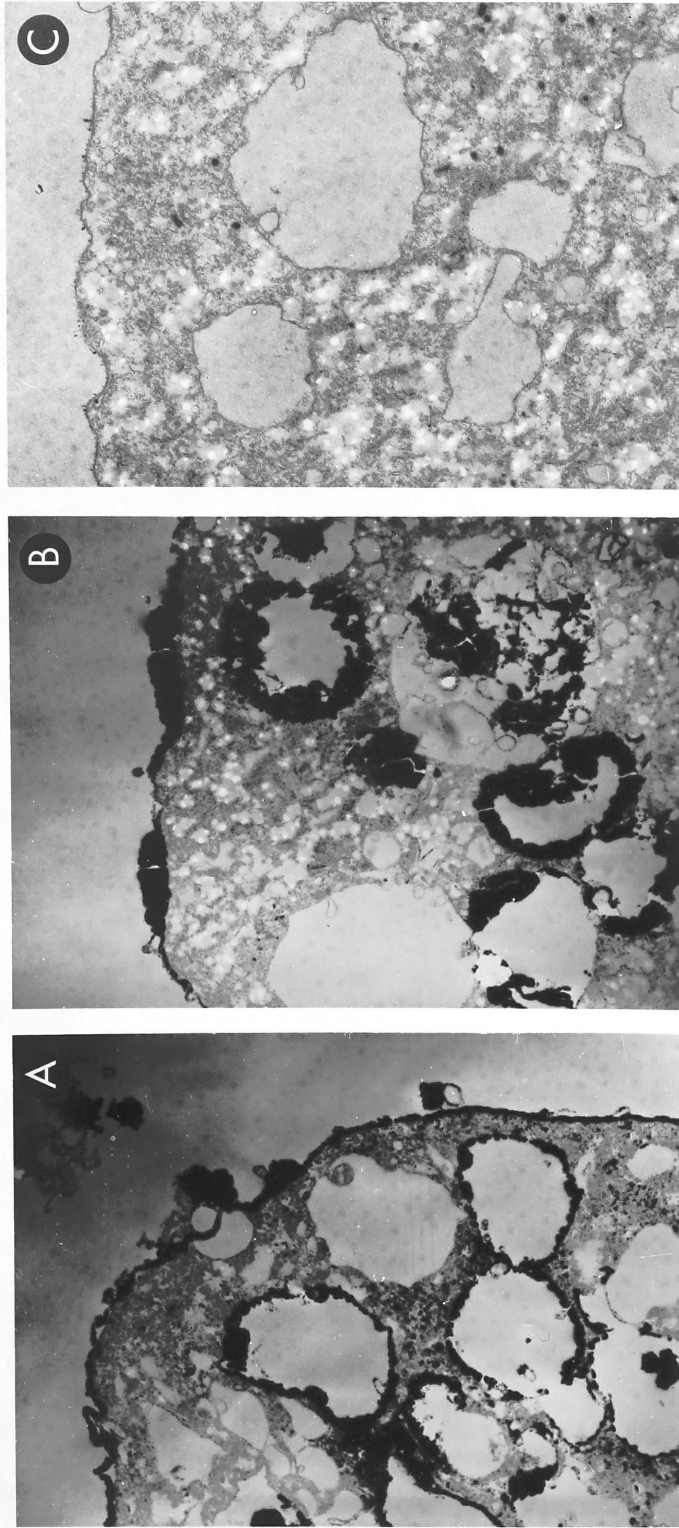


Fig. 8. Localization of acid phosphatase activity in *E. histolytica* strain HM1. Trophozoites were stained for acid phosphatase activity using either beta-glycerol phosphate or p-nitrophenyl phosphate as substrate and processed for electron microscopy. (A) Beta-glycerol phosphate used as substrate. Vacuoles and surface show reaction product. (B) p-Nitrophenyl phosphate used as substrate. Distribution of reaction product is same as for (A). (C) No substrate. (x9,300).

surface of the reptilian parasite E. invadens. The latter authors were able to distinguish surface and interior activities on the basis of substrate specificity. When p-nitrophenyl phosphate was used as substrate, only the cell surface was stained. However, when beta-glycerol phosphate was used as substrate, both the cell surface and the internal vesicles were stained. In contrast, cytochemical staining of E. histolytica using either substrate gave similar subcellular distributions of reaction product (fig 8 a and b).

E. histolytica contains a unique Ca^{++} -dependent alkaline ATPase, described by McLaughlin and Müller (82). When trophozoites were stained for alkaline ATPase, a distribution of staining similar to the distribution of acid phosphatase staining was found (fig 9). Staining was associated with the large vacuoles and with the surface of some, but not all trophozoites. The reason for the non-uniform distribution of these enzymes is not known.

F). DISCUSSION

As a prelude to our biochemical studies we analysed the morphology and characteristic functions of axenically grown trophozoites of E. histolytica. Axenically cultured trophozoites were found to be morphologically identical to trophozoites obtained from human patients with active amebiasis, particularly in reference to diagnostic characteristics. All strains tested were found to avidly ingest human erythrocytes, corresponding to a diagnostic trait of E. histolytica. These strains were also all cytolytic to tissue culture cells in vitro, with HM1 most virulent and H303 least virulent. This cytolytic activity has been

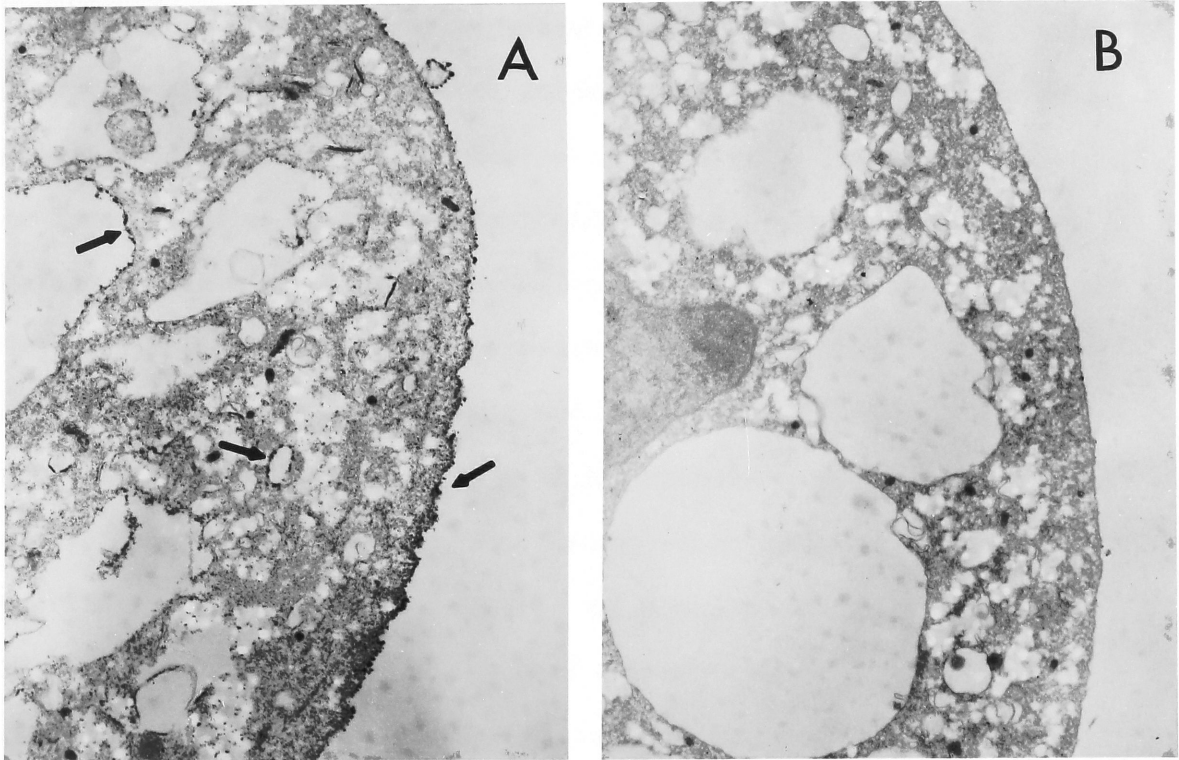


Fig. 9. Localization of Ca^{++} -dependent ATPase activity in E. histolytica strain HM1. Trophozoites were stained for ATPase activity and processed for electron microscopy. (A) ATP used as substrate. Reaction product (indicated by arrows) was visible both in vacuoles and on surface membrane. (B) ATP omitted from reaction mixture. (x11,700).

shown to be related to disease potential of the strains in animal models (40).

Our experiments with cytochemical staining for selected enzymatic activities did not define useful subcellular markers. Both acid phosphatase and alkaline ATPase reactions stained both the cell surface and the large vesicles of the trophozoites. The distribution of acid phosphatase agrees with reports for the related parasite E. invadens (104,105), but is in contrast to reported results for E. histolytica (103), where surface activity was not mentioned. However, we were unable to differentiate surface acid phosphatase activity from that of interior vesicles by the use of different substrates, as previously reported for E. invadens (104,105). The cytochemical distribution of alkaline ATPase has not been reported previously.

Of other potential surface membrane activities, alkaline phosphatase and neutral ATPase were not detected enzymatically, and 5'-nucleotidase was present only in low levels such that residual acid phosphatase activity could not be ruled out.

Results:

Part II

Plasma Membrane Isolation

and

Characterization

IODINATION OF TROPHOZOITE SURFACE PROTEINS

As discussed in the previous section, plasma membrane associated enzymatic activities of a variety of cell types were found to be either widely distributed or not present in E. histolytica. To overcome this lack of a defined plasma membrane enzymatic activity or marker, we elected to create an unambiguous marker for the surface of trophozoites. The radio-iodination of cells by LPO and GO, when performed on intact, viable cells under conditions where endocytosis is inhibited, has been shown to label only the externally disposed peptides of a number of cell types (78,106). We harvested, washed, and surface radiolabeled trophozoites of E. histolytica as described. When these surface labeled amoebae were lysed by freeze/thawing and the lysates were spun at 40,000 x g for 1 hour, over 80% of the total TCA precipitable radioactivity was recovered in the membrane pellet, suggesting that only membrane associated peptides were radiolabeled. Surface labeled amoebae were solubilized in 4% SDS and the total cell peptides were separated by SDS-PAGE (fig 10). A total of 12 major surface labeled peptides, ranging in molecular weights from 12,000 to 200,000, were visualized by autoradiography. These peptides presented a pattern distinct from that of the Coomassie stained protein of the total cell lysate (fig 10 a and b) and also distinct from the major serum proteins of the culture medium. These surface labeled peptides, assayed by measuring TCA precipitable ^{125}I , were used as a marker for the cell surface membrane throughout the plasma membrane isolation procedure.

To determine which of these peptides were glycoproteins, surface labeled amoebae were solubilized in non-denaturing detergent (Zwittergent 3-12) and applied to a concanavalin A-Sepharose column. Peptides

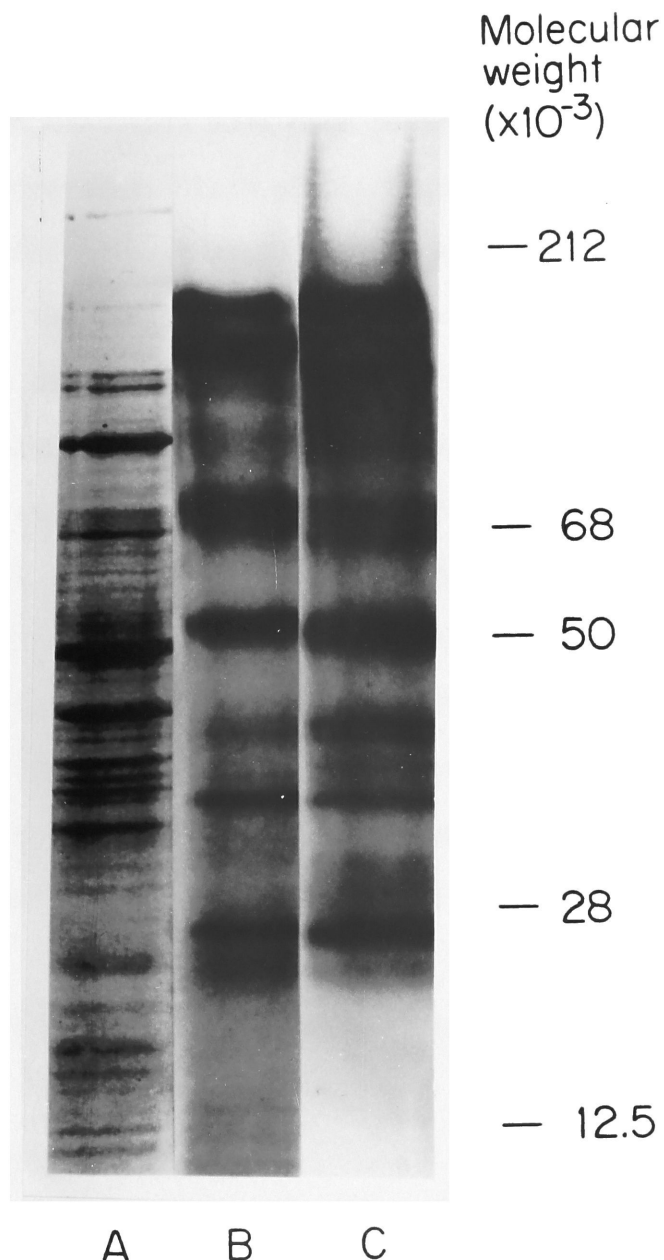


Fig. 10. Peptides of *E. histolytica* strain HK9 separated by SDS-PAGE. (A) Total peptides of amoebae solubilized in SDS, visualized by Coomassie blue staining. (B) Iodinated surface proteins. Intact trophozoites were iodinated by the glucose oxidase-lactoperoxidase procedure at 4°C and solubilized in SDS. The radiolabeled peptides were then separated by SDS-PAGE and visualized by autoradiography. (C) Iodinated surface glycoproteins. Iodinated trophozoites 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 alpha-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 (28,000), and cytochrome c (12,500).

eluted by alpha-methyl mannoside were then separated by SDS-PAGE and visualized by autoradiography. Each of the high molecular weight, surface radiolabeled peptides was present in this isolated glycoprotein fraction (fig 10 c). Independent identification of glycoproteins was obtained by enzymatic oxidation of galactose residues, followed by reduction with tritiated borohydride (79). This treatment resulted in the labeling of each of the major surface radiolabeled peptides of the trophozoite (fig 11 b). Prior treatment of trophozoites with neuraminidase had no effect on the level of tritium incorporation, implying that terminal sialic acid is not a major component of the trophozoite surface glycoconjugates (fig 11 a). The low molecular weight portion of the gel contains a smear of incorporated tritium, presumably in glycolipid, which may obscure lower molecular weight glycoproteins.

ISOLATION OF THE PLASMA MEMBRANE

A). Initial Fractionation Attempts

Initially, isolation of the plasma membrane was attempted by hypotonic lysis of surface radiolabeled trophozoites in a Dounce homogenizer followed by either differential centrifugation or sedimentation on a continuous sucrose gradient. ^{125}I was found in equal enrichment in low speed (200 x g, 5 min) and high speed (32,000 x g, 1 hr) pellets of homogenates of surface radiolabeled trophozoites. On sucrose gradients, the ^{125}I surface marker migrated as a broad smear across the gradient, without evidence of discrete bands. Dr. D. Lindmark and Dr. J. McLaughlin, in the laboratory of Dr. M. Müller, had made earlier attempts to

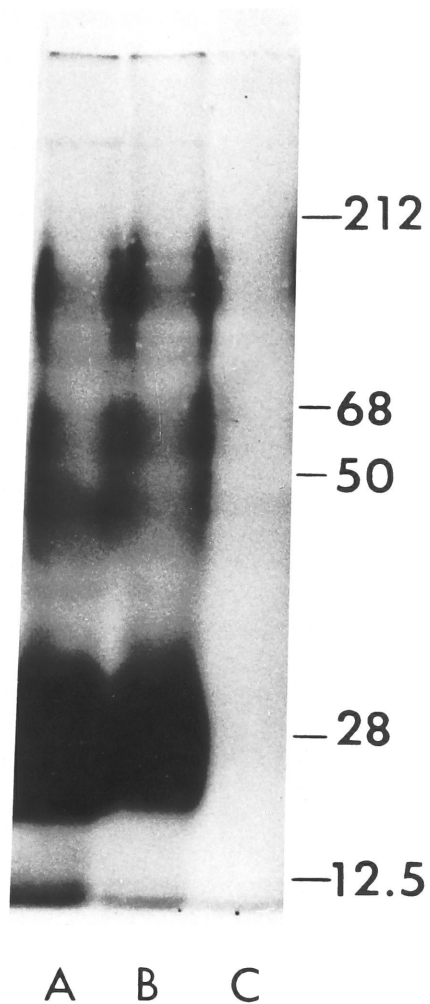


Fig. 11. Surface glycopeptides of *E. histolytica*, strain 200, labeled with tritiated borohydride and separated by SDS-PAGE. (A) Trophozoites treated with galactose oxidase before reductive labeling. (B) Trophozoites treated with both neuraminidase and galactose oxidase before reductive labeling. (C) Trophozoites reductively labeled without prior enzyme treatment.

isolate lysosomes from E. histolytica. They found that each of the membrane associated activities, including both acid and alkaline hydrolases, migrated as a broad smear on isopycnic centrifugation (personal communication). The fact that surface bound ^{125}I behaved in similar fashion convinced us that a different approach to plasma membrane isolation was desirable.

B). Scarborough Membrane Fractionation Procedure

One strategy in plasma membrane isolation has been to maintain the large size of the surface membrane through the homogenization process, followed by concentration of all large membrane fragments by low speed centrifugation (107). Unfortunately, many of the surface stabilizing agents have drastic effects on the surface membranes, including irreversible crosslinkage of surface proteins or inhibition of enzymatic activities (108).

Scarborough, in work on the fungus Neurospora crassa, developed a novel technique in which the plant lectin concanavalin A was used as the surface stabilizing agent (109). Concanavalin A, a multivalent lectin specific for mannose residues, can crosslink the surface glycoproteins of the cell and reinforce the plasma membrane structure. In addition, the binding of large amounts of concanavalin A to the surface membrane increases the density of that organelle. The concanavalin A bound to the cell surface can be partly removed by treatment with alpha-methyl mannoside, thus reversing the density and structural effects. As a result, this procedure avoids chemical modification of the cell membranes, and it does not interfere with most enzyme activities.

C). Isolation of the Plasma Membrane of E. histolytica

Concanavalin A bound avidly to the surface of trophozoites of E. histolytica. However, prolonged exposure to high levels of the plant lectin were toxic, even at low temperatures. Scatchard analysis of concanavalin A binding to intact trophozoites at 4°C showed a maximal binding of 2.6×10^8 molecules of concanavalin A / trophozoite (fig 12). For purposes of membrane reinforcement, we treated 10^7 trophozoites/ml with 0.5 mg/ml of concanavalin A in PD supplemented with 10 mM $MgCl_2$, for 5 minutes on ice. This protocol resulted in approximately 50% of maximal binding of concanavalin A. Under these conditions, amoebae were agglutinated within 30 sec and remained fully viable for up to 15 min.

Observations at light and electron microscope levels confirmed that concanavalin A did indeed help preserve the large sheet structure of the plasma membrane upon lysis of amoebae (fig 13). Trophozoites lysed under hypotonic conditions in a Dounce homogenizer displayed a range of small vesicles when viewed by the electron microscope. When trophozoites were first pretreated with concanavalin A and then homogenized a substantial number of large membrane sheets or scrolls were evident.

Our protocol for the isolation of plasma membranes from trophozoites of E. histolytica is shown in fig 14. Trophozoites, usually containing trace numbers of surface radiolabeled amoebae, were treated with concanavalin A. Excess concanavalin A was removed through centrifugation and the cells were resuspended in Tris buffer supplemented with 1mM $MgCl_2$. Cells were incubated in this buffer for 10 min and then homogenized in a Dounce homogenizer with a tight fitting pestle until microscopic observation showed lysis to be complete-- 6 to 8 strokes. The

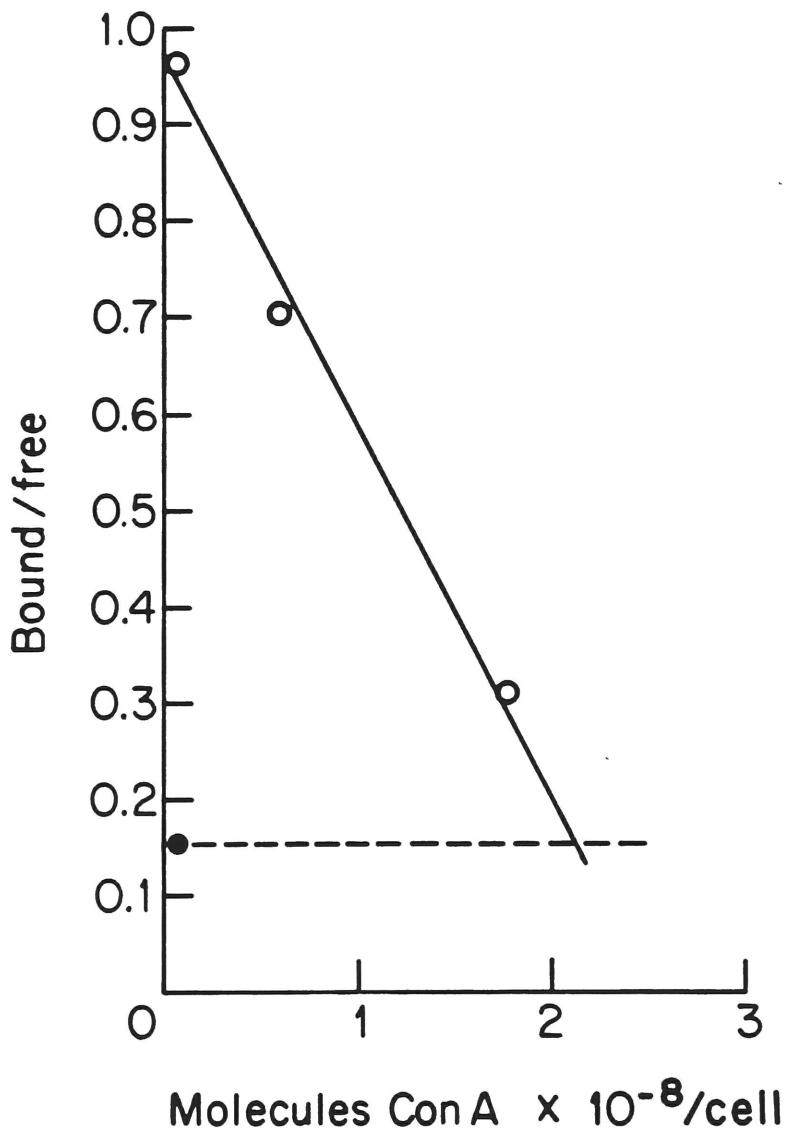


Fig. 12. Scatchard analysis of concanavalin A binding to *E. histolytica* strain 200. (—○—) Trophozoites incubated at 4°C with 5 to 500 µg/ml of concanavalin A. (---●---) Trophozoites incubated with 3 mg/ml of concanavalin A, a 5-fold excess.

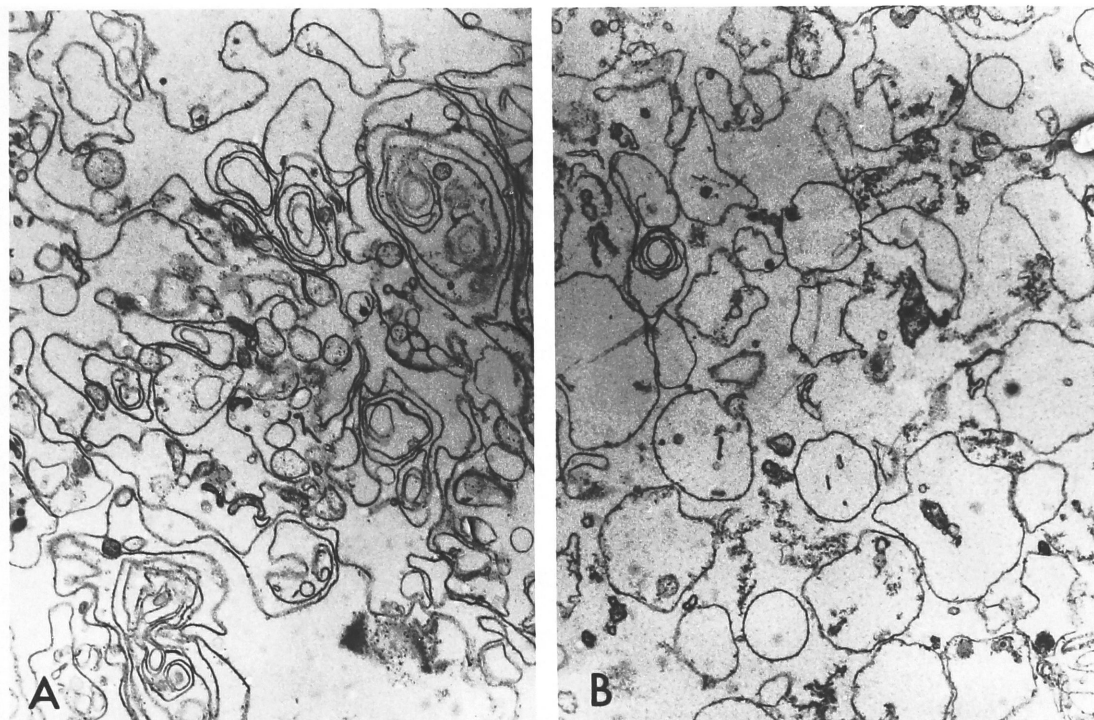


Fig. 13. Morphology of membranes in lysates of concanavalin A-treated amoebae, strain HK9, as determined by electron microscopy. (A) Trophozoites were treated with 0.5 mg/ml concanavalin A, homogenized, and pelleted in a Beckman microfuge. (B) No concanavalin A. (x5,300).

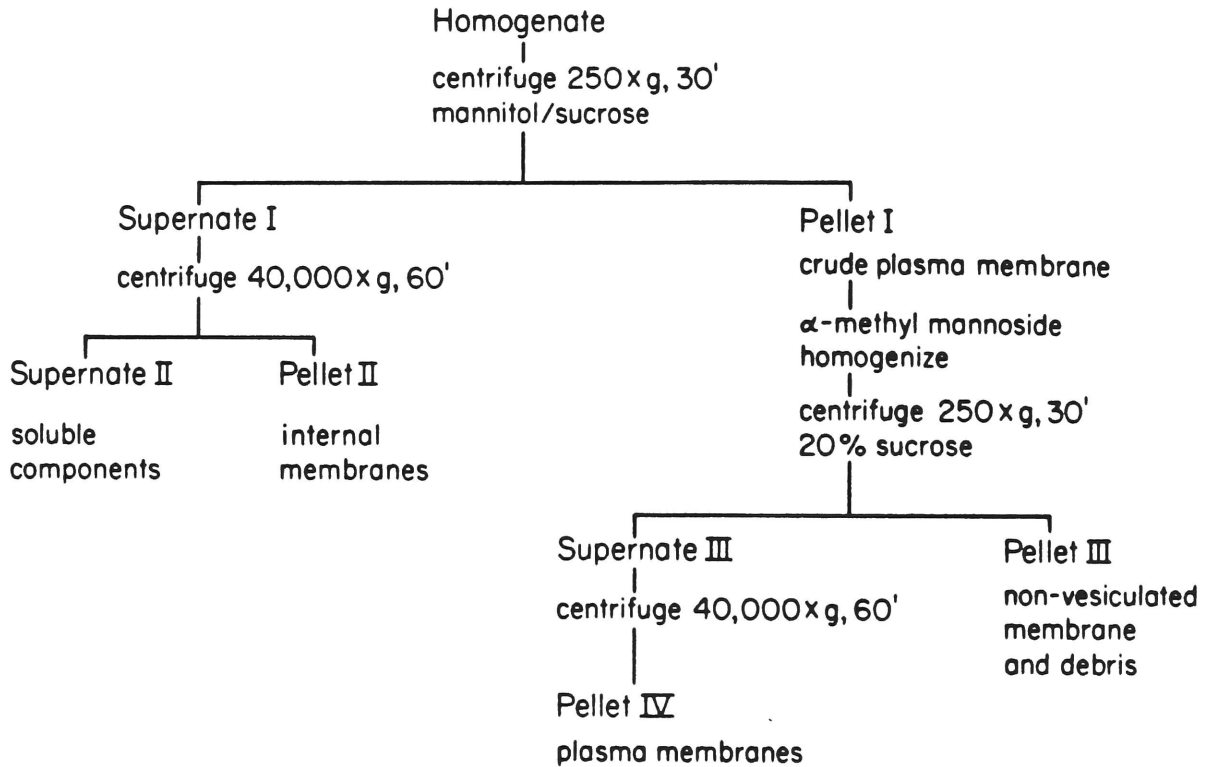


Fig. 14. Isolation procedure for E. histolytica plasma membranes.

surface membrane remained largely intact, forming large sheets or scrolls (fig 15 a). The cell homogenate was then spun at 250 x g for 30 min through a step gradient consisting of 0.5 M mannitol layered over 0.58 M sucrose, both in Tris buffer. Observations of the various sub-cellular fractions by electron microscopy demonstrated that while the bulk of the membrane, comprised of small vesicles, was retained above the mannitol phase (fig 15 b), large membrane sheets were concentrated in the pellet at the bottom of the gradients (fig 15 c). For purposes of analysis, this initial supernate, Supernate I, was centrifuged at 40,000 x g to obtain a soluble molecule fraction, Supernate II, and an internal membrane fraction, Pellet II. Over 90% of the total cell protein and nucleic acids were recovered in the soluble molecule fraction.

When TCA precipitable ^{125}I was monitored (Table I), 67% of the radiolabel was associated with the large membrane sheets in the crude plasma membrane fraction, Pellet I. Only 10% of this surface label was associated with the internal membranes of Pellet II. 22% of the precipitable ^{125}I was recovered in Supernate I. However, the major portion of TCA precipitable radiolabel in this fraction was shown by separation on SDS-PAGE not to be covalently associated with protein (data not shown).

Further purification of plasma membrane was achieved by treating Pellet I with 1 M alpha-methyl mannoside in Tris buffer on ice for 40 min. Alpha-methyl mannoside treatment resulted in the release of more than 50% of the concanavalin A, as revealed by addition of trace amounts of ^3H -concanavalin A, and resulted in a substantial density change in the membranes. Removal of concanavalin A also resulted in the loss of the crosslinking reinforcement of the plasma membranes. Upon homogenization, the alpha-methyl mannoside treated membranes were fragmented.

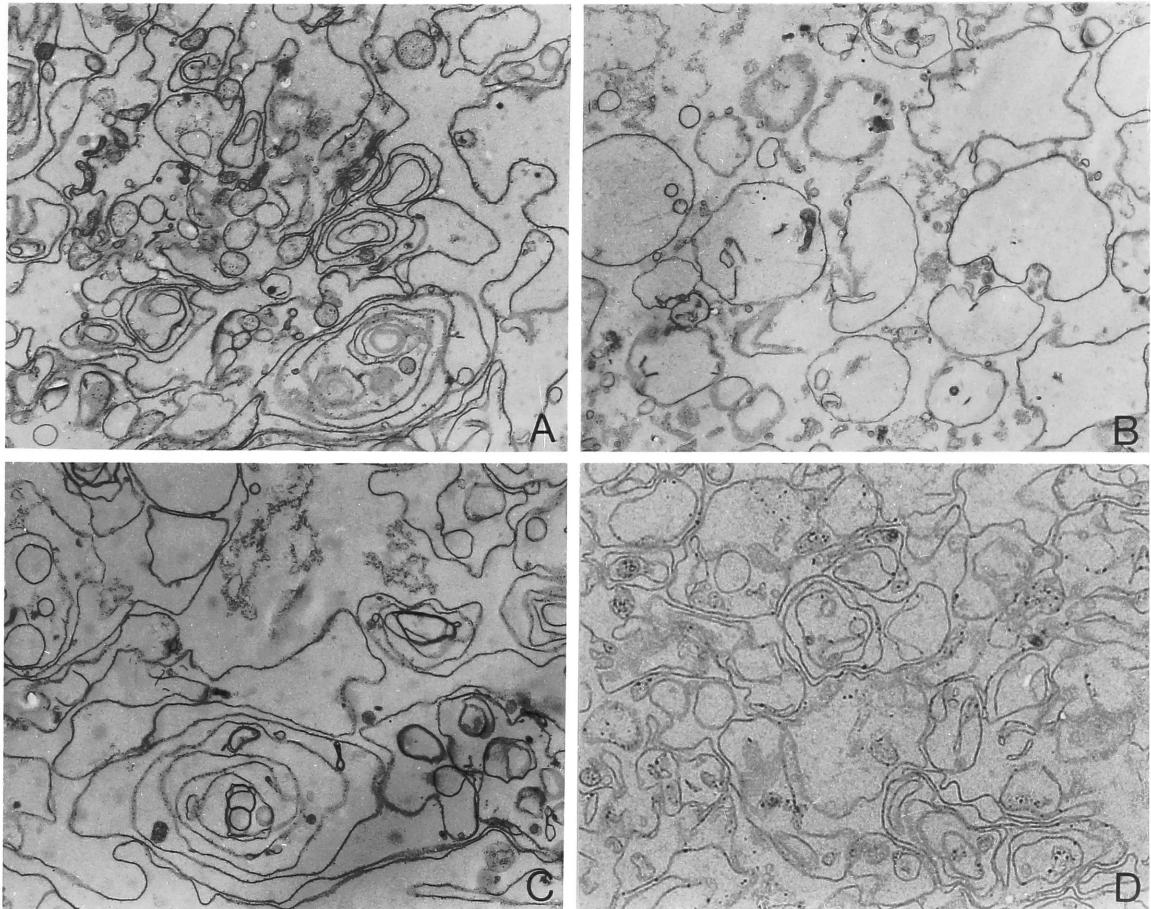


Fig. 15. Morphology of subcellular fractions of concanavalin A-treated amoebae as determined by electron microscopy. Trophozoites of strain HK9 were homogenized and membranes were fractionated as in fig 14. Subcellular fractions were pelleted in a Beckman microfuge and processed for electron microscopy. (A) Cell homogenates. Scrolls and large membrane sheets are visible. (B) Internal membrane vesicles of supernate I. Few scrolls and membrane sheets are seen. (C) Crude plasma membranes of pellet I. 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. (x6,700).

TABLE I

Summary of *E. histolytica* Plasma Membrane Isolation

Fraction	Protein recovery	¹²⁵ I		ATPase		Acid Phosphatase		NAGase [#]	
	%	Recovery	Sp Act	Recovery	Sp Act	Recovery	Sp Act	Total	Latent
Homogenate	100	100	1.0	100	1.0	100	1.0	100	100
Soluble proteins (Supernate II)	87±1.3	22.2±8.1	0.3	3.8±2.1	0.1	7.0±1.3	0.1	55.5	
Internal membranes (Pellet II)	10.2±1.3	10.5±1.7	1.0	59.6±4.1	5.8	70.0±7.8	5.7	30.4	80.0
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	3.5	9.2
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	2.8		
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	2.1	5.5

*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, 93.8, and 87.6 for protein, ¹²⁵I, ATPase, acid phosphatase, and NAGase, 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 protein.

[#]Values for NAGase assay are typical results from a representative experiment. Latency was calculated as (nonsedimentable activity in 0.1% Nonidet NP-40) - (nonsedimentable activity in untreated fraction).

The alpha-methyl mannoside treated crude plasma membranes were then centrifuged at 200 x g for 30 min onto a cushion of 20% sucrose. The resulting pellet, Pellet III, consisted of non-vesiculated plasma membrane and aggregated material. The material retained above the sucrose, Supernate III, consisted of small scrolls and vesiculated plasma membrane. The membranes in Supernate III were concentrated by centrifugation at 40,000 x g for 1 hr, both to remove unbound concanavalin A and to concentrate the membranes for analysis (fig 15 d). 28% of the ^{125}I surface label was associated with this purified plasma membrane fraction at an enrichment of over 30 fold (Table I). Overall recovery of the ^{125}I marker was 84%. Electron microscopy of a cross section of an oriented pellet of the purified plasma membranes revealed a uniform mat of vesicles (fig 16). We take these data to indicate that we have indeed separated the surface plasma membrane from both the soluble components of the cell and the majority of internal membranes.

COMPOSITION OF SUBCELLULAR FRACTIONS

A). Distribution of Peptides

Surface radiolabeled trophozoites were homogenized, and the membranes fractionated as described. A sample containing 50 μg of protein was taken from each of the subcellular fractions and solubilized in SDS. The peptides were separated by SDS-PAGE and visualized by staining with Coomassie blue (fig 17). A peptide comigrating with concanavalin A (see arrow, fig 17) was seen as a distinct band in the crude plasma membrane fraction (fig 17 d) but was not detected in the internal membrane frac-

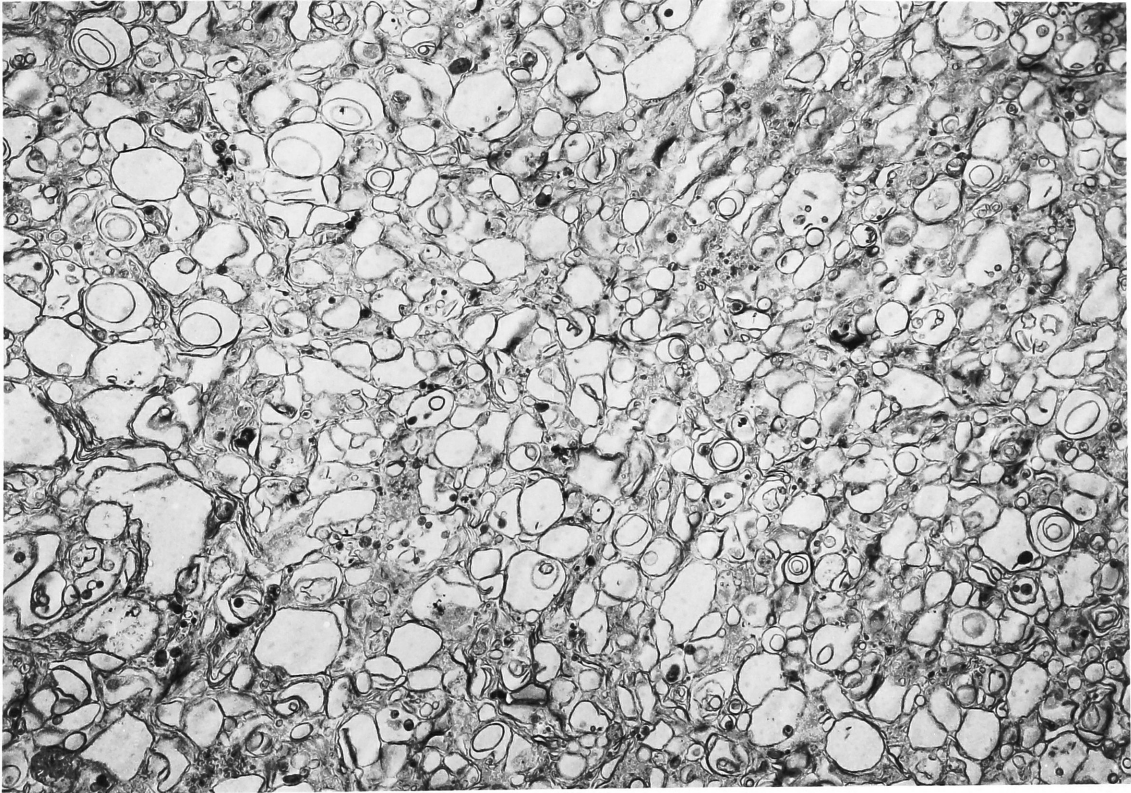


Fig. 16. Oriented pellet of purified plasma membranes. Membrane vesicles from pellet IV were centrifuged at 80,000 x g for 1 hr. Pellet was removed and fixed for electron microscopy. Sections were cut through the entire thickness of the pellet. Left is bottom and right is top of the pellet. The plasma membrane fraction appears homogeneous by this criterion. (x3,000).

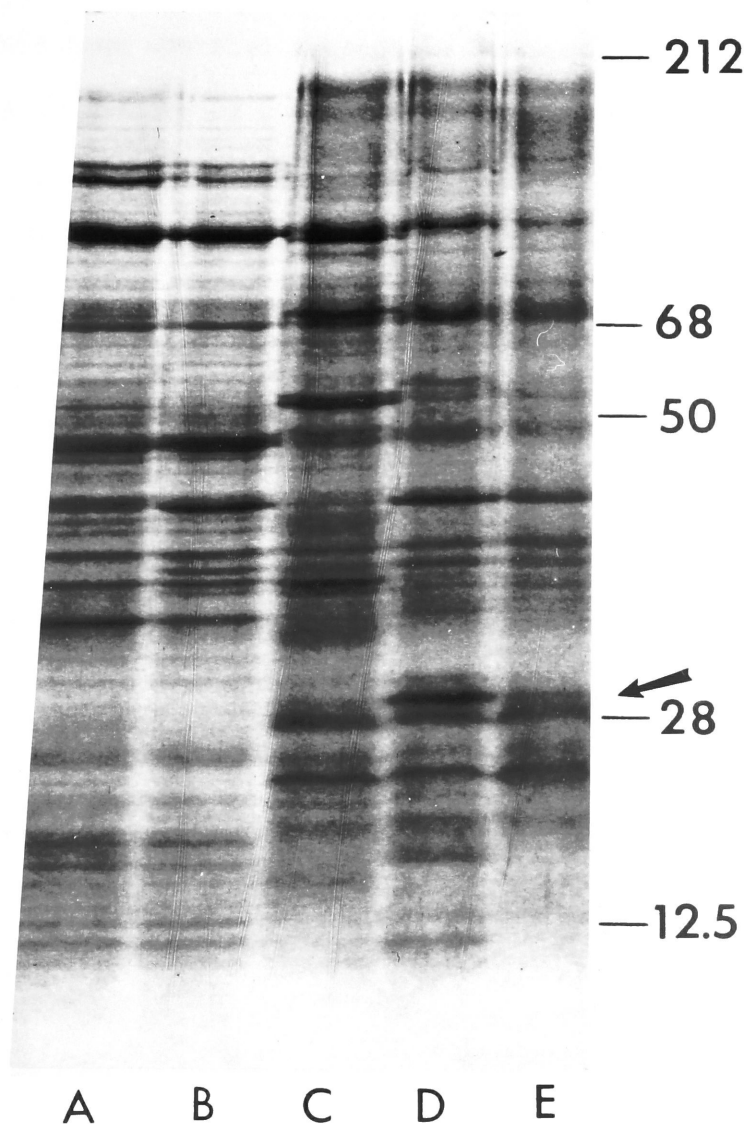


Fig. 17. Total peptide composition of the various subcellular fractions obtained by the plasma membrane isolation scheme outlined in fig 14. Intact trophozoites of strain HK9 were iodinated and homogenized, and the membranes fractionated as in fig 14. 50 μ g of protein from each fraction was separated by SDS-PAGE and stained for protein with Coomassie blue. (A) Homogenate. (B) Cytosol (Supernate II). (C) Internal membranes (Pellet II). (D) Crude plasma membranes (Pellet I). (E) Purified plasma membranes (Pellet IV). Arrow indicates migration position for concanavalin A.

tion (fig 17 c). This was consistent with the predicted distribution of concanavalin A. This band was also present in the purified plasma membrane fraction (fig 17 e), but at a much lower level than for Pellet I -- consistent with the partial release of concanavalin A by alpha-methylmannoside. One could visualize two general peptide patterns among the subcellular fractions. In particular, the peptides of the Homogenate and Supernate II (figs 17 a and b, nearly 90% of the homogenate protein was recovered in the soluble fraction, Supernate II) presented a pattern quite distinct from the pattern of the three membrane containing fractions. In contrast, the membrane containing fractions from both the surface and from the interior of the cell showed a strong similarity, especially in the high molecular weight portion of the gel pattern (figs 17 c, d, and e).

When this same gel was subjected to autoradiography, the ^{125}I labeled peptides could be visualized in each fraction (fig 18). A faint general pattern was visible in the Homogenate (fig 18 a), but few if any radiolabeled peptides were visualized in the soluble fraction (fig 18 b) or among the internal membranes (fig 18 c). In contrast, the peptides in the plasma membrane fractions were heavily radiolabeled (figs 18 d and e). Each of the major surface radiolabeled peptides of whole cells was present in the final plasma membrane fraction (fig 18 e), indicating that a representative portion of the plasma membrane had been isolated.

We further compared the surface radiolabeled peptides of intact cells to the Coomassie stained peptides of the final plasma membrane fraction (fig 19). While co-migration on SDS-PAGE cannot be considered proof of identity, we were encouraged by the close agreement between the two patterns. In particular, each of the surface radiolabeled peptides

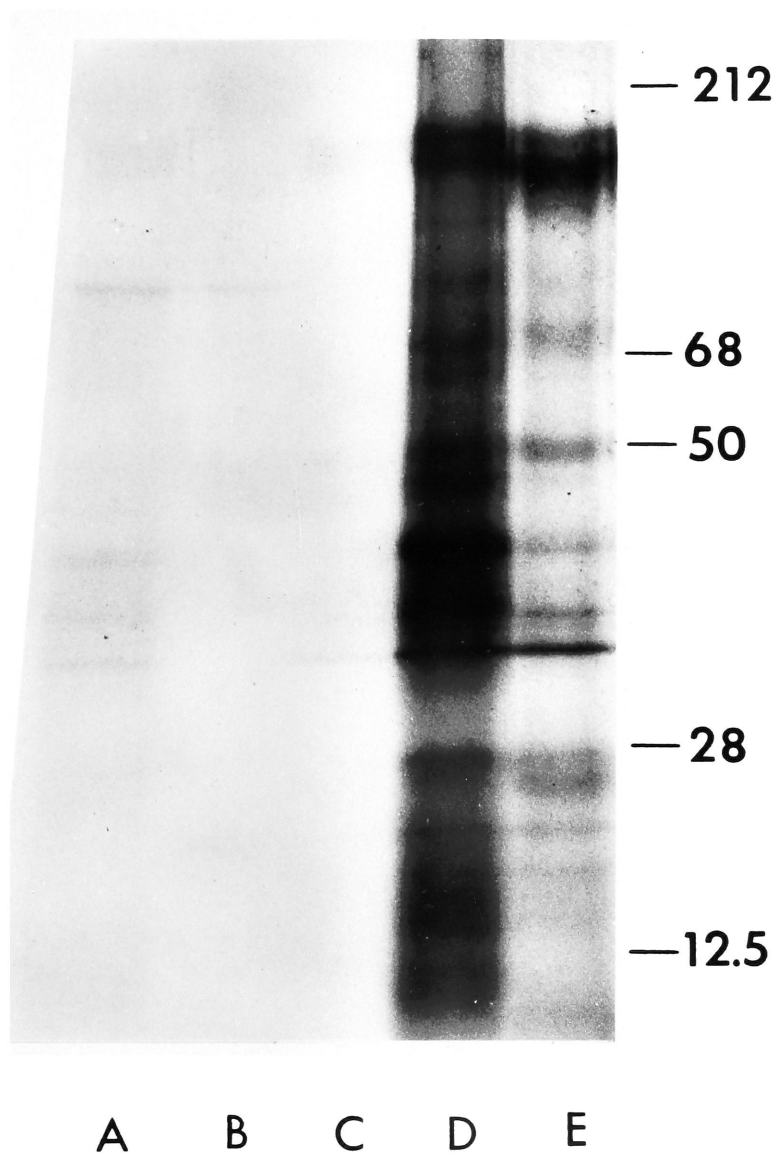


Fig. 18. Autoradiogram of the radiolabeled peptides from the SDS-PAGE shown in fig 17. (A) Homogenate. (B) Cytosol (Supernate II). (C) Internal membranes (Pellet II). (D) Crude plasma membranes (Pellet I). (E) Purified plasma membranes (Pellet IV).

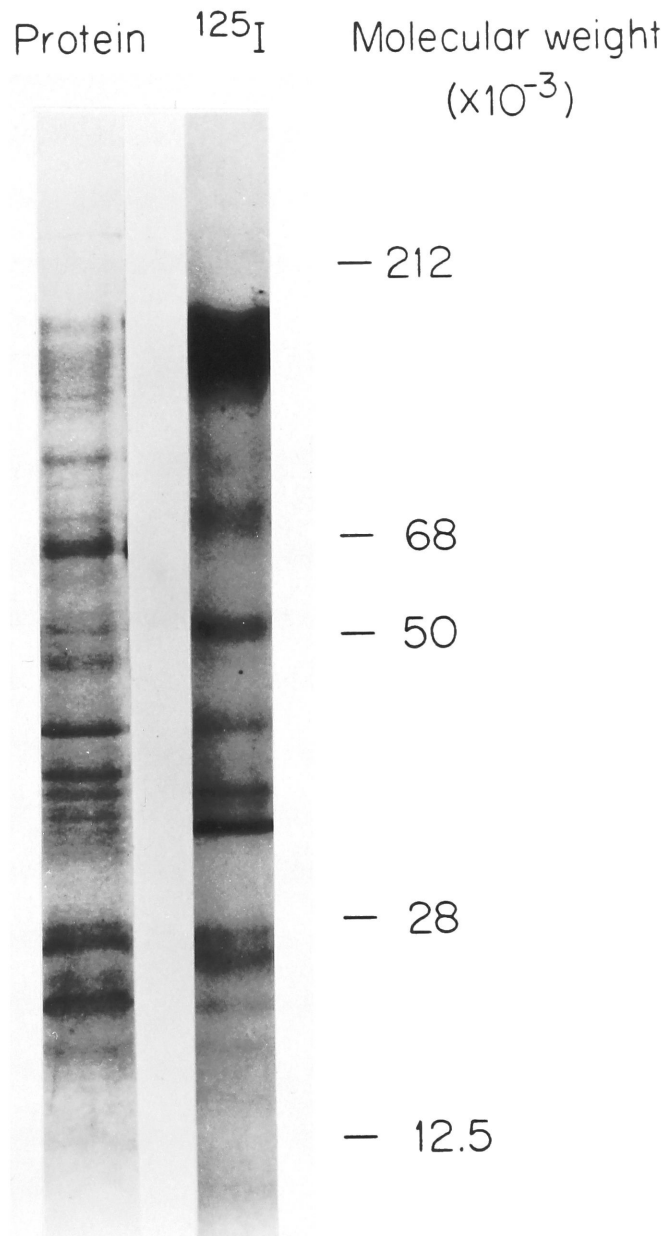


Fig. 19. Peptides of *E. histolytica* plasma membranes. Plasma membranes or iodinated intact trophozoites were solubilized in SDS and subjected to SDS-PAGE. [¹²⁵I]: Autoradiogram of the plasma membrane peptides. Each of the major iodinated peptides was isolated in this fraction. [Protein]: Total peptides of the plasma membrane visualized by Coomassie blue staining. Note that the position of each radiolabeled band corresponded to a Coomassie blue-stained peptide. Molecular weight standards are the same as those in fig 10.

had a Coomassie stained counterpart in the final plasma membrane fraction. An additional 6 Coomassie stained bands were associated with our plasma membrane fraction, but were not surface radiolabeled. This was not unexpected, as lactoperoxidase - glucose oxidase catalysed iodination can label only those peptides with externally disposed tyrosine residues.

B). Distribution of ^{35}S -Cystine Labeled Peptides

Labeling of surface peptides with ^{125}I does not indicate the origin of the radiolabeled peptides. To prove unambiguously that these peptides were of amoebic origin, we attempted to radiolabel peptides metabolically by adding radiolabeled amino acids to the culture medium. Unfortunately, the TYI culture medium is complex, containing a variety of amino acids. Radiolabeled peptides were not detected after 24 hr of incubation in medium containing 5 $\mu\text{Ci/ml}$ of a mixture of ^3H -amino acids or with 5 $\mu\text{Ci/ml}$ of ^{35}S -methionine. Significant incorporation was obtained only with ^{35}S -cystine, and then only in a modified TYI medium in which cysteine was omitted. Trophozoites could be maintained in cysteine free medium containing ^{35}S -cystine for 15 hr before the growth rate of the amoebae slowed. Viability, as assessed by trophozoite motility and trypan blue exclusion, remained at normal levels (>95%). We normally incubated cells with radiolabel for 12 hr, or just under one cell division, in medium containing 5 $\mu\text{Ci/ml}$ ^{35}S -cystine.

The proportion of cystine in known proteins varies widely. As we were limited to this single amino acid, it was important to determine how representative of total cell proteins our radiolabeled peptides

were. The uniformity or asymmetry of this radiolabeling procedure was readily assessed by SDS-PAGE analysis. If radiolabel were incorporated to a similar degree in all cell peptides, we would expect the distribution of radiolabel detected by autoradiography to approximate the distribution of protein detected by Coomassie stain. When peptides from ^{35}S -cystine labeled trophozoites were analysed in this manner (fig 20), it was apparent that the distribution of radiolabel was not representative of the distribution of protein. In particular, a number of peptides of 100,000 to 150,000 molecular weight were labeled far more heavily than one would predict from the intensity of the Coomassie blue staining (compare figs 20 c and g). As these gels were run under conditions of protein denaturation and in the presence of a reducing agent, a covalent, non-disulfide linkage can be assumed.

^{35}S -cystine labeled trophozoites were homogenized, and membranes fractionated as outlined in fig 14, except that the concanavalin A release steps were not performed. A comparison of radiolabel and protein distributions (Table II) again emphasize that labeling with ^{35}S -cystine was not uniform. Membrane containing fractions (Pellets I and II) contained only 15% of the total cell protein, yet they accounted for over 25% of the total incorporated radiolabel. The specific activities, relative to the starting homogenate, increased 1.4-fold for internal membranes and 3.2-fold for plasma membranes, with the specific activity of soluble proteins decreasing to only 0.75 of the amoebic homogenate. SDS-PAGE analysis of the subcellular fractions (fig 20 e - h) indicated that the heavily radiolabeled 100,000 to 150,000 molecular weight peptides were membrane associated. These ^{35}S -cystine containing peptides co-migrated with the major ^{125}I high molecular weight surface

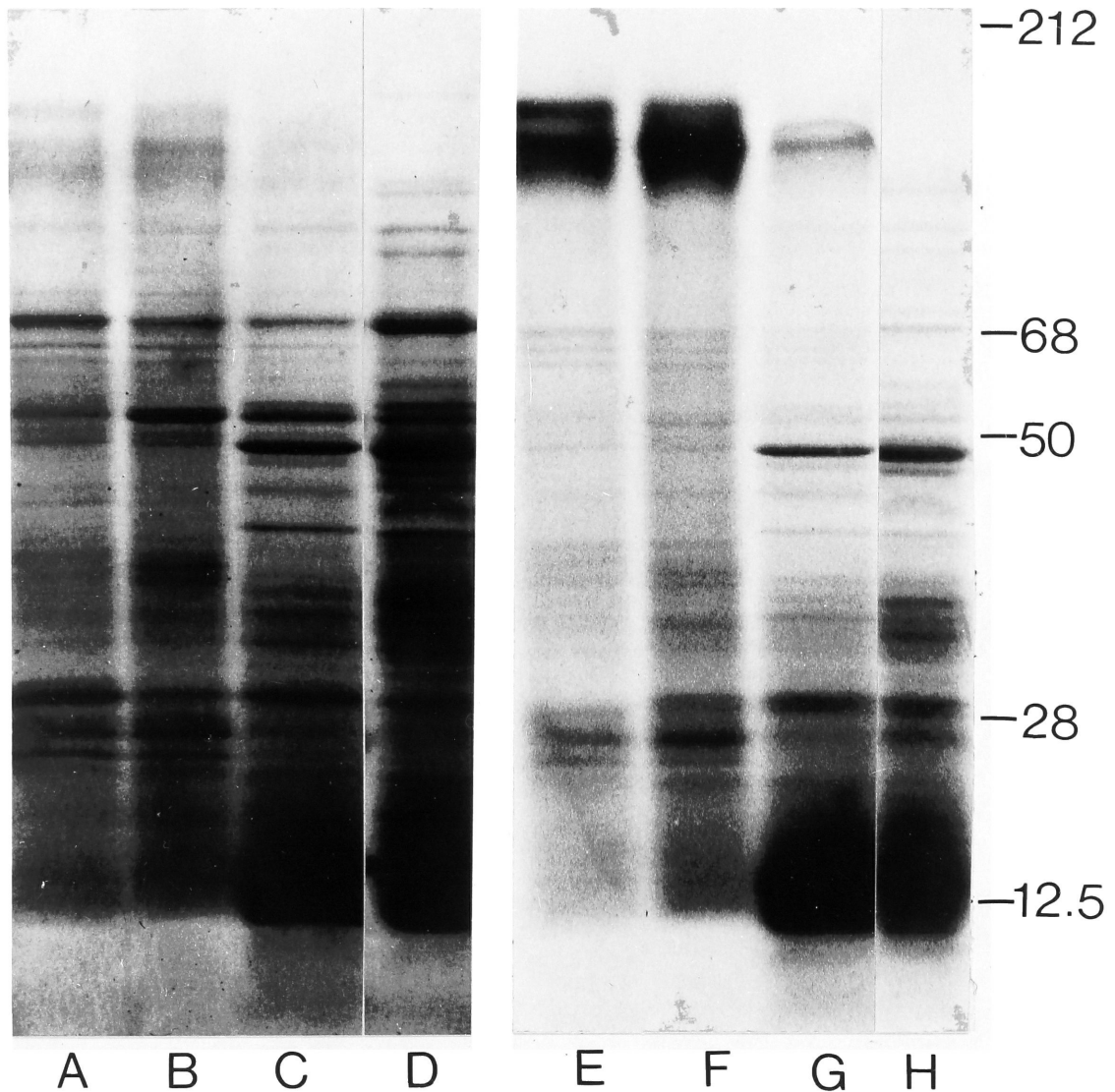


Fig. 20. Distribution of total and ^{35}S -cystine labeled peptides of *E. histolytica* strain HM1. Trophozoites were incubated in $5\ \mu\text{Ci/ml}$ of ^{35}S -cystine for 15 hr, then washed and lysed. Membranes were fractionated as outlined in fig 14, and peptides separated by SDS-PAGE. (A-D) Total peptides visualized by Coomassie blue staining. (E-H) autoradiography of ^{35}S -cystine labeled peptides from the same gel. (A, E) Crude plasma membranes (Pellet I). (B, F) Internal membranes (Pellet II). (C, G) Whole cell homogenate. (D, H) Cytosol (Supernate II). Molecular weight markings are $\times 10^{-3}$.

Table II

Distribution of Metabolically incorporated ^{35}S -Cystine
among proteins of *E. histolytica**

Sample	Protein		TCA Ppt		Sp Act
	μg	%	dpm	%	
Homogenate	5940	100.0	970	100.0	1.0
Soluble Protein	5450	91.8	669	69.0	.75
Internal Vesicles	780	13.1	177	18.2	1.4
Plasma Membranes	130	2.2	69	7.1	3.2

*Overall recoveries were 107.1 for protein and 94.3 for ^{35}S -cystine. Specific activity was calculated as counts per minute per mg protein. The specific activity of the homogenate was arbitrarily set equal to 1.0.

radiolabeled peptides of the trophozoite.

C). Distribution of Enzyme Markers

Protein, nucleic acid, and four enzymatic activities were monitored throughout the plasma membrane fractionation procedure. Alcohol dehydrogenase, a soluble enzyme (81), was found solely in the cytosol (Supernate II). No activity was found in any membrane containing fraction. Likewise, all of the detectable nucleic acid was recovered in the soluble fraction, and no intact nuclei were seen nor was nucleic acid 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 is strongly membrane associated (110). Greater than 85% of the activity was pelleted by centrifugation of frozen and thawed cells at 40,000 x 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 5% of the total activity at a 3-fold 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 beta-glycerol phosphate (111) as substrate equally well. We also assayed subcellular fractions for a second lysosomal hydrolase, N-acetyl-glucosaminidase, which is a soluble intralysosomal enzyme both in mammalian cells and in E. histolytica. N-acetyl-glucosaminidase levels, corrected for latency, indicated that the final

plasma membrane fraction was contaminated with 6-12% of total lysosomes (Table I).

A calcium-dependent ATPase recently described by McLaughlin and Müller (82) was included in this analysis (Table I) as it was 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, over 19% of the activity was localized in the plasma membrane fraction, with an enrichment of almost 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.

D). Sterol Composition

Cholesterol is a required growth factor in the TYI culture medium (Sawyer) and was the only sterol detected in E. histolytica. Each of the strains we tested had similar overall amounts of cholesterol, 2.2 μg / mg protein. We could detect no cholesterol esters. We analysed the molar ratio of cholesterol to lipid phosphorus in the cell homogenate, the internal membranes, and in the plasma membrane fraction. Both the cell homogenate and the internal membranes had cholesterol to phospholipid ratios of 0.30. The plasma membrane fraction, however, was enriched with cholesterol to a ratio of 0.87. Enrichment of sterol is characteristic of the plasma membranes of a number of cell types

(104,112,113).

E). Identification and Distribution of Phospholipids

Nine phospholipid species could be distinguished when trophozoite lipids were extracted and separated by two-dimensional thin layer chromatography (fig 21). The major phospholipid in extracts of amoebae was identified as phosphatidylcholine (PC) by co-migration with standards and a positive reaction with Dragendorff's reagent. Phosphatidic acid (PA), phosphatidylinositol (PI), and phosphatidylserine (PS) were further identified by co-migration with standards. PS was also identified by its reaction with ninhydrin. Ceramide aminoethyl phosphonate (CAEP), which was also ninhydrin positive, was resistant to digestion by mild alkali, consistent with a ceramide linkage (93). 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 (94,104,112).

Two phosphatidylethanolamine species (PE_1 and PE_2) were tentatively identified. Both were ninhydrin positive, and both had R_f values appropriate for phosphatidylethanolamine. Two PE species have also been characterized in the related reptilian parasite E. invadens (114).

Sphingomyelin was identified by co-migration with a standard and by its resistance to mild alkaline digestion. The minor phospholipids X and Y were not identified.

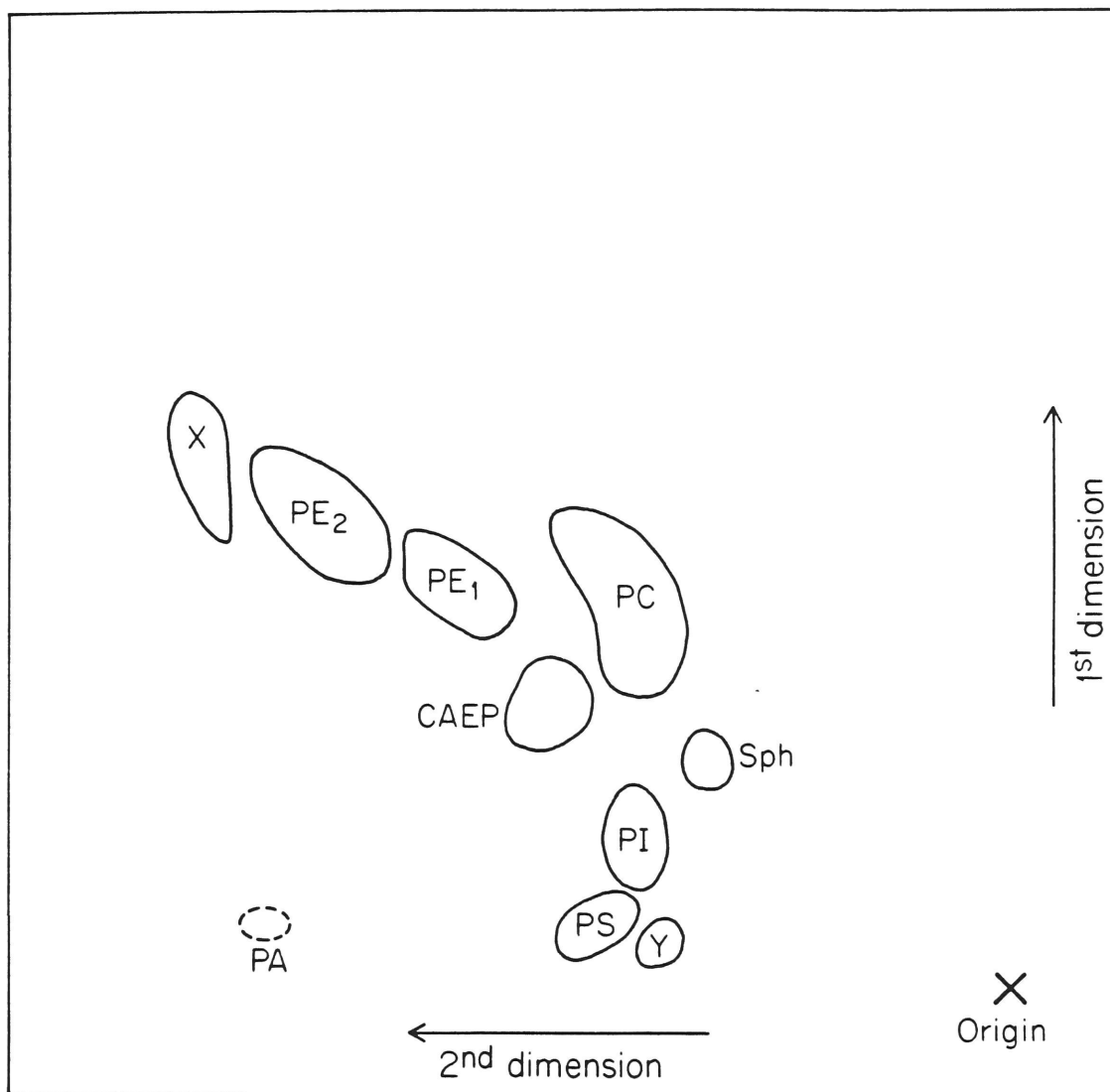


Fig. 21. Separation of whole cell phospholipids from *E. histolytica* strain HK9 by two-dimensional thin-layer chromatography.

The phospholipid composition of whole cells, internal vesicles, and the plasma membrane are compared in fig 22. Whole cells and internal vesicles have similar compositions, with PC as the dominant phospholipid, followed by the two classes of PE. Plasma membranes, however, were greatly enriched in CAEP and PE₂ and correspondingly deficient in PC and PE₁.

F). Incorporation of ³²P into *E. histolytica* Phospholipids

It is known that Entamoeba can take up fatty acids from the surrounding medium (115), and that cholesterol is required in the medium as a growth factor (116). The bovine serum used in the culture medium of *E. histolytica* is also a potential source of phospholipid. Upon analysis we found that bovine serum contained substantial amounts of the phospholipid species, phosphatidylcholine. Because the greatest differences in phospholipid distribution involved PC, we wanted to be sure that possible incorporation of phospholipid from the medium by *E. histolytica* could not account for the asymmetrical distribution. To this end we incubated cultures with 5 to 10 $\mu\text{Ci/ml}$ ³²PO₄ for 4 days, to label phospholipids de novo, and extracted cell phospholipids as before. The separated phospholipids were visualized by autoradiography of the chromatograms. The radiolabel content of each phospholipid was determined by liquid scintillation counting after elution from the gel by chloroform : methanol (2:1). The autoradiogram of the separated phospholipids of whole cells (fig 23 a) revealed each of the major species detected by chemical methods, however, the relative quantity of PC was reduced. This implied that *E. histolytica* trophozoites can take up

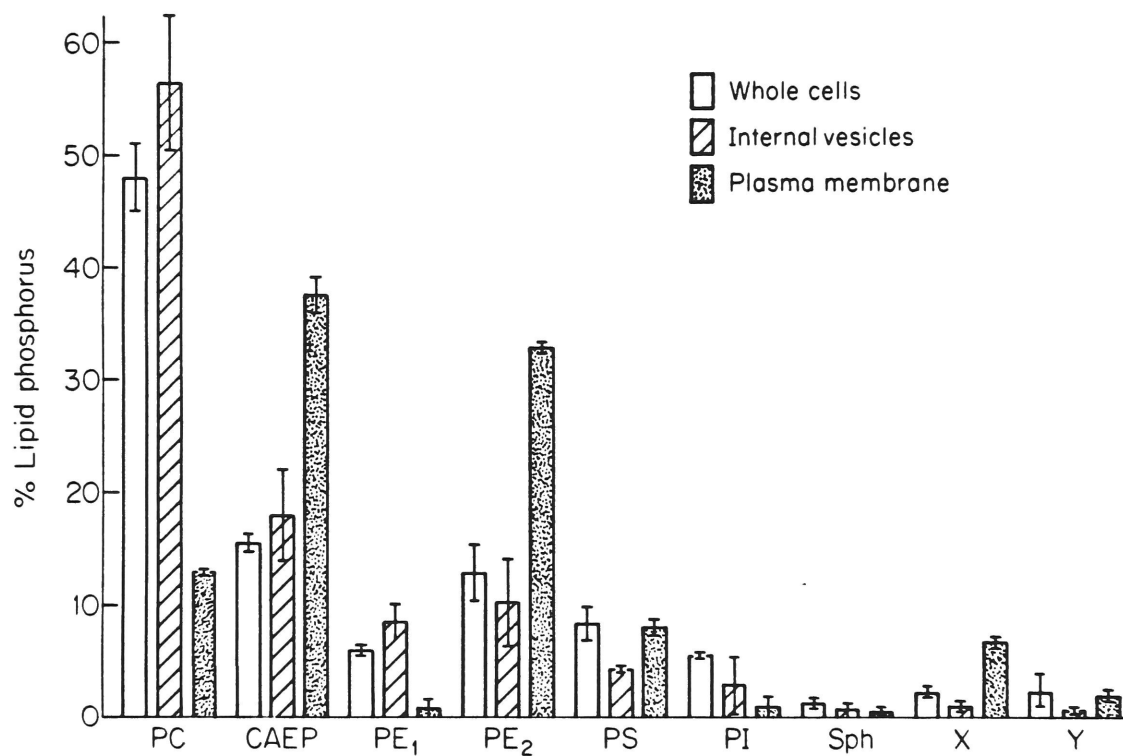


Fig. 22. Phospholipid composition of whole cells, internal vesicles (Pellet II), and plasma membranes (pellet IV). The lipids of whole amoebae and the isolated membrane fractions were extracted and subjected to two-dimensional thin-layer chromatography, and the phosphorus content of individual phospholipids was determined. Values are means \pm SE of three experiments.

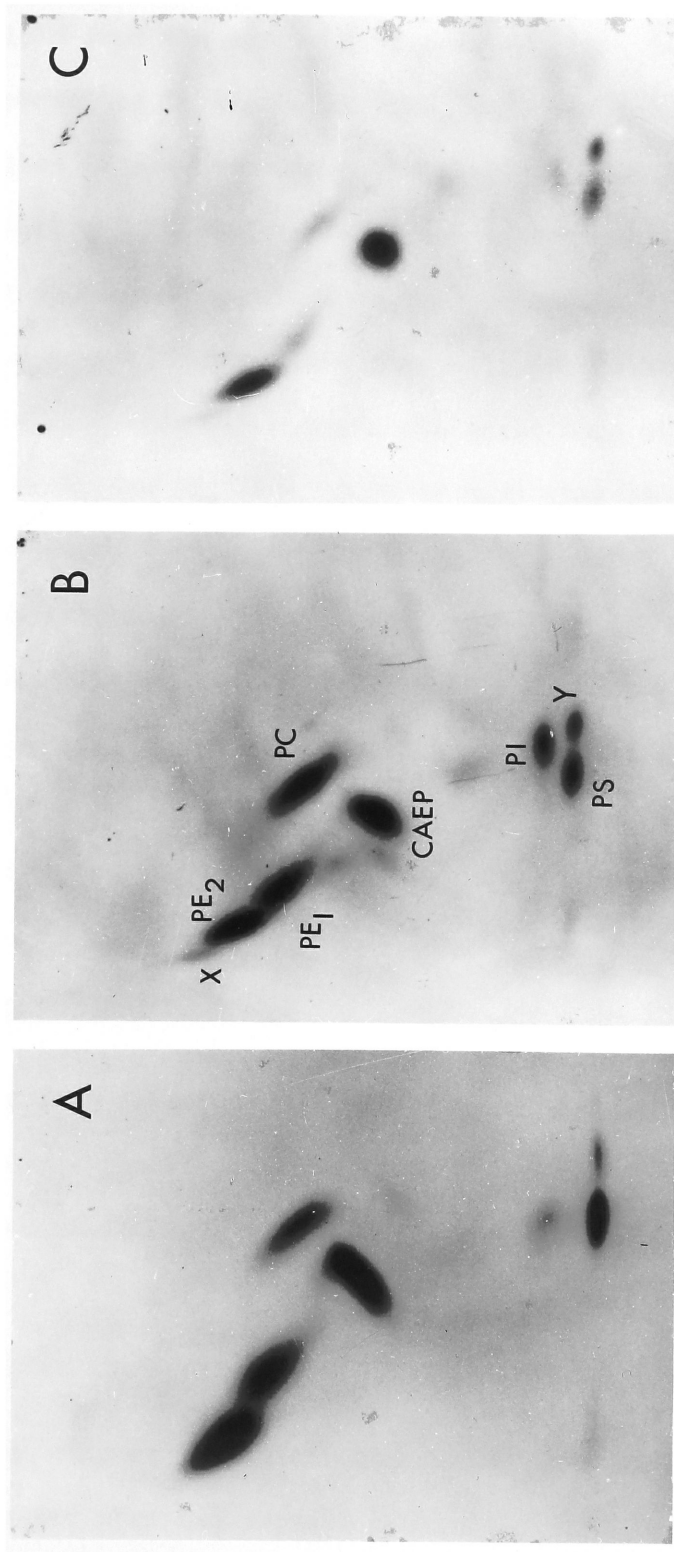


Fig. 23. Two-dimensional thin-layer chromatograph of ^{32}P labeled phospholipids from E. histolytica strain HK9. Trophozoites were incubated with $5\text{ }\mu\text{Ci/ml}$ of ^{32}P for three days. Subcellular membranes were obtained as outlined in fig 14, and the phospholipids extracted and separated. (A) Whole cell homogenate. (B) Internal membranes (Pellet II). (C) Crude plasma membranes (Pellet I).

phospholipid from the surrounding medium.

Trophozoites labeled with $^{32}\text{PO}_4$ were homogenized and fractionated as described before, and the radiolabeled phospholipid distribution of the internal and plasma membranes was determined (fig 23 b and c, Table III). As mentioned for whole cell phospholipid, PC was radiolabeled to a lesser degree than the other phospholipid species. However, the asymmetry seen with chemical methods was again apparent. PC varies from 20.9% to 8.0%, and PE_1 from 18.9% to 8.1% when comparing internal and plasma membranes. CAEP and PE_2 change from 22.9% to 35.6% and 18.1% to 29.0%, respectively. These results indicated that regardless of whether one considers total phospholipid or de novo synthesized phospholipid, the trophozoites maintained a definite asymmetry in phospholipid distribution. This asymmetry involved the substantial enrichment of both PE_2 and the phosphonolipid CAEP in the plasma membrane of this organism.

G). Fatty acid composition

Fatty acid composition of whole amoebae consisted mainly of fatty acids found in the serum (Table IV). No differences were discerned among extracts of whole cells or plasma membranes.

H). DISCUSSION

In our efforts to isolate the plasma membrane of E. histolytica, we were presented with two initial problems. First was the lack of a defined surface enzyme activity which could be used as a marker for the plasma membrane. Second was a problem of insufficient resolution of plasma membrane from other cell membranes when either differential or

Table III

Distribution of ³²P Labeled Phospholipids from Membrane
Fractions of *E. histolytica* Trophozoites

Phospholipid Species	Whole Cells %	Internal Vesicles %	Plasma Membrane %
PS	11.2	7.2	7.6
PI	2.7	5.5	2.8
Sph	0.5	0.7	0.3
PC	10.4	20.9	8.0
CAEP	23.7	22.9	35.6
PE1	19.2	18.9	8.1
PE2	28.5	18.1	29.0
X	0.6	2.0	2.5
Y	2.2	2.2	3.8
PA	1.0	1.5	2.4

Table IV

Distribution of Fatty Acids in Membrane
Fractions of *E. histolytica*

Fatty Acid	Homogenate mass %	Plasma Membrane mass %
16:0	38.8	39.9
16:1	4.7	6.5
18:0	7.2	9.1
18:2	23.3	19.1
18:3		
+20:1	8.7	9.5
20:4	4.7	3.2

isopycnic centrifugation methods were used as separation techniques.

To provide an unambiguous marker for the plasma membrane, we radiolabeled cell surface proteins with ^{125}I using lactoperoxidase and glucose oxidase. The relatively mild conditions of this coupled enzyme system did not modify the viability or functions of amoebae. Surface iodination of intact amoebae radiolabeled approximately 12 peptides, ranging from 12,000 to 200,000 molecular weight. These radiolabeled peptides were distinct from the major soluble proteins of E. histolytica lysates, as judged by migration on SDS-PAGE. Furthermore, they remained associated with the trophozoite membranes throughout the fractionation procedure. The major surface radiolabeled proteins were shown to be glycoproteins by their specific adherence to and elution from a concanavalin A lectin column. The peptides of 90,000 and 150,000 molecular weight were directly shown to have sugar residues by periodate reduction and tritiated borohydride. Glycosylation of peptides is consistent with both secreted and membrane associated proteins.

Our procedure for the isolation of the trophozoite plasma membrane was based on a novel procedure first described by Scarborough (109). This procedure takes advantage of membrane crosslinking and density change by surface bound concanavalin A to greatly enhance the resolving power of classical centrifugation techniques. It was first described for use on the fungus Neurospora crassa, and has subsequently been applied to yeast (117) and astrocytoma cells (118).

We have demonstrated that preincubation with concanavalin A stabilized the plasma membrane of E. histolytica and maintained it in large sheets during homogenization. It was then separated from internal vacuoles and vesicles by low-speed centrifugation. Subsequent removal of

the lectin by treatment with alpha-methyl mannoside resulted in changes in membrane density and loss of the crosslinking reinforcement. The treated membranes were homogenized. The resulting changes in membrane size and density permitted further separation of plasma membrane from aggregated material and debris.

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 radiolabeled peptides evident in whole cell lysates were found in the enriched plasma membrane preparation, suggesting that the entire surface membrane had been isolated. A total of approximately 18 peptides were recovered with the plasma membrane. Of these, 12 co-migrated with the radiolabeled peptides demonstrated by autoradiography. This complex pattern of surface peptides greatly contrasts with that of the free living amoeba, Acanthamoeba castellanii. Korn and Wright (119) demonstrated that a single peptide of 15,000 mol wt constituted >80% of plasma membrane-associated protein in this protozoan. The reptilian parasite E. invadens is reported to have 5-9 peptides associated with its plasma membrane (112). The complexity in range and number of peptides of the surface of E. histolytica compares favorably with many mammalian cells.

Because of this unexpected complexity in peptide composition, we were concerned about the possibility of adsorption of serum proteins from the culture medium onto the surface of the trophozoite. Our surface radiolabeling and Coomassie staining techniques could not distinguish adsorbed proteins from amoeba derived peptides. We made several attempts to have amoebae incorporate radiolabeled amino acids into protein. However, we were unable to detect significant amoebic

incorporation of radiolabel into trophozoite peptides with most amino acids tried, presumably because of the complex nature of the TYI-S medium. The one exception was cystine, provided in the form of ^{35}S -cystine. When this radiolabeled amino acid was added to cysteine deficient TYI medium, trophozoites incorporated sufficient radiolabel into protein to permit analysis by SDS-PAGE.

An exceptionally high proportion of ^{35}S -cystine was incorporated into the membrane peptides in the molecular weight range of 100,000 to 150,000 in both the plasma membrane and internal membrane fractions. While this unambiguously demonstrates the amoebic origin of these peptides, the origin of the other peptides in the plasma membrane fraction remains unknown. We will again address the question of peptide origin as one aspect of the antigenic composition of trophozoites.

There are three possible mechanisms for the asymmetric incorporation of ^{35}S -cystine: (1) a significantly higher proportion of cystine residues occurs in the heavily labeled peptides, (2) the heavily labeled peptides turn over more rapidly relative to the time of incubation, or (3) a large pool of unlabeled cystine is available for the synthesis of all peptides except those heavily labeled.

The time of labeling covers almost one cell cycle. It would thus seem unlikely that either accelerated turnover or a large amino acid pool could account for the greater than four fold difference in specific activity. This leads us to believe that these peptides in the molecular weight range of 100,000 to 150,000 contain a relatively high proportion of cystine. As these peptides constitute a substantial proportion of the plasma membrane protein (by SDS-PAGE analysis), the potential for extensive inter- and intra-molecular crosslinking of such cystine rich

proteins could have important implications as to trophozoite surface structure and motility as well as to parasite defense and parasite host interactions. These cystine rich surface peptides might also help explain the culture requirements both for thiols and reducing conditions (29).

A substantial portion of an Ca^{++} -dependent ATPase was found to co-purify with the E. histolytica 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. This finding was in complete accord with the cytochemical localization of this activity.

An acid phosphatase activity has been proposed as a surface marker in E. invadens (104,105). 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 beta-glycerol phosphate, was found associated with internal vesicles. The second acid phosphatase activity, assayed with p-nitrophenyl phosphate, was found associated with both the plasma membrane and 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 latent N-acetyl glucosaminidase activity. This finding was also consistent with the cytochemical demonstration of surface acid phosphatase on some but not all trophozoites. 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 in E. histolytica.

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 (113) and E. invadens (104,112).

The phospholipid components of E. histolytica were similar to those reported for E. invadens and included one unusual phosphonolipid, CAEP. The phospholipid composition of the plasma membrane differed substantially from the composition of both whole cells and internal vesicles. In particular, PC and PE₁ levels were substantially lower in the plasma membrane, and both CAEP and PE₂ were correspondingly increased. PS may also be increased in the plasma membrane.

Because of the obvious asymmetry, we felt it important to demonstrate the amoebic origin of the analyzed phospholipid. To this end we added $^{32}\text{PO}_4$ to cultures and analyzed the distribution of radiolabeled phospholipids. We found that PC was radiolabeled to a lower specific activity than the other phospholipid species. As PC was also the major phospholipid species in bovine serum, this observation suggests that amoebae were able to incorporate at least the head group of exogenous PC. However, when the radiolabeled phospholipids of internal and plasma membrane fractions were determined, the same asymmetric distribution was found. Plasma membranes were low in PC and PE₁ and enriched in CAEP and PE₂.

Similar enrichment of PE and depletion of PC has been reported in membrane fractions of other protozoa, including the plasma membrane of A. castellanii (113) and the phagolysosomes (104) and plasma membrane (112) of E. invadens. The high proportion of the phosphonolipid, CAEP,

was more unusual, with similar levels reported only for ciliary sheaths of Tetrahymena (120). CAEP is a known inhibitor of most phospholipases (94,120). Thus, the surface localization of this species would be an important consideration in cytotoxic mechanisms involving phospholipase activity.

In contrast to the asymmetric distribution of the phospholipid classes, no differences were found in the distribution of fatty acids among the various membrane containing fractions.

Results:

Part III

Antigenic Analysis

ANTIGENIC COMPOSITION OF SURFACE MEMBRANE

Previous work on the antigenic composition of E. histolytica has centered on serology based diagnostic techniques, including gel diffusion techniques (121), derivatized red cell agglutination (122,123), and complement fixation (123,124). Of these procedures, only immunoelectrophoresis (IEF) has provided direct analysis of the antigens. The dependence of this procedure of gel diffusion, however, limits its use to the analysis of soluble antigens only. While analysis of the soluble antigens can be useful for strain or species differentiation, their relationship to host immunity is doubtful. Soluble antigens involve only secreted products or cytoplasmic components released following cell disruption. Protective antibodies would have to be directed against surface components of the trophozoite. Our studies focused on the amoebic surface antigens, through precipitation of surface radiolabeled peptides or of isolated plasma membrane peptides.

We had two goals in our studies of the antigenic composition of E. histolytica. The first was to identify which of the major surface proteins are derived from the amoebae, as opposed to the medium. The use of antigenic determinants to answer this question was made necessary by the inconclusive results of metabolic labeling of protein, presented in the previous section. The second goal was to identify the antigenic determinants on the surface of E. histolytica trophozoites and to compare those determinants with antigens recognized by the immune sera of infected patients. We have used two methods to analyze the surface antigens of E. histolytica. In the first procedure, either surface radiolabeled or metabolically radiolabeled proteins were precipitated by

a complex of formalin fixed Staphylococcus aureus and specific antibody. In the second procedure, unlabeled membrane proteins were separated on SDS-PAGE, transferred to nitrocellulose paper by the Southern blot procedure, and reacted with radiolabeled specific antibody. Both techniques can be performed in the presence or absence of competing unlabeled antigens.

A). Precipitation of ^{125}I Surface Labeled Peptides

To analyse the antigenic composition of the trophozoite surface proteins, intact cells were surface radiolabeled with ^{125}I and solubilized in NP-40. Antibody - staph reagent complexes were added, either in NET buffer or in neat bovine serum to precipitate specific antigens. These precipitated proteins were solubilized in SDS and separated on SDS-PAGE, and radiolabeled peptides were revealed by autoradiography (fig 24).

Amoebic lysates precipitated with pre-immune rabbit serum contain a slight background pattern of radiolabeled peptides, presumably due to non-specific adsorption (fig 24 a). Addition of unlabeled bovine serum to the precipitation mixture had no effect on the background pattern.

Amoebic lysates precipitated with a commercial goat antiserum against bovine serum proteins contain, in addition to the background pattern, three radiolabeled peptides at molecular weights of 72,000, 68,000, and 50,000 (fig 24 b). The two peptides of lower molecular weight were most likely the bovine serum proteins albumin and immunoglobulin G — presumably adsorbed from the culture medium. Together these two proteins comprise over 74% of serum proteins and would be the major

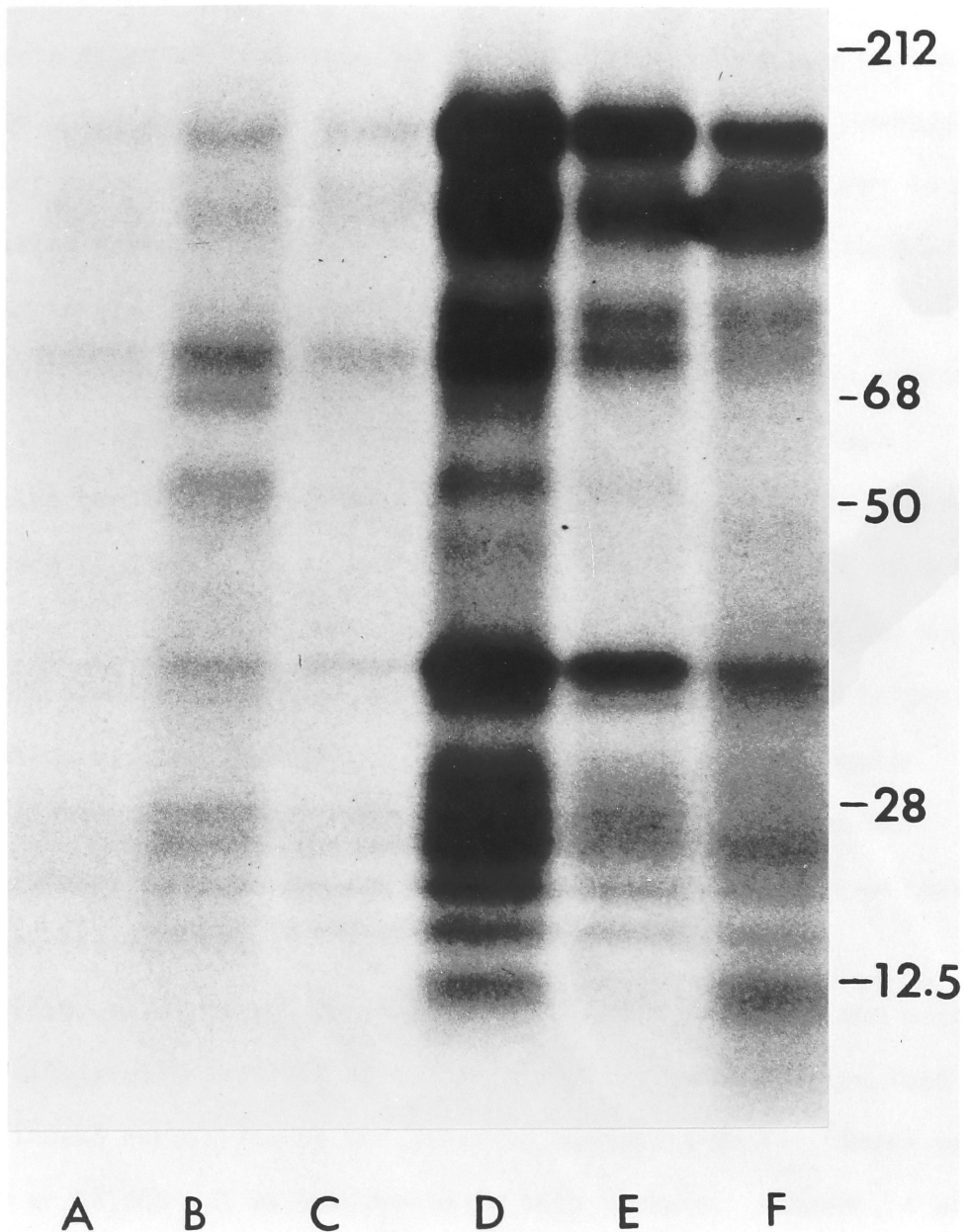


Fig. 24. Immunoprecipitation of E. histolytica surface peptides. Trophozoites were iodinated, and detergent solubilized peptides were precipitated by Staph reagent and various antisera. (A) Normal rabbit serum. (B) Goat immunoglobulin prepared against bovine serum proteins. (C) Same as (B) except that 0.5 ml neat bovine serum was added to the precipitation reaction. (D) Rabbit antiserum prepared against membranes of Pellet IV. (E) Same as (D) except that 0.5 ml neat bovine serum was added to the precipitation reaction. (F) Human convalescent serum HC-1. Molecular weight markings are $\times 10^{-3}$.

contaminants expected from non-specific adsorption of culture medium components. The peptide at 72,000 molecular weight does not correspond to any such major serum protein. When bovine serum was included in the precipitation mixture, the levels of all three peptides were reduced to background levels (fig 24 c).

Amoebic lysates precipitated with rabbit antiserum raised against purified trophozoite plasma membrane contained all major surface radiolabeled peptides (fig 24 d). It is important to note that this pattern does include radiolabeled peptides of molecular weight 72,000 and 50,000 molecular weight with possible trace amounts of 68,000 molecular weight species. When unlabeled bovine serum is included in the precipitation mixture, the peptides at 50,000 and 68,000 molecular weight are reduced to background levels while the 72,000 molecular weight component -- and all other major radiolabeled peptides -- remain unaffected (fig 24 e).

Precipitates of human convalescent serum HC-1 contained the major surface radiolabeled peptides of the trophozoite, demonstrating that they are indeed recognized by the infected host (fig 24 f). There were no 50,000 or 68,000 mol wt components in this pattern. However, a major 72,000 molecular weight peptide was present. Inclusion of unlabeled bovine serum in the precipitating mixture did not alter this pattern.

Not surprisingly, human sera showed some variation in their reactivities to E. histolytica. Comparison of two convalescent sera, HC-1 and HC-2, revealed similar reactivities but at very different relative intensities (fig 25 b and c). In both of these cases, however, the high molecular weight membrane polypeptides were major components.

It is interesting to note that while normal exposure times showed

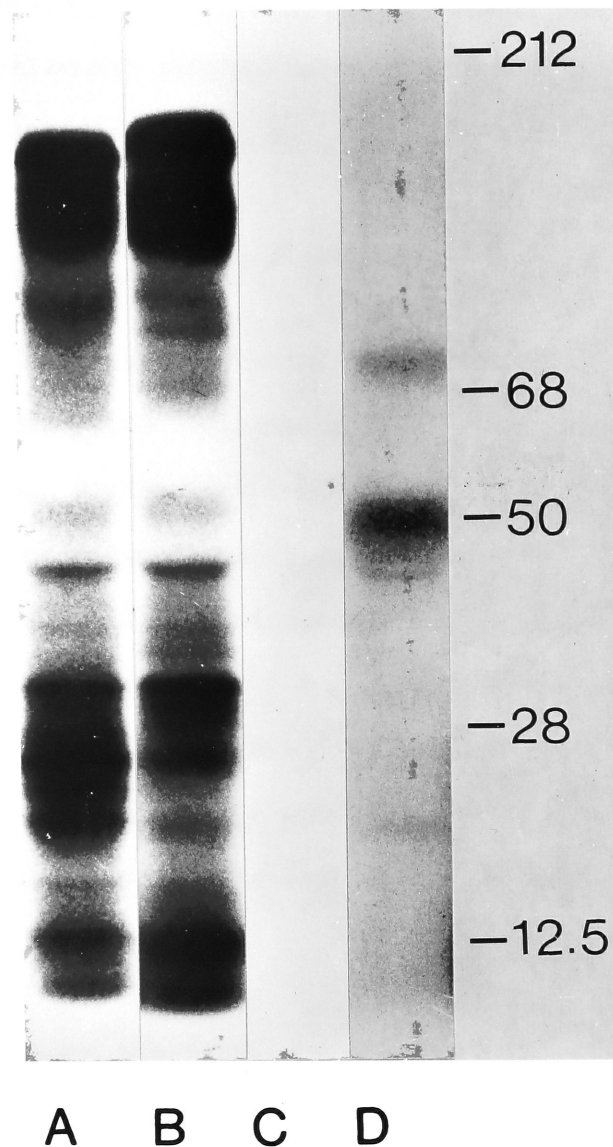


Fig. 25. Immunoprecipitation of E. histolytica surface peptides. Intact trophozoites were iodinated, and the detergent solubilized peptides were precipitated by Staph reagent and various human sera. (A) Human convalescent serum HC-1. (B) Human convalescent serum HC-2. (C) Normal human serum. (D) Enhanced exposure of normal human serum, lane C. Molecular weight markings are $\times 10^{-5}$.

no radiolabeled peptides precipitated by normal human serum (fig 25 a), increased exposure demonstrated the enrichment of one radiolabeled peptide at 50,000 molecular weight, corresponding to IgG bound to the staph reagent, and an unexplained peptide at 72,000 molecular weight.

B). Precipitation of ^{35}S -Cystine Labeled Peptides

For antigenic analysis of metabolically labeled amoebic proteins, trophozoites labeled with ^{35}S -cystine were solubilized and reacted with antisera as above. Neither pre-immune rabbit serum nor anti-bovine serum proteins precipitated substantial amounts of ^{35}S -radiolabeled peptides (fig 26 a and d). In contrast, both rabbit anti-plasma membrane and HC-1 human serum strongly precipitated the high molecular weight membrane proteins (fig 26 b and c). The anti-plasma membrane serum contains no apparent reactivity against soluble proteins of E. histolytica. HC-1 human serum precipitated one major soluble peptide of 33,000 molecular weight.

C). Antigen Identification on Southern Protein Blot

In precipitation experiments, rabbit and human sera each precipitated all of the major surface radiolabeled peptides. Two explanations for this observation are possible. The host immune system could be responding to each of the many proteins on the trophozoite surface. Alternatively, only one or a few peptides would be recognized, but in detergent solutions all of the surface peptides would exist in complexes with these few antigenic peptides. To distinguish between these possibilities, we subjected the membrane peptides of E. histolytica to

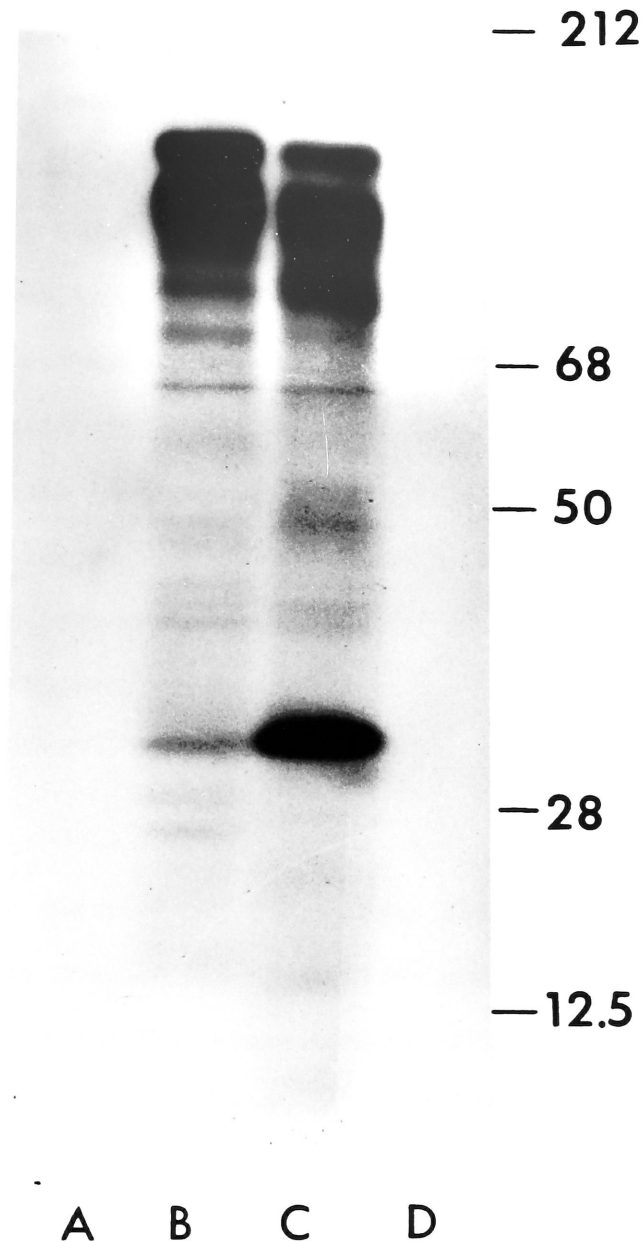


Fig. 26. Immunoprecipitation of endogenously synthesized proteins of E. histolytica. Trophozoites were incubated with ³⁵S-cystine, and detergent solubilized peptides were precipitated with specific antisera. (A) Preimmune rabbit serum. (B) Rabbit antiserum prepared against proteins in pellet IV. (C) Human convalescent serum HC-1. (D) Goat immunoglobulin prepared against bovine serum proteins. Molecular weight markings are x 10⁻³.

analysis by Southern blot of SDS-PAGE.

Trophozoites were homogenized and a total membrane fraction obtained by centrifugation at 40,000 x g for 40 min. The membrane peptides were separated by SDS-PAGE and blotted onto nitrocellulose filters. The filter was then incubated with radiolabeled IgG isolated from either normal rabbit serum or from rabbit anti-trophozoite plasma membrane serum. Autoradiography of the blots (fig 27) demonstrated that rabbit IgG against plasma membrane proteins reacted with a spectrum of peptides ranging from 30,000 to 200,000 molecular weight. Thus, each of the major membrane peptides was immunogenic and not merely in complexes with a few selected antigens.

D). DISCUSSION

Our first goal in the study of the surface antigens of E. histolytica was to determine whether plasma membrane associated peptides were of amoebic origin. This question arose because of the apparent complexity, in comparison with other protozoa, of the peptide composition of the plasma membrane of E. histolytica. In particular, we had to consider the possibility of adsorption of bovine serum proteins from the culture medium onto the surface of the trophozoite. In support of this possibility is the reported interaction of fluorescein-labeled antibodies against bovine serum proteins with the surface of trophozoites grown in TYI-S culture medium (125).

In our experiments with surface radiolabeled trophozoites, antiserum against bovine serum proteins precipitated three radiolabeled peptides. Two of these peptides, of 68,000 and 50,000 mol wt, were most

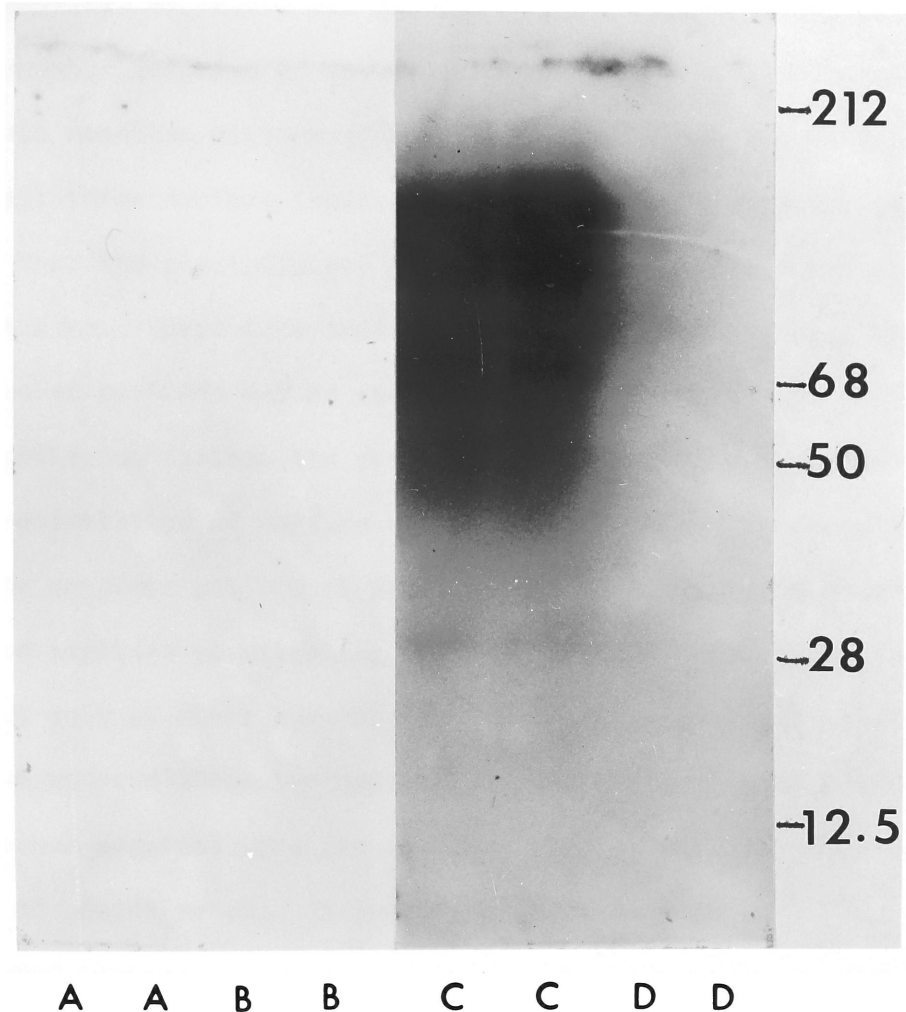


Fig. 27. Southern blot of E. histolytica membrane peptides. A total membrane preparation was made by centrifugation of homogenates at 40,000 x g for 40 min, and the membrane peptides were separated by SDS-PAGE and blotted onto nitrocellulose filters. (A, C) Total membrane peptides. (B, D) Rabbit serum peptides. (A, B) Blot was reacted with radiolabeled normal rabbit IgG. (C, D) Blot was reacted with radiolabeled rabbit IgG against purified plasma membrane peptides.

likely the serum proteins albumin and immunoglobulin G, respectively. The third peptide, at 72,000 mol wt, did not correspond to any major serum component. Addition of bovine serum to this goat anti-bovine serum protein reaction mixture effectively competed the immune precipitation of all three surface labeled peptides down to background levels--indicating that the precipitation reaction was specific for bovine serum protein antigens. These data indicated that a maximum of three of the surface labeled peptides may be serum derived.

When antiserum against the purified plasma membrane fraction was used for precipitation of surface radiolabeled peptides in amoebic lysates, the complete pattern of surface radiolabeled peptides was seen. Radiolabeled peptides co-migrating with albumin and immunoglobulin were visible, but only as minor components. Competition with unlabeled bovine serum reduced those two radiolabeled peptides to background levels. All other peptides were precipitated equally well in the presence or absence of bovine serum. In comparing these results with the aforementioned reports, we must emphasize that less stringent washing of cells did result in albumin and immunoglobulin G being the two major surface radiolabeled peptides of the trophozoite surface. Rabbit anti-plasma membrane antiserum precipitated a major radiolabeled peptide at 72,000 mol wt, corresponding to the third peptide precipitated by anti-bovine serum protein reagent. With anti-plasma membrane antiserum, however, this peptide was precipitated equally well in the presence or absence of bovine serum. Experiments using immune serum from human patients with symptoms of amoebic dysentery bore out these findings. No peptides were precipitated in the region of albumin and immunoglobulin G, although a major peptide of 72,000 mol wt was precipitated.

This peptide(s) at 72,000 mol wt deserves special consideration. It or they were precipitated by anti-bovine serum protein reagent in a manner that is competitively inhibited by the addition of unlabeled bovine serum. Both rabbit anti-plasma membrane antiserum and human immune serum precipitated a 72,000 mol wt peptide that was insensitive to competition by unlabeled bovine serum. Furthermore, long exposure of a precipitation by normal human serum revealed a single peptide at 72,000 mol wt.

Three explanations are consistent with the first two findings. Two, or more, peptides might be co-migrating on SDS-PAGE -- one derived from the bovine serum and one synthesized by the amoeba. Alternatively, a single peptide synthesized by the amoeba could have some antigenic determinants in common with one or more bovine serum components. Finally, an amoeba derived surface peptide at 72,000 mol wt might form a non-covalent complex with either albumin or immunoglobulin G as either a deliberate or accidental receptor. The observation of a 72,000 mol wt peptide weakly precipitated with normal human serum favors this third explanation. In this case the precipitation of the peptide would result from the weak interaction of a nominal receptor with the staph A/normal IgG complex.

Our analysis of amoeba derived antigens is at a more general level. Several conclusions may be drawn from our data. Each of the surface radiolabeled amoebic peptides are antigenic under experimental conditions, in that all can be precipitated by rabbit antiserum against the purified plasma membrane. Human immune serum from two patients was also found to precipitate all the major amoebic peptides, though autoradiograms appeared to be quantitatively different (fig 5.2). Normal

human serum showed no reaction except for a low level precipitation of a 72,000 mol wt peptide. All antisera tested reacted equally well with each of the four strains of E. histolytica maintained in our laboratory.

Experiments with ^{35}S -cystine radiolabeled peptides demonstrated that human serum HC-1 reacted strongly with amoebic surface peptides, yet contained little activity against soluble amoebic proteins. This clearly demonstrated the potential for separation of surface directed antibodies from soluble protein reactive antibodies.

SDS-PAGE separation of peptides, followed by reaction with radiolabeled rabbit immunoglobulin, has the theoretical advantages of eliminating the artifact of co-precipitation caused by non-covalent interaction of antigen with other peptides. It can also permit a more detailed analysis of which antigenic determinants are actually recognized. By reacting the blot with radiolabeled immunoglobulin directed against plasma membrane fraction, we demonstrated that each of the major membrane peptides were antigenic. Addition of bovine serum to this latter reaction had no effect on the resulting pattern.

Results:

Part IV

Endocytosis

ENDOCYTOSIS IN E. HISTOLYTICA

A substantial portion of membrane in E. histolytica is associated with the internal vesicles and vacuoles (fig 5). The vacuoles, as large as 5 μ m in diameter, are presumed to be largely endocytic in origin (104). Because of the size of the membrane compartment and its potential for interaction with the cell surface membrane, we considered it important to investigate the rate and extent of endocytosis in this organism. To this end we used two pinocytic markers, horseradish peroxidase (HRP) and fluorescein isothiocyanate-dextran (FITC-dextran) to study the rate and extent of fluid uptake, localization of interiorized markers, and fate of endocytosed material. Uptake of both of these markers was temperature dependent, with no uptake or surface binding detected at 4°C. Uptake was also not saturable, indicating that these compounds behave as true fluid phase markers for trophozoites of E. histolytica. Phagocytic studies were performed using latex beads and human erythrocytes as particles.

A). Uptake of Fluid Phase Markers

The uptake of 1 mg/ml fluid phase FITC-dextran by trophozoites (fig 28) was rapid and linear for about 60 min at 37°C. After 60 min the rate of uptake slowed, reaching a steady state value at about 2 hr. This steady state value was not due to receptor saturation or other absolute limit on the quantity of dextran able to be ingested, as increasing the initial concentration of FITC-dextran from 1 to 10 mg/ml increased the amount of interiorized FITC-dextran 10 fold. In each case the amount of marker endocytosed corresponds to about 2 μ l of fluid

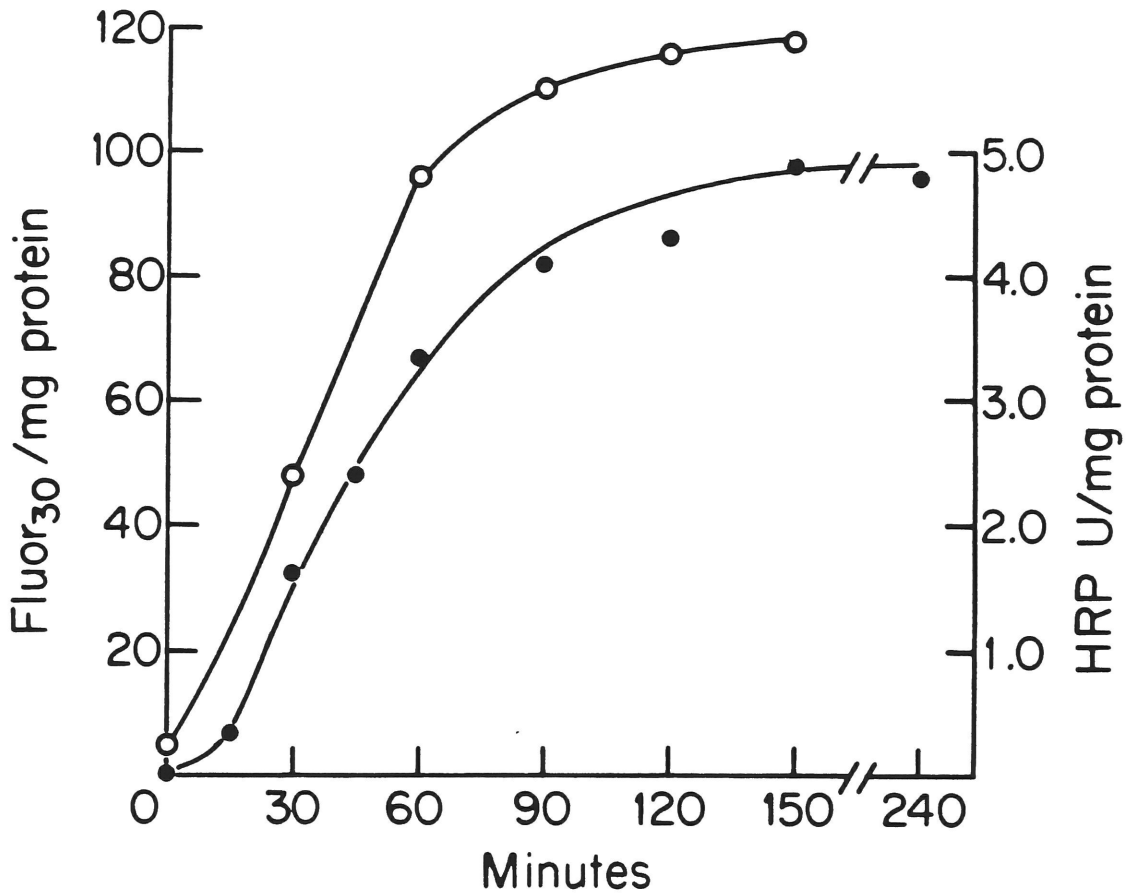


Fig. 28. Uptake of fluid phase markers by *E. histolytica*. (—○—) Trophozoites were incubated in 1 ml/mg FITC-dextran, and cell associated fluorescence was determined at various times. (—●—) Trophozoites were incubated in 1 mg/ml HRP, and the cell associated peroxidase activity was determined at various times.

taken up by 10^6 amoebae, or about 10% of the total cell volume.

When 1 mg/ml HRP was used as a marker, similar results were found (fig 28). Uptake of enzyme was linear for about 60 min, but then leveled off at a steady state level equivalent to 2 μ l of fluid interiorized by 10^6 trophozoites.

Furthermore, the reduction in rate of uptake was not due to a cessation of endocytosis. This was shown by incubating trophozoites in medium containing FITC-dextran (1 mg/ml) until a steady state was reached (fig 29, 2 hr). At this time, HRP was added to the medium to a final concentration of 1 mg/ml. The trophozoites proceeded to take up HRP with normal kinetics for 60 min while maintaining the steady state level of FITC-dextran (fig 29). Thus, the steady state value appeared to be a state of dynamic equilibrium between uptake of marker and either destruction or efflux, or both, of our fluid phase markers.

B). Localization of Pinocytic Markers

Both FITC-dextran and HRP were readily visualized in cells by fluorescence microscopy and cytochemical staining, respectively. Trophozoites were incubated in FITC-dextran until a steady state of uptake was reached (2 - 3 hr). They were then chilled on ice, washed twice, and treated with 1% formalin for 30 min in the dark. After washing, the cells were mounted for viewing in a fluorescence microscope. The photomicrograph in fig 30 shows the fluorescence to be confined predominantly in large vacuoles inside the cell, although some smaller vesicles are also labeled.

To further define the localization of our endocytosed fluid phase

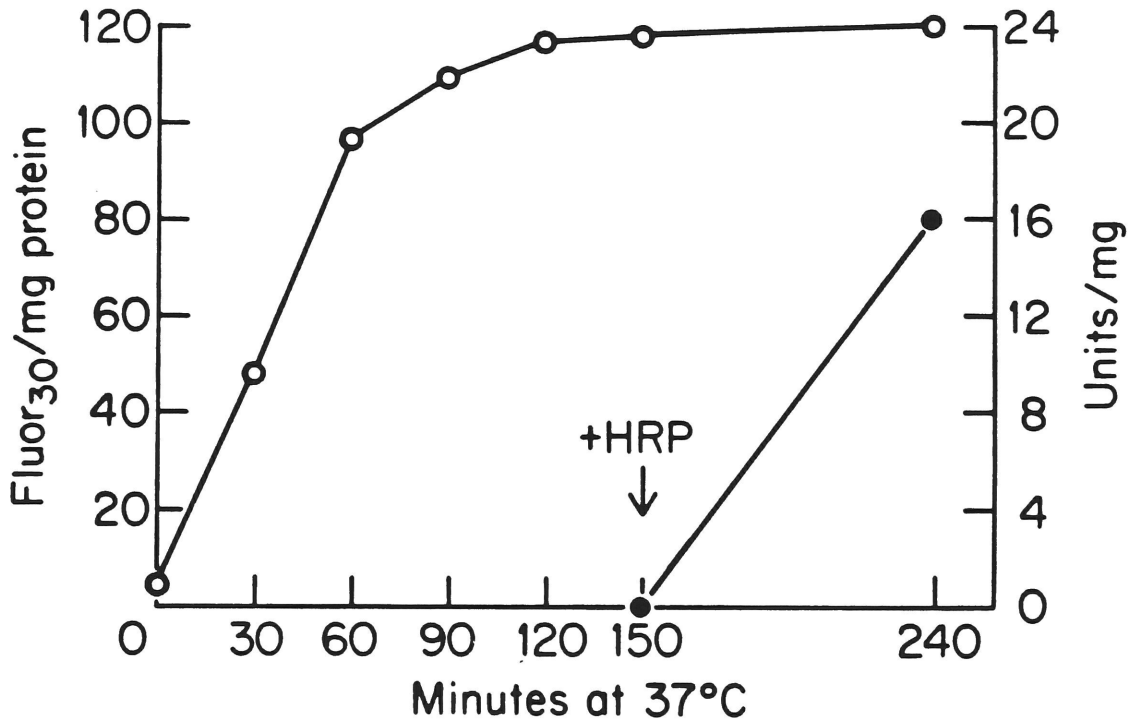


Fig. 29. HRP uptake by trophozoites containing maximal levels of FITC-dextran. (—○—) Cells were incubated in 1 mg/ml FITC-dextran and cell associated fluorescence was determined at various times. At 150 min, 1 mg/ml HRP was added to the culture medium. (—●—) Cell associated peroxidase activity determined at 150 and 240 min. HRP was taken up normally while intracellular FITC-dextran remained at steady state levels.

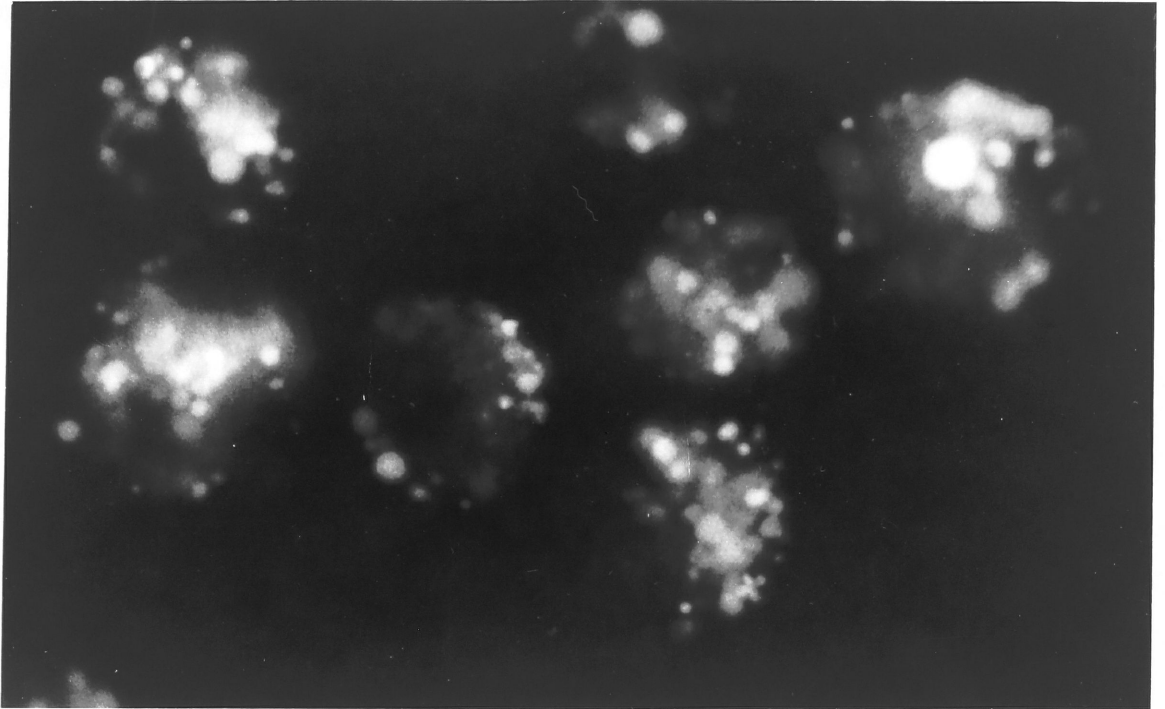


Fig. 30. Intracellular localization of FITC-dextran in E. histolytica as visualized by fluorescence microscopy. Trophozoites were incubated in 1 mg/ml FITC-dextran for 3 hr. Fluorescence was confined predominately within the large vacuoles of the cells. (x1,120).

markers, trophozoites were incubated with 10 mg/ml HRP until maximal uptake was reached. The trophozoites were washed, fixed, and stained for peroxidase activity. Stained trophozoites were then prepared for electron microscopy. Figs 31 a and b are electron micrographs of trophozoites incubated in the presence and absence of HRP. Nearly all of the vacuoles and some, but not all, of the small vesicles contained reaction product in the HRP incubated cells. Vacuoles and vesicles of trophozoites not incubated with HRP were vacant.

C). Fate of Endocytosed Markers

The results in part (A) of this section suggested that an equilibrium existed between the interior vesicles of E. histolytica trophozoites and the external milieu. This equilibrium implied a loss of marker, either through digestion or efflux, sufficient to balance uptake of that marker. We used a "wash out" type experiment to demonstrate loss of marker and to assess the relative importance of degradation and efflux.

Trophozoites were incubated with 1 or 10 mg/ml FITC-dextran until equilibrium was established (4 hr). Amoebae were then chilled, washed with cold TYI, and spun gently (50 x g) onto the sides of a culture tube. These cultures were then carefully transferred to a 37°C water bath, maintaining tube orientation. These manipulations were necessary to insure rapid warming of amoebae on reincubation. Amoebae returned to culture in medium not containing FITC-dextran showed a steady reduction in cell associated fluorescence over a period of 90 min (fig 32). Furthermore, 70 to 80% of this fluorescence could be recovered in the

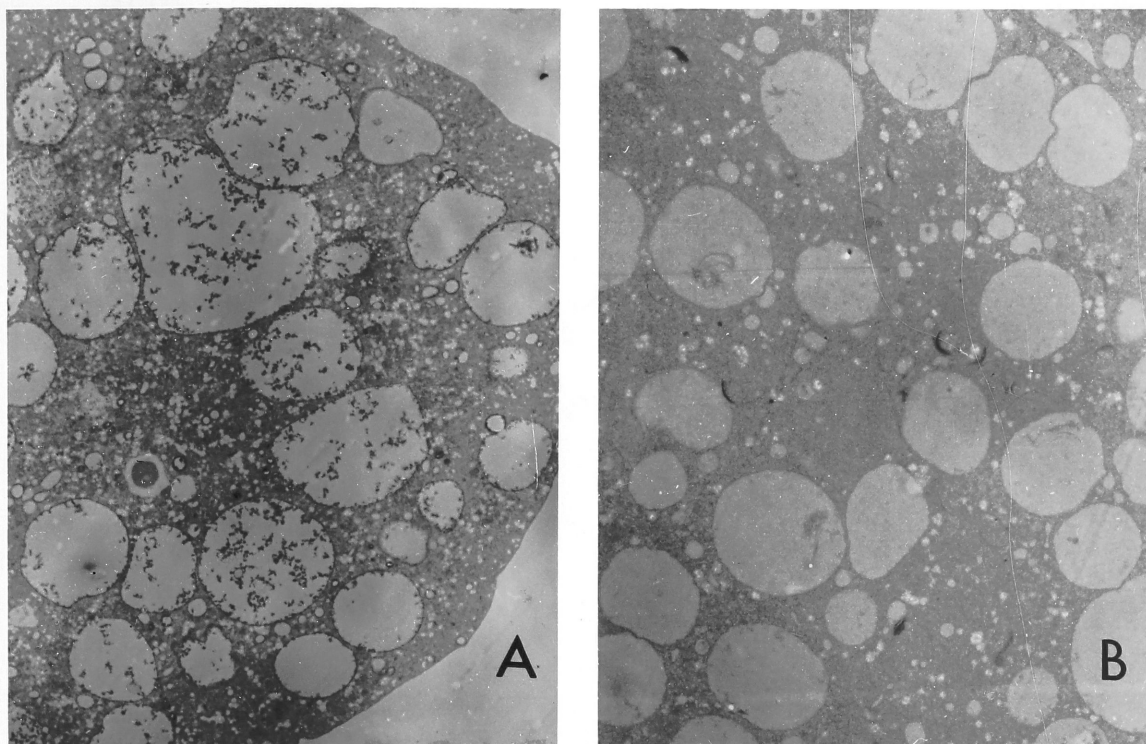


Fig. 31. Intracellular localization of HRP as visualized by electron microscopy. Trophozoites of strain HM1 were incubated with or without 10 mg/ml of HRP for 6 hr and stained for peroxidase activity. (A) HRP. Note the localization of reaction product within the large vacuoles. (B) No HRP. (x4,000).

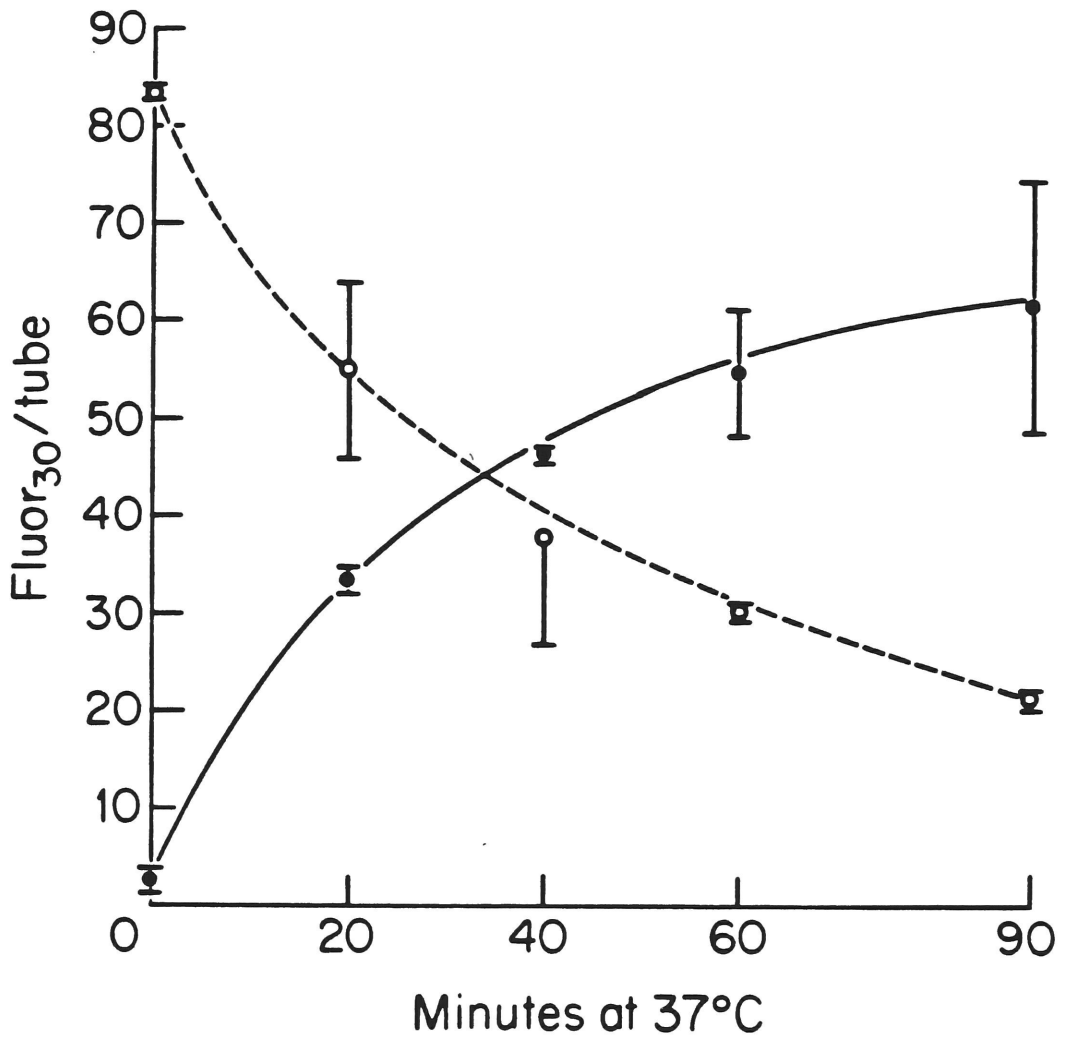


Fig. 32. Release of intracellular FITC-dextran by trophozoites, and recovery in the culture medium. Trophozoites were incubated in 10 mg/ml FITC-dextran for 3 hrs, then washed and returned to culture in fresh medium. (—○—) Cell associated FITC-dextran, determined at various times. (—●—) Total FITC-dextran recovered from the culture medium at various times of incubation.

culture medium. At each stage of the operation we solubilized the fluorescent material and checked for degradation of the dextran backbone by chromatography on Sephadex G-200 (fig 33). Native FITC-dextran in TYI and fluorescent material recovered from the washout medium (fig 33 a and c) contained only a small amount of low molecular weight fluorescent material derived from the culture medium (fig 33 d). Fluorescence activity recovered from washed cells following detergent lysis (and hence free of any medium components) did not have any fluorescent material in the included volume of the column (fig 33 b). Thus, fluorescent material from the original culture medium was the same size as material recovered from cell endosomes and that released from the cells. At no stage in this procedure was there any significant degradation of the FITC-dextran backbone, indicating that the entire 70,000 molecular weight FITC-dextran was being first taken up and then released intact by the trophozoites.

The efflux of pinocytic marker could also be demonstrated using HRP as the fluid phase marker. 10^7 trophozoites were incubated in 1 mg/ml HRP for three hours, washed twice, and resuspended in fresh TYI-S medium. Aliquots were distributed among 18 tubes of fresh medium and recultured in a 37°C water bath. At regular intervals paired cultures were harvested and centrifuged. The medium was saved and the cells were washed twice and resuspended in 1 ml of Tris buffer containing PMSF and 0.1% NP-40. Media and cell lysates were assayed immediately for peroxidase activity. Cells were also assayed for protein content. As can be seen in fig 34, peroxidase activity was progressively lost from the cells and recovered in the culture medium. Recoveries varied from 40 to 100% in separate experiments. This behavior is in marked contrast to

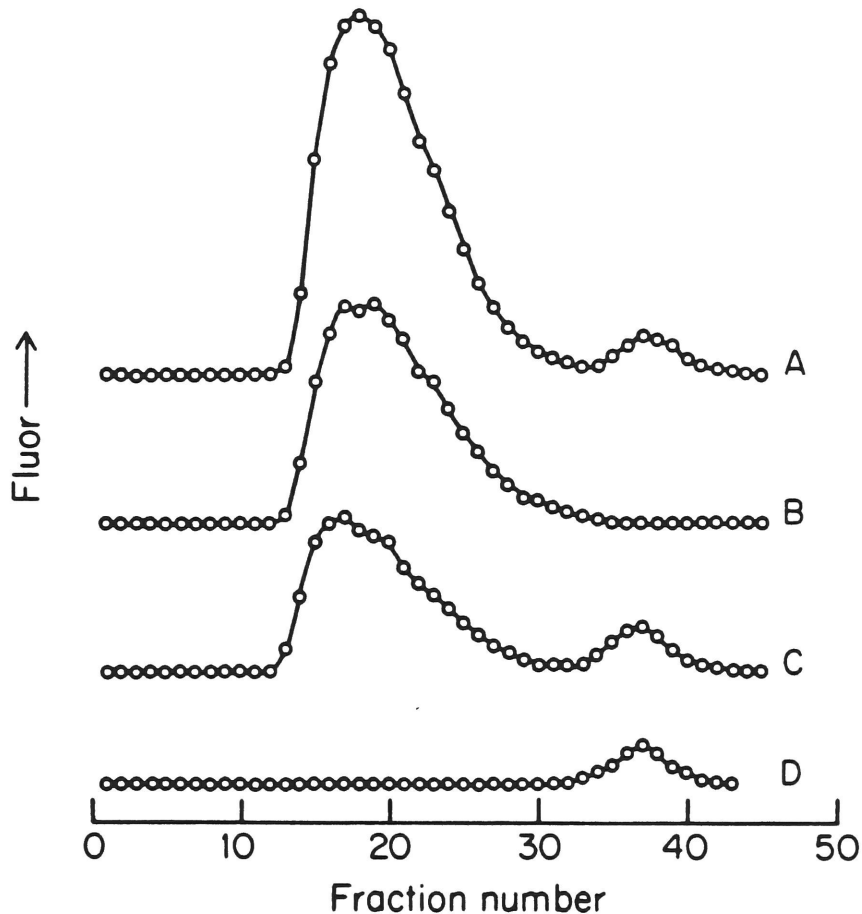


Fig. 33. Lack of degradation of FITC-dextran after uptake by *E. histolytica*. Solubilized cells or medium from cultures incubated with FITC-dextran were chromatographed on an 80 cm Sephadex G-200 column. (A) FITC-dextran dissolved in fresh culture medium. (B) Trophozoites incubated with FITC-dextran, then washed free of medium and solubilized with nonionic detergent. (C) Medium containing FITC-dextran released from cells pre-incubated with the marker. (D) Fresh culture medium showing low molecular weight fluorescence inherent in the medium.

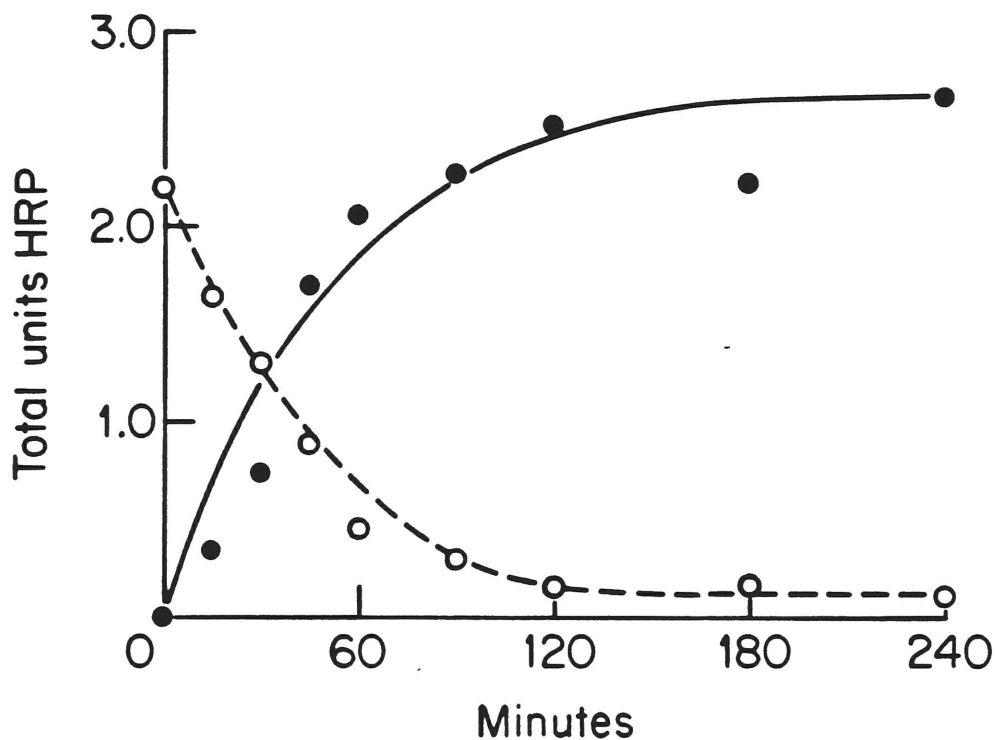


Fig. 34. Release and recovery of HRP by *E. histolytica*. Trophozoites were incubated in 10 mg/ml HRP for 3 hr, then washed and returned to culture in fresh medium. (—○—) Cell associated peroxidase activity determined after various times of incubation. (—●—) Total peroxidase activity recovered from the medium after various times of incubation.

that of mammalian cells where endocytosis appears to be a one way process, leading to concentration and/or degradation of the endocytic marker.

D). Phagocytosis by Trophozoites

We used two particulate markers, latex particles and human erythrocytes, to investigate phagocytosis by trophozoites of E. histolytica. In each case the test particles were added to the culture medium and allowed to settle onto the monolayer of trophozoites. Human erythrocytes were ingested actively. Erythrocytes were first bound to the surface of the trophozoites. They were then either ingested immediately or cause to migrate along the trophozoite surface to the uroid region and then ingested. If trophozoites were layered onto erythrocytes bound to coverslips coated with poly-L-lysine, the trophozoites could be seen to actually pry up bound erythrocytes from the surface and then to ingest them. At temperatures of 4°C, erythrocytes bound to the trophozoite surface, but were not ingested, resulting in rosette like structures.

In contrast, latex particles seemed to be taken up accidentally. No binding of latex to the trophozoite surface could be observed. Trophozoites could be observed to move through latex particles on the bottom of the culture tubes, pushing them aside with no apparent binding. Latex particles could be found inside amoebae in low numbers, but this could easily have occurred through vacuole formation by a food cup. Food cup formation interiorizes a relatively large volume of medium, equivalent to a sphere 2 to 5 μm in diameter. Any particle in the medium small enough to be contained in that volume could easily be

interiorized by that fluid uptake mechanism.

E). Fate of Phagocytosed Particles

When trophozoites were allowed to ingest erythrocytes and returned to culture overnight, no intact erythrocytes could be found either in the medium or within the cells. Instead the vesicles of the trophozoites appeared to be filled with membranous material. No intracellular hemoglobin was seen. When either glutaraldehyde fixed human erythrocytes or latex particles were ingested, the phagocytosed particles remained visible inside the trophozoites through at least three days of incubation at 37°C.

In order to determine the fate of the contents of erythrocytes, we set up a system analogous to our analysis of fluid uptake of HRP. Human erythrocytes were lysed and then resealed in the presence of HRP. These peroxidase containing erythrocyte ghosts were then washed and added to trophozoites as for intact erythrocytes. Ingestion of erythrocyte ghosts was as rapid and directed as ingestion of intact erythrocytes.

After an initial ingestion period of 1 hr, non-adherent erythrocyte ghosts were removed by rinsing the culture tubes with warm medium, and the phagocytically loaded trophozoites were incubated overnight in fresh medium. Little or no peroxidase activity could be detected in the cultured trophozoites. Peroxidase activity, however, could be recovered in the culture medium. Because of interference from an endogenous erythrocyte peroxidase activity, quantitation of HRP release was not possible.

F). pH of Endocytic Vacuoles

The fluorescence of FITC-dextran has been shown to be an excellent indicator of pH over a broad range of concentrations and environments (99). We used this property to estimate the pH of the endocytic vacuoles of E. histolytica trophozoites. A standard curve of 495 nm / 450 nm excitation fluorescence ratios was constructed by varying the pH of TYI medium containing 0.1 mg/ml FITC-dextran between pH 4 and pH 8. This curve was found to agree with published standards in less complex solutions (fig 35) (99). When this ratio was measured in cells the pH of FITC-dextran containing vacuoles was found to be 6.3 ± 0.2 (Table V). This is only slightly more acidic than the culture medium pH of 6.8.

Trophozoites were cultured in TYI plus FITC-dextran, adjusted to pH 7.5 or 8.0, for 3 hr, and the pH of the FITC-dextran containing vacuoles was determined as before. The pH of these vacuoles was found to vary with the pH of the medium. We found vacuolar pH values of 7.5 ± 0.5 and >8.0 for trophozoites cultured in media of pH of 7.5 and 8.0, respectively (Table V). Trophozoites labeled with FITC-dextran in medium of pH 6.8 and recultured in fresh medium of the same pH for 30 min showed no detectable acidification of the FITC-dextran containing vacuoles (Table V).

G). Acridine Orange Staining

Acridine orange has been used as a vital stain in the detection of primary and secondary lysosomes (126). It is a metachromatic dye, fluorescing red when concentrated and green in dilute solutions. It is also a weak base, and is thus concentrated by the low pH of the

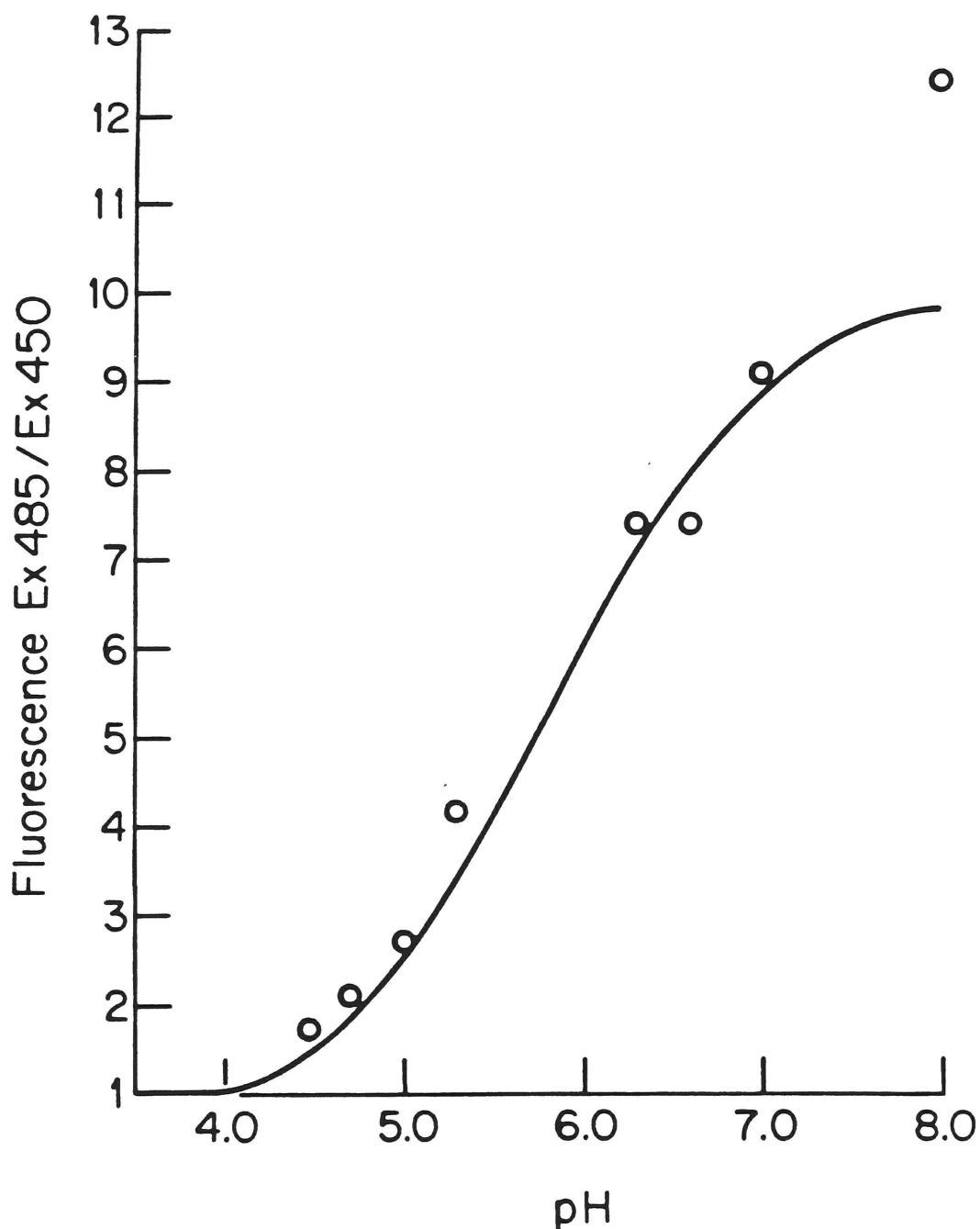


Fig. 35. Spectrum of FITC-dextran fluorescence as a function of pH. (○) FITC-dextran was dissolved in culture medium adjusted to various pH values, and the excitation 485/450 was determined. (—) Excitation fluorescence 485/450 of FITC-dextran in simple buffer solutions, from Ohkuma and Poole (99).

Table V

Relationship of the pH of Endocytic Vesicles
of *E. histolytica* to length of exposure and
pH of medium*

Sample	pH of Vesicles	pH of Medium
FITC-dextran, 3 hr	6.3 ± 0.2	6.8
FITC-dextran, 3 hr	7.7 ± 0.3	7.5
FITC-dextran, 3 hr	> 8.0	8.0
FITC-dextran, 3 hr 30 min chase	6.2 ± 0.4	6.8
FITC-dextran, 3 days 10 hr chase	5.1 ± 0.4	6.8

*Vesicle pH is the mean \pm standard deviation of three determinations.

lysosomes (127). As a result of this concentration, lysosomes of viable cells are seen to fluoresce red-orange on a yellow green background.

Trophozoites were cultured in 5 μ g/ml acridine orange for 60 min, washed, and observed by fluorescence microscopy. Numerous small vesicles of uniform size were seen to fluoresce red, indicating dye concentration by lysosomes (fig 36). All other areas of the cell, including the large vesicles of the endocytic pool, fluoresce either green or not at all.

H). Identification and pH of Secondary Lysosomes

The data from the acridine orange experiments indicated that there existed a population of acidic vesicles in E. histolytica. To determine if these vesicles constituted part of the endocytic apparatus of the trophozoite, we again used FITC-dextran as a pinocytic marker. The pH of solutions of FITC-dextran affected not only the spectrum but also the quantum yield of fluorescence. Thus in our measurements, the fluorescence of a small portion of FITC-dextran in low pH vesicles would not have been detected by the averaging process of the spectrofluorimeter. However, if these small acidified vesicles were to behave more like secondary lysosomes of mammalian cells and turnover their contents more slowly, it would be possible to preferentially label these vesicles. We designed an experiment to maximize the uptake of FITC-dextran into slowly exchanging vesicles and to minimize the fluorescence of the rapidly exchanging vacuoles. Trophozoites were incubated in 10 mg/ml FITC-dextran for 3 days at 37°C. Cells were then washed free of external dextran and incubated in fresh medium for 10 hr. This second period of incubation permitted the wash out of FITC-dextran in the vacuolar

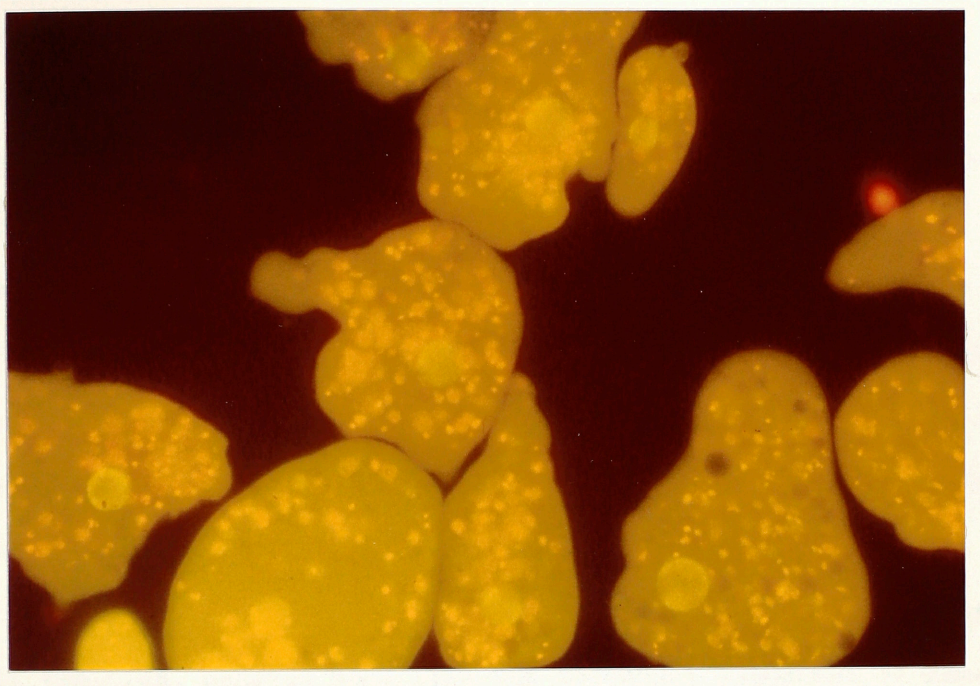


Fig. 36. Acridine orange staining of E. histolytica. Trophozoites were incubated in 1 mg/ml acridine orange for 60 min and observed in a fluorescence microscope. Numerous small vesicles and some larger vacuoles fluoresce red-orange, indicating concentration of dye within those vesicles. (x560).

compartment of the trophozoites, as shown in section F. A portion of the FITC-dextran remained associated with the amoebae. When these cells were viewed in the fluorescence microscope, numerous small vesicles and some vacuoles were seen to be strongly fluorescent (fig 37). This contrasted with the findings in fig 30, where primarily the vacuoles were labeled during the rapid uptake phase of FITC-dextran. When the fluorescence spectrum of the residual FITC-dextran was analyzed, it was found that the pH of these slowly exchanging vesicles was about 5 (Table V). Thus the vacuolar compartment of E. histolytica is segregated into at least two compartments. One compartment consists mainly of large vacuoles. The contents of these rapidly exchanges with the external milieu and are not acidified. The second compartment, consisting mainly of small vesicles, behaves more like the secondary lysosomes of mammalian cells. Endocytic material can also enter this compartment, but it exchanges out only slowly, if at all. The small vesicle compartment was acidified, with a pH at least as low as 5.

I). DISCUSSION

While it was not surprising that trophozoites of E. histolytica actively took up fluid and particles, the kinetics of these processes and the fate of internalized material contrasted strongly with the results of similar studies in mammalian cells. Trophozoites took up fluid phase markers in a linear fashion for only about 60 min. By 90 min an equilibrium was established with the equivalent of about 2 μ l of culture fluid internalized in 10^6 cells, or 10% of the total cell volume. This same level of fluid internalization was found regardless

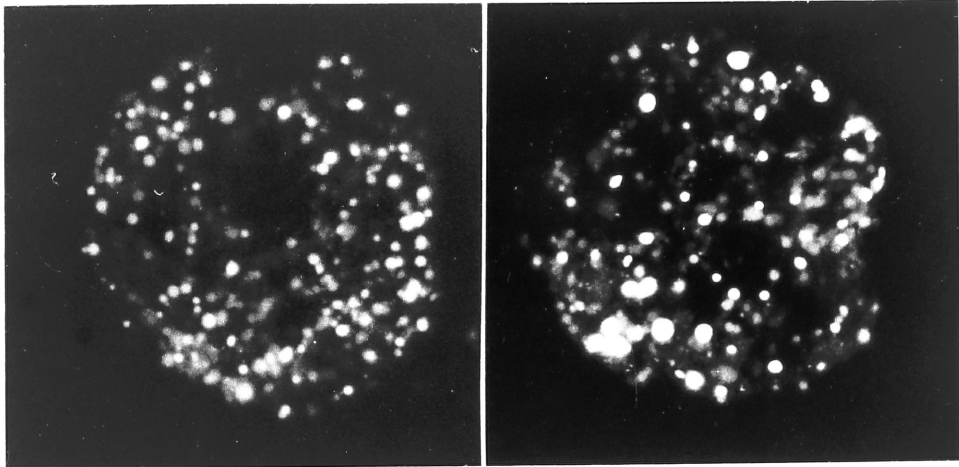


Fig. 37. Distribution of residual FITC-dextran in E. histolytica as determined by light microscopy. Trophozoites were incubated in 10 mg/ml FITC-dextran for 3 days, washed, and incubated in fresh medium for 10 hr to eliminate marker from the rapidly exchanging vacuolar pool. Residual FITC-dextran appeared to be mostly confined to the small vesicles of the cell. (x800).

of the marker or concentration of marker used, ruling out receptor saturation as a cause of cessation of interiorization. A general cessation of endocytic activity was also not a possibility. If a second, independent fluid marker, HRP, was added after the uptake of the initial marker, FITC-dextran, had reached equilibrium, the HRP was taken up normally. The FITC-dextran, however, remained at its equilibrium value. Fluid phase markers were localized by both light and electron microscopy to the large vesicles of the trophozoite, with minimal involvement of the small vesicles.

By way of comparison, uptake of these markers by mouse peritoneal macrophages continues in a linear manner for hours, with the marker being continually concentrated in the secondary lysosomes (128). Published reports on Acanthamoeba show linear uptake of soluble markers for 60 min, but unfortunately data for longer incubation times were not available (129).

When trophozoites at equilibrium with a fluid phase marker are transferred to fresh medium, the level of internalized marker decreases steadily with kinetics similar to those of uptake. The marker lost from cells could be recovered in the culture medium, apparently unchanged. The efflux of pinocytic markers by E. histolytica does not resemble the exocytosis process of the free living amoebae. Exocytosis in Chaos occurs 2 - 9 days after ingestion, generally involves phagocytosed particles, and occurs only after extensive degradation of the phagocytosed particle (130a). Exocytosis in Tetrahymena can occur within hours of ingestion of either fluid or particles, but only after extensive digestion (130b). No efflux of internalized marker has been reported for mammalian cells.

E. histolytica trophozoites thus appeared to have a fixed intracellular endocytic pool, corresponding to the large vacuoles of the trophozoite and amounting to 10% of total cell volume. This pool was in dynamic equilibrium with the extracellular milieu. Any fluid phase marker added to the culture medium becomes equilibrated with this interior pool, passing both in and out with a halflife of about 30 min. No significant degradation of the two markers used, HRP and FITC-dextran, was detected. This would appear to imply a pathway and perhaps a purpose of endocytosis that is distinct from that of either mammalian cells or the freeliving amoebae. In these latter cells, material is internalized by either pinocytosis or phagocytosis and the endosomes rapidly fuse with lysosomes. Material in these secondary lysosomes (lysosomes fused with other membrane vesicles) is generally degraded. In the case of a non-degradable marker, a mammalian cell will concentrate the marker within these structures, but is seldom if ever will the marker be released again into the medium. Other protozoans routinely exocytose indigestible remains, but only after extensive digestion. In contrast, the non-degradative cycling of fluid components by E. histolytica might be an attempt to increase the effective surface area of the cell. This increased surface area could enhance the uptake of low molecular weight components, such as glucose (110), or simply provide a larger membrane area for a second, degradative endocytic mechanism.

In studies of phagocytosis by trophozoites of E. histolytica we found it necessary to distinguish between true phagocytosis, binding of a particle followed by ingestion of that particle, and accidental phagocytosis via food cup formation. Phagocytosis of human erythrocytes was rapid and complete. Binding of erythrocytes was clearly evident, even

at temperatures which inhibited endocytosis. In contrast, phagocytosis of latex particles required high concentrations of particles, with no evidence of binding and only relatively few particles interiorized. E. histolytica trophozoites have been reported to interiorize other particles, including bacteria (131), starch (132), and trypanosomes (38). Of these three, only trypanosomes were reported to actually bind to the trophozoites as a prelude to ingestion. Both the starch particles and the bacteria of monoxenic cultures were small enough to be internalized as fluid components via the food cup of trophozoites.

In contrast to soluble components, ingested erythrocytes remained inside the trophozoites. After overnight incubation of phagocytically loaded trophozoites, intact erythrocytes could not be visualized. Instead, trophozoite vacuoles appeared to be full of membranous components. However, no hemoglobin coloration remained inside trophozoites.

In order to investigate the fate of soluble components of erythrocytes, we lysed human erythrocytes and resealed them in the presence of HRP. These HRP loaded erythrocyte ghosts were ingested in a manner similar to intact red blood cells. After overnight incubation no peroxidase activity was detected in trophozoite lysates, however, activity was detected in the culture medium. We take this to indicate that soluble components of ingested erythrocytes are exchanged with the external medium as pinocytic markers. In contrast, the erythrocyte membranes remain in the internal vesicles, possibly still bound to the membranes there, and do not participate in this cycling of vesicle contents. Indigestible particles, such as latex or glutaraldehyde fixed erythrocytes, also do not appear to cycle outside the trophozoite.

In our cytochemical studies we showed that many of the large vacuoles of the trophozoite contained acid phosphatase activity. Evidence presented in this section identifies these vesicles as endocytic in nature and suggests that these vesicles may function as secondary lysosomes. The recovery of HRP activity from this compartment was not entirely consistent with this notion, as HRP is irreversibly inhibited within mammalian cell lysosomes.

To resolve this issue, we measured the pH of the contents of the endocytic vacuoles directly, using FITC-dextran. Ohkuma and Poole have shown that the fluorescent spectrum of FITC-dextran is sensitive to pH and relatively independent of buffer composition (99). They used these characteristics to determine the pH of mammalian cell secondary lysosomes. Our studies on trophozoites of E. histolytica showed that the pH of the endocytic vacuoles was directly related to the pH of the surrounding medium. In standard culture medium, at pH 6.8, the pH of the endocytic compartment was 6.3 ± 0.2 . When the pH of the culture medium was increased to 7.5 or 8.0, the pH of the endocytic vesicles was found to be 7.5 ± 0.5 and >8.0 respectively.

Because the fluorometric measurement of intracellular FITC-dextran is an averaging process and because the fluorescence intensity was biased in favor of higher pH, it was possible that acidification of a small proportion of endocytic vesicles would be overlooked. To test for this possibility we used the fluorescent dye acridine orange as a qualitative indicator of vesicles of low pH. Acridine orange is a metachromatic dye, changing its fluorescent spectrum with concentration of dye. As it is concentrated in lysosomes as a weak base, it causes low pH vesicles (lysosomes) to fluoresce at a wavelength different from the

surrounding environment -- a bright red on a background of yellow green (126). When trophozoites of E. histolytica were incubated with acridine orange, numerous small fluorescent red vesicles of uniform size are visible. However, few of the large vacuoles of the trophozoite were seen to fluoresce red. We take these results to indicate that the large vacuoles of the trophozoite, in particular those large vacuoles involved in endocytosis, are not acidified to any extent.

To investigate the possibility that these acidic vesicles are involved in the endocytic process or at least interact with the endocytic vesicles, we first loaded trophozoites with FITC-dextran for 3 days. Then FITC-dextran was "washed out" of the rapidly exchanging endocytic compartment by a 10 hr incubation in fresh medium. After the "wash out" incubation, FITC-dextran was still readily visible in the small vesicles of the trophozoite. This residual FITC-dextran was found to be in an acidic environment.

Thus E. histolytica appears to have 2 classes of internal vesicles. The small vesicles correspond to secondary lysosomes, taking up fluid phase markers into an acidic environment and releasing markers only slowly, if at all. The large vacuoles mainly belong to an endocytic network, rapidly exchanging with the environment outside the cell and not generally acidified. The purpose of the rapid and extensive turnover of outside contents is uncertain. It could, however, be a mechanism whereby a large cell with an inherently low surface/volume ratio can increase its effective surface area.

Results:

Part V

Dynamics

of the

Surface Membrane

DYNAMICS OF THE SURFACE MEMBRANE OF *E. histolytica*

A). Internalized Plasma Membrane

In a cell undergoing endocytosis, plasma membrane is continually interiorized (133). "Plasma membrane" can thus be considered as being divided between two pools of membrane -- one pool consisting of surface plasma membrane, the other pool consisting of surface-derived membrane associated with endocytic vesicles inside the cell body. With the demonstration of extensive endocytosis in *E. histolytica*, the question of the extent of involvement of the surface membrane with this interior pool became increasingly important.

B). Redistribution of Surface Radiolabel

We used our plasma membrane isolation procedure to directly assess the proportion of plasma membrane in this interior pool. Cells were chilled on ice to inhibit membrane turnover, and the externally disposed peptides were radiolabeled using the lactoperoxidase - glucose oxidase procedure. Our earlier results had demonstrated that the radiolabel was confined to the cell surface and that our plasma membrane fractionation procedure, based on interaction of surface membrane with concanavalin A, separated the surface membrane from the internal membranes of the cell. However, when surface labeled amoebae were returned to culture at 37°C, normal membrane flow resumed. Following various periods of incubation, amoebae were chilled to stop endocytosis, diluted with unlabeled carrier cells, and subjected to our plasma membrane fractionation procedure. Because concanavalin A was added after inhibition of endocytosis, we

could still separate the surface and interior membranes. If some of our surface radiolabel had been internalized on return of amoebae to culture, we should be able to detect an increase in radiolabel associated with the internal membrane compartment. The rate of appearance of surface radiolabel in the internal membrane fraction would provide an estimate of the rate and extent of plasma membrane exchange from the surface into the internal membrane pool.

When surface labeled trophozoites remained on ice for periods of time up to 1 hour, 85% of the TCA precipitable ^{125}I was found to segregate with the crude plasma membrane fraction. 15% was found in the internal membrane fraction, and no TCA precipitable ^{125}I was found in the soluble fraction. With increasing periods of incubation at 37°C , the percentage of ^{125}I associated with the internal membranes gradually increases to a plateau at about 2 hr (fig 38). At this plateau, only 37% of the surface radiolabel was still found on the surface of the cell, with over 60% associated with the internal membranes. The kinetics of this redistribution resembled those for the uptake and release of fluid phase markers and were consistent with the cycling of surface membrane into and out of the endocytic compartment of the trophozoite. However, the proportion of internalized plasma membrane, 60%, was larger than we had expected. We proceeded to determine next to what extent normal turnover of surface proteins might have affected these results.

C). Degradation of Surface Radiolabel

In order to examine the rate of degradation of ^{125}I surface label, we surface radiolabeled 10^7 cells and divided them among 10 tubes.

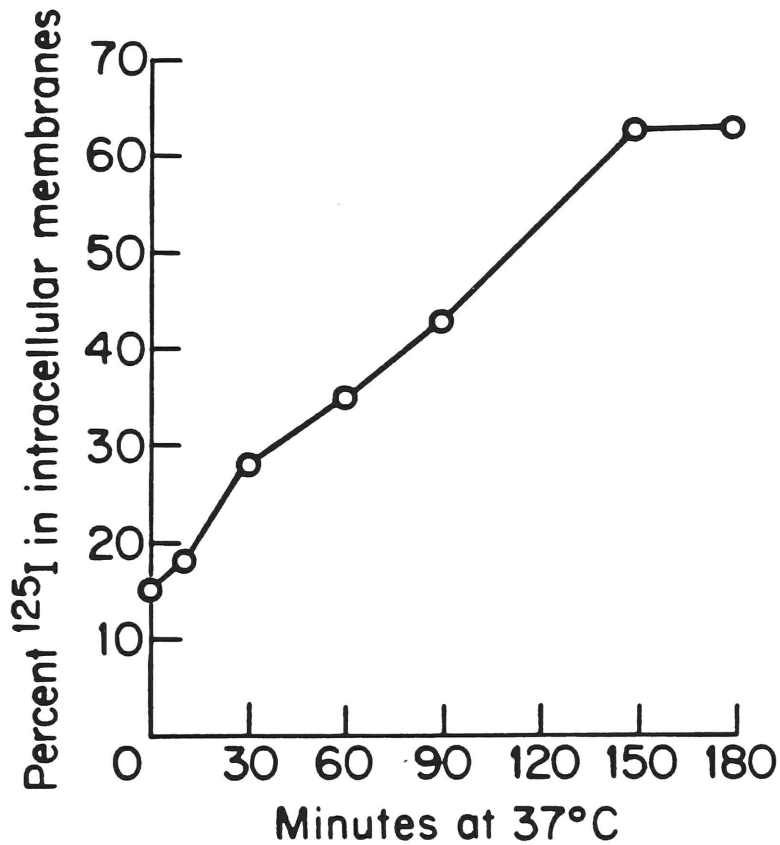


Fig. 38. Redistribution of ^{125}I surface radiolabel on trophozoites of E. histolytica, strain HM1. Intact trophozoites were radiolabeled with ^{125}I and returned to culture. At various times cells were harvested and the subcellular membranes fractionated as in fig 14. TCA precipitable radiolabel was determined for both crude plasma membranes (Pellet I) and internal membranes (Pellet II). Percent of ^{125}I associated with intracellular membranes was plotted with time.

These cultures were incubated at 37°C for varying periods of time before harvest. The cells were harvested and washed, and the TCA precipitable ¹²⁵I and total cell protein were determined. The specific activity of the radiolabel was found to vary only slightly over a period of 4 hours (fig 39). It is important to note that a substantial portion of the TCA precipitable ¹²⁵I associated with the cells at 0 min was shown by SDS-PAGE analysis not to be covalently linked to protein, and thus was an overestimate of the initial incorporated radiolabel in the internal membrane fraction. These data suggest that degradation of surface peptides does not occur at a sufficiently rapid rate to account for the internalization of surface peptides and the subsequent steady state distribution of surface radiolabel.

D). Redistribution of Surface Radiolabeled Peptides

Samples of internal (Pellet II) and crude plasma membranes (Pellet I) from radiolabeled cells recultured for various times were analysed by SDS-PAGE (fig 40). The addition of large amounts of unlabeled protein, in the form of carrier cells, was required to facilitate the subsequent isolation of plasma membrane and resulted in a very low specific activity in these samples. This low specific activity limits the resolution and detail of the resulting autoradiograms. For each time point, approximately equal amounts of TCA precipitable ¹²⁵I were added to each gel lane. However, TCA precipitation overestimated the amount of protein associated ¹²⁵I which was contained in the internal membrane vesicles at time 0. This overestimation resulted in the comparatively faint autoradiographic pattern for internal vesicles at time 0 and in an

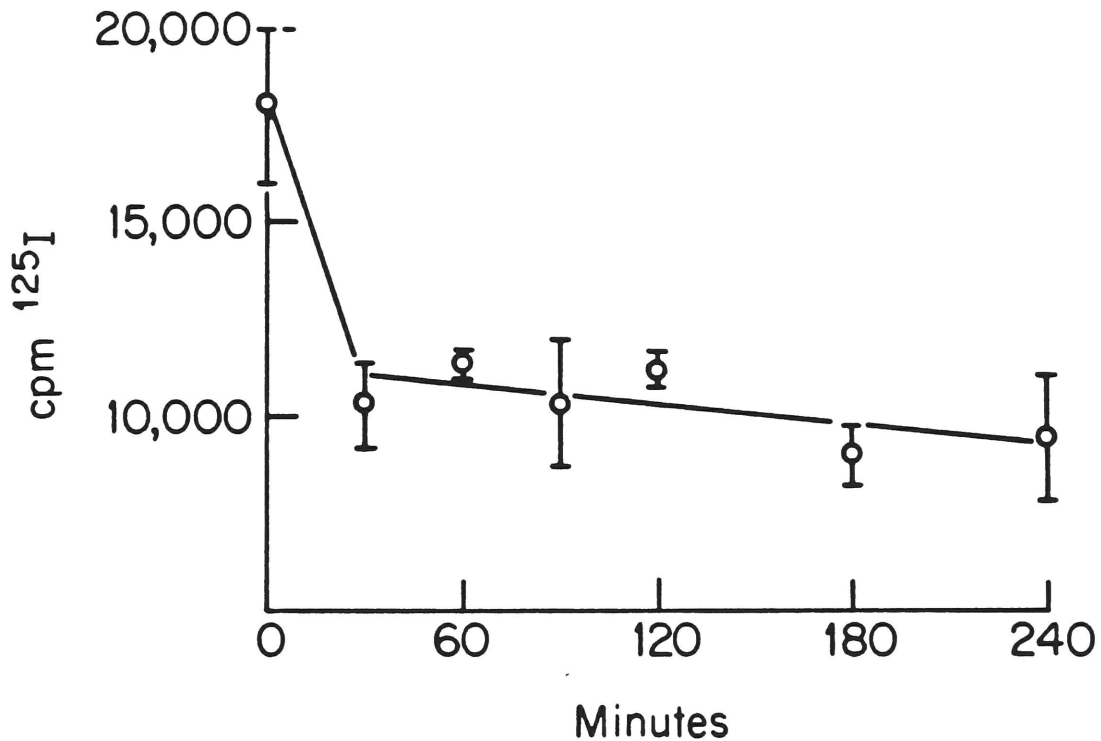


Fig. 39. Degradation of surface ^{125}I radiolabel in E. histolytica. Intact trophozoites were radiolabeled with ^{125}I and returned to culture. Total cell protein and TCA precipitable ^{125}I were determined at various times of incubation.

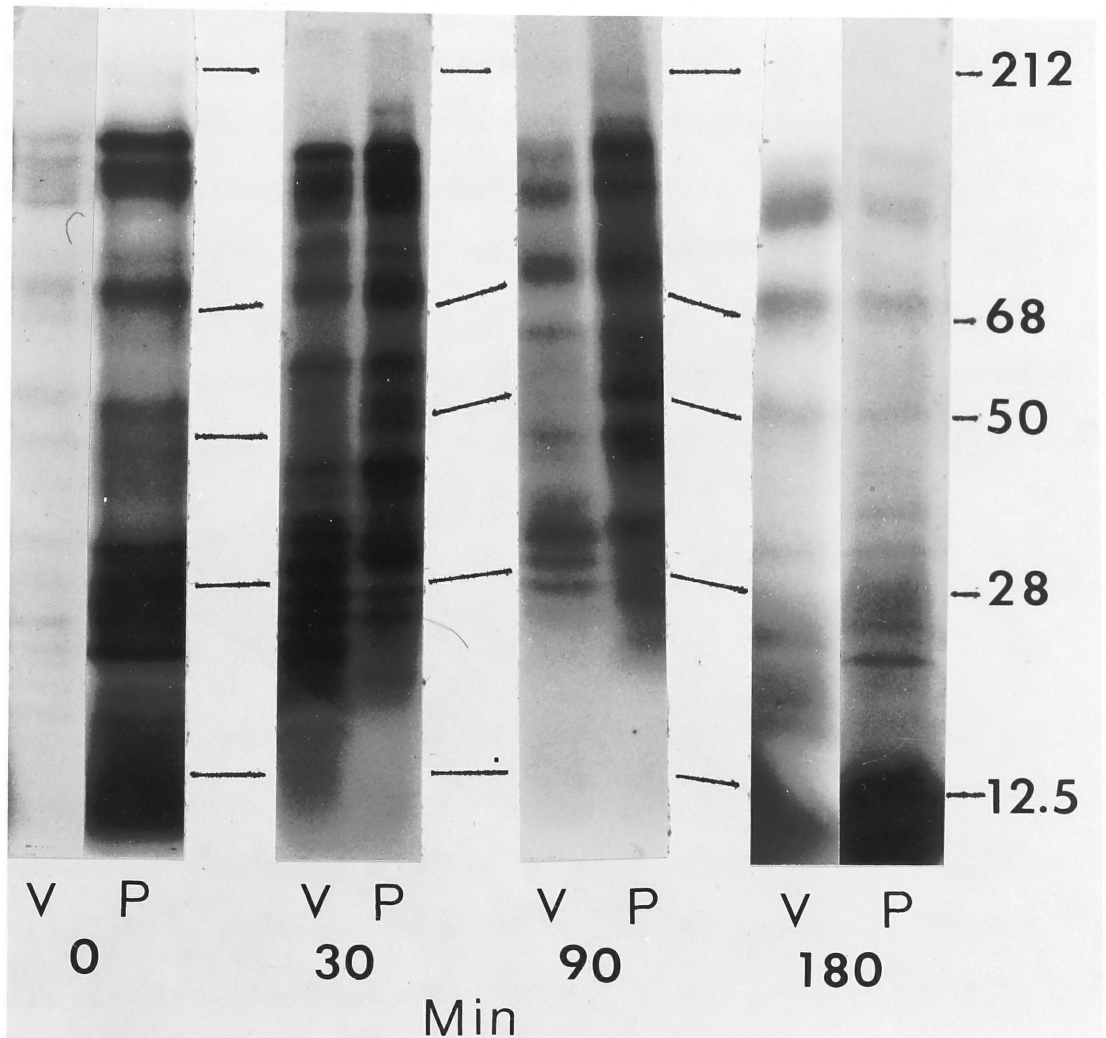


Fig. 40. Redistribution of surface ^{125}I radiolabeled plasma membrane peptides of *E. histolytica*. Trophozoites were prepared as for fig 38, and the peptides separated by SDS-PAGE. (P) Plasma membranes. (V) Interior vesicle membranes. Each time point is from an independent experiment and cannot be directly compared.

overestimate plasma membrane contamination of the internal membrane fraction. It is also important to realize that each internal membrane -- plasma membrane pair is from a separate experiment, with some variation in initial labeling. This means that intensities of bands from different time points cannot be directly compared. In these experiments, the majority of trophozoite surface peptides were found to distribute equally between the two membrane fractions. Individual exceptions were found (eg. fig 40 90 min, 50,000 and 200,000 mol wt), but no consistent pattern was observed. We take these data to indicate that all the major trophozoite surface peptides participate in the redistribution of surface membrane both into and out of the endocytic compartment.

E). DISCUSSION

It has been known for some time that cells internalize a portion of their surface membrane during endocytosis (133). Considering the extent of endocytosis constantly underway in trophozoites of E. histolytica, it was important to determine the extent of plasma membrane internalization in this organism. We found that over 60% of the proteins of the surface of trophozoites was internalized after only 2 hours of incubation at 37°C. At that time the redistribution of surface membrane reached an equilibrium analogous to the steady state level of fluid uptake that we found in our experiments on endocytosis. Proteolytic degradation of ¹²⁵I surface radiolabeled peptides did not occur at rate sufficient to account for this internalization and subsequent steady state. Separation of the radiolabeled peptides of internal and plasma membranes

showed that each of the major amoebic surface peptides partitioned between the two membrane pools.

Because of the similarity in the kinetics of pinocytic uptake and membrane internalization and the steady state condition reached after two hours, it is tempting to postulate that these two processes are closely linked. With the endocytic process, markers were transported in as well as out of the vacuolar compartment, accounting for the equilibrium condition. The redistribution of surface membrane can be explained in similar terms. A portion of the surface membrane is assimilated into the vacuolar compartment upon internalization of fluid or particles, accounting for the influx. Upon efflux of fluid marker from the vesicles, a portion of the vacuolar membrane would be returned to the trophozoite surface. The steady state level of internalized surface proteins represents the dynamic equilibrium of these two processes.

Such cycling of membrane between vesicular and plasma membrane pools has been demonstrated in mouse macrophages by Muller et al (134). In their work on mouse peritoneal macrophages, they developed a technique to label the membrane proteins of secondary lysosomes and demonstrated the movement of these peptides from the lysosomes to the surface of the cells. Studies of plasma membrane enzymes in fibroblasts (135) and sterology of membrane compartments of Acanthamoeba during phagocytosis (136) are also consistent with this interpretation.

We were initially surprised at the large proportion of surface membrane found, under steady state conditions, inside the trophozoite. Studies by Steinman and Cohn had measured the endocytic compartment of mouse peritoneal macrophages at only 10% of total membrane (133). Even the sum of endocytic and secondary lysosomal vesicle membranes could

only account for 23% of total vesicle and surface membrane. This same report also demonstrated that the combined vesicle pool of mouse fibroblasts could account for only 10% of the total vesicle and surface membrane pool.

By comparison, 60% of plasma membrane found inside the cell at any time seemed rather high. Several more recent reports, however, indicate that E. histolytica is not alone in this characteristic. An electron micrographic study of Acanthamoeba demonstrated that 60% of the total membrane of this organism was involved with the endocytic vesicles (136). Furthermore, studies on the "plasma membrane" enzymatic activity 5'-nucleotidase in fibroblasts suggested that as much as 50% of that activity was inside the cell at any given time and that this interior pool could exchange with the surface pool of enzyme (135). These latter studies are in complete concert with our findings on E. histolytica.

As a final note, this high proportion of interiorized plasma membrane has important ramifications in our initial efforts to identify a specific enzymatic marker for the plasma membrane. The model that the data suggest, a surface membrane constantly exchanging with an interior pool of similar material, implies that most plasma membrane proteins will be distributed throughout the surface and vacuolar membrane compartments. In particular, the surface/interior membrane distribution of ATPase, and possibly acid phosphatase, was completely consistent with those activities being exclusively plasma membrane associated-- with 60% of their activity inside the cell.

Summary

As a prelude to the biochemical analysis of the plasma membrane of E. histolytica, we developed a simple and rapid procedure to isolate that organelle in high yield and enrichment. To accomplish this we relied on the ability of the plant lectin concanavalin A to crosslink the cell surface glycoproteins, a technique first described by Scarborough (109). Crosslinking of the surface glycoproteins helped to maintain the integrity of the surface membrane during cell disruption and modified the density of the surface membrane, allowing isolation by differential centrifugation. The plasma membrane could be further enriched by a second differential centrifugation following the removal of concanavalin A with alpha-methyl mannoside. To demonstrate the isolation of this organelle, we surface radiolabeled intact trophozoites with ^{125}I and used the radiolabeled surface peptides as a marker for the plasma membrane. Approximately 28% of the radiolabel was recovered at over 30-fold enrichment. Additional evidence of purification was provided by electron microscopic analysis of subcellular fractions and by a high cholesterol to phospholipid ratio in the plasma membrane fraction. Two membrane associated enzymatic activities, Ca^{++} -dependent ATPase and acid phosphatase, showed substantial enrichment in this fraction.

Analysis by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of both surface labeled peptides and total peptides in the plasma membrane fraction demonstrated a peptide distribution which was more complex than had been described for free living amoebae (119). A total of about 18 peptides were associated with the cell surface, ranging in molecular weights from 20,000 to 200,000. This complexity compares favorably with the composition of mammalian cell surfaces. The majority of the surface peptides on E. histolytica were

glycoproteins, as demonstrated by specific binding to a concanavalin A column and by labeling of constituent sugar residues by tritiated borohydride.

To demonstrate that these peptides were synthesized by the trophozoite, we attempted to metabolically label amoebic proteins in culture. After unsuccessful attempts using several amino acids, we found that trophozoites cultured in a reduced cysteine medium could incorporate ^{35}S -cystine into protein. On SDS-PAGE analysis it was apparent that the radiolabeled peptides were not representative of the total protein composition. The soluble peptides were uniformly labeled. However, the membrane peptides in the 150,000 molecular weight range incorporated far more label than was predicted from their protein content. This finding suggested an abnormally high proportion of cystine was covalently linked in those peptides. The high cystine content implies the potential for extensive disulfide linkages among the major surface peptides. The disproportionate labeling of a few membrane peptides, however, limited the value of this labeling procedure in the analysis of membrane peptides.

There was a close correlation between the total number and size of plasma membrane peptides, as revealed by Coomassie stain of SDS-PAGE, and by ^{125}I surface radiolabeled peptides from whole cells. Radiolabeled peptides were found only in the plasma membrane fraction. However, unlabeled peptides of identical mobility were found to be major components of the internal membranes. This finding suggested that some "surface membrane" proteins might also be associated with membranes of internal vesicles and vacuoles. This dual distribution led to speculation that a substantial portion of the surface membrane might be

associated with the vacuolar compartment of the cell. This hypothesis was demonstrated by direct experiment in the final section of this thesis.

The phospholipids of E. histolytica showed a definite asymmetry in their distribution between surface and internal vesicles. In particular, the surface membrane was deficient in phosphatidylcholine and one species of phosphatidylethanolamine (PE_1) and correspondingly enriched in a second species of phosphatidylethanolamine (PE_2) and in ceramide aminoethylphosphonolipid (CAEP). These studies were confirmed in experiments on trophozoites metabolically labeled with $^{32}PO_4$. This enrichment of CAEP in the E. histolytica plasma membrane may be an important factor in protection of the trophozoite against enzymatic action, as CAEP is a known inhibitor of most phospholipases (94,120).

Our studies on the surface antigens of trophozoites of E. histolytica focused on two questions. The first was a return to the question of which, if any, of the plasma membrane peptides were not of amoebic origin. We reasoned that any contaminating proteins would have to have come from the serum components of the culture medium. To identify bovine serum proteins, we used a goat IgG against bovine serum proteins to precipitate solubilized peptides from ^{125}I surface radiolabeled trophozoites. Three peptides were precipitated, two of which were tentatively identified as albumin and gamma globulin. These two proteins were present in low levels on washed trophozoites and did not correspond to any of the major surface labeled peptides. The third precipitated peptide, of 70,000 molecular weight, did not correspond to any major serum protein. Competition of immune binding with bovine serum demonstrated that this 70,000 molecular weight peptide was antigenically

distinct from serum proteins. This peptide may represent an amoebic peptide which can bind serum protein(s). Our results indicated that serum proteins are not major constituents of the trophozoite plasma membrane.

The second question we asked concerning antigenic structure of E. histolytica was which of the many surface peptides were recognized by the immune system of either laboratory animals or human hosts. Studies with an antiserum raised against the purified plasma membrane fraction of trophozoites demonstrated that each of the peptides on the surface of the amoeba was antigenic and that antisera raised against one strain crossreacted with each of the strains used in this study. Moreover, sera from two human patients with amoebic dysentery also precipitated the major surface peptides on each of the four strains used in this study. To demonstrate that each of the peptides were antigenic and not merely complexed with other antigenic peptides, we showed that radiolabeled rabbit serum against trophozoite plasma membrane proteins reacted with the major surface peptides in an SDS-PAGE "Southern blot." Immune precipitation of solubilized ³⁵S-cystine labeled cells showed that, with the sera used, high molecular weight membrane antigens comprise a major portion of the immune targets in both animal and human systems.

Our studies on endocytosis in trophozoites suggested that there were two vacuolar compartments in E. histolytica. The first compartment is in rapid equilibrium with the external milieu, constantly exchanging the bulk of its contents for external medium. Fluid phase markers such as FITC-dextran and HRP were readily moved into and out of this compartment, but were not concentrated within the vacuoles. The large vacuoles (2-5 μ m) of the trophozoite, presumably including the food vacuoles,

appeared to comprise the bulk of this compartment. The contents of these vacuoles were not generally acidified, clearly differentiating them from the lysosomal vacuoles of higher eucaryotes. These vacuoles made up about 10% of the total volume of the cell. The second vacuolar compartment consisted mainly of small vesicles ($<2\ \mu\text{m}$). These vesicles were first visualized by vital staining with acridine orange, implying that they were acidified and that they corresponded to the lysosomes of higher eucaryotes. We demonstrated that these vesicles represented an endocytic compartment by first preincubating the cells for two days in FITC-dextran and then washing out the contents of the large vacuoles by a 10 hour incubation in fresh medium. Spectral analysis of the fluorescence of the residual FITC-dextran demonstrated that this second compartment was acidified, with a pH of about 5.

In conjunction with these endocytic experiments, we found that the surface membrane of the trophozoite was rapidly internalized. The kinetics of this process are best explained in terms of a cycling of surface membrane into the vacuolar compartment(s) and then back to the cell surface. As external fluid is taken up into the compartment, plasma membrane buds off the surface to fuse with the internal vacuoles. With the efflux of internalized material, a portion of the vacuolar membrane fuses with the trophozoite surface. The result is an equilibrium distribution of membrane components in both the surface and vacuolar compartments, with over 60% of the membrane of these compartments inside the cell. This model could also explain the apparent dual distribution of membrane peptides and enzymatic activities. A protein or enzymatic activity which is entirely plasma membrane associated would be found inside the trophozoite 60% of the time.

Our studies demonstrated that the E. histolytica plasma membrane contains no fewer than 18 peptides, a composition and range of molecular sizes reminiscent of mammalian cell plasma membrane peptides and more complex than had been anticipated from studies on other protozoa. The majority of the trophozoite surface peptides are glycoproteins, despite the absence of obvious endoplasmic reticulum or Golgi apparatus in E. histolytica. We showed that the externally disposed glycoproteins are antigenic and that they are recognized by the host immune system. Using sera from animal models and from human patients with amoebic dysentery, trophozoites of four strains were found to have crossreacting antigens on the cell surface. However, previous exposure to E. histolytica does not seem to protect the host against subsequent infection. Finally, our studies revealed the plasma membrane to be in dynamic equilibrium with the vacuolar system. Through a unique cycle of non-degradative endocytosis and efflux these two pools of membrane are continually exchanging, resulting in a general distribution of "surface" components in both compartments. In contrast, we were able to differentiate among various intracellular membranes, as demonstrated by the asymmetric distribution of polar lipids. Likewise, we could distinguish at least two vacuolar compartments, one consisting of small, acidic vesicles and the other of larger, neutral vacuoles. In each of the aspects studied, E. histolytica was grossly similar to mammalian cells. Yet on further investigation, the variations developed by E. histolytica proved to be both striking and unique. With these studies we have dissected some of those variations, but the promise of future research is that of more surprises, and contradictions, to come.

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