

MEMBRANE STRUCTURE AND SURFACE COAT OF *ENTAMOEBEA HISTOLYTICA*

Topochemistry and Dynamics of the Cell Surface: Cap Formation and Microexudate

PEDRO PINTO DA SILVA, ADOLFO MARTÍNEZ-PALOMO, and
ARTURO GONZALEZ-ROBLES

From The Salk Institute, San Diego, California 92112 and the Department of Cell Biology, Centro de Investigación del I. P. N., Mexico 14, D. F. Mexico

ABSTRACT

Treatment of living *Entamoeba histolytica* cells with low concentrations of concanavalin A (con A) and peroxidase results in redistribution of the plasma membrane con A receptors to one pole of the cell where a morphologically distinct region—the uroid—is formed. Capping of con A receptors is not accompanied by parallel accumulation of ruthenium red-stainable components. In capped cells, the pattern of distribution of acidic sites ionized at pH 1.8 (labeled by colloidal iron) at the outer surface and of membrane particles (integral membrane components revealed by freeze-fracture) is not altered over the cell body, but acidic sites and particles are not observed over the uroid region. Cytochemistry of substrate-attached microexudate located in regions adjacent to *E. histolytica* cells demonstrates the presence of con A binding sites and ruthenium red- and alcian blue-stainable components and the absence of colloidal iron binding sites. In a previous report we demonstrated that glycerol-induced aggregation of the plasma membrane particles is accompanied by a discontinuous distribution of colloidal iron binding sites, while con A receptors and acidic sites ionized at pH 4.0 remain uniformly distributed over the cell surface. Taken together, our experiments show that, in *E. histolytica* cells, peripheral membrane components may move independently of integral components and, also, that certain surface determinants may redistribute independently of others. These results point to the complexity of the *membrane structure-cell surface* relationship in *E. histolytica* plasma membranes relative to the membrane of the erythrocyte ghost where integral components (the membrane-intercalated particles) contain all antigens, receptors, and anionic sites labeled so far. We conclude that fluidity of integral membrane components (*integral membrane fluidity*) cannot be inferred from the demonstration of the mobility of surface components nor, conversely, can the fluidity of peripheral membrane components (*peripheral membrane fluidity*) be assumed from demonstration of the mobility of integral membrane components.

Among the human diseases caused by protozoan parasites, amebiasis produced by *Entamoeba histolytica* is one of the most important, due to its high incidence in large areas of the world and the severity of the clinical manifestations (1). In spite of this relevance, the mechanisms which mediate the pathogenic activity of *E. histolytica* remain largely unknown (5). In the last few years, however, the development of axenic cultures (10) has greatly facilitated the experimental study of the biological and pathogenic properties of *E. histolytica*. The understanding of the host-parasite interactions which determine the invasive behavior of the amoeba requires knowledge of its cell surface properties. Experiments in this laboratory showed that concanavalin A (con A) induces extreme agglutination in pathogenic strains of *E. histolytica*, whereas weak agglutination is obtained in strains isolated from asymptomatic carriers (21).

The concept which envisages cellular membranes as fluid domains is now generally accepted. Fluidity of integral membrane components (here termed *integral membrane fluidity*) has been established by the demonstration of the molecular motion of phospholipid molecules (14), and the rotational and translational freedom of membrane lipids and integral membrane proteins (4, 7, 8, 25, 33). Alternatively, fluidity of peripheral membrane components (*peripheral membrane fluidity*) has been demonstrated by the observation of the intermixing of surface antigens in heterokaryons (12) and the polar accumulation of surface receptors in lymphoid cells—"cap formation" (35). While both lines of evidence clearly illustrate the dynamic state of cellular membranes, the inference of integral membrane fluidity does not necessarily follow from data on the dynamic nature of the cell surface nor, conversely, the implication that peripheral membrane fluidity is a necessary consequence of results which demonstrate the redistribution of integral membrane components. The inference is possible, however, when integral membrane components traverse the hydrophobic matrix of the membrane and expose antigens and receptors at the outer surface. At present, this relationship has only been established in erythrocyte ghost membranes (27, 29, 30, 37). It is clear, however, that conclusions obtained from the erythrocyte ghosts cannot be freely extrapolated as a general model for the relationship between membrane structure and cell surface in eukaryotic cells.

We have recently obtained evidence which indicates that the aggregation of integral components in *E. histolytica* plasma membranes—the membrane particles revealed by freeze-fracture—while accompanied by aggregation of acidic sites ionized at pH 1.8 (as labeled by colloidal iron) does not result in clustering of con A surface receptors, or of surface anionic sites ionized at pH 4.0 (28). In view of these results, we have induced cap formation of con A binding sites in *E. histolytica* plasma membranes, in order to establish whether polar redistribution of con A sites is accompanied by rearrangement of other surface components detected by ultrastructural cytochemistry, and/or by redistribution of membrane particles, as revealed by freeze-fracture. We have extended our observations to the comparison of the cytochemical properties of the membrane surface coat and the substrate-attached microexudate of *E. histolytica* cultures.

MATERIALS AND METHODS

Cell Cultures

Trophozoites of *E. histolytica* HK9, a pathogenic strain cultured under axenic conditions (10), were used. Cells were harvested at 72 h of cultivation, after 5-min incubation in an ice bath, by centrifugation in a clinical centrifuge at 1,200 rpm for 5 min. The cells were washed three times in phosphate-buffered saline (PBS) at 34°C.

Treatment of Cells with Con A-Peroxidase

Washed cells were incubated for 15 min at 34°C with 10, 20, or 100 µg/ml con A (Miles Yeda) in PBS. After washing twice with PBS some cells were fixed (1.5% glutaraldehyde in PBS, 30 min at 34°C), while others were incubated with 50 µg/ml horseradish peroxidase (Sigma Chemical Co., St. Louis, Mo.) in PBS, for 15 min at 34°C and fixed as above. Fixed cells were washed twice in PBS and reacted with 3,3'-diaminobenzidine (0.5 mg/ml) in 0.1 M Tris buffer, pH 7.4, containing 0.01% H₂O₂ for 15 min (2). After washing, cells were postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer. Subsequently, cells were dehydrated and embedded in Epon. In other experiments, cells were fixed after con A treatment and incubated subsequently with peroxidase and benzidine and postfixed with osmium tetroxide.

Trophozoites incubated with 10 µg/ml con A and 50 µg/ml peroxidase, a treatment which resulted in cap formation, were processed for freeze-fracture, as described below, or incubated with ruthenium red (18) or colloidal iron (13) to stain surface coat components of the trophozoites.

Freeze-Fracture

Fixed, glycerol-impregnated cells were rapidly frozen in the liquid phase of partially solidified Freon 22 cooled by liquid nitrogen and were stored in liquid nitrogen until used. Freeze-fracture was carried out at -100°C in a Balzers 300 apparatus equipped with a turbomolecular pump (Balzers High Vacuum Corp., Santa Ana, Calif.). The relatively high temperature was used to minimize the danger of contamination. In order to increase the probability of having large fracture faces of the plasma membranes, the knife was manually lowered by approximately one-fifth of a turn for the last cut to produce a deeper cut than that which could be obtained by the automatic lowering device of the Balzers 300 machine. Replicas were produced by evaporation from a platinum-carbon source. The specimens were shadowed at 2×10^{-6} mm Hg within 2 s of fracturing.

Detection of Microexudate

In order to detect the presence of the microexudate attached to the substratum, *E. histolytica* trophozoites were grown on 30-ml Falcon tissue culture flasks. After washing three times with PBS at 34°C , cells were fixed *in situ*, according to the technique developed by one of us to study fibroblasts in culture (20). Fixation with 1.5% glutaraldehyde was carried out at 34°C in order to minimize morphological changes of the amoeba. Cells attached to the substratum were reacted with ruthenium red (18), colloidal iron at pH 1.8 (13), and alcian blue-lanthanum nitrate (34), or stained *in situ* with uranyl acetate after fixation with glutaraldehyde and osmium tetroxide (16). In order to maintain the relationship between cultured cells and their substratum, dehydration and embedding were carried out directly on the plastic flasks. For infiltration, mixtures of Epon 812 with absolute ethanol were used. After polymerization of the resin, the whole culture was easily separated from the plastic flask after a brief immersion in boiling water. Thin sections perpendicular to the plane of the culture were obtained with diamond knives and stained for 5 min with lead citrate. Observations were carried out with a Zeiss EM 10 electron microscope.

RESULTS

Native Distribution of Con A Receptors

Incubation of amoebae from the pathogenic strain HK9 with various concentrations of con A produced an intense agglutination of the cells, in contrast with the weak agglutination induced by the lectin on amoebae originally isolated from asymptomatic carriers (21). When pathogenic cells agglutinated by con A were fixed with glutaraldehyde and incubated with peroxidase and benzidine, the surface distribution of con A receptors detected

by the con A-peroxidase method (2) was uniform at all lectin concentrations used (Fig. 1).

Con A-Induced Cap Formation

We attempted to establish whether con A-peroxidase treatment of unfixed agglutinated cells would induce short-range changes in receptor distribution similar to those which have been observed in tumoral cells (6, 22, 23, 32), or might result in accumulation of the receptors in one pole of the cell, as observed in lymphocytes during cap formation (35). We found that con A treatment ($10 \mu\text{g/ml}$, 15 min at 34°C) followed by incubation with peroxidase ($50 \mu\text{g/ml}$, 15 min at 34°C) induced a striking accumulation of the reaction product at one focal region of the cell surface (Figs. 2 and 3). Caps were easily identified in 3–5- μm thick Epon unstained sections observed by light microscopy (Fig. 3). In a given section, about 30% of the amoebae clearly showed a cap of con A-peroxidase-benzidine reaction product. The region where con A receptors accumulated upon treatment with con A and peroxidase showed a noticeable morphological differentiation when observed with the electron microscope. This region, which may correspond to the uroid or "tail" end described for *Entamoeba invadens* (39), was characterized, as illustrated in Figs. 2 and 4, by a complex accumulation of vesicular and tubular structures, some of which could be seen in thin sections to originate from the plasma membrane (Fig. 4). The uroid region was generally slightly raised in relation to the adjacent cell surface. It contained numerous microfilaments and was usually devoid of cytoplasmic vesicles. Cellular debris could be found in close proximity to the uroid, as seen near the uropod of lymphocytes (3).

Freeze-Fracture Morphology of *E. histolytica* Plasma Membranes

Observation of fracture faces of *E. histolytica* plasma membranes revealed a marked heterogeneity in the size of membrane particles. As in other cells, fracture face A (which represents the outer aspect of the inner membrane half) exhibits a distinctly larger density of membrane particles than that observed on fracture face B (which corresponds to the inner aspect of the outer membrane half) (26). This difference is illustrated in Fig. 5 which shows a face A and a face B of two adjacent cells. At higher magnification, the heterogeneity of the particle population became espe-

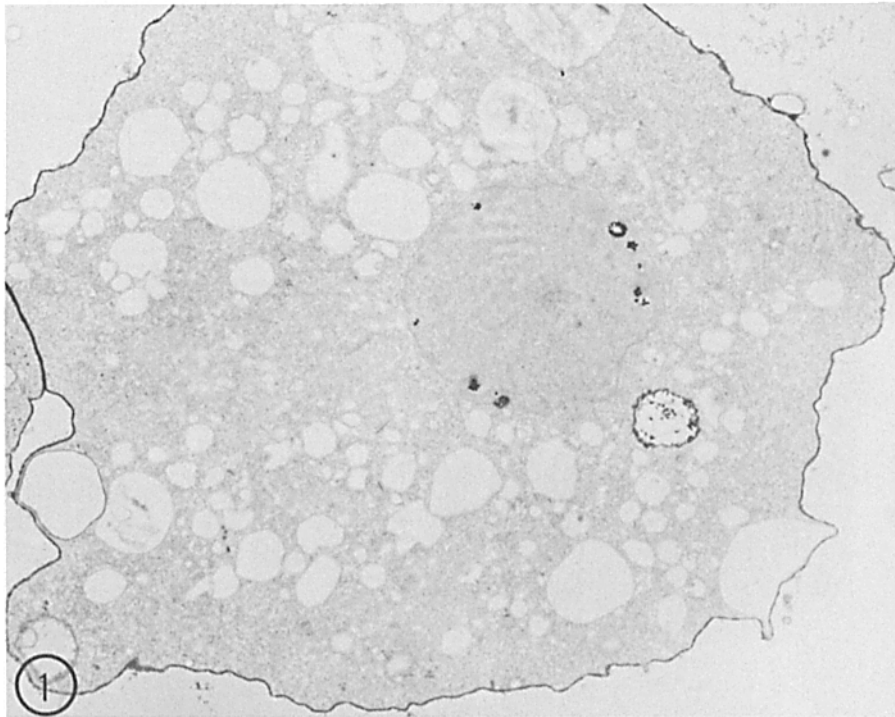


FIGURE 1 Normal distribution of con A receptors in an *E. histolytica* trophozoite treated with con A, peroxidase, and benzidine after glutaraldehyde fixation. $\times 5,000$.

FIGURE 2 Polar accumulation of con A receptors (cap formation) in an amoeba treated with con A and peroxidase, fixed with glutaraldehyde, and reacted subsequently with benzidine. $\times 5,000$.

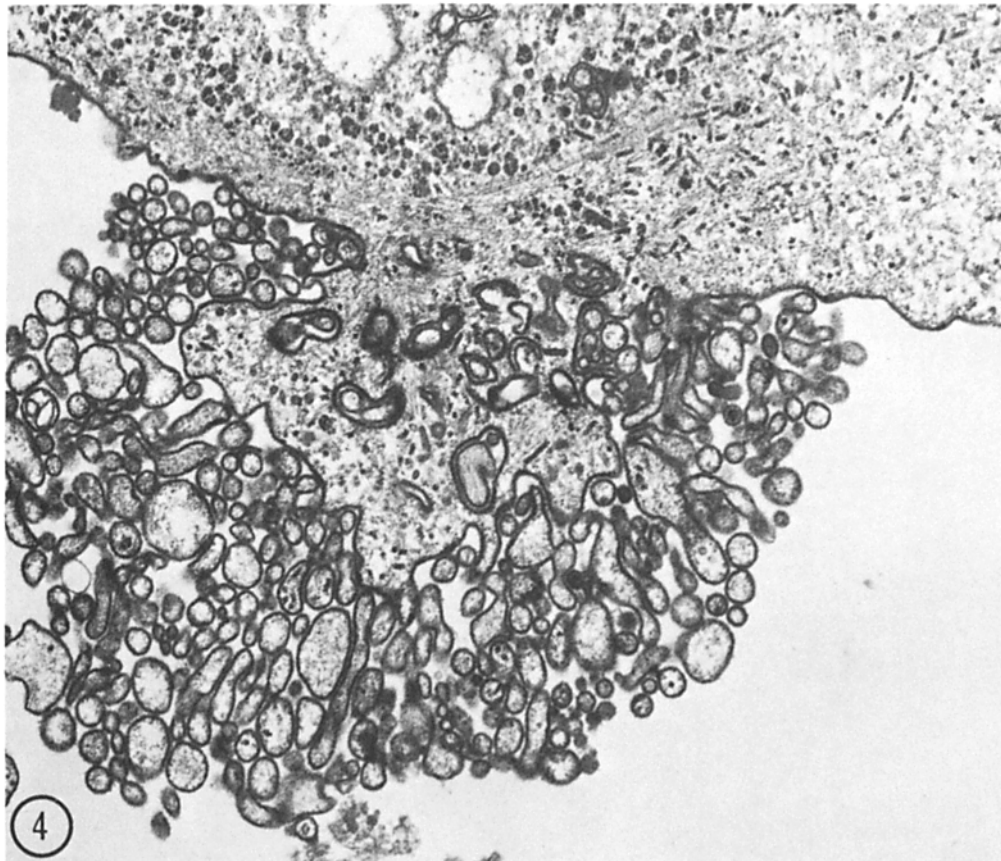
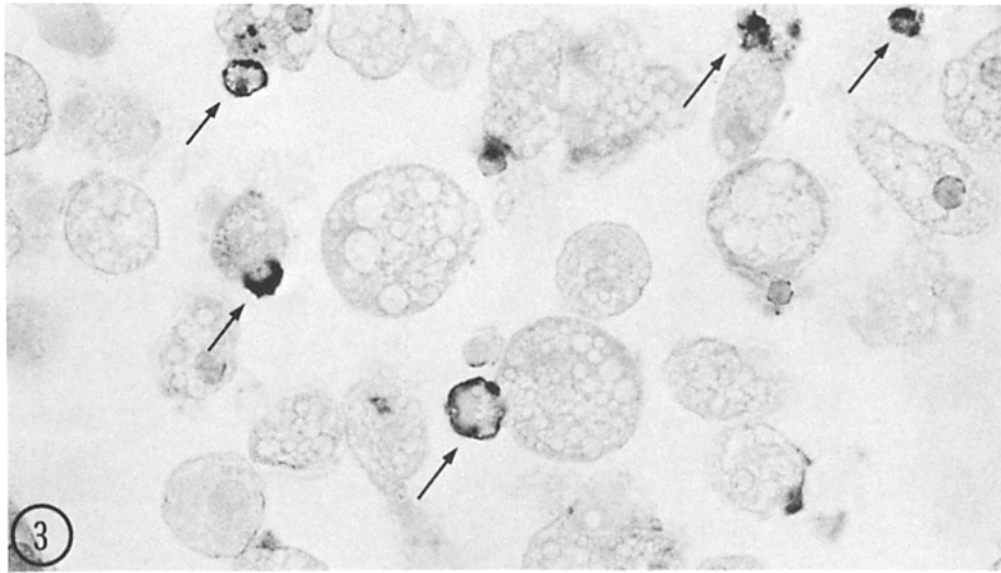


FIGURE 3 Light micrograph of *E. histolytica* cells treated with con A and peroxidase before fixation. Notice the prominent caps of con A-peroxidase-benzidine reaction product in various cells (arrows). Uncontrasted 5- μ m thick section. Bright-field optics. $\times 650$.

FIGURE 4 Electron micrograph of the uroid region in an amoeba treated with con A and peroxidase but without incubation with benzidine. The uroid is characterized by a complex array of vesicular and tubular structures. $\times 20,000$.

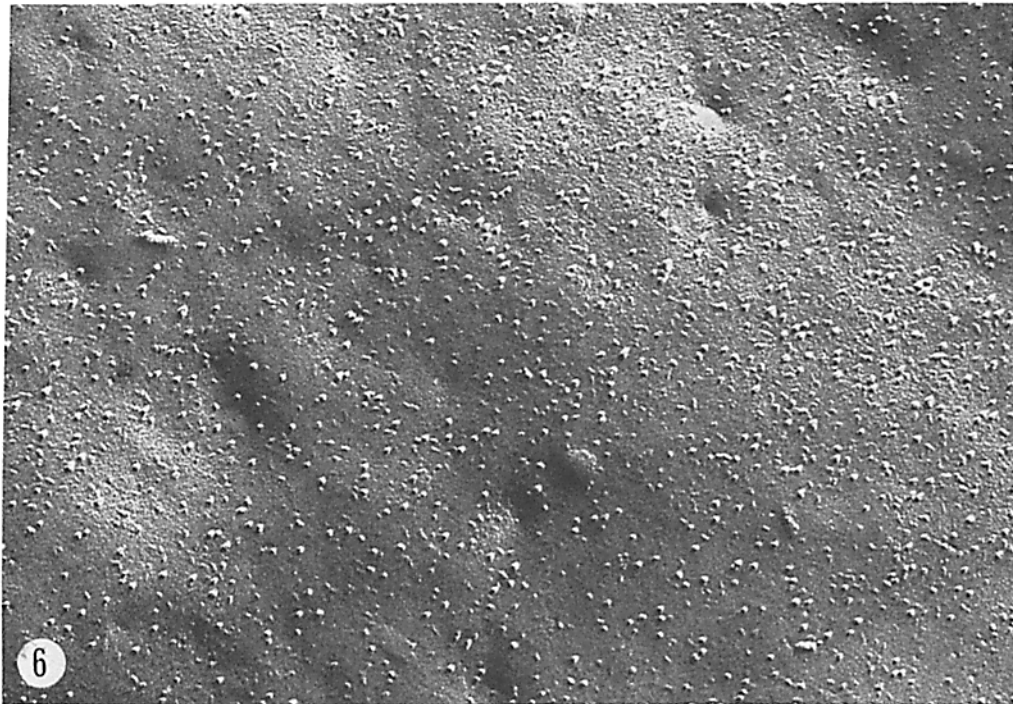
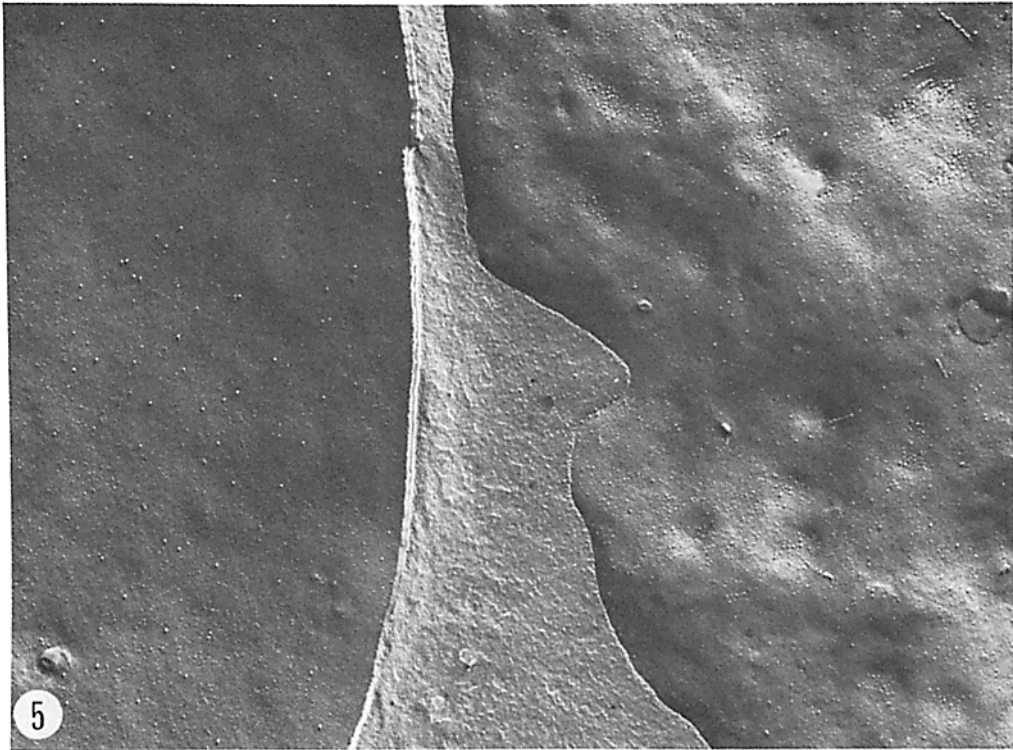


FIGURE 5 Fracture face A (particle rich, right side) and fracture face B (particle poor, left side) of two *E. histolytica* HK9 trophozoites prefixed before glycerol impregnation. $\times 15,000$.

FIGURE 6 High magnification of a fracture face A of prefixed *E. histolytica* plasma membrane. Size heterogeneity of membrane particle population is seen. $\times 85,000$.

cially clear over membrane regions which received a lower angle of shadow (Fig. 6, upper right). The smaller rugosities ("subparticles") are not an artifact of the technique since glycerol treatment induces coaggregation of particles and subparticles into a single, random network (28).

Distribution of Membrane Particles and Surface Components in "Capped" Cells

Because our previous experiments showed that glycerol induced coaggregation of all membrane particles and "subparticles" as well as a discontinuous surface distribution of colloidal iron sites (i. e. acidic sites ionized at pH 1.8) but not of con A receptors or of acidic sites ionized at pH 4.0 (28), we attempted to establish whether capping of con A receptors resulted in a redistribution of membrane particles and surface coat components.

Our results demonstrate that membrane particles did not follow the polar accumulation of con A surface receptors induced by con A-peroxidase treatment. The uroid region was easily identified on replicas of amoebae treated with con A and peroxidase (Fig. 7). No alteration of the pattern of distribution of membrane particles was detected in either A or B fracture faces of plasma membranes of capped cells (i. e. of cells where the uroid could be positively identified) (Fig. 8). Remarkably, however, the fracture faces of membranes at the uroid region were always devoid of particles (Fig. 7). Observation of the pattern of distribution of colloidal iron binding sites on the surface of capped cells revealed that, *like the membrane particles*, acidic sites were uniformly displayed over the cell body but absent from the uroid region (Fig. 10). The distribution of ruthenium red-stained components was uniform over the entire surface of capped or uncapped cells (Fig. 9).

Cytochemistry of Microexudate

In thin sections of *E. histolytica* cultures that were fixed, dehydrated, and embedded *in situ*, cells were found to be located on top of a thin layer invariably found attached to the substrate. In cultures stained with uranyl acetate before dehydration, the layer was seen as a nearly uniform dense coat approximately 5 nm in thickness (Fig. 11). Cells were separated from the thin layer by a clear space of about 15 nm in width (Fig. 11). In order to establish whether this layer contains surface coat components of *E. histolytica*, we applied several cytochemical techniques which have been used for the detection of cell coat

components (19). When alcian blue-lanthanum nitrate, ruthenium red, or con A-peroxidase-benzidine was used, the layer showed a reaction entirely similar to that found at the cell surface of immediately adjacent cells (Figs. 12 and 13). However, when anionic sites ionized at pH 1.8 were labeled, colloidal iron particles were seen over the cell surface, whereas essentially no reaction was observed at the substrate-attached layer (Fig. 14). The possibility that the microexudate might represent a thin layer of culture medium components adsorbed to the substrate was ruled out by the observation of the absence of microexudate reactions in regions of the substrate separated several millimeters from the nearest accumulation of cells. Also, no microexudate reactions were observed in culture dishes incubated with culture medium alone. In consequence, our results enable us to ascribe the thin layer to the microexudate, detected in cultures of other eukaryotic cells by means of electron microscopy (38), ellipsometry (31), and biochemical assay (9, 15, 36).

DISCUSSION

In the present article we study the effect of the redistribution of con A surface receptors on the distribution of membrane particles and other cell surface components. In a previous report (28) we have studied the effect of redistributing integral membrane components (particle aggregation induced by glycerol) on the distribution of con A receptors and other surface components. Our results are complemented here by the comparative cytochemistry of the cell coat and the microexudate. The following table summarizes our data.

TABLE I
Experimental Redistribution of Integral and Peripheral Membrane Components and Microexudate Cytochemistry

Experiment	Positive	Negative
Con A-perox	Con-A	MP* CI* RR
Glycerol (28)	MP CI	Con-A RR F4
Microexudate	Con-A RR AB	MP† CI

MP = membrane particles. CI = colloidal iron binding sites (acidic sites labeled at pH 1.8). RR = ruthenium red-stained components. Con A = concanavalin A binding sites. AB = Alcian blue stained components. F4 = acidic sites labeled by ferritin at pH 4.0.

* No membrane particles or colloidal iron binding sites are detected on the uroid region ("cap").

† Membrane particles are assumed to be absent from the microexudate.

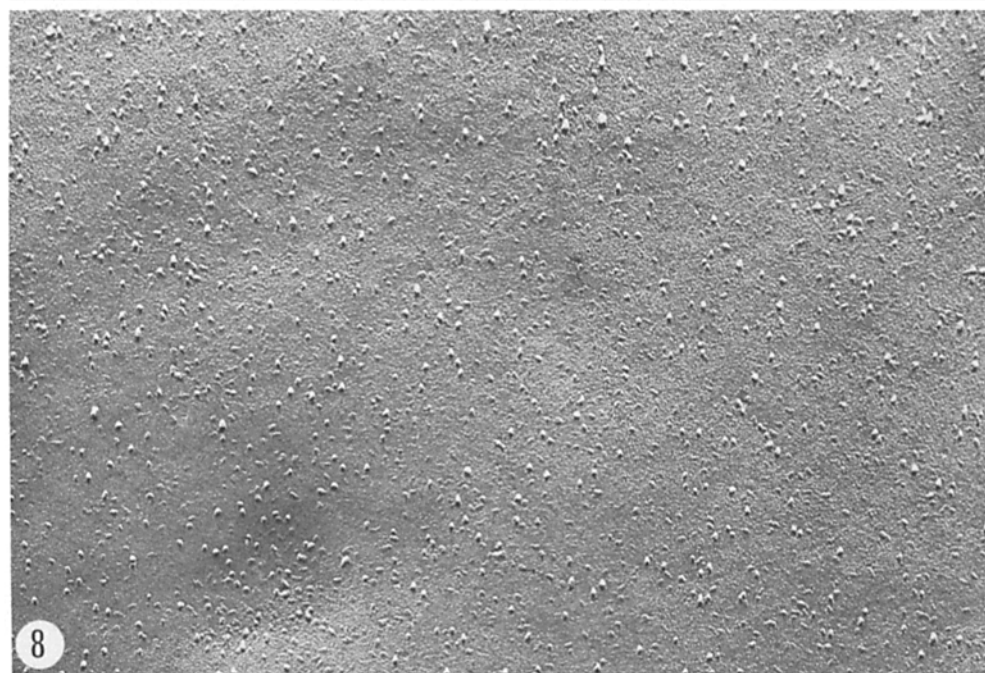
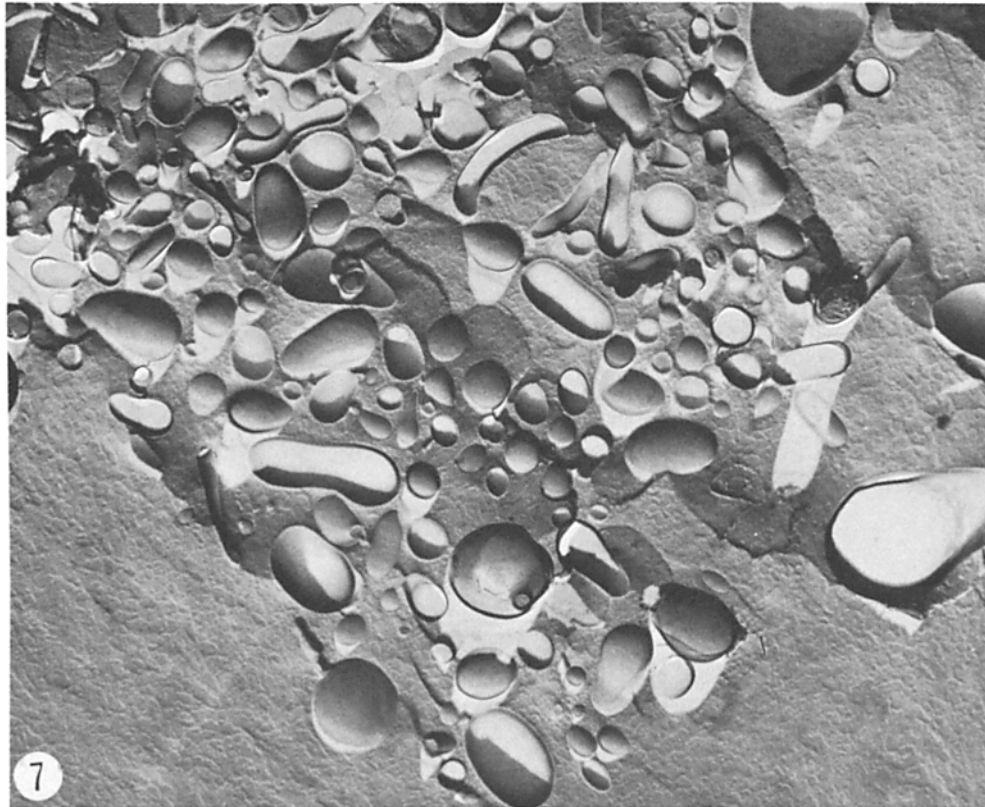


FIGURE 7 Freeze-fracture replica from the uroid region of an amoeba treated with con A and peroxidase. The uroid can be easily identified in replicas as numerous vesicles located at one pole of the cell. No membrane particles are seen at the uroid region. $\times 18,000$.

FIGURE 8 Fracture face A from an amoeba treated with con A and peroxidase. No clustering or polar accumulation of membrane particles may be seen in cells where con A receptors form caps. $\times 80,000$.

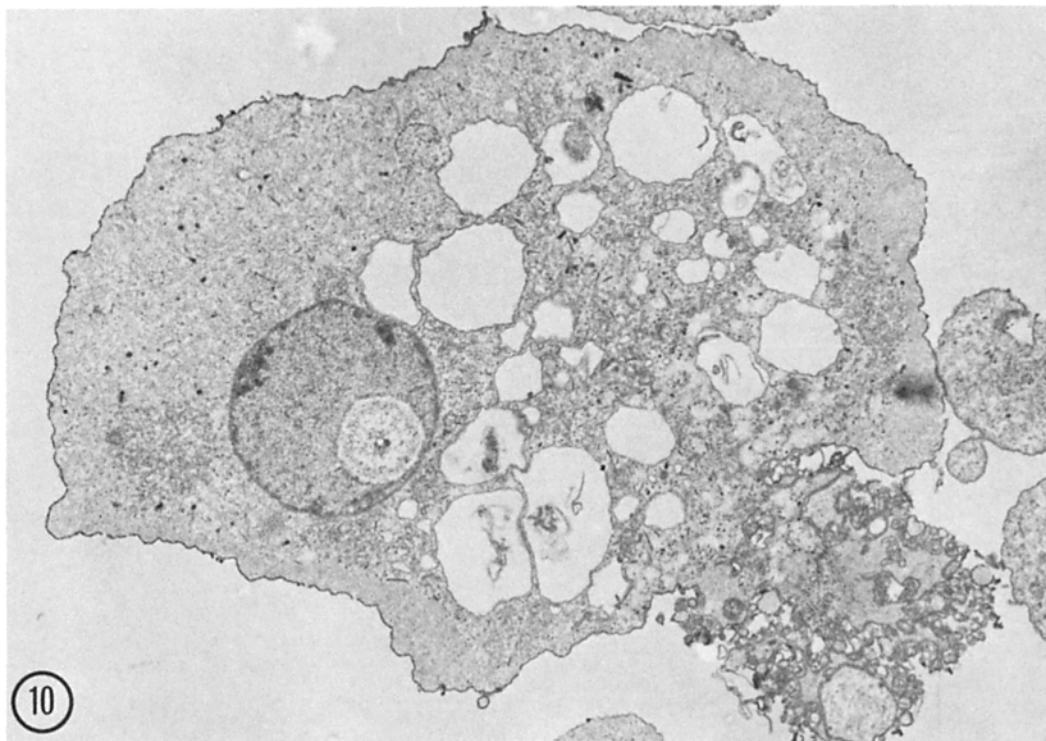
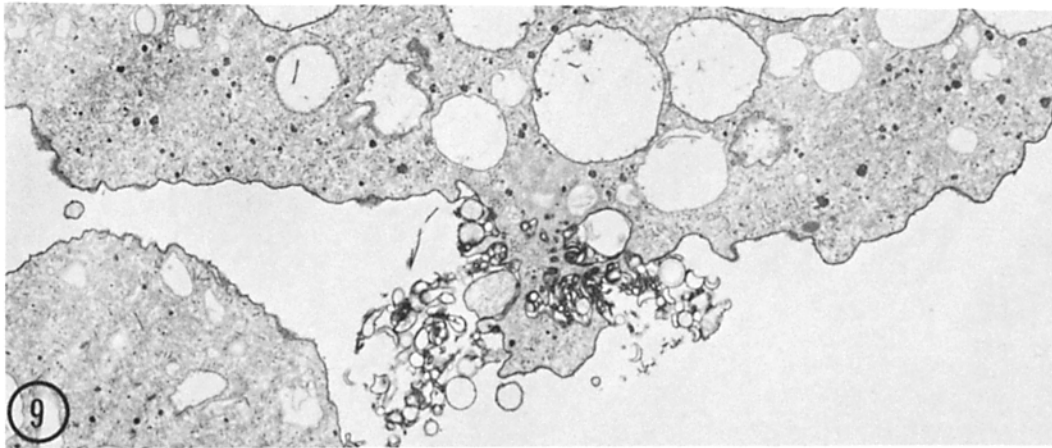
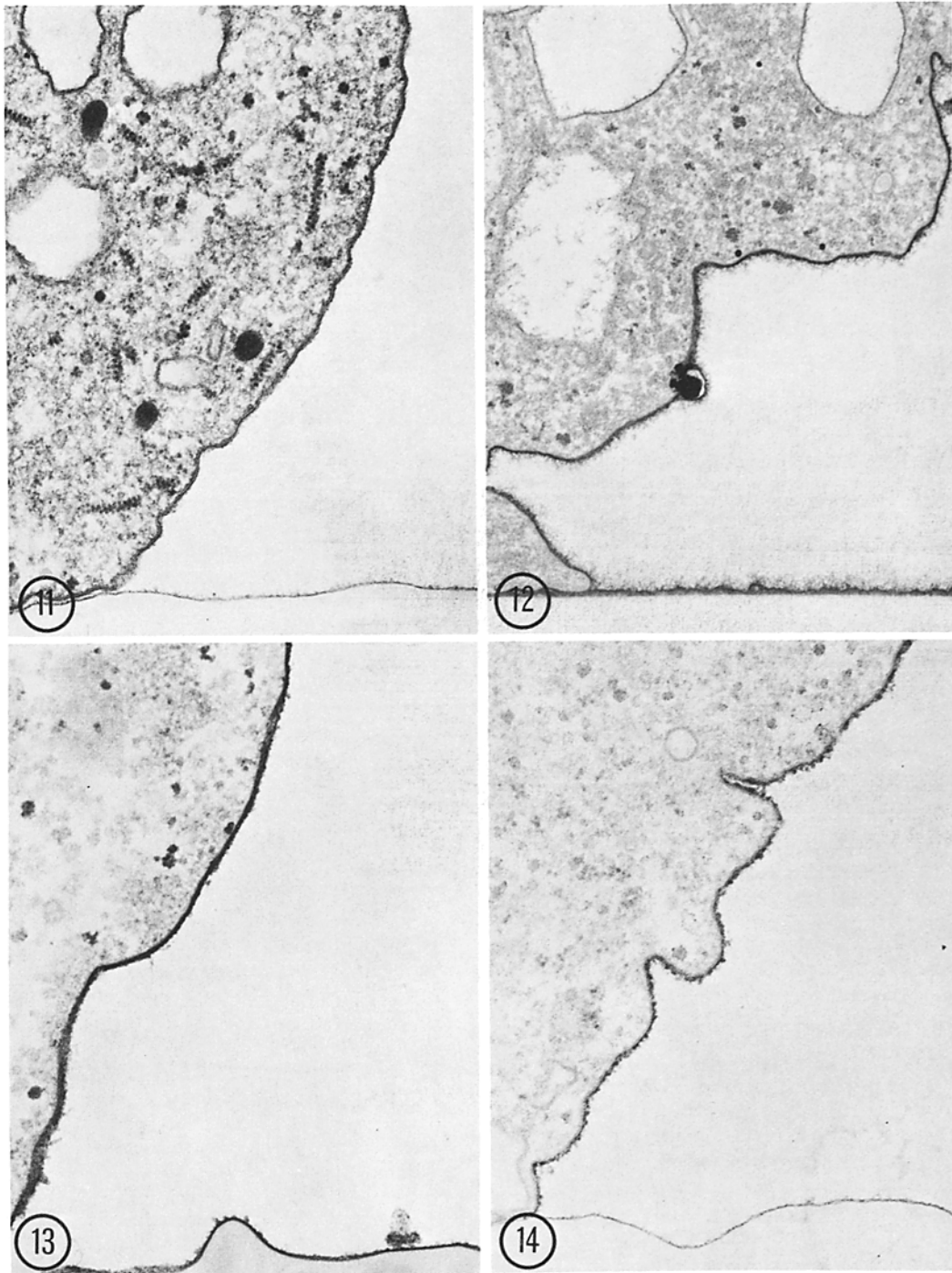


FIGURE 9 Uniform distribution of ruthenium red-stainable components at the surface of an amoeba treated with con A and peroxidase before fixation. $\times 20,000$.

FIGURE 10 Uniform distribution of colloidal iron binding sites at the surface of an *E. histolytica* trophozoite treated with con A and peroxidase before fixation, except at the uroid region where the acidic sites are virtually absent. $\times 6,000$.

As seen in Table I, con A-peroxidase treatment of living cells, while inducing generalized capping of con A receptors, does not result in accumulation of membrane particles, colloidal iron binding sites, or even of surface components stained by ru-

thenium red (a nonspecific saccharide reagent). Remarkably, membrane particles and colloidal iron binding sites are absent from the uroid region of capped cells. Lack of redistribution of membrane particles at the lymphocyte uropod where



FIGURES 11-14 Cytochemistry of cell surface and microexudate in *E. histolytica* cultures. All electron micrographs represent sections vertical to the plane of the culture; the microexudate is seen as an horizontal layer at the right side of the cells. Fig. 11, uranyl acetate staining after fixation. Fig. 12, alcian blue-lanthanum nitrate. Fig. 13, ruthenium red. Fig. 14, colloidal iron. Figs. 11-14, $\times 40,000$.

various surface markers form a cap has been the object of a preliminary report¹ (17). On the other hand, glycerol treatment of living *E. histolytica* cells results in a pattern of extreme aggregation of membrane particles and discontinuous distribution of acidic sites ionized at pH 1.8, but does not result in any apparent alterations of the distribution of Con A receptors, anionic sites ionized at pH 4.0 (28), or of components stained by ruthenium red (our unpublished observations). In addition, comparative cytochemistry of the microexudate and of adjacent areas of *E. histolytica* plasma membrane shows that microexudate and surface coat share common cytochemical properties, with the notable exception of colloidal iron binding sites.

Taken together, our experiments show: (a) parallel behavior of membrane particles and colloidal iron binding sites, which strongly suggest that these acidic sites represent the surface expression of an integral membrane component, possibly one included in the membrane particles²; (b) a negative association between membrane particles and con A receptors, which indicates independent dynamics of integral and peripheral membrane components; and (c) independent distribution of con A receptors, colloidal iron sites, and ruthenium red-positive components, which indicates a structural dissociation of the con A receptors from other component(s) of the surface coat.

In summary, our results reveal the complexity of

¹ Lack of correspondence between membrane particles and surface sites has been claimed for lentil-phytohemagglutinin receptors on human platelet membranes (11). These membranes, which are derived from an intracellular membrane of the megakaryocyte, exhibit reversed distribution of the membrane particles, most particles being present on fracture face B. In our opinion, however, lack of published high resolution micrographs of this fracture face make it impossible to decide whether or not a membrane particle-surface receptor relationship does or does not exist.

² Although the combination of cytochemistry and freeze-etch is, in principle, adequate to solve this problem, our attempts to visualize the relative distribution of aggregates of membrane particles in relation to colloidal iron micelles on the etched surface were unsuccessful. We were able to observe the continuous distribution of peroxidase-benzidine precipitates on etched surfaces of *E. histolytica* cells, but attempts to detect colloidal iron receptors were not successful because the harsh conditions involved in the staining procedure (treatment with 20% acetic acid), and freezing in the absence of a cryoprotectant caused disruption of fracture faces.

the relationship *membrane structure-cell surface* in *E. histolytica* trophozoites relative to erythrocyte ghosts where integral components (the membrane-intercalated particles) exclusively contain all antigens, receptors, and anionic sites labeled so far (27, 29, 30, 37). While our observations are restricted to the freeze-fracture morphology of the plasma membrane and the cytochemistry of the *outer surface coat*, recent work has also shown the probable importance of peripheral membrane components at the *inner surface* in controlling membrane dynamics and the distribution of sites at the outer surface (24). Our results illustrate structural and organizational complexity of plasma membranes and suggest prudence in the extrapolation of concepts derived from studies of specialized, structurally simpler membranes to the plasma membranes of living eukaryotic cells. It is also clear that the fluidity of integral membrane components (*integral membrane fluidity*) cannot be inferred from the demonstration of the mobility of surface components, nor, conversely, can the fluidity of peripheral membrane components (*peripheral membrane fluidity*) be assumed from demonstration of the mobility of integral membrane components.

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