"NEW MEMBRANE" FORMATION IN *AMOEBA PROTEUS* UPON INJURY OF INDIVIDUAL CELLS

Electron Microscope Observations

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ABSTRACT

Changes in the plasma membrane complex following the injury of single cells of *Amoeba proteus* were examined with the electron microscope. Two types of injury were employed in this study; cells were either pinched ("cut") in half or speared with a glass microneedle, and quickly fixed. Speared cells, when fixed in the presence of the ruthenium violet (a derivative of ruthenium red), revealed the presence of an extra trilaminar structure outside of each cell. This structure, called the "new membrane," was separated from the plasma membrane complex by a distance of less than a micron. The trilaminar structure of the new membrane strikingly resembled the image of the plasma membrane in all cells examined, except for its increased width (30%). This new membrane appeared nearly to surround the injured amebae. Attempts were made to demonstrate the possible origin of the new membrane, its reality, and its sensitivity to calcium. Also, some evidence is shown concerning the role of the small dense droplets (100–1200 A in diameter) normally present in the cytoplasm of amebae. Their frequent contact with the plasma membrane of the cell as the result of injury is interpreted as indicating their involvement in the formation and expansion of the plasma membrane.

INTRODUCTION

Living cells have been explored by a variety of approaches which are, of necessity, dependent upon the tools and techniques available. The majority of information about cells, however, comes from the examination of dead material. Fine structure cytologists, by the nature of the electron microscope, find themselves limited to the study of dead, preserved cells. Under these circumstances, the most desirable situation would be to correlate, as far as possible, the characteristics of living material with those details seen in sections of fixed cells. Especially useful, therefore, should be any technique which permits experiments to be done under continuous observation directly upon living cells, and subsequently allows these same cells to be examined at the ultrastructural level.

In fact, such a method is available. Abundant and interesting observations about the properties and behavior of living cells come from the era when micromanipulation was popular. This field was pioneered by Robert Chambers, who made important contributions to our understanding of living cells under a variety of conditions. Of particular interest were Chambers' experiments indicating that certain cells had the ability to heal wounds or repair tears in their plasma membrane that were deliberately inflicted by microneedles, provided that calcium was present (Chambers and Chambers, 1961). Many other papers on a

similar theme appeared. For example, Heilbrunn (1927) and Costello (1932) demonstrated in eggs of many marine species the formation of a membrane or surface film following injury. The reaction which underlies this membrane formation has been termed the "surface precipitation reaction" by Heilbrunn (1927).

Curiously enough, very few of such interesting experiments done in the past have been repeated with the aid of the electron microscope, and none as far as regeneration of the plasma membrane is concerned.

These intriguing phenomena, and particularly the ability of the cells to repair quickly their torn plasma membranes, led to the work reported here. In these experiments amebae were injured in two different ways. Cells were either pinched in half or speared with a glass microneedle. In addition to the conventional EM techniques used, ruthenium violet was employed to show fine details in the plasma membrane and outer coat of the ameba.

Surprisingly enough, the results suggested that amebae have the ability to create what appears to be a "new membrane" around themselves within a few seconds after injury.

MATERIALS AND METHODS

Culture

Amoeba proteus, obtained from Turtox,[1] was grown on 1% agar (Bacto, Difco Labs., Detroit, Mich.) in Prescott's medium (1956). Stacking dishes of 11 cm diameter were used as culture containers. *Tetrahymena pyriformis* was added periodically to the cultures, plus a half grain of precooked brown rice per dish. The amount of food was controlled carefully and new medium was added when necessary. About 100 amebae were used on inoculation of the new culture in each dish, and cultures were never grown for longer than a period of 3 wk in the same dish. Within that period of time, a large population of actively streaming and star-shaped amebae with long pseudopods was obtained. Cultures were grown in dim light at 20-22°C. Amebae were cultured in this manner successfully for over 2 yr.

Method of Handling Cells

For all observations, as well as electron microscope preparations, amebae were handled individually with

a braking pipette, while being constantly observed under a dissecting microscope with transmitted light. The desired number of cells with about 30 µl of the medium was transferred from the culture dish into a depression slide filled with 1 cc of new Prescott's medium. It is important to mention that, on transferring the cells to the new medium, the cells did not change their shape nor did the active cytoplasmic streaming stop, provided that a braking pipette with a hole of suitable diameter was chosen. The medium was changed twice in order to remove any excess food particles.

Afterward, each ameba was placed separately in about 10 µl of medium into a polyethylene cup (caps of "BEEM" polyethylene embedding capsules)[2] where the rest of the procedure was performed, e.g. injuring cells, fixation, dehydration, embedding, and polymerization, as described below. Replacement of the solutions in polyethylene cups was done carefully, since the air-water interface destroys the amebae. Therefore, solutions were withdrawn slowly, leaving the ameba immersed in a minute drop, and then the new solution was added slowly from the pipette placed at the side of the drop.

In this study, intact as well as injured cells were examined. Both groups of amebae were fixed either with ruthenium violet in the fixatives or without it.

Cell Injury

Amebae placed in a drop of Prescott's medium in a polyethylene cup were allowed to settle for 10 min. Usually within that period of time they adhered tightly to the bottom, which made it possible to perform the injury.

Cells were injured in two different ways. They were either pinched ("cut") in half, or were speared so that one or two holes were produced in them. Both types of injury were done by hand with fine glass microneedles (3-4 µ tip diameter) under the dissecting microscope. Microneedles were pulled by hand from solid rods of soft glass according to the method described in the literature (Chambers and Chambers, 1961). In the first instance, amebae were cut with the smooth and round portion of the microneedle by a sawing motion. In contrast, spearing was done with the sharp, fine tip of the microneedle, by inserting it deep into the cell and removing it in the same direction as the original stroke. Injuries were made gently and all precautions were taken not to detach amebae from the bottom of the polyethylene cup. Generally, such operations were successful; if not, the cells were discarded. Usually cells adhered quite tightly to the bottom and did not change posi-

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tion during the fixation and embedding procedure. This made it possible to recognize the same regions which were cut or speared. Although holes were not visible, usually there were some changes at the surface of the cell that are associated with this type of injury. In the case of larger holes, most of these changes were due to the reaction of the cell in response to the wound. Very small holes which did not produce any reaction on the part of the cell could not be located later.

Special attention was paid to the artifacts produced during injuries. When the microneedle was withdrawn from a speared ameba, occasionally material adhered to the needle and was pulled out from the ameba. This behavior during wounding produced artifacts that were visible in the electron microscope, such as pulled portions of plasma membrane, and so these amebae were also discarded. When the microneedle was withdrawn cleanly, the electron microscope showed a neat hole about 5-10 μ in diameter, with the plasma membrane on both sides curled inward but with no other distortion. These injuries were checked later by using a micromanipulator,[3] which allowed us to assume with confidence that the original work done by hand was valid.

Several experiments were done by spearing amebae in low calcium solutions. Two solutions were used: a "calcium-free" Prescott solution, made by omitting the calcium phosphate; and a solution of 2 mM ethylenediaminetetraacetic acid (EDTA). The 2 mM EDTA was prepared from a stock solution of 0.1 M EDTA (brought to pH 7.0 with NaOH) by dilution with distilled water. All solutions were made with reagent grade salts and glass-distilled water, and stored in polyethylene bottles. For experiments in which the calcium-free Prescott solution was used, the amebae were rinsed with it several times and kept for 2 hr. When EDTA was used, the amebae were rinsed for only 2 min. In both cases, the cells were handled in plastic Petri dishes, but were injured, as usual, in polyethylene cups.

Fixation, Dehydration, Embedding

Injured cells were fixed quickly, within 35-45 sec. All cells were fixed without being touched. Fixatives were delivered from the braking pipette directly to the polyethylene cups where amebae were already immersed in very small drops (10 μl each) of medium. The quantity of fixative added per cup was 0.25 ml.

Fixation was carried out in 2.5% acrolein buffered with 0.067 M cacodylate buffer at pH 7.2-7.4 for 15 min. Then the cells were postfixed in 1.3% OsO_4, buffered as before, for 30 min. Several other fixative

combinations were tried (glutaraldehyde, osmium tetroxide, and various buffers), but acrolein (Luft, 1959) proved to be most successful due to its rapid action, and cacodylate was the best of those buffers compatible with ruthenium violet.

When ruthenium violet was used in the fixatives, the aqueous solutions of ruthenium were prepared at sufficiently high concentration so that diluting them with fixatives would give a final concentration of 500 ppm. Ruthenium violet is a derivative of ruthenium red, and both dyes have many properties in common. Both have a strong affinity for acid mucopolysaccharides associated with plasma membranes of a variety of cells, and permit the visualization of cell membranes together with their surface coats in the electron microscope. It has also been shown that ruthenium red interacts strongly with acidic phospholipids, particularly those which actively form myelin figures. Details will be published elsewhere (Luft, 1971 a, b). The use of ruthenium violet on amebae is also described (Szubinska, 1964 a; Szubinska and Luft, 1971).

After being fixed in acrolein, all cells were washed briefly in cacodylate buffer before osmium tetroxide was added. This rinse was particularly important in the experiments with ruthenium violet because the dye precipitated in the osmium tetroxide fixative when much acrolein was carried over in transfer.

Dehydration was carried out in 35, 70, and 95% ethanol (5 min in each) followed by two changes of 100% ethanol, each for 10 min. Changing the alcohols was carried out in the same manner as before, i.e., removing and adding with a braking pipette. The entire procedure through embedding was carried out at room temperature in the original polyethylene cups. Previous experiments revealed the types of distortion which cells undergo during fixation, dehydration, and embedding (Szubinska, 1964 b). For embedding, a new experimental epoxy resin based on diglycidyl ether (Szubinska and Luft, to be published) of low viscosity was used to reduce cell shrinkage. Those precautions gave reproducible results with minimum distortion in all steps of the procedure.

The fixed and embedded cells were split from the polyethylene cups, carefully oriented, and sections for both light and electron microscopy were cut with a Huxley ultramicrotome. The sections were collected on carbon-coated grids, and examined in a Siemens Elmiskop I. Sections were examined both unstained and double-stained with a saturated aqueous solution of uranyl acetate followed by Reynolds' lead citrate (1963) for 5 and 10 min, respectively.

OBSERVATIONS

Normal Amoeba proteus

Unusual changes in amebae are seen following injury, and the most efficient way to describe

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[3] The author is grateful to Dr. Edward L. Chambers for receiving her as a guest in his laboratory at Miami, and for his help and the use of his facilities in checking the procedure used in this paper.

these alterations is by comparison with the normal. Thus, a survey is presented of the structure of intact *Amoeba proteus*. Regardless of the techniques used, electron microscopists find reproducibly the same components in amebae, the fine structure of which has been well documented (Pappas, 1959; Brandt and Pappas, 1960, 1962; Roth et al., 1960; Bhowmick and Wohlfarth-Bottermann, 1965; Nachmias, 1968; Flickinger, 1968; Bowers and Korn, 1969; Pollard and Ito, 1970; and others). Therefore, only a brief description will be given, with particular attention paid to those structures thought to be involved in the alterations produced during injury.

A single, intact ameba gives the typical appearance seen in Fig. 1. The boundary of the cell is recognized as a single black line with multiple reflections and infoldings, and this is accepted as the morphological counterpart of the plasma membrane. The interior is uniformly filled with a matrix containing cell organelles such as mitochondria, Golgi apparatus, rough endoplasmic reticulum (ER), and fibrils. However, of special interest are small, very dense droplets (arrows, Fig. 1) spread throughout the interior of the cell. Their diameter varies from 100 to 1200 A. Occasionally, one sees them very close to, or even in contact with, the plasma membrane. They have been described in the literature (Cohen, 1957; Mercer, 1959; Brandt and Pappas, 1960; and Pollard and Ito, 1970), but their function, origin, and composition are unknown. Their presence in the normal controls is emphasized because their importance in the ameba following injury will become apparent later. At higher magnifications of the same ameba (Fig. 2) the familiar trilaminar appearance of the plasma membrane (unit membrane) can be recognized. The extraneous coat is composed of two layers: a thin, compact "amorphous layer" which is adherent to the outer leaflet of the plasma membrane, and, external to this, a filamentous layer composed of irregular, branching filaments. The above description is common and reproducible, both in this laboratory and in others.

Injury—Light Microscope Observations

In this study, the amebae were injured in two different ways as described above, being either pinched in half or speared once or twice with a glass microneedle. The amebae reacted quite differently to these two types of injury. When the actively streaming ameba was pressed with the shaft of the microneedle for cutting, the streaming stopped suddenly and the two portions on either side of the needle rounded up gradually. As the further pressure on the needle thinned the cytoplasm between it and the polyethylene surface, gentle streaming reappeared in the cytoplasm near the needle and spread away from it to involve each half. This outward streaming continued as the needle was moved back and forth to cut the cytoplasm, leaving the two halves adherent to the polyethylene with ineffective streaming activity directed away from the cut. At the site of the cut, the membrane surfaces appeared to be smooth, and after a minute or two, streaming movements were more organized and small pseudopods became visible on the surface away from the cut, although both portions still remained attached to the polyethylene. Both halves behaved in the same way, despite the presence of the nucleus in one half and not in the other. The different activities for the nucleated and nonnucleated portions of the ameba, as reported in the

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All amebae were fixed in acrolein and postfixed in osmium tetroxide, both buffered with cacodylate buffer. Most sections were unstained; a few sections were double stained with uranyl acetate and lead citrate. Abbreviations repeated through the figures and captions are *D*, droplets; *I*, inside; *N*, "new membrane"; *O*, outside; *P*, plasma membrane.

FIGURE 1 Cross-section, stained, of an intact *Amoeba proteus*. Note at arrows dense droplets (*D*), which are present normally in the cytoplasm of these amebae. × 5170.

FIGURE 2 Portion of the plasma membrane complex of the same ameba as in Fig. 1, seen at higher magnification; stained section. This region comes from the place marked with double arrows in Fig. 1. The plasma membrane complex is composed of plasma membrane (*P*), an amorphous layer (*A*), and a filamentous layer (*F*). The filamentous layer is coarser and therefore more visible when acrolein is used with buffers other than cacodylate (e.g. phosphate), or when different fixatives are employed. × 118,000.
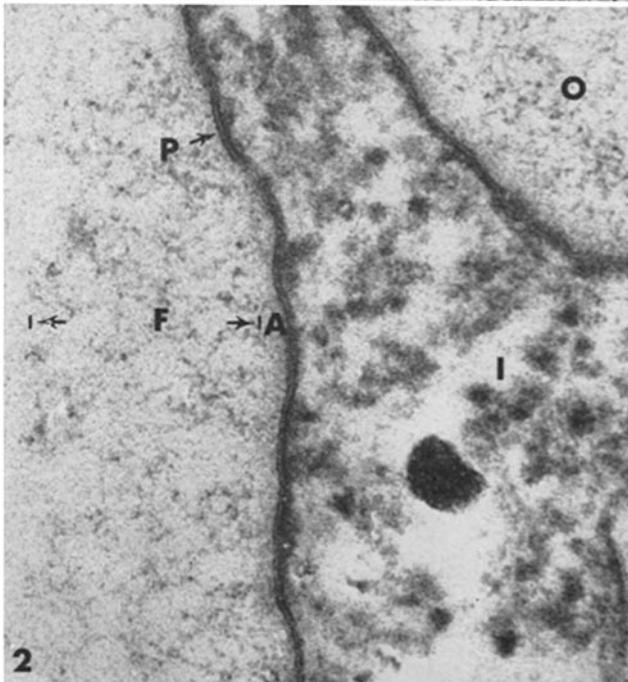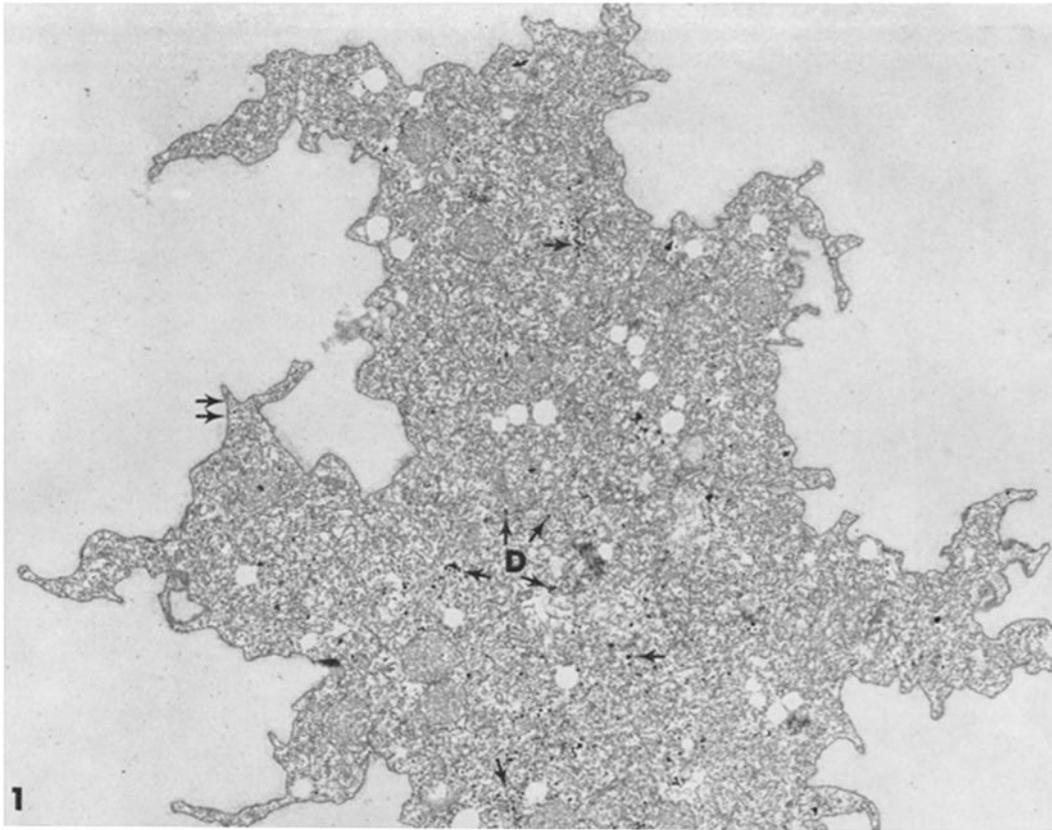
literature, did not develop in the brief interval between cutting and fixation (Comandon and de Fonbrune, 1939; Prescott and Carrier, 1964).

On the other hand, when amebae were speared their behavior was much more variable. The amebae rounded up, but without the sudden freezing of cytoplasmic movement which was characteristic of them when they were cut with the needle. Instead, contraction toward sphericity began, in some amebae slowly but in others very rapidly. A few amebae contracted to spheres too rapidly to be followed by the eye, and appeared as a ball on the end of the needle in the next moment. These amebae would then squeeze themselves off the needle point, still remaining attached to the polyethylene. Occasionally, at the site of the wound after withdrawal of the needle, very rapid undulation and activity could be observed on the surface of the ameba. Within a minute or so after spearing, the ameba could stream vigorously in a direction away from the wound. Some amebae, however, continued streaming even when impaled with the needle, rounding

up only when the needle was withdrawn, while others continued directed streaming after they were injured and the needle was withdrawn, apparently as if nothing had happened.

Injury—Electron Microscope Observations

CUT AMEBAE: Two remarkable findings emerged when the cut pair of amebae were examined in thin sections. The first was that although the ameba had been cut in two by the needle, the two halves were found to be limited by plasma membranes which were not ragged or torn but, on the contrary, were intact, continuous, and seemingly undamaged by the pinching and sawing. The second finding was a concentration of the dark droplets at the plasma membrane in patches along the cut and in neighboring areas as well. Instead of being occasionally in contact with the plasma membrane, as in the controls, these droplets appeared to be inserted into the plasma membrane which, together with their abundance, gave them the appearance of beads in a string as
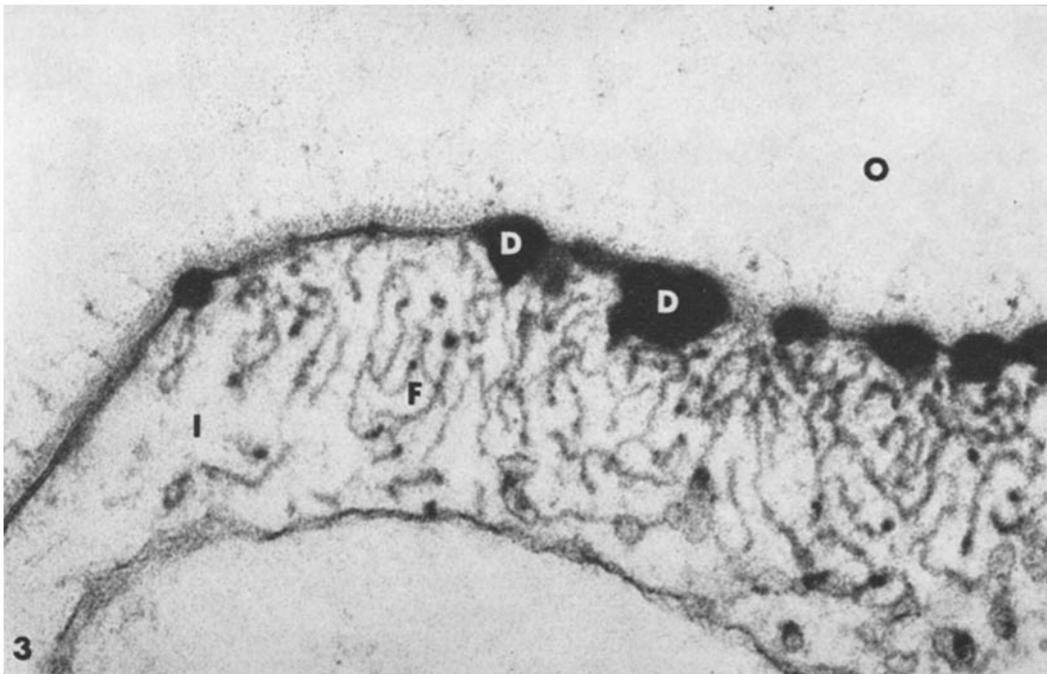

FIGURE 3 Portion of the plasma membrane complex from an ameba which was pinched (cut) in half, showing the region of the pinch itself; stained section. Note the intact extraneous coat and plasma membrane complex, as well as dense droplets (D) inserted in the plasma membrane. Filaments (F) extend from the dense droplets toward the interior. × 130,000.
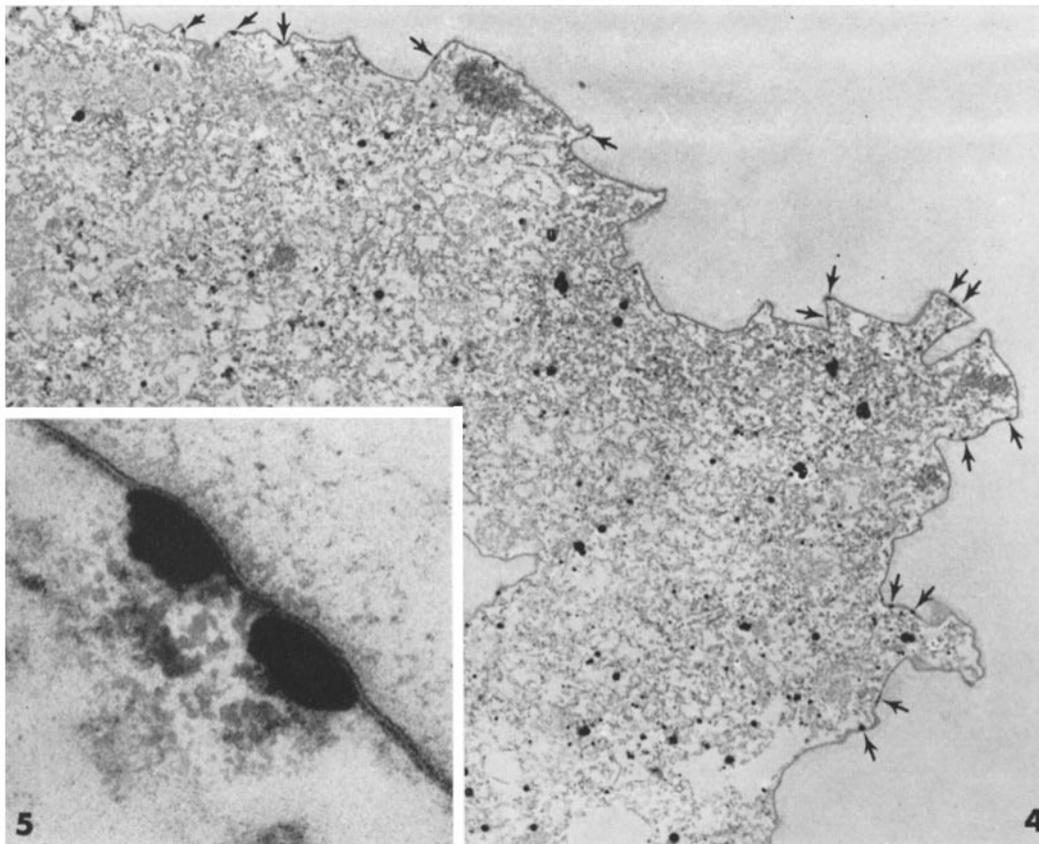
FIGURE 4   Cross-section of an ameba which was speared by a microneedle; stained section. Note the abundance of dense droplets in the cytoplasm and their frequent contact with the plasma membrane at the arrows. × 5440.

FIGURE 5   Higher magnification micrograph of the region marked with double arrows in Fig. 4; stained section. Here, dense droplets are shown in contact with the inner leaflet of the plasma membrane. × 159,000.

shown in Fig. 3. Both portions of the extraneous coat could be recognized external to the droplets as well as elsewhere along the cut surfaces of the amebae. From both the droplets and the inner leaflet of the plasma membrane between droplets, dense strands could be seen extending into the cytoplasm. These filaments were 50-100 A in diameter, they frequently terminated in a dense knob about twice their diameter, and in several instances they were clearly continuous with the larger dense droplets.

SPEARED AMEBAE: In several amebae, the hole produced by the microneedle could be recognized. A hole is not depicted in this paper because it was so buried in cytoplasmic debris that a

sequence of micrographs at low and high magnifications would be required to illustrate it adequately for publication. The hole was identified where the plasma membrane was interrupted and its edges were curled back into the cytoplasm on both sides of the hole, a situation similar to that shown by O'Neill for plasma membranes isolated from *Amoeba proteus* (O'Neill, 1964, Fig. 1). Clearly there was no membrane visible to cover the injury, and cytoplasmic granules and organelles appeared to be open to the exterior, contrary to the appearance of the cut amebae.

Near the injury, as well as in other regions of the ameba, dense droplets were found in an unusually high concentration, although not to the
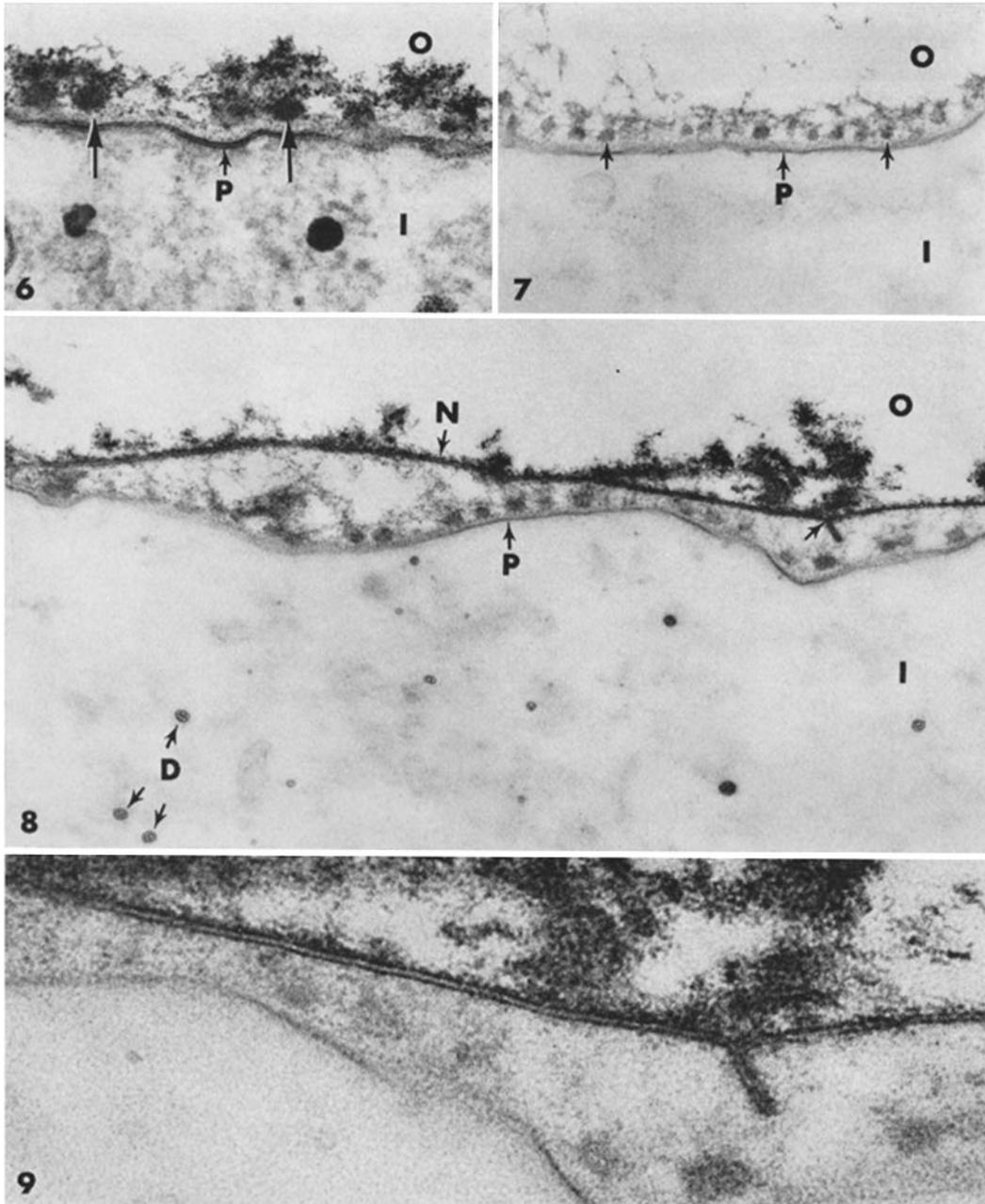
FIGURE 6 Plasma membrane complex of intact ameba fixed in the presence of ruthenium violet; stained section. Spherical globules (marked with arrows) are present in the extraneous coat. × 118,000.

FIGURE 7 Plasma membrane complex of intact ameba fixed in the presence of ruthenium violet; unstained section. Spherical globules (arrows) are seen in the extraneous coat at regular intervals. × 41,000.

FIGURE 8 Plasma membrane complex of speared ameba fixed in the presence of ruthenium violet; unstained section. On the outside of the plasma membrane complex, new membrane is present as a dense layer branching at one point (arrow). Its trilaminar structure is apparent. Small dense droplets (D) showing a foamy appearance are scattered in the cytoplasm. × 41,000.

FIGURE 9 Same region of the new membrane as seen at arrow in Fig. 8, but at higher magnification, showing more clearly its trilaminar structure; unstained section. × 159,000.
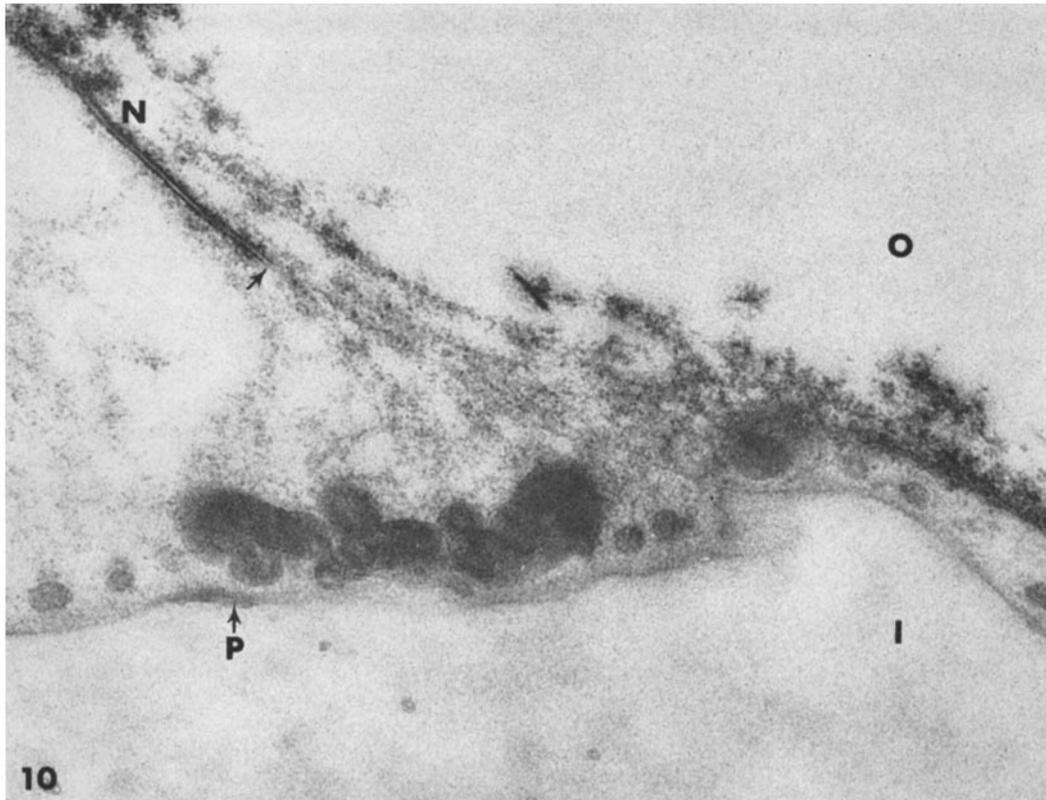
FIGURE 10 Illustrates breaks in the new membrane of a speared ameba; unstained section; ruthenium violet. At the point marked with an arrow there can be seen the gradual transition of the new membrane into strands of flocculent material. Below, at the plasma membrane complex, large dark bodies are present showing myelinated figures. × 90,000.

extent of forming a string of beads. Fig. 4 shows a region near the wound where the abundance of dense droplets in the cytoplasm is evident. There is likewise an unusually high frequency of contact between these droplets and the plasma membrane, as shown at the arrows in Fig. 4. At the double arrows in Fig. 4 is a pair of droplets which are further magnified in Fig. 5. Here it can be seen that the droplets are closely apposed to or even fused with the inner leaflet of the plasma membrane, the middle and outer leaflets continuing over the droplets without interruption. The dense strands which were abundant near the droplets in the cut amebae (Fig. 3) were not seen in the speared amebae.

Ruthenium Violet

In order to obtain further information concerning the response of the plasma membrane and

the extraneous coat in the injured ameba, some of the previous experiments were repeated with the use of ruthenium violet in the fixative solutions (Szubinska and Luft, 1971).

When the normal, uninjured ameba was treated with ruthenium violet, the appearance of the plasma membrane, and particularly the extraneous coat, was quite different from the usual image illustrated in Fig. 2. As shown in Fig. 6, spherical globules were seen at the base of the filamentous layer separated from the plasma membrane by a distance equal to the thickness of the amorphous layer. The globules were about 400–800 A in diameter and rather uniformly spaced with respect to one another. Even in unstained sections and at lower magnification (Fig. 7), the globules and the filaments were visible as well as the plasma membrane. This configuration and staining pattern was consistent and reproducible without

variation in many normal amebae treated with ruthenium violet (Szubinska and Luft, 1971).

When the speared ameba was fixed in the presence of ruthenium violet, the appearance was dramatically different from anything seen previously, as illustrated in Fig. 8. Here, the plasma membrane with the globules in the extraneous coat is visible as expected, but less than half a micron external to it is a densely staining, thick layer which has no precedent. This layer is continuous over large areas and has fuzzy or flocculent surfaces, although the fuzz appears to be thicker on the outer than on the inner face. Occasional branches are seen (Fig. 8, arrow). The same region at higher magnification (Fig. 9) reveals that the layer is trilaminar, reminiscent of the dark-light-dark sandwich structure of the unit membrane, although with a larger spacing (black line center-to-center distance, 69 A). Still more unexpectedly, the trilaminar structure continues unbroken into the side branch (Fig. 9). It appeared that this dense layer, which henceforth will be referred to as a "new membrane," was found over a large part of the ameba in cross-section, although there were frequent breaks. Sections at intervals through 130 μ showed that this new membrane persisted in depth as well as in circumference and covered large areas of the surface of the ameba. However, so far the new membrane has not been observed to cover the holes in amebae.

The breaks in the new membrane provided additional detail about its components, as depicted in Fig. 10. Still from the same ameba, a break is visible in the dense lamina, but continuity is retained across the gap by threads of the fuzzy or flocculent material adherent to both surfaces of the dense layer at the upper left. These threads fan out to contact extraneous coat material and the adjacent segment of new membrane extending to the lower right. Also, some further features of the new membrane are visible. The trilaminar

structure is clearly apparent, but it terminates with both dense laminae becoming pale and delicate and fading away entirely over a distance of about 500 A, although their spacing is retained precisely until they vanish completely. This observation has no counterpart elsewhere in the description of membrane behavior.

Figs. 11–14 complement the previous observations. Fig. 11 shows more irregularities in the new membrane, with two regions (A and B) shown enlarged in Figs. 12 and 13. Fig. 12 clearly illustrates branching, the central light component of the trilaminar structure forming a Y-shaped figure (arrow). The branch extends for about 1000 A and then terminates abruptly, and both of its surfaces as well as its end are covered by the flocculent dense material. Nearby are three more or less parallel lamellae showing the same trilaminar structure and existing in apparent isolation from the main layer of new membrane, in which the trilaminar appearance is lost, presumably due to the obliquity of the section. In Fig. 13, the new membrane follows the surface into a pit where a branch occurs. Because the section was cut obliquely, the trilaminar structure is obscured in all but a few regions, however the entire segment of membrane as well as the branch is anchored by radial strands of material which extend from the flocculent material to the extraneous coat. Fig. 14 shows dramatically the ability of the new membrane to follow pits and invaginations in the surface of the ameba. This figure shows not a fold following a pit but rather a single branch of membrane which extends into a deep invagination and which itself branches many times and maintains roughly equidistant spacing from the walls by a mechanism similar to that shown in Fig. 13. Although not illustrated, the trilaminar appearance of the new membrane appears throughout the branch where the geometry permits visualization.

FIGURE 11 Cross-section of speared ameba showing a variety of different forms of the new membrane; unstained section; ruthenium violet. It can be seen that new membrane is branching at various places but particularly at A. It can also be traced into a pit at B. Dark bodies are present at the plasma membrane complex. $\times$ 23,000.

FIGURE 12 High magnification of the region marked A in Fig. 11, showing, at arrow, branching of the light component of the new membrane; unstained section; ruthenium violet. To the right are seen three lamellae showing a trilaminar structure. Myelinated figures can be distinguished within the dense bodies located at the plasma membrane complex. $\times$ 116,000.
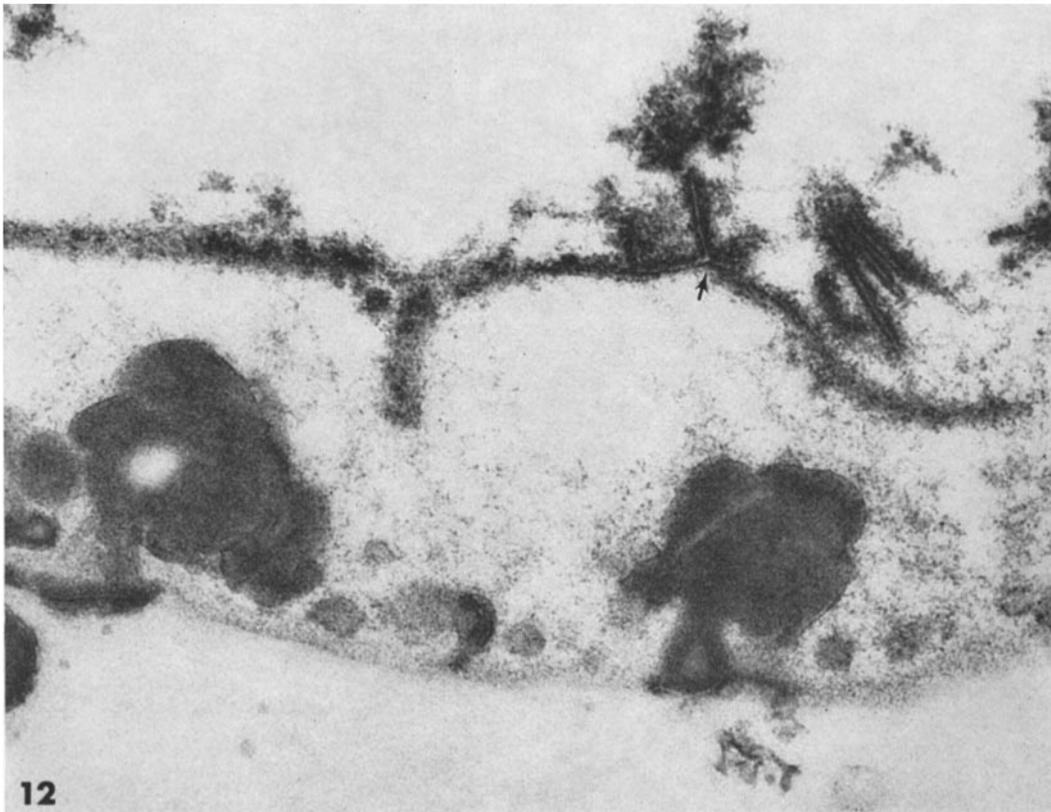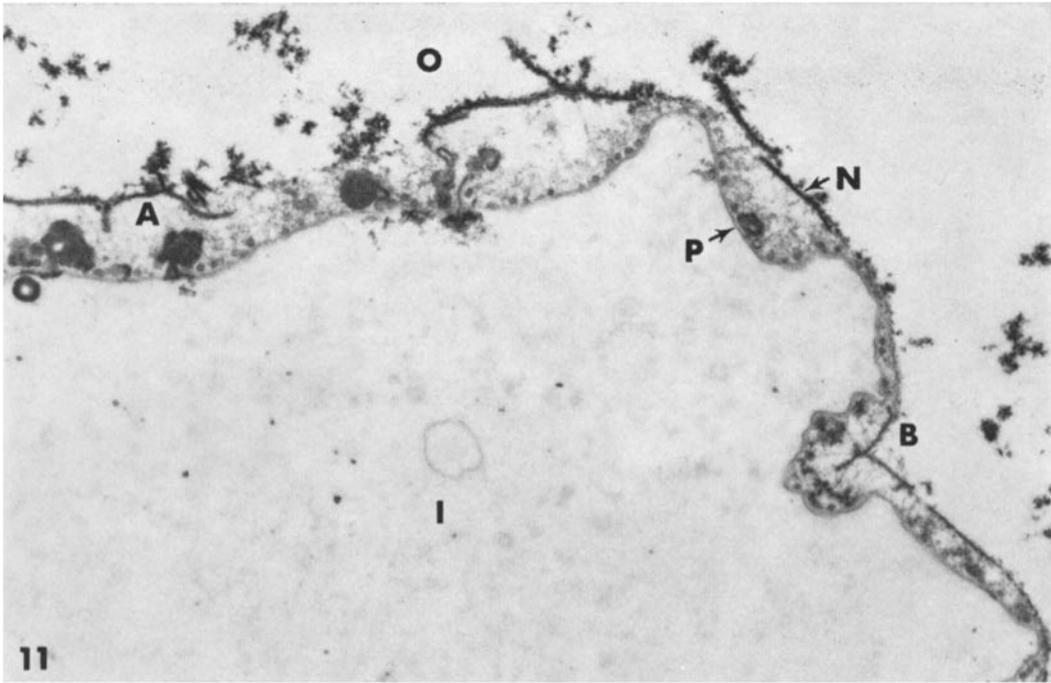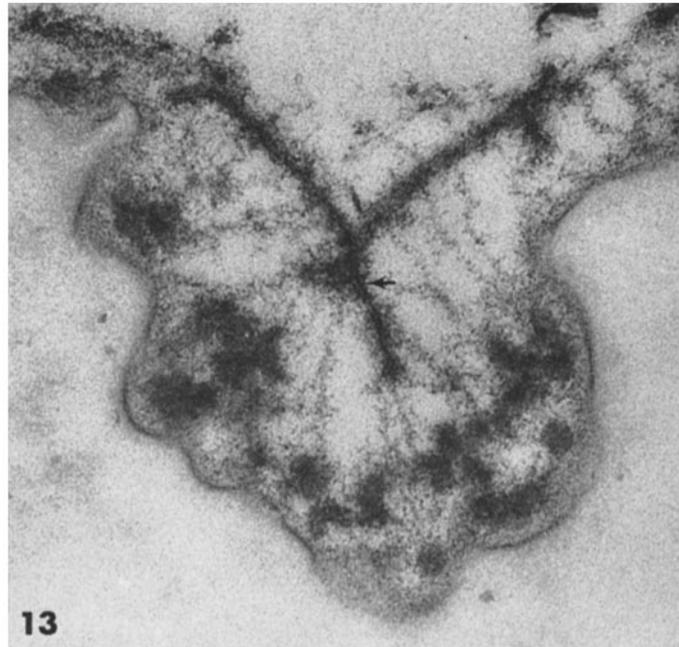
FIGURE 13 Illustrates the region *B* from Fig. 11 at higher magnification; unstained section; ruthenium violet. Here new membrane is descending from both sides into a pit. At the arrow, where branching takes place, the trilaminar structure can easily be seen. Note the radial arrangement of strands which are in contact with both the extraneous coat and new membrane. × 116,000.

Origin of the New Membrane—A Hypothesis

Since the new membrane appears to develop very rapidly, it has not been feasible to trace its origin in a verified sequence, and the details are too delicate to follow in the light microscope. Nevertheless, electron micrographs of different regions of the same ameba (Figs. 15, 18, 19) and other amebae show modifications of the new membrane and the plasma membrane, and can be arranged to form a plausible sequence. In the following description the electron micrographs are so ordered, accepting the hypothetical nature of the sequence in exchange for the advantage of logical exposition.

Although the new membrane covered large areas of the ameba as described earlier, these membrane segments merged into other regions which were less distinct than those regions where the trilaminar membrane was obvious, as if the membrane were still condensing (Fig. 15). In Fig. 15 the plasma membrane and the globules have their normal configuration, but the new membrane is flocculent and irregular, although at the arrow there is the suggestion of a linear core upon

which it may be organizing. The flocculent material is very similar to that which adheres to the surfaces of the presumably more mature new membrane (Figs. 8, 9), and for convenience we have called this material "nascent membrane." The new membrane together with the nascent membrane nearly covered the entire ameba.

As mentioned earlier, when amebae were speared vigorous activity or "undulation" sometimes occurred near the wound. In the electron microscope these amebae were seen to possess small projections near the wound as shown in Figs. 16 and 17. These projections were like pseudopods but much smaller (only 2–4 μ in diameter). The plasma membrane of these projections often presented a remarkable appearance, as seen in Fig. 16. A row of black dots 300–500 A in diameter adheres to the cytoplasmic surface of the plasma membrane, the disposition of the dots having unusual regularity and frequency. The dots are like those in Figs. 4 and 5, but much greater in number. The larger black spheres (about 1000 A in diameter) dispersed in the cytoplasm of the projection are more numerous than
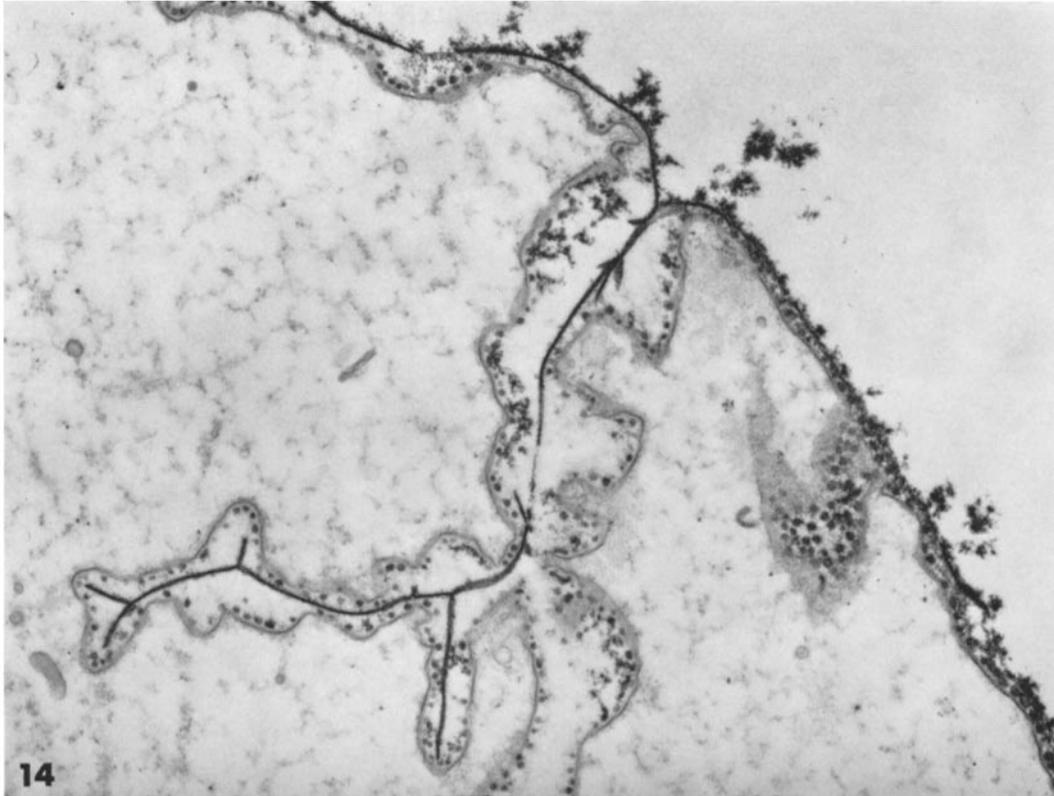
FIGURE 14 Cross-section through a large invagination in the surface of ameba showing new membrane as it follows an elaborate channel, branching several times along the way; speared ameba; unstained section; ruthenium violet. Note that the new membrane remains approximately in the center of the channel. × 23,000.

those seen in control amebae but otherwise are no different. Fig. 17 shows a projection from a different speared ameba in which the density is spread as plaques instead of regular droplets. The density is similar in both cases, since some droplets in Fig. 16 are similarly spread, and again, the large dense drops are visible in the center of the projection. In both Figs. 16 and 17, there is as yet no new membrane formation, and the appearance of the extraneous coat is closer to that of the extraneous coat of an uninjured, control ameba than it is to even that of the very early stage depicted in Fig. 15 as nascent membrane.

An enlargement of a region like Fig. 17 is illustrated in Fig. 18. The dense layer is irregular internally but spreads to within 50 A of the inner leaflet of the plasma membrane. At least two components can be distinguished: a dense amor-

phous substance (A), and another material occurring as strips or islands with a characteristic foamy appearance (B). This foamy appearance can be identified also within the dense droplets (D) in the cytoplasm of the projection. It is an artifact produced during irradiation of the droplets in the electron microscope, since the foamy texture can be observed to develop when a fresh region of the section is moved into the electron beam. However, it has the virtue of providing a valuable label for identifying the dense cytoplasmic droplets, because their foamy appearance is unique among the various organelles in ameba.

This digression implies some relationship between the dense material beneath the plasma membrane in these speared amebae, and the new membrane which presumably would have condensed a few moments later outside the ameba.
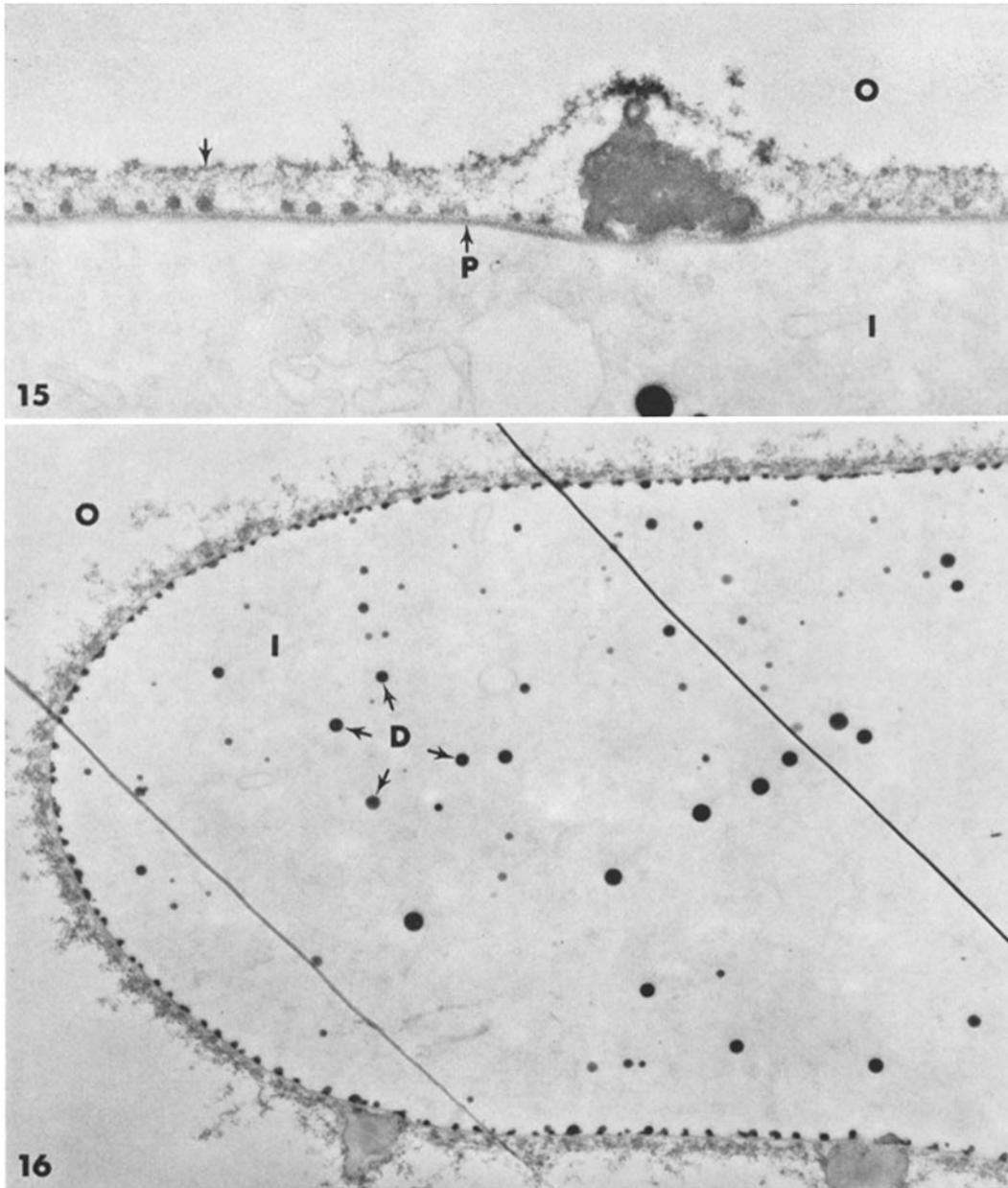
FIGURE 15 Portion of the plasma membrane complex which shows a layer of flocculent material on top of the filamentous layer as marked with the arrow; speared ameba; unstained section; ruthenium violet. The large dark body located in the extraneous coat seems to be lifting (and in contact with) the forming layer. The dark body is seen at higher magnification in Fig. 21. × 42,000.

FIGURE 16 Small projection from the region of vigorous surface activity near the wound; speared ameba; unstained section; ruthenium violet. Numerous dense droplets are seen adhering everywhere to the cytoplasmic surface of the plasma membrane with remarkable frequency and regularity. An abundance of droplets of the same density is present in the cytoplasm. (The two diagonal lines are folds in the section.) × 23,000.

If one contributes materially to the other, how does the dense substance penetrate the plasma membrane? Figs. 19–23 suggest an answer. Figs. 19 and 20 show dense droplets fused with the plasma membrane. That portion of the density still in the cytoplasm can be identified in Fig. 19, because of its foamy appearance, as being derived from the cytoplasmic droplets described earlier. However, the density transforms at the plasma membrane (which here is oblique) into a voluminous, but less dense, cloud without sharp demarcation. Fig. 20 shows the same phenomenon without the foamy characteristic; instead, Fig. 20 shows concentric lamellae, reminiscent of myelin figures, in the voluminous material external to the plasma membrane, and an inner dense component as well. Both Figs. 19 and 20 show an increased density on the external surface of the voluminous material which varies from a flocculent layer in Fig. 19 to a single discrete lamella in Fig. 20. In Fig. 21, the voluminous material is completely external to the plasma membrane and the myelin lamellae are well developed throughout. A nascent membrane has a tenuous existence nearby, but its density is strikingly increased near the apex of the voluminous material, and a suggestion of lamellae appears at that point.

Fig. 22 shows another collection of myelinated figures completely external to the plasma membrane. However, less than a quarter of a micron above the myelinated figures, instead of flocculent material, new membrane is present. The trilaminar structure of this new membrane is quite apparent, as well as its connections at several points with the material beneath.

Figs. 19–22 suggest that the dense droplets and the foamy material pass through the plasma membrane, but as is so often the case, the plasma membrane is seen obliquely and the details of the presumptive penetration are obscured. Fig. 23 illustrates this more clearly, and shows the overall relationship of the dense material to the cytoplasm, to the plasma membrane, and to the nascent membrane. There are two dense elements of interest in Fig. 23: a large wormlike element labeled A, and a smaller one shaped like an inverted T and labeled B. These two elements appear to be continuous at the arrow. The trilaminar structure of the plasma membrane is clearly visible (P) with element A exterior to it. Element B is intracellular, facing the cytoplasm on one surface and being close to or in contact

with the cytoplasmic leaflet of the plasma membrane at the other surface. Even the stem of the inverted T is covered with trilaminar membrane as the stem rises to fuse with element A, at which point the plasma membrane is no longer traceable. The image is compatible with the assumption that element A was produced as an extrusion from B through a fissure in the plasma membrane.

Membrane Survival

From the previous experiments, it became apparent that the new membrane could not be demonstrated without the use of ruthenium violet. It was possible, therefore, that the new membrane was not a real structure, but instead was some sort of precipitation artifact produced by contact of ruthenium violet with some substance released by the ameba at the moment of injury. To explore this possibility, further experiments were designed in which ruthenium violet was withheld until fixation with acrolein was well advanced (5 and 20 min). Low magnification pictures (not illustrated here) obtained from a speared ameba fixed 5 min before ruthenium violet was added reveal that the new membrane is still present. However, the new membrane is broken more than would have been expected upon immediate application of ruthenium violet, but the new membrane still penetrates deep channels. Higher magnification (Fig. 24) verifies the typical structure of the new membrane which has sufficient strength to bridge an infolding. The trilaminar structure of the new membrane can be seen in this material (arrows).

Even after 20 min delay in applying ruthenium violet, the new membrane still survives. The regions of the ameba that are covered with new membrane are smaller than were seen previously, and the flocculent material appears to be detached from the new membrane. Despite these alterations, however, Fig. 25 demonstrates the new membrane following and branching into a pit, one branch of which (arrow) shows the trilaminar structure more clearly than others. On the surface, the new membrane lies external to the globules of the extraneous coat and has the typical high density seen in earlier micrographs; it likewise shows the trilaminar structure, in segments at least.

The ameba in the 5 min delay experiment provided a fortuitous micrograph which not only confirmed the persistence of the new membrane, but provided further evidence as to the nature of the

trilaminar core and the flocculent coat. Low magnification pictures of the surface of this ameba showed regions in which the new membrane had certain segments which were more dense than others. A particularly interesting segment of density is shown at high magnification in Fig. 26. Here the trilaminar character of the new membrane is easily appreciated, the outer leaflet being much thicker and denser than the inner leaflet as well as being coated with flocculent material. However, a finding which is very unusual is that the outer leaflet terminates abruptly (right) or gradually (left) but the inner leaflet continues on both sides alone, and by itself then constitutes the low density portions of the new membrane.

The central, light component of the trilaminar membrane is visible only where it separates the outer from the inner leaflet; it is not possible to discern whether it continues together with the inner leaflet, or stops with the outer leaflet. This phenomenon may be related to the odd membrane termination noted in Fig. 10. This discontinuous element of outer leaflet material in Fig. 26 may prove to be important in the explanation of new membrane formation in the ameba.

Dependence of the New Membrane on Calcium

Because there is an extensive literature concerning the effects of calcium on the plasma membrane, preliminary experiments were designed to test the sensitivity of the new membrane to calcium concentration in the medium. One group of amebae was rinsed several times and kept for 2 hr in calcium-free Prescott's medium. Another group of amebae was rinsed for 2 min in 2 mM EDTA (longer times caused disintegration upon injury). Both groups of amebae were speared in these same solutions and quickly fixed as previously described with acrolein containing ruthenium violet. In the

light microscope it was clear that there was greater than normal leakage of cytoplasm after the amebae were speared in calcium-free medium, and this was greater still in the EDTA solution. Both groups of amebae seemed to be unable to contract around the wound, contrary to the behavior of amebae speared in normal Prescott's solution.

In the electron microscope, it was obvious that the amount of new membrane formed in the calcium-depleted medium was much less than that in the controls speared in medium with the usual calcium content. It was convenient to measure the length of plasma membrane visible in an electron micrograph and to compare this value with the length of new membrane visible in the same micrograph. The ratio of the two is easily expressed as the percentage of plasma membrane covered by new membrane. (For this comparison, only the regions of trilaminar new membrane were counted, and the areas of nascent membrane were disregarded.) The values give an average of 51% of plasma membrane covered by new membrane for the control amebae exposed to a solution of usual calcium content, in contrast to only 6.5% for those amebae speared in calcium-free Prescott's solution. No new membrane was visible in the three amebae speared in EDTA solution, except for a single patch in one region of one of the amebae; this singularity is unexplained. Essentially no nascent membrane was visible in the amebae exposed to either of the calcium-depleted solutions, and the entire plasma membrane complex was well preserved, including the unit membrane. However, there was considerable alteration and degradation of the cytoplasm in the amebae wounded in EDTA solution.

DISCUSSION

The new membrane described in this report is so unusual that many questions have arisen as to its

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FIGURE 17 Another projection from a region similar to that in Fig. 16, but from a different ameba; speared ameba; unstained section; ruthenium violet. Dense material underlying the plasma membrane, although spread in the form of plaques instead of droplets, shows the same density as droplets (D) which are spread throughout the cytoplasm. X 23,000.

FIGURE 18 High magnification of a plaque like those shown in Fig. 17; speared ameba; unstained section; ruthenium violet. The micrograph reveals the identical foamy structure of the dense droplets (D) in the cytoplasm as well as of the material (B) which is fused with the dark substance (A) underlying the plasma membrane. X 124,000.

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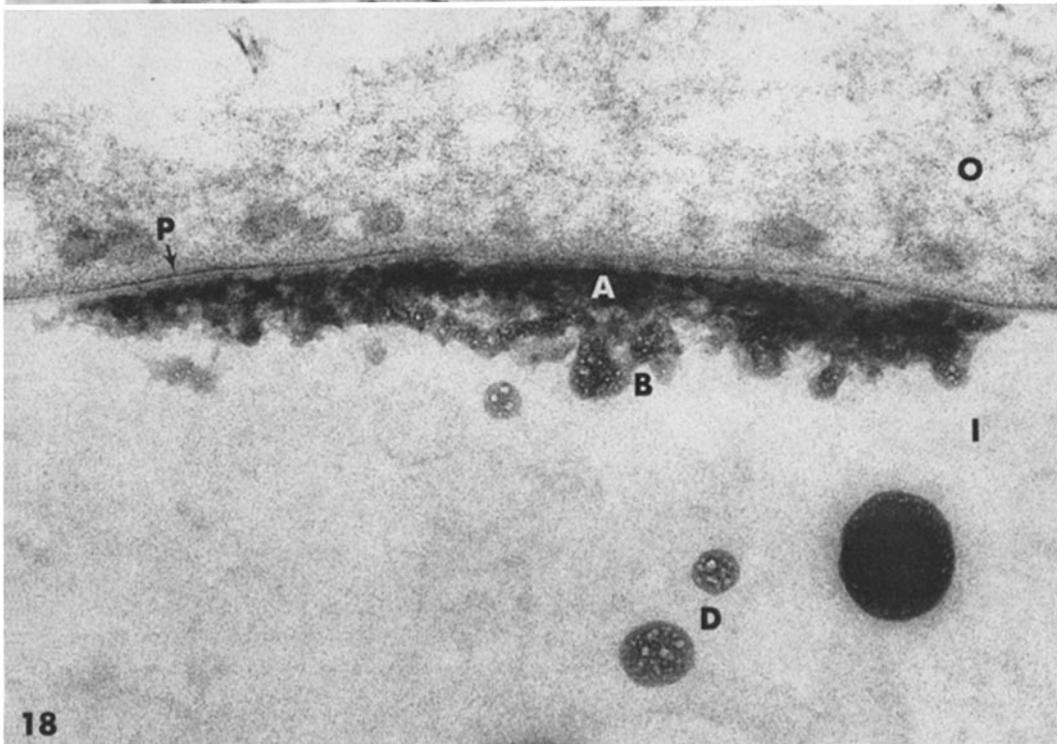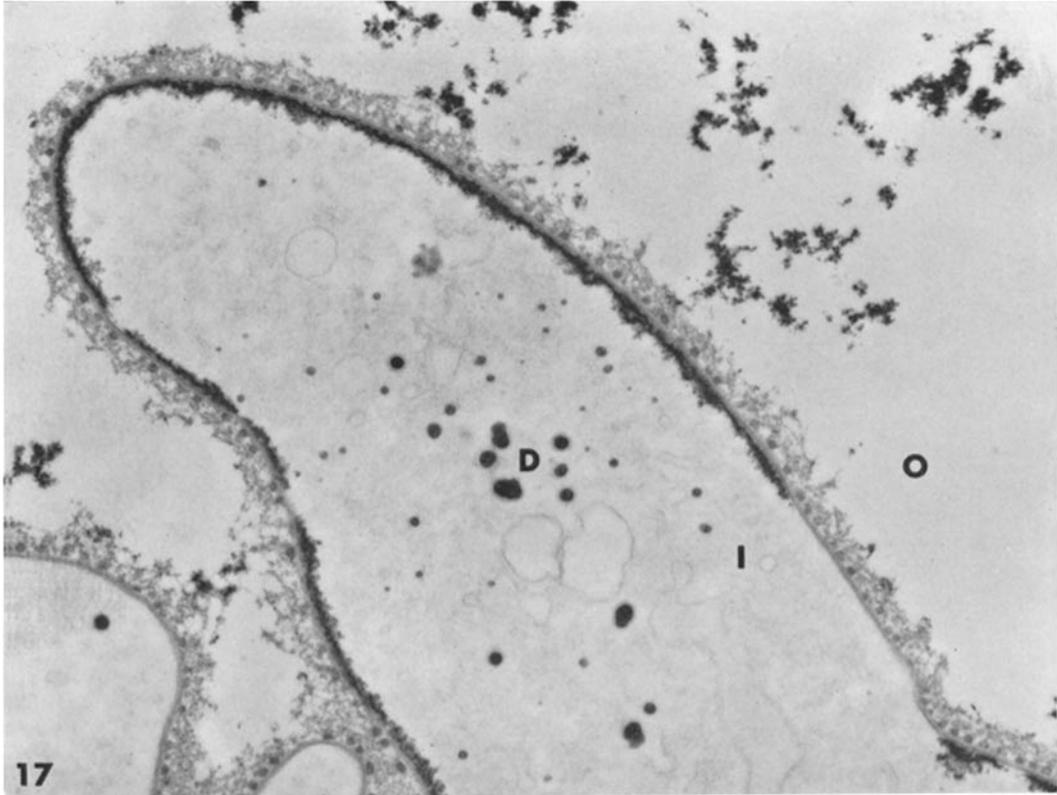
nature and even as to its existence. The experiments described here offer a partial answer to some questions, but others remain unanswered.

Of primary importance is whether the new membrane is real or an artifact, since it can be demonstrated thus far only by the use of ruthenium violet. Two of the experiments reported in this paper were designed to deal with this question. In the first set of experiments, the ruthenium violet was withheld for 5 min and again for 20 min after fixation was begun with buffered acrolein. By visual observation, acrolein fixation is fast, with all cytoplasmic movement stopping within a few seconds. Thus the ameba should have been thoroughly fixed and "dead" by the time the ruthenium violet was introduced. The observation of the new membrane after a 5 or 20 min delay makes unlikely the possibility that the ruthenium violet precipitates with something diffusing outward from the ameba at the time of death. Figs. 24 and 25 indicate that the new membrane persists essentially unchanged for at least this long after the onset of fixation in the absence of ruthenium violet. Alternatively, an explanation for the existence of the new membrane could be that some hypothetical material diffuses passively from the ameba and is precipitated as a membrane by the acrolein but does not stain or is otherwise not demonstrable except by the use of ruthenium violet.

The second set of experiments was based on the well-established fact that most biological membranes, particularly plasma membranes, are sensitive to, if not dependent upon, calcium ion in the external environment (Höber, 1945; Morrill et al., 1964; Cole, 1968). The experiments, which employed both a calcium-free medium and a dilute EDTA solution in which amebae were first wounded and then fixed, indicated that the formation of the new membrane was defective without calcium. In control amebae in a medium with the usual calcium content, 51% of the plasma membrane surface was covered with the new membrane. In the calcium-free medium the coverage dropped to only 6.5%, and in the presence of EDTA there was virtually none. Therefore, it may be concluded that, like more familiar biological membranes, the new membrane is affected by low concentrations of ionic calcium. However, calcium-dependence is not a property unique to membranes.

Another question which arises is why the new membrane (plus nascent membrane) is found only after injury, and generally around the cell instead of at the site of the wound. There is evidence in the literature that free-living cells especially have a remarkable ability to make emergency repair of tears in their plasma membranes, provided that the hole is not too large. This ability varies from one type of cell to another, with some cells being able to survive relatively large perforations, whereas other cells disintegrate after even small wounds. Damage to the plasma membrane with survival of the cell can even reach the extreme case cited by Chambers and Chambers (1961) in the fresh water ameba, *Amoeba dubia*. This ameba, apparently, is able to regenerate a completely

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FIGURE 19 Dense droplet (with its characteristic foamy appearance) still located in the interior of the cell is fused with the plasma membrane. Speared ameba; unstained section; ruthenium violet. Dark material at the plasma membrane level transforms gradually into a less dense, voluminous substance on the outside. × 144,000.

FIGURE 20 Illustrates situation similar to that in Fig. 19; speared ameba; unstained section; ruthenium violet. However, both the inner dense component and the voluminous material on the outside show distinct concentric (myelin) lamellae. × 159,000.

FIGURE 21 Voluminous material located entirely on the outside of the cell shows well-developed myelinated figures; speared ameba; ruthenium violet. Of special interest is the connection of this material at the apex with the forming membrane (nascent membrane). This picture is a higher magnification of Fig. 15. × 120,000.

FIGURE 22 Voluminous material like that in Fig. 21 is seen here also on the outside of the cell and shows well-developed myelin figures; speared ameba; unstained section; ruthenium violet. But above it, instead of a flocculent layer, new membrane is present which remains in contact with the voluminous material at several points. × 40,000.
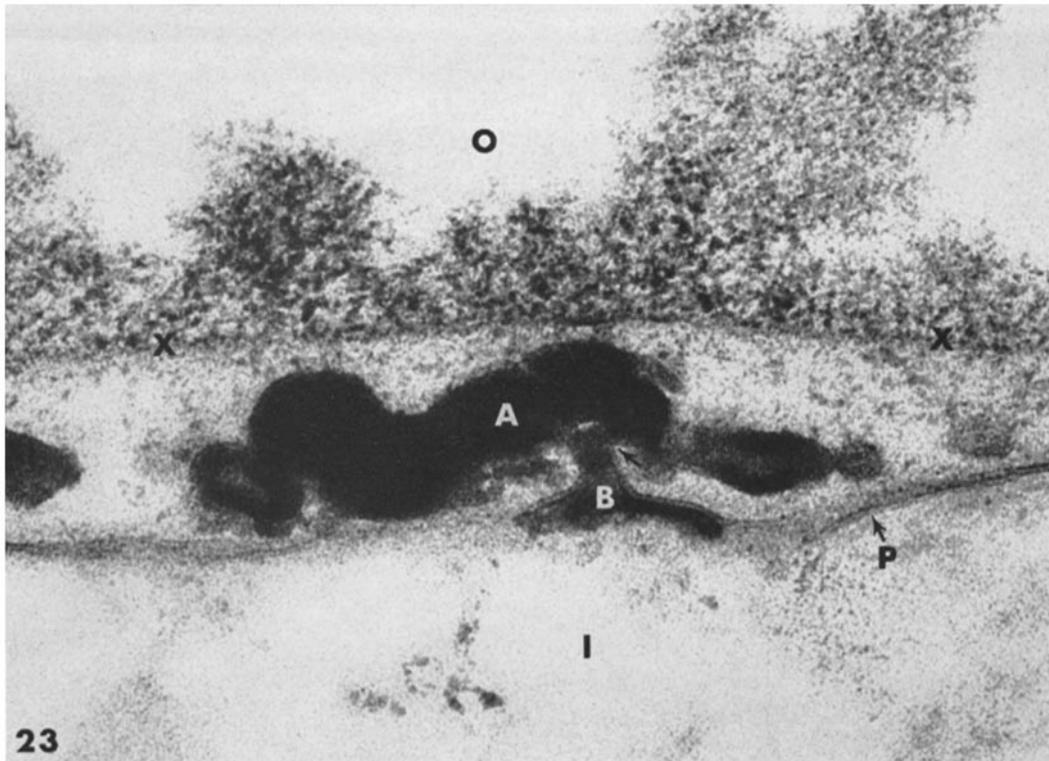
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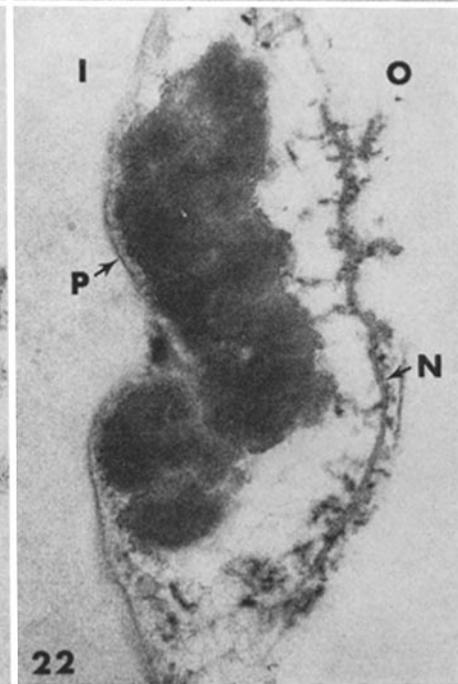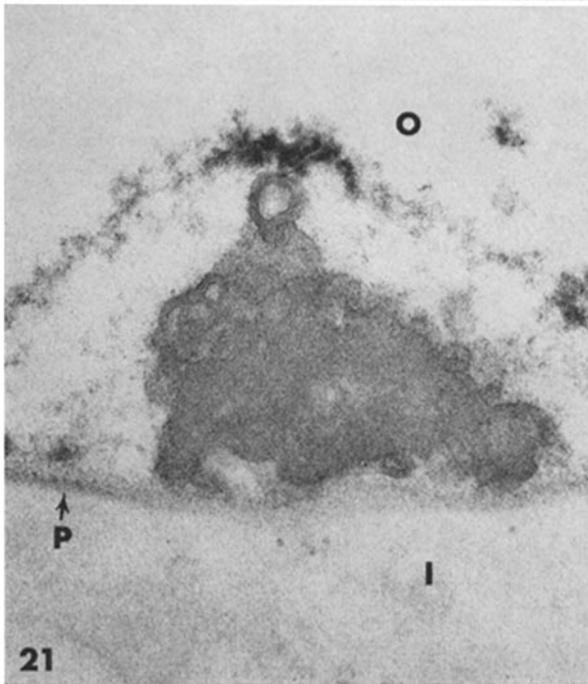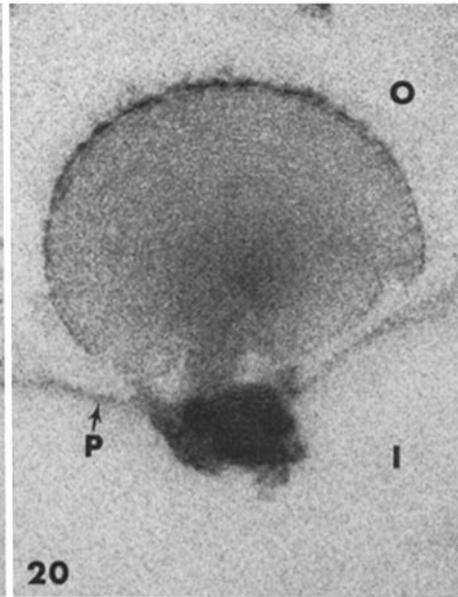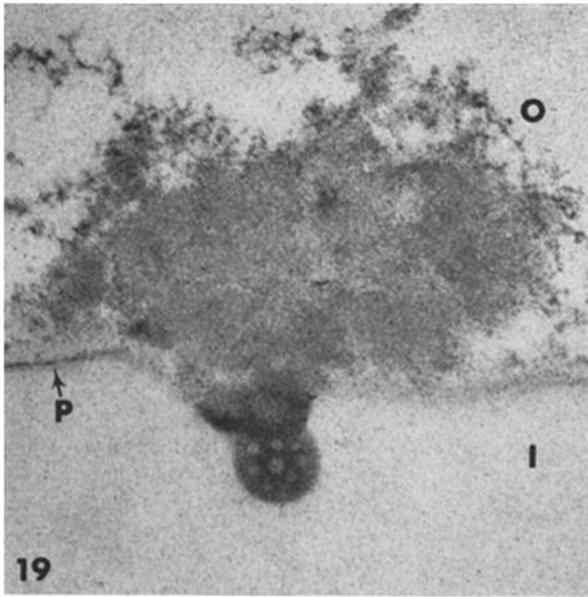
FIGURE 23 This picture illustrates possible passage of the dense material through the plasma membrane; speared ameba; unstained section; ruthenium violet. The arrow indicates continuity between the intracellular (B) and extracellular (A) portions of this dense material. Intracellular density (B) is in contact with the inner leaflet of the plasma membrane. The center and the outer leaflet of the plasma membrane can be traced easily on both sides covering the density (B). Above the extracellular density (A) extends the layer of flocculent material which condenses to a distinct line (X). × 159,000.

new surface film in a matter of seconds if pond water is available. Their experiment was carried out with the ameba in a hanging drop of the pond water on a cover glass. The ameba was detached from the cover glass by a microneedle and brought into contact with the air-water interface where its delicate pellicle was ripped off by surface tension, leaving an adhering granular mass of cytoplasm and nucleus. If the mass was returned quickly enough into the deeper region of the hanging drop, a "protoplasmic film" immediately regenerated at the surface and the ameba recovered completely. Wounding experiments performed by the same authors on other cells (starfish eggs, Arbacia eggs, etc.) indicated that these cells also were able to repair accidental damage to their plasma membranes and cell walls. On the basis of the above data, the presence of a new structure, such as the

new membrane after the injury, can be understood. However, its location and distribution remains unclear. Although the new membrane appears to cover large areas of the surface of the ameba (and together with the nascent membrane more or less surrounds the cell), so far it has not been found to cover the hole, where it would seem to be most necessary. Further experiments may reveal whether the time between wounding and fixation in the present studies is too brief (35–45 sec) for the new membrane to spread completely. However, it is evident, even from current experiments, that some kind of barrier is present over the wound, since ruthenium violet is arrested along a line and does not enter the ameba cytoplasm.

In view, then, of the reports in the literature together with the present experiments, perhaps it
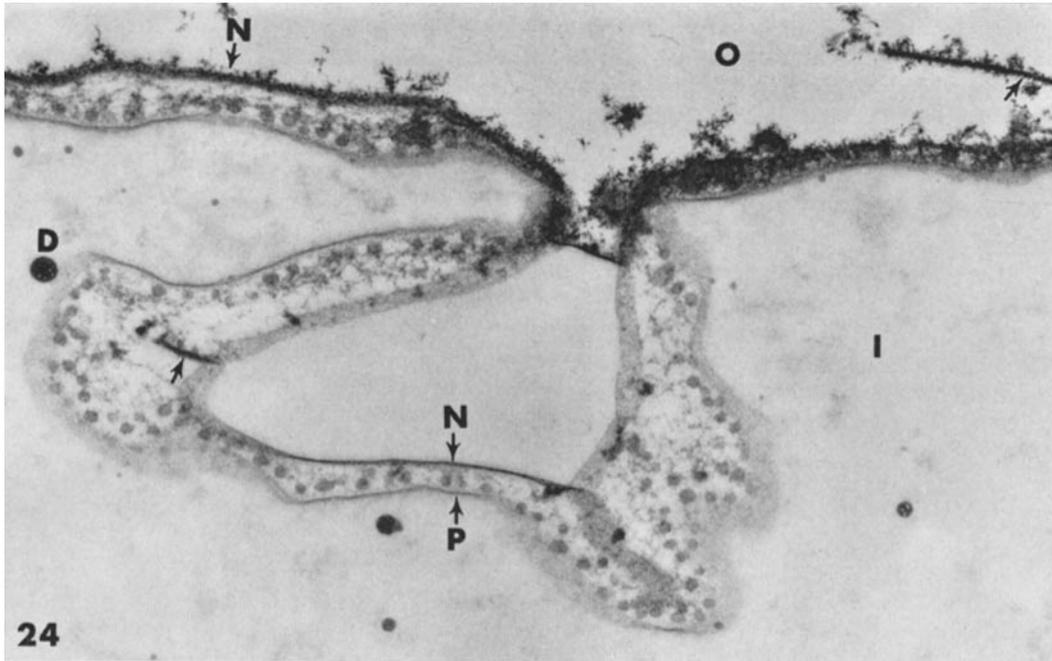
FIGURE 24 Portion of the plasma membrane complex covered with the new membrane obtained from an ameba which was fixed for 5 min before ruthenium violet was added to the fixative; speared ameba; unstained section; ruthenium violet. New membrane is seen bridging the infolding, as well as completely lining the cavity, although it is obliquely cut at times. A trilaminar structure can be seen at arrows. × 43,000.

FIGURE 25 Portion of the plasma membrane from an ameba which was fixed for 20 min before ruthenium violet was added; speared ameba; unstained section; ruthenium violet. New membrane is present on the surface as well as in the infolding, and it branches several times. A trilaminar structure can be seen at the arrow. × 43,000.
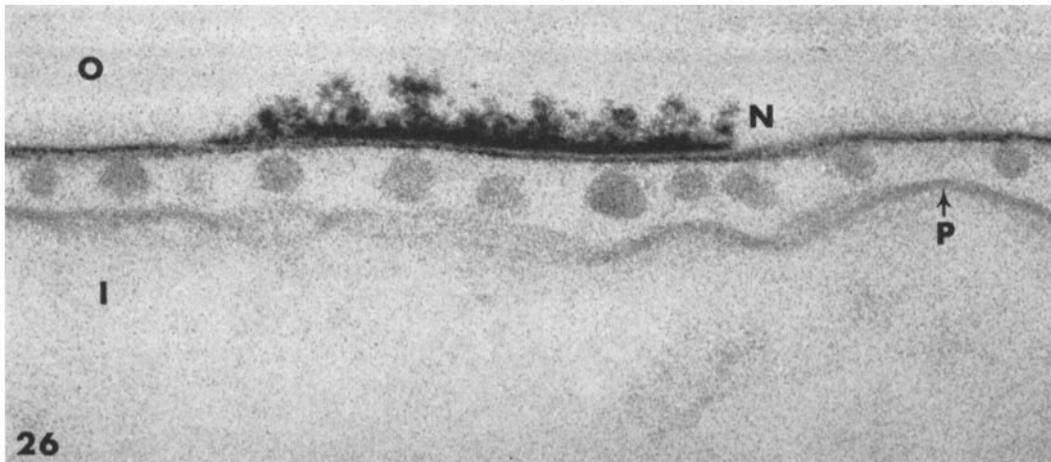
FIGURE 26 Micrograph from same section as Fig. 24; speared ameba; unstained section; ruthenium violet. The trilaminar character of the new membrane is separated into at least two components. The outer leaflet exists as a discontinuous layer, whereas the inner leaflet continues unaltered. The central light component, upon which the discontinuous element appears to float, may or may not spread over the inner leaflet. × 160,000.

is not unreasonable to propose that cells, particularly free-living cells, have some protective mechanism against sudden, large injuries, such as those which were made by hand in this study. It is plausible that a successful mechanism would be general, nonspecific, and automatic. A new membrane which would "crystallize" quickly around the entire stricken ameba and isolate it temporarily from its environment would seem to meet these requirements. It is important to note that these events are triggered by large punctures; small punctures, which can be made by hand with the same microneedles that were employed for the larger punctures, in no way alter the behavior of the ameba, which continues streaming as if nothing had happened. The electrophysiological work which has been done on various amebae by means of microelectrodes (Bruce and Marshall, 1965; Bingley, 1966) certifies that these micropunctures produce little if any disturbance in behavior. On the other hand, the large wounds described in this study cause reactions which supersede all other types of behavior.

Another important question is why this new membrane has not been reported before, despite the numerous, careful experiments on amebae in the past. There seem to be several reasons for this deficiency. Before 1955, the light microscope was the only instrument capable of detecting the mem-

brane. The optical conditions which prevail during micromanipulation are not ideal, as indicated by Kopak's efforts (1935) to adapt dark-field illumination to micromanipulation. However, since the electron micrographs in this paper show that the new membrane is never more than 0.5 μ from the plasma membrane and is usually much less, it is very unlikely that the new membrane could be recognized as a distinct layer over the large, rounded ameba, even under the best of conditions with the light microscope.

After 1955, the electron microscope was available, but evidence is accumulating that the initial methods of fixing and handling tissues did not preserve everything. This is particularly true of cell coat material (Luft, 1966). New techniques are being developed to reveal this type of material, among them the ruthenium dyes (Luft, 1971 a, b; Szubinska and Luft, 1971). So far, the new membrane has been seen only with the aid of ruthenium violet, and there has not been time to explore other procedures.

As implied earlier, if the new membrane has a protective function, the question arises as to whether it has a precedent elsewhere in the animal world. Beament (1968) has described a monolayer of lipid outside the cuticle of insects. His experiments in insect physiology have shown that this monolayer is essential to protect the insects against

dehydration. Another line of evidence comes from electron microscopy with the use of conventional methods. Petrik and Riedel (1968) have shown, in lungs of birds, a trilaminar membrane above the plasma membrane of the alveolar epithelial cells. Their trilaminar membrane is similar to the new membrane illustrated here.

A further question concerns the speed with which the new membrane is formed: How can the cell produce the layer so fast? The same question can be asked about the plasma membrane, since there is evidence (Chambers and Chambers, 1961) that it can be formed with considerable speed. Also, experiments with laser irradiation of the plasmodium *Physarum polycephalum* indicate that there is rapid formation of new membranes within 2–10 sec after laser injury (Griffin et al., 1969); however, from those experiments it is not clear how these membranes are formed. From the sequence of observations in the present paper, it seems not unreasonable to suspect that the new membrane is formed from material normally present in the cytoplasm and which, under certain circumstances, is extruded to the outside. This material, which is normally present in the cytoplasm in the form of dense droplets, is presumably some sort of lipid, perhaps phospholipid. When extruded, this material appears to hydrate, forming myelin figures, and to spread over the surface of the ameba. In some way, perhaps by condensing with another component, it is converted into the new membrane configuration. Fig. 26 seems to indicate that at least two components are involved in the formation of the new membrane. The discontinuous element of external dense material is uniformly separated from the inner leaflet material beneath it, and seems to float on a layer of invisible substance. It is possible that the invisible layer (perhaps lipid) has spread in advance of condensation of the second component and would have remained undetected here except for its capture of a fragment of external coating. The myelin figures on the outside of the plasma membrane (e.g., Figs. 20, 21), like the new membrane itself, are not seen when the injured amebae are fixed without the use of ruthenium violet. However, as long as the dense droplets remain within the cytoplasm, they are visible by conventional procedures. Thus, in speared cells fixed in the conventional way, a great number of dense droplets appear to be fusing with the inner leaflet of the unit membrane, and this is the main difference

between the injured and the control cells. In uninjured controls, the dense droplets occasionally are seen attached to, or closely associated with, the unit membrane. This phenomenon was also noticed by other scientists as mentioned previously. Mercer (1959), for example, points out that dense droplets are "very common against the membrane in the tail of the amoeba." Brandt and Pappas (1960) have also mentioned the frequent association of such droplets with the membranes in amebae. Cohen (1957) shows them arranged against a vacuole membrane in *Amoeba proteus*, as has also been observed by the author. Injury appears to increase the frequency of contact of these droplets with the plasma membrane, so that a reasonable inference is that the droplets are fusing with the membrane and, in this way are contributing material to it and expanding its surface area.

Support for this concept comes from experiments in which amebae were pinched or cut in half with a microneedle. The plasma membrane of the ameba remains intact in each of the two halves at the site of the pinch, and it appears quite normal except for having an abundance of dense droplets in contact with it near the pinch. From Chambers' work, it is clear that this kind of injury is quite different from injuries in which the plasma membrane is torn. He states: "An increase in area of the protoplasmic surface film, which occurs when cells divide or when cells are stretched or cut into fragments with microneedles, is explained not only by the stretching of a pre-existing surface layer, but also by the addition of surface layer material from the underlying cytoplasm. As long as there is no rupture of the surface, this type of surface formation occurs readily in isosmotic solutions of calcium chloride or in isosmotic solutions of monovalent salts in the complete absence of calcium."

Other findings also reinforce this concept. Experiments done by Nachmias (1966), who carried out electron microscope studies on *Chaos chaos* with Alcian blue as a marker, indicate that the new, unlabeled membrane appears to be randomly inserted in between the patches of older plasma membrane complex labeled with Alcian blue.

Both the dense droplets in contact with the plasma membrane and the dense droplets dispersed throughout the cytoplasm have an unusual morphological characteristic in common, i.e., a foamy appearance in the electron microscope. (Even though this characteristic appears to be an

artifact produced during electron bombardment, its rarity and uniqueness make it a convenient marker for identifying the dense droplets.) Therefore, it is reasonable to propose that both types of droplets are identical, in which case the material for plasma membrane formation would be synthesized and stored in the cytoplasm as an emulsion of spherical droplets. It would seem that these droplets could be drawn upon at any time and be incorporated into membrane. In emergencies, this would seem to be an especially efficient way to mobilize large quantities of material for repair. The experimental observation that various cells, including amebae, are able also to rebuild plasma membrane in a few seconds requires that the mechanism elaborated here be considered seriously.

The question that naturally arises is whether these dense droplets are found in other cells, or whether they are unique to amebae. There is evidence that they are present in other cells, not only in *Amoeba proteus* and indeed not only in *Protozoa*. The studies by Cohen (1957), Mercer (1959), Pappas (1959), Brandt and Pappas (1960), Brandt and Freeman (1967), and Pollard and Ito (1970), in which different methods were used, confirm the presence of dense droplets in *Amoeba proteus* and *Chaos chaos*. Among these authors, Cohen and Mercer remark upon the foamy appearance of the droplets. The droplets also are present in the micrographs of *Hyalodiscus simplex* published by Wohlfarth-Bottermann (1960). They have been seen in *Chaos chaos* and in the slime mold *Physarum polycephalum* (Szubinska, unpublished data), where they not uncommonly contact the plasma membrane. In both *Chaos chaos* and *Physarum*, they show also the same foamy pattern as in *Amoeba proteus*. They have also been seen in the fungus *Neurospora crassa* (Tsuda, 1958, unpublished data). They are shown in Fig. 2 in the recent paper of Pollard and Ito (1970), but they do not have the foamy characteristic.

The presence of these dense foamy droplets in a variety of cells strongly suggests their participation in a common function. Membrane formation is a necessary requirement of all of these cells, although it probably proceeds normally at a slow and more or less continuous rate. This constancy makes it difficult to identify the association between the droplets and their function in membrane formation. The injuries reported in this paper appear to place on the ameba a maximum demand to form new surfaces, a dramatic circumstance which emphasizes the new distribution of the dense droplets, and which has permitted accumulation of the evidence presented here.

This hypothesis of membrane formation is not new. As early as 1899, Pfeffer proposed that "any part of the protoplasmic matrix is capable of forming new surface film as long as the cell is exposed to the appropriate environment." Recently, Beament (1968) has come to similar conclusions about plasma membrane formation. He says: "Even with the evidence on the rate of the synthesis of protein and lipid molecules of which the cells are capable, it is unlikely that cytoplasm can manufacture new membrane at the rate at which it appears over the surface of naked cellular material when, for example, an amoeba is broken up. The major problem envisaged is how the protein and the lipid, which can certainly be present in the cytoplasm, are assembled, and especially how the lipid is transported in the aqueous medium of the cell and then released in a form that will produce organized membrane material. The lipid will occur in the cytoplasm as spherical emulsion droplets, or as bimolecular leaflets with the polar groups outward."

There are many problems which need to be examined further in order the better to document the present findings. To mention only one, it is not clear why the same material which appears to give rise to the new membrane can be visualized by conventional methods when it is in the cytoplasm but not when it is released to the outside. The work reported here explores only the earliest events in formation of the new membrane. Studies are in progress to investigate the later stages of its development. However, the present findings, if correct, may not be restricted to amebae only, and may have a more general application in cell biology.

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