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Form and function of the glycocalyx on free cell surfaces

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[Plates 17–22]

Selected examples of the glycocalyx or cell coat on rickettsiae, bacteria, amoebae, sea-urchin eggs and the cat intestinal microvilli are illustrated and their functional roles are discussed. The differences in the form of various surface coats are noted; while many surface components are truely extraneous expendable coatings, others are so firmly attached that they seem to be a permanent part of the cell. The fuzzy surface coat on the cat intestinal microvilli have been considered in some detail and some new observations on the form of the glycocalyx are presented. The enteric surface coat is not readily visualized in fractured surface replicas of glycerinated tissue but fixed cells frozen in distilled water when replicated after freeze-etching reveal a flamboyant array of a filamentous meshwork attached to the microvilli. This fuzzy coat layer is at least twice as thick in the freeze-etched preparations when compared to thin sectioned material. Fresh tissue frozen without fixation or glycerin treatment did not have a thick fuzzy coat. In its place a thin amorphous blanket-like layer was found.

Introduction

The diversity of papers being presented in this discussion meeting attests to the fact that our knowledge of the plasma membrane is rapidly expanding. Investigations on cell surface coats have attracted increasing numbers of investigators and numerous excellent papers and reviews have been published. Among the more recent of these are the comprehensive morphological analysis of the polysaccharide coat of mammalian cells by Parsons & Subjeck (1972), the extensive review of the structure and histochemistry of glycoproteins at the surface of animal cells by Rambourg (1971), and the excellent review of the structure of surface coats of cells by Martinez-Palomo (1970). A notable early contribution on this subject was the stimulating discussion by Bennett (1963), who consolidated a number of early morphological observations, directing attention to the probable importance of these coats, and presenting a number of challenging hypotheses. In large measure we are still trying to verify and extend Bennett's penetrating analysis of more than ten years ago. In the intervening years widespread occurrence of cell coatings on mammalian cells has been surveyed by Rambourg & Leblond (1967); and more specialized reviews have been published by Fawcett (1965), Revel & Ito (1967), Curtis (1967), Ito (1969), Bennett (1969) and Winzler (1970). Numerous other workers have directed their studies towards specific aspects of the glycocalyx and even larger numbers have referred to the cell surface coats incidental to observations on other aspects of cell biology.

At the outset we should define the glycocalyx. It is a term proposed by Bennett (1963) and is based on its location and chemical nature. The term, which means 'sweet husk', was deemed appropriate because it always contains carbohydrate. It embraces all components and products external to the plasmalemma, including such structures as the cork cell wall described over 300 years ago by Robert Hooke as well as cell walls of bacteria and higher plants, invertebrates cuticles or slime coats of protozoa, and the diverse coats of animal cells. Indeed there is reason to believe that all cells have a glycocalyx on the outer surface of their plasma membrane. Many

of the properties of the plasma membrane can be ascribed to the glycocalyx or may be influenced by it. Some of these properties of membranes include the surface antigens and hormone binding sites; substances involved in cell recognition, adhesiveness, adsorption, protection, cell movement and maintenance of form. The cell surface obviously plays an important role too in a great number of metabolic activities, including synthetic activity, transport and secretion. Whether the glycocalyx should be considered as an extracellular component or whether it should be considered an integral part of the cell itself is largely semasiological. Some readily removed surface carbohydrates are obviously extraneous, but others cannot be easily removed without altering cell viability and therefore should not be considered extraneous. Furthermore, isolated cell membranes that have been chemically analysed are known to contain between 2 and 10% carbohydrates (Winzler 1970). Although proteins and lipids represent the major component of plasma membranes, it is clear that the carbohydrate component must also be considered when discussing its structure and function.

The visual demonstration of the surface coat on cells requires the development of methods that first stabilize the coat material and secondly render it visible. Methods for studying the fine structure and cytochemistry of these coats have involved chemical fixation and staining with coloured dyes for light microscopy or with electron-dense heavy metals for electron microscopy. More recently, rapid freezing followed by fracturing and surface replication has been introduced. All of these methods have their limitations. To date, only Parsons & Subjeck (1972) have pioneered in attempts to examine wet and physiologically active living cells in hydration chambers by high-voltage electron microscopy, and these efforts have yet to yield interpretable results.

A discussion of the form and function of the glycocalyx or cell coat includes such a wide variety of specializations that only a few selected examples from diverse cell types can be considered in this presentation. The glycocalyx on the intestinal absorptive cell are discussed in some detail and results of recent studies by freeze-fracturing and freeze-etching techniques are presented.

Conspicuous surface coats or the fuzzy mantle

In addition to the cell coats that are obviously extraneous and those that are ordinarily invisible, some cell types have a prominent glycocalyx which is readily observed by routine electron-microscopic methods. This filamentous type of coat is known by various names but of these the most descriptive is *antennular glycocalyx* (Bennett 1969). On intestinal microvilli it was designated the *enteric surface coat* (Ito 1965). It was first recognized by Yamada (1955), who noted a thin layer of fine filaments radiating upward from the surface of mouse gall bladder epithelium and called them the *antennulae microvillares*. Other colourful descriptive terms include 'hirsutulous', 'hispiditious', 'hairy coats', or simply the fuzz or fuzzy coat. Despite their dubious etymological origin and lack of linguistic elegance these two latter terms seem to have become firmly established as the common designations for this type of glycocalyx.

For reasons that are yet unclear, the degree of development of the surface coat on the intestinal striated border is tremendously variable from species to species (Ito 1965). These differences seem to be unrelated to the taxonomic affinities or dietary habits of the animals. The cat small intestine has been the subject of several studies because of its luxuriant surface coat but several other species possess a glycocalyx of equal thickness on the microvilli of their intestinal

absorptive cells. These include humans, monkeys, and certain bats. In other species examined to date the degree of development of this layer on the same cell type varies from an invisible layer in some species to a moderately thick coat. A recent investigation of the enteric surface coat of mice by Rao, Mukherjee & Wynn Williams (1972) noted a marked quantitative variation of glycocalyx from cell to cell. These differences were interpreted as being related to the degree of cell maturity and to the functional state of the intestinal absorptive cell. This study served to substantiate the earlier interpretation that the glycocalyx is a product of the cell on which it is present.

Light-microscope observations

Although the striated border of intestinal cells has long been recognized, the detection of its enteric surface coat at the light-microscope level had to await the development of special staining methods for carbohydrates such as the periodic acid–Schiff (PAS) reaction. A positively reacting layer at the luminal margin of the striated border was noted soon after introduction of this method. The determination of just what element of the striated border was responsible for this staining required the resolution of electron microscope.

Use of the cationic dyes alcian blue, ruthenium red or colloidal iron at low pH has permitted further characterization of the enteric surface coat. The striated borders of all intestinal absorptive cells stain vividly by these methods and the well-organized apical band is prominent. In species where a prominent fuzzy coat is present, it stains much more intensely than the striated border and can be recognized as a thin band above the tips of the microvilli.

Electron-microscopical observations

Our recognition and understanding of the structure of the fuzzy coat are due to the electron-microscope techniques and technical developments in tissue preparation. The ultrastructure of microvilli has been studied by numerous investigators. After Yamada's (1955) description of the glycocalyx on mouse gall-bladder epithelium Peachy & Rasmussen (1961) described a similar coat on toad-bladder epithelium and interpreted the filamentous substance as a product of certain secretory cells that was adsorbed on to the plasmalemma of the epithelium. It has since been demonstrated quite convincingly that this type of glycocalyx is a product of the cell itself and not an extraneous or adsorbed surface component.

The following illustrations and discussion will be devoted to the fuzzy coat of the cat intestinal absorptive cell but most of its structural features and staining characteristics will apply equally well to similar fuzzy surface coats on other cells.

In electron micrographs of cat intestinal microvilli (figure 1) a prominent, thick surface coat is observed routinely. The coat is a closely packed layer of fine filamentous material that is at least 0.1 μm in thickness and sometimes more than 0.5 μm thick. Although these variations in thickness may reflect differences in the differentiation or functional state of the cell this has not been proven. There seems to be no striking difference in the prominence of this coat in the cat intestine whether it is seen on young cells near the base of the villus or on mature cells at the villus tip.

Evidence that the cell manufactures its own surface coat is based on the observation that there may be abrupt differences in thickness of the layer on adjacent cells. Further evidence indicating that the cell itself is the source of its fuzzy coat derives from the radioautographical studies of Ito & Revel (1968), Bennett (1970) and Bennett & Leblond (1970). These studies indicate that the initial incorporation of sugars in the biosynthesis of the glycocalyx is in the

Golgi complex followed by movement of glycoprotein to the cell surface. In the cat intestinal absorptive cell it was found that incubation with tritiated acetate, glucose, galactose or mannose resulted in accumulation of label in the striated border and fuzzy coat after an initial lag period of 30 to 60 min. When the tissues were pulse-labelled there was a continued decrease in the radioautographic label in the surface coat after 6 h, indicating that it is indeed a dynamic cell component constantly being replaced.

The actual process by which the newly synthesized surface coat material is transferred to the outer surface of the cell is not at all clear. Mukherjee & Williams (1967) observed what they interpreted to be apparent continuity of the core filaments of the microvilli through the unit membrane and into the surface coat. However, this interpretation has not gained acceptance. Histochemical staining reactions of the coat substance are quite distinct from those of the microvillus core filaments and extrusion of preformed filaments through the plasma membrane seems quite unlikely.

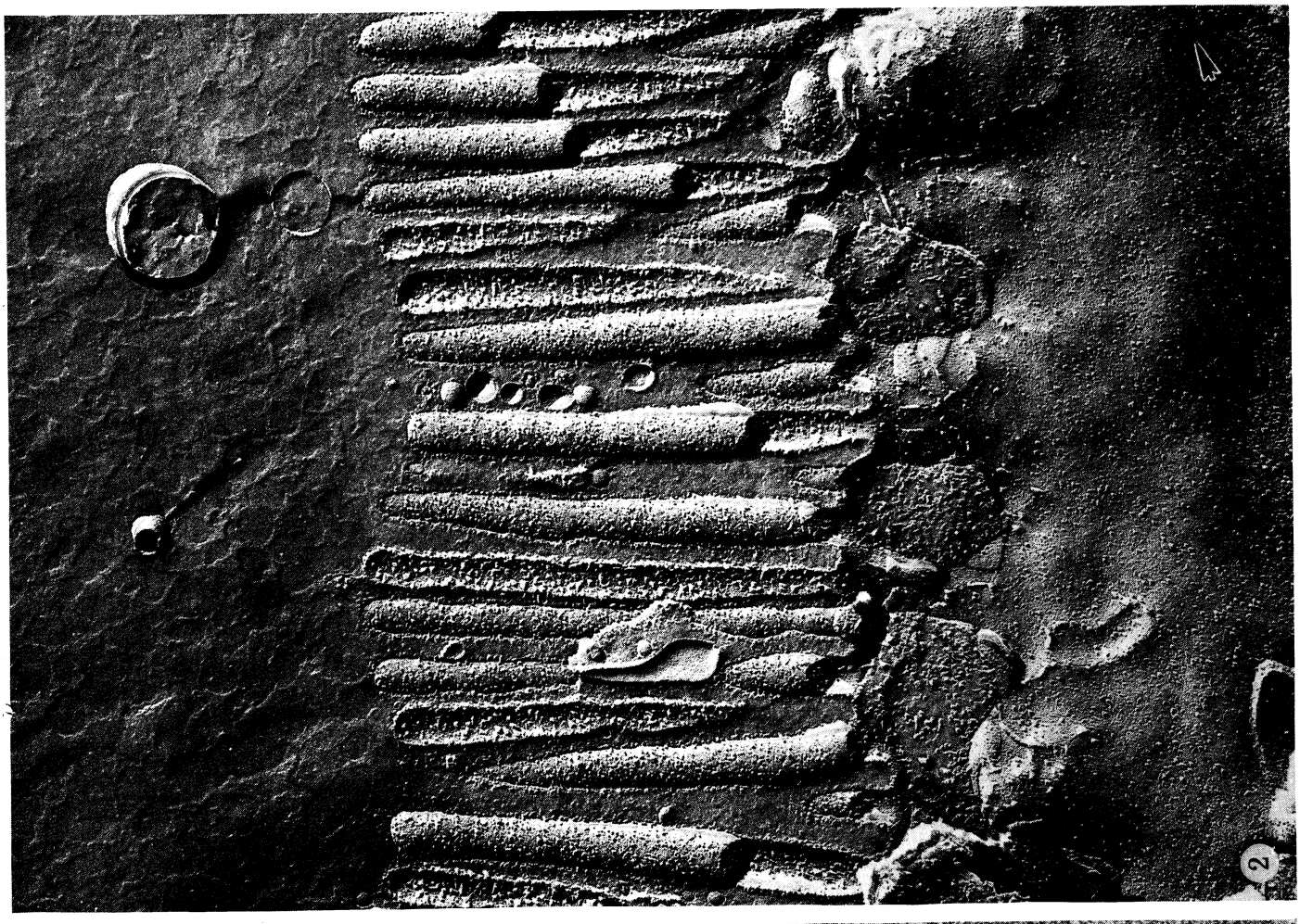
Different methods of tissue preparation markedly affects the appearance of the surface coat. A completely different texture is seen in images obtained by negative staining with potassium phosphotungstate. Isolated hamster intestinal brush border negatively stained shows small knob-like particles about 6 nm in diameter in place of the delicate filaments seen by other methods (Johnson 1969). Parsons & Subjeck (1972) have suggested that these images may be due to the clumping of the delicate filaments by the potassium phosphotungstate treatment. Since the hamster intestinal epithelium has a relatively small amount of fuzzy coating this may be a reasonable explanation. The surface coat of the cat intestine is so much thicker that one might expect a larger number or larger size of the particles. However, when prepared by the same negative-staining technique, cat intestinal microvilli have surface knobs (figure 2) quite indistinguishable in size and number from those reported for the hamster. In order to explain these observations it seems necessary to postulate that a large amount of the surface coat of the cat intestine must be lost during negative staining and that only the 6 nm knobs remain.

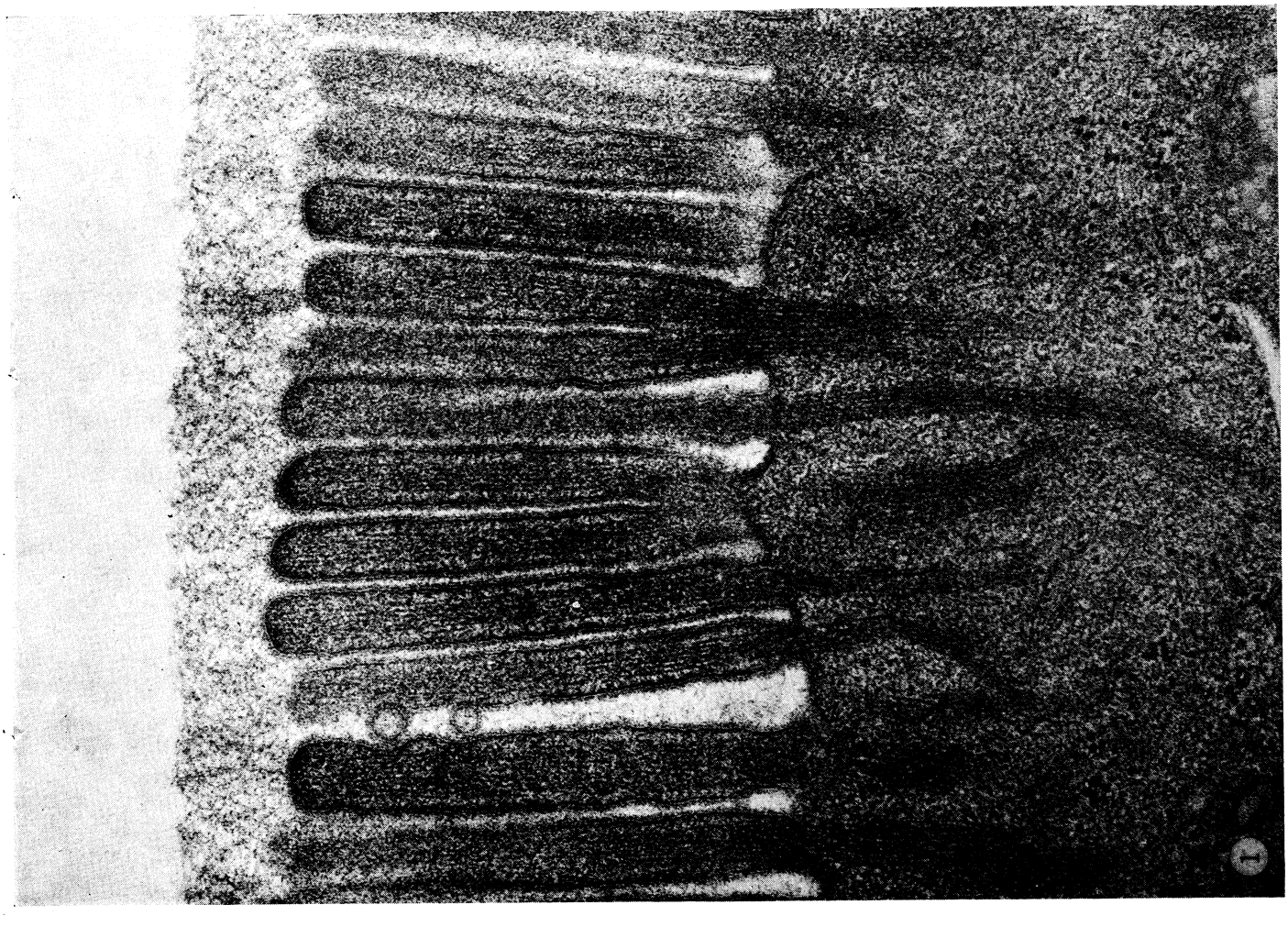
Even though these small particles are not seen in thin-sectioned material, further evidence suggesting that they are real structures is based on convincing isolation studies by Johnson (1969). He found that papain digestion would denude the microvilli of the 6 nm particles and they could be sedimented separately from the microvillus membranes. Furthermore, the enzymes invertase and maltase were found in the fraction rich in these particles. Inasmuch as these particles contain enzymes and no carbohydrates have yet been demonstrated, an alternative explanation is that they have little to do with the fuzz itself but happens to be a common tenant on the cell surface. In other words, it may be an additional cell-specific surface enzyme retained in the surface coat for digestion of sugars.

Description of plate 17

Figure 1. A micrograph of cat intestinal absorptive cell microvilli and its fuzzy coat. The filamentous glycocalyx appears to be attached to the outer surface of the plasma membrane. The smooth outer limits of the surface coat may be due to the partial loss of the glycocalyx. This coat is about 20 nm thick. (Fixed with glutaraldehyde-paraformaldehyde followed by osmication. Section stained with uranyl acetate and lead citrate. Magn. × 90 000.)

Figure 2. A freeze-fracture replica of cat intestinal brush border. This tissue was fixed with buffered aldehyde, treated with 20% glycerol and shadowed after fracturing. Occasional tiny extrusions from the tips of the microvilli presumably represents the glycocalyx. The reticular meshwork in the lumen cannot be the fuzzy coat for it extends indefinitely from the microvilli. (Magn. × 70 000.)





FIGURES 1 and 2. For description see opposite

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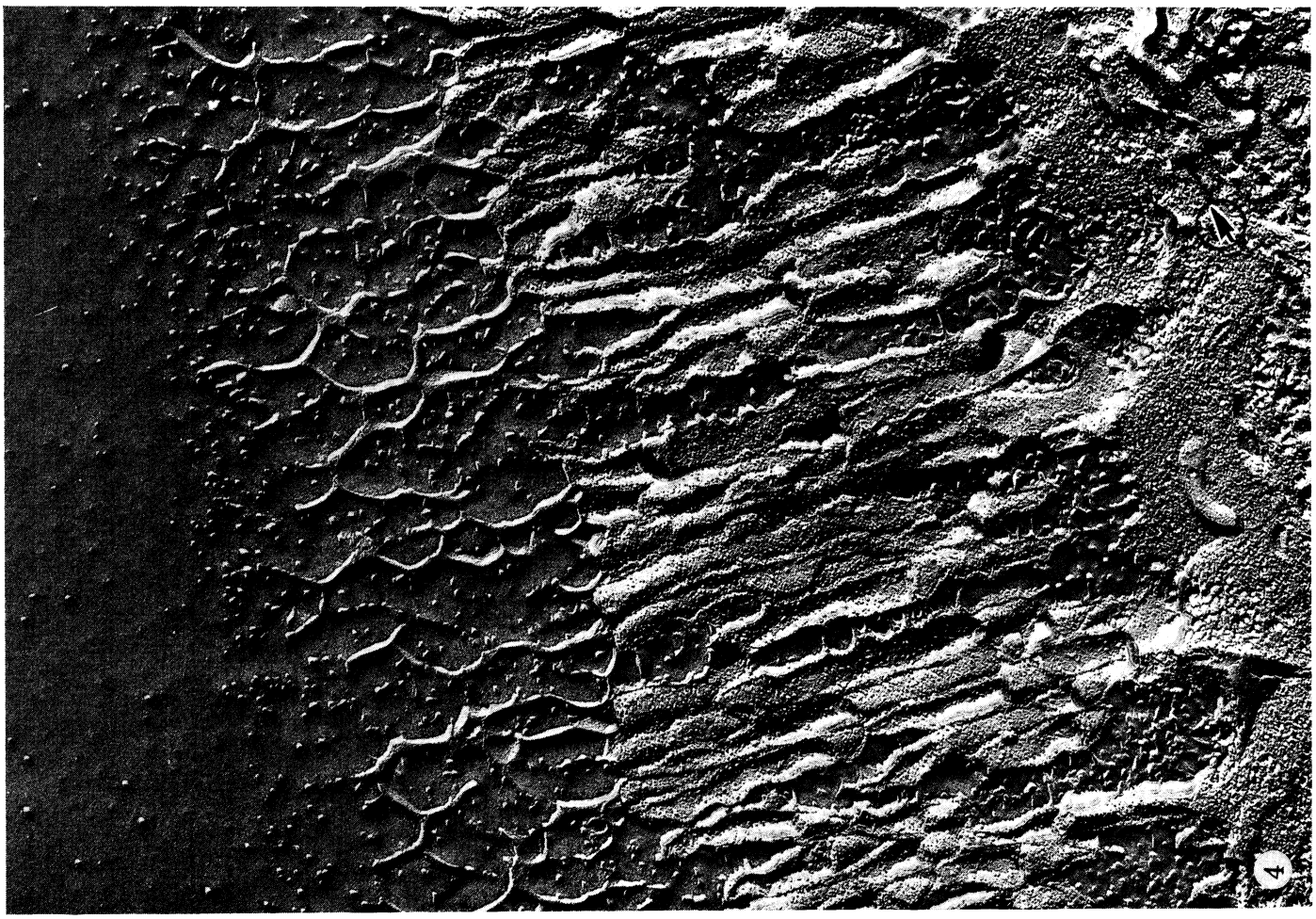

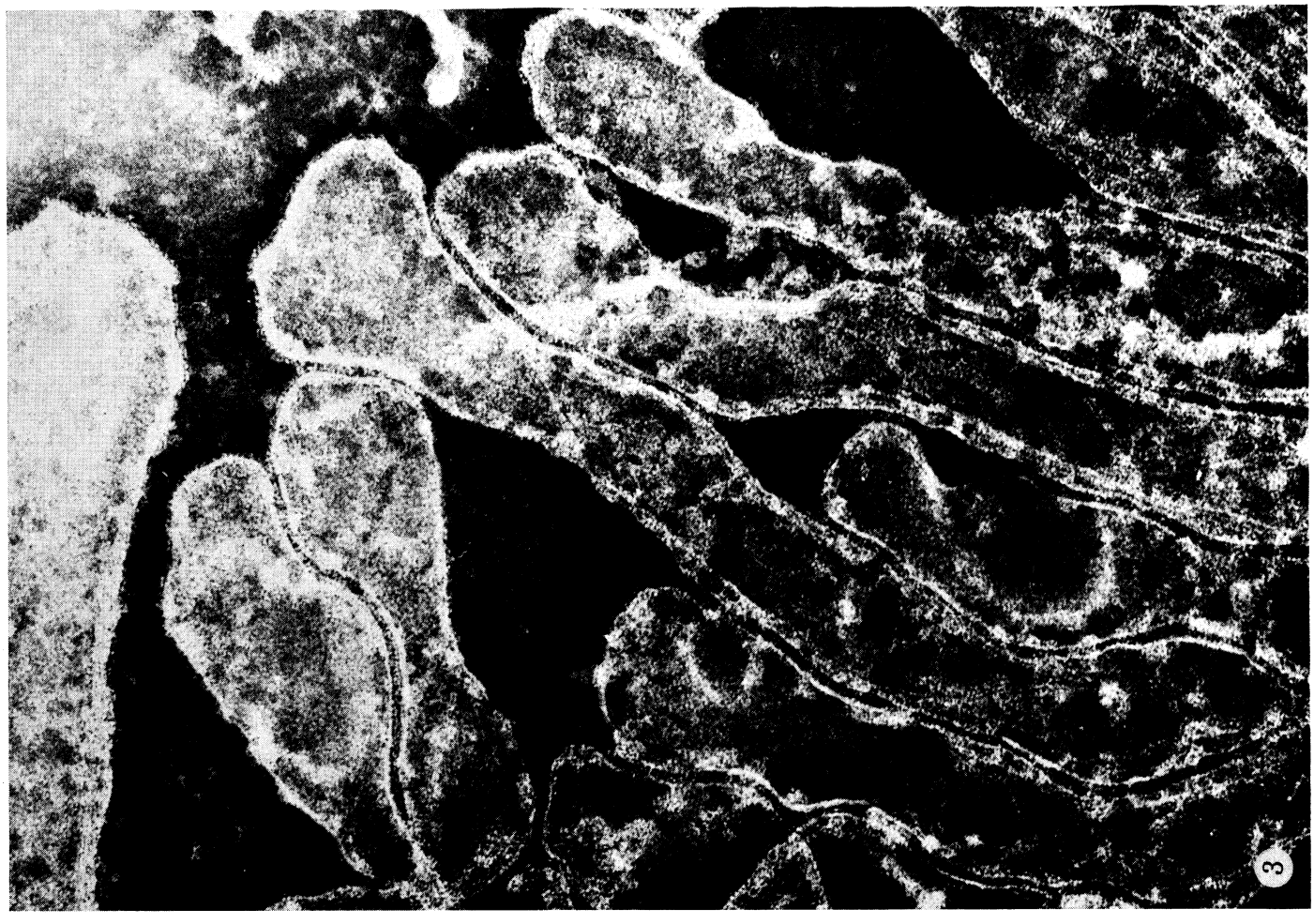

FIGURES 3 and 4. For description see opposite

The presence of enzymes in the intestinal brush border has been localized histochemically as well as biochemically. Alkaline phosphatase has been convincingly demonstrated in the intestinal brush border. By isolating brush borders, Miller & Crane (1961) found that almost all of the alkaline phosphatase, sucrase, maltase, isomaltase and lactase of the cell was in the brush border fraction. It was speculated that these and other enzymes present on or possibly within the plasma membrane were involved in the terminal digestion of carbohydrates and protein. The extensive studies of Ugolev (1965) points out the importance of the zone just external to the plasma membrane as being the most important site for terminal digestion and absorption.

Further investigations on the form of the fuzzy coat

In view of the completely different appearance of the enteric surface coat after negative staining, the morphologist is confronted with the problem of determining what is the true structural configuration of this layer in the living state. The structures at issue are too small for light microscopy of living cells to be helpful. Electron microscopy of living tissue in wet chambers for high-voltage microscopy is still in the developmental stages (Parsons & Subjeck 1972). Thus some form of tissue preparation cannot be avoided.

Scanning electron microscopy would seem appropriate for studies of surface topography but the necessary freezing or fixation followed by drying and the evaporation of 20 nm or more of metal on the surface so alters and obscures detail in the coat that there is little hope for this approach. Furthermore, the resolution of the conventional scanning electron microscope is limited to about 10 nm.

A related technique which seems to be more promising is carbon replication of fixed and dried cell surfaces after metal shadowing. This is an old technique but one which has been very little used by biologists. To date, none of these methods have provided us with significant new information on the form of the fuzzy coat.

One of the most promising of the newer techniques for studying membrane structure is the freeze-etching method. Mukherjee & Staehelin (1971) have presented the first observations on the intestinal surface coat of mice. In this comprehensive study of the brush border, they found that freeze-etching did not reveal the glycocalyx very satisfactorily. They concluded that the glycoprotein coat probably exists in an extremely hydrated state and that when frozen it is difficult to distinguish from the surrounding aqueous media.

Our own attempts to visualize the surface coat of cat intestinal epithelium by the freeze-etch techniques were also initially unsuccessful. Results such as shown in figure 3 were typical. Faint suggestions of some surface material could occasionally be seen attached to the outer

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Description of plate 18

Figure 3. Cat intestinal microvilli negatively stained with potassium phosphotungstate. Along the border of the microvilli numerous small particles are present. These are similar to those reported in the hamster intestine by Johnson (1969). The fuzzy coat is not seen in negative-stained preparations. (Magn. × 130 000.)

Figure 4. A freeze-etched replica of cat intestinal microvilli. This tissue was fixed with aldehydes, washed with distilled water, and frozen without glycerine treatment. The fractured specimen was etched for 1 min at −100 °C. The prominent fuzzy coat formed by a meshwork of filaments extends about 100 nm above the microvillus tips. Numerous intermicrovillus filaments of the glycocalyx may be seen particularly near the base of the microvilli. (Magn. × 60 000.)

leaflet of the membrane but nothing comparable to the thick fuzzy coat we were accustomed to seeing. This was particularly puzzling because samples of the same aldehyde-fixed and glycerinlated tissue that was used for freeze-etching showed a prominent surface coating when processed for conventional thin sectioning.

Our next step was to freeze aldehyde-fixed cat intestinal mucosa in distilled water after thorough washing in distilled water. This was followed by fracturing and etching at −100 °C.

Results of this preparative procedure are shown in figures 4 and 5. In striking contrast to the glycerinated specimen a very prominent surface coat is seen. The thickness of the freeze-etched glycocalyx is consistently thicker than that seen in thin sections. Not infrequently it is as thick as the microvilli are tall. The usual cat intestinal microvilli are about 10 nm in diameter and 100 nm tall and the thickness of the freeze fractured fuzzy-coat layer is between 50 and 100 nm, compared to the 10 to 50 nm of coat material seen in thin sections. The explanation for this dimensional difference still eludes us but it may possibly be due to further hydration of the surface coat when the glutaraldehyde–paraformaldehyde fixed tissue is placed in distilled water just before freezing. Alternatively the embedding techniques may tend to shrink and dislodge some of the surface coat.

Further evidence suggesting the partial loss of fuzzy-coat material during tissue processing is the following observation. If aldehyde-fixed cat intestinal epithelial is stained with colloidal thorium without osmication a thicker surface coat is retained (figure 6). The preserved glycocalyx is not as thick as after distilled-water freeze-etch preparations but more prominent than in the routine preparations. Colloidal thorium staining is relatively coarse and the particles obscure the filaments of the fuzzy coat.

In the freeze-etched preparations the glycoprotein coat seems to consist of a loose meshwork of fine filaments. These branching filaments originate from the outer leaflet of the membrane of the microvilli and form an interwoven meshwork with other filaments from the same or adjacent microvilli. The three-dimensional configuration of this glycocalyx can be revealed by deep etching and examination of stereo pairs. The lateral or intervillus space contains an abundance of shorter filaments that seem to bridge the space between microvilli. How the filaments of the glycocalyx are attached to the plasma membrane is still not clear. In areas where the etching has revealed the outside surface of the microvillus as shown in figure 5, the attachment of filaments to the microvillus can be clearly seen. It is not yet established whether they penetrate the lipid bilayer and are continuous with the protein particles seen on the A face of cleaved membranes of the microvilli.

On the basis of these observations, it seems reasonable to conclude that the filamentous

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Description of plate 19

Figure 5. A freeze-etched replica of aldehyde-fixed cat intestinal mucosa washed in distilled water and frozen. The fractured specimen was etched for 1 min at −100 °C. Much of the field is occupied by a goblet cell and its microvilli with its meshwork of filamentous glycocalyx. Of particular interest are the several small areas in the centre of the illustration where there are microvilli with some etched free surface. Several fuzzy coat filaments appear to be attached to the microvillus membrane. (Magn. ×75000.)

Figure 6. Cat intestinal brush border stained with colloidal thorium. The heavy accumulation of thorium on the fuzzy coat obliterates its fine filaments. The coat in this illustration is more than 50 nm thick and was consistently thicker than in the routine preparations (see figure 1). (This tissue was fixed with glutaraldehyde–paraformaldehyde and stained with colloidal thorium in 3% acetic acid and embedded without osmication. Section stained with uranyl acetate and lead citrate. (Magn. ×50000.)

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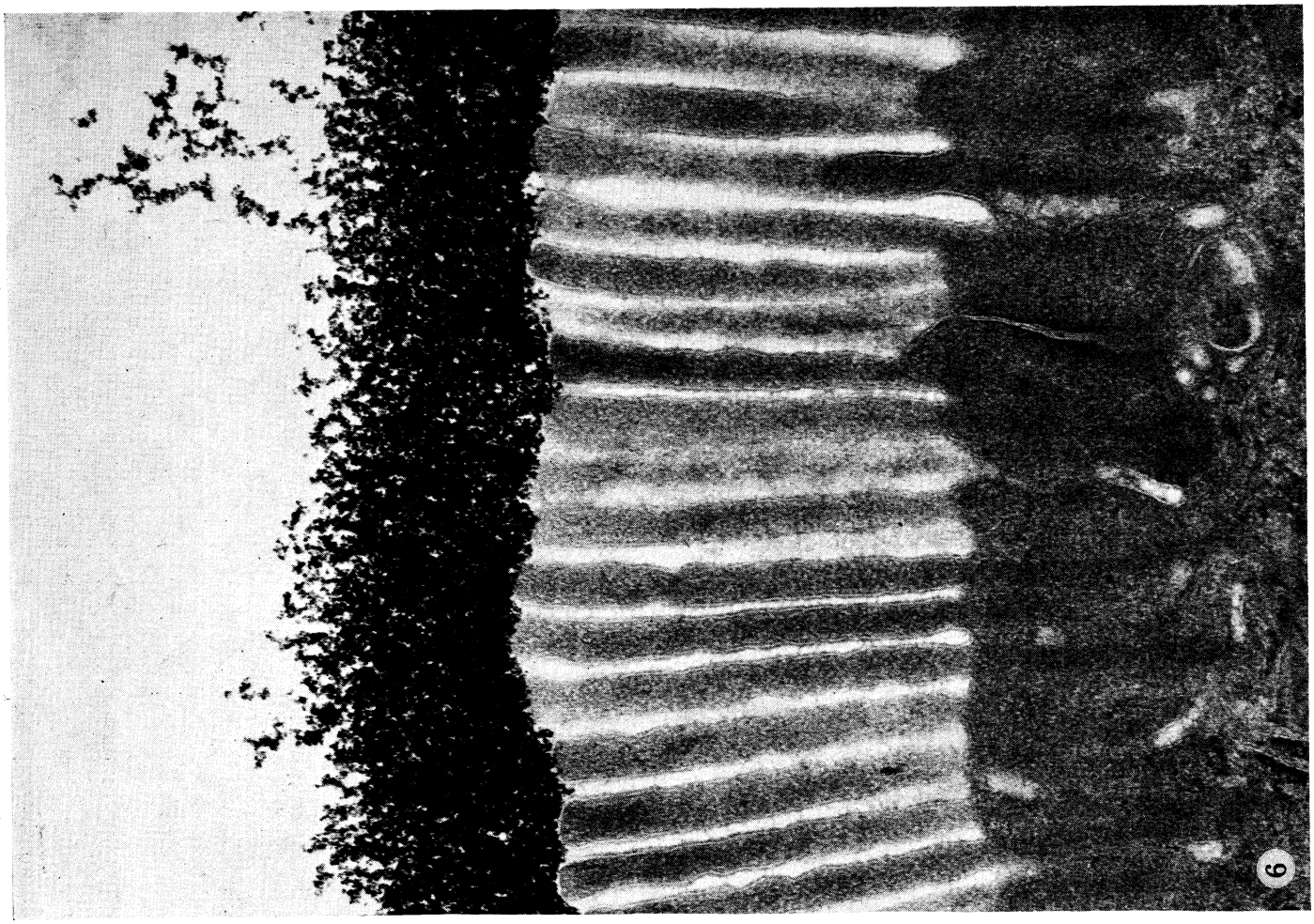

FIGURES 5 and 6. For description see opposite

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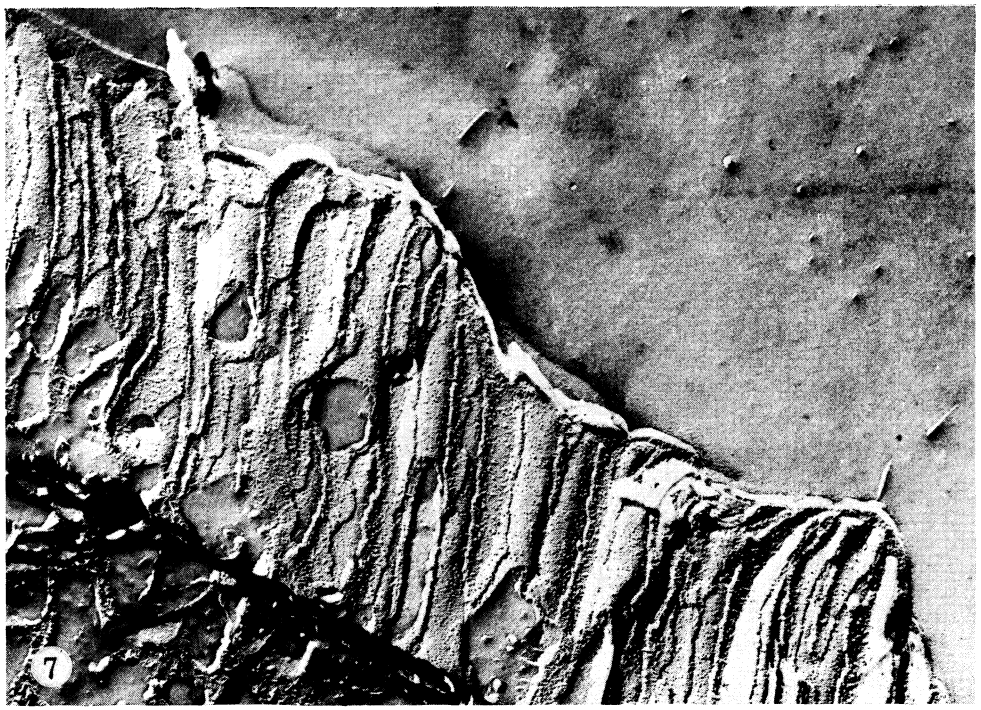

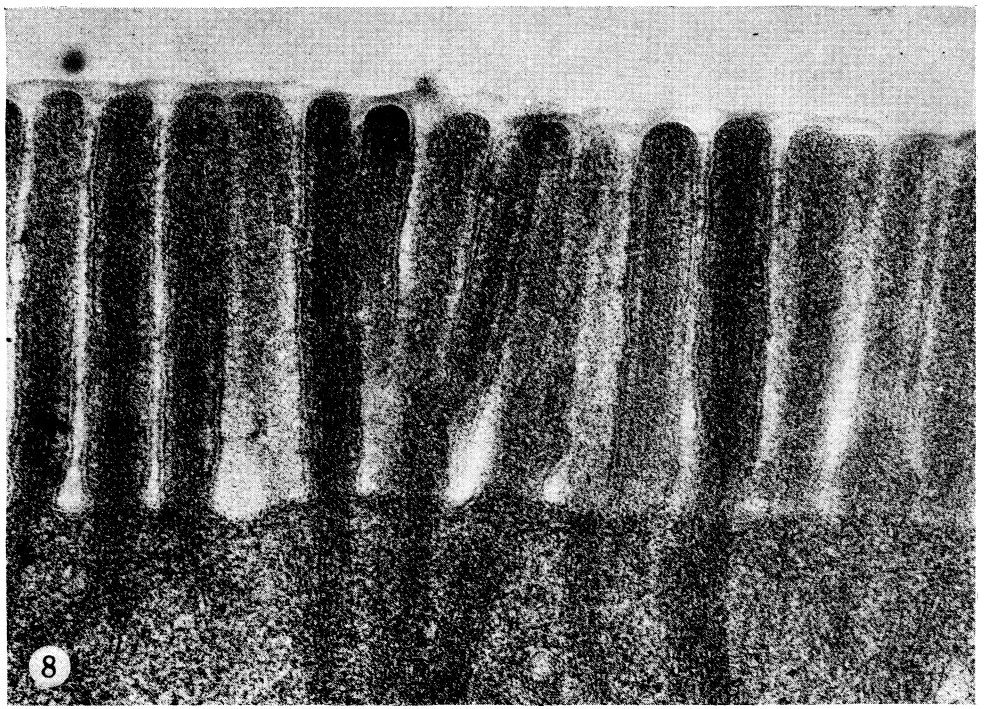

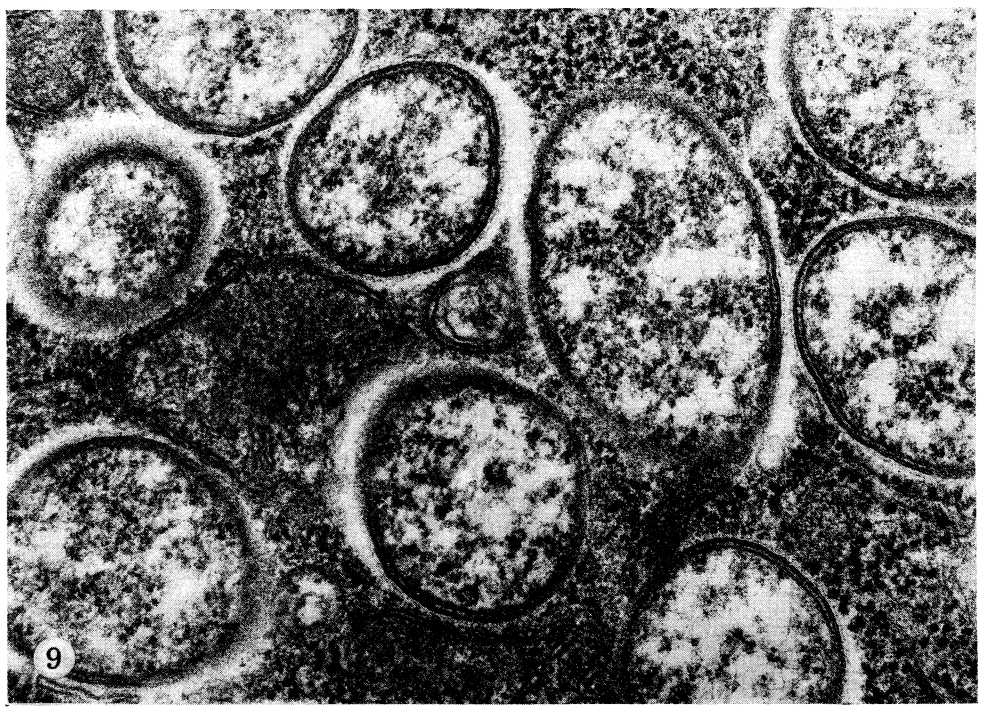

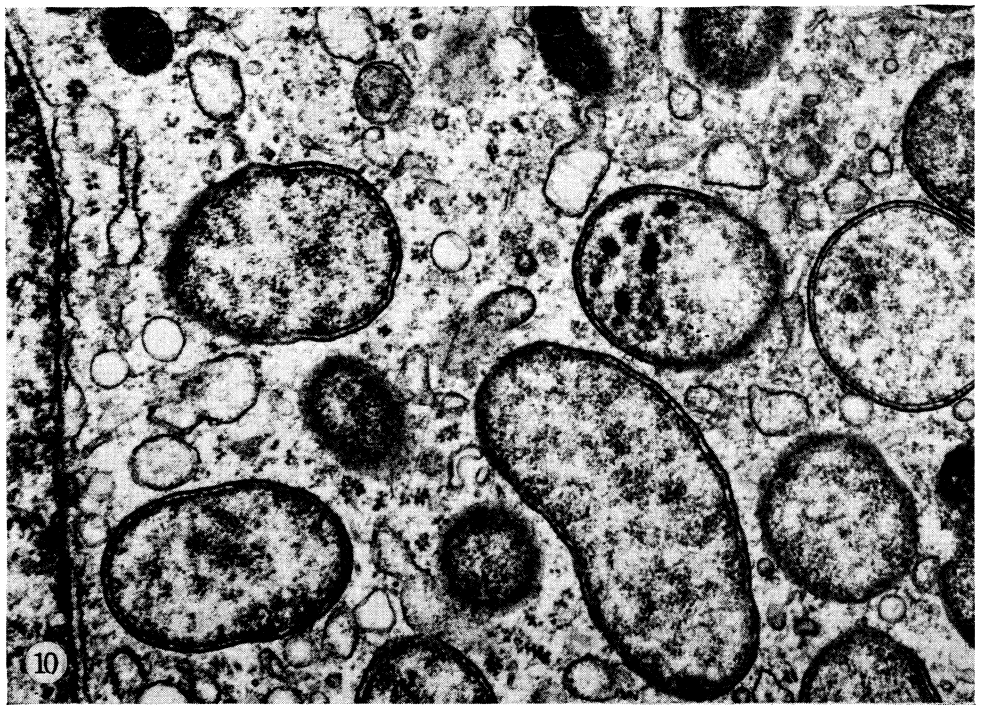

FIGURES 7–10. For description see opposite

appearance of the fuzzy coat on the cat intestinal microvilli is not an artefact produced by action of fixatives on a structureless, gelatinous or mucus-like layer. Instead it seems that the images seen in thin sections accurately reflect its true nature. In fact, the imaginative depiction of the antennular glycocalyx presented by Bennett (1969) seems a remarkably accurate representation of the freeze-etch images reported here. Its only short-coming is that Bennett's drawing is more conservative and far less elaborate than nature.

This would be a most appropriate place to end this discussion of the glycocalyx with a positive evaluation of its probable natural state. Instead, an attempt is made to correlate these observations with freeze-etch preparations of fresh, unfixed and unglycerinated cat intestinal villi. What we expect to see is some semblance of the filamentous meshwork found in freeze-etched preparations of fixed intestinal mucosa. However, all of our attempts have so far resulted in images such as that shown in figure 7. The microvilli in these replicas resemble those made from fixed tissues, but there is little that can be recognized as the filamentous glycocalyx material. In occasional areas a structureless mantle of material seems to overlie the microvilli and this is presumed to represent the surface coat.

An observation that may be related to the absence of the glycocalyx in fresh tissue is the as yet unexplainable absence of the thick fuzzy coat in aldehyde-fixed tissue embedded for thin sectioning *without post fixation* in osmium tetroxide. As shown in figure 8, there is a thin blanket of material similar in appearance to that found in the fresh frozen, etched replicas of similar tissue. These findings are somewhat different than that of Pratt & Napolitano (1969), who reported a completely clear and low-density zone just apical to the cat intestinal microvillus tips. The reason for the discrepancy with the present observations is not known.

The glycocalyx of micro-organisms and protozoa

The glycocalyx of micro-organisms is represented by their cell wall and other components external to the plasma membrane and differs from the surface coat of animal cells in appearance as well as composition. However, it shares with surface coats of other cells its strategic peripheral location interposed between the cell membrane and the environment. In bacteria the

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Description of plate 20

Figure 7. A freeze-etch preparation of the brush border from a sample of fresh cat intestinal mucosa frozen without prior fixation or glycerin treatment. The microvillar membrane is clearly depicted but the surface coat is not visible. A narrow zone of material immediately adjacent to the tips of the microvilli may be elements of the fuzzy coat. (Magn. × 60000.)

Figure 8. A thin section of the microvillus border from a cat intestinal absorptive cell that was fixed with the same aldehyde containing fixative used to prepare the freeze-etch preparations. This tissue was embedded in plastic without osmication. Most of the fuzzy coat has been lost and the thin remnant remaining over the microvilli no longer appears filamentous. Section stained with uranyl acetate and lead citrate. (Magn. × 75000.)

Figure 9. An electron micrograph of *Rickettsia prowazekii* in the cytoplasm of a louse intestinal epithelial cell. The trilaminar nature of the cell wall and the plasma membrane is apparent. In routine preparations that are embedded in the usual epoxy resins a clear halo of varying width surrounds each micro-organism. Although some of this clear space may be due to extracted glycocalyx material, most of the space is due to shrinkage of the micro-organism. (Magn. × 90000.)

Figure 10. An example of *Rickettsia tsutsugamushi* in the cytoplasm of an infected tissue culture cell. This micro-organism is similar to other rickettsiae in its ultrastructural features. Note that these rickettsiae do not have a clear halo around them. The only significant difference between figure 9 and this example is that a low-viscosity embedding media was used for this example. (Magn. × 60000.)

glycocalyx is not an essential to life, for viable micro-organisms can be maintained without their cell walls. Electron micrographs of rickettsia and a gastric spirillum will be shown as examples.

The cell wall of *Rickettsiae prowazeki* (figure 9) closely resembles that surrounding other Gram-negative micro-organisms. Sections perpendicular to the plasmalemma and cell wall reveal a total thickness of this complex which includes the interspace between them to be about 20 to 25 nm. The cell wall is approximately 8 nm thick and the plasma membrane slightly thinner (about 7 nm). Each of these structures appears to be trilaminar in fixed and embedded specimens and resembles the unit membrane described by Robertson (1959). When rickettsia are located extracellularly, they sometimes have a fuzzy coating on the outer aspect of the cell wall but it is not a constant feature and the possibility that this may be adsorbed extraneous material cannot be excluded. Negative staining of *Rickettsia quintana* (Ito & Vinson 1965) reveals no pili, flagella, or other cell-wall specializations. When these bacteria-like micro-organisms are located in the cytoplasmic matrix of the host cell, usual methods of tissue preparation show a clear halo of varying width immediately surrounding their cell wall. This appearance has sometimes been attributed to presence of a glycocalyx or surface coat revealed in negative images. However, if special precautions are taken in embedding the cells this halo is lacking (figure 10). Thus it is probably an artefact and the rickettsial cell wall probably has little or nothing external to the cell wall.

Another micro-organism, a gastric spirillum, and its specialized surface are illustrated in figures 11 and 12. This bacterium is unusual in several respects. It lives in the gastric gland o mammals in an environment at pH 1 and rich in powerful hydrolytic enzymes. The spirillum has a thin cell wall from which tufts of 10 to 12 flagella project at either end of the organism. These flagella seem to be modifications of the cell wall and have no visible connexion with the plasma membrane or the cytoplasm. These terminal tufts are remarkable examples of an activity of a glycocalyx. Although they lack the customary fibrillar structure of bacterial flagella they execute rapid swirling movements that propel the micro-organism on its long axis in a rapid rotatory, corkscrew motion.

Among the most thoroughly studied of cell surface coats is the glycocalyx of the amoeba (figure 13). Its histochemical staining for polysaccharides was demonstrated by Bairati & Lehman (1953) and by Pappas (1954) some 20 years ago. These workers also noted meta-

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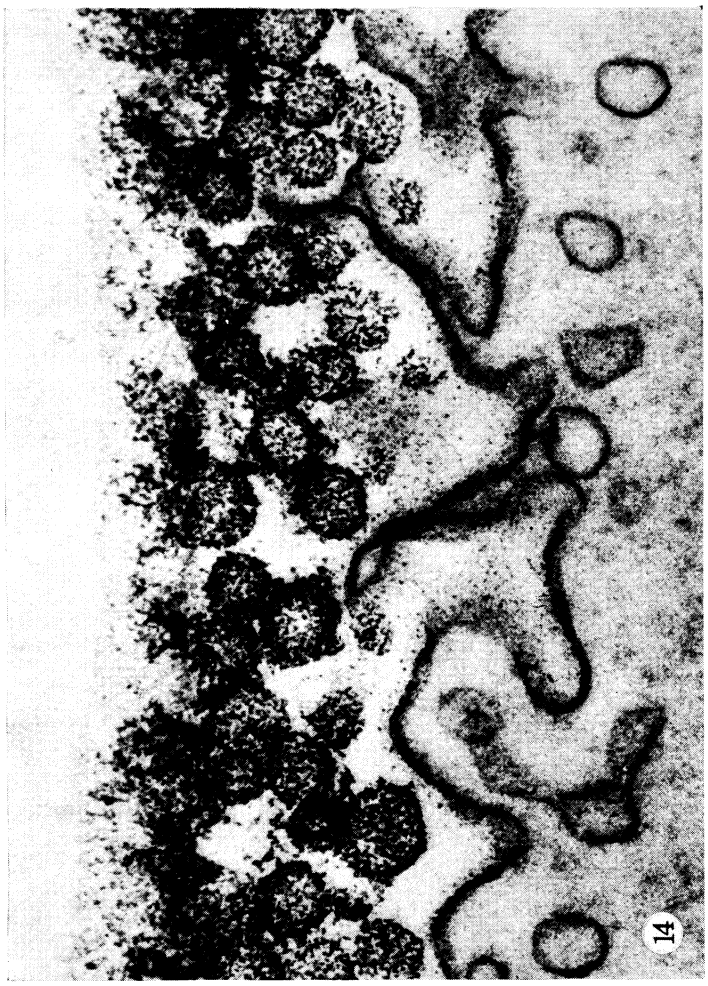
DESCRIPTION OF PLATE 21

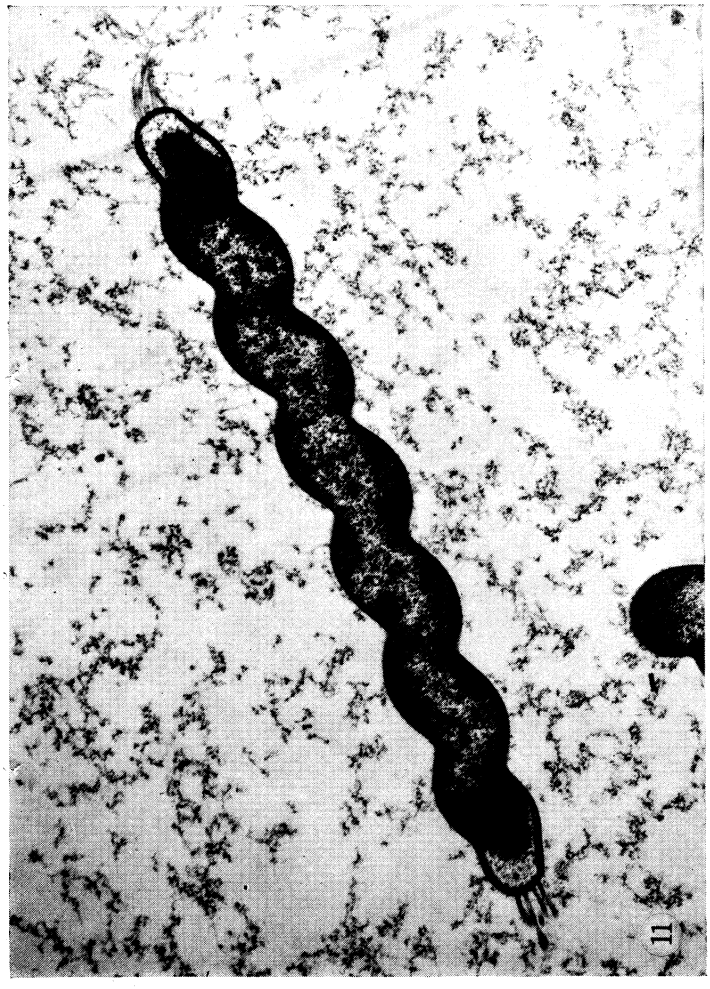
FIGURE 11. An electron micrograph of a gastric spirillum in the gastric gland of a cat. At either end of the cell there is a tuft of 10 to 12 bacterial flagella which rotate like a propeller and moves the bacteria in a rapid corkscrew fashion. This motile appendage is a part of the cell wall, and therefore part of the glycocalyx. (Magn. × 17000.)

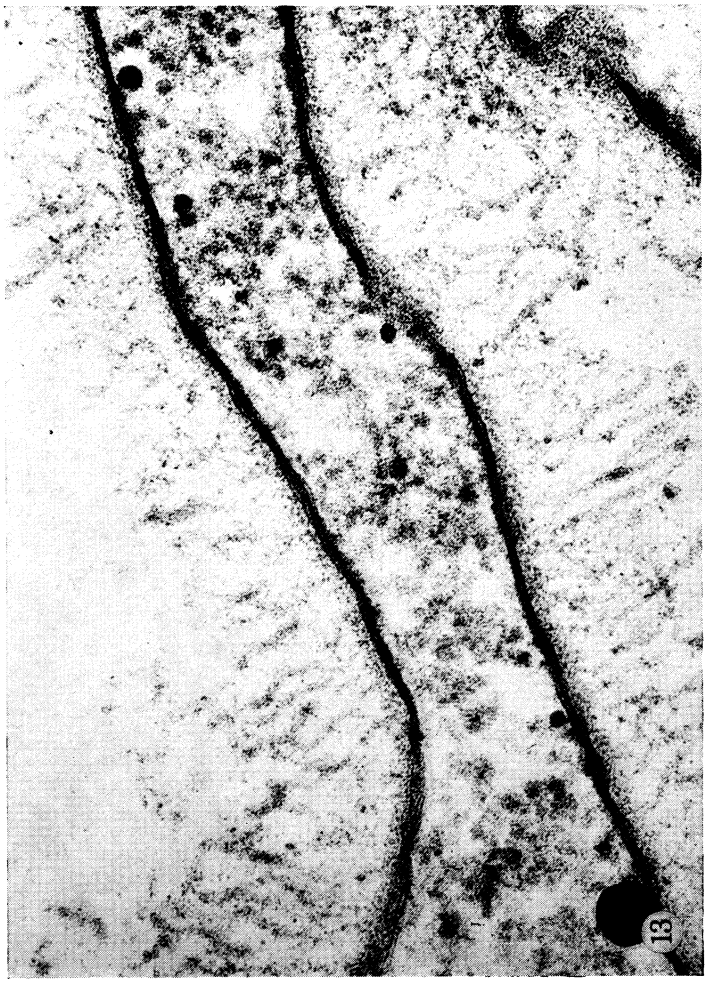
FIGURE 12. A higher magnification of the terminal tuft region of a gastric spirillum. The flagella are formed by an extension of the outer leaflet of the cell wall. On the outer surface of the cell wall there is a small amount of fuzzy material which is much less prominent than the glycocalyx on the gastric epithelial cell microvilli. (Magn. × 140000.)

FIGURE 13. Part of a pseudopodium from the amoeba *Chaos chaos* showing the trilaminar cell membrane, its adjacent amorphous layer, and the fuzzy coat layer. This preparation illustrates the typical glycocalyx of the amoeba after conventional preparation methods. (Magn. × 95000.)

FIGURE 14. The plasma membrane and the glycocalyx of *Amoeba proteus* exposed to ruthenium violet while still alive and subsequently fixed and embedded. The glycocalyx which ordinarily appears filamentous is now seen as a series of globules 100 to 150 nm in diameter. The surface of the globules is covered by particles 3 to 10 nm in diameter. (Magn. × 100000.) (After Szubinska & Luft 1971.)







FIGURES 11–14. For description see opposite

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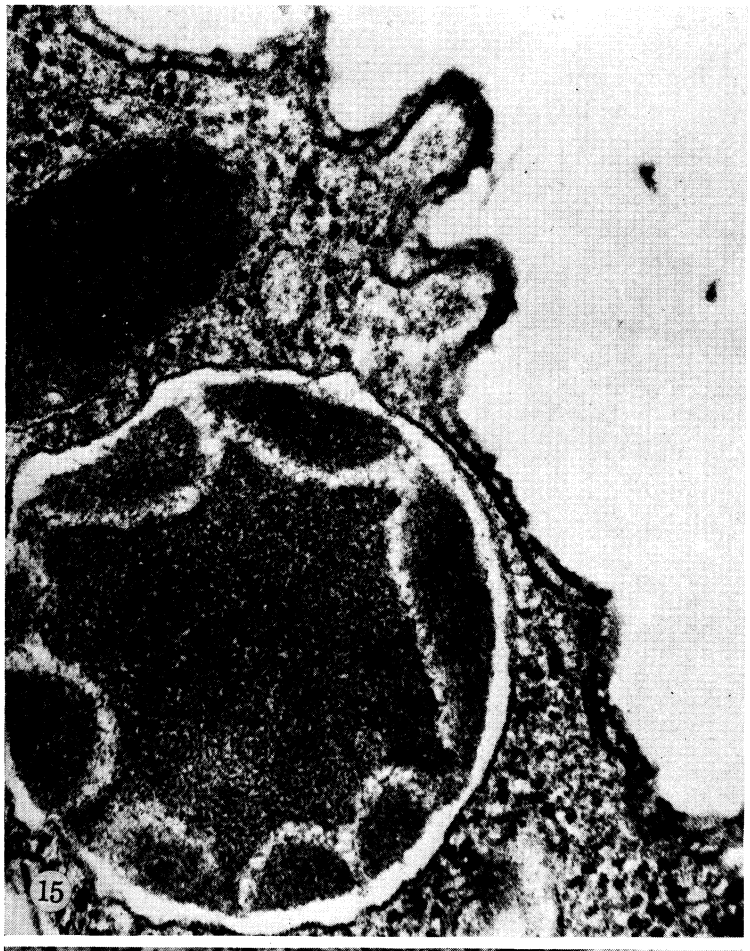

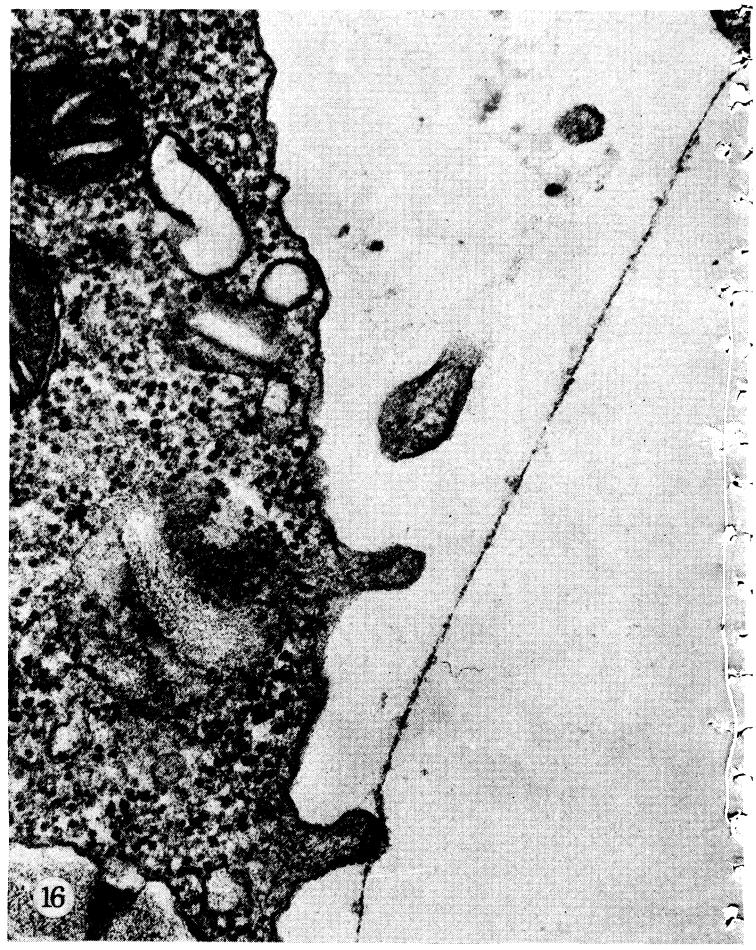

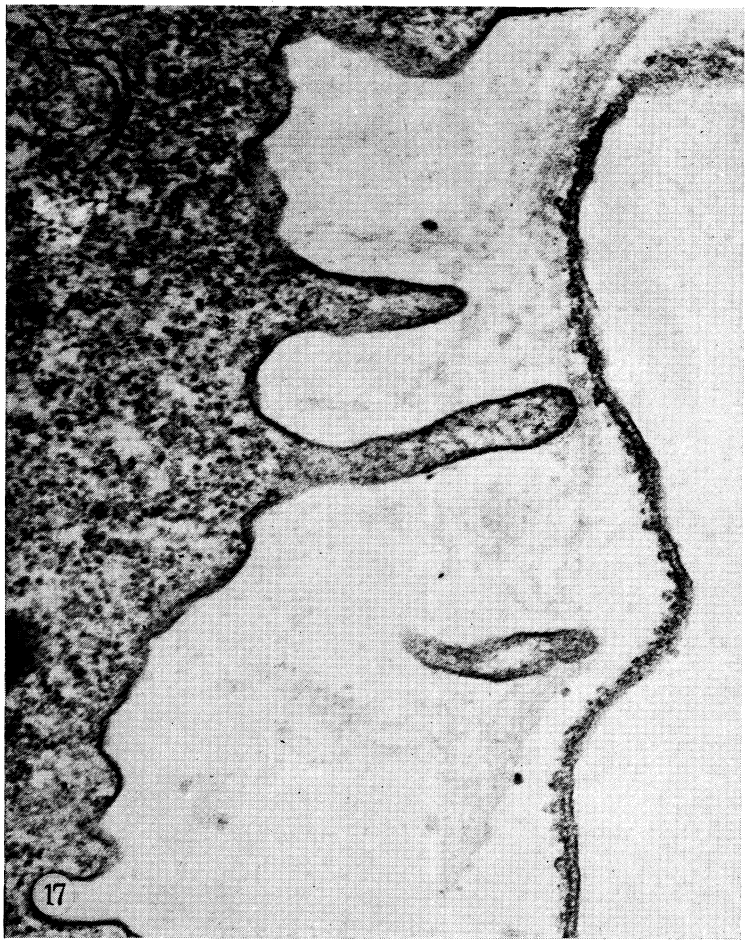

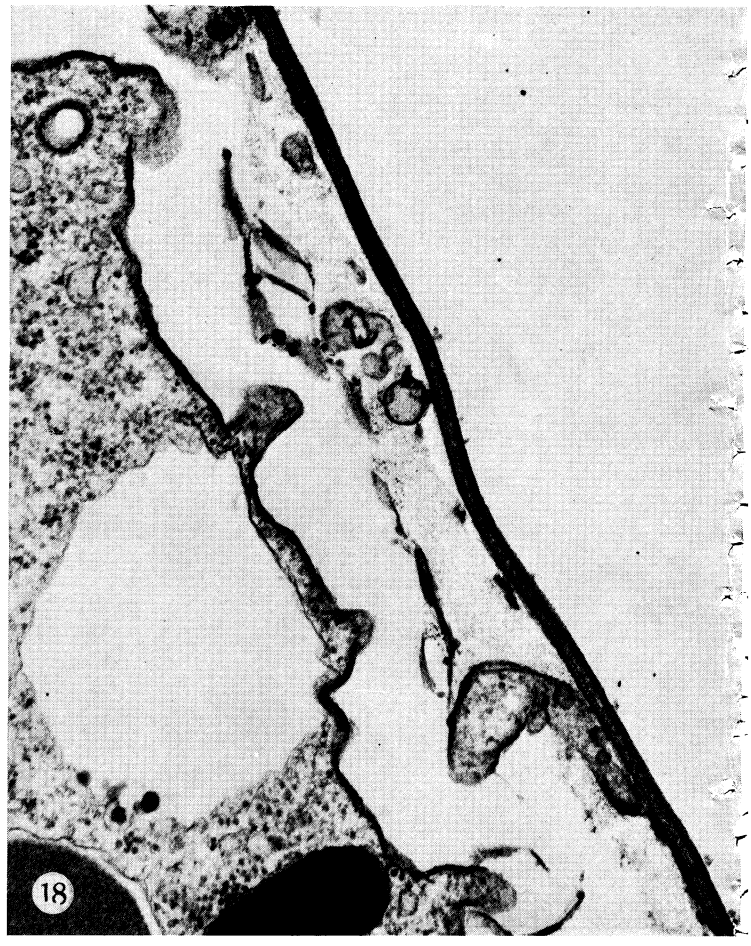

FIGURES 15–18. For description see opposite

chromasia of the surface coat with toluidine blue as well as its staining with a cationic dye, alcian blue. The surface coat of amoeba avidly binds colloidal thorium at pH 2.0, staining it well for the electron microscope (Revel & Ito 1967). The ultrastructure of this mucoid slime coat has been studied by Pappas (1959), Brandt & Pappas (1962) and numerous others. It has been variously described by different authors as 'hairy', filamentous', or 'fuzzy', and Bennett (1969) has designated this type of surface coat as the '*antennular glycocalyx*'. Szubinska & Luft (1971) in a recent study of surface coats of amoeba used ruthenium violet, which binds to polyanionic surface coats and report some interesting observations. When they exposed living *Amoeba proteus* or *Chaos chaos* to ruthenium violet they found that the cell coat appeared to be arranged in the form of regularly spaced 120 μm globules (figure 14). These arrays of globules were found under these conditions in the areas normally invested by a filamentous glycocalyx. Although the ruthenium dyes are acknowledged to be toxic to living amoeba, these investigators have seriously questioned the reality of the widely accepted filamentous nature of the surface coat. This interpretation derives support from an earlier observation of Nachmias (1966), who noted similar spherical globules on the surface of amoeba treated with alcian blue, a dye which evidently is not toxic and is often used to induce pinocytosis.

The staining affinity of ruthenium dyes for the surface coat of amoeba is believed by Szubinska & Luft (1971) to be due to the presence of sulphated polysaccharides. Early work by Marshall & Nachmias (1965) demonstrated that this glycocalyx contains sulphate, and O'Neill (1964) has identified galactose, mannose and glucose as well as amino acids in the isolated cell membranes. The surface coat of amoeba binds substances in a manner suggestive of strong ion-exchange properties and this binding is followed by pinocytosis or phagocytosis. Once internalized, the surface coat is detached from the plasma membrane and aggregates into dense masses within vacuoles.

The sea-urchin egg glycocalyx

Surface coats of sea-urchin eggs have long been recognized and studied extensively, Chambers (1940). (See Chambers & Chambers (1961) for a review of the early literature as well as Monroy (1965) and Austin (1968).) The shed sea-urchin egg has a thick jelly coat which is obviously an extraneous coating secreted by the ovary. This coat contains about 20% protein and the

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Description of plate 22

Figures 15 to 18. Electron micrographs of the surface of the *Arbacia punctulata* oocytes at various stages of fertilization membrane formation.

Figure 15. The oolemma and glycocalyx of an unfertilized egg. The surface coat of these eggs are very inconspicuous in routine preparations. This specimen was fixed with ruthenium red in the fixative solution and the glycocalyx or primary coat of Anderson (1968) is distinct. A mature cortical granule occupies much of the cytoplasm. (Magn. × 70000.) (Courtesy of E. Anderson.)

Figure 16. The surface of a recently activated egg with its newly forming fertilization membrane or activation calyx. This structure is formed by the lifting of the primary coat (figure 15). The newly created space is the perivitelline space. (Magn. × 60000.) (Courtesy of E. Anderson.)

Figure 17. A section of an egg fixed 2 min after fertilization. The fertilization membrane has thickened markedly and in certain areas has already reached its near maximal width. By this time most of the cortical granules have released their contents into the perivitelline space. (Magn. × 50000.)

Figure 18. The surface of a 4-cell stage embryo fixed 75 min after fertilization. The prominent fertilization membrane is about 50 nm thick and has a complex internal structure. The perivitelline space contains some flocculent, filamentous material which is the hyaline layer. (Magn. × 50000.)

remainder is polysaccharide esterified with $-SO_4$ groups. The *jellycoat* is a very highly hydrated glycoprotein that seems to be involved in normal fertilization by the transient agglutination of specific spermatozoa which is followed by increased motility. Although the volume of this jelly is some five times greater than the egg, it contains less than 1 % of the total dry mass of the egg. It is not preserved in the usual electron-microscopical preparations but may be seen on ovarian eggs (Ito 1969).

Another part of the glycocalyx associated with the sea-urchin egg is the *fertilization membrane*, which forms immediately following sperm penetration. This structure is quite distinct from the jelly and its formation can be readily visualized. Comprehensive studies of the events leading to formation of this glycocalyx have been reported by Anderson (1968) and by Millonig (1969) and will be summarized here.

Since jelly coat of the sea urchin is lost in preparation, the outer boundary of the fixed egg is the plasmalemma. Attached to the external surface of the trilaminar oolemma is a thin and irregular layer of cell coat material which is often invisible, but can be seen to better advantage if ruthenium red is used to stabilize and stain it (figure 15). Immediately upon fertilization this thin layer of material (the vitelline or primary envelope) rapidly elevates from the egg surface (figure 16), creating a space beneath it. The cortical granules in the peripheral cytoplasm release their contents into this 'hyalin space'. The glycocalyx called the vitelline envelope then quickly becomes appreciably thickened and hardens to form the tough resistant fertilization membrane or chorion. Stages of the formation of this glycocalyx and some features of its structure are illustrated in figures 17 and 18.

The role of the fertilization membrane is not completely understood but its formation reduces the chances of polyspermy and it obviously has a protective role during early embryonic development. Numerous mechanical, chemical or enzymic methods have been devised for removing or preventing the formation of this glycocalyx. Removal of the layer does not seem to affect development of the egg. In the course of normal development a 'hatching enzyme' secreted by the blastula ultimately lyses and the fertilization membrane ruptures.

Recently Lallier (1972) treated sea-urchin eggs with Concanavalin A, a globulin of plant origin which reacts with specific polysaccharide groups and clearly demonstrated reduced fertility. This was presumed to be due to blockage of fertilization as a result of the binding of Concanavalin A to oligosaccharides on the egg surface. Similarly, mammalian fertilization Oikawa, Yanagimachi & Nicholson (1973), have shown that wheat-germ agglutinin specifically binds to oligosaccharides in the zona pellucida and prevents sperm penetration.

Inconspicuous and invisible surface coats

It was stated earlier that there was no cell known to lack completely a carbohydrate-rich coating of some type even though the coat may be so tenuous as to be invisible in routine microscopy. Indeed, after the usual methods of tissue preparations for electron microscopy the glycocalyx is either inconspicuous or invisible. The demonstration of such thin coats requires special markers of staining methods such as the periodic acid–Schiff reaction (Rambourg, Neutra & Leblond 1967), periodic acid–silver methenamine reaction (Rambourg & Leblond 1967), phosphotungstic acid staining at low pH (Rambourg 1971), or the cationic dyes Alcian blue (Mowry 1963), colloidal iron (Gasic & Berwick 1963), colloidal thorium (Revel 1964) or ruthenium red (Luft 1971) (see Rambourg (1971) for a detailed discussion of different staining

methods for cell coats). Papers using one or another of these techniques have appeared in ever increasing numbers. The most comprehensive study of this kind is that of Rambourg *et al.* (1967), who demonstrated cell coats stainable by the PAS and alcian blue method covering the entire surface of more than 50 different cell types in the rat. These light-microscopic observations were extended to the electron-microscopic level by the same investigators (Rambourg & Leblond 1967) using a modified silver staining method and the colloidal thorium technique. Revel & Ito (1967), using the colloidal thorium stain on thin sections, demonstrated a distinctive pattern of localization of the electron-dense particles directly on the red-cell membrane, whereas in neutrophils the particles were often found at a distance of 20 to 30 nm from the membrane. These images were interpreted to indicate that the charged groups of the glycocalyx were very close to the surface of the red-cell membrane but that the glycocalyx may extend some distance from the surface of the neutrophil.

In one of the early histochemical studies on cell surfaces Gasic & Berwick (1963) demonstrated the presence of sialic acid at the surface of Ehrlich ascites tumour cells by staining intact cells with colloidal iron at low pH. However, when intact cells rather than sections are used, the intramembranous or intracellular localization of acidic carbohydrates cannot be determined due to the poor penetration of the colloidal reagents.

Another cationic dye useful for demonstrating surface carbohydrates by electron microscopy is ruthenium red (see Luft (1971) for an extensive evaluation of this stain). This dye is most attracted to polyanions and does not react with neutral polysaccharides. Its mechanism of staining is not well understood but it is a sensitive method for the ultrastructural localization of surface polyanions.

The elegant studies of Singer & Nicolson (1972) using labelled lectins (plant agglutinins) that bind to specific sugar residues on cell surfaces has yielded exciting results indicating the presence of oligosaccharides on the outer surface of cell membranes but not on their cytoplasmic surface. This very sensitive method for labelling particular carbohydrates on cell surfaces will undoubtedly contribute greatly to our understanding of the chemical nature and specificity of the glycocalyx.

In addition to the direct staining and demonstration of the cell coat, the labelling of cell surface antigens by employing antibodies labelled with antigens that are in turn coupled to fluorescent dyes or electron-dense markers has become a widely used technique combining sensitive immunological techniques with morphological methods of visualization. It is beyond the scope of this presentation to consider this topic except to acknowledge these surface antigens as important components of the glycocalyx that can be exploited to achieve a better understanding of its varied biological functions.

Thus it is clear that if appropriate methods are used, a glycocalyx can be demonstrated on virtually all plasma membranes. The precise relations of the carbohydrate to the protein components and to the lipid bilayer are aspects of the problem that remain to be clarified.

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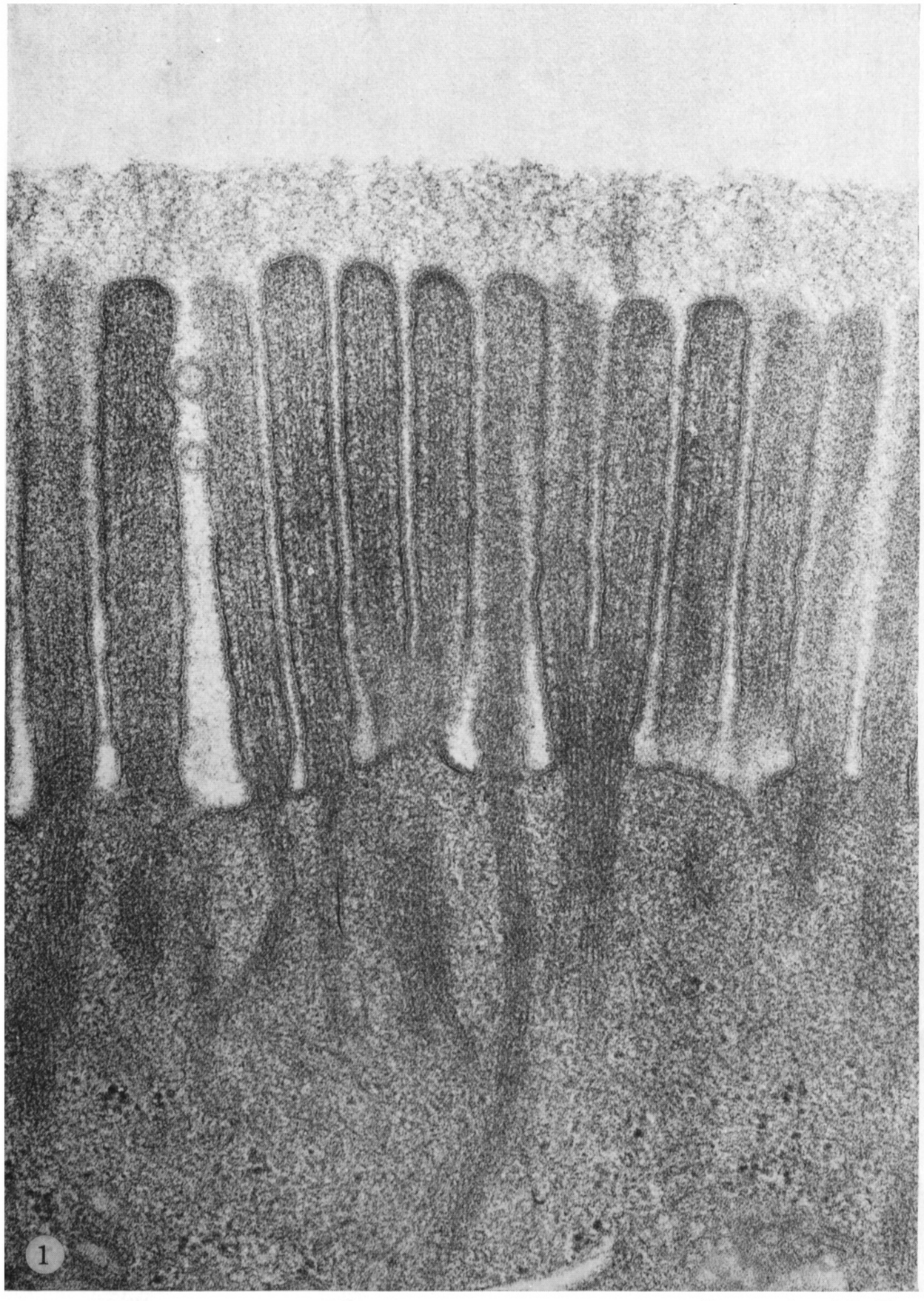

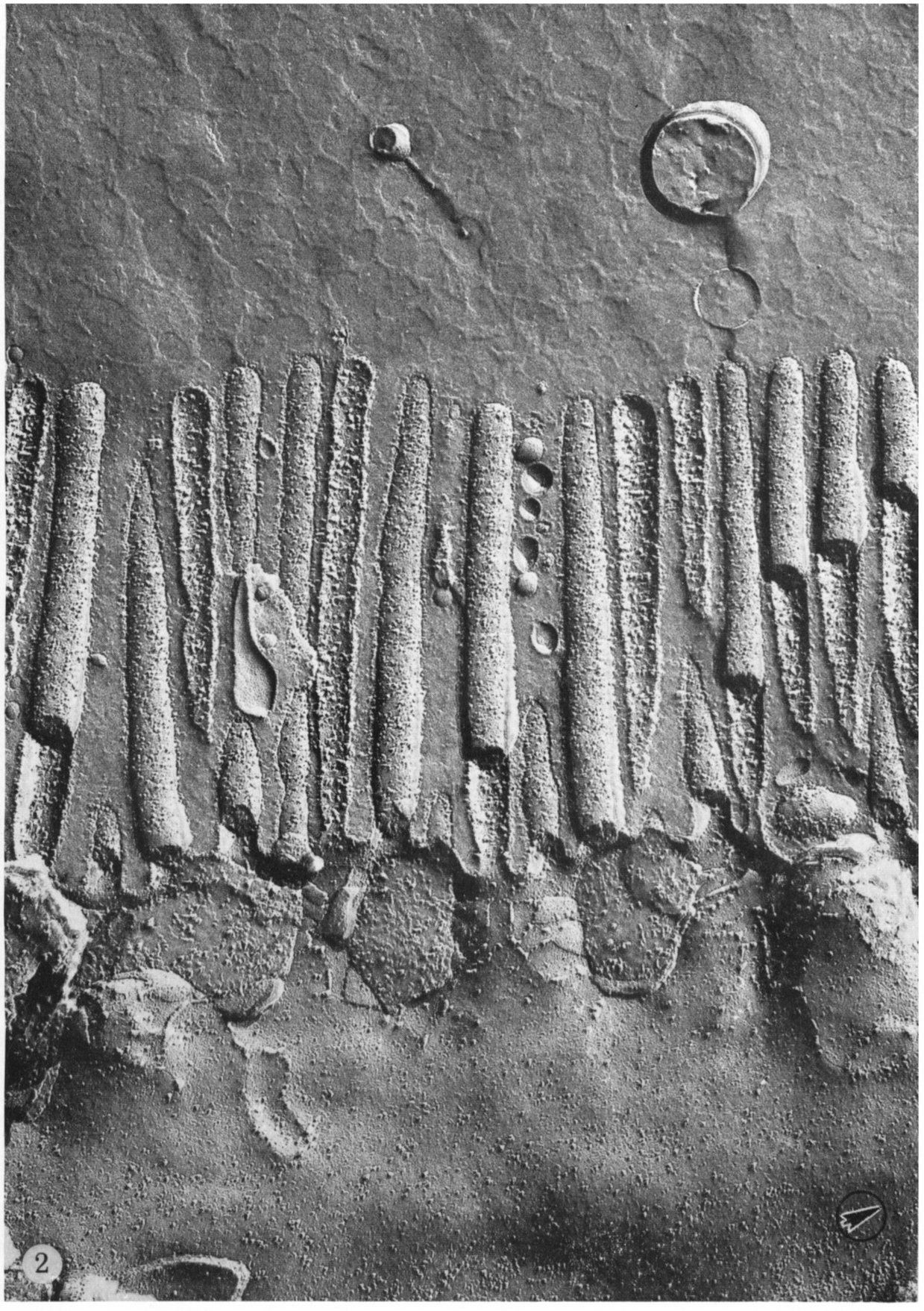

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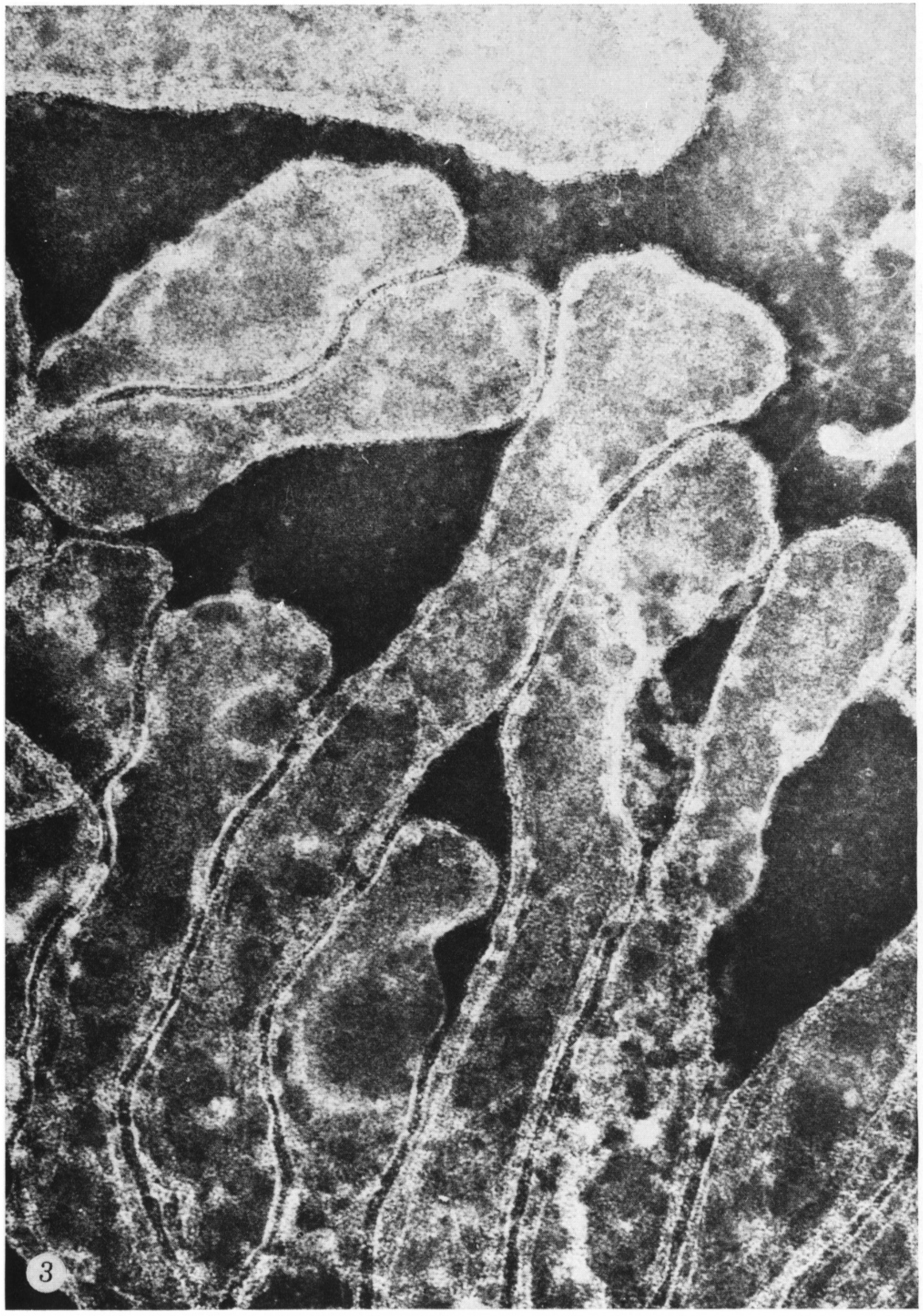

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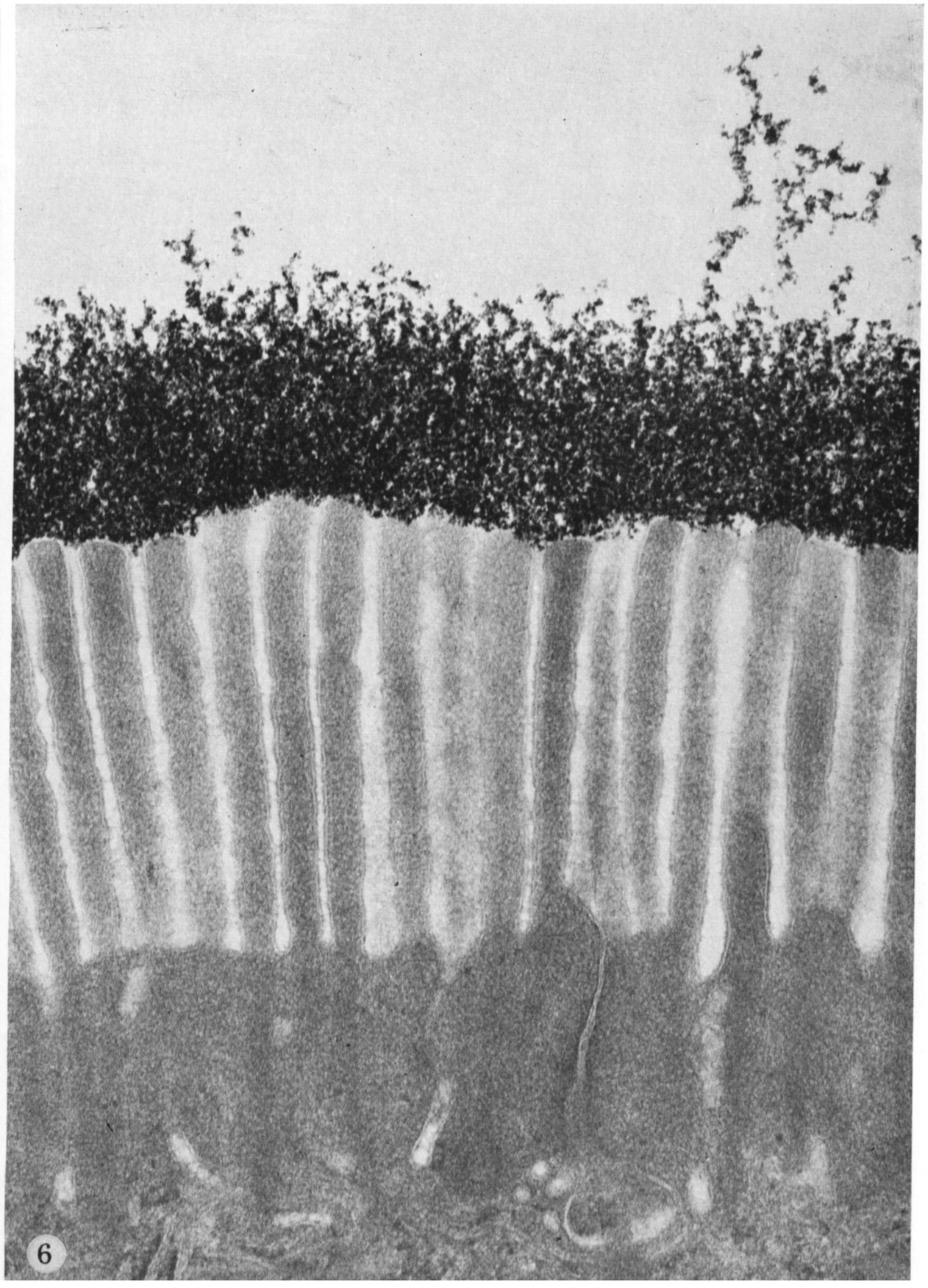

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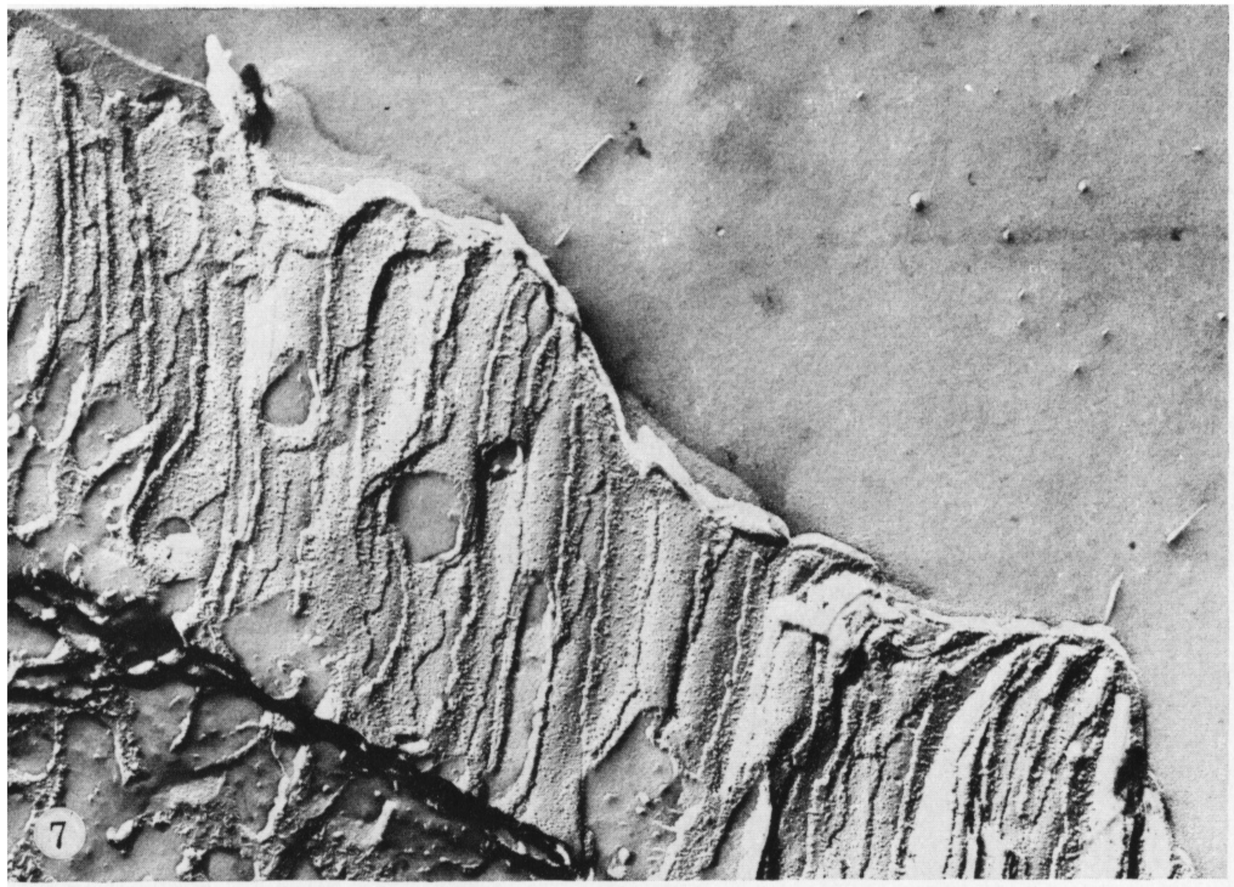

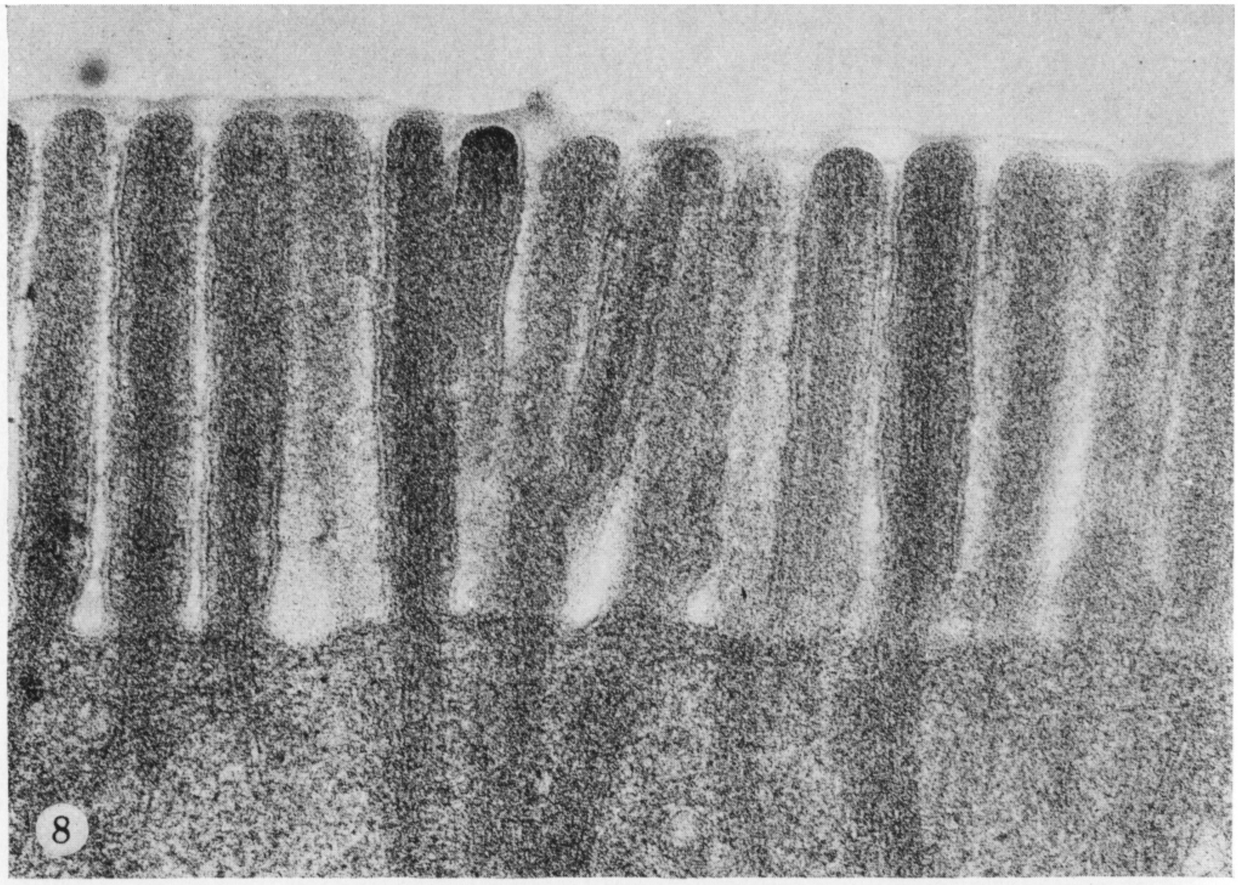

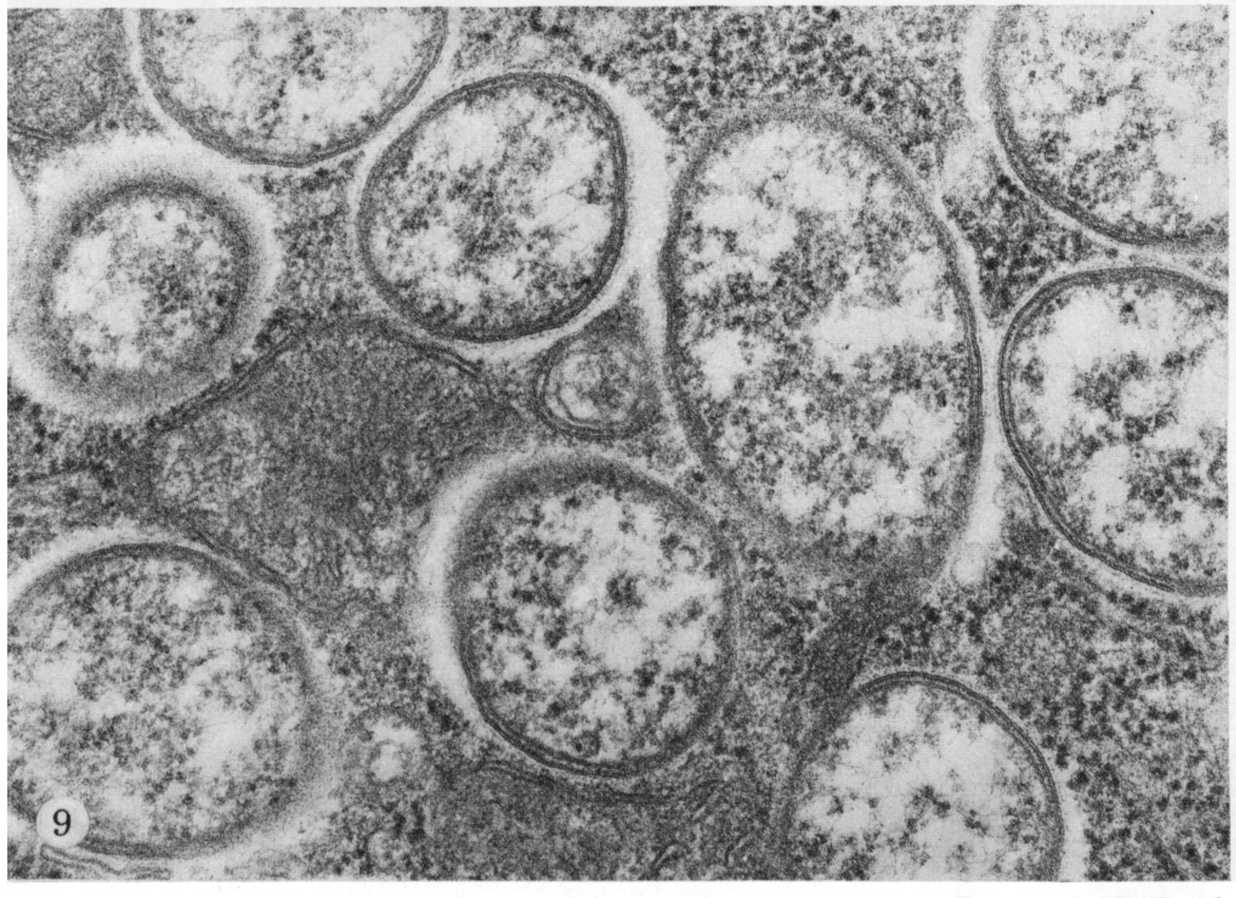

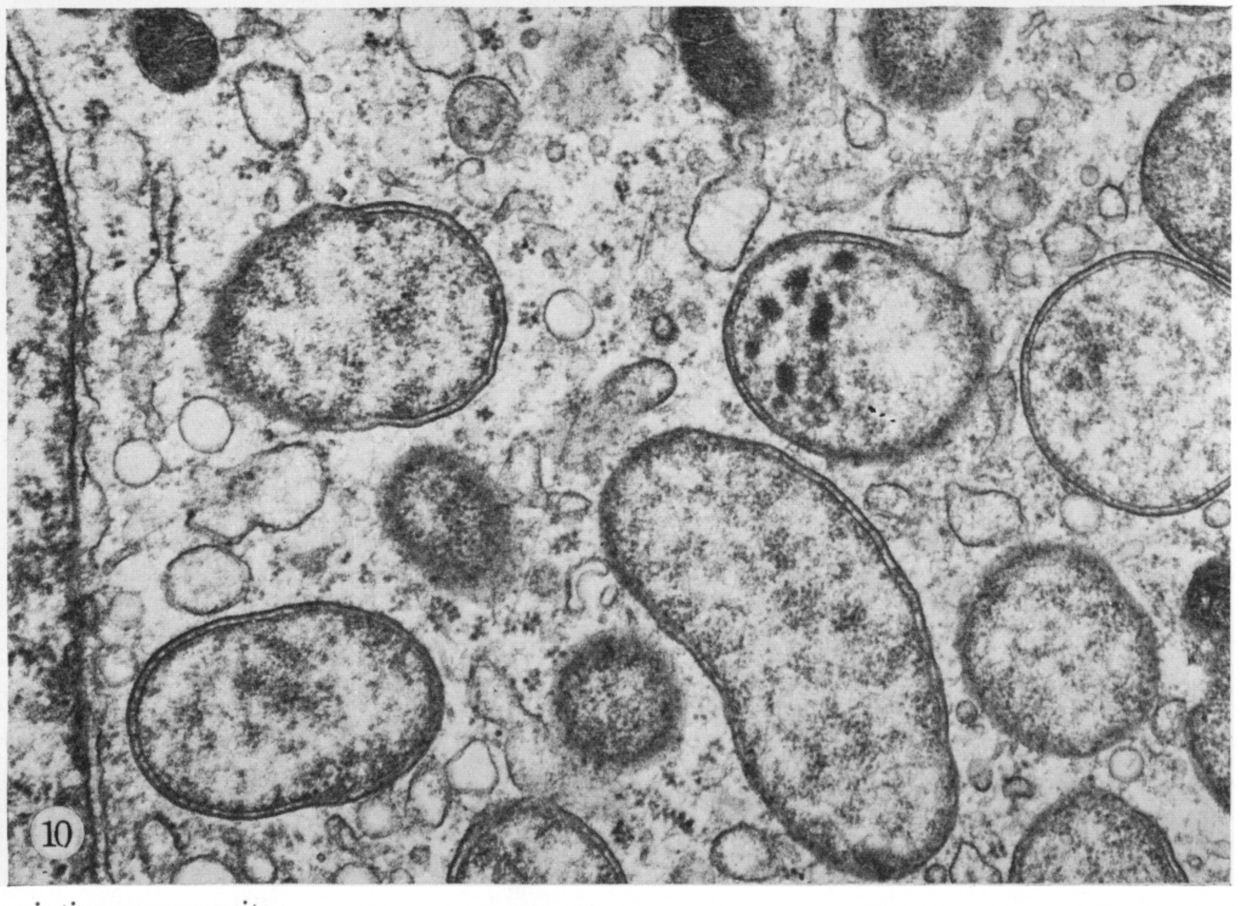

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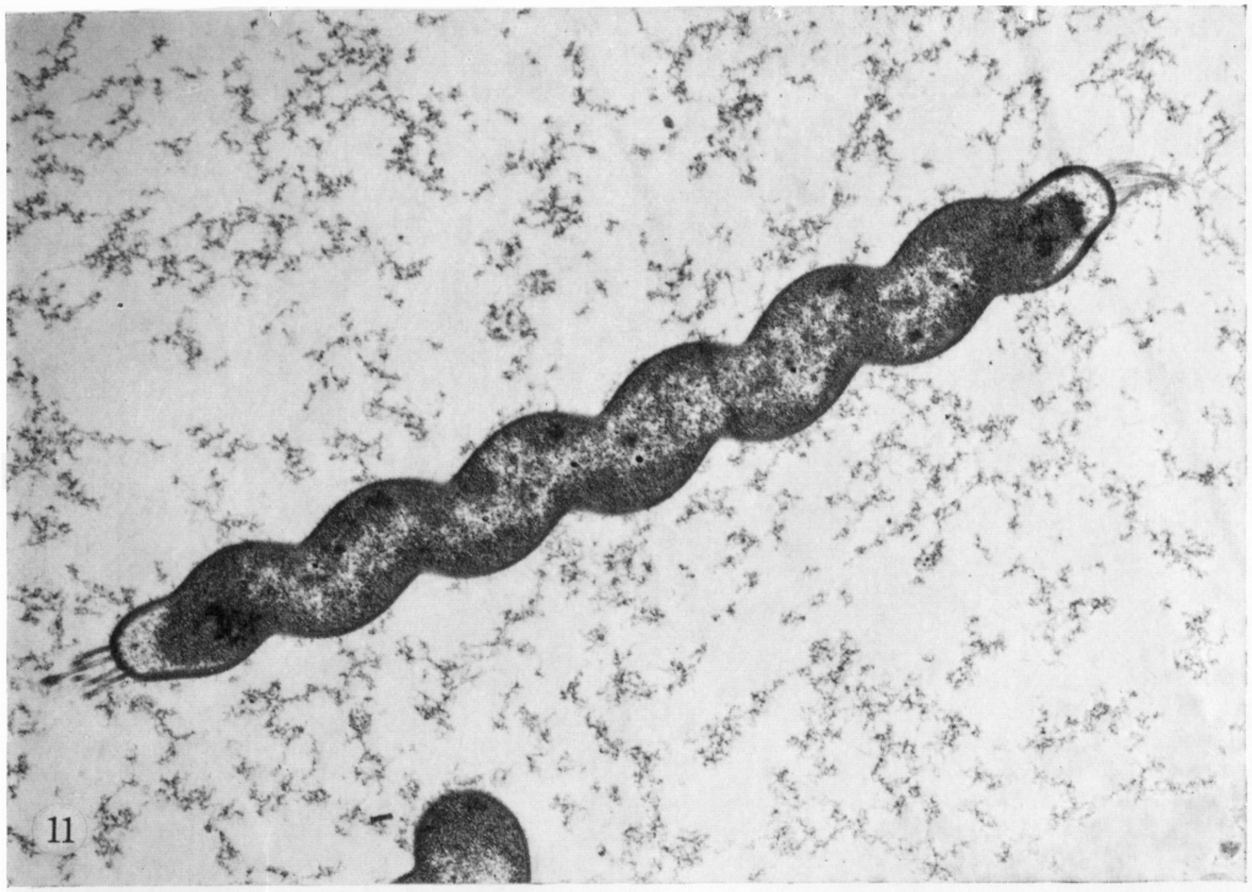
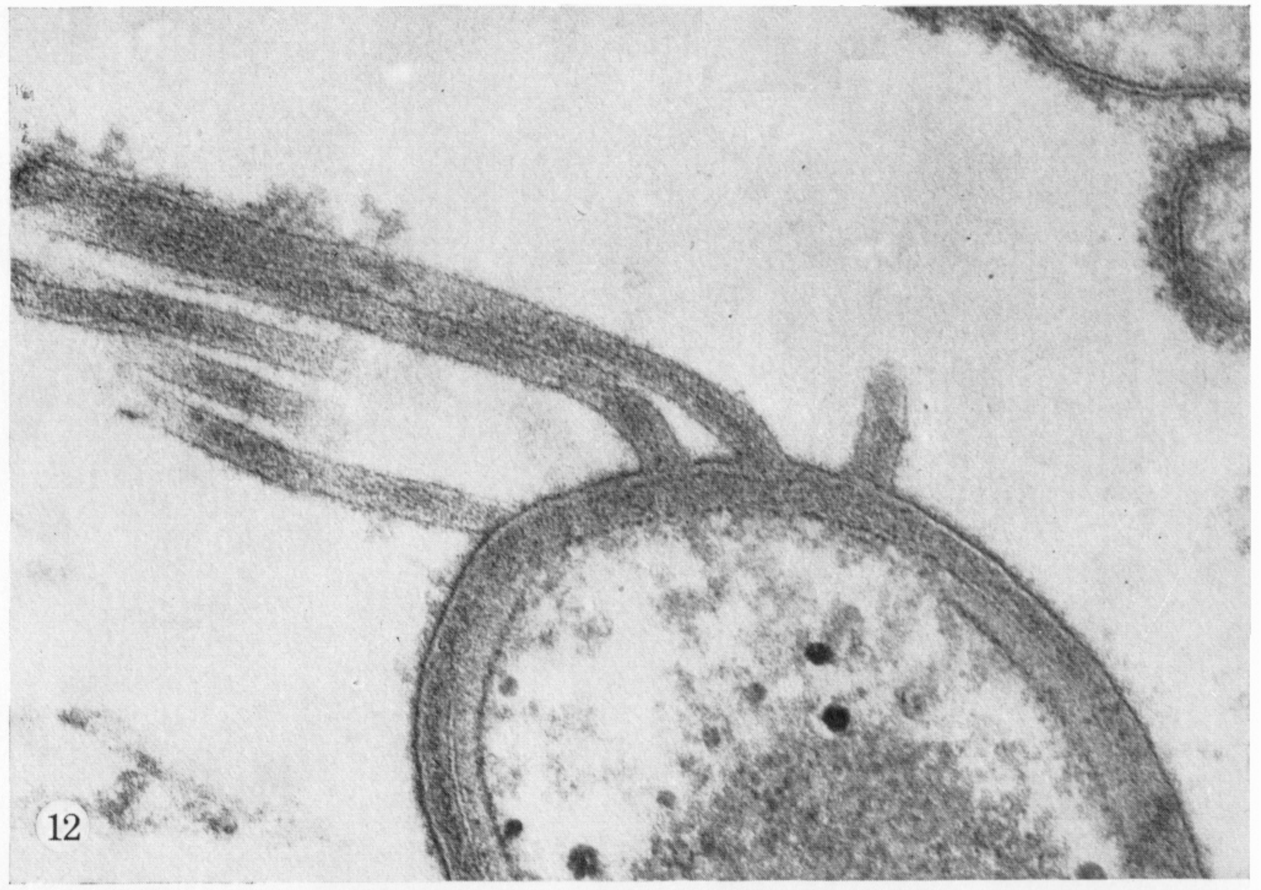
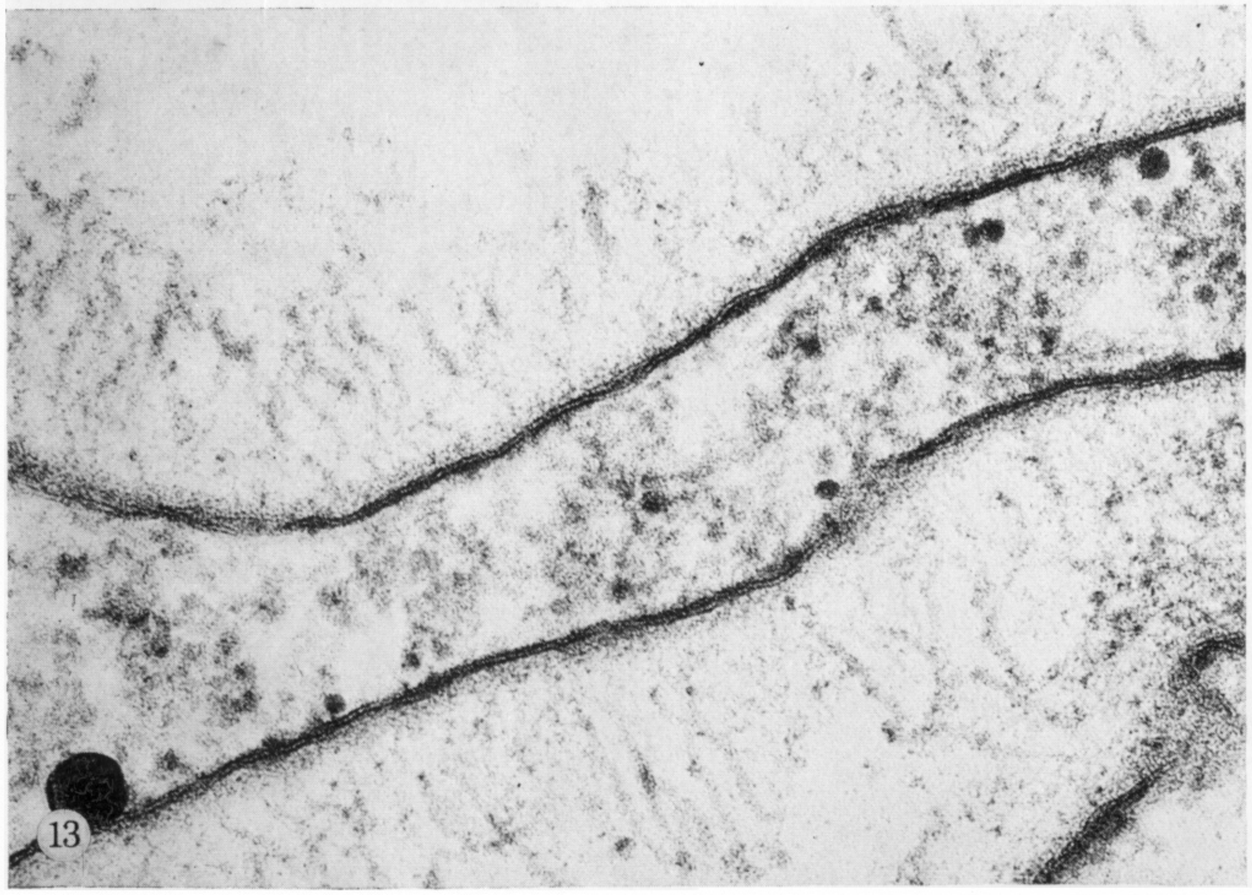
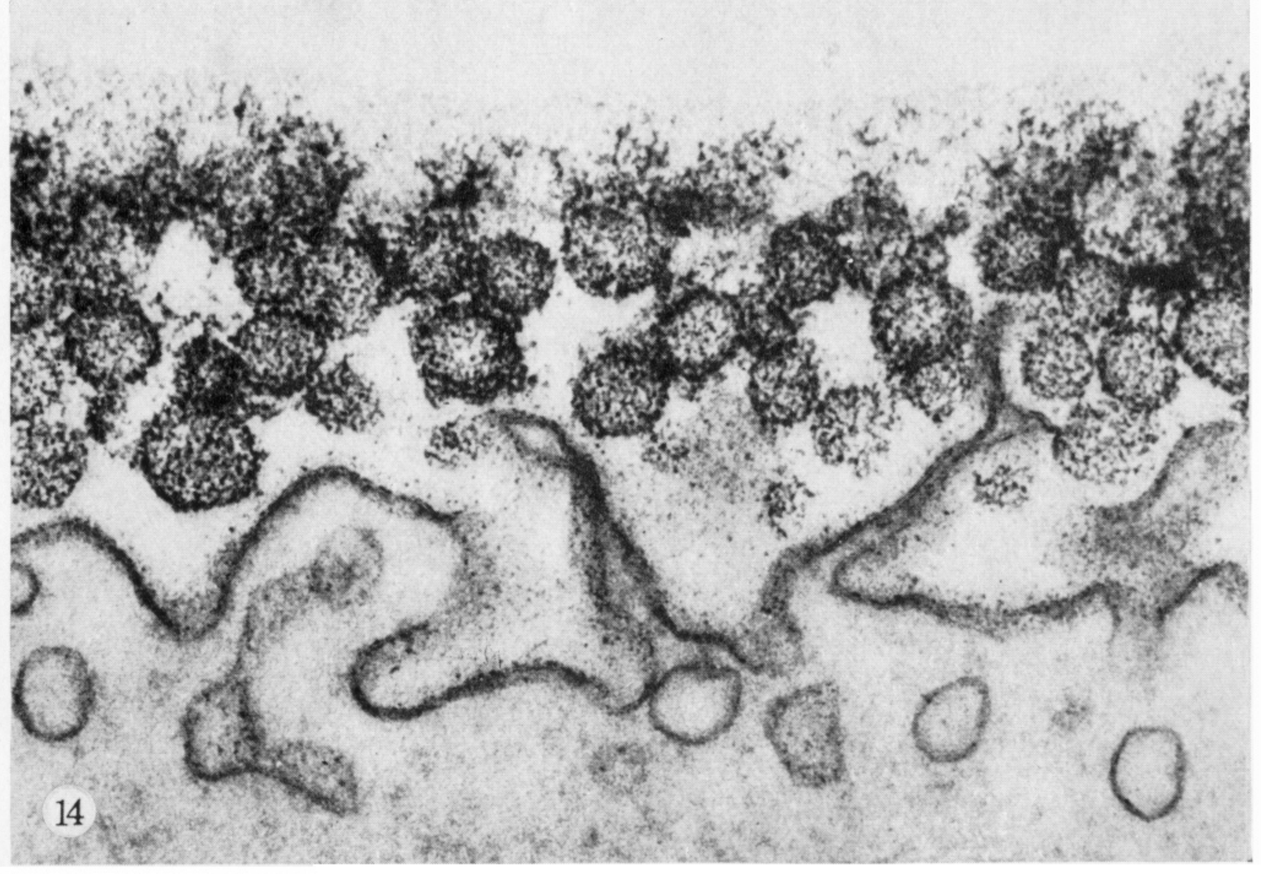
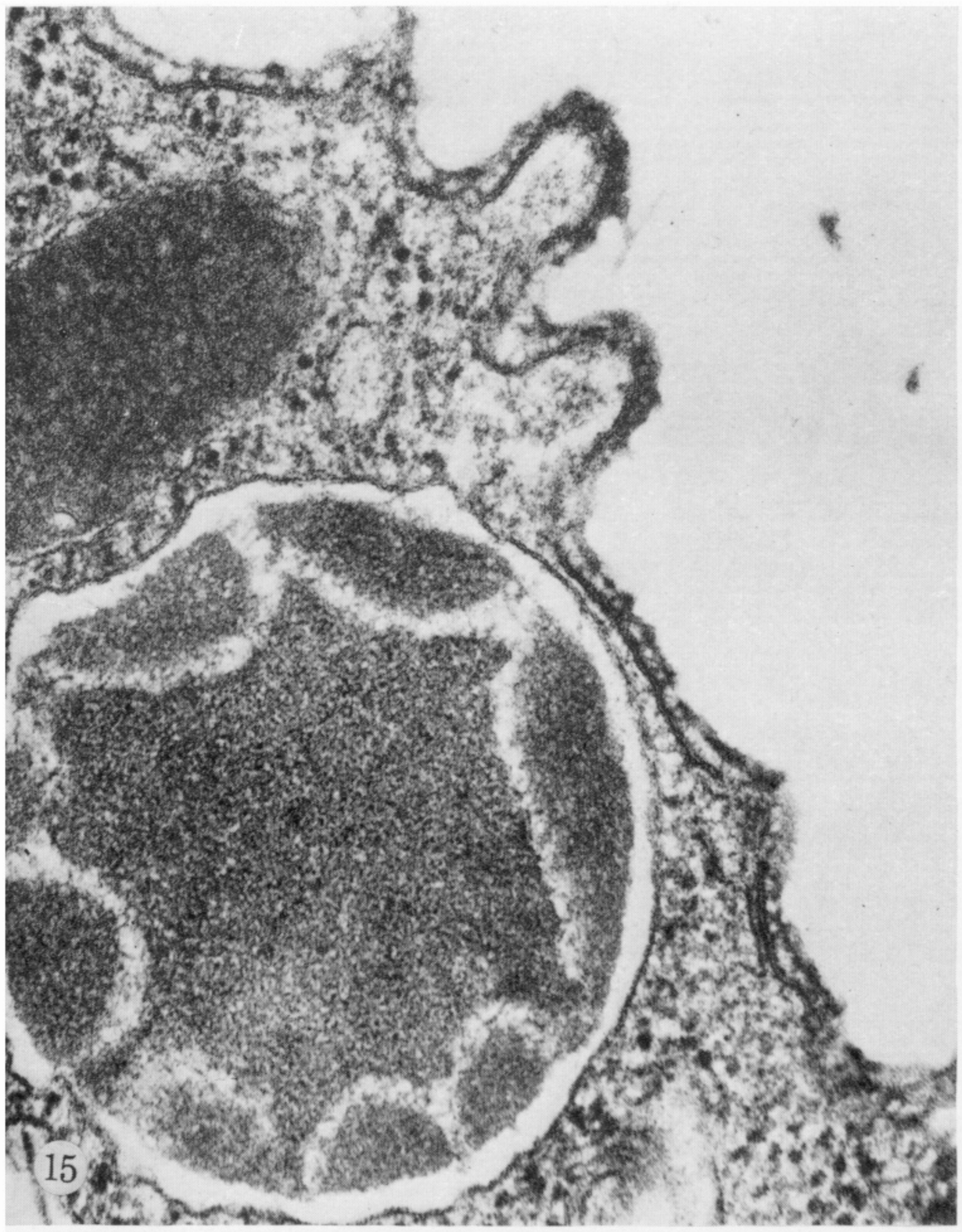


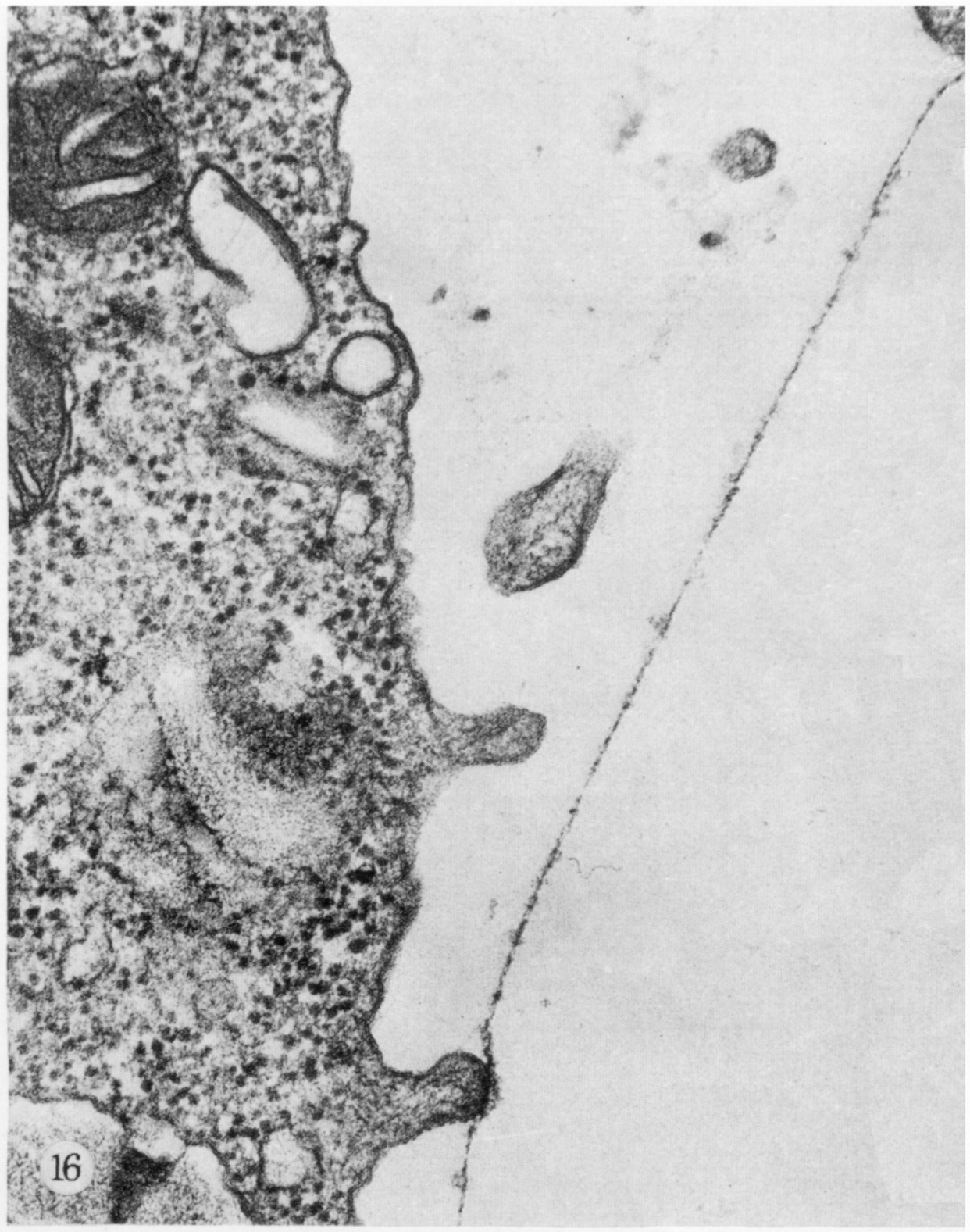


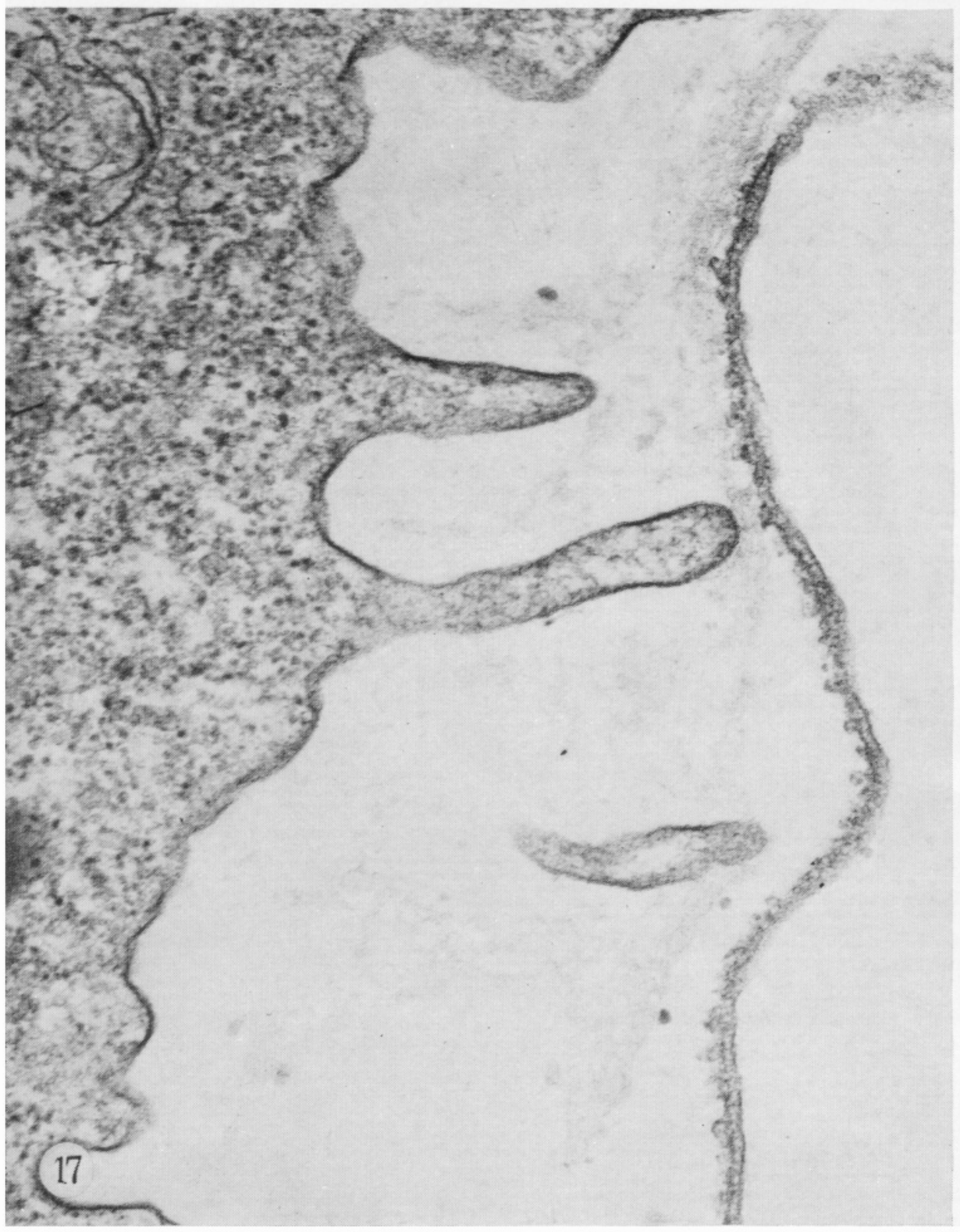


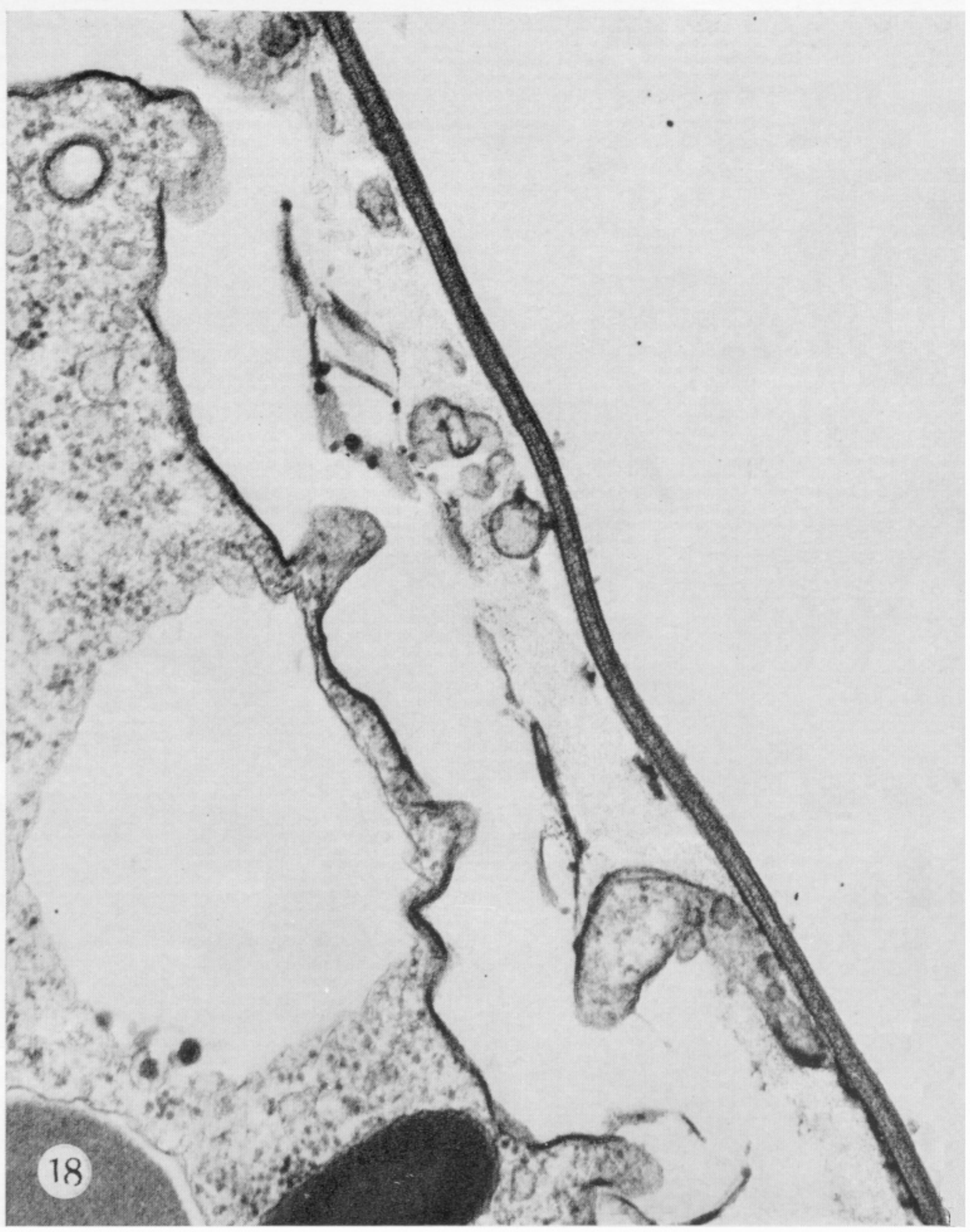


FIGURES 11–14. For description see opposite









FIGURES 15–18. For description see opposite