FREEZE-ETCH STUDIES OF THE PLASMA
MEMBRANE OF PULMONARY ENDOTHELIAL CELLS

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ABSTRACT

Pulmonary endothelial cells are capable of metabolizing a variety of circulating hormonal substances. Indirect evidence indicates that some of the relevant enzymes are located on the plasma membrane. The associated caveolae are of special interest as globular subunits, possibly enzyme clusters, are evident in their membranes. In the present study, freeze-etch techniques were used to improve understanding of the fine structure of endothelial cells and to extend our investigations of possible sites of enzymes capable of metabolizing circulating vasoactive agents. As in other cells studied by freeze-etching, intramembranous particles are found on both inner aspects of the plasma membrane. In undifferentiated areas of plasma membrane, the particles appear to have a random distribution. These areas fracture such that approximately equal proportions of the particles adhere to the cytoplasmic aspect of the outer leaflet and the extracellular aspect of the inner leaflet. However, the particles organize into rosettes and plaques at the base of caveolae, and, after fracture, the rosettes and plaques adhere predominantly to the cytoplasmic aspect of the outer leaflet. The peculiar organization of particles in association with caveolae supports the concept that caveolae have a stomal skeletal structure and raises the possibility that the organization may be in some way related to pinocytosis.

INTRODUCTION

Endothelial cells, especially those of the lung, appear to be capable of metabolizing a variety of circulating vasoactive substances, such as bradykinin, angiotensin I, and the adenine nucleotides (see references in Ryan et al., 1972 a). Current evidence indicates that the relevant enzymes are located on the plasma membrane or within the attached caveolae intracellulares (Smith and Ryan, 1972 a; Ryan et al., 1972 b).

Cytochemical studies have shown that a 5'-nucleotidase enzyme is located within the caveolae (Smith and Ryan, 1971; Ryan and Smith, 1971 a). Further, there is evidence of globular subunits, possibly enzyme clusters, within the membrane of the caveolae (Smith and Ryan, 1972 b). At present

there is less information on the subcellular location of the enzymes that metabolize bradykinin and angiotensin I. However, as the kinetics of their disappearance are similar to those of the adenine nucleotides (Ryan et al., 1972 a, 1972 b), and as bradykinin and angiotensin I are metabolized by isolated plasma membrane/caveolae fractions of lung (Ryan and Smith, 1971 b; Ryan et al., 1972 a), it is likely that their metabolic enzymes are located near the sites of 5'-nucleotidase.

Freeze-etch studies of other cell types have shown particles within the plasma membranes, and it has been suggested that the frequency of these particles may relate to physiological activity (Branton, 1966, 1971). The variety of enzymes
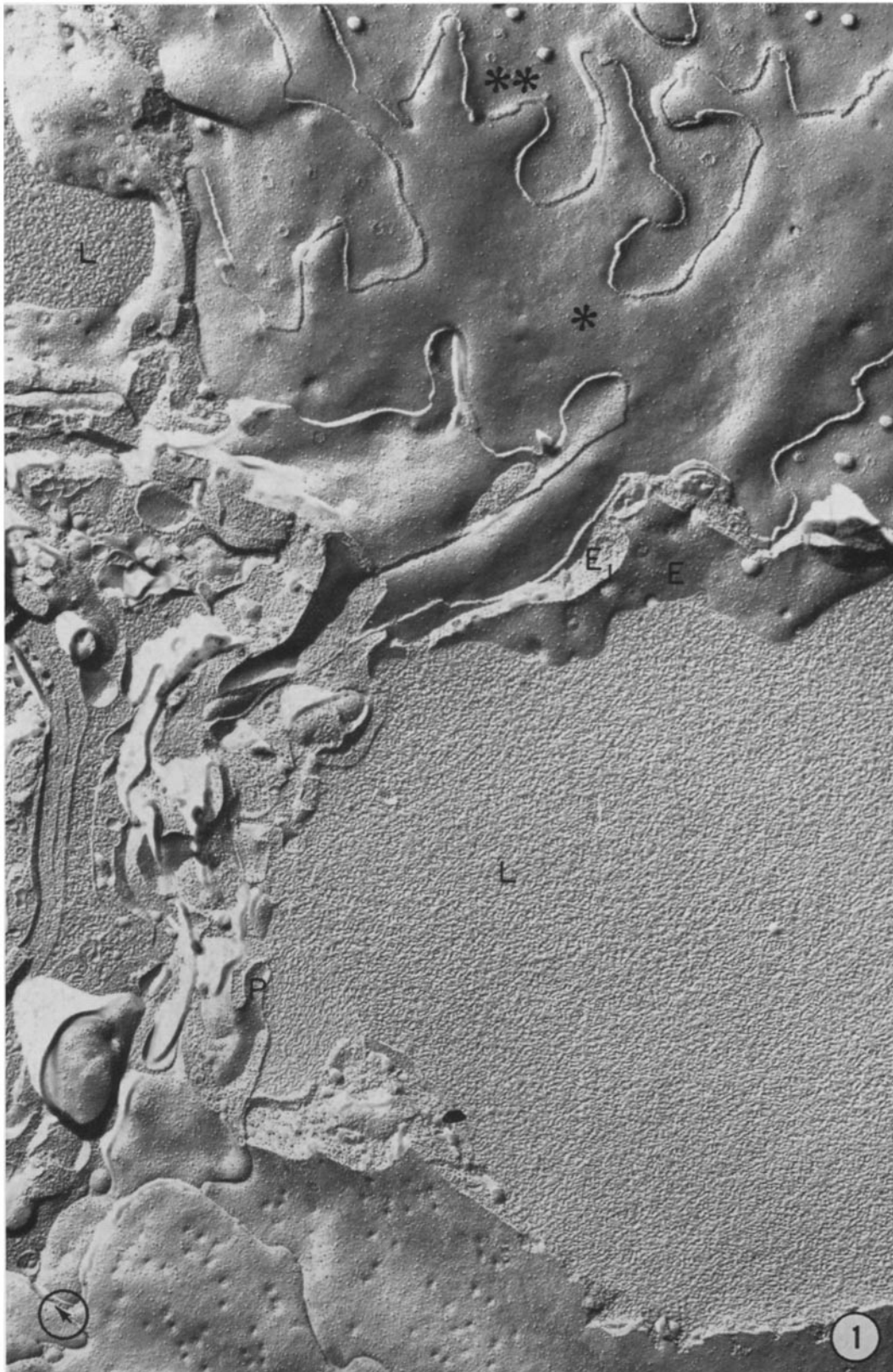
FIGURE 1 Survey micrograph of freeze-etched rat lung showing a small vessel with lumen (*L*). Endothelial cells are fractured through their plasma membranes (*E*) and across the cytoplasm (*E₁*). Note endothelial projections at *P*. The large areas of fractured membrane (*) may represent the inner leaflet of a septal (pericyte) cell membrane. The dome-studded area (**) above may represent the outer leaflet of a closely apposed cell. The circled arrow indicates the direction of shadowing. × 29,000.
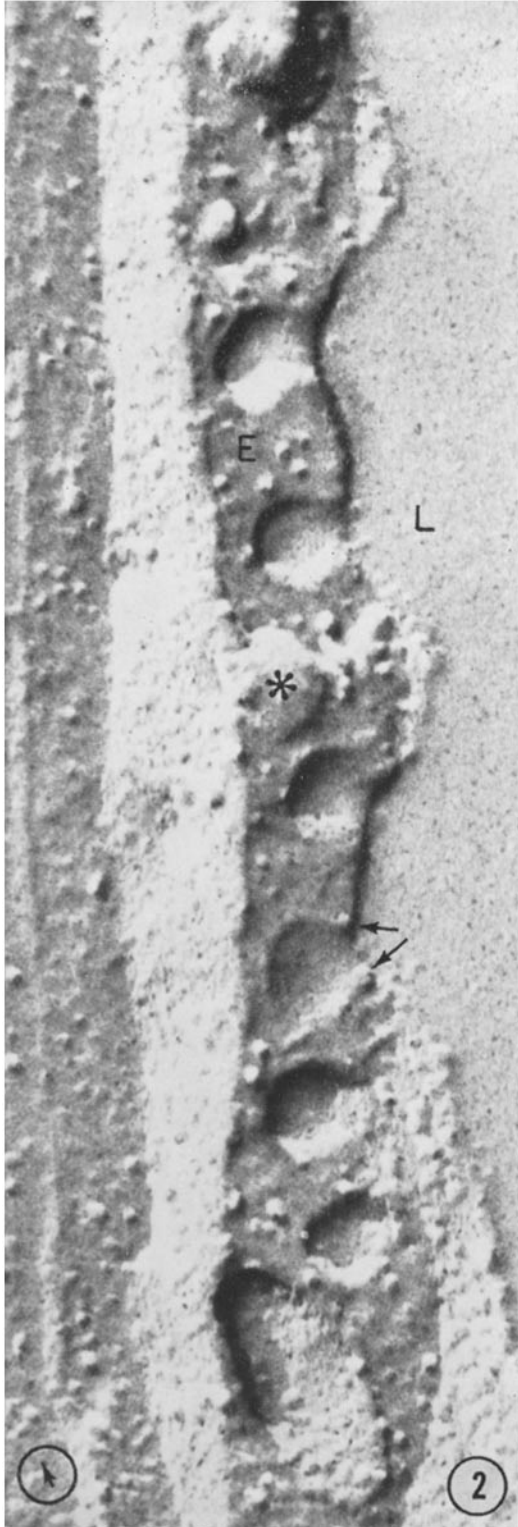
known to be associated with plasma membrane (for references see Benedetti and Emmelot, 1968) raises questions about the relationship between intramembranous particles and enzymic activities. We have, therefore, begun a survey of pulmonary endothelial cells using freeze-etch techniques to extend our investigation of possible sites of enzymes which metabolize circulating vasoactive substances, and further to define substructural features of endothelial caveolae.

MATERIALS AND METHODS

Rat lungs were prepared for freeze-etching by two techniques. First, lungs were perfused with Krebs-Henseleit solution until the venous effluent was free from blood (approx. 3 min) and then with 0.25 M glutaraldehyde buffered at pH 7.4 with 0.05 M cacodylate buffer (Smith and Ryan, 1970, 1972 b). The fixed lungs were cut into small pieces (approx. 2 mm per side) and were transferred to 30% glycerol for 3 h. In a second series, small pieces of unperfused lung tissue were dissected, then fixed for 3 h in a mixture of 30% glycerol and 70% buffered glutaraldehyde solution (prepared as above), and were thus ready for immediate freeze-etching.

Tissue blocks prepared by either method were mounted on nickel-gold holders, frozen in "Freon 12" chilled by liquid nitrogen, and placed in a Balzer's BA 360 M freeze-etch microtome. After cleaving, etching was continued for 2 min before evaporation of platinum and carbon. The replicas were cleaned in Chlorox and distilled water, and examined in a Philips EM 200. As an aid to interpretation, stereo pairs were taken at a tilt angle of ±6°.

RESULTS

The membrane of the caveola is of unit membrane construction similar to the plasma membrane while the diaphragm is composed of a single lamella (Bruns and Palade, 1968). The diaphragms which span the stomata of the caveolae may vary in exact position. Occasionally, transverse fractures through the endothelial cell are encountered but most

FIGURE 2 Transverse fracture showing an endothelial cell (E) with many caveolae facing the vascular lumen (L). In this field most of the caveolae appear as pits representing the extracellular aspect of the inner leaflet (IFF). One appears as a dome (*) formed by the cytoplasmic aspect of the outer leaflet. Particles are scattered in the cytoplasm. The luminal stoma of one of the caveolae is bounded by two knobs (arrows) which may represent the stomal rim. The circled arrow indicates the direction of shadowing. × 237,000.
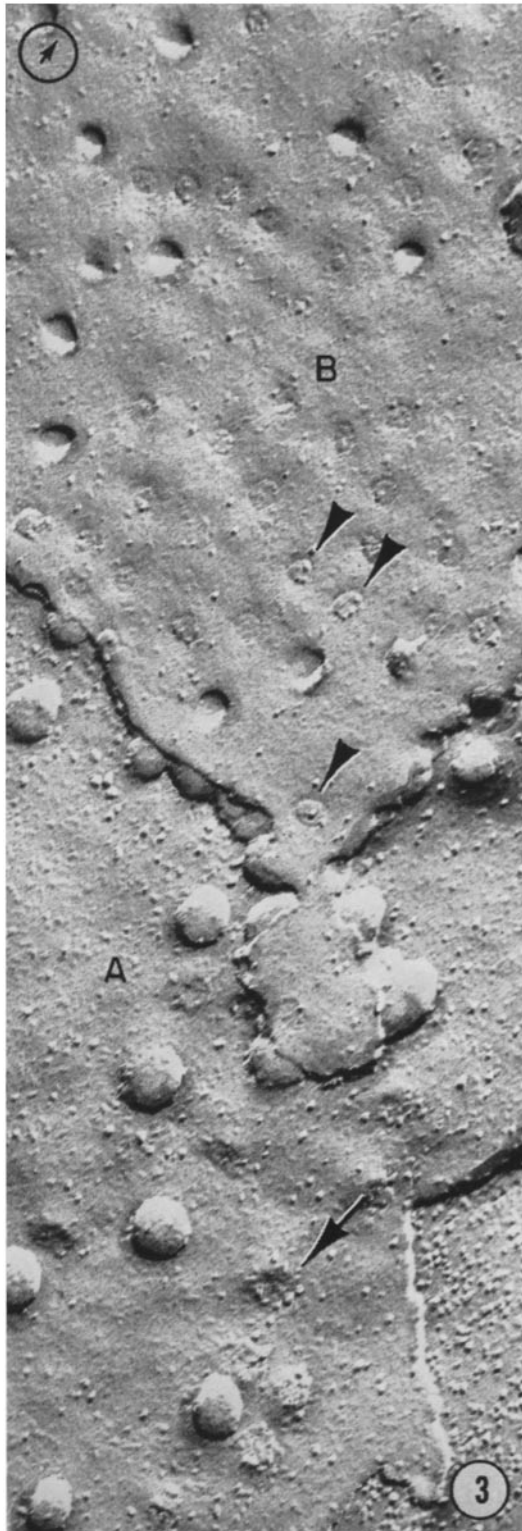
fractures appear to occur in the plane of the plasma membrane itself, exposing extensive areas of particle-studded membrane surfaces. It is possible to picture four different membrane faces (Mühlethaler, 1972): (a) The extracellular aspect of the outer leaflet (OS), (b) the cytoplasmic aspect of the outer leaflet (OFF), (c) the extracellular aspect of the inner leaflet (IFF), and (d) the cytoplasmic aspect of the inner leaflet (IS). In the present study the unfractured surfaces (a) and (d) were not identified; we interpret the fractures as occurring predominantly between the hydrophobic ends of the bimolecular phospholipid leaflet (Branton, 1966, Smith and Aldrich, 1971). Fractures generally followed the contours of the caveolae which then appeared as pits or domes.

Fig. 1 is a survey micrograph of freeze-etched rat lung showing small vessels and surrounding cells. In some instances the fracture exposes large sheets of the inner aspects of plasma membrane but may also traverse the cytoplasm.

Fig. 2 shows a transverse fracture through an endothelial cell. Particles approximately 85 Å in diameter occur in the cytoplasm. Generally, the fracture plane follows the contours of the caveolae, leaving smooth depressions (IFF) or domes (OFF). The latter surface shows attached particles. The two knobs delineating the stoma of one of the caveolae may represent the skeletal rim or ring of

Figure 3 Replica showing the inner surfaces of both leaflets of the plasma membrane of an endothelial cell. Surface *A* represents the cytoplasmic aspect of the outer leaflet (OFF) while surface *B* shows the extracellular aspect of the inner leaflet (IFF). Surface *A* shows an extensive area of plasma membrane without caveolae but covered with randomly distributed particles. The domes represent the cytoplasmic side of the outer leaflet of the caveola membrane; particles also occur on this aspect of the domes. A circular constellation of particles can be seen (arrow) and is interpreted as the stomal rim of an avulsed caveola. The rough, circular plaques may be the underside of the diaphragm or a break at some level through the caveola. The domes seen near to and under surface *B* do not appear to open to the inner leaflet, indicating that caveolae do not normally open on both sides of the cell simultaneously. Surface *B* (IFF) shows scattered particles on undifferentiated plasma membrane. The circular plaques (arrowheads), most of which are slightly depressed, may represent the face of diaphragms covered with particles (one centrally placed). The circled arrow indicates the direction of shadowing. × 34,000.

beads which has been described previously (Smith and Ryan, 1972 b).

Fig. 3 shows two distinct views of endothelial plasma membrane: *A*, OFF, and *B*, IFF. These probably represent two faces of the plasma membrane of the same cell. The surface replicated in *A* shows areas of plasma membrane which are devoid of caveolae. Both surfaces are studded with particles, again approximately 85 Å in diameter. Surface *A* shows the domelike cytoplasmic aspect of the outer leaflet of caveolae (OFF) illustrated in more detail in Figs. 4–7. In some instances, the particles are attached to the domes (see Fig. 6). Circular constellations of particles occur around the stoma of some caveolae. Other areas show circular, rough plaques, where the dome has been avulsed (see Fig. 6). There is one clear circle of particles which may represent a stomal ring (Smith and Ryan, 1972 b). Conceivably, the rough surface of the plaques represents the underside of the diaphragm. Caveolae arising on one side of the cell (see transition from area *A* to area *B*) do not appear to open to the other side of the cell. The latter point is consistent with the view obtained from thin sections of pulmonary endothelium that caveolae do not open to both sides of the cell simultaneously, even where the endothelium is highly attenuated. Surface *B* shows excavated caveolae (see also Fig. 9); in this field the IFF of the caveola membrane appears to be smooth but this may be because particles in pits are sometimes not revealed by shadowing (contrast Fig. 9). We interpret the rough, circular plaques to be diaphragms covered with particles (see Fig. 8).

Figs. 4–7 (comparable to surface *A* in Fig. 3) show the OFF. Fig. 4 illustrates an area having caveolae with a density of 37 per μm^2. In Fig. 4 the particles appear to be randomly distributed on areas of undifferentiated plasma membrane and their relationship to caveolae is not clear. However, in Fig. 5 a high degree of organization of particles is evident where caveolae have been fractured at their bases. Particles adhering to this surface occur as circular constellations and may represent the

skeletal rim described previously (Smith and Ryan, 1972 b). Other circular plaques show minute depressions, presumably sockets of particles removed by fracture. Figs. 6 and 7 are higher magnifications giving evidence of particle attachment to the domes and a better view of the circumference of the domes.

Figs. 8 and 9, like surface *B* in Fig. 3, represent replicas of the IFF. In Fig. 9 the caveolae membranes appear as pits, two of which contain particles. However, in our experience most excavated caveolae do not show particles. Possibly particles tend to adhere to the outer leaflet of the caveola; alternatively particles in the excavated caveolae may be poorly revealed by current shadowing techniques. The shallower depressions (Fig. 8) correspond to diaphragms which are also covered with particles.

DISCUSSION

The findings illustrated here by freeze-etching confirm and extend previous studies of thin-sectioned material. The previous studies showed that dense knobs occur at the place where caveola membrane, diaphragm, and plasma membrane fuse. In the present study (cf. Figs. 2 and 5) circular constellations of particles are evident on the cytoplasmic aspect of the outer leaflet where caveolae have been fractured at their bases. This finding reinforces the concept that caveolae have a circular skeletal structure (Smith and Ryan, 1972 b). In addition, the globular substructures of the caveola membrane seen in thin sections may correspond to the particles seen on the dome-shaped OFF of fractured caveola membranes.

Despite the ubiquity of small particles (75–100 Å in diameter) on freeze-etched membranes, our studies suggest that there is a relationship between the structure of endothelial caveolae and the distribution and organization of intramembranous particles. Undifferentiated areas of plasma membrane fracture such that approximately equal proportions of randomly distributed particles are found attached to the OFF and to the extracellular

Figures 4–7 The OFF with scattered intramembranous particles. In Fig. 4 the plane of fracture follows the contours of the caveolae which appear as domes. Clusters of caveolae reach a density of 37 per μm^2 in some areas. In Fig. 5 some of the caveolae have been broken off, leaving rough plaques, some of which appear as circular constellations of particles (arrow) while others show depressed sockets (arrowhead). Fig. 6 shows that particles also occur on the dome of the caveola. Fig. 7 illustrates the far side of the caveola dome (arrow) which is ordinarily obscured by the shadow. The circled arrow indicates the direction of shadowing. Fig. 4, × 89,000; Fig. 5, × 169,000; Fig. 6, × 242,000; Fig. 7, × 419,000.
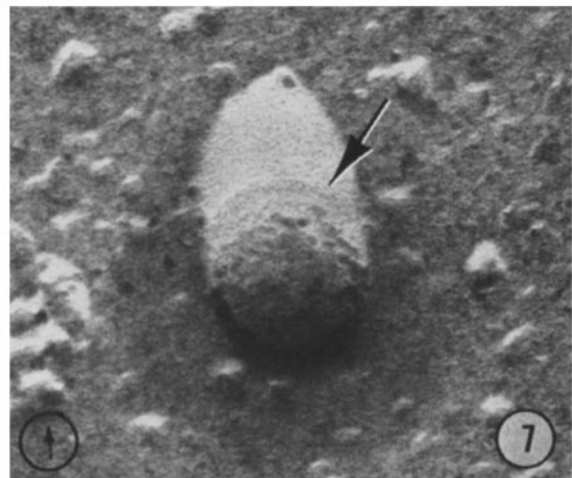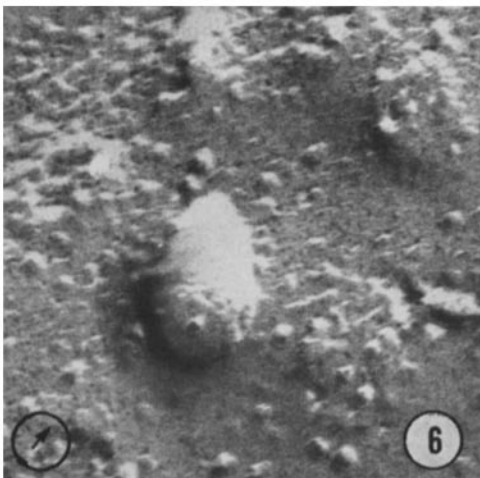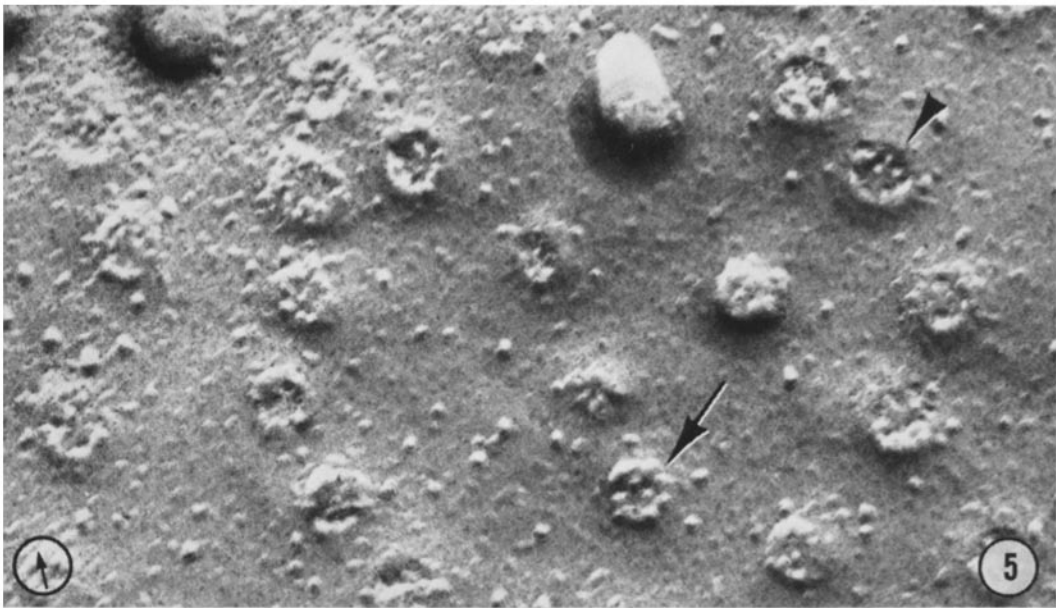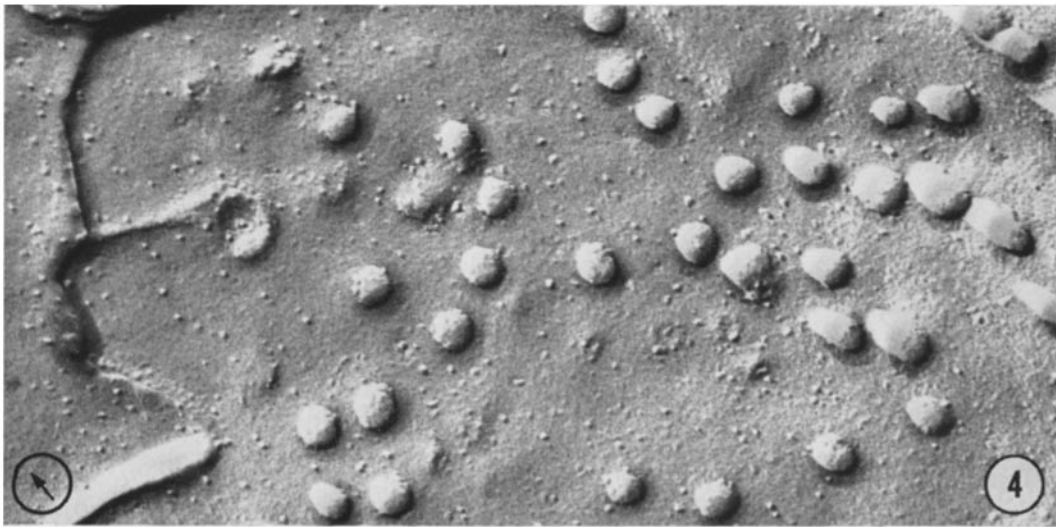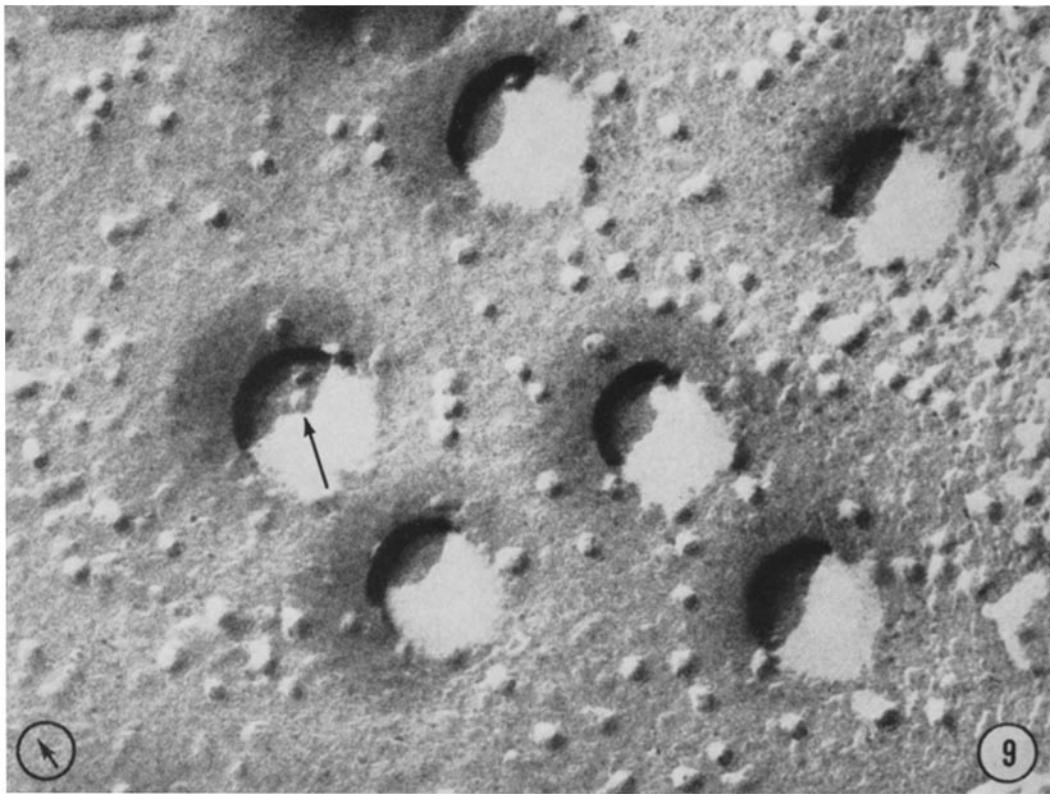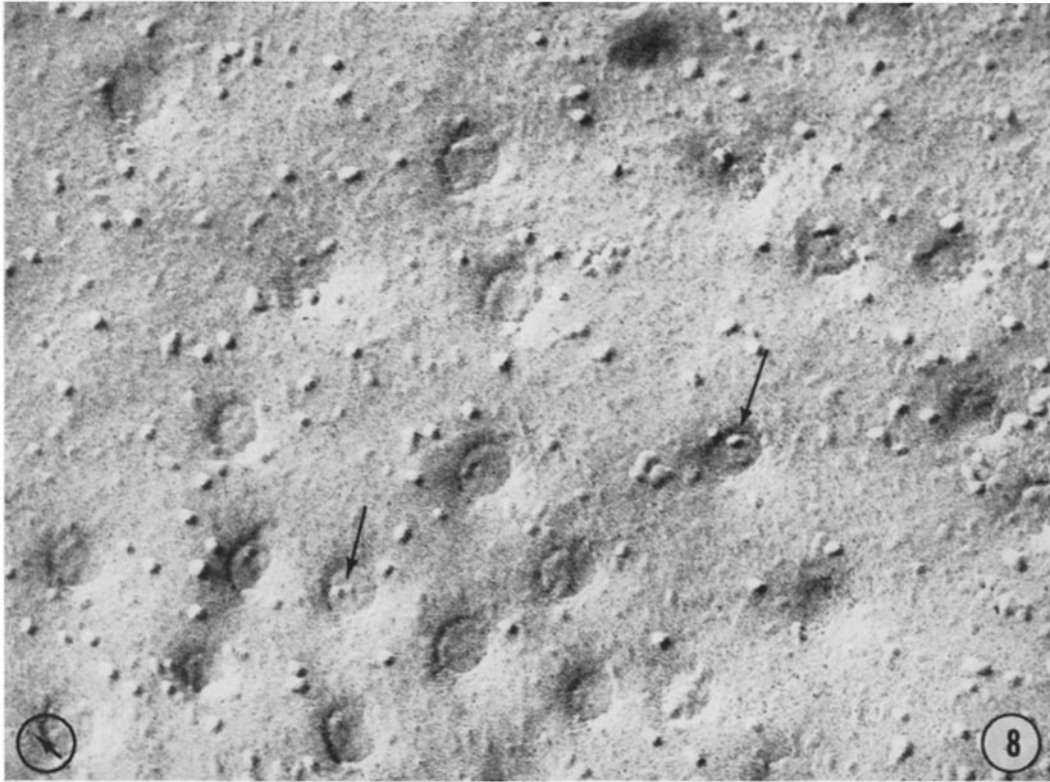
FIGURES 8-9 The IFF is also studded with particles. Fig. 8 shows the diaphragms which span the apertures of the caveolae, and these too show particles (arrows). In Fig. 9 the fracture follows the contours of the caveolae which appear as pits. Particles can be discerned at the base of the pits (arrow). The circled arrow indicates the direction of shadowing. Fig. 8, × 169,000; Fig. 9, × 210,000.

aspect of the inner leaflet (IFF). However, there appears to be some tendency for those particles, which are organized, to adhere to the outer leaflet. For example, the rings and circular plaques are readily identified on the outer leaflet but are seldom found on the inner leaflet.

Possibly the organization of the particles is in some way related to the formation and function of caveolae. On this point there is no direct evidence. Branton (1966) has suggested that the frequency of particles may be related to the physiological activity of the membrane. In addition, there is reason to believe that some of the particles have reactivities like blood groups (Marchesi and Andrews, 1971). Furthermore, the particles may migrate within the plane of the plasma membrane (Pinto da Silva and Branton, 1972). The recent description of 'cap' formation on reaction of multivalent antibodies with lymphocyte surface immunoglobulin implies an antigen-antibody migration, and evidence has been presented that cap formation is followed by pinocytosis (Taylor et al., 1971). Whether intramembranous particles, as a response to external stimuli, organize into rings and circular plaques and whether these rings and plaques are functionally related to pinocytosis are intriguing possibilities. The sequence of events could have analogies with the discharge of mucocysts in *Tetrahymena*, where intramembranous particles (150 Å in diameter) organize as rosettes. Specific rosettes fuse with individual mucocysts and cooperatively form a channel communicating with the exterior of the cell (Satir et al., 1972).

REFERENCES

BENEDETTI, E. L., and P. EMMELOT. 1968. *In* Ultrastructure in Biological Systems. A. J. Dalton and F. Haguenau, editors. Academic Press, Inc., New York. 4:33.

BRANTON, D. 1966. *Proc. Natl. Acad. Sci. U. S. A.* 55:1048.

BRANTON, D. 1971. *In* New Developments in Electron Microscopy. H. E. Huxley and A. Klug, editors. The Royal Society, London. 133.

BRUNS, R. R., and G. E. PALADE. 1968. *J. Cell Biol.* 37:244.

MARCHESI, V. T., and E. P. ANDREWS. 1971. *Science (Wash. D. C.).* 174:1247.

MÜHLETHALER, K. 1972. *Chem. Phys. Lipids* 8:259.

PINTO DA SILVA, P., and D. BRANTON. 1972. *Chem. Phys. Lipids.* 8:265.

RYAN, J. W., and U. SMITH. 1971 a. *Trans. Assoc. Am. Physicians Phila.* 84:297.

RYAN, J. W., and U. SMITH. 1971 b. *Biochim. Biophys. Acta.* 249:177.

RYAN, J. W., U. SMITH, and R. S. NIEMEYER. 1972 a. *Science (Wash. D. C.).* 176:64.

RYAN, J. W., R. S. NIEMEYER, and D. W. GOODWIN. 1972 b. *Adv. Exp. Med. Biol.* 21:259.

SATIR, B., C. SCHOOLEY, and P. SATIR. 1972. *Nature (Lond.).* 235:53.

SMITH, D. S., and H. C. ALDRICH. 1971. *Tissue Cell.* 3:261.

SMITH, U., and J. W. RYAN. 1970. *Adv. Exp. Med. Biol.* 8:249.

SMITH, U., and J. W. RYAN. 1971. *Chest.* 59:12S.

SMITH, U., and J. W. RYAN. 1972 a. *Adv. Exp. Med. Biol.* 21:267.

SMITH, U., and J. W. RYAN. 1972 b. *Tissue Cell.* 4:49.

TAYLOR, R. B., W. P. H. DUFFUS, M. C. RAFF, and S. DEPETRIS. 1971. *Nat. New Biol.* 233:225.