FREEZE-FRACTURE STUDIES OF THE DEVELOPING CELL SURFACE

II. Particle-Free Membrane Blisters on Glutaraldehyde-Fixed Corneal Fibroblasts Are Artefacts

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ABSTRACT

We describe, in sections and by freeze-fracture, four classes of intramembrane particle (IMP)-free membrane blebs or "blisters" associated with glutaraldehyde-fixed embryonic corneal fibroblasts: (*a*) Single blisters attached to the cell membrane; (*b*) free (detached) vesicles; (*c*) myelin figures; (*d*) multivesicular protrusions which resemble the "mounds" described by others on nerve growth cones. The IMP-free, membrane-bounded blisters contain no ground cytoplasm or organelles, in contrast to blebs on trypsin-isolated fibroblasts, which we show here do contain cytoplasm and IMP-rich membranes. That the IMP-free membrane blisters in embryonic corneas are artefacts of fixation is demonstrated by (*a*) their absence in replicas of fibroblasts frozen and fractured without prior aldehyde fixation and (*b*) their absence in sections of fibroblasts fixed in a combination of glutaraldehyde and osmium tetroxide. We suggest that the addition of osmium prevents postfixation movement of membrane lipids, especially the negatively charged "fluid" lipids which others have shown are capable of considerable mobility after aldehyde fixation alone. Recent literature has implicated membrane blistering in secretory processes and in growth of nerves, but before the functional significance of such IMP-free blisters is assessed, membrane mobility of the type shown here should be taken into consideration.

KEY WORDS freeze-fracture · particle-free blebs · cell surface · membrane structure · glutaraldehyde fixation · developing avian cornea · membrane blisters

In a recent freeze-fracture study of the changing structure of the avian corneal fibroblast plasmalemma during differentiation (14), we observed numerous cell-associated and extracellular membranous structures that were free of intramembrane particles (IMP). In this report, we show that these IMP-free structures correspond to the cytoplasm-free blebs and vesicles commonly seen in thin sections of glutaraldehyde-fixed tissues and thought to be artefacts of aldehyde fixation (4, 27, 38). Since we find that trypsin-induced cytoplasmic blebs are bounded by a membrane that contains IMP, we will use the name "blister," sug-

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gested by McIntyre et al., (25) instead of "bleb" for the IMP-free structure that is the main subject of this paper, in order that the distinction be clear.

It is especially important at this time to raise the question of the possible artefactual nature of IMP-free blisters viewed by freeze-fracture because recent freeze-fracture studies of aldehyde-fixed cells have attached physiological significance to similar vesicular and multivesicular structures on the cell surface (8, 23, 31, 35). In order to explore the possibility that these structures arise artefactually after aldehyde fixation in the embryonic cornea, we examined replicas of freeze-fractures of unfixed tissue and thin sections of tissue fixed simultaneously in glutaraldehyde and OsO_4 instead of glutaraldehyde followed by OsO_4. The results have implications for our understanding of lipid mobility in membranes.

MATERIALS AND METHODS

Corneas that were used in this study were ~200 μm thick. They were removed from White Leghorn chicken embryos (SPAFAS, Inc., Norwich, Conn.), cut into pie shaped pieces 1 mm thick at the base, and fixed by immersion by one of the two following methods: (*a*) The first fixative (19) consisted of formaldehyde (1% from paraformaldehyde) and glutaraldehyde (2.5%) in a sodium cacodylate buffer (0.1 M, pH 7.4, 0.01 M $CaCl_2$) as the primary fixative. Tissue was fixed for 30–60 min at room temperature and was then washed several times in the same buffer, with or without calcium. Tissues to be used for thin sections were fixed secondarily in 1.5% OsO_4 for 1 h at 4°C, while tissues which would subsequently be used for freeze-fracture were glycerinated (20% glycerol, 0.1 M cacodylate buffer, 0.4% $CaCl_2$, pH 7.4) for 1 h at room temperature. (*b*) Another fixative solution (adapted from references 16, 20, 29, and 40) consisted of a 1:1 mixture of a 1% solution of OsO_4 containing 7.5 mg of potassium ferricyanide/ml and a 5% solution of glutaraldehyde in cacodylate buffer (0.2 M, 0.01 M $CaCl_2$, pH 7.4). These two solutions were mixed immediately before adding to the tissues. Freshly prepared osmium-glutaraldehyde solution was added to the tissue every 10 min, an important step that needs to be adapted for tissue size and temperature. The tissue was fixed for 30–60 min and was then washed several times in buffer, all at 25°C. These tissues were not fixed secondarily in OsO_4. Uniformly fixed blocks were selected by light microscopy for thin sectioning. We also tried adding ferrocyanide (20) to the glutaraldehyde-osmium mixture, with variable results.

The tissues used for thin sections were stained en bloc with 1% uranyl acetate, dehydrated, and embedded in Epon or Araldite. Sections were stained with lead citrate. An area 0.2 × 0.5 mm from three blocks of each preparative method was examined *in toto* (except for grid bars) by transmission electron microscopy. No significant differences in blister formation were noted between center and periphery of tissue.

It should be emphasized that the osmolality of the fixative carrier was the same in all experiments (0.1 M cacodylate, 300 mosM) and thus was not the variable involved in eliminating cytoplasm-free membrane blebs and vesicles (see reference 2). Moreover, omission of calcium from the fixative did not prevent blister formation.

Tissues to be used for freeze-fracture were frozen in liquid nitrogen-cooled Freon 22 either immediately after dissection from the chicken embryos (unfixed), or after fixation with or without subsequent glycerination (14). The tissue was fractured in a Balzers apparatus (Balzers High Vacuum Corp., Santa Ana, Calif.) at −115°C. The platinum-carbon replicas were left in 100% methanol overnight and then cleaned by floating over a commercial bleach. Replicas and sections were examined in a JEOL 100-B electron microscope.

Fibroblasts which were to be examined after enzymatic dissociation procedures were isolated from 14-day corneas by four sequential 30-min incubation periods in trypsin-collagenase (1 mg/ml in Ca^{++}- and Mg^{++}-free Hanks's solution). Enzyme solution was made up fresh and stored at 4°C until immediately before use. Cell pellets were collected by low-speed centrifugation. The trypsin-collagenase solution was removed from each pellet and was replaced by culture medium containing fetal calf serum (10%). The isolated cells were fixed for 15–20 min in formaldehyde-glutaraldehyde (*a*, above). Cell pellets were prepared for both thin sectioning and freeze-fracturing as described above.

RESULTS

Freeze-fracture replicas of the embryonic avian corneal stroma reveal the elongated, irregularly shaped fibroblasts within their collagenous extracellular matrix. Numerous filopodia (*f*, Fig. 1) and larger cell processes (*cp*, Fig. 1) extend from the cell body into the extracellular space. There are depressions on the P face of the fibroblasts (*p*, Fig. 1) that may represent exo- or endocytotic phenomena. Both P and E fracture faces of the developing corneal fibroblast plasmalemma are rich in IMP, which increase in number as differentiation proceeds (14).

Appearance of Blisters in Freeze-Fracture Replicas and in Sections

During all stages of development, IMP-free membrane blisters were so common that, in most of our replicas, it was difficult to find a grid square that did not contain at least one or two such

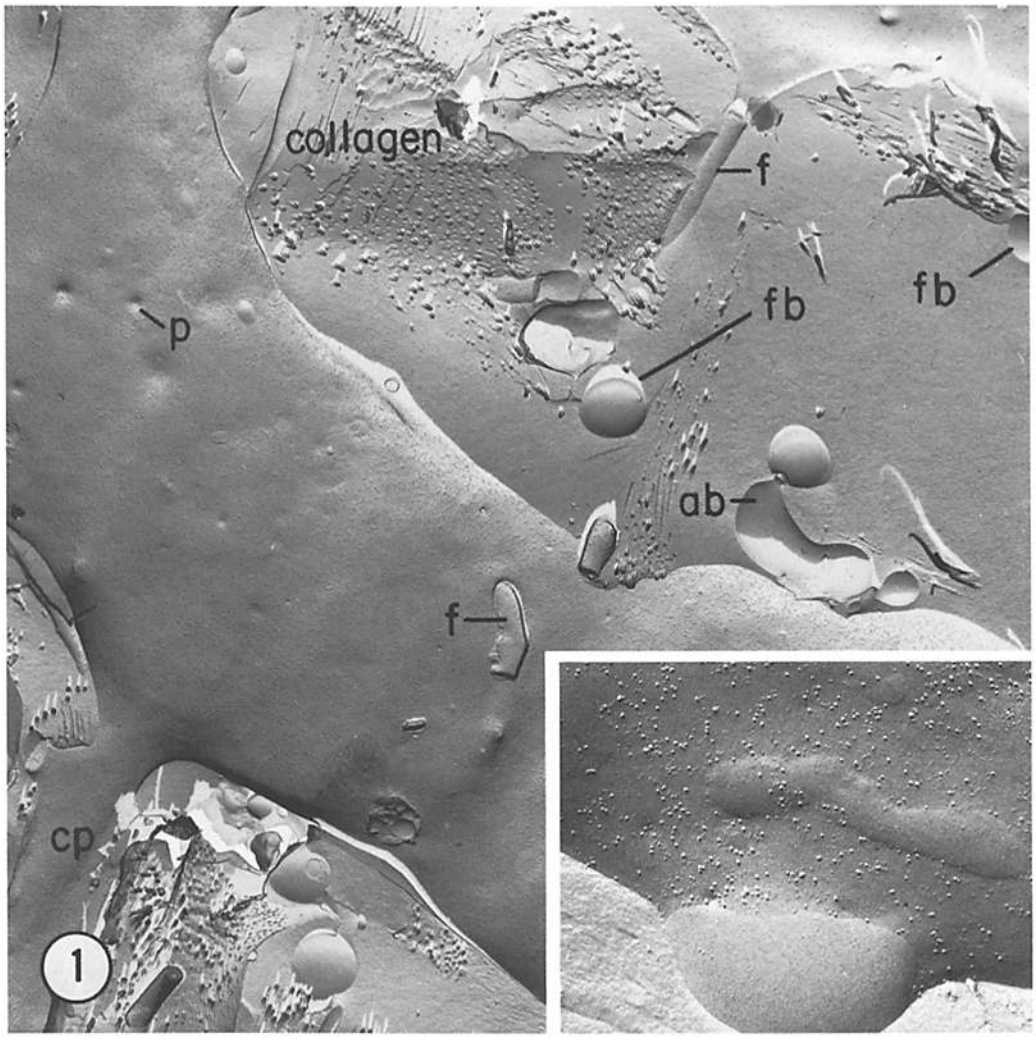

FIGURE 1 An electron micrograph of a replica of a freeze-fracture through the corneal stroma of a 14-day-old embryo fixed in glutaraldehyde before freezing. Numerous IMP-free blisters float free in the extracellular space (*fb*). One is seemingly attached to the fibroblast (*ab*). The P face of the plasmalemma contains pits (*p*) which probably represent exocytotic or endocytotic events. The *inset* shows at higher magnification the P face of an attached blister; it is virtually particle-free. × 8,300. *Inset*, × 57,800.

structures (Fig. 1). This was true whether or not the tissue was glycerinated. IMP-free surface membrane protrusions took several configurations in freeze-fracture replicas, each corresponding to similar structures in section.

Many of the blisters are smooth, round structures floating free, or seemingly so, in the extracellular space, apparently not connected to the fibroblasts (Fig. 1). Both P and E fracture faces are virtually devoid of the typical IMP. The walls of such vesicles seem to be bilayers of lipid, because their fracture faces are extremely smooth and resistant to etching and because they have a trilaminar bounding membrane in sections.

In thin sections, the unattached or "free" blisters (*fb*, Fig. 2) can be seen to correspond to the empty, membrane-bounded structures that are present in embryonic corneas after glutaraldehyde fixation. Free blisters, in either freeze-fracture replicas or thin sections, measure anywhere from several hundred to several thousand nanometers in diameter and are usually quite round, though

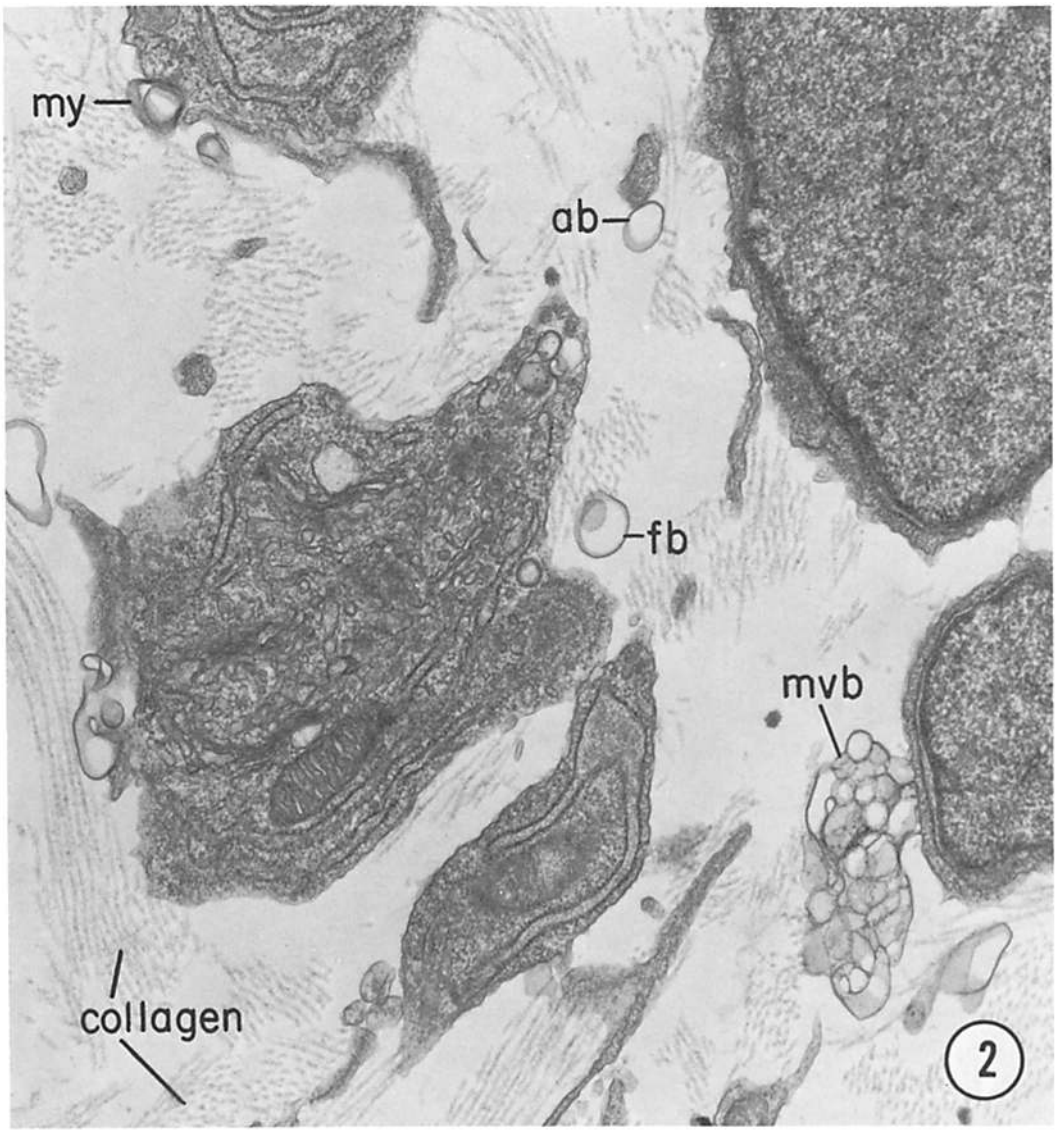

FIGURE 2 An electron micrograph of a section through the corneal stroma of a 14-day-old embryo fixed in glutaraldehyde followed by osmium. The membrane blisters seen in thin sections after aldehyde fixation correspond to those visualized in freeze-fractures. Free blisters (*fb*) and attached blisters (*ab*) are the most numerous, but myelin figures (*my*) and multivesicular blisters (*mvb*) may also be present. × 24,000.

they can be somewhat irregular.

In some sections, blisters with the same structure as the so-called free blisters appear to be connected to the fibroblast plasmalemma (*ab*, Fig. 2). As observed by freeze-fracture, these smooth, round structures attached to the fibroblast surface are also seen to be IMP-poor (*inset*, Fig. 1). The continuity of the blister surface with the plasmalemma again indicates that the blisters are membranous structures (Figs. 1–3). Although the plasmalemma and blister membranes are drastically different in terms of IMP content, both appear to have the same trilaminar structure in thin sections.

There are several more elaborate, but less common, kinds of membrane blisters, each of which seems to involve protrusion of surface membrane and loss of IMP. One type is composed of multiple bilayers which give the terraced, onion-

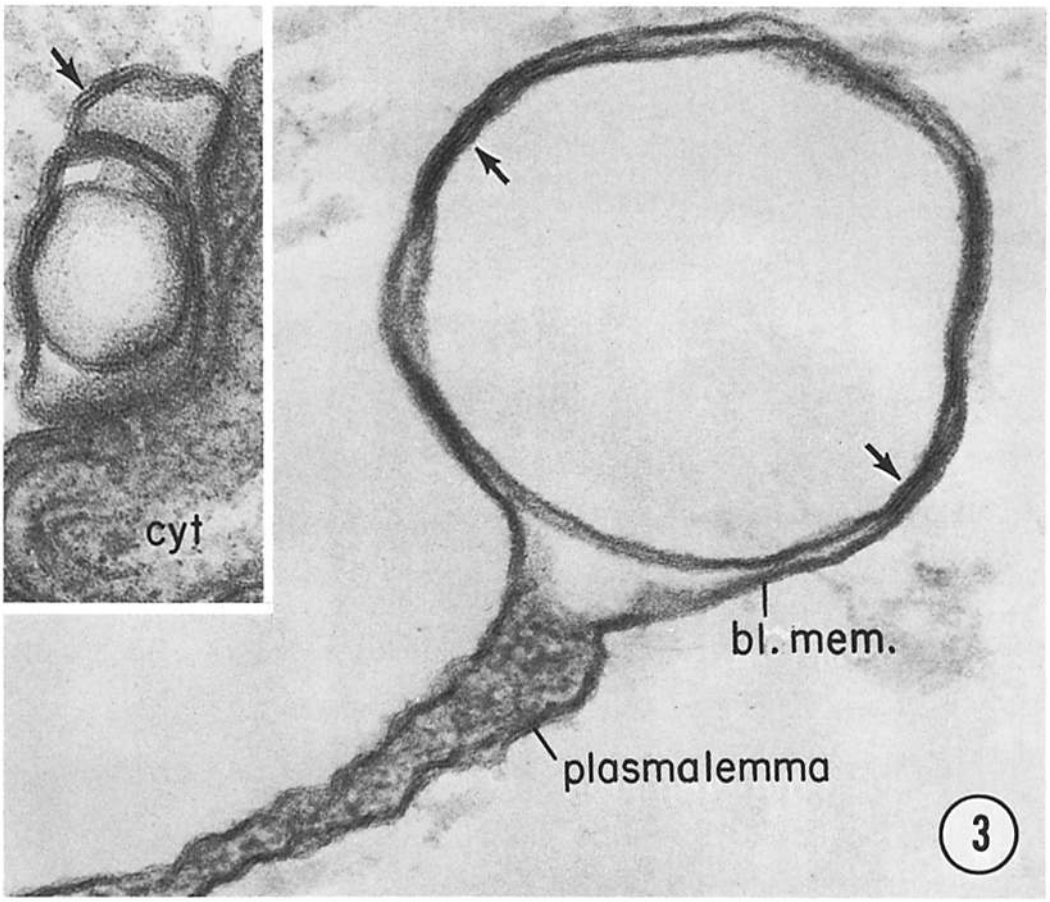

FIGURE 3 An electron micrograph of a thin section showing a fibroblast process in a 14-day-old cornea fixed in aldehyde followed by osmium. The membrane of the blister forming at the tip of this cell process (*bl. mem.*) is continuous with the plasmalemma of the fibroblast. At some points (arrows, see *inset* also), the membranes of the blister are closely adherent. These blisters could be called early myelin figures. *cyt*, cytoplasm. × 133,700. *Inset*, × 136,000.

like appearance of myelin in freeze-fracture replicas (Figs. 4 and 5). These blisters look somewhat like typical myelin figures in sections, but, in contrast to true myelin (30, 33), the period line in these myelin figures does not alternate in density as viewed in sections, and these structures have virtually no IMP. Transitions from simple blisters to myelin figures occur (Fig. 3). The tendency of the blister membranes to adhere to one another may be due to the fact that, except for an occasional particle (Fig. 5), they are virtually IMP-free, a condition which might reduce their repulsive charge.

Another type of blister is multivesiculated. Large membrane-bounded sacs occur that in thin sections are seen to contain numerous, smaller (100–200 nm in diameter), empty-appearing vesicles (*mvb*, Fig. 2; Figs. 6 and 7). What may be early stages in the formation of these multivesiculated blisters appear in freeze-fracture replicas as groups of round IMP-poor membrane protrusions (100–200 nm in diameter) on the P face of the fibroblast plasmalemma (Fig. 8). The IMP which, presumably, are left behind on the plasmalemma are usually not concentrated around the bases of the IMP-free protrusions. Occasionally, blisters seem to be surrounded by pits or small inward protrusions (arrows, Fig. 9) that are similar to those surrounding the mounds observed in nerve growth cones (31).

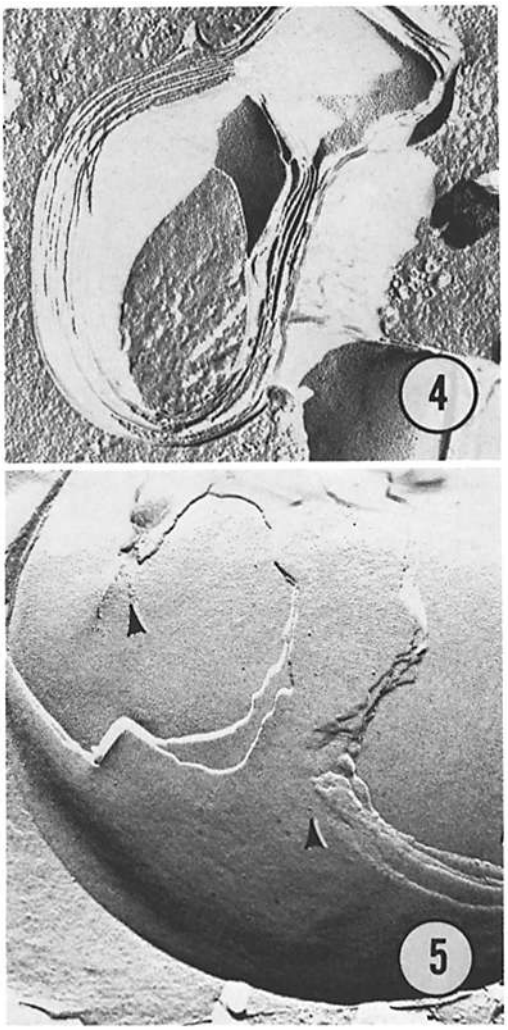

FIGURE 4 Electron micrograph of a freeze-fracture replica showing a myelin figure from a 9-day-old cornea fixed in glutaraldehyde before freezing. The multilamellar organization of the myelin figure is evident. × 60,300.

FIGURE 5 Electron micrograph of a tangential fracture of a myelin figure in a 9-day-old cornea fixed in glutaraldehyde before freezing. The membranes are virtually devoid of IMP (arrowheads). × 52,000.

Evidence That Blisters are Artefacts Produced during or after Glutaraldehyde Fixation

The membrane protrusions described above have been suspected to be artefactual, at least as viewed in sections (4, 27, 38). Now we are able to present two lines of evidence which add considerable weight to this argument, at least for our material. The first line of evidence comes from study of freeze-fracture replicas of unfixed corneas. Since the unfixed tissue must be frozen without cryoprotectants, freezing damage results in irregular membranes, clumped IMP and eutectic artefact in the extracellular spaces. However, we have not observed any of the IMP-free blisters (Fig. 10), such as are seen when aldehyde fixation is used before freezing the tissue (Fig. 1). In scanning several grid squares, one might observe an elevated, IMP-free area which could be interpreted as a very early stage in blister formation, but these structures were rare and could have been caused by ice crystal formation. In any case, we never observed any of the large, IMP-free blisters which are so conspicuous after aldehyde fixation. At the very least, there is a drastic reduction in blisters when unfixed tissue is observed with freeze-fracture.

A second line of evidence that blisters are artefactual comes from adding OsO_4 to the glutaraldehyde used as the initial fixative. This fixative (16, 29, 40) was employed in the hope that both lipids and protein would be stabilized simultaneously and that blisters, if they form artefactually during aldehyde fixation, would not develop. Examination of glutaraldehyde-OsO_4-fixed tissues reveals that blisters are virtually eliminated. Cell membranes are well preserved, and we have seen no myelin figures or multivesiculated mounds and only rare examples of very small unilamellar blisters in any of the sections we have inspected, whereas after the ordinary glutaraldehyde fixation blisters are common in every field (Fig. 2). Extracellular matrix and cytoplasmic components also are usually well preserved by this fixative (Fig. 11).

Lack of Relationship to Fibroblast Age, IMP Concentration, and Matrix Differentiation

Membrane blisters such as those described in this paper have seemed in the past to be particularly common in sectioned embryonic tissue. To determine whether the occurrence of these artefacts decreases as the plasmalemma matures, we compared aldehyde-fixed corneal fibroblasts at 19 days of development with those at 9 and 15 days of development. At 19 days, the morphogenesis of the cornea and cytodifferentiation of the fibro-

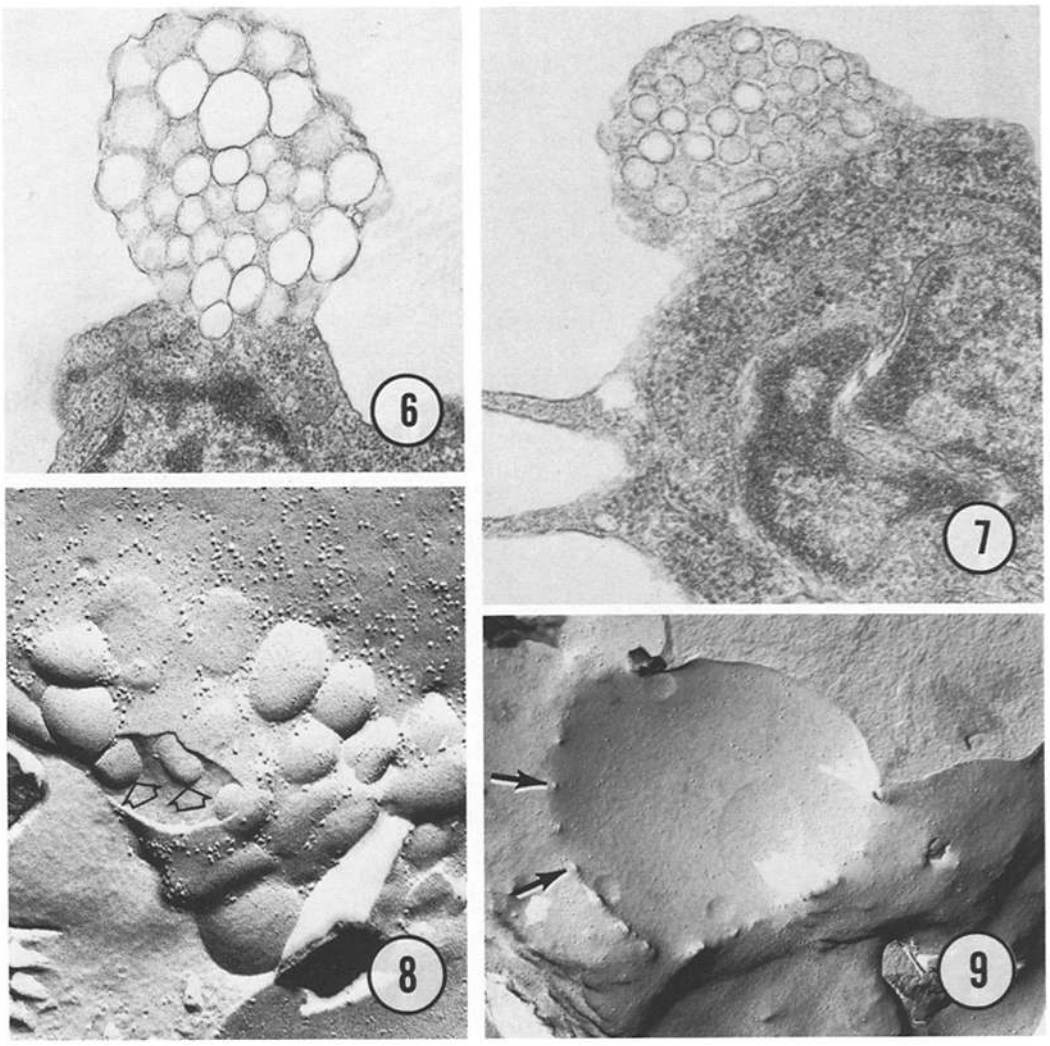

FIGURE 6 Electron micrograph of a thin section of a 14-day-old cornea fixed in glutaraldehyde followed by osmium, showing a multivesicular bleb. × 27,000.

FIGURE 7 Electron micrograph showing a multivesicular blister in a thin section of a fibroblast fixed in aldehyde followed by osmium. Several cell processes are nearby. × 43,500.

FIGURE 8 Freeze-fracture replica of aldehyde-fixed cornea demonstrating what is probably an early stage in the development of a multivesicular blister. Several IMP-free cytoplasmic vesicles appear beneath the plasmalemma (open arrows). × 72,000.

FIGURE 9 Electron micrograph of a 14-day-old corneal fibroblast fixed in aldehyde and freeze-fractured. Pits (arrows) surrounding the base of this elevation are quite similar to pits reported around the bases of "mounds." × 21,400.

blasts are nearly completed. The fibroblasts are tightly embedded between the now compacted collagenous lamellae, and their plasmalemmas contain a greater concentration of IMP than at 15 days (14). Examination of fibroblasts in 19-day corneas fixed in aldehyde followed by osmium reveals that blisters still occur. Thus, neither the increased collagenous matrix nor the increased

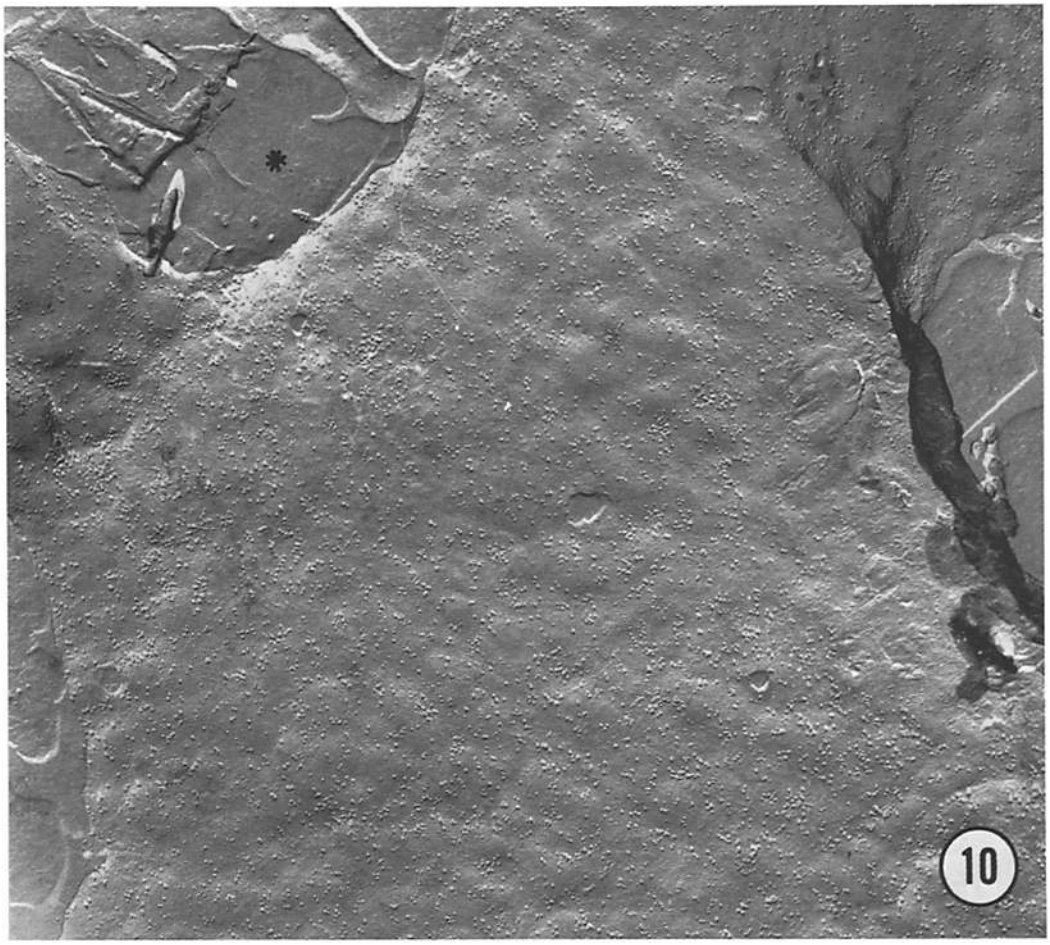

FIGURE 10 Electron micrograph of a freeze-fracture replica of an unfixed 9-day-old fibroblast. The surface is uneven and some IMP clumping occurs, but no blisters are observed. Freezing damage caused by ice crystals (*) may distort cellular structure in unfixed preparations. × 39,100.

IMP concentration of the plasmalemma afford it sufficient stability to prevent membrane mobility during or after fixation.

Trypsin-Induced Blebs

The dramatic blebbing of cell surfaces that occurs upon trypsinization of cells and tissues is a process that has received some attention in the past (11). Unlike the empty and the vesicle-filled blisters described here, the projections formed during trypsinization contain cytoplasmic components, such as ground cytoplasm, free ribosomes and small segments of rough endoplasmic reticulum (*inset*, Fig. 12). In freeze-fracture replicas, the plasma membrane over most of these cytoplasm-filled projections is seen to have the usual complement of IMP (Fig. 12), unlike the particle-free membrane of the aldehyde-induced blisters (Fig. 1).

DISCUSSION

In this paper we have described the thin-section and freeze-fracture appearance of membrane protrusions we call "blisters" (25), which occur on corneal fibroblasts fixed with standard aldehydes. Evidence is presented that these membrane blisters and the extracellular vesicles derived from them are artefacts of aldehyde fixation. Although they are very common after primary fixation in aldehydes, the blisters and vesicles are virtually

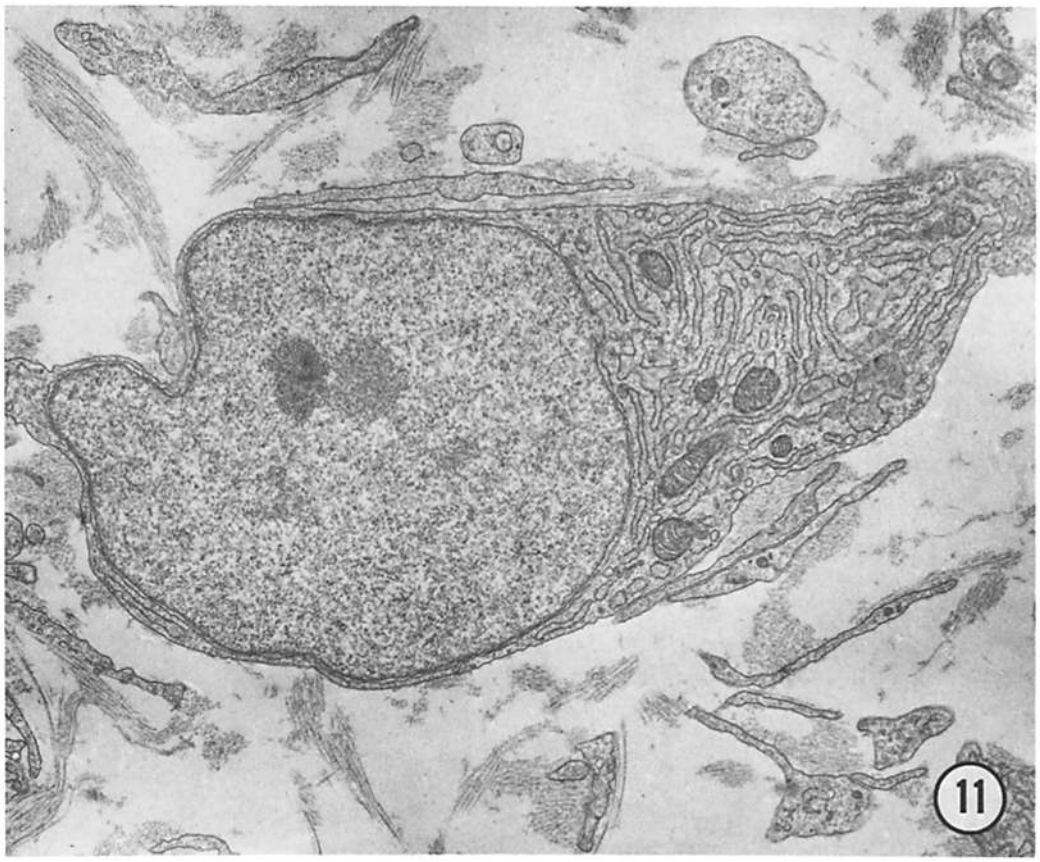

FIGURE 11 An electron micrograph of a thin section demonstrating the effect of fixation of the cornea with a combined glutaraldehyde-OsO_4 mixture. There are no membranous blisters in preparations treated with osmium before or with aldehyde, although in some areas (not shown) cell processes appear exploded. 14 day old. × 10,000.

absent in freeze-fracture replicas of corneal fibroblasts that are frozen without aldehyde fixation and in sections of corneas that are fixed with a combined glutaraldehyde-OsO_4 mixture. These artefacts offer dramatic proof of the fluidity of the lipid bilayer of the plasmalemma and of the restriction of movement of intramembrane particles. They also draw attention to one of the major alterations in cellular ultrastructure that can take place during or after aldehyde fixation and urge caution in the interpretation of blisters and vesicles that appear on the surfaces of cells seemingly as a result of a particular treatment (6, 23, 31, 35, 41).

The blisters, vesicles, and multivesicular structures under discussion here do not contain ground cytoplasm or filaments and so can be distinguished from the so-called "blebs" that arise on enzyme-isolated cells and on mitotic cells in culture (3, 11). On enzyme-isolated cells, such blebs may arise as a result of removal of peripheral membrane proteins. Manual removal of extracellular matrix in preparation of tissue for scanning electron microscopy produces similar blebs (3). Blebs of this general type (short, round cell processes containing cytoplasm) have also been reported on the free surface of invaginating embryonic epithelia (12). We show here that the membranes of trypsin-induced blebs contain IMP and it seems reasonable to predict that other cytoplasm-rich blebs will also contain IMP in their bounding membranes. In tissues prepared for scanning electron microscopy, of course, cytoplasmic blebs cannot be readily distinguished from membrane blisters unless the material is also sectioned. Shelton and Mowczko (38) recently called attention to

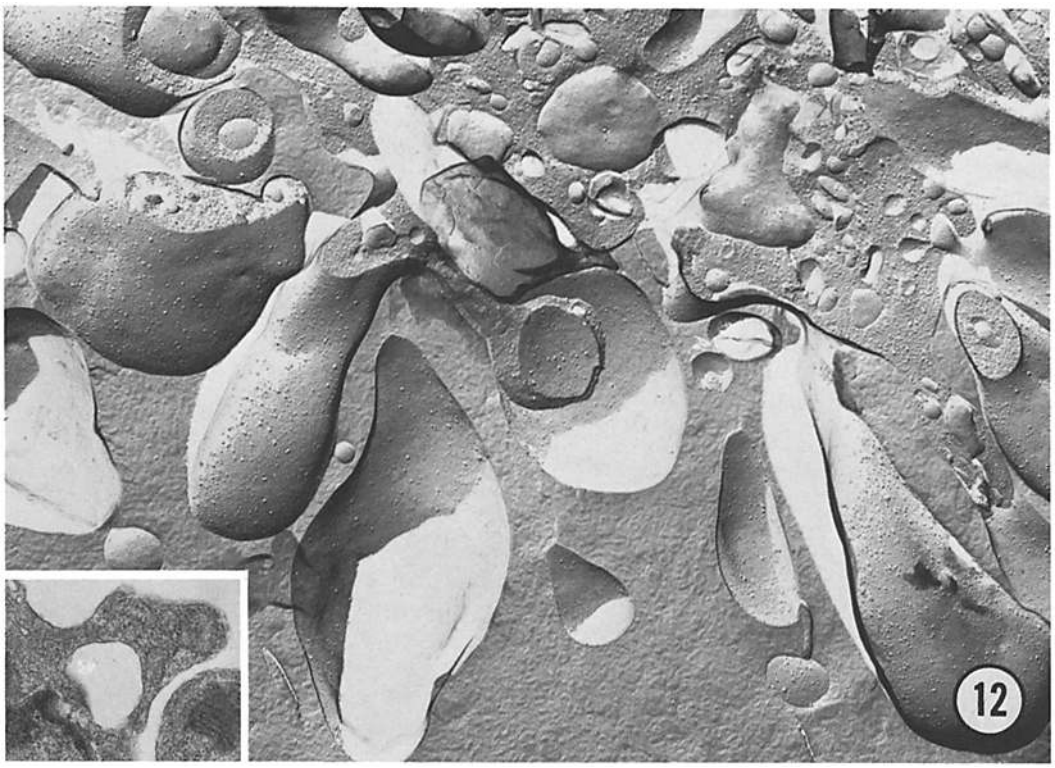

FIGURE 12 Electron micrograph of a freeze-fracture replica of a 14-day-old corneal fibroblast isolated by trypsin-collagenase digestion. Many bulbous projections extend from the cell body, but these blebs contain the usual complement of IMP. In thin-section (*inset*), trypsin-induced blebs can be seen to contain cytoplasmic matrix and organelles. × 25,000. *Inset*, × 19,500.

cell surface blebs viewed by scanning electron microscopy, which they concluded were artefacts because the membrane-bounded blebs seemed empty in thin sections of the same material.

Elgsaeter and Branton (9) have studied the formation of protein-free lipid vesicles from erythrocyte plasmalemmas and have suggested a possible mechanism of formation of IMP-free membranes that should be considered here. If a cytoplasmic protein such as spectrin is attached to IMP, aggregation of this protein could cause IMP-free lipid vesicles to bleb off the membrane by exerting a tangential pressure on the phospholipid component (9). Possibly, the blistered membrane we see here originally contained IMP that were attached to a cytoplasmic protein which contracted during aldehyde fixation. However, Elgsaeter et al. (10) illustrate IMP aggregation around developing membrane vesicles of erythrocytes, and "collars" of particles have occasionally been noted around the base of the particle-free bulges on mast cells (8, 23), though not around dimethyl sulfoxide (DMSO)-induced blisters on lymphocytes (25). We did not observe prominent collars of IMP at the base of the blisters seen here, but this does not rule out the protein aggregation theory.

Another mechanism of formation of IMP-free vesicles can be suggested, however, that might better explain mounds and myelin figures of the type described here as well as the single-layered blisters. It is well known that pure phospholipid can associate into myelin figures resembling intracellular membranes arranged as single-layered and multilayered vesicles (38). During glutaraldehyde fixation, lipids in the cytoplasm as well as in the plasmalemma that are not cross-linked by the aldehyde may be free to move around to form not only single and multilayered vesicles, but also multivesicular mounds. Possibly, this occurs because the lipid molecules in the membrane are in continual flow (5), in such a way that aldehyde-

immobilization of protein and certain lipids (18, 34) without concurrent osmium fixation of remaining lipid may in itself be sufficient to cause both intracytoplasmic vesiculation (mounds) and blebbing into the extracellular space (1, 2, 3, 39). Poste and Papahadjopoulos (34) report that negatively charged vesicles composed of "fluid" phospholipids (phosphatidylserine/phosphatidylcholine) can fuse with cells that have been fixed in glutaraldehyde and aldehyde fixation does not impair the ability of Sendai virus to fuse cells (28, 39). Also, labeled lipid can be transferred between fixed cells.[1] Thus, certain components of the lipid bilayer are still able to move and mingle dramatically even after aldehyde fixation. Presumably, it is such lipids that are involved in the membrane alterations demonstrated in the present study.

Membrane mobility after aldehyde fixation could seriously affect the interpretation of certain recent observations on cell secretion and growth. Lawson et al. (23) speculate that the particle-free blebs they see on stimulated mast cells are the result of fusion of intracellular granules with the plasma membrane and they argue that this fusion is a natural intermediate leading to degranulation (see also Chi et al., reference 8). Lawson et al. (23) suggest that specific fixation conditions might be required for the induction or preservation of such blebs, but they did not investigate the question. Interestingly, Schneeberger et al. (37) observed blebs and myelin figures on aldehyde-fixed mitochondria from fibroblasts fed an excess of polyunsaturated acids but not on normal fibroblasts. These membrane peculiarities were prevented from forming by osmium or simultaneous osmium-aldehyde fixation.

Pfenninger and Bunge (31) and Pfenninger and Maylié-Pfenninger (32) have suggested that the empty, particle-free membrane vesicles they observed in mounds beneath the plasmalemma of nerve growth cones in vitro may be the source of new membrane for these growing cell processes. Wessells et al. (41) observed that cationic ferritin does not bind to such mounds, which might be expected if their bounding membrane is protein-poor, whether that be an artefact or not. Bunge (7) found that peroxidase and ferritin are incorporated into vesicles within the mound, provided these tracers are present in sufficient concentration around the growth cone at the time of fixation. She reported that these mounds occur in areas otherwise displaying filopodia, and she suggested that they might take up extracellular tracer whenever filopodia retract during life. On the other hand, the mounds on growth cones could arise during or after fixation, as appears to be the case for corneal fibroblasts, and it would still be possible to explain the labeling observed by Bunge (7).

One could argue that even if multivesiculated mounds and other forms of blisters are an artefact of aldehyde fixation, they are telling us something meaningful about the stability and structure of cell membranes, as seems to be the case for the lipid-altered fibroblast mitochondria described by Schneeberger et al. (37). The tendency to form mounds characterizes certain parts of the nerve growth cone (6, 7, 31). We found no correlation in this study between membrane blistering and the stage of corneal development. We also compared fixation with and without calcium (13, 23), but found no correlation with the presence or absence of blisters.

No single method of morphological preservation is completely adequate, but it should be possible to answer questions such as these by manipulating the method of fixation or, in the case of freeze-fracture, by examining unfixed tissues. Simultaneous osmium-aldehyde fixation (16, 22, 29, 40) for cell membranes has the advantage, although tricky to use, that osmium interacts well with lipid and glutaraldehyde with protein. Glutaraldehyde alone seems to cross-link membrane proteins possibly via lysine (17), without reducing the fluidity of the lipid bilayer (18). Most neutral lipids are lost after glutaraldehyde fixation (reference 21; footnote one). OsO_4 seems to fix the relative positions of amphipathic proteins in membranes and to immobilize molecular motion in the lipid bilayer (18); it may build bridges between the aliphatic chains of lipids and the peptide bonds of certain membrane proteins (24, 26). It definitely interferes with membrane cleavage (26) and membrane fusion (34) and reduces postfixation loss of neutral lipids (21). Osmium alone (37, 38), osmium-aldehyde followed by aldehyde (22), and rapid freezing followed by freeze-substitution osmium fixation (15) would also be expected to reduce or eliminate membrane blisters, if the reasoning above is correct.

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