PARTICLES WITHIN MEMBRANES: A FREEZE-ETCH VIEW

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SUMMARY

Particles are commonly present on the membrane faces revealed by freeze-etching. The number, distribution and size of these particles vary considerably both between different membranes and, in many cases, between the 2 fracture faces found in individual membranes. Many of the larger particles appear to be too large to fit totally within smooth-surfaced membranes, so raising the question of how particles, especially the larger ones, are contained within membranes. This could be accomplished by a local reorganization of the membrane's internal structure such that small particles would be totally enclosed within smooth-surfaced membranes, while large particles would protrude from the membrane surface. Alternatively, all sizes of particles could be contained within membranes by a bulging of the 2 component lamellae such that protuberances, having a larger diameter than the underlying particles, would arise on the membrane surface.

Evidence is presented to show that in the case of specialized particles, which are located in rows around the base of flagella in the mollusc Cominella maculosa, protuberances are present on the membrane surface. However, it is possible that particles could be accommodated within membranes from other tissues by a different mechanism, and only further work will decide whether or not the present findings can be applied to these other membrane surfaces.

INTRODUCTION

The development of the freeze-etch technique by Moor & Mühlenthaler (1963) has allowed more direct observation of membranes than was possible by sectioning techniques. Recent studies (Chalcroft & Bullivant, 1970; Mühlenthaler, 1969; Sleytr, 1970; Steere, 1970; Wehrli, Mühlenthaler & Moor, 1970) of the complementary replicas obtained by fracturing specimens and replicating both fracture faces have all supported Branton's hypothesis (Branton, 1966) that membranes fracture internally during freeze-etching. Thus, in the conditions used during freeze-etching, membranes appear to have an internal region of weakness and so behave as bilaminar structures. Such a conclusion suggests that the particles which are observed on the membrane faces exposed during freeze-etching must in some way be located within membranes. Since the diameters of these particles appear to be comparable with the average thickness of membranes, any membrane model must make special provision to accommodate them. This may be in the form either of a local reorganization of the membrane's internal structure so that the particles can be enclosed within smooth-surfaced membranes (Branton, 1966; Wrigglesworth, Packer & Branton, 1970) or a bulging of the 2 component lamellae such that protuberances arise on the membrane surface.
Deep etching of freeze-etched specimens has been shown to reveal membrane surfaces (Branton, 1966, 1967) and so should allow one to differentiate between these two possibilities. However, it is probable that any surface bulging occurring over randomly arrayed particles would not be readily identifiable. Possibly the most favourable conditions for observing any protuberances on the surface of membranes would occur if a readily located and recognizable array of particles were present in a membrane, especially if the particles in this array were sufficiently well separated so that the surface bulging caused by individual particles did not merge. Such an array of particles was discovered during a freeze-etch investigation of flagellated cell surfaces, where readily recognizable and well separated rows of particles were found around the base of flagella. As these flagella could be surrounded by distilled water prior to freezing, it was believed that they would provide good subjects for deep etching and, because of the pattern of the particles, for observing any reorientation in the surface of the membrane.

MATERIALS AND METHODS

Whelks of the species *Cominella maculosa* were obtained locally and the egg-capsule gland excised from adult females. Small pieces of the gland containing areas of the flagellated luminal surface were dissected out and placed in either distilled water or 25% glycerol. The samples were rapidly frozen in Freon 12 at −150 °C and freeze-etching carried out on a Balzers BA500 apparatus as described by Moor & Mührlethaler (1963). The glycerol-infiltrated specimens were etched for 1 min at −100 °C, while those frozen in distilled water were etched at −100 °C for 4 min in order to remove between 50 and 100 nm of the surrounding ice.

RESULTS

The flagellated luminal surface of the egg-capsule gland is readily recognizable, and so could be quickly located when present in replicas. In glycerol-infiltrated specimens many particles are found on the membrane face exposed when the fracture plane passes over a flagellum so as to reveal an extracellular, or external, view of its membrane (e in Fig. 1). Most of these are randomly distributed, but near the base of each flagellum 3 or 4 regularly arranged wavy rows of particles can be identified, each particle being approximately 7 nm in diameter. The rows are separated from each other by about 30 nm.

In specimens infiltrated with water and deeply etched during the freeze-etch procedure the cytoplasm was badly damaged and little internal structure could be recognized (Fig. 2). However, most membranes and in particular flagellar membranes were readily recognizable after this treatment and did not appear to be badly damaged. In most of the replicas prepared in this way, the bases of flagella are surrounded by rows of particles (p in Fig. 2), identical with those identified on flagella in glycerol-infiltrated specimens. Often, however, the deep etching of these specimens has revealed on the flagella a new surface which can be recognized by its boundary ridge, r (Fig. 2). The shadows cast by the ridges show that these new surfaces stand above the surfaces revealed during normal freeze-etching. When this ridge is suitably
located (r in Fig. 3) the rows of particles can be seen on the lower-lying surface revealed by normal freeze-etching, while on the new upper-lying surface shallow domes of a larger diameter can be identified. Careful examination of a large number of such flagella has confirmed that the rows of particles on the lower-lying surface are continuous with these protuberances on the upper-lying surface, indicating that the protuberances are associated with the underlying particles. The protuberances can also be seen on flagella whose membranes have not been fractured in this basal region, but have subsequently been exposed by deep etching (f in Fig. 3).

**DISCUSSION**

As well as the membrane faces exposed by normal freeze-etching, 2 further features can be recognized on deeply etched flagellar membranes. These are a clearly defined step approximately 5 nm in height and a new surface which runs parallel to the face exposed during normal freeze-etching and terminates at the step.

This new surface is only present in large enough areas to be readily identifiable after deep etching when the surrounding water has been removed from around the flagella to a depth of 50–100 nm. Normal etching of glycerol-infiltrated specimens removes only a small amount of water from the water–glycerol mixture covering the flagella so that little, if any, of this new surface is usually revealed. As this new surface is external to the face revealed during freeze-etching, it is reasonable to interpret it as the true outer surface of the flagellar membrane. This is in agreement with the conclusions reached in other recent deep-etching studies (Branton, 1966; Meyer & Winkelmann, 1969; Tillack & Marchesi, 1970; Wrigglesworth et al. 1970). Thus it appears that the particles which are present on the membrane faces revealed by normal freeze-etching must be contained within the membranes.

When the true membrane surfaces of flagella, as revealed by deep etching, are examined (Figs. 2, 3) in the regions where rows of particles are present in normal freeze-etch replicas it can be seen that rows of large protuberances are present. The latter can be positively identified as being due to the underlying particles, since not only are they present in wavy lines matching those of the particles, but also in many instances the rows of particles can be seen to be continuous with the rows of protuberances across the step between the membrane fracture face and surface. Close examination of the protuberances suggests that they have a much larger diameter than the underlying particles and, therefore, that it is unlikely that they could represent the tips of particles protruding through the surface of flagellar membranes. The only satisfactory explanation of the protuberances appears to be that they are formed by the outer layers of the flagellar membrane, which are split away during normal freeze-etching, folding over the particles so as to enclose them totally within the membrane.

Little support for this conclusion is contained in other studies of membrane surfaces as revealed by deep etching (Branton, 1966, 1967; Meyer & Winkelmann, 1969; Park & Pfeifhofer, 1969; Pinto da Silva & Branton, 1970; Tillack & Marchesi, 1970; Wrigglesworth et al. 1970). This could indicate that particles are accommodated
within membranes in a number of different ways, depending on the type of membrane. However, the membrane systems used in these studies were probably not optimal for positively identifying surface changes over underlying particles, either because the membranes lacked regular arrays of particles or because the particles were too closely packed (Park & Pfeifhofer, 1969). Thus it would not be possible to identify positively any surface structure as being associated with an underlying particle if the particles were randomly arrayed within the membrane. Similarly, protuberances above a densely packed array of particles would tend to merge and, therefore, may not be readily recognizable, especially as the present investigation shows that protuberances have a much larger diameter than the underlying particles. Such an effect could explain the results obtained by Park & Pfeifhofer (1969) during a study of deeply etched thylakoid membranes. Although bulges were observed on the membrane surfaces overlying fracture faces which contained large particles, they were interpreted as representing the protrusion of the tops of the large particles through the membrane surface. It appears to be just as likely that the protuberances represent the outer layers of the thylakoid membranes folding over the particles. Unfortunately, the close apposition of the particles precludes any chance of differentiation between these 2 possibilities.

Thus the present investigation indicates that, in the basal regions of flagella, particles are accommodated within membranes by the external layers of the membranes folding over them and so giving rise to protuberances on the membrane surface. It also suggests possible reasons why this phenomenon has not been observed in previous deep-etch studies of membranes. Before any conclusion can be reached as to whether particles observed on freeze-etch fracture faces in other membrane systems are contained within membranes in this way, or whether it is unique to this system, other suitable membrane systems will have to be examined. Unfortunately, membrane systems containing readily recognizable arrays of particles in which the individual particles are well separated are not common. Most occur in junctional structures between cells, such as the septate junction (Flower, 1970; Gilula, Branton & Satir, 1970), gap junction (Chalcroft & Bullivant, 1970), and tight junction (Staehelin, Mukherjee & Williams, 1969). As these occur in situations where 2 cells appose each other they cannot be directly observed by deep etching.

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REFERENCES

Particles within membranes


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Fig. 1. Fracture through a flagellated epithelial cell illustrating the appearance of flagellar membrane faces after infiltration with 25% glycerol. Near the base of each flagellum rows of particles are present on the external membrane faces (e). \( \times 75,000 \).

Fig. 2. Epithelial cell infiltrated with distilled water before deep-etching. Rows of particles (p) can be identified on some flagella as after normal freeze-etching. Deep-etching has revealed 2 new features on many flagella: a new membrane surface external to the face revealed by normal freeze-etching, and a ridge (r) which delineates the transition between these 2 planes. \( \times 65,000 \).

Fig. 3. Deeply etched specimen in which the ridge (r) can be seen on several flagella. Protuberances are present on the true membrane surfaces in the region where rows of particles are present on the normal fracture face. These protuberances have a considerably larger diameter than the particles. That the protuberances are caused by the accommodation of particles within the membrane can be seen from the way that the wavy lines followed by the protuberances and particles are concurrent across the ridge. Protuberances can also be seen on flagella (f) whose membranes have not been fractured in the basal region but which have been revealed by deep-etching. \( \times 75,000 \).

Note. The encircled arrow in each micrograph indicates the shadowing direction.
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