FREEZE-FRACTURE REPLICATION OF ROUGH ENDOPLASMIC RETICULUM OF MOUSE LIVER CELLS

A. S. BREATNACH, C. STOLINSKI AND M. GROSS

Departments of Anatomy and Biophysics, St Mary's Hospital Medical School, London, W.2, England

SUMMARY

Fresh, chemically unfixed, glycerinated specimens of mouse liver were examined by the technique of freeze-fracture replication without sublimation (i.e. they were not 'etched'). Where extensive areas of fractured lamellar membranes of the rough endoplasmic reticulum are revealed en face, 2 types of fracture face are distinguishable. One of these fracture faces (A) is directed towards the cytoplasm, and the other (B) towards the cisternal cavity. A characteristic mosaic, or patchwork pattern of flat areas circumscribed by particles, is evident on both faces, and more clearly so on face B, due to a greater number of more prominent particles. Similar mosaic patterns are revealed on convex faces of the nuclear membrane, and on concave fracture faces of mitochondrial membranes, but are not evident on fracture faces of the plasma membrane.

Uncertainty in establishing the exact plane of fracture of membranes in this material, since glycerol is virtually non-sublimable, makes it difficult to assess the significance of these mosaic patterns. The fact that ribosomes are not identifiable on either face of fractured endoplasmic reticulum membranes, gives no certain indication of the plane of fracture.

INTRODUCTION

The technique of freeze-fracture replication, with or without sublimation ('freeze-etching'), is being employed with increasing frequency in the analysis of the functional morphology of biological membranes (Moor, 1966; Koehler, 1968; Branton, 1971). Several investigators (Bullivant, 1969; Orci, Matter & Rouiller, 1971) have described the general appearance of the rough endoplasmic reticulum in freeze-etch replicas of chemically fixed tissue, and the picture which has emerged is one of stacked membranes revealing 2 types of fracture face. One of these faces, relatively smooth, is directed towards the cytoplasm, and the other, which carries particles 10–15 nm high, is directed towards the cisternal space. Ribosomes have not been identified with certainty on either face. The present report describes regular patchwork-like or mosaic patterns of flat areas and particles on fracture faces of non-chemically fixed membranes of the rough endoplasmic reticulum of intact mouse liver cells. This type of pattern bears a certain, though not complete resemblance to patterns previously observed by Wrigglesworth, Packer & Branton (1970), on fractured membranes of mitochondria isolated from rat liver cells, and it is also similar to a pattern revealed on fracture faces of the nuclear membrane. Bullivant & Ames (1966) illustrated cytoplasmic membranes of a presumed Paneth cell which exhibited 'a granulated mosaic' pattern, but they did not specifically identify the membranes.
MATERIALS AND METHODS

Fresh, chemically unfixed pieces of mouse liver, approximately 10 mm³ in size, were immersed for 1 h in 20% glycerinated Ringer-Tyrode solution, and for a further 2 h in 25% glycerinated Tyrode at a temperature of 4°C. Tissue was then rapidly frozen in an isopentane bath to −160°C and transferred to a dewar vessel filled with liquid nitrogen. Specimens were then fractured in a freeze-fracture apparatus designed in this laboratory (by C.S.) and built into a standard Genevac coating unit. The fracture faces exposed were next shadowed with platinum carbon at 45° to the plane of fracture, and a further backing layer of carbon was evaporated normal to the surface. Sublimation or 'etching' was not performed since this is not really practicable with glycerinated non-chemically fixed, organized tissue.

Following replication, tissues were thawed in 30% glycerinated Ringer solution, and immersed in 2% glutaraldehyde for 1 h at room temperature. This last procedure has been found to lessen disintegration of the replica while the tissue is being digested away from it. Washing of specimens in distilled water followed, and they were then placed in 20% nitric acid at 20°C for 0.5 h, and in concentrated nitric acid overnight. This treatment usually resulted in complete digestion of the tissue, and replicas were finally placed in concentrated nitric acid at 60°C for 15 min, then washed 3 times in distilled water, from which they were picked up on uncoated copper grids. They were examined with a Philips EM 300 electron microscope fitted with a goniometer stage.

RESULTS

Rough endoplasmic reticulum

Stacked lamellae of the rough endoplasmic reticulum, fractured at different angles, present a characteristic and readily identifiable appearance in replicas (Figs. 1, 2). Where extensive areas of the lamellar membranes are revealed en face, 2 types of fracture face can be seen (Figs. 3, 4). One of these faces, which may be referred to as face A, is overlain by cytoplasm, and is frequently visualized as a layer which rests on top of another face, face B, the junction between the 2 being outlined by a step; fracture face B, in turn, covers the cytoplasm. Examination of a number of replicas, and consideration of appearances in thin sections, leads to the conclusion that the step includes the cistemal cavity. It would follow from this that fracture face A is seen as if one were looking into the cisterna from the cytoplasmic matrix, and fracture face B as if one were looking from within the cisterna towards the cytoplasm.

Both types of fracture face present a patchwork, or mosaic, pattern of flat areas circumscribed by rows of particles (Figs. 3, 4). This pattern is somewhat less evident, or clear-cut, on face A, because the particles here are less prominent, more variable in size, and fewer, than on face B. The flat areas on both faces vary somewhat in size, and also in shape, being either circular in outline or 5- or 6-sided. The manner of deposition of carbon–platinum grains on the flat areas indicates an underlying substructure, which causes the grains frequently to be arranged in parallel array. This feature is evident only at narrow angles of shadowing, and is just resolvable with present techniques (Fig. 4).

As can be seen in Fig. 3, rows of particles on face A carry over directly across the cisternal step into rows of particles on face B, so that particles and flat areas on both faces coincide. It can also be seen that the edge of the cisternal step, where the fracture plane leaves face A to reveal face B, frequently coincides with lines of particles on
Freeze-fracture of endoplasmic reticulum

both faces. This indicates, where face A at any rate is concerned, that cross-fracture
of the membrane tends to occur more easily, though not invariably, along the margin
of a flat area, rather than within it. No particles equivalent in size or arrangement to
ribosomes as seen in thin sections are evident on either fracture face.

Nuclear membranes

Similar mosaic patterns of particles and smooth areas are frequently seen on fracture
faces of the outer and inner nuclear membranes (Fig. 5). The convex face of the outer
membrane (Fig. 5) closely resembles the A-type face of the endoplasmic reticulum as
described above, in that the particles are not very prominent. Particles are quite
prominent, however, on the convex fracture face of the inner nuclear membrane, and
the overall mosaic pattern is particularly evident here. It is also evident, though not
as clearly, on the concave fracture face of the outer membrane, and barely evident, if
present at all, on the concave face of the inner membrane (Fig. 1).

Mitochondrial membranes

Wrigglesworth et al. (1970) described faces exposed during fracture of unfixed
isolated mitochondria, and similar fracture faces were observed in the present replicas.
One of these faces, which is concave (Figs. 5, 6), bears some resemblance to face B
of fractured endoplasmic reticulum (Fig. 3). It exhibits a similar pattern of flat areas
circumscribed by particles, though the mosaic arrangement may be obscured to a
varying extent by plaques lying above the plane of the particles (Fig. 6). The other type
of exposed mitochondrial face, which is convex (Figs. 5, 7), and evidently comple-
mentary to the above face, also exhibits plaques and particles.

DISCUSSION

Interpretation of features revealed in freeze-fracture replicas is beset by difficulty in
defining the exact path of the fracture plane in relation to membranes. This is at
present a matter of uncertainty. Some workers (e.g. Moor, 1966) have concluded that
the fracture passes along, or very close to, the true surfaces (outer and inner) of
membranes, while the majority (e.g. Branton, 1966, 1971; Scott McNutt & Weinstein,
1970) maintain that it invariably passes within them, thereby revealing internal or
'split' faces; still others attempt to describe replicas without paying any attention to
this very important point. 'Etching' of membranes following fracture (and before
replication), i.e. subliming away surrounding ice by raising the temperature, reveals an
additional face, which those who support the 'split' theory claim to be the true surface
of the membrane, never exposed in 'unetched' specimens. This 'etching' procedure
is only applicable to specimens suspended in water, such as isolated cells or organelles,
or to organized tissue pre-treated with chemical fixatives before freezing. It is difficult
to apply to non-chemically fixed organized tissue, such as that studied here which was
impregnated with glycerol in order to prevent damage during freezing, since glycerol
is virtually non-sublimable under the conditions obtaining. There is evidence
(Wrigglesworth et al. 1970) that whereas general morphological features of membranes
are preserved by chemical fixation, there is considerable loss of fine detail, and it also seems that sublimation per se can introduce artifact, the degree and nature of which is not yet fully documented (Moor, 1971; Staehelin & Bertaud, 1971). The truest picture of membrane fracture faces exposed, therefore, is probably seen on replicas of non-chemically fixed, unsublimated material, such as that studied here, but, in seeking to interpret the significance of the details presented, the difficulty of defining the plane of fracture must be borne in mind. As regards the membranes of the rough endoplasmic reticulum, there is some collateral evidence which might be relevant in this connexion.

The fact that fracture faces of mitochondrial membranes identical in appearance to those described by Wrigglesworth et al. (1970) are revealed in our equally non-chemically fixed material, indicates that the plane of fracture was the same in both instances. Exposure of an additional face by etching their isolated mitochondria led Wrigglesworth et al. (1970) to conclude that the fracture plane probably follows the interior of the membranes. The same, by analogy, might be thought to apply here, not only with regard to mitochondria but to the membranes of the rough endoplasmic reticulum as well. If this be the case, the particles revealed on the fracture faces would then represent components embedded within the membranes, rather than true surface elements. The fact that ribosomes are not evident on face A of the membrane (directed towards the cytoplasm) could further be taken to indicate that it is an internal 'split' face, rather than the true surface, though the possibility remains that it does, in fact, represent the latter, and that the ribosomes were removed from it with the tissue which was fractured away or at some other stage of the processing. One might say that this difficulty concerning the plane of fracture in intact organized tissues can only be solved by sublimation. This requires the employment of cryo-protective agents other than glycerol, which are sublimable, and experiments directed towards finding suitable ones are in hand.

The fact that the rows of particles revealed on fracture faces of the rough endoplasmic reticulum of the present material are not usually evident in chemically fixed material (Bullivant, 1969; Orci et al. 1971) can probably be attributed to the effects of this type of fixation, which, as already stated, is known to lead to some loss of fine detail. As regards the nature and significance of the particles, it is not possible to advance points for realistic discussion beyond those speculatively considered by Wrigglesworth et al. (1970) in connexion with mitochondria. It is of interest, however, to note that a similar type of mosaic pattern of particles and flat areas is associated with mitochondrial, rough reticular, and nuclear membranes. This certainly suggests the existence of common structural and/or functional components. Such a pattern is not evident on fracture faces of the plasma membrane of the liver cell or of other cells, and in our experience is also absent from Golgi membranes. This further suggests that these latter 2 membranes are structurally different or that the fracture proceeds in a different plane in relation to them. There is some evidence (Staehelin, 1968; Watson & Remsen, 1970; Koehler, 1970) that all membranes do not fracture in an identical manner.

There is one further point which merits brief discussion. This concerns the
observation that particles on both fracture faces of membranes of the rough endoplasmic reticulum lie opposite each other, and that the fracture when traversing the cisternal space frequently departs from particles on one face (face A) and meets the opposite face (face B) likewise at the site of particles. This suggests the existence of some sort of bridge connecting the opposing membranes across the cisternal space which influences or conditions the path of the fracture, and which may be related to the disposition of particles upon, or within the membranes. Orci et al. (1971) illustrate apparent ‘fusions’ of membranes enclosing cisternal spaces of rough endoplasmic reticulum in thin sections of rat liver, and it is possible that this provides some evidence for the existence of a bridging element.

We gratefully acknowledge the support of the Wellcome Trust, which provided the electron microscope used in this study. We are also grateful to the Peel Medical Trust, the Fitton Trust and the Joint Standing Research Committee of St Mary’s Hospital, for grants to purchase items of ancillary equipment.

REFERENCES


(Received 7 January 1972)
Fig. 1. Replica of mouse liver cell showing cross-fractured lamellae of the rough endoplasmic reticulum (er). *nu*, concave fracture face of nuclear membrane. Encircled arrow in this and succeeding micrographs indicates direction of platinum shadowing. $\times 52,700$.

Fig. 2. Replica of mouse liver cell showing *en face* view of a stack of fractured membranes of the rough endoplasmic reticulum (er) $\times 43,000$. 

A. S. Breathnach, C. Stolinski and M. Gross
Freeze-fracture of endoplasmic reticulum

1

2

nu

er

er
Fig. 3. Replica showing the 2 types of face revealed on fracture of membranes of the rough endoplasmic reticulum. A, fracture face directed towards the cytoplasm, cy; B, fracture face directed towards the cisternal cavity, included in the step s. Note mosaic pattern of particles circumscribing flat areas on both faces; the pattern is more evident on face B, due mainly to a greater number and prominence of particles. At various points it can be seen that rows of particles on face A are in line across the cisternal step with rows of particles on face B. Also, in some situations (f), the fracture plane has evidently passed from face A to face B at the site of a row of particles. × 66 500.

Fig. 4. Fracture faces of membranes of rough endoplasmic reticulum. In this higher-power micrograph the fact that particles on face B are taller or more prominent than those on face A is clearly evident. At the situations arrowed, it is just evident that carbon-platinum grains on flat areas are arranged in parallel array, suggesting an ordered underlying substructure. cy, cytoplasm; s, cisternal step. × 136 000.
Fig. 5. Replica showing convex fracture faces of the outer (ou) and inner (in) nuclear membranes. Note mosaic pattern of particles and flat areas on both faces. The pattern is more evident on the inner membrane due to the more prominent particles in this situation. Compare with Fig. 3, showing fracture faces of membranes of rough endoplasmic reticulum. Similar fracture faces (A, B) are also present in this micrograph. Note also, mosaic pattern evident on concave fracture faces of mitochondria (mcx). mcx, convex fracture face of mitochondrion (see also Fig. 7). × 39,000.
Fig. 6. Replica showing concave fracture face of mitochondrion, see. In this instance, the mosaic pattern is partially obscured by overlying plaques (compare Fig. 5). × 66500.

Fig. 7. Replica showing convex fracture face of mitochondrion, mex. g, fractured gap junction. × 66500.
Freeze-fracture of endoplasmic reticulum