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MEMBRANES

Freeze-etching techniques applied to biological membranes

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[Plates 1–5]

Basic freeze-etching methods are described.

When biological membranes are freeze-fractured the fracture plane is smooth, but interrupted to a greater or lesser extent by numbers of small (8.5 nm) particles. The evidence that the fracture occurs in the interior of the membrane and that the particles represent proteins within the membrane is reviewed.

A problem of interpretation of freeze-fracture replicas is that the two 'complementary' faces, produced by the fracture of a single membrane, do not match exactly. In particular, particles on one face are often not matched by corresponding depressions on the other. Work in the author's laboratory using the complementary replica technique is described. One conclusion from this work is that plastic deformation of the intra-membrane protein particles may occur, and that this may be responsible for the lack of small-scale complementarity.

Introduction

The end-product of the freeze-etching technique is a platinum–carbon shadowed replica of the fracture face produced in a frozen biological specimen. This replica has two advantages over specimens produced by other techniques. First, it can show the morphology of a cell which only differs from the living state by the changes brought about by rapid freezing. No chemical fixation need be done, and dehydration and embedding are not required. Secondly, and more importantly from the standpoint of an interest in membranes, the passage of the fracture through frozen tissue results in membrane splitting, thus revealing the interior of membranes. Because of this, the technique is unique in electron microscopy in that it reproducibly shows structures modifying the lipid bilayer. The freeze-etch image is compatible with the fluid mosaic model of Singer & Nicolson (1972), which proposes that biological membranes in general consist of a lipid bilayer with various proteins intercalated and floating in it.

In this paper I will briefly describe the freeze-etching method, review the evidence that it does reveal membrane interiors, and discuss the interpretation of these interior views. I will finally describe some experiments which throw light on the physical events which occur when membranes are fractured in the frozen state and the resulting faces are replicated by shadow-casting-in a vacuum.

Freeze-etching methods

As already stated, the aim of the technique is to produce a replica of the fracture face through frozen material. A brief description of the technique will be given here but for details the recent reviews of Koehler (1972) and Bullivant (1973) should be consulted. The technique was originated by Meryman & Kafig (1955) and Steere (1957). There are various kinds of equipment now available, and these fall into two main classes.

The first is an apparatus in which the cold specimen is kept at a fixed low temperature (usually – 100 °C), its surface is cut with a liquid-nitrogen cooled knife in a vacuum, and then

replicated by shadowing with platinum-carbon and backing with carbon (Moor, Mühlethaler, Waldner & Frey-Wyssling 1961; Steere 1966, 1969). If the specimen is left for a short while between the completion of the cutting and the beginning of replication, then ice sublimes from the fracture face. If there is no interval between the two operations, or if the specimen temperature is below −120 °C, then no sublimation occurs. If there is sublimation of ice, the technique is referred to as freeze-etching; if not, as freeze-fracturing (freeze-cleaving). The technique, with or without sublimation, is often loosely referred to as freeze-etching, presumably in deference to the originators of the method, who invariably used an etching step. I will use the term 'freeze-etching' for the technique as a whole, reserving the term 'freeze-fracturing' for the technique without sublimation, and also using it with reference to membrane fracture faces, which are unchanged by etching.

The second apparatus is simpler (Bullivant & Ames 1966; Bullivant 1973). The frozen specimen is fractured at −196 °C under the surface of liquid nitrogen and is subsequently carried, suitably shielded against condensation of contaminants by a covering of liquid nitrogen and enclosure in a cold metal block, into the evaporator. After a suitable vacuum is achieved, a protecting lid is lifted and the specimen is shadowed and backed through tunnels in the cold block. The temperature is so low that sublimation does not occur. All the micrographs in this paper are of freeze-fracture replicas produced by this latter technique.

Membrane splitting

It is now generally accepted that the majority of membranes, when freeze-fractured, split along an interior plane to produce two fracture faces, rather than along a surface to reveal that surface and the apposed ice surface. It is conventional to use the term 'face' to describe an internal fracture face, and the term 'surface' to describe either of the two natural surfaces of a membrane. The novel idea that membranes split was proposed by Branton (1966). His evidence rested on the appearance of a small ridge at the base of membrane fracture faces, and on the fact that membrane fracture faces would not etch. Deamer & Branton (1967), using radioactive labelling, produced evidence that artificial membranes split when freeze-fractured. On theoretical grounds splitting is to be expected, because the hydrophobic bonds in the interior of the membrane are weaker than the bonds in the surrounding ice (Branton 1966; Branton & Park 1967; Branton 1971). Experimental evidence in favour of Branton's proposal can be described under three headings:

Deep etching

If membranes are frozen in distilled water, or in very dilute buffer, the preparation can be deep-etched, revealing a membrane surface additional to the fracture face. The evidence that this surface was really the membrane surface was provided by experiments in which a recognizable marker was attached to the surface before freezing (Pinto da Silva & Branton 1970; Tillack & Marchesi 1970). The marker was seen on the etch surface, but not on the fracture face. Pinto da Silva & Branton labelled both outer and inner surfaces of red cell ghost membranes. They showed the fracture face to be below the labelled surface, whichever aspect of the membrane was seen. This provides quite unequivocal evidence, in the case of this particular membrane, that the fracture face is situated between the outer and inner surfaces, and hence within the membrane.

Thin sectioning

If tissue is freeze-fractured, allowed to warm up and then fixed and embedded, sections can be cut at right angles to the fracture plane. They provide information on the way the membranes fracture. Early work with this technique is now thought to be unreliable because of the possibility of reformation of complete membranes after fracturing and before final fixation (Bullivant 1973). Most recent work shows a 'half membrane' at the fracture face (Nanninga 1971; Hereward & Northcote 1972; Bullivant 1973). However, Hereward & Northcote (1973) have evidence that the plant plasmalemma may fracture to show either an interior face or a surface.

Complementary replicas

If both halves produced by freeze-fracturing a specimen are replicated, then examination of these complementary replicas shows that membranes split along a unique plane. This in itself does not prove that membranes split along an interior plane, but does prove that the split cannot reveal both surfaces at one point or another, for this would have resulted in two identifiable fracture planes per membrane. Complementary replica experiments are now rather numerous in the literature and I will only refer to some of the early ones. They were by Steere & Moseley (1969) on nerve myelin; Wehrli, Muhlethaler & Moor (1970) on chloroplasts; Chalcroft & Bullivant (1970) on liver cell plasma membranes; and Weinstein, Clowes & McNutt (1970) on red-cell membranes.

Most experimental evidence favours splitting of biological membranes when freeze-fractured. This certainly applies where a membrane is surrounded at both surfaces by water (ice). However, there are reports that certain membranes may fracture along a surface. This may occur where the membranes are closely apposed to each other as in chloroplast grana stacks (Branton & Park 1967) or where the plasma membrane is closely apposed to a cell wall as in yeast or plants (Branton & Southworth 1967; Hereward & Northcote 1973). This unusual type of fracture may thus occur when the membrane is so close to another structure as to exclude unbound water from the intervening space, leading to weaker bonding at the surface than in the membrane interior.

Interpretation of membrane fracture faces

The convention that I will use to refer to fracture faces is that of McNutt & Weinstein (1970). This is shown diagrammatically in figure 1. For a plasma membrane, the A face is the face of that portion of membrane left frozen to the cytoplasm. Conversely, the B face is the face of that portion left frozen to the extracellular space. The use of this convention is illustrated by figure 2, plate 1, which shows the fracture faces of plasma membranes bounding surface extensions of a tissue culture cell. It will be noted that the A face appears convex and the B face concave. It can also be seen that the A face has many small particles, typically around 8.5 nm in diameter; whereas the B face has few particles and is relatively smooth. This predominance of particles on the A face holds for most membranes, although there are exceptions (see Dempsey, Bullivant & Watkins 1973, for details). Using the same convention, that the A face is the face of that portion left attached to the cytoplasm, then we see from figure 1 that the A face of the limiting membrane of an intracellular organelle now appears concave and the B face convex. However, the A face still has most particles. As pointed out by Branton (1969) and Bullivant (1969), this would follow

if internal organelles are assumed to have formed by involution of the plasma membrane. It should be emphasized that, for any particular membrane, only one face (A or B) is seen in a single replica. However, representative A and B faces from different membranes can be seen in the same replica. To see both A and B faces of a particular membrane, then both replicas of a complementary pair have to be examined.

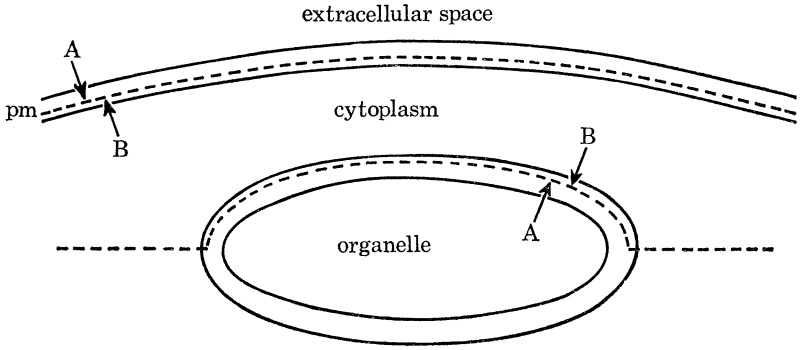


FIGURE 1. Diagram showing the convention used to describe fracture faces. The A face is the face of that portion of membrane left frozen to the cytoplasm, the B face of that portion left frozen to extracellular space. For the plasma membrane (pm) the A face appears convex, the B face concave. The reverse is true for the outer membrane of an intracellular organelle.

Interpretation of membrane fracture faces was discussed three years ago at a Royal Society Meeting by Branton (1971). He gave the evidence that the smooth areas of the fracture face represented the hydrophobic regions within the lipid bilayer, and that the particles represented proteins intercalated within that bilayer. This evidence, and more that has accumulated in the intervening years, will now be reviewed.

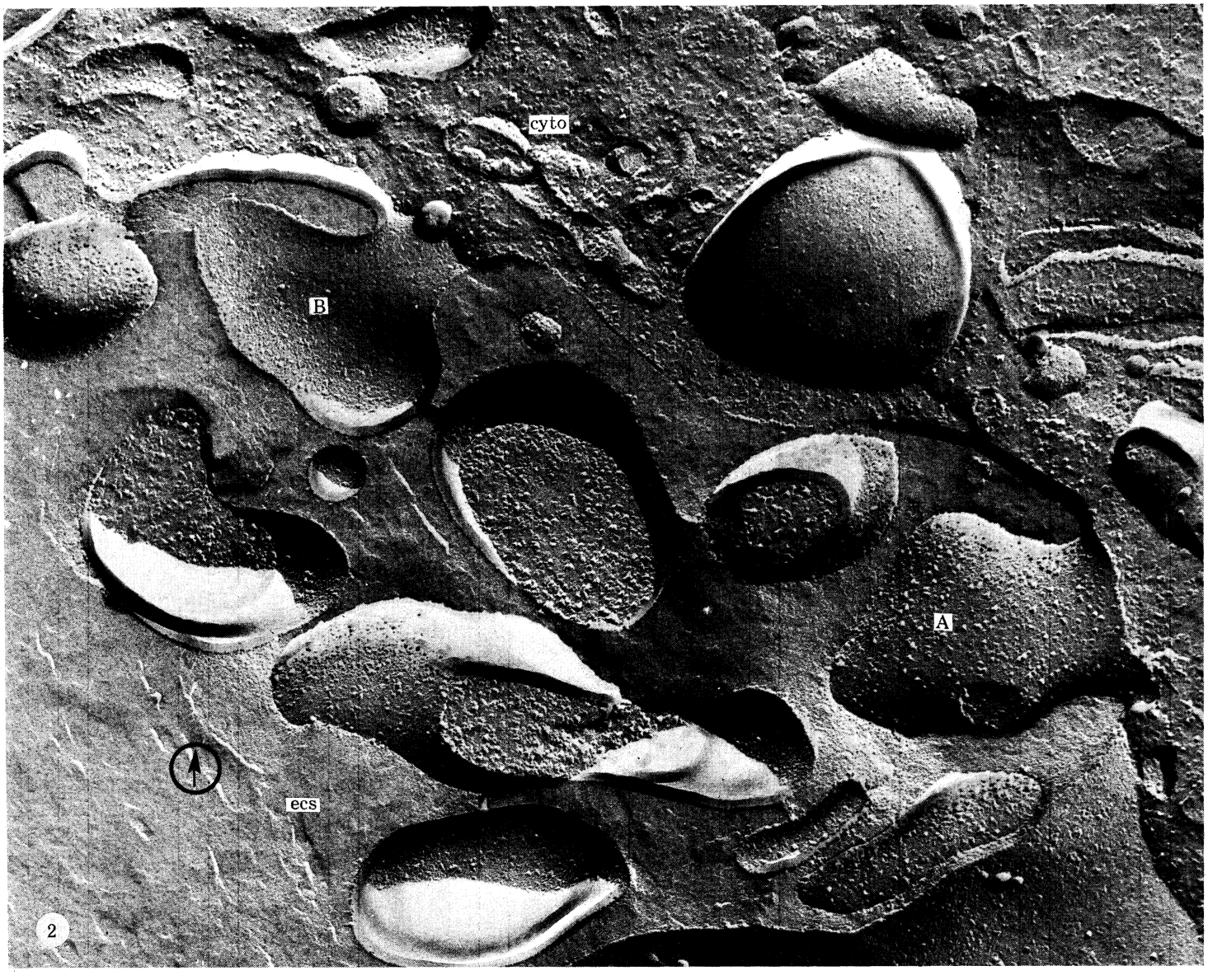
Lipid component

The best evidence that the smooth areas of membrane fracture faces represent lipid came from the finding of completely smooth, particle-free fracture faces in myelin (Branton 1967) and in artificial membranes made only of lipid (Deamer & Branton 1967; Staehelin 1968).

There is good evidence that freeze-fracturing shows the lipids of membranes in the physical state they were in at the commencement of freezing (Deamer, Leonard, Tardieu & Branton 1970; James, Branton, Wiesnieski & Keith 1972). This being so, it is reasonable to look at changes in membrane structure brought about by changes in the lipid.

Acetone extraction of the lipid of chloroplasts abolishes membrane splitting (Branton & Park 1967). A similar loss of fracture faces of red-cell membranes is brought about by the action of saponin, sodium dodecyl sulphate or lysolecithin (Speth, Wallach,Weidekamm & Knüfemann 1972). However, Seeman, Chang & Iles (1973) found different effects with saponin, possibly due to experimental differences. They showed that it produced 4–5 nm wide pits in the extracellular surface of red cell membranes, as revealed by deep etching. The appearance of the membrane fracture face itself was not affected.

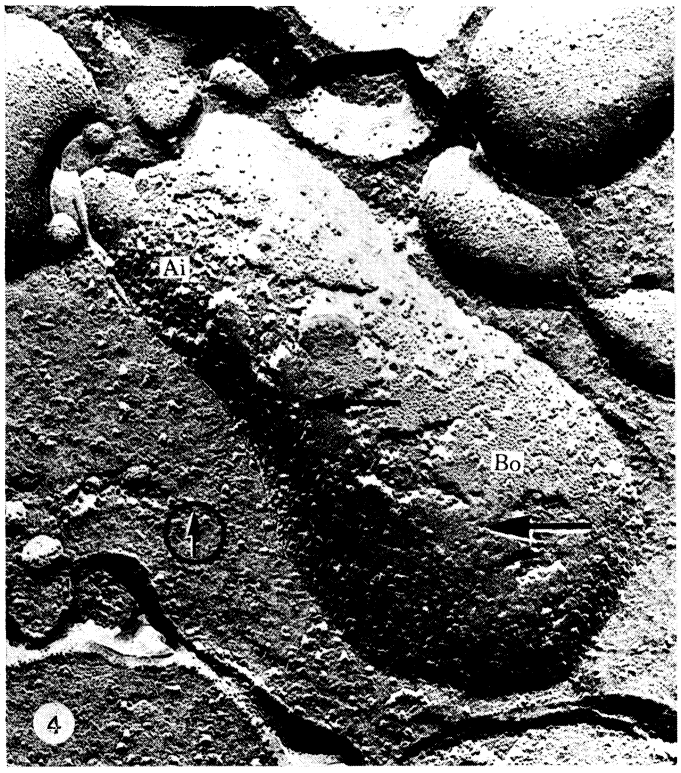
Some cells can be grown so that their membrane lipids are enriched with respect to certain fatty acids. If the proportion of unsaturated fatty acids is high, the particles appear evenly dispersed, while if it is low, the particles are aggregated, as if they had been pushed into regions between the large smooth-faced patches of lipid (Tourtellotte, Branton & Keith 1970; James *et al.* 1972). Another effect, depending on the proportion of unsaturated fatty acids, is the change



All electron micrographs are of freeze-fracture replicas and they are printed as positives with the shadowing direction indicated by an encircled arrow.

FIGURE 2. The edge of an L cell in tissue culture. The A face of the plasma membrane is convex and covered with particles. The B face is concave and much smoother. Cyto, cytoplasm, ecs, extracellular space (Magn. × 76 000).

(*Facing p.* 8)



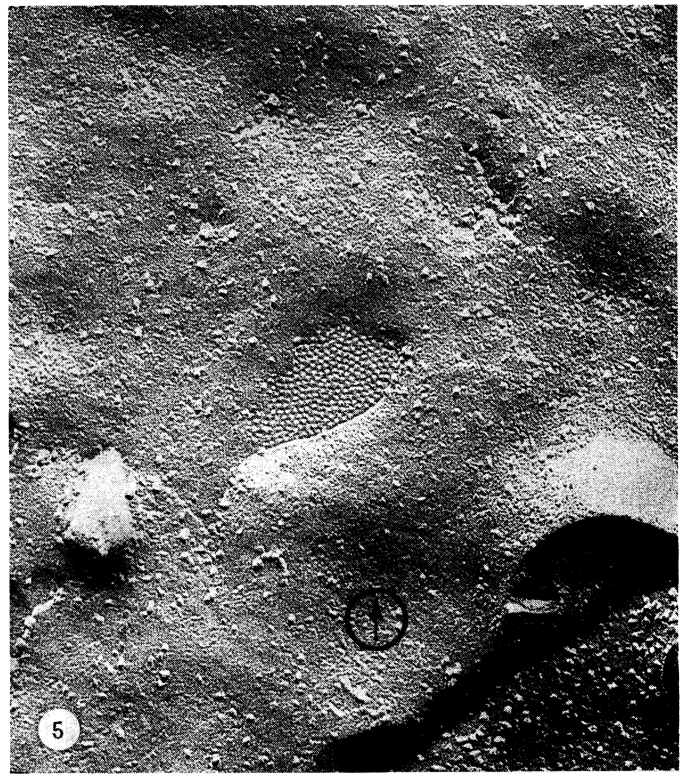
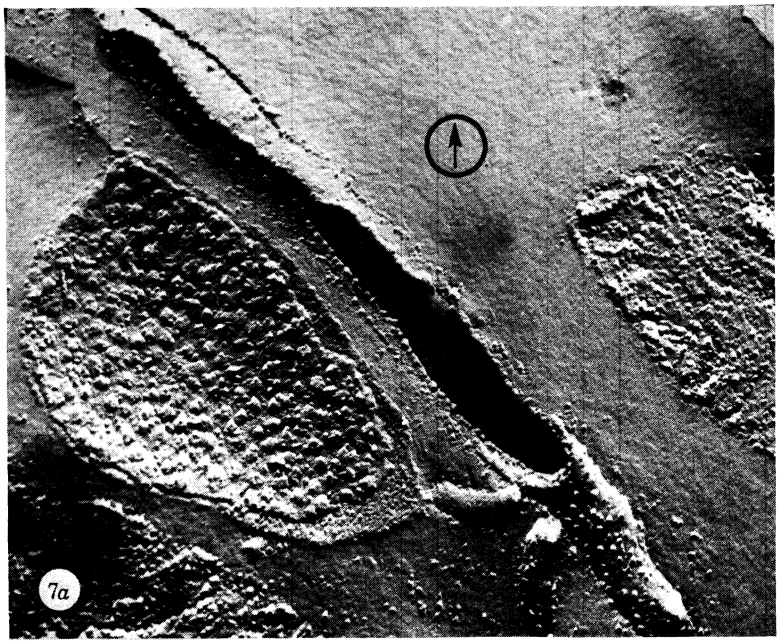
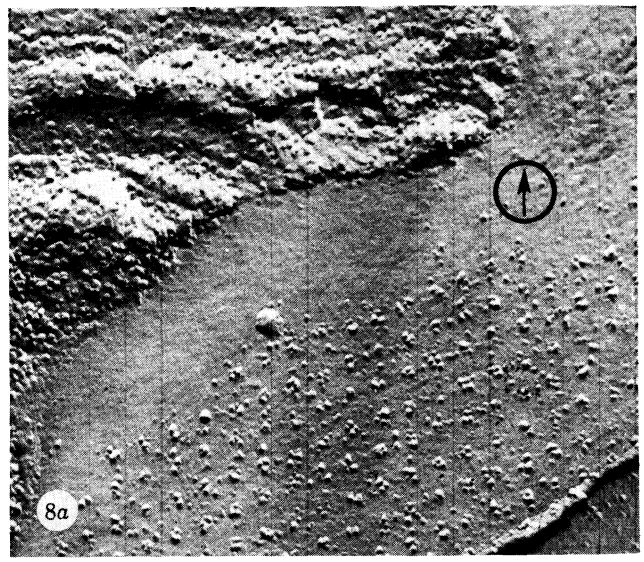


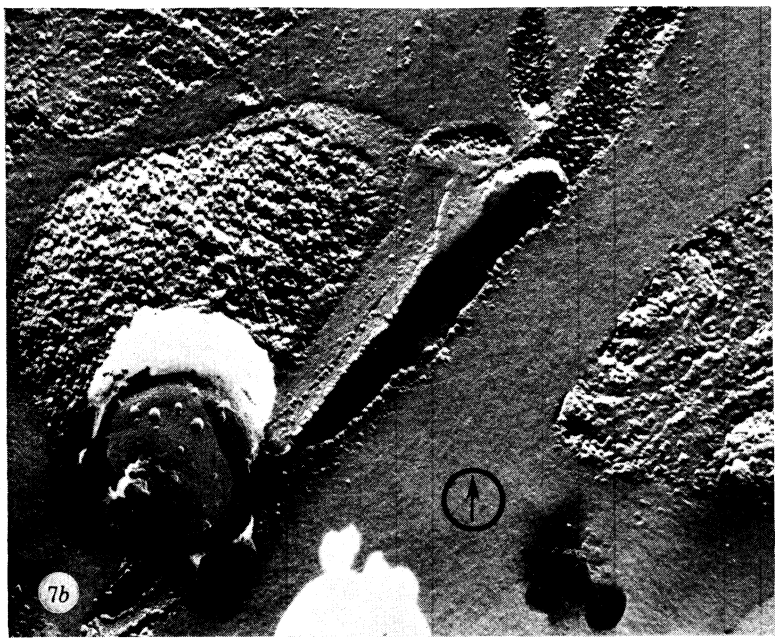
FIGURE 4. Fracture faces of mitochondrial membranes in L cell. The B face of the outer membrane (Bo) is relatively smooth, but does show some depressions (arrowed). The A face of the inner membrane (Ai) is covered with particles (magn. × 70 000).

FIGURE 5. The B face of a liver cell membrane showing the ordered array of depressions of a gap junction (magn. × 86 000).

FIGURE 6. The B face of the cell wall of *Proteus vulgaris*, which exhibits many small depressions (magn. × 193 000).







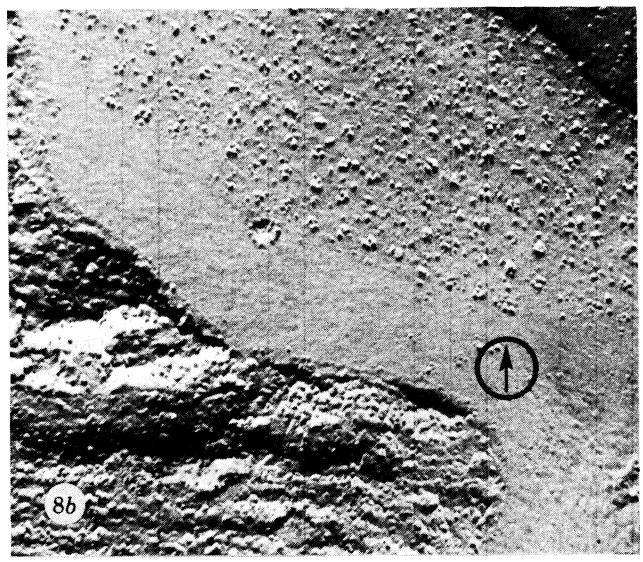


FIGURE 7. Complementary replicas of mitochondrial membranes from insect flight muscle. (*a*) Mostly inner membrane B face, (*b*) inner membrane A face (magn. × 68 000).

FIGURE 8. Complementary replicas of insect flight muscle. Part of a mitochondrion, and the hexagonal array of cross-fractured myosin filaments can be seen. The filaments appear as broken-off projections on both faces (magn. × 80 000).

FIGURES 7 and 8 are printed as mirror image complements and have to be folded about a line to give coincidence.)

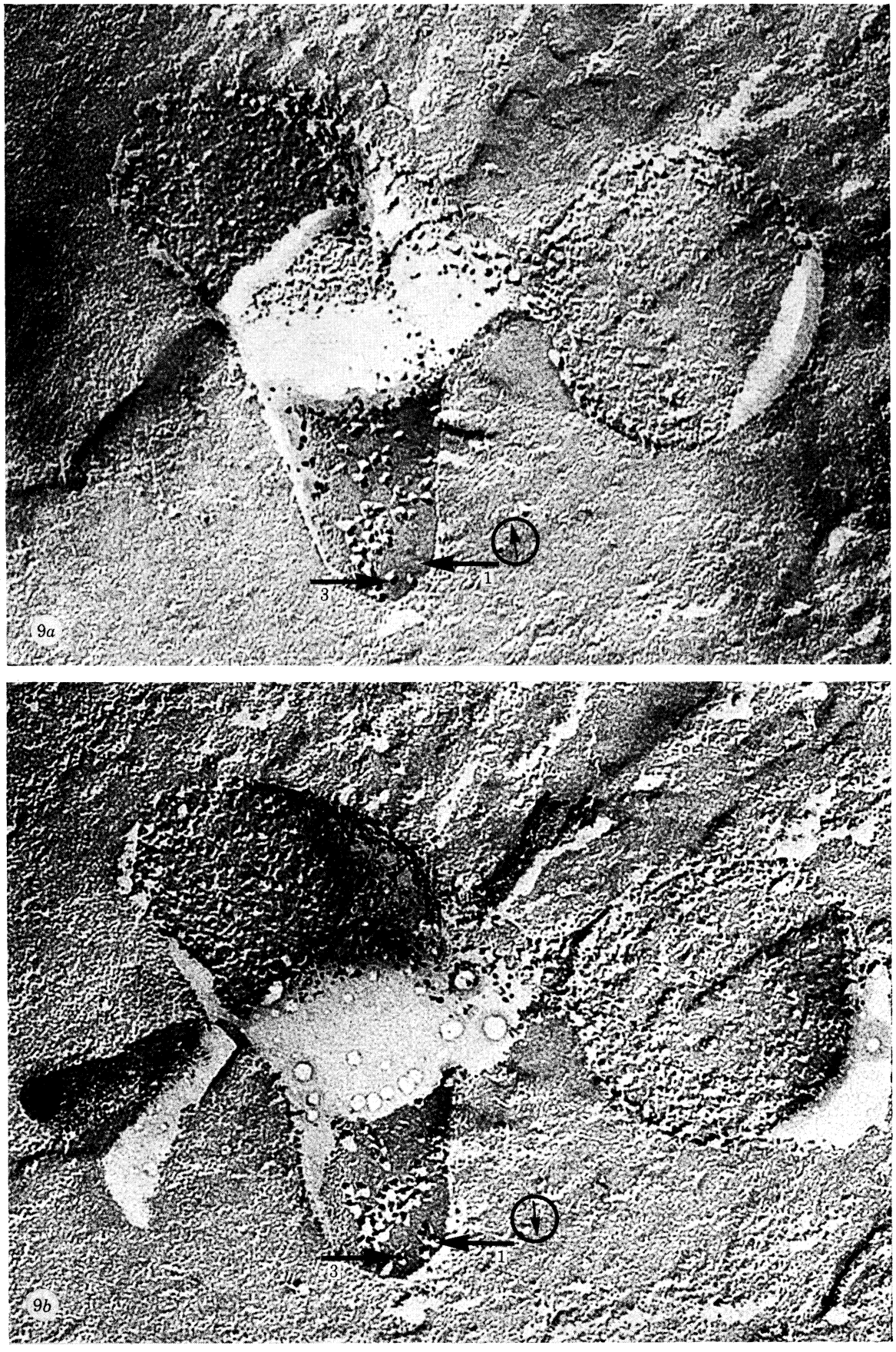


FIGURE 9. Complementary replicas of pig red cell membrane vesicles (magn. × 180 000). In this figure and in figure 10 a particle opposite a depression is indicated with an arrow labelled with the number 1; a particle opposite a smooth region with one labelled 2; and a particle opposite a particle with one labelled 3. Figures 9 and 10 are not shown as mirror images. One grid of the complementary pair was photographed inverted with respect to the other.

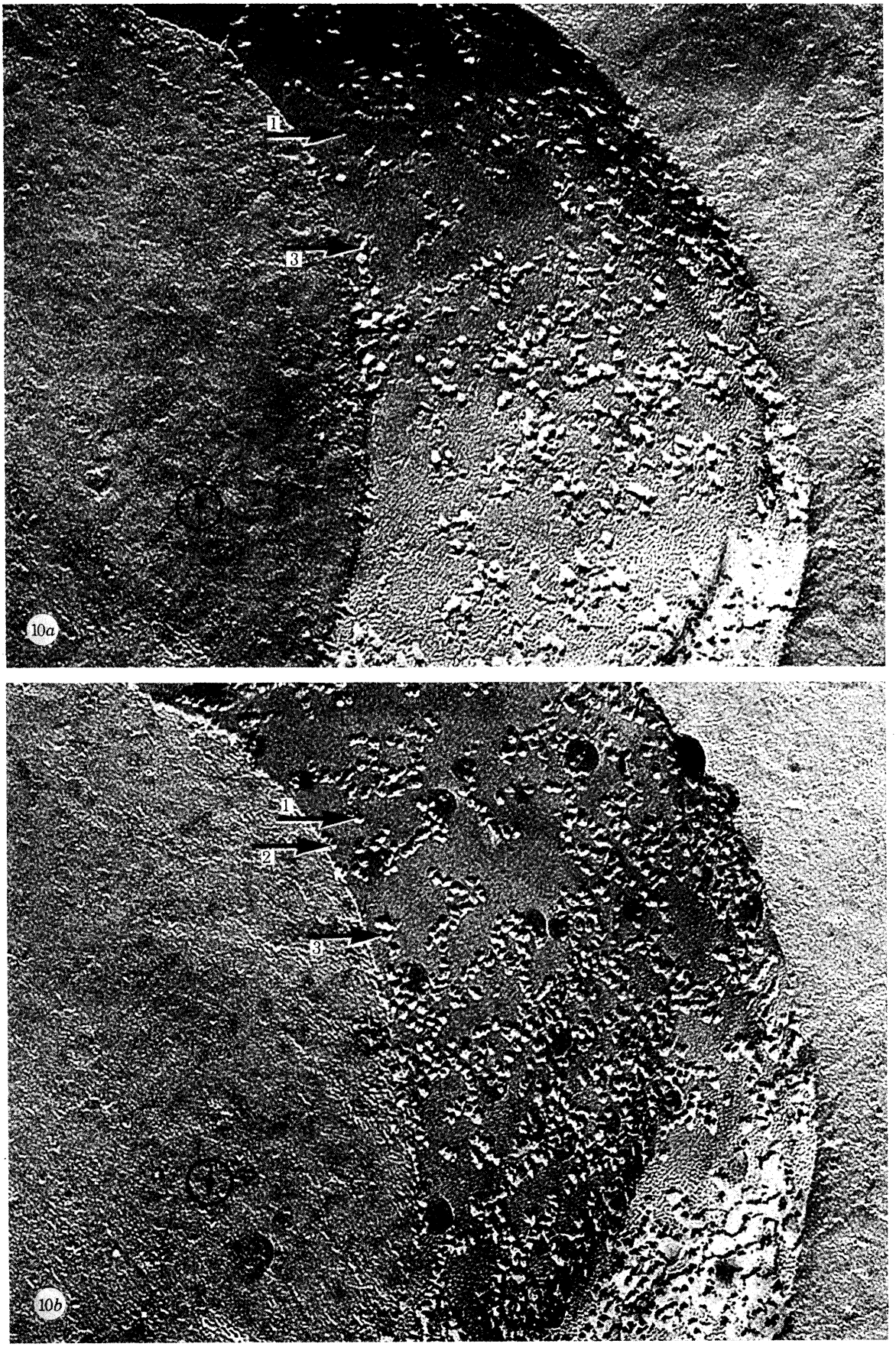


FIGURE 10. Complementary replicas of a piece of pig red-cell membrane. For details of labelling see the legend to figure 9 (magn. × 180 000).

in fracturing properties brought about by osmium tetroxide fixation. In membranes with a low proportion of unsaturated fatty acids, the membrane fracture plane is not affected by osmium fixation, whereas in those with a high proportion of unsaturated fatty acids, osmium fixation leads to the abolition of membrane splitting (James & Branton 1971). This effect is presumably related to the known reaction of osmium tetroxide with double bonds.

Membranes frozen from above their phase transition point show dispersed particles, while from below, they show aggregated particles (Verkleij, Ververgaert, Van Deenan & Elbers 1972; Speth & Wunderlich 1973). Presumably the lipid is normally in the liquid crystalline state, but cooling below the transition point before the freezing for freeze-fracturing causes areas to crystallize, thereby moving the particles into regions analogous to the eutectic observed in frozen solutions.

Verkleij *et al.* (1973) have shown that filipin interacts with the cholesterol of membranes to give 'bumps' in the fracture plane corresponding to the cholesterol–filipin complexes.

In the above work the freeze-fracture appearance of the membranes is generally compatible with the known properties of the lipids. The freeze-etch technique is much more useful than conventional techniques in demonstrating changes in membrane lipids. Early freeze-etch work concentrated on the newly discovered protein particles, regarding the smooth lipid face almost as a background. The recent work on lipids signifies a more balanced approach to the structure of the whole membrane.

Protein component

As already mentioned, particles are seen on the fracture faces of membranes, usually more on the A than on the B face. The particles are typically about 8.5 nm in diameter, but may be more in some membranes. Considerable evidence has accumulated that they are, or at least represent the site of, proteins intercalated within the lipid bilayer of the membrane. A very good argument in favour of the protein nature of the particles is the correlation by Branton (1969) of the particle population density in a particular membrane with the functional complexity and protein content of that membrane. At two extremes are the myelin membrane with no particles, and the chloroplast membrane, sometimes with particles so packed that they are in regular array. Apart from this circumstantial evidence, there is more direct experimental evidence linking the particles with the protein component of membranes. This evidence will now be described.

A seemingly obvious way to demonstrate that the particles are protein would be to remove them with protein digesting enzymes. This type of experiment has not given a straightforward answer, possibly because the proteins are in the hydrophobic interior of the membrane and thus not readily accessible to the enzyme. Branton (1971) reports experiments by Engstrom (1970) in which red-cell ghosts were digested with pronase. Removal of up to 45 % of the original membrane protein caused aggregation of the particles but no diminution in their number. It was necessary to remove 70 % of the protein before there was any decrease in particle number. Somewhat different results were obtained by Tillack, Scott & Marchesi (1970). They showed that the particles could only be removed by treatment with lithium diiodosalicylate, which appears to dissociate lipid–protein complexes. Proteolytic enzymes or neutral salts only caused clumping of the particles.

Pinto da Silva, Douglas & Branton (1971) showed that sites on the etch surface of red-cell membranes, ferritin-labelled specifically for A antigen, were in areas confluent with regions

showing particles on the fracture face. The ferritin was presumed to be attached to the particle where it protruded to the surface of the membrane.

Toutellotte & Zupnik (1973) freeze-fractured *Acholeplasma laidlawii* cells grown either in the presence of puromycin or in media lacking amino acids. In both cases there was a lower population density of particles in the membranes than in those from control cells.

Another approach to the problem is to reconstitute membranes from lipid and protein fractions. Tillack, Carter & Razin (1970) did this for *Acholeplasma* membranes and found that the fracture faces of the reconstituted membranes showed no particles, even though the membranes contained all the lipid and more than 85% of the original protein. On the other hand, Maclennan, Seeman, Iles & Yip (1971) were able to reconstitute the membranes of sarcoplasmic reticulum so that particle-covered fracture faces were produced.

All the above evidence indicates that the particles do represent the sites of lipid protein interaction in the membrane, but that the protein must be complexed with the lipid in a specific way, presumably by hydrophobic bonding, in order to show up as a particle. No definite function can be ascribed to these protein particles, although in the case of those in the red-cell membrane which protrude to the surface, it is tempting to equate them with transport functions. As membranes are multifunctional, it is likely that many classes of particles exist. Glycophorin (Segrest, Kahane, Jackson & Marchesi 1973), a glycoprotein of the red-cell membrane, has been shown to extend through the membrane and to be hydrophobically bonded in the interior. It would be an ideal candidate to appear as a particle on freeze-fracturing, although of course there is as yet no way of identifying it in freeze-fracture preparations. The freeze-etch image of membranes with the smooth lipid regions and the particulate proteins correlates very well with the fluid mosaic model (Singer & Nicolson 1972) derived from other biophysical and biochemical experiments.

In this section on the interpretation of membrane fracture faces there has been no mention of specialized junctional regions, where the particles are organized into regular structures or arrays. It was decided not to discuss junctions in this relatively brief review concerned with membranes in general, particularly as the freeze-etch structure of junctions has been adequately treated recently (McNutt & Weinstein 1973).

Matching of complementary faces

If a membrane containing protein particles fractured without deformation, and subsequent exposure to vacuum and shadowing did not lead to any addition or subtraction of material from the two resulting faces, then there would be absolute complementarity between them. In particular, there would be depressions on one face to match protruding particles on the other. This is shown in a highly schematic form in figure 3 (*a*). In the majority of instances membrane faces appear as shown in figure 3 (*b*) with particles on one face not matched by depressions on the other.

Perfect matching

There are a number of reports relating to non-junctional areas of membranes where sufficient depressions were seen on the B faces to match the number of particles seen on the A faces (Staehelin 1970; Steere & Sommer 1972; Flower 1973). Mitochondrial membranes show many depressions (figure 4, plate 2). The B faces of gap junctions (figure 5, plate 2) show a hexagonal array of depressions matching the A face array of particles, as shown in complementary replicas

by Chalcroft & Bullivant (1970). It is interesting that what could be called the B face of a Gram-negative bacterial cell wall also shows many depressions (figure 6, plate 2). The cell wall is lipopolysaccharide in nature rather than lipoprotein.

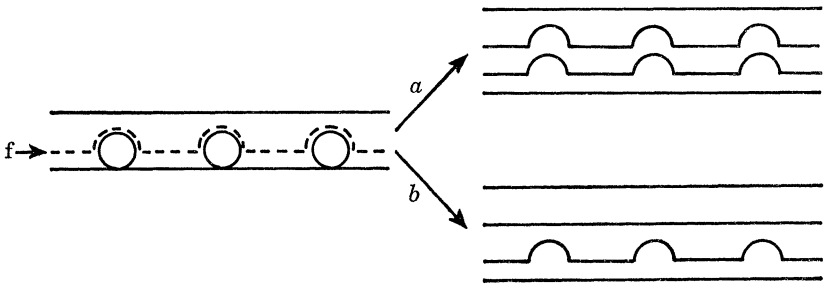


FIGURE 3. Schematic diagram to show that a membrane may fracture (f) in an idealized way (*a*) to give exact complementarity; or with some distortion (*b*) during fracturing or subsequently.

Imperfect matching

Examination of figure 2 will show that there are very few depressions visible on the B faces. Complementary replicas of the non-junctional plasma membrane reinforce this point (Chalcroft & Bullivant 1970). The complementary replicas of a mitochondrial inner membrane (figure 7, plate 3) show that the large particles on one face do fit in between groups of smaller particles on the other, but that particle to depression matching is still far from perfect.

The absence of depressions on most B faces has been a problem, because it could be construed to cast doubt on the reality of the A-face particles. There now seems no doubt that the particles do represent protein, but it is not certain that their structure as seen on fracture is the same as that in the intact membrane. In attempting to extrapolate back to the real structure, it is useful to consider the processes which could lead to imperfect matching. They are:

(*a*) *Shadowing.* By its nature, shadowing enhances the size of a particle and tends to fill in a depression.

(*b*) *Contamination.* Contamination condensing on the fracture face would also lead to increase in particle size and decrease in depression size, before shadowing. A two-stage replica experiment, which showed that particles were enhanced relative to depressions was described by Bullivant (1973). This cannot, of course, differentiate between shadowing and contamination effects.

(*c*) *Plastic deformation.* Clark & Branton (1968) showed that polystyrene latex spheres were considerably distorted during freeze-fracturing. Bullivant *et al.* (1972), using the complementary replica technique, demonstrated that myosin filaments plastically deform during fracture. An example of such a complementary replica is shown in figure 8, plate 3. There is now evidence that the particles of membranes can also plastically deform. Recently I have made complementary replicas of vesicles derived from red-cell membranes and they show that particles can be seen opposite particles, indicating that single particles in the intact membrane must have been plastically deformed and finally pulled into two parts by the fracture (figures 9 and 10, plates 4 and 5). These same replicas also show other particles opposite depressions, and yet others opposite apparently smooth spots on the complementary face.

(*d*) *Removal or collapse of material.* It seems unlikely that much material would be removed from the hydrophobic interior of a membrane by fracturing at − 196 °C. There is a possibility that the lipid tails could collapse, either during fracturing or shadowing, leading to loss of

depressions and enhancement of particles (Chalcroft 1971). Examination of complementary replicas of vesicle necks indicates a small difference in the diameter of a neck between the two faces (T. Hatta, S. Bullivant & R. E. F. Matthews 1972, unpublished observation). This could be explained by loss of lipid material.

There thus seems to be some evidence in favour of each of the processes which might account for the imperfect matching of faces. The evidence is strongest for plastic deformation, but all the processes may play a part. A model of membrane fracturing involving particle deformation was advanced by Meyer & Winkelmann (1969) some years ago. Flower (1973) has argued that plastic deformation must be the major process accounting for imperfect matching. He believed that this was so, because in the same preparations he saw one type of membrane exhibiting perfect complementarity while another did not. He argued that if other processes, such as shadowing and contamination, had operated, they would have done so irrespective of the membrane type and all depressions would have been lost.

Particles can apparently fracture to leave a depression in the opposite face, leave no depression at all, or leave a particle. The mode of fracture of a particle frozen in a membrane is determined entirely by the path of least resistance, which depends on the level at which the fracture strikes it, and on the physical properties of the particle and the nature of its bonding to the surrounding lipid in the frozen state. Different particles behave differently. For example, those in the gap junction and those described by Flower (1973) in the membranes of the microvilli of the light organ of the New Zealand glow-worm fracture so that they are completely pulled out of the B face. Others plastically deform so that the particle is pulled into two parts, as in the red-cell membranes described in this paper. The model of Singer & Nicolson (1972) depicts particles sunk to different depths in the membrane and even penetrating it completely. The way a particle fractures may be determined by its level in the membrane and hence the position at which the fracture strikes it. In addition, its other physical properties, when it is frozen, may play their part. There may prove to be several types of particles, possibly functionally defined, which can be classified according to the way they fracture.

Conclusions

The freeze-etching technique has proved invaluable in membrane structure studies and a new field of correlated biochemical, biophysical and electron microscopical investigations has been opened up. With such correlated efforts it may be possible to characterize biological membranes completely with respect to their structure and function.

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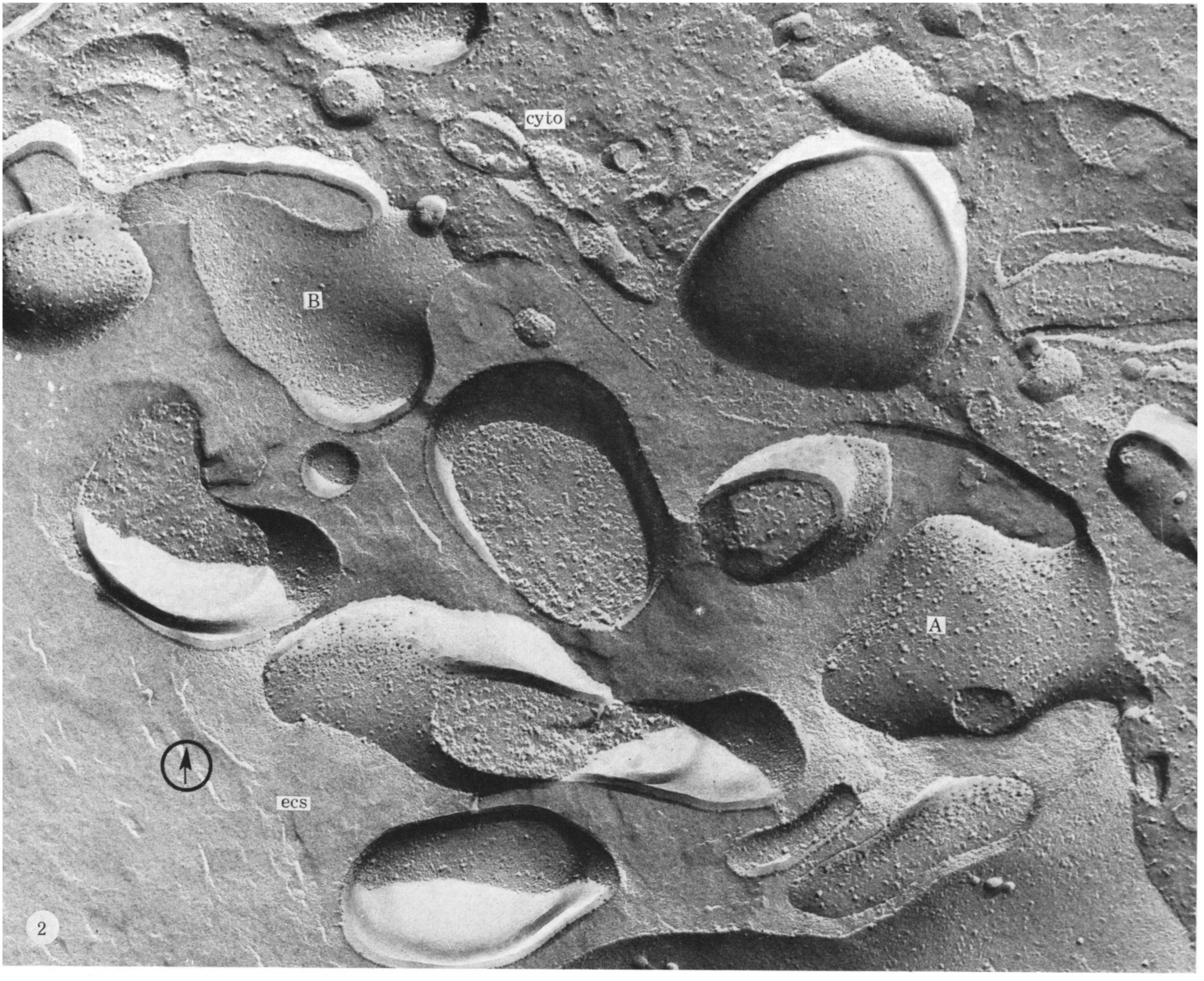
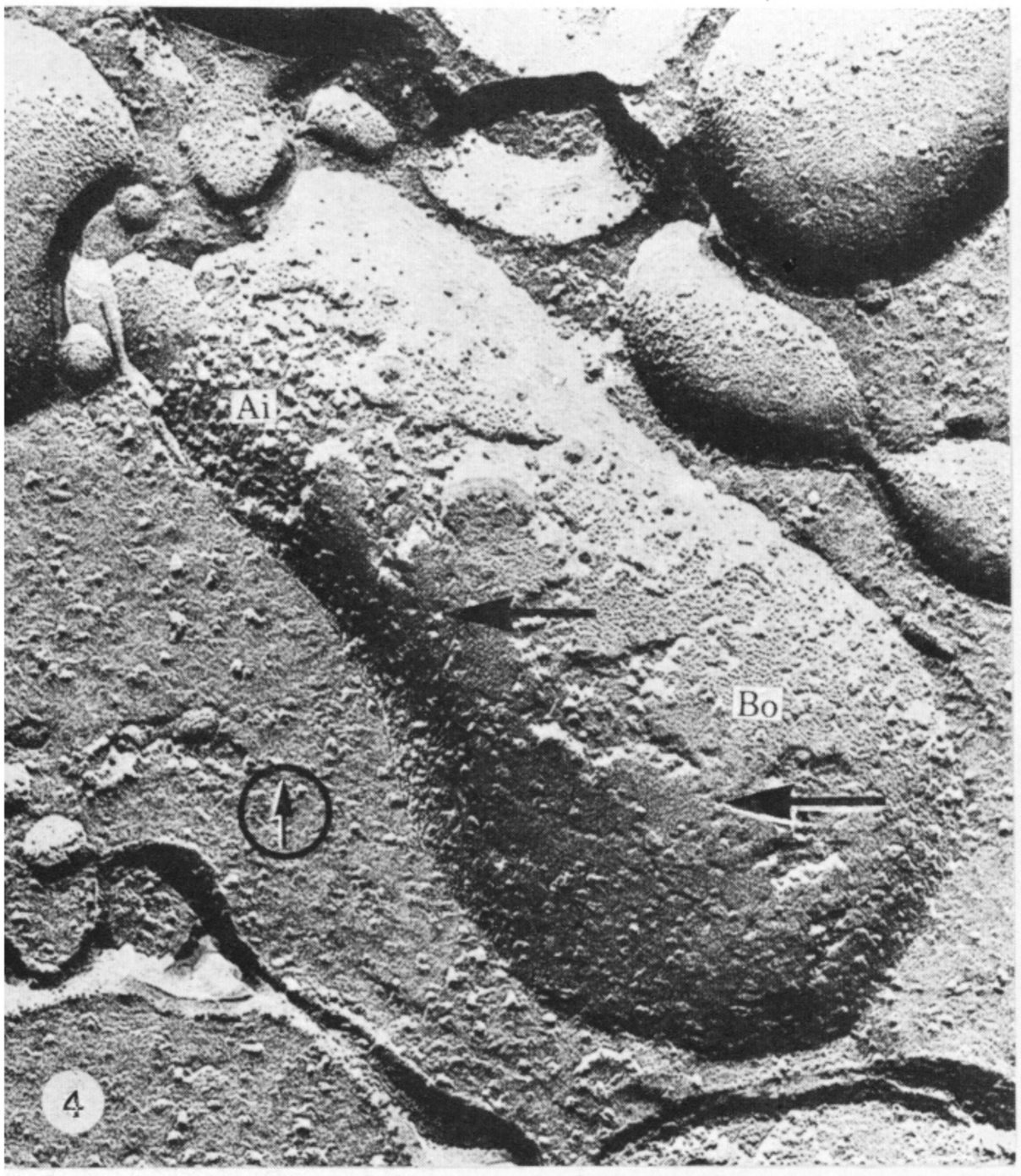


All electron micrographs are of freeze-fracture replicas and they are printed as positives with the shadowing direction indicated by an encircled arrow.

FIGURE 2. The edge of an L cell in tissue culture. The A face of the plasma membrane is convex and covered with particles. The B face is concave and much smoother. Cyto, cytoplasm, ecs, extracellular space (Magn. $\times 76\,000$).



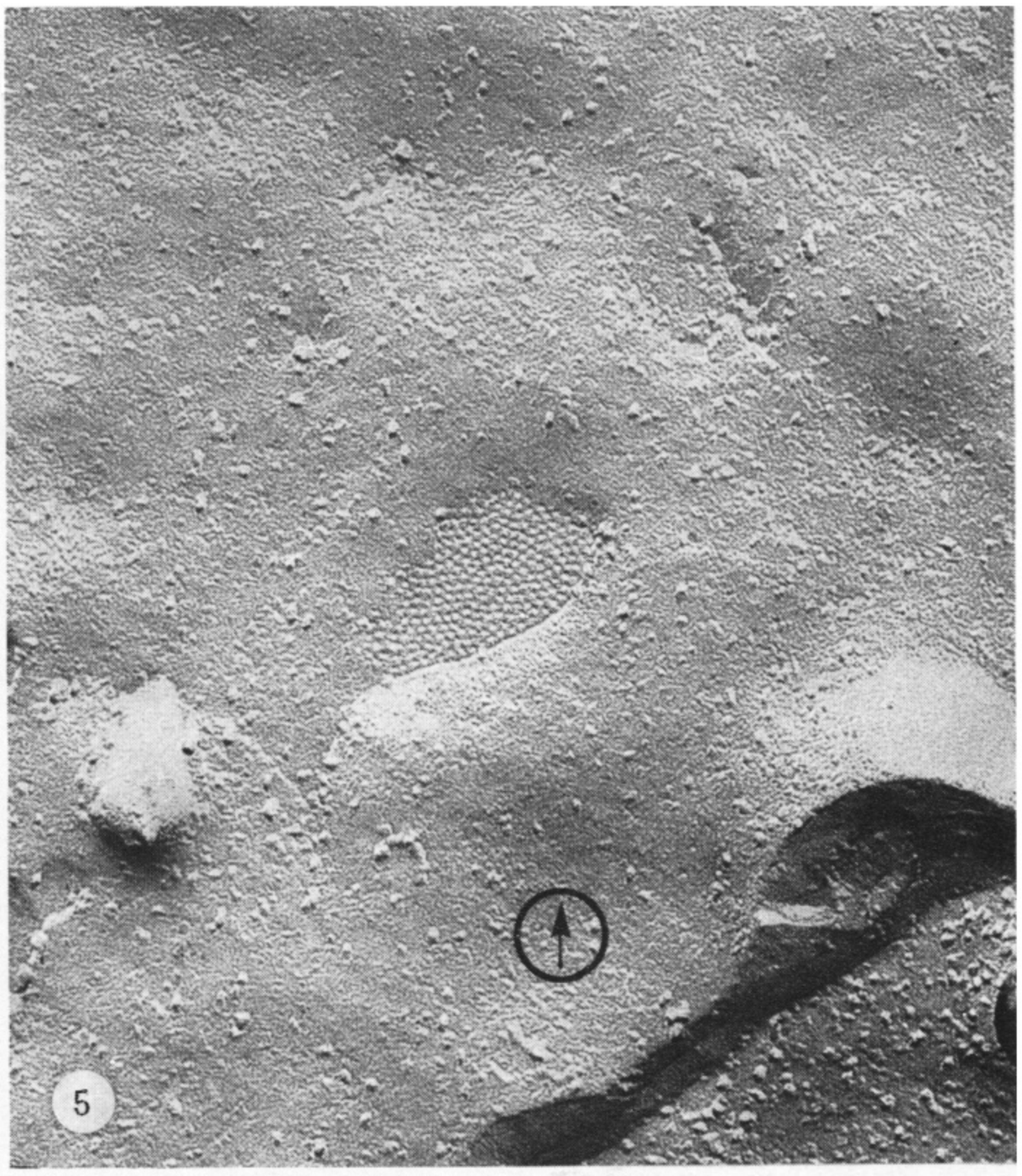
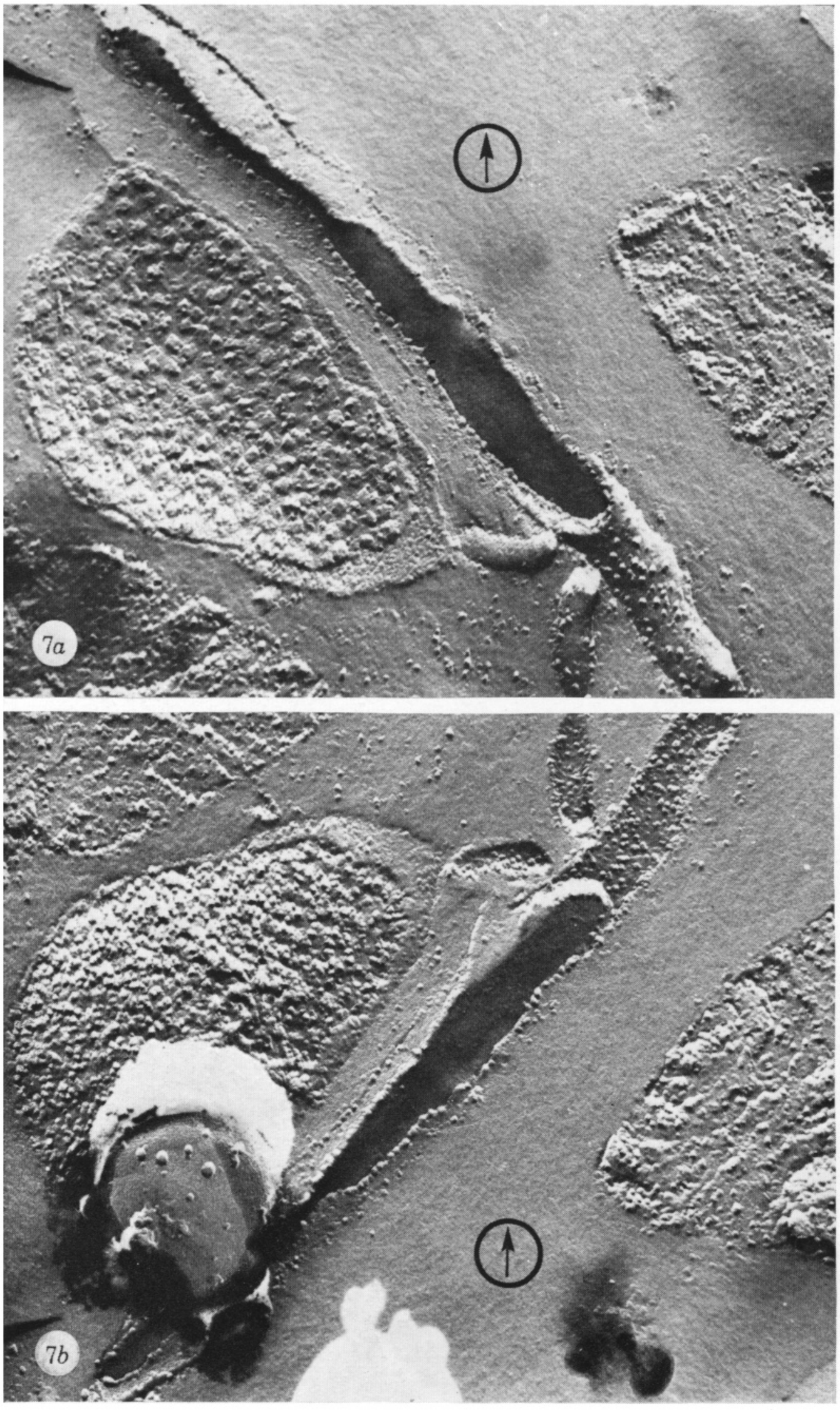
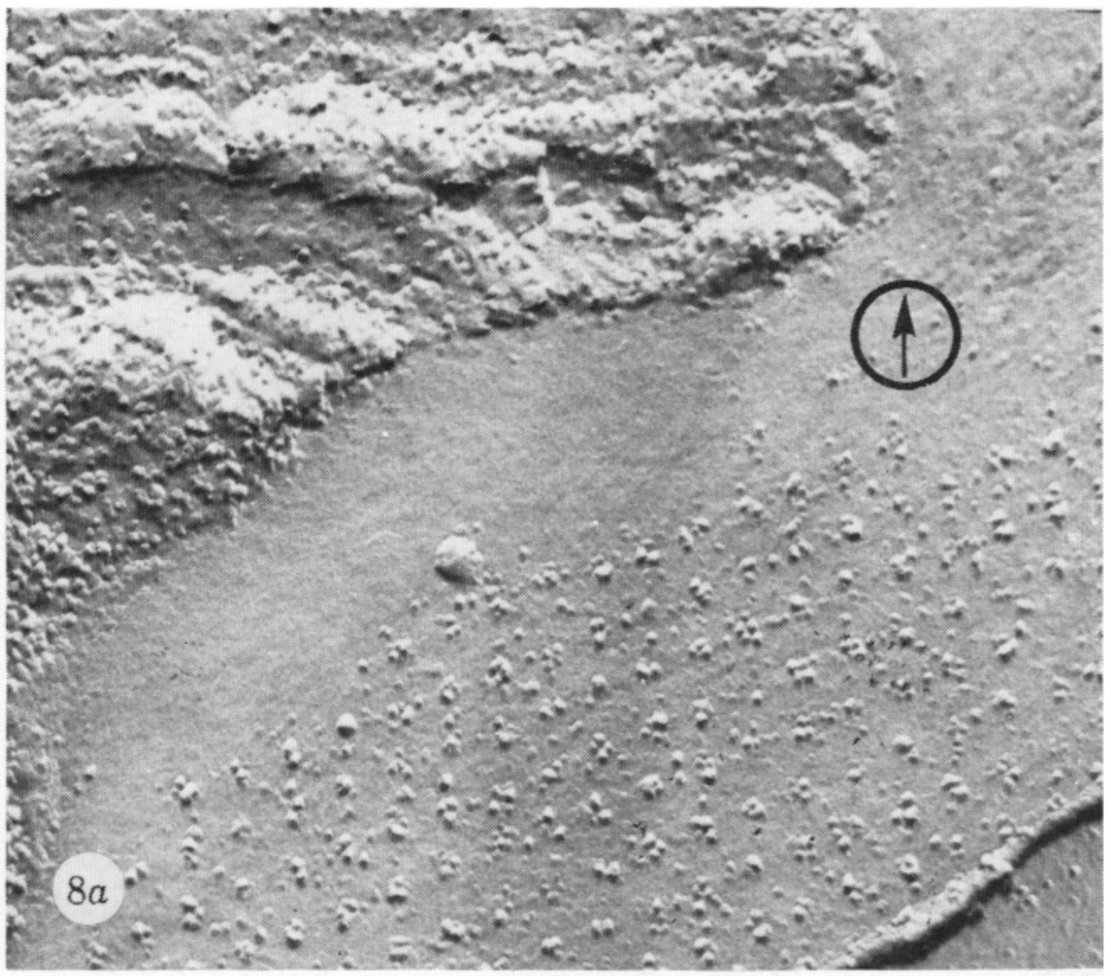


FIGURE 4. Fracture faces of mitochondrial membranes in L cell. The B face of the outer membrane (Bo) is relatively smooth, but does show some depressions (arrowed). The A face of the inner membrane (Ai) is covered with particles (magn. $\times 70\,000$).

FIGURE 5. The B face of a liver cell membrane showing the ordered array of depressions of a gap junction (magn. $\times 86\,000$).

FIGURE 6. The B face of the cell wall of *Proteus vulgaris*, which exhibits many small depressions (magn. $\times 193\,000$).





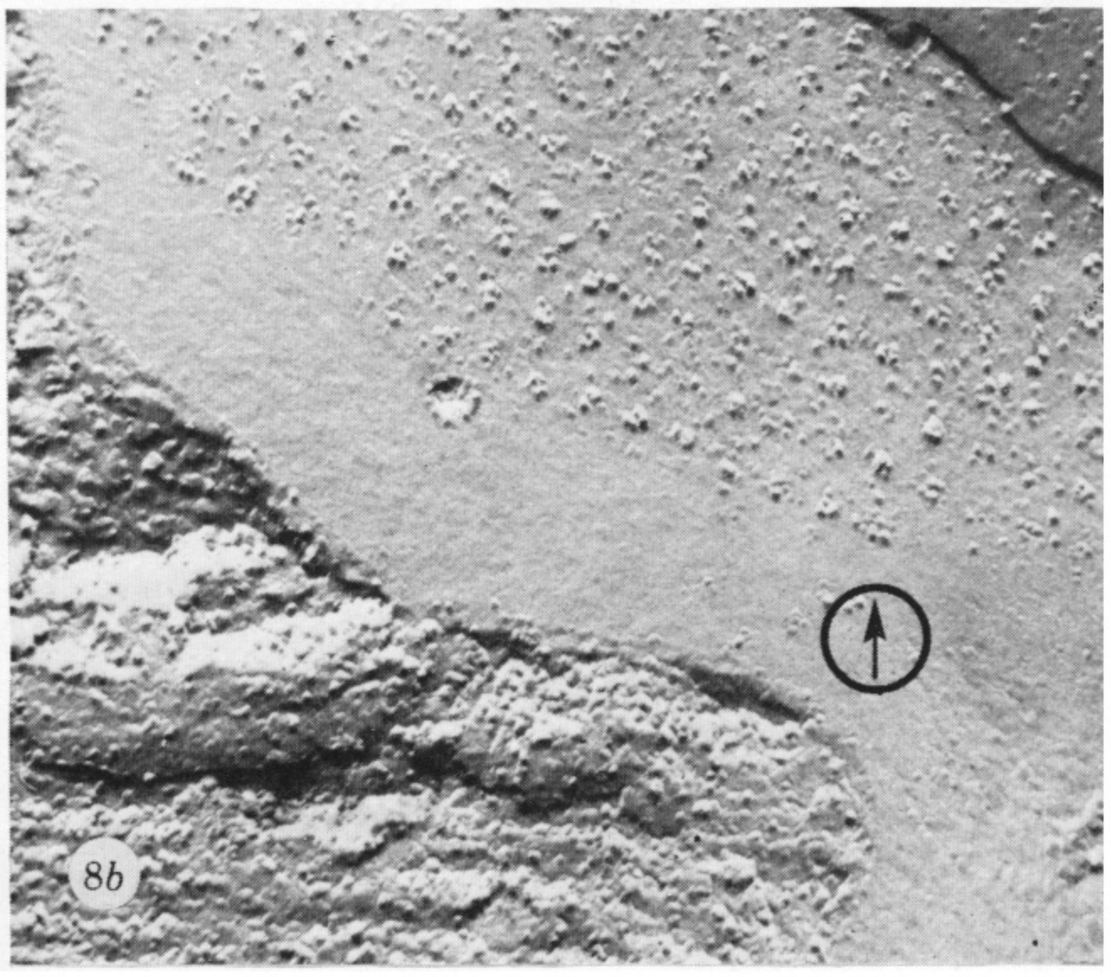


FIGURE 7. Complementary replicas of mitochondrial membranes from insect flight muscle. (*a*) Mostly inner membrane B face, (*b*) inner membrane A face (magn. × 68 000).

FIGURE 8. Complementary replicas of insect flight muscle. Part of a mitochondrion, and the hexagonal array of cross-fractured myosin filaments can be seen. The filaments appear as broken-off projections on both faces (magn. × 80 000).

FIGURES 7 and 8 are printed as mirror image complements and have to be folded about a line to give coincidence.)

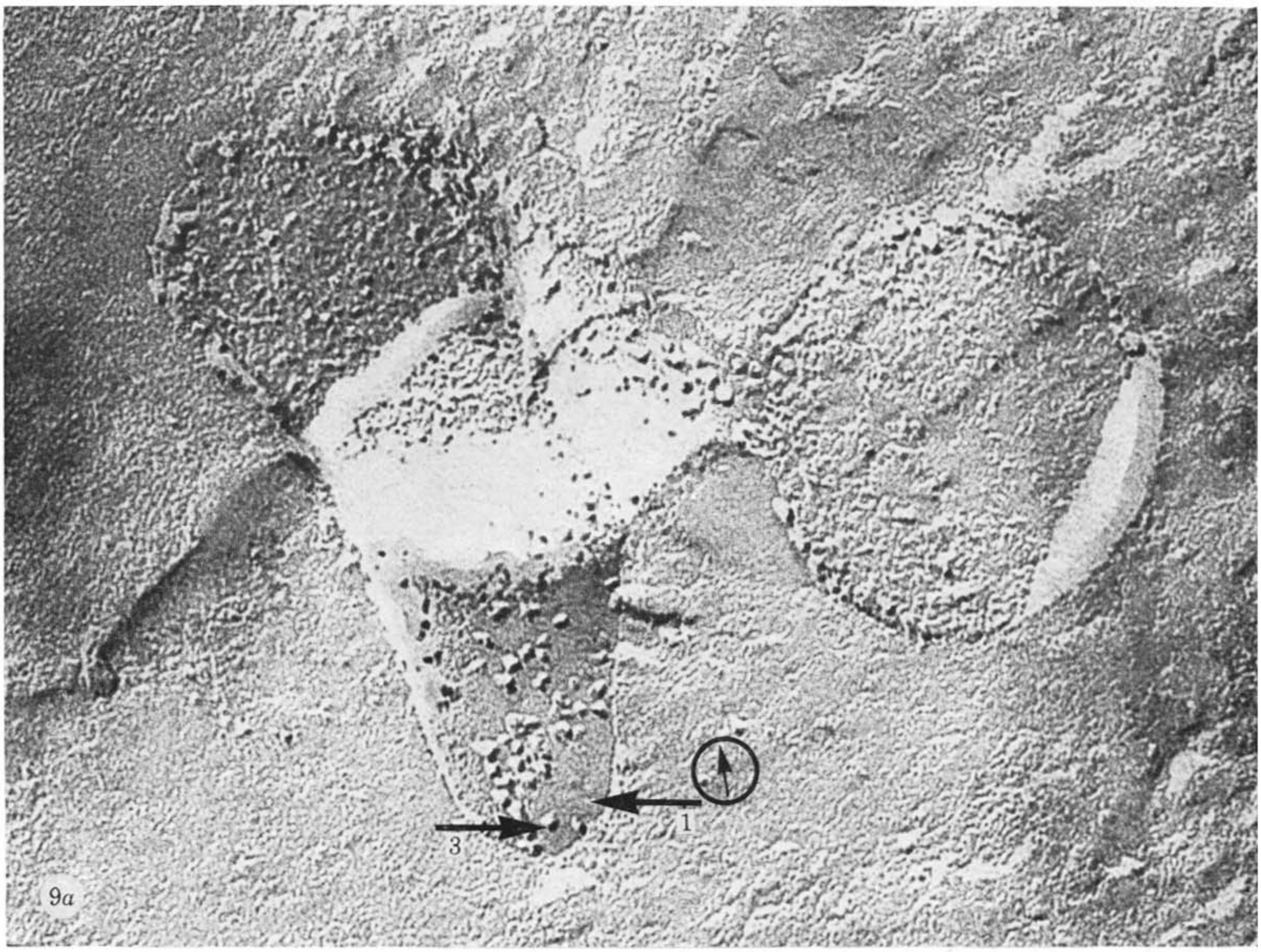


FIGURE 9. Complementary replicas of pig red cell membrane vesicles (magn. × 180000). In this figure and in figure 10 a particle opposite a depression is indicated with an arrow labelled with the number 1; a particle opposite a smooth region with one labelled 2; and a particle opposite a particle with one labelled 3. Figures 9 and 10 are not shown as mirror images. One grid of the complementary pair was photographed inverted with respect to the other.

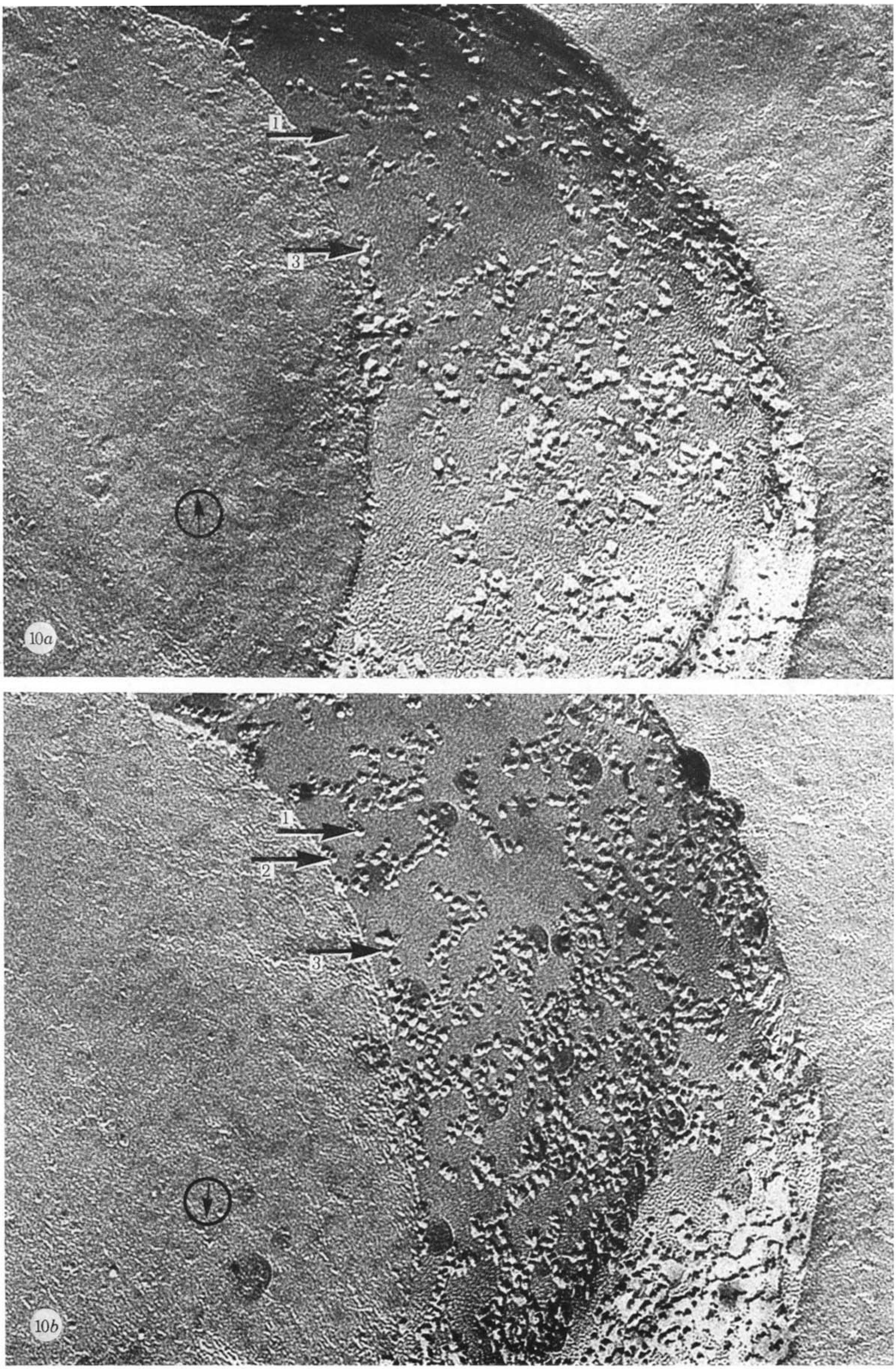


FIGURE 10. Complementary replicas of a piece of pig red-cell membrane. For details of labelling see the legend to figure 9 (magn. × 180 000).