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Freeze-Etching Studies of Membrane Structure

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Freeze-etching studies of membrane structure

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[Plates 23 to 25]

Thin sections of biological membranes examined by electron microscopy appear as two dark lines separated by a lighter space. This observation, first made in the 1950s, has been interpreted as confirmation of the Danielli–Davson model of the biological membrane (Robertson 1959). Proponents of this interpretation have equated the dark lines to proteins and other polar groups in the membrane and the intervening light space to the lipid fatty acids (Stoeckenius 1960). However, it has become clear that other interpretations are possible (Korn 1966; Branton & Park 1968) and that the electron microscope observations do not in fact prove the validity of any one molecular model of the biological membrane (Stoeckenius & Engelman 1969). During the last few years a number of biochemical and physical probes have given us more direct information regarding the composition and molecular configurations within biological membranes. Differential scanning calorimetry (Steim *et al.* 1969), X-ray diffraction (Engelman 1969), nuclear magnetic resonance (Kaufman, Steim & Gibbs 1970), and electron paramagnetic resonance (Hubbel & McConnel 1969; Tourtellotte, Branton & Keith 1970) have made it clear that a major portion of the lipids in a variety of membrane systems is in a bimolecular layer. In view of the evidence which these techniques have provided, what role can the electron microscope play in current and future studies of biological membranes?

One of the unique advantages of microscopic approaches is the possibility of examining individual components in a mixed system. Unlike most physical probes, which are essentially averaging techniques, the electron microscope makes it possible to examine specific sites within a specific membrane. Although averaging techniques provide information about the organization of components in the bulk of the membrane, they do not necessarily tell us anything about the molecular order in limited regions of a membrane. It is here that electron microscopy, and particularly the relatively new freeze-etch method, may help. Whereas thin sectioning methods provide information about the structure in a 40 to 60 nm thick section of a membrane, the freeze-etch technique allows us to examine structure within a 2 nm region in the plane of the membrane.

The freeze-etch technique was first applied to biological materials by Steere (1957) and later developed by Moor, Muhlethaler, Waldner & Frey-Wyssling (1961). Descriptions of freeze-etching have been published (Moor & Muhlethaler 1963; Branton 1966), but it may be helpful to emphasize the distinction between the fracture process and the etching process used in this technique. Fracturing breaks the specimen along planes of weak bonding; etching sublimes away volatile materials, usually water, from between the non-volatile components of the sample. The fracture process is a critical and necessary part of the procedure; the etching process is a useful but optional operation whose results have been separately evaluated (Davy & Branton 1970).

Electron microscopic examination of the replica produced by freeze-etching reveals extensive structures which represent face views of membranes. Initially, we compared structure in several

functionally differentiated membranes. Two important observations emerged from this survey. The first is that different membrane systems appear structurally different when examined by freeze-etching. For example, myelin membranes appear as extensive smooth sheets (Branton 1967), but a variety of other membrane systems appear as smooth sheets interrupted by particles, which usually average about 8.5 nm in diameter (Branton 1966). The number and distribution of these particles seems to be a fixed characteristic of a given membrane type. Comparison of various biological systems shows that the number of particles associated with the membrane is greatest in physiologically active membranes such as the chloroplast lamellae (Branton & Park 1967), which perform the light reactions of photosynthesis, and least in inactive membranes

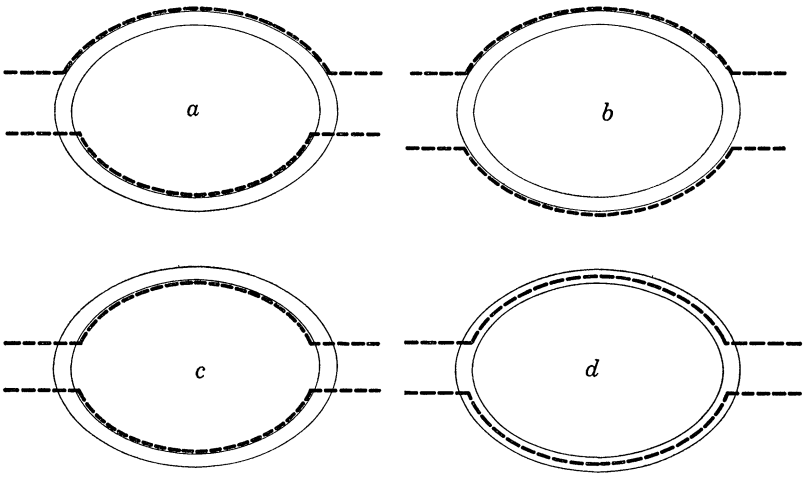

FIGURE 2. Hypothetical fracture schemes. (*a*) Both membrane surfaces provide a fracture plane. Fracture exposes one or the other of these membrane surfaces, but never the previously apposed ice. (*b*) and (*c*) A unique membrane surface—either the outside surface (*b*) or the inside surface (*c*) depending upon membrane type—provides the fracture plane. Fracture exposes either the membrane surface or the previously apposed ice. (*d*) Fracture occurs within the membrane. Neither membrane surface is exposed.

such as the myelin layers, which function primarily as metabolically inert insulators around the nerve axon. Thus one of the first observations to arise from comparison of different membrane systems was that the degree of functional complexity of a membrane may be directly related to the number of particles seen in freeze-etched specimens (Branton 1969).

The second important observation to arise from these comparisons is that in every membrane system there is a marked asymmetry in the distribution of particles between the two membrane faces seen after fracture (Branton 1969). This is clearly illustrated if one examines the membrane of a single red blood cell ghost (figure 1, plate 23). The membrane face seen from the outside of the cell looking inward appears as a smooth layer interrupted by numerous particles, whereas the membrane face seen from the inside of the cell looking outward appears as a more roughened background interrupted by far fewer particles. Initially, the differences between these two faces were taken to indicate that the fracture process exposed either the outer surface or the inner surface of the red blood cell ghost membrane (Weinstein & Bullivant 1967). According to this hypothesis, one fracture exposes the outer surface of the membrane whereas another fracture exposes the inner surface of the membrane (figure 2*a*). But recent work in several laboratories has shown that the differences between the two characteristic faces of a given membrane cannot be the result of fracture along two different surfaces. For any one membrane there is but a single unique plane along which all fractures occur (Wehrli, Muhlethaler & Moor 1970; Chalcroft &

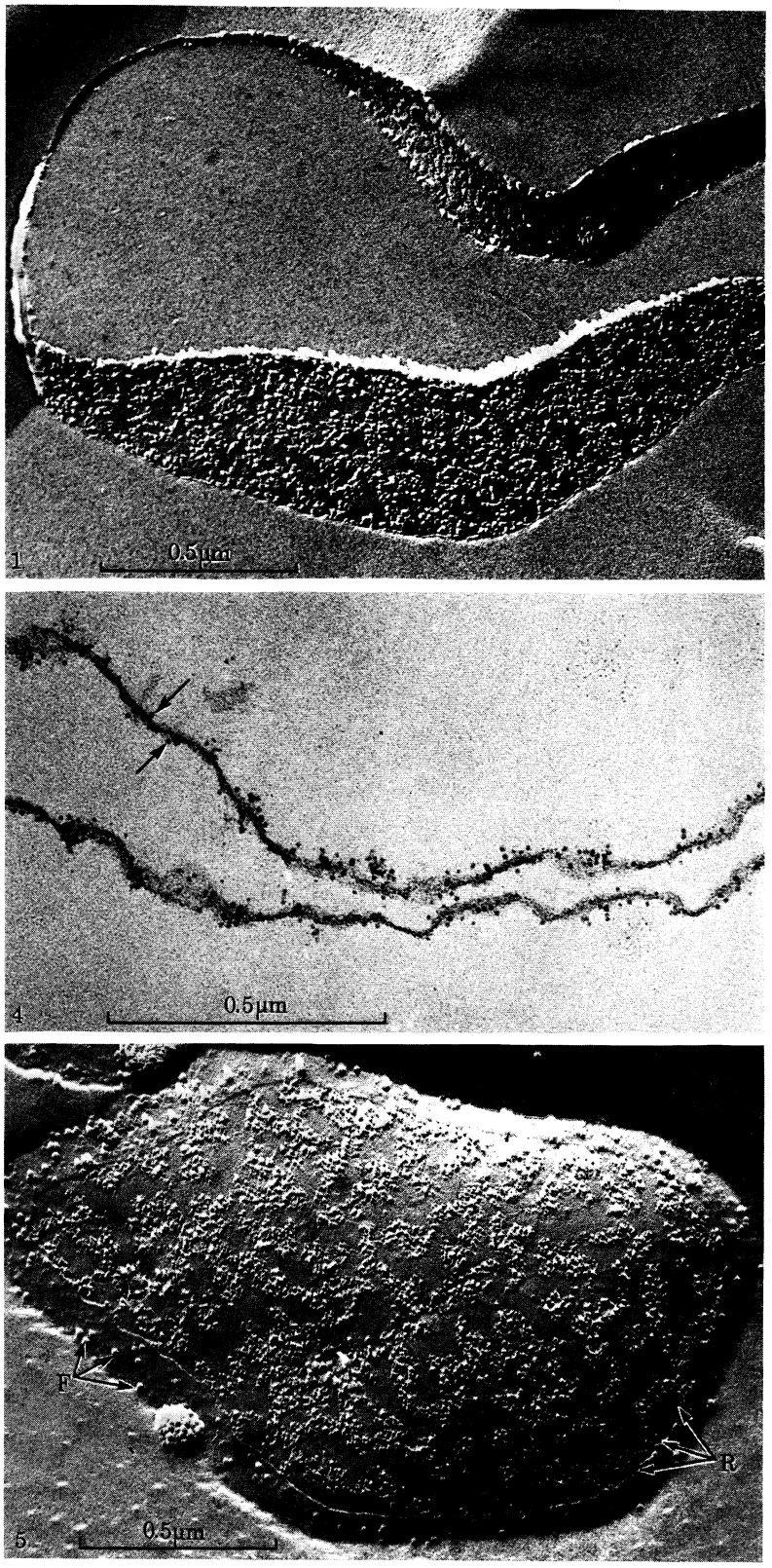

FIGURE 1. Fracture faces of a red blood cell membrane. (Magn. × 52 500.) (Figures 1 to 5 are from the work of Pinto da Silva & Branton 1970.)

FIGURE 4. Section of a red blood cell ghost conjugated with ferritin. Ferritin (arrows) is associated with both surfaces of the membrane. (Magn. × 75 000.)

FIGURE 5. Membrane face in etched, ferritin conjugated cell. Ferritin molecules (F) are associated with the etched face but not visible on the fracture face. As predicted by figure 3, a ridge (R) separates the fracture face from the etch face. (Magn. × 52 500).

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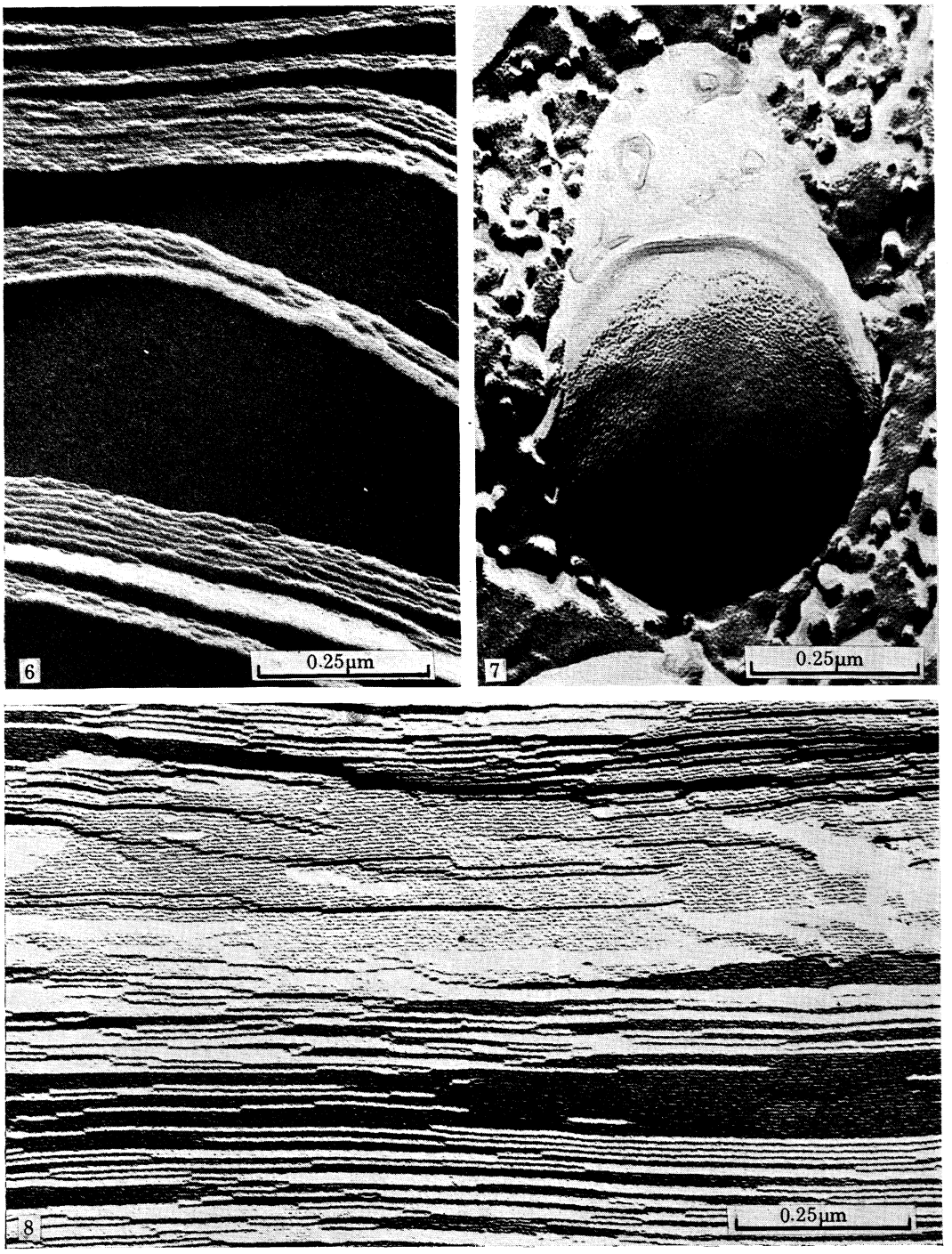

FIGURE 6. Dipalmitoyl phosphatidyl choline in a lamellar phase. (Magn. × 120 000.) (This and figure 8 are from the work of Deamer *et al.* 1970.)

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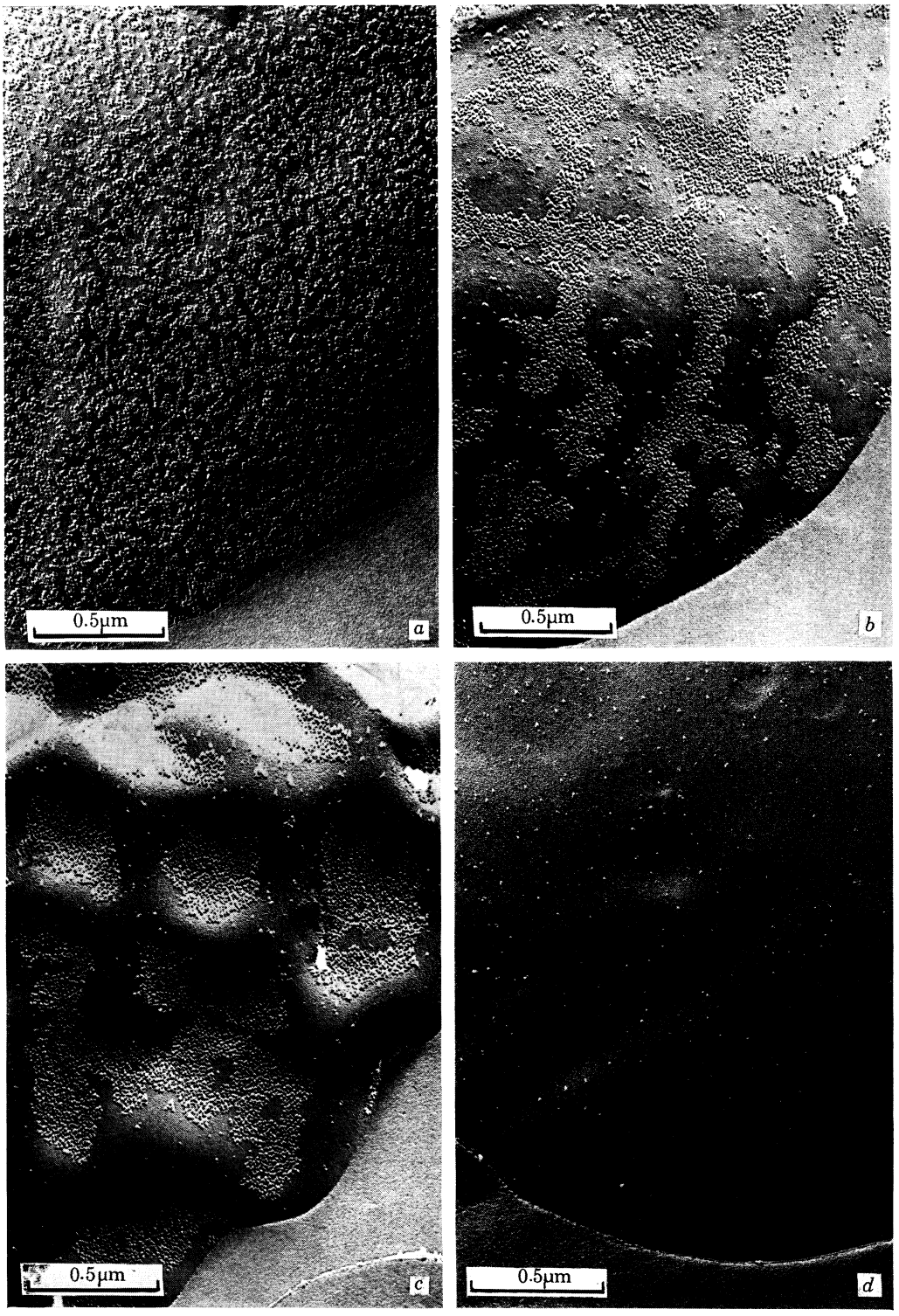

FIGURE 10. Particle aggregation and removal during pronase digestion of red blood cell ghosts. (*a*) Control cell incubated in buffer only; (*b*), (*c*) and (*d*) digested cells incubated in pronase so as to remove (*b*) 30%, (*c*) 45% or (*d*) 70% of the original membrane protein. (All magn. ×40 000.) (From Engstrom 1970.)

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Bullivant 1970). Hence both membrane faces are the product of one fracture. Moor & Muhlethaler (1963) proposed that the two faces represent the surface of the membrane and the surface of the previously apposed ice matrix (figures 2*b* and 2*c*). I (Branton 1966) suggested that in most cases fracture splits the membrane along some inner hydrophobic region (figure 2*d*). Deamer & Branton (1967) used radioisotope labelling techniques to show that the fracture process can split model membranes, and recently Pinto da Silva & Branton (1970) used a morphological label to show that fractures in frozen cellular membranes also occur within the membrane matrix and do not expose the features of the membrane surface.

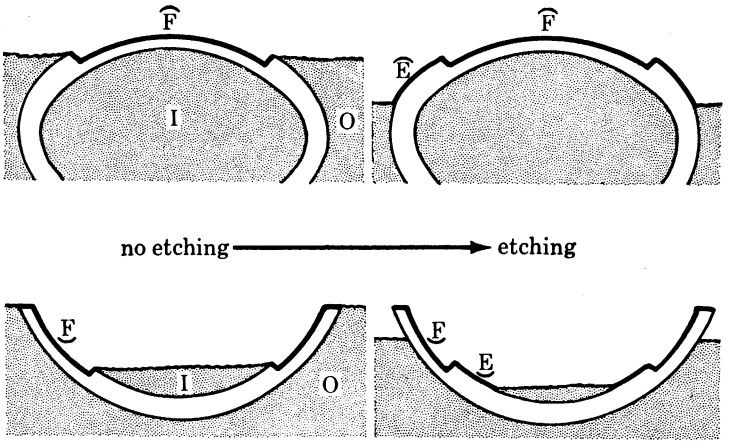

FIGURE 3. Rationale of fracturing and etching red blood cell ghosts assuming membrane splitting. Without etching, fracture exposes convex ($\widehat{F}$) and concave ($\underset{\frown}{F}$) fracture faces. As the ice inside (I) and outside (O) the cell is lowered by etching, convex ($\widehat{E}$) and concave ($\underset{\frown}{E}$) etch faces are exposed.

The rationale of Pinto da Silva & Branton's experiments is explained in figure 3. We reasoned that if the fracture passes through the hydrophobic interior of a membrane, neither the membrane surface nor any surface attached markers would be exposed. If fracturing were followed by etching to sublime away the surrounding ice, both the fracture face as well as the surface with any attached markers would be exposed. A ridge representing a portion of the membrane thickness would form the border between the fracture face and the etch face.

With this reasoning in mind, etching experiments were performed with red blood cell ghosts to which ferritin had been conjugated using a bifunctional organic reagent. Because of the electron scattering properties of the iron in the ferritin core, we could demonstrate by standard thin sectioning techniques that the ferritin was attached to the surface of the cell (figure 4, plate 23). When the cells with attached ferritin were freeze-etched, ferritin was clearly visible on the etch faces (figure 5, plate 23). On the other hand, no ferritin was detected on the fracture faces which were identical to those of control cells without conjugated ferritin. Thus only the etch faces and not the fracture faces show the morphological features associated with the membrane surface. These observations together with analogous ones on the inside concave faces of ferritin-labelled red blood cell ghosts (not shown) show that the structural detail seen on fracture faces cannot be that of the membrane surface. The fracture process must expose structure within the membrane matrix itself.

Having demonstrated that membranes split during the freeze-etching, we must ask: what are the extended smooth regions of the membrane face seen by freeze-etching? What are the particles which interrupt these smooth faces?

One approach we have used to answer these questions is the study of model systems composed of individual membrane components. Several years ago we studied stearic acid bilayers and were able to demonstrate that when these bilayers were frozen, fracture caused them to split between the methyl end groups of the fatty acid molecules (Deamer & Branton 1967). Believing that similar fracture between the methyl ends of membrane phospholipids might account for the extended smooth regions seen in membranes, Deamer, Leonard, Tardieu & Branton (1970) examined a range of bulk phase fatty acids and phospholipids. In all cases where the existence of a lamellar phase structure was indicated by an independent observation such as X-ray diffraction, the freeze-etch results (figure 6, plate 24) showed extensive smooth faces similar to the smooth regions seen in biological membranes. When synthetic phospholipids with saturated and unsaturated fatty acid groups were compared, no differences in the fracture

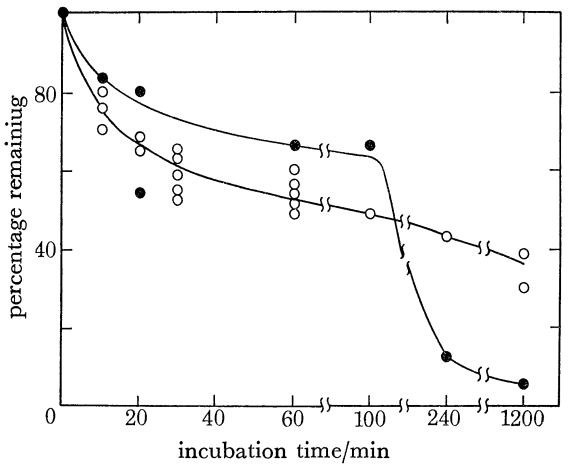

FIGURE 9. Protein (○) and particles (●) remaining after incubation of red blood cell ghosts in pronase. (This and figure 10 are from the work of Engstrom (1970).)

faces were apparent. Examinations of phospholipids with differing polar groups showed that substitutions in this hydrophilic portion of the membrane did not alter the appearance of the fracture faces. Neither the degree of hydration (figure 7, plate 24) nor the admixture of other components such as cholesterol altered the freeze-etch images. In all cases where a lamellar phase was examined, extended smooth sheets were seen.

Before concluding that these extended smooth sheets were diagnostic for the lamellar phase lipids, Deamer *et al.* (1970) showed that other phases would give rise to different appearances. Several lipid systems occur in hexagonal phases as defined by X-ray diffraction (Luzzati 1968). In these hexagonal phases the lipid molecules are arranged in rods of indefinite length; the packing of the rods is hexagonal. The freeze-etch images of all the hexagonal phase lipids examined were distinctly different from those of lamellar phase lipids. The fracture faces of hexagonal phase lipids were composed of indefinitely long parallel lines, giving the fracture face a ribbed appearance (figure 8, plate 24). Furthermore, each specimen contained at least two fracture planes at approximately 120° to each other.

These observations show that consistent and recognizable features distinguish different lipid phases when prepared by the freeze-etch technique. Hence, many aspects of the long range molecular order detected by X-ray diffraction can be visualized by freeze-etching bulk phase lipids. In biological membranes it is probable that the smooth regions represent fracture

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through lamellar lipid regions. Because the bulk phase lipids, even when hydrated, did not contain any discontinuities which might contribute to the particulate appearance of fractured biological membranes, it was suggested (Deamer *et al.* 1970) that the particles in biological membranes may be due to proteins or localized and specific protein–lipid interactions.

We have investigated this suggestion by using freeze-etching to follow the course of proteolytic digestion in red blood cell ghosts (Engstrom 1970). If the particles represent proteins within the membrane matrix, they should disappear when protein is removed from the ghosts before freeze-etching. Red blood cells were incubated in the proteolytic enzyme pronase for varying time periods. The course of protein hydrolysis was monitored by simultaneously measuring protein loss and particle loss (figure 9). Preliminary experiments indicated that pronase attacked all of the cells in our preparation with equal vigour. Furthermore, the digested ghosts remained intact and retained their osmotic properties. Loss of membrane protein initially caused extensive particle aggregation with little decrease in particle number (figures 10*a–c*, plate 25). The initial resistance of the particles to pronase digestion is consistent with the view that the particles are buried within the hydrophobic region of the membrane matrix into which pronase diffusion would be greatly retarded. Most of the particles were lost after more prolonged pronase treatment (figure 10*d*). This eventual removal of particles from extensively digested ghosts is consistent with the hypothesis that the particles are due to protein.

Although we are encouraged by these preliminary attempts to identify the nature of the smooth areas and the membrane particles, it is clear that complete characterization of structures seen in freeze-etch preparations will require investigation by more direct experimental approaches. Ideally, one would want to isolate membrane fractions enriched for particles or enriched for smooth areas. Indeed, it may be here that the freeze-etch technique offers its greatest potential in future studies of membrane structure. This technique should allow us to monitor fractionation procedures designed to separate the membrane into morphologically homogeneous preparations enriched for the smooth or particulate regions. These morphologically homogeneous preparations could in turn be analysed with the physical probes required to determine the details of molecular orientation and interaction.

Our observations of the structural heterogeneity within the membrane matrix warn us that investigations which assume homogeneity can only produce an incomplete picture of the totality of membrane structures. Such assumptions may in fact overlook the most important components of membranes. Indeed, one would expect the compositional and functional diversity of membranes to have a structural counterpart. By displaying this structure, freeze-etching provides the background we need to apply the physical and biochemical averaging techniques to a heterogeneous system.

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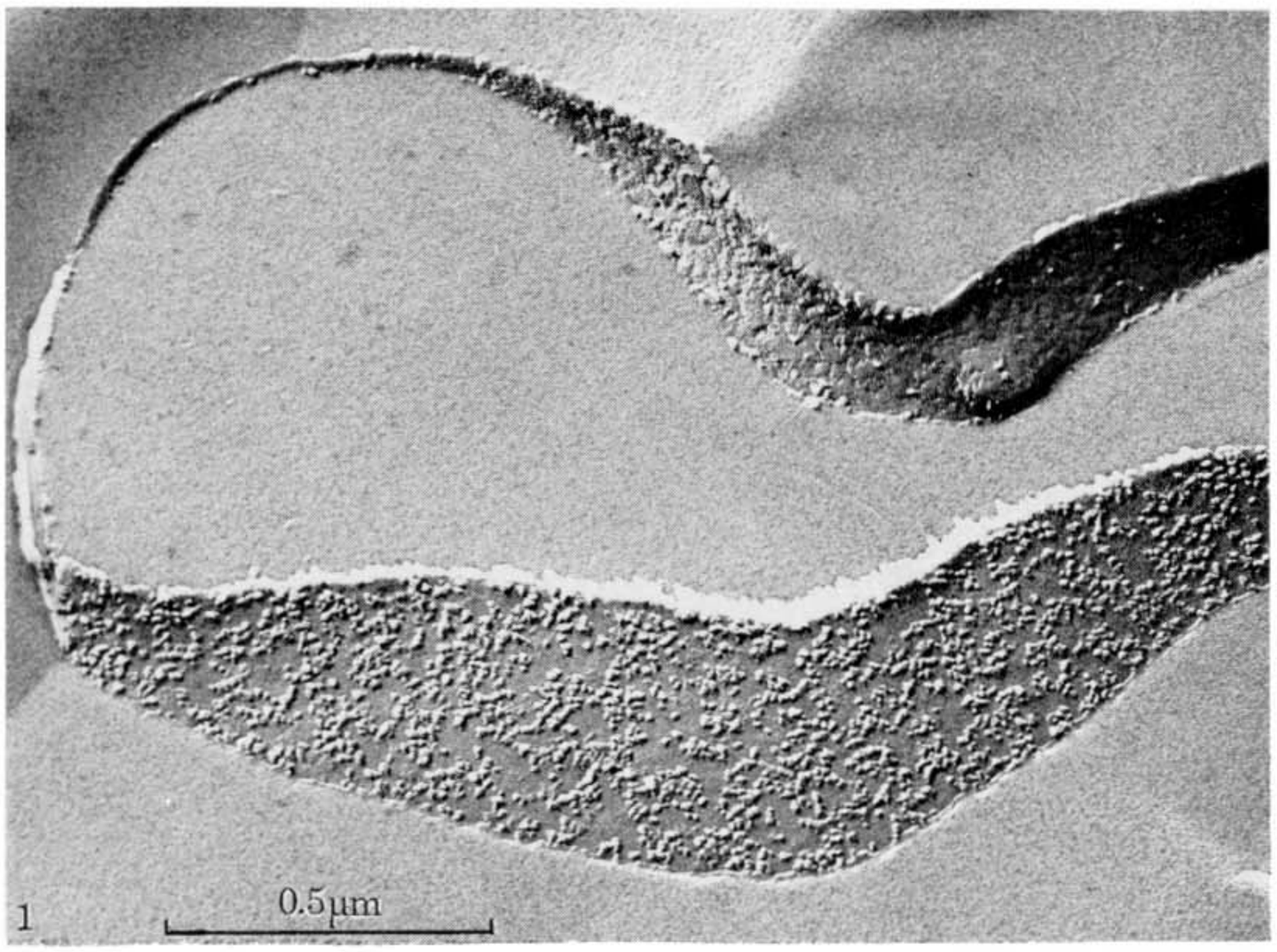

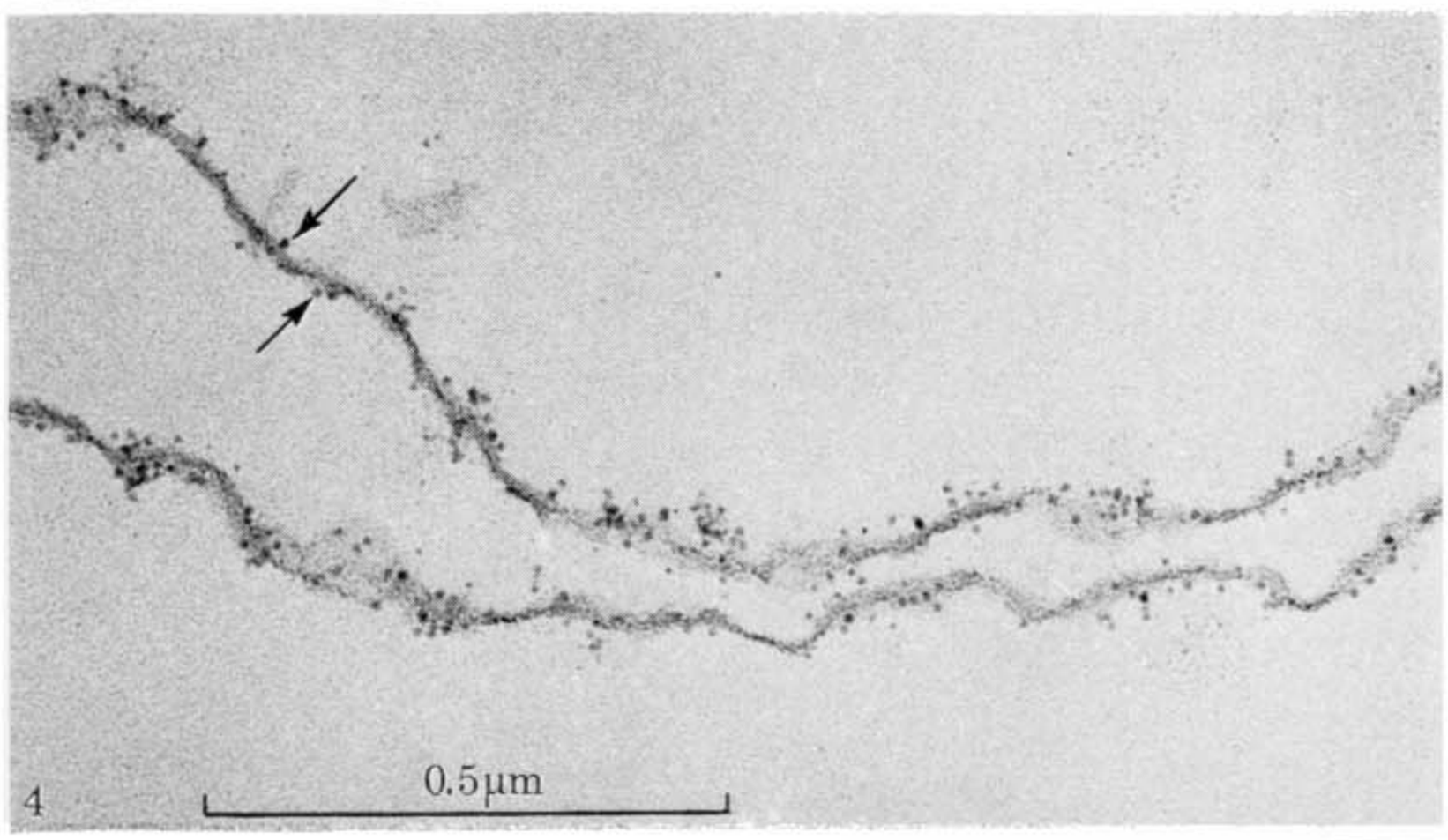

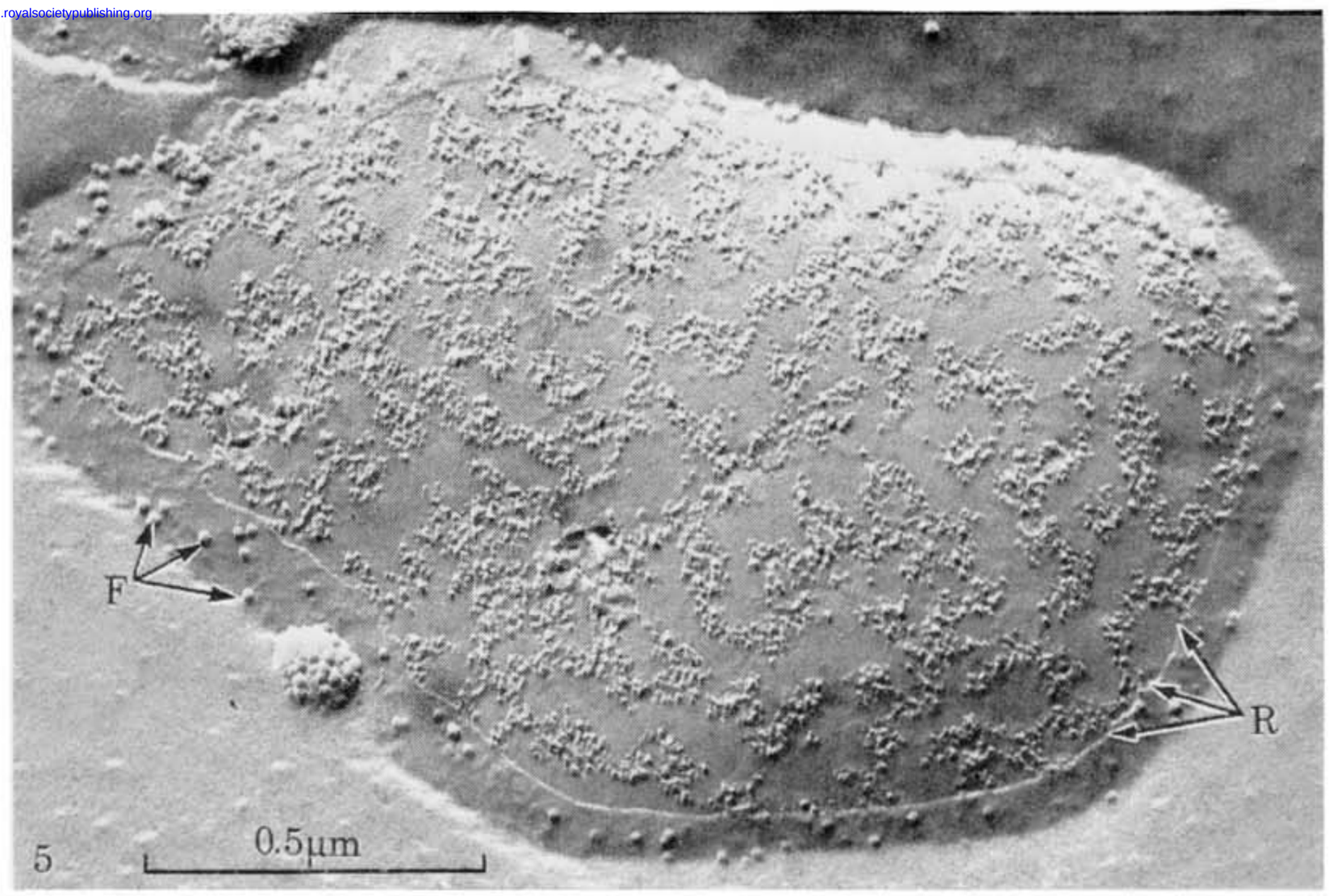

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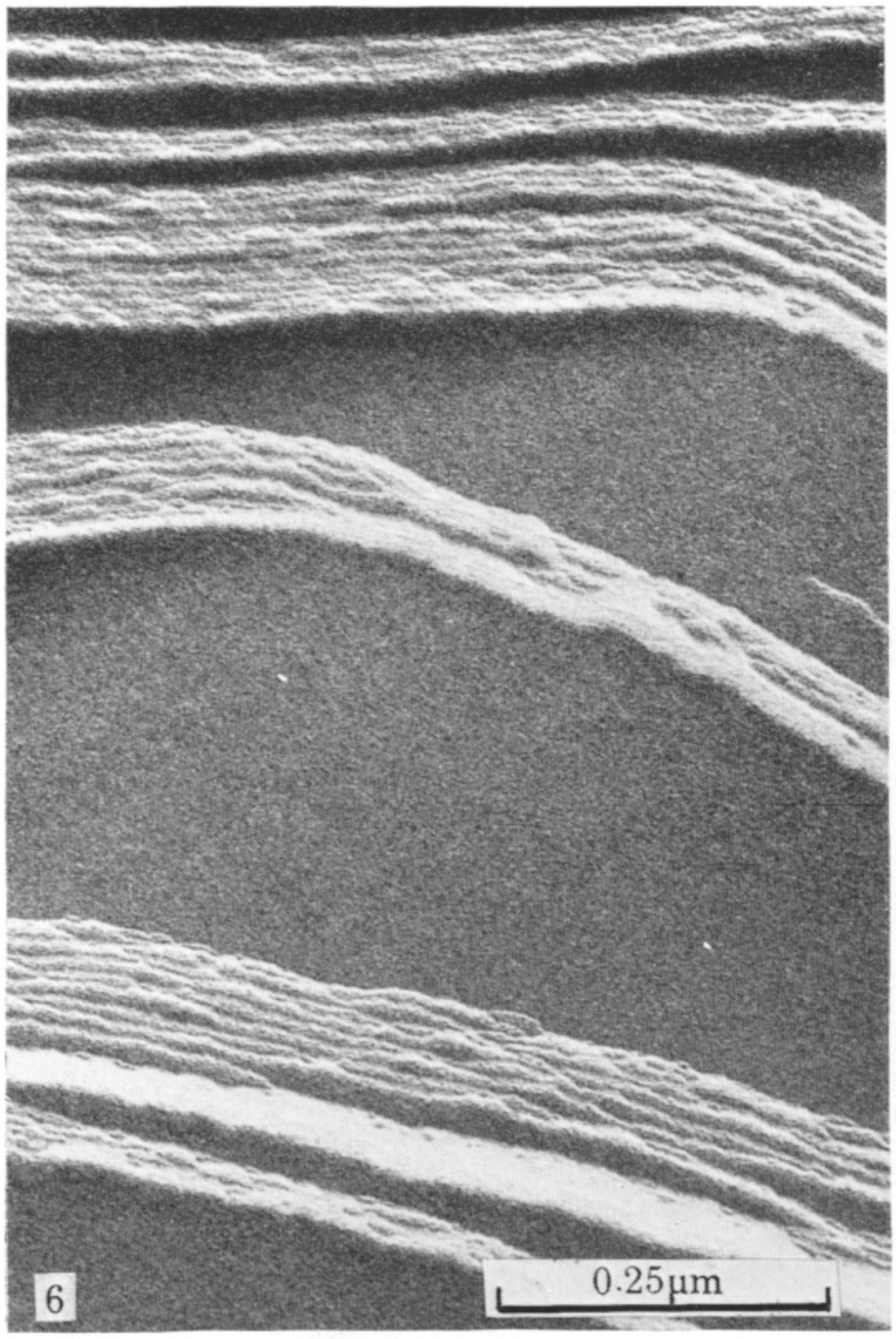

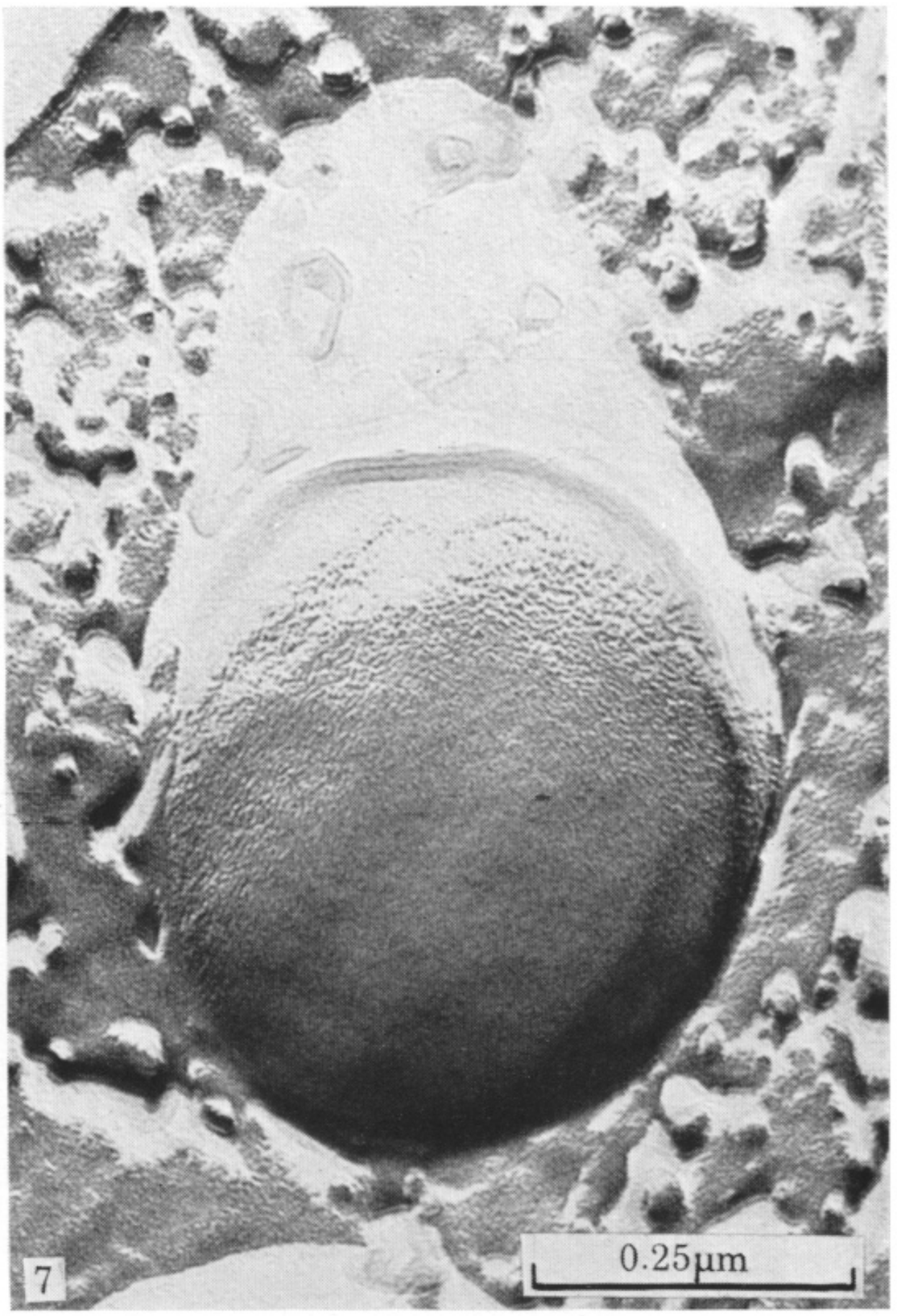

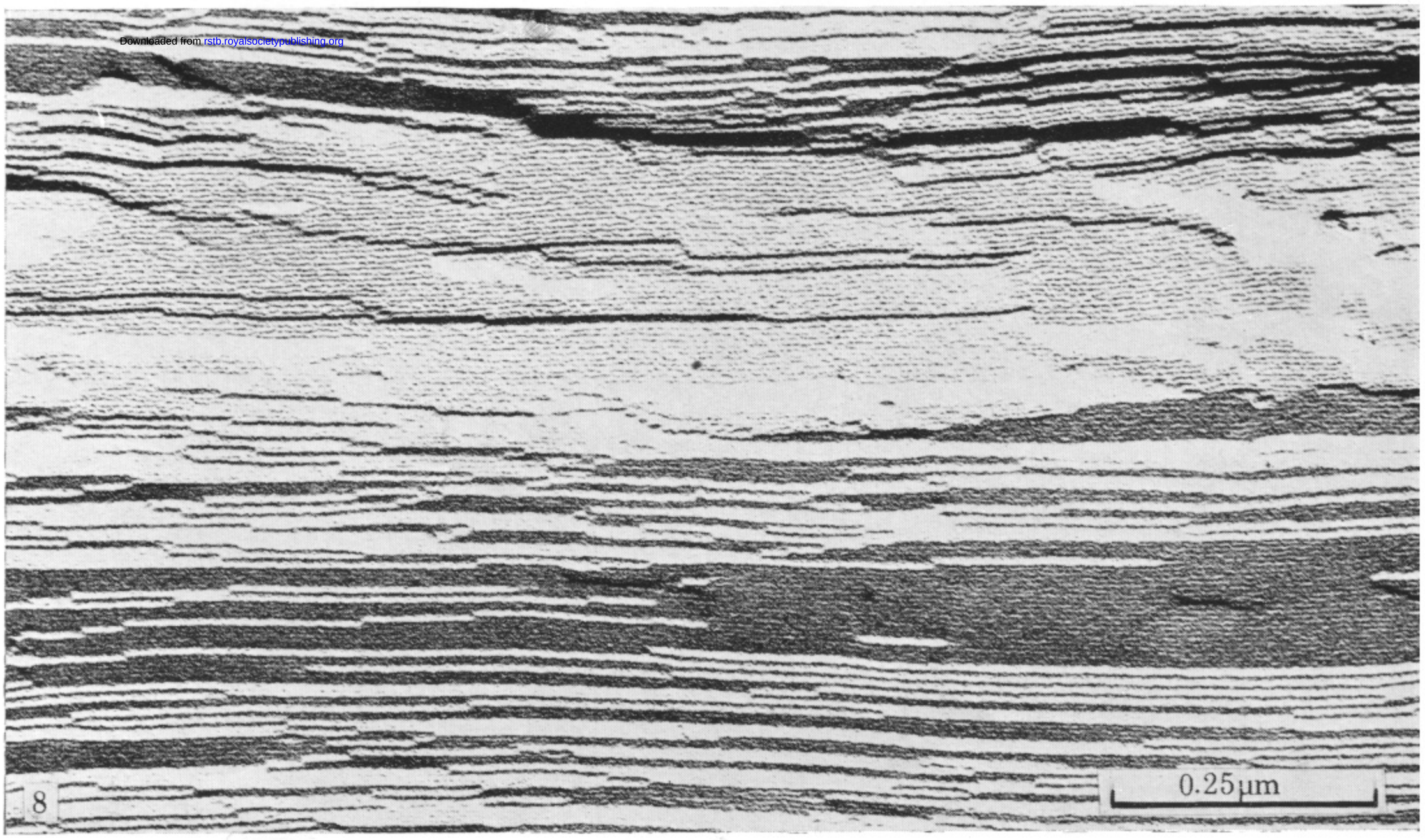

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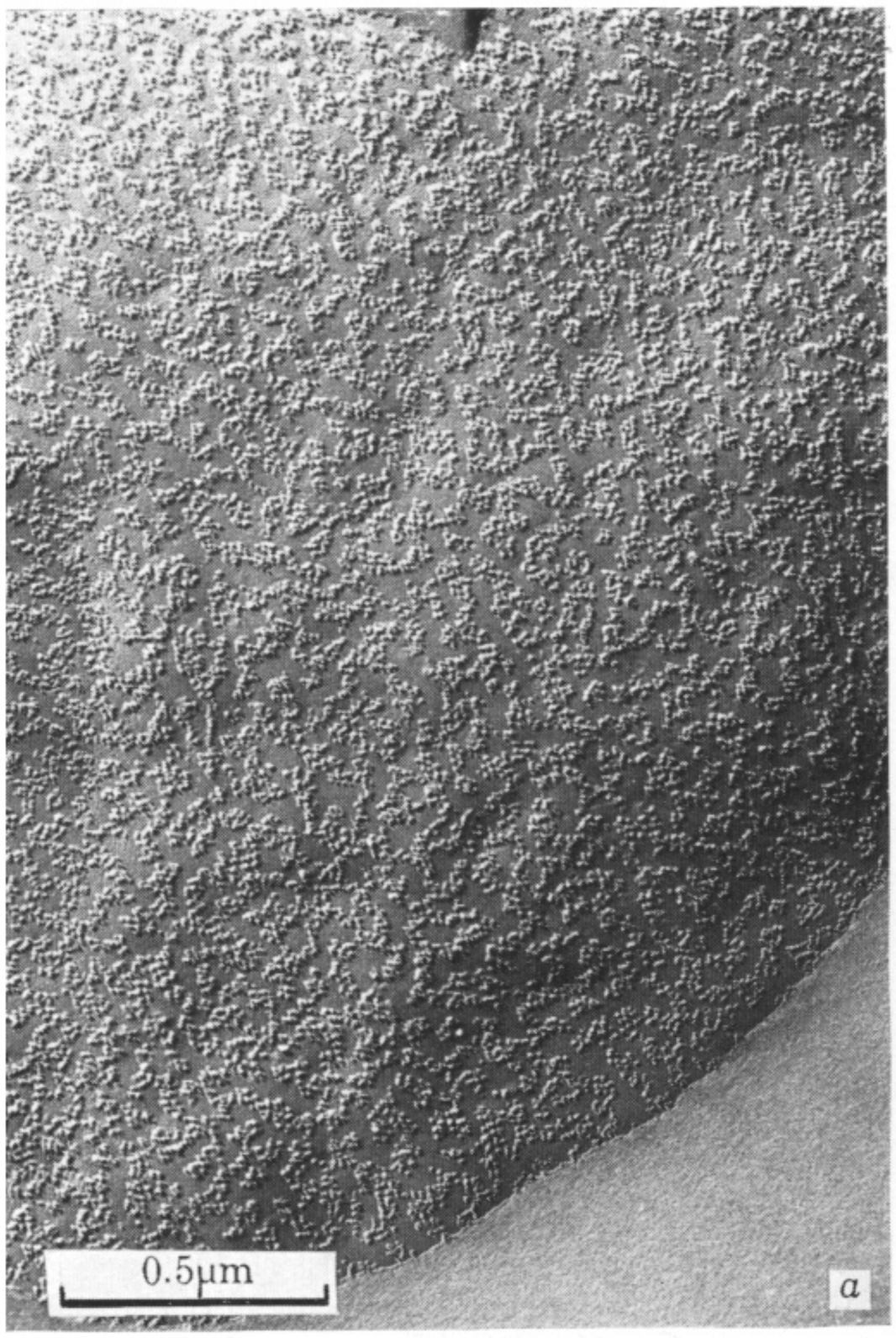

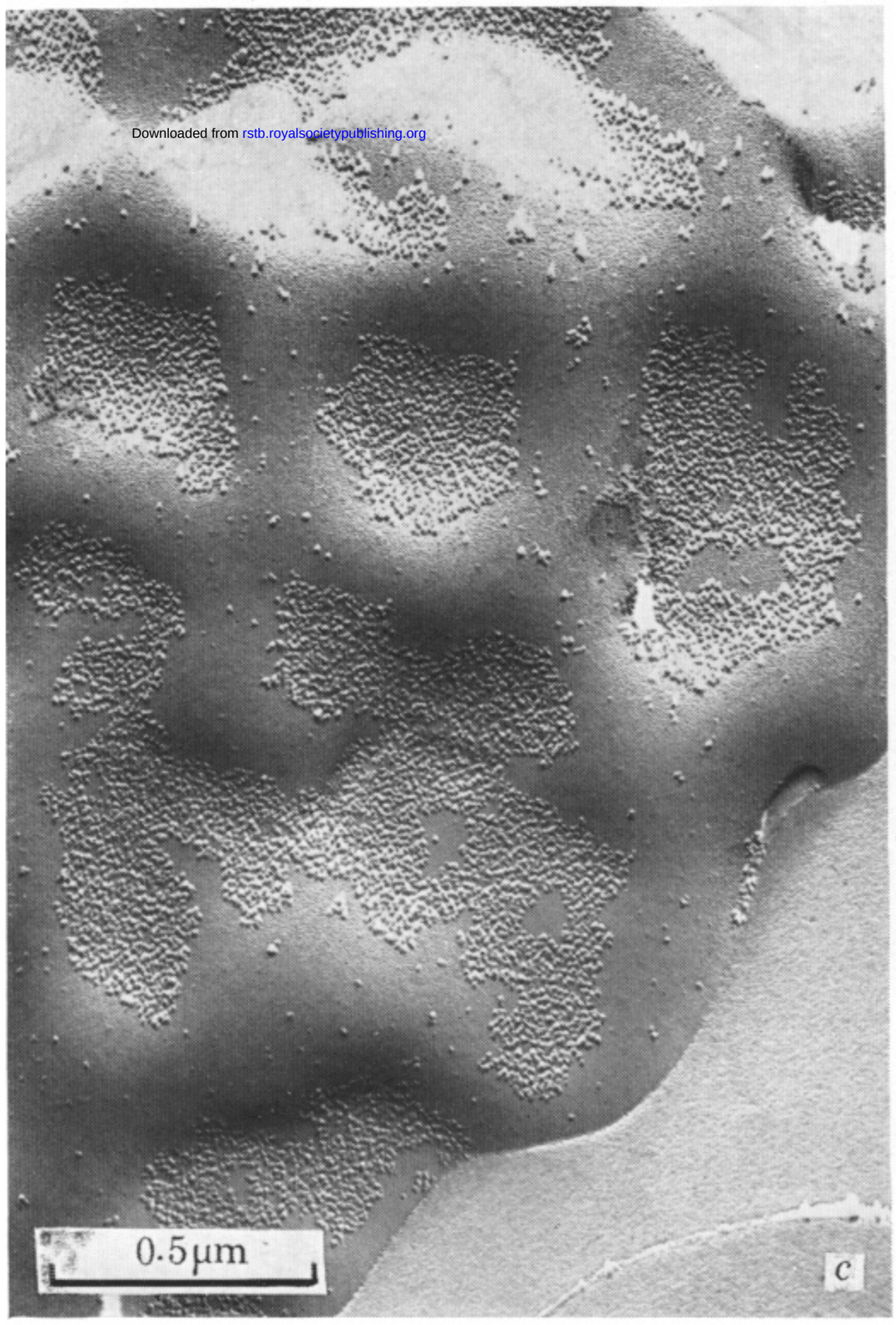

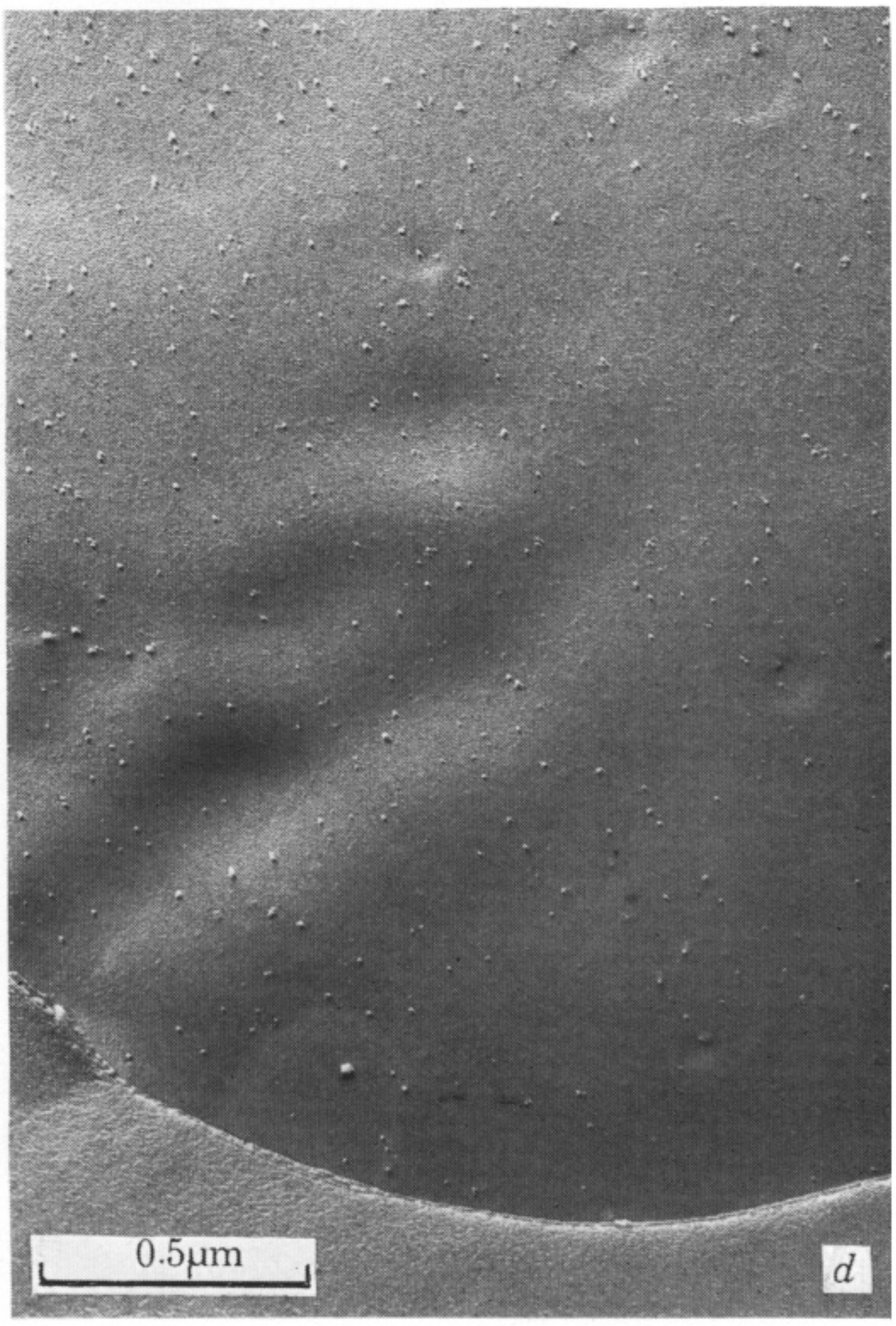

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