The Fine Structure of Freeze-Fractured Blood Platelets

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The present study has examined the fine structure of freeze-fractured human platelets. Advances in methods used for cell preparation and freezing, together with better instrumentation, have resulted in improved preservation of platelet ultrastructure. Aspects of surface membrane topography and the number, depth of penetration, tortuosity and fenestration of channels belonging to the open canalicular system were revealed in a manner which cannot be fully appreciated in thin sections of platelets. Elements of the dense tubular system of channels and masses of glycogen were identified in the platelet replicas. The technic may provide an important approach for defining the structural physiology and pathology of blood platelets (Am J Pathol 70:45–56, 1973).

THE TECHNIC of freeze-fracture and etching has provided an exciting new approach for evaluating fine structure in cells and tissues.[1–3] Details revealed in replicas obtained from freeze-cleaved surfaces contain structural features which were relatively obscure or absent in cells prepared for study in the electron microscope by other methods. The procedure has been particularly useful for the characterization of plasma membrane topography, as well as inter- and intracellular membrane systems.[4–6] Although the technic is not free from artifact,[7] it adds an important new dimension to the continuing effort to identify the macromolecular organization of cell structures.

Freeze-fracture has been used in previous investigations of blood platelets.[8–13] The results of our initial efforts, however, were disappointing. We have been encouraged to employ the procedure again because of improvements in cell preparation, instrumentation and appreciation of the advantages and disadvantages of the method.[4–6,14–16] Our particular interests concern changes in the surface structure of activated platelets, the relationships of membrane systems within the cell and the interaction of membranous channels and organelles during platelet secretion. The present report will emphasize general features of freeze-cleaved platelets, some of which were not clearly defined in the

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Supported by Grants HE-1180, AM-15010 and CA-08832 from the US Public Health Service and the Cardiovascular Clinical Research Program Project.

Accepted for publication Sept 1, 1972.

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past, and will also serve as a background for detailed explorations of altered platelets in subsequent reports.

Materials and Methods

The technics used in this laboratory to obtain blood from normal donors in 3.8% trisodium citrate, separate platelet-rich plasma (C-PRP), and prepare control and experimental samples for study in the electron microscope were described in detail in several recent reports.[17–19] Initial steps in the preparation of platelets for freeze-fracturing were similar to the procedures used routinely for preservation of ultrastructure. An equal volume of 0.1% glutaraldehyde buffered to pH7.3 with White's saline was added to samples of C-PRP maintained at 37 C. After 15 minutes, the samples were sedimented to buttons, the supernatant discarded, and freshly prepared 3% glutaraldehyde in buffered White's saline was added to the pellets. The platelets were incubated in the fixative solution at room temperature for 15 minutes, then chilled to 4 C for 1½ to 2 hours. At that time, the fixative was discarded and replaced with 20% glycerol in White's saline for 10 minutes. The pellet was then cut into small pieces, transferred to special holders and dropped immediately into receptacles containing liquid freon suspended in liquid nitrogen. After the initial step of freezing, the samples were placed in liquid nitrogen until ready for fracturing. Holders with the frozen platelet samples were transferred quickly to the stage of a Balzer's freeze-fracture unit cooled with liquid nitrogen and exposed immediately to high vacuum. The frozen platelets were fractured at −120 C, allowed to etch at that temperature for 30 seconds and then freed from tissue by soaking the samples in 30% chlorine bleach. The replicas were washed in distilled water, mounted on uncoated 200 or 300 mesh grids and examined in a Phillips 200 electron microscope.

Results

At first glance the appearance of freeze-cleaved platelets was bewildering and difficult to compare with platelet fine structure viewed in thin sections.[11] After experience with the technic, most of the structures identified in thin sections could be found in replicas of freeze-fractured cells. The fracture plane may crack through the platelet interior, pass up and over the tops of the cells or shell out the contents, leaving an empty membrane embedded in the ice (Figure 1). Platelet surfaces revealed by the fracture were relatively smooth in appearance. Small particles, approximately 40 to 50 Å in diameter, were randomly dispersed over most of the surfaces, but not on every cell. In some examples the exposed surface appeared very smooth and free of particles. Granules were as common on surfaces interpreted as the inner side of the cell wall as on the outside face. They also covered the inner and outer surfaces of membrane systems and organelles in the platelet interior (Figure 2). Small granules were more abundant on some surfaces than on others, and in some examples they resembled short filaments rather than particles. Whether the particles represent

intrinsic structures of the membranes or artifacts of the technic will be determined in subsequent investigations.

The problem of determining which face of a membrane is exposed by the fracture has stirred considerable discussion.[3,14,20–22] In freeze-cleaved erythrocytes the fracture has been shown to split the lipid bilayer of the cell wall.[22] As a result, the similarity in size and distribution of particles on what appear to be outer and inner sides of a membrane may be due to the fact that they are really on the inner surface of the outer layer or the outside of the inner portion of the lipid bilayer.

This rationale, however, may not be applicable to freeze-fractured platelets.[8,13] Examination of replicas, in which contents of the cell were exposed, revealed a system of interior channels which communicated directly with the platelet surface (Figure 2). Membranes of the open canalicular system were continuous with the membrane of the cell wall (Figure 3). If the plane of fracture splits the lipid bilayer of the platelet surface, then it should have broken the continuity between open channels and the cell wall at some point. In some examples, the platelet surface membrane was fractured in such a manner that inner and outer layers were revealed (Figure 1). This did not occur, however, at junctions between membranes of the channel system and the surface (Figures 2 and 3). The finding suggested that the fracture did not split the lipid bilayer of the platelet membrane as it does in the red blood cell.

The open canalicular system was clearly revealed in replicas of freeze-cleaved platelets (Figures 2 and 3). Channels could be followed from one or more sites of communication with the cell wall along tortuous courses deep into the cytoplasm. The fracture plane often passed over the top of a channel and then broke into it, revealing the inner and outer surfaces and the amorphous contents. Separate elements of the canalicular system communicated with each other in the platelet interior. Fenestration of the open canalicular system was well demonstrated (Figures 2 and 3), and individual canaliculi in certain areas split into separate channels which separated and then fused together at another site. This honeycombing of the cytoplasm greatly increased the total surface area of canalicular membrane in contact with the platelet interior. The unusual arrangements of fenestrated channels closely resembled the membrane complexes recently described in thin sections of platelets.[23]

Elements of the dense tubular system of channels were distinguished by their narrow diameter, angular shape and relative absence of surface granularity (Figure 4A). They were found most easily in their

usual location at the polar ends of the cells and, occasionally, in small groups deep in the cytoplasm. Interaction of fenestrated channels of the open canalicular system and canaliculi of the dense tubular system occurred most often in the area of the membrane complex (Figure 3). In other areas of the cytoplasm, it was difficult to distinguish channels of the dense tubular system or separate them clearly from elements of the open canicular system.

Characterization of specific organelles in the interior of freeze-fractured platelets presented a difficult problem (Figures 2, 3 and 4). The surface granularity of the membranes covering most organelles resembled the particles on the platelet surface membrane and channels of the open canalicular system. When the fracture plane cracked open an organelle, the presence of nucleoids identified granules, and the presence of cristae identified mitochondria (Figure 4B). Some of the granules with nucleoids may have been dense bodies, since the replicated interior of the two organelles would be similar.[19,24] An occasional organelle in the cytoplasm was covered by a more concentrated coat of small particles or spike-like projections (Figures 2A and 4A). These may be dense bodies, since they occur in about the same frequency as serotonin storage organelles in thin sections. The identification, however, was based on exclusion rather than certainty.

Microtubules and microfilaments were rarely identified in our preparations, probably because the samples were not etched at a higher temperature after fracturing at −120 C (Figure 4C). Masses of glycogen particles, however, were apparent in some of the replicas (Figure 4D). The particles were packed tightly together and had dimensions similar to glycogen examined by the thin section or whole mount technics.[11]

Discussion

The fine structure of freeze-fractured platelets was described initially by Ruska and Schulz [8] and subsequently by Behnke,[9,10] Hoak,[12] Mason and Reddick,[13] and White.[11] Although the number of detailed reports is limited, it is evident from previous work and the present investigation that replicas of freeze-cleaved platelets are very similar to the appearance of platelets examined in thin sections from plastic-embedded samples. Platelet discoid shape is maintained in replicas of freeze-fractured cells. The organelles are similar in size, number and distribution; membrane systems are preserved in the relationships found in sectioned platelets.[11] The similarity in ultrastructure supports

the validity of many findings made in studies of platelets by the thin section technic.

Identification of specific types of platelet organelles presented a difficult problem. When the fracture passed over the surface of an organelle or scooped it out, it was not possible to distinguish granules, mitochondria, or dense bodies; nor was it certain that some of the structures resembling organelles were not, in fact, dilated elements of the open canalicular system. Only when the fracture cleaved through an organelle could a characterization be made. Even then, separation of granules from the serotonin storage organelles was not clear, because of their expected similarity in freeze-fractured replicas.[19,24] These difficulties are not impossible to solve, and application of several experimental procedures employed previously to characterize organelles in thin sectioned platelets may answer the problem in the near future.

The technics of freeze-fracture offers a particular advantage for the study of membrane surfaces.[3–6] Large areas of membrane are revealed and small particles randomly dispersed or gathered in regular geometrical arrangements have been identified.[4–6,14–16] The most serious problem in evaluating surface topography by this method has been to determine which face of the membrane has been exposed. In studies on erythrocytes it has been clearly demonstrated that the fracture splits the lipid bilayer of the cell wall.[20,22] As a result, the surface revealed in the replica is most likely the inner face of the outer layer or the outside of the inner lamella. Deep etching reveals the exterior aspect of the outer layer of the red cell membrane, and fortuitous cleavage exposes the cytoplasmic face of the inner layer. A similar exposure of different faces of the lipid bilayer has been observed in replicas of freeze-fractured surface membranes of other cell types[6,21] and may prove to be a universal finding.

The platelet, however, may be an exception to this rule.[8,13] Surfaces revealed in replicas of freeze-cleaved platelets were either covered by a fine layer of randomly dispersed 40 to 50 Å particles or appeared smooth. Some of the smooth surfaces were secondarily fractured, revealing a second smooth face underlying the first. This relationship suggested a bilayered structure of the surface. The unit membrane of the cell wall of the platelet is continuous with channels of the open canalicular system which penetrates deep into the cytoplasm of the cell. Channels were frequently fractured in a manner which revealed an outer surface, material lying in the lumen, and, when substances filling the lumen had been scooped out, the interior floor of the channel. Both the outer surface of the fractured canaliculus and the in-

terior face were continuous with the cell wall. The nature of that continuity suggested that the fracture did not split the bilayer of the membrane, but revealed either the exterior face of the outer leaflet or the cytoplasmic aspect of the inner lamella. If the true exterior surface of the platelet was revealed, as suggested by these observations, then a large number of studies related to molecular aspects of platelet stickiness will be possible. The use of surface markers and deep etching will be required to determine if the platelet is truly an exception to the rule established in red blood cells.[22]

Replicas of freeze-fractured platelets provide a dramatic demonstration of the number, depth of penetration, tortuosity and complex arrangements of channels of the open canalicular system. The findings have been described previously on the basis of observations made on thin sections,[9,11,23,24] but the tendency of the fracture to follow channels for considerable distances provides a clearer picture of the extent of this system. Replicas reveal the fenestrated organization of canaliculi in some areas of platelet cytoplasm. Previous studies have indicated the importance of the open channels and their interaction with elements of the dense tubular system to form membrane complexes at the sites of fenestration.[23] Freeze fracture and etching combined with other methods may provide answers to the function of this unusual arrangement of membranes which could only be speculated upon in the past.[11]

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Acknowledgments

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Legends for Figures

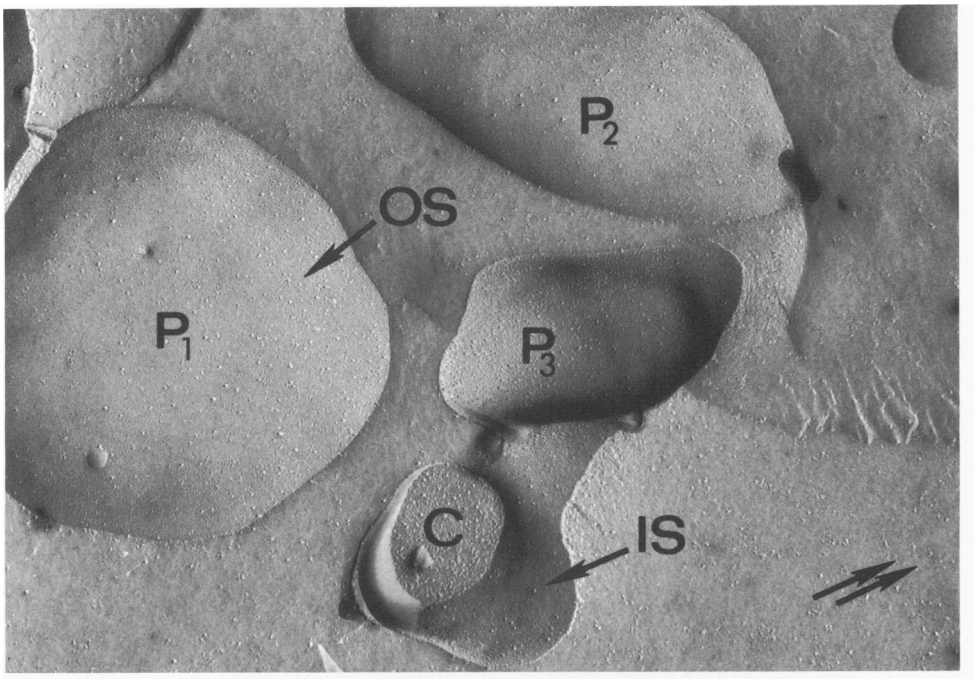
Fig 1A—Platinum-carbon replica of freeze-fractured blood platelets. The fracture has passed over the surfaces of two cells (P_1, P_2) and partially shelled out the contents of a third platelet (P_3), leaving a small fragment of cytoplasm (*C*). Small particles, 40 to 50 Å in diameter, are randomly dispersed over the outer surface (*OS*) of platelets P_1 and P_3 and on the inner surface (*IS*) of the third cell. The *double arrows* in the lower right corner of this and subsequent micrographs indicate the direction of metal shadowing (× 33,200). **B**—The platelet surface revealed in this replica is buried in the ice and appears to be the inner surface of the cell wall. It is nearly free of the small particles which coated the surfaces of cells in the previous illustration. The fracture has torn through the lipid bilayer, revealing the interior aspect of the inner lamella (*1*) and the inner face of the outer membrane leaflet (*2*) (× 56,500). **C**—The plane of fracture in this replica has cleaved into the cytoplasm of a platelet, revealing some of the internal contents. The outer and inner surfaces of granules(*G*) and channels (*C*) of the open canalicular system are apparent. In one area, cytoplasm has been scooped out, revealing the inner surface (*IS*) of the cell wall. A

membrane (*M*) in the midst of the organelles has been fractured in the same manner as the cell wall in the previous illustration. The double layered structure is characteristic of membranes. However, it is not possible in this example to determine if the membrane originates from a granule, an element of the channel system or the cell wall (× 49,800).

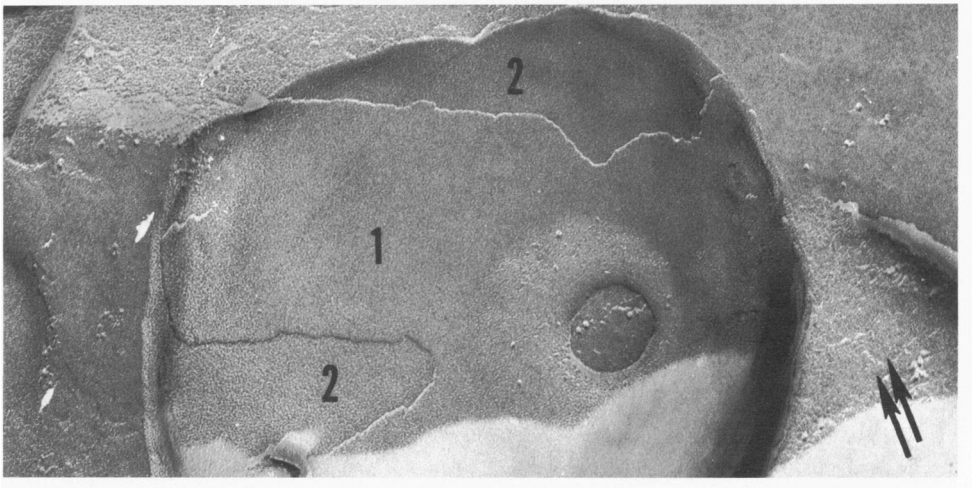
Fig 2A—The platelet in this replica has been fractured in the equatorial plane. The bewildering array of surface features revealed in this replica are difficult to relate to the organization observed in thin sections of platelets. However, granules are the most numerous structures in the cytoplasm of sectioned cells, and the majority of the surfaces revealed in the replica are oval or round, similar in size, and randomly dispersed. Most of these profiles are granules (*G*), although some may be mitochondria or serotonin storage organelles. One type of organelle found in the replica is densely coated with small particles or spike-like projections. The frequency of this type of organelle in replicas is similar to the frequency of dense bodies in sectioned cells, and the possibility that this form represents the dense body (*DB*) is suggested. Myriads of short membrane segments are distributed randomly or clustered together in the cytoplasm. The sites where they are clustered resemble membrane complexes (*MC*) (× 37,000). **B**—The platelet in this replica has been fractured in the vertical plane, revealing the typical discoid appearance of the cell. Channels (*C*) of the open canalicular system are readily identified because of their continuity with the surface membrane of the platelet. The tortuous course of the channels as they tunnel into the cytoplasm is also revealed (× 41,500). **C**—The relationships of channels from the open canalicular system to the cell surface and cytoplasm is well demonstrated in this example. A channel (*C*) continuous with the outer surface (*OS*) of the cell wall splits into multiple canaliculi in the cytoplasm. The fenestrated organization of the channels maximizes contact with the cytoplasm and elements of the dense tubular system in membrane complexes (*MC*) (× 49,800).

Fig 3A—The fracture plane in this replica has passed over the surface (*OS*) of one channel communicating with the platelet surface (C_2), and broken into the lumen of the channel, revealing the inner surface (*IS*) of the membrane and a second communication (C_1) with the cell wall. Projections of this same channel (*C*) can be followed deep into the cell substance. Particles covering the surface of granules (*G*) and elements of the channel system are indistinguishable (× 46,500). **B**—The similarity of small particles covering the inner and outer membranes of granules (*G*) and channels is apparent in this example. Particles are also present on the cytoplasm; interpretation of their importance in the structure of membranes is not possible. The fracture plane in this replica has revealed the floor of a short segment of a channel (C_1), the outer surface as it penetrates into the cytoplasm, and a connection to another channel (C_2) deep inside the cell. Without such relationships it is difficult to distinguish elements of the open channel system from granules (× 40,000). **C**—The extensive interaction of the open channel system with the cytoplasm and dense tubular system is revealed in this example. An element of the open channel system extending into the cytoplasm from the upper left develops a fenestrated appearance as it approaches the central region of the cell. At this point the membrane is extensively revealed. The stub-like projections in the channel floor are fenestrations where elements of the dense tubular system interact with the channels of the open canalicular system to form membrane complexes (*MC*) (× 31,500).

Fig 4A—The platelet in this replica demonstrates to advantage the inner and outer surfaces of granules and the heavily coated organelles, which may be serotonin organelles, referred to as dense bodies (*DB*). This possibility is suggested because the organelles covered with particles occur in about the same frequency as dense bodies in thin sections of platelets. The short segments of narrow membranes are strikingly similar to elements of the dense tubular system (*DTS*) (× 37,350). **B**—In this replica the fracture has passed through an element of the open channel system (*C*), over a platelet granule (G_2), and through another (G_1). The nucleoid of the organelle (G_1) is revealed clearly in this example (× 70,000). **C**—The replica in the inset reveals an area of platelet cytoplasm with two thick filaments or microtubules. These structures were rarely encountered in our material for reasons discussed in the text (× 56,200). **D**—The fracture plane in this example has cleaved into a channel of the open canalicular system (*C*) with two sites of communication with the outer surface (*OS*) of the cell wall. Uniform profiles packed tightly together in one area of the cytoplasm are similar in size and arrangement to masses of glycogen particles (*Gly*) (× 41,500).



1A



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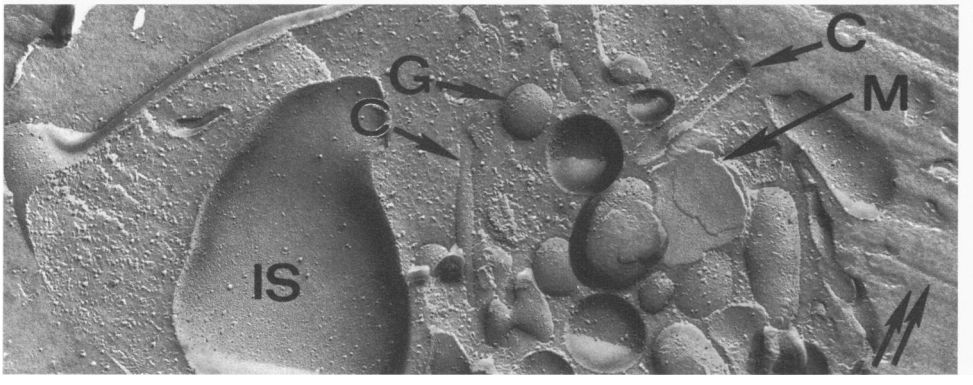
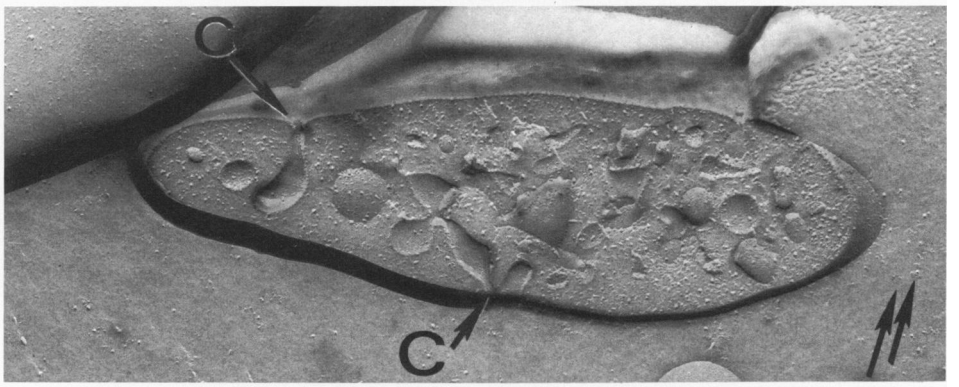


1C

2A



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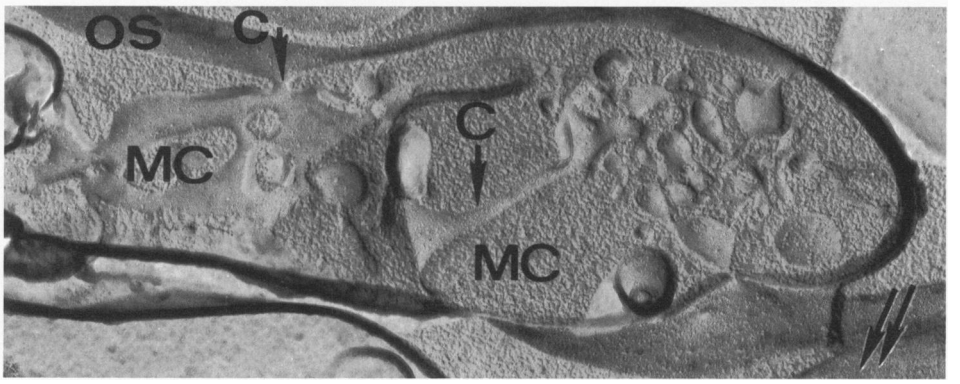

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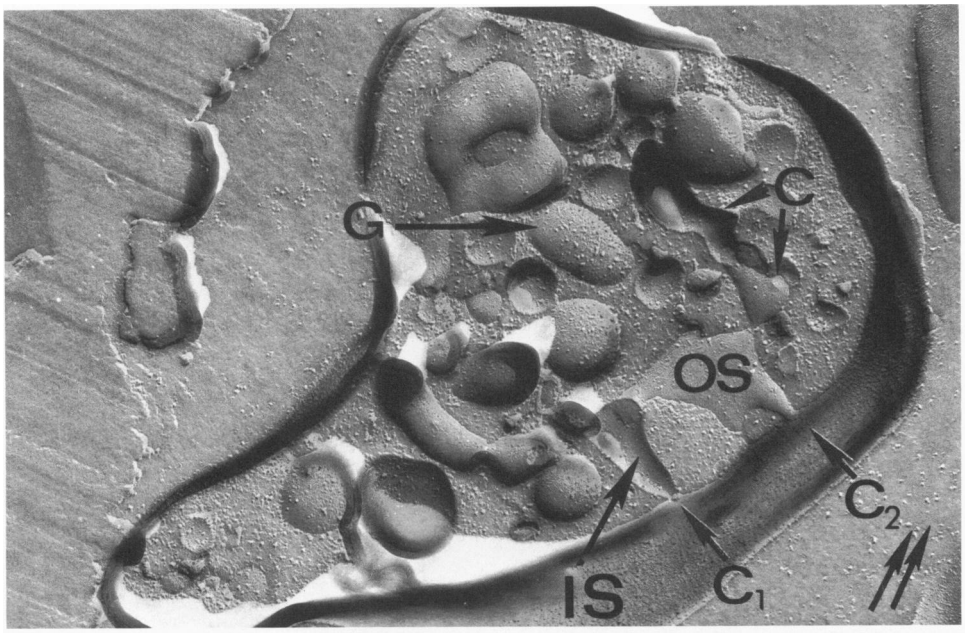

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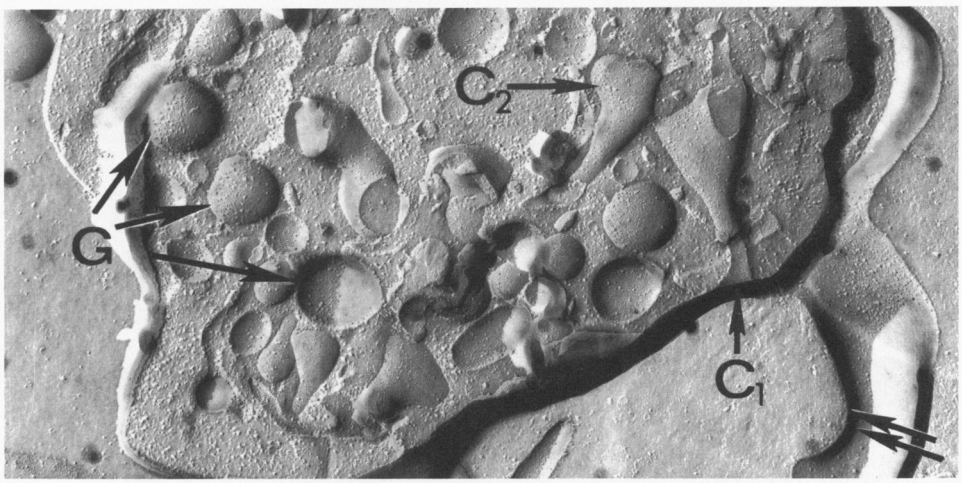

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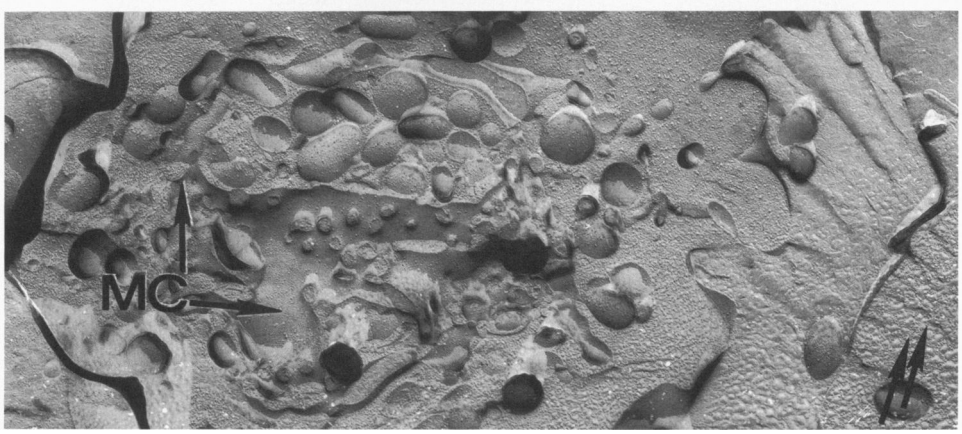

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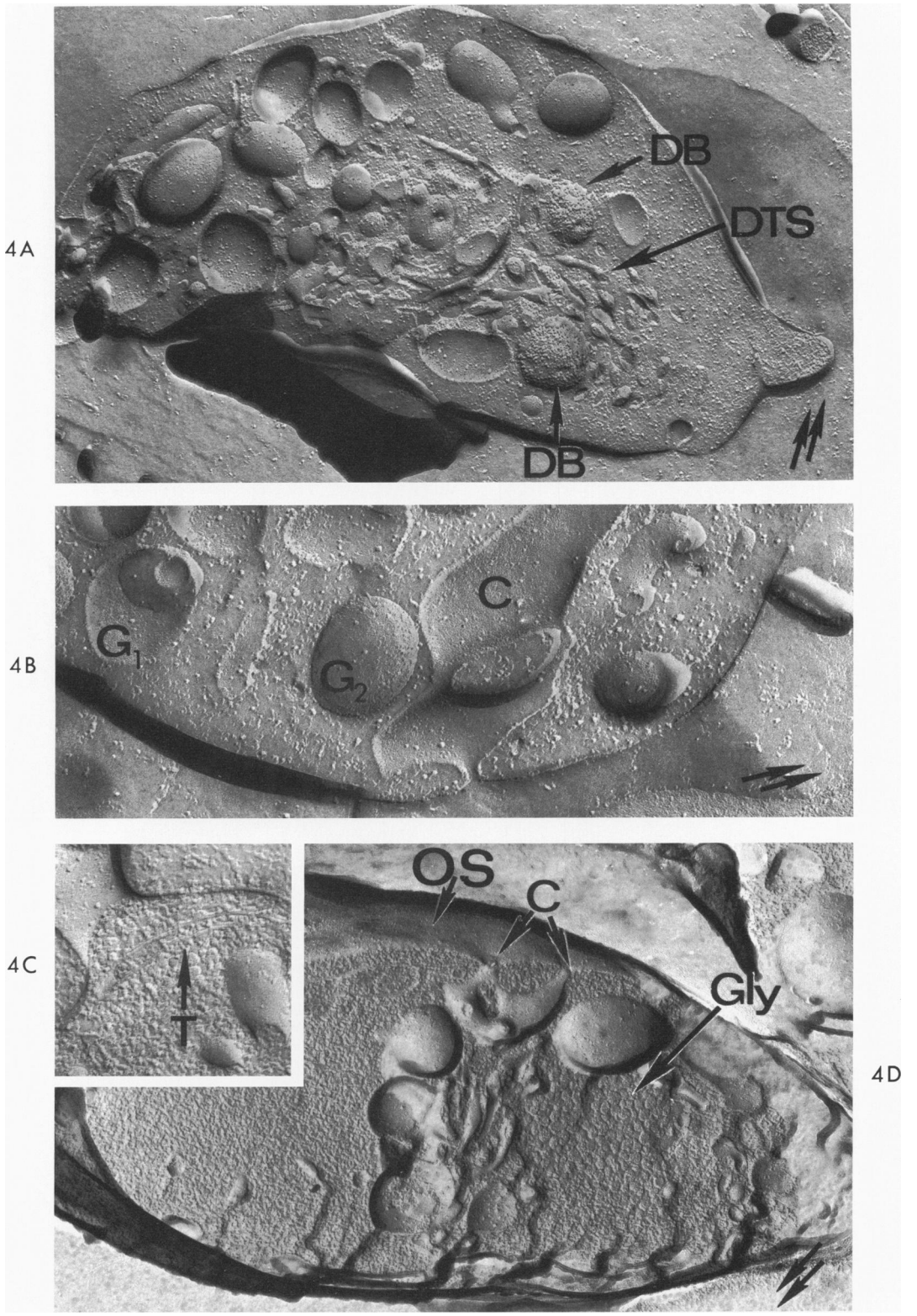

4A

4B

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4D