Interaction of Membrane Systems in Blood Platelets

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The existence of two separate groups of membrane-enclosed channels in platelets, the open canalicular system (OCS) and the dense tubular system (DTS), has been recognized for several years. The present study has employed ultrastructural observations on normal platelets, cells from a child with the gray platelet syndrome and cytochemical localization of peroxidase in platelets to define the origin of the DTS and point out a specialized membrane complex in platelets formed by interaction of channels from the OCS and DTS. The similarities of membrane complexes in platelets to the organization of membrane systems in muscle is pointed out, and their potential function as the sarcoplasmic reticulum of platelets is discussed. (Am J Pathol 66:295–312, 1972)

BLOOD PLATELETS contain two distinct channel systems.[1–4] The open canalicular system (OCS) is composed of tortuous, tubular invaginations of the platelet surface coursing in serpentine fashion through the platelet hyaloplasm. Elements of the dense tubular system (DTS) are also distributed randomly in the platelet substance, but appear to be associated regularly with the circumferential band of microtubules supporting the discoid form of the cell. Channels of the DTS occur in short segments demarcated by an irregular membrane, and, in contrast to clear elements of the OCS, contain an amorphous or filamentous material similar in density to the surrounding matrix of platelet hyaloplasm. Close relationships between channels of the two systems have been noted, but were usually regarded as chance associations.

Investigations of the OCS have demonstrated that canaliculi are continuous with surrounding plasma, that particles taken up by the system can be transferred to apparently intact granules, and that channels remain open before, during and after the physical alterations of aggregation and release of secretory products.[5,6] These findings suggest that the OCS serves as a conduit for uptake of plasma-borne substances, and as a pathway for extrusion of endogenous chemicals secreted during the

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platelet release reaction. The DTS is considered to be a product of the Golgi apparatus[7] or a residual of endoplasmic reticulum from the megakaryocyte,[8] but definite proof of its origin has not been advanced. Investigations of the association between the DTS and circumferential microtubules have suggested that the system may serve as a template for organization and provide filamentous elements for construction of the annular bundle of tubules.[9]

The purpose of the present communication is to provide new evidence for the origin of the DTS from endoplasmic reticulum, and to focus attention on a physical association between channels of the OCS and DTS that may serve a critical role in platelet physiology.

Materials and Methods

General

The methods used to obtain blood from normal donors in 3.8% trisodium citrate anticoagulant, to separate samples of citrated platelet-rich plasma (C-PRP) by two steps of centrifugation, and to prepare the platelets by fixation in glutaraldehyde and osmic acid for study in the electron microscope were described in detail in recent publications.[5,6,10,11] Technics employed in the present investigation were identical.

Gray Platelets

A male child with hemorrhagic problems associated with thrombocytopenia and abnormal platelets was recently reported by Raccuglia.[12] The bleeding problem and low platelet count improved after splenectomy, but the abnormal platelets persisted. Due to the unusual coloration of the patient's cells on Wright-stained smears, the term *gray platelets* was selected, and his condition referred to as the gray platelet syndrome. Dr. Raccuglia kindly permitted us to see the patient and obtain blood samples for study in the electron microscope. The child's clinical history, laboratory analysis and special platelet studies were described in detail. Platelets from the patient were selected for the present investigation because they revealed a process which was not evident in normal cells.

Cytochemical Localization of Peroxidase in Normal Platelets

A variation in our usual procedure of preparation was required in order to secure regular demonstration of peroxidase activity in platelets. Samples of C-PRP were fixed initially at 37 C with an equal volume of 0.1% glutaraldehyde in 0.1 M cacodylate buffer containing 5% sucrose. After 15 minutes, the suspension of platelets was sedimented to a button, and refixed for 30 minutes in a mixture of 2.5% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M cacodylate buffer without sucrose at room temperature. The fixed samples were washed three times in Tris-HCl buffer at either pH 6 or pH 9, then incubated in a modification of the media described by Graham and Karnovsky for demonstration of endogenous peroxidase[13] and adapted by others for localizing catalase.[14]

Our contribution consisted of adding 1% dimethylsulfoxide to the incubation system in order to improve penetration of substrate into the platelets. The final media contained the following reagents:

diaminobenzidine	25 mg
3% hydrogen peroxide	0.1 ml
0.05 M Tris buffer	10 ml
dimethylsulfoxide	1%

Samples of platelets fixed in the manner described were incubated in the solution for 60 minutes at room temperature. Reacted platelets were then washed twice in Tris-Hcl buffer, pH 6 or pH 9, and fixed for 1½ hours in 2% osmic acid in 0.1 M cacodylate buffer. The fixed samples were dehydrated and embedded in the routine manner.

Controls to determine the specificity of the enzyme reaction included the following:

1. Fixing platelets in O.1% glutaraldehyde in White's saline [10] and then in 3% glutaraldehyde in White's saline before washing and exposure to complete media.
2. Incubating platelets fixed in glutaraldehyde-paraformaldehyde in media without hydrogen peroxide (H_2O_2), without diaminobenzidine (DAB), with excess H_2O_2 (3%) or with reduced amounts of H_2O_2 (0.003% or 0.0003%).
3. Incubating of glutaraldehyde-paraformaldehyde fixed platelets with 0.03 M aminotriazole in the presence or absence of 0.03% H_2O_2 for 16 hours before exposure to the complete media.
4. Incubation of glutaraldehyde-paraformaldehyde fixed platelets in complete media containing 0.02 M potassium cyanide for 1–2 hours.

Results

Membrane Systems of Normal Platelets

Platelets prepared for electron microscopy after fixation in glutaraldehyde and osmium tetroxide retained structural features that were poorly preserved or not evident in cells exposed to osmic acid alone. The open canalicular system (OCS) was particularly sensitive to fixation damage. Continuity of membranes lining channels of the OCS with the surface membrane was easily identified in doubly fixed cells (Fig 1A and B), but could not be found in platelets incubated at 4 C in osmium tetroxide.[15] The tubular invaginations of the platelet cell wall extended deeply into the matrix of the platelet, but were difficult to follow for long distances in thin sections because of their tortuous course (Fig 1C). As a result, the channels often appeared to be isolated clear vesicles or vacuoles. However, previous investigations with electron-dense tracers added before or after fixation and serial sectioning have established that vesicles and vacuoles are portions of continuous OCS channels connected to the platelet surface.[4–6] Osmic acid fixation alone converted the tortuous channels into vesicles and vacuoles which were not connected to each other or to the platelet cell wall.

In one or two areas of the platelet cytoplasm in doubly fixed cells, the canaliculi of the OCS were gathered into groups or clusters (Fig 1A–D and 2A–B). The closely approximated masses of sectioned channels were observed frequently, and probably occur in every platelet. They were absent when the plane of section failed to pass through the area of platelet cytoplasm in which they were located.

The dense tubular system (DTS) is not as susceptible to fixation damage, though it is affected.[17] In doubly fixed cells, canaliculi of the DTS were most easily identified in association with the circumferential band of microtubules (Fig 1C and D). Membranes enclosing the amorphous or filamentous contents of the channels were usually irregular, yielding a twisted appearance. Channels of the DTS were also widely dispersed in the platelet cytoplasm, and could be identified more easily in swollen or slightly altered cells.

A relationship between elements of the DTS and channels of the OCS was apparent in areas where the open canaliculi were gathered in clusters (Fig 1C and D, 2A and B). Even though open channels were closely approximated, small canaliculi of the DTS were interspersed between them. Membranes of the two channel systems did not appear to fuse or communicate with each other, even though the distance separating them was slight. Microtubules were occasionally identified in the complex of channels formed by the OCS and DTS, but other components of the platelet hyaloplasm were rarely observed.

Origin of the Dense Tubular System Based on Evaluation of Gray Platelets

Behnke's study of developing megakaryocytes with electron-dense tracers established that membranes lining the OCS and covering the platelet surface were formed by invaginations of the parent cell wall during maturation of the bone marrow.[4,8] Elements of the DTS were believed to originate from endoplasmic reticulum or components of the Golgi apparatus.[7,8] In order to understand the nature of the complex formed in normal platelets by interdigitation of channels from the OCS and DTS, it was important to identify the origin of the DTS.

Studies of platelets from a patient with an unusual bleeding disorder termed the gray platelet syndrome by Raccuglia [12] have helped to resolve this question. Unusual features noted by Raccuglia included marked anisocytosis, many giant cells, frequent occurrence of large intracellular vacuoles and a paucity, or complete absence of granules in a significant number of platelets. The cytoplasm of the abnormal platelets had an immature appearance which may account for their gray coloration on stained slides.

In our evaluation of gray platelets, the elements suggesting immatur-

ity included ribosome-studded rough endoplasmic reticulum (RER) and an abundance of channels similar to smooth endoplasmic reticulum (SER) (Fig 2C and 3A). The continuity between channels of the RER and SER was clearly evident in the gray platelets. It was also apparent that channels of the SER were identical in morphology and distribution to elements of the DTS in gray platelets and normal cells. These findings suggested that the DTS of platelets is residual SER derived from RER after detachment of ribosomes.

Peroxidase Activity in the Platelet Dense Tubular System

Selective staining of the platelet DTS helped identify the distribution of the channels and their interaction with elements of the OCS. Thin sections of control and experimental samples were always examined without the enhanced contrast provided by staining with uranyl acetate and lead citrate. The reaction product produced after incubation of platelets in the complete media described above was localized specifically to channels of the DTS (Fig 3A, 4A and B, 5A and B). Osmiophilic benzidine was apparent in the channels after incubation of platelets at pH 6 or pH 9, but appeared more intense at the lower pH. Elements of the DTS filled with electron-dense material were randomly dispersed in the platelet, but appeared to form specific associations with circumferential microtubules and the clustered group of transected channels of the OCS. Short segments and cross sections of the DTS were regularly interspersed between the clustered channels of the OCS, yielding a membrane complex. In other areas of the cytoplasm, components of the OCS and DTS were occasionally found in close association, or completely separated from each other.

Control experiments indicated that the dense product deposited in the DTS was due to endogenous peroxidase activity. No reaction product was observed in platelets incubated in the media without DAB, without H_2O_2, with added potassium cyanide or with a minute amount or a marked excess of H_2O_2. Aminotriazole reduced the intensity of staining in the DTS when combined with the incubation media, and blocked deposition in channels completely when added with H_2O_2 16 hours before exposure to complete media. These findings are compatible with those of prior studies suggesting that platelets contain catalase but not peroxidase.[18]

However, some caution is required in differentiating between catalase and peroxidase on the basis of histochemical or cytochemical technics and inhibition experiments.[19] Inconsistent results have been obtained with inhibition by aminotriazole and potassium cyanide of endogenous peroxidases in other tissues, suggesting that their effects cannot be re-

lied on for definitely characterizing the type of enzyme. In most reported studies of catalase localization, the staining reaction is intense when the tissues are incubated in DAB media buffered to pH 9, and less intense or absent when the same media is buffered to pH 6.[20] Glutaraldehyde fixation was not reported to affect the reaction. Since platelets appeared to react most intensely at pH 6 and the reaction was sensitive to the usual concentrations of glutaraldehyde, it is possible that the peroxidase in the DTS was not catalase. Although this question was not solved in the present study, the highly selective nature of the staining reaction has served the purpose of delineating the association between elements of the DTS and OCS in a specialized membrane complex in blood platelets.

Discussion

The existence of two distinctly different membrane systems in the cytoplasm of blood platelets was recognized by Behnke several years ago.[1–3] His investigations established that one of the specialized groups of membranes, the open canalicular system (OCS), was physically linked to the platelet surface.[3] The second group of membranes, the dense tubular system (DTS), was not connected to the cell wall of the platelet, and did not appear to communicate with channels of the OCS. Morphologic differences between the two membrane systems were also pointed out.[2,3,8] The OCS consisted of tortuous invaginations of the platelet surface membrane tunneling throughout the hyaloplasm. An amorphous material similar to the platelet exterior coat covered the membranes lining the OCS, but the channels were otherwise clear. Elements of the DTS, on the other hand, appeared to be filled with amorphous or filamentous material resembling the matrix of the hyaloplasm.

Subsequent investigations have confirmed and extended Behnke's observations. The OCS greatly expands the total area of the platelet surface exposed to plasma, and provides a route for chemical or particulate substances to reach the deepest recesses of the cell.[5,11] It remains open even after the aggregation of platelets,[6] and may act as a conduit for substances secreted during the platelet release reaction. Investigations of the DTS have suggested that it may serve an important role in establishing the circumferential band of microtubules responsible for maintaining the asymmetric form of unaltered platelets.[1,9,21] However, despite advances in our knowledge of the two specialized channel systems, basic aspects of their function in platelet physiology remain obscure.

Previous investigations have emphasized the morphologic and physiologic segregation of the two membrane systems in platelets. The results of the present study indicate that the OCS and DTS are not completely isolated membrane systems. It was evident on close inspection of many well-preserved normal platelets that channels of the DTS and OCS had an intimate physical relationship in nearly every cell. The association of the channels was usually restricted to one or two areas of the hyaloplasm where elements of the OCS were gathered into clusters or groups. Segments of the DTS regularly interdigitated with transected open canaliculi, and appeared to be the only major structures combined into the membrane complex. Absence of the membrane complex from some platelets was probably due to eccentric location, so that the plane of thin section simply failed to pass through it. The presence of one or more of the membrane complexes in most platelets suggests that it is a normal constituent of the cell.

Elements of the OCS and DTS present elsewhere in the platelet hyaloplasm do not necessarily manifest a particular degree of association. The intimate physical contact was prominent mainly in the membrane complexes. Impressive features of the complex were the large surface area of membrane that the two systems exposed to each other and the close proximity of the separate channels. The regular occurrence and highly organized nature of the membrane interaction suggested an important role in platelet physiology, but it was essential to know the origin of the DTS before assessing a particular function.

The study of platelets from the child with the gray platelet syndrome has helped to define the origin of the DTS. Raccuglia has described the physical and biochemical abnormalities of the child's unusual platelets in a recent report.[12] As is frequently the case, a considerable amount of information on the structure of normal cells can be gained through observations on those which are abnormal. The principle defect evident in gray platelets is the paucity or nearly complete absence of granules in many cells. A significant percentage of the patient's circulating platelets are very large, suggesting that they are young platelets.[22] Examination of the cytoplasm of the large cells frequently revealed areas containing channels studded with ribosomes similar to rough endoplasmic reticulum (RER), and masses of smooth-surfaced channels resembling smooth endoplasmic reticulum (SER). Sites of continuity between channels of RER and SER were easily identified. The distribution and appearance of the SER in gray platelets was identical to that of the DTS in patient and normal cells. This finding supports the concept that the DTS is residual SER,[8] and indicates also that the DTS is

a descendent of RER. Other aspects of the abnormality in gray platelets will be dealt with in a separate report.[23]

Thus, the membrane complex formed by the OCS and DTS in normal platelets is a specialized arrangement of membranes derived from the platelet surface and elements of smooth endoplasmic reticulum. The cytochemical demonstration of a peroxidase in the DTS of platelets supports the idea that this system of channels originates from endoplasmic reticulum, and identifies more clearly the extent of interaction between the OCS and DTS in the membrane complexes. Problems in defining the precise enzyme responsible for the oxidation of diaminobenzidine in the presence of hydrogen peroxide were discussed in the results. Although the activity may be due to catalase, other enzymes known to be present in platelets could also be responsible for deposition of the reaction product.[18] The critical point, however, is that an enzyme or enzymes with peroxidase activity are localized specifically within the DTS. This finding indicates that the DTS is endoplasmic reticulum, for when this activity is found outside of specific organelles in other tissues, it is nearly always confined to endoplasmic reticulum.[19,20,24]

The extent of the network formed by channels in the membrane complex was more clearly defined in samples of platelets that underwent the reaction for peroxidase. Elements of the DTS in routinely fixed platelets contained an amorphous material similar in density to the cytoplasm, and recognition was often difficult. Deposits of electron-opaque reaction product in the DTS sharply delineated the small channels throughout the platelet cytoplasm. The contrast between stain-filled channels of the DTS and open canaliculi of the OCS provided a clear picture of the extensive interweaving and proximity of elements making up the membrane complex.

The physiologic significance of the complexes formed by the interaction of two separate membrane systems in platelets is not known with certainty. They appear to be normal constituents of the cell's anatomy, and are unique in the sense that similar organizations of membranous channels have not been observed in other blood cells. In fact, the only other type of cell with an arrangement of channel systems resembling the complexes in platelets is muscle.[25,26] The differentiation of the sarcoplasmic reticulum and transverse tubular (T) system in embryonic muscle yields a model of membrane organization essentially identical to that described for the interaction of OCS and DTS in platelets.[25] Channels of the T system are formed by invaginations of the sarcolemma (muscle cell membrane) which pursue a tortuous course into the sar-

comere cytoplasm. Elements of the sarcoplasmic reticulum evolve by conversion of rough endoplasmic reticulum into smooth, irregular channels after detachment of ribosomes. The tubules of the T system are empty except for an amorphous exterior coat covering the lining membrane. Channels of the sarcoplasmic reticulum contain an amorphous matrix often more dense than the surrounding cytoplasm. Extensive surface-to-surface relationships are formed between elements of the two channel systems in developing muscle. In early stages of differentiation, the intimate membrane associations are simple, and resemble those observed in complexes of the OCS and DTS in platelets. Later, as the organization of the sarcomere advances, the interaction between transverse tubules and terminal cisternae of sarcoplasmic reticulum becomes more sophisticated, resulting in formation of dyadic and triadic junctions. Complex junctions of this type have not been identified in platelets as yet.

The parallels are very close. In both platelets and embryonic muscle, the membrane interaction involves two separate systems, one derived from the cell wall and the other from endoplasmic reticulum. The channels derived from the surface in both cell types are very susceptible to fixation damage by osmic acid, follow tortuous courses into the cytoplasm and have no internal matrix except the exterior coat covering the lining membranes. Elements of the second system in the two types of cells represent residual smooth endoplasmic reticulum, are relatively resistant to fixation damage, and contain a matrix composed of amorphous or filamentous material. The similarities suggest that the OCS of platelets may be comparable to the T system of embryonic muscle, and the DTS to a form of sarcoplasmic reticulum.

The analogy made between platelets and muscle is not new.[27–29] Previous investigations have demonstrated that platelets contain substantial amounts of contractile protein resembling muscle actomyosin, that the metabolic response of platelets to stimulation is similar to that of muscle, that platelets participate in phenomena that are clearly contractile in nature, and that the physical changes that develop in platelets after exposure to aggregating agents are manifestations of contractile activity in the cell. The suggestion that platelet membrane systems might serve as a sarcoplasmic reticulum was made several years ago,[30] and definitive experiments were carried out demonstrating that a membrane fraction from platelets was as efficient as sarcotubular vesicles from muscle in sequestering calcium ions.[31] Our conclusions from many investigations indicate that platelets are not merely similar to muscle cells,

but are muscle cells, and that contractile physiology dominates the functional activity of platelets in hemostasis.[29]

The present work has carried this thesis a step farther. Our previous concept held that the surface membrane and channels of the OCS were most likely the sarcoplasmic reticulum of the platelet. The results of this study indicate that the sarcoplasmic reticulum in platelets may be a more sophisticated structure, involving an interaction of two membrane systems, the OCS and the DTS. By focusing on the membrane complexes formed by the two channel systems, which resemble so closely the interaction of surface membrane and sarcoplasmic reticulum in muscle, it may at last be possible to define the trigger mechanism for platelet contraction.

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[*Illustrations follow*]

Fig 1A and B—Low-power photomicrographs of platelets from samples of citrated platelet-rich plasma (C-PRP) fixed in glutaraldehyde at 37 C and then in osmic acid at 4 C. Tortuous elements of the open channel system appear to form clusters or groups in the platelet cytoplasm (*arrows*) (A, × 12,300; B, × 12,500). **C**—A platelet from C-PRP prepared for study in the same manner as the cells in Fig 1A and B. General features of platelet morphology include the circumferential band of microtubules (*MT*), granules (G), dense bodies (*DB*), mitochondria (*M*) and glycogen particles (*Gly*). Elements of the dense tubular system (*DTS*) are usually observed in close association with circumferential microtubules. Channels of the open canalicular system (*OCS*) are randomly dispersed in the cytoplasm. In addition, they are frequently observed in a group or cluster suggesting a membrane complex (*MC*). Components of the DTS are present between the open channels, but are difficult to identify because of their density (× 41,500). **D**—Young platelet from the patient with the "gray platelet syndrome." Gross architectural features are similar to those of normal cells except for large size, frequent vacuolization, relative immaturity and paucity of granules. Elements of the DTS are closely associated with developing microtubules (*MT*), and with clustered channels of the OCS in a membrane complex (*MC*). Cross sections of the DTS canaliculi (*arrows*) are evident in the walls separating channels of OCS in the complex (× 42,100).

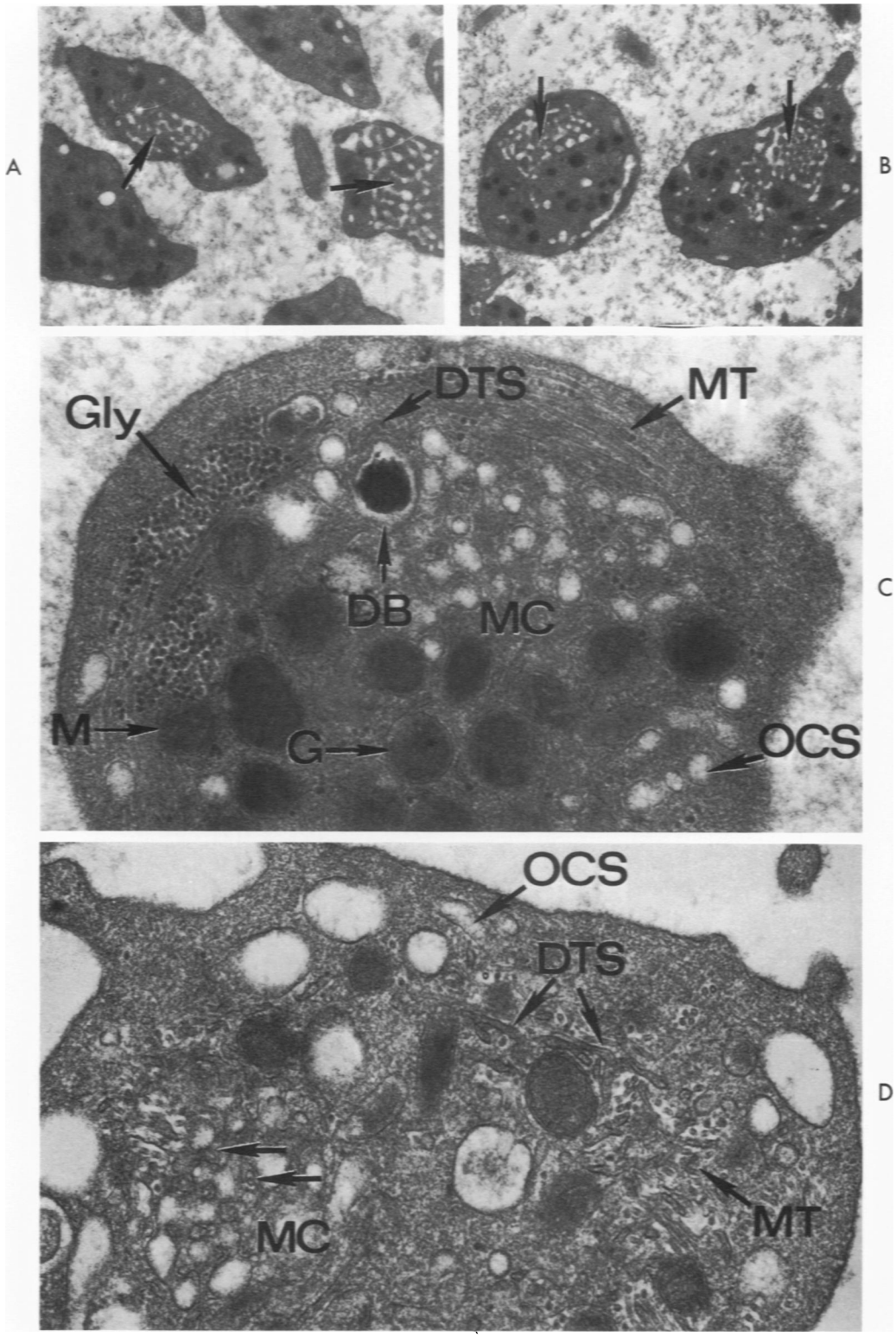
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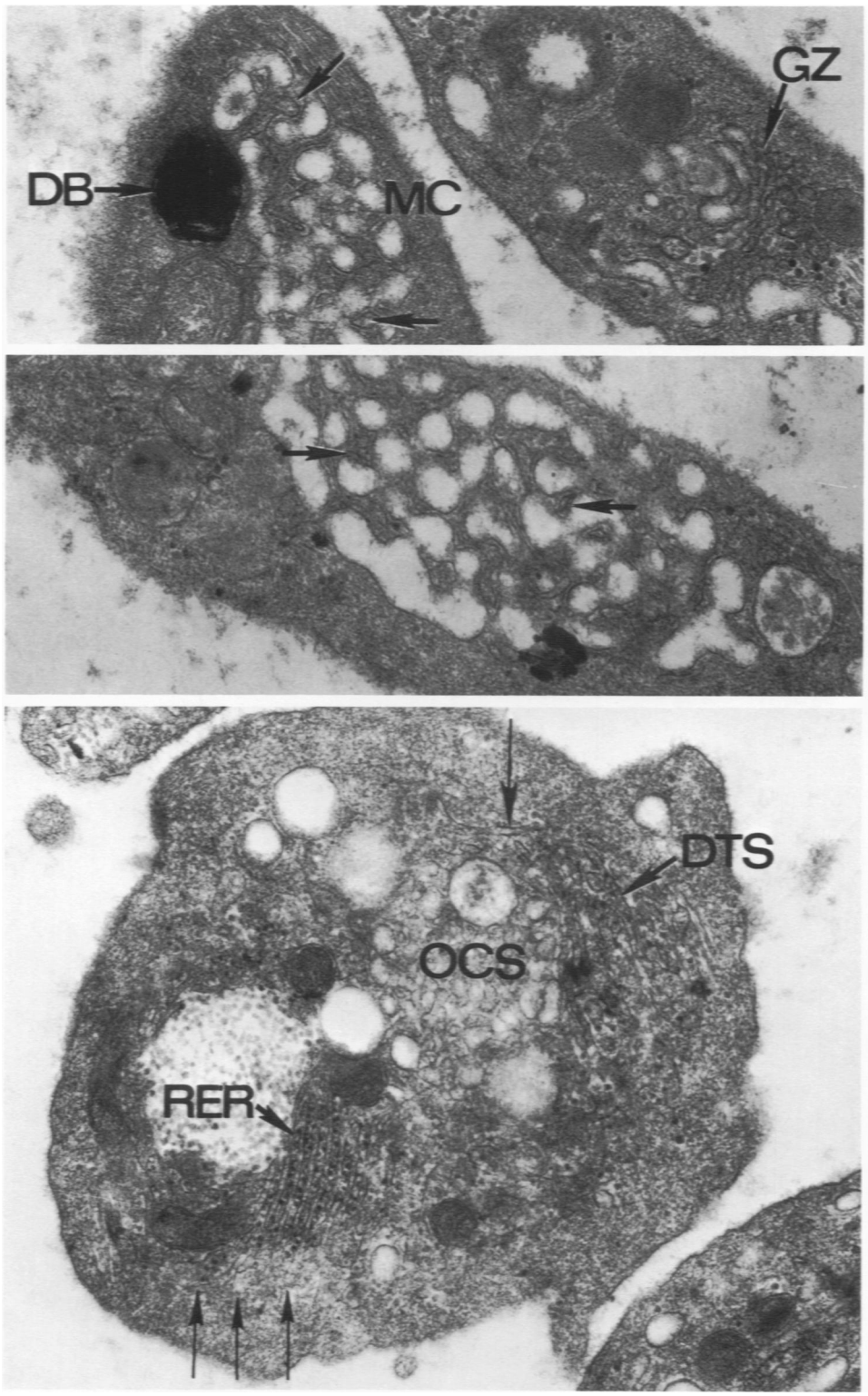
Fig 2A and B—Platelets from samples of normal C-PRP. Intermural interwoven membrane complex (*MC*) containing clustered elements of OCS and intramural channels of DTS in platelet with dense body (*DB*) contrasts with flattened saccules making up the Golgi zone (*GZ*) in the adjacent cell. In Fig 2B, the close association of channels of the OCS and DTS (*arrows*) can be identified with ease (A, × 49,800; B, × 53,900). **C**—Large, immature platelet from the child with gray platelets. Ribosome-studded channels of rough endoplasmic reticulum (*RER*) are evident in this cell. The channels of RER are continuous with smooth-surfaced channels (*triple arrows*) on which an occasional ribosome can still be identified (*arrow*). Glycogen particles in the pale zone adjacent to the RER are larger than the ribosomes. The smooth-surfaced channels derived from RER are identical in appearance to elements of the DTS (× 38,200).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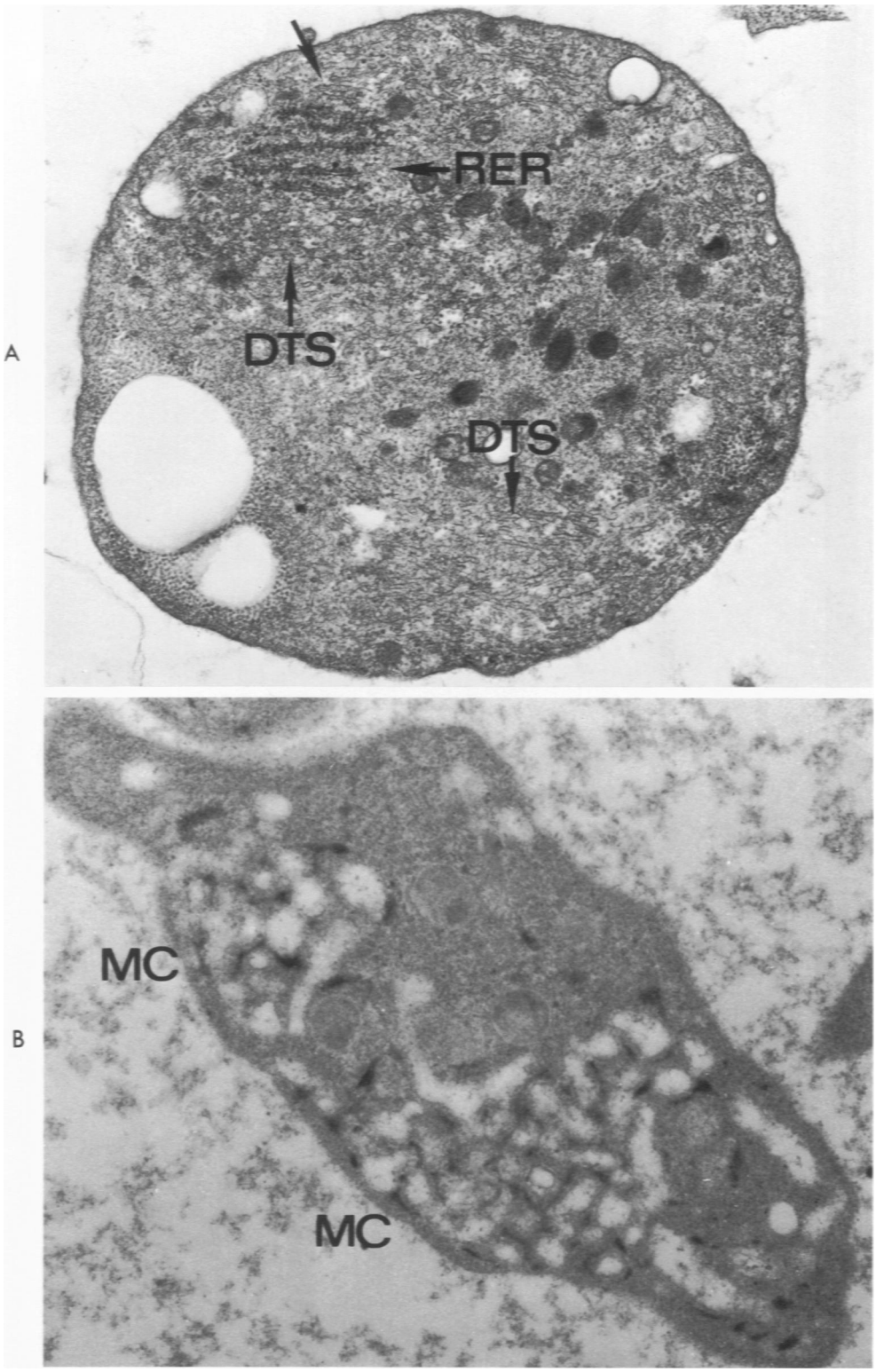

Fig 3A—Gray platelet containing masses of smooth-surfaced channels identical in appearance to elements of the DTS, and an area of RER. Channels above and below the RER retain some surface-associated ribosomes (*arrow*) (× 21,500). **B**—Platelet from sample of normal C-PRP incubated for peroxidase activity. Enzyme reaction product is deposited specifically in channels of the DTS. A membrane complex (*MC*) formed by elements of OCS and DTS is distributed in two areas of the cytoplasm in this platelet (× 53,900).

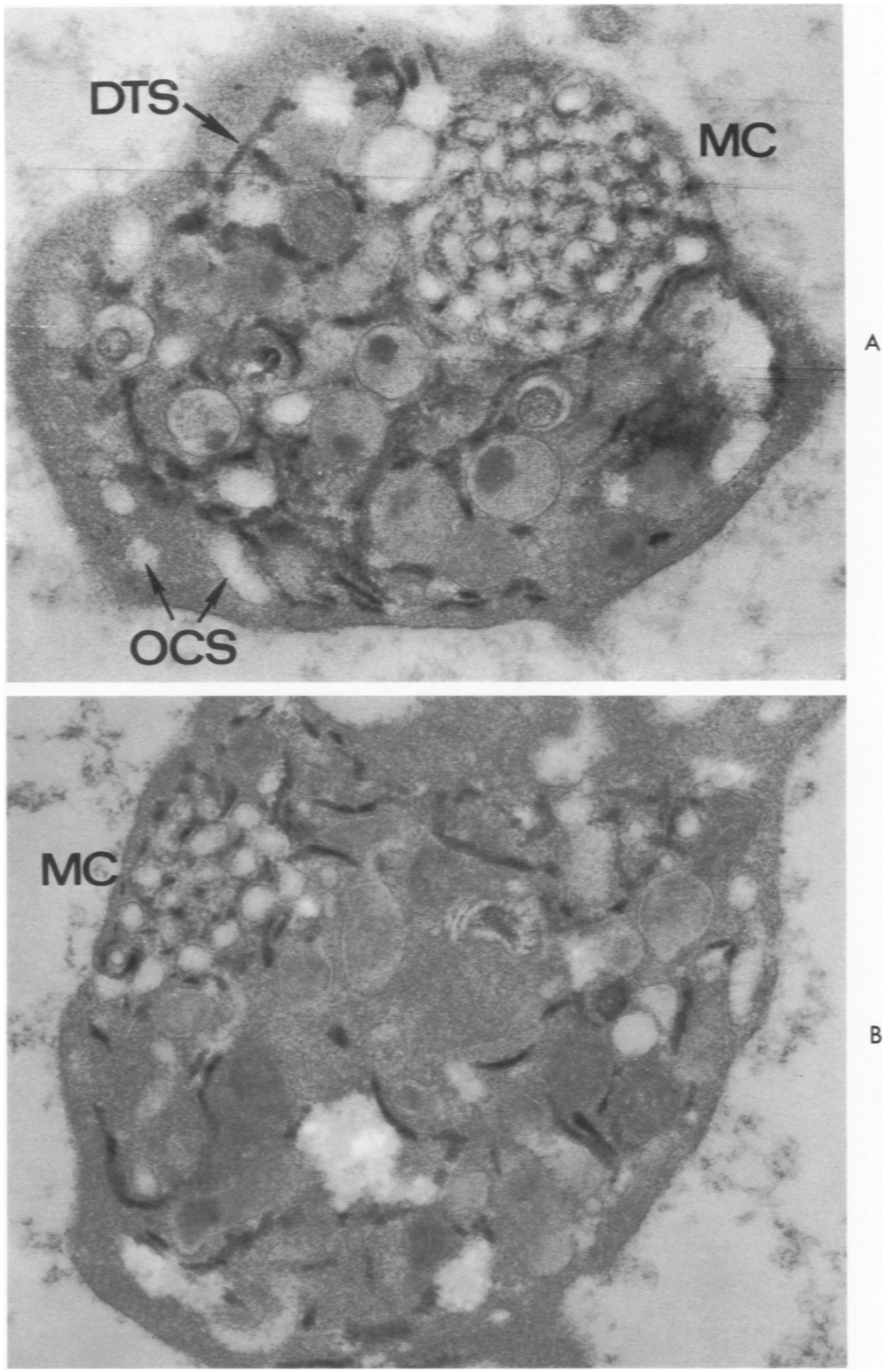

Fig 4A—Platelet stained for peroxidase activity. Elements of the OCS and stain-filled DTS occur independently as well as in close association in the membrane complex (*MC*). The reaction product in the DTS permits clearer identification of the tubules lying between the closely grouped channels of OCS (× 54,400). **B**—Platelet incubated for peroxidase activity. The membrane complex is often eccentric in location, as in this example, and may be missed in some platelets when it is out of the plane of section. An element of the open-channel system in the membrane complex of this cell is in continuity with the platelet surface (× 53,900).

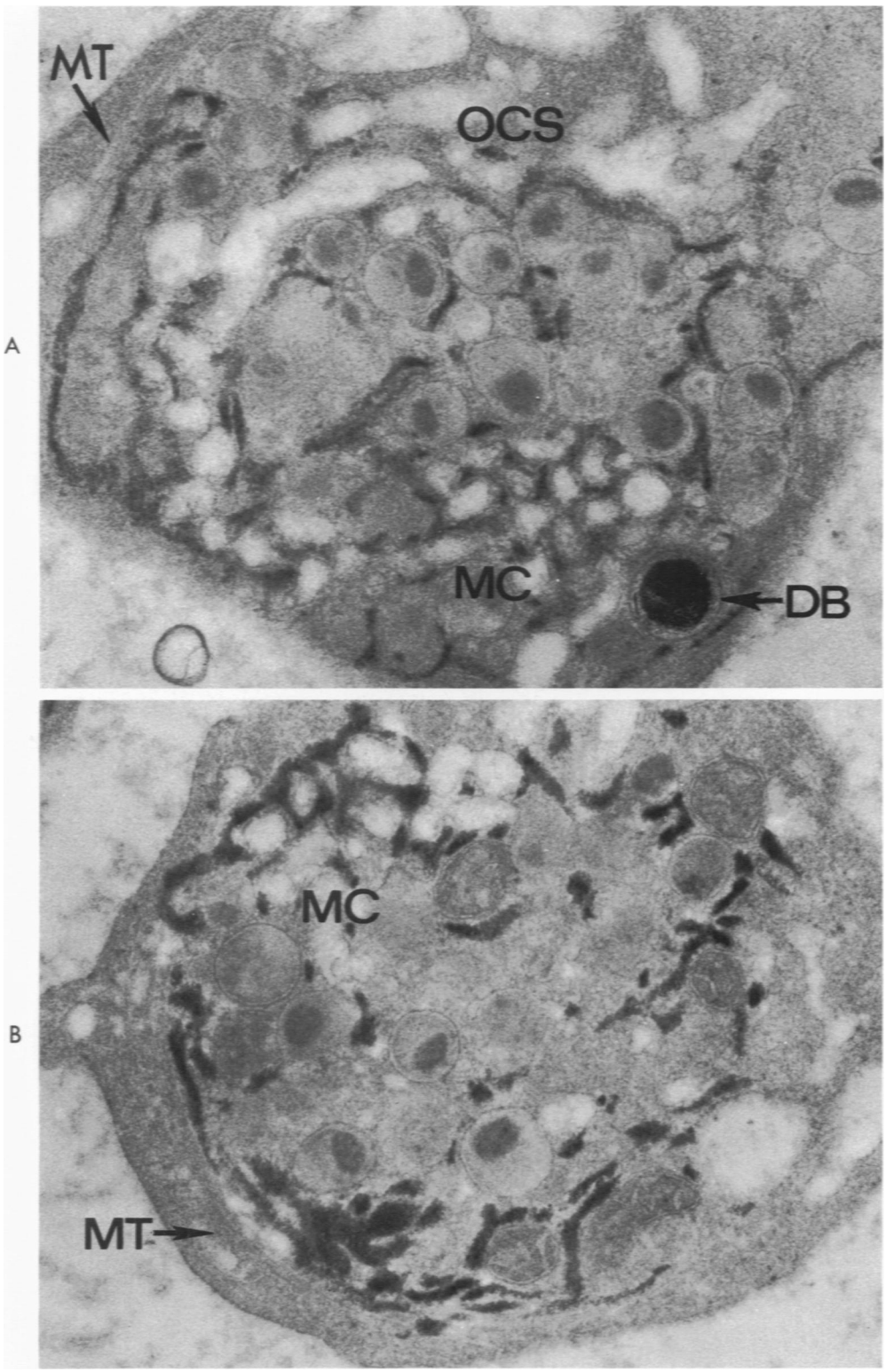

Fig 5A and B—The platelets stained for peroxidase activity in A and B reveal the close association of the stain-filled DTS with circumferential microtubules (*MT*), as well as with open channels in the membrane complex (*MC*). (A, × 56,500; B, × 53,000).