Ultrastructural Localization of Peroxidase Activity in Human Platelets and Megakaryocytes

Janine Breton-Gorius, PhD and Josette Guichard, BS

Normal human platelets and megakaryocytes were examined for peroxidase activity by the diaminobenzidine (DAB) cytochemical technic. When the fixation and the incubation were adequate, a strong reaction was present in the dense tubular system of platelets suspended in plasma or spread on carbon. The black reaction product was ascribed to enzyme activity, since the reaction was completely eliminated when H_2O_2 or DAB were omitted, or when H_2O_2 was in excess. In addition, the reaction was inhibited by aminotriazole, cyanide and azide. In the human megakaryocytes, the reaction was localized in the endoplasmic reticulum including the perinuclear envelope. The Golgi complex and the clear vacuolar system were negative for the reaction. After platelet release, the reaction was always seen in the perinuclear space. The nature and function of the enzyme, as well as its possible relationships with catalase, are discussed. (Am J Pathol 66:277–286, 1972)

GRAHAM AND KARNOVSKY [1] have made it possible to detect the ultrastructural localization of endogenous peroxidases in a variety of animal and plant cells.[2–15] In addition, other enzymes possessing peroxidase activity, such as catalase and some of the cytochromes, can be detected by the diaminobenzidine tetrachloride (DAB) method.[16–24]

Biochemical studies have shown that normal human blood platelets contain no peroxidase at all, whereas they do contain a small quantity of catalase.[25] Catalase activity of platelets is equal to about one-twelfth that of erythrocytes. Studies of human platelet granules and membranes separated by gradient ultracentrifugation have indicated that only 8% of the catalase was associated with the pellet and 63% was released into the soluble fraction.[26] Other oxidoreductases were also found in platelets, including $NADPH_2$ dehydrogenase, cytochrome oxidase, $NADH_2$ cytochrome c reductase and dopa oxidase.[27]

The present study concerns the demonstration, under certain conditions of fixation and incubation in DAB, of the presence of peroxidase-like activity in the platelet dense tubular system (DTS) described by Behnke.[28] In megakaryocytes, the black reaction was seen only in the mature stage localized to the nuclear envelope and the endoplasmic

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reticulum. After the release of platelets from megakaryocytes, the enzyme was always seen in a very narrow rim of residual cytoplasm in ergastoplasmic cisternae and the nuclear envelope. A preliminary report has been published.[29]

Materials and Methods

Collection and Fixation of Platelets

Blood samples of 9 ml were taken from normal donors by venipuncture into warm (37 C) glass syringes containing heparin (300 IU, Liquemine Roche). Platelet-rich plasma (PRP) was prepared by differential centrifugation and divided into two parts. The first part was used to study the cytochemical reaction of platelets in suspension. After sedimentation of platelets at 1500 rpm, the plasma was replaced by a 1.25% solution of distilled glutaraldehyde (TAAB Laboratories) in 0.1 M phosphate buffer at pH 7.2 for 10 to 30 minutes. (In some cases, cells were washed in a Tris buffer before fixation to eliminate the proteins adhering to the cell coat.) The sediment was then washed three times in phosphate buffer. The second part of the PRP was spread on glass slides, as previously described.[30] After 20 minutes at 37 C, the platelets adhering to the slides were fixed with the 1.25% buffered glutaraldehyde solution for 15 minutes.

Two different methods of washing after fixation were used: either 12 hours at 4 C in a phosphate buffer, or ten successive washings of 10 minutes each in 0.1 M phosphate buffer.

Collection and Fixation of Bone Marrow Cells

Marrow was obtained by sternal aspiration from patients whose myelogram was normal according to Pappenhein-stained smears. The marrow blocks were immediately fixed in glutaraldehyde[31] [either 3% glutaraldehyde (Light Laboratories) or 1.25% distilled glutaraldehyde (TAAB Laboratories)], in phosphate buffer as above. The fixation was done at 4 C or 25 C for 30 minutes. The marrow blocks were then left in the same buffer overnight at 4 C or washed three times.

Cytochemistry

The platelet pellets, marrow samples, and the platelets spread out on the glass slides were incubated in the medium of Graham and Karnovsky[1] or the media modified by Novikoff *et al*[18] and Fahimi.[26]

The following concentrations were used:

DAB, 20 mg, (Sigma Chemical Company, St Louis, Mo) dissolved in 10 ml of 0.05 M Tris-HCl buffer at pH 9 or 7.6 to which 0.02% H_2O_2 was added.

DAB, 5 mg, dissolved in 10 ml of 0.05 M Tris-HCl buffer at pH 7.6, to which 0.01% H_2O_2 was added.

DAB, 20 mg, dissolved in 8.90 ml of acetate buffer at pH 6 to which 0.1 ml of freshly prepared 0.1% H_2O_2 and 1 ml 0.05 M manganese chloride were added.

Cells were incubated for 10–90 minutes at 25 C or at 37 C. In some experiments, the incubation was done at 4 C for 12 hours before the incubation at 37 C to obtain a better penetration of substrate.[6] Control blocks of tissue and platelets were incubated in media without DAB or without H_2O_2.

Inhibitors

Various compounds were tested for their inhibitory effect upon the cytochemical

reaction. In each case, blocks of bone marrow cells and platelets were premcubated for 10 minutes in 0.05 M Tris-HCl buffer containing the inhibitor. They were then placed into complete media containing the same concentration of the inhibitor. The following substances were tested: 3-amino-1,2,4-triazole (AMT) in a final concentration of 0.02 M and 0.001 M; potassium cyanide, 0.1 M and 0.01 M; sodium azide, 0.1 M and high concentrations of H_2O_2 (2%) in medium containing DAB. To prevent a false positive reaction caused by nonspecific binding of oxided DAB, cells were incubated in DAB oxidized by potassium ferricyanide.[20]

Postfixation and Embedding

After incubation, the blocks of tissue cells in suspension and cells spread out on slides were rinsed in phosphate buffer and postfixed in 1% osmium tetroxide in phosphate buffer for 30 minutes before being dehydrated in ethanol and embedded in Epon.

Spread platelets fixed in glutaraldehyde, incubated for peroxidase reaction and dehydrated, were embedded by turning a capsule filled with Epon upside down on the slide. After partial polymerization (3–6 hours at 60 C), the slide was detached by means of a thermic shock with dry ice. Thus the cellular layer adhering to the carbon was at the surface of the capsule. Polymerization was then continued for 12 hours at 60 C. Sections parallel to the plane of spreading were made, with great care being taken to have the block and knife parallel. All sections including the first one (which has to be thin) were mounted on 75-mesh grids coated with formvar and carbon.

In order to find the megakaryocytes, sections of 1–2 μ made on the blocks of bone marrow were examined in the light microscope before making the thin sections. The latter were examined either stained with lead citrate or unstained, in a Philips EM 300 at 60 or 40 kV with objective aperture of 25 μ.

Results

Peroxidase Activity of Circulating Platelets Fixed by 1.25% Glutaraldehyde

Examination by electron microscopy revealed an electron-opaque reaction product in the dense tubular system (DTS) described by Behnke.[28] This system consists of narrow tubules and vesicles. Some tubules are localized along the interior of the marginal bundle of microtubules, and others are scattered in the granulomere between granules and mitochondria (Fig 1). Sometimes these tubules branch (Fig 3 and 4). They are often seen in close proximity to the clear vacuoles of the surface-connected system (SCS) (Fig 6 and 10). This system of channels is composed of clear canaliculi that are continuous peripherally with the cell surface. Elements of the two-channel systems do not communicate with each other.[28] The black product of oxidized DAB is seen best in unstained sections. When tubules of the DTS are dilated, the reaction product appears as dense fine particles. The intensity of the reaction and the extent of the tubules in individual platelets are highly variable from cell to cell. Granules in platelets incubated at pH 9 and stained only with lead showed a zone of increased density (bull's-eye or alpha granules) (Fig 1, 4 and 10), but less prominent than the

opaque reaction of the DTS. With substances (see below) that inhibit the reaction of the DTS, the granules retained their dense zones (Fig 7). A few very electron-dense bodies seen in human platelets [33–35] were also darkly stained with media containing DAB. When the peroxidase reaction was inhibited by AMT, however, the density was the same (Fig 7).

Peroxidase Activity of Blood Platelets Spread on Glass or Carbon

Ultramicrotomy of spread-out platelets allows complete sectioning of the hyalomere in four to five thin sections. The first section contains the zone adhering to the surface of the slide. At this level, the only visible organelles are microfibrils and microtubules arranged concentrically when the contour of the spread platelet is exactly circular. In cells having pseudopods, the microtubules and microfibrils are parallel out to the extremity of the long surface extensions (Fig 8).

Generally, the second section contains some particles of glycogen and sometimes the section of a contractile vacuole. The shape of this vacuole is very often elliptic, as it has been shown in phase-contrast microscopy.[36] Groups of microtubules are along the cell membrane. Some profiles of the DTS, recognized by the dense reaction with DAB, are interspersed between the microtubules (Fig 8).

The next two sections are cut through the granulomere, composed of granules and mitochondria. Channels stained with the reaction product are found between these organelles (Fig 9). In some platelets, reactive canaliculi are more numerous at this level, extending through the whole hyalomere (Fig 11).

In the next section, only the granulomere is visible. The marginal band of microtubules is often accompanied by a dense tubule on each side. The latter appear continuous when the section is parallel to the plane of spreading (Fig 12). At this level, clear elements of the SCS are visible, closely associated with one or several dense tubules (Fig 10).

Localization in Megakaryocytes and Bone Marrow Platelets

As the substrate diffuses slowly in marrow blocks, megakaryocytes were examined only in zones where erythrocytes and granulocytes exhibited intense peroxidase activity.[8]

In megakaryocytes, a reaction is visible in the perinuclear space and endoplasmic reticulum (Fig 16), but only in mature cells having granules and demarcation membranes. Ribosomes are never stained. After platelet liberation, the reaction is still present in the perinuclear space (Fig 14), and in the endoplasmic reticulum of the cytoplasmic remnant

surrounding the nucleus. It is even possible to identify some multilobulated and pycnotic megakaryocyte nuclei showing a perinuclear reaction. Some of them have only a very thin rim of cytoplasm with a few mitochondria. During platelet liberation in the elongated aggregates detaching from the megakaryocyte (Fig 13), microtubules and a part of the DTS are oriented parallel to the axis of the filaments (Fig 15). Marrow platelets are similar to circulating ones. No peroxidase activity is seen in sacs probably representing remnants of the Golgi apparatus (Fig 5).

Technical Considerations

The reaction was never seen in platelets if the concentration of distilled glutaraldehyde was more than 2%. Provided the incubation time in DAB was the same, fixation of 10 minutes gave a stronger reaction than did a 1-hour fixation, but cellular preservation was less satisfactory. Fixation for 3 hours inhibited the reaction. Washing the blocks in buffer after glutaraldehyde fixation is imperative in order to eliminate excess fixative. Similar precautions are not necessary to demonstrate peroxidase in the primary granules of neutrophils.[4,5] The optimum time of incubation appeared to be 1 hour. The pH (between 6 and 9) and the incubation temperature (23 C and 37 C) between the limits indicated, did not influence the intensity of reaction in the DTS. With 0.2% DAB, the results were better than with 0.05%. Incubation of cells during 12 hours at 4 C in the complete media [6] seemed to improve the penetration of DAB into the cellular membrane, especially in young megakaryocytes.

Despite all these precautions, results on blood platelets fixed in pellets were not totally reproducible. The reaction was repeated on blood obtained from 12 healthy donors and 3 patients with hematologic diseases; in 2 cases no reaction could be obtained on platelets fixed in pellets from healthy donors, whereas the same platelets examined after spreading showed an intense reaction.

Controls and Inhibitors

In the absence of DAB, in sections stained only by lead citrate, the DTS is never black, the density being similar to that found after double fixation.[28,32] The center of the granules is slightly denser than their periphery. In the absence of H_2O_2 or at a H_2O_2 concentration lower than 0.001%, the reaction in the DTS is totally absent. Hydrogen peroxide in excess (2%) inhibits DTS staining without influencing the dense zone of the granules. AMT (0.001 M) totally inhibits the reaction in the DTS, but the "bull's-eye" of the granules remains electron

dense (Fig 7). A complete inhibition in DTS is also observed with potassium cyanide (0.01 M) and sodium azide (0.1 M). These inhibitors, at these concentrations, had no effect on the reactivity of granulations in monocytes, eosinophils and neutrophils.

At pH 6 with AMT (0.02 M), the tubules of the DTS were negative but the membranes of mitochondria were very strongly stained (Fig 2), whereas with KCN (0.1 M) no reaction was observed. These observations are similar to those of Novikoff and Goldfischer.[18] When DAB was oxidized by potassium ferricyanide,[20] no reaction was present in DTS.

Discussion

Our observations indicate that peroxidase activity is present in the DTS of human platelets, and in the endoplasmic reticulum and nuclear envelope of megakaryocytes. The question arises whether the black deposit results from enzymatic activity. Behnke, in fact, described a nonspecific lead staining of the DTS after incubating controls in the Gomori reaction medium at pH 5 for acid phosphatase.[37] However, the black reaction product in our study is ascribed to enzyme activity, since the reaction is completely eliminated by the omission of DAB or H_2O_2, and by an excess of H_2O_2. In addition, the reaction is inhibited by cyanide, azide and aminotriazole, and DAB oxidized by potassium ferricyanide does not stain the DTS.

The nature of the enzyme is uncertain. Histochemical procedures that use DAB detect, in addition to peroxidase, other enzymes that also have peroxidase activity.[18,20,24] In some cases, where biochemical analyses have not been made, the histochemical inhibition methods are too imprecise to allow identification of the enzyme.[11,12] In platelets, several oxidoreductases have been described including $NaDPH_2$ dehydrogenase, dopa oxidase and catalase.[27] Cytochrome c oxidase is found only in mitochondria.[26] Our localization on membrane mitochondria at pH 6 is consistent with the biochemical analysis of Marcus *et al.*[26]

Biochemical studies have indicated that a small proportion of platelet catalase (8%) is associated with the granules.[26] The platelet granules contain two zones of differing electron opacity, and the central, more dense zone was clearly apparent in cells incubated with DAB even in thin sections that were not stained with uranyl acetate. However, the same degree of opacity was evident in the dense zone of granules in platelets incubated in DAB medium containing AMT (Fig 7). Since AMT completely inhibited the deposit of reaction product in the DTS, it is unlikely that enhanced opacity of granule dense zones is

due to the same peroxidase activity present in the system of tubules. Platelet granules are similar morphologically to organelles in other cells and tissues where DAB procedures have revealed the presence of catalase. Hepatic microbodies (peroxisomes),[40] for example, have two zones of differing electron opacity and react strongly when incubated in DAB medium at pH 9. The persistence of opacity in the central zone of platelet granules after treatment with AMT, however, contrasts sharply with the complete absence of reaction product in hepatic peroxisomes treated in a similar manner. It is possible that the area in platelet granules that remains opaque despite inhibition of catalase is caused by an inherent electron opacity. Cytochemical studies have indicated that the dense area of the granule is rich in acid mucopolysaccharide (AMPS),[38] and the binding of divalent cations by AMPS may inhibit transmission of electrons through the dense zone.

Is catalase implicated in the reaction of the DTS? In liver, the catalase present in peroxisomes is very sensitive to inhibitors such as AMT but not sensitive to cyanide,[16,18] as we observed in platelets. The platelet DTS, in contrast to the liver peroxisome, gave an intense reaction at pH 6. Moreover, in liver, the catalase is never associated with endoplasmic reticulum, not even during microbody regeneration [39] or recovery of synthetic processes after AMT inhibition *in vivo*.[49] It follows that neither the nature nor the function of the enzyme responsible for the peroxidase activity can be determined from these histochemical reactions.

Behnke previously pointed out the existence of two unconnected systems of channels in platelets, which are morphologically [28] and cytochemically different.[37] He showed that the DTS is often seen in close proximity to SCS.[41,42] The channels are also intimately associated with the circumferential band of microtubules.[28,32] This relation of DTS to microtubules and SCS is clearly demonstrated when the DTS is darkly stained by the peroxidase reaction.

A hypothetic function of DTS was advanced by White.[43] The channels of DTS may act as a reservoir for subunits of filaments of microtubules and guide the formation of circumferential band microtubules. Various concepts have also been offered concerning the origin of DTS. Jean and Racine suggested an origin from the Golgi complex or the endoplasmic reticulum.[44] Behnke [41,42] supposed that ergastoplasmic elements lose their attached ribosomes, the remaining membrane becoming the DTS. Our histochemical results corroborate the endoplasmic reticulum origin of the dense tubules since in the most mature megakaryocytes all

the cisternae of the endoplasmic reticulum, including the nuclear envelope, are stained. The Golgi zone is never involved in the reaction.

It remains to be seen whether the synthesis of this enzyme appears as late in the maturation as the reaction indicates. It may be that in an early stage where the demarcation system is not yet well developed, the substrate fails to penetrate into the cell. Indeed, preliminary experiments with 12 hours of incubation indicate a reaction in young pathologic megakaryocytes. As in platelets, the black deposit is never seen if AMT is added to DAB medium.

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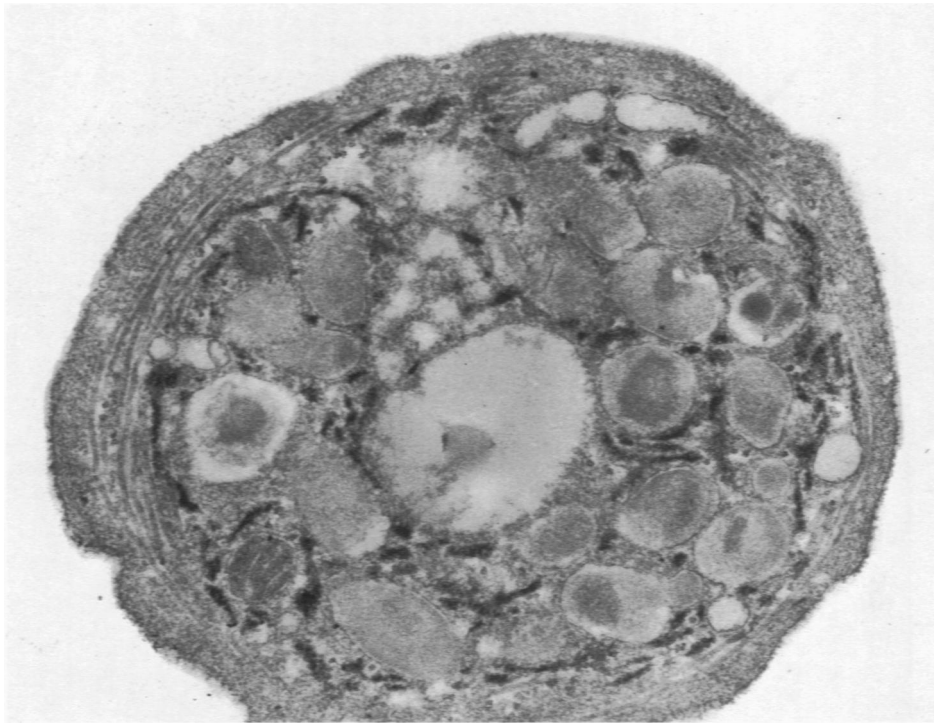
Legends for Figures

Fig 1—Spread platelets from sample of heparinized PRP fixed in glutaraldehyde for 15 minutes and incubated for 60 minutes at pH 9 in 0.2% DAB medium. The section stained with lead is made at the level of the granulomere. A marginal bundle of microtubules encircles the group of granules and mitochondria. A contractile vacuole is seen in the center, and a fragment of a submarginal reactive tubule is visible inside it. DTS is scattered between granules and mitochondria. Some clear vacuoles of the SCS are present at the periphery and near the contractile vacuole. Granules contain two zones in their matrix, one of which is more electron dense than the other (× 38,990).

Fig 2—Platelet fixed as in Fig 1, but incubated in 0.02 M AMT before incubation at pH 6. The tubules of DTS are completely negative. The dense zone of the granules has the same opacity as in Fig 1. Membranes of mitochondria are strongly stained by the DAB media (× 29,750).



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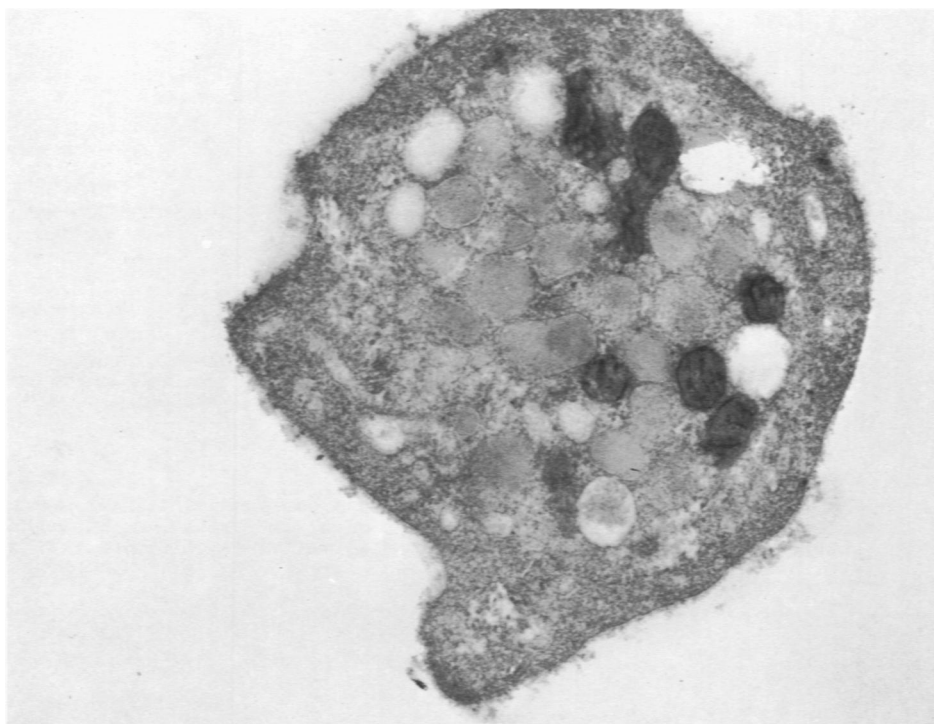

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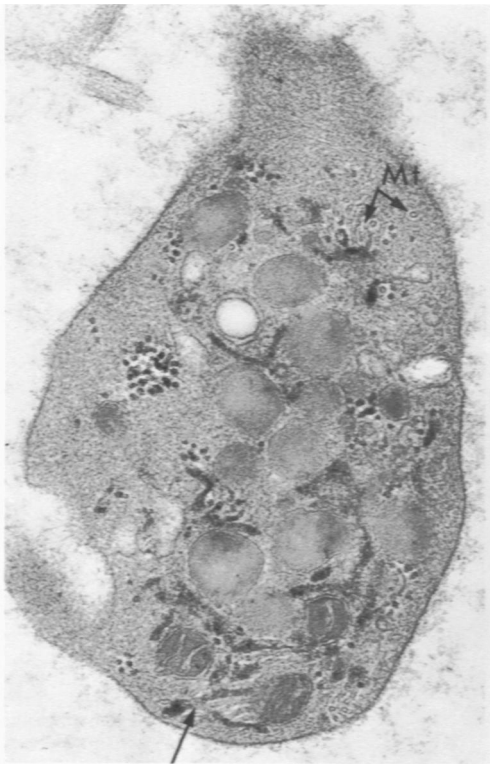
Fig 3—Platelet from human bone marrow fixed for 30 minutes in glutaraldehyde and incubated for 30 minutes at pH 7.6 in 0.05% DAB medium (sections stained with lead only). Granules have a homogeneous appearance. The DTS is filled with the opaque product of the reaction. Above and below, microtubules (*Mt*), are cut in various planes. A dense, branching tubule (*arrow*) is accompanied, in the lower part of the figure, by a group of three microtubules. Other sections of DTS are interspersed between the granules, mitochondria and clear vacuoles (× 33,520).

Fig 4—Spread platelet from sample of heparinized PRP fixed for 15 minutes in glutaraldehyde (1.25%) and incubated 60 minutes at pH 9 in 0.2% DAB medium. The DTS is darkly stained. A branching tubule (*arrow*) is seen with the band of microtubules. The center of the granules is more dense than in Fig 3, but is less stained than DTS (× 33,520).

Fig 5—Platelet from human bone marrow incubated as in Fig 3. The DTS is well developed and very reactive. Golgi apparatus (*GA*) is negative. Granules are very clear (× 15,140).

Fig 6—Part of platelet from PRP fixed in sediment for 30 minutes, incubated in DAB medium at pH 7.6. The section of DTS is seen in close proximity to the surface-connected system, giving the impression of being interwoven with it (× 35,910).

Fig 7—Platelet fixed as in Fig 2, incubated for peroxidase activity at pH 9, in presence of 0.02 M AMT. The DTS appears without reaction. The "bull's-eye" and very dense granules have the same appearance as with DAB medium (× 21,550).



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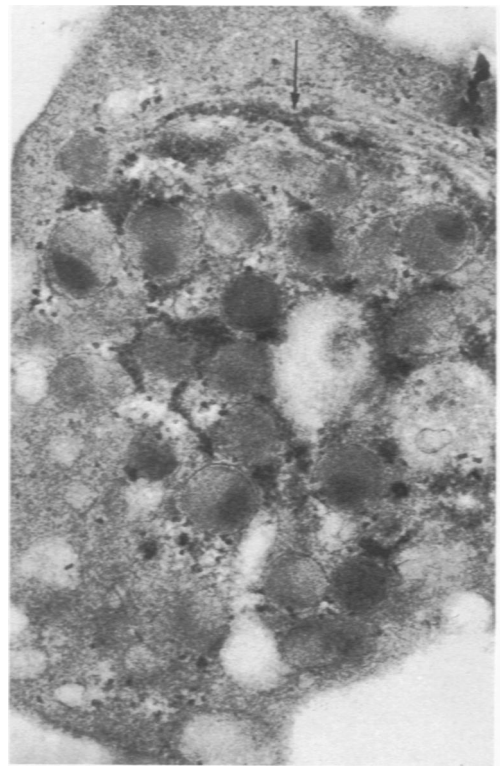

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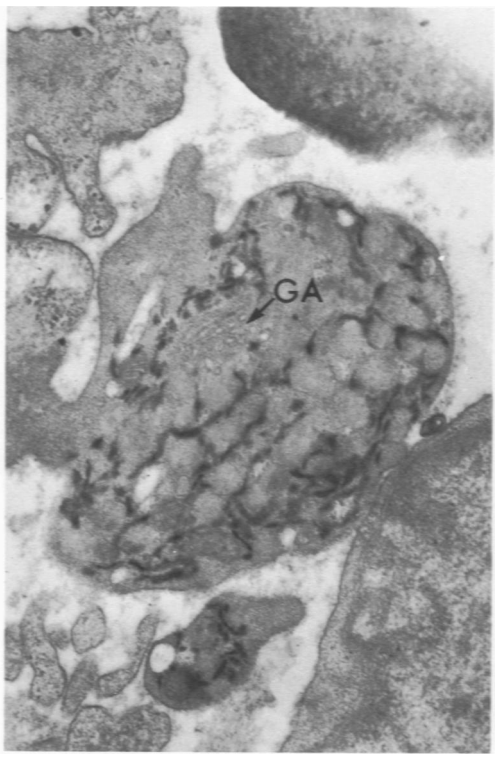

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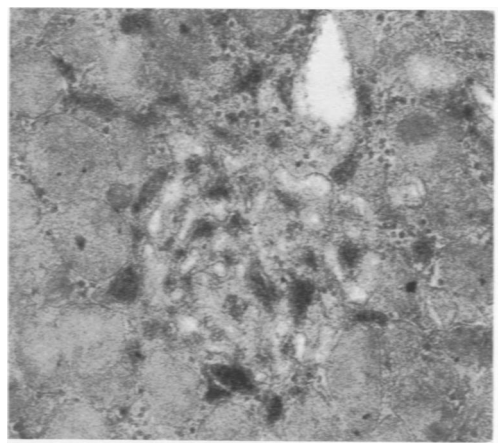

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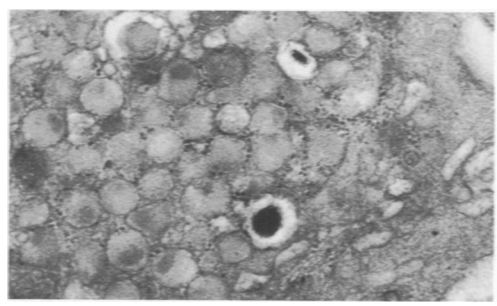

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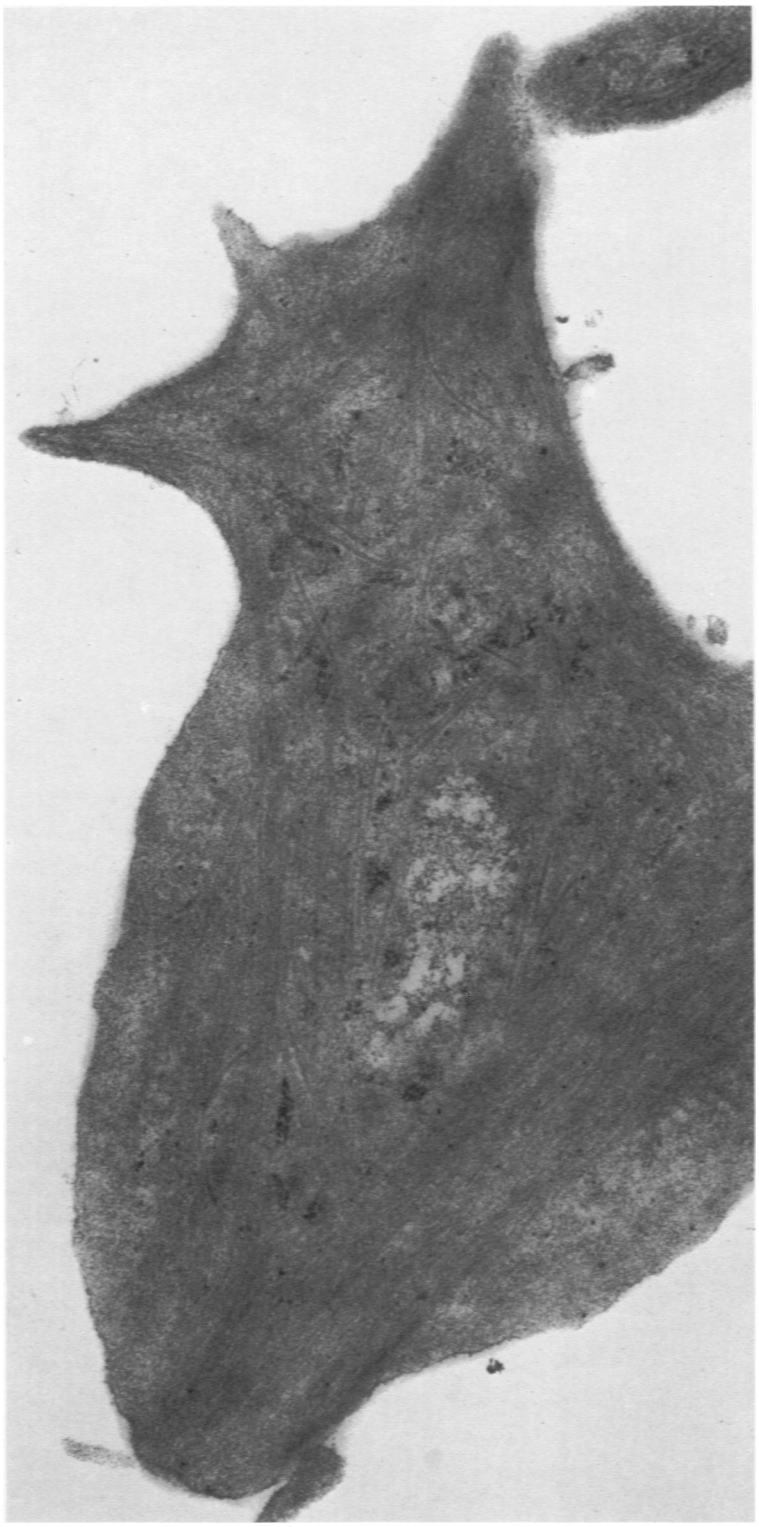
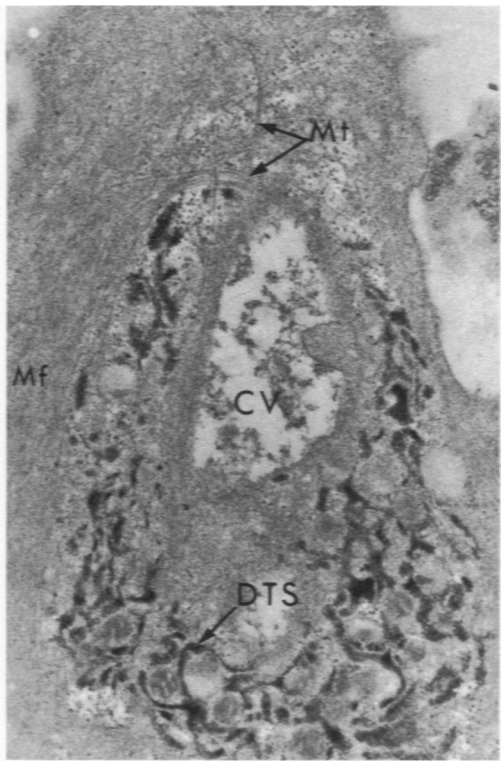


Fig 8—Second section of platelet spread on carbon, fixed as in Fig 1. At this level, many microfibrils are seen. Microtubules run in all directions. In the middle, a contractile vacuole is seen with flocculent material inside. Tubules identified by the black reaction are present everywhere in the cytoplasm (× 25,650).



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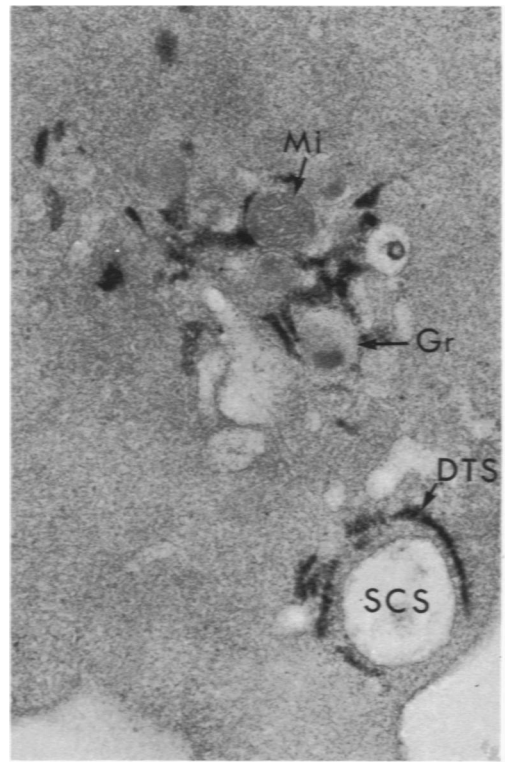

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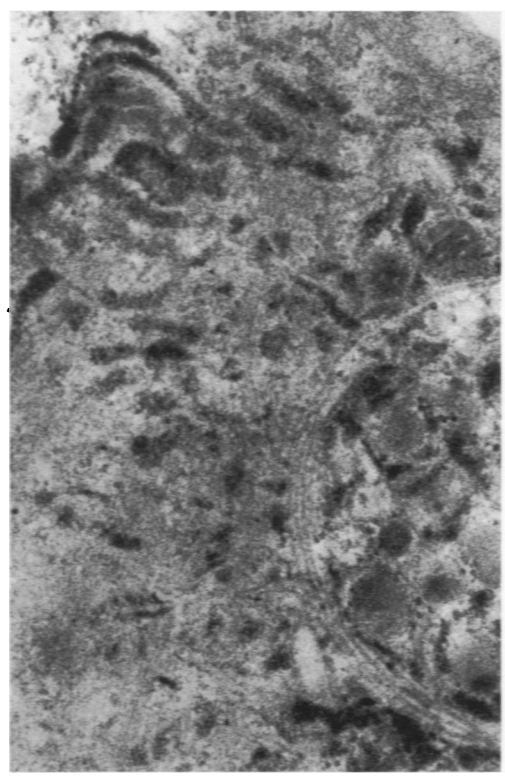
Fig 9—Spread platelet fixed as in Fig 1 and cut at level of granulomere. A contractile vacuole (*CV*) is in the center. The tortuous DTS is dispersed between mitochondria and granules. The microfibrils (*Mf*) and microtubules (*Mt*) are dispersed in the hyalomere, along with particles of glycogen (× 17,890).

Fig 10—Part of spread platelet cut near the surface in contact with air. Some mitochondria (*Mi*) and granules (*Gr*) are present in the middle with the reactive DTS. A clear vacuole (*SCS*) is surrounded by tubules (*DTS*) (× 30,780).

Fig 11—Spread platelet very rich in DTS. Incubation at pH 9. The circular band of microtubules surrounds the granulomere. DTS is localized in the granulomere and hyalomere (× 32,600).



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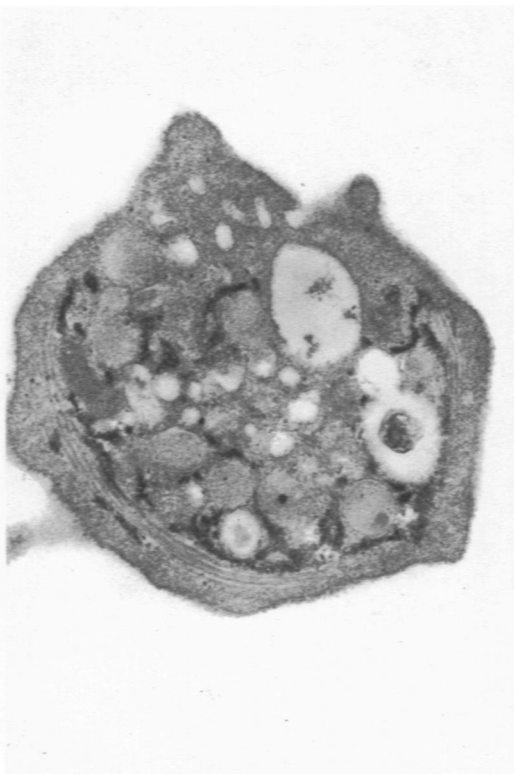

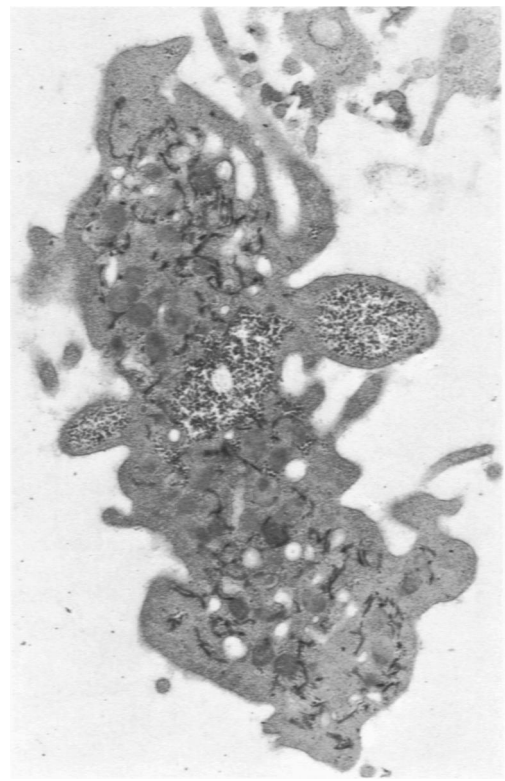

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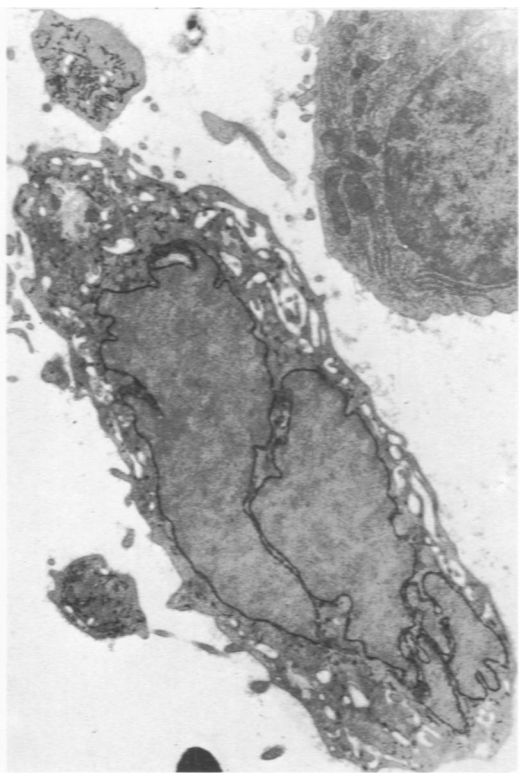

Fig 12—Spread platelet cut at the level of the granulomere. The marginal bundle is sandwiched between two tubules, with peroxidase activity. Other DTS tubules are in the granulomere. Clear vacuoles of SCS are negative. A lipid inclusion is seen in a clear vacuole (× 25,500).

Fig 13—Fragment of megakaryocyte cytoplasm. Fixation and incubation as in Fig 3. DTS is regularly distributed. SCS vacuoles are clear. Glycogen is in the center in a clump (×12,940).

Fig 14—Pycnotic megakaryocyte. The nucleus is deeply indented. The perinuclear space is darkly stained. In the narrow rim of the cytoplasm, between clear organelles, some parts of DTS are reactive. Two platelets probably from this cell are especially rich in DTS. At the right, a plasma cell with negative endoplasmic reticulum (× 6840).

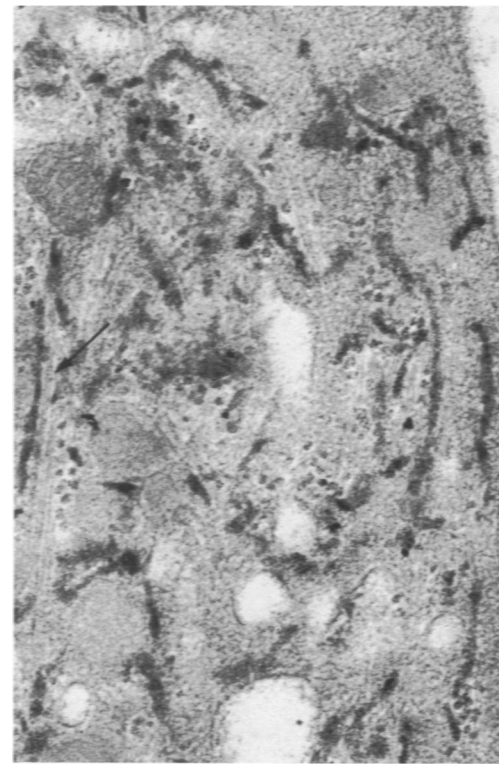

Fig 15—Fragment of thrombopoietic megakaryocyte. In this part of elongated cytoplasm, tubules of DTS are parallel to microtubules (*arrows*) (× 45,140).

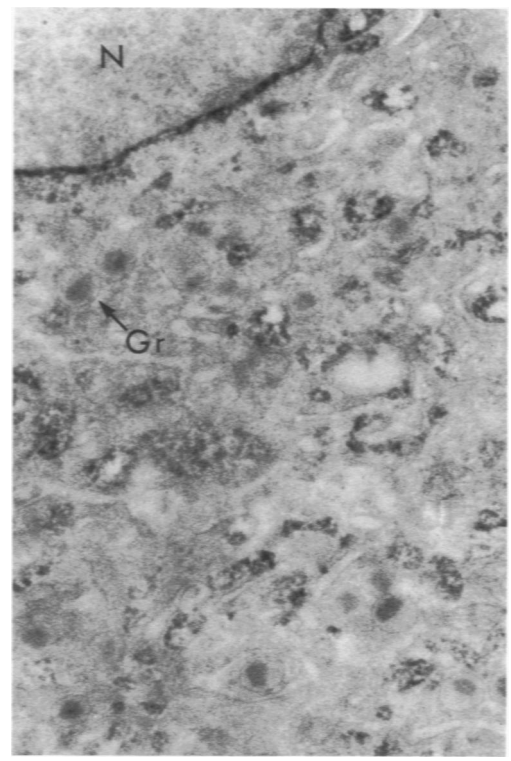

Fig 16—Part of megakaryocyte in bone marrow. Fixation and incubation are as in Fig 3. The nucleus (*N*) is at the top. The granular reaction is seen in the nuclear envelope and endoplasmic reticulum. In the granules (*Gr*), the center is dense but less so than in endoplasmic reticulum (× 31,920).

[End of Article]