Cytochemical Localization of Lysosomal Enzymes in Rat Megakaryocytes and Platelets

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ABSTRACT Platelets secrete lysosomal enzymes during the "platelet release reaction" early in clot formation. This study was undertaken to identify primary lysosomes of platelets and to determine their origin in megakaryocytes. Using electron microscopy and cytochemistry, we localized two lysosomal enzymes, arylsulfatase and acid phosphatase, in megakaryocytes and platelets of normal and thrombocytopenic rats. In platelets and mature megakaryocytes, reaction product for both enzymes is confined to vesicles measuring 175-250 nm. These vesicles, which are primary lysosomes, first appear in the earliest recognizable megakaryocytes and increase in number during cellular maturation. In immature and maturing megakaryocytes, arylsulfatase and acid phosphatase can also be demonstrated in an organelle similar to GERL (Golgi-endoplasmic reticulum-lysosome), i.e., a single smooth-surfaced cisterna with associated vesicles near the stacked Golgi cisternae. Scant reaction product for acid phosphatase is also sometimes seen in Golgi cisternae and endoplasmic reticulum. No reaction product was found in α-granules at any stage of megakaryocyte maturation, nor in α- or serotonin granules of platelets. Thus, our findings indicate that the primary lysosomes of megakaryocytes and platelets are small vesicles derived from GERL early in megakaryocyte differentiation. They can be identified only after cytochemical staining and are distinct from both α- and serotonin granules.

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INTRODUCTION

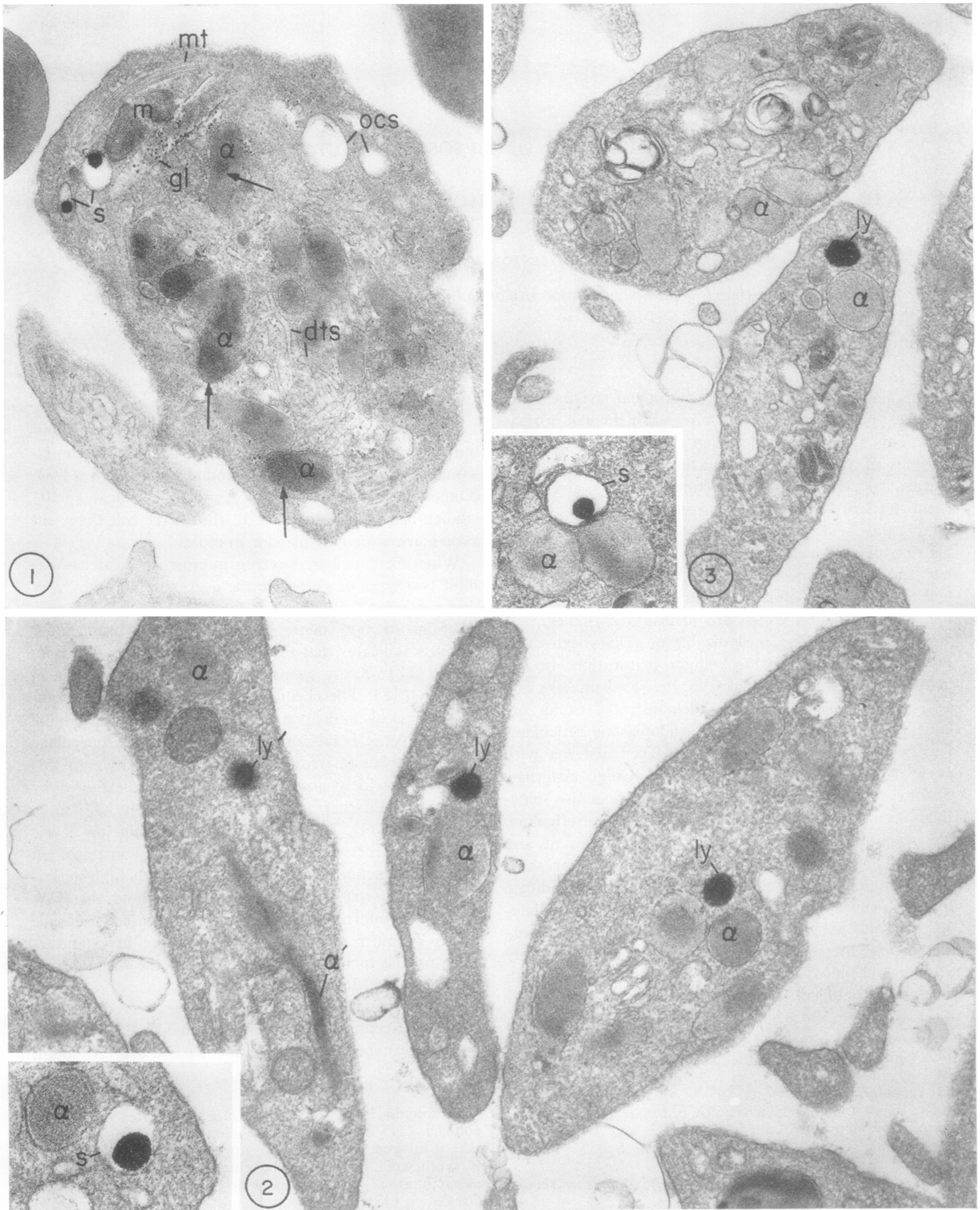
During the "platelet release reaction" induced by thrombin or collagen, mammalian blood platelets secrete lysosomal enzymes into the surrounding medium (1-3). Numerous histochemical (4-7) and biochemical (8-10) studies have suggested that in the intact cell these enzymes are stored in platelet granules.

When examined by electron microscopy, platelets exhibit two types of granules: the α-granule and the "very dense granule." Biochemical analysis of relatively pure fractions of very dense granules isolated from rabbit platelets indicates that they contain serotonin and nucleotides (11); X-ray spectroscopy reveals the presence of calcium (12). The chemical composition of the α-granule is less well defined than that of the "serotonin" granule, yet it is the former granule which is purported to be the platelet lysosome (4-7, 9, 10). Although this opinion is widely accepted, it has been questioned by Siegel and Lüscher (13) and more recently by Broekman et al. (14). In both these studies, only low levels of acid hydrolase activities could be demonstrated in α-granule fractions, whereas fractions with high activities were morphologically heterogeneous and therefore difficult to interpret. While their data present evidence that α-granules are not lysosomes, these investigators were unable to identify with certainty the organelle in which the acid hydrolases reside.

Clearly, platelets originate from megakaryocytes, and with the exception of serotonin granules[1] all platelet organelles can be identified in the parent cell. Although

(*Text continues on page 1639.*)

[1] Serotonin is not found in megakaryocytes of normal animals (11) but only in the circulating platelets, which take it up from the plasma for storage. Reportedly, serotonin is synthesized mainly by the enterochromaffin cells of the gastrointestinal tract, from which it is released into the circulation (15).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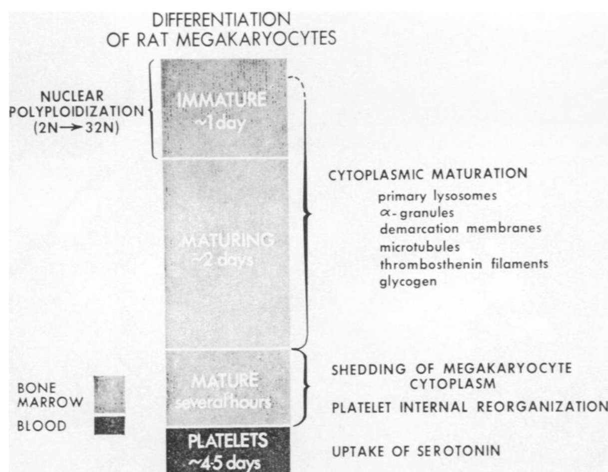

FIGURE 4 Schematic summary of megakaryocyte maturation in the rat. Although megakaryocytes constitute only 0.1–0.5% of the nucleated cells of the bone marrow, they are conspicuous because of their large size and multilobulated nuclei. During a short maturation period of 2–4 days (30, 31), the small (~15 μm) basophilic megakaryocyte, frequently containing a bilobed nucleus, differentiates to a very large cell, with cytoplasm filled with fine azurophilic granules and a highly lobulated nucleus. The nucleus becomes polyploid by a series of endomitoses; ploidy values of mature cells range from 8N to 32N (31). Because attainment of nuclear polyploidy and cytoplasmic differentiation overlap (29, 32), the stages of maturation are difficult to classify and are generally based upon cytoplasmic characteristics. The nomenclature used here is that of Paulus (29). (Paulus defines an "immature" cell as one still synthesizing DNA. Since we have no marker for DNA synthesis, we consider as immature, cells with a large nuclear/cytoplasmic ratio and a paucity of cytoplasmic organelles.) Our observations indicate that primary lysosomes begin to form with the onset of cytoplasmic maturation. α-Granules and demarcation membranes also emerge early but become predominant only later in differentiation. Randomly oriented microtubules, filaments of thrombosthenin, an actomyosin-like protein, and glycogen are abundant by the end of maturation (33, 34). The mature cell becomes irregular in shape as it sheds large portions of cytoplasm. These cast-off segments fragment further, and after internal reorganization, assume the form of definitive platelets which circulate for 4–5 days (35). The time span given for platelet liberation is based on in vitro observations of human megakaryocytes by Thiéry and Bessis (36); the kinetics of in vivo release have not been clarified for either species—i.e., rat or man. Only after platelets enter the blood do they normally exhibit the dense serotonin granules.

FIGURES 1–3 Platelets from normal rat blood. FIGURES 5–15 Megakaryocytes from the bone marrow of rats stimulated by anti-rat-platelet serum. The cells in Figs. 2, 6, 9, and 15 were fixed for 30 min in glutaraldehyde, incubated for 2 h at 25°C in Goldfischer's AS medium, and treated with $(NH_4)_2S$ before further processing. Those in Figs. 5, 7, 8, and 10–14 were fixed in the same way and incubated for 4 h at 25°C in the Barka-Anderson AcPase medium. In all cases except Fig. 1, thin sections were stained with alkaline lead citrate.

FIGURE 1 Platelet from normal blood illustrates the morphology of typical organelles. About 10 α-granules (α) of diverse size and shape are depicted; most are elongated, with nucleoids of variable size (arrows). This cell also contains two serotonin granules (s), identifiable by their eccentric dense cores surrounded by clear halos. Part of the circumferential band of microtubules (mt) is visible near the cellular periphery adjacent to two mitochondria (m). The two membrane systems of platelets are shown here: the dense tubular system (dts) and the open canalicular system (ocs) (26, 27). Small particles of glycogen (gl) are associated with the open canalicular system as well as being scattered throughout the cell (28). Tissue was fixed in paraformaldehyde-glutaraldehyde at 25°C, postfixed in OsO_4, and stained en bloc with uranyl acetate, followed by thin-section staining with uranyl acetate and lead citrate. × 25,000.

FIGURE 2 Circulating platelets reacted for the lysosomal enzyme AS. One vesicle (ly) in each cell contains homogeneously dense lead sulfide reaction product. The larger α-granules (α) nearby are devoid of reaction product. An elongated form, believed to be an atypical α-granule, is labeled "α'." × 37,000. *Inset:* Serotonin granule (s) in another platelet incubated for AS. Its core appears mottled, presumably due to exposure to ammonium sulfide during the AS procedure. Although the density of reaction product in the lysosomal vesicle and that in the core of the serotonin granules are similar, the characteristic reaction-product-free halo encircling the core of the granule usually allows one to distinguish between the two organelles. However, a grazing section through either organelle might be difficult to interpret (see ly' for example). This limitation in interpretation is discussed further in the legend to Fig. 6. × 51,000.

FIGURE 3 Circulating platelets incubated for another lysosomal enzyme, AcPase. One vesicle (ly), containing dense lead phosphate reaction product, is of similar size and shape as those illustrated in Fig. 2 after incubation for AS. Again, note that the numerous α-granules are nonreactive. × 33,000. *Inset:* A portion of another platelet incubated for AcPase to illustrate the appearance of the serotonin granule (s), which is identical to those in Fig. 1 prepared for morphological observation alone. Again, the typical halo around the serotonin core usually distinguishes it from the lysosome. Tissue was fixed for 10 min in glutaraldehyde at 4°C and incubated for 2 h at 37°C in Barka-Anderson's AcPase medium. × 45,000.

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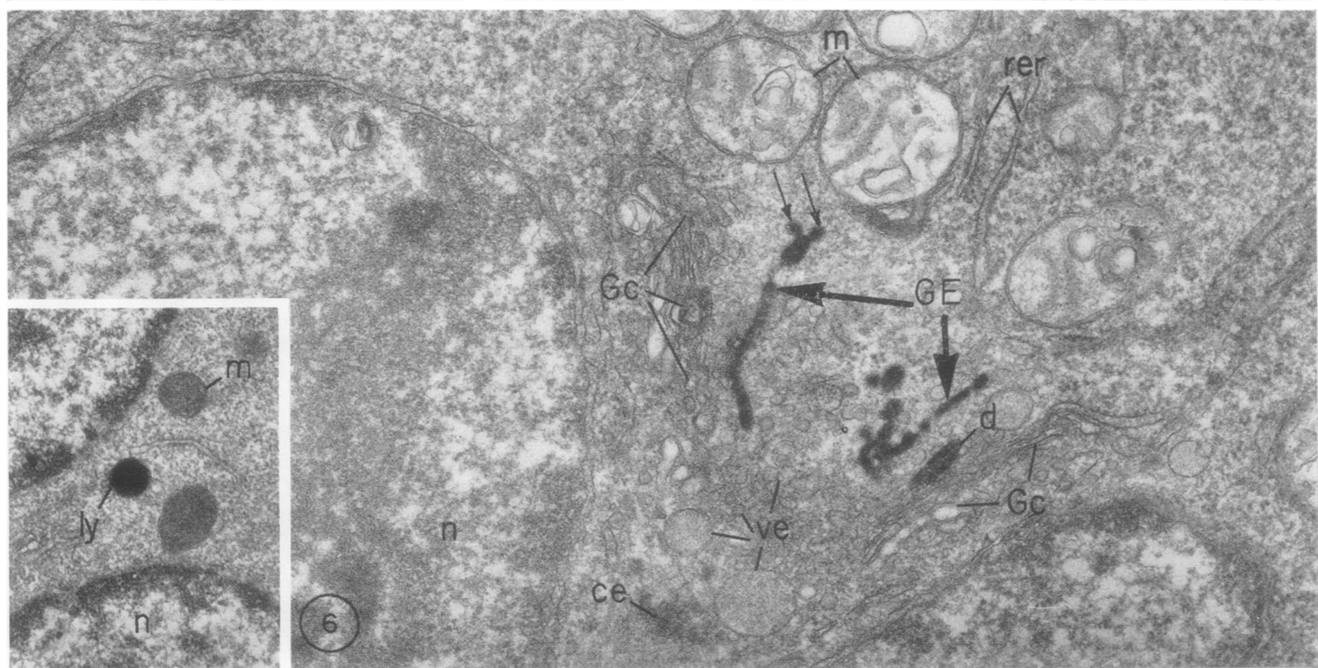
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megakaryocytes of several species have been studied by electron microscopy, the localization of their lysosomal enzymes has not been systematically investigated. Examination of maturing megakaryocytes is essential for a comprehensive understanding of the relationship between α-granules and lysosomal enzymes. Therefore, we have applied electron microscopic and cytochemical techniques to rat bone marrow and blood to determine when and where lysosomal enzymes can first be detected in developing megakaryocytes and to identify the organelles containing them in the mature megakaryocyte and platelet.

METHODS

Materials. A total of 35 young male Sprague-Dawley rats weighing 160–200 g were used in these studies. Some were rendered thrombocytopenic by the injection of antiplatelet serum. Since prolonged thrombocytopenia is known to increase the number of megakaryocytes and augment the proportion of younger cells 2–3-fold (16), we concentrated on the stimulated animals for analyzing lysosome formation.

The production, collection, and processing of rabbit anti-rat platelet antiserum were accomplished according to the procedures of Ebbe et al. (17). Substrates employed for enzyme reactions were β-glycerophosphate (grade I) for acid phosphatase (AcPase),[2] and *p*-nitrocatechol sulfate for arylsulfatase; these were purchased from Sigma Chemical Co., St. Louis, Mo.

Induction of thrombocytopenia. Over a period of 9 days, rats were given five or six i.p. injections of 0.2–0.7 ml antiserum. Platelet counts were less than 3% of normal 24 h after the first injection. The antiserum-treated animals were sacrificed on days 10–13, 24 h to 4 days after the last dose.

[2] *Abbreviations used in this paper:* AcPase, acid phosphatase; AS, arylsulfatase; GERL, Golgi-endoplasmic reticulum-lysosome; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum.

Tissue preparation. With the animal under ether anesthesia, the heart was exposed; blood was obtained by cardiac puncture and collected into a plastic syringe containing 50–100 U of heparin (Invenex Pharmaceuticals, San Francisco, Calif., 10,000 U/ml) per ml blood. It was centrifuged in Kaplow tubes (18) at ~2,000 rpm for 10 min. Platelet-rich plasma was then pipetted into 4–5 vol of fixative. Methods for the collection and handling of bone marrow have already been described (19).

For morphological examination, tissues were fixed for 4 h at 25°C in a mixture of 1% freshly prepared paraformaldehyde and 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, with 0.05% $CaCl_2$ (20). Subsequently, they were washed three times in the same buffer, but with the addition of 7% sucrose, and after being concentrated by centrifugation at 10,000 rpm in a Microfuge 152 (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.), the tissues were immersed overnight at 4°C in 1% OsO_4 in acetate-Veronal buffer.

For cytochemical procedures, tissues were fixed for 10 min–4 h at 4°C in 1.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, with 1% sucrose added. They were then washed three times with the same buffer, with 7% sucrose added.

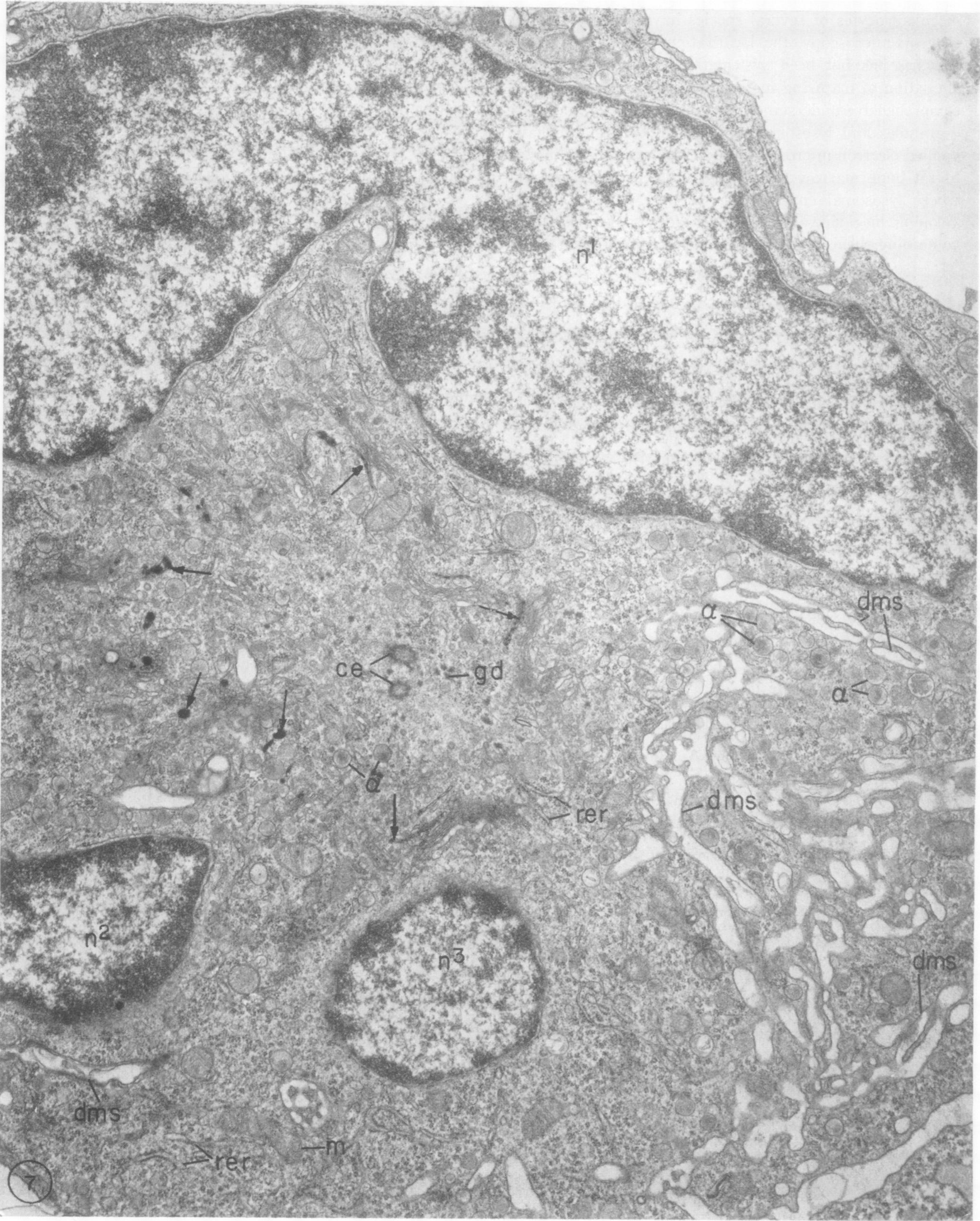
Cytochemical methods. To determine arylsulfatase (AS) activity, tissues were incubated for 2–4 h at 25°C in Goldfischer's medium (21), with the addition of 5% sucrose. Cells were then washed three to five times in acetate-Veronal buffer (pH 5.5) with 7% sucrose, treated for 10 min with 2% $(NH_4)_2S$ (22), and washed again for 2 h in the same buffer with several changes.

For detecting AcPase activity, cells were incubated either for 90–120 min at 37°C or for 4 h at 25°C in the modified Gomori medium of Barka and Anderson (23), with 5% sucrose. At the end of the reaction, cells were washed three times in 0.1 M acetate-Veronal buffer at pH 7.4 with 7% sucrose.

Control preparations and special treatment. Controls for AS consisted of tissues incubated without substrate or cells heated at 90°C for 10 min before incubation. AcPase controls were either incubated without substrate in the medium

FIGURE 5 A very immature megakaryocyte (~12 μm on its long axis) incubated for AcPase. The centrally placed, bilobed nucleus (n^1–n^2) contains large nucleoli (nu) and widely dispersed chromatin. The compact Golgi complex (G) is composed of three individual stacks of about four cisternae each, distributed around the centrioles (ce). Although lead phosphate reaction product is present in the Golgi area (arrows), it is difficult to detect at this low magnification. The cell is rich in free polyribosomes (p) (seen to better advantage in the inset), the chief cytoplasmic component at this stage. A few profiles of RER (rer) are visible. Some of the mitochondria (m) in this cell appear swollen, probably as a result of the short fixation at 4°C. × 17,500. *Inset:* Peripheral cytoplasm of a very early megakaryocyte incubated for AcPase. Faint reaction product in all RER profiles, as seen here, is encountered only occasionally; most cells show none. × 17,000.

FIGURE 6 Portion of another immature megakaryocyte incubated for AS. In this cell, the Golgi complex consists of two stacks of parallel cisternae (Gc) located near a centriole (ce) in an indentation of one of the two nuclear lobes. On one side of each stack is another smooth-membrane cisterna, containing dense lead sulfide reaction product (large arrows). Two coated vesicles (small arrows) appear to be budding from the reactive cisterna. We consider these reactive elements to be part of GERL (GE). The Golgi cisternae (Gc) and numerous vesicles (ve) of varying size not associated with GERL, as well as the nearby RER (rer), are nonreactive. Mitochondrion (m); dirt (d). × 24,700. *Inset:* A larger vesicle (ly), ~250 nm in diameter and containing AS reaction product, is seen in the cytoplasm of a similar cell. These reactive vesicles appear identical to those in the circulating platelets in Fig. 2. Unlike those in the platelet, the densities seen in megakaryocytes can be interpreted unequivocally as reaction product for lysosomal enzymes, since the dense serotonin granules are not present in megakaryocytes of normal animals. × 20,000.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or with 0.01 M NaF added. To assess the amount of possible histochemical latency (19, 24, 25), incubation of some bone marrow samples was preceded by three cycles of rapid freezing in liquid nitrogen and thawing at 37°C.

Subsequent processing. After the postincubation wash, cell suspensions were packed by centrifugation as described above, postfixed as blocks of tissue for 1 h at 4°C in 1% OsO_4 in acetate-Veronal buffer with 5% sucrose, treated with buffered 0.5% uranyl acetate containing 4% sucrose for 30–60 min at 25°C, dehydrated in graded ethanols, and embedded in either Spurr's medium or Epon. Sections were cut on a Sorvall MT-2 ultramicrotome (DuPont Instruments, Sorvall Operations, Newtown, Conn.), stained briefly with alkaline lead citrate (in some cases, preceded by uranyl acetate), and examined with a Siemens 1a or 101 electron microscope (Siemens Corp., Medical/Industrial Groups, Iselin, N. J.) operating at 80 kV.

RESULTS

Platelets

Platelet granule morphology. Rat platelets are disk-shaped, enucleate derivatives of megakaryocyte cytoplasm, measuring 2-3 μm in diameter (Fig. 1). Since their fine structure has been thoroughly described in recent publications (see, e.g., references 26-28), only platelet granules will be characterized in detail here. As in most species, α-granules are abundant in the rat. They are spherical or oval, measuring 300-500 nm in diameter, although in the oval form they may be 700 nm long. These granules often have a moderately dense nucleoid within a diffuse, finely granular matrix. In contrast, the serotonin granules have distinctly different features. They are smaller (200-300 nm), and can be consistently demonstrated only in platelets fixed in glutaraldehyde and osmium or dichromate (6). Under our fixation conditions, they consist of an eccentric, extremely dense core surrounded by a clear halo, seen in 5-15% of platelet profiles.

AS activity. When platelets are incubated for AS, no reaction product is associated with either of the two granule types. Rather, it is consistently present in 175-250-nm vesicles (observed in 20-25% of platelet sections) (Fig. 2). These reactive vesicles are smaller and fewer than the nonreactive α-granules, which retain their characteristic morphology after incubation for AS. Serotonin granules which survive the acidic conditions of the AS medium have a mottled, dense appearance (Fig. 2, inset).

AcPase activity. Reaction product for AcPase is likewise demonstrable in 175-250-nm vesicles (Fig. 3), although it is found here far less frequently than AS. Both the α-granules and serotonin granules, also easily identifiable after incubation for AcPase (Fig. 3, inset), lack this activity.

Since both of the above-mentioned lysosomal enzymes are present in small vesicles, and since there is no evidence that they have previously participated in a digestive event, these enzyme-containing vesicles can be considered true primary lysosomes.

Megakaryocytes

On the basis of a thin electron microscopic section, it is difficult to assign a given megakaryocyte to one of the maturation stages recognizable in bone marrow smears. Therefore, we adopted the broad categories used by Paulus (29) and summarized in Fig. 4, dividing the maturation period into (*a*) immature (Fig. 5), (*b*) maturing (Fig. 7), and (*c*) mature (Fig. 15) stages. The bare megakaryocyte nuclei remaining after platelet liberation is complete have been excluded from this study.

Lysosomal enzymes are detectible in the earliest recognizable cell of the megakaryocyte series, even before α-granules and demarcation membrane system formation are appreciable (Fig. 5). No differences in time of appearance or localization of enzymes are apparent between normal and stimulated animals. Because the locations of AS and AcPase in the immature and maturing stages are similar, these two stages will be considered together.

AS. After incubation of bone marrow for AS, reaction product is found exclusively in two sites in immature and maturing megakaryocytes: (*a*) near the Golgi complex in a single cisterna apparently comparable to Golgi-endoplasmic reticulum-lysosome (GERL) as described in several other cell types (37), and (*b*) in a

FIGURE 7 A maturing megakaryocyte incubated for AcPase. The cell measures ~18 μm in diameter and has numerous cytoplasmic features not present in the immature cell in Fig. 5. Here the centrioles (ce) are located in the center of the cell, still surrounded by the Golgi complex, which has expanded to at least eight stacks of cisternae. A few cisternae and vesicles in the Golgi region (arrows) contain AcPase reaction product. The RER (rer) is increased. The three nuclear lobes encircle the Golgi region, thereby dividing the cytoplasm into inner (perinuclear) and outer (peripheral) zones. Demarcation membranes (dms) form irregular channels in the peripheral cytoplasm on one side of the cell. α-Granules (α), visible in both perinuclear and peripheral zones, are quite uniform in size and shape, and some contain dense nucleoids. Also note the discrete granular densities (gd), frequently seen near the centrioles of immature and maturing megakaryocytes. They resemble the centriole-associated structures described by Dirksen (39) which are believed to be centriole precursor material. Mitochondrion (m). × 15,000.

population of 175-250-nm vesicles, which increase in number throughout maturation. Deposits were never found in the cisternae of the Golgi complex itself, even in tissues fixed for only 10 min and incubated for 4 h to decrease enzyme inactivation and increase cytochemical reactivity (Figs. 6 and 9).

Late in maturation, AS is only occasionally demonstrable in GERL. At this time, its most obvious and consistent localization is within the population of vesicles which initially appear early (Fig. 6, inset). These organelles, often diverse in size and shape at first, but distinct from the larger, more abundant α-granules,[3] now increase in number and become more uniform. They are identical to those in circulating platelets (Fig. 2), and we consider them to be primary lysosomes.

AcPase. AcPase localization in immature and maturing megakaryocytes is similar to, but more extensive than, that of AS. The heaviest and most consistent reaction product is localized in GERL and adjacent coated vesicles (Figs. 5, 7, 8, 10–14). In addition, about half the immature and maturing cells contain small amounts of lead phosphate in the Golgi cisternae (Figs. 8, 13, and 14) and occasionally in rough (RER) (Fig. 5, inset) and smooth (SER) endoplasmic reticulum (Figs. 13 and 14).

Whether this lack of uniformity in reaction reflects a true variability in the cells or an experimental variable is uncertain. It is not related to the temperature or length of fixation or incubation, nor to the duration of the postfixation wash. Within the same experiment, cells containing reaction product for AcPase in the Golgi cisternae may be found with other megakaryocytes at the same stage of maturation but lacking the enzyme.

Besides occasional 175–250-nm vesicles resembling those in AS preparations, other vesicles of diverse size, containing loose, flocculent reaction product, are sometimes found (Fig. 14, inset). Perhaps these represent immature forms which later condense to form primary lysosomes.

Mature megakaryocytes

AS. In mature cells, AS is confined to the population of vesicles–i.e., the primary lysosomes, which remain distinct from the nonreactive α-granules (Fig. 15). Like the α-granules, the lysosomes are distributed among the prospective platelets, evidently persisting as specific entities when the platelets are shed (see Fig. 2).

AcPase. AcPase is likewise demonstrable in small lysosomal vesicles of mature megakaryocytes, although it is observed less consistently than AS. Golgi regions are rarely encountered; when seen, they are devoid of reaction product.

Technical variations. One serious drawback of metal-salt techniques such as we have used is the problem of "histochemical latency." Because of this phenomenon, an enzyme may be contained within an organelle where it cannot be demonstrated (19, 24, 25, 40). Since AcPase was only occasionally observed in mature cells, we attempted to overcome such possible latency by rapidly freezing and thawing tissues before incubation. In this experiment, the number of reactive vesicles was increased at least 5-10-fold, but even after this treatment, no reaction was apparent in α-granules.

Shortening fixation times seemed to result in more reaction product for AcPase, but little difference could be detected with respect to AS. Variations in the duration or temperature of incubation did not alter the intracellular localization of either enzyme. However, since incubation at 37°C tended to produce a nonspecific, fine sprinkling of reaction product throughout many cells, most experiments were performed at 25°C.

Control preparations

The omission of substrate from AS medium caused a loss of deposits in GERL, but the vesicle reaction in both megakaryocytes and platelets was only slightly reduced, presumably because of endogenous substrate (19). Heating cells to destroy enzyme activity completely abolished the reaction in all organelles. No reaction product was evident after incubation for AcPase when the substrate was omitted or when 0.01 M NaF was added to the medium.

DISCUSSION

The new findings we have obtained here by electron microscopy and cytochemistry clarify the structure, time of appearance, and site of origin of primary lysosomes in rat megakaryocytes and platelets. In immature and maturing megakaryocytes, two lysosomal enzymes, AS and AcPase, appear in small (∼ 70 nm) vesicles in the Golgi region. During maturation, these vesicles apparently fuse to form larger ones (175-250 nm), which increase in number, disperse throughout the mature megakaryocyte cytoplasm, and ultimately reside in circulating platelets. We found AcPase and AS restricted to such vesicles, which, like the primary lysosomes of most other cells, require cytochemical staining procedures for identification (41).[4] Since there is no evidence that the

[3] Although it is widely quoted that α-granules originate from the Golgi complex (38), we are not aware of any conclusive evidence that they are formed by this organelle. In this study, we were unable to visualize α-granule formation.

[4] Primary lysosomes in the form of morphologically recognizable granules are found in relatively few cell types, notably eosinophils, neutrophils, and monocytes of the blood. In other tissues, small Golgi- or GERL-derived vesicles presumably function as primary lysosomes (see reference 42 for discussion).

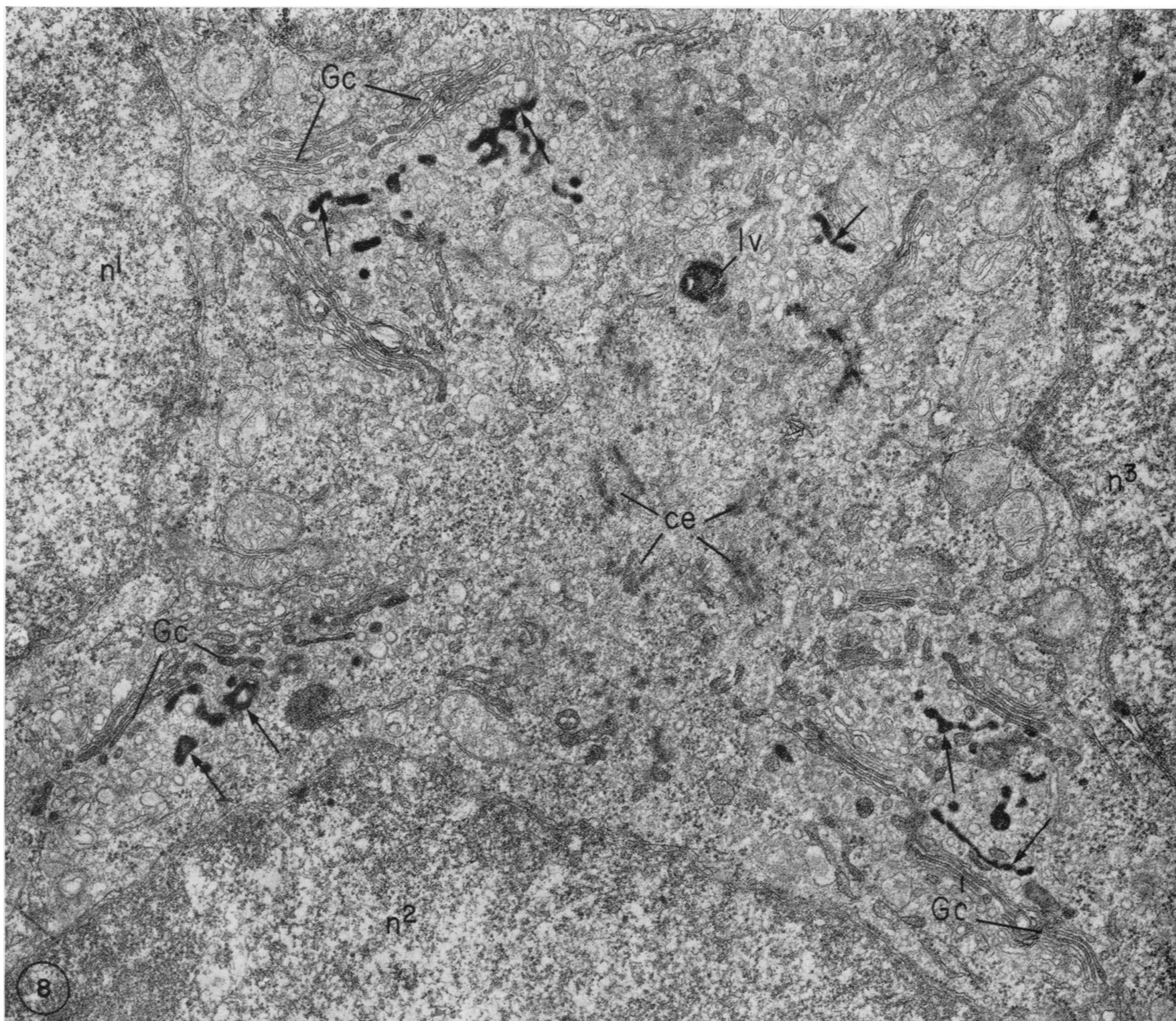
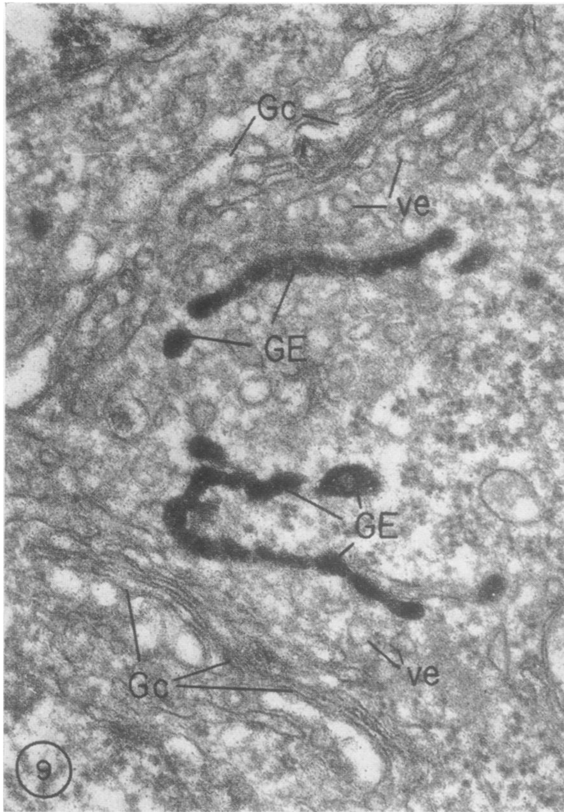


FIGURE 8 AcPase reaction in the Golgi region of a megakaryocyte slightly larger than those in Figs. 5 and 6. The extensive Golgi complex surrounds the centrioles (ce), filling most of the cytoplasm framed by the nuclear lobes (n^1–n^3). Flat cisternae or irregularly-shaped components of GERL lie close to one side of most Golgi stacks depicted here. GERL has dense AcPase reaction product (arrows). In this specimen, some Golgi cisternae (Gc) react weakly. One large vesicle (lv) contains heavy deposit. (See Figs. 13 and 14 for higher magnifications of two of these Golgi areas.) × 20,000.

acid hydrolases in these vesicles have participated in a digestive event, we conclude that they represent primary lysosomes. In megakaryocytes and platelets, they appear to be distinct from both α- and serotonin granules.

Although megakaryocyte lysosomes clearly originate in the Golgi region, the exact mode of their formation is not obvious because in early cells the two enzymes were not identically localized. Both AS and AcPase were present in high concentrations in GERL. According to Novikoff's concept of GERL (37), materials synthesized in RER move directly into the specialized tubules and cisternae of SER which constitute GERL. But in this study, we frequently observed AcPase in the stacked Golgi cisternae as well. Several explanations may possibly account for this difference in site: (*a*) Low levels of enzyme activity could have escaped detection by our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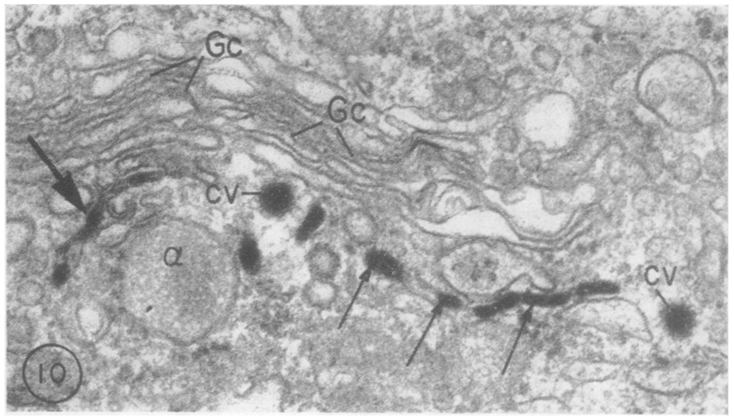
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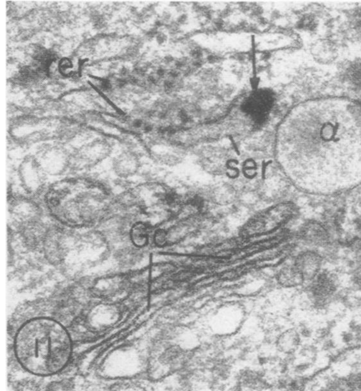
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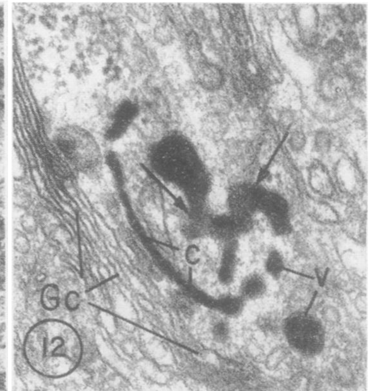
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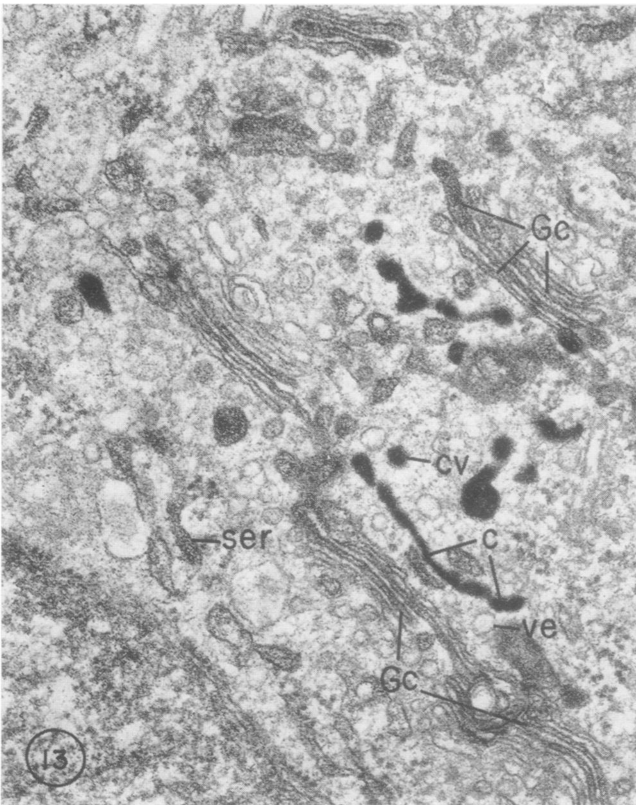
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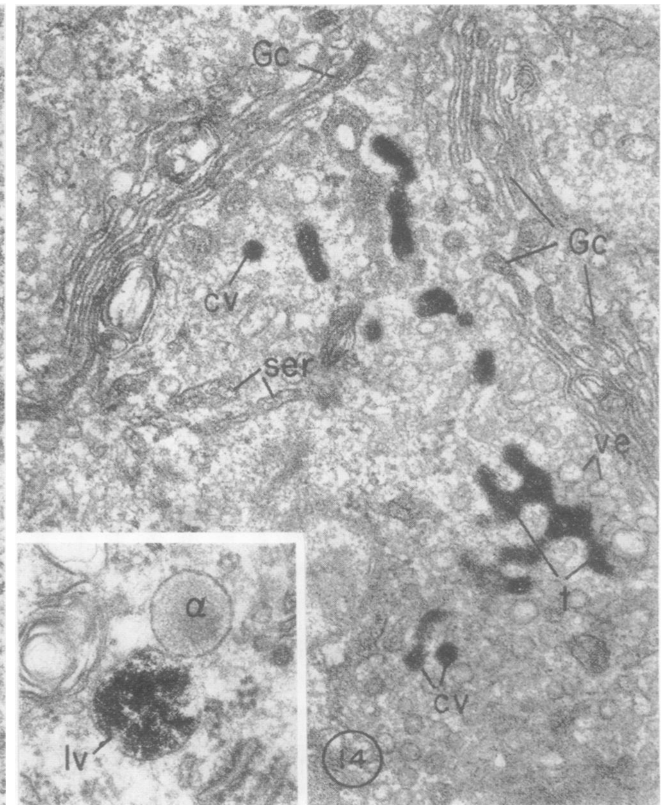
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methods, and thus, AS might be present but not demonstrable in the Golgi cisternae. (*b*) Perhaps the two lysosomal enzymes enter the Golgi area by dissimilar routes and are united and concentrated by GERL into a single organelle–the primary lysosome. (*c*) Another consideration deserving serious thought is that we may have demonstrated two AcPases–one lysosomal and another related to some other function of the Golgi complex (see reference 43 for discussion). Several other investigators have found this dual localization of AcPase (22, 40, 44), and some have suggested that primary lysosomes may originate from either GERL or the Golgi cisternae under varying physiological circumstances (22, 44). In our study, we were unable to correlate this variation in AcPase localization with any developmental stage or physiological condition (e.g., thrombocytopenia). Thus, the significance of such a dual localization remains unknown.

To our knowledge, only two previous studies, both by Behnke (45, 46), have involved the fine-structural localization of lysosomal enzymes in rat megakaryocytes and platelets. In rat megakaryocytes, he reported AcPase-positive vesicles similar to those we have described, but did not investigate the time of their appearance or place of origin. However, in platelets, he found solely nonspecific lead deposits and so judged lead-salt techniques inappropriate for studying lysosomal enzyme localization in these particular cells (45). Since we observed no lack of specificity in our material, we cannot concur with Behnke's conclusion.

Most previous cytochemical investigations of megakaryocytes and platelets have pertained to other species, and in some cases, with quite different methods of cell preparation, making direct comparisons difficult (4-7). In a study of rabbit blood and bone marrow, Wetzel et al. (47) reported AcPase in the Golgi region of megakaryocytes and in rare granules distinct from α-granules. In platelets, AcPase activity occurred as densities within vacuoles. These workers pointed out the morphological similarity of the AcPase-reactive granules to the serotonin granules of unincubated platelets, suggesting that the two might be identical. However, biochemical analysis of the purified serotonin granule fraction of rabbit platelets (11) does not support this hypothesis.

As mentioned in our Introduction, the majority of earlier investigators (4-7, 9, 10) have claimed that lysosomal enzymes are contained in α-granules. This consensus is not universal. On the basis of cell fractiona-

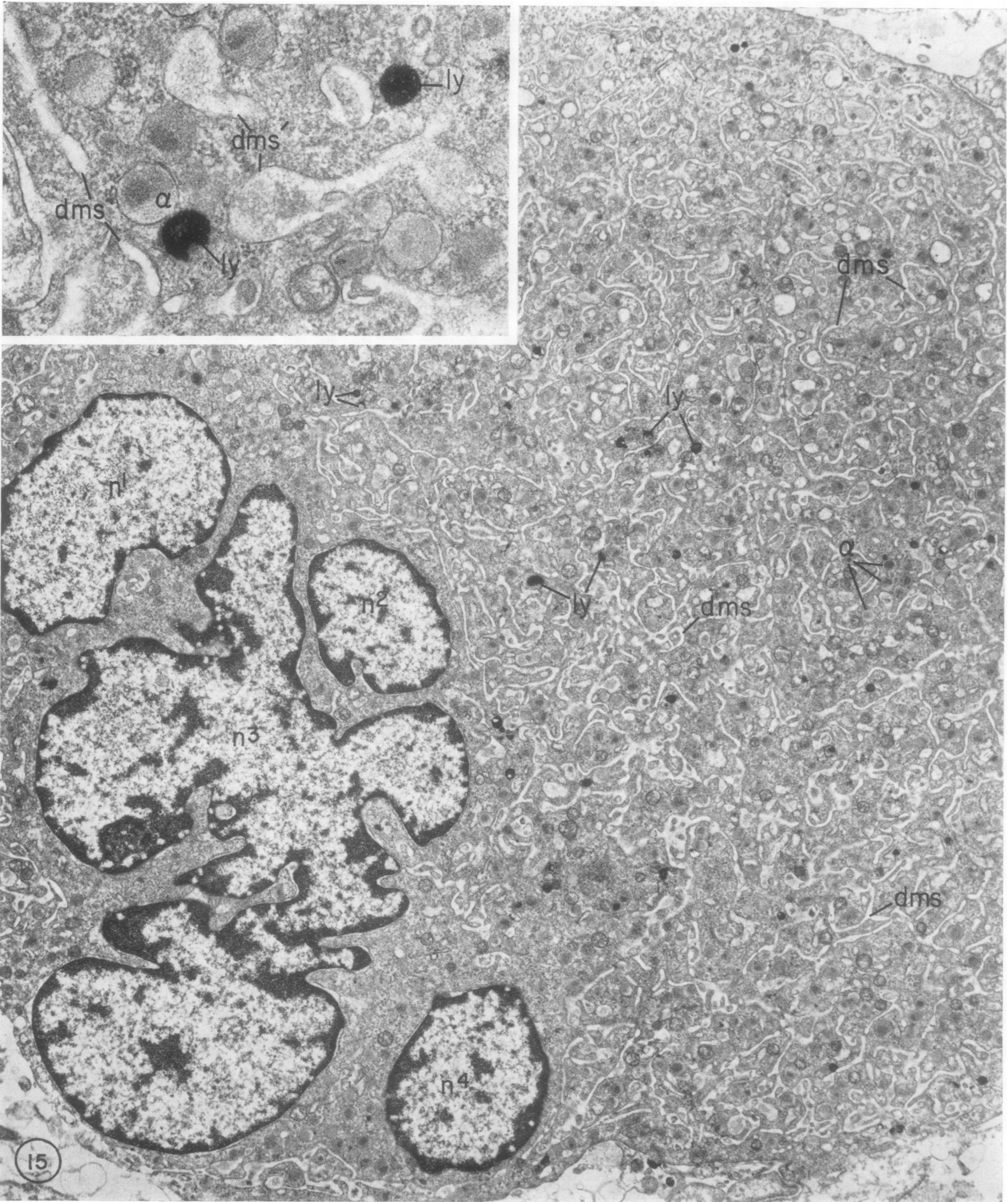
FIGURES 9–14 Golgi regions of several immature and maturing megakaryocytes incubated for AS (Fig. 9) or AcPase (Figs. 10–14). These micrographs illustrate the structure typical of GERL in this system, the spatial relationships between Golgi cisternae and GERL, and the variations in enzyme distribution which we encountered.

FIGURE 9 Golgi zone of a very immature megakaryocyte incubated for AS. This figure emphasizes the distinction between the stacked Golgi cisternae (Gc) and the GERL cisterna (GE). The latter is separated from the Golgi stack by a space containing vesicles (ve). This space is several times the width of the Golgi intercisternal space. Only the GERL cisterna contains reaction product for AS. × 44,200.

FIGURES 10 and 12 Golgi areas of two maturing megakaryocytes reacted for AcPase. In these cells, the distribution of AcPase reaction product in GERL is similar to that of AS. The α-granules (α) and the bulk of the Golgi cisternae (Gc) are nonreactive. In Fig. 10, the identity of the reactive cisterna on the left (large arrow) is uncertain, but the major reactive elements are tubular and vesicular profiles (small arrows). Reactive coated vesicles (cv) are prominent, while many other vesicles of varying sizes, both coated and uncoated, contain no reaction product. Fig. 12 illustrates a reactive GERL cisterna (c), an *en face* view of an irregularly-shaped saccule (arrows), and vesicles (v). (It is possible that some of the small "vesicles" may actually represent cross-sections of tubules.) Fig. 10, × 59,500; Fig. 12, × 49,000.

FIGURE 11 Part of the Golgi region of another maturing megakaryocyte reacted for AcPase. Reaction product (arrow) is visible in a smooth-membrane portion of a cisterna (ser) (presumably part of GERL), which is continuous with the RER (rer). Golgi cisternae (Gc) and an α-granule (α) are nonreactive. × 66,000.

FIGURES 13 and 14 Higher magnifications of two Golgi regions of the cell in Fig. 8, which was incubated for AcPase. The heaviest reaction product is apparent in GERL. In Fig. 13, a cisternal portion (c) of GERL is clearly evident near the stacked Golgi cisternae, while Fig. 14 illustrates a branching tubular (t) profile. Coated vesicles (cv) are also present. In contrast to Figs. 10 and 12, flattened Golgi cisternae (Gc) and segments of SER (ser) contain scant amounts of reaction product. Both figures show nonreactive vesicles (ve) in the space between Golgi cisternae and GERL. Figs. 13 and 14, × 44,500. *Inset:* Large vesicle (lv), ~330 nm in diameter, containing loose, flocculent AcPase reaction product, perhaps formed by the fusion of two or more smaller GERL-derived vesicles. Note the adjacent nonreactive α-granule. × 46,500.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tion studies, Siegel and Lüscher (13) were the first to suggest that α-granules are not "typical lysosomes." Nevertheless, in a subsequent publication, they stated that because of possible artifacts produced by their fractionation procedure and incomplete separation of subcellular particles, the question remained unresolved (48). The recent and more extensive work of Broekman et al. (14) provides additional evidence for the nonlysosomal nature of the α-granule. Their remarkably pure α-granule fraction revealed no peak for any enzyme tested (lysosomal or otherwise). Activities of AcPase (with β-glycerophosphate as substrate), as well as three other lysosomal enzymes, peaked in a morphologically heterogeneous fraction containing vesicles, mitochondria, anisometric bodies, and a few α-granules. Our cytochemical data and the biochemical results of these workers point to the existence of lysosomes as entities distinct from both α- and serotonin granules in megakaryocytes and intact platelets.

Since AcPase is often the only enzyme used for identifying lysosomes, the question of platelet AcPase activity warrants further discussion. Zucker and Borrelli (49) first demonstrated that all the acid β-glycerophosphatase activity and some of the *p*-nitrophenylphosphatase activity (50) present in normal human serum were derived from platelets. Furthermore, Walter et al. (51) and Kaulen and Gross (52) also found both these AcPases in human platelet homogenates. The latter investigators, after thoroughly characterizing the two enzymes with respect to pH optima, rates of hydrolysis, and sensitivity to inhibitors, concluded that only the β-glycerophosphatase of platelets is lysosomal. In another blood cell type, the neutrophilic leukocyte, it is likewise clear that only β-glycerophosphate demonstrates the reactivity of lysosomal AcPase, whereas *p*-nitrophenylphosphate reveals a totally different, membrane-associated enzyme, which is not localized in lysosomes (53). Nevertheless, in many enzyme characterizations of platelet fractions, as well as in studies of the release reaction, *p*-nitrophenylphosphate alone has been used as the substrate for demonstrating AcPase. Thus, the localization of lysosomal enzymes in platelet fractions becomes more difficult to interpret, and the already clouded issue of the fate of AcPase in the release reaction is still further complicated[5] (3, 54).

Although the results of our analysis lead us to believe that both α- and serotonin granules are nonlysosomal in intact platelets, we have no data on lysosomal enzyme localization during or after the release reaction. It is quite possible, then, that a redistribution of lysosomal enzymes may occur when platelets are treated with thrombin or other aggregating agents (see reference 6).

The function of the released lysosomal enzymes is still enigmatic. Apparently, acid hydrolase release is complete by 60 s after exposure to thrombin (2), and it has been postulated that lysosomal enzymes trigger coagulation in the platelet environment (2) or participate in platelet lysis (55). Whether platelet lysosomes have any intracellular function is also unknown. Although extensively studied, the question of platelet phagocytic and digestive capacities remains unresolved (56-58).

Thus far, our studies afford no new clues to the content of the α-granule. Presently, there is evidence for calcium (59) and an acid mucosubstance (60) in the α-granule nucleoid, and a sulfated mucopolysaccharide has been demonstrated by autoradiography (61). Fibrinogen, too, has been reported within this organelle (62), and the disappearance of α-granules has been correlated with the appearance of fibrin in the platelet open canalicular system (54, 63). Obviously, new approaches are needed to define the full composition, origin, and function(s) of this most prominent platelet organelle.

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[5] Holmsen and Day (3) reported the release of β-*N*-acetylglucosaminidase, β-galactosidase, and arylsulfatase; *p*-nitrophenylphosphatase was retained, but with their assay conditions, these investigators were unable to demonstrate β-glycerophosphatase, either intra- or extracellularly.

FIGURE 15 A mature megakaryocyte incubated for AS. At this stage, the nuclear lobes (n^1–n^4) are close together and eccentrically located. The vast demarcation membrane system (dms) dominates the cytoplasm forming "platelet fields"; RER is scarce, and the once-extensive Golgi complex is now diminutive. AS is limited to small vesicles (ly), which, along with the more numerous α-granules, become platelet constituents. × 9,000. *Inset:* Higher magnification of a small part of the cell. The primary lysosomes (ly), identified by their content of AS reaction product (not as homogeneous here as in Fig. 2), are shown together with the nonreactive α-granules. Numerous membranes of the demarcation membrane system (dms), occasionally filled with a fuzzy material (dms′), are also present. × 35,000.

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