

ULTRASTRUCTURAL LOCALIZATION OF DIALYZED IRON-REACTIVE MUCOSUBSTANCE IN RABBIT HETEROPHILS, BASOPHILS, AND EOSINOPHILS

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ABSTRACT

For ultrastructural localization of acid mucosubstances in rabbit granulocytes, bone marrow and buffy coat specimens were fixed with formalin, glutaraldehyde, or osmium tetroxide, sectioned at 40 μ , and stained with the Rinehart and Abul-Haj solution of dialyzed iron (DI). Heterophils revealed DI staining on the outer surface of the plasma membrane, in the Golgi complex involved in primary granulogenesis, and in primary granules. The intragranular distribution of DI-stained material varied at different stages in the maturation of primary granules. Immature granules of heterophils fixed by any of the three methods contained a peripheral concentric band of DI-positive material; however, fully mature primary granules possessed a core of DI-reactive material in heterophils fixed with osmium tetroxide, but they contained little or no staining in heterophils fixed with formalin or glutaraldehyde. Secondary granules of rabbit heterophils failed to stain with DI. Tertiary granules, observed only in late heterophils, contained distinct DI-positive particles. Basophil granules exhibited intensely DI-stained material distributed in an orderly pattern throughout the granule. In eosinophils, DI staining was localized in the Golgi complex and in the rims of a few immature cytoplasmic granules.

Bone marrow actively incorporates radioactively labeled sulfate (14, 46, 52). Radioautographic studies (24, 28, 35, 39) have shown that much of this sulfate is incorporated into developing granular leukocytes, and a sulfated mucosubstance has been localized by specific cytochemical methods in cytoplasmic granules of rabbit (28) and human (16) neutrophil, basophil, and eosinophil leukocytes. In addition, biochemical evidence has been presented for the existence in leukocytes of several mucosubstances, including chondroitin sulfate, hyaluronic acid, and glycoprotein (9, 11, 33, 40). Hyaluronic acid and chondroitin sulfate have also been isolated from a granule-rich fraction obtained from rabbit peritoneal exudate leukocytes (20).

In the present study, the acid mucosubstances of rabbit heterophils, basophils, and eosinophils

were investigated with the Rinehart and Abul-Haj solution of dialyzed iron (DI) (42), which is a specific reagent for ultrastructural visualization of acid mucosubstance (56). The specific objective of the study was to determine, with the high resolution of electron microscopy, which granules or other cytoplasmic structures contained acid mucosubstance, the site of biosynthesis of this component, and the intragranular distribution of this component during various stages of cell development. A preliminary report of this investigation appeared previously (27).

MATERIALS AND METHODS

Bone marrow specimens were obtained from femurs of young albino male rabbits anesthetized with sodium pentobarbital. For preparing buffy coat specimens,

blood was drawn into heparinized syringes from the young rabbits by cardiac puncture. After its transfer to segments of 8 mm diameter glass tubing which had been sealed at one end, the blood was centrifuged for 10 min at 8°C and 2,000 rpm in a refrigerated International centrifuge with a swing-out rotor. When the plasma had been removed by breaking the tubing immediately above the level of the buffy coat, a small amount of 1% thrombin was injected gently into the buffy coat layer so as to promote clotting. The glass tubing was broken below the level of the buffy coat after it had clotted, and intact pieces of buffy coat were expressed from the tubes and separated from the erythrocytes.

For visualizing acid mucosubstance, the bone marrow and buffy coat specimens were first fixed at 4°C for 1 hr in a solution containing 10% formalin and 2% calcium acetate, for 1 hr in cacodylate-buffered, 6.25% glutaraldehyde (44), or for 1 hr in collidine-buffered, 2% osmium tetroxide. After fixation, these specimens were rinsed in 7.5% sucrose and then minced with a razor blade into small blocks or sectioned at 40 μ in a cryostat or with a Smith-Farquhar tissue sectioner. The small blocks or sections were rinsed in 7.5% sucrose and stained 1–24 hr with the Rinehart and Abul-Haj solution (42) of dialyzed iron (DI) at room temperature. The dialyzed iron stock solution was prepared by dissolving 75 g of powdered anhydrous ferric chloride in 250 ml of distilled water, adding 100 ml of glycerin, and then slowly adding, with constant stirring, 55 ml of concentrated (28%) ammonium hydroxide. This stock solution was then poured into dialyzing tubing (Scientific Products, Charlotte, N.C.), with a pore size of ca. 2.4 μ , and dialyzed against distilled water, which was changed twice a day for 3 days. In contrast to the original procedure (42), the dialyzing tubing was almost completely filled with the stock solution, thereby allowing very little dilution. This stock solution could be stored in brown bottles at 4°C for several months. Just before use, 1 volume of glacial acetic acid was added to 4 volumes of the dialyzed stock solution, yielding a staining solution with a pH of 1.8–2.0. Staining with this solution in this pH range has been shown to demonstrate selectively acid mucosubstances rich in carboxyl groups and sulfate esters by specific blockage of these reactive groups and by comparison of DI-staining results with those obtained by light microscope cytochemical methods (56). The high acidity of this staining solution probably accounted for the relatively poor cytological preservation of most of the specimens examined. Control sections were placed in a nondialyzed solution prepared as above but containing magnesium chloride in place of the ferric chloride.

In an attempt to identify the groups reacting with DI, cryostat sections were subjected to either methylation or hyaluronidase digestion prior to DI staining.

Methylation was carried out by incubating cryostat sections for either 6 or 18 hr at 60°C in absolute methanol containing 0.1 N HCl (21); this procedure should abolish DI staining of free carboxyls and sulfate esters by blocking the former (22) and removing the latter (32). Control sections were treated in a similar manner, except that isopropanol was substituted for methanol; such treatment does not esterify the carboxyls (22) or hydrolyze the sulfate groups (21). Cryostat sections of mouse rectosigmoid colon were used as positive controls for evaluating the effectiveness of the methylation procedure. As reported previously (56), 18-hr but not 6-hr methylation blocked DI staining of sulfated mucosubstances of the goblet cells in the colon.

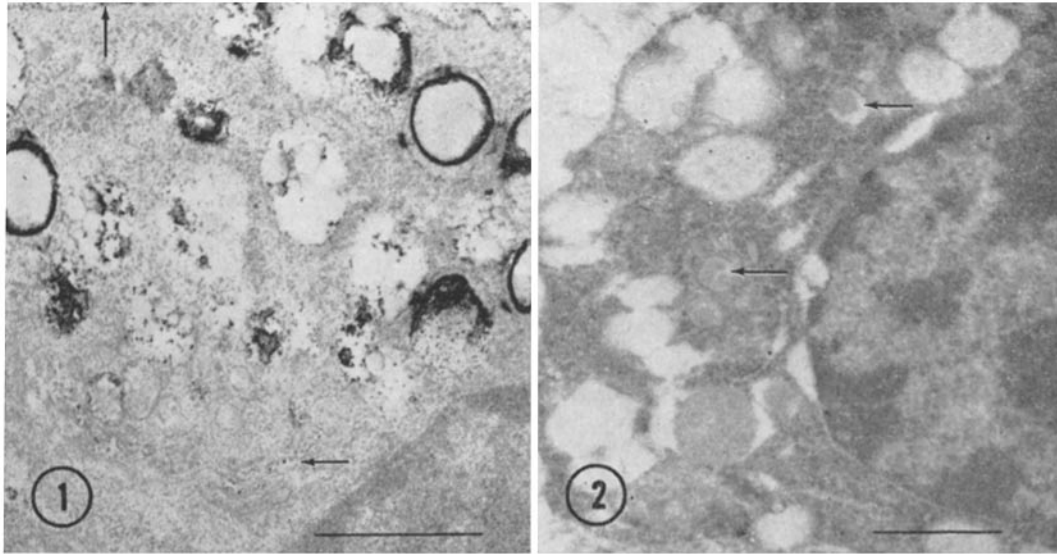
Hyaluronidase digestion, which is known to block basic dye staining of sites rich in hyaluronic acid and chondroitin 4- and 6-sulfates at the light microscope level (49), was carried out by incubating cryostat sections at 37°C for 6 hr in pH 5.5, 0.1 M sodium phosphate buffer, containing 0.05% testicular hyaluronidase (Worthington Biochemical Corp., Freehold, New Jersey). Control sections were treated in a similar fashion except that hyaluronidase was omitted from the phosphate buffer. Previous studies have shown that hyaluronidase digestion eliminates DI reactivity in the hyaluronic acid-rich mucoid stroma in the dermoid layer of the cock's comb (Wetzel, M. G., and S. S. Spicer, unpublished observations).

All specimens were rinsed with buffer and post-fixed 1 hr in either 2% collidine-buffered osmium tetroxide or in Millonig's osmium tetroxide (37). Specimens were then rinsed in water, dehydrated with graded alcohols, embedded in Maraglas (Polysciences, Inc., Rydal, Pa.), and sectioned with an LKB ultratome. The ultrathin sections, with and without prior staining with lead citrate, were examined in an AEI-6B electron microscope.

RESULTS

Sections stained with DI showed electron opacity that was confined to the outer surface of the plasma membrane, certain cytoplasmic granules, and, in some instances, the Golgi complex of rabbit heterophils, basophils, and eosinophils (Figs. 1, 3–30). This electron opacity was apparently due to the presence of an acid mucosubstance, since sections exposed to the control $MgCl_2$ solution (Fig. 2) or processed by routine procedures showed no such density in these sites.

The DI reactivity in heterophils was not altered by digestion with testicular hyaluronidase or a 6-hr methylation. An 18-hr exposure to acidified methanol abolished all DI reactivity in



FIGURES 1 and 3-21 illustrate rabbit heterophils from specimens which were sequentially fixed with glutaraldehyde, sectioned in a cryostat, stained 24 hr with DI, and postfixed with osmium tetroxide.

Figs. 1-18 and 20 were obtained from bone marrow specimens, and Figs. 19 and 21 came from buffy coat preparations. The heterophil in Fig. 2 was from a specimen treated similarly, except that a control solution was employed instead of DI. Figs. 3-8 and 11-21 show heterophils from thin sections stained with lead citrate; the remaining figures illustrate heterophils from unstained sections. The scale line in all figures equals 1 μ .

FIGURE 1 Early heterophil showing intense DI staining in the rims of some primary granules, lightly stained granular material within Golgi lamellae (horizontal arrow), and light staining on the outer surface of the plasma membrane (vertical arrow). $\times 20,000$.

FIGURE 2 Early heterophil exposed to a control solution containing magnesium chloride instead of ferric chloride. Vacuoles with one or several spherules (arrows) and the empty-appearing primary granules appear no more dense than nuclei or other cytoplasmic structures. $\times 15,000$.

heterophil leukocytes. In specimens treated 18 hr with acidified isopropanol, primary granules of many but not all immature heterophils contained scattered DI-stained particles but disclosed less DI reactivity than granules of heterophils without such treatment.

Staining of Heterophils after Glutaraldehyde Fixation

As will be described, the type of fixation did not radically affect the affinity of mucosaccharide-rich sites for DI in heterophils at an early stage of development but markedly influenced such reactivity in the mature cells. Since cell structure survived the staining procedure best in

specimens fixed with glutaraldehyde prior to DI staining, results obtained with this fixative are described in greater detail. After glutaraldehyde fixation, DI staining was observed in all heterophils in rabbit marrow and buffy coat specimens (Figs. 1, 3-21). With the exception of the outer surface of the plasma membrane and certain Golgi elements, the only sites with DI affinity in heterophils fixed with glutaraldehyde were intensely stained primary and tertiary granules.

Primary Granules

Previous ultrastructural studies (4, 5, 7, 26, 29, 53-55) have shown that early developmental stages of rabbit heterophils (promyelocytes) con-

tain a population composed only of primary (azurophil) granules. The formation of these granules involves budding from the Golgi saccules of small vacuoles with a central spherule (54) or core (7). Fusion of several of these small vacuoles leads to the formation of larger multi-cored vacuoles (7) containing many discrete spherules (54). The spherules themselves fuse to form a morula (54) or multi-lobulated mass (7), which then becomes a central nucleoid (7, 54) enclosed by a less dense cortex. The nucleoid form apparently condenses to form a granule with homogeneous dense content.

Early heterophils engaged in formation of primary granules revealed small DI-stained particles in some Golgi lamellae (Fig. 1) and in the periphery of many vacuoles associated with the Golgi complex (Fig. 1) and located elsewhere in the cytoplasm (Figs. 3, 4). In more mature vacuoles, in which the spherules were in a process of fusion, the DI staining was more intense and appeared homogeneous (Fig. 5).

The primary granules of the early heterophils varied markedly in their staining with DI apparently in relation to stages in their maturation. On the basis of differences in distribution of the DI-stained material, four general subclasses of primary granules, designated the post-morula, A, B, and C varieties, could be distinguished in rabbit heterophils. These designations were adopted so as to assign, as closely as could be determined, the same term to each chronological stage in granule maturation when visualized by DI staining and when distinguished by fixation with pyroantimonate-osmium tetroxide (26).

The term "*post-morula*" designates a granule variety that possibly represents a stage following the morula in granule maturation. This granule contained a large, usually central, rounded-to-irregular area, which consisted of DI-reactive and moderately dense unreactive substances and was enclosed by low-density flocculent material (Figs. 6, 7). Post-morula granules were present only in early heterophils (Table I).

A granules exhibited three distinct zones (Figs. 8-11): (a) a nearly central, unstained area with contents of very low density, (b) an intervening, concentric band of DI-stained substance, and (c) a low-density, unstained cortex, which closely resembled the flocculent material in the periphery of post-morula granules. The structure and relative abundance of the three zones varied among

the A granules. In some A granules, the peripheral concentric rim of DI reactivity appeared partially interrupted as though in an early phase of disruption (Fig. 17). The A granules were the most numerous variety of primary granules in early heterophils (Fig. 15); they were present in intermediate heterophils (Figs. 16, 17) and absent from late heterophils (Fig. 18) (Table I). These granules appeared to correspond with the nucleoid form observed morphologically and cytochemically (5, 7, 54, 55).

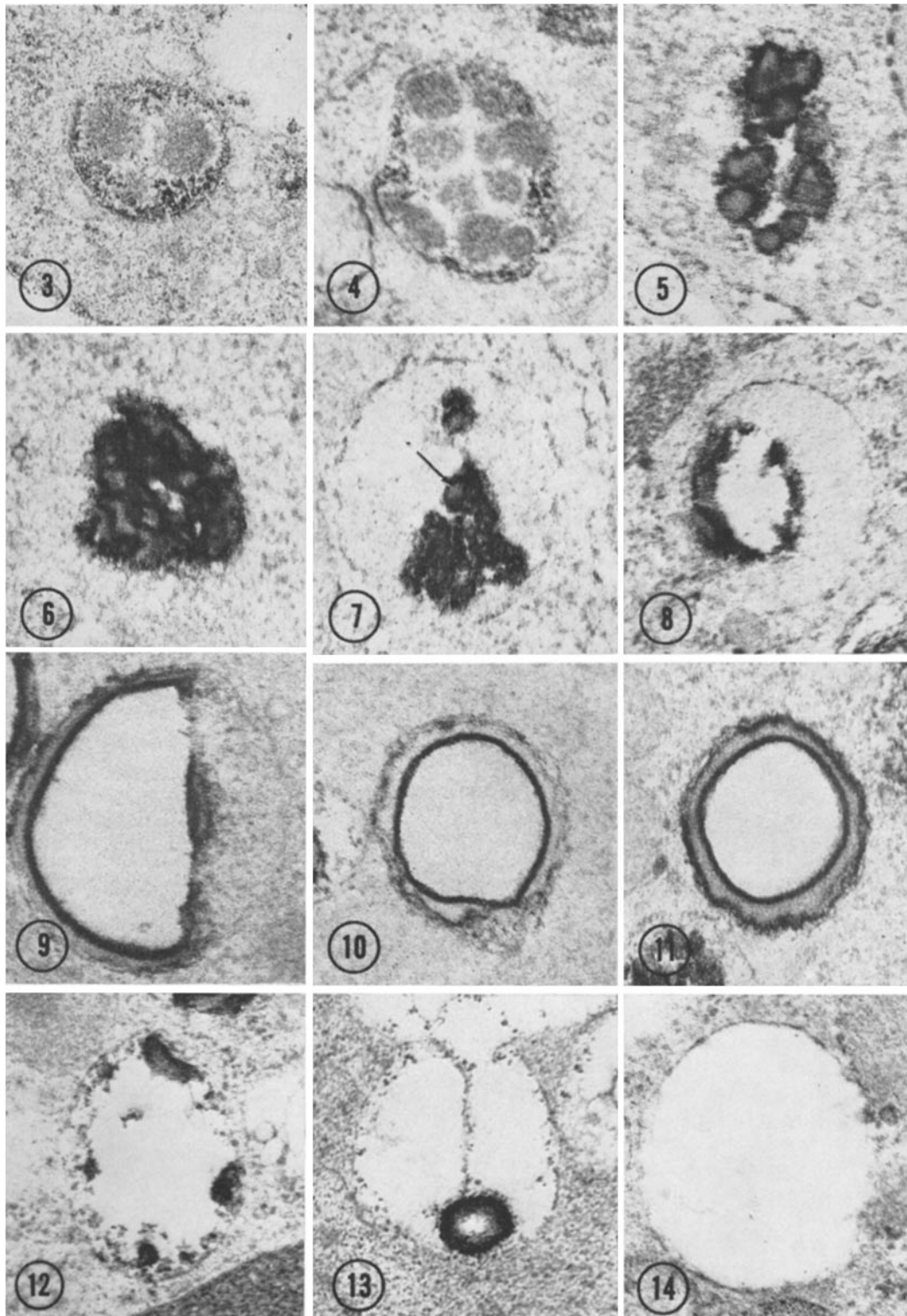
B granules consisted of a uniform, very electron-translucent material which enclosed one or more DI-stained bodies (Figs. 12, 13). Although these bodies varied, they were most often roughly spherical, about 100-200 μ in diameter, and peripherally located within the granule. In addition, small, stained particles were scattered unevenly in some B granules (Fig. 12). On the average, B granules comprised about a third of the granule population in early and intermediate (Figs. 16, 17) heterophils but only a small percentage of the granules in late cells (Fig. 18) (Table I).

C granules lacked DI staining and consisted of very low-density material enclosed by a unit membrane (Fig. 14). Occasional profiles included a few moderately dense strands or irregular aggregates in the translucent matrix. Some C granules were artifactually distorted and fused with one another. The percentage of C granules in an average cell profile increased progressively from less than 10% in early heterophils (Fig. 15) to about 90% in late heterophils (Fig. 18) (Table I).

Secondary Granules

As shown in the earlier ultrastructural studies (4, 5, 7, 26, 29, 53-55), heterophils at an intermediate stage of development (myelocyte to band forms) differ from early heterophils in containing secondary (specific) as well as primary granules in the cytoplasm and in displaying Golgi configurations characteristic of secondary rather than primary granulogenesis.

In the present study, no DI staining was seen in the nucleoid-containing (5), immature, secondary granules (Fig. 16) of early intermediate heterophils, in the homogeneous mature secondary granules (Figs. 16-18) of later stage heterophils,



or in the Golgi elements involved in secondary granulogenesis (Fig. 20).

Tertiary Granules

As observed in previous morphological investigations (26, 54, 55), a third granule type, desig-

nated the tertiary granule, is observed only in late heterophils.

In bone marrow and buffy coat specimens exposed to DI, most late heterophils (Fig. 18) disclosed a variable number of round or ellipsoid, membrane-limited organelles, the largest profiles

FIGURES 3-14 illustrate primary granules arranged in a sequence which may show, in a general manner, progressive changes in the distribution of DI-stained material during maturation. Figs. 3-11 are $\times 50,000$; Figs. 12-14 are $\times 40,000$.

FIGURE 3 DI-stained particulate material partially fills the space around the unstained moderately dense spherules in this small Golgi-associated vacuole.

FIGURE 4 DI-stained particles lie beneath a portion of the limiting membrane of this large vacuole, which was probably formed by fusion of smaller vacuoles, as the one in Fig. 3. The discrete spherules, although inherently dense, are not DI reactive and are surrounded by unstained, less dense material.

FIGURE 5 The spherules in this immature granule apparently were in a process of fusion at the time of fixation and have a morula-like appearance. Intensely DI-reactive, homogeneous substance coats the unstained spherules. Unstained flocculent material of low density surrounds the spherules.

FIGURE 6 In some post-morula granules, such as this one, strands of moderately dense material, which closely resemble the spherules in Fig. 5, are coated by DI-reactive substance. Such a glomerular configuration could have developed from the morula in Fig. 5 through fusion of spherules into strands which remain immiscible with and coated by DI-reactive substance. Flocculent material of low density partially surrounds the stained compartment.

FIGURE 7 In this post-morula granule, strongly DI-positive substance surrounds numerous, very small foci of moderate density and a large spherule (arrow) roughly similar to those in Fig. 5.

FIGURE 8 In this A granule, the nearly central low-density area is relatively small, the intervening band of DI-stained substance is interrupted, and the cortical zone of low-density flocculent material is relatively thick.

FIGURE 9 The left half of this A-granule profile resembles the granule profile in Fig. 10; the right half, having an interrupted DI-positive band between the low-density center and the thick cap of moderately dense flocculent matter, resembles the profile in Fig. 8. This granule might, therefore, be in transition from the maturational stage of Fig. 8 to that of Fig. 10.

FIGURE 10 This A granule consists mainly of very electron-translucent material in the center, surrounded by a continuous thin band of DI-reactive substance and more peripherally by a rim of intermediate density. Light DI staining is evident also in foci on the inner surface of the limiting membrane.

FIGURE 11 This A granule differs from that in Fig. 10 in its content of a second, nearly complete DI-positive layer immediately inside the limiting membrane.

FIGURE 12 In this early B granule, DI-reactive bodies and smaller particles form an interrupted rim that surrounds a central area of low density. These bodies and particles probably are remnants of the DI-reactive band of A granules.

FIGURE 13 Most of the DI-reactive bodies in B granules appeared solid, but the one remaining body in this B granule consists of a heavily DI-stained periphery and an unstained low-density center.

FIGURE 14 Very low-density material fills this C granule. The lack of DI staining in this C granule contrasts with the DI-stained core of C granules fixed with osmium tetroxide (cf. Figs. 24, 25).

Percentage of Primary Granule Varieties in Various Stages of Heterophil Development

Stage of heterophil development	No. of profiles	Mean percentage of primary granule population composed of each variety:			
		Post-morula	A	B	C
Early	50	7.1	52.4	31.9	8.6
Intermediate	50	0.0	21.2	38.8	40.0
Late	25	0.0	0.0	11.8	88.2
Buffy coat	50	0.0	0.0	8.2	91.8

of which measured between 200 and 250 $\mu\mu$ (Figs. 19, 21). The size, shape, and prevalence of these organelles and their exclusive presence in late heterophils strongly indicated that they were tertiary granules. Particles measuring 20–40 $\mu\mu$ completely filled some profiles and partially and unevenly filled other profiles of tertiary granules.

These particles were apparently stained by DI, since they were very electron opaque and were not evident in routine morphological specimens or in specimens treated with the control $MgCl_2$ solution.

Staining of Heterophils after Osmium Tetroxide or Formalin Fixation

Several differences were observed between DI-stained rabbit heterophils fixed with osmium tetroxide and heterophils fixed with glutaraldehyde prior to DI staining. In heterophils of osmium tetroxide-fixed specimens (Figs. 22–25), cell structure was less well preserved, DI staining was generally more intense, and Golgi saccules of early heterophils were more frequently stained (Fig. 23). Peripheral staining was observed in morulas (Fig. 22), but configurations comparable to the post-morula granules were not encountered

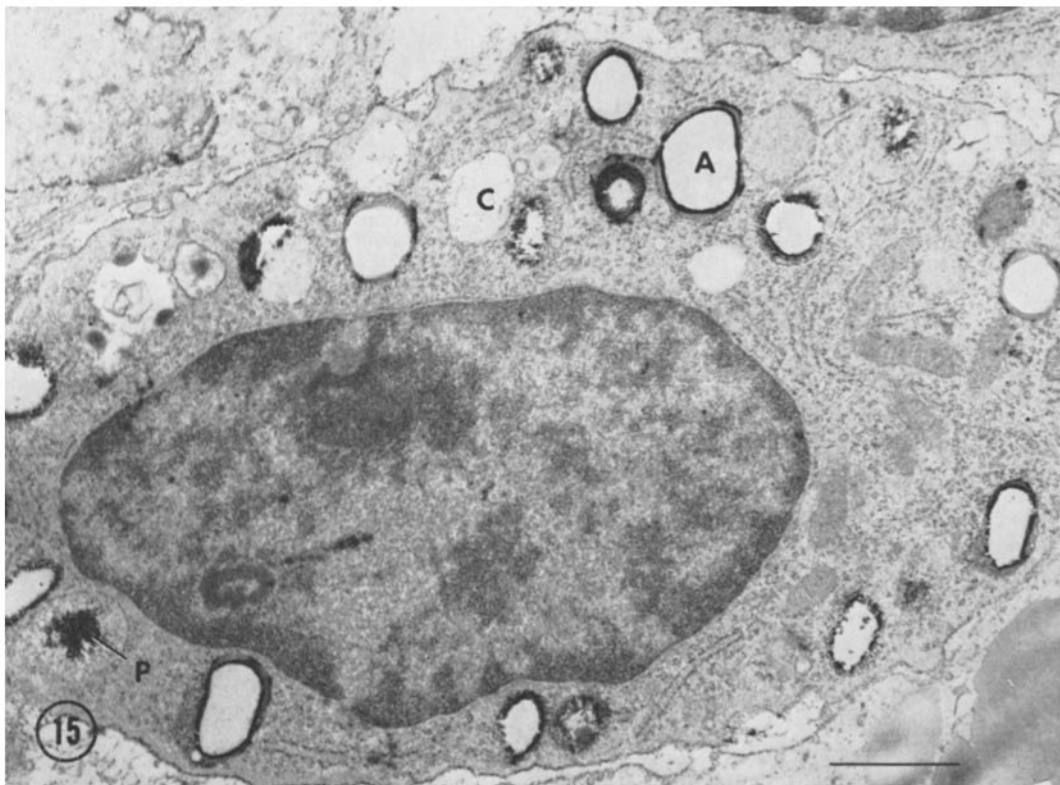


FIGURE 15 The granule population in this early heterophil (promyelocyte) consists entirely of primary granules. Most of these are A (*A*) granules, but a few post-morula (*P*) and C (*C*) granules are also present. The outer surface of the plasma membrane is the only other site with affinity for DI. $\times 15,000$.

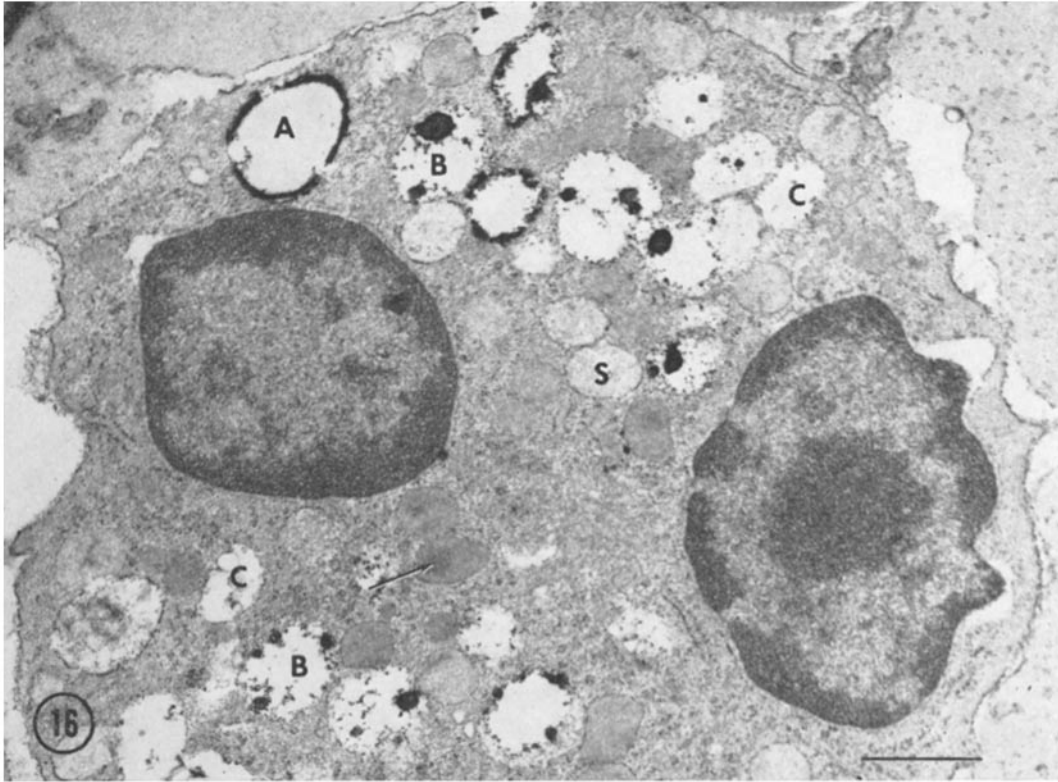


FIGURE 16 This early intermediate heterophil (myelocyte) contains about an equal number of primary and secondary granules, and most of the primary granules contain some DI reactivity. Most of the primary granules are the B (B) variety, but one A (A) granule and several C (C) granules are also present. Secondary granules (S), some of which contain a nucleoid (arrow), lack DI reactivity. $\times 16,000$.

in these specimens. The A variety of primary granules in osmium tetroxide-fixed heterophils differed from those in glutaraldehyde-fixed cells as follows: the unstained central area was usually more dense, the DI-stained band more frequently exhibited an irregular or partially collapsed contour, and the cortex usually contained small, loosely scattered, DI-reactive particles (Fig. 22). Early (Fig. 22) and intermediate (Fig. 24) heterophils fixed with osmium tetroxide disclosed some granules which were uniformly filled with small or variable-sized, DI-reactive particles and other granules which showed constriction, irregularity, or partial collapse of the DI-positive band. Possibly one or both of these variants was the counterpart of the B granule of glutaraldehyde-fixed cells. Late heterophils fixed with osmium tetroxide (Fig. 25) possessed primary granules with an intensely DI-stained solid or occasionally hollow core and

a low-density cortex, which sometimes contained several small DI-reactive particles. These granules probably corresponded with the DI-negative C granules in late heterophils fixed with glutaraldehyde.

Secondary granules, although not preserved in many heterophils after osmium tetroxide fixation, were evident in a few such cells (Fig. 24) and lacked DI affinity; the Golgi complex of intermediate heterophils involved in secondary granulogenesis was also negative.

Mature heterophils fixed with osmium tetroxide possessed DI-stained cytoplasmic organelles (Fig. 25) which closely resembled the tertiary granules seen in mature heterophils fixed with glutaraldehyde.

Although formalin did not preserve cell structure as well as did glutaraldehyde, the DI reactivity at most phases of primary granule forma-

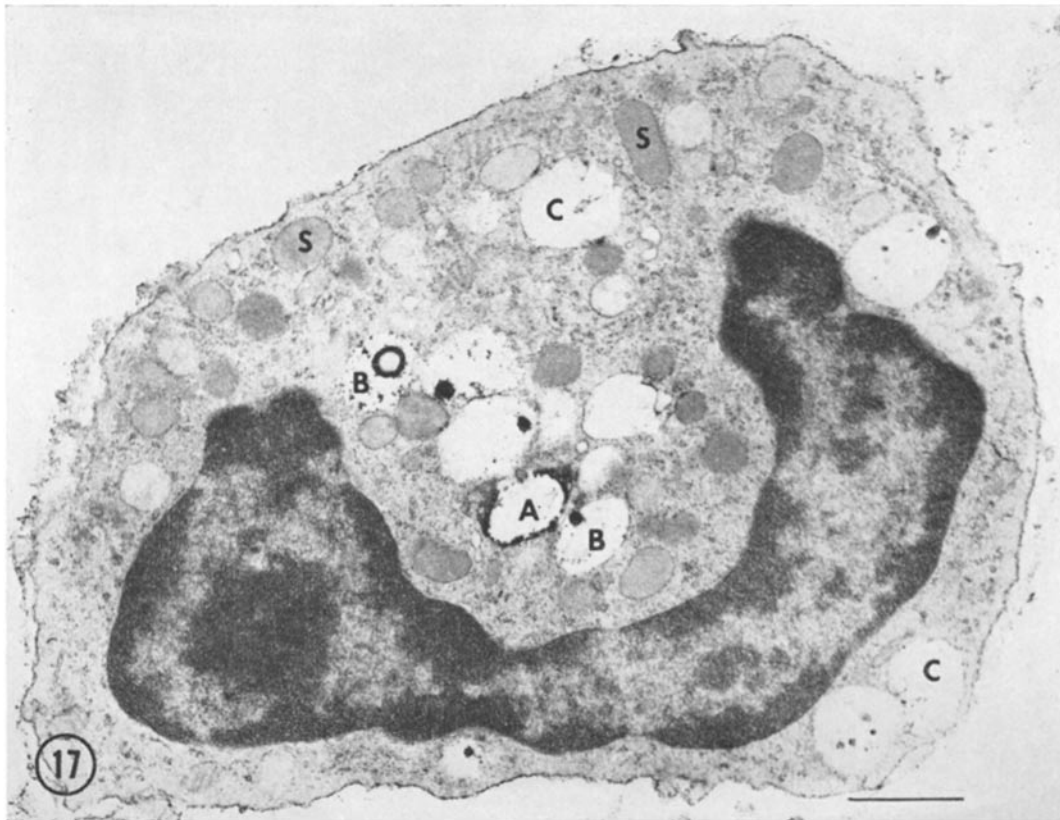


FIGURE 17 The advantageous plane of section through the nucleus of this heterophil provides a means of correlating the degree of maturation of the cell with that of the cytoplasmic granules. In this late intermediate heterophil (band form), the primary granules are only about one-half as numerous as the secondary granules (*S*), and they contain less DI reactivity than those in Fig. 16. One *A* (*A*) granule with an interrupted rim of DI-stained material remains, but most of the primary granules are the *B* (*B*) and *C* (*C*) varieties. The plasma membrane also exhibits DI-positive material. $\times 15,000$.

tion was generally comparable after either type of fixation. In formalin-fixed specimens, DI staining was not seen in secondary granules but was observed in tertiary granules.

Staining of Basophils

Previous morphological study (54) has indicated that basophilic leukocytes of rabbits produce only one type of cytoplasmic granule. DI reactivity in basophils fixed with glutaraldehyde was localized on the outer surface of the plasma membrane and within cytoplasmic granules (Fig. 26). Although moderately dense, spherical bodies were observed in Golgi vacuoles, as previously described (54), conclusive evidence was not

obtained for DI reactivity in these bodies or in Golgi lamellae. Although most granules in buffy coat basophils survived the cytochemical procedures adequately, those in the bone marrow basophils usually suffered extractive loss and marked disruption. The fragmented granules of bone marrow basophils disclosed DI-reactive filamentous or irregular particles in a low-density matrix. The better preserved granules in buffy coat basophils (Fig. 26) measured up to $800 \text{ m}\mu$, and usually had a rounded profile. These granules were filled with DI-reactive particles, which lay crowded together with a regular arrangement in a translucent matrix. The particle profiles were rounded or elongated and measured $20\text{--}40 \text{ m}\mu$ in width. The appearance of this stained component

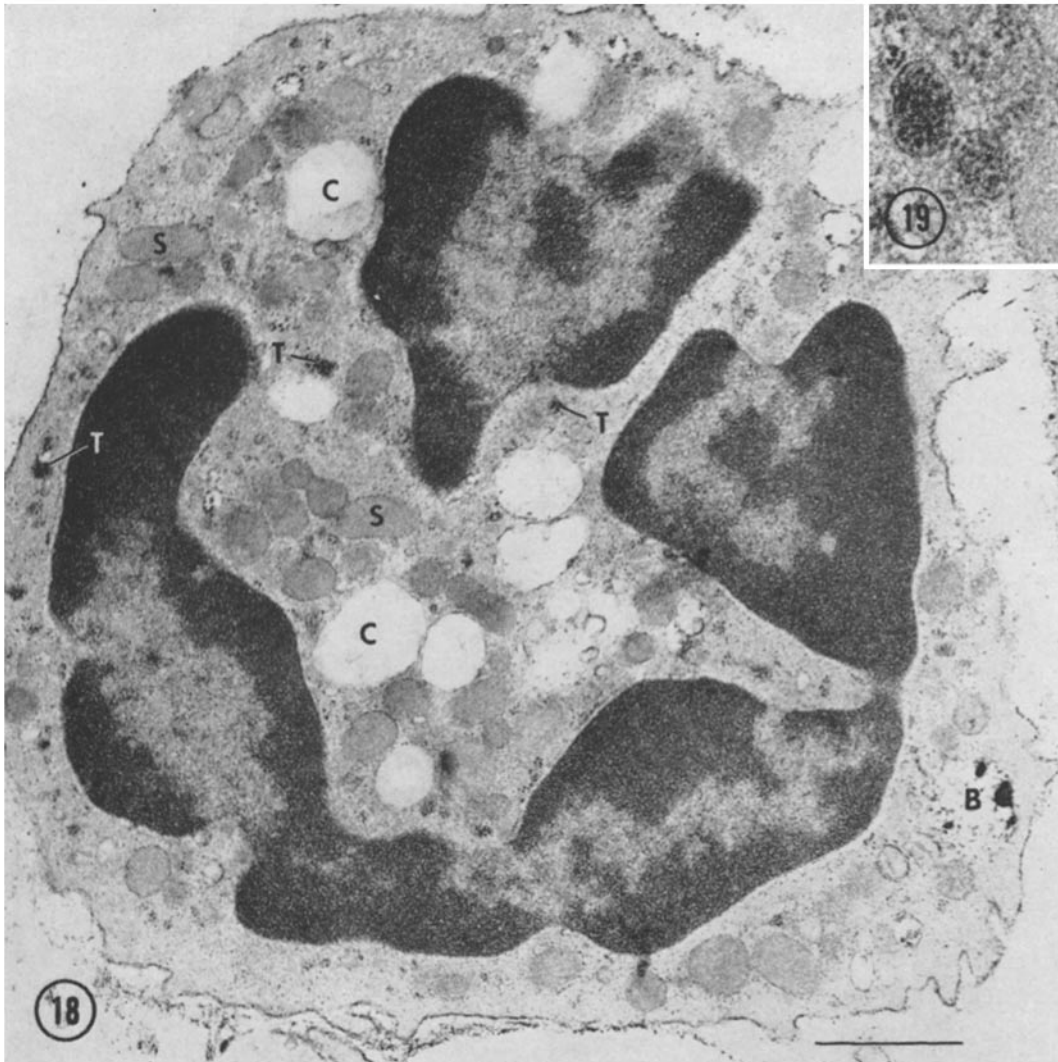


FIGURE 18 This late (segmented) heterophil discloses a primary granule population composed of one B (*B*) and several C (*C*) granules. Secondary granules (*S*) are more numerous and more dense than primary granules and lack affinity for DI. A few small DI-reactive tertiary granules (*T*) are also present in the cytoplasm. $\times 20,000$.

FIGURE 19 High magnification of a tertiary granule with limiting membrane and DI-reactive particles. $\times 50,000$.

in these granule profiles resembled that of a component visualized in rabbit basophil granules with standard morphological processing (54) and seemed consistent with the structure of a coiled thread. Occasional granules of buffy coat cells appeared disrupted and extracted (Fig. 26).

Some small granules contained a homogeneous body which was very dense, presumably as a result of DI staining, and which was partially or completely surrounded by a space with very low density. Whether these granules constituted development or fixation-dependent variants of the

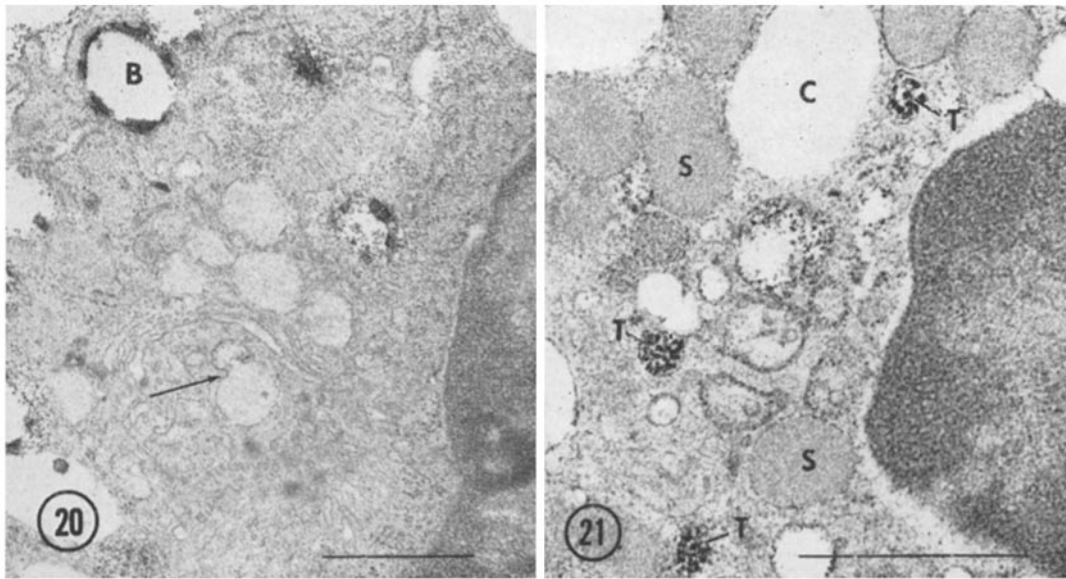


FIGURE 20 Portion of intermediate stage heterophil exhibiting lack of DI staining in Golgi lamellae and vacuoles involved in formation of secondary granules. A configuration in the concavity of the lamellae suggests partial fusion of a nascent secondary granule with a small vacuole (arrow). The cytoplasm contains a few B (*B*) primary granules. $\times 20,000$.

FIGURE 21 Area in a late heterophil showing tertiary granules (*T*) limited by a membrane and filled with DI-reactive particles. C-stage primary granules (*C*) and secondary granules (*S*) are unstained with DI. $\times 30,000$.

larger cytoplasmic granules or a distinct entity was not evident.

Staining of Eosinophils

Previous ultrastructural study (54) revealed that rabbit eosinophilic leukocytes at certain stages of development contain two morphologically distinct types of granules: (*a*) a round homogeneous granule, which is an immature form of the (*b*) angulated granule with crystalloids.

Eosinophils, like neutrophils and basophils, revealed DI reactivity on the outer surface of the plasma membrane. After glutaraldehyde fixation, DI-reactive material, having a granular appearance, was present within many Golgi-associated vacuoles and also within a few, small, immature cytoplasmic granules which lacked crystalloids (Fig. 27). DI staining was not evident in either the larger, homogeneous granules which lacked crystalloids and possessed a fine-textured sub-

stance or in the more mature crystalloid-containing granules (Fig. 27).

After osmium tetroxide fixation, cytological structure was poorly preserved, but DI reactivity was more extensive. Golgi lamellae and associated vesicles stained with DI and, in contrast with those in glutaraldehyde-fixed eosinophils, a few spherical immature granules displayed a thin outer rim of DI reactivity (Figs. 28, 29). DI staining was absent around the many immature granules which had lost their limiting membrane during processing, or it was present only where adjacent granules lay in contact with one another. Mature crystalloid-containing granules lacked staining.

Following formalin fixation, the Golgi lamellae and entire content of small, Golgi-associated vacuoles disclosed strong affinity for DI (Fig. 30). In a few cell profiles, DI staining was also evident in a thin rim subjacent to the limiting membrane of immature granules lacking crystalloids. In other profiles, similar staining was seen only at

the points at which immature or mature granules contacted one another.

DISCUSSION

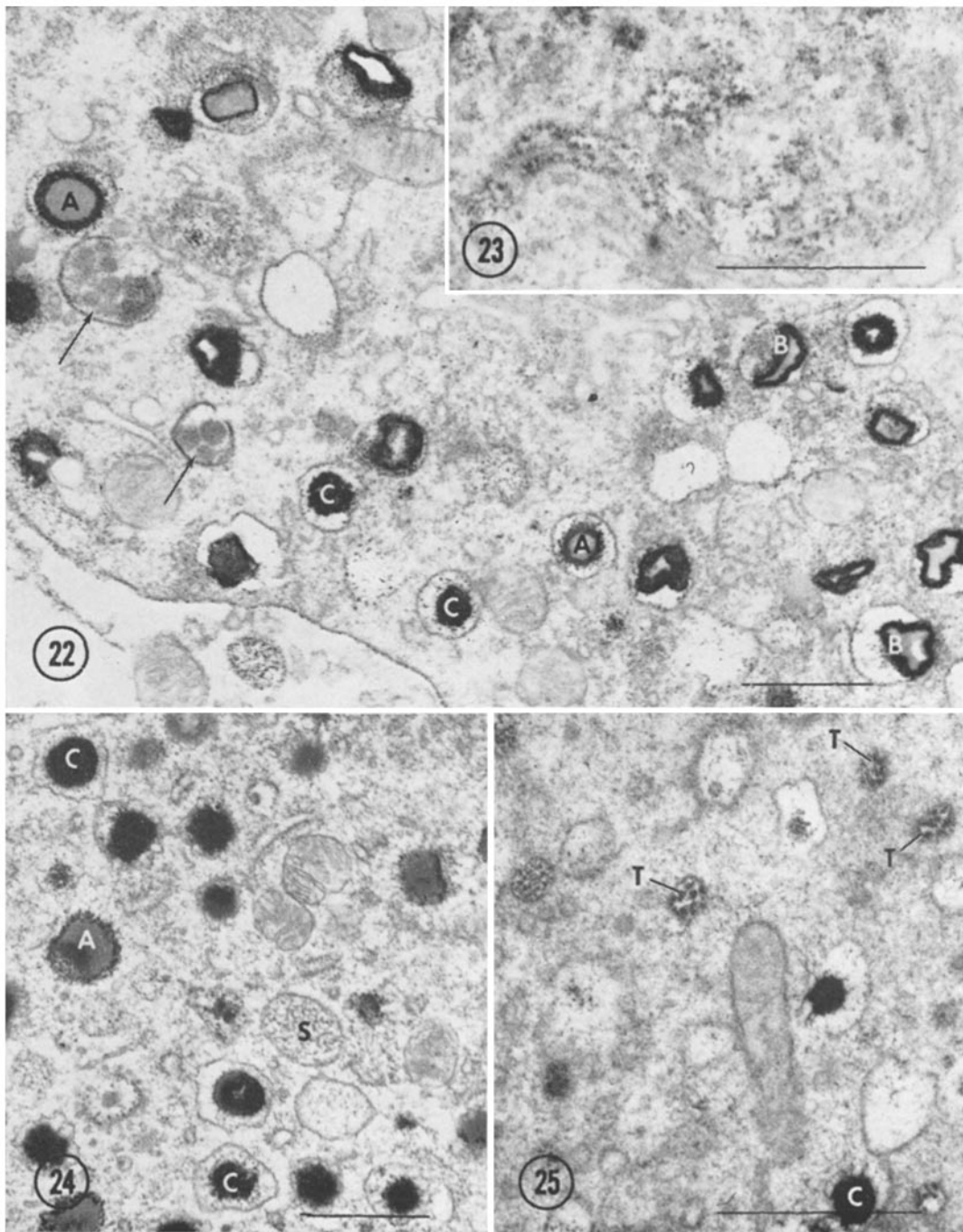
DI staining of certain granules in rabbit heterophils, basophils, and eosinophils, visualized in the present study at the ultrastructural level, confirms previous light microscope cytochemical and radioautographic observations for the presence of sulfated mucosubstance in these sites (28). The higher resolution obtained by electron microscopy provides additional information about the localization and stainability of the acid mucosubstances. Thus, the present investigation has shown: (a) the presence of mucosubstance in cytoplasmic organelles involved in its biosynthesis, (b) which granule within each granulocyte contains acid mucosubstance, (c) the intragranular distribution of mucosubstance, and (d) the variability in the distribution of mucosubstance during different stages of granule maturation.

Heterophils

The present study confirms light microscope evidence that an acid mucosubstance is present in primary but not in secondary granules of rabbit heterophils (28) and provides new evidence for acid mucosubstance in tertiary granules in these cells. In the course of primary granule development, DI-reactive mucosubstance is first seen in the Golgi lamellae and associated vacuoles involved in formation of these granules (Figs. 1, 23). This observation is in agreement with previous cytochemical (41, 50) and radioautographic (38) studies demonstrating synthesis of mucosubstance in this organelle. The mechanism by which the mucosubstance coating the spherules of the morula-containing granules (Fig. 5) comes to occupy a peripheral rim in the A granules (Figs. 9–11) is uncertain. This transition, perhaps involving a post-morula stage (Figs. 6, 7), is more difficult to envision than the more readily apparent sequence from A to B to C granules in which the rim of mucosubstance undergoes disruption and disappears (Figs. 11–14). The characteristic distribution of the A, B, and C varieties in heterophils at various stages of development strongly suggests that they represent sequential stages in the maturation of the primary granule (Table I). Additional evidence supporting this proposed sequence comes from the apparent possibility that

each could derive from the preceding phase as a result of minor rearrangement or change in granule components (Figs. 8–14). The mechanisms responsible for these intragranular changes in the distribution of mucosubstance are unknown. The immiscibility of the rim mucosubstance with the core in the A granules may be the result of the physical-chemical properties of some core component, as perhaps a lipid material thought to be present in neutrophils (2). A change in this component with further maturation would permit the reorganization of the rim and core material of primary granules which apparently ensues in the B and C stages of maturation. The marked variation in distribution and stainability of acid mucosubstance during primary granule maturation could be the result of intragranular chemical reactions which may or may not involve addition or deletion of some component. However, no morphological evidence was obtained for transport to or from any primary granule.

The lack of DI staining of mature primary granules, observed in the present ultrastructural study in specimens fixed with formalin and glutaraldehyde (Figs. 14, 17, 18), concurs with the weak basic dye staining of mature heterophils observed at the light microscope level (28). This study then explains the discrepancy between the large number of primary granules observed ultrastructurally in late heterophils (5, 7, 15, 26, 54) and the small number seen stained by basic dyes with the light microscope (16, 28). Since radioautographic studies have demonstrated ³⁵S-labeled sulfate in mature rabbit heterophils (28) and since chemical studies have found acid mucosubstances in granules isolated from mature rabbit leukocytes (20), mucosubstance is probably present in the fully mature primary granules as seen in the osmium tetroxide-fixed specimens (Figs. 24, 25). The reason for lack of affinity of the mucosubstance for DI in the C granules after glutaraldehyde and formalin fixation is not known but may result from obstruction to the cationic DI reagent imposed by cationic protein, which has been cytochemically (28) and radioautographically (19) demonstrated in primary granules. Inadequate preservation of this basic protein in specimens fixed with osmium tetroxide could explain the presence of DI staining in the C granules of heterophils preserved with this fixative. Cytoplasmic granules of other cells, for example, mouse Paneth cells (50), mast cells (47),



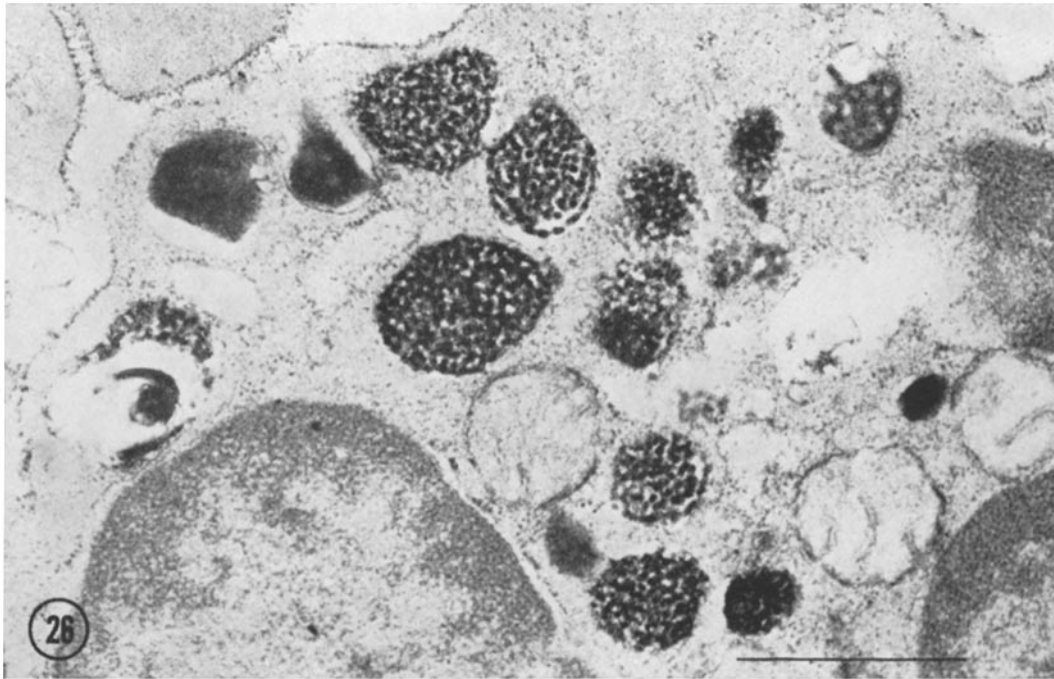


FIGURE 26 Basophil from rabbit buffy coat fixed with glutaraldehyde, sectioned with a Smith-Farquhar tissue slicer, stained 3 hr with DI, and postosmicated. The cytoplasmic granules enclose DI-positive particles arranged uniformly throughout the granule. The outer surface of the plasma membrane of the basophil, like that of adjacent platelets, is DI reactive. Lead citrate staining of thin section. $\times 30,000$.

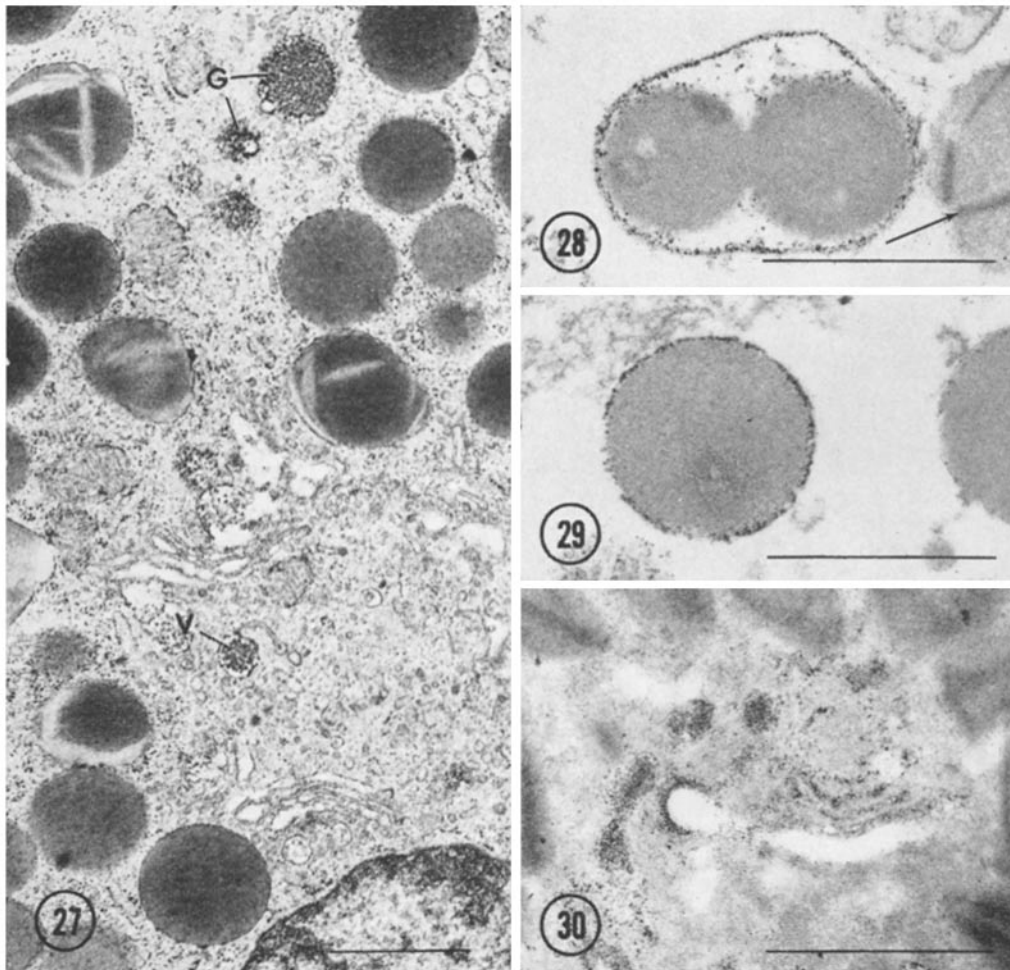
FIGURES 22-25 show portions of bone marrow heterophils from specimens fixed with osmium tetroxide sectioned with a Smith-Farquhar tissue slicer, and stained 1, 3, 24, and 1 hr, respectively, with DI. The heterophils in Figs. 22, 23, and 25 were from unstained thin sections; the heterophil in Fig. 24 was from sections stained with lead citrate.

FIGURE 22 Three varieties of primary granules are evident in this early heterophil: those with round or spherical DI-reactive rims (A), those with irregular or collapsed rims (B), and those which contain solid DI-stained cores (C); these three varieties probably represent, respectively, the counterparts of the A, B, and C primary granules seen in heterophils fixed with glutaraldehyde. The unstained cores of most A and B granules appear more dense than in the granules of heterophils fixed with glutaraldehyde. This heterophil also contains two Golgi vacuoles with unstained discrete spherules and a faint rim of DI-stained material (arrows). $\times 19,000$.

FIGURE 23 Golgi zone of early heterophil. DI-reactive particles are evident in some Golgi lamellae and in associated small vacuoles. $\times 30,000$.

FIGURE 24 Most of the primary granules in this intermediate heterophil are C (C) granules, which possess intensely DI-stained cores. One A (A) granule is present. Secondary granules (S), although usually not preserved in these specimens, show a finely granular content with no DI affinity. $\times 19,000$.

FIGURE 25 All primary granules in this late heterophil are of the C variety (C). Small tertiary granules (T) are membrane limited and contain DI-positive particles. $\times 30,000$.



FIGURES 27-30 illustrate granules from immature rabbit bone marrow eosinophils stained with DI.

FIGURE 27 DI-stained granular material is evident within a small Golgi vacuole (*V*) and larger precursor granules (*G*) of this immature eosinophil. Presumably, more mature granules, with and without crystalloids, lack DI staining. Fixed with glutaraldehyde, sectioned in a cryostat, stained 24 hr with DI, and postosmicated; thin sections stained with lead citrate. $\times 20,000$.

FIGURE 28 These two immature eosinophil granules were apparently in the process of fusion at the time of fixation. A loosely fitting limiting membrane with subjacent DI-positive material surrounds both granules. DI staining was not seen in mature crystalloid-containing granules (arrow). Fixed with osmium tetroxide and stained 1 hr in block with DI; unstained thin section. $\times 30,000$.

FIGURE 29 DI-stained material partially surrounds the contents of the eosinophil granule at the left; in most eosinophil granules of osmium tetroxide-fixed specimens, the limiting membrane was not intact, and little or no DI staining was evident. Processed as described in Fig. 30. $\times 30,000$.

FIGURE 30 Intensely DI-stained material is present within Golgi lamellae and vacuoles of this eosinophil. Little staining was seen within granules. Fixed with formalin, stained 1 hr in block with DI, and postosmicated; unstained thin section. $\times 30,000$.

basophils, and eosinophils (16, 28), are known to contain acid mucosubstance and basic protein.

A close correlation exists in the distribution of DI-reactive mucosubstance and antimonate-precipitable cation (26) in primary granules of rabbit heterophils; and the degree of maturation of the A, B, and C granules defined by DI reactivity closely corresponds with that of the A, B, and C granules defined by antimonate reactivity. The presence of these two components in rims of A and throughout B granules suggests that antimonate-reactive cation may neutralize the sulfate esters of the mucosubstance without blocking their DI affinity. The lack of DI reactivity (after aldehyde fixation) and of antimonate deposits in C granules suggests that the sulfate groups in these granules are not neutralized by antimonate-precipitable cation but rather may be neutralized by basic protein. However, the presence of antimonate-precipitable cation does not correlate invariably with that of acid mucosubstance; for example, secondary granules do and tertiary granules do not disclose antimonate deposits (26).

The distribution of DI-reactive mucosubstance in primary granule precursors and in immature primary granules also generally coincides with that of several enzymes, including acid phosphatase (7, 55), peroxidase, aryl sulfatase, and 5-nucleotidase (7). With the exception of peroxidase, these enzymes are either not demonstrable in mature primary granules or, because of poor preservation, their precise intragranular localization is questionable. The observation that enzyme activity is reduced or abolished in mature primary granules correlates with the lack of DI reactivity in these granules in glutaraldehyde-fixed specimens. These correlations in distribution and reactivity of acid mucosubstance and acid hydrolases in immature and mature primary granules suggest some form of complex between these two types of components.

Evidence was not previously available from light microscope histochemistry for acid mucosubstance in tertiary granules because of their small size (Figs. 18, 19, 21, 25). This finding points to the presence of a third mucosubstance in neutrophils, in addition to the primary granule mucosaccharide and the surface mucous coat. The tertiary granules provide another instance of close topographical association within the granule of acid mucosubstance and acid phosphatase (55).

Basophils

In contrast to heterophils and eosinophils, the basophils disclosed DI-stained mucosubstance in all of the mature granules. The distribution of this mucosubstance within granules (Fig. 26) corresponded with that of filamentous material observed morphologically in some rabbit basophil granules (54) and was unique for this granule type. Determination of the distribution of mucosubstance in guinea pig and human basophil granules would be interesting, since granules of rabbit basophils differ morphologically from those of guinea pig (18, 51) but roughly resemble those of human basophils (57).

Differences observed in the intensity and distribution of DI staining were less apparent among basophil granules than among eosinophil and heterophil primary granules; the relationship of these differences to degree of granule maturation could not be assessed because of the relatively poor preservation of the bone marrow basophils.

Eosinophils

The presence of DI-stained material in some immature eosinophil granules lacking crystalloids but not in any of the mature granules possessing crystalloids (Figs. 27-30) confirms and extends previous light microscope observations that granules of early but not late rabbit eosinophils stain with basic dyes (28). The previous observations that early eosinophils incorporate ^{35}S -sulfate (24, 28, 35, 39) indicates that at least part of the DI-stained material in eosinophil granules is a sulfated mucosubstance. The lack of DI staining in most of the immature granules lacking crystalloids was probably due to inadequate preservation of the cortical mucosubstance. The absence of DI-stained material in all mature eosinophil granules possessing crystalloids does not necessarily indicate that these granules lack a mucosubstance but may be due to combination of the acid mucosubstance with basic protein, which is known to be present in eosinophil granules (17, 28).

The distribution of the DI-stained acid mucosubstance in the rims of immature eosinophil granules closely corresponds to the distribution of acid phosphatase (8, 36, 55), peroxidase (8, 15, 36), aryl sulfatase (8), phosphotungstic acid-stained material (31), and pyroantimonate-precipitable cation (25).

Significance of Acid Mucosubstance in Leukocytes

The comparable intragranular localization of acid mucosubstance and acid hydrolases in rabbit heterophil primary granules (7, 55) and eosinophil granules (8, 36, 55) suggests a possible role of the mucosubstance in regulating the activity of the enzymes in these lysosomal (3, 6, 12, 30) structures. The hydrolases in these granules, unlike most enzymes, survive long periods of inactivity in granules subjected to strong physical and perhaps chemical influences. A complex of mucosubstance with enzyme, as suggested earlier, would perhaps stabilize or, alternatively, might reversibly inactivate the enzyme during the prolonged storage. The action of one enzyme such as a protease on another enzyme in the granule during storage could be avoided by either a mucosaccharide-protease complex which inactivates the protease or a mucosaccharide-protein complex which protects the protein from enzymatic action. The acid mucosubstance could also buffer the reaction of the granule content at a pH level away from the optimum for hydrolase activity, or, on the other hand, it might influence the final pH level in phagocytic vacuoles in heterophils toward the acid level which is known to prevail in some phagocytic vacuoles (43). Antibacterial activity of the mucosubstance against phagocytized microorganisms and/or protection of the host cell against bacterial enzymes or toxins constitute other possible functions for the mucosaccharide in heterophil and eosinophil granules. Knowledge of the chemical nature of the granule mucosubstances would contribute to understanding their possible reactions with other granule components. Participation in cell mechanisms controlling granulogenesis seems a possible role of the mucosubstance, but an unlikely one in view of the probable absence of such a component from granules in many cell types.

The failure of hyaluronidase to abolish DI staining of rabbit heterophil primary granules confirms light microscope observations that hyaluronidase does not destroy staining of these granules with methods specific for sulfated mucosubstance (49). These observations are surprising in view of the report that rabbit heterophil granules contain hyaluronic acid and chondroitin sulfate (20), both of which are DI reactive and hyaluronidase digestible. Although the possibility

exists that hyaluronidase did not reach the intragranular site of hyaluronic acid or chondroitin sulfate, it seems more likely that the DI method is visualizing some acid mucosubstance other than these two components. The failure of rabbit heterophil primary granules to stain with DI after 18-hr methylation confirms light microscope observations that methylation blocks high iron diamine staining of these granules (Hardin and Spicer, unpublished observations). The reduced staining seen in heterophil primary granules from specimens treated with acidified isopropanol was probably due to extraction of the mucosubstance during this treatment rather than to specific removal of sulfate esters. Since methylation apparently blocks DI staining of mucosubstances rich in sulfate but not in carboxyl groups (56), and since high iron diamine staining is specific for sulfated mucosubstances (48), the DI method is probably demonstrating a sulfated mucosubstance in rabbit heterophil primary granules.

Since basophils are not known to be so active as neutrophils and eosinophils in phagocytosis (45), and since basophil granules are apparently not rich in hydrolytic enzymes (1), the significance of the mucosubstance in heterophil and eosinophil granules probably has little relation to the significance of this component in basophil granules. The DI-stained component in basophil granules may be carboxyl groups and sulfate esters of heparin, which is thought to be present in these granules (1, 10) and has the known function of activating serum lipoprotein lipase (34).

Knowledge is lacking as to whether the affinity for DI of the mucosubstance on the plasma membrane of the three types of leukocytes is attributable to carboxyl or sulfate groups or both. Since a sialic acid-rich glycoprotein has been localized on the plasma membranes of ascites tumor cells (23), erythrocytes, and other cells (13), the DI staining of the leukocyte plasma membranes may be due to the carboxyl groups of this component.

The authors express their appreciation to Miss Joanne Wright, Mrs. Kathryn Cowart, Mr. George Malanos, and Mr. W. B. Greene for technical assistance.

Dr. Hardin was supported by a Postdoctoral Fellowship from the National Institute of General Medical Sciences.

This study was supported by Research Grants AM-10956 and AM-11028 from the National Institutes of Health.

Received for publication 14 January 1970, and in revised form 31 August 1970.

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