

# THE DEVELOPMENT OF NEUTROPHILIC POLYMORPHONUCLEAR LEUKOCYTES IN HUMAN BONE MARROW

## ORIGIN AND CONTENT OF AZUROPHIL AND SPECIFIC GRANULES\*

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Previous electron microscopic and cytochemical studies from this laboratory (1-3) have demonstrated that rabbit neutrophilic polymorphonuclear leukocytes (PMN)<sup>1</sup> contain two types of granules, azurophil and specific, which have separate origins and are different in nature. The large, dense azurophil granules represent a special type of primary lysosome containing peroxidase and lysosomal or digestive enzymes. They are produced early in development, during the promyelocyte stage,<sup>2</sup> and arise from the concave face of the Golgi complex. The smaller, less dense specific granules represent an entirely different secretory product which contains alkaline phosphatase and lacks lysosomal enzymes and peroxidase. They are produced later in development, during the myelocyte stage, and arise from the opposite or convex face of the Golgi complex. Recently Baggiolini, Hirsch, and de Duve have prepared fractions of azurophil and specific granules from rabbit PMN by zonal sedimentation (4) and isopycnic centrifugation (5) and have confirmed by biochemical assay our cytochemical findings concerning the enzyme content of the two granule types.

It now remains to be determined whether the findings obtained in rabbit PMN pertain to other species, especially the human. To date there has been no

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<sup>1</sup> *Abbreviations used in this paper:* ER, endoplasmic reticulum; PMN, neutrophilic polymorphonuclear leukocytes.

<sup>2</sup> In our earlier papers, the term "progranulocyte" was used to designate this stage of development, but we will employ the equivalent and currently more widely used term "promyelocyte" hereafter.

general agreement on the nature or numbers of granule types present in human PMN; two, three, and even four granule types have been described in various electron microscopic studies (6–20). The present study represents an attempt to establish the nature and number of PMN granule types in the human using an approach similar to that used previously for the rabbit, with the following modification: in this work we have taken advantage of the fact that myeloperoxidase is localized exclusively in azurophil granules and we have utilized this enzyme as a cytochemical marker to facilitate identification of azurophils. This modified approach was necessary when it became apparent that morphologic criteria (size, shape, density) were not sufficient to distinguish granule types in the human. Our results, already reported in abstract form elsewhere (21–23), indicate that although human PMN granules are morphologically more heterogeneous than those of the rabbit, the situation is basically the same in the two species with respect to the existence, mode of origin, and enzyme content of azurophil and specific granules.

#### Materials and Methods

*Collection of Tissues.*—Bone marrow was obtained from the sterna or ribs of two normal volunteers and 30 hematologically normal patients undergoing cardi thoracic surgery. Venous blood was obtained in polyethylene syringes wet with aqueous heparin, and the leukocytes were concentrated in one of four ways: (a) dextran sedimentation (24), (b) ammonium chloride lysis of red blood cells (25), (c) buffy coat disc of Anderson (26), or (d) the buffy coat tubes of Kaplow (27). Cover glass smears and cell suspensions were processed as previously described (2, 3) unless otherwise stated.

*Methods for Morphologic Studies.*—Fixation was carried out in one of the following solutions: (a) 1.5% distilled glutaraldehyde (28) in 0.1 M sodium cacodylate-HCl buffer (pH 7.4) with 1% sucrose for 16 hr at 4°C or 6 hr at 22°C, (b) 1% formaldehyde-3% glutaraldehyde with CaCl<sub>2</sub> (29) for 4 hr at 22°C, or (c) glutaraldehyde-OsO<sub>4</sub> for 1 hr at 4°C (15). The cells were then packed by centrifugation at ~10,000 g, postfixed for 1 hr at 4°C in 1% OsO<sub>4</sub> in acetate-Veronal buffer (pH 7.4) with 5% sucrose added, treated with buffered 0.5% uranyl acetate (30) for 1 hr at 22°C, dehydrated in ethanol, carried through propylene oxide, and embedded in Araldite. In some cases propylene oxide was avoided by infiltrating specimens with increasing concentrations of Araldite mixed with 100% ethyl alcohol (31), or embedding according to Spurr's technique (32).

*Methods for Cytochemical Studies.*—Fixation was carried out for 10 min–16 hr in either glutaraldehyde at 4°C or formaldehyde-glutaraldehyde at 22°C as described above. Cells were

#### Abbreviation for figures:

<i>ag</i> , azurophil granule (spherical)	<i>M</i> , mature PMN or band
<i>ag'</i> , azurophil granule (ellipsoid)	<i>m</i> , mitochondrion
<i>ce</i> , centriole	<i>n</i> , nucleus
<i>er</i> , rough-surfaced endoplasmic reticulum	<i>nu</i> , nucleolus
<i>g</i> , glycogen	<i>P</i> , promyelocyte
<i>G</i> Golgi complex	<i>pn</i> , perinuclear cisterna
<i>Gc</i> , Golgi cisternae	<i>r</i> , ribosome
<i>ia</i> , immature azurophil granule	<i>sg</i> , specific granule
<i>is</i> , immature specific granule	<i>ve</i> , vesicles
	<i>Y</i> , myelocyte

incubated in the following media containing 5% sucrose: (a) modified Gomori's medium (33), pH 5.0, for acid phosphatase, using  $\beta$ -glycerophosphate (Sigma Chemical Co., St. Louis, Mo.; Grade I) as substrate; (b) Goldfischer's medium (34), pH 5.5, for arylsulfatase, using *p*-nitro-catechol sulfate, followed by treatment with 2% ammonium sulfide (35); (c) Graham and

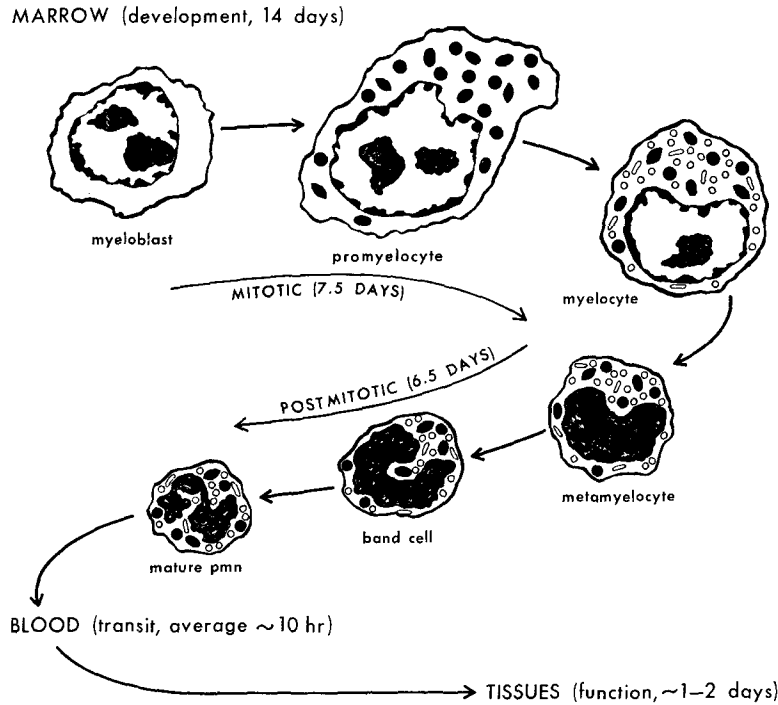


FIG. 1. Diagrammatic representation of PMN life cycle and stages of PMN maturation. The myeloblast is a relatively undifferentiated cell with a large oval nucleus, large nucleoli, and cytoplasm lacking granules. It originates from a precursor pool of elusive stem cells and is followed by two secretory stages: the promyelocyte and the myelocyte. During each of these stages a distinct type of secretory granule is produced: azurophils (solid black) formed only during the promyelocyte<sup>2</sup> stage, and specific granules (light forms) produced during the myelocyte stage. The metamyelocyte and band forms are nonproliferating, nonsecretory stages which develop into the mature PMN. The latter is characterized by a multilobulated nucleus and cytoplasm containing primarily glycogen and granules. Out of every 100 nucleated cells in bone marrow, 2% are myeloblasts, 5% promyelocytes, 12% myelocytes, 22% metamyelocytes and bands, and 20% mature PMN, giving a total of ~60% developing PMN (39). The times indicated for the various compartments were determined by isotope labeling techniques (see refs. 40-42).

Karnovsky's medium (36), pH 7.6, for peroxidase; (d) Gomori's medium for alkaline phosphatase (pH 9.2), using  $\beta$ -glycerophosphate followed by treatment with 2% lead nitrate (3, 37). Methods for the other enzyme incubations and controls, as well as those for subsequent processing and microscopy, were as previously described (2, 3), with the exception that KCN

inhibition of peroxidase activity was tested in a closed system after preincubation of the cells with KCN (0.01 M) at pH 6.0 for 30 min (38).

*Histochemical Staining on Smears of Bone Marrow.*—Cover glass smears were prepared, fixed, stained, and incubated and processed for enzyme histochemistry exactly as described in a previous paper (2) unless otherwise stated.

## RESULTS

### *Background Information*

The PMN leukocyte of the blood is a highly specialized, nondividing “end” cell with a short life-span (see Fig. 1). The bulk of its life cycle is spent in the marrow, where it proliferates, differentiates, and is stored for a few days. The mature cell is then released into the blood and circulates briefly before migrating into the tissues where it functions as a mobile phagocyte.

PMN development in the bone marrow has classically been divided into six stages (myeloblast, promyelocyte, myelocyte, metamyelocyte, band, and mature PMN) distinguished in Romanovsky-stained smears of bone marrow (see Fig. 1) on the basis of cell size, nuclear morphology, and granule content. Two of these stages are recognized mainly by the nature of their granules: the *promyelocyte*, which contains a single population of large, reddish-purple “azurophil”<sup>3</sup> granules, and the *myelocyte*, which contains a mixed granule population consisting of smaller, pinkish “specific” granules, along with azurophils which become less conspicuous. Up to now, it has not been established whether human PMN contain a single type of granule which alters its staining properties during maturation, or whether, as is now known for the rabbit, the granules represent entirely different products formed in two successive waves of secretory activity. To resolve this question, we have focussed our attention on the promyelocyte and myelocyte, the stages of development during which the granules appear.

### *Effects of Preparatory Techniques on Granule Morphology*

Our initial electron microscopic studies revealed that human PMN granules cannot be divided into distinct types on the basis of morphology alone. We tried numerous variations in methodology (see Materials and Methods), of which none was entirely satisfactory, and in accord with the experience of others (12–14), several were found to affect granule morphology profoundly. In particular, after propylene oxide, the otherwise dense content of the granules of the promyelocyte (azurophils) appeared irregularly extracted, and after uranyl acetate (in block or on grid), the smaller, generally less dense granules that arise in the myelocyte (specific) appear darker. Cells fixed in formaldehyde-glu-

<sup>3</sup> The term “azurophil,” introduced by Michaelis, refers to an affinity for an “azure” or “sky-blue” dye and not to the color of the granules, which stain metachromatically, i.e., reddish-purple. They have also been called “unripened,” “undifferentiated,” or “nonspecific” granules.

taraldehyde usually contain a higher proportion of elongated or dumbbell-shaped granules than those fixed in glutaraldehyde alone, presumably owing to better preservation of the elongated forms. Fixation with a mixture of glutaraldehyde and  $\text{OsO}_4$  (15) gave results similar to those seen with glutaraldehyde followed by  $\text{OsO}_4$ .

The best results in tissue prepared for morphology alone were obtained by fixation in glutaraldehyde followed by  $\text{OsO}_4$ , and by omission of both uranyl acetate and propylene oxide during subsequent processing. With this method, two main types of granules could be distinguished: one larger and denser, and the other smaller and less dense. However, in mature PMN there were many granules of intermediate size and density which were difficult to classify (see Fig. 2). Moreover, such embeddings (carried out without propylene oxide) were difficult to section except in the case of those embedded according to Spurr's technique (32).

We were able to avoid the above-mentioned difficulties and obtain excellent differential contrast among granules by fixing cells in glutaraldehyde and reacting them for peroxidase, after which they could be processed routinely (uranyl acetate staining in block, alcohol-propylene oxide dehydration, and Epon or Araldite embedding). Since, as in the case of the rabbit, peroxidase is present in all the granules of promyelocytes (i.e. in all azurophils), the peroxidase reaction product renders the azurophil granules very dense and easily distinguishable from granules formed during the myelocyte stage, which are peroxidase-negative. Accordingly, the following observations on developmental stages of human PMN are based on such preparations reacted for peroxidase in which the presence of enzyme reaction product serves as a marker and stabilizer of the azurophils.

#### *Stages of Neutrophil Maturation*

*Myeloblast.*—The earliest cell of the neutrophilic series is the myeloblast, a relatively small ( $\sim 10 \mu$ ) undifferentiated cell with a high nuclear:cytoplasmic ratio and prominent nucleoli. The scant cytoplasm is devoid of granules but contains abundant free polysomes and mitochondria. Annulate lamellae, considered characteristic of embryonic cells and reported in blasts from mouse marrow (43), are occasionally seen. Very early promyelocytes resemble the myeloblast, but contain a few azurophil granules and a larger Golgi complex (Fig. 3).

*PMN Promyelocyte (Figs. 4-8).*—The promyelocyte can be recognized by its large size ( $\sim 15 \mu$ ), rounded nucleus, and population of variable but frequently large numbers of peroxidase-positive granules which correspond to the azurophil granules seen by light microscopy. It has a large Golgi region and moderate amounts of rough endoplasmic reticulum (ER). Fig. 4 illustrates a typical cell profile.

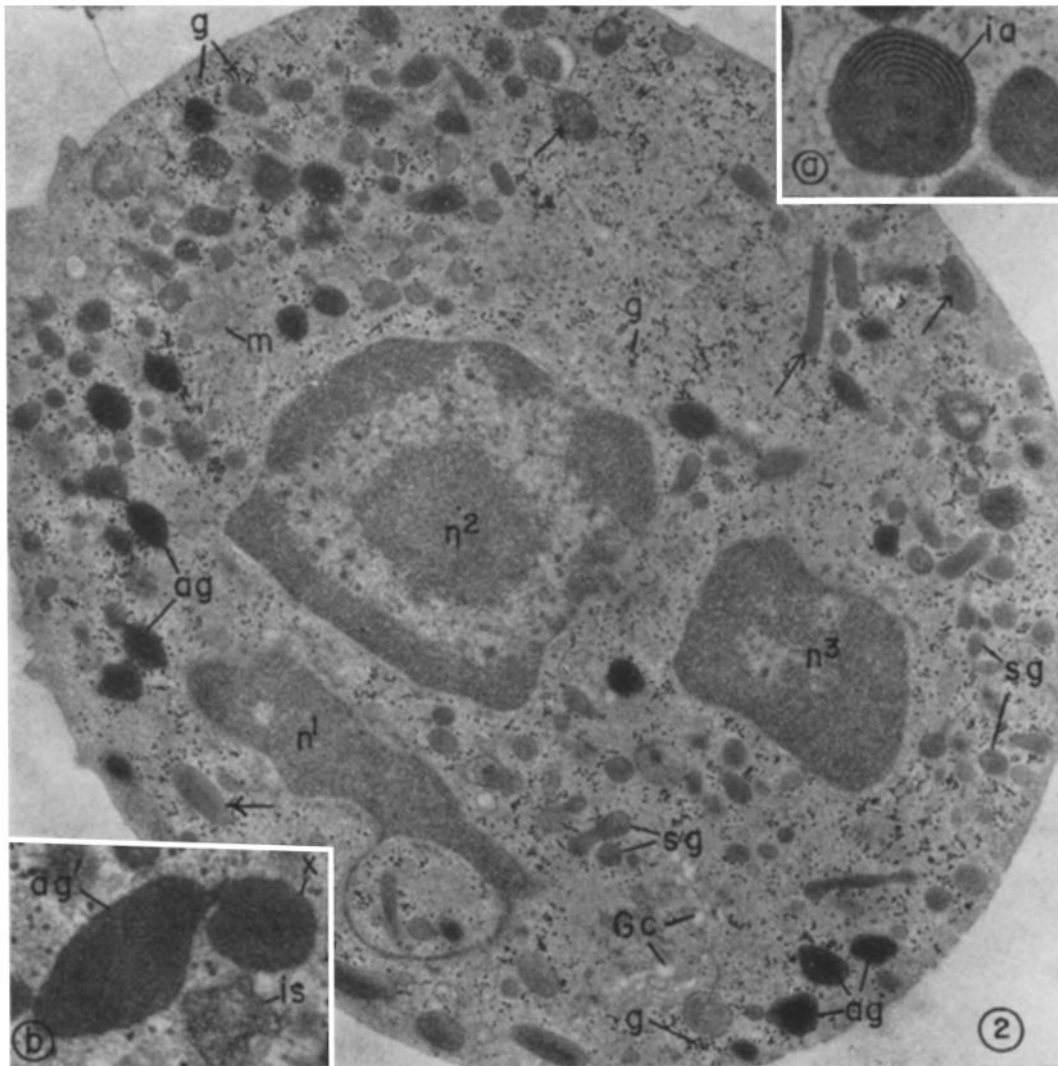
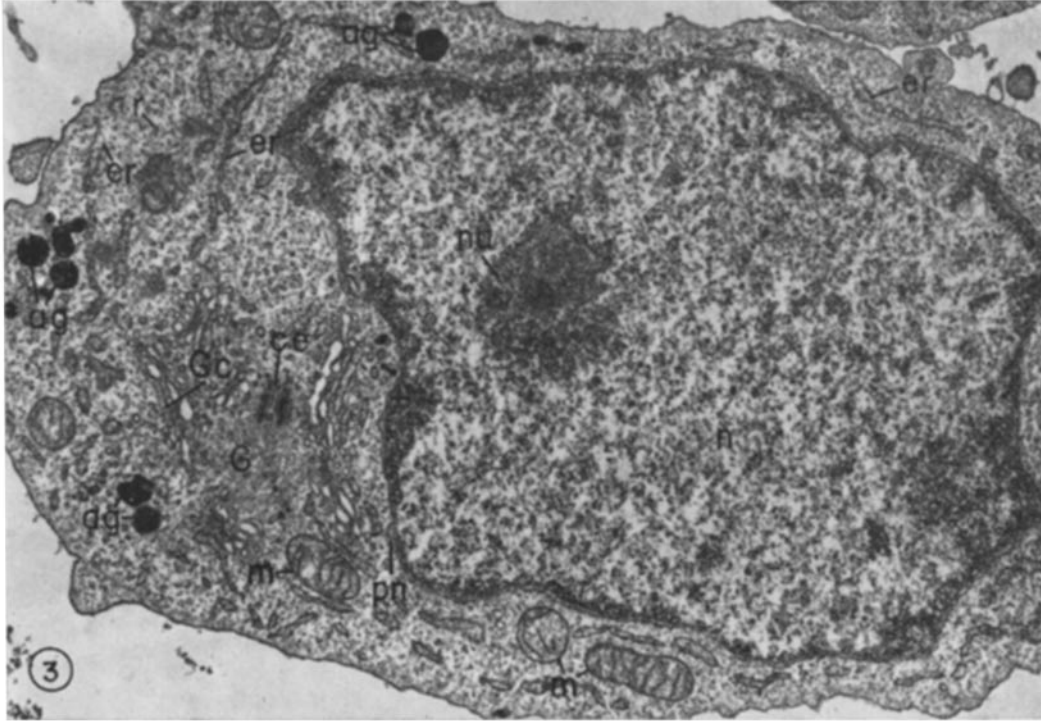


FIG. 2. Mature PMN from human bone marrow. Several lobes of the nucleus are seen ( $n^1 - n^3$ ), and numerous granules, as well as beta particles of glycogen ( $g$ ), are scattered throughout the cytoplasm. A few mitochondria ( $m$ ) and a small Golgi complex ( $Gc$ ) are also visible. Some of the granules present are large and dense ( $ag$ ), whereas others are small and less dense ( $sg$ ). However, many granules are intermediate in size and density. Elongated forms, including football and dumbbell shapes, are also present (arrows). This great variability in size, shape, and density renders identification of granule types by morphologic criteria difficult and unreliable. The glycogen is well preserved but membranes show poor contrast in this type of preparation in which there was no staining with uranyl acetate in block (cf. Fig. 11).

The insets depict internal structure within the large, dense (azurophil) granules. Inset *a* shows a spherical granule (*ia*) containing concentric half-rings. This type of internal structure was only observed occasionally in azurophil granules of normal mature PMN. Inset *b* illustrates the crystalline lattice with periodicity of  $\sim 80$  A which is commonly seen in football or ellipsoid forms ( $ag'$ ). A cross-section (*X*) of the ellipsoid form and an immature specific granule (*is*) are also present in this field taken from a PMN myelocyte. Tissue was fixed in glutaraldehyde, 22°C for 6 hr, postfixed in osmium, dehydrated in alcohol, and embedded in Araldite with omission of propylene oxide. The sections were stained with uranyl acetate and alkaline lead. Fig. 2,  $\times 17,000$ ; *a*,  $\times 45,000$ ; *b*,  $\times 45,000$ .



Figures 3-11 are electron micrographs of developing PMN from normal human bone marrow reacted for peroxidase.

FIG. 3. Early PMN promyelocyte. The nucleus (*n*) with its prominent nucleolus (*nu*) occupies the bulk of this very immature cell. The surrounding cytoplasm contains a few azurophil granules (*ag*), a large Golgi complex (*G*), several mitochondria (*m*), scanty rough endoplasmic reticulum (*er*), and many free polysomes (*r*). A centriole (*ce*) is present in the Golgi region.

All of the azurophil granules (*ag*) appear dense, since they are strongly reactive for peroxidase. The secretory apparatus [i.e. the perinuclear cisterna (*pn*), rough endoplasmic reticulum (*er*), and some of the Golgi cisternae (*Gc*)] is also reactive, although less so than the granules, a point which is better illustrated in Figs. 4 and 5.

Specimen fixed in glutaraldehyde for 16 hr at 4°C, incubated in the peroxidase medium of Graham and Karnovsky for 1 hr at 22°C, postfixed in OsO<sub>4</sub>, treated in block with uranyl acetate, dehydrated in ethanol, infiltrated with propylene oxide, and embedded in Araldite. Section stained for 1 min with lead citrate. × 21,000.

Although all the azurophil granules are reactive for peroxidase, they vary in size and shape and in the amount and degree of compaction of peroxidase reaction product. Two main shapes can be identified: round and football-shaped. The predominant form is round (diameter = ~ 500 mμ); its contents appear flocculent and less compact in immature granules, but become more homogeneous and dense as the granules mature and undergo condensation. Football-

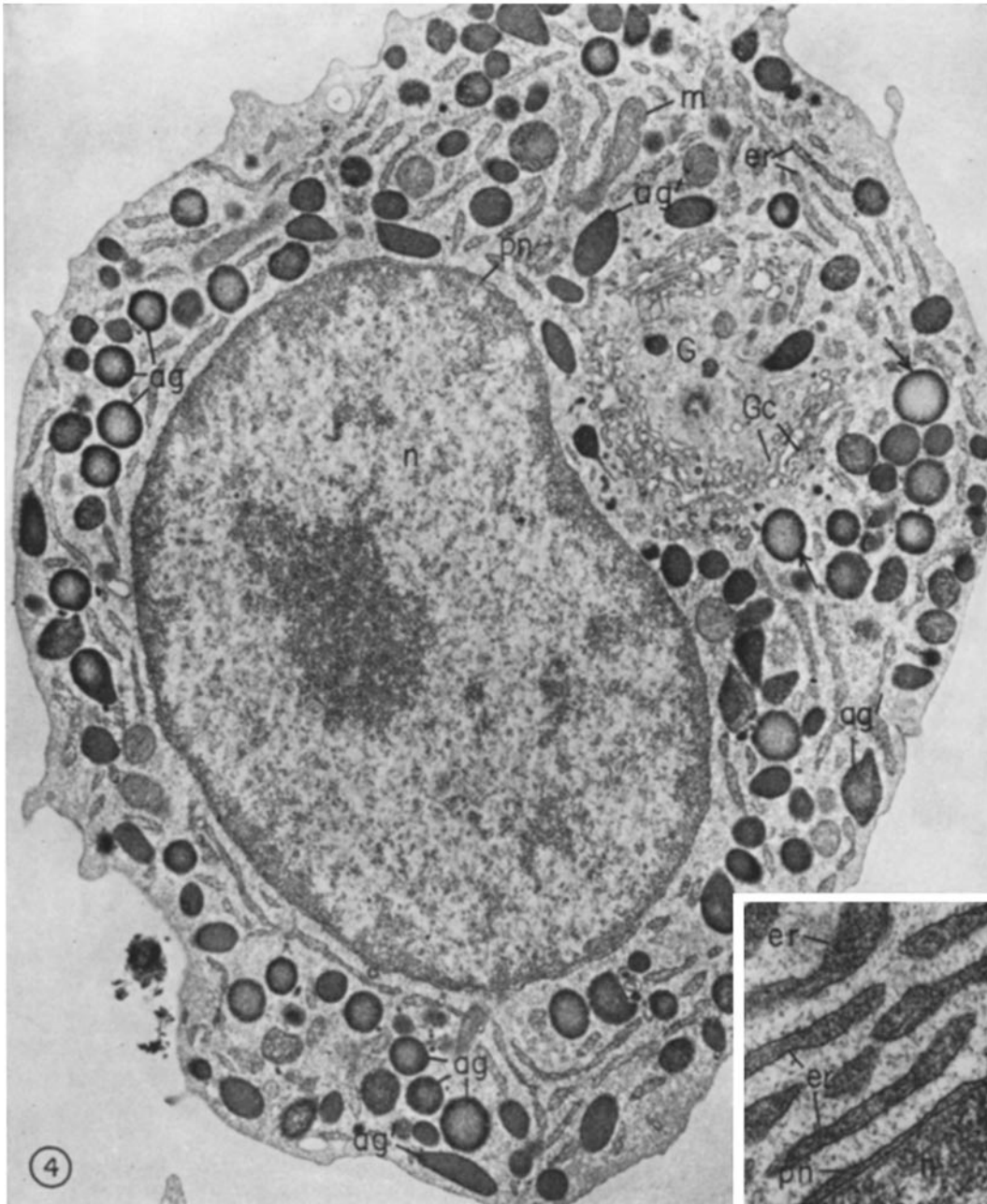


FIG. 4. PMN promyelocyte, reacted for peroxidase. This cell is the largest ( $\sim 15 \mu$ ) of the neutrophilic series. It has a sizable, slightly indented nucleus ( $n$ ), a prominent Golgi region ( $G$ ), and cytoplasm packed with peroxidase-positive azurophil granules ( $ag$ ). Note the two general shapes of azurophil granules, spherical ( $ag$ ) or ellipsoid ( $ag'$ ). The majority are spheri-



shaped forms ( $300 \times 900 \text{ m}\mu$ ) are less common. They frequently contain crystalline inclusions with a periodicity of  $\sim 80 \text{ \AA}$ , oriented parallel to their long axis (Figs. 6–7). Round profiles with a central core also showing a periodic structure (Fig. 8) are presumed to represent “footballs” cut in cross-section. The secretory apparatus of the promyelocyte (i.e. rough ER, including the perinuclear cisterna, and Golgi cisternae) also contains peroxidase reaction product which is less concentrated than that in the azurophil granules (Fig. 5). At times the innermost Golgi cisterna along the concave surface of the Golgi stack is more reactive than the others, as seen in Fig. 5. Promyelocytes are frequently observed in various stages of mitosis during which reaction product is present throughout the entire ER.

*PMN Myelocyte (Figs. 9 and 10).*—The myelocyte is smaller ( $10 \mu$ ) than the promyelocyte and can be recognized by its indented nucleus, prominent Golgi complex, and mixed population of granules, which includes variable numbers of smaller, peroxidase-negative granules, as well as the large peroxidase-positive azurophil granules. These new granules, which accumulate throughout the myelocyte stage and eventually come to outnumber the azurophils, correspond to the specific granules. They vary in size and shape but typically occur as spheres ( $\sim 200 \text{ m}\mu$ ) or rods ( $130 \times 1000 \text{ m}\mu$ ); the latter are often cigar- or dumbbell-shaped. Despite their variability in shape, they all have a similar content which is homogeneous, of low density, and devoid of peroxidase activity. Hence in these respects they appear to represent a single granule population.

The Golgi complex of the myelocyte is large, and in contrast to that of the promyelocyte, is not reactive for peroxidase. Immature specific granules appear to bleb off its outer cisternae along the convex Golgi surface (Fig. 10). The presence of a prominent Golgi complex containing forming specific granules distinguishes the myelocyte from later, nonsecretory stages.

It should be emphasized that elements of the secretory apparatus of the myelocyte were never seen to contain peroxidase. The only peroxidase-positive elements present at this and subsequent stages are the azurophil granules.

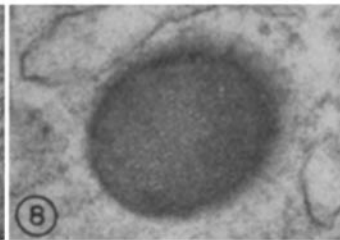
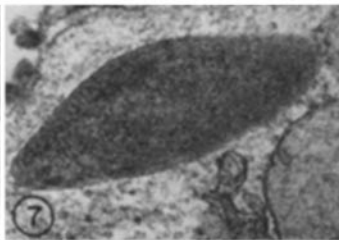
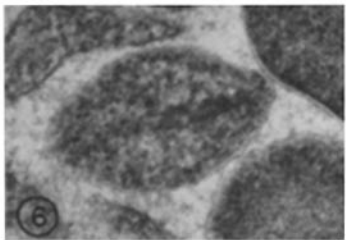
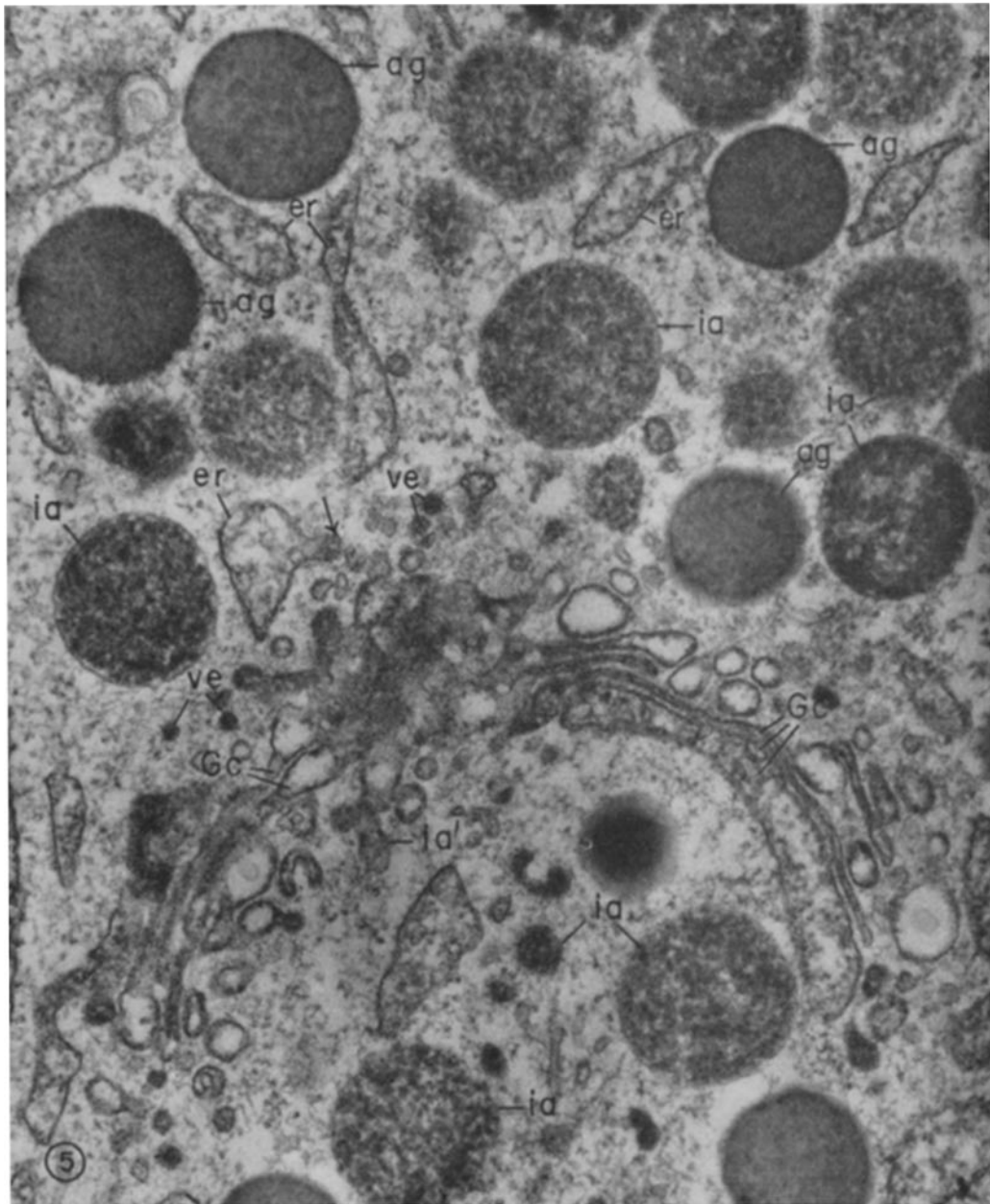
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cal, with a homogeneous matrix, but a few ellipsoid forms containing crystalloids (best seen in Figs. 6–8) are also present. Many of the spherical forms (arrows) have a dense periphery and a lighter core, owing presumably to incomplete penetration of substrate into the compact centers of mature granules.

Peroxidase reaction product is visible in less concentrated form within all compartments of the secretory apparatus [endoplasmic reticulum (*er*), perinuclear cisterna (*pn*), and Golgi cisternae (*Gc*)]. No reaction product is seen in the cytoplasmic matrix, mitochondria (*m*), or nucleus (*n*).

The inset depicts a portion of another promyelocyte at higher magnification, showing to better advantage flocculent deposits of peroxidase reaction product in the rough ER (*er*) including the perinuclear cisterna (*pn*). Ribosomes do not show up in this section, which was lightly stained with lead.

Specimen fixed in glutaraldehyde for 10 min at  $4^\circ\text{C}$  and subsequently processed exactly as in Fig. 3. Fig. 4,  $\times 15,000$ , inset,  $\times 34,000$ .



Among the latter, in addition to the usual large azurophil forms, occasional clusters of much smaller (200  $m\mu$ ) peroxidase-positive granules can be found (see inset, Fig. 9).

It appears that, as in the case of the rabbit, production of peroxidase-positive azurophil granules ends in the promyelocyte, and the beginning of the myelocyte stage is marked by the production of a second population of granules which are peroxidase negative. It is of interest that cells are frequently observed which contain mature azurophils but no specific granules, have the indented nuclear configuration of the myelocyte, lack peroxidase in the ER or Golgi cisternae, and have a large Golgi apparatus which does not contain forming granules of either type. Such cells are presumed to be in a hiatus between the two waves of secretory activity. Similar cells have been described by Ackerman (44) in the cat. Mitoses have also been observed during the myelocyte stage; it can be seen that the granules are distributed in approximately equal numbers to daughter cells.

*Later Stages (Bone Marrow).*—The metamyelocyte, band, and mature PMN are nondividing, nonsecretory stages which are identified by their nuclear morphology, mixed granule population, inactive Golgi region (Fig. 11), and accumulation of glycogen particles (Fig. 2). The number of granules present at these stages is quite large; granule counts carried out on sections passing through the Golgi region indicated the presence of an average of 200–300/cell profile, with approximately twice as many specifics as azurophils. As in the myelocyte, peroxidase activity is present only in azurophil granules. During these stages the azurophils become somewhat smaller and more oval (compare azurophils in Fig. 4 to those in Figs. 9 and 11), a change which probably occurs due to progressive concentration of the granule contents, and which may be related to the diminished azurophilia noted in Wright's-stained smears (see

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Figs. 5-8 illustrate portions of PMN promyelocytes.

FIG. 5. Golgi region of PMN promyelocyte. At this stage, peroxidase reaction product is present within [a] all cisternae of the rough ER (*er*), including transitional elements (arrow), and the perinuclear cisterna (not shown here); [b] clusters of smooth vesicles located at the periphery of the Golgi complex (*ve*); [c] all cisternae of the Golgi complex (*Gc*); and [d] all immature (*ia*) and mature azurophil granules (*ag*). Most of the mature granules (*ag*) appear uniformly dense due to the presence of reaction product throughout. Immature granules are larger and their contents less compact (*ia*). Note that peroxidase is more concentrated in the cisternae along the concave surface of the Golgi complex, suggesting that, as in the case of the rabbit (1), azurophil granules arise from this Golgi face (see *ia'*).

FIGS. 6-8. Azurophil granules of promyelocytes occur in two main forms. The majority are spherical, with a dense, homogeneous matrix (*ag* in Fig. 5). Others are ellipsoid or football-shaped (Figs. 6 and 7) with a crystalline substructure which may be partially obscured in mature granules by the dense peroxidase reaction product (Fig. 7) but is clearly visible in the immature ellipsoid (Fig. 6) or in preparations not incubated for peroxidase (Fig. 2 *b*). Round granule profiles with a central periodicity (Fig. 8) are presumed to represent "footballs" cut perpendicular to the crystal axis.

Specimen preparation as in Fig. 4. Fig. 5,  $\times 56,000$ ; Figs. 6-7,  $\times 63,000$ ; Fig. 8,  $\times 90,000$ .

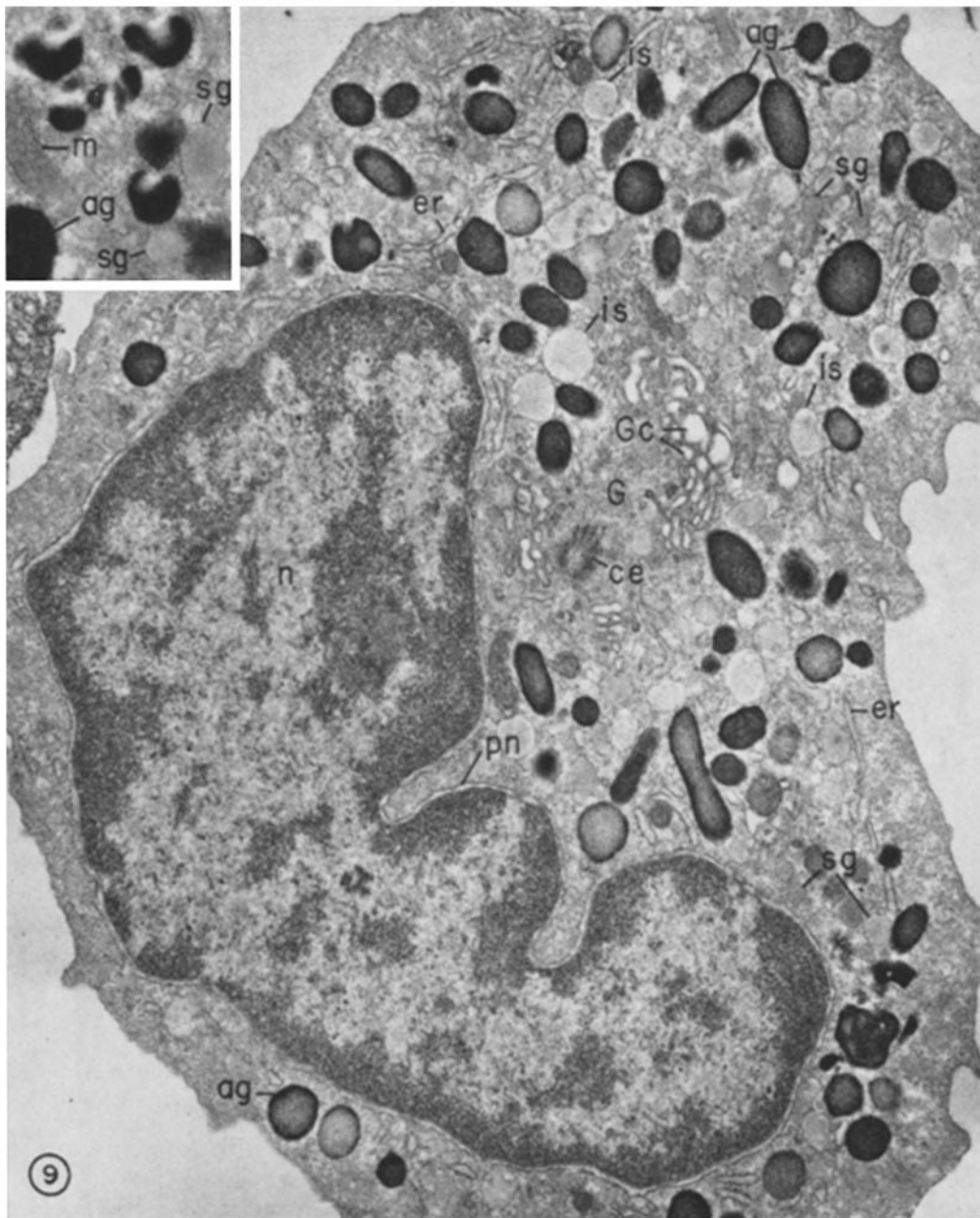


FIG. 9. PMN myelocyte. At this stage the cell is smaller ( $\sim 10 \mu$ ) than the promyelocyte (see Fig. 4), the nucleus is more indented, and the cytoplasm contains two different types of granules: [1] large, peroxidase-positive azurophils (*ag*), and [2] the generally smaller specific granules (*sg*), which do not stain for peroxidase. A number of immature specifics (*is*), which are larger, less compact, and more irregular in contour than mature granules, are seen in the Golgi region (*G*). Note that peroxidase reaction product is present only in azurophil granules,

Fig. 12). A few of the azurophils take on a rough dumbbell-shape (see Figs. 9 and 11) similar to the form common among specifics, emphasizing the necessity of using peroxidase to identify the azurophils in more mature neutrophils.

*Blood PMN.*—The mature PMN of the blood appear generally similar to the mature PMN in the marrow when cells are collected using the Kaplow buffy-coat tube, a method involving a minimal delay in fixation ( $\sim 5$  min) and minimal trauma to the cells. When other currently available methods of isolating blood PMN are used, which involve longer delays before fixation as well as chemical or osmotic trauma (heparin, ethylenediaminetetraacetic acid [EDTA],  $\text{NH}_4\text{Cl}$  lysis, or dextran), more variation is observed in the size, shape, and numbers of granules present, and in the intensity of the peroxidase reaction in azurophils.

#### *Cytochemical Studies on Granule Contents*

The results with peroxidase preparations indicated clearly the existence of two distinct types of granules which are produced at different times during the development of human PMN. Hence the next step was to establish the nature of their contents other than peroxidase. Accordingly, we carried out combined histochemical staining on bone marrow smears and electron microscopic cytochemistry on bone marrow cells in suspension, as in our earlier studies on the rabbit (2, 3).

*Light Microscopy of Bone Marrow Smears.*—Tests for six enzymes and basic protein were carried out on smears of bone marrow cells. When available, both azo-dye and metal-salt techniques were used. As in the rabbit (2), we compared the distribution and intensity of each reaction to the distribution of azurophil and specific granules in developing neutrophils. Thus, if a given granule enzyme is confined to the azurophils, promyelocytes should contain numerous reactive granules and mature cells, progressively fewer. Conversely, if an enzyme is present only in specifics, mature cells should show many reactive granules and promyelocytes, none. If the enzyme is present in both granule types, all stages after the myeloblast should contain numerous reactive granules.

Our results are given in Table I and are illustrated in Figs. 12–17. Smears

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and is not seen in the rough ER (*er*) (which nonetheless has a content of moderate density), perinuclear cisterna (*pn*), or Golgi cisternae (*Gc*) (which appear empty). This is in keeping with the fact that azurophil production has ceased, and only peroxidase-negative specifics are produced during the myelocyte stage.

The inset, a portion of a myelocyte, depicts a cluster of peroxidase-positive granules, most of which are smaller and more pleomorphic than the surrounding specifics (*sg*) and azurophils (*ag*). These are presumed to represent azurophil variants, since they appear during the promyelocyte stage. The presence of such granule variants emphasizes the need for using criteria other than size and shape for identifying PMN granules in the human. Specimen preparation as in Fig. 4. Fig. 9,  $\times 20,000$ ; inset,  $\times 41,000$ .

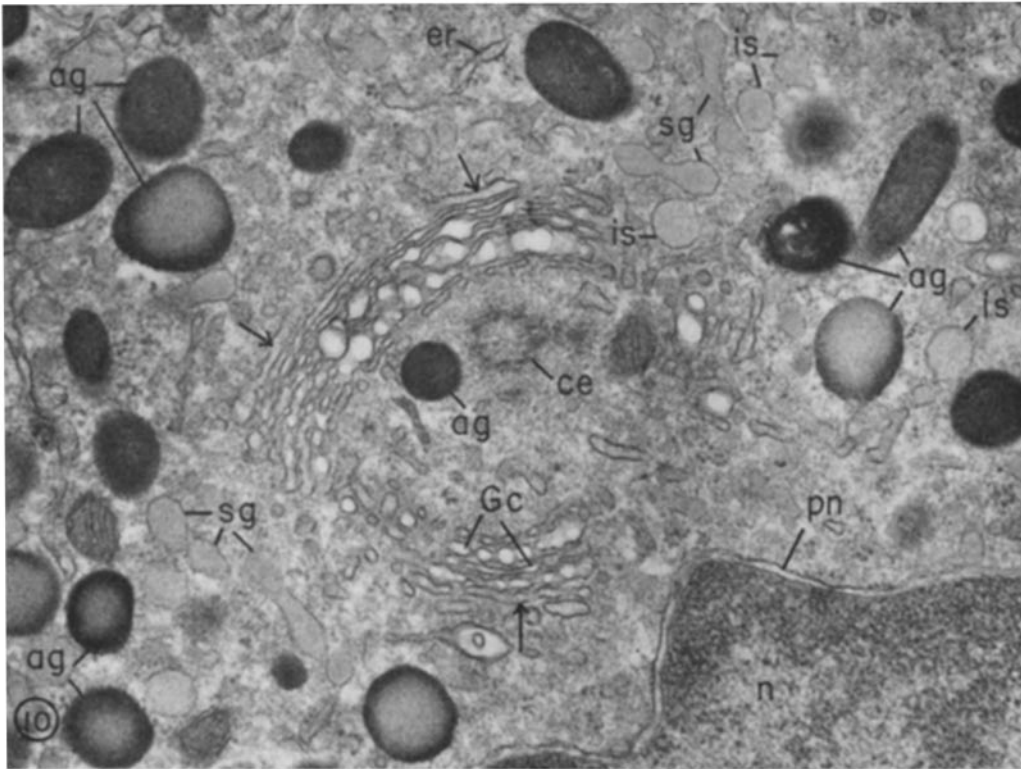


FIG. 10. Higher power view of the Golgi region of PMN myelocyte similar to the cell shown in Fig. 9. As in the preceding figure, peroxidase reaction is seen in azurophilic (*ag*) but not in specific granules (*sg*). No reaction product is seen in the ER (*er*), perinuclear cisterna (*pn*), Golgi cisternae (*Gc*), or newly formed granules (*is*), but the ER has a content of moderate density. The stacked, smooth-surfaced Golgi cisternae (*Gc*) are oriented around the centriole (*ce*). Note that the outer cisternae have a content of intermediate density (arrows) which is similar to the content of the specific granules. The images are less suggestive than in the rabbit (1), but they are compatible with the view that specific granules arise from the convex face of the Golgi complex in both species. Specimen fixed in formaldehyde-glutaraldehyde for 1 hr at 22°C and thereafter prepared as that in Fig. 3.  $\times 33,000$ .

incubated for peroxidase, pH 7.6 (Fig. 13), acid phosphatase, pH 5.0 (Fig. 15), arylsulfatase, pH 5.5 (Fig. 16),  $\beta$ -galactosidase, pH 4.5, or 5'-nucleotidase, pH 4.0, showed numerous reactive granules in promyelocytes, whereas later stages contained fewer reactive granules. Hence these four lysosomal enzymes, like peroxidase, appear to be confined to azurophil granules.

A different pattern of distribution was found for one enzyme tested, alkaline phosphatase, pH 9.2 (Fig. 14). Promyelocytes showed no reactive granules,

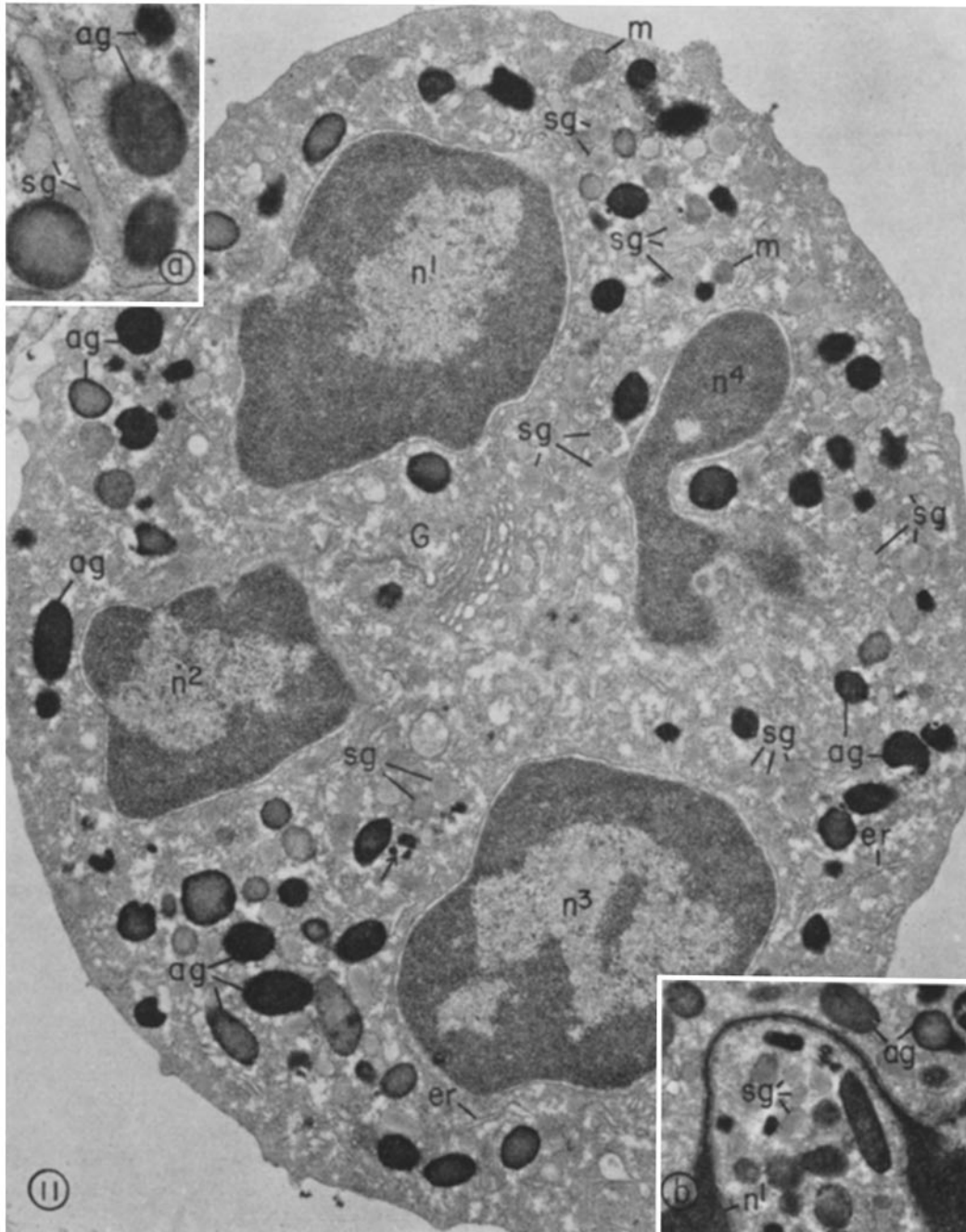


FIG. 11. Mature PMN, reacted for peroxidase. The cytoplasm is filled with granules; the smaller peroxidase-negative specifics (*sg*) are more numerous, azurophils (*ag*) having been reduced in number by cell divisions after the promyelocyte stage. Some small, irregularly-shaped azurophil granule variants are also present (arrow) (see Fig. 9). The nucleus is condensed and lobulated ( $n^1 - n^4$ ), the Golgi region (*G*) is small and lacks forming granules, the ER (*er*) scanty, and mitochondria (*m*) few. Note that the cytoplasm of this cell has a rather ragged, moth-eaten appearance due to the fact that the glycogen, which is normally present (cf. Fig. 2), has been extracted in this preparation by staining in block with uranyl acetate.

The insets depict portions of the cytoplasm of mature PMN reacted for peroxidase. Inset *a* demonstrates that the peroxidase-positive azurophils (*ag*) can be easily distinguished from the unreactive specifics (*sg*). Note that one of the specifics is quite elongated ( $\sim 1000 \text{ m}\mu$ ). Inset *b* illustrates the narrow connection between two lobes ( $n^1 - n^2$ ) of the PMN nucleus. Specimen preparation as in Fig. 3. Inset, specimen preparation as in Fig. 4. Fig. 11,  $\times 21,000$ ; inset *a*,  $\times 36,000$ ; inset *b*,  $\times 14,000$ .

TABLE I  
*Distribution of Reactive Granules in Human Promyelocytes and Mature PMN*

Test	pH	Number of reactive granules	
		Promyelocyte	Mature PMN
Peroxidase	7.6	Many	Few
Acid phosphatase	5.0	Many	Few
5'-nucleotidase	4.0	Many	Few
$\beta$ -galactosidase	4.5	Many	Few
Arylsulfatase	5.5	Many	Few
Alkaline phosphatase	9.2	None	Many
Fast green FCF	8.1	Many	Many
Biebrich scarlet	9.9	Many	Many

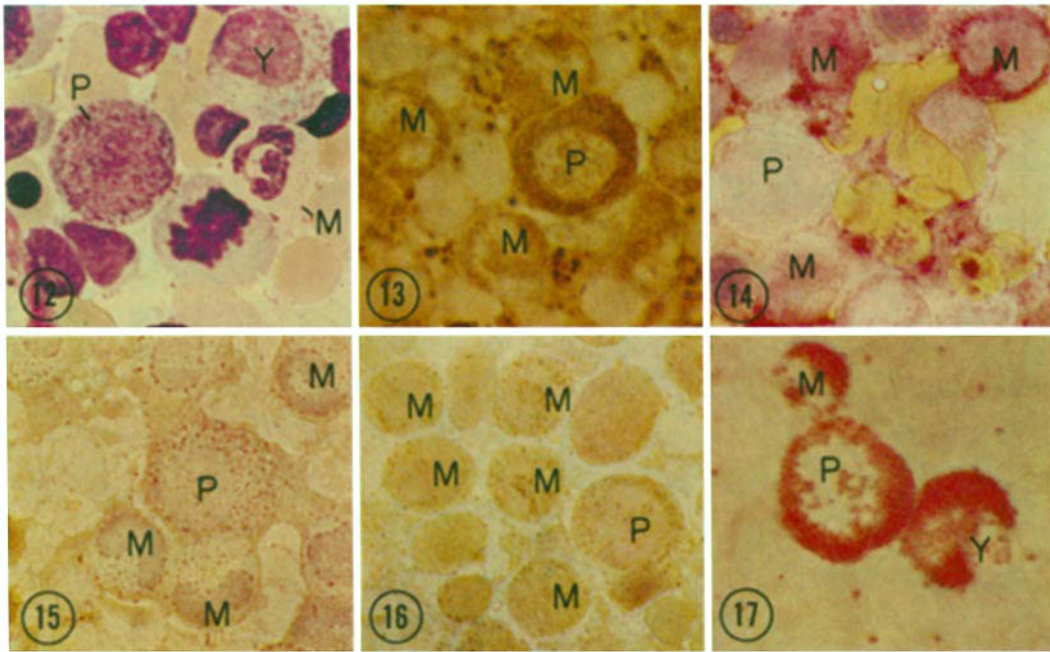
myelocytes contained a variable number, and later stages contained many. This distribution indicates that alkaline phosphatase is localized in specific granules.

Two tests for basic protein, fast green FCF (pH 8.2) and Biebrich scarlet (pH 9.9), yielded intense staining of all stages (promyelocytes, myelocytes and more mature PMN) (Fig. 17), indicating that both azurophil and specific granules contain basic proteins.

*Electron Microscopy.*—Cytochemical techniques were used to determine directly the intracellular localization of those of the above enzymes for which ultrastructural methods are available, i.e., arylsulfatase, alkaline phosphatase, and acid phosphatase. As in the case of the rabbit, we found that the enzyme activity present within mature PMN granules was latent to demonstration by metal-salt techniques (3). However, the presence of a given enzyme within a given granule population was inferred when reaction product was demonstrable in immature granules and in the secretory apparatus at the stage during which granules in question are being formed. Thus, enzyme reactivity within ER and Golgi elements at the promyelocyte stage indicates that the enzyme is being packaged into azurophils, whereas localization of reactivity in these compartments during the myelocyte stage suggests packaging into specifics.

*Arylsulfatase.*—Reaction product was generally scanty but was found consistently in promyelocytes, where it appeared as a punctate density and was localized in immature azurophils (both round and football forms) and within occasional rough ER and Golgi cisternae (Fig. 18). After the promyelocyte (myelocyte and later stages), reaction product was restricted to some of the granules and did not occur in the secretory apparatus. The identity of the reactive granules could not be established with certainty in this type of preparation (not reacted for peroxidase), but they were presumed to be azurophils. The fact that arylsulfatase activity was absent from immature specifics and from ER and Golgi cisternae of myelocytes suggests that this enzyme is restricted to azurophil granules.





Figs. 12-17 are light micrographs of bone marrow smears  $\times 950$ . Fig. 12 shows the various stages of maturation with Wright's stain. Figs. 13-16 show the results of tests for various granule enzymes which appear to be confined to either azurophil (Figs. 13, 15, 16) or specific (Fig. 14) granules.

FIG. 12. Wright's stain, air-dried. Azurophil granules appear large and stain reddish-purple in the promyelocyte (*P*), but their numbers diminish and staining characteristics are altered (see Discussion) in the mature cells (*M*). Compare the size and color of the "lilac" granules seen in the mature cell (*M*) to the larger reddish-purple granules in the promyelocyte (*P*). Note the pink blush of the cytoplasm of the mature PMN (*M*). The large pink area in the cytoplasm of the PMN myelocyte (*Y*) marks the beginning of specific granule formation and is called "the sunrise of neutrophilia."

FIGS. 13, 15-16. In all these figures, promyelocytes (*P*) contain more reactive granules than do mature cells (*M*). Fig. 13, peroxidase, fixed in glutaraldehyde and incubated in the medium of Graham and Karnovsky (pH 7.6). Fig. 15, acid phosphatase, fixed in acetone and incubated in Burstone's medium (pH 5.0) and counterstained with hematoxylin. Fig. 16, arylsulfatase, fixed in glutaraldehyde and incubated in Goldfischer's medium (pH 5.5). This distribution of reactivity corresponds to that of azurophil granules.

FIG. 14. Alkaline phosphatase, fixed in formol-calcium, incubated in Kaplow's medium (pH 9.2), and counterstained with hematoxylin. The large promyelocyte (*P*) is unreactive, but mature cells (*M*) stain intensely. This distribution of reactivity corresponds to that of the specific granules.

FIG. 17. Biebrich scarlet (pH 9.9), methyl alcohol fixation. All stages after the myeloblast, i.e. promyelocytes (*P*), myelocytes (*Y*), and mature cells (*M*), contain numerous stained granules.

*Alkaline Phosphatase.*—Reaction product for phosphatase activity at pH 9.2 was confined to the myelocyte stage, where it appeared in Golgi cisternae (Fig. 19) and within scattered specific granules, both rounded (Fig. 20) and elongated (Fig. 21) forms. No reaction product was seen in promyelocytes,

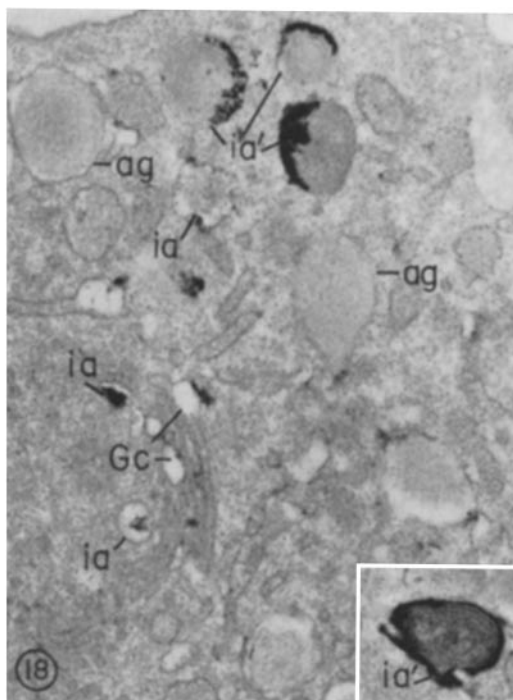
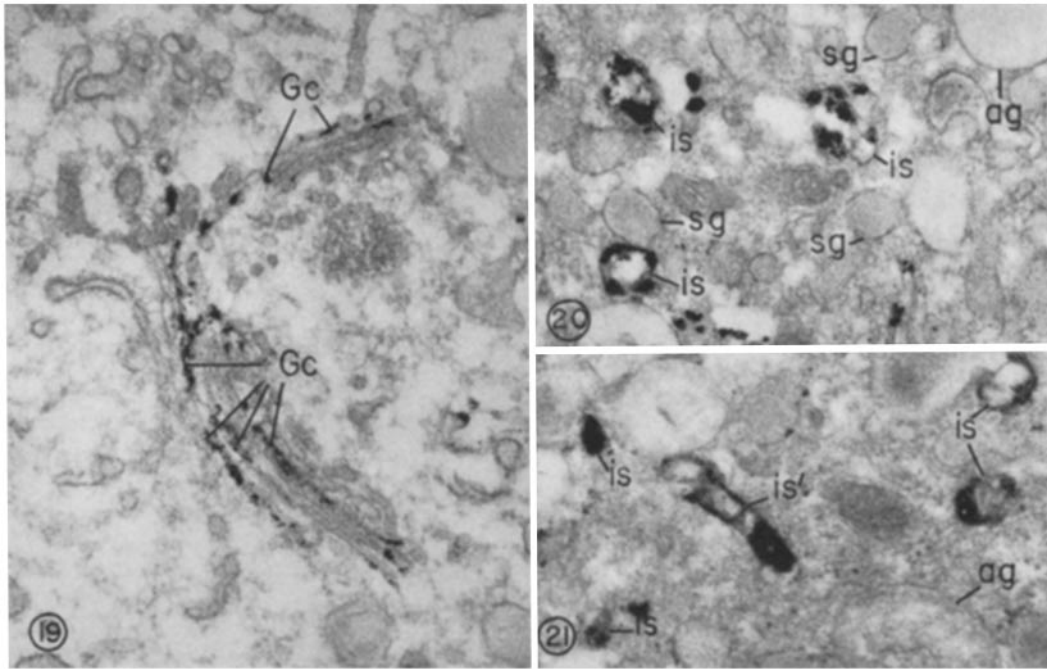


FIG. 18. PMN promyelocyte reacted for arylsulfatase. Deposits of dense lead sulfide are present in small amounts in the Golgi cisternae (*Gc*) and in some adjacent immature azurophil granules (*ia*). Three of the larger immature azurophil forms (*ia'*) are rimmed by dense reaction product. Two of these granules also appear to contain concentric rings; this aspect can be seen better in the inset, which is also an immature azurophil granule (*ia'*). Two mature azurophil granules (*ag*) do not contain reaction product. Note that fine particles cover the background in this preparation, which was treated in block with  $(\text{NH}_4)_2\text{S}$ . Specimen fixed for 30 min at 4°C in glutaraldehyde, incubated for 4 hr at 37°C in Goldfischer's medium (pH 5.5), washed in 2%  $(\text{NH}_4)_2\text{S}$ , postfixed in  $\text{OsO}_4$ , treated for 10 min in block with uranyl acetate, and embedded in Araldite. Section stained 1 min with lead citrate. Fig. 18 and inset  $\times 29,000$ .

confirming the observations obtained by light microscopy that alkaline phosphatase appears relatively late in neutrophil development and is restricted to specific granules. The enzyme was not demonstrable in any organelles, including the granules in stages after the myelocyte. As already mentioned, the enzyme activity present in mature granules is latent to demonstration by metal-salt techniques in this type of preparation (3).

*Acid Phosphatase.*—Distribution of reaction product for this enzyme was more complex than the above. In accordance with the light microscopic findings, the strongest reaction was found in promyelocytes, in which all compartments of the secretory apparatus, as well as some immature azurophil granules



FIGS. 19-21. Alkaline phosphatase preparations of PMN myelocytes. The morphologic preservation is generally poor due to the high pH (9.2) of the incubation medium. Lead phosphate reaction product is present in most of the Golgi cisternae (*Gc*) and in several of the immature specific granules (*is*). The somewhat denser, mature specific granules (*sg*) are unreactive, as are the azurophil granules (*ag*), which commonly appear pale and extracted. Reaction product can be seen in both round (*is*) and elongated (*is'*) immature specific granules. Specimen preparation as for Fig. 4, except that incubation was carried out in Gomori's medium, pH 9.2, for 2 hr (Figs. 19 and 20) or 16 hr (Fig. 21) at 37°C, and was followed by treatment with lead nitrate. Sections stained 1 min with lead citrate. Fig. 19,  $\times 46,000$ ; Fig. 20,  $\times 40,000$ ; Fig. 21,  $\times 44,000$ .

(including the ellipsoid forms), were reactive. In the myelocyte stage, reaction product was far less abundant but was nevertheless present in a few fragments of rough ER, in the Golgi cisternae, and in small vesicles (presumably immature specific granules) which are located near the Golgi region. We found a similar reaction previously in PMN myelocytes of the rabbit (3) and suggested that this phosphatasic activity may represent an enzyme different from lyso-

somal acid phosphatase, since such activity has been demonstrated in Golgi cisternae and around forming secretory granules in many different types of cells (see references 3, 45, 46). Stages beyond the myelocyte, including blood PMN, did not contain reaction product.

Table II summarizes our conclusions on the localization of enzymes within PMN granules, based on the combined results of histochemical staining of bone marrow smears and electron microscopic cytochemistry. Acid hydrolases, as well as peroxidase, appear to be localized in azurophil granules and absent from specifics. Alkaline phosphatase, which is not a lysosomal enzyme, is found in the specifics. The number of reactive granules and the amount of reaction product present vary with the enzymes tested. This variability may reflect actual differences in the amount of enzyme present, or could be explained on the basis of technical difficulties associated with the cytochemical techniques.

TABLE II  
*Results of Analysis of Enzyme Content of Human PMN Granules*

Azurophil granules	Specific granules
Lysosomal enzymes	No lysosomal enzymes*
Acid phosphatase	
Arylsulfatase	
$\beta$ -galactosidase	
5'-nucleotidase	
Peroxidase	No peroxidase
	Alkaline phosphatase

\* Acid phosphatase reaction product was seen in some immature specific granules. See text for discussion.

*Controls and Comments.*—Peroxidase activity within the secretory apparatus of promyelocytes was heaviest after brief (10–15 min) fixation in glutaraldehyde at 4°C. With longer fixation, or with formaldehyde-glutaraldehyde, this activity was diminished but the reaction in azurophil granules was not affected. Reaction product appears more dense if staining with uranyl acetate in block is omitted, but membranes, particularly those of the Golgi complex, are less well defined.

No reaction product was observed in marrow or blood PMN incubated in peroxidase medium lacking either H<sub>2</sub>O<sub>2</sub> or diaminobenzidine (36). Addition of KCN and/or aminotriazole (pH 7.4) (inhibitors of peroxidase) to the incubation medium eliminated the reaction in ER and Golgi cisternae but did not affect the reaction in azurophils. However, preincubation in 0.01 M KCN at pH 6 for 30 min, followed by incubation in peroxidase medium in a closed system, pH 7.6, with 0.01 M KCN added (38), inhibited the reaction in azurophil granules as well as the secretory apparatus.

Control preparations incubated with substrate omitted from the medium were consistently unreactive. NaF added to the acid phosphatase medium inhibited the reaction completely.

#### DISCUSSION

The present study of human bone marrow has shown that developing PMN undergo two successive waves of secretory activity resulting in the production and accumulation of two types of granules with different contents. The azurophils, which represent the first generation of granules, are formed during the promyelocyte stage. They occur in two different forms, rounded and football-shaped, the latter with crystalline inclusions, and contain peroxidase and several acid hydrolases. The second generation of granules, the specifics, are formed later in development, during the myelocyte stage. They are on the average smaller than the azurophils, with homogeneous content, and are spherical or elongated. They contain alkaline phosphatase, but lack lysosomal enzymes and peroxidase, and therefore represent a different type of secretory product. The specifics are the predominant granule of mature PMN, since the number of azurophil granules per cell is reduced during the myelocyte stage by mitoses, whereas the specifics are formed continually throughout this stage and progressively accumulate.

These findings indicate that the situation in the human is basically similar to that in the rabbit with respect to the existence of two types of granules, their mode of formation, and nature of their contents. The differences between the two species seem to be largely in the granule packaging: the human makes more, smaller, and more heterogeneous granules than does the rabbit. Thus, rabbit azurophils are large ( $\sim 800 \text{ m}\mu$ ) spherical granules, whereas those of the human are smaller ( $\sim 500 \text{ m}\mu$ ), more variable in size, occur in two different shapes (spherical or ellipsoid), and have a content which is easily extracted during processing. Specific granules of rabbit and man are more similar in density and content and in the fact that they occur in two different shapes (round or elongated), except that in the human they are much smaller in diameter and are frequently more elongated. In both species, specifics outnumber azurophils, but the human averages 150 specifics and 75 azurophils/cell profile for a ratio of 2:1; whereas, the corresponding figures in the rabbit are smaller, i.e. 54 and 16 specific and azurophil granules, respectively, for a ratio of 3:1. It should be pointed out, however, that since the diameter of the rabbit granules is much larger, the estimated collective volume of each type of secretory product in the two species is similar. Observations carried out so far on other species [rat (47), cat (44), guinea pig (48), and horse (49)] indicate that PMN granule morphology, particularly that of the azurophils, is characteristic for a given species. PMN are similar in this respect to certain other cell types [eosinophilic (50) and basophilic (48) leukocytes and pancreatic beta cells (51)]

in which granule morphology is so distinctive that it serves to identify the species of origin.

Our findings are in accord with the reports of others that strong peroxidase activity is characteristic of the human azurophil (10, 16-19) at all stages of PMN development. We have also stressed the necessity of using peroxidase as a marker for azurophils because of the difficulty of distinguishing azurophil granules from specifics in the human and in some other species, e.g., the cat (44) and, according to our preliminary observations, the rat, and the Minnesota miniature pig. Moreover, in the human the density, size, and shape of the granules appear to be readily influenced by the preparatory methods such as fixation, dehydration, and staining. Some of the additional "granule types" reported in other studies of human PMN (6-9, 11-15, 20) may be produced artifactually by variations in methodology. Most investigators have described one granule type that is large and commonly pale, and another smaller and denser. The large, pale granules are undoubtedly azurophils which have been extracted by propylene oxide. Elongated variants with crystalline inclusions corresponding to our "footballs" have also been described (9, 12-14, 16, 17); Daems and van der Ploeg (9) and Dunn et al. (16) found peroxidase activity in such granules, whereas Breton-Gorius and Guichard (17) reported negative results.

One further species difference is that in the human, unlike the rabbit, it is possible to demonstrate peroxidase within the entire secretory apparatus of the promyelocyte from the onset of granule production, and thus identify the compartments involved in the segregation of azurophil granule contents (17, 22, 23). The fact that reaction product is present in progressively increasing concentration within the ER, Golgi cisternae, and azurophil granules indicates that the pathway for secretion and condensation of peroxidase conforms in general to that described for secretory proteins in the pancreas (52) and other cell types (see ref. 53). However, in this instance, as in the neutrophilic promyelocyte of the cat (44) and in the rabbit and rat eosinophilic myelocyte (53, 54), the participation of the Golgi cisternae in the concentration and packaging process has been demonstrated. The abrupt disappearance of peroxidase from the secretory apparatus at the end of the promyelocyte stage in the human marks the point at which azurophil production ceases and the myelocyte stage begins. This provides clear cytochemical evidence of the change in secretory product (from azurophil to specific) that takes place during neutrophilic maturation.

Up to now, the relationship between the azurophil and specific granules has been unclear, because the large reddish-purple azurophils so prominent in the early neutrophil precursors in smears of normal marrow are not observed after the myelocyte stage. The electron microscopic demonstration that two distinct granule types persist in mature PMN suggests that the relatively large "lilac"

granules visible in mature cells with the light microscope are, in fact, azurophils with altered staining characteristics. Maturation and increasing concentration of azurophil granule contents may lead to decreased adsorption of dye molecules and lessened metachromasia, particularly if maturation is associated with complex formation between stainable acid mucosubstances and basic enzymes, as suggested by Spicer and his coworkers (55, 56).<sup>4</sup> Since most of the granules are in a size range ( $\sim 200 \text{ m}\mu$ ) at the limit of resolution of the light microscope, they probably cannot be distinguished individually, but are responsible for the pink background color of neutrophils during and after the myelocyte stage.

Heterogeneity in the enzyme content of PMN granules is well documented in the rabbit and suggested by previous histochemical work in the human. Ackerman (57) noted that peroxidase, acid phosphatase, and arylsulfatase appear early in PMN development, whereas alkaline phosphatase appears later. Our studies confirm these findings and demonstrate that the early and late appearance of these enzymes is associated with their packaging into azurophil and specific granules, respectively. Just as peroxidase reaction is used in clinical hematology to mark an earlier stage of development, i.e. differentiation into the granulocytic series, alkaline phosphatase may serve as a further useful index of neutrophil maturation (differentiation into myelocytes) as well as a marker for the specific granule.

Biochemical assays of a total "granule fraction" prepared from human PMN have indicated that these granules contain a complement of enzymes similar to that of rabbit PMN [i.e. peroxidase (58), acid phosphatase,  $\beta$ -glucuronidase (59, 60)]. Recently Olsson (61), using a silica-gel density gradient, was able to separate this common granule fraction from human neutrophils into two subfractions: a heavy fraction composed of denser and larger granules which contained acid phosphatase,  $\beta$ -galactosidase, and  $\beta$ -glucuronidase; and a more heterogeneous, lighter fraction containing all the alkaline phosphatase. Our results indicate this same distribution of enzymes between azurophils and specifics, suggesting that these granule types constitute Olsson's "heavy" and "light" fractions, respectively. It should be emphasized that there is at present no evidence that human PMN contain a third granule population with distinctive chemical contents, as has been suggested in the case of the rabbit (4, 5, 37).<sup>5</sup> However, the existence of subpopulations of the two main granule types

<sup>4</sup> In previous work on rabbit PMN, we were able to stain azurophil granules at all stages of PMN development, including the mature PMN of blood and exudate, by using azure A at pH 5 after heat fixation or brief fixation in acetone (see Fig. 1 of ref. 2). Thus far, we have been unsuccessful in applying the same stain to human azurophils.

<sup>5</sup> Even in the rabbit the existence of a third granule population is open to question since we did not find any evidence of such granules in our morphological (1) or cytochemical (3) PMN preparations. In addition, in collaboration with Baggiolini and de Duve, we recently have examined PMN granule fractions by electron microscopical cytochemistry and have found that

cannot be ruled out on the basis of data obtained by either cell fractionation or cytochemical staining, since according to our present findings, except in the case of peroxidase, only a portion of the granules of either type gave a positive reaction with any of the tests.

The demonstration of distinct chemical differences between azurophil and specific granules, coupled with the persistence of both types in mature PMN, suggests that the different granules may have independent functions. This implication is supported by recent evidence (63) that azurophil and specific granules of the rabbit discharge their contents into phagocytic vacuoles in sequence, the specifics preceding the azurophils. Since as far as we have been able to determine, granules of human PMN parallel those of the rabbit in enzyme content, though differing in morphology, it seems likely that the more extensive data available on the rabbit may eventually prove applicable to the human. Thus, lysozyme is known to be present in human granules (59), and the recent studies of rabbit granules by Baggiolini et al. have shown that the bulk of the lysozyme activity (4, 5) and all of the lactoferrin (64) are associated with the specifics. If this proves to be the case with human specifics as well, present evidence suggests that azurophils have both antibacterial and digestive functions and therefore qualify as modified primary lysosomes, whereas specifics lack lysosomal enzymes and have so far been demonstrated to have only antibacterial activity and alkaline phosphatase. In fact, their contents and their exact role in phagocytosis remain largely unknown.

#### SUMMARY

Neutrophilic leukocytes (PMN) and their precursors from normal human marrow and blood were examined by histochemical staining and by electron microscopy and cytochemistry in order to determine the origin and nature of their cytoplasmic granules. Human neutrophils contain two basic types of granules, azurophils and specifics, which differ in morphology, contents, and time of origin. Azurophils are large and may be spherical or ellipsoid, the latter with a crystalline inclusion. They are produced in the first secretory stage (promyelocyte), contain peroxidase and various lysosomal enzymes, and thus correspond to modified primary lysosomes. Specifics are smaller, may be spherical or elongated, and are formed during a later secretory stage (myelocyte). They lack lysosomal enzymes and contain alkaline phosphatase and basic protein; their contents remain largely undetermined. Specifics outnumber

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the acid phosphatase activity in their C fraction (which is supposed to contain tertiary granules [5]) is localized in at least three different organelles: Golgi cisternae, phagocytic vacuoles, and small pleomorphic granules. The Golgi cisternae appear to be derived largely from PMN, but the latter two elements resemble phagocytic vacuoles and azurophil granules (62), respectively, of monocytes which are present in small numbers (~2%) in the starting material (peritoneal exudates).



azurophils in the mature PMN because of reduction in numbers of azurophils per cell by cell division in the myelocyte stage. The findings indicate that the situation is basically the same as described previously in the rabbit, insofar as the origin, enzymic activity, and persistence in the mature cell of the two types (azurophil and specific) of granules are concerned. The main difference between PMN of the two species is in the morphology (size, shape, and density) of the granules, especially the azurophils.

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