

ORIGIN OF GRANULES IN POLYMORPHONUCLEAR LEUKOCYTES

Two Types Derived from Opposite Faces of the Golgi Complex in Developing Granulocytes

DOROTHY FORD BAINTON and MARILYN G. FARQUHAR

From the Department of Pathology, University of California School of Medicine, San Francisco

ABSTRACT

The origin, nature, and distribution of polymorphonuclear leukocyte (PMN) granules were investigated by examining developing granulocytes from normal rabbit bone marrow which had been fixed in glutaraldehyde and postfixed in OsO_4 . Two distinct types of granules, azurophil and specific, were distinguished on the basis of their differences in size, density, and time and mode of origin. Both types are produced by the Golgi complex, but they are formed at different stages of maturation and originate from different faces of the Golgi complex. Azurophil granules are larger ($\sim 800 \text{ m}\mu$) and more dense. They are formed only during the progranulocyte stage and arise from the proximal or concave face of the Golgi complex by budding and subsequent aggregation of vacuoles with a dense core. Smaller ($\sim 500 \text{ m}\mu$), less dense specific granules are formed during the myelocyte stage; they arise from the distal or convex face of the Golgi complex by pinching-off and confluence of vesicles which have a finely granular content. Only azurophil granules are found in progranulocytes, but in mature PMN relatively few (10 to 20%) azurophils are seen and most (80 to 90%) of the granules present are of the specific type. The results indicate that inversion of the azurophil/specific granule ratio occurs during the myelocyte stage and is due to: (a) reduction of azurophil granules by multiple mitoses; (b) lack of new azurophil granule formation after the progranulocyte stage; and (c) continuing specific granule production. The findings demonstrate the existence of two distinct granule types in normal rabbit PMN and their separate origins from the Golgi complex. The implications of the observations are discussed in relationship to previous morphological and cytochemical studies on PMN granules and to such questions as the source of primary lysosomes and the concept of polarity within the Golgi complex.

INTRODUCTION

The granules of polymorphonuclear leukocytes (PMN) are primary lysosomes (1, 2) which store hydrolytic enzymes and antibacterial agents (3) until needed to digest phagocytized particles. When particulate matter such as a microorganism is engulfed by a PMN, the enzymes contained in

the granules are discharged into the phagocytic vacuole (4) by fusion of the granule with the vacuole (5, 6). Thus, both the nature and fate of PMN granules are clear. Their mode of origin, however, has not been definitely established; moreover, the question as to whether or not hetero-

geneity exists within the granule population has not been resolved (cf. references 7-9).

Previous electron microscope studies of developing granulocytes from bone marrow (9-16), in which the granules originate, have failed to provide conclusive evidence on how PMN granules are formed. These and similar morphological studies on mature PMN from peripheral blood (15, 17-19) or inflammatory exudates (5, 6, 20, 21) have also varied widely in their descriptions of the number and appearance of granule types. Attempts to separate granule fractions prepared from exudate PMN have so far been unsuccessful in the rabbit (8), the species on which most such studies have been carried out, although a partial separation has recently been reported in the guinea pig (22).

In order to investigate these problems, we have undertaken systematic electron microscope and cytochemical studies on granulocytes from bone marrow, peritoneal exudates, and peripheral blood in a number of species. In this paper we report our morphologic observations on developing granulocytes from bone marrow of normal rabbits. For this study we have taken advantage of the improved preservation, particularly of the granules, afforded by prolonged glutaraldehyde fixation. The findings provide some new information on the origin and nature of PMN granules and also contribute evidence on the source of primary lysosomes.

MATERIALS AND METHODS

Observations were made on leukocytes obtained from the bone marrow of 9 normal adult, New Zealand rabbits. During the course of this investigation, various methods of collecting, handling, and fixing PMN were tried. Fixation with OsO_4 (regardless of variations in concentration, buffer, additives, etc.) yielded preparations of widely varying quality in which granule preservation was generally poor. By far the most satisfactory preservation of PMN structure was obtained by utilizing primary glutaraldehyde fixation followed by postfixation in OsO_4 according to the procedure described.

Preparation and Fixation of Bone Marrow Cells

Femoral marrow, obtained from animals under deep ether anesthesia, was placed immediately in chilled fixative; it was then teased into small pieces, filtered through coarse surgical gauze to remove fat globules, and finally transferred to a vial containing

fresh fixative. The cells usually aggregated into clumps and remained partially suspended. Fixation was carried out at 4° C for 16 to 24 hr in 1.5% glutaraldehyde¹ buffered at pH 7.4 with 0.067 M sodium cacodylate (24) containing 1% sucrose. The osmolality of the final fixative, determined with an Advanced Osmometer (Model 63-31), was 290 milliosmols.

After fixation, the cell suspension was poured into a 15 ml conical tube and centrifuged at 3000 RPM for 5 min. The glutaraldehyde was carefully pipetted off and replaced with cold 1% OsO_4 buffered at pH 7.4 with 0.05 M acetate-Veronal containing 5% sucrose, and the tubes were placed in the refrigerator for 1 hr. The cell pack, which becomes firm after osmification, was subsequently cut with a scalpel into small (1 mm³) blocks which could be conveniently handled during further processing. Some specimens were dehydrated directly after osmification. The majority were stained in block for 1 hr at room temperature with 0.5% uranyl acetate in Michaelis buffer (final pH 5.3), prior to dehydration, to enhance membrane contrast (cf. reference 25). Others were dehydrated in acetone and stained in block for 5 min with 0.5% KMnO_4 in absolute acetone (26). Araldite was used for embedding (27).

Preparation of Sections for Electron Microscopy

Sections were prepared on a Sorvall MT-2 microtome equipped with a diamond knife; they were stained either with lead alone (28) or doubly stained with lead preceded by uranyl acetate (29).

Micrographs were taken at original magnifications of 3000 to 40,000 with a Siemens Elmiskop I, operating at 80 kv, with a double condenser and a 50 μ objective aperture.

Processing of Leukocytes for Light Microscopy

Two types of specimens were prepared for correlative light microscope studies: (a) Coverslip smears were air-dried, fixed briefly with methyl alcohol, and stained for 5 min with Wright's stain buffered at pH 6.4 (30); (b) Thick sections (1 to 2 μ), cut from Araldite-embedded blocks, were affixed by low heat to glass slides, and stained with azure II and methylene blue (31). Cell measurements were obtained directly from Araldite sections, which provided large numbers of leukocytes for sampling purposes and had the advantage of showing more detail than could be seen in smears.

¹ The glutaraldehyde stock solution, obtained from Union Carbide Corp., was purified by distillation prior to use, as described in reference 23.

RESULTS

This study was concerned primarily with the structure, distribution, and mode of formation of granules in developing PMN or heterophil² leukocytes of normal rabbit bone marrow. The detailed description of cells in each of the developmental stages is prefaced by a general description of the maturation process and associated nomenclature.

General Description of Granulocyte Maturation

Granulocytes develop from less differentiated elements by a process of progressive maturation. Since the classical work of Pappenheim (33), there has been general agreement on the over-all nature of the process,³ although some conflict has arisen in regard to terminology. The terminology used here is that recommended by the Committee for Clarification of Nomenclature of Diseases of the Blood and Blood-Forming Organs (36).

Six developmental stages are recognized in smears of bone marrow stained with Wright's stain (cf. reference 37). The most immature cell of the series is the *myeloblast*, recognized by its large oval nucleus and strongly basophilic cytoplasm, lacking granules. Upon the acquisition of metachromatic, red-to-purple-staining azurophil granules, the developing cell is designated a *progranulocyte*. It becomes a *myelocyte* with the appearance of so called specific granules which can be of the eosinophilic, basophilic, or heterophilic variety in a given cell. When the nucleus appears distinctly indented and cell size is reduced, the cell is designated a *metamyelocyte*; with marked nuclear indentation, it is called a *band cell*; and with nuclear segmentation into distinct lobes, a *mature granulocyte*.

² The general term, heterophil, introduced by Kyes (32), is used interchangeably with PMN leukocyte to denote the cell type analogous to the neutrophil in man. Heterophil granules differ in size and staining reaction according to species. In the rabbit and guinea pig, they have a predilection for acid stains and are sometimes referred to as "pseudo-eosinophilic."

³ The accepted sequence of events, originally proposed on the basis of morphologic observations (33), has been substantiated by numerous investigators using a variety of approaches, including supravital (34), histochemical (34), and isotopic (cf. reference 35) techniques.

cyte. Classical light microscope studies have established that, in addition to the appearance of cytoplasmic granules, the maturation process is characterized by a decrease in cell size, development of nuclear lobulation, and a decrease in cytoplasmic basophilia and in the number of mitochondria (38).

Under normal conditions, the total maturational process of PMN takes about 4 days (39), during which the cells undergo 4 to 5 mitotic divisions (40, 41). Mitoses occur only during the first three stages; most take place during the myelocyte stage. Metamyelocytes, band cells, and mature PMN apparently do not divide (cf. references 35, 40, 41).

It is usually assumed that a developing granulocyte is multipotential (36, 42) (i.e. can develop into an eosinophil, basophil, or heterophil (PMN)) through the progranulocyte stage. Our own observations indicate, however, that progranulocytes are already differentiated into distinct subtypes (see below).

Stages of PMN Maturation

In the detailed description of the stages of PMN maturation that follows, emphasis is placed on the granules and on the Golgi complex from which they are formed.

MYELOBLAST

The myeloblast (cf. Fig. 12) is a relatively small ($\sim 10 \mu$), rounded, or slightly elongated cell, characterized by a large ovoid nucleus and sparse cytoplasm lacking granules. The general organization of the cytoplasm is that typical of many undifferentiated cells: there are abundant free ribosomes, only a few isolated cisternae of the rough-surfaced endoplasmic reticulum, a small centrosphere or Golgi region located near the nucleus, and numerous mitochondria which are frequently aggregated at the cytoplasmic pole opposite the Golgi zone. The nucleus usually contains several nucleoli and has a relatively light background density, except peripherally where the chromatin is concentrated along the nuclear membrane.

GOLGI COMPLEX: The Golgi complex consists of a few stacks of 3 to 4 relatively empty-appearing, flattened cisternae. In contrast to later stages, centrioles are not seen and there is no evidence of secretory activity. The first indication

that a myeloblast is beginning to differentiate into a progranulocyte is the appearance in the Golgi zone of vacuoles with a dense core, which represent early stages in the formation of azurophil granules (see below).

PROGRANULOCYTE

The progranulocyte nucleus is less regularly round, an indentation being frequently seen near the centrosphere region (Figs. 1 and 2). During this stage the cytoplasm becomes increasingly voluminous owing to an increase in the amount of rough-surfaced endoplasmic reticulum, enlargement of the Golgi complex, and especially to the accumulation of azurophil granules. The latter, which have a maximal diameter of ~ 800 $m\mu$ and are uniformly dense (Figs. 1 and 2), can be clearly distinguished from granules of eosinophilic and basophilic progranulocytes.⁴ Hence

⁴ Granules of the eosinophilic progranulocyte are larger (~ 1200 $m\mu$) and more regularly round;

their presence serves to identify the developing cell as a prospective PMN. Since this stage extends from the beginning of azurophil granule accumulation until the appearance of specific granules, it follows that cell size and granule content differ widely depending on whether the cell is an early or late progranulocyte. Early cells are smaller and contain fewer granules. Fully developed, more heavily granulated progranulocytes measure up to 16μ , and are the largest cells of the PMN series; they have a large centrosphere region (Figs. 2 and 3) which characteristically contains many profiles of immature or forming granules. The remaining cytoplasm, exclusive of the Golgi zone, contains abundant free ribosomes and rough-surfaced endoplasmic reticulum. The latter consists primarily of single, relatively long, flattened cisternae disposed

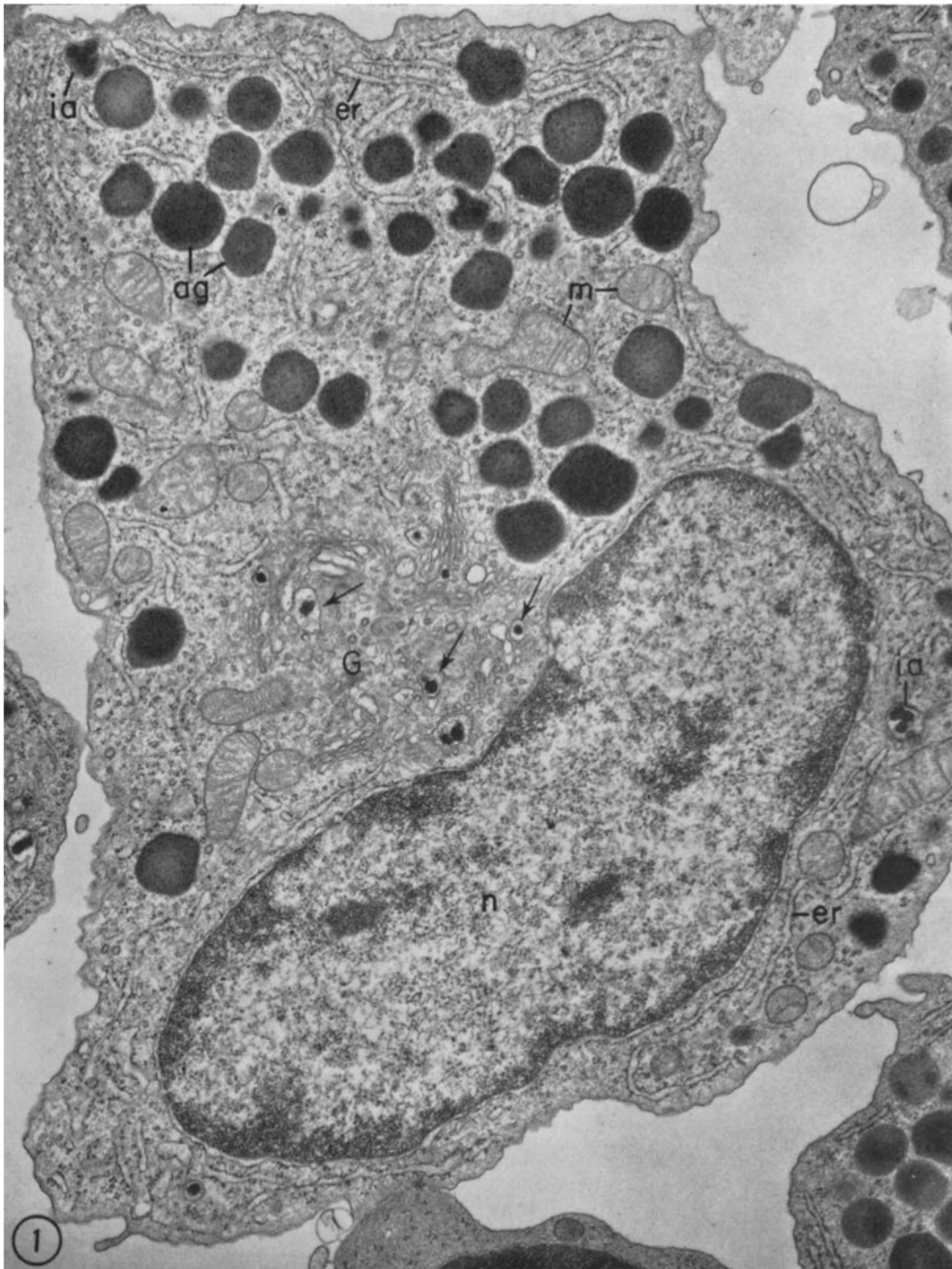
those of the basophilic progranulocyte are smaller (~ 700 $m\mu$) and the content of some of the immature forms shows a regular crystalline lattice structure with a repeating period of ~ 1500 A.

Abbreviations for Figures

<i>G</i> , Golgi complex	<i>mt</i> , microtubule
<i>ag</i> , azurophil granule	<i>n</i> , nucleus
<i>ce</i> , centriole	<i>oc</i> , outer Golgi cisterna
<i>er</i> , rough-surfaced endoplasmic reticulum	<i>p</i> , plaque along outer membrane leaflet
<i>g</i> , glycogen	<i>pn</i> , perinuclear cisterna
<i>ia</i> , immature azurophil granule	<i>r</i> , ribosome
<i>is</i> , immature specific granule	<i>s</i> , satellite of centriole
<i>ic</i> , inner Golgi cisterna	<i>sg</i> , specific granule
<i>m</i> , mitochondria	<i>v</i> , dense-cored vacuole
<i>me</i> , membrane	<i>ve</i> , vesicle

Figs. 1 to 10 are electron micrographs of developing granulocytes from normal rabbit bone marrow fixed overnight in glutaraldehyde, postfixed in OsO_4 , stained in block in buffered uranyl acetate, dehydrated in ethanol, and embedded in Araldite. Fig. 11 is from similar tissue fixed overnight in glutaraldehyde, postfixed in OsO_4 , dehydrated in acetone, and stained in block with $KMnO_4$.

FIGURE 1 A large PMN progranulocyte, showing its content of azurophil granules (*ag*), abundant free ribosomes, flattened, rough-surfaced cisternae of the endoplasmic reticulum (*er*), and prominent supranuclear Golgi complex (*G*). Mature azurophil granules are large (maximal diameter = ~ 800 $m\mu$), vary somewhat in shape and have a uniform content of high density; 40 to 50 such granules are present in the field, concentrated mainly at the pole opposite the nucleus (*n*). Two smaller, less condensed immature granules (*ia*) are seen on the upper left and far right. The Golgi region is cut in grazing section and hence does not include the centrioles; associated with it are several vacuoles (arrows) with a dense core and lighter periphery, which, as shown in detail in subsequent figures, represent early stages in the formation of azurophil granules. $\times 17,000$.



at random (Figs. 1 and 2). Mitotic figures are occasionally encountered.

GOLGI COMPLEX: The centrosphere region (Figs. 2 and 3) occupies a large, roughly spherical area of the cytoplasm near one pole of the nucleus. It is circumscribed by stacks of 4 to 9 smooth-surfaced, slightly curved cisternae oriented around two more-or-less centrally located centrioles. Clusters of ($\sim 600 \text{ \AA}$) smooth-surfaced vesicles occur in close association with the ends of the cisternae (Fig. 3), and small numbers of so called "coated" vesicles (cf. references 43, 44) are seen scattered throughout the Golgi zone. The core of cytoplasm outlined by the cisternae also contains a few ribosomes and vesicular profiles of rough-surfaced endoplasmic reticulum, abundant microtubules which converge from all directions onto the centriolar satellites, and numerous dense-cored vacuoles, described in detail below. Mitochondria and mature granules are effectively excluded from this region (Fig. 2), a feature noted previously by Policard et al. (45).

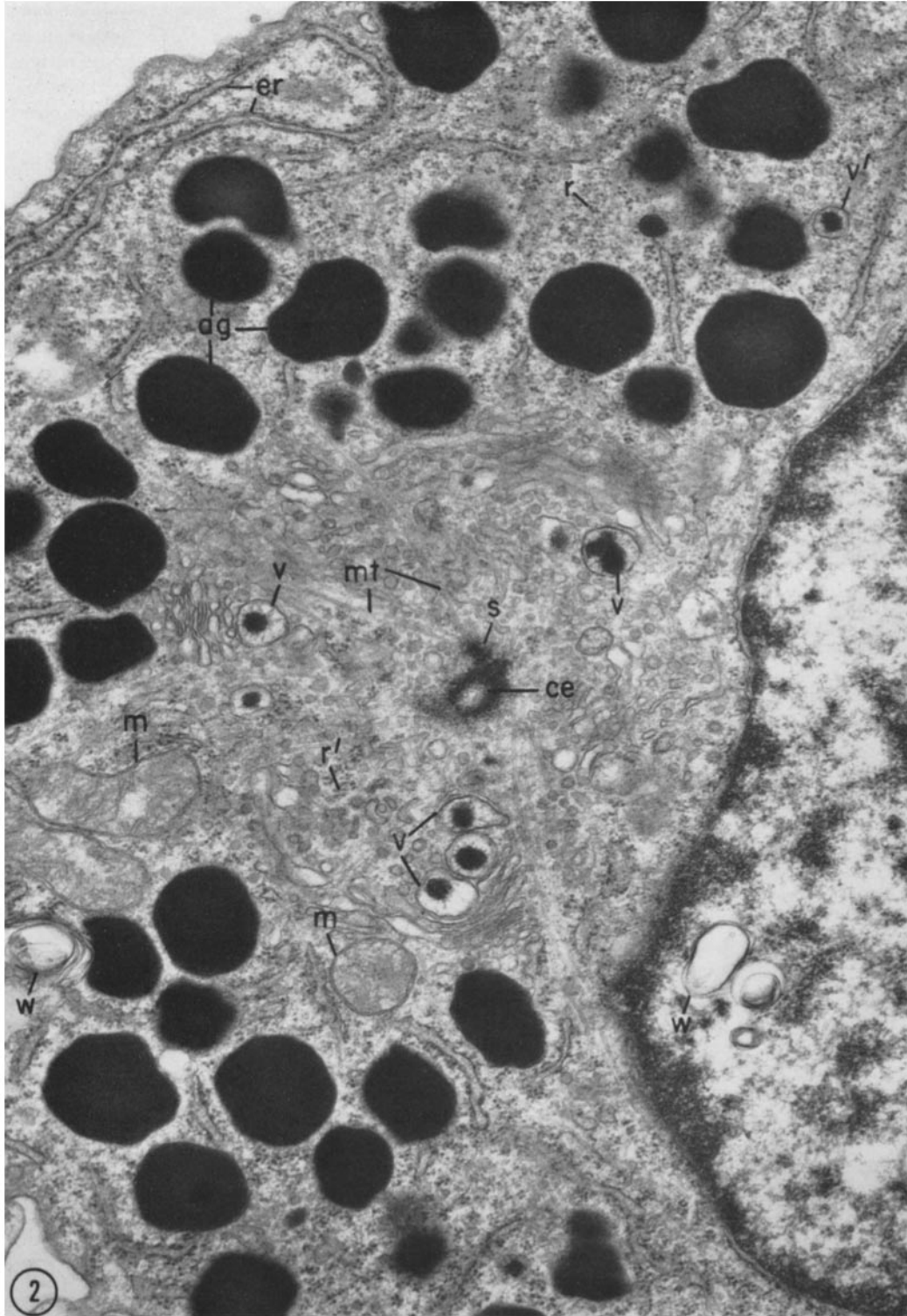
Because of the characteristic circular orientation of successive cisternae around the centriole, the Golgi complex can be divided topographically into a proximal or concave surface, which faces the centriole, and a distal or convex surface facing away from it. All the cisternae have a content of lower density than the surrounding cytoplasmic matrix and show irregular dilations along their course, but the proximal or inner ones tend to be relatively more dilated and the distal or outer ones more flattened (Figs. 2, 3, and 5).

As already mentioned, a characteristic and even diagnostic feature of the Golgi zone of the pro-

granulocyte is the presence of numerous vacuoles, 200 to 400 $m\mu$ in diameter, each with a dense central core (100 to 150 $m\mu$) and a lighter periphery composed of finely flocculent material of low density (Figs. 2 to 5). Sometimes, the periphery appears partially or completely "empty," a portion of its content presumably having been extracted during the preparative procedures. These biphasic vacuoles can be found anywhere in the Golgi zone and even at some distance from it; they are particularly frequent along the proximal face of the Golgi complex (Figs. 2, 3, and 5). Sometimes the membrane of one of these vacuoles is continuous with that of a Golgi cisterna. Continuity is frequently observed with the inner one or two cisternae (Figs. 3 and 4) but has not been seen in association with the remaining Golgi cisternae.⁵ It appears, therefore, that such vacuoles are formed by condensation of dense material within the proximal Golgi cisternae from which they subsequently pinch off. In addition to those with a single dense core, larger (250 to 900 $m\mu$) vacuoles, containing from 2 to as many as 20 cores, frequently occur in the Golgi region (Fig. 5) or elsewhere in the cytoplasm (Figs. 1 and 6). The diameter of the vacuoles increases with the number

⁵ It should be emphasized that conclusions concerning the relationship between forming granules and Golgi cisternae are based on the analysis of sections which cut through the center of the Golgi complex and include one or both centrioles (e.g. Figs. 2 and 3). When only part of the Golgi complex is included and centrioles are not present in the plane of section (e.g. Fig. 1), these relationships are not clear.

FIGURE 2 Portion of a PMN progranulocyte including the centrosphere region. In the peripheral cytoplasm, there are abundant large azurophil granules (*ag*), ribosomes (*r*), rough-surfaced cisternae of the endoplasmic reticulum (*er*), and a few mitochondria (*m*). All these elements are effectively excluded from the centrosphere region or are present in very small numbers (*r'*). The Golgi complex consists of stacks of 5 to 8, slightly curved cisternae which encircle a centriole (*ce*). Microtubules (*mt*) converge from several directions onto the centriolar satellites, one of which is indicated at *s*. A number of $\sim 240 m\mu$ vacuoles (*v*), with a dense, spherical $\sim 140 m\mu$ core and lighter periphery, are present along the proximal or concave surface of the Golgi complex in close association with the inner cisternae. One such vacuole (*v'*) is also present above and to the right in the peripheral cytoplasm. These vacuoles, which represent early or forming azurophil granules, are illustrated to better advantage in Figs. 3 to 5. Note the irregular contours and high density of the mature azurophil granules (*ag*). The membranous whorls (*w*), present on the right in the nucleus and to the left in the cytoplasm, are thought to be artifacts produced by glutaraldehyde fixation. $\times 27,000$.



of cores they contain, which suggests that small vacuoles fuse to form larger aggregates. Some of the larger ones have a denser, finely granular periphery (Figs. 5 and 6). Occasionally, a dense plaque of fuzzy, segmented material is seen along a portion of the cytoplasmic surface of the vacuole membrane (Fig. 5); such plaques resemble those seen previously in association with membranes of multivesicular bodies (46, 47) and secretion granules (48). Vacuoles with more than 1 core have not been seen in direct continuity with Golgi cisternae; hence aggregation must take place after separation of single units from Golgi cisternae. The biphasic vacuoles apparently represent the basic subunits from which, by aggregation and subsequent condensation, mature azurophil granules are formed.

GRANULES: All granules of the progranulocyte represent mature or immature forms of a single population of granules, which by definition are azurophil. In sections cut parallel to the long axis of the cell, up to 60 mature granules, as well as a number of immature forms (Fig. 1), can be counted in a given cell (see Table I). Mature azurophil granules appear ovoid, irregularly spherical, or slightly angular (Fig. 2). They have a finely granular, homogeneous content of high density⁶ (Figs. 2 and 6) and are limited by a typical triple-layered unit membrane which measures ~ 70 A in thickness; this membrane appears highly asymmetrical in ordinary preparations fixed in glutaraldehyde-OsO₄ but is nearly symmetrical after staining in block with uranyl acetate or KMnO₄ (Fig. 6). In this respect it is similar to Golgi membranes (Fig. 4) from which it is presumably derived.

⁶ Azurophil granules have an inherent electron opacity in unstained specimens fixed in glutaraldehyde alone.

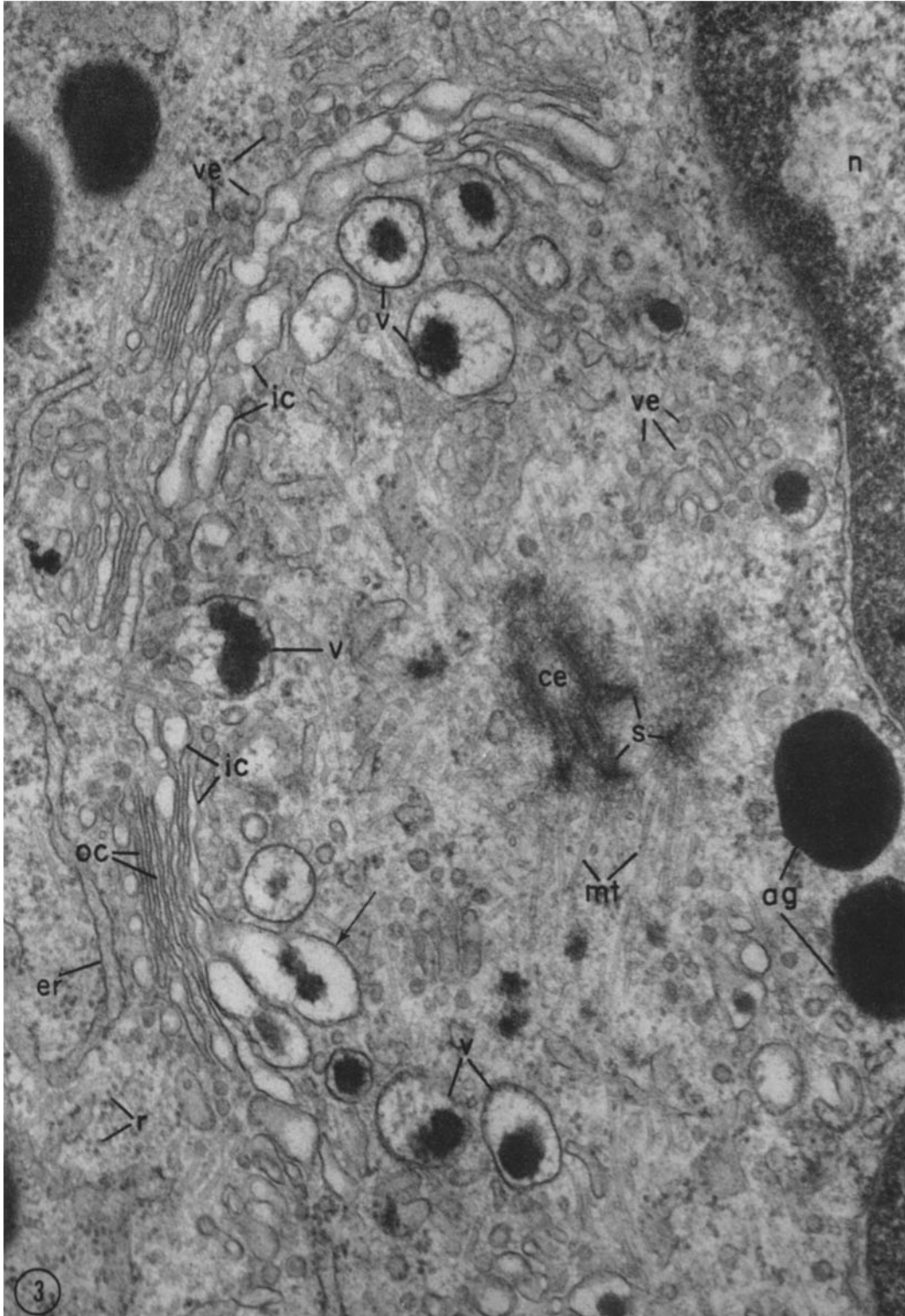
The appearance of immature granules varies with their state of aggregation and condensation. The formation of azurophil granules appears to proceed in four main steps: (a) condensation of secretory material takes place within the inner Golgi cisternae; (b) the condensed material buds off the Golgi cisterna to form a "dense-cored vacuole"; (c) several vacuoles fuse to form a multicored aggregate; and finally, (d) the content of the aggregate undergoes progressive condensation. Steps (a) through (c) occur primarily or exclusively in the Golgi region and are described in detail above. Step (d) occurs in the peripheral cytoplasm and consists, as illustrated in Fig. 6, of a gradual condensation of both the core and periphery of the granule. A large nucleoid with angular contours is formed during the condensation of the core material (Fig. 6, inset). The diameter (~ 900 m μ) of this immature form is greater than that of the more condensed, mature azurophil granule.

MYELOCYTE

The myelocyte (Fig. 7) is distinguished from the progranulocyte by the more variable shape of its nucleus, smaller cell size (8 to 12 μ), and particularly by its content of two types of granules; in addition to azurophil granules, there are variable numbers of smaller (~ 500 m μ), less dense, specific granules. The nucleus appears more distinctly indented, and its chromatin is more condensed than that of the progranulocyte. Mitotic figures are occasionally seen. In the cytoplasm, ribosomes are numerous, but there is a marked decrease in the amount of rough-surfaced endoplasmic reticulum and mitochondria compared to the progranulocyte. The centrosphere region, usually located at the nuclear "hof," is also smaller (Fig. 7).

GOLGI COMPLEX: The general organization

FIGURE 3 Golgi region in another PMN progranulocyte shown at higher magnification. The characteristic arrangement of the cisternae around the centrioles (one of which is seen at *ce*) and the concentration of dense-cored vacuoles (*v*) along the concave or proximal face of the Golgi complex are well shown. The outer Golgi cisternae (*oc*) are relatively more flattened and the inner ones (*ic*) more dilated. Clusters of smooth-surfaced vesicles (*ve*) occur primarily near the ends of the cisternae. Most of the dense-cored vacuoles have peripheral content of finely flocculent material and are closely associated with the inner cisternae; the membrane of one (arrow) appears to be continuous with the innermost cisterna, suggesting that these vacuoles are formed by condensation of dense material within the inner cisterna and subsequently pinch off (see also Fig. 4). Several mature azurophil granules (*ag*) occur in the cytoplasm outside the Golgi zone. $\times 50,000$.



of the Golgi complex (Figs. 8 and 9) is similar to that of the progranulocyte; however, the total area of cytoplasm it occupies is smaller (about half that in the progranulocyte), the cisternae are more flattened, and fewer cisternae (3 to 5) comprise the stacks. In addition, the outer cisternae have a finely granular content which is increasingly dense towards the distal face. Biphasic vacuoles of the type seen along the proximal face of the Golgi complex of the progranulocyte, and believed to represent forming azurophil granules, are not present. However, numerous small granules, with a content similar in density and texture to that of the outer Golgi cisternae, are frequently seen along the distal face of the Golgi complex (Figs. 8 and 9). In addition, bleblike expansions sometimes occur



FIGURE 4 Stack of Golgi cisternae in a PMN progranulocyte. The triple-layered structure of the Golgi membranes, which appear nearly symmetrical in this type of preparation, is visible in a number of places. A dense-cored vacuole (*v*), cut in grazing section, is marked above. A spherical dense mass (arrow), resembling the core material of dense-cored vacuoles, is present within a dilated cisterna (*ic*) along the proximal face of the Golgi complex. Images such as this suggest that dense-cored vacuoles are formed by budding from the ends of the inner one or two Golgi cisternae. $\times 100,000$.

along the outer 1 or 2 cisternae (Fig. 9). From the close association between the small granules and the outer cisternae, the similarity of their content, and the existence of bleblike cisternal expansions, it can reasonably be assumed that the small granules are formed by budding from the outer cisternae. Sometimes, a whole series of granules, ranging from 90 to 500 $m\mu$ in diameter can be seen in a single field (Fig. 9). Some have a denser central, or slightly eccentric spot. All these structures are considered to be immature specific granules. It is noteworthy that specific granules are apparently formed along the distal face of the Golgi complex whereas azurophil granules are formed along its proximal face, and that no azurophil granules are produced during the myelocyte stage.

GRANULES: Two distinct populations of granules, azurophil and specific, are seen in the myelocyte. Both the ratio and absolute numbers of the two types of granules vary widely from cell to cell (see Table I). Most of the azurophils present are fully condensed or mature (Figs. 7 and 8); sometimes those with a central dense nucleoid are found, but other immature forms, i.e. vacuoles with single or multiple dense cores, are not seen, as a rule. Like the azurophils, the mature specific granules have a distinct limiting membrane and a finely granular, homogeneous content (Fig. 10); however, they are smaller (maximal diameter = $\sim 500 m\mu$), more regularly spherical, vary more in size, and are decidedly less dense (Figs. 8 and 9) than azurophil granules. Their lower density is readily discerned in ordinary glutaraldehyde- OsO_4 preparations stained only with lead or preceded by staining in block with $KMnO_4$. It is less pronounced, however, after prolonged staining with uranyl acetate, particularly in the block. Compare, for example, the tissue shown in Fig. 7, which was stained in block with uranyl acetate, with that shown in Fig. 11, which was not. The majority of the specific granules are spherical, but rod or dumbbell forms, measuring up to 750 $m\mu$ in length (see Fig. 11), are occasionally seen.

Immature specific granules have a content similar in texture to that of the mature ones, but they are generally less dense and sometimes contain a central denser spot (Fig. 9). It appears that specific granules are formed in the same general way as azurophil granules: secretory material condenses within, and subsequently pinches off Golgi cisternae, smaller units aggregate to form larger

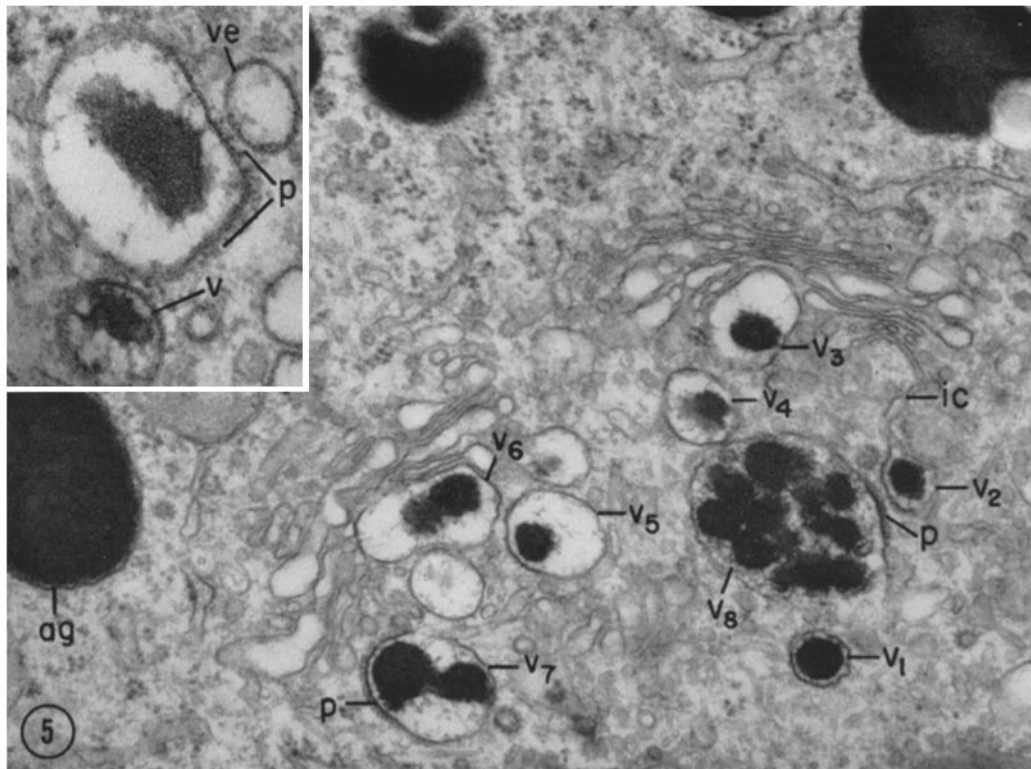


FIGURE 5 Golgi region of a PMN progranulocyte, illustrating early stages in the formation of azurophil granules. Numerous dense-cored vacuoles (v_1 to v_8) occur along the proximal face of the Golgi complex. One of these (v_2) appears to be in continuity with the innermost Golgi cisterna (*ic*). Most of the vacuoles (v_1 to v_6) have a single core; however, several (v_6 and v_7) have two, and one (v_8) has multiple (10 to 13) cores. The peripheral content of the latter, exclusive of the core material, is finely particulate and denser than that of the smaller forms. After budding from Golgi cisternae, single vacuoles apparently fuse to form larger aggregates.

Dense plaques (*p*) occur along part of the cytoplasmic surface of the limiting membranes of v_7 and v_8 . Another dense-cored vacuole, illustrated at higher magnification in the inset, shows a similar plaque (*p*) which is composed of a 200 Å fuzzy layer with repeating bands of greater density oriented perpendicular to the membrane. Also shown in the inset are a portion of another dense-cored vacuole (*v*) and a vesicle (*ve*). Fig. 5, $\times 40,000$; inset, $\times 90,000$.

TABLE I
Number of Granules Per Cell Section During Stages of PMN Maturation*

Stage	Azurophil granules			Specific granules		
	Mean	Range	% of total granules	Mean	Range	% of total granules
Progranulocyte	45	5-64	100	0		0
Myelocyte	16	2-45	37	27	2-70	63
Metamyelocyte and band cell	7	1-13	14	45	17-70	86
Mature PMN	7	2-14	14	47	23-94	86

* For each stage, 50 to 100 cell profiles were counted.

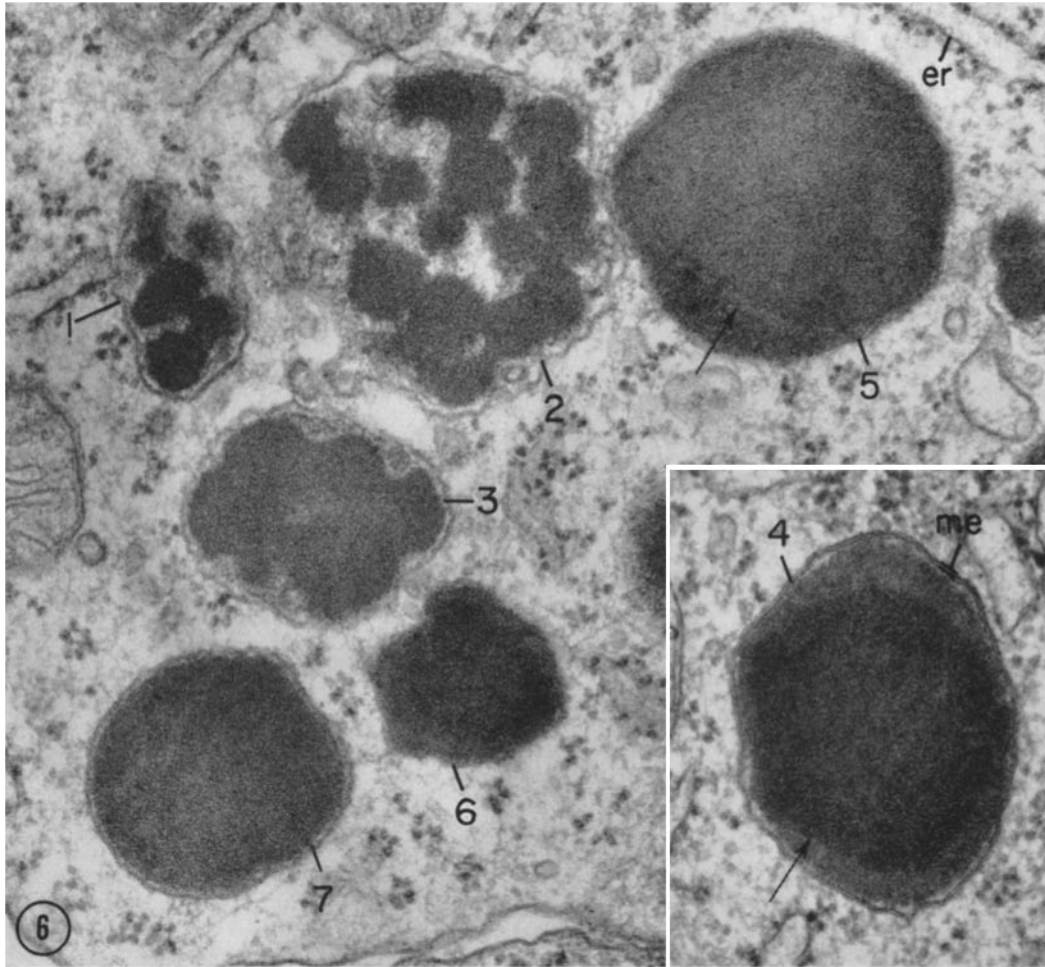


FIGURE 6 Small field from the cytoplasm of a PMN progranulocyte illustrating later stages in the formation and condensation of azurophil granules. Several multicored vacuoles (1 and 2) formed by aggregation of single-cored subunits are seen. The granule content surrounding the core material is moderately dense and finely granular. In a subsequent step, the core material apparently aggregates to become a multilobulated mass (3), which undergoes further condensation to form a more compact, nucleoid structure (arrows, 4 and 5). At the same time, the finely granular content around the core material also condenses, becoming more dense and compact until it is barely distinguishable from the core material. In fully condensed, mature granules (6 and 7) the entire content is uniformly dense and the core material is no longer recognizable. The trilaminar structure of the granule membranes (*me*) can be clearly discerned around the granule (4) shown in the inset. $\times 65,000$.

ones, and, finally, condensation of the aggregate's content takes place (Fig. 10). Admittedly, however, the aggregation and condensation process is more difficult to follow in specific granules because of the lack of a definitive marker comparable to that provided by the core material of azurophil granules.

METAMYELOCYTE AND BAND CELL

These two forms are described together because they represent arbitrary stages in the development of nuclear indentation and segmentation, and, in contrast to previous stages, they merge into one another without precise limits. Moreover, assignment of a given cell to one of these cate-

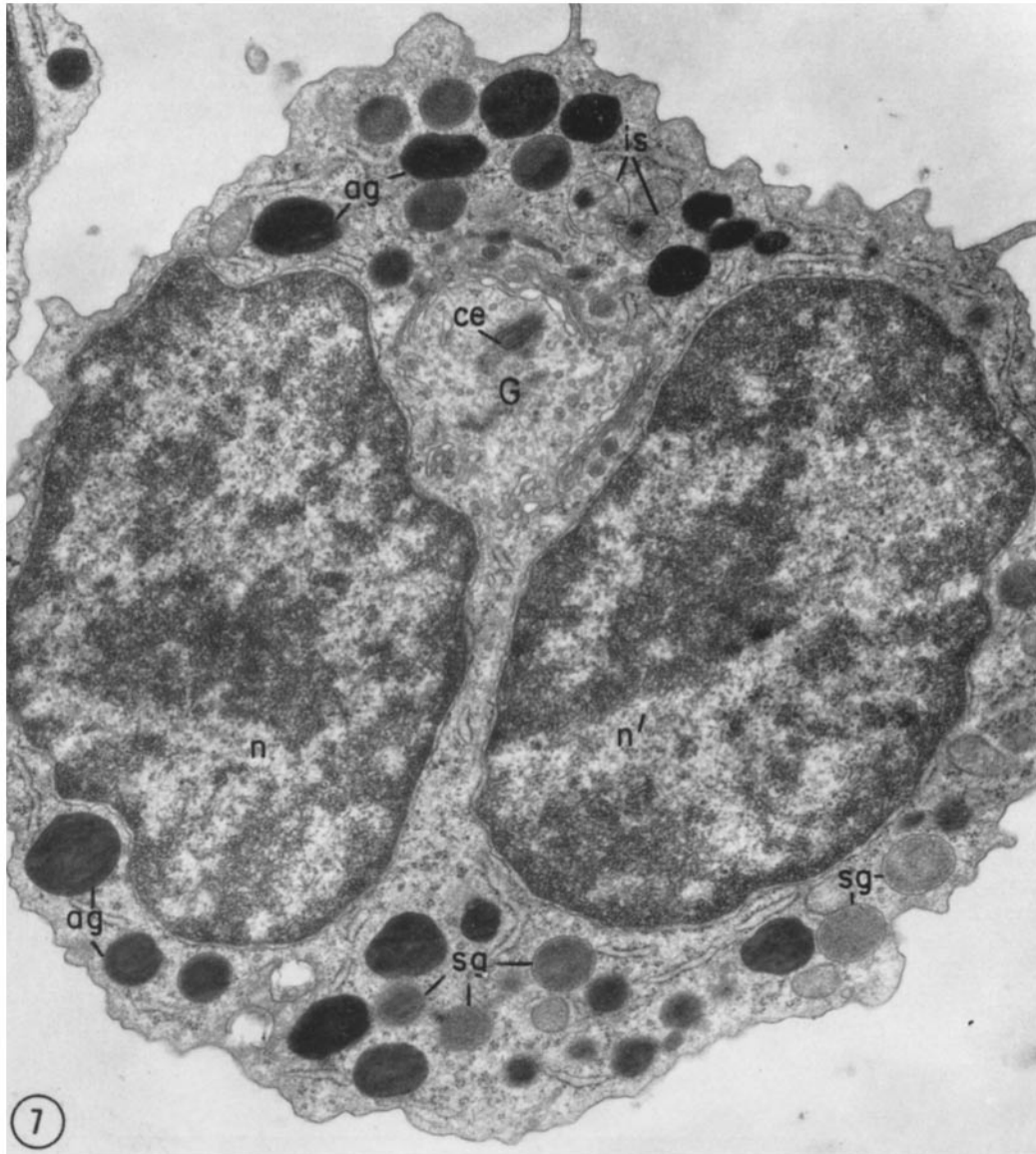


FIGURE 7 PMN myelocyte; cell size is smaller, the nucleus more indented, the Golgi region more compact, and the granule population more heterogeneous than in the progranulocyte. Here the nucleus is cut so that it appears as two separate lobes (*n* and *n'*) with the Golgi region located at the indentation. Cytoplasmic granules are of two distinct types, present in approximately equal numbers: large (maximal diameter = ~ 800 m μ), dense azurophil granules (*ag*) and smaller (maximal diameter = ~ 500 m μ), less dense, specific granules (*sg*). Several immature specific granules (*is*) are present near the Golgi zone (*G*). These are seen to better advantage at higher magnification in Fig. 8. The density difference between the two types of granules has been diminished here by prolonged (2 hr) staining in block with uranyl acetate. (Compare with Fig. 11). $\times 20,000$.

gories, which has classically been based on nuclear shape as determined in smears of whole cells, is frequently difficult since relatively few of the thin (~ 500 A) sections used for electron micros-

copy cut completely through the plane of the nucleus.

In addition to progressive nuclear indentation and reduction in cell size, these two stages are

characterized by a predominance of specific granules and a decline in cytoplasmic organelles (ribosomes, rough-surfaced endoplasmic reticulum, mitochondria, and Golgi complex); the nuclear chromatin is more condensed and nucleoli are not seen. There is no evidence of either mitotic or secretory activity (i.e., granule formation).

A "typical" metamyelocyte measures about 7.5 to 8 μ , has an indented nucleus, and a Golgi complex whose over-all size is only slightly less than that of the myelocyte. The band cell is smaller (6 to 7 μ) and has a horseshoe-shaped nucleus and a smaller Golgi complex (see Fig. 12.)

GOLGI COMPLEX: The area occupied by this organelle, which is composed of 2 to 4 relatively short, flattened cisternae, gradually diminishes during these stages. No secretory material is seen within the cisternae and no immature, aggregating granules are associated with it.

GRANULES: As in the case of the myelocyte, the granule population during these two stages is mixed, i.e., it is composed of both azurophil and specific granules. However, the distribution of the two granule types and their absolute and relative numbers present per cell are much more uniform; specific granules invariably predominate, comprising 80 to 90% of the total granule population, and relatively few azurophil granules are present (Table I). Among the latter a few (1 to 3) immature "nucleoid" forms persist in about half the cells. As in the case of the myelocyte, most of the specific granules are spherical and measure 300 to 500 $m\mu$ in diameter; however, unusually small ($\sim 100 m\mu$) forms are occasionally seen, and rod or dumbbell shapes are frequently present.

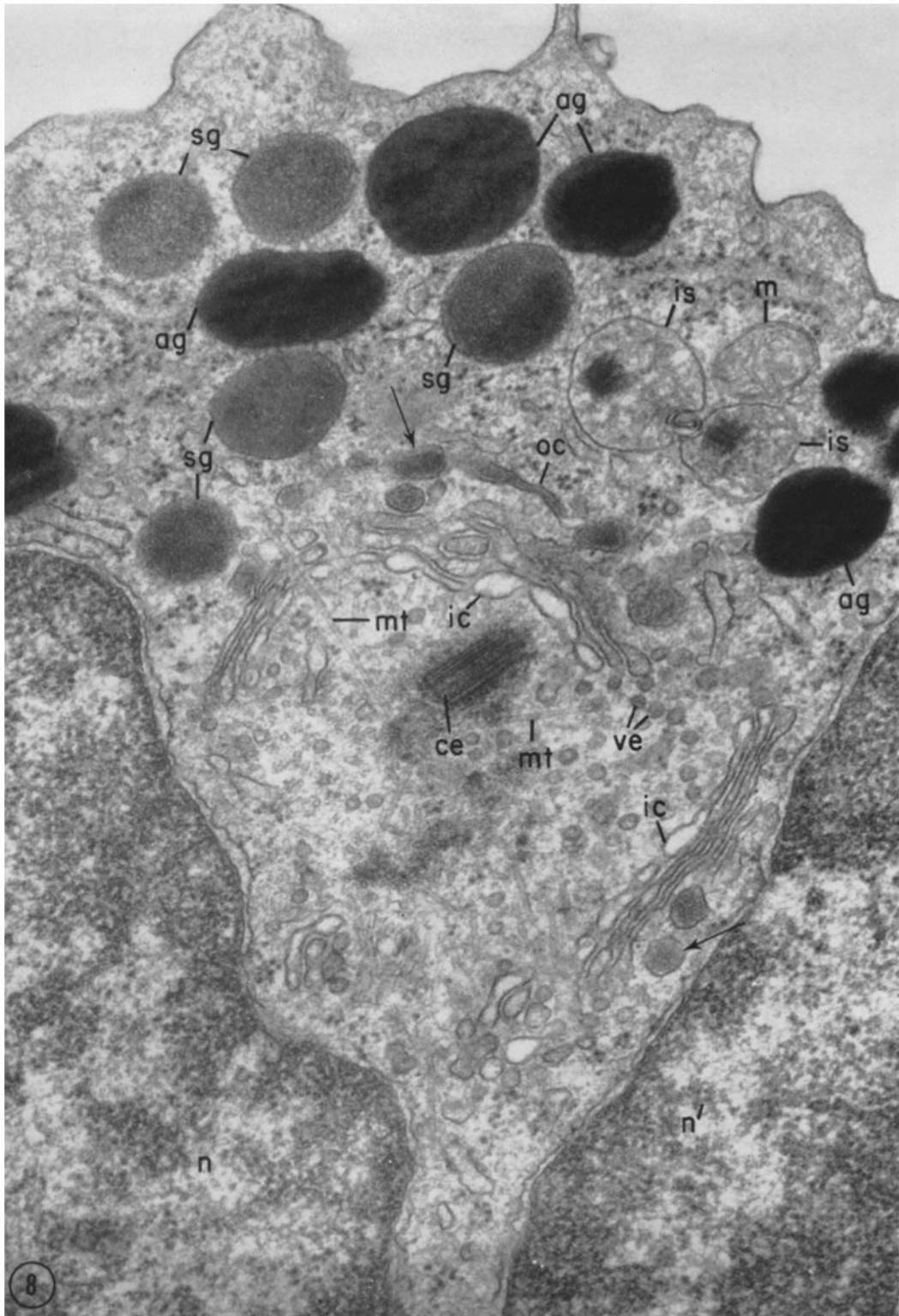
MATURE PMN

The mature cell (Fig. 11) differs from preceding stages in its smaller size (6 to 7 μ), multilobulated nucleus, and darker, more condensed cytoplasm. The latter contains particulate glycogen and granules of both types but lacks significant quantities of most other cell organelles (i.e., mitochondria, microtubules, ribosomes, and endoplasmic reticulum). The nucleus usually appears as one or more profiles of seemingly isolated lobes, for the connections between the lobes are so thin ($\sim 40 m\mu$) that they are seldom included in the plane of section. Glycogen occurs in the form of $\sim 250 A$ particles, corresponding to the "beta" particles of Drochmans (49), composed of smaller $\sim 25 A$ ("gamma") subunits. A characteristic feature of the mature cell is the presence of a dilated perinuclear cisterna which, owing to the low density of its content, stands out sharply against the dark background provided by the surrounding nucleoplasm and cytoplasm and gives the appearance of a perinuclear "halo" (Fig. 11).

GOLGI COMPLEX: This organelle is small and rudimentary. There is a further reduction in the number and average length of the cisternae comprising the stacks compared to preceding stages: only 2 to 3 short cisternae, which have lost their circular orientation around the centriole, are present. Centrioles are seen less frequently.

GRANULES: The distribution of granules and the number and ratio of the two types remain the same as during the metamyelocyte and band stages, with 80 to 90% specific and 10 to 20% azurophil (Table I). A profile of a typical mature cell contains 5 to 11 azurophil and 30 to 50 specific granules. The incidence of "nucleoid" forms of

FIGURE 8 Higher power view of the Golgi region of the myelocyte shown in Fig. 7. Note that, as in the case of the progranulocyte, the Golgi complex is spherical in shape and is oriented around the centriole (*ce*); however, it is composed of fewer (3 to 5) stacks of flatter, more elongated cisternae. The content of the inner cisterna (*ic*) is less dense than the cytoplasmic matrix whereas that of the outer cisterna (*oc*) is finely granular and denser than the surrounding cytoplasm. The remaining cisternae have a content of intermediate density. Numerous small granules (arrows) similar in density and texture to the content of the outer cisterna are seen all along the distal or convex face of the Golgi complex. Specific granules are apparently formed by budding and subsequent aggregation of small granules along the distal face of the Golgi complex. Steps in the formation of specific granules are illustrated to better advantage in Fig. 9. Several immature (*is*) and mature (*sg*) specific granules are present in the field. A few azurophil granules (*ag*), distinguished by their larger size and denser, more compact content, are also seen. $\times 50,000$.



the former and rod, dumbbell, and diminutive forms of the latter is the same as for the metamyelocyte and band stages.

Mitotic Figures

During the course of this investigation, 44 micrographs of dividing cells were collected; 29 of these mitotic figures were in myelocytes and 15 in progranulocytes. In keeping with previous work which indicates that no divisions occur after the myelocyte stage (40, 41), mitotic figures were not seen in metamyelocytes, band cells, or mature forms. A detailed description of the changes in cell organization which occur during mitosis is beyond the scope of this study. Relevant to the present investigation, however, and to the discussion that follows, is the observation that, during cell division, granules are distributed in approximately equal numbers to daughter generations.

Granule Counts

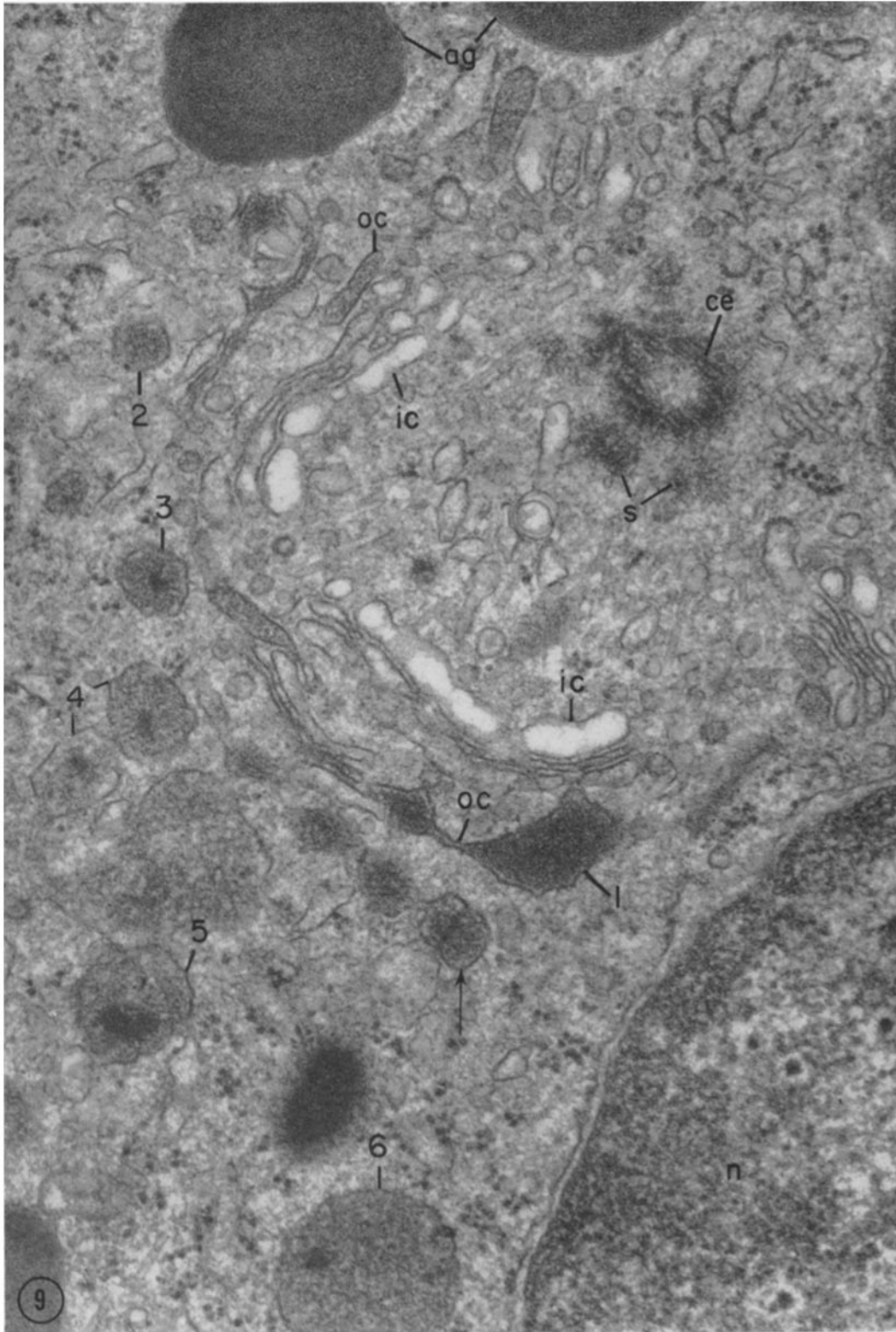
The numbers of azurophil and specific granules were counted on low magnification electron micrographs in 50 to 100 cell profiles for each developmental stage. In all cases, only cells sectioned in a plane which included most of the nucleus were counted. For the myelocyte, metamyelocyte, and band forms, only cells sectioned through the Golgi complex were counted. The criteria used for cell identification were as follows: *progranulocyte*, any cell with exclusively azurophil granules; *myelocyte*, both specific and azurophil granules present plus an active Golgi complex with one or several forming granules; *metamyelocyte* and *band forms*, both granule types present plus an inactive Golgi

complex and absence of either perinuclear halos or glycogen; *mature PMN*, more than one nuclear lobe, dense cytoplasm, and presence of perinuclear halos and glycogen. Nuclear shape is not a reliable criterion for cellular identification in sectioned material, since it is more irregular and variable than in preparations of whole cells.

The results (Table I) show that, whereas only azurophil granules are present in the progranulocyte, approximately 86% of the granules in the metamyelocyte, band, and mature forms are of the specific variety. Both the actual and relative numbers of azurophil granules decrease between the progranulocyte stage, where an average of 45 per cell profile are found, and the metamyelocyte and band stages, where only 7 are present. At the same time, the number of specific granules, which do not occur in the progranulocyte, increases up to an average of 45 per cell profile in the metamyelocyte and band cell. Inversion of the azurophil/specific granule ratio therefore occurs during the myelocyte stage, and the granule population is fixed or stable thereafter. These results are in keeping with the morphologic observations which indicate that no granules are formed after the myelocyte stage and that specific granule formation is restricted to the myelocyte.

The reduction in the number of azurophil granules that takes place during the myelocyte stage is in keeping with the finding that no new granules of this type are formed after the progranulocyte stage; yet the myelocyte is known to undergo several mitotic divisions. Since our observations indicate that granules are distributed more or

FIGURE 9 Centrosphere region of another PMN myelocyte illustrating the organization of the Golgi complex and presumed steps in the formation of specific granules. Stacks of 4 to 5 curved cisternae are oriented around the centriole (*ce*). The inner cisterna (*ic*) appears dilated and relatively empty; the remaining cisternae are flatter and show a finely granular content of increasing density towards the distal or convex face of the Golgi complex. Numerous small granules (*g* to *h*), with a content similar to that of the outer cisternae (*oc*) and believed to represent forming specific granules, are seen along the distal face of the Golgi complex. Specific granules appear to be formed in several steps (*1* to *6*): small granules bud from the outer Golgi cisternae (*1* and perhaps also at the arrow) and accumulate along the distal Golgi face (*2* and *3*); several of these (*4*) merge to form larger aggregates (*5* and *6*), which further condense to form mature granules (see Fig. 10). Note that many of the immature forms (*3* to *6*) show a denser central or slightly eccentric spot. The content of specific granules can be contrasted to that of the larger, denser azurophil granules (*ag*) above. $\times 60,000$.



less equally to daughter generations, it can reasonably be assumed that the number of azurophil granules present per cell would be reduced by approximately one-half with each mitotic division. Hence the reduction in azurophil granules from the large number (about 60 per cell profile) present in the late progranulocyte, to the small number (7 to 8) found in the metamyelocyte and following stages, could be explained by the occurrence of three consecutive divisions which decrease the azurophil granule number from 60, to an average of 30, 15, and 7.5, respectively, in successive myelocyte generations. Results of granule counts indicate a distribution of azurophil granules within the myelocyte population which is compatible with this hypothesis: myelocytes can be divided into three groups (Table II) with azurophil granule counts closely approximating those predicted. Since the number of azurophil

granules in the myelocyte is always less than that in the fully granulated progranulocyte, the data further suggests that one division, the first of the three proposed, occurs prior to the initiation of specific granule formation, i.e., in the late progranulocyte, with the remainder occurring during the myelocyte stage. The wide range in the number of specific granules present in the three groups of myelocytes is compatible with the assumption that specific granules are produced continually by each daughter generation.

Summary of Findings on PMN Maturation

The sequence of events that occurs during PMN maturation and the characteristic features of each developmental stage are summarized diagrammatically in Figure 12. With the exception of the progranulocyte, for each stage the number of granules of either type depicted corresponds to one half the mean determined by counts.

DISCUSSION

Significant Findings

Our results demonstrate that the granules of rabbit PMN, fixed in glutaraldehyde-OsO₄ and examined by electron microscopy, can be divided into two distinct types based on differences in size, density, and time and mode of origin. Mature granules of both types are membrane-limited and have a finely particulate, homogeneous content. One type is large and more dense, occurs in small numbers in mature PMN, and corresponds to the azurophil granules seen after Wright or Romanovsky-type staining. Granules of the second type are smaller and less dense, predominate in mature PMN, and correspond to the specific or pseudocoinophilic granules of Wright preparations.

Both populations of granules are produced by the Golgi complex of maturing granulocytes in bone marrow, but they are formed at different maturational stages and originate from different faces of the Golgi complex. *Azurophil granules* are formed only during the progranulocyte stage; they arise from the proximal or concave face of the Golgi complex (towards the centriole) by budding and eventual aggregation of vacuoles with a dense core. *Specific granules* develop later; they are formed by the myelocyte and arise from the distal or convex face of the Golgi complex by budding and confluence of small granules of moderate density.

The findings have a direct bearing on the gen-

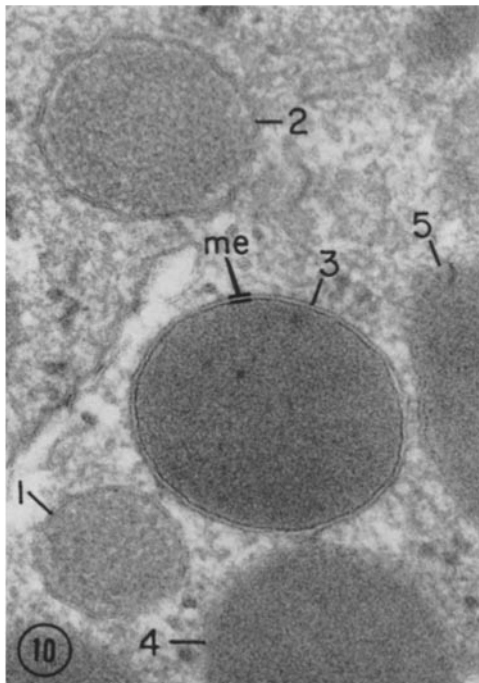


FIGURE 10 Small field from the cytoplasm of a PMN myelocyte showing two immature (1 and 2) and several mature (3 to 5) specific granules. All the granules have a uniform, finely punctate content of moderate density and are limited by a distinct unit membrane, well shown at *me*. The mature granules are larger and their content is denser and more compact than that of the immature forms. $\times 100,000$.

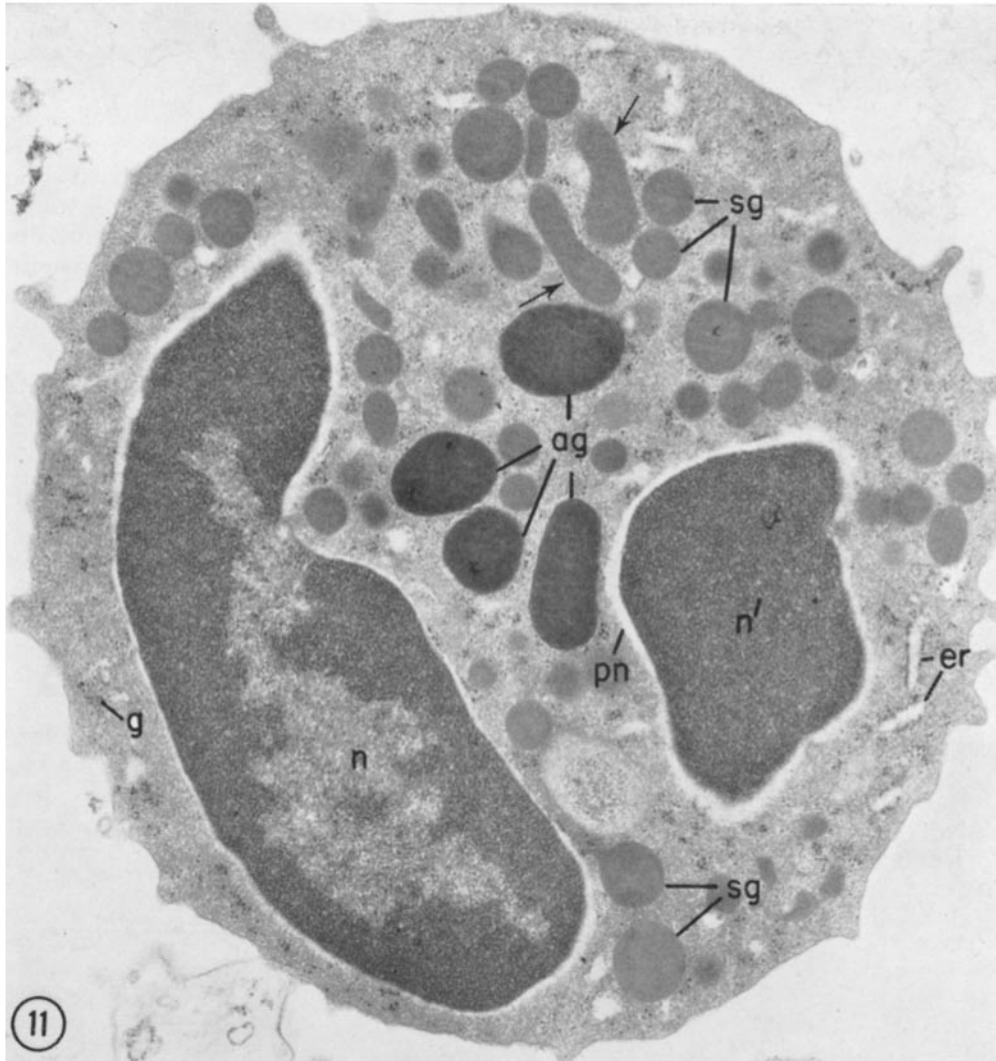


FIGURE 11 Mature PMN, showing two lobes (*n* and *n'*) of its dense, multilobulated nucleus and its rounded shape with occasional cytoplasmic projections. The cytoplasm, which is more condensed than at previous stages, contains primarily granules and glycogen particles (*g*); the only other organelles present are a few profiles of rough-surfaced endoplasmic reticulum (*er*). Each nuclear lobe is surrounded by a prominent light "halo" created by the presence of a dilated perinuclear cisterna (*pn*) which, owing to the low density of its content, stands out sharply against the dark background provided by the dense nucleoplasm and cytoplasm.

About sixty granules are present. Most (90%) are of the smaller, less dense specific type (*sg*). Note that the majority are spherical with a diameter of 300 to 500 μ , but a few rod or dumbbell forms (arrows), up to 750 μ in length, are seen. Four azurophil granules (*ag*), distinguished by their larger size and greater density, are also present between the nuclear lobes. $\times 25,000$.

eral problem related to the origin of "primary" lysosomes, i.e., hydrolase-containing granules manufactured by the cell, in contrast to "secondary" lysosomes which develop as a result of pino-

cytic, phagocytic, or autolytic events (1). Since PMN granules represent the best documented (1, 2) example available of lysosomes of the primary variety, their origin from the Golgi complex,

in keeping with evidence discussed elsewhere (7, 23), suggests that primary lysosomes in other cells may be similarly derived.

Morphological Heterogeneity Among PMN Granules

Previous electron microscope studies have called attention to considerable variation in the over-all size and shape of PMN granules as well as in the form and density of their content (3, 5, 6, 9-12, 14-18, 20, 21), but have not agreed on the number or character of the granule types present. Some of the disagreement, particularly with respect to size, can be attributed to well known species differences; however, interpretations have differed even in regard to observations on the same species. With respect to previous morphological studies on rabbit PMN, most investigators have been consistent in describing three granule types (5, 6, 20, 21), but interpretations as to their fine structural characteristics have differed.

One source of discrepancy between previous findings on rabbit PMN and our observations is the difference in fixatives employed; all previous studies except one (16) were carried out largely or exclusively on PMN fixed in OsO₄. Our experience indicates that OsO₄ does not adequately preserve PMN granules, especially the less condensed, immature forms. With this fixation, they often appear

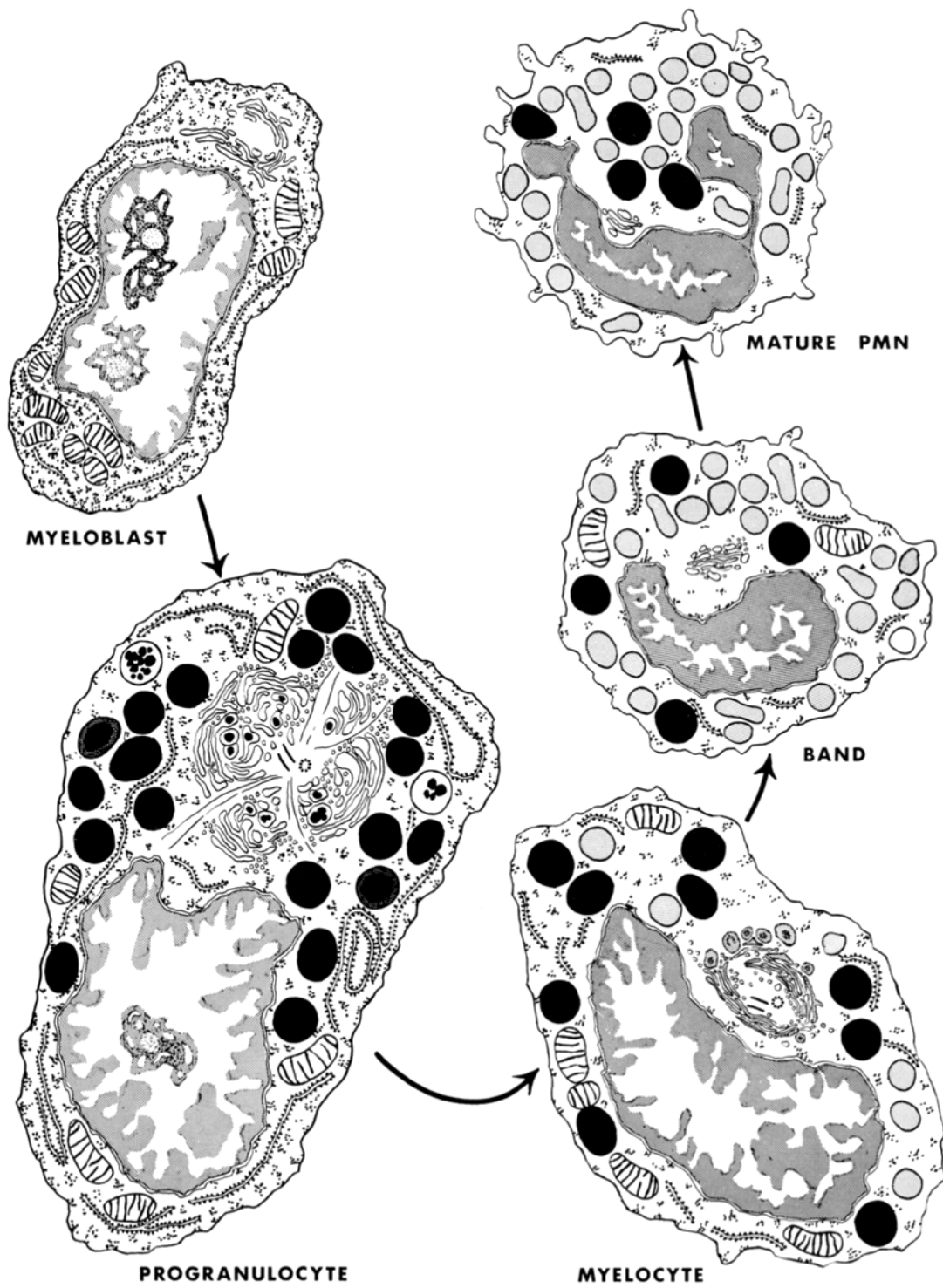
partially extracted or crenated, and their membranes are disrupted. Hence many of the so called granule types described in previous studies are believed to be created artificially, by inadequate preservation. Some difficulty in preserving azurophil granules might be anticipated in view of their high acid mucopolysaccharide content (50, 51), easy extractability (50), and the well known lack of affinity of osmium for many complex carbohydrates (52).

Another possible source of discrepancy is the fact that much of the past work (3, 5, 6, 20, 21) has been concerned with PMN obtained from exudates. According to our observations, which will be reported in a subsequent paper, such PMN have a greatly modified granule population in that there is a higher azurophil/specific granule ratio (mentioned also by Horn et al. (21)), and atypical granules are present.

Relationship Between Azurophil and Specific Granules

Large, dense granules have previously been seen in progranulocytes with the electron microscope and identified as azurophil granules (9, 10, 14, 16). Up to now, however, electron microscope studies have not resolved the long-standing controversy (53-55) regarding their relationship to specific granules. In order to explain the well known shift

FIGURE 12 Diagrammatic representation of stages in the maturation of PMN leukocytes. Granules are drawn $1\frac{1}{2} \times$ scale, and only half the average number determined by actual counts are included for a given stage. The myeloblast is a relatively undifferentiated or embryonic cell with a large oval nucleus and a cytoplasm lacking granules but containing abundant ribosomes, mitochondria, and a small, rudimentary Golgi complex. The progranulocyte and myelocyte are stages of intense secretory activity associated with the formation of azurophil and specific granules, respectively; these cells show elaborate development of cytoplasmic organelles involved in protein synthesis, segregation, and concentration (i.e., ribosomes, rough-surfaced endoplasmic reticulum, and Golgi complex). The larger azurophil granules are formed by condensation of secretory material along the proximal or concave face of the Golgi complex of the progranulocyte. Smaller, less dense specific granules are formed by a similar process occurring along the distal or convex face of the Golgi complex of the myelocyte. The metamyelocyte (not shown) and band cell are nonsecretory stages during which there is a progressive decrease in cell size associated with changes in the shape of the nucleus and a gradual diminution in most cytoplasmic organelles. The final product of the maturation process, the mature PMN, has a multi-lobulated nucleus and a cytoplasm containing primarily glycogen and granules. The progranulocyte contains azurophil granules exclusively; the myelocyte has both specific and azurophil granules in varying numbers; and the metamyelocyte, band, and mature forms contain 80 to 90% specific and only 10 to 20% azurophil granules. Inversion of the azurophil/specific granule ratio occurs during the myelocyte stage. (For explanation, see text.)



in the granule population which occurs between the progranulocytes, where azurophils occur exclusively, and mature PMN, where specific granules predominate, it has been proposed that azurophil granules degenerate (14) or are transformed into specific granules (53-55). However, no evidence was obtained in the present study to indicate that azurophil granules change into specific granules or that they degenerate or are discharged. Our results clearly indicate that azurophil and specific granules represent two separate lines which are distinct morphologically from the time of their formation. A more likely explanation for the shift in the granule population is the occurrence of multiple mitoses just prior to or during the myelocyte stage, after azurophil granule formation has ceased. Thus azurophil granules are reduced in number with each successive mitotic division, whereas specific granules, which are produced

same granule, or whether heterogeneity exists in the enzyme content of the granule population. Utilizing cytochemistry and electron microscopy, Wetzel, Horn, and Spicer (16) found acid phosphatase activity localized in azurophil granules and alkaline phosphatase in specific granules of developing granulocytes.⁷ Our own cytochemical studies, reported briefly elsewhere (57) and to be described in detail in a subsequent paper, indicate that acid phosphatase activity is present in both granule populations; however, with the Gomori technique, it is demonstrable in only the immature or less condensed forms. Hence our results indicate that there is no heterogeneity insofar as acid phosphatase is concerned, at least during the formative stages. Heterogeneity in respect to the other numerous enzymes present in PMN granules is, of course, still possible. The fact that the two types of granules are so different in their morphology,

TABLE II
Granules Per Cell Section in Myelocytes

	Azurophil granules			Specific granules			No. cells counted	Cell size μ
	Range	Mean	% of total granules	Range	Mean	% of total granules		
Group I	21-45	29	59	2-70	20	41	20	8.1
Group II	11-20	15	34	5-70	29	66	49	7.3
Group III	2-10	7.5	21	6-48	28	79	24	6.9

throughout the myelocyte stage, continue to accumulate in the cytoplasm and become preponderant. Our results suggest that there are three such divisions, one late in the progranulocyte stage and two during the myelocyte stage. Kinetic studies carried out on other species (40, 41) indicate that a total of four to five divisions take place during PMN development. This difference is best explained by the occurrence of one or two additional divisions during the myeloblast and/or progranulocyte stages.

Enzymatic Heterogeneity Among PMN Granules

In addition to lysosomal or hydrolytic enzymes, PMN granule fractions also contain considerable peroxidase (2), alkaline phosphatase (3), and lipase (56) activities, as well as antibacterial agents (3). It is not known, however, whether all these substances are segregated together in the

mode of origin, and staining properties, taken together with histochemical evidence indicating that different enzymes appear at different stages in development (55), suggest that this may well be the case.

Origin of PMN Granules: Polarity of the Golgi Complex

The origin of PMN granules has been a matter for considerable speculation for some time (58). In studies with the electron microscope, mitochondria (19), the endoplasmic reticulum (9, 12, 14), and the Golgi apparatus (11, 13, 54) have all been implicated as sites of origin at one time or another. Although a close association between developing granules and Golgi elements has been

⁷ In exudate PMN these authors found acid phosphatase activity localized in "small pleomorphic dense granules" as well as in typical azurophil granules (21).

mentioned in several previous studies of bone marrow (11, 13, 54, 55), the source of PMN granules is still generally regarded as unsettled (7-9), probably because no convincing micrographs illustrating their mode of origin have been published up to now.

The derivation of both azurophil and specific granules from the Golgi complex and the sequential steps in their formation are clearly documented by our results. The Golgi complex apparently functions as a condensing and packaging station for many protein secretory products (e.g., pancreatic enzymes (59), pituitary hormones (60), milk (61), and neurosecretory material (62, 63), all of which are normally exported from the cell. In the case of PMN, the manufactured secretory products are not exported but are utilized intracellularly, since the granule content is ultimately discharged into phagocytic vacuoles (4-6). Regarding the mechanism of PMN granule formation, the findings clearly demonstrate, in the case of azurophil granules, that large numbers of small units made in the Golgi complex come together to form the secretory granule, and that subsequent condensation of the granule content occurs not only in the Golgi region, but also in the peripheral cytoplasm.

Perhaps more interesting and novel, however, is the finding that the two granule populations originate from different faces of the Golgi complex. Here, as in the case of zooflagellates (64), plant

cells (65), amebae (66), and epithelial cells of Brunner's glands and the epididymis (67), functional as well as morphological (45) polarity of the Golgi complex has been demonstrated. In granulocytes, however, the concept of a continuous membranous system (64, 65), with an incoming or "forming" face along which membrane is added, and an outgoing or "mature" face from which secretory products emerge, is not tenable unless it is assumed that this organelle "reverses" its polarity between the progranulocyte and myelocyte stages. Assuming that the contents of the two granule populations are different, their origins from different parts of the Golgi complex imply instead functional compartmentalization within this organelle, with certain cisternae specializing in the condensation of specific secretory products. The validity of these assumptions and the exact nature of any functional specialization existing within elements of the Golgi complex remain, however, to be established by future research.

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