

SEGREGATION AND PACKAGING OF GRANULE ENZYMES IN EOSINOPHILIC LEUKOCYTES

DOROTHY FORD BAINTON and MARILYN G. FARQUHAR

From the Department of Pathology, University of California School of Medicine,
San Francisco, California 94122

ABSTRACT

During their differentiation in the bone marrow, eosinophilic leukocytes synthesize a number of enzymes and package them into secretory granules. The pathway by which three enzymes (peroxidase, acid phosphatase, and arylsulfatase) are segregated and packaged into specific granules of eosinophils was investigated by cytochemistry and electron microscopy. During the myelocyte stage, peroxidase is present within (*a*) all rough ER cisternae, including transitional elements and the perinuclear cisterna; (*b*) clusters of smooth vesicles at the periphery of the Golgi complex; (*c*) all Golgi cisternae; and (*d*) all immature and mature specific granules. At later stages, after granule formation has ceased, peroxidase is not seen in ER or Golgi elements and is demonstrable only in granules. The distribution of acid phosphatase and arylsulfatase was similar, except that the reaction was more variable and fully condensed (mature) granules were not reactive. These results are in accord with the general pathway for intracellular transport of secretory proteins demonstrated in the pancreas exocrine cell by Palade and coworkers. The findings also demonstrate (*a*) that in the eosinophil the stacked Golgi cisternae participate in the segregation of secretory proteins and (*b*) that the entire rough ER and all the Golgi cisternae are involved in the simultaneous segregation and packaging of several proteins.

INTRODUCTION

Eosinophilic leukocytes are phagocytes with an appetite for antigen-antibody precipitates (1, 2). The mature eosinophil found in the blood and tissues represents a nonsecretory, nondividing stage in the life cycle of the cell and consists mainly of stored granules. These granules have been isolated and found to contain large quantities of peroxidase and lysosomal hydrolytic enzymes (3). As is the case with all the granulocytes, protein synthesis and granule formation occur during differentiation of immature cells in the bone marrow. In this study,¹ we have utilized

cytochemistry and electron microscopy to investigate granule formation and to determine the pathways by which three enzymes—peroxidase, acid phosphatase, and arylsulfatase—are segregated and packaged into specific granules in developing eosinophils. The results are compared to those obtained on the secretory process in other systems, particularly the guinea pig pancreas (5, 6).

MATERIALS AND METHODS

Materials

Observations were made on eosinophilic leukocytes from bone marrow and blood of 40 normal young adult New Zealand rabbits and 18 normal Sprague-

¹ A preliminary report of the findings was published previously (4).

Dawley rats, 90 days of age. In addition, observations were made on bone marrow from several rabbits and rats sacrificed 17 days after intragastric administration (7) of freshly harvested larvae of *Trichinella spiralis*, which induces a severe eosinophilia (8).

Substrates and catalase (Stock No. C-10) were obtained from Sigma Chemical Company, St. Louis, and 3-amino-1,2,4-triazole from Mann Research Laboratory, New York.

Methods

COLLECTION AND FIXATION OF BONE MARROW CELLS: Cells were fixed in 1.5% distilled glutaraldehyde in 0.1 M sodium cacodylate-HCl buffer (pH 7.4) with 1% sucrose at 4° or 25°C for 10 min–16 hr. The cell suspension was subsequently either (a) processed directly, or (b) washed in cacodylate-HCl buffer with 7% sucrose and incubated in the enzyme media described below before further processing. Detailed methods for collecting and handling bone marrow cells were described previously (9, 10).

PEROXIDASE: Cells were incubated for 30 sec–1 hr at 25°C in Graham and Karnovsky's medium (11) containing 5 mg of 3,3'-diaminobenzidine tetrahydrochloride (DAB) in 10 ml of 0.05 M Tris-HCl buffer, pH 7.6, to which 0.01% H₂O₂ was added. In addition, a few incubations were also carried out at pH 6 and pH 8.6. Controls consisted of incubations in which (a) DAB or H₂O₂ was omitted, or (b) 0.01 M KCN, sodium azide, 3-amino-1,2,4-triazole, or catalase was added to the medium, prior to the addition of H₂O₂; or (c) cells were washed in three changes of buffer after incubation in DAB medium and then incubated for 5 min in 0.003 M potassium ferricyanide. In addition, some incubations were carried out for 4 hr at 37°C in a modified medium, at pH 8.6, with 10 mg of DAB and without added H₂O₂ (12, 13).

ACID PHOSPHATASE: Cells were incubated in a modified (14) Gomori medium with β -glycerophosphate (Sigma, Grade I) and lead nitrate at pH 5.0 for 90 min at 37°C. Controls were carried out by adding 0.01 M NaF to the medium, or by omitting the substrate.

ARYLSULFATASE: Cells were incubated in Goldfischer's medium (15) using *p*-nitrocatechol sulfate and lead nitrate at pH 5.5 for 90 min at 37°C. They were then washed five times in 0.05 M acetate-Veronal buffer (pH 7.4) with 7% sucrose and subsequently resuspended for 5 min in 2% (NH₄)₂S in the same buffer (16). Controls consisted of incubations in which the substrate was omitted.

DOUBLE INCUBATIONS: Cells were first incubated as described above for arylsulfatase or acid phosphatase, washed three times in acetate-Veronal buffer, and then incubated for peroxidase. In addition,

some cells were incubated first for peroxidase and then for acid phosphatase.

SUBSEQUENT PROCESSING: After incubation, cells were washed three times in 0.05 M acetate-Veronal buffer, pH 7.4, with 7% sucrose and packed by centrifugation into blocks, postfixed in OsO₄, stained in block in uranyl acetate, dehydrated, and embedded. Detailed techniques for preparation of tissues for electron microscopy were given previously (10).

RESULTS

General Description of Eosinophil Maturation

During maturation, granulocytes develop from less differentiated elements in the marrow and undergo a continuous sequence of events which has been arbitrarily divided into a number of stages: the first two (progranulocyte and myelocyte) are those during which granule formation occurs, and the last two (metamyelocyte and band cell) are associated primarily with changes in nuclear and cell size and shape. The details of the maturation process, the nomenclature used, and pertinent literature references are given elsewhere (9).

It is usually assumed that a developing granulocyte is multipotential (i.e., it can develop into either an eosinophil, basophil, or heterophil [PMN]) through the progranulocyte stage. However, as reported previously (9), in the rabbit progranulocytes can be differentiated into three distinct subtypes, one for each of the granulocyte lines. Our observations indicate further that the eosinophil, like the PMN, produces two types of granules in two separate generations—one during the progranulocyte and the other during the myelocyte stage. The process by which primary granules are formed in the eosinophilic progranulocyte of the rabbit resembles closely that by which primary or azurophil granules are formed in the PMN progranulocyte. However, there are distinct morphological differences between the two types of progranulocytes and the granules formed (see Figs. 1–4); moreover, considerably fewer primary granules (about ten per cell profile) are accumulated by the eosinophilic progranulocyte, whereas many more (up to 60 per cell profile) are accumulated by its PMN counterpart. These first granules formed by the developing eosinophil are large (600–1200 m μ) and spherical and have a homogeneous dense content; they undoubtedly correspond to the few spherical,

blue-staining granules which are seen in Romanovsky-stained preparations of rabbit marrow (see reference 17).

The second type of granule, formed during the myelocyte stage, is the well known specific granule which stains a brilliant pink with Romanovsky stains and shows a characteristic crystalline fine structure. The size and shape of the granules and the nature and arrangement of the crystals vary from species to species (see reference 18 and Figs. 20 and 21). It is the specific or secondary granules, the processes involved in their formation and in the segregation of enzymes therein, with which this study is concerned. The primary granules will not be considered further.

Specific Granule Formation by Myelocytes

MORPHOLOGICAL OBSERVATIONS: Eosinophil myelocytes show considerable development

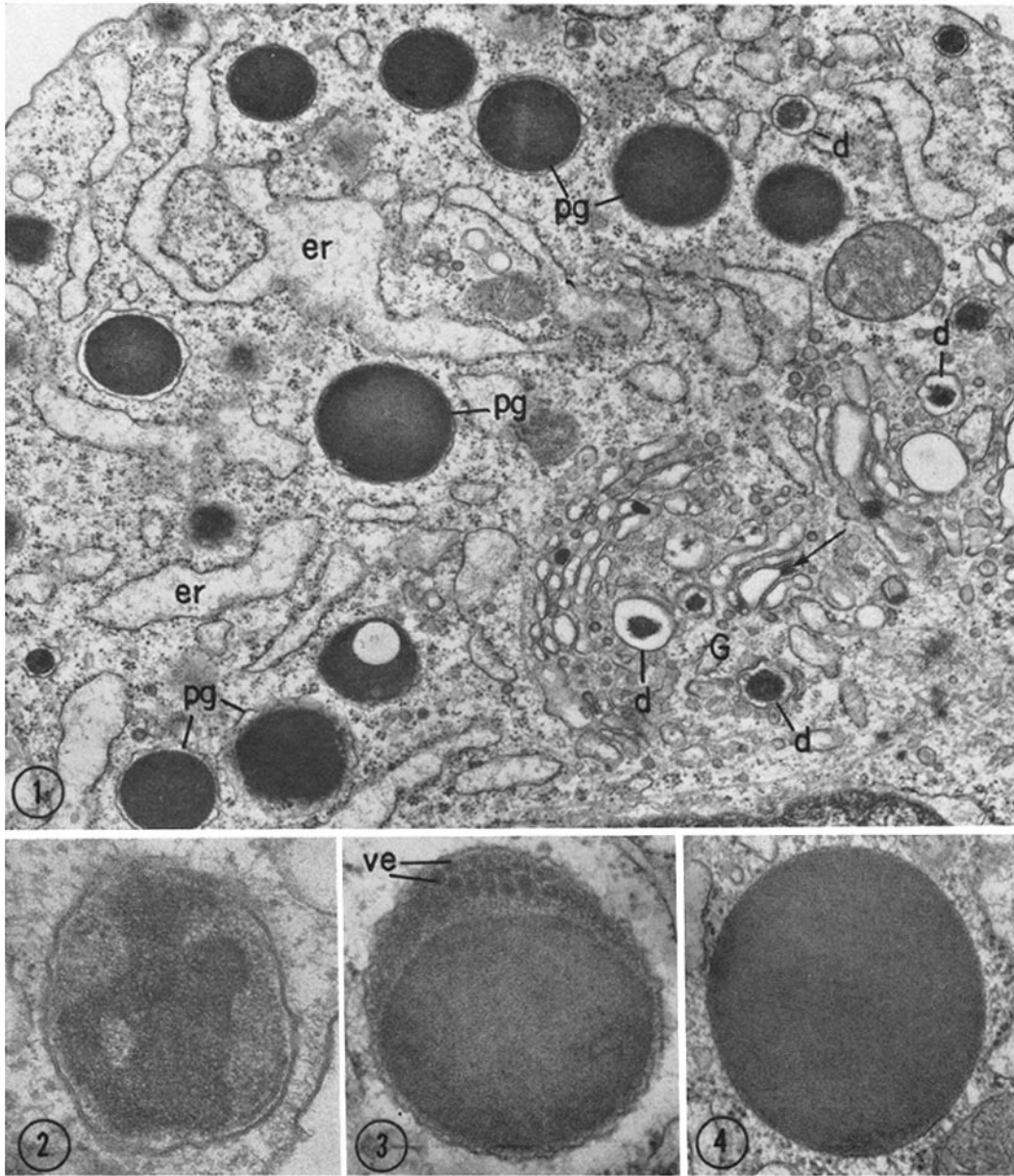
of cytoplasmic organelles involved in protein synthesis, segregation, and concentration, i.e., ribosomes, rough-surfaced ER, and Golgi complex (Fig. 5). The specific granule appears to be formed in several steps as follows: (a) dense material arises within Golgi cisternae (Fig. 5); (b) vacuoles containing a stringy material pinch off from the Golgi cisternae (Fig. 5 a); (c) several of these vacuoles aggregate to form larger immature granules (Figs. 5 and 6); and (d) the content undergoes condensation and needle-like crystals appear (Fig. 7). In the rabbit (Fig. 5), the crystals are smaller than in the rat (Fig. 9), and the crystal is often seen to lie immediately beneath the membrane of the partially condensed granule (Fig. 8). It should be mentioned that some of the immature forms of the specific granule are large and spherical and difficult to distinguish from primary granules (see Fig. 5). In addition to the usual organelles, myelocytes frequently contain

Abbreviations

<i>G</i> , Golgi region	<i>m</i> , mitochondrion
<i>Gc</i> , Golgi cisterna	<i>me</i> , membrane
<i>cē</i> , centriole	<i>n</i> , nucleus
<i>d</i> , dense-cored vacuole	<i>pg</i> , primary granule
<i>er</i> , rough endoplasmic reticulum	<i>pn</i> , perinuclear cisterna
<i>f</i> , filamentous matrix	<i>sg</i> , mature specific granule
<i>g</i> , glycogen	<i>te</i> , transitional element
<i>ig</i> , immature specific granule	<i>ve</i> , vesicle

All the figures except Figs. 1, 20, and 20 a show developing eosinophil granulocytes from normal rabbit or rat bone marrow. Fig. 1 is from the bone marrow of a rabbit with experimental eosinophilia, and Figs. 20 and 20 a are mature eosinophils from normal rabbit blood. Tissues were fixed in glutaraldehyde, postfixed in OsO₄, and all except those in Figs. 20 and 20 a were stained in block in buffered uranyl acetate. The sections from which Figs. 1-8, 20, and 20 a were taken were doubly stained with aqueous uranyl acetate and lead; all others were stained with lead alone.

FIGURES 1-4 Fields from eosinophilic progranulocytes in rabbit bone marrow, illustrating the steps involved in the formation of the large (600-1200 mμ) primary eosinophil granules. There are numerous dilated cisternae of the rough ER (*er*), and a prominent Golgi region (*G*) with condensing secretory material. Granule formation appears to occur by condensation of dense material within Golgi cisternae (arrow) with subsequent budding of dense-cored vacuoles (*d*) from the inner Golgi cisternae. Several vacuoles then merge into a form with a dense center and lighter periphery containing small vesicles (*ve*) (Fig. 3). The mature or fully condensed granule (*pg*) is spherical with a homogeneous dense content (Figs. 1 and 4). The formation of these large primary granules by the eosinophilic progranulocyte resembles the process by which azurophil granules are formed in the PMN progranulocyte (see reference 9). However, the eosinophilic progranulocyte differs from its PMN counterpart in that: (a) its ER cisternae (*er*) are shorter and more dilated; (b) its granules are larger and more regularly spherical; and (c) immature granules frequently contain small (~ 300 Å) vesicles which are not seen in PMN azurophil granules. Fig. 1, × 19,000; Fig. 2, × 78,000; Fig. 3, × 62,000; Fig. 4, × 41,000.



bundles (500 m μ) of fine fibrils (\sim 50 A) located near the cell periphery. When cut in cross-section, each bundle is seen to be composed of about 70 fibrils which appear punctate.

PEROXIDASE RESULTS: During the myelocyte stage, peroxidase reaction product was seen within (a) all cisternae of the rough ER (Fig. 9), including transitional elements (Figs. 10 and 12), and the perinuclear cisterna (Figs. 9–11); (b) clusters of smooth vesicles located at the periphery of the Golgi complex (Figs. 9 and 12); (c) all cisternae of the Golgi complex (Figs. 9 and 10); and (d) all immature and mature specific granules (Fig. 9). The latter were completely filled with reaction product except for areas occupied by crystals. No ribosomal staining was seen. The reaction was equally intense in all of the above compartments in specimens which were fixed for 10 min and incubated for 30 min or more. When cells were fixed for longer periods (4 hr) and incubated for 30 min, the reaction was faint in the ER, and heavier in the Golgi cisternae and granules (Fig. 13), suggesting that the enzyme is more concentrated in these last elements. When the incubation time was very brief (30 sec), reaction product was present in ER and Golgi cisternae and in immature granules but none was seen in mature granules, suggesting that there is rapid penetration of substrate into the former, and slower penetration into the latter compartments. The over-all reaction was generally less intense in the rabbit than in the rat. It should be noted that all the compartments mentioned—the perinuclear cisterna, rough ER, and Golgi cisternae—were reactive, even in the earliest myelocytes, and none of these

elements appeared to become reactive earlier in cellular differentiation than the others.

Cells fixed for short periods (10–60 min) at 4°C showed a more intense reaction than those fixed for longer periods (4 hr), particularly at 25°C, but morphological preservation was better in the latter. In cells fixed for only 10 min, there was frequently aggregation between ER and immature granules. The results of incubations carried out at pH 6 and pH 8.6 were the same as described above for pH 7.6.

ACID PHOSPHATASE RESULTS: Lead phosphate reaction product was found in (a) some of the rough ER cisternae including the perinuclear cisterna (Fig. 15), (b) some of the Golgi cisternae (Fig. 14), and (c) some immature granules (Figs. 14 and 16) excluding the area occupied by the crystals (Fig. 16). Only a few individual cisternae of the ER and portions of the perinuclear cisterna contained deposits. Within the Golgi complex, reaction product could be found within one to six of the stacked cisternae. Reaction product could not be seen in the fully condensed or mature specific granules (Fig. 22). The reaction was more variable from cell to cell and less intense than that seen with peroxidase, but was greatest in material fixed briefly (10 min) at 4°C. The ER reaction was seen more frequently in specimens fixed at 37° and was always sparse (Fig. 15). Structural preservation was poor, even after prolonged fixation (4 hr).

ARYLSULFATASE RESULTS: The distribution of the reaction product (lead sulfide) was similar to that for acid phosphatase and was found in (a) some rough ER cisternae (Figs. 18

FIGURES 5–8 Fields from eosinophilic myelocytes in rabbit bone marrow, depicting stages in the formation of specific granules. There is abundant dilated rough ER (*er*) and a large Golgi region (*G*) containing many early or immature specific granules (*ig*). At the periphery of the Golgi complex there are a number of transitional elements of the ER (*te*) and clusters of small vesicles (*ve*).

Granule formation begins with the appearance of dense (arrows) or stringy material within Golgi cisternae. Here numerous immature granules (*ig*) with a flocculent content are present in the Golgi region (Figs. 5 and 5 *a*). As granule formation proceeds, several of these immature granules coalesce into larger forms (Fig. 6 and *ig'*, Fig. 5), condensation occurs, and crystals appear (Fig. 7). Fig. 8 shows that the forming crystals frequently are found adjacent to the granule membrane (*me*) and running parallel to it. Here the membrane follows the contour of the crystal. Fig. 5 includes a primary granule (*pg*) which appears denser and more regularly round than the condensing specific granules and lacks crystals. Fig. 5, \times 19,000; Fig. 5 *a*, \times 57,000; Fig. 6, \times 66,000; Fig. 7, \times 38,000; Fig. 8, \times 48,000.

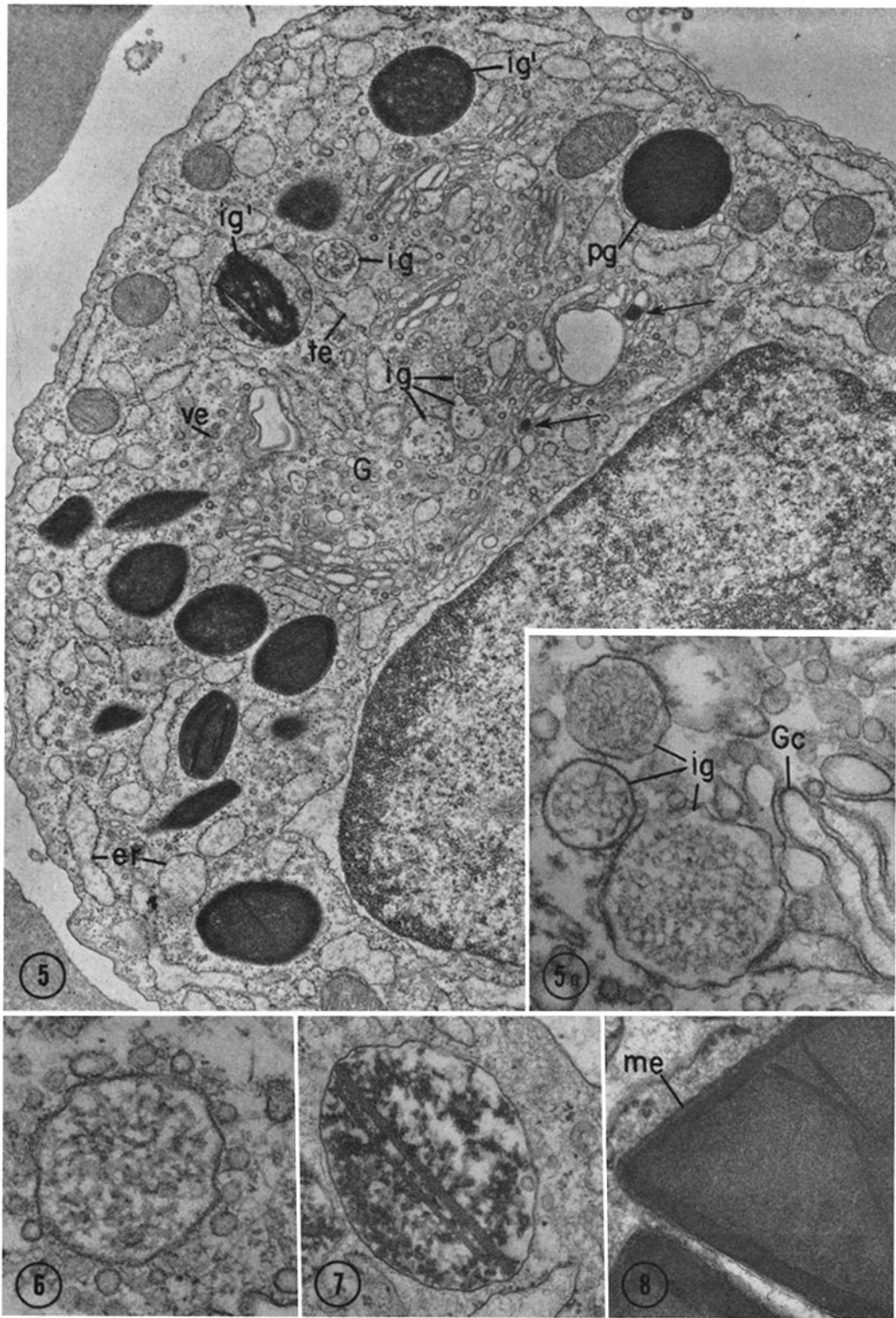




FIGURE 9 Rat eosinophil myelocyte from a preparation incubated for peroxidase. Reaction product appears as a dark, flocculent precipitate which fills the entire rough ER (*er*) including the perinuclear cisterna (*pn*), clusters of smooth vesicles (*ve*) at the periphery of the Golgi complex, all of the cisternae of the Golgi complex (*Gc*) (shown at a higher magnification in Fig. 10), all the immature granules located both in the Golgi region (*ig*) and in the peripheral cytoplasm (*ig'*), and mature granules (*sg*). In mature granules, reaction product is not present in the area occupied by the crystalline bar which stands out sharply against the dark background provided by the remainder of the reactive granule. Since the section was only lightly stained with lead, ribosomes do not show up and the density of the granules is due almost entirely to reaction product. Note that no reaction product is seen in the cytoplasmic matrix, the nucleus (*n*), or in mitochondria (*m*). Specimen fixed 3 hr at 4°C in glutaraldehyde and incubated 1 hr at 25°C in Graham and Karnovsky's medium (pH 7.6). $\times 15,000$.

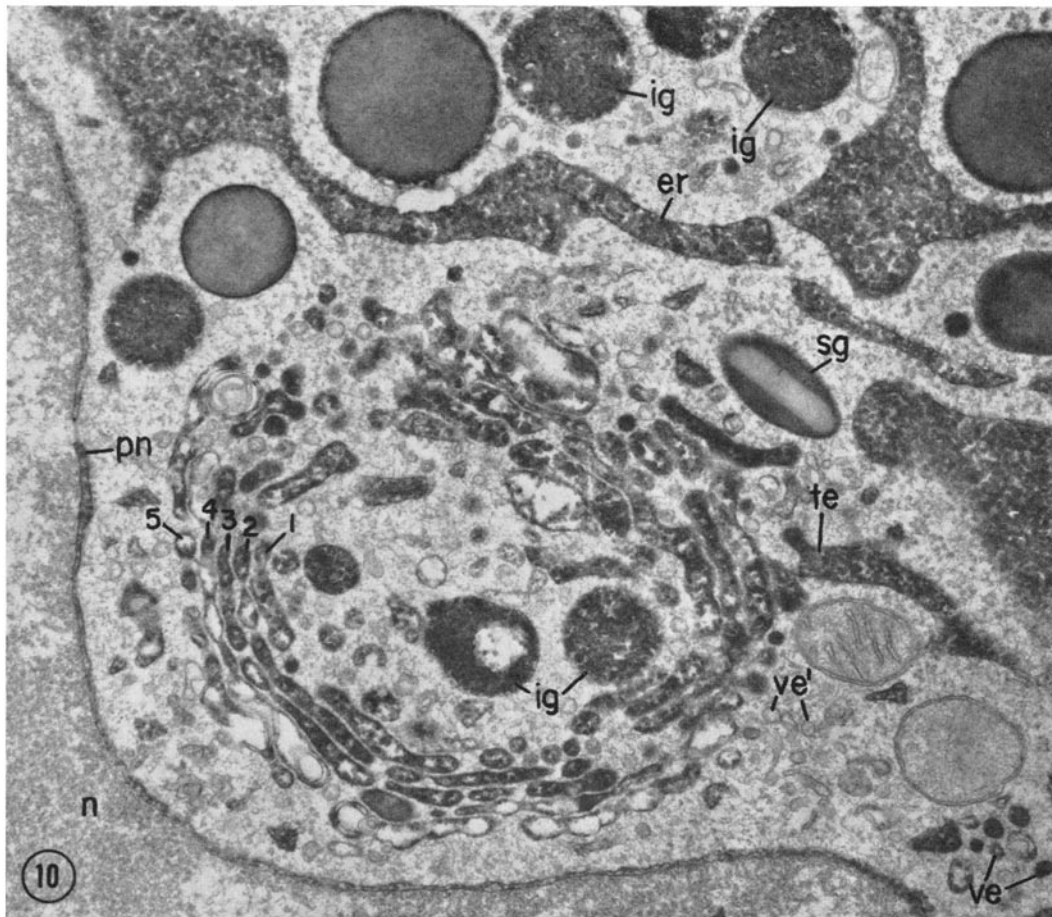


FIGURE 10 Higher magnification of the Golgi region of the eosinophilic myelocyte shown in Fig. 9. The localization of peroxidase reaction product in Golgi cisternae and in transitional elements at the periphery of the Golgi complex (*te*) can be seen to better advantage. Note that all five cisternae in the Golgi stack (1-5) are reactive. Reaction product can also be seen in the rough ER (*er*), perinuclear cisterna (*pn*), and in immature granules (*ig*). Many of the small vesicles (*ve*) in the Golgi region contain reaction product, but others (*ve'*) do not. $\times 43,000$.

and 19), including the perinuclear cisterna (Fig. 19), (b) some Golgi cisternae (Fig. 17), and (c) some immature granules (Figs. 17 and 18). Only individual cisternae of the ER and portions of the perinuclear cisterna contained deposits. Within the Golgi complex, reaction product was limited to one to three cisternae along the inner (concave) surface of the membrane stacks (Figs. 17 and 17 a). As with peroxidase and acid phosphatase, reaction product was restricted to that part of the granule exclusive of the crystal (Figs. 17 and 18), and no reaction was seen in mature specific granules. No difference was noted in the

distribution or intensity of the reaction in material fixed for different times (10 min-4 hr) or temperatures (4° or 25°C). Structural preservation was distinctly better than that of preparations incubated for acid phosphatase; however, a diffuse precipitate, which is apparently produced by the $(\text{NH}_4)_2\text{S}$ treatment (see reference 10) was frequently seen in the background.

DOUBLE INCUBATIONS: When cells were doubly incubated for arylsulfatase or acid phosphatase followed by peroxidase, both peroxidase reaction product and that for arylsulfatase or acid phosphatase could be demonstrated within the

same (immature) granule. However, the reaction was quite variable, and no peroxidase reaction product was present in ER or Golgi cisternae under these conditions. Hence, two enzymes could not be demonstrated simultaneously in these last compartments. When specimens were incubated first for peroxidase and then for acid phosphatase, the localization of peroxidase reaction product was similar to that in preparations incubated for peroxidase alone, but little or no acid phosphatase reaction product could be visualized.

Later Stages (Metamyelocyte, Band, and Mature Cell)

In contrast to the situation at the earlier stages already described, at later stages of development after granule formation has ceased, the eosinophil contains few organelles associated with synthesis and packaging of secretory proteins: rough ER is sparse or virtually nonexistent, and the Golgi complex is small and inconspicuous. The cytoplasm of the mature eosinophil from blood (Fig. 20) or bone marrow contains primarily granules and glycogen. Most of the granules present (95%) are specific granules with crystals which are usually centrally located. Relatively few (<5%) of the spherical, homogeneously dense primary granules are present (Fig. 20 *a*); hence they do not occur in every cell profile.

ENZYME LOCALIZATIONS: After the myelocyte stage, i.e., after specific granule formation has ceased, reaction product is not seen in the ER

or Golgi elements with any of the enzyme procedures. It is demonstrable only in the granules. With peroxidase, all immature and mature granules are reactive (Fig. 21). With acid phosphatase (Fig. 22) and arylsulfatase, only a few of the immature specific granules were reactive, and no reaction product was seen in fully condensed mature granules. It appears that the enzyme activities present in the mature or fully condensed eosinophil granules, like those in PMN leukocyte granules (10), are latent to demonstration by lead techniques in this type of preparation.

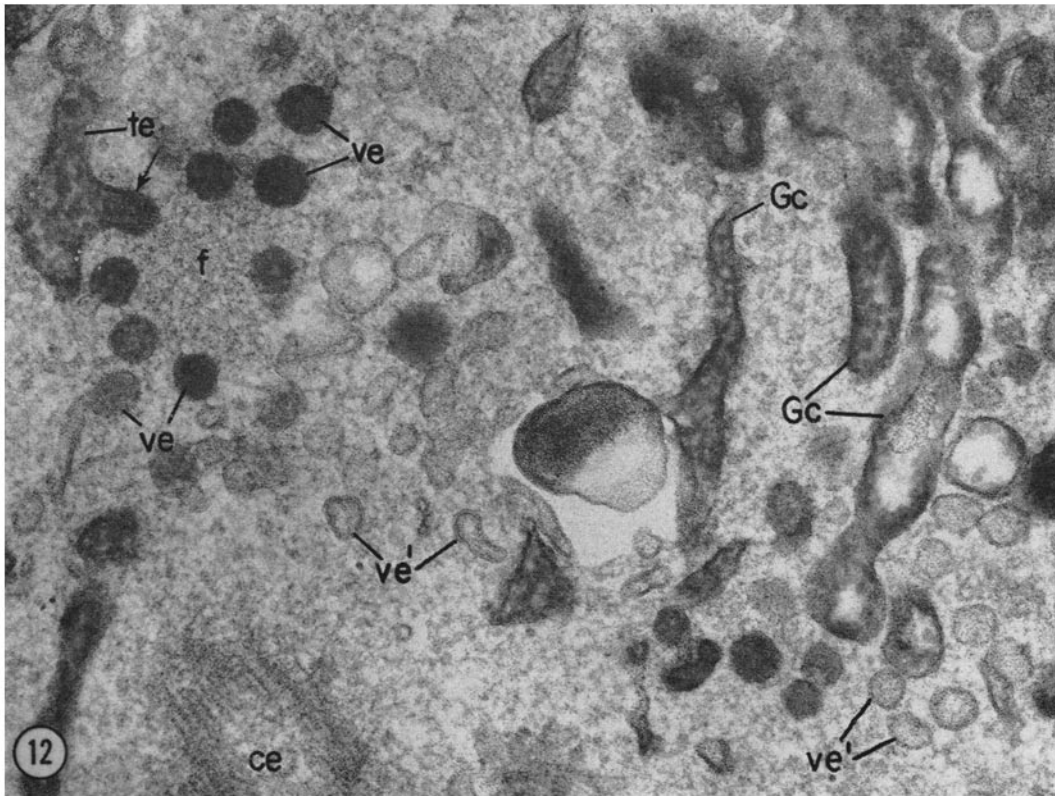
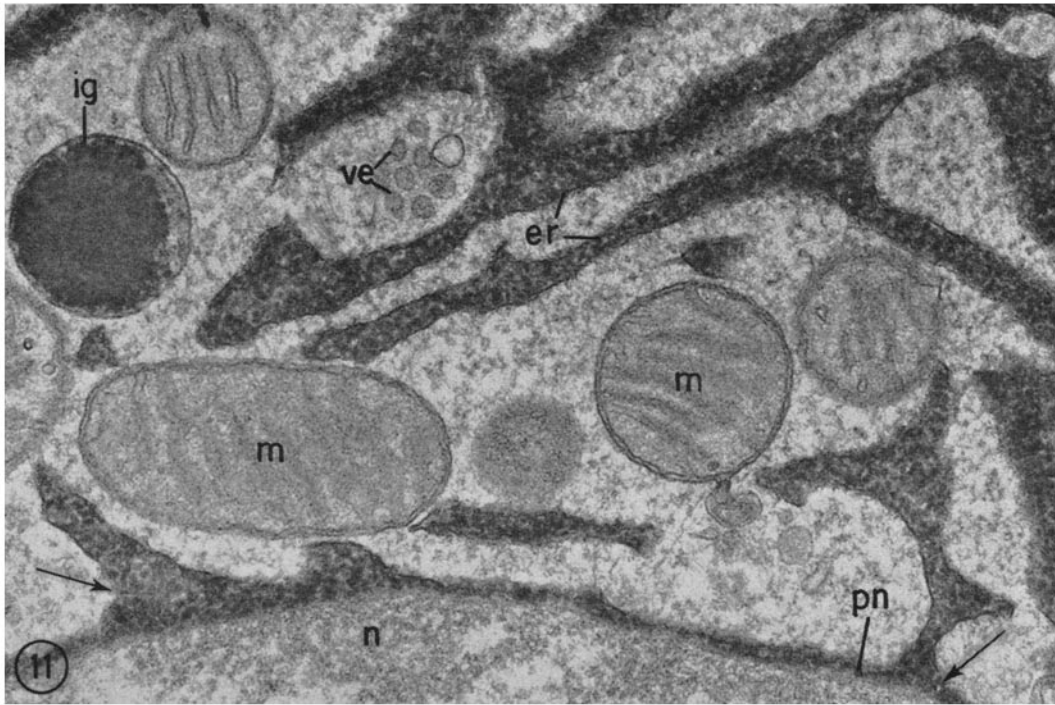
Controls

PEROXIDASE²: No reaction was observed in any organelles of eosinophils when (*a*) H₂O₂ or DAB was omitted from the incubation medium, or (*b*) catalase was added; (*c*) cells were incubated for 4 hr at 37°C in the alkaline DAB medium without H₂O₂, or (*d*) cells were incubated and then washed in potassium ferricyanide, a procedure designed to rule out artificial absorption of DAB (11). The addition of sodium azide,

² Eosinophilic peroxidase has been isolated and partially purified by Archer et al. (19) and found to be a hemoprotein distinct from neutrophilic peroxidase. However, no specific inhibitors of this enzyme are available. The above control experiments were performed because they were suggested by Graham and Karnovsky (11), or because they had been used previously in light microscopic investigations of peroxidase in leukocytes.

FIGURES 11 and 12 Portions of rabbit eosinophil myelocytes incubated for peroxidase. In Fig. 11, the rough ER (*er*), including the perinuclear cisterna (*pn*) with which it is continuous (arrows), is filled with dense reaction product. An immature granule (*ig*) is also reactive. No peroxidase is seen in the cytoplasmic matrix, the nucleus (*n*), in mitochondria (*m*), or in a cluster of smooth vesicles (*ve*). The ribosomes do not show up in this section which was lightly stained with lead. Specimen preparation as for Fig. 9. × 52,000.

Fig. 12 shows a stack of Golgi cisternae (*Ge*) and cluster of smooth vesicles (*ve*) located at the periphery of the Golgi stack. Just to the left of the cluster is what is believed to be a transitional element (*te*) of the ER in continuity with a vesicle (arrow) which appears to be pinching off therefrom. The transitional element, the vesicle pinching off from it, the cluster of vesicles, as well as the Golgi cisternae, all contain peroxidase reaction product. Note that the cluster of reactive vesicles is surrounded by a finely filamentous matrix (*f*) and that not all of the vesicles in the Golgi region contain reaction product. Some vesicles (*ve'*) located mainly near the ends of the cisternae are not reactive. × 87,000.



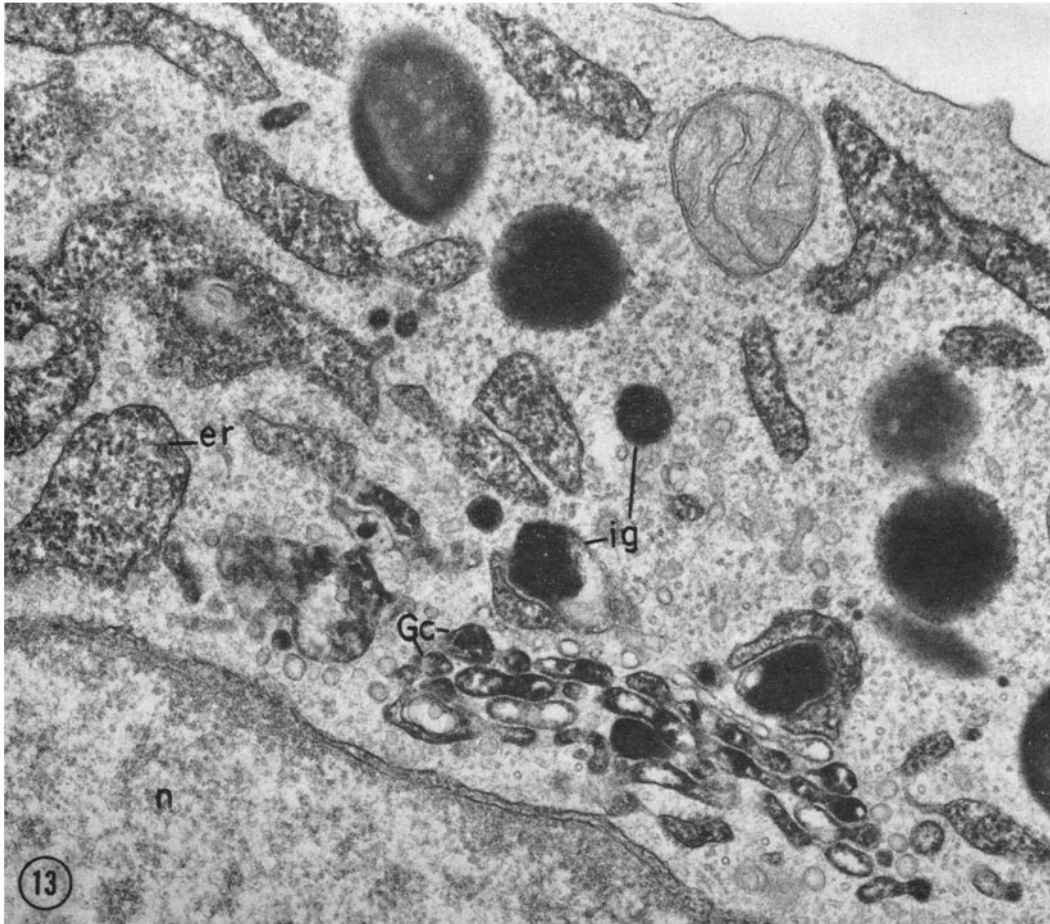
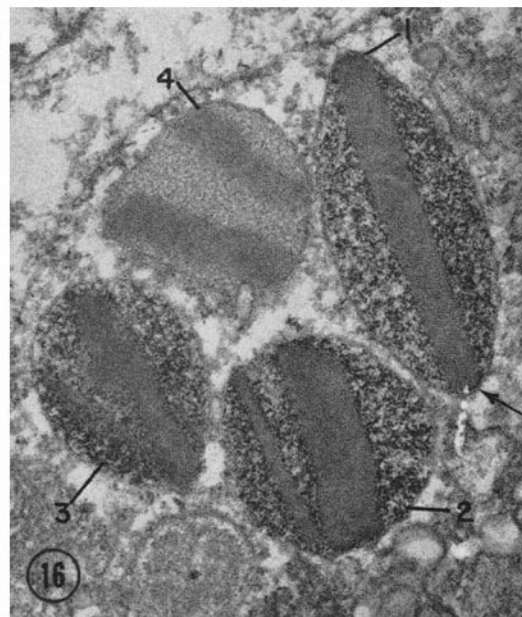
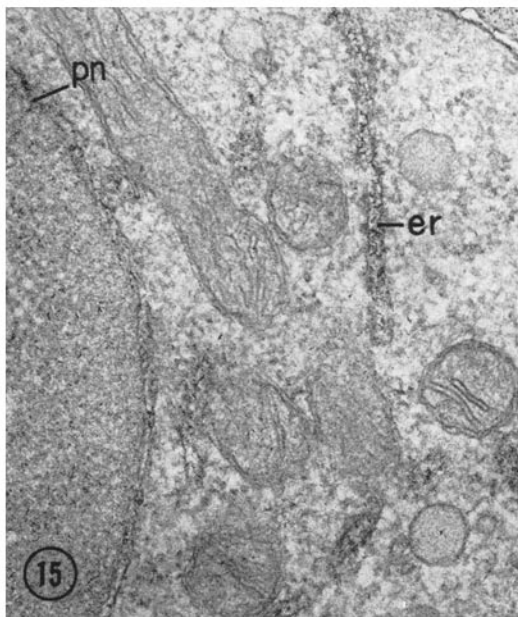
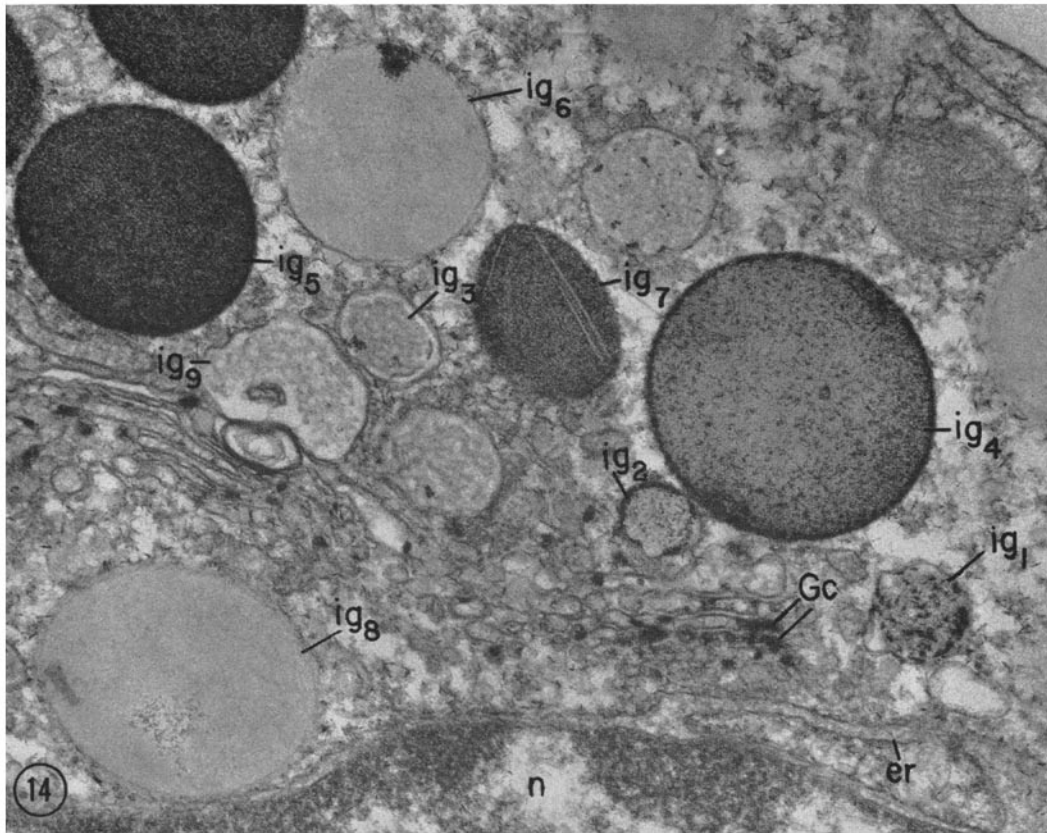


FIGURE 13 Portion of a rabbit eosinophil myelocyte incubated for peroxidase. In cells fixed for long periods, the reaction is less intense in the rough ER (*er*) and heavier in the Golgi cisternae (*Gc*) and granules (*ig*), suggesting that the enzyme is more concentrated in these last compartments. Specimen fixed 4 hr at 25°C in glutaraldehyde and incubated as for Fig. 9. $\times 37,000$.

FIGURES 14-16 Developing eosinophils from preparations incubated for acid phosphatase. Fig. 14 shows the Golgi region of a rabbit myelocyte. Reaction product can be seen in several Golgi cisternae (*Gc*) and in some of the small (*ig₁-ig₃*) and large (*ig₄-ig₇*) immature granules found near the Golgi cisternae. One of the latter (*ig₇*) contains a needle-like crystal. Note that the reaction is quite variable. Some immature granules (*ig₁*, *ig₂*, *ig₄*, *ig₅*) contain a large amount of reaction product; others contain little (*ig₃*, *ig₆*) or none (*ig₃*, *ig₅*). No reaction is recognizable in the ER (*er*). Fig. 15 shows a small portion of rat eosinophil myelocyte. Sparse deposits of reaction product can be seen in the ER (*er*) and perinuclear cisterna (*pn*). Fig. 16 shows a group of immature eosinophil granules in a rat myelocyte. Three of the immature granules (1-3) are heavily reactive for acid phosphatase and one (4) is not. Note that no reaction product is seen in the crystalline part of the granule (arrow). The specimen in Fig. 15 was fixed for 10 min at 25°C, and those in Figs. 14 and 16 for 4 hr at 4°C in glutaraldehyde, and incubated for 2 hr in a modified Gomori medium (pH 5.0). Fig. 14, $\times 53,000$; Fig. 15, $\times 46,000$; Fig. 16, $\times 24,000$.



KCN, or aminotriazole did not affect the reaction of mature granules, but completely inhibited the reaction in ER and Golgi cisternae.

ACID PHOSPHATASE: No reaction product was seen in specimens incubated without substrate or with NaF added to the medium.

ARYLSULFATASE: No reaction product was seen in specimens incubated without substrate.

DISCUSSION

The granulocytes represent a system of cells which provides a variation on the usual secretion theme seen in most glandular epithelia. Their life cycle is such that protein synthesis and packaging of granules are separated in time and location from granule discharge. The former processes occur during differentiation of immature cells in the bone marrow. When differentiation is complete, the protein synthetic apparatus is dismantled, and the cells enter a very long storage phase which lasts several days (during the latter part of their stay in the marrow and while they are circulating in the blood) until they reach the tissues where they function and degranulation occurs. In this situation, in contrast to that in most glandular epithelia in which secretion is continuous or intermittent, synthesis of secretory proteins occurs only once during the life cycle of the cell, and one can follow a secretory cycle by following steps in cellular differentiation.

In the present work, we have established (a) the steps involved in the differentiation of eosinophilic leukocytes and in the formation of their secretory granules, particularly the specific granules, and (b) the distribution during eosinophil differentiation of three enzymes—peroxidase, acid phosphatase, and arylsulfatase—which are ultimately pack-

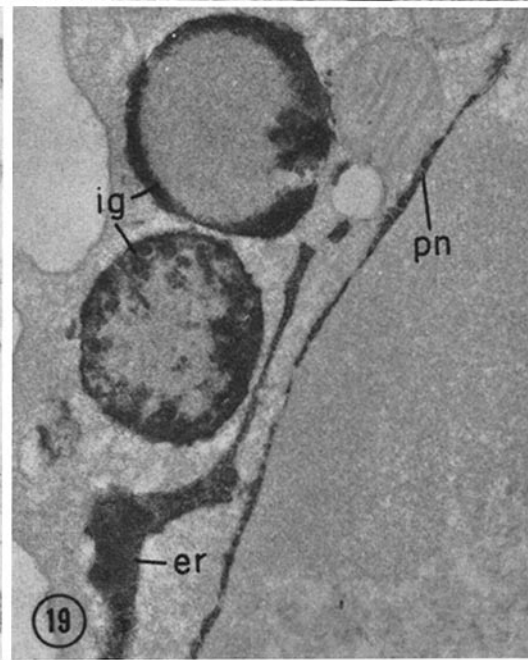
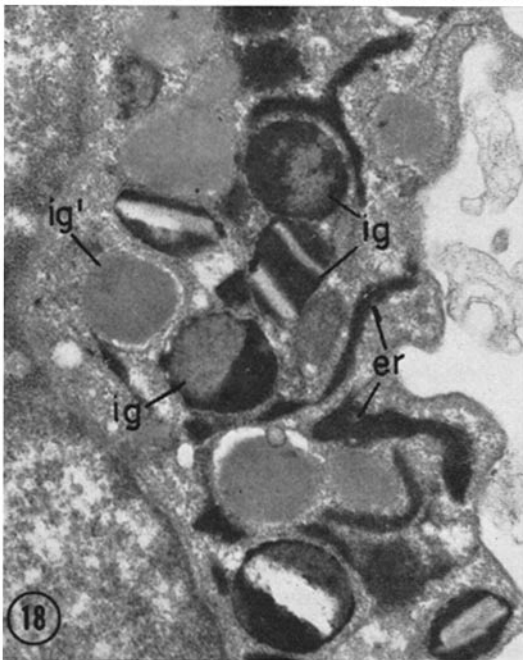
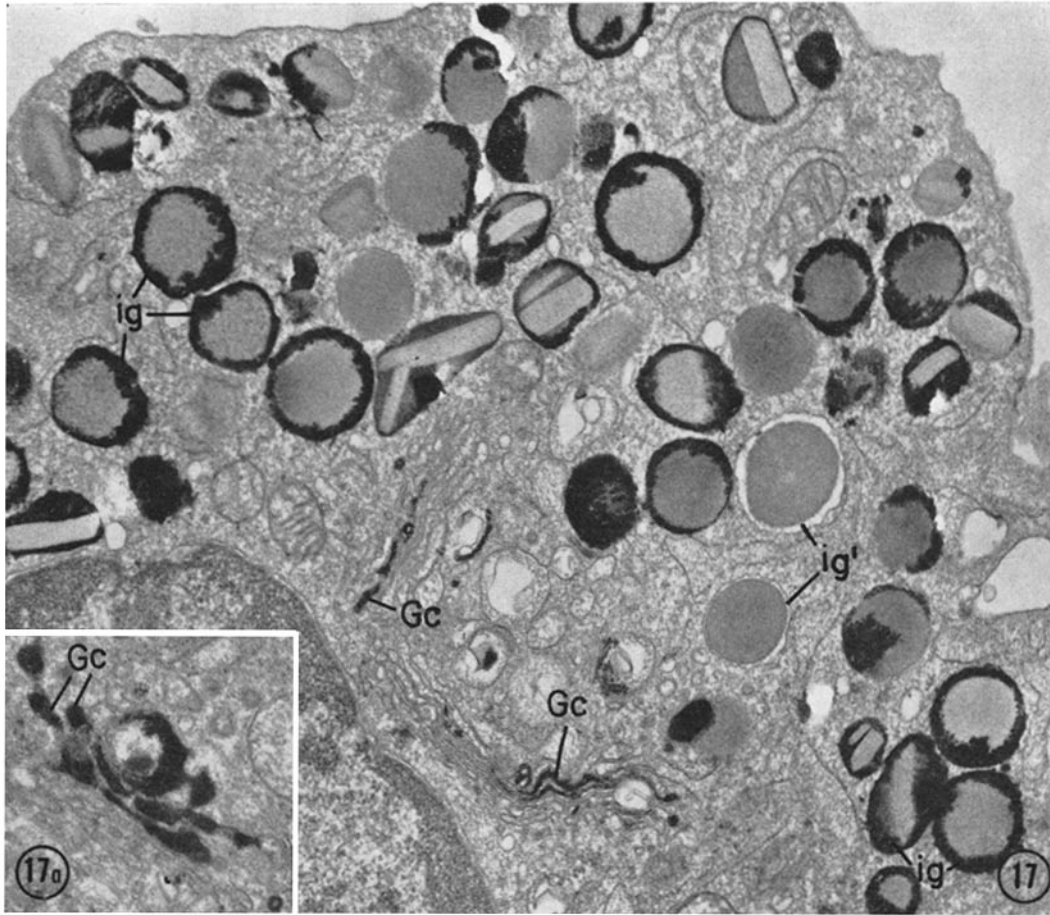
aged into specific granules.³ Our observations have shown that reaction product for all three enzymes is found throughout the secretory apparatus (rough ER, Golgi complex, and granules) during the stage of differentiation (myelocyte) when these granules are being formed and their contents are being synthesized. At later stages in maturation after granule formation has ceased, these secretory proteins are not found in ER and Golgi elements and are demonstrable only in the granules.

Current concepts of events in the secretory process are based on the extensive work carried out on the exocrine cell of the guinea pig pancreas by Palade and coworkers (5, 6, 20, 21). In this cell, which is producing large quantities of zymogens for the digestive juice, secretory proteins are probably synthesized exclusively on bound polyribosomes attached to the membranes of the ER and transferred across the same membranes into the cavities of the ER. From there they are transported via small vesicles which pinch off the ER cisternae to a condensing vacuole located in the Golgi region where condensation of the content takes place, forming the mature granule.

The results of the present work on the eosinophil are in accord with the general pathway for intracellular transport of secretory proteins worked out in the pancreas, since reaction product for the

³ Whereas the morphological findings indicate clearly that the developing eosinophil produces two types of granules (primary and specific), further work is needed to determine whether or not, as in the case of PMN (10), differences exist in the chemical content of the two granule types. Preliminary findings indicate that the primary granules contain peroxidase, but results with acid phosphatase and aryl sulfatase are incomplete.

FIGURES 17-19 Rat eosinophil myelocytes incubated for arylsulfatase. Fig. 17 shows deposits of reaction product in segments of some Golgi cisternae (*Gc*) and in many immature specific granules (*ig*). As with peroxidase and acid phosphatase, reaction product is absent from the crystalline part of the granule. The *inset* (Fig. 17 *a*) depicts a Golgi region with reaction product in two or three Golgi cisternae (*Gc*). Fig. 18 shows reaction product in several cisternae of the rough ER (*er*) and a number of immature granules (*ig*); Fig. 19 shows it in the ER, including the perinuclear cisterna (*pn*) and several immature granules (*ig*). Note that not all of the immature granules (*ig'*) in Figs. 17 and 18 contain reaction product. Specimens fixed 10 min in glutaraldehyde, incubated 90 min at 37°C in Goldfischer's medium, and treated in (NH₄)₂S before further processing. Fig. 17, × 23,000; *inset*, × 33,000; Fig. 18, × 21,500; Fig. 19, × 46,000.



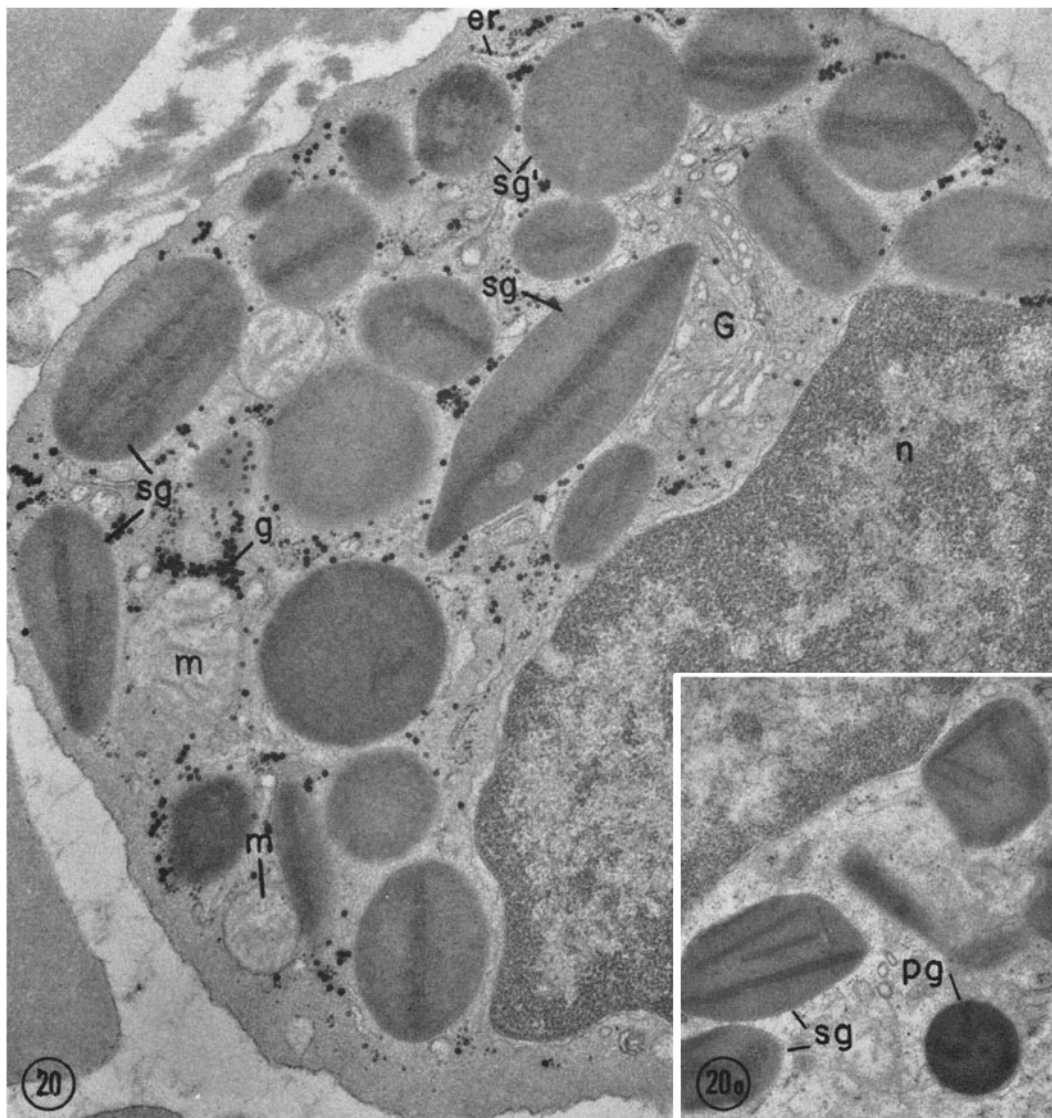


FIGURE 20 Mature eosinophil from rabbit blood, showing its rounded shape and one lobe of its dense nucleus (*n*). The cytoplasm contains mainly granules and glycogen particles (*g*). The Golgi apparatus (*G*) appears small and inactive. Rough ER (*er*) and mitochondria (*m*) are sparse. Most of the granule profiles are identifiable as specific granules (*sg*) since they contain thin, needle-like crystals. Others (*sg'*) do not contain crystals, but are believed to represent cross-sections through specific granules in an area not occupied by crystals. No primary granules are seen in this field. The *inset* (Fig. 20 *a*) is from another, similar cell and depicts a group of granules in which a single primary granule (*pg*) is included. The primary granule appears more regularly round and denser than the specific granules (*sg*) and does not contain crystals. Fig. 20, $\times 27,500$; Fig. 20 *a*, $\times 20,000$.

three enzymes tested was found in the appropriate compartments (rough ER, vesicles at the periphery of the Golgi complex, and immature granules corresponding to condensing vacuoles) during

periods of known protein synthesis and packaging and was absent from these compartments during stages in the life cycle of the cell when granule formation and synthesis of secretory protein has

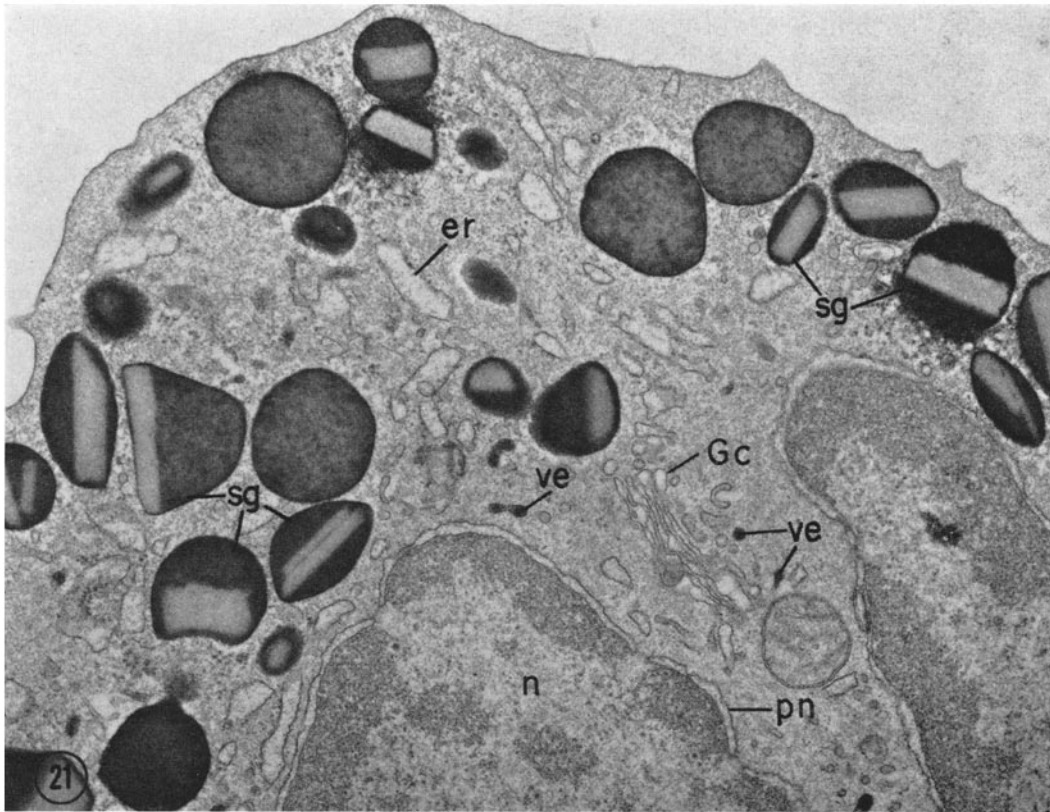


FIGURE 21 Portion of rat eosinophil metamyelocyte reacted for peroxidase. Reaction product is demonstrable only in granules (*sg*) and in a few small vesicles (*ve*) in the Golgi region. The rough ER (*er*), including the perinuclear cisterna (*pn*), and the Golgi cisternae (*Gc*) do not contain reaction product. Specimen preparation as for Figs. 9-13. $\times 53,000$.

presumably ceased. Hence our work corroborates in another tissue, studied by a different approach, certain aspects of the pancreas scheme. The cytochemical technique has the disadvantage that one cannot follow the kinetics of synthesis and transport of secretory proteins. It is superior, however, to cell fractionation procedures as far as localization is concerned, for the secretory protein can be visualized in the appropriate compartments in the intact cell. In particular, with peroxidase it can be seen that the entire secretory apparatus of the eosinophilic myelocyte is labeled. It is clear that in this system, (a) condensation of the secretory product begins in the stacked Golgi cisternae in which the first condensing secretory material becomes visible, and continues in the cytoplasm in something analogous to a condensing vacuole formed by the fusion of several small Golgi-derived elements; and (b) that all the ER cisternae, including

the perinuclear cisterna and transitional elements, and all the Golgi cisternae are involved in the segregation and concentration, respectively, of secretory proteins, since all these elements contained peroxidase-reaction product. In addition, since two other enzymes (acid phosphatase and arylsulfatase) can also be demonstrated regularly, though less consistently, in the same compartments, the ER and Golgi cisternae must be involved in the simultaneous segregation and concentration of more than one protein. It should be emphasized, however, that it cannot be determined whether (a) the whole rough ER system is involved in synthesizing a given enzyme or (b) production is limited to a few cisternae and the whole space is available to diffusion after segregation.

The fact that concentration of secretory proteins occurs in the stacked Golgi cisternae is an important point, because in the work on the guinea pig

pancreas there is no indication that under normal conditions these cisternae are involved in the concentration process. This could indicate a difference in the activities of the elements of the Golgi complex in the two cell types, or it could be explained on the basis of methodological differences, for the histochemical procedures we have used, owing to their amplification effect, are capable of detecting lower concentrations of secretory proteins than can be detected by direct morphological examination or by radioautography. From observations on other systems, it appears that the occurrence of condensation in Golgi cisternae is rather general (see references 9, 22–25).

The cytochemical staining also gives us some important information not available from cell fractionation data—it indicates not only where secretory proteins are found, but also where they are *not* found, for within the limits of detection of

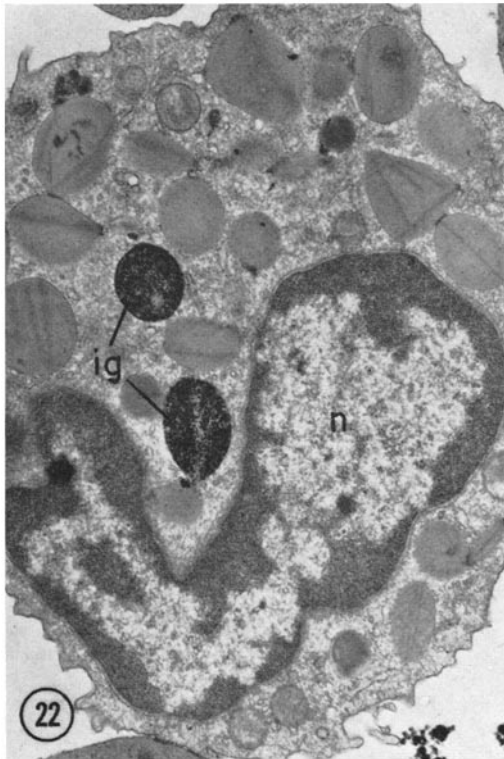


FIGURE 22 Band form of a rabbit eosinophil reacted for acid phosphatase. The only elements which contain reaction product are two immature granules (*ig*). The remainder of the granules, most of which appear fully condensed or mature, are not reactive. Specimen preparation as for Fig. 14. $\times 11,500$.

the technique, no peroxidase was seen in the cytoplasmic matrix, mitochondria, the nucleus, or in some of the cytoplasmic vesicles.

Work Carried out by Others

Wetzel et al. (26) and Hudson (27) have investigated eosinophil differentiation in the rabbit and guinea pig, respectively, and Fedorko (28) has studied amino acid incorporation into developing human eosinophils by electron microscopic radioautography. Cytochemical studies similar to, but less extensive than ours have been carried out on eosinophils by several workers. Acid phosphatase has been localized in the Golgi complex of developing eosinophils (29–31) and in eosinophil specific granules (29–33). Peroxidase has previously been localized in rough ER, Golgi cisternae, and granules of the rat (34) and rabbit (35) eosinophil as well as developing cat (36) and human (37) PMN leukocytes.⁴ In addition, Leduc (38) has localized another secretory protein (immunoglobulin) within rough ER and Golgi cisternae of developing plasma cells using the peroxidase-labeled antibody technique.

General Comments on Secretion

From the present work, the studies cited above, and other work carried out up to now in a variety of cell systems, it appears that some of the operations which occur during the production of proteins for export are rather general, such as synthesis on ribosomes and segregation in the rough ER (6, 10, 34–38). Other aspects, however, are more variable. We have already stressed the fact that the sites of condensation vary, for in the eosinophilic leukocyte and the majority of other situations investigated up to now, condensation takes place in the piled Golgi cisternae, whereas in the exocrine cell of the guinea pig pancreas and probably also the rat parotid (39), specialized condensing vacuoles exist for this purpose. Our previous work on developing PMN leukocytes (9) indicates that concentration can vary in different regions of the Golgi complex, with one area specializing in the concentration of one secretory product and another area specializing in the concentration of another secretory product. It should be recognized,

⁴Since this paper was submitted for publication, Miller and Hertzog (48) have published findings very similar to ours on the localization of peroxidase and acid phosphatase in rabbit and rat eosinophils.

however, that some of these variations may be due to the fact that, in addition to transport and condensation, the Golgi complex is apparently also involved in the conjugation of proteins, especially glycoproteins (40-41).

Variations are also encountered in the packaging or storage phase of the secretory process. The extent of storage varies with the cell type; the size, density, and shape of the granules vary with the product, and some granules such as those of eosinophilic leukocytes in most species (18) and basophilic leukocytes of some species (24) are crystalline, whereas others are noncrystalline. Finally, the discharge operation can vary. In many cells, discharge occurs by exocytosis or reversed pinocytosis, i.e., by fusion of the granule membrane with the plasma membrane, as originally described by Palade (42) in the pancreas. In other cells (e.g., fibroblasts [43], osteoblasts [44], and plasma cells [45]), the mechanism of discharge of the secretory product is less clear, and alternative methods of discharge have been considered. Among those in which exocytosis has been demonstrated, the eosinophilic (46) and PMN (47) leukocytes provide an interesting variant, for discharge occurs intracellularly into phagocytic vacuoles instead of extracellularly into ducts as is the case in the pancreas.

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In still other instances, such as in cells of the anterior pituitary gland (22, 25), an option exists in this regard, for discharge may occur either extracellularly (into pericapillary spaces) or intracellularly (into lysosomes).

It should be stressed that the foregoing applies primarily to situations in which the secretory product is a protein. Considerably less is known about the localization and nature of the various steps in secretion in systems in which the product is of a different nature, such as a polypeptide, steroid, or biogenic amine. Undoubtedly, as such systems are dismantled and investigated, other variations in the secretory process will emerge.

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