INTRODUCTION

Recent work carried out on rabbit polymorphonuclear leukocyte (PMN) granules in situ (1–5) and in fractions (6, 7) has established the existence of two types of granules in mature PMN—azurophils and specifics. The azurophils represent a special type of primary lysosome containing acid hydrolases as well as peroxidase (4, 5, 6–8) and acid mucosubstance (9). The specifics are not lysosomal in nature since they lack acid hydrolases and so far are known to contain alkaline phosphatase,
lactoferrin (10), and considerable lysozyme activity (6). The cytotoxic and biochemical data are in close agreement concerning the nature and enzyme content of these two granule types but have left unresolved the question of the existence of a third type. Wetzel et al. (2, 3) reported finding an additional population of "tertiary" granules, which arose late in PMN development, appeared smaller and more variable in shape than the other two types, and contained acid phosphatase (AcPase) activity. However, no such granules were detected by Bain ton and Farquhar (1, 4, 5). In their fractionation studies, Baggiolini et al. (7) identified a third group of particles, lysosomal in character and smaller and lighter than the azurophil and specific, which they suggested might correspond to tertiary granules. However, the fraction (C) in which these particles were concentrated, was quite heterogeneous. They also considered the possibility that the C particles were derived from mononuclear cells, that is monocytes and macrophages, which are present in small numbers in the starting material (peritoneal exudates).

The aim of this study was to obtain further information on the nature and source of the C particles by carrying out a combined morphological, biochemical, and cytotoxic inquiry. 2 Cytotoxic tests for AcPase were conducted on PMN granule fractions, and the distribution of reaction product was compared to that in the intact cells of the starting material. Our results indicate that (a) the distribution of AcPase in the C fraction is heterogeneous (being localized in Golgi cisternae, secondary lysosomes, and small pleomorphic granules), and (b) the bulk of the cytotoxic demonstrable AcPase activity present in this fraction is derived from mononuclear cells rather than from PMN.

**Materials and Methods**

**Materials**

Shellfish glycogen was obtained from Amend Drug and Chemical Co., Inc., New York; o-tolidine from MC&B Manufacturing Chemists, Norwood, Ohio, 8-glycerophosphate (grade I), and p-nitrophenyl-N-acetyl-6-glucosaminide from Sigma Chemical Co., St. Louis, Mo., and p-nitrophenyl phosphate (dissodium salt 5 H2O, A grade) from Calbiochem, La Jolla, Calif. Glutaraldehyde was obtained either as a 25% solution (biological grade) from Fisher Scientific Co., Pittsburgh, Pa., and redistilled (12) before use, or as an 8% solution of "pure glutaraldehyde" from Electron Microscopy Sciences, Fort Washington, Pa. Both glutaraldehyde solutions showed a single peak at 280 nm when analyzed by ultraviolet spectrophotometry (13).

**Methods**

**Production of Exudates:** Peritoneal exudates were induced in adult New Zealand rabbits according to the method of Hirsch (14). In brief, each animal was injected with 250 ml of a 0.1% solution of shellfish glycogen in sterile saline. After 4 hr, the exudate was collected by gravity drainage either directly into a 15 ml conical centrifuge tube containing fixative (in the case of cells to be examined by electron microscopy for morphology and cytochemistry) or into a bottle containing 5000 units/ml heparin (in the case of cells to be fractionated).

**Procedures for Exudate Cells:** Specimens prepared for morphologic studies were fixed for 4-6 hr at 25°C in 1.5% glutaraldehyde in 0.1 M cacodylate (pH 7.4) or phosphate (pH 7.6) buffer, placed in 0.4 ml polyethylene tubes, packed by centrifugation for 4 min at ~ 10,000 g with a Microfuge 152 (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), postosmicated, and processed as described previously (5). Specimens collected for cytochemistry were fixed 10 min in 1.5% glutaraldehyde in 0.1 M cacodylate buffer at 4°C, and washed three times (by repeated spinning and resuspension) in 0.1 M cacodylate containing 0.2 M sucrose. The cells were incubated in suspension for 90 min to 17 hr in modified Gomori medium, pH 5.0 (15), with 8-glycerophosphate as substrate, and then washed, postosmicated, stained in block with uranyl acetate, and embedded in Epon as previously described (5).

The controls were incubated without substrate.

**Fractionation:** Cells were washed three times in 0.34 M sucrose (by centrifugation for 10 min at 400 g and resuspension) and homogenized by mild shearing on a Vortex mixer (Scientific Industries, Inc., Queens Village, N. Y.). The homogenate was spun at 400 g for 10 min (to remove intact cells, nuclei, gross debris, and erythrocytes). The pellet was then fixed in glutaraldehyde and prepared for examination by electron microscopy. The resulting supernatant was fractionated by zonal differential centrifugation through a sucrose gradient in a B-XIV rotor as described by Baggiolini et al. (6, 7). All spins were carried out at 6800-7500 rpm for 15 min.

**Biochemical Assays:** The fractionations were monitored by assaying for peroxidase, alkaline phosphatase, N-acetyl-6-glucosaminidase, and acid -
ni trophenyl phosphatase, which served as marker enzymes for azurophils, C particles (in addition to azurophils), and membranes, respectively. Assays for $\beta$-glycerophosphatase were also performed in most experiments. These enzymes were measured as described previously (6, 7). The four fractions in which marker enzymes showed a peak of activity were selected for morphological examination, and labeled A, B, C, and D. The A and B fractions essentially correspond to those previously labeled as such (6). Fractions C and D represent adjacent cuts in the previous C fraction Isopycnic centrifugation, which affords better resolution between the C and D particles, was not used here because of the higher contamination of the C zone by C particles (7), and the longer preparation time involved (2-3 hr) with resulting delays in obtaining the fractions.

PROCEDURES FOR GRANULE FRACTIONS:
Samples (7.5-10 ml) were collected from peak regions of the A, B, C, and D fractions and placed in No. 30 cellulose nitrate centrifuge tubes (capacity, 38.5 ml) containing 10 ml of 0.5% glutaraldehyde in 0.1 M cacodylate (pH 7.4). After 10 min, the tubes were filled with cacodylate buffer and spun at 30,000 rpm for 1 hr in a No. 30 rotor on a Spinco Model L ultracentrifuge (Beckman, Spinco Div.). The supernatant was decanted, the surface of the resulting pellets was rinsed three times in the same buffer, and ACPase incubation medium (15 ml) was added to each tube. Incubation was carried out for 90-120 min at 37°C. After incubation, the pellets were rinsed three times in 0.1 M acetate-Veronal buffer (containing 0.2 M sucrose), postosmicated (60 min), treated with 0.5% uranyl acetate (60 min), rinsed briefly in 70% and 95% alcohol, and placed in absolute alcohol. All preceding steps were accomplished directly in the centrifuge tubes, since the pellets usually remained adherent to the tubes. During dehydration (95% or absolute alcohol), the pellets and tubes were cut into strips of 1 mm X 3 mm X 0.1-0.2 mm which were placed in vials for subsequent processing. The strips were then infiltrated with propylene oxide (in which the cellulose nitrate backing dissolved) and embedded in Epon in flat embedding molds (16) (Ladd Research Industries, Burlington, Vt.) to facilitate orientation during sectioning.

SPECIAL STAINS AND CELL COUNTS:
Drops of peritoneal exudate and granule fractions were placed on glass slides, rapidly dried, and heat-fixed. Some were processed and stained with Wright's stain or azure A as previously described (4). Others were stained for 30 min with 1% eosin Y in 0.01 M Na-Veronal buffer (pH 7.6), and washed briefly in the same buffer. Differential counts of 500 exudate cells were performed on Wright's-stained smears of peritoneal exudate and granule fractions were placed on glass slides, rapidly dried, and heat-fixed. Some were processed and stained with Wright's stain or azure A as previously described (4). Others were stained for 30 min with 1% eosin Y in 0.01 M Na-Veronal buffer (pH 7.6), and washed briefly in the same buffer. Differential counts of 500 exudate cells were performed on Wright's-stained smears.

RESULTS

EXUDATE CELLS

DIFFERENTIAL CELL COUNTS:
Differential counts indicated an average of 97% PMN, 2% mononuclear cells, and 0.5% each eosinophils and lymphocytes in all exudates. These percentages are very similar to those obtained previously by Baggiolini et al (7).

MORPHOLOGICAL STUDIES:
The mature PMN (Fig 1), which constitute the major component of the exudate, showed features identical to those described previously (1) for mature PMN in the bone marrow. Nuclei were typically condensed and multilobulated and the cytoplasm contained numerous granules and large lakes of glycogen but very few other structural elements (endoplasmic reticulum [ER] was scanty, mitochondria were few, and Golgi complexes small). The granules were of two types: large ovoid azurophil or primary granules and smaller, less dense specific or secondary granules, which were more numerous. Most of the azurophils were mature and appeared ovoid or spherical, with homogeneous contents. A few immature "nucleoid" forms (with lamellated cores) (5) were also present (Fig. 3). The majority of the granules were spherical with diameters of 300-500 nm, but a few rod or dumbbell forms could usually be seen. The main difference noted between mature PMN found in the bone marrow and those in exudates was in the amount of glycogen present, which is massive (occupying as much as one-third of the cell volume) in the case of the exudates. PMN glycogen deposits have the distinction of being composed of unusually large (30-40 nm) $\beta$-particles (17).

The mononuclear cells in the exudate differed in size and content. Some undoubtedly had recently immigrated from the blood. These had the characteristic features of monocytes (18), with a moderate amount of rough ER, a small Golgi complex, and clusters of azurophil granules varying in size and shape located near the hof of the horsehoe-shaped nucleus (Figs 4-6). Other, larger cells were typical macrophages, exhibiting a more elaborate rough ER, a larger Golgi complex, and a spectrum of lysosomal elements (phagocytic vacuoles, dense bodies of variable size and shape, and coated vesicles [18]). Some of these cells appeared to be actively phagocytizing the fibrin present in the exudates.

CYTOCHEMICAL STUDIES:
In preparations
Figure 1. PMN leukocyte from a rabbit peritoneal exudate. The section cuts through three lobes (n₁-n₃) of its multilobulated nucleus. Its cytoplasm is packed with granules and huge lakes of glycogen (g) but contains little else. The granules are of two types: the azurophils (ag) and the specifics (sg). The azurophils are larger and denser than the specifics which are twice as numerous. The glycogen deposits consist of unusually large (80-40 nm) β-particles (17). Specimen fixed in 1½% glutaraldehyde in 0.1 M cacodylate (pH 7.4) for 4 hr and postfixed in OsO₄ in the same buffer. Section doubly stained in uranyl and lead. × 12,000.


cell incubated for up to 90 min for AcPase, only some of the PMN showed reaction product; in fact, most of the cells were unreactive. In cells where reaction product was present (Fig. 3), it was found in two sites: (a) Golgi cisternae, and (b) immature azurophil granules. Mature azurophil granules were not reactive. In preparations incubated for longer periods (3½ hr), more PMN contained reaction product, and the amount in Golgi cisternae and in immature azurophils was increased. Deposits were also seen in the perinuclear cisternae of a few cells and, rarely, around immature specific granules (sg) were not reactive. In either preparation. Nucleus (n). Specimens fixed 10 min in 1½% glutaraldehyde in 0.1 M cacodylate (pH 7.4), incubated in Gomori medium at pH 5.0 with β-glycerophosphate, and postfixed in OsO₄. Sections stained in lead alone. Fig. 2, × 32,000; Fig. 3, × 37,000.

Figures 2 and 3. AcPase preparations of exudate cells, illustrating the distribution of AcPase reaction product in PMN and the histochemical latency (to heavy metal techniques) of AcPase activity in azurophil granules. Among the granules only the immature azurophils (Fig. 3) or mature azurophils that have been activated by disruption (Fig. 2) are reactive. Mature, intact azurophils (ag) are not reactive. Fig. 3, incubated for 90 min, shows reaction product in the Golgi complex (Gc) and in a single immature azurophil granule (ag) identifiable as a “nucleoid” form (see reference 5) by virtue of its lamellated central core. The cell in Fig. 2, which was subjected to prolonged incubation (6 hr), contains several reactive azurophil granules (arrows) that have been partially disrupted (as evidenced by their partly extracted content) and thereby activated by the prolonged incubation at acid pH. The specific granules (sg) are not reactive in either preparation. Nucleus (n). Specimens fixed 10 min in 1½% glutaraldehyde in 0.1 M cacodylate (pH 7.4), incubated in Gomori medium at pH 5.0 with β-glycerophosphate, and postfixed in OsO₄. Sections stained in lead alone. Fig. 2, × 32,000; Fig. 3, × 37,000.
cific granules When the incubation time was pro-
longed (6-17 hr) (Fig. 2), the deposits were heavier
in immature azurophil granules and in Golgi and
perinuclear cisternae, but there was increased non-
specific background (i.e., a fine sprinkling of lead
phosphate) over the entire cell. In addition, we
observed increased numbers of reactive azurophil
granules, usually appearing partially damaged or
extracted. Thus our findings confirm previous
studies on P1VIN from bone marrow (5) and from
peritoneal exudates (19) indicating that the AcPase
activity present in mature azurophils is latent to
demonstration in this type of preparation. Only
immature azurophils are reactive, whereas the
mature granules require disruption (such as occurs
after prolonged incubation or smearing [4]) for
activation.

In mononuclear cells, there were typically a
number of AcPase-positive elements in essentially
every cell after incubation for only 90 min, and the
amount of reaction product visible increased after
longer incubation. In confirmation of previous
work (18), in those cells which apparently repre-
sented monocytes recently immigrated from the
blood, some but not all of the azurophil granules
as well as the Golgi complex contained reaction
product (Figs. 4-6). In macrophages, reaction
product was observed in the Golgi complex and in
many of the lysosomal elements (coated vesicles,
phagocytic vacuoles, and dense bodies), and occa-
sionally in the rough ER, including the perinuclear
cisterna.

In the few eosinophils present, reaction product
was found in some but not all of the granules, with
the exact number of reactive granules varying from
cell to cell.

CONTROLS: No reaction product was seen in
intact exudate cells or in fractions incubated up to
90 min without substrate After 90 min, there was
an increasing (nonspecific) background of fine lead
phosphate precipitate within the cells.

PMN Fractions

BIOCHEMICAL RESULTS: The biochemical
results were identical to those reported earlier (6,
7). As mentioned, fractions A, B, C, and D corre-
spond to peaks of activity for peroxidase, alkaline
phosphatase, N-acetyl-β-glucosaminidase, and
acid p-nitrophenyl phosphatase, respectively.

SPECIAL STAINS: Fraction A contained many
large round or oval granules which stained reddish-
purple with azure A, thus confirming their azuro-
philic nature in that of azurophil or pri-
mary granules. With Wright's stain or with eosin,
these granules stained pink, since as already indi-
cated (4), they do not stain azurophilic in mature
PMN with the routine Wright's procedure. Fraction
B, in which specific granules are concentrated,
contained many granules which were smaller than
A granules, did not stain with azure A, and stained
pink with Wright's stain or eosin. Fraction C con-
tained relatively few granules. Some of those pres-
ent stained pink with Wright's stain or eosin, while
others, which were relatively small, were azuro-
philic with azure A. No granules were seen in frac-
tion D with any of the staining procedures.

400 g PELLET: The pellet obtained after ini-
tial centrifugation of the total homogenate (400 g
for 10 min) contained primarily nuclei, most of
which were disrupted, and miscellaneous cell de-
bris (granules, primarily azurophils, mitochon-
dria, nuclear envelopes, vesicular profiles), as well
as fibrin, red blood cells, and intact PMN. It is of
interest that numerous undisrupted PMN were
always present, but we could not identify any in-
tact mononuclear cells, indicating that these ele-
ments are quite effectively disrupted by the homog-
ization procedure.

A FRACTION: The bulk of this fraction con-
ists of the large A or azurophil granules (5) (Fig.
7), which were most concentrated at the bottom
of the pellet. Miscellaneous cell debris (occasional

Figures 4 and 5 Mononuclear cell from a peritoneal exudate reacted for AcPase (90 min). The cell in
Fig. 4 contains a large phagosome (ph) and a number of monocyte azurophil granules which vary in size
(300–400 nm) and shape. Rounded (1), elongated (2), and dumbbell (3) forms are seen. The Golgi com-
plex (Gc) and some of the granules contain AcPase reaction product. Fig. 5 is an enlargement of the
Golgi region showing the localization of reaction product within Golgi cisternae (Gc) and granules
(arrow) to better advantage. The fact that many of the monocyte azurophils (a) are not reactive is
due presumably to their histochemical latency. As in the case of the PMN it is the smaller, less con-
densed immature forms that react (18). Nucleus (n); endoplasmic reticulum (er). Specimen preparation
as for Figs. 2 and 3. Fig. 4, X 20,000; Fig. 5, X 54,000

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Acid Phosphatase in PMN Granule Fractions

Farquhar et al.
Another mononuclear cell from a peritoneal exudate. AcPase reaction product is seen within a small azurophil granule (1) and within several large dense bodies (2-4) which correspond to secondary lysosomes, as well as in the cisternae of the Golgi complex (Gc). Several other small, monocyte azurophil granules (a) and structures (d) which could represent either unusually large azurophil granules or dense bodies are not reactive. Specimen preparation as for Figs. 2 and 3. X 40,000.

nuclei, red blood cells, pieces of granule-containing cytoplasm, membranous profiles, and aggregated chromatin) was also present. In addition, occasional eosinophil granules could be identified. The distribution of AcPase reaction product was very limited. In accordance with the findings in situ, most of the azurophil granules were not reactive, but reaction product was occasionally noted in eosinophil granules (Fig. 7, inset). It was also observed within circular profiles which have the same diameter as azurophil granules and which most probably represent "ghosts" of azurophils disrupted by the prolonged incubation at acid pH. A fine sprinkling of lead phosphate crystals was seen sticking to the outer leaflets of azurophil granule membranes and some (but not all) of the other elements present in the fraction. Such deposits were not found in the controls or in the B or C fractions. A result of this sort could obtain if the granule membrane were permeable to \( \beta \)-glycerophosphate and inorganic phosphate but not to lead ions, in which case inorganic phosphate would be released enzymatically inside the granules, but be trapped by lead only as it diffuses out. The fact that reaction product is not found on the outside of disrupted ghosts or of some of the smaller vesicular profiles present favors this interpretation. An alternative possibility is that the deposits are due to
A fraction, AcPase test. This fraction consists primarily of PMN azurophil granules. Most of the granules are intact and do not show AcPase reaction product owing to their histochemical latency (see Figs. 2 and 3). Reaction product is present within two membranous profiles (arrows) which most probably represent ghosts of disrupted azurophil granules and within an eosinophil granule (eo, inset). Lead phosphate crystals are also seen adhering to the outside of the membranous of intact azurophil granules. Such deposits could represent enzyme activity released from disrupted azurophil granules, and reabsorbed to the outside of intact azurophils, or could result from differential permeability of the membrane (see text). Note that many of the azurophils (ag') show a central denser core and a lighter periphery. Specimen incubated in pellet (90 min) in Gomori medium, pH 5.0, with β-glycerophosphate. × 80,000.
enzyme molecules that became adsorbed onto the membrane after disruption of azurophils during the separation or incubation procedures.

**B fraction:** The main components of the B fraction are the B or specific granules (Fig. 8), which were concentrated near the bottom of the pellet. As in the case of the A pellet, the top of the pellet contained a certain amount of cell debris (mitochondria, vesicular profiles, and chromadn). This pellet displayed the least cytochemically demonstrable AcPase activity of all the fractions. Reaction product was found only rarely and when present was located either inside membranous profiles (possibly representing the ghosts of azurophil granules or phagocytic vacuoles) or within dense bodies (Fig. 8).

**C fraction:** As already reported (6, 7), this fraction is quite heterogeneous and contains glycogen, large and small vesicles, smooth cisternae, specific granules, and other small granules of varied size and shape (Figs. 9-13). The granules were concentrated at the bottom of the pellet while the cisternal and vesicular elements were located at the top. Lead phosphate deposits indicative of AcPase activity were found in several different structures: (a) in smooth cisternae, (b) in small pleomorphic granules, (c) in larger dense bodies, and (d) in membrane-limited vacuoles. The smooth cisternae can be identified as Golgi cisternae based on their tendency to remain in stacks (Fig. 10) or networks (Fig. 12) and on the basis of the thickness of their membranes (greater than that of ER membranes). Moreover, there is little or no smooth ER in the starting cells. It should be emphasized that under the conditions studied, only some of each of these elements in a given preparation contained reaction product. As far as their source is concerned, the Golgi cisternae could come from either PMN or mononuclear cells, since AcPase reaction product was found in the Golgi complexes of both cell types in the starting material. In fact, there is some evidence that they come from both, since some of the Golgi cisternae remain stacked (Fig. 10), resembling those commonly seen in intact PMN, whereas others (Fig. 12) resemble those found in mononuclear cells. Of the remaining AcPase-positive elements, the small pleomorphic granules are similar in size (100–400 nm) and shape to the azurophil granules seen in mononuclear cells. In our material, no granules of this type were seen in the PMN of the starting exudate. The dense bodies appear to be secondary lysosomes again of mononuclear cell origin since such bodies were not found in PMN of the exudate.

**D fraction:** The D fraction consists primarily of vesicular profiles of various sizes, along with a few Golgi cisternae which often occur as single flattened cisternae. Very few granules are present. AcPase activity was very limited; it was seen occasionally within Golgi cisternae and within rare profiles which appeared to correspond (on the basis of size) to azurophil granule ghosts.

**DISCUSSION**

**Origin of the C Particles**

The purpose of these studies was to obtain further information on the nature of the C fraction previously isolated from rabbit peritoneal exudate cells by zonal sedimentation (6) and isopycnic centrifugation (7). This fraction consists of particles lower in density than those of the A and B fractions (with azurophil and specific granules, respectively), contains considerable activity for a number of acid hydrolases including acid phosphatase, and is morphologically quite heterogeneous. Our present goal was to establish the source of the C particles, and in particular, to determine whether they are derived from PMN or mononuclear cells.

Examination of the peritoneal exudates that constitute the starting material for the fractionation procedure confirmed the presence of significant numbers (2%) of mononuclear cells in the exudates, which contain primarily (97%) PMN. Upon examination of the pellets obtained after centrifugation of the initial homogenate at 400 g, we were unable to identify any intact mononuclear cells, which indicates that these elements were quite effectively disrupted by the homogenization procedure and thus, their components must be accounted for in the fractionation scheme. Moreover, such components would represent a reasonable source for the C fraction because, as is well known, monocytes (18) and macrophages (20, 21, 18) contain a whole spectrum of lysosomal elements. In addition, as recently reported by Nichols et al. (18), monocytes contain a population of lysosomal granules, the azurophils, which vary in size and shape and are generally smaller than PMN granules. Examination of the C fraction by light microscopy indicated that it contains some azurophil granules, smaller than PMN azurophils, and in the same size range as monocyte azurophil granules.

Electron microscopy confirmed the heterogeneity
Figure 8. B fraction, AcPase test. This fraction consists primarily of PMN specific granules (sg), of varying density, which are not reactive for AcPase. A number of the granules are partially disrupted as evidenced by their interrupted membranes and less homogeneous content (sg'). Very little AcPase reaction product is found in this fraction. The only reactive element in this field is a dumbbell-shaped dense body (d) or lysosome. Specimen incubated as in Fig. 7. X 30,000
This fraction is quite heterogeneous and contains small (re) and large (re') vesicular profiles, small specific granules (sg), smooth-surfaced cisternae corresponding to Golgi cisternae (Gc), dense bodies (d), small granules (gr) of various sizes and shapes, occasional mitochondria (m), and glycogen particles (not seen here). AcPase reaction product is present within several of these structures: (a) within the Golgi cisterna, (b) within a dense body (d), and (c) within some of the small pleomorphic granules (arrows). Specimen incubated as in Fig. 7. X 60,000.
Figures 10–13 Fields from the C fraction, showing the distribution of AcPase reaction product within Golgi cisternae (Gc), within a dense body (d), and within small pleomorphic granules (gr). The dense body and small granules resemble the secondary lysosomes and azurophil granules, respectively, of intact mononuclear cells (Figs. 4–6). The Golgi complex in Fig. 10 consists of 3–4, short, stacked cisternae such as those commonly found in intact PMN leukocytes. The Golgi complex in Fig. 12 consists of several more elaborate, elongated cisternae such as those commonly found in mononuclear cells. Note that the stacked membranes in Fig. 10 appear fused. Most probably fusion occurs during the fractionation procedure. Fig. 10, × 90,000; Fig. 11, × 75,000; Fig. 12, × 36,000; Fig. 13, × 60,000.
of the C fraction which consists of a variety of vesicular and granular elements (glycogen particles, large and small vesicles, Golgi cisternae, specific granules, dense bodies, and small pleomorphic granules). Cytochemical tests further demonstrated that the distribution of AcPase activity in the C fraction is heterogeneous being found in Golgi cisternae, small pleomorphic granules, and dense bodies. Comparison with the AcPase-positive structures in the starting material indicates that the Golgi elements are probably derived from both PMN and mononuclear cells, whereas the pleomorphic granules and dense bodies correspond to azurophil granules and secondary lysosomes, respectively, of mononuclear cells. It appears, therefore, that the bulk of the cytochemically detectable AcPase activity present in the C fraction is derived from mononuclear cells, since they contain elements comparable to those found in the C fraction whereas (except for the Golgi cisternae) PMN do not. Of special interest is the presence in this fraction of granules which resemble monocyte azurophil in their staining (azurophil), morphology (small, pleomorphic), and cytochemically detectable enzyme content (AcPase). These monocyte granules were recently characterized by electron microscopy and cytochemistry and shown to be primary lysosomes containing AcPase and aryl sulfatase activity in all species investigated (rabbit, guinea pig, and human) and peroxidase in some (guinea pig and human). The separation of a homogeneous azurophil granule population from monocytes now becomes a project of considerable interest for the future comparison of their enzyme content to that of the C fraction could explain the unusual spectrum of hydrolytic activities of the C fraction and will help provide definitive evidence for or against our interpretation of the present results.

Number of PMN Granule Types

There is at present agreement among workers in the field (1-10, 22, 25) concerning the existence of the two main types—azurophil or primary granules, and specific or secondary granules—in rabbit PMN. Their distinctive morphology (1, 2), separate origins (1, 2), and different nature, i.e. one lysosomal and the other nonlysosomal (4-7), are firmly established. Beyond this, Wetzel et al. (2, 3) have described a third or tertiary granule type in rabbit PMN. According to their findings, such granules appeared late in development, were smaller than the other two types, variable in shape, and contained AcPase. However, no such granules were observed by Bainton and Farquhar (1, 4, 5) or Miller (22), and they were not found in the PMN of the exudates used as starting material for these experiments.

As pointed out above, the cytochemical findings in situ and the biochemical results on fractions obtained by Baggiolini et al. (6, 7) are in close agreement as far as the azurophil and specific granules are concerned. However, the fractionation data left unresolved the question of the existence of a third granule type. Baggiolini and his coworkers suggested that the small pleomorphic granules found in the C fraction might correspond to the tertiary granules of Wetzel et al., but they also considered the possibility that such particles were derived from contaminating mononuclear cells.

The present data provide strong circumstantial evidence against this concept. Using sucrose density gradient fractionation, these authors have obtained three fractions from rabbit exudate PMN: (a) a band of large, heavy particles which contained the antibacterial proteins plus most of the acid mucopolysaccharides and myeloperoxidase, (b) a middle band of intermediate-sized particles containing most of the alkaline phosphatase, and (c) a lighter band with much of the p-nitrophenyl-phosphatase. All fractions contained acid hydrolases with the distributions varying from one enzyme to another. The authors concluded that their heaviest fraction corresponds to specific granules, a conclusion which is based largely on the staining of the fraction with routine Wright's stain and therefore, in our opinion, is not justified (see Special Stains section in Results). Moreover, examination of their supporting electron micrographs and distribution data for peroxidase and alkaline phosphatase suggests that their heaviest fraction consists largely of azurophil or primary granules and their intermediate fraction of specific or secondary granules. However, the inadequate basis of the azure A staining, the lack of computed enzyme recoveries, and the evidence that enzyme leakage occurred from damaged particles (as suggested by the very high amounts of soluble activities recovered in the loading zone) render further interpretation of their data difficult. The results of their fractionation of rabbit bone marrow (24) are even more difficult to interpret due to the multiplicity of cell types present in the starting material (e.g., mononuclear cells, eosinophils, megakaryocytes, and reticuloendothelial cells as well as PMN).
evidence that the small granules in question are azurophil granules derived from mononuclear cells. Accordingly, the existence of the C fraction can no longer be considered as supportive evidence for the existence of a third type of PMN granule. As pointed out earlier (5), it is impossible to rule out the existence of heterogeneity among the two main PMN granule types on the basis of evidence currently available. However, the existence of a special class of tertiary granules (2, 3, 25) is open to question since it has not been generally confirmed and since the evidence is limited in extent and not beyond doubt technically.

In summary, the existence of azurophil and specific granules with the general characteristics described above is established beyond reasonable doubt, but the existence of more than two kinds of PMN granules (or subtypes of these) lacks convincing supporting evidence.

Comments on the AcPase Reaction

Our observations have confirmed a number of points reported previously (5, 19) concerning the histochemical latency of the enzyme activity present in PMN granules to demonstration by lead techniques: (a) intact, mature PMN azurophil granules are not reactive; (b) granules must be disrupted (e.g., by freezing and thawing, smearing, or prolonged incubation at acid pH) to be activated; (c) immature (not fully condensed) PMN azurophils are reactive; and (d) eosinophil granules and monocyte azurophil granules (18) exhibit the same latency phenomenon, but are more readily activated than PMN azurophils, i.e., they do not require such extensive disruption for activation.

The histochemical latency, like the biochemical latency, could be due to lack of permeability of the granule membrane to one or more of the components of the incubation medium (5, 19). As mentioned above, in the case of glutaraldehyde-fixed, isolated azurophils, this impermeability could be restricted to the lead ions, accounting for the appearance of lead phosphate deposits on the outer face of the membrane.

Finally, the finding of AcPase in Golgi and ER (perinuclear) cisternae of some exudate PMN should be mentioned. A similar localization of AcPase activity was observed previously in developing PMN (5) in which it was presumed to be associated with secretion. However, there is no clear explanation for the presence of reactive Golgi in mature PMN from exudates. This situation requires further investigation.

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REFERENCES

1. Bainton, D. F., and M. G. Farquhar. 1966 Origin of granules in polymorphonuclear leukocytes. Two types derived from opposite faces of the Golgi complex in developing granulocytes. J. Cell Biol. 28:277.

2. Wetzels, B. K., R. G. Horn, and S. S. Spicer 1967 Fine structural studies on the development of heterophil, eosinophil, and basophil granulocytes in rabbits. Lab. Invest. 16:349.

3. Wetzels, B. K., S. S. Spicer, and R. G. Horn 1967 Fine structural localization of acid and alkaline phosphatases in cells of rabbit blood and bone marrow. J. Histochem. Cytochem. 15:311.

4. Bainton, D. F., and M. G. Farquhar. 1968 Differences in enzyme content of azurophil and specific granules of PMN leukocytes. I. Histochemical staining of bone marrow smears. J. Cell Biol. 39:286.

5. Bainton, D. F., and M. G. Farquhar. 1968 Differences in enzyme content of azurophil and specific granules of polymorphonuclear leukocytes. II. Cytochemistry and electron...
microscopy of bone marrow cells. *J. Cell Biol.* 39:299.

6. Baggiolini, M., J. G. Hirsch, and C. de Duve. 1969. Resolution of granules from rabbit heterophil leukocytes into distinct populations by zonal sedimentation. *J. Cell Biol.* 40:529.

7. Baggiolini, M., J. G. Hirsch, and C. de Duve. 1970. Further biochemical and morphological studies of granule fractions from rabbit heterophil leukocytes. *J. Cell Biol.* 45:386.

8. Dunn, W. B., J. H. Hardin, and S. S. Spicer. 1968. Ultrastructural localization of myeloperoxidase in human neutrophil and rabbit heterophil and eosinophil leukocytes. *Blood.* 32:935.

9. Hardin, J. H., and S. S. Spicer. 1971. Ultrastructural localization of dialyzed iron-reactive mucin in rabbit heterophils, basophils, and eosinophils. *J. Cell Biol.* 48:368.

10. Baggiolini, M., C. de Duve, P. L. Masson, and J. F. Heremans. 1970. Association of lactoferrin with specific granules in rabbit heterophil leukocytes. *J. Exp. Med.* 131:559.

11. Farquhar, M. G., D. F. Bainton, M. Baggiolini, and C. de Duve. 1971. Number of granule types in rabbit PMN granulocytes. Proceedings of the 11th Annual Meeting of the American Society for Cell Biology. 90.

12. Smith, R. E., and M. G. Farquhar. 1966. Lysosome function in the regulation of the secretory process in cells of the anterior pituitary gland. *J. Cell Biol.* 31:319.

13. Anderson, P. J. 1967. Purification and quantitation of glutaraldehyde and its effect on several enzyme activities in skeletal muscle. *J. Histochem. Cytochem.* 15:652.

14. Hirsch, J. G. 1956. Phagocytosis: A bactericidal substance from polymorphonuclear leukocytes. *J. Exp. Med.* 103:539.

15. Barka, T., and P. J. Anderson. 1962. Histochmical methods for acid phosphatase using hexazonium pararosanilin as coupler. *J. Histochem. Cytochem.* 10:741.

16. Rockwell, A. F., P. Norton, J. B. Caulfield, and S. I. Roth. 1966. A silicone rubber mold for embedding tissue in epoxy resin. *Sci. Tools.* 13:19.

17. Wanson, J., and L. Tielemans. 1971. Morphological and biochemical characteristics of glycogen particles isolated from rabbit polymorphonuclear leukocytes. *J. Cell Biol.* 49:816.

18. Nichols, B. A., D. F. Bainton, and M. G. Farquhar. 1971. Differentiation of monocytes. Origin, nature, and fate of their azurophil granules. *J. Cell Biol.* 50:498.

19. Seeman, P. M., and G. E. Palade. 1967. Acid phosphatase localization in rabbit eosinophils. *J. Cell Biol.* 34:754.

20. Corn, Z. A., M. E. Fedoreko, and J. G. Hirsch. 1966. The in vitro differentiation of mononuclear phagocytes. V. The formation of macrophage lysosomes. *J. Exp. Med.* 123:737.

21. Corn, Z. A. 1966. The structure and function of monocytes and macrophages. *Adv. Immunol.* 9:163.

22. Miller, F. 1966. Electron microscopic cytochemistry of leucocyte granules. In Sixth International Congress for Electron Microscopy, Kyoto, Japan. R. Uyeda, editor. Maruzen Co., Ltd., Tokyo. 2:71

23. Zeya, H. I., and J. K. Spitznagel. 1971. Characterization of cationic protein-bearing granules of polymorphonuclear leukocytes. *Lab. Invest.* 24:229.

24. Zeya, H. I., and J. K. Spitznagel. 1971. Isolation of polymorphonuclear leukocyte granules from rabbit bone marrow. *Lab. Invest.* 24:237.

25. Wetzler, B. K. 1970. The fine structure and cytochemistry of developing granulocytes with special reference to the rabbit. In Regulation of Hematopoiesis. A. S. Gordon, editor. Appleton-Century-Crofts, New York. 2:769.

26. Bainton, D. F., J. L. Ulliyot, and M. G. Farquhar. 1971. The development of polymorphonuclear leukocytes in human bone marrow. Origin and content of azurophil and specific granules. *J. Exp. Med.* 134:907.