RESOLUTION OF GRANULES FROM RABBIT HETEROPHIL LEUKOCYTES INTO DISTINCT POPULATIONS BY ZONAL SEDIMENTATION

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ABSTRACT

Postnuclear supernates from homogenates of essentially pure rabbit heterophil leukocytes were fractionated by means of zonal differential centrifugation through a discontinuous sucrose gradient at various speeds. Three distinct groups of granules were characterized biochemically and morphologically. They were, in order of decreasing sedimentation coefficient: (*a*) Large, relatively dense granules, identified morphologically as the azurophil or primary granules, and containing essentially all of the myeloperoxidase activity of the preparations, about one-third of their lysozyme activity, and between 50 and 80% of their content in five acid hydrolases typically associated with lysosomes in other cells; (*b*) smaller, less dense granules, with the morphological appearance of the specific or secondary granules, and carrying most of the alkaline phosphatase and the remainder of the lysozyme activity of the preparations; (*c*) a second group of lysosome-like particles, associated with a morphologically heterogeneous fraction, and containing the remainder of the acid hydrolases, but little or no myeloperoxidase. When *p*-nitrophenyl phosphate was used instead of β-glycerophosphate for the assay of acid phosphatase, only small proportions of the total activity accompanied the two main lysosomal bands, and considerable activity was found in a zone slightly retarded with respect to the slowly moving band of acid hydrolases.

INTRODUCTION

Particulate fractions from heterophil leukocytes were first isolated in 1960 by Cohn and Hirsch (9), who found them to possess many properties characteristic of lysosomes. Other investigations by the same authors have shown that leukocyte granules fuse with phagocytic vacuoles and that the resulting release of their contents into these vacuoles initiates changes, believed to be of digestive nature, of the phagocytized materials (10, 16, 17, 33). Similar observations have since been made on numerous other cell types (for a review, see reference 13), and the concept that the leukocyte granules are typical lysosomes, involved mainly in the intracellular digestion of the materials engulfed by these phagocytic cells, has been widely accepted.

However, granule preparations from heterophil leukocytes contain, in addition to the regular lysosomal acid hydrolases, several components not usually found in the lysosomes of other cells, for instance peroxidase (31), alkaline phosphatase (9), and a number of cationic proteins having bactericidal properties (15, 32). Furthermore, there is good morphological evidence that at least two distinct types of granules (2, 6, 24, 28, 29) or possibly more (29) are present in heterophil leukocytes. Attempts at subfractionating the granules by various centrifugal procedures have met so

far with limited success, but they have at least provided strong indications that the preparations may be biochemically, as well as morphologically, heterogeneous (23, 27).

In the present investigation, preparations of rabbit heterophil leukocytes were fractionated by zonal differential centrifugation through a discontinuous sucrose gradient to yield two relatively homogeneous fractions, clearly resolved from each other and having distinct morphological and biochemical properties. A third, more heterogeneous fraction, containing at least one additional group of particles, was isolated also.

MATERIALS AND METHODS

Fractionation Techniques

Peritoneal exudates were obtained from rabbits as described by Hirsch (15). The collected exudates were checked by differential count in stained smears and found to contain more than 98% heterophil leukocytes. Exudates with less than 3×10^6 cells per ml or contaminated by large numbers of erythrocytes were discarded. The cells were separated and homogenized in 0.34 M sucrose by the procedure of Cohn and Hirsch (9). Intact cells, gross debris, erythrocytes, and nuclei were removed by centrifugation for 10 min at 400 *g*, and the resulting supernate was fractionated by zonal differential sedimentation through a discontinuous sucrose gradient in a B-XIV rotor (1) operated by a Spinco Model L ultracentrifuge (Spinco Div., Beckman Instruments, Inc., Fullerton, Calif.). The gradient, resting on a 100 ml cushion of 60% (w/w) sucrose, extended from 4.4 to 6.15 cm radial distance, and from 0.45 to 0.80 M sucrose by 0.05 M increments. It was established by 35 ml portions of 0.45, 0.50, 0.55, 0.60, 0.65, and 0.70 M sucrose, followed by 45 ml each of 0.75 and 0.80 M sucrose. The preparation (20 ml in 0.34 M sucrose) was sandwiched between the gradient and a 230 ml overlayer of 0.25 M sucrose. The rotor was filled and emptied, at a rate of 25–30 ml/min, while rotating at 2,300 rpm. All operations were conducted at 0°. The centrifugation conditions given in the protocols are those applied between filling and emptying. The speed of the rotor was checked with a stroboscopic flash (Strobotac Type 1531-A, General Radio Company, West Concord, Mass.).

Biochemical Assays

Protein (22), lysozyme (9), and glycosidases (7) were assayed according to published methods, which were found to give good results with leukocyte preparations. For the enzymes, 0.04–0.5% BRIJ-35 (Technicon Corporation, Ardsley, N. Y.) was included in the incubation mixture to release any latent activity.

Myeloperoxidase was determined automatically with a Technicon Autoanalyzer unit. In this method, the sample, pumped at a rate of 0.42 ml/min, is mixed with a substrate solution (1.60 ml/min) containing 10 parts of 0.1 M citric acid-Na citrate buffer pH 5.0, one part of 0.1% *o*-tolidine in ethanol, one part of 1.5 mM H_2O_2, and 0.05% BRIJ-35, segmented with air (0.80 ml/min) by means of a HO connector. Washing is done with 0.34 M sucrose pumped at a rate of 1.20 ml/min, and the sampling rate is 20 per hr, with a one-to-one sampling/washing ratio. The reaction mixture passes through a 105-87 glass mixing coil at 25° (incubation time about 2 min), and is then inactivated with 1.7 M H_2SO_4 (0.60 ml/min), added through a D1 connector into a 105-87 glass mixing coil. The mixture then enters a C5 debubbler, and is pumped through the flow cell of a Technicon colorimeter at the rate of 2.00 ml/min. Transmission is recorded on log paper, at 440 nm with a flow cell of 15 mm light path. A solvaflex pumping tube (Technicon) is used for the substrate solution. Glass tubing is used for the substrate line and for all connections up to the flow cell.

Alkaline phosphatase was also assayed automatically. The manifold, similar in design to the preceding one, had the following specifications: Sample (0.32 ml/min) is mixed with substrate solution (1.20 ml/min), segmented by air (0.80 ml/min). The substrate solution contains 1.5 mM *p*-nitrophenyl phosphate (disodium salt) and 0.05% BRIJ-35, in 0.1 M diethanolamine buffer pH 9.75. Washing (1.2 ml/min) is done with 0.05% BRIJ-35 in water The sampling rate is 30 per hr, with a two-to-one sampling/washing ratio. The incubation time, at 25°, is extended to about 15 min by means of two 105-87 glass mixing coils and a 4 mm (outer diameter) delay coil, and the reaction is then stopped with 2 N NaOH (0.8 ml/min.). After debubbling, the mixture is pumped through a 15 mm flow cell at the rate of 1.6 ml/min. Transmission at 40 0nm is recorded on log paper. A 5 μM solution of *p*-nitrophenol in 10 mM NaOH is used as standard.

In a number of experiments, acid phosphatase was measured in exactly the same manner, with a substrate solution containing 5 mM *p*-nitrophenyl phosphate, 0.2 M KCl, and 0.05% BRIJ-35, in 0.2 M acetate-acetic acid buffer pH 4.5. The automated assay with β-glycerophosphate as substrate worked out by Leighton et al. (22) could not be applied to the leukocyte gradient fractions owing to their low acid phosphatase activity. However, the sensitive inorganic phosphate method of Chen et al. (8) allowed the development of a reliable assay for acid β-glycerophosphatase activity. In this method,

incubation is carried out for 4–6 hr at 37° in a total volume of 2 ml containing 0.05 M β-glycerophosphate and 0.05 M acetate-acetic acid buffer adjusted to pH 4.2. The reaction is stopped with 0.4 ml of 35% trichloracetic acid, the mixture is filtered, and 1 ml of the filtrate is mixed with 2 ml of the ascorbic acid-molybdate reagent of Chen et al. (8). The color is developed by a 20 min incubation at 45°, and the absorbance is read at 820 nm in a Zeiss (Carl Zeiss, Inc., N. Y.) spectrophotometer.

The methods described above were adopted as a result of kinetic experiments in which the effects of pH, substrate concentration, and other variables were investigated systematically for the determination of optimum assay conditions. Linearity over the incubation period adopted and proportionality with enzyme concentration were verified in all cases.

Morphological Techniques

0.5 ml samples from peak regions (see Fig. 4) were diluted with 3 ml of a mixture of glutaraldehyde and osmium tetroxide (18). After 10 min at 0° the samples were centrifuged into a pellet in the cold (10,000 *g* × 20 min). The pellet was suspended for 5 min in a fresh batch of mixed fixatives, and was then washed, exposed to uranyl acetate, embedded in agar, and processed into Epon for electron microscopy, as described elsewhere (18). Sections from the top, middle, and bottom of each pellet showed similar morphology.

Materials

Reagents used in the present work were obtained from the following sources: Calbiochem (Los Angeles, California), *p*-nitrophenyl phosphate (disodium salt 5 H_2O, A grade); Matheson, Coleman and Bell (Norwood, Ohio), *o*-tolidine; Pierce Chemical Co. (Rockford, Illinois), *p*-nitrophenyl-N-acetyl-β-D-glucosaminide, *p*-nitrophenyl-α-D-mannopyranoside and *p*-nitrophenyl-β-D-galactopyranoside; Sigma Chemical Co. (St. Louis, Missouri), DL-β-glycerophosphate (grade I), phenolphthalein-β-glucosiduronic acid, and *p*-nitrophenol (crystalline, spectrophotometer grade). *Micrococcus lysodekticus* 4698 was obtained from the American Type Culture Collection (Rockville, Maryland).

RESULTS

Biochemical Results

Altogether 15 experiments were performed, with centrifugation speeds varying between 4,000 and 7,500 rpm maintained during 15 min. The results were very reproducible, and yielded distribution patterns of the type illustrated in Figs. 1–4.

In the interpretation of these results, it is best to focus first on the behavior of myeloperoxidase and alkaline phosphatase, since these enzymes gave essentially unimodal distributions, and could be disassociated from each other almost completely under suitably chosen experimental conditions. After centrifugation at 4,000 rpm, most of the peroxidase has reached the lower half of the gradient, beginning already to accumulate against the cushion; except for a tiny amount of activity, probably carried down with cytoplasmic clumps or granule aggregates, the alkaline phosphatase has only started sedimenting, forming a symmetrical zone in the upper half of the gradient (Fig. 1). After centrifugation at 6,500 rpm (Fig. 2), which corresponds to an approximately two-fold increase in centrifugal force (taking into account the force developed during filling and emptying of the rotor at 2,300 rpm), practically all of the peroxidase is packed against the cushion, whereas the alkaline phosphatase zone is now found to straddle the middle of the gradient; only very small amounts of the two enzymes remain in the starting zone, presumably as free molecules released from damaged particles.

These results establish the existence of two distinct populations of particles in mature heterophil leukocytes, characterized respectively by myeloperoxidase (A particles) and by alkaline phosphatase (B particles). It may be estimated from the median positions of the two groups with respect to the starting zone that the A particles sediment, on an average, about four times faster than the B particles in a centrifugal field. It is likely that this difference is due mostly to a difference of size. Therefore, the A particles must be about twice as large in diameter, or 8 times as large in volume, as the B particles. Broadening of the two zones in the course of sedimentation indicates a certain spread of sizes within each population, but this spread must be relatively restricted since separation of the two populations can be accomplished almost quantitatively by zonal sedimentation alone (Fig. 2).

Among the other enzymes assayed, lysozyme is the only one to show a peak coinciding with that of alkaline phosphatase. However, only about two-thirds of the total lysozyme activity seem to be associated with the B particles. The remainder comes down with the A particles, accompanied by such small amounts of alkaline phosphatase as to make it very unlikely that an agglutination artifact is involved. Thus, it appears that lysozyme has a true dual localization. It occurs predominantly in

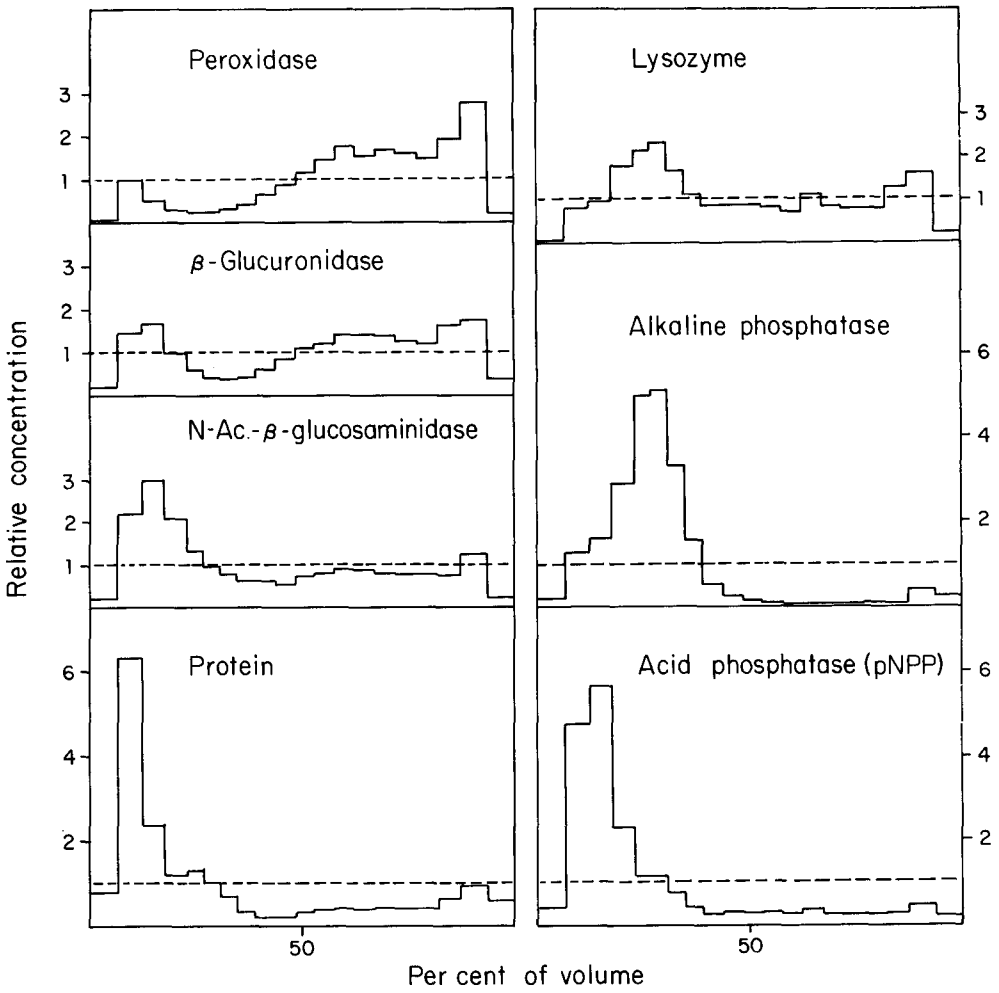


FIGURE 1 Fractionation of leukocyte granules by zonal differential centrifugation at 4,000 rpm for 15 min. Graphs are normalized distribution histograms as a function of the volume collected. Radial distance increases from left to right. Ordinate is concentration in fraction relative to concentration corresponding to uniform distribution throughout the gradient. Acid phosphatase was assayed with *p*-nitrophenyl phosphate (pNPP). Percentage recoveries were 105 for protein, 97 for myeloperoxidase, 92 for alkaline phosphatase, 86 for lysozyme, 87 for acid phosphatase, 88 for β-glucuronidase, and 89 for N-acetyl-β-glucosaminidase.

the B particles, but also, though in smaller amounts, in the A particles.

Looking now at the distributions of α-mannosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, and β-galactosidase, we see that large amounts of each of these four acid glycosidases sediment together with peroxidase, and are presumably associated likewise with the A particles. However, this association is not quantitative. It is of the order of 70–80% for α-mannosidase and β-glucuronidase, somewhat less for β-galactosidase, and only 50% for N-acetyl-β-glucosaminidase. The remainder of these activities spreads out into the upper part of the gradient in the form of a sloping edge adjacent to the starting zone containing soluble proteins. This distribution is particularly obvious for N-acetyl-β-glucosaminidase, but characterizes also the smaller amounts of the other glycosidases that do not sediment with the A particles. These glycosidase activities in the upper zone of the gradient reflect for the most part the presence of particles rather than of

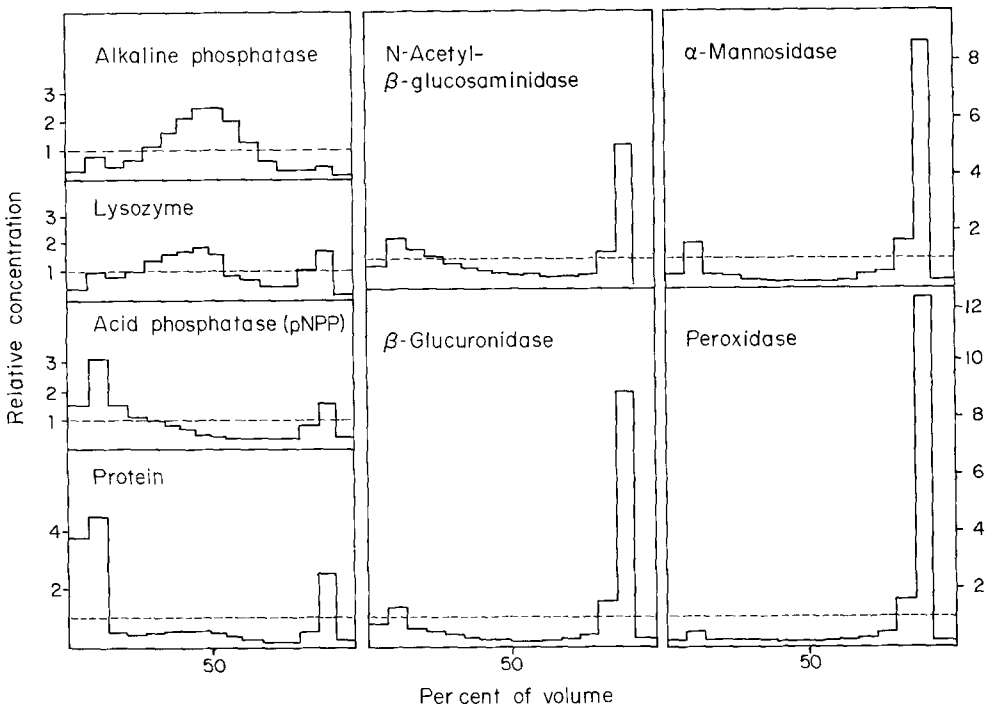


FIGURE 2 Fractionation of leukocyte granules by zonal differential centrifugation at 6,500 rpm for 15 min. Results represented as in Fig. 1. Acid phosphatase was assayed with *p*-nitrophenyl phosphate (pNPP). Percentage recoveries were 94 for protein, 82 for myeloperoxidase, 82 for alkaline phosphatase, 76 for lysozyme, 92 for acid phosphatase, 93 for β-glucuronidase, 86 for N-acetyl-β-glucosaminidase, and 100 for α-mannosidase.

soluble material, since high speed centrifugation of these fractions leaves only a very small proportion of the enzyme activities in the supernatant. These glycosidase-rich particles sediment much more slowly than do the B particles, and may therefore be taken to form a third population (C particles). Unfortunately, this population displays considerable heterogeneity in sedimentation coefficient, resulting in a fair amount of overlapping between their distribution and those of the B particles on one hand, and of the soluble enzymes arrested in the starting zone on the other. Thus, we cannot rule out entirely the possibility that the C particles may contain small amounts of lysozyme and alkaline phosphatase, or even of peroxidase, or that some glycosidase activity may be present in the B particles. However, the shapes of the observed distributions render these possibilities rather unlikely.

The distribution of acid phosphatase, determined with *p*-nitrophenyl phosphate as substrate, is surprisingly different from that of the glycosidases. Only some 10–15% of the total activity comes down with the A particles, and a much higher proportion is found in the upper part of the gradient, especially in or near the starting zone. In contrast, when β-glycerophosphate is used as substrate (Fig. 3), the distribution of acid phosphatase resembles much more closely that of the glycosidases, being very similar to that of N-acetyl-β-glucosaminidase. The reasons for this difference is under investigation. Present indications are that heterophil leukocytes contain at least two acid phosphatases, one of which accompanies the other acid hydrolases and has the usual properties of the lysosomal enzyme, whereas the other, attached to a slowly sedimenting component of microsomal type, rapidly hydrolyzes *p*-nitrophenyl phosphate, but has little or no activity on β-glycerophosphate.

Our results also give some idea of the amount of protein associated with each group of particles. As shown in Fig. 2, the maximum purification achieved for myeloperoxidase in the sediment con-

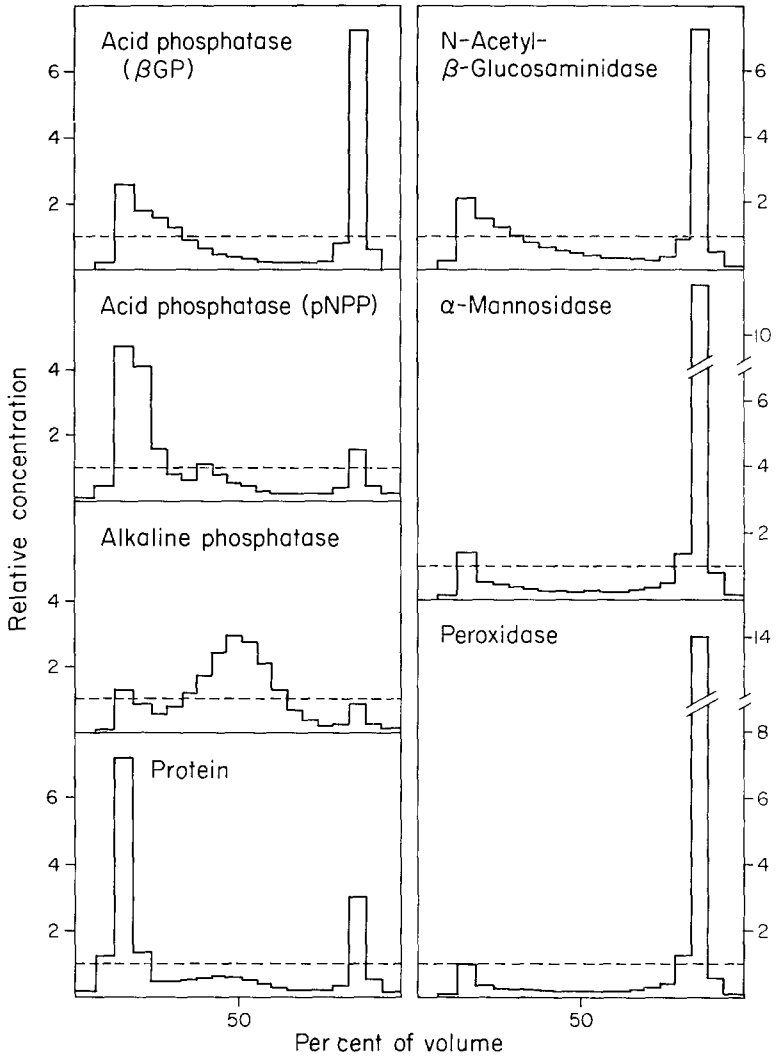


FIGURE 3 Fractionation of leukocyte granules by zonal differential centrifugation at 6,500 rpm for 15 min. Results represented as in Fig. 1. Acid phosphatase was assayed with *p*-nitrophenyl phosphate (pNPP) and β-glycerophosphate (βGP). Percentage recoveries were 83 for protein, 90 for myeloperoxidase, 79 for alkaline phosphatase, 80 for acid phosphatase (pNPP), 94 for acid phosphatase (βGP), 92 for N-acetyl-β-glucosaminidase, 92 for α-mannosidase.

taining the A particles is five-fold, and the area under the protein histogram in this region represents about 20% of the total area of the histogram. Unless this sediment is significantly contaminated by other particles, this finding indicates that the A particles contain about 20% of the total proteins of the extract. The protein histogram also shows a significant hump, corresponding to about 20% of the total proteins, under the alkaline phosphatase zone. But, in this case, there is some skewness on the left side due to the penetration of C particles and possibly of other slowly sedimenting components. Taking this fact into account, we may estimate that the B particles contain some 10–15% of the total proteins of the extract. The protein content of the C particles could be of the same order of magnitude, or it could be much lower, depending on the amount of other components contaminating the top gradient fractions. As indicated above, the A particles appear to be, on an

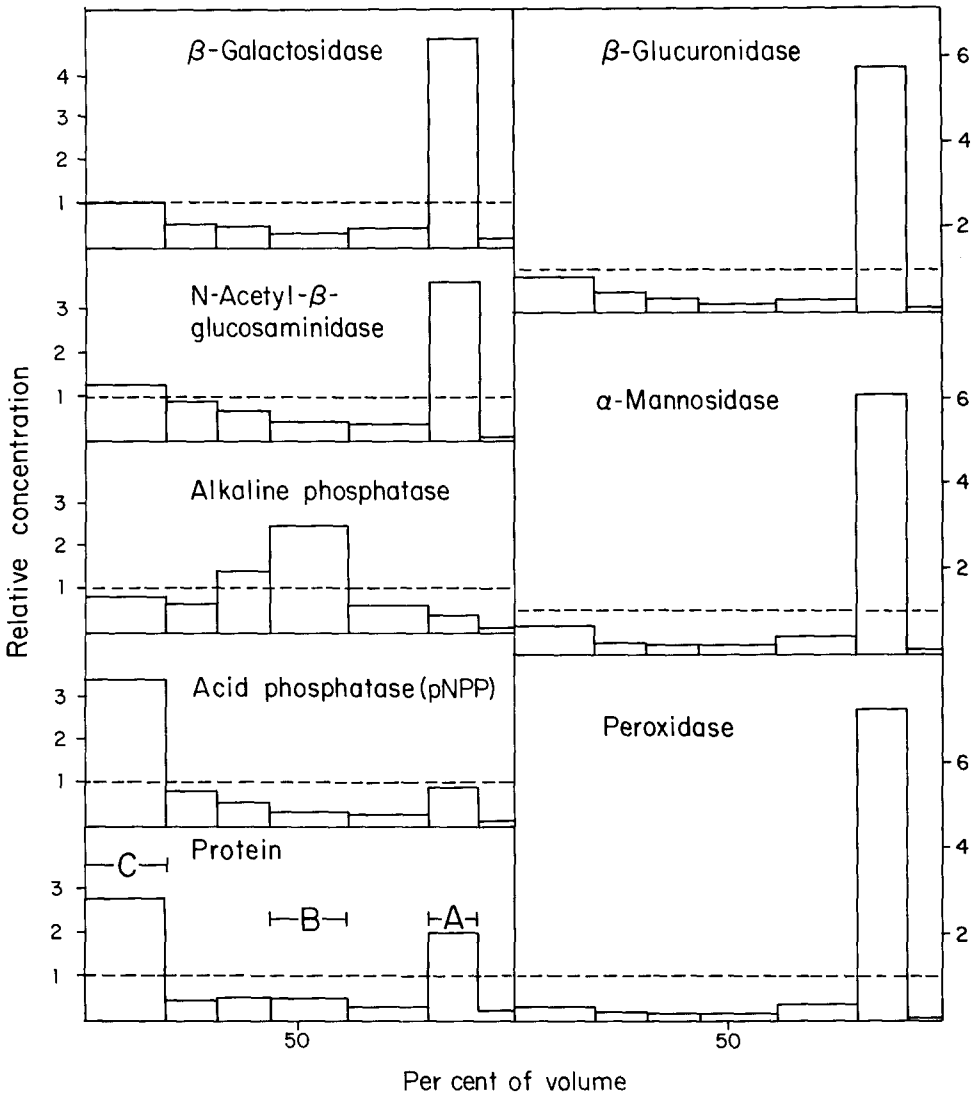


FIGURE 4 Fractionation of leukocyte granules by zonal differential centrifugation at 6,500 rpm for 15 min. Results represented as in Fig. 1. Acid phosphatase was assayed with *p*-nitrophenyl phosphate (pNPP). Fractions A, B, and C are those illustrated in Figs. 5–10. Percentage recoveries were 97 for protein, 107 for myeloperoxidase, 87 for alkaline phosphatase, 88 for acid phosphatase, 96 for β-glucuronidase, 83 for N-acetyl-β-glucosaminidase, 79 for α-mannosidase, and 80 for β-galactosidase.

average, eight times larger in volume than the B particles. If the total protein content of the latter is 50–75% that of the A particles, they should be 4–6 times more numerous than are the A particles, and give 2–3 times as many profiles as do the A particles in ultra-thin sections (since they are only half as wide in diameter). As will be shown below, these predictions are in agreement with the morphological observations.

Heterophil leukocyte granules can be separated also by isopycnic centrifugation in a sucrose gradient. The A and B particles equilibrate in two slightly overlapping bands, with modal densities of 1.26 and 1.22 respectively. The C particles form a somewhat broader band around a density of 1.20, whereas the major peak of acid *p*-nitrophenyl phosphatase activity occurs at a density of 1.14. These experiments, which are still being

pursued, will be described in a subsequent publication.

We have examined also the bactericidal action of some of our fractions. Preliminary studies show that citric acid extracts of the A and the B granule fractions are both highly active in killing various bacteria in vitro. These extracts appear furthermore to differ significantly in their relative activities on Salmonella and Staphylococcal organisms, thus suggesting that the antibacterial agents studied by Zeya and Spitznagel (32) are distributed in these two granule types in a specific manner.

Morphological Results

In Figs. 5–10 electron micrographs of fractions A, B, and C of Fig. 4 are shown. A micrograph of an intact cell fixed by the same method is shown at slightly lower magnification in Fig. 11 for comparison. Both A and B fractions are homogeneous, containing mostly relatively large particles surrounded by a membrane. The most striking difference between the two populations is one in size, the diameter of sharp circular profiles varying between 0.5 and 0.8 μm for the A particles, and between 0.3 and 0.5 μm for the B particles. In addition, the B particles appear to stain somewhat less intensely and to suffer manipulative damage more easily than do the A particles. These morphological properties, as well as the relative frequencies derived from centrifugation data, identify the A particles with the azurophil, or primary granules, and the B particles with the specific, or secondary granules.

The appearance of an unusual double membrane approximately 12 nm thick in the B particles after separation may well reflect alteration induced by sucrose or processing agents, since both primary (azurophil) and secondary (specific) granules are limited by typical 7 nm unit membranes after fixation of intact cells (see reference 2). The morphological difference between the membranes

FIGURE 5 shows elements present in the uppermost zone (fraction C of Fig. 4) after differential centrifugation of a leukocyte homogenate. This fraction is quite heterogeneous containing small opaque round or irregularly shaped granules, mitochondria, smooth and rough membranous components, and numerous vesicular structures varying widely in their internal content. × 24,000

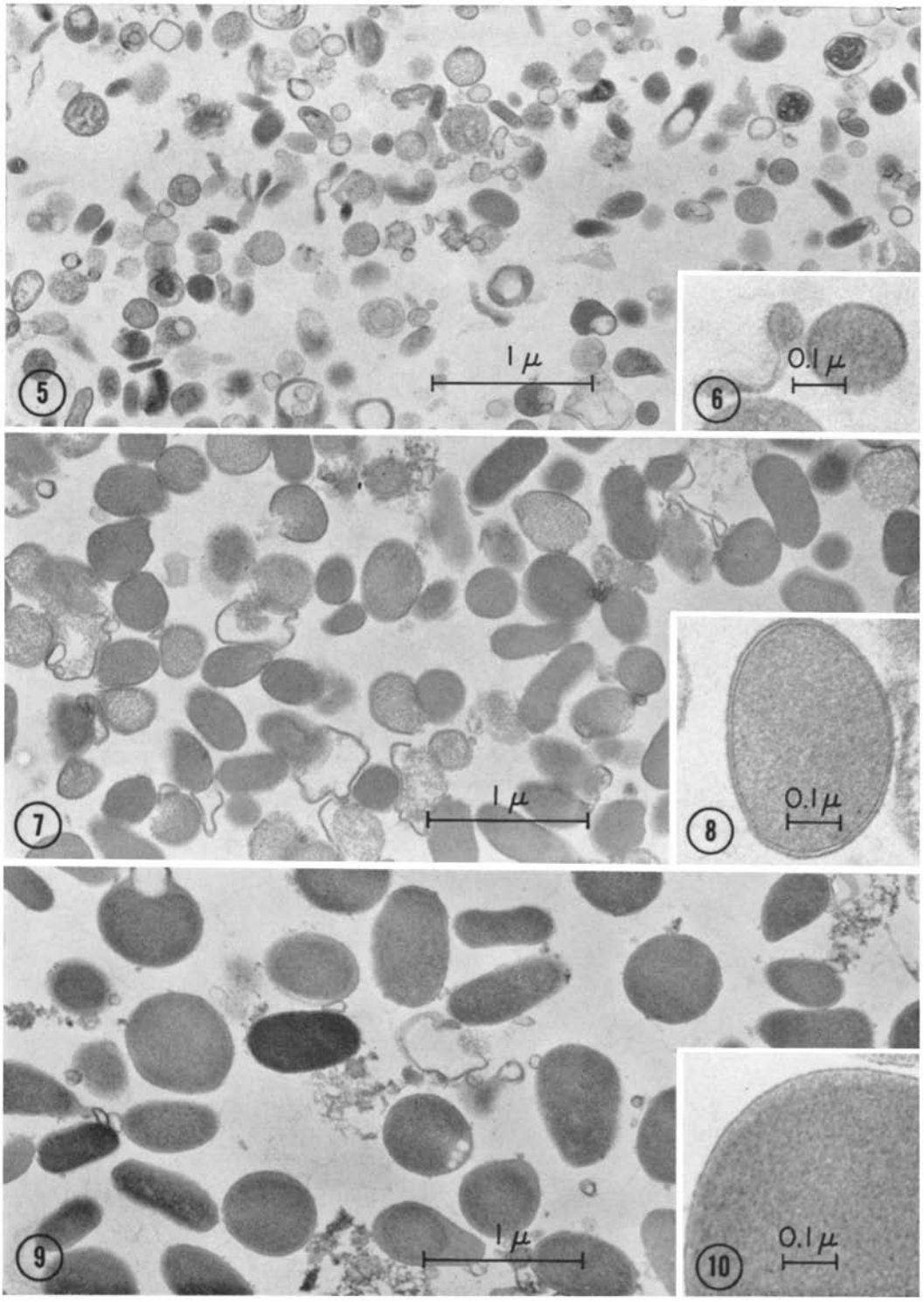
FIGURE 6 is a high power view of two of the small granules present in this upper fraction. × 80,000

FIGURE 7 shows the elements present in the mid-zone (fraction B of Fig. 4) after differential centrifugation of a leukocyte homogenate. The fraction contains granules ranging from 0.3 to 0.5 μm in diameter. Many of the granules appear to have been damaged or disrupted in the processing. Well-preserved granules show a homogeneous, moderately electron-opaque matrix and clearly defined limiting membranes. × 24,000

FIGURE 8 is a high power view of one of these midzone B granules. The membrane is composed of two dark bands and an intervening light zone with over-all dimensions of 12 nm. Since these specific or secondary granules exhibit typical 7 nm unit membrane in the intact cell (see reference 3), the appearance seen in the isolated granules may reflect alterations subsequent to exposure to sucrose or some other agent in the course of processing. The granule matrix at high power appears structureless. × 80,000

FIGURE 9 shows the elements present in the lower, most rapidly sedimenting portion (fraction A of Fig. 4) after differential centrifugation of leukocyte homogenates. The fraction consists predominantly of large granules (0.5 to 0.8 μm in diameter) which are quite electron-opaque. Some granules show a thin band of lesser opacity beneath the limiting membrane. × 24,000

FIGURE 10 is a high power view of a portion of one of these rapidly sedimenting A granules. A thin limiting membrane, a peripheral narrow light staining zone, and a homogeneous electron-opaque matrix are seen. × 80,000



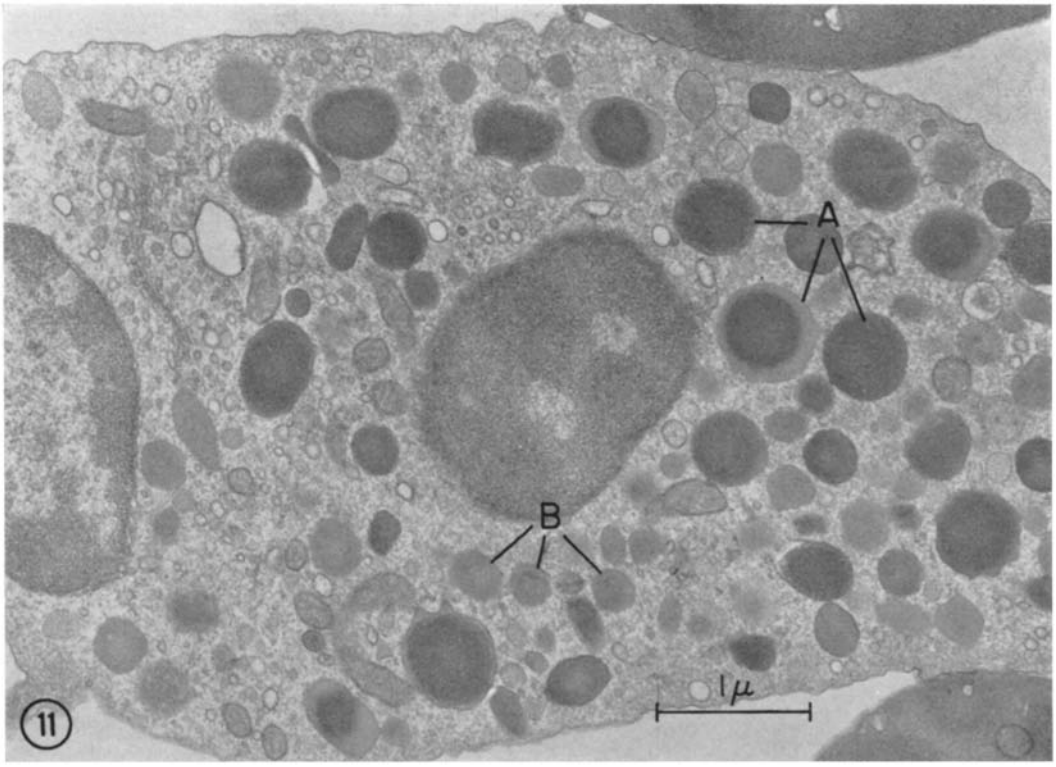


FIGURE 11 illustrates two types of granules in the cytoplasm of a rabbit blood heterophil leukocyte. The A granules (azurophil, primary) are approximately 0.6 μm in diameter and are quite electron-opaque; often they show a large dark core and a lighter thin peripheral zone. The B granules (specific, secondary) are smaller (diameter 0.3–0.4 μm) and show a uniform matrix that is generally less electron-opaque than that of the A granules. This particular section shows almost equal numbers of A and B granules, but as a rule many more B than A granules are present (see 11). Other organelles seen in the area between the two nuclear lobes include a poorly developed Golgi zone, a few small mitochondria, and scattered vesicular and vacuolar elements. × 20,000

limiting the A and B particles seen here is nevertheless of special interest in relation to the fact that the two granule types arise from opposite faces of the Golgi apparatus (2).

The C fraction is much more heterogeneous than the A and B fractions. It contains membranes, vesicular structures of variable size and content, small round or irregularly shaped dense particles, and mitochondria. This heterogeneity is not surprising since the starting material for centrifugal fractionation was a postnuclear supernate, containing cytoplasmic particles other than granules. Until better separations are achieved, identification of the C particles containing acid hydrolases and of the component bearing the greater part of the acid *p*-nitrophenyl phosphatase cannot be accomplished. It may, however, be pointed out that the small dense particles seen in the C fraction resemble the tertiary granules described by Wetzel et al. (29, 30) and shown by these authors to stain positively for acid phosphatase (with β-glycerophosphate as substrate).

DISCUSSION

It has long been known that heterophil leukocytes contain two main groups of granules which differ from each other by their size and staining properties, and also by their mode of formation and by the time at which they appear during the maturation of the cells (2, 29). In the present investigation, a relatively good separation of these two groups has been achieved, allowing accurate biochemical determinations on the two populations of granules.

According to our results, the azurophil or

primary granules contain substantial amounts of five acid hydrolases which are characteristic components of lysosomes in numerous other cell types. Thus they may be considered true lysosomes, probably of the primary type. They contain also significant amounts of lysozyme, which has been found in lysosomes in at least some other cells, for instance macrophages (11) and kidney cells (26). In addition, they include bactericidal agents and large quantities of peroxidase, which have not been found so far in other lysosomes. Peroxidase is known to be absent in alveolar macrophages (11) and we have failed to detect any peroxidase activity in the purified lysosomes isolated by Leighton et al. (22) from the livers of rats treated with Triton WR-1339.

It is always possible, in experiments of this sort, that two distinct species of granules may be separated together by the method used. The morphological homogeneity of the A fraction argues against this possibility. So do the results of our recent isopycnic centrifugation experiments, which indicate the same association of peroxidase with acid hydrolases in a fraction of relatively high density. The clinching argument is provided by the recent cytochemical and electron microscopical results of Bainton and Farquhar (3, 4), who, in complete agreement with our observations, have found that the azurophil granules stain positively for peroxidase and for six acid hydrolases. Further confirmation of these observations has been provided recently by Dunn et al. (14). There can be no doubt, therefore, that we are dealing here with a special kind of lysosome, having peroxidase in addition to the usual digestive system. The role of this peroxidase could be related to the special bactericidal activity of leukocytes, as indicated by the interesting results of Klebanoff (20, 21). In our kinetic experiments we have found that, with *o*-tolidine as substrate, myeloperoxidase shows a clear optimum of activity at pH 5.2. It is therefore well adapted to acting in the acid medium believed to prevail in secondary lysosomes or digestive vacuoles.

Our B fraction, containing the specific or secondary granules, is unfortunately not quite as pure biochemically as the A fraction, even though it appears relatively homogeneous morphologically. All that can be stated with certainty is that the specific granules contain essentially all the alkaline phosphatase activity of the preparations, in confirmation of the results of Wetzel et al. (30), and of Bainton and Farquhar (4), and considerable amounts of lysozyme, corresponding to about two-thirds of its total activity. Thus, the latter enzyme appears to be a true component of both the azurophil and the specific granules. Even our purest subfractions in the B region of the gradient contain detectable quantities of acid hydrolases. But, on the basis of the distribution patterns of these enzymes, we are inclined to conclude that the specific granules are devoid of acid hydrolases, and therefore do not qualify as lysosomes. This conclusion agrees with the cytochemical results of Wetzel et al. (30), but not with those of Bainton and Farquhar (4), who have found some specific granules to stain positively for acid phosphatase. However, these authors find little evidence for the presence of acid hydrolases in specific granules in experiments performed on mature leukocytes at the light microscope level (3). Our conclusions as well as those of other workers (4, 14, 30) fail to support the claim by Daems and van der Ploeg (12) that only the small, peroxidase negative granules of heterophil leukocytes are lysosomes. It is, of course, possible that the human leukocytes studied by these authors differ radically from rabbit leukocytes. But it appears more probable that their identification of lysosomes, which depends on staining with phosphotungstic acid after periodic acid bleaching, may not be reliable.

The distributions of the acid hydrolases indicate that heterophil leukocytes contain a third population of particles, apparently smaller and also more heterogeneous in size than the specific granules. Biochemically, these particles qualify as lysosomes. They differ from the azurophil granules by the virtual absence of peroxidase and by a higher content in acid β-glycerophosphatase and N-acetyl-β-glucosaminidase relative to their content in β-glucuronidase and α-mannosidase. They also contain little, if any, lysozyme. We have no decisive morphological identification of these small lysosomes, but it is tempting to assume that they correspond to the small, dense pleomorphic particles described by Wetzel et al. (29) as tertiary granules, and shown by these authors to stain positively for acid phosphatase (30). Particles resembling these tertiary granules were abundant in our C fraction.

In addition, the C fraction contains also a component, which sediments even more slowly than the small lysosomes, and is characterized by the acid phosphatase acting preferentially on

p-nitrophenyl phosphate, but showing little or no activity on β-glycerophosphate. This component which is separated more easily by isopycnic centrifugation thanks to its low equilibrium density in sucrose could be associated with some of the membrane structures seen in the C fraction. Its possible relationship to glucose 6-phosphatase, which is known to act on phenyl phosphate (5) is under investigation.

Finally, a word must be said about mitochondria. We have seen such particles only in the C fraction, as would be expected from their small size in rabbit heterophil leukocytes (see: Fig. 11). However, these cells contain very few mitochondria, and we have been unable to detect sufficient cytochrome oxidase in them to allow reliable assays for this enzyme in the gradient subfractions.

Several other workers have attempted to subfractionate leukocyte granules. The design of these experiments has not permitted unequivocal conclusions, but the results obtained in them are essentially in agreement with our own findings. Working with horse blood leukocytes fractionated by conventional differential centrifugation, Vercauteren (27) has found that peroxidase, alkaline phosphatase, and acid phosphatase tend, in this order, to sediment at increasing centrifugal forces. This is consistent with our results on the rabbit leukocytes, since this author used phenyl phosphate as a substrate for acid phosphate. In his studies on horse blood leukocytes, Ohta (23) has found, again in agreement with our results, that β-glucuronidase, and also acid ribonuclease and acid protease, sediment more rapidly than do alkaline phosphatase and lysozyme. Acid phosphatase, measured with *p*-nitrophenyl phosphate, showed an intermediary distribution, in contrast with Vercauteren's data (27) and with our own. Schultz and co-workers have combined differential centrifugation with density gradient centrifugation in sucrose (25) or Ficoll (19) gradients to subfractionate granule fractions from human white blood cells. In these experiments, peroxidase was largely concentrated in a fraction of high density, together with considerable amounts of acid phosphatase and β-glucuronidase; relatively high acid hydrolase activity occurred also in a fraction of lower density in which several mitochondrial enzymes were concentrated. Interpretation of these results is complicated by the heterogeneous cellular composition of the starting material and by lack of quantitative data on the distribution of the enzymes between the fractions isolated by the initial differential centrifugation procedure.

In our opinion, three important technical factors contributed to the satisfactory resolution achieved in the present experiments: (*a*) starting cell suspensions were in every instance composed of 98% or more heterophil leukocytes, thus eliminating the possibility that one or more of the particle population recovered might be derived from other cell types; (*b*) care was taken in homogenization and centrifugation procedures so that little granule breakage and no granule clumping occurred; (*c*) zonal sedimentation, minimally complicated by artifacts, allowed full advantage to be taken of the differences in size (and possibly density) between the various granule populations present in the starting preparations.

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BIBLIOGRAPHY

1. Anderson, N. G., D. A. Waters, W. D. Fisher, G. B. Cline, C. E. Nunley, L. H. Elrod, and C. T. Rankin, Jr. 1967. *Anal. Biochem.* **21**:235.
2. Bainton, D. F., and M. G. Farquhar 1966. *J. Cell Biol.* **28**:277.
3. Bainton, D. F., and M. G. Farquhar. 1968a. *J. Cell Biol.* **39**:286.
4. Bainton, D. F. and M. G. Farquhar. 1968b. *J. Cell Biol.* **39**:299.
5. Beaufay, H., and C. de Duve. 1954. *Bull. Soc. Chim. Biol.* **36**:1525.
6. Bessis, M., and J. Thiery. 1961. *Int. Rev. Cytol.* **12**:199.
7. Bowers, W. E., J. T. Finkenstaedt, and C. de Duve. 1967. *J. Cell Biol.* **32**:325.

8. CHEN, JR., P. S., T. Y. TORIBARA, and H. WARNER. 1956. *Anal. Chem.* **28:**1756.
9. COHN, Z. A., and J. G. HIRSCH. 1960. *J. Exp. Med.* **112:**983.
10. COHN, Z. A., and J. G. HIRSCH. 1960. *J. Exp. Med.* **112:**1015.
11. COHN, Z. A., and E. Wiener. 1963. *J. Exp. Med.* **118:**991.
12. DAEMS, W. TH., and M. VAN DER PLOEG. 1966. *In* Sixth International Congress for Electron Microscopy, Kyoto, Japan, 1966. R. Uyeda, editor. Maruzen Co. Ltd., Tokyo. **2:**83.
13. DE DUVE, C., and R. WATTIAUX. 1966. *Annu. Rev. Physiol.* **28:**435.
14. DUNN, W. B., J. H. HARDIN, and S. S. SPICER. 1968. *Blood.* In press.
15. HIRSCH, J. G. 1956. *J. Exp. Med.* **103:**589.
16. HIRSCH, J. G. 1962. *J. Exp. Med.* **116:**827.
17. HIRSCH, J. G., and Z. A. COHN. 1960. *J. Exp. Med.* **112:**1005.
18. HIRSCH, J. G., and M. E. FEDORKO. 1968. *J. Cell Biol.* **38:**615.
19. JOHN, S., N. BERGER, M. J. BONNER, and J. SCHULTZ. 1967. *Nature.* **215:**1483.
20. KLEBANOFF, S. J. 1967. *J. Exp. Med.* **126:**1063.
21. KLEBANOFF, S. J. 1968. *J. Bacteriol.* **95:**2131.
22. LEIGHTON, F., B. POOLE, H. BEAUFAY, P. BAUDHUIN, J. W. COFFEY, S. FOWLER, and C. DE DUVE. 1968. *J. Cell Biol.* **37:**482.
23. OHTA, H. 1964. *Acta Haematol. Jap.* **27:**555.
24. PAESE, D. C. 1956. *Blood.* **11:**501.
25. SCHULTZ, J., R. CORLIN, F. ODDI, K. KAMINKER, and W. JONES. 1965. *Arch. Biochem. Biophys.* **111:**73.
26. SHIBKO, S., and A. L. TAPPEL. 1965. *Biochem. J.* **95:**731.
27. VERCAUTEREN, R. E. 1964. *Enzymol.* **27:**369.
28. WATANABE, Y. 1957. *J. Electronmicroscopy.* **5:**46.
29. WETZEL, B. K., R. G. HORN, and S. S. SPICER. 1967. *Lab. Invest.* **16:**349.
30. WETZEL, B. K., S. S. SPICER, and R. G. HORN, 1967. *J. Histochem. Cytochem.* **15:**311.
31. WOODIN, A. M. 1962. *Biochem. J.* **82:**9.
32. ZEYA, H. I., and J. K. SPITZNAGEL. 1968. *J. Exp. Med.* **127:**927.
33. ZUCKER-FRANKLIN, D., and J. G. HIRSCH. 1964. *J. Exp. Med.* **120:**569.