

FURTHER BIOCHEMICAL AND MORPHOLOGICAL STUDIES OF GRANULE FRACTIONS FROM RABBIT HETEROPHIL LEUKOCYTES

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ABSTRACT

Fractionation of rabbit heterophil leukocyte homogenates by isopycnic centrifugation as well as by zonal sedimentation has helped to characterize further the particulate components of these cells. Four classes have been identified: (A) Large (0.5–0.8 μm) and dense (1.26) azurophil or primary granules, containing all the myeloperoxidase, one-third of the lysozyme, and a major proportion of the lysosomal acid hydrolase activities of the cells. (B) Smaller (0.25–0.40 μm) and less dense (1.23) specific or secondary granules, containing 90% of the alkaline phosphatase and the remainder of the lysozyme activities, but very little if any acid hydrolases. (C) Particles of low density (1.20), containing the remainder of the lysosomal acid hydrolases. This fraction was heterogeneous, but showed abundant small rod- or dumbbell-shaped particles of moderate electron opacity, surrounded by a single membrane (tertiary granules?). The possible origin of these lysosomes from contaminating macrophages could not be ruled out but appeared unlikely. (D) Slowly sedimenting material of very low density (1.14), made up of large, empty vesicular membrane structures, and containing 10% of the alkaline phosphatase, and all of a thiol-dependent acid *p*-nitrophenyl phosphatase, an enzyme clearly different from the lysosomal acid phosphatase.

INTRODUCTION

In a previous publication, we described the separation by means of zonal differential centrifugation of the two major classes of cytoplasmic granules found in rabbit heterophil leukocytes (2). The particles sedimenting most rapidly were identified morphologically as the azurophil or primary granules. They contained essentially all of the peroxidase activity of the cells, about one-third of their lysozyme activity, and a major part, varying from 50 to more than 90% depending on the enzyme, of the total cell content of a number of acid hydrolases known to be associated with lysosomes in other cells. The specific or secondary granules formed a more slowly sedimenting band,

which contained the bulk of the alkaline phosphatase and the remainder of the lysozyme activity of the cells, but only small amounts of acid hydrolases, probably belonging to contaminating particles. A third fraction, morphologically heterogeneous, was located in the region between the soluble proteins retained in the starting zone and the tail portion of the band containing the specific granules. This third fraction contained the remaining portion of the acid hydrolases and also an unusual acid phosphatase highly active on *p*-nitrophenyl phosphate, but having little or no activity on β -glycerophosphate. Better resolution and a more precise characterization of the compo-

nents of this third fraction has now been achieved based on the discovery of specific inhibitors for the two phosphatases and on the use of isopycnic density gradient centrifugation in addition to zonal sedimentation. The results of these new experiments are reported in the present paper.

MATERIALS AND METHODS

Fractionation Techniques

Rabbit heterophil leukocytes were obtained from glycogen-induced peritoneal exudates, homogenized, and fractionated by a preliminary centrifugation to yield a postnuclear supernate, exactly as described in our previous publication (2). The supernates were further fractionated, either by zonal differential sedimentation according to the procedure followed in our earlier experiments or by isopycnic density gradient centrifugation in the automatic rotor of Beaufay (5). The use of this rotor is described by Leighton et al. (11). In our experiments, 14 ml of starting material brought to a density of 1.10 by the addition of 60% (w/w) sucrose, were layered over a 19 ml sucrose concentration gradient extending linearly with respect to volume between densities 1.18 and 1.32; the gradient itself rested on a 6 ml sucrose cushion of density 1.32. Centrifugation was carried out at 35,000 rpm for 60 min. The distribution histograms of protein content and enzyme activities are presented as a function of the gradient volume for the zonal differential centrifugation and as a function of the equilibrium density for the isopycnic centrifugation experiments. The results were calculated and plotted with the help of a CDC 160 G computer (Control Data Corp., Minneapolis, Minn.) (11). The data were converted to distribution histograms following the method described by Beaufay et al. (7).

Biochemical Assays

Most of the biochemical assays are identical with those described previously (2). The protein content of each fraction was determined by the Lowry method (11), and should be considered as a reasonable estimate rather than an absolute value, since there may be differences in the relative reactivity of the proteins in the various fractions. The lysozyme assay was made more sensitive by adoption of the following conditions: 0.1 ml of enzyme was added to a cuvette (1 cm light path) containing 2.5 ml of a fresh suspension of heat-killed *Micrococcus leiodykticus* 4698 (American Type Culture Collection, Rockville, Md.) in 50 mM citric acid-phosphate buffer, pH 6.0, having an initial OD of 0.3 at 450 nm. After mixing, OD at 450 nm was recorded in a Cary 14 recording spectrophotometer (Cary Instruments, Monrovia, Calif.), full

scale being equivalent to 0.1 OD. The initial slope of the OD curve is proportional to enzyme concentration under these conditions. Glucose 6-phosphatase was determined according to de Duve et al. (10). The inorganic phosphate released in the assay of phosphatases was measured automatically in the trichloroacetic acid filtrate with the help of a Technicon Autoanalyzer unit (Technicon Corporation, Tarrytown, N. Y.). In this method, based on the procedure of Chen et al. (8), a solution of 0.42% ammonium molybdate·4H₂O, in 1 N H₂SO₄ (1.20 ml/min), segmented with air (1.20 ml/min), is mixed with a 10% ascorbic acid solution (0.23 ml/min) in a 105-87 glass mixing coil. The sample (0.23 or 0.80 ml/min) and water (0.80 or 0.23 ml/min) are then added to the reagents and mixed in a 105-83 glass mixing coil. The two lines for sample and water are interchangeable to allow a suitable adjustment of the assay sensitivity. The sampling rate is 50 per hr, with a two-to-one sampling-washing ratio. The reaction mixture is incubated for 15 min at 60° C in a 4 mm (outer diameter) glass delay coil. It 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 815 nm with a flow cell of 15 mm light path.

Morphological Examinations

The fractions were prepared for electron microscopy and examined as in our previous experiments (2).

RESULTS

Enzyme Activities and Inhibition of Acid Phosphatases

Table I summarizes the enzyme activities observed in homogenates subjected to fractionation in this and in our earlier work.

Table II illustrates the effect of inhibitors on the activity of *p*-nitrophenyl phosphatase and of β -glycerophosphatase, assayed in 0.04 M sodium acetate-acetic acid buffer, pH 4.5, on homogenates from rabbit heterophil leukocytes. Hydrolysis of β -glycerophosphate is completely suppressed by relatively low concentrations of fluoride and of L-(+)-tartrate, which, however, inhibit only slightly the hydrolysis of *p*-nitrophenyl phosphate. In contrast, sulfhydryl blocking agents inhibit the hydrolysis of *p*-nitrophenyl phosphate much more strongly than they do that of β -glycerophosphate.

These results indicate the presence of more than one acid phosphatase in the leukocyte homogenates, and can be explained entirely on the basis of two

TABLE I

Specific Activities of Enzymes in Homogenates of Rabbit Heterophil Leukocytes

Values give mean specific activity in milliunits per mg of protein \pm sd. When not specified, 1 unit of activity is defined as the amount of enzyme necessary to split 1 μ mole of substrate in 1 min under the conditions used (2).

Enzyme	Temperature	Specific activity	No. of experiment
Acid β -glycerophosphatase	37°	8.4 \pm 1.0	10
Acid <i>p</i> -nitrophenyl phosphatase	25°	65.5 \pm 9.0	10
Alkaline phosphatase	25°	403.0 \pm 21.0	10
<i>N</i> -Acetyl- β -glucosaminidase	37°	42.0 \pm 6.5	8
β -Glucuronidase	37°	1.0 \pm 0.4	8
α -Mannosidase	37°	18.0 \pm 4.0	5
Lysozyme	20°-22°	0.12 \pm 0.03*	3
Peroxidase	25°	2.45 \pm 0.22*	10

* The activities of lysozyme and peroxidase are given in arbitrary units.

Lysozyme units are defined as the amount of enzyme causing a decrease in OD of 1.0 in 1 min. Peroxidase units are defined as the amount of enzyme giving an OD value of 1.0 in 1 min under the experimental conditions described previously (see Reference 2).

TABLE II

Effect of Inhibitors on Activity of Acid Phosphatases of Rabbit Heterophil Leukocytes

Compound	Concentration	Inhibition	
		<i>p</i> -Nitro-phenyl-P	β -Glycero-P
	mM	%	%
KF	0.5	0	50
	2.0	4	100
	5.0	4	100
L-(+)-Tartrate	1.0	—	92
	5.0	7	100
<i>p</i> -Chloromercuribenzoate	0.002	46	—
	0.01	88	10
	0.04	90	22
	0.1	90	37
<i>N</i> -Ethylmaleimide*	4.0	96	0

* Enzyme incubated with *N*-ethylmaleimide for 10 min at 37° before addition of substrate.

distinct enzymes having the following properties. One enzyme, acting only on *p*-nitrophenyl phosphate and accounting for 93-96% of the total activity on this substrate, is unaffected by fluoride or tartrate and is very sensitive to *p*-chloromercuribenzoate, and especially to pretreatment with *N*-ethylmaleimide. The other, responsible for all of the activity on β -glycerophosphate and for 4-7%

of the activity on *p*-nitrophenyl phosphate, is completely inhibited by fluoride or tartrate, partially inhibited by high concentrations of *p*-chloromercuribenzoate, and totally resistant to *N*-ethylmaleimide under the conditions used. Since homogenates are almost eight times as active on *p*-nitrophenyl phosphate, at 25° C, as they are on β -glycerophosphate, at 37° C (Table I), we may take it that this second enzyme acts about equally well on the two substrates.

The possibility that an enzyme similar to the microsomal glucose 6-phosphatase of liver, which acts on phenyl phosphate, but not on β -glycerophosphate (6), may actually be responsible for most of the hydrolysis of *p*-nitrophenyl phosphate has been explored, with negative results. We have found that leukocyte homogenates split glucose 6-phosphate even more slowly than they do β -glycerophosphate, and that this activity is strongly inhibited by 5 mM KF. The fluoride-sensitive, unspecific acid phosphatase is most likely involved here, and there is no reason to believe that rabbit heterophil leukocytes contain any true glucose 6-phosphatase.

Zonal Differential Sedimentation

Fig. 1 shows the distribution of four enzymes and of protein in six zonal differential centrifugation experiments carried out as in the previous investigation but extended over a wider range of angular velocities. As a very rough approximation, the integrated centrifugal force applied to

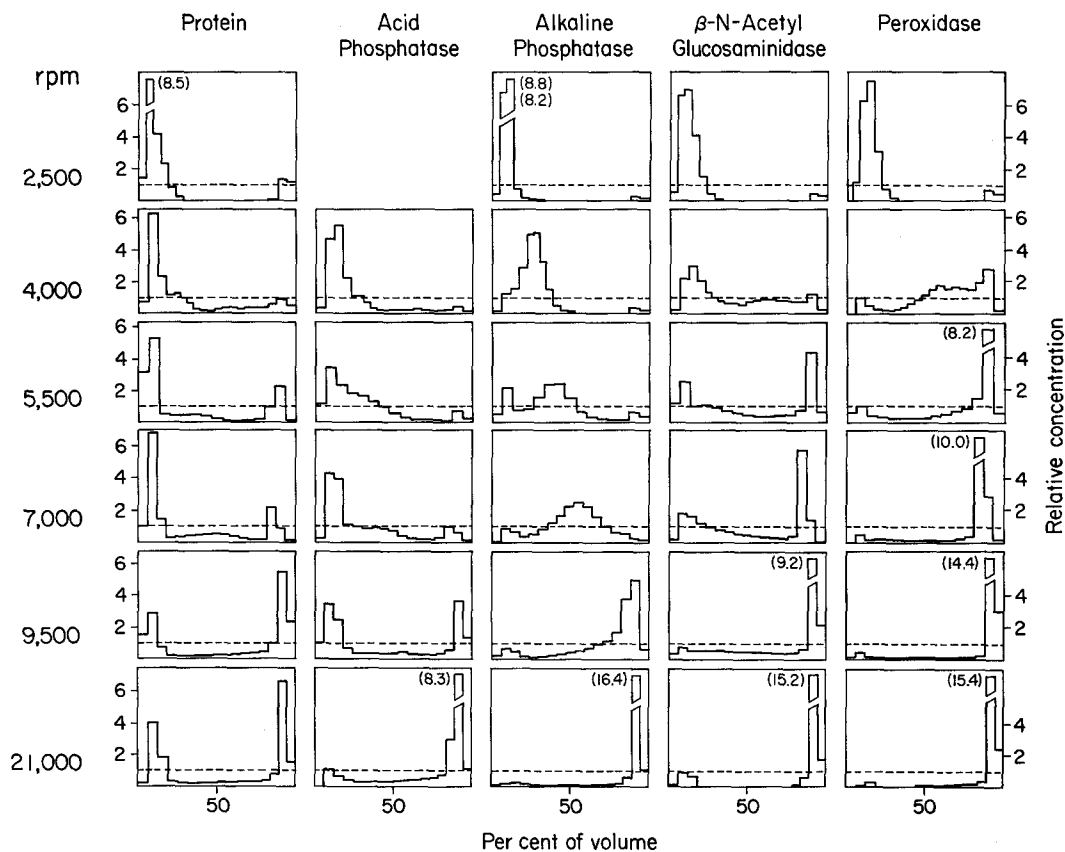


FIGURE 1 Fractionation of subcellular components of rabbit heterophil leukocytes by zonal differential centrifugation at different speeds (2,500–21,000 rpm) for 15 min. Graphs are normalized distribution histograms as a function of the volume collected. Radial distance increases from left to right. Ordinate is concentration in fraction relative to concentration corresponding to uniform distribution throughout the gradient. Acid phosphatase was assayed with *p*-nitrophenyl phosphate. Percentage recoveries (\pm sd) were 97.0 ± 15.0 for protein, 78.5 ± 6.5 for acid phosphatase, 89.3 ± 9.0 for alkaline phosphatase, 91.0 ± 2.9 for *N*-acetyl- β -glucosaminidase, 93.5 ± 4.0 for myeloperoxidase.

the particles may be taken to double with each increase in angular velocity, except at the last step, from 9,500 to 21,000 rpm, where the increase is about 4.5-fold. The four enzymes were selected as markers of the four zones that can be completely or partially resolved by differential sedimentation. The effect of centrifugal force is best shown by the position of the specific granules marked by alkaline phosphatase, which provides a convenient internal sedimentation standard in this type of experiment.

At 2,500 rpm, only the granules containing peroxidase show significant displacement from the starting zone, except for a small mixed sediment almost certainly made up of agglutinated particles.

The granules containing peroxidase are almost completely sedimented at speeds higher than 5,500 rpm, and they are best resolved from the other particles at 7,000 rpm. The association with these granules of about half the *N*-acetyl- β -glucosaminidase activity and of a small fraction of the acid *p*-nitrophenyl phosphatase activity is clearly apparent at this speed. Most of the remaining *N*-acetyl- β -glucosaminidase, together with an additional small fraction of the acid *p*-nitrophenyl phosphatase activity, is seen to be associated with a polydisperse group of particles, which lag behind the specific granules at 7,000 rpm, and follow in their wake at 9,500 rpm. Much of the acid *p*-nitrophenyl phosphatase is still close to the starting

zone at 9,500 rpm, but is almost completely sedimented at 21,000 rpm. A small proportion of the alkaline phosphatase behaves in the same way.

While confirming our earlier separation of the A (azurophil) and B (specific) granules, these results establish more clearly the presence of two distinct groups of particles in our former C fraction, which was found to be very heterogeneous morphologically (2). We can now distinguish in this fraction a polydisperse group of small particles containing acid hydrolases, henceforth to be referred to as C particles, and a more slowly sedimenting D group, uniquely characterized by its high content in acid *p*-nitrophenyl phosphatase. The D particles are largely sedimented at 21,000 rpm. Only traces of the measured enzymes, probably released

from damaged particles, remain in the starting zone after centrifugation at this speed.

Isopycnic Density Gradient Centrifugation

Figs. 2 and 3 show the enzyme distribution histograms obtained in two density equilibration experiments. Eight other experiments of this type gave closely comparable results.

Peroxidase equilibrates in a single band (A), with a modal density of 1.26. This band is skewed on the side of lower densities, but there is no clear evidence of a second peak for peroxidase.

Alkaline phosphatase forms a major peak (B) with a modal density of 1.23, and a secondary peak, containing about 10% of the total activity, and coinciding with the major peak of acid *p*-

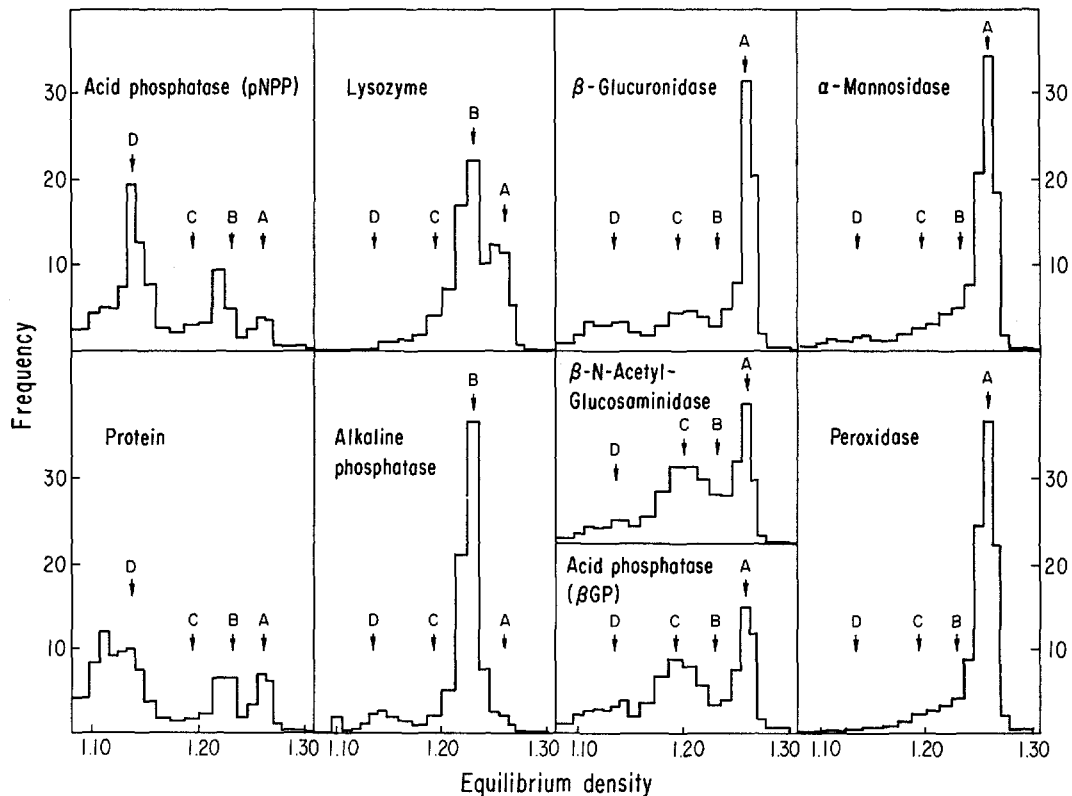


FIGURE 2 Isopycnic equilibration of subcellular components of rabbit heterophil leukocytes. Graphs are frequency distributions as a function of density. Acid phosphatase was assayed with *p*-nitrophenyl phosphate (pNPP) and β -glycerophosphate (β GP). Percentage recoveries were 11% for protein, 85 for acid phosphatase (pNPP), 82 for lysozyme, 85 for alkaline phosphatase, 88 for β -glucuronidase, 91 for *N*-acetyl- β -glucosaminidase, 120 for acid phosphatase (β GP), 91 for α -mannosidase, 92 for myeloperoxidase. Differential count of 500 cells gave 97.8% heterophils, and about 2% mononuclear cells, in the original exudate.

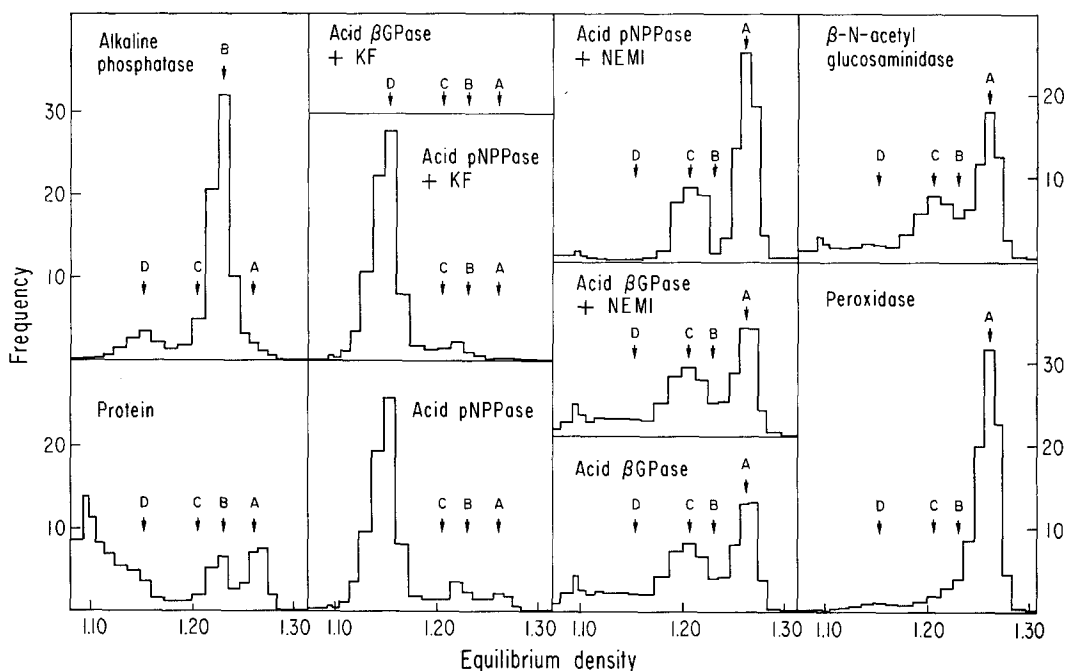


FIGURE 3 Isopycnic equilibration of subcellular components of rabbit heterophil leukocytes. Graphs are frequency distributions as a function of density. Acid *p*-nitrophenyl phosphatase and acid β -glycerophosphatase were both assayed without addition, in the presence of 2 mM KF, and after preincubation with 4 mM *N*-ethylmaleimide (NEMI). Percentage recoveries were 104 for protein, 83 for peroxidase, 75 for alkaline phosphatase, 102 for *N*-acetyl- β -glucosaminidase, 79 for acid *p*-nitrophenyl phosphatase, 75 for acid *p*-nitrophenyl phosphatase + KF, 88 for acid *p*-nitrophenyl phosphatase + NEMI, 103 for acid β -glycerophosphatase + NEMI. In the presence of KF, no acid β -glycerophosphatase activity was found. Differential count of 500 cells gave 96% heterophils, 2.5% lymphocytes, and 1.5% monocytes. Preparation showed some cell clumping.

nitrophenyl phosphatase activity (D). Since a similar association between the two phosphatases is found after zonal sedimentation (Fig. 1), it appears that the D particles contain alkaline as well as acid *p*-nitrophenyl phosphatase activity, in amounts that are about equivalent in absolute terms (see Table I). However, we cannot exclude the possibility that the alkaline phosphatase activity of the D fraction may belong to contaminating membrane fragments originating from disrupted B particles. There is evidence that these particles may be relatively fragile (2), and we have found that their disruption by freezing and thawing releases only negligible amounts of alkaline phosphatase in soluble form, suggesting a membrane localization of this enzyme.

After density equilibration, acid β -glycerophosphatase, *N*-acetyl- β -glucosaminidase, β -glucuronidase, and α -mannosidase are found in a sharp peak coinciding with that of peroxidase (A)

and, with the possible exception of α -mannosidase, in a second, slightly broader peak (C) with a modal density of 1.19–1.20. This second peak carries 40–50% of the total acid β -glycerophosphatase and *N*-acetyl- β -glucosaminidase activities, but only about 20% of the β -glucuronidase; it obviously contains the particles designated above as C and characterized by a relatively slow rate of sedimentation in the zonal centrifugation experiments presented in Fig. 1. The A and C bands of acid hydrolases overlap in the B region, which thereby contains small, but significant amounts of these enzymes. The possibility that some of the acid hydrolase activities found in the B region may actually belong to the B particles cannot be entirely excluded on the basis of these results, but the over-all shape of the distribution patterns, which show a minimum in the fraction with highest alkaline phosphatase activity, renders this possibility unlikely. A small proportion of the acid

hydrolases spreads from the starting zone to that occupied by the D particles, probably representing enzyme molecules released from damaged particles and entering the gradient either in soluble form or adsorbed to membrane fragments.

About 70% of the lysozyme equilibrates with the major alkaline phosphatase band, whereas the remainder is found in the peroxidase-rich zone. This distribution pattern confirms the dual localization of lysozyme in A and B particles, as found in zonal differential centrifugation experiments (2). In the C zone of the density distribution histograms, there is an excess of lysozyme over alkaline phosphatase. Since a similar excess of lysozyme activity over alkaline phosphatase was also observed in the upper zone of sedimentation histograms, this may indicate that the C particles contain small amounts of lysozyme.

The density distribution of acid *p*-nitrophenyl phosphatase shows a predominant peak (D) with a modal density of 1.14, accounting for 60–80% of the total activity and accompanied by only small proportions of other enzymes. The remaining acid *p*-nitrophenyl phosphatase activity is irregularly distributed in the gradient, with a small peak coinciding with the position of the A particles, and another, usually larger, situated between the B

and C peaks. As shown in Fig. 3, potassium fluoride, which inhibits completely the activity on β -glycerophosphate (Table II), leaves unaffected the major D peak but suppresses entirely the A peak and lowers somewhat the activity in the B and C regions. On the other hand, the activity remaining after preincubation with *N*-ethylmaleimide is distributed almost exactly like that of acid β -glycerophosphatase, which is unaffected by this treatment. These results thus confirm our interpretation of the inhibition data of Table II, and show, in addition, that the *N*-ethylmaleimide-sensitive acid *p*-nitrophenyl phosphatase is associated almost exclusively with the low density particles equilibrating in the D region. It may also occur in small amounts in particles with a density of 1.22. However, the size of this 1.22 peak was found to vary from experiment to experiment (see Figs. 2 and 3), and it was particularly large when an unusual degree of agglutination was observed. It could reflect an artifactual aggregation of some D particles with denser granules. The small portion of acid *p*-nitrophenyl phosphatase activity that is resistant to *N*-ethylmaleimide and sensitive to fluoride belongs to the unspecific acid phosphatase, revealed with β -glycerophosphate, which has the bimodal A-C

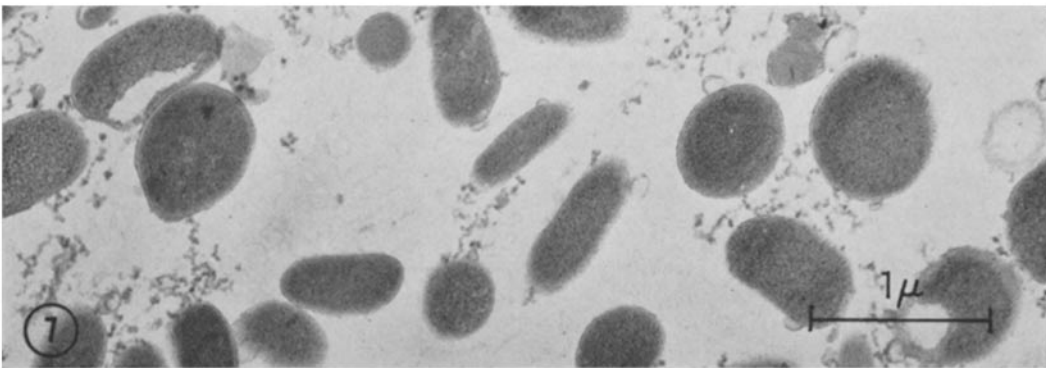
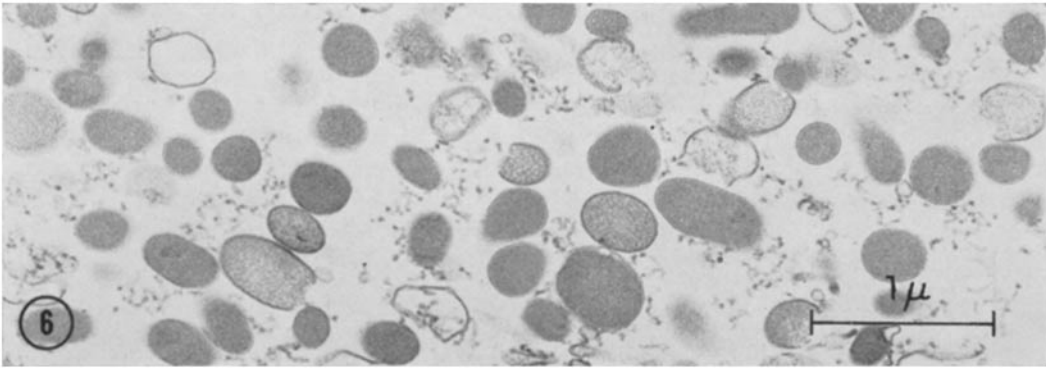
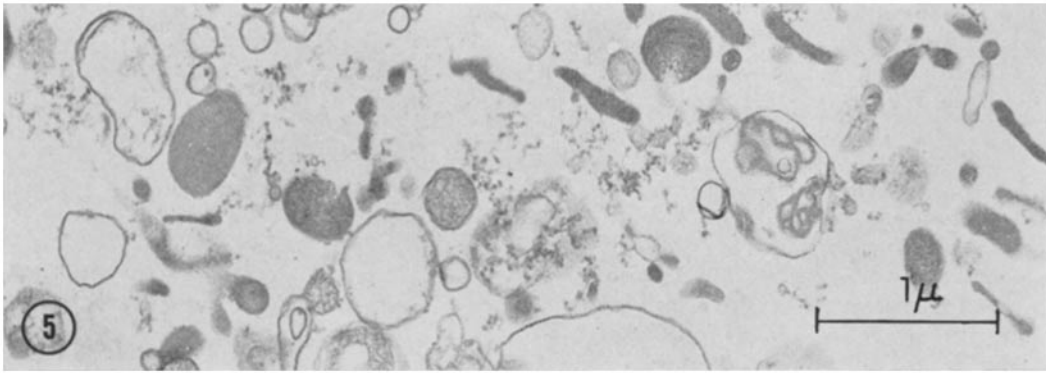
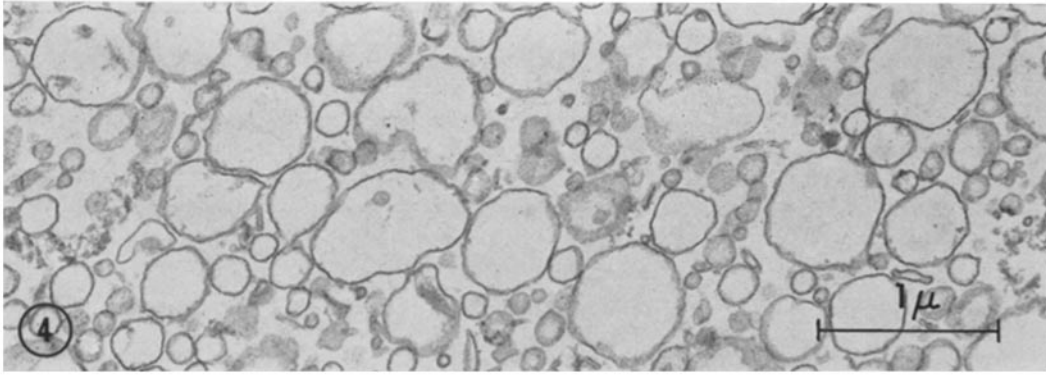
FIGURES 4–7 Electron micrographs of fractions separated by isopycnic centrifugation having the highest specific activity in a given marker enzyme (see Figs. 2 and 3).

FIGURE 4 Fraction D, rich in acid *p*-nitrophenyl phosphatase ($d = 1.14$). This fraction appears quite homogeneous, consisting almost exclusively of morphologically empty, smooth-surfaced vesicles of variable size. Oblique or grazing cuts through the membrane probably account for the fuzzy contour of many of the smaller profiles.

FIGURE 5 Fraction C, rich in acid β -glycerophosphatase ($d = 1.19$). This fraction is very heterogeneous. It contains vesicular structures of the type seen in fraction D, fairly large granules resembling those concentrated in fraction B, damaged mitochondria, and clusters of glycogen particles. The most conspicuous components not seen in other fractions are the small, rod- or dumbbell-shaped particles, surrounded by a membrane, with an electron-opaque, homogeneous matrix. The small ellipsoidal or circular profiles of similar opacity probably belong to the same particles sectioned more or less transversely.

FIGURE 6 Fraction B, rich in alkaline phosphatase, ($d = 1.23$). Except for clusters of glycogen particles and a few empty membrane profiles, this fraction is made up largely of round or oval granules, about 0.25–0.40 μm in diameter, surrounded by a distinct membrane, and containing a moderately opaque, slightly granular matrix. Several of these granules are disrupted and have partially released their contents.

FIGURE 7 Fraction A, rich in myeloperoxidase ($d = 1.26$). This fraction consists essentially of large, membrane-bounded granules, with a homogeneous, very electron-opaque matrix. The diameter of round profiles ranges between 0.5 and 0.8 μm . Glycogen is the only contaminant.



distribution typical of other acid hydrolases. There are slight differences between the distributions of the activities on the two substrates after *N*-ethylmaleimide treatment, but these may not be significant since the activities measured are very low.

The distribution of protein shows two distinct peaks, corresponding to the A and B particles, respectively. This is in agreement with the results of zonal sedimentation, which indicate that these two groups of particles account each for a significant fraction of the total proteins. In contrast, the amount of protein equilibrating in the C region is very low and shows no peak coinciding with that of the acid hydrolases. Therefore, the C particles can represent only a small fraction of the total protein. The amount of protein associated with the D particles is difficult to assess, in view of the unknown contribution of soluble proteins present in the D region. It is probably relatively small.

Morphological Results

Four fractions from an isopycnic centrifugation experiment were selected for morphological examination. Their position in the gradient is indicated in Figs. 2 and 3 by the letters A–D. Each corresponds to the peak of specific activity for one of the marker enzymes: peroxidase, alkaline phosphatase, acid β -glycerophosphatase, and acid *p*-nitrophenyl phosphatase. Electron micrographs of these four fractions are shown in Figs. 4–7.

The A and B fractions are identical in appearance to the corresponding fractions isolated by zonal sedimentation (2) except for a higher degree of contamination by clusters of glycogen particles. The A fraction is made up essentially of azurophil or primary granules, recognizable by their large size and electron-opaque contents, whereas the B fraction contains only the smaller, less opaque, and more fragile, specific or secondary granules. Sections through different levels of the pellets showed each fraction to be very homogeneous.

The C fraction is much more heterogeneous. It contains small, electron-opaque, rod- or dumbbell-shaped bodies surrounded by a single membrane, a few larger granules resembling those seen in fraction B, some damaged mitochondria, vesicular membrane structures similar to those found in fraction D, and glycogen granules. Except for the mitochondria, which hardly qualify as lysosomes, the only components that appeared to be concentrated in the C fraction are the small,

opaque particles. They were also present in large number in the C fraction separated by zonal sedimentation (2), and most likely represent the small lysosomes or C particles identified in these fractions by the biochemical measurements.

Fraction D is very homogeneous and contains exclusively smooth membranes in the form of morphologically empty vesicles of various sizes. These structures must be the bearers of the specific acid *p*-nitrophenyl phosphatase.

DISCUSSION

The results reported in this paper provide additional support to the main conclusions reached in our earlier investigations. They also help to clarify a few points that could not be settled by zonal sedimentation alone. The interpretation of the results has already been covered in the preceding section; the discussion will therefore be restricted to a summary of the properties that may be attributed with some measure of confidence to the four particle populations characterized in this work.

Azurophil or Primary Granules

These particles are both larger and denser than any other cytoplasmic particle found in the leukocytes and can thus be separated either by zonal differential sedimentation or by density equilibration. While the former technique gives a better resolution than the latter, it suffers from some contamination by agglutinated material. In our experiments, a sixfold purification of myeloperoxidase was regularly achieved in the best fraction isolated by isopycnic centrifugation, whereas zonal sedimentation never gave more than a fivefold purification for this enzyme. We must therefore lower somewhat our estimate of the total amount of protein associated with these granules, from 20 to about 15% of the proteins present in the starting material, which, it will be remembered, does not include the nuclei. The fractions isolated by either method are morphologically identical, except for the presence of glycogen particles in the isopycnic fractions. They also have the same biochemical properties, indicating that the azurophil granules contain essentially all of the myeloperoxidase, a part varying between 50 and more than 90% of the various lysosomal acid hydrolases that were assayed, and about one-third of the total lysozyme activity. All these conclusions are in perfect agreement with those derived from

cytochemical staining results (4, 12, 14). As mentioned before (2), the azurophil granules also contain bactericidal agents, but these have not yet been characterized in detail.

Specific or Secondary Granules

In agreement with our earlier results and with cytochemical observations, these particles are found to include at least 90% of the alkaline phosphatase activity and most of the lysozyme that is not present in the azurophil granules. It has recently been found that they are also the exclusive bearers of the lactoferrin present in rabbit heterophil leukocytes (1). They contain bactericidal agents which still await further characterization (2). Unfortunately, isopycnic centrifugation did not prove more efficient than zonal sedimentation as a means of separating the specific granules completely from the A and the C granules. The possibility, suggested by cytochemical staining results (4), that the specific granules may contain some acid phosphatase can therefore not be ruled out. However, the shape of the distribution patterns obtained by both separation techniques for this and the other acid hydrolases makes it clear that only traces of these enzymes could belong to the specific granules. As yet, we see no reason to amend our former conclusion that the specific granules do not qualify as true lysosomes.

As in the case of the azurophil granules, isopycnic centrifugation gave a somewhat better purification of the specific granules than did zonal sedimentation. Despite some contamination by neighboring particles, up to sixfold purification of alkaline phosphatase was reached in the B peak, thus confirming our earlier estimate that the specific granules account for 10–15% of the total proteins of the starting material.

Tertiary Granules

The existence of these granules as a second group of lysosomal particles is clearly confirmed by the present results, which show that the same characteristic partition of each acid hydrolase between the A and the C fraction is obtained with either of the separation techniques used. The C fraction isolated by isopycnic centrifugation is cleaner than that separated by zonal sedimentation, but it is still heavily contaminated, as shown by its heterogeneous morphological composition and by the flatness of the protein distribution in the C region.

The most likely bearers of acid hydrolases in the C fraction are the small, dense, rod- or dumbbell-shaped particles that are concentrated in this fraction by either technique. The other main components of the C fraction are either mitochondria or structures, such as large empty vesicles or specific granules, that are more abundant in neighboring fractions. Most probably, the C lysosomes correspond to the "tertiary granules" of Wetzel et al. (13, 14), which show a great similarity to the small dense bodies concentrated in our C fractions and were found by these authors to stain positively for acid phosphatase.

The significance of these tertiary granules is obscure. One possibility is that they represent secondary lysosomes or residual bodies that were functionally active during the developmental stages of the leukocytes, as opposed to the primary or azurophil granules which are believed to be simple storage forms or primary lysosomes that become active only after the onset of phagocytosis in the mature cells. However, there are no indications of lysosomal activity during leukocyte ontogenesis, and doubts have even been raised as to the existence of tertiary granules in rabbit heterophil leukocytes by Bainton and Farquhar (4), who have found no evidence of the occurrence of such granules in their material.

In view of these uncertainties, we have considered the alternative possibility that the C lysosomes may be derived, not from the leukocytes themselves, but from contaminating macrophages. According to our cell counts (see legends, Figs. 2 and 3), mononuclear phagocytes represent only 2% or less of the total cells in our exudates. However, since C particles appear to account for only a small proportion of the total protein in the homogenates, the possibility that they originate from macrophages cannot be ruled out simply on the basis of cell counts. It remains to be seen whether this possibility is compatible with the enzymic data. Cohn and Wiener (9) have analyzed rabbit peritoneal macrophages by means of techniques very similar to ours and have found them to contain about 10 milliunits/mg protein of acid phosphatase and 1.4 milliunits/mg protein of β -glucuronidase. On the other hand, the C particles, which contain about 50% of the total acid phosphatase and 20% of the total β -glucuronidase of our preparations, account for specific activities in the exudates of approximately 4 and 0.2 milli-

units/mg protein for acid phosphatase and β -glucuronidase, respectively (Table I). Thus, if the C particles belonged to macrophages, these cells should represent 40% of the total proteins of the exudates on an acid phosphatase basis, or 14% on a β -glucuronidase basis. These estimates are incompatible with each other as well as with the cell counts. However, this does not entirely settle the argument, since the enzyme levels of macrophages are known to vary within very wide limits. If, for instance, glycogen induced the presence in peritoneal exudates of macrophages resembling the BCG-induced alveolar macrophages studied by Cohn and Weiner (9), both enzymic activities would lead to comparable estimates of about 10% for the contribution of the macrophages to the exudate proteins. The protein content per cell of the macrophages need be only 5.5 times that of the leukocytes (or their diameter about 1.75 times that of the leukocytes) for this value to be compatible with a 2% contamination in terms of cell number. We are back in the realm of possibility, but we still have to assume in addition that the contaminating macrophages are quantitatively disrupted by the mild homogenization procedure used, and that their whole lysosome population is composed of very small particles having a low equilibrium density in sucrose gradients [1.19–1.20, as against 1.27 for the lysosomes from BCG-induced alveolar macrophages (9)]. In summary, we cannot draw any firm conclusions as to the structure and source of the C particles, but it seems unlikely that they are derived from macrophages.

Membrane Fraction

Isopycnic centrifugation proved far superior to zonal sedimentation for the isolation of the struc-

tures containing the thiol-dependent acid *p*-nitrophenyl phosphatase activity. Characterized by a low equilibrium density in aqueous sucrose, these structures appear morphologically as relatively large, empty looking vesicles, made up essentially of membrane material. Their cytological origin is unknown but could possibly be ascertained by cytochemical staining procedures. The natural substrate and physiological function of the acid phosphatase associated with these membranes also are intriguing questions. We have no information concerning the significance of the small peak of acid *p*-nitrophenyl phosphatase activity found between the B and C fractions, at density 1.22. As mentioned above, it could result from an agglutination artifact. The membrane fraction shows also alkaline phosphatase activity in amounts that are comparable in absolute terms to its content in acid phosphatase. Unfortunately, the high level of alkaline phosphatase in the specific granules and the possible location of this enzyme in the membrane of these granules render this observation difficult to interpret.

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