BIOCHEMICAL AND MORPHOLOGICAL CHARACTERIZATION OF AZUROPHIL AND SPECIFIC GRANULES OF HUMAN NEUTROPHILIC POLYMORPHONUCLEAR LEUKOCYTES

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ABSTRACT

Postnuclear supernates from homogenates of purified neutrophil polymorphonuclear leukocytes (PMNs) from human blood were fractionated by zonal sedimentation and isopycnic equilibration in sucrose gradients. The fractions were characterized biochemically by measuring protein content and the activities of eight enzymes. Selected fractions were further analyzed by electron microscopy. In both centrifugation systems, azurophil and specific granules could be resolved almost completely. Azurophil granules sediment three to four times faster than the specifics and have an average density of 1.23. They contain all the peroxidase of the cells, large portions of four lysosomal hydrolases, and about half of the total lysozyme, and therefore appear to be, in biochemical terms, very similar to the azurophil granules of rabbit PMNs. The specific granules, which have an average density of 1.19, contain the remaining half of the lysozyme but appear to be free of the other components of the azurophil granules, and of alkaline phosphatase. Isopycnic equilibration disclosed a minor lysosomal population, which strongly overlaps the specific granules, and made possible the identification of a membrane-fraction which is characterized by the presence of the thiol-sensitive acid 4-nitrophenyl phosphatase and of alkaline phosphatase.

Electron microscope and cytochemical investigations (1-6) as well as cell fractionation studies (7-9) have established that the polymorphonuclear leukocytes (PMNs) of the rabbit contain two morphologically and biochemically distinct types of granules, azurophils and specifics. More recently, in an extensive ultrastructural and cytochemical study on human bone marrow (10), Bainton et al. have shown that developing human PMNs produce and accumulate two types of granules which are similar to the azurophil and specific granules of rabbit PMNs with respect to their time of formation and cytochemical properties. In size, shape, and electron density, however, the granules of human PMNs differ considerably from those of the rabbit.

In the present study, postnuclear supernates from human PMN homogenates were fractionated by zonal sedimentation and zonal isopycnic equilibration. The experimental conditions were almost identical with those adopted for the separation of azurophil and specific granules from rabbit PMNs (7, 8). The results obtained, already reported in abstract elsewhere (11, 12), largely confirm the

above conclusions of Bainton et al. (10) and indicate that nature and function of the vacuolar apparatus of human and rabbit PMNs are very similar.

MATERIALS AND METHODS

Preparation of PMNs from Human Blood

PMNs were purified from the pooled buffy coats of donor blood, which was anticoagulated by the addition of one-fifth of its volume of ACD solution (see Materials) and stored at 4°C for 16–24 h. The pooled buffy coats from 4 to 8 500-ml blood bottles (Swiss Red Cross Laboratory, Berne) were diluted with one-fifth of their volume of a solution containing 6 g of dextran T-500 per 100 ml of isotonic saline, and kept in 100-ml cylinders at room temperature for 30–60 min in order to allow sedimentation of the aggregating erythrocytes. The white blood cells of the upper layer were centrifuged in 35-ml glass tubes at 5,500 *g*-min. The supernate was discarded and the cell pellet was washed three times in isotonic saline by centrifuging at 300 *g*-min for complete removal of the remaining erythrocytes and of the platelets. The washed white cell pellet was then resuspended in half the original volume of 0.34 M sucrose and the PMNs were further enriched according to the procedure described by Böyum (13). 10-ml aliquots of the white cell suspension were layered over 18 ml of an aqueous solution containing 9.6 g Triopac and 6.4 g Ficoll per 100 ml in 35-ml glass tubes and centrifuged at 4,500 *g*-min at 20°C. The sample and one-third of the Triopac-Ficoll layer were discarded. The pellet cells were resuspended in 0.34 M sucrose, counted, centrifuged at 3,300 *g*-min, and resuspended in 0.2M sucrose at a concentration of 5×10^7 cells/ml. This suspension was then subjected to homogenization. All centrifugations described in this section were carried out in an MSE Mistral 6-L refrigerated centrifuge (Measuring & Scientific Equipment, Crawley, Sussex, England) using a no. 59560 swing-out rotor. The *g*-min values were calculated for the average radius of the tube content. Where not stated, the centrifuge temperature was set at 4°C.

Fractionation Techniques

HOMOGENIZATION: 15 ml of the PMN suspension (5×10^7 cells/ml) in 0.2 M sucrose were homogenized for 3 min in a Teflon-glass homogenizer (no. C-39605, Arthur H. Thomas Co., Philadelphia, Pa.) kept in ice. The pestle was driven at 2,000 rpm by a drilling machine which was moved automatically and was set to perform one full up-and-down stroke in 1 min. After homogenization, the suspension was centrifuged at 3,300 *g*-min at 4°C as described in the above section. The supernate (postnuclear supernate) was transferred into a graduated tube and its density was adjusted, by the addition of 60% (wt/wt) sucrose, either to 1.05 or to 1.10 g/ml, depending on the type of fractionation experiment.

ZONAL SEDIMENTATION: These experiments were performed in a B-XIV Titanium rotor (MSE no. 59144) operated by an MSE SS-65 ultracentrifuge. The initial conditions were identical with those described by Baggiolini et al. (7), the density of the sample layer being 1.05 g/ml. The rotor was filled and emptied by means of a Vario Perpex II peristaltic pump (Guldener, Zürich, Switzerland) at a rate of 25–30 ml/min while rotating at 2,000 rpm. Sedimentation was carried out at different rotor speeds in 15 min at an average temperature of 8°C.

ISOPYCNIC EQUILIBRATION: These experiments were performed in a Beaufay rotor (14) which was modified in our laboratory for manual operation in an MSE SS-65 ultracentrifuge. The initial conditions were again identical with those previously described (8). The rotor was filled and emptied while rotating at angular velocities between 2,000 and 5,000 rpm, the centrifuge chamber being open, and the equipment used being similar to that described by Leighton et al. (15). Equilibration was obtained after 2 h at 30,000 rpm.

PRESENTATION OF THE FRACTIONATION DATA: The fractionation results were calculated and plotted with a Siemens 4004-35-64K computer (Siemens Corp. Medical Industrial Div., Iselia, N. J.) according to the method described by Beaufay et al. (16).

Biochemical Assays

Peroxidase (EC 1.11.1.7) was assayed in 0.1 M citric acid-sodium citrate buffer, pH 5.5 in the presence of 0.08 mM H_2O_2, 0.32 mM *o*-dianisidine and 0.05% (vol/vol) of Triton X-100. The assay was started by adding 0.1 ml of sample to 1.0 ml of substrate solution containing the above reagents. Incubation was carried out at room temperature for 1 min, and stopped by the addition of 1.0 ml of 35% (vol/vol) perchloric acid. Absorbance was measured at 560 nm in a Zeiss PM 4 spectrophotometer (Carl Zeiss, Oberkochen, FRG). Since no standard is available, the molar extinction coefficient for the oxidized *o*-dianisidine was assessed with known amounts of glucose in a glucose oxidase-peroxidase assay and found to be 20,040 ± 400 (12 determinations). This value was used for calculating the enzyme activity in international units.

Acid 4-nitrophenyl phosphatase (EC –) was assayed in 0.1 M acetic acid-sodium acetate buffer, pH 4.5 in the presence of 4.5 mM 4-nitrophenyl phosphate. The assay was started by adding 0.1 ml of sample to 1 ml of the substrate solution. Incubation was carried out at room temperature for 10 min, and stopped with 1.0 ml of 1 N NaOH. Absorbance was measured at 405 nm in a Zeiss PM 4 spectrophotometer. Standard assays contained 0.04 μmol of 4-nitrophenol.

Acid β-glycerophosphatase (EC 3.1.3.2) was determined in 0.05 M acetic acid-sodium acetate buffer, pH 4.5 in the presence of 7.5 mM sodium β-glycerophosphate and 0.05% (vol/vol) of Triton X-100. The assay was started by adding 0.5 ml of sample to 0.5 ml of the

substrate solution. Incubation was carried out for 1 h at 37°C, and stopped with 0.5 ml of 2.5 M trichloroacetic acid containing 13.4 mM EDTA and 0.56 M ascorbic acid. The precipitate was removed by filtration through Whatman GF/C filters, and the released inorganic phosphate determined according to Baginski et al. (17). Absorbance was measured at 850 nm in a Zeiss PM 4 spectrophotometer equipped with an infrared-sensitive photocell. Blanks were run for each fraction by adding the substrate after the stopping solution. Standard assays contained 0.05 μmol potassium phosphate.

α-Mannosidase (EC 3.2.1.24), *N*-acetyl-β-glucosaminidase (EC 3.2.1.30), and β-glucuronidase (EC 3.2.1.31) were determined in 0.1 M acetic acid-sodium acetate buffer pH 4.0 (β-glucuronidase), or 4.5 (the others), containing 0.1 mM of the corresponding 4-methylumbelliferyl substrate (see Materials) and 0.05% (vol/vol) of Triton X-100. The assay was started by adding 0.1 ml of sample to 0.1 ml of the substrate solution. Incubation was carried out for 45 min at 37°C, and stopped with 2 ml of a 0.05 M glycine-NaOH buffer pH 10.4 containing 5 mM EDTA. The 4-methylumbelliferone formed was measured fluorimetrically in a Hitachi-203 fluorimeter (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.) (18). Standard assays contained 0.5 nmol of 4-methylumbelliferone.

Alkaline phosphatase (EC 3.1.3.1) was determined either with 4-methylumbelliferyl phosphate, 4-nitrophenyl phosphate, or sodium β-glycerophosphate, as described by Bretz and Baggiolini (19).

Lysozyme (EC 3.2.1.77) (8), and protein (15) were assayed according to previously described methods.

Determination of Enzyme Latency

Latency measurements were carried out with the postnuclear supernates diluted in 0.34 M sucrose to a protein content of 0.07 mg/ml. Enzyme activities were determined in the absence and in the presence of various concentrations of detergents. Peroxidase, α-mannosidase, and lysozyme were assayed in citric acid-sodium phosphate buffer pH 5.5. Alkaline phosphatase was assayed with 4-nitrophenyl phosphate at pH 7.5 (triethanolamine-HCl) and at pH 9.75 (diethanolamine-HCl). The tonicity of the substrate solution was always adjusted to 340 mosM by the addition of sucrose with the help of an osmometer (Knauer, Berlin, FRG). Other details concerning the assay conditions are given in the Results.

Morphological Techniques

The material was processed according to the random-sampling procedure of Baudhuin et al. (20) under the conditions described for subcellular fractions of rat brain (21). The fractions were adjusted to a protein content of approximately 0.5 mg/ml and 0.1 ml aliquots were added to 1.9 ml of ice-cold 1.5% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 made isotonic with sucrose. The suspension was filtered through Millipore membranes with pore size of 0.025 μm (VSWP filters, Millipore Ltd., Kloten, Switzerland) under nitrogen pressure of 1–2 atm/cm^2. The particle pellicles were postfixed for 2 h in 1% osmium tetraoxide buffered with 50 mM sodium phosphate made isotonic with sucrose, and subsequently stained en block with uranyl acetate as described by Bolender and Weibel (22). After dehydration in graded ethanol solutions, the pellicles were released in propylene oxide and embedded in Epon (23). Silver to gold sections, cut at right angle to the pellicle surface, were examined in a Zeiss EM 9 S electron microscope.

Materials

Reagents used in the present work were obtained from the following sources: Triopac, (Cilag Chemie Ltd., Schaffhausen, Switzerland); osmium tetraoxide, (Fluka Ltd., Buchs, Switzerland); Emasol 4130, (KAO Atlas Ltd., Tokyo, Japan); 4-methylumbelliferone, 4-methylumbelliferyl-β-D-glucuronide, 4-methylumbelliferyl-β-D-mannopyranoside, 4-methylumbelliferyl-2-acetamido-2-desoxy-β-D-glucopyranoside, and 4-methylumbelliferyl dihydrogen phosphate, (Koch-Light Laboratories, Colnbrook, Buckinghamshire, England); 4-nitrophenol, 4-nitrophenyl phosphate (disodium salt, 5 H_2O), Perhydrol, *o*-dianisidine, sucrose for density gradient centrifugation, digitonin (crist.), and sodium deoxycholate, (E. Merck A.G., Darmstadt, FRG); Triton X-100, (Packard Instruments Co. Inc., Downers Grove, Ill.); dextran-T-500. Ficoll, (Pharmacia Ltd., Uppsala, Sweden); EDTA: ethylenediamine tetraacetic acid (disodium salt). (Siegfried Ltd., Zofingen, Switzerland); DL-β-glycerophosphate (grade I), (Sigma Chemical Co., St. Louis, Mo.); glutaraldehyde, (TAAB Laboratories, Reading, Sussex, England). The ACD (acid citrate dextrose) solution used for anticoagulation of donor blood contains 7.4 g citric acid, 22.0 g sodium citrate and 24.5 g dextrose per liter, according to the U. S. Pharmacopeia XV.

RESULTS

Purification of the PMNs

Table I shows the differential counts of 15 PMN preparations that were used for fractionation, before and after sedimentation through a Triopac-Ficoll layer. This last purification step eliminated almost entirely the mononuclear cells and resulted in a relative enrichment of the PMNs. No separation between PMNs and eosinophil leukocytes could be achieved by this purification procedure.

Homogenization of the PMNs

The homogenization procedure described in the Materials and Methods has been adopted as a

Table I
*Differential Counts of Leukocyte Preparations Before and After Centrifugation through Triopac-Ficoll**

	Leukocytes		
	Neutrophil	Eosinophil	Mononuclear
Before	75 ± 5.8	4.5 ± 2.0	21 ± 5.1
After centrifugation through Triopac-Ficoll	94 ± 3.1	5.7 ± 2.7	0.7 ± 0.7

* See Materials and Methods. Mean percent value ± standard deviation from 15 separate preparations. Each value was obtained by counting 500 cells.

Table II
*Homogenization of Human PMNs**

Measured component	Minutes at 2,000 rpm	Percent of total† activity in:		Free activity in PN (percent of total)
		PN	N	
Peroxidase	1	56	43	10
	3	60	37	11
	10	65	35	11
	20	71	30	12
	30	70	28	14
α-Mannosidase	1	55	46	24
	3	61	39	25
	10	68	31	26
	20	73	26	27
	30	71	28	27
Protein	1	53	47	
	3	58	42	
	10	68	33	
	20	68	31	
	30	70	29	

* Cell suspensions (5×10^7 cells/ml 0.2 M sucrose) were homogenized at 2,000 rpm in a Teflon-glass homogenizer (see Materials and Methods).
† N is the pellet and PN the supernate after centrifugation at 3,300 *g*-min. The sum of the values of PN and N gives the percent recovery.

result of experiments in which the pestle rotation speed and the molarity of the sucrose medium were varied systematically. The high pestle speed of 2,000 rpm was found necessary for disrupting more than half of the cells. Disruption was facilitated by slight cell swelling induced by hypotonic sucrose. In the presence of NaCl, the PMNs were almost totally resistant to mechanical disruption. Repeated washing in sucrose was therefore found necessary. Table II shows the homogenization yield in dependence of time under the conditions adopted. Progressive cell disruption was observed up to 20 min. The average homogenization yields of seven different PMN preparations are given in Table III. It is noteworthy (Table II) that the latency of two particle-bound enzymes, peroxidase and α-mannosidase, remains nearly constant upon homogenization up to 30 min in 0.2 M sucrose. The latency data of Table IV give an indication of the osmotic stability of the particles containing peroxidase and α-mannosidase. In all latency experiments (see also Fig. 5), the free activity of α-mannosidase measured at pH 5.5 (see Materials and Methods) was considerably higher than that of peroxidase. We have recently found that human PMNs contain an apparently nonparticulate α-mannosidase with pH optimum between 5 and 6 which could account for the high free activity

Table III
*Average Homogenization Yield of Seven PMN Preparations**

Measured	Percent of total activity (mean ± SD)	
	PN	N
Peroxidase	64 ± 5.0	33 ± 3.3
α-Mannosidase	64 ± 5.0	30 ± 1.7
β-Glucuronidase	70 ± 1.3	29 ± 2.0
Protein	69 ± 5.4	31 ± 5.0

* See Table II for explanations.

Table IV
Osmotic Resistance of Peroxidase- and α-Mannosidase-Containing PMN Granules in Sucrose

Sucrose molarity	Free activity in percent of total*	
	Peroxidase	α-Mannosidase
M		
0.2	5.4	17.5
0.1	27.7	31.1
0.05	41.7	46.0
0.01	77.0	100.0

* The sucrose concentration was adjusted by dilution with H_2O, and the free activity determined after 10 min at 0°C.

observed. Nonlysosomal α-mannosidases have been described in other tissues by Marsh and Gourlay (24) and by Dewald and Touster (25). The average specific activities of the measured enzymes in the postnuclear supernate which was used as starting material in the fractionation experiments are given in Table V.

Differentiation between Acid β-Glycerophosphatase and Acid 4-Nitrophenyl Phosphatase in the Postnuclear Fraction

The presence of a nonlysosomal, thiol-dependent acid 4-nitrophenyl phosphatase in PMNs was first

TABLE V

Specific Activities of Enzymes in the Postnuclear Fraction of Human PMNs

Enzyme	Assay conditions			Specific activity
	Temperature	pH	Substrate concentration	
			mM	
Peroxidase	20–22	5.5	0.08	3,876± 560
α-Mannosidase	37	4.5	0.1	0.9± 0.1
N-Acetyl-β-glucosaminidase	37	4.5	0.1	0.5± 0.1
β-Glucuronidase	37	4.0	0.1	1.8± 0.2
Acid β-glycerophosphatase	37	4.5	7.5	25.2± 3.4
Lysozyme	20–22	6.0	—	33.7± 4.8*
Acid 4-nitrophenyl phosphatase	20–22	4.5	4.5	111.4± 13.3
Alkaline phosphatase‡	37	9.75	0.1	6.1± 0.8 (*a*)
	37	9.75	7.5	13.8 (*b*)
	25	9.75	4.5	23.2 (*c*)

Values give mean specific activity in milliunits per milligram of protein ± SD from seven preparations. When not specified, 1 unit of activity is defined as the amount of enzyme that splits 1 μmol of substrate in 1 min under the conditions given (see also Materials and Methods).

* Equivalents of μg of crystalline hen egg-white lysozyme.

‡ Alkaline phosphatase was assayed with 4-methylumbelliferyl phosphate (*a*), β-glycophosphate (*b*), and 4-nitrophenyl phosphate (*c*). The specific activity value with the two last substrates is the mean of three and two determinations, respectively.

TABLE VI

*Effects of Inhibitors on the Activity of Two Acid Phosphatases in Human and Rabbit PMNs**

Inhibitor		Relative activity‡			
		4-NPPase		β-GPase	
		Human	Rabbit	Human	Rabbit
	mM				
Fluoride	5	96.7 ± 3.1	96	4.8 ± 5.5	0
L-(+)-Tartrate	5	91.7 ± 5.0	93	4.2 ± 3.8	0
p-Chloromercuribenzoate	0.04	5.5 ± 2.9	10	86.6 ± 7.9	78
N-Ethylmaleimide	4	29.7 ± 3.0	4	97.1 ± 5.6	100

* Assays were carried out in postnuclear supernates at 25°C. *N*-Ethylmaleimide was added to the preparations 10 min before the substrate. Experiments with postnuclear supernates of rabbit PMNs were performed according to the previously described methods (7, 8).

‡ Data are mean ± SD from five to seven experiments (human), and mean from three experiments (rabbit).

demonstrated in the rabbit (8). The results shown in Table VI strongly suggest that human PMNs also contain this enzyme. The 4-nitrophenyl phosphate hydrolysis in postnuclear supernates of human PMNs at pH 4.5 is nearly unaffected by fluoride and tartrate concentrations that almost entirely block the lysosomal acid β-glycerophosphatase. On the other hand, the acid 4-nitrophenyl phosphatase is very sensitive to the thiol reagents *p*-chloromercuribenzoate and *N*-ethylmaleimide which are weak inhibitors of the lysosomal phosphatase. Similar results have been published recently by Avila and Convit (26).

Zonal Sedimentation

Figs. 1 and 2 show the distribution profiles of protein content and of a number of enzyme activities upon fractionation of postnuclear supernates by zonal differential sedimentation during 15 min at 9,500 and 13,500 rpm, respectively. We have carried out a total of eight experiments of this type, three at the low, and five at the high speed, and the results were very consistent. After sedimentation at 9,500 rpm, virtually all of the peroxidase activity has reached the outer half of the gradient and is already beginning to accumulate against the cushion. A strikingly similar profile is shown by the three glycosidases measured. The distribution of α-mannosidase is nearly identical with that of peroxidase while those of β-glucuronidase and *N*-acetyl-β-glucosaminidase show a minor relative activity excess over peroxidase in the inner half of the gradient. Part of the lysozyme activity also coincides with that of peroxidase, but at least 50% of the total is found in a well-defined peak in the inner half of the gradient. This slow-sedimenting lysozyme band is well resolved from the activity patterns of all other enzymes measured. Alkaline phosphatase, which shows identical profiles when assayed with 4-nitrophenyl phosphate or β-glycerophosphate, is found in a narrow peak just resolved from the starting zone, and appears therefore to be almost completely resolvable from lysozyme. Most of the activity of the acid 4-nitrophenyl phosphatase coincides with that of the alkaline phosphatase. The distribution profiles of the two enzymes, however, are not identical: the acid 4-nitrophenyl phosphatase seems to belong to particles that sediment on average slightly faster than those containing the alkaline phosphatase. Finally, the distribution of protein shows two predominant peaks, one in the

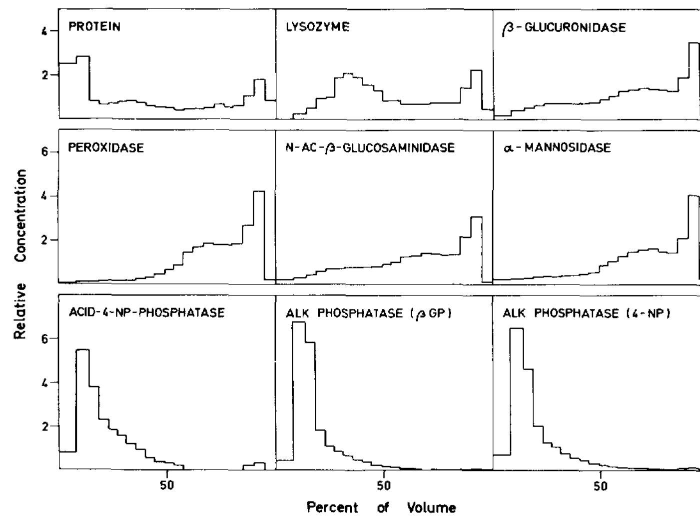


FIGURE 1 Fractionation of subcellular components of human PMNs by zonal sedimentation at 9,500 rpm for 15 min. Graphs are normalized distribution histograms as a function of the volume collected. Radial distance increases from left to right. Ordinate is concentration in fraction relative to concentration corresponding to uniform distribution throughout the gradient. Alkaline phosphatase was assayed with β-glycerophosphate (β-GP) and 4-nitrophenyl phosphate (4-NP-phosphate). Percentage recoveries were 103 for protein, 94 for lysozyme, 87 for β-glucuronidase, 94 for peroxidase, 72 for *N*-acetyl-β-glucosaminidase, 78 for α-mannosidase, 73 for acid 4-nitrophenyl phosphatase, and 65 and 75 for alkaline phosphatase.

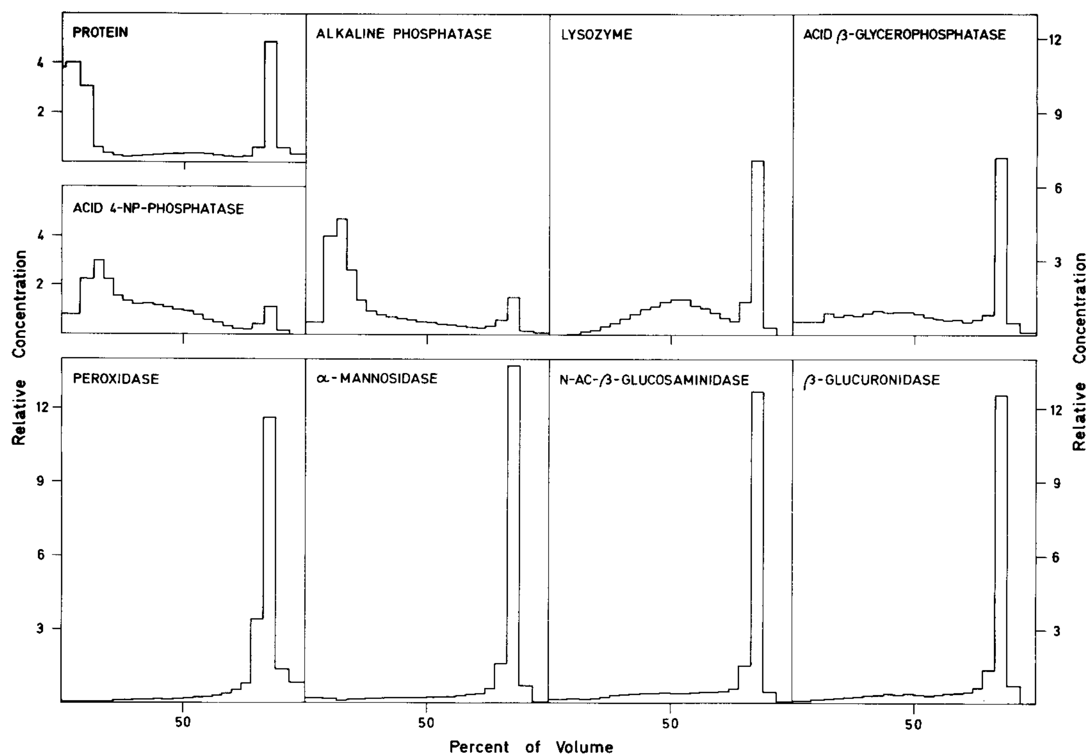


FIGURE 2 Fractionation of subcellular components of human PMNs by zonal sedimentation at 13,500 rpm for 15 min. Results represented as in Fig. 1. Alkaline phosphatase was assayed with 4-methylumbelliferyl phosphate. Percentage recoveries were 101 for protein, 87 for acid 4-nitrophenyl phosphatase, 74 for alkaline phosphatase, 94 for lysozyme, 63 for acid β-glycerophosphatase, 79 for peroxidase, 73 for α-mannosidase, 85 for *N*-acetyl-β-glucosaminidase, and 88 for β-glucuronidase.

starting zone, mainly representing the bulk of the soluble material, and one at the cushion paralleling the peroxidase profile. In between these zones, a very flat protein peak appears to correspond to the slow-sedimenting peak of lysozyme. If the total centrifugal force is doubled by running the experiments at 13,500 instead of 9,500 rpm, the sedimentation profiles shown in Fig. 2 are obtained. These patterns are perfectly consistent with those described above. The peroxidase activity, which is completely accumulated against the cushion, is accompanied by high percentages of the total activities of the glycosidases. As in the low-speed experiments, however, some of the β-glucuronidase and of the *N*-acetyl-β-glucosaminidase activities are retarded with respect to peroxidase. A similar distribution is found for the acid β-glycerophosphatase, but, in this case, the portion of slow-sedimenting activity is more prominent. The bimodal distribution of lysozyme is clearly confirmed. Its slow-sedimenting portion stands out as a broad, symmetrical peak covering a large zone of the gradient, while the remnant is concentrated against the cushion together with peroxidase. Here again, lysozyme is almost completely resolved from alkaline phosphatase, and the slight dissociation between the alkaline phosphatase and the acid 4-nitrophenyl phosphatase is also apparent.

These results establish the existence in our starting material of at least three distinct populations of particles which are marked by the two peaks of lysozyme and that of alkaline phosphatase, respectively. The population with the highest sedimentation rate, which fully accumulates against the cushion upon centrifugation at 13,500 rpm, contains, in addition to lysozyme, all the peroxidase of the preparations and between 60 and 90% of their total activity of four lysosomal hydrolases. By contrast, the other lysozyme-containing particle population appears to be free of peroxidase. In the same zones of the gradient, we

also found sizable activities of three lysosomal hydrolases. The shape of their distribution profiles, however, markedly differs from that of lysozyme, thus indicating that they are likely to be associated with structures which are poorly resolvable from the lysozyme particles by sedimentation. Most remarkably, the slow-sedimenting lysozyme-containing particles appear to be devoid of alkaline phosphatase, which, together with lysozyme and lactoferrin, is a typical constituent of the specific granules in the PMNs of the rabbit (7–9). In human PMNs, alkaline phosphatase is bound to structures that sediment on average at about the same speed as those containing the acid 4-nitrophenyl phosphatase, and which are therefore likely to be membrane fragments. In Fig. 2, the alkaline phosphatase profile shows a small peak at the outer limit of the gradient, which most probably reflects the presence of some aggregated, and therefore fast-sedimenting, material. The lack of corresponding peaks in other experiments of this kind (see Fig. 1) precludes the possibility that the peroxidase-containing particles may also contain alkaline phosphatase.

Zonal Isopycnic Equilibration

Fig. 3 shows the density equilibration profiles obtained in two out of 10 fractionations performed in Beaufay's rotor. The two sets of patterns, A and B, are the extremes among those obtained in this series of experiments. They illustrate the degree of variability observed in the activity distributions of the lysosomal hydrolases. Data from another experiment are given in Fig. 7.

Nearly all of the peroxidase equilibrates in a very narrow band with modal density of 1.23. In the fraction with the highest relative activity, peroxidase is usually enriched more than 10 times over the value it would have upon equal distribution throughout the gradient. As it emerges from the rotor, this fraction is easily recognized by its green color. The peroxidase band always contains a large portion of the total activity of three lysosomal glycosidases and of acid β-glycerophosphatase. A minor part of these activities is "tailing" or forming a small peak at lower density. As stressed by the data of Fig. 3, this part of the hydrolase profiles varies considerably from experiment to experiment quite in contrast to the other equilibration patterns which are far better reproducible. Two features of the peroxidase peak will be noted: the comparatively high activity of its peak fraction and its slight asymmetry in some of the experiments. The remarkable concentration of activity into a very narrow zone of the gradient, which is common to the peroxidase and the glycosidases, suggests an unusually tight packing and a possible aggregation of the particles that carry these enzymes (see for comparison the broader equilibration profiles of rabbit azurophil granules, reference 8). The slight asymmetry of the peroxidase peak suggests the possibility of physical heterogeneity among the particles of this band. It should also be considered that granules from eosinophil leukocytes, which contaminate our cell preparations (Table I), might be enriched in the densest fractions of the band. On the basis of the equilibration experiments performed, however, we have no indication that the asymmetry of the peroxidase peak may correlate with the percentage of eosinophil leukocytes originally present.

The distribution of lysozyme shows two narrow and well-resolved peaks each containing about half of the total activity of the starting material. The densest peak coincides with peroxidase and has a modal density of 1.23, the other has a modal density of 1.19. Density equilibration proved unsatisfactory in resolving the light lysozyme band from the varying portion of acid hydrolase activities that do not accompany peroxidase. Nevertheless, the distinct shapes of the peaks and the slight difference in modal density suggest a different localization for lysozyme and the acid hydrolases. The evidence for this statement is summarized in Fig. 4 which shows, together with the equilibration data, the rate sedimentaion profiles discussed in the above section. Both lysozyme peaks are clearly resolved from the alkaline phosphatase. About 90% of this activity equilibrates in a narrow band with modal density of 1.16. As expected (see Fig. 1), the equilibration profile of alkaline phosphatase is identical when β-glycerophosphate instead of an aryl phosphate ester is used as the substrate. Upon isopycnic centrifugation, the alkaline phosphatase and the acid 4-nitrophenyl phosphatase show very similar distributions. In addition to the major peak at low density, both activity profiles frequently exhibit a small peak coinciding with that of peroxidase. In interpreting these data, the possibility should be considered that, upon homogenization, some of the structures carrying the phosphatases may aggregate with the peroxidase particles. This, in our opinion, explains the small, and fairly variable amount of alkaline phosphatase

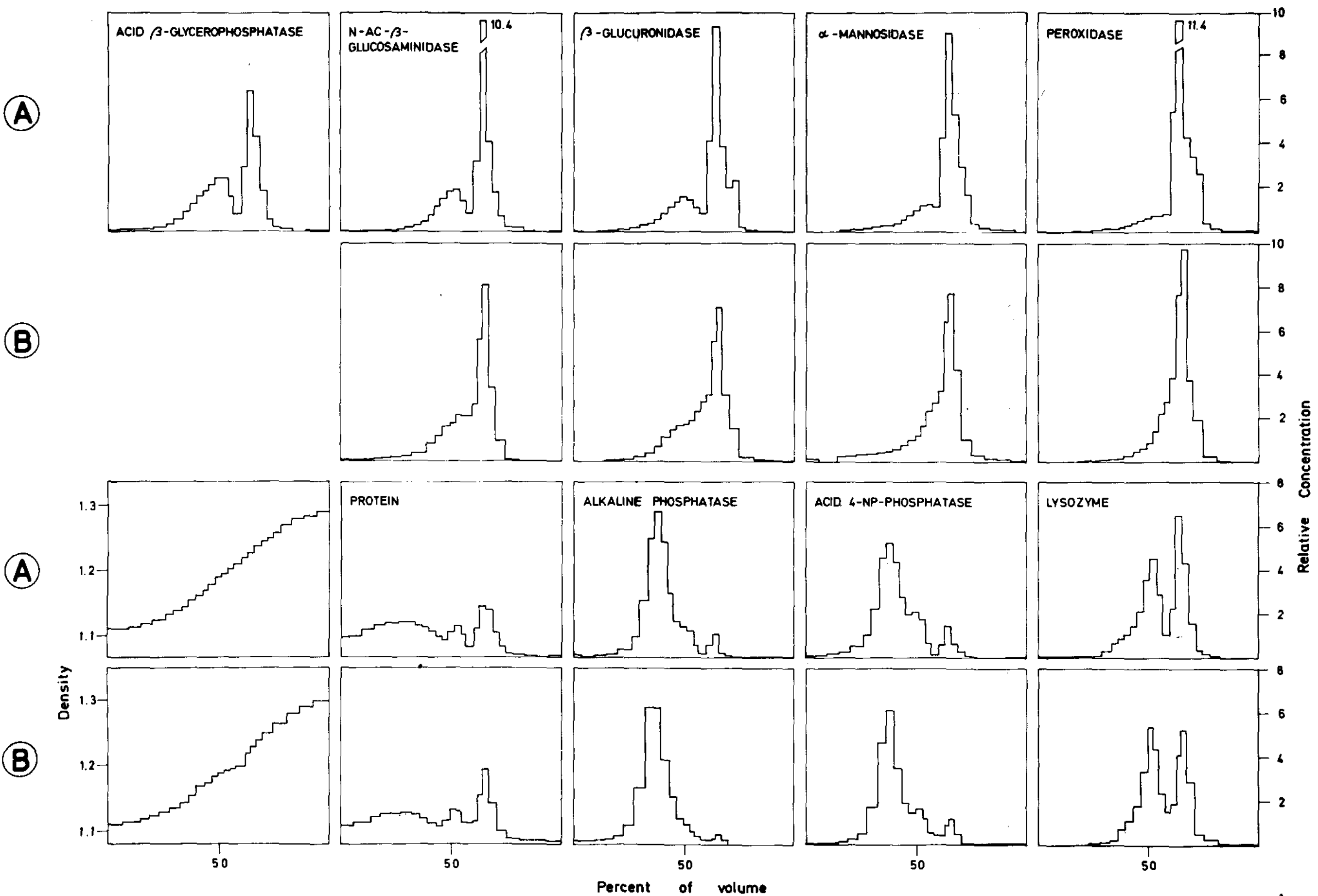


FIGURE 3 Isopycnic equilibration of subcellular components of human PMNs. Results from two experiments, A and B, represented as described in Fig. 1. The two lower left-hand graphs show the average density for each fraction. Percentage recoveries were 93 for acid β-glycerophosphatase, 91 and 94 for *N*-acetyl-β-glucosaminidase, 100 and 111 for β-glucuronidase, 79 and 86 for α-mannosidase, 75 and 94 for peroxidase, 92 and 95 for protein, 62 and 79 for alkaline phosphatase, 79 and 66 for acid 4-nitrophenyl phosphatase, and 80 and 91 for lysozyme.

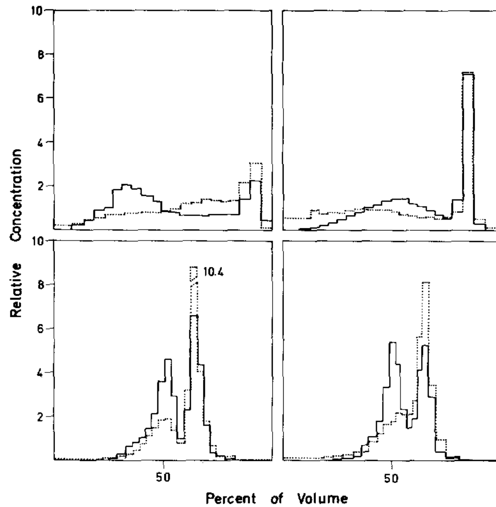


FIGURE 4 Subcellular distribution of lysozyme (solid line) and of a lysosomal hydrolase (dotted line) obtained in the four experiments of Figs. 1–3. The lysosomal hydrolase is acid β-glycerophosphatase in the upper right-hand graph and N-acetyl-β-glucosaminidase in the others.

which equilibrates at high density. As for the acid 4-nitrophenyl phosphatase profile, however, the comparatively more prominent activity in this zone reflects at least in part the presence of the lysosomal acid phosphatase which is known to hydrolyze 4-nitrophenyl phosphate (8).

The distribution of protein shows two distinct peaks with modal densities of 1.23 and 1.19, respectively, thus corresponding to the two main populations of particles marked by lysozyme. As in the sedimentation experiments, the bulk of the protein is spread over the starting zone.

The results of the density gradient centrifugation experiments are fully compatible with the conclusions drawn from the sedimentation experiments. We are able to separate, on the basis of their specific gravity, two classes of moderately dense lysozyme-containing particles, one of which exhibits the biochemical characteristics of azurophil granules (7–10), and a membrane fraction with acid and alkaline phosphatase activities. These results clearly confirm the resolution between lysozyme and alkaline phosphatase shown by the sedimentation experiments, and indicate that the specific granules of human PMNs may be devoid of alkaline phosphatase.

Determinations of Enzyme Latency

These experiments were prompted by the observation that in human PMNs alkaline phosphatase does not seem to be localized in either of the two main granule populations. Determinations of peroxidase and α-mannosidase activity of postnuclear fractions in the presence and in the absence of Triton X-100 show that these enzymes are largely inaccessible to substrates (Tables II and IV). Fig. 5 shows the latency curves of peroxidase, α-mannosidase, and lysozyme obtained in identical buffer-sucrose solutions (see Materials and Methods). With increasing amounts of Triton X-100 or digitonin, all three activities increase up to a maximum-value plateau. The three sigmoidal curves are very similar. The reasons for the relatively high initial free activity of α-mannosidase has already been discussed. The effects of detergents on alkaline phosphatase activity are much less pronounced. As shown in Table VII, increasing concentrations of either Triton X-100 or digitonin moderately enhance the activity of the enzyme both at pH 7.5 and at the optimum pH 9.75. Little if any effect, however, is seen with

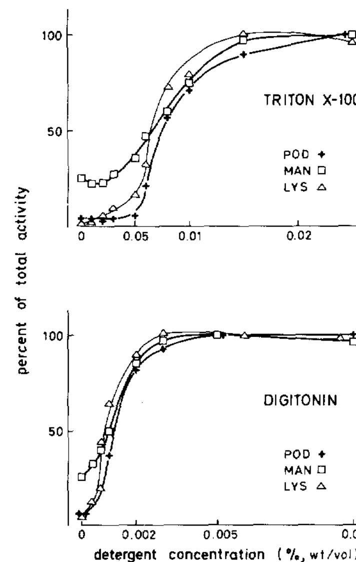


FIGURE 5 Effect of detergents on the activity of peroxidase (POD), α-mannosidase (MAN), and lysozyme (LYS) in postnuclear supernates of human PMNs. Symbols represent mean values from two to three single experiments. Protein concentration in the assay was always adjusted to 0.007 mg/ml.

TABLE VII
*Effect of Detergents on Phosphatase Activity at pH 7.5 and 9.75**

Detergent	Concentration in assay	pH 7.5		pH 9.75	
		MUP	4-NPP	MUP	4-NPP
	mg/ml				
None	—	100	100	100	100
Triton X-100	0.05	105	95	99	91
	0.10	116	112	106	114
	0.25	124	120	146	123
	0.50	129	122	145	129
Digitonin	0.01	99	100	97	95
	0.02	110	111	99	101
	0.05	127	121	115	116
	0.10	128	125	120	116
	0.20	130	142	124	116
Emasol-4130	0.10	112	94	95	94
	0.50	111	96	100	94
	1.00	102	94	99	93
	1.50	102	90	98	93
	2.00	98	90	97	92
Deoxycholate	0.01	92	91	98	91
	0.05	90	94	95	89
	0.10	89	92	91	84
	0.25	85	86	83	72
	0.50	78	100	71	63

* Activities are given in percent of the control values obtained without detergent. Alkaline phosphatase was assayed with 4-methylumbelliferyl phosphate (MUP) and 4-nitrophenyl phosphate (4-NPP).

another detergent, Emasol-4130 (27), up to the final concentration of 0.2%. Sodium deoxycholate, which was also tried, appears to inhibit the enzyme. We studied the effect of Emasol-4130 on lysozyme and peroxidase activity in two experiments. In concentrations that do not alter alkaline phosphatase activity, this detergent clearly reduced lysozyme latency. Unfortunately, under these experimental conditions, Emasol-4130 partially inhibited lysozyme and completely inactivated peroxidase, thus making quantitative assessments difficult. Obviously, these data do not provide a clear-cut answer to the question of whether in postnuclear supernates of human PMNs alkaline phosphatase is latent or not. However, the following facts offer some evidence suggesting that the activity increase induced by Triton X-100, or digitonin may reflect enzyme activation rather than suppression of latency: (*a*) The rise in activity is considerably smaller than that obtained with granule-bound enzymes upon granule lysis; (*b*) No rise in activity is obtained with Emasol-4130; (*c*) The effect of Triton X-100 is independent of the protein concentration in the assay, as illustrated in Fig. 6.

Morphological Results

Fractions from isopycnic experiments were repeatedly examined by electron microscopy. Fig. 7 shows the equilibration profiles of peroxidase, lysozyme, and alkaline phosphatase from one of such experiments. Figs. 8–12 represent, in this order, micrographs of the fractions designated A–E in Fig. 7. Samples of each of these fractions were processed according to the random-sampling technique of Baudhuin et al. (20). The micrographs represent vertical sections of the particle pellicles

obtained, which allow a true morphological assessment of the purity of the fractions.

Micrographs of fraction A (Fig. 8), which has the highest relative peroxidase activity, show this to consist mainly of round and oval profiles of particles with a single membrane and a uniform matrix of medium electron density. Numerous particles show different degrees of extraction. In a number of extracted particles, only a small, core-like portion of the matrix is seen. The well-preserved profiles are similar in size and overall appearance to the oval or football-shaped azurophil granules of intact PMNs (10). Some glycogen particles and clusters of amorphous material, which presumably represent released granule content, are present between the granules. In this and other experiments, micrographs of the peroxidase peak fraction hardly contain any granules from

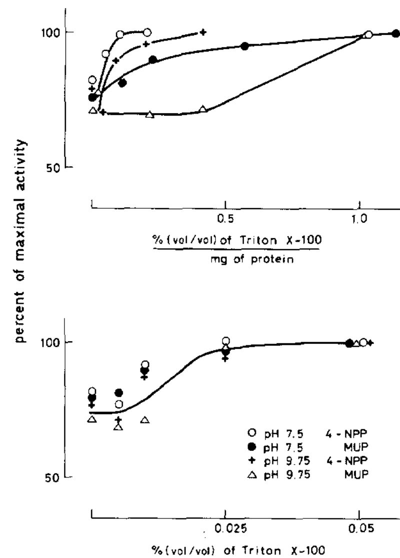


FIGURE 6 Effect of Triton X-100 on the phosphatase activity of postnuclear supernates at pH 7.5 and 9.75. The phosphatase was assayed with 4-nitrophenyl phosphate (4-NPP) and 4-methylumbelliferyl phosphate (MUP).

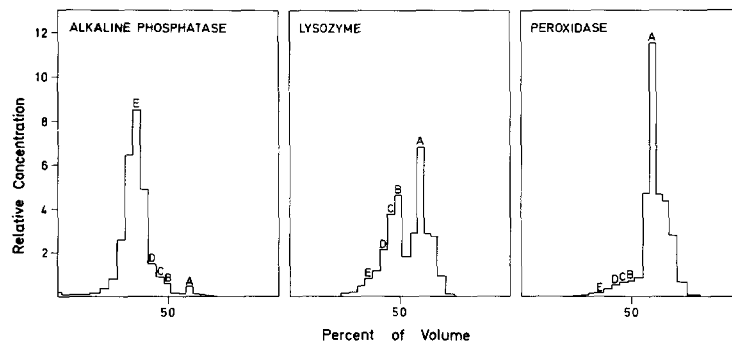


FIGURE 7 Isopycnic equilibration of subcellular components of human PMNs. Results represented as in Fig. 1. Alkaline phosphatase was assayed with 4-methylumbelliferyl phosphate. Fractions A-E are those which were analyzed by electron microscopy. See Figs. 8-12. Percentage recoveries were 73 for alkaline phosphatase, 68 for lysozyme, and 84 for peroxidase.

eosinophil leukocytes. Because of their size, many of these particles may be eliminated from the starting material through sedimentation into the nuclear pellet. The relatively large number of extracted granule profiles has been a disappointing characteristic of all preparations of peroxidase-rich fractions. It is interesting to note, however, that azurophil granules of intact PMNs seem to be equally difficult to preserve (10).

The three following micrographs (Figs. 9–11) represent three adjacent fractions B, C, and D of the less dense lysozyme peak (see Fig. 7). Fractions B and C (Figs. 9 and 10) are virtually identical. They consist mainly of round, ellipsoidal, sausage- or dumbbell-shaped profiles of particles with a single membrane and a uniform, moderately electron-dense content. As compared to the main components of fraction A (Fig. 8), these profiles are on average considerably smaller, much more varied in shape, and rarely show signs of extraction. These features enable us to identify the main components of fraction B and C as the specific granules (10). In addition, these micrographs show a large amount of glycogen particles, few mitochondria, occasional large membrane profiles, and small numbers of vesicle-like structures which may represent granule ghosts. As expected, fraction D (Fig. 11) had a much more heterogeneous aspect. It still contains great numbers of the particles which we have identified as the specific granules in fraction B and C. However, many morphologically empty, vesicular membrane structures of different sizes are also present together with a few mitochondria, and a large amount of glycogen particles. The average volume of the specific granules seems to be smaller than in the fractions B and C. Also, the rod- and dumbbell-shaped profiles seem to prevail.

In fraction E (Fig. 12), which is characterized by the highest relative activity of alkaline phosphatase and of the thiol-sensitive acid 4-nitrophenyl phosphatase, membrane profiles clearly predominate. We see a great number of small- and medium-sized profiles of empty, round vesicles and some more irregular, large membrane figures. The number of mitochondria, which are all badly preserved, is definitely larger than in the fractions described above. There are also many thin, dumbbell-shaped membrane-bound particle profiles with moderately dense content. The layer of amorphous material which is packed at the filter face of the pellicle is most likely made up of components of the cytosol since fraction E, in this type of experiments, always slightly overlaps the sample zone.

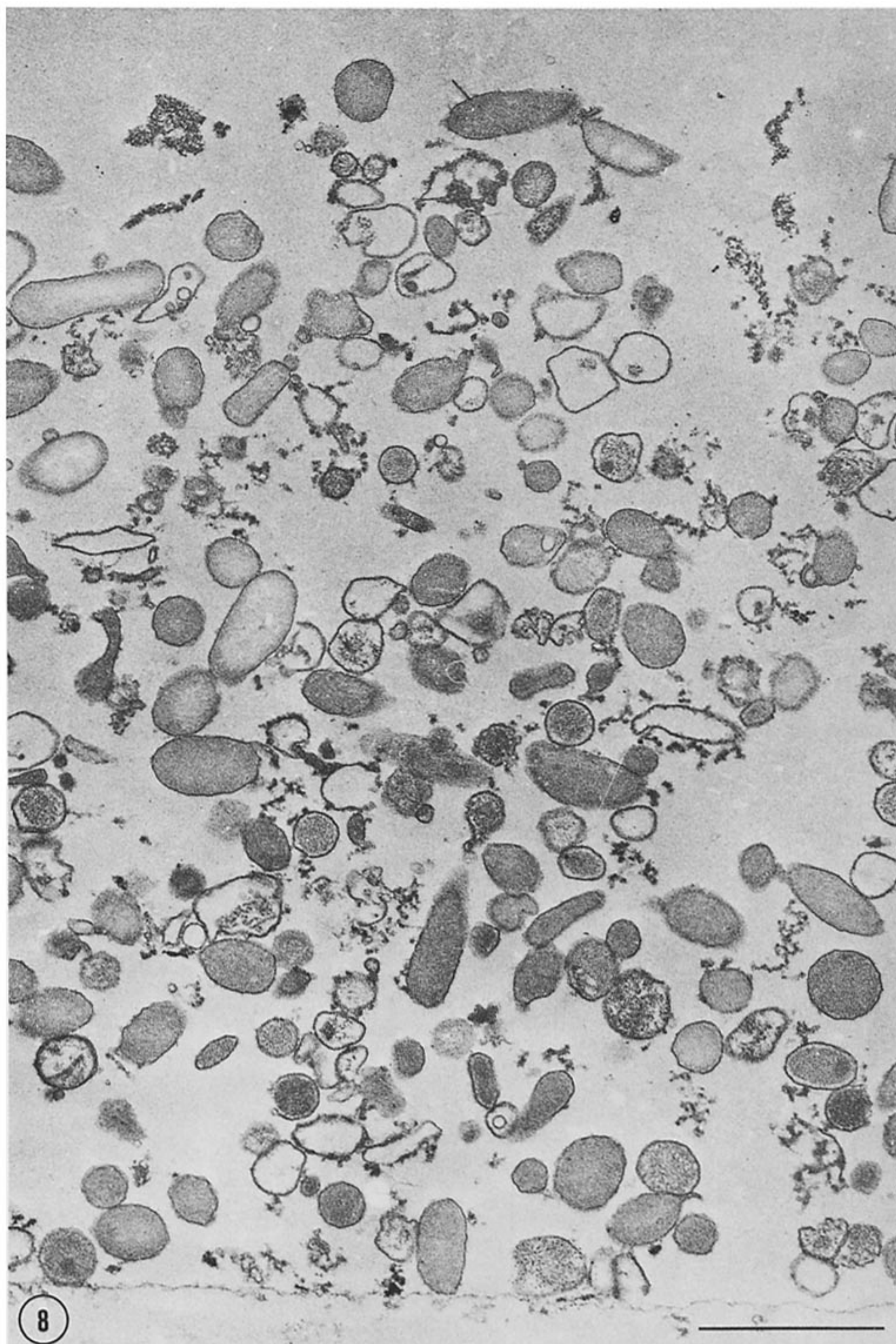
DISCUSSION

In this work, we describe the separation of two main populations of granules from human PMNs both by sedimentation and isopycnic equilibration. On the basis of biochemical and electron microscope analyses, these two particles have been identified as the azurophil and the specific granules. Two further populations of particles, a minor population of lysosomes and a membrane fraction, were also resolved and partially characterized. Since the results have already been interpreted in detail, this section will be restricted to a discussion of the properties of the four particle populations.

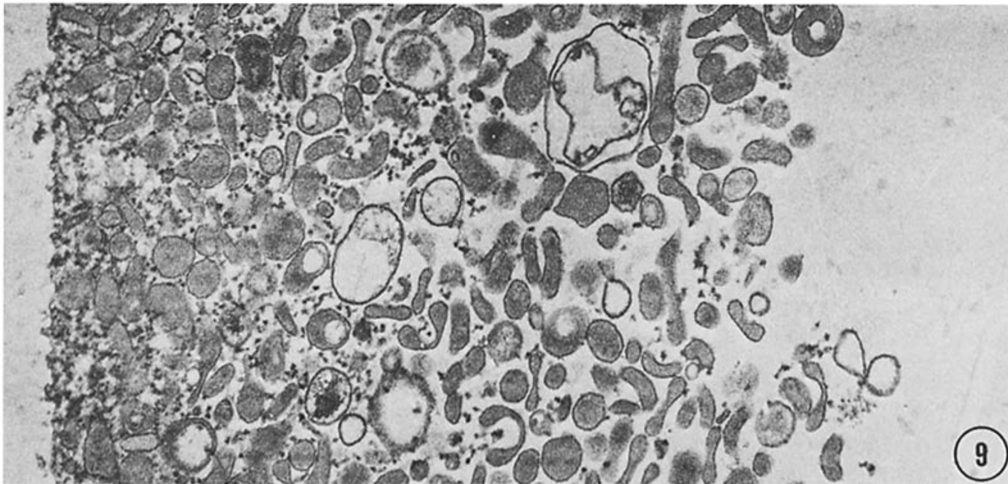
Azurophil Granules

These particles can be easily separated from other PMN structures because they are both larger and denser than any other component of the postnuclear fraction. Their sedimentation velocity is about three to four times faster than that of the specific granules, and their modal equilibrium density is 1.23. According to our results, the azurophil granules contain all the peroxidase, a major portion of four lysosomal hydrolases, and about half of the lysozyme of the postnuclear supernate. Their biochemical properties appear therefore to be strikingly similar to those of the corresponding granules of rabbit PMNs (7, 8). In both species, azurophil granules must be considered a special kind of primary lysosome which contains microbicidal agents in addition to the characteristic digestive enzymes (7, 8, 28). These conclusions are in full agreement with those drawn from cytochemical studies which demonstrate that azurophil granules of maturing human PMNs contain peroxidase and four acid hydrolases (10). Earlier, less extensive cytochemical work also indicated the presence of a population of peroxidase-positive granules in circulating human PMNs (29–34).

We were surprised by the virtual absence of granules from eosinophil leukocytes in micrographs of peroxidase-rich subfractions. Because of their size, such particles could largely be destroyed during homogenization or sediment into the nuclear pellet. On the other hand, they could also be enriched in the heaviest subfractions of the peroxidase peak which were not examined in the electron



FIGURES 8–12 Survey electron micrographs of the fractions A (Fig. 8), B (Fig. 9), C (Fig. 10), D. (Fig. 11), and E (Fig. 12) obtained in the isopycnic equilibration experiment represented in Fig. 7. The filter face of the pellicles (see Materials and Methods) in recognizable at the bottom of Fig. 8, on the left side of Figs. 9–11 and in the left-hand lower corner of Fig. 12. The morphology is described in the text. The bar in Fig. 8 corresponds to 1 μm. $\times$ 27,000 in all figures.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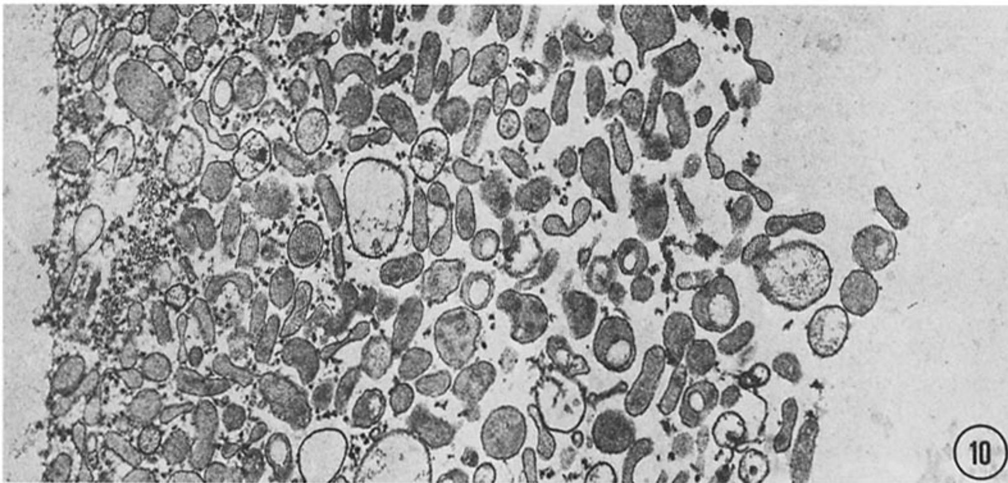
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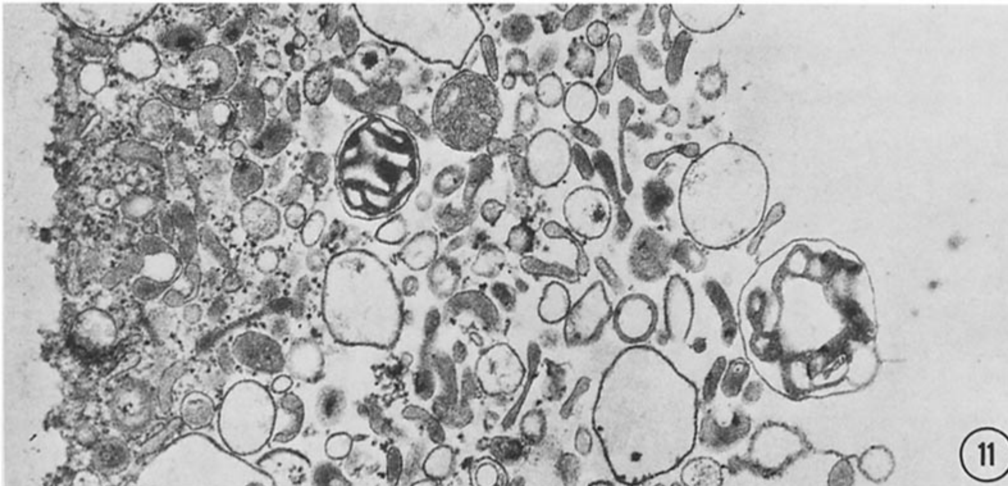
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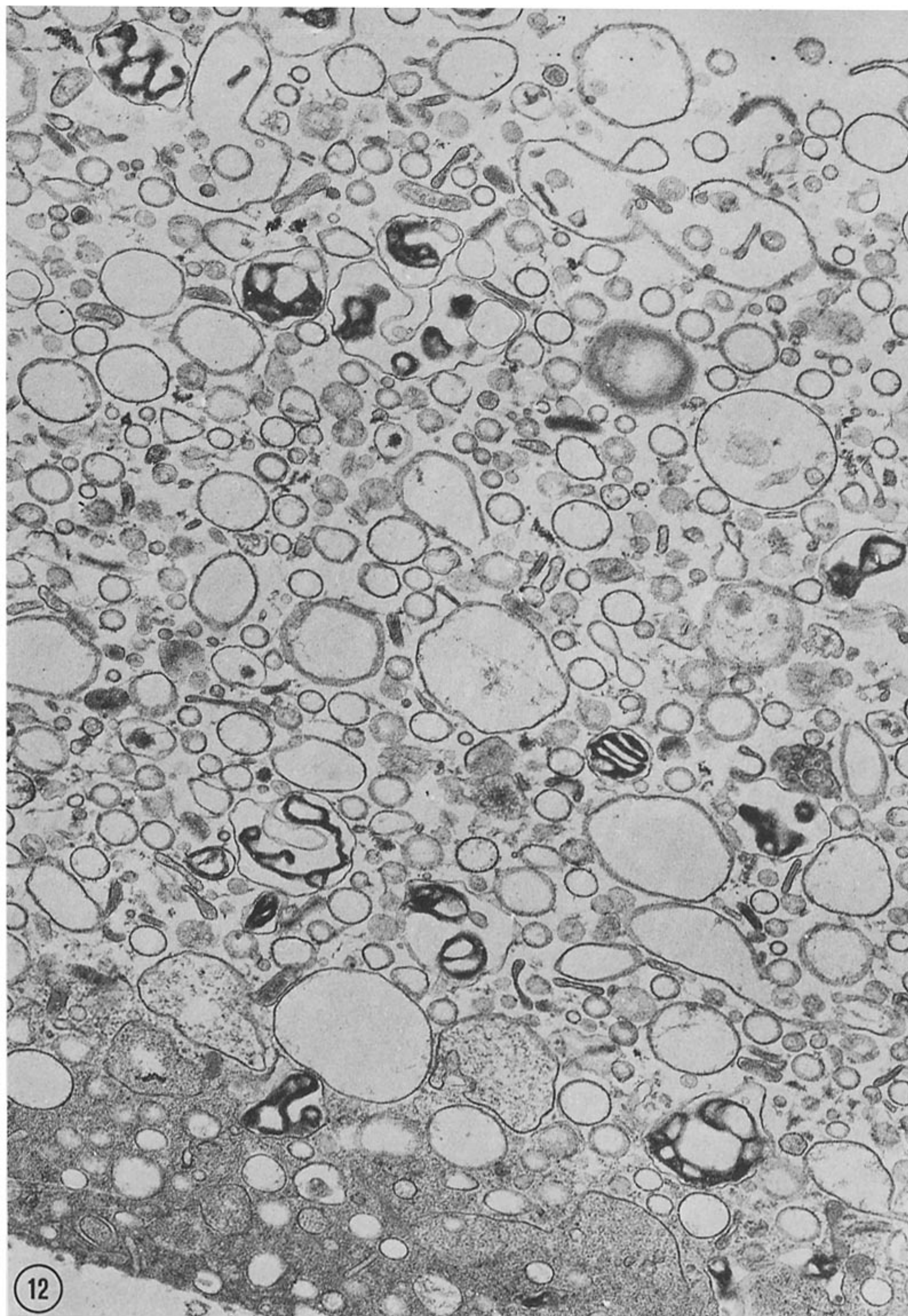
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microscope. Even in the latter event, however, our conclusions concerning the biochemical nature of the azurophil granules are not invalidated, as they are based on data of subfractions which are free of morphologically detectable eosinophil granules.

Specific Granules and Small Lysosomes

The specific granules are smaller, less dense, and apparently more complex in shape than the azurophils. They equilibrate at an average density of 1.19. Our data suggest that they contain the remaining half of the lysozyme activity but none of the other components of azurophil granules mentioned above. Furthermore, the specific granules of PMNs appear to be devoid of alkaline phosphatase. In this respect, therefore, they differ from the corresponding particles of the rabbit PMN (7, 8).

As shown by the distribution profiles of lysozyme activity, the resolution between the two main granule populations is virtually complete. Unfortunately, the specific granules are not resolved as well from an elusive small population of lysosomes reminiscent of the C particles of rabbit PMNs (8, 35). In both of the centrifugation systems used, the peak of lysozyme activity corresponding to the specific granules overlaps to various extents the activity profiles of at least three lysosomal hydrolases. These profiles, however, are not congruent with that of lysozyme, thus suggesting that the lysosomal enzymes belong to particles other than the specific granules. This interpretation is supported by the cytochemical results of Bainton et al. (10), who found the lysosomal enzymes to be restricted to the azurophil granules. We have not been able to identify, in electron micrographs of specific granule fractions, structures that may qualify as the carriers of the lysosomal hydrolases.

Another finding of interest was the clear-cut resolution between lysozyme and alkaline phosphatase. Alkaline phosphatase activity was almost totally resolved from the specific granule peak, both by rate sedimentation and isopycnic equilibration, irrespective of the substrate used for its assay. In this respect, biochemical and cytochemical results do not seem to agree. Bainton et al. (10) found alkaline phosphatase actvity in Golgi cisternae of myelocytes, as well as in immature specific granules. Accordingly, in light microscopy preparations, alkaline phosphatase appears at late stages of neutrophil development. The obvious interpretation of these observations, i.e., that alkaline phosphatase is confined in the specific granules, may not be correct, and the lack of activity in mature specific granules (10) may reflect the absence rather than latency of the enzyme. It is conceivable that the enzyme which appears to be packed into the developing specific granules of myelocytes is eventually inactivated.

Membrane Fraction

This fraction is characterized by the thiol-dependent acid 4-nitrophenyl phosphatase, which has a similar localization in rabbit PMNs, and by the alkaline phosphatase. The structures seen in micrographs of this fraction presumably arise from various membrane compartments of the cell, but it is likely that a large portion of them represents fragments of the plasmalemma. The profiles of the two phosphatases are very similar but not identical. In all experiments, the acid 4-nitrophenyl phosphatase is slightly heavier than alkaline phosphatase. This indicates that the two enzymes may belong to distinct particles which are barely resolved under the present experimental conditions. In the attempt of learning more about the localization of alkaline phosphatase, we have investigated the effects of several detergents on its activity. According to our results this alkaline phosphatase appears to be nonlatent, as opposed to that present in the specific granules of rabbit PMNs (19). The enzyme is presumably associated with a membrane. Whether it is localized in the vesicle-like structures or at the outer face of the rod-shaped particles found in fraction E (Fig. 12) might be determined by cytochemical methods.

In isopycnic fractionations of rabbit PMN homogenates (8, 19), a minor alkaline phosphatase peak regularly appeared in the equilibrium zone of the membrane fraction, and was thought to reflect the presence of fragments of damaged specific granules. In the light of the present results, however, it is tempting to consider that minor alkaline phosphatase peak as a separate entity, and to conclude that both human and rabbit PMNs may have an extragranular, membrane-bound alkaline phosphatase. Rabbit PMNs contain a further, much more active alkaline phosphatase in their specific granules, which appears to be lacking in human PMNs. In absolute terms, the alkaline phosphatase of human PMNs and the putative extra-granular alkaline phosphatase of the rabbit cells have about the same activity.

Fractionation of Human PMN Homogenates in Other Laboratories

The first attempt to isolate granule fractions from human PMNs was made by Schultz and co-workers (36, 37). These authors have submitted granule preparations obtained by differential centrifugation to isopycnic equilibration in sucrose (36) and in Ficoll (37). They found that peroxidase was largely concentrated in a band with average density of 1.24, together with large amounts of β-glucuronidase and acid phosphatase. Lysozyme activity, which was assayed in the Ficoll fractionations, exactly paralleled that of peroxidase, thus suggesting that, under these conditions, both azurophil and specific granules may have banded together. The granule band was resolved in both systems from a less dense particulate peak showing a relative enrichment in cytochrome oxidase but also containing sizable portions of the granule enzymes. More recent work by Olsson (38) showed for the first time that alkaline phosphatase activity is easily dissociated from that of lysosomal hydrolases, both by differential centrifugation and by density equilibration in colloidal silica. Unfortunately, in the latter experiments, the density distribution of alkaline phosphatase varied considerably, depending on whether the starting material was layered on top of, or incorporated into, the gradient-forming solution. Nevertheless, these data strongly suggest that the alkaline phosphatase-containing particles have a much smaller sedimentation coefficient and are considerably less dense than those containing the lysosomal hydrolases. This unexpected behavior of alkaline phosphatase was subsequently confirmed by Welsh et al. (39) and by West and Kimball (40). Using a five-step differential centrifugation scheme, Welsh and Spitznagel (41) found that peroxidase, β-glucuronidase, lysozyme, and acid 4-nitrophenyl phosphatase tend, in this order, to sediment at increasing centrifugal forces. Their conclusion that human PMNs contain three or even four types of lysosomes was perhaps premature, and seems to be in contradiction with more recent data from the same laboratory. A much better resolution was obtained by Leffell and Spitznagel (42) in a study on the subcellular localization of lactoferrin, using density gradient centrifugation. The distribution histograms of the illustrated experiment are consistent with those obtained in the present study. The lysozyme shows a bimodal distribution. Its two peaks correspond to small peaks of protein, and are well resolved from the bulk of the alkaline phosphatase which bands at low density. Most of the peroxidase accompanies the dense lysozyme peak, but nearly one-third of its total activity appears to be retained at the top of the gradient as does alkaline phosphatase. This is in contradiction with our results which show that peroxidase is totally confined to the densest and fastest sedimenting particulate band comprising the azurophil granules. Unfortunately, withou data on the distribution of lysosomal hydrolases, which could be taken as a reference, the significance of this bimodal distribution of peroxidase is difficult to assess. A major portion of the lactoferrin is found together with the light lysozyme peak. This important observation strongly supports our conclusion that the light lysozyme-containing particles represent the specific granules of human PMNs, since lactoferrin has been found to be an exclusive constitutent of specific granules in the PMNs of the rabbit (9). In a recent abstract, Spitznagel et al. (43), while confirming most of the data discussed above (42), claim that the azurophil granules may be resolved into two subpopulations which differ in their content of lysozyme, a neutral protease, and peroxidase. A resolution between acid 4-nitrophenyl phosphatase, three lysosomal hydrolases, and peroxidase has also been reported by Avila and Convit (26). These authors also studied the effects of various membrane-disrupting treatments on the activity of several particle-bound enzymes in postnuclear supernates from human PMN homogenates (44). The behavior of the lysosomal hydrolases, under the various experimental conditions adopted, is consistent with that observed in the present work, and equally fits with results obtained with postnuclear supernates of rabbit PMNs (Baggiolini, unpublished observations, and reference 45). The same cannot be said for the results on alkaline phosphatase, which appear to suffer from imperfections of the experimental design. The authors assume that alkaline phosphatase is a constituent of the specific granules, and, therefore, consider as soluble the portion of its activity that does not pellet at approximately 600,000 *g*-min. Under these conditions, as indicated from the data discussed in the present paper, a large portion of the alkaline phosphatase-containing particles cannot be expected to sediment into a pellet. This, however, would happen upon particle aggregation, which probably accounts for the reported increase

of alkaline phosphatase activity in the 600,000 g-min pellet when KCl is added to the medium. Furthermore, it is difficult to conceive of alkaline phosphatase being largely latent when its measurable activity does not increase with decreasing tonicity of the medium. We tend therefore to adhere to our interpretation of the detergent effects as some sort of enzyme activation, but it is clear that more work will be needed in order to clarify this issue.

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