ASSOCIATION OF THE ALKALINE PHOSPHATASE

OF RABBIT POLYMORPHONUCLEAR LEUKOCYTES

WITH THE MEMBRANE OF THE SPECIFIC GRANULES

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ABSTRACT

The localization of alkaline phosphatase in the specific granules of rabbit polymorphonuclear leukocytes was investigated. The results obtained suggest very strongly that alkaline phosphatase is a component of the granule membrane. The enzyme remains attached to the membrane upon disruption of the granules by the use of detergents or by hypotonic shock and subsequent extraction with sodium sulfate, and can be isolated together with fragments of the granule membrane by isopycnic equilibration. Treatment of the granules with high amounts of Triton-X-100, sodium deoxycholate, or hexadecyltrimethylammonium bromide releases the enzyme in soluble form. In polymorphonuclear leukocyte homogenates, lysis of the granules is needed in order to render alkaline phosphatase fully accessible to substrates. This suggests that the catalytic site of the enzyme is exposed at the inner face of the granule membrane.

INTRODUCTION

Earlier cytochemical (1–4) and biochemical (5–7) studies on rabbit polymorphonuclear leukocytes (PMNs) have demonstrated that alkaline phosphatase is entirely confined to the specific or secondary granules. In addition, the fractionation experiments had shown that the enzyme, unlike the other granule-bound hydrolases, was not released in soluble form following granule damage (6). A minor proportion of the total alkaline phosphatase activity was regularly present in fractions containing membrane fragments, but virtually none was recovered together with the soluble material. This indicated that the enzyme might be a component of the granule membrane or might become attached unspecifically to membranes upon release from the granules.

In this paper, we present multiple evidence suggesting that alkaline phosphatase is localized exclusively in the membrane of the specific granules.

MATERIALS AND METHODS

Fractionation Techniques

Rabbit PMNs were obtained from glycogen-induced peritoneal exudates, homogenized, and fractionated by differential centrifugation to give a postnuclear (4,000 g-min) supernate as described previously (5). Bovine serum albumin (BSA, 0.5 mg/ml) and $MgSO_4$ (5 mM), which were found to prevent inactivation of alkaline phosphatase, were added in most of the experiments to the 0.34 M sucrose medium after the preparation of the postnuclear supernate. BSA-free aliquots were kept for the determination of the protein content. The sucrose-BSA-$MgSO_4$ solution was also used for diluting the fractions obtained from isopycnic centrifugation of detergent-treated samples.

The postnuclear supernates were exposed to different treatments (see Results) and then further fractionated either by differential centrifugation or by

isopycnic equilibration. A particulate and a soluble fraction were separated by subjecting the postnuclear supernate to an integrated average centrifugal force of 3 × 10⁶ g-min. Portions of 1.5 ml of the appropriately treated postnuclear supernates containing 0.15–0.3 mg of protein/ml were centrifuged in 2 ml polycarbonate tubes (no. 59439) using a no. 59593 titanium angle rotor fitted with no. 59445 adaptors and operated by an MSE SS-65 ultracentrifuge (Measuring & Scientific Equipment Ltd., Crawley, Sussex, England) at the maximum speed of 40,000 rpm. The supernate was collected by aspiration using a disposable pipette, and the pellet was carefully resuspended in 1.5 ml of the medium, using a Teflon pestle tightly fitting the polycarbonate tubes. Isopycnic equilibration was carried out in a Beaufay rotor (8, 9) adapted to the MSE SS-65 ultracentrifuge under conditions (see Results) similar to those applied in previous experiments (6). The distribution histograms of protein content and enzyme activities are presented as a function of the gradient volume. The results were calculated and plotted with a Siemens 4004-35-64K computer (Siemens Corp., Iselin, N. J.) according to the method described by Beaufay et al. (10).

Biochemical Assays

Alkaline phosphatase was determined in 0.1 M diethanolamine-HCl buffer, pH 9.75, in the presence of 0.05% of ·Triton-X-100, and 50 mM β-glycerophosphate (β-GP), 5 mM adenosine 5'-monophosphate (5'-AMP), 5 mM adenosine 3'-monophosphate (3'-AMP), or 0.5 mM 4-methylumbelliferyl phosphate (4-MUP), respectively. The assays were started by adding 0.1 ml of sample to 0.1 ml of a substrate solution containing the above reagents. Incubation was carried out at 25°C for 30 min, and stopped by the addition of 2 ml of either a trichloroacetic acid solution or a glycine-NaOH buffer, pH 10.4. When inorganic phosphate was determined, the incubation was stopped with a solution containing 40 g of trichloroacetic acid, 10 g of ascorbic acid, and 1 g of ethylenediaminetetraacetate in 100 ml. The acidified mixture was left for 15 min in ice and then filtered through a Whatman GF/C filter (W. and R. Balston, Ltd., Maidstone, Kent, England). Phosphate was determined in the filtrate according to the method of Baginski and Zak (11) and measured at 850 nm in a Zeiss PM 4 spectrophotometer (Carl Zeiss, Ober Kochen, W. Germany) equipped with an infrared-sensitive photocell. 4-Methylumbelliferone was measured fluorometrically in the incubation mixture after stopping the reaction with a 0.05 M glycine-NaOH buffer, pH 10.4, using a Hitachi 203 fluorimeter (Perkin-Elmer Corp., Norwalk, Conn.) (12). In appropriate experiments it was established that under the described conditions no further hydrolysis of

4-methylumbelliferyl phosphate occurred within 1 h after addition of the glycine stopping solution.

Alkaline 4-nitrophenyl phosphatase was determined in 0.1 M diethanolamine buffer, pH 9.75, in the presence of 0.05% of Triton-X-100 and of 1.25 mM 4-nitrophenyl phosphate (4-NPP). The assay was started by adding 0.1 ml of sample to 1.0 ml of a substrate solution containing the above reagents. Incubation was carried out at 25°C for 30 min, and stopped by the addition of 1 ml of 2 N NaOH. Optical density was then measured at 405 nm in a Zeiss PM 4 spectrophotometer. Myeloperoxidase was determined in 0.1 M citric acid-sodium citrate buffer, pH 5.5, in the presence of 0.05% of Triton-X-100, of approximately 0.08 mM H_2O_2, and of 0.32 mM o-dianisidine (13). The assay was started by adding 0.1 ml of sample to 1.0 ml of a substrate solution containing the above reagents. Incubation was carried out at room temperature for 1 min, and stopped by the addition of 1 ml of 35% (vol/vol) perchloric acid. Optical density was then measured at 560 nm in a Zeiss PM 4 spectrophotometer.

β-Glucuronidase, α-mannosidase, and N-acetyl-β-glucosaminidase (β-acetyl glucosaminidase) were determined in 0.1 M acetic acid-sodium acetate buffer, pH 4.5, in the presence of 0.05% Triton-X-100, and 0.1 mM of the 4-methylumbelliferyl substrate (see Materials, following). The assay was started by adding 0.1 ml of sample to 0.1 ml of a substrate solution containing the above reagents. Incubation was carried out at 37°C for 30 min and stopped by the addition of 2 ml of a 0.05 M glycine-NaOH buffer, pH 10.4. The 4-methylumbelliferone formed was measured fluorimetrically in a Hitachi 203 fluorimeter (12).

Lysozyme and protein were determined according to published methods (5, 6) which were adapted to the available equipment.

The radioactivity of fractions from centrifugation experiments was measured in 0.1 ml aliquots of the samples mixed with 10 ml of 12% (vol/vol) ethanolamine in methanol and 10 ml of a scintillator solution containing 6 g of PBD (see Materials) per liter of toluene, using a Beckman LS-233 liquid scintillation counter (Beckman Instruments International S.A., Geneva, Switzerland).

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 in order to establish optimum assay conditions. Linearity over the period of incubation adopted and proportionality with amount of sample were verified in all cases. Specific activities of the enzymes were calculated on the basis of the measurement of appropriate internal standards. Blank values for the enzyme assays were obtained by adding to the incubated substrate solution, in this order, the stopping solution and the sample.

Determination of Alkaline Phosphatase Latency

The free activity of alkaline phosphatase was assayed in 0.1 M triethanolamine-HCl buffer, pH 7.5, whose tonicity was made up to 340 mosM by the addition of sucrose, in the presence of 0.1 mM 4-methylumbelliferyl phosphate. The assay was started by adding 0.1 ml of sample in 0.34 M sucrose to 0.1 ml of the isotonic substrate solution. Incubation was carried out at 25°C for 20 min, and stopped as described in the preceding section. The total alkaline phosphatase activity was measured under the same conditions with an excess of detergent added to the substrate solution. Latency is the difference between total and free activity expressed as percent of total activity. The effect of detergents on the latency of alkaline phosphatase was determined by measuring the enzyme activity in the presence of increasing amounts of detergents.

Negative Staining for Electron Microscopy

For the preparation of negatively stained specimens, the biological material was adsorbed on carbon-coated, UV-treated copper grids and stained with a 2% (wt/vol) solution of phosphotungstic acid adjusted to pH 6.8. Micrographs were taken in a Siemens Elmiskop 101 microscope (Siemens, Berlin, W. Germany).

MATERIALS

Reagents used in the present work were obtained from the following sources: Amend Drug & Chemical Co. Inc. (New York)-glycogen, chemically pure; Armour Pharmaceutical Co. (Chicago, Ill.)-bovine albumin powder (fraction V from bovine plasma); Fluka A. G. (Buchs, Switzerland)-2-(4-tert-butyl-phenyl)-5-(4-biphenyl)-1,3,4-oxadiazole (PBD), D-(+)-sucrose (for bacteriology, puriss.) hexadecyl-trimethylammonium bromide (purum); Koch-Light Laboratories (Colnbrook, Buckinghamshire, England)-4-methylumbelliferone, 4-methylumbelliferyl-β-D-glucuronide, 4-methylumbelliferyl-α-D-manno-pyranoside, 4-methylumbelliferyl-2-acetamido-2-des-oxy-β-D-glucopyranoside, and 4-methylumbelliferyl dihydrogen phosphate; E. Merck A. G. (Darmstadt, W. Germany)-4-nitrophenol, 4-nitrophenyl phosphate (disodium salt, 5 H$_2$O), o-dianisidine, digitonin (cryst.), and sodium deoxycholate; NEN Chemicals GmbH (Frankfurt a. M., Germany)-[^{14}C]sucrose; Packard Instruments Co., Inc. (Downers Grove, Ill.)-Triton-X-100; Sigma Chemical Co. (St. Louis, Mo.)-DL-β-glycerophosphate (grade I), adenosine 5'-mono-phosphoric acid (type IV), and adenoside 3'-mono-phosphoric acid.

RESULTS

Subcellular Distribution of Alkaline Phosphatase Assayed with Different Phosphate Esters

SPECIFIC ACTIVITIES: The alkaline phosphatase activity of postnuclear supernates of rabbit PMN homogenates was determined with various concentrations of five different substrates, 4-nitrophenyl phosphate, β-glycerophosphate, 4-methylumbelliferyl phosphate, adenosine 5'-mono-phosphate and adenosine 3'-monophosphate. As expected, in all cases high concentrations of substrate were inhibitory (14). Table I shows the specific activities obtained at optimum substrate concentration and the corresponding apparent K_m values determined from the linear part of the Lineweaver-Burk plot.

SUBCELLULAR DISTRIBUTIONS: The distribution of alkaline phosphatase assayed with the above substrates after zonal density gradient centrifugation of postnuclear supernates of rabbit PMN homogenates is shown in Fig. 1. The profiles are almost identical, irrespective of the substrate used. They are characterized by a predominant peak with modal density of 1.23, accounting for

TABLE I

Specific Activities and Apparent K_m Values of Alkaline Phosphatase Determined with Different Substrates in Post-nuclear Supernates of Rabbit PMN Homogenates

Substrate	Specific activity*	Apparent K_m
		mM
4-Nitrophenyl phosphate	0.300	0.27
β-Glycerophosphate	0.053	7.14
4-Methylumbelliferyl phosphate		
(pH 9.75)	0.073	0.26
(pH 7.5)‡	0.007	—
Adenosine 5'-monophosphate	0.042	1.92
Adenosine 3'-monophosphate	0.042	1.28

*Values give specific activity (mean from two to three experiments) in IU per mg of protein. One unit of activity is defined as the amount of enzyme necessary to split 1 μmol of substrate in 1 min at 25°C at optimum substrate concentration and optimum pH.

‡ The specific activity of 4-methylumbelliferyl phosphatase was also determined at pH 7.5, under the conditions used for measuring enzyme latency.
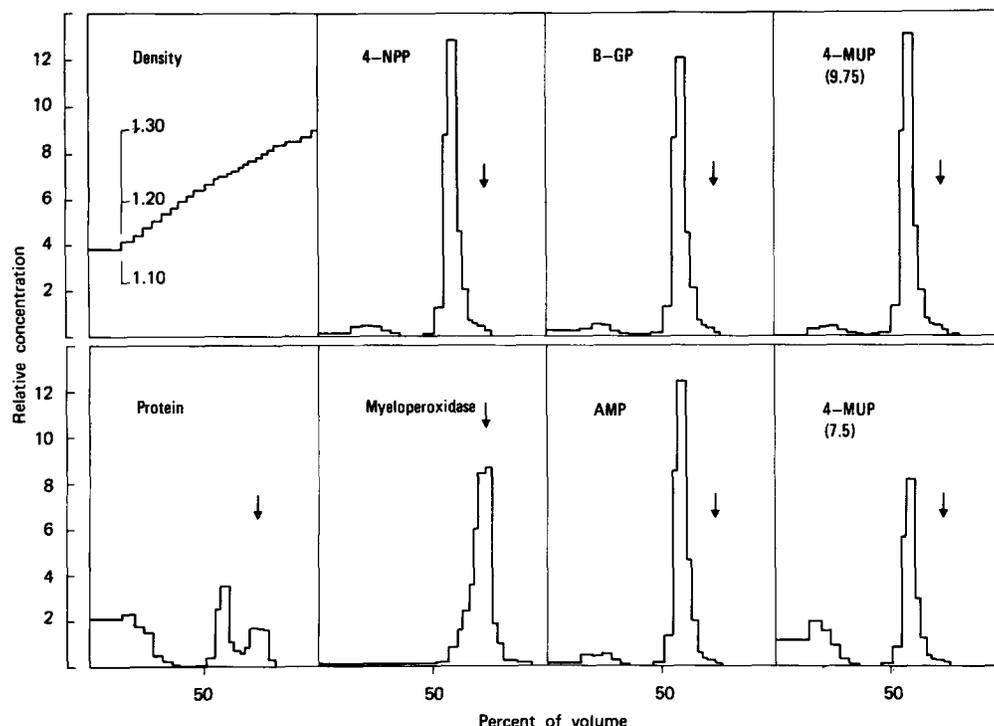
FIGURE 1 Isopycnic equilibration of subcellular components of rabbit PMNs. Distribution of alkaline phosphatase assayed with various substrates. 10 ml of a postnuclear supernate, brought to a density of 1.10 by the addition of 60% (wt/wt) sucrose, were layered over a 30-ml sucrose concentration gradient extending linearly with respect to volume between the densities 1.18 and 1.32. The gradient rested on a 5-ml sucrose cushion of density 1.32. Centrifugation was carried out at 30,000 rpm for 2 h. Graphs are normalized distribution histograms as a function of the volume collected. Average density of each fraction is given in the upper left-hand plot. Percentage recoveries were 114 for protein, 100 for myeloperoxidase, 92, 94, 96, and 93 for alkaline phosphatase assayed with 4-nitrophenyl phosphate (4-NPP), β-glycerophosphate (β-GP), adenosine 5'-monophosphate (5'-AMP), and 4-methylumbelliferyl phosphate (4-MUP) respectively, and 99 for 4-methylumbelliferyl phosphatase activity at pH 7.5. Arrows indicate the modal equilibrium density of myeloperoxidase.

almost all the alkaline phosphatase activity of the preparations. In this peak, alkaline phosphatase is enriched up to 13 times with respect to uniform activity distribution throughout the gradient. Less than 10% of the total activity is found in a small peak with modal density of 1.14–1.15. This peak is clearly resolved from the starting zone which is almost free of enzyme activity. These results confirm our previous findings (5–7) that in rabbit PMNs alkaline phosphatase activity is confined to the specific granules. It seems likely that we are dealing with one enzyme acting on different phosphate esters, although the above fractionation data could also suggest that the specific granules contain more than one alkaline phosphatase. Using 4-methylumbelliferyl phosphate, we also deter-

mined the distribution of phosphatase activity at pH 7.5, under the conditions used for measuring enzyme latency. In this case, 60–70% of the total coincide with the alkaline phosphatase peak, while the remaining 30–40% are recovered in the sample zone and might reflect the presence of a soluble phosphatase inactive at high pH. As indicated in Table I, the absolute 4-methylumbelliferyl phosphatase activity at pH 7.5 is nearly one-tenth of that found at the optimum pH of 9.75. Myeloperoxidase, which was determined as a marker for the azurophil granules (5, 6), equilibrates as expected at the density of 1.26 and is almost completely resolved from the alkaline phosphatase. Both the myeloperoxidase and the alkaline phosphatase bands coincide with a protein peak.

TABLE II

Distribution of Alkaline Phosphatase and Lysozyme between the 3 × 10⁶ g-min Pellet (P) and Supernate (S) of Suspensions of Rabbit PMN Granules Treated with Detergents

Detergent	mg/ml of detergent mg of protein	Relative enzyme distributions*					
		Alkaline phosphatase			Lysozyme		
		P	S	(recovery)	P	S	(recovery)
None (controls)	—	98	2	(88)	89	11	(110)
Digitonin	0.66	100	0	(80)	7	93	(107)
Triton-X-100	2.50	93	7	(75)	2	98	(106)
Sodium deoxycholate	0.62	98	2	(68)	9	91	(88)

*The sum of the enzyme activities of supernate and pellet were taken to be 100% and the relative amounts in both fractions were calculated. The recovery of enzyme activity in the two fractions, expressed as percent of the corresponding activity in the unfractionated material, is given in brackets. The data represent mean values from two to four different experiments.

Distribution of Alkaline Phosphatase after Lysis of the Granules

Postnuclear supernates were treated with various membrane-disrupting agents and the distribution of alkaline phosphatase between a 3×10^6 g-min supernate and the corresponding pellet was measured, using 4-nitrophenyl phosphate as the substrate (see Table II). In selected experiments, the distribution of lysozyme, two-thirds of which are found in the specific granules (5, 6), was also determined. After the addition to the postnuclear supernates of amounts of detergents which lyse both the specific and the azurophil granules, as indicated by the complete release of lysozyme, nearly all of the alkaline phosphatase activity remains bound to structures that are sedimentable at 3×10^6 g-min. As shown in Fig. 2, treatment of postnuclear supernates with digitonin releases very little, if any, alkaline phosphatase into the soluble fraction. In contrast, lysozyme as well as a number of enzymes which are localized in the azurophil granules (5, 6) are released almost completely by digitonin in the presence of high concentrations of salt. The effect of salt is particularly impressive in the case of myeloperoxidase which is completely sedimentable in the absence, and completely soluble in the presence of 0.4 M sodium sulfate. As upon density equilibration of intact granules (Fig. 1), in these experiments we obtained almost identical distributions of alkaline phosphatase with different phosphate esters as substrate, which strongly suggests that rabbit PMNs contain only one alkaline phosphatase with broad substrate
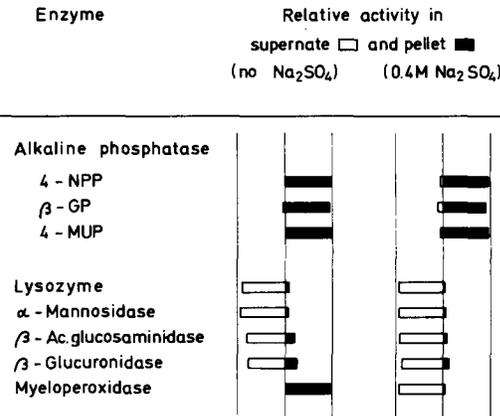

FIGURE 2 Relative distribution of alkaline phosphatase and of other granule-bound enzymes between the 3×10^6 g-min pellet and supernate from suspensions of rabbit PMN granules treated with digitonin. Postnuclear supernates containing on average 2.0–3.5 mg of protein per ml were diluted 1:20 with a 0.05% solution of digitonin in 0.34 M sucrose or in 0.34 M sucrose containing 0.4 M sodium sulfate. The sum of the enzyme activities in the pellet and in the supernate was taken to be 100%, and the relative amounts in both fractions were calculated. The average recoveries (mean ± standard deviation) of enzyme activity in the two fractions in percent of the corresponding activity in the unfractionated material were 85 ± 6 for alkaline phosphatase (with different substrates), 106 for lysozyme, 102 for α-mannosidase, 100 ± 6 for β-acetyl-glucosaminidase, 86 for β-glucuronidase, and 93 ± 9 for myeloperoxidase. The following symbols were used for the substrates of alkaline phosphatase: 4-NPP, 4-nitrophenyl phosphate; β-GP, β-glycerophosphate; 4-MUP, 4-methylumbelliferyl phosphate.
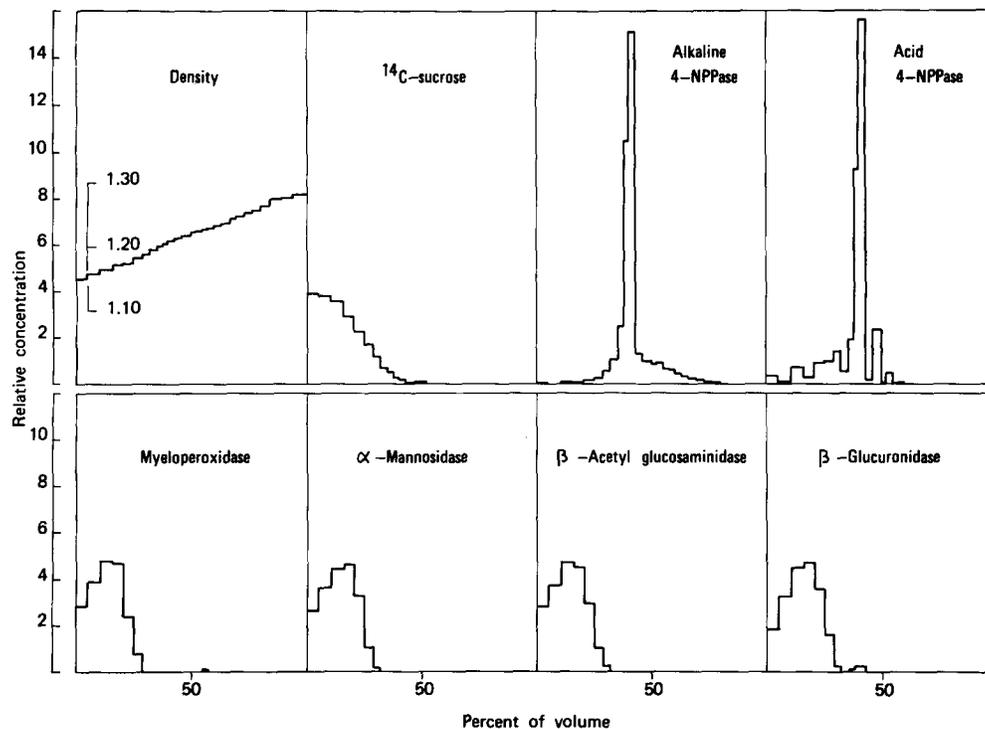
FIGURE 3 Isopycnic equilibration of subcellular components of rabbit PMNs after exposure to digitonin and sodium sulfate. 10 ml of a postnuclear supernate containing 25 mg of protein, 0.02% (wt/vol) digitonin, 0.4 M sodium sulfate, 5 mM magnesium sulfate, 0.05% (wt/vol) BSA, and approximately 0.05 μCi of [^{14}C]sucrose, were brought to a density of 1.13 by the addition of sucrose and layered over a 30-ml sucrose concentration gradient. The gradient was linear with respect to volume between the densities 1.18 and 1.29, contained 5.0 mM magnesium sulfate and 0.05% (wt/vol) BSA, and rested on a 5-ml sucrose cushion of density 1.29. Centrifugation was carried out at 30,000 rpm for 2 h. Graphs are normalized distribution histograms as a function of the volume collected. Average density of each fraction is given in the upper left-hand plot. Percentage recoveries were 101 for [^{14}C]sucrose, 75 for alkaline phosphatase, 64 for acid 4-nitrophenyl phosphatase, 93 for myeloperoxidase, 106 for α-mannosidase, 98 for β-acetyl glucosaminidase, and 116 for β-glucuronidase.

specificity. Fig. 3 shows the distribution patterns of alkaline phosphatase (assayed with 4-nitrophenyl phosphate) and other particulate enzymes of a postnuclear supernate treated with digitonin and 0.4 M sodium sulfate, and fractionated by zonal isopycnic equilibration. As one would expect in view of the data of Fig. 2, the glycosidases and myeloperoxidase, which are solubilized by the treatment, are retained in the starting zone, marked here by the distribution of radioactive sucrose added to the sample. Alkaline phosphatase, on the contrary, is completely resolved from the sample zone. It is found well below the density of intact specific granules, very close to the thiol-dependent acid 4-nitrophenyl phosphatase (6), where membranes of disrupted specific granules

are likely to equilibrate. The equilibrium density of nearly 1.20, which may appear rather high for membrane fragments, could be brought about by the binding of digitonin to membrane cholesterol, a phenomenon which apparently occurs in membranes of rat hepatocytes (15).

In one experiment, the distribution of alkaline phosphatase after hypotonic disruption and saline extraction of purified specific granules was examined. A postnuclear supernate was fractionated by zonal differential sedimentation in a B-XIV rotor at 7000 rpm for 15 min under the conditions established in previous experiments (5), and about one-third of the specific granules was collected within three fractions of approximately 20 ml each (Fig. 4, shaded area of the left-hand graphs). This

specific granule fraction contained 32% of the alkaline phosphatase, together with 6% of the β-acetyl glucosaminidase and 0.8% of the myeloperoxidase, and was therefore only minimally contaminated by C particles and by azurophil granules (16). The specific granule fraction was concentrated to about 2 ml by ultrafiltration through a XM-100 membrane in a 60 ml Amicon Model 12 ultrafiltration cell (Amicon Corp., Lexington, Mass.) under nitrogen at a pressure of 2 atm. The material remaining in the ultrafiltration cell was collected quantitatively with approximately 30 ml of distilled water, and homogenized at 0°C in an automatically operated Teflon-glass homogenizer (Arthur H. Thomas Co., Philadelphia) at 1,800 rpm and four up-and-down strokes per minute. By resuspending the ultrafiltration residue in water, the osmolarity of the medium containing the granules was lowered to about 30 mosmol per liter, a tonicity sufficiently low to osmotically disrupt more than 90% of the specific granules. 12 ml of the hypotonic suspension of

granule constituents were mixed with 3 ml of 2 M sodium sulfate, and brought to a density of 1.10 by the addition of sucrose. Approximately two-thirds of this mixture was then fractionated by isopycnic centrifugation in a Beaufay rotor under the conditions given in the legend of Fig. 4. As shown in Fig. 4 (right-hand graphs), alkaline phosphatase equilibrates in a single, slightly asymmetrical peak with modal density of 1.18, accounting for approximately 90% of the enzyme activity in the starting material. The alkaline phosphatase peak coincides with a small, well-resolved protein peak. Most of the protein, however, is retained in the sample zone and most likely represents granule constituents released in soluble form by hypotonic shock and salt extraction. Micrographs of negatively stained preparations of the starting material and of the two fractions with the highest relative concentration in alkaline phosphatase (Fig. 5) consist essentially only of membrane fragments. This shows that the starting material was virtually free of intact specific granules, and provides further
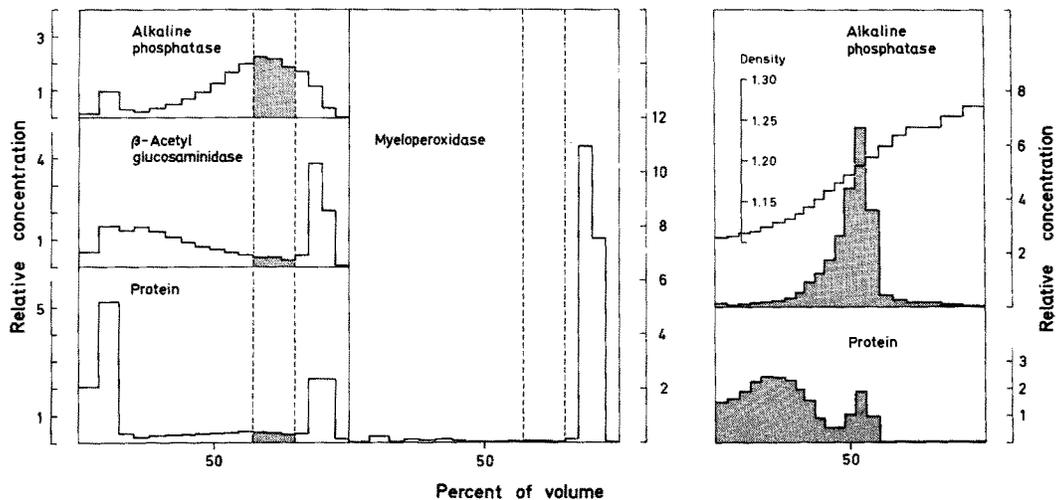
Figure 4 Isolation of membranes from specific granules of rabbit PMNs by zonal sedimentation and isopycnic equilibration. Distribution of alkaline phosphatase. The left-hand graphs show enzyme distribution histograms after zonal sedimentation of a postnuclear supernate. The shaded areas indicate the specific granule preparation collected. The experimental conditions are given in the text. The right-hand graphs show the density-equilibration histograms of alkaline phosphatase and protein after isopycnic centrifugation of a suspension of osmotically lysed and sodium sulfate-extracted specific granules (see text) in a Beaufay rotor. 14 ml of sample with a density of 1.10 were layered over a 20-ml sucrose concentration gradient extending linearly with respect to volume between the densities 1.16 and 1.29 and resting on a 5-ml sucrose cushion of density 1.29. Centrifugation was carried out at 30,000 rpm for 2 h. Graphs are normalized distribution histograms as a function of the volume collected. Average density of each fraction of the density-equilibration experiment is given in the upper right-hand graph. Percentage recoveries were 93 and 77 for protein, 91 and 78 for alkaline phosphatase, 91 for myeloperoxidase, and 72 for β-acetyl glucosaminidase.
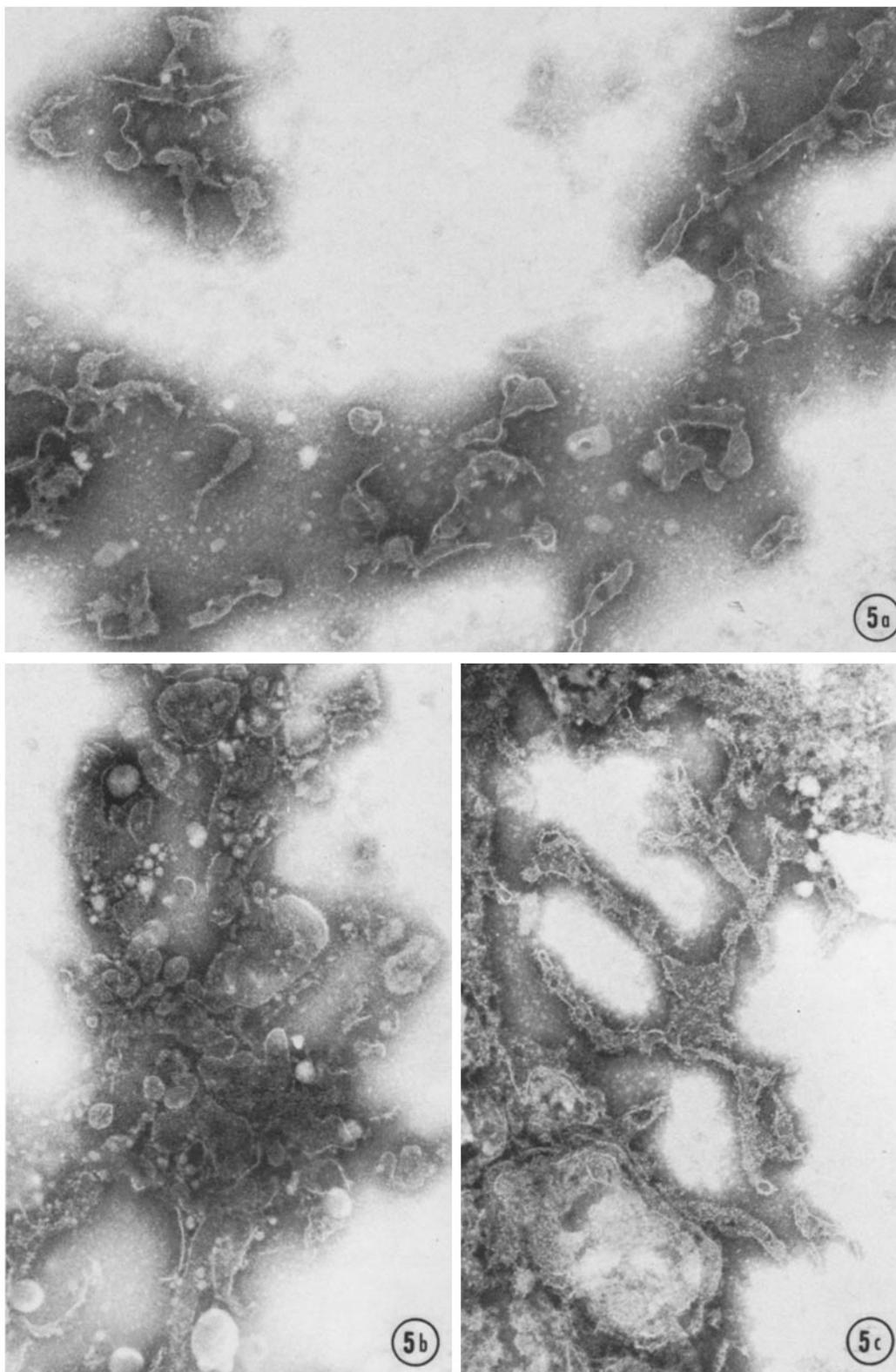
FIGURE 5 Negatively stained samples of the starting material (a) and of the two fractions with the highest relative concentrations in alkaline phosphatase (b and c) from the experiment of Fig. 4, right-hand graphs. All three preparations consist virtually of membrane fragments only. × 70,000.

evidence for the localization of alkaline phosphatase within the granule membrane.

Latency of Alkaline Phosphatase in Intact Specific Granules

Determinations of alkaline phosphatase activity in fresh PMN homogenates at nearly neutral pH in the presence and in the absence of detergents show that, in intact granules, the enzyme is not fully accessible to substrates that do not diffuse through the granule membrane. In postnuclear supernates, the alkaline phosphatase activity increased with increasing concentrations of digitonin, hexadecyltrimethylammonium bromide, or Triton-X-100 up to a maximum-value plateau. In these experiments, however, the enzyme activity in the absence of detergents (free activity) was on average as high as 50% of the total. Since this rather high proportion of free activity seemed to result from the fact that at pH 7.5 the substrate used was hydrolyzed by both the alkaline phosphatase and by another, nonlatent, enzyme, as suggested by the different subcellular distributions of 4-methylumbelliferyl phosphatase activity at pH 7.5 and 9.75 (Fig. 1 and Table III), the latency experiments were repeated with isolated

TABLE III

Isolation of a Granule Fraction from a Postnuclear Supernate of Rabbit PMN Homogenates by Zonal Sedimentation with Isopycnic Banding in a Swinging Bucket Rotor

Volume collected from top to bottom	Protein	Alkaline phosphatase pH 9.75	Alkaline phosphatase pH 7.5	Myeloperoxidase	Acid 4-NP-phosphatase
ml					
8.9	57.1	2.8	33.8	2.1	18.4
3.8	2.1	2.4	3.7	0.3	26.1
3.3	40.1	94.0	61.8	96.0	54.5
3.1	0.7	0.8	0.7	1.6	1.0

Relative distributions of

Five ml of a postnuclear supernate were layered onto a discontinuous density gradient consisting of 8, 2 and 5 ml of sucrose solutions with densities of 1.08, 1.23 and 1.32 respectively. Values (mean from two experiments) represent the amount of component in the fraction expressed as percent of the total amount recovered. The percentage recoveries were: 97 for protein, 80 and 76 for alkaline phosphatase, 90 for myeloperoxidase and 106 for acid 4-nitrophenyl phosphatase.

granules. Granule preparations were obtained by zonal sedimentation with isopycnic banding in a swinging bucket rotor (MSE No. 59590, Measuring & Scientific Equipment Ltd., London). 5 ml of a postnuclear supernate were layered in a 25-ml tube over a discontinuous sucrose density gradient consisting of three steps of 8, 2, and 5 ml of density 1.08, 1.23, and 1.32 respectively, and centrifuged at 30,000 rpm for 90 min. The granules were collected by aspiration in the thin zone of density 1.23 and diluted five to ten times with 0.34 M sucrose. Table III shows that the granule fraction accounts for almost all of the 4-methylumbelliferyl phosphatase activity at pH 9.75 but only for about ⅔ of the corresponding activity at pH 7.5, the remainder being soluble. The latency curves
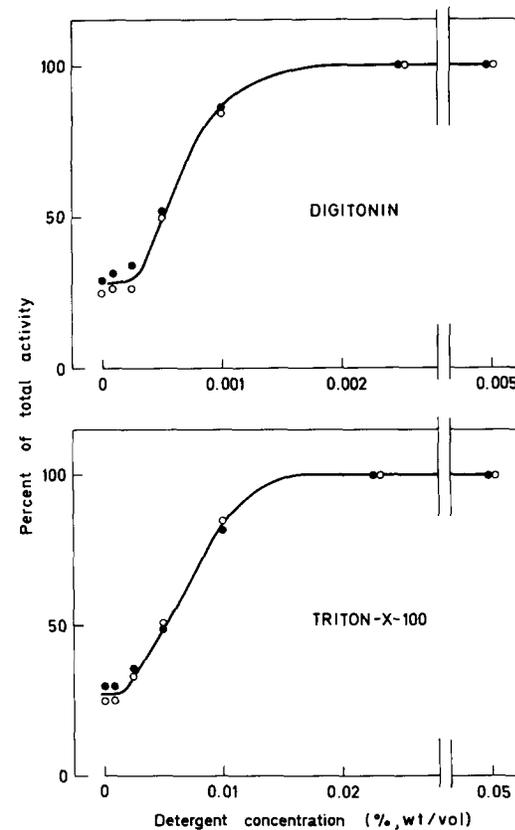

FIGURE 6   Latency of alkaline phosphatase in granule fractions of rabbit PMNs. Results from two experiments (white and black symbols) with two different granule preparations. In both experiments, the protein concentration in the assay mixtures was 0.12 mg/ml. Note that the concentrations of Triton-X-100 used are 10 times higher than those of digitonin.

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obtained with the granule fraction are shown in Fig. 6. Both with digitonin and Triton-X-100 typical sigmoidal curves were obtained. The free activity was about 25% of the total. These results demonstrate that the membrane of the specific granules must be damaged in order to render alkaline phosphatase accessible to its substrates. The enzyme, or at least its catalytic site, seems therefore to be localized at the inner face of the granule membrane.

Solubilization of Alkaline Phosphatase in PMN Granule Preparations

Suitably diluted postnuclear supernates from PMN homogenates were treated with various detergents in the presence of different concentrations of sodium sulfate. A particulate and a soluble fraction (3 × 10⁶ g-min pellet and supernate, respectively) were then separated as described in the

section on methods, and the distribution of alkaline phosphatase was determined.

As shown in Fig. 7, Triton-X-100, hexadecyltrimethylammonium bromide, and sodium deoxycholate all release alkaline phosphatase into the soluble fraction while digitonin does not. In the presence of relatively high concentrations of salt, some differences in the solubilizing activity of the detergents became apparent. The solubilization of enzyme in the presence of Triton-X-100 was linearly proportional to the salt concentration of the medium, while being nearly independent of the concentration of the detergent over a rather wide range. The reverse was observed with hexadecyltrimethylammonium bromide, whose solubilizing activity was completely independent of the amount of added salt. Finally, sodium deoxycholate seemed to take an intermediate position: the solubilizing effect of detergent and sodium sulfate seemed to be additive.
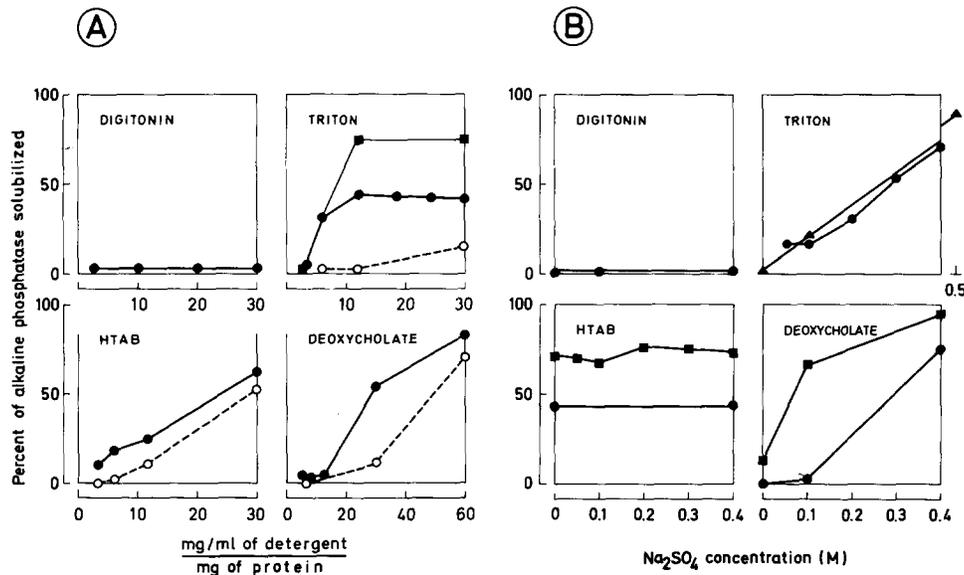
FIGURE 7   Solubilization of alkaline phosphatase from postnuclear supernates of rabbit PMN homogenates. (A) White symbols and broken lines represent values obtained in the absence of salt. Black symbols and solid lines represent values obtained in the presence of 0.4 M Na₂SO₄. Two such experiments (● and ■) with different granule preparations are shown for the solubilization with Triton-X-100. (B) The following concentrations of detergents were used 0.2 (▲), 0.5 (●), and 1.0 (■) mg/ml. The amount of protein per assay was 0.017 mg. Triton means Triton-X-100, and HTAB means hexadecyltrimethylammonium bromide. The sum of the soluble and particulate enzyme activities (activities in the 3 × 10⁶ g-min supernate and pellet, respectively) were taken to be 100%, and the percentage of solubilized enzyme calculated. The average recoveries of enzyme activity in the soluble and particulate fractions, expressed as percent of the corresponding activity of the starting material, were between 78 and 92% under all experimental conditions with the exception of one. Solubilization by HTAB in the absence of salt apparently resulted in enzyme inactivation. In this case, the percentage recovery was 46 ± 7.

DISCUSSION

The results presented suggest very strongly that the alkaline phosphatase of rabbit PMNs is a component of the membrane of the specific granules. The enzyme is not released in soluble form after disruption of the granules by osmotic shock, or by appropriate concentrations of detergents, and it is not extractable from disrupted granules by salt alone even in high concentrations. Upon density gradient centrifugation of specific granule samples disrupted by detergents or osmotic shock and extracted with salt, the alkaline phosphatase activity equilibrates together with the granule membranes. Very high amounts of Triton-X-100, of sodium deoxycholate, or of hexadecyltrimethyl-ammonium bromide are needed to solubilize the enzyme. In intact specific granules, alkaline phosphatase was found to be latent to a great extent, thus suggesting that the catalytic site of the enzyme is exposed at the inner face of the granule membrane.

On the basis of these results, it is possible to explain the apparently anomalous redistribution of alkaline phosphatase in phagocytosing rabbit PMNs described by Bainton (17, 18) and by Henson (19). These authors observed that alkaline phosphatase activity, visualized cytochemically by the deposition of lead phosphate, is found almost exclusively at the inner face of the membrane of phagocytic vacuoles, quite in contrast to myeloperoxidase, for instance, which can be demonstrated in the lumen of the vacuole, and often surrounds the phagocytosed material (18, and D. F. Bainton, unpublished micrographs). In addition, Henson (20) observed that rabbit PMNs exposed to nonphagocytosable particles do not release alkaline phosphatase into the medium together with lysozyme and other granule components. The cytochemical data discussed suggest that alkaline phosphatase, which is tightly bound to the membrane of the specific granules under the conditions of our experiments, is not released in soluble form when, after granule fusion with the phagosome, the granule membrane becomes exposed to the medium of the digestive vacuole.

Alkaline phosphatase is, so far, the only enzyme which has been shown with a high degree of probability to be localized in the membrane of the specific granules of PMNs. This is a further, important demonstration of the biochemical difference between the membrane of the azurophil and that of the specific granules of rabbit PMNs, as

suggested by previous experiments (21). As a component of the membrane of the specific granules, which have been shown to arise from the convex face of the Golgi apparatus of myelocytes (3, 4), alkaline phosphatase should be present in the membranes of the outer Golgi cisternae in the later stages of PMN maturation and could perhaps be used as a cytochemical marker for membrane flow during granule formation.

REFERENCES

1. WETZEL, B. K., R. G. HORN, and S. S. SPICER. 1963. Cytochemical localization of non-specific phosphatase activity in rabbit myeloid elements. *J. Histochem. Cytochem.* 11:812.
2. WETZEL, B. K., S. S. SPICER, and R. G. HORN. 1967. Fine structural localization of acid and alkaline phosphatases in cells of rabbit blood and bone marrow. *J. Histochem. Cytochem.* 15:311.
3. BAINTON, D. F., and M. G. FARQUHAR. 1968. Differences in enzyme content of azurophil and specific granules of polymorphonuclear leukocytes. I. Histochemical staining of bone marrow smears. *J. Cell Biol.* 39:286.
4. BAINTON, D. F., and M. G. FARQUHAR. 1968. Differences in enzyme content of azurophil and specific granules of polymorphonuclear leukocytes. II. Cytochemistry and electron microscopy of bone marrow cells. *J. Cell Biol.* 39:299.
5. BAGGIOLINI, M., J. G. HIRSCH, and C. DE DUVE. 1969. Resolution of granules from rabbit heterophil leukocytes into distinct populations by zonal sedimentation. *J. Cell Biol.* 40:529.
6. BAGGIOLINI, M., J. G. HIRSCH, and C. DE DUVE. 1970. Further biochemical and morphological studies of granule fractions from rabbit heterophil leukocytes. *J. Cell Biol.* 45:586.
7. BAGGIOLINI, M., C. DE DUVE, P. L. MASSON, and J. F. HEREMANS. 1970. Association of lactoferrin with specific granules in rabbit heterophil leukocytes. *J. Exp. Med.* 131:559.
8. BEAUFAY, H. 1966. La centrifugation en gradient de densité. Thèse d'aggrégation de l'enseignement supérieur, Université Catholique de Louvain, Louvain, Belgium. Ceuterick S.A., Louvain, Belgium.
9. LEIGHTON, F., B. POOLE, H. BEAUFAY, P. BAUD-