ISOZYMES OF ACID PHOSPHATASE IN NORMAL AND CALMETTE-GUÉRIN BACILLUS-INDUCED RABBIT ALVEOLAR MACROPHAGES*

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The discovery by de Duve (1) that lysosomes contain the major portion of intracellular hydrolytic enzymes has opened the way for new insights into the digestive processes of phagocytic cells. Hirsch and Cohn (2, 3) have shown that lysosomes are particularly large and numerous in macrophages and polymorphonuclear leukocytes and have clearly demonstrated lysosomal participation in bacterial digestion by phagocytic cells. However, the enzymatic changes that accompany phagocytosis and the specific role of these enzymes remain to be clarified. In this initial paper on the chemistry of phagocytosis we have investigated acid phosphatase, a principle lysosomal enzyme, of activated phagocytic cells. There have been no reports indicating the existence of isoenzymes of acid phosphatase in phagocytic cells although there have been several studies of total enzyme activity in mononuclear phagocytes (4–6). The demonstration of multiple forms of acid phosphatase in alveolar macrophages, a partial characterization of the isoenzymes, and analysis of quantitative changes in the various forms following phagocytic stimulation form the subject of this report. Our experimental system consists of Calmette-Guérin bacillus (BCG)–stimulated and nonstimulated rabbit alveolar macrophages. A preliminary report describing the multiple forms of macrophage acid phosphatase was presented to the American Association of Immunologists (7).

Materials and Methods

Normal Alveolar Macrophages.—The general methods of Myrvik et al. (8) and Cohn and Wiener (4) were used to obtain alveolar macrophages from normal New Zealand albino

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rabbits weighing 2–4 kg. The yield from each normal rabbit was 1–2 × 10^7 cells of which 95% were large macrophages. Only preparations that were erythrocyte-free were used for analysis.

BCG-Induced Alveolar Macrophages.—The procedure of Cohn and Wiener (4) was used to obtain alveolar macrophages from rabbits following BCG stimulation. BCG (Aranson), kindly supplied by Dr. Z. Cohn of The Rockefeller University, was injected into the marginal ear vein of the rabbits on 2 consecutive days. Each injection contained 20 mg of the water-washed, lyophilized, killed BCG suspended in 1 ml of saline containing 0.01% Tween 80. The alveolar macrophages were harvested in the same manner as for normal rabbits 28–35 days after BCG injection. The yield from each rabbit was 1–3 × 10^9 cells of which 80–85% of the cells were large macrophages, 5–10% small mononuclear cells, and the remainder were polymorphonuclear leukocytes.

Macrophage Disruption.—The macrophages were collected by centrifugation at 140 *g* for 15 min at 4°C. The cells were saline washed four times and resuspended in saline to a cell concentration of 1–2 × 10^7/ml.

The saline-washed macrophages were disrupted by one of four methods: (*a*) five cycles of alternate freeze-thaw treatment; (*b*) vigorous homogenization with a Teflon pestle for 4 min in an ice bath; (*c*) French press treatment with exposure to 6000 psi in a Wabash press; or (*d*) nonionic detergent treatment with 5% Triton X-100 for 30 min at 4°C. Disruption of at least 95% of the cells was achieved by each method used.

Disrupted cell suspensions were then centrifuged at 11,000 *g* for 15 min in a refrigerated centrifuge. The resulting supernatant combined with the first wash of the pellet formed the "11,000 *g* supernatant" fraction.

After four washes of the pellet with physiological saline, the 11,000 *g* sediment was treated with 5% Triton X-100 as described above. The suspension was then centrifuged at 11,000 *g* for 15 min and the supernatant was combined with the first wash of the sediment to yield the Triton extract of the "particle" fraction.

Isolation of Macrophage Lysosomes.—Saline-washed macrophages were suspended in 0.25 M sucrose at 0°C to a cell concentration of 5 × 10^7/ml. Cell suspensions were disrupted with a chilled Teflon pestle homogenizer for three 2 min periods with equal intervals in an ice bath. Homogenates were then separated into three fractions by the differential centrifugation method of Cohn and Wiener (4). Homogenates were centrifuged at 500 *g* for 15 min at 0°C, and the cloudy supernatant fraction was decanted. The pellet was resuspended to the original volume in isotonic sucrose and constituted the "nuclear" fraction. The "lysosomal-mitochondrial" fraction was separated from the supernatant fraction by centrifugation at 15,000 *g* for 12 min. The precipitate was gently resuspended in sucrose, washed once, and adjusted to the original volume in sucrose. The supernatant and the washing from the high speed centrifugation were combined and designated the "15,000 *g* supernatant" fraction.

Lysosomal-mitochondrial fractions were then disrupted either by repeated freezing and thawing or by Triton X-100 treatment as described above.

Enzyme Assay.—Acid phosphatase activity was quantitated by measuring the enzymatic liberation of α-naphthol from α-naphthyl acid phosphate at pH 5.0 by the method described by Allen and Gockerman (9). The reaction mixture contained 5 mM sodium α-naphthyl acid phosphate and 50–100 mM sodium acetate–acetic acid buffer at pH 5.0. After incubation at 25°C for 15 min, the coupling reagent was added in a volume equal to that of the reaction mixture. The coupling reagent contained 0.2% diazotized Fast Red ITR, 4% sodium lauryl sulfate, 100 mM sodium acetate, and 10 mM barbital-HCl buffer at pH 8.0. After addition of the coupling reagent, the tubes were incubated for 5 min at room temperature in the dark to permit color development. Optical density was measured with a Gilford spectrophotometer at 545 mμ. The enzyme reactions proceeded linearly with time and the activity was proportional to the amount of enzyme added under the conditions of the test.

The micro-Kjeldahl procedure was used to determine protein content of dialyzed specimens for specific activity studies.

Electrophoresis.—The general method of polyacrylamide gel electrophoresis described by Ornstein and Davis[1] and Raymond and Weintraub (10) was used. Optimal separation could be obtained with electrophoresis at pH 4.0 on a 7% acrylamide gel matrix using a modification of the system developed by Reisfeld et al. (11). Triton X-100 in a final concentration of 0.5% was incorporated into the gels. Both buffer reservoirs contained 35 mM β-alanine and 1.4 mM acetic acid (buffer pH 5.0). Enzyme-containing specimens which had been dialyzed overnight against the β-alanine buffer were layered over the upper surface of the gels in a volume of 5–20 μl. Electrophoresis was carried out at 4°C for 90 min with a current of 4 ma/tube.

Acid phosphatase activity was detected in the acrylamide gels by the coupling diazo dye method described by Barka (12) using sodium α-naphthyl acid phosphate as the substrate. After electrophoresis the gels were incubated for 15–30 min in the reaction mixture containing 0.1% Na α-naphthyl acid phosphate, 0.1% diazo Fast Garnet GBC in 50 mM sodium acetate–acetic acid buffer pH 5.0. The reaction was stopped by immersing the stained gels in 7% acetic acid. The relative activity of each acid phosphatase was estimated from densitometric tracings of stained acrylamide gels. A Canalco model E densitometer equipped with an integrator was used for these studies.

Isoelectric point determinations were obtained by electrophoresis on acrylamide gels. The discontinuous buffer system previously described could not be used for the pI studies. Sodium phosphate or sodium acetate–acetic acid buffers (μ = 0.025) were used in the buffer reservoirs and in the preparation of the gels. The buffer was continuously recirculated during electrophoresis to prevent pH and concentration changes during the run. To further improve the uniformity of electrophoresis conditions a prerun before applying the specimen was employed. The same voltage and current to be used for electrophoresis were applied to the buffer and gel for 45 min to achieve a steady state flux of buffer ions in the gel.

Gel Filtration.—The molecular weight determinations were obtained by gel filtration on Sephadex G-200 by the general method of Whitaker (13). All experiments were carried out on a 1.3 × 60 cm column using 100 mM acetate buffer pH 5.5 containing 5% Triton X-100. Eluate fractions of 1.0 ml were collected with a volumetric siphon. The elution volumes (V_e) of trypsin (Nutritional Biochemical Corp., Cleveland, Ohio), bovine serum albumin (Armour & Co., Chicago, Ill.), human gamma globulin (fraction II, Pentex Inc., Kankakee, Ill.), and urease (kindly supplied by Sigma Chemical Corp., St. Louis, Mo.) were used to standardize the column. The column void volume (V_o) was determined with Blue Dextran (Pharmacia Fine Chemicals, New Market, N. J.). All elution volumes from the addition of specimen to the maximum concentration of solute were corrected for the buffer volume in the tubing between the column and the fraction collector. The straight line plot of V_e/V_o against the logarithm of the molecular weights formed the standard curve. Blue Dextran was estimated by extinction at 280 mμ (610 mμ in the presence of Triton X-100). Trypsin, albumin, and γ-globulin were detected by continuous monitoring of the column eluate for absorption at 280 mμ. The turbidimetric protein assay method of Tappan (14) was used in the presence of Triton X-100. Urease was detected by the activity assay of Gorin et al. (15).

Ion Exchange Chromatography.—CM-Sephadex C-50 (kindly supplied by Pharmacia) was cleaned and prepared for use as described by the manufacturers. A column of CM-Sephadex (1 × 20 cm) was gravity packed and equilibrated with the starting buffer (5 mM sodium phosphate buffer pH 5.6 containing 0.5% Triton X-100). A 1.0 ml. volume of specimen, previously dialyzed overnight against the starting buffer, was applied and the column was

[1] Ornstein, L., and B. Davis. 1962. Disk Electrophoresis. Parts I and II. Distillation Products Industries, Rochester, N. Y.

washed with starting buffer to remove unadsorbed protein. The enzyme was eluted with a linear gradient of 0 to 1.0 M NaCl buffered with starting buffer. Fractions of 0.75 ml were collected with a volumetric siphon. The sodium concentrations of the eluate were determined in an Instrumentation Laboratories flame photometer.

RESULTS

Localization and Liberation of Acid Phosphatase.—The majority if not all of the acid phosphatase activity of macrophages resides in the lysosomal par-

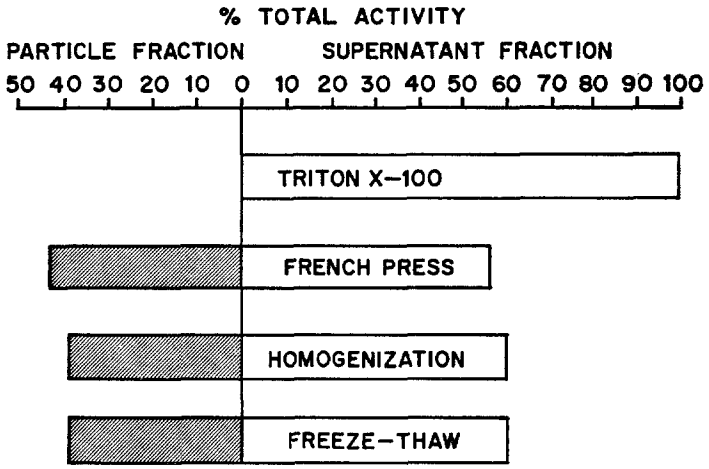

FIG. 1. Distribution of acid phosphatase activity between particle fraction (11,000 *g* sediment) and supernatant fraction of macrophage suspensions disrupted by repeated freeze-thaw treatment, prolonged homogenization, French-press treatment, and extraction with 5% Triton X-100.

ticles and a marked increase in acid phosphatase activity results from lysosomal rupture (4). We first investigated the distribution of acid phosphatase activity of whole cell homogenates following lysosomal rupture.

Macrophage suspensions were disrupted by freeze-thaw treatment, prolonged homogenization, French press treatment, or exposure to Triton X-100. The suspensions were then separated into a supernatant and particle fraction by centrifugation at 11,000 *g* for 15 min. To insure that all the acid phosphatase activity was measured the particle fractions were assayed in the presence of 5% Triton X-100.

Freeze-thaw treatment released 60% of the total activity into the supernatant fraction and the remainder was present in the sediment (Fig. 1). Mechanical means to solubilize acid phosphatase–prolonged homogenization and French press treatment—released no more activity than freeze-thaw treatment. However, extraction of the cell suspension with Triton X-100 released 100% of the total activity into the supernatant fraction.

The observed discrepancy between the supernatant fraction and total acid phosphatase activities could represent retention of the soluble enzyme in lysosomal particles and/or adsorption of the enzyme to other cellular material (16, 17). Alternatively, the lysosomal particle could contain at least two categories of acid phosphatase, one readily released following lysosomal rupture and the other more firmly particle-bound. The demonstrations by Barka (12, 18) and Allen and Gockerman (9), that a detergent soluble acid phosphatase is chromatographically and electrophoretically distinct from an aqueous

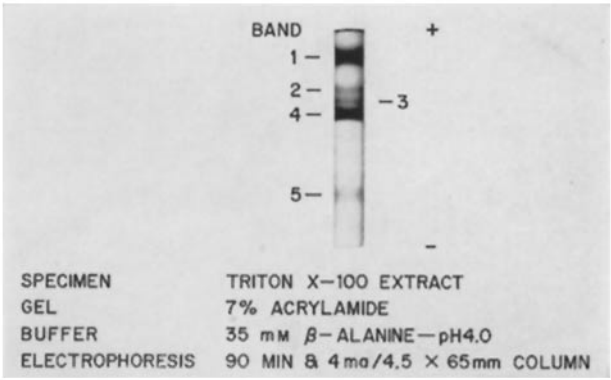

FIG. 2. Acid phosphatase pattern of rabbit alveolar macrophages following acrylamide gel electrophoresis. A 5% Triton X-100 extract of macrophage suspension was applied to the anodal end of the acrylamide gel. Following electrophoresis acid phosphatase activity was detected in the gels by a coupling diazo dye method (12).

soluble acid phosphatase in rat liver lysosome preparations, lend support to the latter hypothesis.

Separation of Acid Phosphatase Isozymes.—To examine this hypothesis we first sought to determine if alveolar macrophages contained more than one form of acid phosphatase. We attempted electrophoretic separation by previously described techniques (9, 11, 19) but found none of them to be entirely satisfactory. The most satisfactory system yielding good protein separation, distinct narrow bands of acid phosphatase activity, and short product-staining times was obtained by electrophoresis for 90 min at 4 ma/tube at 4°C on 7% acrylamide gels containing 0.5% Triton X-100.

Whole cell extracts from both normal and BCG-induced alveolar macrophages showed five bands of acid phosphatase activity (Fig. 2). All enzyme activity migrated toward the cathode at a "running" pH of 4.0 used for these studies. This pattern of phosphatase activity differed from that found in rat liver in which two major bands of activity were found. In rat liver preparations, the slower moving band, corresponding to our band 1, occasionally

formed as a doublet (9). We observed the same phenomenon in some macrophage extracts.

Fig. 3 shows a densitometric tracing of a typical specimen. The numbers for the peaks correspond to the numbered bands of Fig. 2. No acid phosphatase activity remained at the point of application as determined by staining techniques.

We next wanted to determine if the distribution pattern of the acid phosphatases was affected by the means used to solubilize the enzymes. The super-

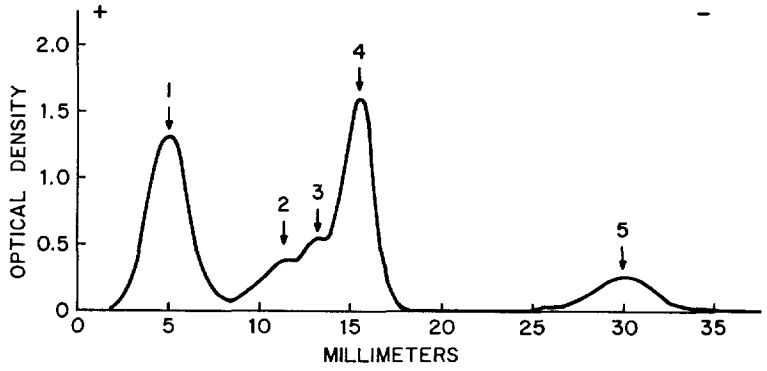

FIG. 3. Densitometric scan of acid phosphatase activity from whole macrophage extract shown in Fig. 2. Distance in millimeters from the anodal end of the gel forms the abscissa.

natant fraction of mechanically ruptured macrophage suspensions contained the major acid phosphatases of the greatest mobility (Fig. 4). Activity bands corresponding to bands 2, 3, 4, and 5 of Fig. 1 were present. Neither mechanical disruption of the cells nor freeze-thaw treatment solubilized the acid phosphatase activity of the lowest mobility. Triton extracts of the particle fraction remaining after mechanical disruption or freeze-thaw treatment were then examined. A small amount of activity corresponding to bands 3 and 4 and occasionally to bands 2 and 5 were present. More importantly, the predominant activity corresponded to band 1, the acid phosphatase activity of lowest mobility, which was not released by mechanical disruption or freeze-thaw treatment. Exhaustive washing of the particle fraction prior to Triton extraction reduced but did not completely eliminate the acid phosphatase activities represented by bands 2, 3, 4, and 5.

It seemed unlikely that the slowest moving fraction of acid phosphatase present in the Triton extracts was due to alteration in electrophoretic mobility of an aqueous soluble enzyme since Triton was incorporated in the acrylamide gels. However, to examine this possibility, supernatant fractions following repeated freezing and thawing were treated with 5% Triton X-100. The elec-

trophoretic patterns of these specimens were identical with those of untreated supernatant fractions eliminating the possibility of a Triton-induced artifact.

The Distribution of Acid Phosphatase Isozymes in Subcellular Fractions.—The distribution of the slowly migrating acid phosphatase (band 1) could be explained by adsorption of a soluble enzyme to nuclear material, undisrupted cells, or cellular debris other than lysosomal membranes present in the particle fraction or macrophage homogenates. To investigate this possibility we examined the distribution of the acid phosphatases in isolated lysosomal

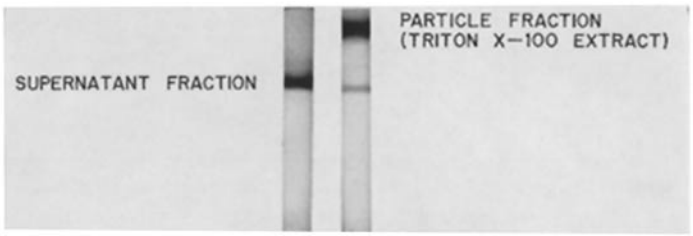

FIG. 4. Comparison of acid phosphatase pattern of supernatant and particle fraction of freeze-thaw treated macrophage suspensions. Acid phosphatase activity of the particle fraction was solubilized by extraction with 5% Triton X-100.

TABLE I

Distribution of Acid Phosphatase Isozymes among Subcellular Fractions

Fractions	Acid phosphatase activity bands				
	1	2	3	4	5
	%	%	%	%	%
Macrophage suspension	40*	15	8	35	2
Lysosomal-mitochondrial					
Whole preparation	43	11	5	37	4
Particle fraction (after freeze-thaw)	62	7	4	25	2
Supernatant fraction (after freeze-thaw)	0	9	4	83	4
Nuclear	44	12	8	34	2
15,000 *g* supernatant	0	5	11	82	2

* Expressed as per cent of total activity for each fraction. Subcellular fractions were obtained by differential centrifugation from cells ruptured by homogenization in 0.25 M sucrose as described in Material and Methods. Triton extracts of each fraction were separated by acrylamide gel electrophoresis and phosphatase activities were quantitated by densitometric scanning.

particles as well as in the "nuclear" fraction and the "15,000 *g* supernatant" fraction.

All five forms of acid phosphatase were found in Triton extracts of the whole lysosomal-mitochondrial preparations (Table I). The predominant phosphatase activity of the particle fraction of freeze-thaw treated lysosomal-mitochondrial preparations was due to the slowly migrating enzyme (band I). Bands 2, 3, 4, and 5 were also present in the particle fraction. The supernatant fraction of freeze-thaw treated lysosomal-mitochondrial preparations contained activity bands 2, 3, 4, and 5 with band 4 the predominant form. Band 1 was not present in the supernatant fraction.

TABLE II

Quantitation of Acid Phosphatase Isoenzymes of Normal and BCG-Stimulated Alveolar Macrophages

Activity site	Total activity (mμmoles phosphate × min^{-1} × mg $protein^{-1}$)			
	29.7 ± 4.1		53.3 ± 7.9	
	Normal		BCG-induced	
	Range	Mean	Range	Mean
1	8.9–11.9	10.4	17.6–24.5	21.9
2	1.5–2.1	1.8	2.7–4.3	3.2
3	1.8–3.0	2.4	0.5–5.3	2.1
4	11.0–12.5	11.9	22.9–26.1	24.0
5	1.8–5.0	3.3	0.5–3.2	2.1

The mean values of total acid phosphatase activity ± standard deviation are shown for the normal and BCG-stimulated alveolar macrophages.

In the course of differential centrifugations used to obtain the lysosomal-mitochondrial fraction a nuclear fraction and a 15,000 *g* supernatant fraction were collected as described in Materials and Methods. Triton extracts of the nuclear fraction contained all five forms of acid phosphatase with the same distribution found in Triton extracts of whole macrophages. The 15,000 *g* supernatant fraction contained phosphatase bands 2, 3, 4, and 5. The distribution of the phosphatases was essentially identical with that of supernatant fractions of freeze-thaw treated, lysosomal-mitochondrial preparations.

The acid phosphatase experiments employing isolated lysosomal fractions and those employing extracts of macrophage suspensions demonstrated that two major groups of acid phosphatase are present in alveolar macrophages. One group of acid phosphatases is soluble in the aqueous phase and is released by physical treatment of the lysosomal particle. The other is firmly membrane bound by the particle and can be extracted by nonionic detergent treatment.

The Influence of BCG Stimulation on Acid Phosphatase Isozymes.—We next compared the total acid phosphatase activities of normal and BCG-stimulated macrophages (Table II) using Triton extracts of cell suspensions. The acid phosphatase activity of normal macrophages was 29.7 ± 4.1 mμmoles of phosphate liberated × min^{-1} × mg $protein^{-1}$ and from BCG-stimulated macrophages was 53.3 ± 7.9 mμmoles of phosphate × min^{-1} × mg $protein^{-1}$. Thus, BCG treatment resulted in an 80% increase in specific activity of the macrophage acid phosphatase. The magnitude of the increase is in close agreement with that found by Cohn and Wiener (4) although the individual specific activities are not comparable due to differences in enzyme and protein assay techniques used.

Having demonstrated a marked effect of BCG stimulation on the acid phosphatase activity, we wanted to determine the contribution due to each of the 5 electrophoretically distinct forms.

Quantitation of the relative activities of the separate phosphatases was obtained by integration of the area under the curve of densitometric tracings of stained acrylamide gels. A linear relationship, with a variation of ±7% was observed between the activities determined by the scanning technique and known activities applied to the gels.

A twofold increase in the two predominant forms of acid phosphatase, band 1 and 4, as well as in a minor form, band 2, resulted from BCG stimulation. The Triton extractable enzyme, band 1, accounted for 35% and 41% of the activity of normal and BCG-stimulated macrophages, respectively. Similarly, the predominant aqueous soluble phosphatase, band 4, accounted for 40% and 45% of the activity before and after BCG stimulation. It is clear that bands 3 and 5 contribute only a small fraction to the total acid phosphatase activity. The effect of stimulation on these two fractions is difficult to interpret because of the low activity of each form and the large variation among specimens. However, the results suggest that no increase of either band 3 or 5 resulted from BCG stimulation.

Properties of Acid Phosphatase Isozymes.—The electrophoretic heterogeneity and solubility differences among the phosphatases could reflect fundamental chemical variations. We examined the isoelectric points, the elution characteristics from ion exchange resins, molecular size differences, pH optima, and enzyme inhibiton characteristics of the principal forms of acid phosphatase to gain further information about the physicochemical background of enzyme multiplicity.

Isoelectric point: Molecular separation by acrylamide gel electrophoresis is a combined function of charge and molecular size differences. To examine charge differences between the predominant acid phosphatase forms, we determined the migration characteristics with varying pH conditions on acrylamide gels of constant pore size.

The mobilities of the two principal forms of acid phosphatase and their

isoelectric points are shown in Fig. 5. The pI of the principal aqueous soluble acid phosphatase (band 4) was 4.9 and the pI for the Triton-extractable acid phosphatase (band 1) was between 4.4 and 5.0. The pI for the Triton-extractable phosphatase could not be determined with precison due to the limited mobility of the enzyme in 7% acrylamide gels. In a sodium acetate–acetic acid buffer system (results not shown) the isoelectric point for both the aque-

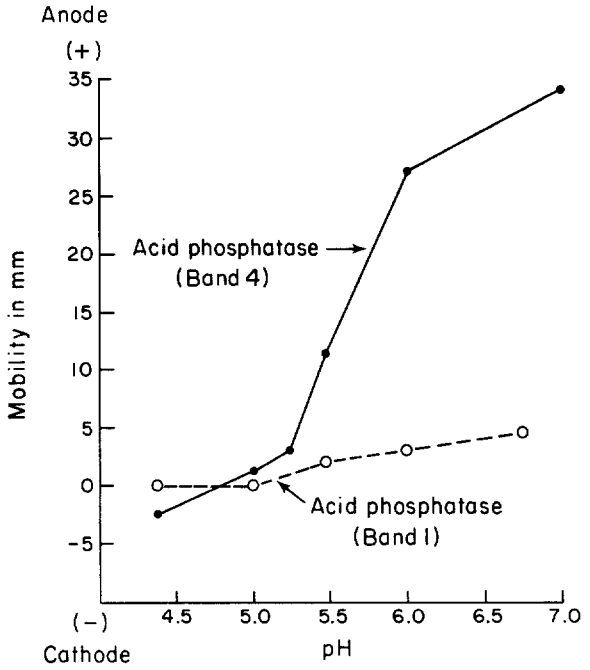

FIG. 5. Electrophoretic mobility of the two principal acid phosphatase forms plotted as a function of pH. •——•, aqueous soluble band 4; o------o, Triton-extractable acid phosphatase band 1.

ous soluble phosphatase (band 4) and for the Triton-extractable phosphatase (band 1) was 5.6. The electrophoretic migration curves were similar to those obtained by electrophoresis in phosphate buffer. Again, the limited mobility of the Triton-extractable phosphatase (band 1) in 7% acrylamide gels prevented a precise determination of the pI.

We further examined charge differences of the two principal enzyme forms by determining their elution characteristics from a cation exchange resin. Both phosphatases were firmly adsorbed to CM Sephadex at pH 5.6 and could be clearly separated by gradient elution column chromatography as shown in Fig. 6. The first acid phosphatase-containing peak, eluted by 0.13 M NaCl,

contained 65% of its activity in the form of band 1 and 35% in the form of band 4. The second acid phosphatase–containing peak, eluted by 0.38 M NaCl, contained 87% of its activity in the form of band 4 and 13% in the form of band 1. The lesser affinity of the Triton-extractable phosphatase for the cation

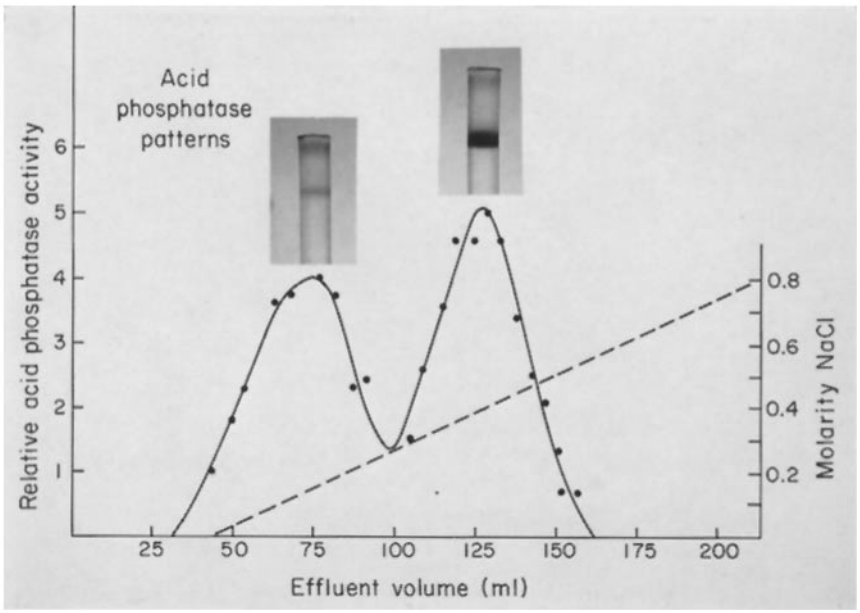

FIG. 6. Separation of the two principal macrophage acid phosphatases by gradient elution chromatography on CM-Sephadex. Acid phosphatase activity not adsorbed to the column had diminished to 0 by fraction 40. Gradient elution was begun at this point. -----, $[Na^+]$ in molarity; •——•, acid phosphatase activity. Electrophoretic characterization of pooled eluate fractions are shown in inserts above graph.

exchange resin suggested that the isoelectric point of the Triton extractable phosphatase was lower than that of the aqueous soluble form band 4.

Additional experiments were performed in which Triton was eliminated from the eluting buffer. The recovery and the elution characteristics of the aqueous soluble phosphatase, band 4, were unaffected by the removal of Triton. By contrast, band 1 could not be eluted by sodium concentrations as high as 1.0 M (NaCl) in the absence of detergent. The addition of Triton to the eluting buffer, however, resulted in prompt elution of an active acid phosphatase with the electrophoretic mobility of band 1. Removal of Triton most likely resulted in precipitation of the slowly migrating phosphatase and subsequent addition of detergent resulted in a resolubilization of the enzyme.

Molecular weight: We next determined the molecular weights of the two predominant forms of acid phosphatase by gel filtration through Sephadex G-200 (Fig. 7). Two standard curves were necessary since the calibration changed when Triton X-100 was present.

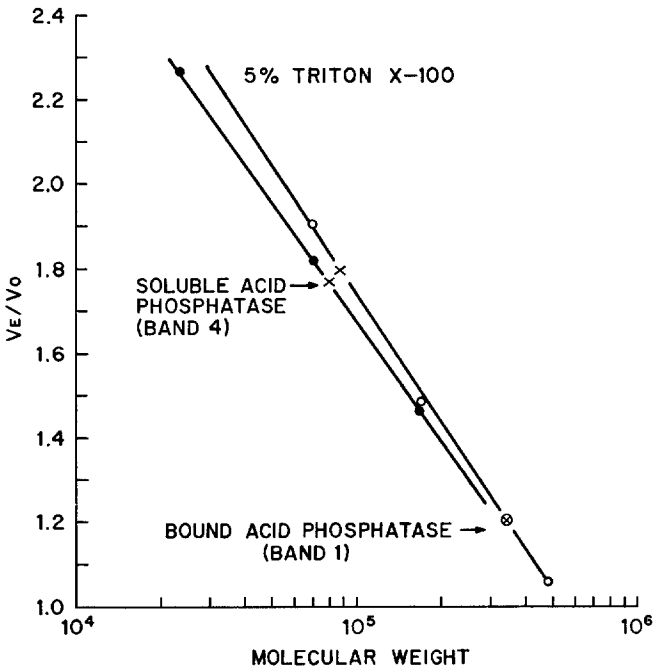

FIG. 7. Molecular weight determination for aqueous soluble acid phosphatase band 4 (×) and Triton-extractable acid phosphatase band 1 (⊗) by gel filtration on Sephadex G-200. Ordinate: ratio of elution volume to column void volume (V_e/V_o); abscissa: molecular weight plotted on logarithmic scale. •——•, standard curve obtained in the absence of Triton; ○——○, standard curve obtained on column equilibrated with buffer containing 5% Triton X-100. Proteins of known molecular weight used to obtain standard curves were trypsin, mol wt = 23,800; bovine serum albumin, mol wt = 68,000; human 7S γ-globulin, mol wt = 154,000; and urease, mol wt = 425,000.

The molecular weight of the predominant aqueous soluble acid phosphatase, band 4, was 80,000. No change in the apparent molecular weight of this phosphatase was observed in the presence of Triton X-100. The molecular weight of the Triton-extractable acid phosphatase, band 1, was 340,000.

pH optima and response to inhibitors: The predominant aqueous soluble phosphatase, band 4, and the Triton extractable phosphatase, band 1, used for these studies were obtained from macrophage suspensions disrupted by repeated freeze-thaw treatment. Band 4 constituted 88% of the acid phospha-

tase activity of the 11,000 *g* supernatant fraction and band 1 constituted 85% of the phsophatase activity of the extract of the "particle" fraction.

The relative activities of the two principal phosphatases are plotted as a function of pH in Fig. 8. The pH optimum for each of the two acid phosphatase forms was 5.2–5.4. The response to enzyme inhibitors was identical for the two prinicpal forms of acid phosphatase. Fluoride (1 mM) inhibited both

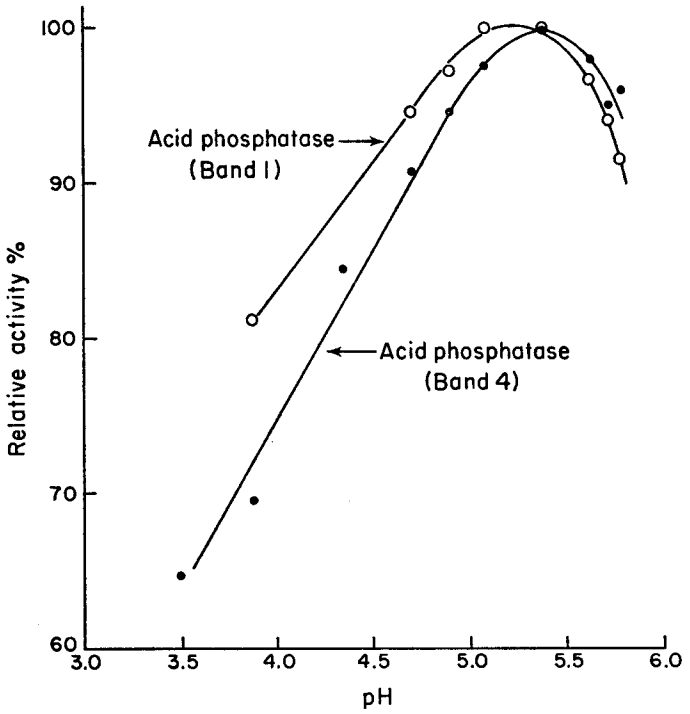

FIG. 8. Acid phosphatase activity of the aqueous soluble band 4 (•——•) and the Triton-extractable band 1 (○——○) are plotted as a function of pH.

phosphatase forms by 90%. Tartrate (10 mM) inhibited the Triton-extractable acid phsophatase, band 1, by 84% and inhibited the aqueous soluble phosphatase, band 4, by 78%. Formaldehyde (0.25 M), EDTA (50 mM), and azide (3 mM) had no inhibitory effect upon either of the two principal acid phosphatases.

DISCUSSION

Localization of nearly 80% of the acid phosphatase activity to lysosomes of rat liver cells was initially demonstrated by de Duve et al. (1). Subsequent evidence from cell fractionation and histochemical studies confirmed the general

nature of the phenomenon for multiple mammalian tissues (20–22). Cohn and Wiener (4) showed that more than 70% of the total acid phosphatase activity of rabbit alveolar macrophages resides in the lysosomal fraction. In all cell fraction studies, 10–20% of the total acid phosphatase activity has been found free in the supernatant fraction. Although this has frequently been considered to be lysosomal enzyme released by trauma during the preparation procedures, Neil and Horner (23) and Tappel et al. (24) suggested that, for liver tissue, the free phosphatase may not be lysosomal in origin. They have demonstrated that the supernatant acid phosphatase differs from the lysosomal form in substrate specificity and in sensitivity to inhibitors. Our observation that the acid phosphatases of the lysosomal fraction are identical in number and electrophoretic mobility with those in whole macrophage extracts suggests that there are no unique nonlysosomal acid phosphatases in rabbit alveolar macrophages.

The data presented here have shown that five electrophoretically distinct forms of acid phosphatase are present in rabbit alveolar macrophages. The phosphatases can be subdivided into two major groups. One group, composed of four electrophoretically distinct forms, is readily soluble in the aqueous phase following prolonged homogenization, French press treatment, or repeated freezing and thawing. The other category, constituting approximately 40% of the total acid phosphatase, is firmly membrane bound and can be solubilized from the lysosomal particle fraction with the nonionic detergent Triton X-100. The predominant component of the particle-bound acid phosphatase is electrophoretically distinct from the readily aqueous soluble forms. In addition, the particle fraction contains a small fraction of acid phosphatase activity electrophoretically identical with that found in the supernatant fraction.

It has previously been observed that not all of the acid phosphatase activity is released from the lysosomal particle following disruption. Berthet, de Duve, and Applemans (16, 17) attributed this discrepancy to nonspecific adsorption of enzyme to collapsed lysosomal vesicles and to other cellular material. This interpretation follows from a view of the lysosome as a sac-like structure containing enzymes which are not bound to membrane or matrix. Nonspecific adsorption may account for our observation that a small fraction of the membrane bound phosphatases are electrophoretically identical with those released into the supernatant fraction. The selective lysosomal membrane binding of the acid phosphatase identified as band 1, however, cannot be explained by nonspecific adsorption.

The firm binding of a large fraction of acid phosphatase to the lysosomal membrane may be representative of a more general phenomenon. Recent investigations by Beck and Tappel (25) have demonstrated that β-glucosidase is firmly membrane bound. Conchie and Hay (26) and Weissman et al. (27) have shown that β-*N*-acetylglucosaminidase is almost completely membrane bound. Conchie and Hay (26) further showed that a significant fraction of α-man-

nosidase and β-galactosidase activities could not be easily removed from the lysosomal membrane. Ugazio and Pani (28) have shown that arylsulfatase A is more firmly membrane bound than arylsulfatase B. Indeed, all lysosomal enzymes may be membrane bound but some are more easily released than others. Membrane binding of lysosomal enzymes as a general phenomenon suggests the possibility of structure-linked enzyme localization. Although multiple forms of acid phosphatase have been identified in human erythrocytes (29–31), there has been no evidence that these nonlysosomal phosphatases are membrane bound.

Any explanation for the structural nature of acid phosphatase multiplicity must take into account the enzyme differences as well as the observed similarities. The two predominant forms of alveolar macrophage acid phosphatase differ in several important respects. They differ significantly in degree of binding to the lysosomal membrane, in solubility, in net charge, and in molecular weight. Despite these differences the two principal phosphatases are identical in pH optima and in their response to enzyme inhibitors. The observed similarities argue against the possibility that the two phosphatases represent entirely different molecular species. The observation that the principal phosphatases differ in molecular weight by a factor of four suggests the possibility that the high molecular weight form is composed of subunits of the lower molecular weight form. Such an explanation is not incompatible with the observed differences in charge. Cytochrome *c* (32), for example, has been shown to form polymers which differ from the monomer in enzymatic properties and in net charge permitting their separation on cation exchange resins. A monomer-tetramer relationship by itself, however, does not adequately explain the requirement for detergent to maintain the high molecular weight form in solution. Triton has in common with other detergents the ability to disperse lipoprotein micelles. It is possible that Triton solubilizes the membrane-bound phosphatase by either breaking lipoprotein complexes between the enzyme and membrane or within the membrane itself. The Triton-solubilized phosphatase could then contain membrane components instead of or in addition to multiple subunits of the low molecular weight aqueous soluble form.

The macrophage has been shown to differentiate in response to multiple environmental stimuli including phagocytosis (4) and pinocytosis (33). An increase in number and size of lysosomal granules accompanies this differentiation. The nearly twofold increase in acid phosphatase activity that we observed after BCG stimulation is in close agreement with that observed by other workers (4–6). In addition we have shown that the increased activity is accounted for by a nearly proportionate increase in the two predominant acid phosphatase forms. Failure to find selective increases in the phosphatase forms would suggest that the enzymatic response to inflammatory stimuli is a nonspecific one. A complete explanation of the changes in the individual phos-

phatase forms, however, must await clarification of the chemical and functional differences of the various isoenzymes.

SUMMARY

The acid phosphatase activity of normal alveolar and BCG-induced alveolar macrophages has been examined. Five electrophoretically distinct forms of acid phosphatase have been identified in both normal and BCG-induced macrophages. The acid phosphatases can be divided into two major categories. One category, containing four distinct forms, is readily solubilized after repeated freezing and thawing or mechanical disruption The second category, containing one form, is firmly bound to the lysosomal membrane and can be solubilized by treatment of the lysosomal fraction with Triton X-100.

The Triton-extractable acid phosphatase and the predominant aqueous soluble acid phosphatase have been shown to differ in the degree of membrane binding, in solubility, in net charge, and in molecular weight. The two predominant phosphatases possess identical pH optimum and do not differ in response to enzyme inhibitors.

BCG stimulation has been shown to result in a nearly twofold increase in acid phosphatase activity. A nearly proportionate increase in the major acid phosphatase forms has been observed.

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BIBLIOGRAPHY

1. de Duve, C., B. C. Pressman, R. Gianetto, R. Wattiaux, and F. Applemans. 1955. Tissue fractionation studies: 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem. J.* **60:**604.
2. Cohn, Z. A., J. G. Hirsch, and E. Wiener. 1963. The cytoplasmic granules of phagocytic cells and the degradation of bacteria. *Ciba Found. Symp. Lysosomes.* 126.
3. Hirsch, J. G., and Z. A. Cohn. 1960. Degranulation of polymorphonuclear leukocytes following phagocytosis of microorganisms. *J. Exptl. Med.* **112:**1005.
4. Cohn, Z. A., and E. Wiener. 1963. The particulate hydrolases of macrophages. I. Comparative enzymology, isolation, and properties. *J. Exptl. Med.* **118:**99.
5. Heise, E. R., Q. N. Myrvik, and E. S. Leake. 1965. Effect of bacillus Calmette-Guerin on the levels of acid phosphatase, lysozyme, and cathepsin in rabbit alveolar macrophages. *J. Immunol.* **95:**125.
6. Mizunoe, K., and A. M. Dannenberg, Jr. 1965. Hydrolases of rabbit macrophages. III. Effect of BCG vaccination, tissue culture, and ingested tubercle bacilli. *Proc. Soc. Exptl. Biol. Med.* **120:**284.
7. Axline, S. G. 1967. Isozymes of acid phosphatase in normal and BCG induced rabbit alveolar macrophages. *Federation Proc.* **26:**702.

8. Myrvik, Q. N., E. S. Leake, and B. Fariss. 1961. Studies on pulmonary alveolar macrophages from the normal rabbit: A technique to procure them in a high state of purity. *J. Immunol.* **86**:128.
9. Allen, J. M., and J. Gockerman. 1964. Electrophoretic separation of multiple forms of particle associated acid phosphatase. *Ann. N.Y. Acad. Sci.* **121**:616.
10. Raymond, S., and L. Weintraub. 1959. Acrylamide gel as supporting medium for zone electrophoresis. *Science.* **130**:711.
11. Reisfeld, R. A., U. J. Lewis, and D. E. Williams. 1962. Biochemistry: Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature.* **195**:281.
12. Barka, T. 1961. Studies of acid phosphatase. I. Electrophoretic separation of acid phosphatases of rat liver on polyacrylamide gels. *J. Histochem. Cytochem.* **9**:542.
13. Whitaker, J. R. 1963. Determination of molecular weights of proteins by gel filtration on Sephadex. *Anal. Chem.* **35**:1950.
14. Tappan, D. V. 1966. A light scattering technique for measuring protein concentration, *Anal. Biochem.* **14**:171.
15. Gorin, G., E. Fuchs, L. G. Butler, S. L. Chopra, and R. T. Hersh. 1962. Some properties of urease. *Biochem. J.* **1**:911.
16. Berthet, J., and C. de Duve. 1951. Tissue fractionation studies: I. The existence of a mitochondria-linked, enzymatically inactive form of acid phosphatase in rat-liver tissue. *Biochem. J.* **50**:174.
17. Applemans, F., and C. de Duve. 1955. Tissue fractionation studies: III. Further observations on the binding of acid phosphatase by rat-liver particles. *Biochem. J.* **59**:426.
18. Barka, T. 1961. Studies on acid phosphatases. II. Chromatographic separation of acid phosphatases of rat liver. *J. Histochem. Cytochem.* **9**:564.
19. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121**:404.
20. Straus, W. 1956. Concentration of acid phosphatase, ribonuclease, desoxyribonuclease, β-glucuronidase, and cathepsin in "droplets" isolated from the kidney cells of normal rats. *J. Biophys. Biochem. Cytol.* **2**:513.
21. Cohn, Z. A., and J. G. Hirsch. 1960. The isolation and properties of the specific cytoplasmic granules of rabbit polymorphonuclear leukocytes. *J. Exptl. Med.* **112**:983.
22. Beaufay, H., A. M. Berleur, and A. Doyen. 1957. The occurrence of lysosome-like particles in rat brain tissue. *Biochem. J.* **66**:32P.
23. Neil, M. W., and M. W. Horner. 1962. The acid-*p*-nitrophenyl phosphatase activity of adult guinea-pig liver subcellular fractions. *Biochem. J.* **84**:32P.
24. Tappel, A. L., P. L. Sawant, and S. Shibko. 1963. Lysosomes: distribution in animals, hydrolytic capacity and other properties. *Ciba Found. Symp. Lysosomes.* 78.
25. Beck, C., and A. L. Tappel. 1968. Rat-liver β-glucosidase: A membrane enzyme. *Biochim. Biophys. Acta.* **151**:159.
26. Conchie, I., and A. J. Hay. 1963. Mammalian glycosidases. IV. The intracellular

localization of β-galactosidase, α-mannosidase, β-*N*-acetylglucosaminidase and α-L-fucosidase in mammalian tissues. *Biochem. J.* **87**:354.

27. Weissmann, B., G. Rowin, J. Marshall, and D. Friederici. 1967. Mammalian α-acetyl-glucosaminidase. Enzymic properties, tissue distribution, and intracellular localization. *Biochemistry.* **6**:207.
28. Ugazio, G., and P. Pani. 1963. Differential release of bound hydrolases from rat-liver lysosomes treated by a non-ionic surface active substance. *Exptl. Cell Res.* **31**:424.
29. Angeletti, P. U., and R. Gayle. 1962. Chromatography of red cell hemolyzate. *Blood.* **20**:51.
30. Hopkinson, D. A., N. Spencer, and H. Harris. 1963. Red cell acid phosphatase variants: A new human polymorphism. *Nature.* **199**:969.
31. Fenton, M. R., and K. E. Richardson. 1967. Isolation of three acid phosphatase isozymes from human erythrocytes. *Arch. Biochem. Biophys.* **120**:332.
32. Margoliash, E. 1962. Some structural aspects of cytochrome C function. *Brookhaven Symp. Biol.* **15**:266.
33. Cohn, Z. A., and B. Benson. 1965. The differentiation of mononuclear phagocytes: morphology, cytochemistry, and biochemistry. *J. Exptl. Med.* **121**:153.