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Purification and Properties of Rabbit Alveolar Macrophage Lysozyme

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Lysozyme was isolated from *Bacillus* Calmette-Guerin-elicited rabbit alveolar macrophages by acid extraction and purified to homogeneity by a single-column procedure. Yields of the purified enzyme averaged between 20 and 30 mg per rabbit, values far in excess of those obtained with previously published methods. Rabbit lysozyme has a molecular weight of 14,300 and exhibits optimal lytic activity against *Micrococcus lysodeikticus* at an ionic strength of 0.04, pH 6.5. Our results indicate that lysozyme and other granule components can be fractionated from elicited alveolar macrophages by using simple techniques, suggesting methods for the bulk purification of lysosomal constituents.

Since the discovery of lysozyme in 1922 by Fleming (9), a great deal of effort has been devoted to the study of the physical, chemical, and biological properties of this enzyme. As a result of the bacteriolytic nature of this protein, which was first described for hen egg white by Laschtschenko in 1909 (28), as well as the widespread occurrence of this enzyme in nature, numerous investigators have attempted to determine the possible significance of lysozyme in resistance to infection. However, much of this work has been done with high concentrations of the hen egg white enzyme and has not taken into consideration the variations in activity which exist between the lysozymes of different species (44). Thus, although a recent communication from this laboratory (45) demonstrated the potent antibacterial activity of lysozyme isolated from human fluids, the role of lysozyme in host defense cannot be generalized from one animal to the next. In view of the ubiquity with which rabbits are used as experimental models for the study of the infectious process, and as a prelude to investigations of the bactericidal components of rabbit serum, a study of the properties of rabbit lysozyme was undertaken.

Lysozyme activity has been detected in a variety of rabbit tissues and fluids, including serum (39), tears (2), and milk (P. S. Prickett, N. J. Miller, and F. G. McDonald, Bacteriol. Proc., p. 61-62, 1933); extremely high concentrations have been found only in tissues of lymphoid origin (10, 42). Previous lysozyme purification procedures have used rabbit spleens (20) or syphilitic rabbit testes (24) as sources for the bulk fractionation of this enzyme, but they have required the processing of large amounts of material and have resulted in exceptionally low yields of the purified enzyme. Although polymorphonuclear leukocytes have been shown to contain substantial quantities of lysozyme (47), Myrvik et al. (37), as well as others (33), have demonstrated significantly higher concentrations in extracts from alveolar macrophages. Specifically, lysozyme has been shown to be associated with the cytoplasmic granules (lysosomes) of both of these phagocytic cells (5, 29, 47). Of interest is the observation that peritoneal macrophages appear to be devoid of this enzyme (36, 39).

More recent observations have demonstrated that, when a variety of cells were maintained under culture conditions in vitro, lysozyme was found in detectable quantities only in cell extracts or culture supernatants of macrophages and polymorphonuclear leukocytes (12). Furthermore, Heise and Myrvik (14) have shown that secretion of lysozyme by rabbit alveolar macrophages in vitro was reduced when cultures were treated with inhibitors of protein or ribonucleic acid synthesis. These results have led to the proposal that it is the macrophages and polymorphonuclear leukocytes residing in lymphoid organs which are responsible for the high concentrations of lysozyme in these tissues (12). In addition, it has been observed that intravenous injection of heat-killed *Bacillus* Calmette-Guerin (BCG) in oil (5, 38) or intratracheal injection of BCG (15) resulted in a tremendous increase in the number of alveolar macrophages, as much as 20-fold, as well as in their content of hydrolytic enzymes, including lysozyme. Taken as a whole, the above results suggest that rabbit alveolar macrophages (i) ac-

tively synthesize and secrete large quantities of lysozyme, and (ii) can be obtained in very large numbers with greater than 80% purity (38), thus making these cells an ideal source material for the purification of this enzyme.

This communication details the purification of rabbit lysozyme from elicited alveolar macrophages and describes some of the properties of the purified enzyme. Generalities and applications of the technique are briefly discussed.

MATERIALS AND METHODS

Stimulation and isolation of rabbit alveolar macrophages. New Zealand white rabbits were given a single intravenous injection of complete Freund adjuvant via the marginal ear vein (30), and after 3 weeks the alveolar macrophages were isolated essentially as described by Myrvik et al. (36). Homogenization of cell suspensions and subsequent isolation of subcellular granules was modified after the method of Leake and Myrvik (29). A more complete description of the methods used for eliciting alveolar macrophages and isolation of granules will be presented by J. Patterson-Delafield and R. H. Lehrer (manuscript in preparation).

Extraction and chromatography. In the procedure routinely employed, the granule-rich fraction obtained from approximately 2×10^9 alveolar macrophages (one to two rabbits) was lysed and extracted by overnight dialysis against 8 liters of 0.01 M citric acid at 4°C, using Spectrapor 1 dialysis tubing (molecular weight cut-off, 6,000 to 8,000; Spectrum Medical Industries). The resulting milky-white precipitate was sedimented by centrifugation at 20,000 × *g* for 20 min at 4°C, and the sediment was reextracted with 10 volumes of cold 0.1 M citric acid for 2 h. After centrifugation, the supernatant fluids were pooled, concentrated by dehydration against 20,000-molecular-weight polyethylene glycol (Aquacide III; Calbiochem), and dialyzed overnight against two changes of 4 liters of cold 0.05 M potassium phosphate buffer, pH 7.4. Insoluble material was removed by centrifugation, and the granule extract was applied to a Sephadex CM-25 column (3 by 30 cm; Pharmacia) cast in a plastic cylinder equipped with a nylon mesh screen and equilibrated at 4°C in phosphate buffer. Loading and elution of the column was maintained at a constant flow rate of 6.4 ml/cm^2 per h; 9-ml fractions were collected. The column was washed with buffer until the absorbance at 280 nm approached 0. A linear 900-ml (total volume) gradient from 0 to 1.0 M NaCl in phosphate buffer was then applied, and elution was monitored by measuring the absorbance at 280 nm, as well as by the ability of the fractions to lyse suspensions of *Micrococcus lysodeikticus* (see below). The single enzymatically active protein peak eluting after the addition of salt (CM peak II) was pooled, dialyzed in Spectrapor 2 dialysis tubing (molecular weight cut-off, 12,000 to 14,000) at 4°C against three changes of 4 liters of 0.01 M ammonium acetate titrated to pH 4.6 with glacial acetic acid, and lyophilized in a flask previously coated with dichlorodimethylsilane (Eastman). The lyophilized powder was stored at −20°C.

Electrophoresis. Samples were subjected to analytical sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, pH 8.3, by following a modification of the method of Laemmli (27), which allows formation of linear polyacrylamide gradient slab gels. Gels were found to be more uniform by imposing a linear gradient of the catalyst (*N, N, N', N'*-tetraethylethylenediamine; Eastman) opposite to the acrylamide gradient (32), causing polymerization to begin at the top of the gel and spread downward. Native polyacrylamide gel electrophoresis at pH 4.5 was performed by using modifications of the method of Reisfeld et al. (43), which include those described by Selsted and Martinez (45), as well as by increasing the concentration of polyacrylamide in the stacking gel to 4%. SDS gels were stained for protein by immersion in 0.1% Coomassie brilliant blue R250 in 30% methanol–10% acetic acid for 20 min. Proteins electrophoresed in native gels were detected by treatment with the above staining solution after soaking for 2 h in 30% methanol–10% acetic acid or by staining with 0.2% amido black in 45% methanol–10% acetic acid. All gels were destained in 10% methanol–10% acetic acid.

Lysozyme assay. Lysozyme activity was detected by using essentially the same assay described by Selsted and Martinez (45); i.e., the initial rate of decrease in the turbidity of suspensions of *M. lysodeikticus* (Difco) was continuously monitored spectrophotometrically at 450 nm for a period of 1 to 2 min (41). Potassium phosphate buffers ranging in concentration from 0.03 to 0.08 M were used for the pH activity profile in order to maintain a constant ionic strength of 0.1 (13). Conductivity measurements were made on a Yellow Springs Instruments model 31 conductivity bridge, and buffers were adjusted accordingly. Ionic strength dependence of the enzyme was determined by using 6 mM potassium phosphate (pH 6.5; ionic strength, 0.01) to which increasing amounts of 1 M NaCl or 1 M KCl in the same buffer were added. The effects of increasing phosphate concentrations at pH 6.5 were also examined. All incubations were done in duplicate at 37°C.

Determination of protein concentration. Based on an amino acid content of four tyrosine and five tryptophan residues per 14,329 daltons (21; see below), and by assuming an ϵ_m of 1.21×10^3 for tyrosine and 5.49×10^3 for tryptophan at neutral pH (6), an $E^{1\%}_{1\,cm}$ (280 nm) of 22.5 was calculated for rabbit lysozyme and used throughout these studies. An $E^{1\%}_{1\,cm}$ (280 nm) of 25.5 (40) was used for hen egg white lysozyme (salt-free; Worthington Biochemicals Corp.). Concentrations of other proteins were determined by the method of Lowry et al. (31), using hen egg white lysozyme or bovine serum albumin (Sigma Chemical Co.) as the standard.

Amino acid analysis. Protein hydrolysates were prepared by the method of Moore and Stein (35) and maintained at 110 ± 1°C in sealed, evacuated tubes for 24, 48, and 72 h. Amino acid analyses were performed in a Spinco model 121 amino acid analyzer. Cysteine, half-cystine, and proline were determined after oxidation with performic acid (16) and hydrolysis. Tryptophan was determined spectrophotometrically by the method of Bencze and Schmid (1) in the presence and absence of 6 M guanidine hydrochloride.

RESULTS

Purification of rabbit lysozyme. Chromatography on Sephadex CM of the granule citric acid extract obtained from approximately 2×10^9 complete Freund adjuvant-elicited rabbit alveolar macrophages is shown in Fig. 1. Lysozyme activity was found to coelute with the single protein peak after the introduction of the NaCl gradient. The active fractions (CM peak II), eluting at approximately 0.42 M NaCl in 0.05 M potassium phosphate buffer, pH 7.4, accounted for more than 95% of the enzymatic activity applied to the column. Reducing the slope of the gradient had no effect on the chromatographic profile nor did increasing the NaCl concentration to as high as 4 M. Preliminary experiments indicated that a large percentage of activity was lost if chromatography was performed in glass columns and/or if glass wool was used in the construction of these columns. As a result, glass surfaces were avoided whenever possible during the purification procedure, or glassware was treated with dichlorodimethylsilane to reduce the hydrophilic nature of the glass surface.

Rechromatography of CM peak II on Sephadex CM, Sephadex G-75, or Bio-Gel P-30 resulted in elution profiles with a single active protein peak. When subjected to electrophoresis on SDS–5 to 20% polyacrylamide gradient slab gels at pH 8.3 (Fig. 2), CM peak II showed a single band, either in the presence or absence of reducing agents, which comigrated with hen egg white lysozyme. Similar results were obtained when samples were electrophoresed on 15% native polyacrylamide gels, pH 4.5. Identical profiles of chromatography and electrophoresis have been observed for all six preparations so far examined. Based on these observations, as well as on the comigration of purified rabbit lysozyme with hen egg white lysozyme on Sephadex G-75 (data not shown), it was concluded that rabbit lysozyme is a basic protein with a molecular weight of approximately 14,300 (40). The data further indicate that lysozyme is the only component of the citric acid extract capable of binding to Sephadex CM under the conditions described, suggesting the applicability of batch procedures.

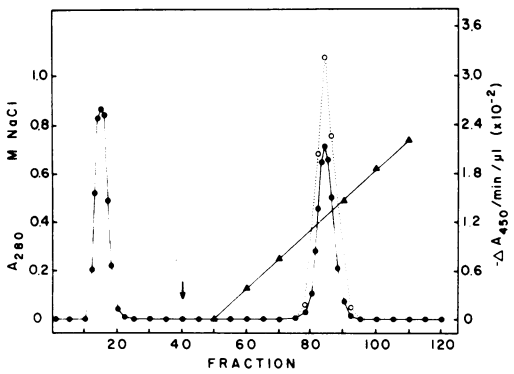

FIG. 1. *Chromatographic profile of granule extract on Sephadex CM. Symbols: ●, absorbance at 280 nm (A_{280}); ○, M. lysodeikticus lysis activity (change in absorbance at 450 nm [ΔA_{450}] per minute per microliter); ▲, NaCl concentration.*

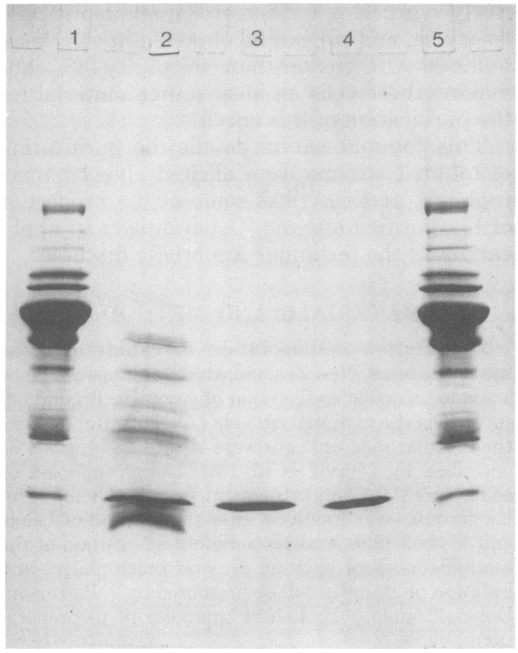

FIG. 2. *SDS-polyacrylamide gel electrophoretic pattern of rabbit serum, alveolar macrophage granule extract, and purified rabbit and hen egg white lysozymes. SDS–5 to 20% polyacrylamide gradient slab gel, pH 8.3. Samples were reduced with 2.5% (vol/vol) 2-mercaptoethanol. Lane 1, 70 µg of normal rabbit serum; lane 2, 35 µg of granule citric acid extract; lane 3, 6 µg of purified rabbit lysozyme (CM peak II); lane 4, 6 µg of hen egg white lysozyme; lane 5, 70 µg of serum from BCG-stimulated rabbits.*

Recovery data for rabbit lysozyme extracted from the alveolar macrophages of several animals are presented in Table 1. The amount of enzyme recovered after citric acid extraction of macrophage granules was found to average 95% of the activity released from whole cells by repeated freeze-thawing and the addition of Triton X-100 to a final concentration of 0.1%. Active material eluting from the Sephadex CM column (CM peak II) accounted for nearly 80% of the lysozyme released from whole cells, resulting in an average final yield of 27 mg/rabbit. After lyophilization (which reduced the yield by 10%)

TABLE 1. *Lysozyme recovery data*

Rabbit	No. of alveolar cells recovered ($\times 10^9$)	Amt of lysozyme (mg/10^9 alveolar cells) in:			% Recovery[c]	Amt of lysozyme (mg per rabbit)[d]
		Freeze-thaw analysis[a]	Acid extraction[b]	CM peak II		
1	1.05	19.92	18.94	16.53	83.0	17.46
2	2.00	22.12	21.24	16.81	76.1	33.78
3	1.95	20.50	19.27	15.38	75.1	29.99
Average	1.67	20.85	19.82	16.25	77.9	27.08

[a] Cell suspensions were freeze-thawed five times in a dry ice–ethanol bath. Triton X-100 was then added to 0.1%, and the suspensions were centrifuged at 15,000 × *g* for 5 min. The supernatants were analyzed for lysozyme content.

[b] Lysozyme was recovered by extraction of alveolar macrophage granules with citric acid as described in the text.

[c] Percentage of enzyme recovered in CM peak II taking freeze-thaw values as 100%.

[d] Total amount of purified lysozyme isolated per rabbit.

or in concentrated solutions, rabbit lysozyme was found to be very stable at −20°C. On the other hand, when stored at low concentrations, this enzyme lost as much as 50% of its activity over a period of several weeks. The addition of bovine serum albumin to a final concentration of 0.1% extended the stability of dilute rabbit (and egg white) lysozyme solutions to several months at −20°C.

Enzymatic activity: lysis of *M. lysodeikticus*. The ability of purified rabbit lysozyme to lyse suspensions of *M. lysodeikticus* was examined by using phosphate buffers of constant ionic strength (ionic strength, 0.1) (Fig. 3). Rabbit lysozyme exhibited a relatively broad pH activity profile centered around pH 6.5, with more than 97% maximal activity observed between pH 6 and pH 7. Assuming optimal activity at pH 6.5, the effect of increasing ionic strength on lytic activity was determined by using phosphate buffer at an ionic strength of 0.01, to which increasing amounts of NaCl were added. The results (Fig. 4) demonstrate a very pronounced sensitivity of the enzyme to ionic strength variations. Maximal activity occurred at an ionic strength of 0.04, with more than 50% inhibition at ionic strengths above 0.15 or below 0.017. Identical profiles were obtained by using KCl or phosphate.

Figure 5 demonstrates the linear relationship between the initial rate of lysis of cell suspensions and enzyme concentration under optimal lytic conditions for rabbit lysozyme (pH 6.5; ionic strength, 0.04). The specific activity of the enzyme determined from the slope of the graph, which under the conditions of our assay is expressed in terms of change in absorbance at 450 nm per minute per microgram of protein, equals 1.61×10^{-1}. This assay is sufficiently sensitive to detect enzyme concentrations below 5 ng/ml. When compared with hen egg white lysozyme

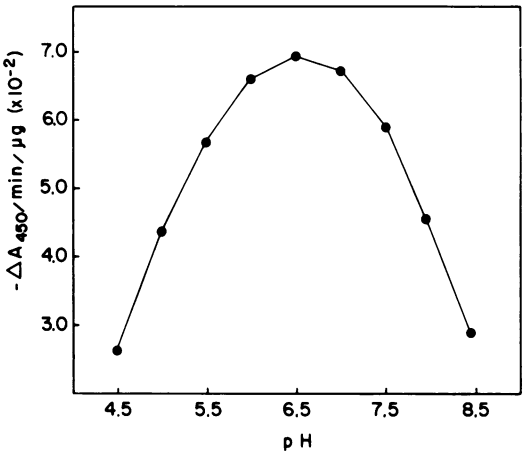

FIG. 3. *pH activity profile for rabbit lysozyme (500 ng/ml). ΔA_{450}, Change in absorbance at 450 nm.*

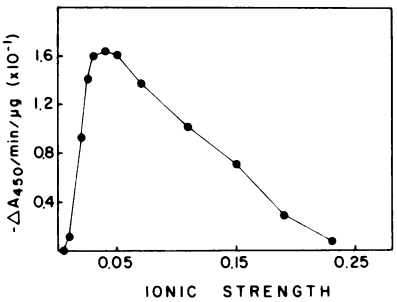

FIG. 4. *Ionic strength activity profile for rabbit lysozyme (250 ng/ml). ΔA_{450}, Change in absorbance at 450 nm.*

under these conditions (Fig. 5), rabbit lysozyme was 1.1 times more active on a specific activity basis. As would be expected from the pH and ionic strength profiles, altering the buffer conditions to approach those of normal serum (pH 7.4; ionic strength, 0.130) reduced the activity of

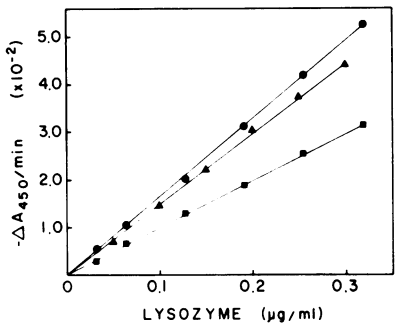

FIG. 5. *Relative activities of rabbit and hen egg white lysozymes. Symbols: ●, rabbit lysozyme at pH 6.5 and an ionic strength of 0.04; ■, rabbit lysozyme at pH 7.4 and an ionic strength of 0.130; ▲, hen egg white lysozyme at pH 6.5 and an ionic strength of 0.04.* ΔA_{450}, *Change in absorbance at 450 nm.*

rabbit lysozyme by 40%, but still resulted in the same linear behavior with respect to enzyme concentrations.

Amino acid composition. Table 2 presents the amino acid composition of rabbit alveolar macrophage lysozyme, as well as the composition determined by Jolles and Fromageot (21) for the enzyme isolated from rabbit spleens. It can be seen that the overall compositions of the two enzymes are quite similar. Further work may prove them identical. Significant differences appear only in the content of aromatic amino acids, half-cystine, and aspartic acid residues. The spectrophotometric method used for the determination of tryptophan in this report (1) gave identical results in the presence or absence of 6 M guanidine hydrochloride. Furthermore, the method was checked by determining the ratio of tyrosine to tryptophan for hen egg white lysozyme; this determination gave values identical to those derived from sequence analysis of this enzyme (3).

TABLE 2. *Amino acid composition of rabbit lysozyme*[a]

Amino acid	Macrophage enzyme		Spleen enzyme no. of residues[c]
	Calculated no. of residues[b]	Assumed no. of residues	
Ala	13.2	13	13
Arg	6.8	7	6
Asx	18.2	18	20
Cys (half)	5.8[d]	6	10
Glx	10.9	11	11
Gly	11.1	11	11
His	1.2	1	1
Ile	6.7[e]	7	7
Leu	8.9[e]	9	10
Lys	6.3	6	6
Met	1.7	2	2
Phe	2.7	3	3
Pro	5.0[d]	5	5
Ser	9.0[f]	9	9
Thr	6.8[f]	7	7
Trp	5.1	5	2
Tyr	4.2	4	3
Val	5.8[e]	6	6

[a] The molecular weight of the macrophage enzyme (assumed number of residues) is 14,329 and that of the spleen enzyme is 14,267.

[b] Calculated by assuming a molecular weight of approximately 14,300 (see text).

[c] From Jolles and Fromageot (20).

[d] Determined after performic acid oxidation and assuming 94% recovery of half-cystine as cysteic acid (34).

[e] Assumes complete release by 72 h.

[f] Extrapolated to zero time hydrolysis.

DISCUSSION

Rabbit lysozyme has been purified to homogeneity, as judged by column chromatography, by SDS-polyacrylamide gel electrophoresis at pH 8.3, and by native polyacrylamide gel electrophoresis at pH 4.5. The enzyme is composed of a single polypeptide chain with a molecular weight of 14,329, based on amino acid composition, and as such closely resembles the other vertebrate lysozymes thus far examined (19). This value is in excellent agreement with the results of Jolles and Fromageot on a muramidase purified from rabbit spleens (21), as well as the results of other investigators who have noted the comigration of rabbit and hen egg white lysozymes on columns (24) and gels (2). The amino acid composition we report for the alveolar macrophage lysozyme compares very favorably with that presented by Jolles and Fromageot for the spleen enzyme, with major differences occurring only in the content of aromatic amino acids. This discrepancy most probably reflects the different methods used for the determination of the ratio of tyrosine to tryptophan, since the tyrosine values differ by only one residue. Comparisons between sequence data and the values determined by the spectrophotometric method used in this report on the hen enzyme produced identical values. Thus, although the content of aromatic residues seems well established, the value for half-cystine represents only a single performic acid oxidation and may be in error. In addition, our values for aspartic acid are two residues below those previously published, but, due to the high content, it is impossible to determine the significance of this discrepancy. Since multiple forms of lysozyme have been isolated from rabbit spleens (20), as well as from the organs and secretions of other animals (23) and birds (22), further anal-

yses need to be performed to determine whether differences exist between the rabbit macrophage and spleen enzymes. The procedures described in this report should allow purification of sufficient quantities of lysozyme for sequence analysis, which will aid in defining the final composition of this enzyme and its relationship to the other lysozymes so far characterized.

The pH activity profile determined for rabbit macrophage lysozyme indicates optimal activity at pH 6.5, which is identical to the value found by Kopec for a muramidase isolated from syphilitic rabbit testes (25). On the other hand, the ionic strength activity profile for the testes enzyme showed little variation between ionic strengths of 0.3 and 0.9, whereas experiments detailed in this report indicate an optimum at an ionic strength of 0.04. We do not believe that these results represent variations in the properties of the two enzymes, but rather they appear to be attributable to the methods used for the determination of enzymatic activity. Although both methods follow the reduction in turbidity of suspensions of *M. lysodeikticus*, the assay used by Kopec was a single point determination which measured percent lysis after 10 min of incubation at 37°C, and the assay used in our laboratory followed the initial rate of lysis at the same temperature. Numerous investigators (17, 18) have reported that lysis of *M. lysodeikticus* by various lysozymes follows second-order kinetics (with respect to the enzyme) only for the first 25 to 35% reduction in absorbance, after which the rate relationship breaks down to pseudo-first order (41). Under such conditions, it has been observed (4) that in order to obtain 50% lysis, twice the amount of enzyme needed to produce 37% lysis must be added, resulting in a plateau at about 60%. As the results of Kopec are all in the 40 to 55% range, his use of too high an enzyme concentration, combined with a relatively insensitive assay, may account for the differences observed.

In terms of specific activity, rabbit lysozyme is 1.1 times more active than hen egg white lysozyme at pH 6.5 and ionic strength of 0.04 (Fig. 5). It should be emphasized, however, that these activity ratios might change if conditions were altered, since the hen enzyme appears to show a much wider pH activity profile (8). Thus, activity of the rabbit enzyme relative to that of hen egg white as described above applies only to the conditions used in this study. On the other hand, as has been demonstrated for the lysozymes of hen egg white (8), guinea hens, ducks (lysozymes II and III), and humans (44), as well as those of papaya latex (18) and fig latex (11), rabbit lysozyme also exhibits a very pronounced inhibition of activity with increased ionic strength (Fig. 4). This inhibition is not the result of a specific ion effect, as KCl and phosphate at equivalent ionic strengths produced results identical to those observed with NaCl. These observations appear to strengthen the argument of Davies et al. (8) that increasing the ionic strength of the medium reduces favorable electrostatic interactions required for the initial absorption of the positively charged lysozyme molecule to the predominantly negatively charged cell wall substrate. Further support for this view comes from experiments which have examined the activity of hen egg white lysozyme after chemical modification of lysine and arginine residues (7), reducing the net positive charge. Under these conditions, the activity profile resembled that of the native enzyme, but was displaced to a much lower ionic strength optimum. Thus, it appears that electrostatic dependence upon activity is a common feature for many lysozymes of diverse origins. However, further detailed examination of enzymes from other sources will be necessary to determine whether this property may be common to muramidases in general.

Extraction of basic proteins with dilute acids has been used extensively for the isolation of the cationic components of many cell types and organelles, including polymorphonuclear leukocytes (17), platelets (46), and nuclei (26). Although the studies of Myrvik et al. (37) and others have demonstrated the exceptionally high levels of acid hydrolases and lysozyme in BCG-elicited alveolar macrophages, little has been done to utilize these cells for the purification of their lysosomal constituents. The procedures presented in this report demonstrate that these enzymes may be fractionated to homogeneity by using simple techniques. Yields of lysozyme, which average between 20 and 30 mg per rabbit, far exceed those of any previously published procedure. For example, starting with 3,000 rabbits, Jolles and Fromageot (21) recovered 167 mg of enzyme, resulting in a yield of 56 μg/rabbit. Thus, in practical terms, this means that one rabbit by our procedure yields as much lysozyme as 480 rabbits by other methods.

From the foregoing discussion, it is obvious that elicited alveolar macrophages are an excellent source for the purification of rabbit lysozyme. In addition, similar procedures are presently being used (Patterson-Delafield and Lehrer, manuscript in preparation) in an effort to purify the low-molecular-weight fungicidal proteins present in alveolar macrophage granule extracts. The purification of these lysosomal constituents may further our understanding of

the mechanisms by which phagocytic cells exhibit their nonoxidative microbicidal activity.

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