LYSOZYME IN EPIPHYSEAL CARTILAGE

IV. Embryonic Chick Cartilage Lysozyme— Its Localization and Partial Characterization

K. E. KUETTNER, R. EISENSTEIN, L. W. SOBLE, and C. ARSENIS

From the Departments of Orthopaedic Surgery and Biochemistry and Division of Pathology, Rush Presbyterian–St. Luke's Medical Center, and Department of Biological Chemistry, University of Illinois College of Medicine, Chicago, Illinois 60612

ABSTRACT

The localization of chick embryonic lysozyme was determined by two techniques: by studying the rate of release from the tissue during sequential enzymatic digestion and by immunocytochemistry. Both techniques indicate that, in this tissue, lysozyme is primarily extracellular. Cartilage lysozyme was isolated and partially characterized and found to be identical with egg white lysozyme in its immunologic and enzymatic behavior. In addition, a method for the isolation of large numbers of viable chondrocytes is described.

INTRODUCTION

The function of animal lysozyme remains unknown. Although it is known to be a muramidase active on bacterial cell walls (1) and to stimulate transglycosylation in cell particulates (2), no substrate which it can catalytically degrade has yet been found in animal tissues (1, 3). In mammals, lysozyme is found in a variety of tissues and body fluids: kidney, cartilage, and blood cells are particularly rich sources (1, 4). In the cells studied thus far, it has generally been considered to be lysosomal (5, 6), although Baggiolini et al. found that in rabbit heterophil leukocytes it is present, along with alkaline phosphatase, in a nonlysosomal particulate fraction (7, 8).

A number of observations suggest that lysozyme has an as yet unidentified role in cartilage metabolism or transformation (9), in mineralization (10), or in bone metabolism (11). It has been suggested that this role involves regulation of the reactivity of certain of the anionic polysaccharide-rich macromolecules of cartilage by the cationic lysozyme molecule (12–14).

As a further step in the study of the role of lysozyme in cartilage, a series of experiments on intact cartilage and isolated chondrocytes from chick embryos was undertaken to determine its precise distribution as well as to partly characterize it. We here report data from this study indicating that over 99% of chick embryonic cartilage lysozyme is extracellular. In addition, certain properties of cartilage lysozyme, including its immunologic identity with hen's egg white lysozyme, are described as well as a digestion procedure for the isolation of chondrocytes in sufficient numbers for biochemical assays.

EXPERIMENTAL PROCEDURE

Materials

Nobel agar (Difco Laboratories, Inc., Detroit, Mich.) was used for the diffusion techniques. Lyophilized *Micrococcus lysodeikticus* cells were purchased from Miles Chemical Company, Clifton, N.J., and from Worthington Biochemical Corp., Freehold,

N.J. Specific antibodies from rabbits (gamma globulins) immunized with egg white lysozyme were isolated according to the procedure of Givol et al. (15). In brief, the lysozyme-antilysozyme complex was precipitated quantitatively, separated from the serum, and dissolved in 0.02 M HCl–0.15 M NaCl (pH 1.8). The antibody was separated from the antigen by column chromatography on Sephadex G-75 (Pharmacia Fine Chemicals Inc., Piscataway, N.J.), then neutralized to pH 7. After dialysis against 0.15 M NaCl the nonspecific proteins were removed by centrifugation, yielding purified anti–egg white lysozyme gamma globulins. Chick egg white lysozyme (EC, 3.2.1.17, muramidase, salt-free, twice crystallized, Worthington Biochemical Corp.) was used as a standard and as an antigen. Fluorescein-labeled antirabbit and antihuman gamma globulins, antihuman fibrinogen, and several nonspecific antisera were kindly provided by Dr. A. S. Markowitz, Hektoen Institute, Chicago. Fluorescein-labeled anti–egg white lysozyme was prepared by the method of Wood et al. (16). Several fluorescein-labeled gamma globulins directed against mammalian protein antigens were purchased from commercial sources. A soluble horseradish peroxidase–rabbit anti-horseradish peroxidase (HAH) complex was generously supplied by Dr. F. Booyse (17). A modified phosphate-buffered saline (MPBS) solution, calcium and magnesium free, pH 7.4, was used for the digestion procedure (NaCl, 8 g; KCl, 0.3 g; $NaHPO_4$, 0.152 g; glucose, 2.0 g/1000 ml H_2O). Trypsin was purchased from Difco Laboratories, Inc. (trypsin 1:250, Control 448631). Collagenase was purchased from Calbiochem, Los Angeles, Calif. (collagenase crude, bacterial, B-grade, Lot 901406). Calf thymus deoxyribonucleic acid (type V), yeast-soluble ribonucleic acid (type XI), and *p*-nitrophenylphosphate disodium salt were all purchased from Sigma Chemical Co., St. Louis, Mo.

Methods

IMMUNOLOGICAL IDENTITY OF EGG WHITE LYSOZYME AND EMBRYONIC CHICK BONE LYSOZYME: Embryonic chick femurs were dissected from 15-day embryos and immediately inserted into 20 ml of melted agar before gelatin (45°C, 1% agar in 0.135 M phosphate buffer, pH 7) in a 100 × 15 mm Petri dish. After allowing the agar to gel, uniform holes (3 mm outer diameter) were punched in the agar to form wells at a distance of 1 cm from the bone. 0.02 ml of the specific rabbit anti–chick egg white lysozyme gamma globulin (2.5 mg/ml), as well as 0.02 ml of egg white lysozyme solution (50 μg/ml), was placed into the wells. The Petri dish was then incubated in a wet chamber at room temperature for 24 hr. The agar was stained for 24 hr in a 0.0025% nigrosin solution in 2% acetic acid and photographed.

IMMUNO-INHIBITION OF THE ENZYMATIC ACTIVITY OF EGG WHITE LYSOZYME AND EMBRYONIC CHICK BONE LYSOZYME BY ISOLATED RABBIT ANTI–CHICK EGG WHITE LYSOZYME GAMMA GLOBULINS: The opposite limb of the embryo was treated identically, except that the agar contained dry *Micrococcus lysodeikticus* (0.3 mg/ml), the substrate for lysozyme, in suspension.

LOCALIZATION OF LYSOZYME BY IMMUNOCYTOCHEMISTRY: Both indirect and direct immunofluorescence techniques with appropriate controls were utilized for the histologic localization of lysozyme in fresh frozen sections of 15½–16–day embryonic chick femurs. The method of Sternberger et al. (18) was also used, in which peroxidase rather than fluorescein is used as a marker.

ISOLATION OF CHONDROCYTES: Femurs and tibias from 15½–16–day embryonic chickens were used. The bones were dissected from the embryos and immediately placed into ice-cold Puck's saline-G (19). As much soft tissue as possible was removed. For all experiments, the terminal cartilaginous cones were severed from the shaft in the area of flattened cells. Histologic study showed that this zone of flattened cells was preserved more or less intact, without marrow or bone rudiments. The wet weight of the cartilaginous rudiments was determined by adding the freshly cut pieces to a preweighed buffer solution. By using approximately 80 eggs in each experiment, between 11 and 12 g of tissue were obtained.

The tissue was then digested in a magnetic stirrer–activated trypsinizing flask for 30 min at 37°C. The dispersing medium consisted of 0.25% trypsin in MPBS. After passing the digest (100 ml) through 4 ply gauze to remove tissue fragments and then rinsing (40 ml) the cartilaginous rudiments in MPBS at 37°C, the remaining tissue was treated for 60 min with 0.4% collagenase–MPBS solution. The freshly liberated chondrocytes were separated from the undigested tissue by passage through 4 ply gauze. The cartilage rudiments were again exposed to 40 ml of fresh collagenase solution. Meanwhile, the chondrocytes were centrifuged (350 *g*, 4°C, 10 min), washed in 40 ml MPBS, and recentrifuged. The washed cells were examined for enumeration and viability in a hemocytometer (Speirs-Levy Chamber, C. A. Hauser & Son, Philadelphia, Pa.), employing the trypan blue dye exclusion test. A sample of 5×10^5 cells/dish was plated for tissue culture viability controls in a tissue culture medium previously described (12). The collagenase treatment of the cartilage rudiment was repeated twice, yielding a total of three chondrocyte fractions and a small undigested residue

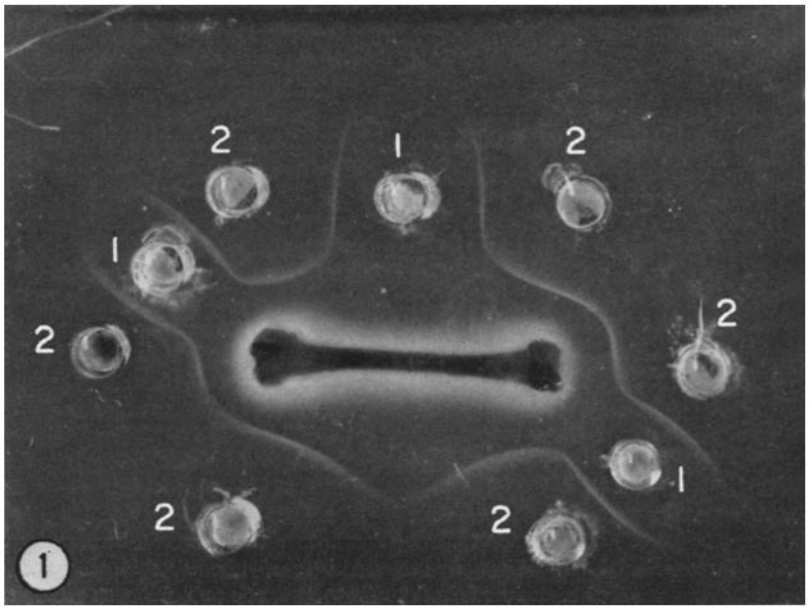

FIGURE 1 Immuno-identity of egg white lysozyme and embryonic chick bone lysozyme. A 16-day embryonic chick bone submerged in agar is shown; purified rabbit anti-chick egg white lysozyme gamma globulin as well as a 5 mg% egg white lysozyme solution were allowed to diffuse freely for 24 hr at room temperature (stained with nigrosin-acetic acid). Note discrete precipitin lines at the antigen-antibody interphase. The precipitin lines formed by egg white lysozyme and cartilage lysozyme are contiguous, indicating immuno-identity. *1*, egg white lysozyme standard; *2*, isolated rabbit anti-chick egg white lysozyme gamma globulins.

representing less than 5% of the initial tissue. In all cases, the digestion media and the MPBS used to wash the cells after centrifugation and removal of the intact cells, as well as the undigested residue, were assayed for nucleic acids, acid phosphatase, lysozyme, and total hexosamine. All three chondrocyte fractions were combined and rewashed. 5-ml samples, containing 5×10^5 cells each, were plated in 60 × 15 mm Falcon plastic tissue culture dishes (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.). The cells were cultured at 37°C, 5% CO_2 in air, 98% relative humidity, for 72 hr.

BIOCHEMICAL ASSAYS: Nucleic acids were determined essentially as described by Schneider (20). Hexosamine released after acid hydrolysis was determined by the method of Boas (21). Lysozyme activity was determined by measuring the decrease in the absorbance at 650 nm in a Gilford recording spectrophotometer (Gilford Instrument Company, Oberlin, Ohio) with enzyme preparations incubated in the presence of cell walls from *M. lysodeikticus* (22). When lysozyme was present in small amounts or was masked by other tissue constituents, it was also assayed by the method of Schumacher (23). Acid phosphatase activity was determined with *p*-nitrophenylphosphatase as substrate (24). Lysozyme which was released from the cartilage during the digestion procedure was concentrated and purified by column chromatography with CM-Sephadex and Sephadex G-10.[1] Protein was determined according to Lowry et al. (25) with bovine serum albumin as standard. Alkaline phosphatase activity with *p*-nitrophenylphosphate as substrate was measured by following the production of *p*-nitrophenolate ion at pH 9.9 in a Gilford recording spectrophotometer (26).

RESULTS

Immunological Identity of Cartilage and Egg White Lysozyme and the Inhibition of its Enzymatic Activity by Rabbit Anti-Egg White Lysozyme Gamma Globulins

The results of these experiments are illustrated in Figs. 1 and 2. Fig. 1 shows a 16 day chick embryonic femur inserted into hypotonic agar in a Petri dish. As the egg white lysozyme from the well and the tissue lysozyme from the bone rudiment diffused into the agar, precipitin lines formed

[1] Guenther, H. L. Personal communication.

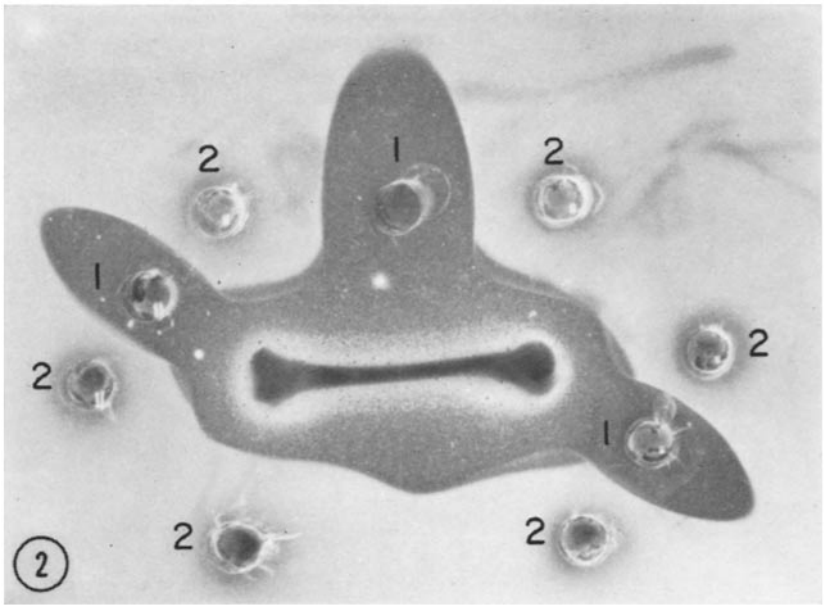

FIGURE 2 Immuno-inhibition of the enzymatic activity of egg white lysozyme and embryonic chick bone lysozyme by isolated rabbit anti-chick egg white lysozyme gamma globulin. As in Fig. 1, however, *Micrococcus lysodeikticus* was suspended in the agar. Note discrete precipitin lines as in Fig. 1. The areas of clearance due to the lytic activity of lysozyme, (which appear dark because the photograph was taken with a dark background), are significantly inhibited at the antigen-antibody interphase, sometimes extending beyond the precipitin lines, indicating either an excess of antigen with respect to available antibody or the possible presence of a second species of lysozyme., *1* egg white lysozyme standard; *2*, gamma globulins.

where these antigens and the inserted specific antibody met in proper concentration. The two precipitin lines—one formed by the interaction of gamma globulins and lysozyme diffusing from the bone rudiment and the other formed by the interaction of gamma globulin and the egg white lysozyme—fused at their contiguous ends. The immunological identity of these two lysozymes derived from egg white and embryonic cartilage was thus established.

Fig. 2 illustrates a similar experiment except that the agar contained *Micrococcus lysodeikticus* in suspension. Here, both the precipitin lines between lysozyme and gamma globulin and lysis of the *Micrococcus* are shown. Once again, precipitin lines form as the diffusing lysozyme and gamma globulin meet in the agar. In addition, it is obvious that the radial area of lysis of the substrate is indented where the precipitin lines form, indicating that the enzymatic activity of lysozyme is inhibited by the antibody, an observation in agreement with those of others (27–29). At the interface between limb lysozyme and gamma globulin, some clearing of the agar extends beyond the precipitin line, suggesting an excess of antigen diffusing beyond the zone of precipitation or the presence of a second species of lysozyme in cartilage as described in other sites (11, 30).

Localization of Lysozyme

Specific antilysozyme fluorescence, whether tested by indirect (Fig. 3 *a*) or direct (Fig. 3 *b*) immunofluorescence technique, was seen only in sections exposed to antilysozyme antiserum. The only anatomic sites where bright, specific, antilysozyme fluorescence was found were in the matrix. Here fluorescence was more intense in a rim about the chondrocytes, roughly corresponding to the anatomic lacunae. There was no such fluorescence in any of the various control specimens, although nonspecific fluorescence of nuclei and bone was seen. With the HAH method, which is more sensitive than immunofluorescence, there was a diffuse staining in the matrix which was somewhat more intense in the lacunar area (Fig. 3 *c*). In addition, in all three techniques small amounts of intracellular immuno-staining were seen.

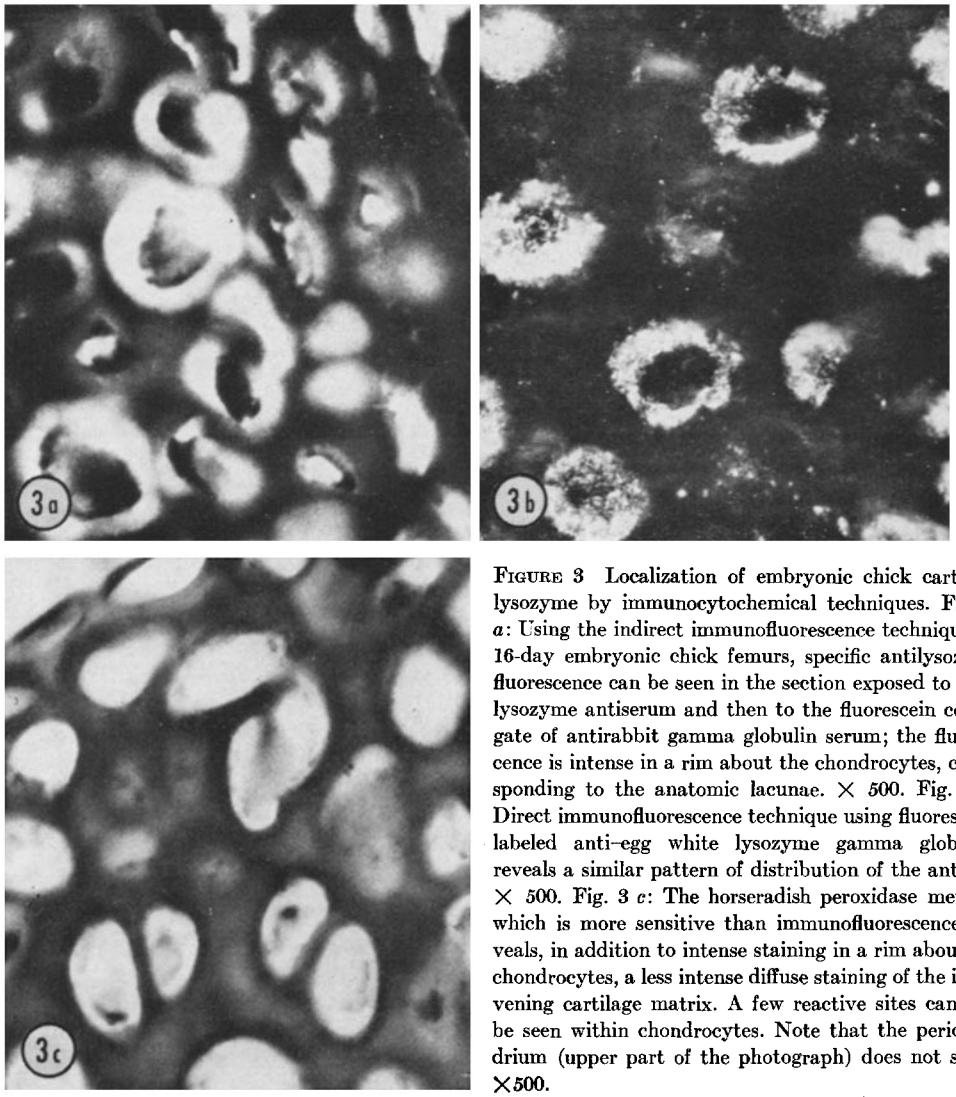

FIGURE 3 Localization of embryonic chick cartilage lysozyme by immunocytochemical techniques. FIG. 3 *a*: Using the indirect immunofluorescence technique on 16-day embryonic chick femurs, specific antilysozyme fluorescence can be seen in the section exposed to antilysozyme antiserum and then to the fluorescein conjugate of antirabbit gamma globulin serum; the fluorescence is intense in a rim about the chondrocytes, corresponding to the anatomic lacunae. × 500. Fig. 3 *b*: Direct immunofluorescence technique using fluorescein-labeled anti-egg white lysozyme gamma globulins reveals a similar pattern of distribution of the antigen. × 500. Fig. 3 *c*: The horseradish peroxidase method, which is more sensitive than immunofluorescence, reveals, in addition to intense staining in a rim about the chondrocytes, a less intense diffuse staining of the intervening cartilage matrix. A few reactive sites can also be seen within chondrocytes. Note that the perichondrium (upper part of the photograph) does not stain. ×500.

Digestion of Cartilage and Isolation of Chondrocytes

By using 11–12 g of cartilage, a total of 4×10^9 chondrocytes was obtained as determined by trypan blue viability counts in a hemocytometer and confirmed by DNA analysis. DNA determinations on the cultured cells after 72 hr showed a 2½–3-fold increase, indicating good viability of the plated cells.

All of the solutions resulting from the digestion and washing of cartilage during the digestion period were assayed for lysozyme, nucleic acids, hexosamine, alkaline phosphatase, and acid phosphatase. The results of the assays plotted as cumulative percentages released into the medium during the various steps of the isolation procedures are shown in Fig. 4. This figure illustrates the percentages of cartilage components present in the supernatant solutions from low speed centrifugation to separate intact cells. The percentages in the graph represent ratios of amounts present in the supernatant when compared to the amount in the entire cartilage. It can be seen that practically all of the lysozyme activity of the cartilage is released from the tissue during the initial trypsin treatment and the subsequent washing of the cartilage residue with MPBS. Only trace amounts of

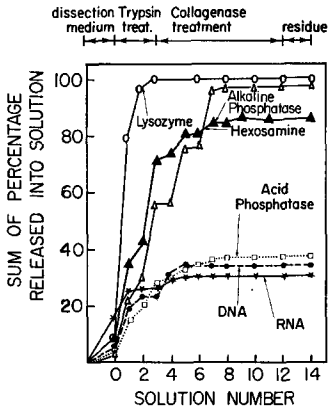

FIGURE 4 Release of chick cartilage components during digestion for cell isolation. Ordinate: Cumulative sum of percentages of compounds released into the medium in soluble form are shown. The residual components are present in either intact isolated cells or the undigested tissue residue. The figure of 100% equals the total amount present in the original tissue. Abcissa: The numbers indicate the number of treatment solution changes. Note that lysozyme (open circles) is rapidly released into the medium during and after the initial trypsin treatment, which does not significantly disrupt chondrocytes, thus suggesting that it is primarily extracellular. Hexosamine, also an extracellular material, was largely solubilized during the earlier part of the extraction, as was alkaline phosphatase, thus suggesting that this enzyme is also at least partially extracellular in location. In contrast, only about 40% of the intracellular markers, nucleic acids and acid phosphatase, were released into the medium during the entire digestion procedure. The remaining nucleic acids were recovered in the intact chondrocytes and the final undigested residue which, by both morphologic and chemical analyses, represented a small portion of the entire tissue.

lysozyme were found in homogenates of the intact cells. Total hexosamine was also released, but at a different and slower rate. Release of nucleic acids into the medium occurred only when cartilage was initially treated with trypsin. No further significant release of nucleic acids was observed when the trypsin treatment was followed by subsequent treatments with collagenase. The nucleic acids released during the trypsin treatment (about 37% of the total) may well be a result of dissolution of adherent perichondrial tissues, residual bone marrow cells, and possibly disruption of some chondrocytes. The remaining nucleic acids (63%) were recovered in the intact chondrocytes and the final undigested cartilage residue which, by both morphologic and chemical analyses, represented a small proportion of the entire tissue (less than 5% of the initial tissue). Acid phosphatase was released in a pattern similar to that of nucleic acid, suggesting that it is an intracellular enzyme. The release of alkaline phosphatase paralleled that of hexosamine, suggesting that this enzyme, too, is primarily extracellular as recently described (31). Of the total activity of alkaline phosphatase, however, approximately 15% was associated with the intact chondrocytes and, to a much lesser extent, with the final undigested residue. By comparison, over 60% of the nucleic acids and acid phosphatase were retained mostly with the chondrocyte fraction, and a small amount with the undigested cartilage residue.

The supernatant solutions from the digestion, containing practically all of the cartilage lysozyme, were lyophilized, and the lysozyme was purified through CM-Sephadex C-50. The results of such purification are tabulated in Table I and illustrated in Fig. 5 *a*. The activity of the highly purified lysozyme was studied as a function of pH, enzyme concentration, and substrate concentration (Fig. 5). Chick embryonic cartilage lysozyme shows maximum activity at pH 7.8 in 0.04 M potassium phosphate buffer (Fig. 5 *b*). There was a linear dependence of activity on the protein concentration when less than 4 μg of protein were used (Fig. 5 *c*). When the concentration of *Micrococcus lysodeikticus* cells was 200 μg/ml of incubation mixture, maximum activity was observed. Higher concentration of this substrate caused inhibition (Fig. 5 *d*).

DISCUSSION

The procedure utilized for the isolation of chondrocytes offers the advantage of providing sufficient numbers of cells for biochemical analyses. Because of the extent of the enzymatic digestion procedure, cellular integrity and survival from various stages of the isolation procedures were checked in the tissue culture system. The ability of all batches of cells to propagate in vitro is evidence that the data obtained on such cells are valid.

The proof of the immunologic identity of egg white and cartilage lysozymes was required for the accomplishment of the immunocytochemical studies for the localization of lysozyme in cartilage, since cartilage lysozyme has not yet been isolated in bulk. Aside from the immunologic similarity or, better, identity, the inhibition of the enzyme ac-

TABLE I

Purification of Chick Embryonic Cartilage Lysozyme

Lysozyme diffused out of cartilage was purified with CM-Sephadex C-50 as described in text. Activity is expressed as the decrease in the absorbance at 650 nm per min. Specific activity is the activity per mg of soluble protein.

Fraction	Volume	Protein	Total activity	Specific activity	Yield
			$\frac{-\text{Absorbancy}}{\text{min}}$	$\frac{-\text{Absorbancy}}{\text{min} \times \text{mg}}$	
	ml	*mg*			%
Homogenized cartilage	40	370.0	28.0	0.075	100
Digestion solutions	5.0	55.0	25.0	0.45	89
Column tube No. 28	10.0	0.72	11.2	15.5	40

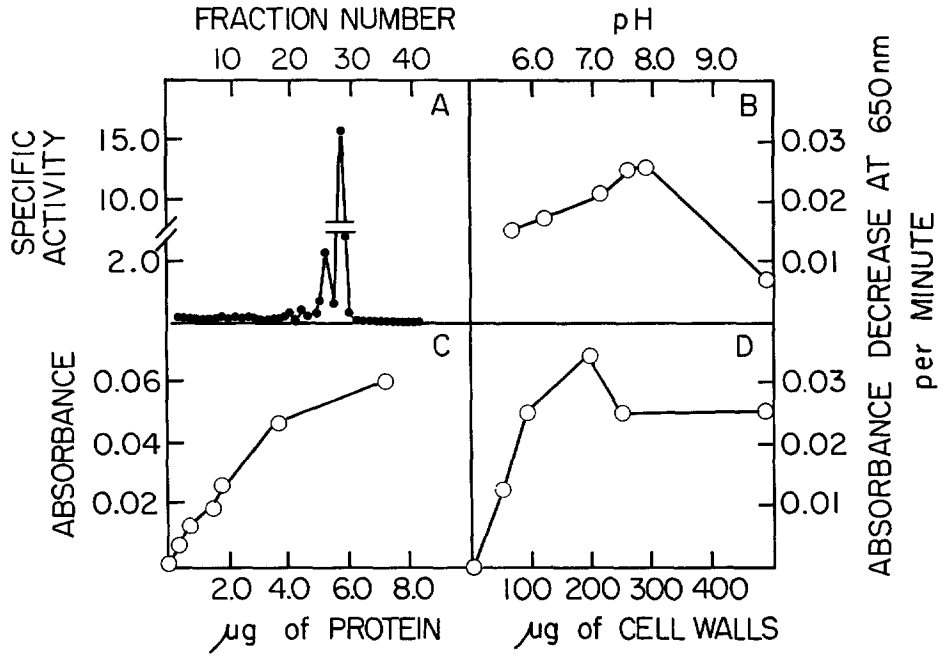

FIGURE 5 Properties of chick embryonic cartilage lysozyme. Fig. 5 A: Effluent pattern of pooled digestion media on CM Sephadex C-50. The sharp protein peak contains all of the lysozyme activity. Fig. 5 B: pH-dependent activity of purified cartilage lysozyme. Maximum activity appears to occur at pH 7.8–0.04 M potassium phosphate. Fig. 5 C: Activity as a result of protein concentration. This relationship appears to be linear up to a concentration of 4 μg of protein. Fig. 5 D: Substrate inhibition of lysozyme activity. At concentrations of *Micrococcus lysodeikticus* above 200 μg/ml, lytic activity is decreased.

tivity by the gamma globulins as well as the similarity of the pH optimum and enzyme kinetics of the lysozyme from the two sources (cartilage and egg white) suggests that the function may be similar at these two sites. There are other observations which lead to the same tentative conclusions. The immunocytochemical data, as well as the observation that virtually all the lysozyme was released into the digestion media, are strong indications that cartilage lysozyme is primarily extracellular in location. Egg white lysozyme is also extracellular. Both egg white and preosseous cartilage are particularly rich in lysozyme, and both calcify in specific anatomic locations. It should be pointed out that no substrate for lysozyme has been found in vertebrate tissues, despite specific search (32), that lysozyme is a highly charged cation, and that the polysaccharides of cartilage

are strong anions. This suggests that one function of lysozyme at these sites may be to interact with polysaccharides or other anions, such as glycoproteins, thus altering their reactivity with calcium and perhaps other ions in a manner promoting calcification. In the accompanying communication (33), in which a detailed biochemical analysis of the isolated chondrocytes is described, it will be pointed out that in cartilage, unlike some other tissues, lysozyme is not a lysosomal enzyme, nor is it a detectable part of any other cytoplasmic particulate fraction.

The immunocytochemical studies also point out that, even in cartilage matrix, lysozyme is heterogeneously distributed since the immunologic markers were particularly concentrated in the area corresponding to the chondrocyte lacunae. This locus appears to be particularly specialized since not only lysozyme, but also specific protein-polysaccharides (34, 35) and chlorides, are concentrated there (36). It is also the area where exogenous lysozyme is specifically bound (12, 14). The functional significance of this anatomic specialization remains obscure. It is of interest that alkaline phosphatase, another enzyme implicated in the process of calcification and whose precise role in this process is imperfectly understood, also appears to be primarily extracellular in cartilage (31).

We wish to thank Doctors R. D. Ray and G. Hass for their continued support, A. S. Markowtiz for help with the immunofluorescence studies; and Mrs. E. B. Schroeder, Mrs. P. Lisk, and Mr. R. Croxen for their skilled technical assistance. We are particularly grateful to Miss A. Laurick for developing and performing the agar diffusion work. The cooperation of the Photography Department, Presbyterian-St. Luke's Hospital, is also gratefully acknowledged.

This work was supported by Grant AM-09132 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland and in part by the Illinois Chapter of the Arthritis Foundation.

Received for publication 8 May 1970, and in revised form 7 December 1970.

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