ENZYME ACTIVITIES IN CHICK EMBRYONIC CARTILAGE

Their Subcellular Distribution in Isolated Chondrocytes

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ABSTRACT

Some characteristic enzymatic activities were determined in chick embryonic cartilage and compared with the analogous activities in bone and liver. Chondrocytes were isolated, broken by sonication, and subjected to subcellular fractionation to yield a nuclear pellet, the mitochondrial, lysosomal, and microsomal fractions, and the high speed supernatant solution. It was established that these fractions are characterized by enzymatic activities usually associated with similar fractions in other tissues, but with some quantitative differences. Lysozyme, a particulate-associated enzyme in other tissues, was not detected in any subcellular fraction even by the sensitive technique of microzone electrophoresis and is therefore considered to be primarily extracellular in cartilage.

INTRODUCTION

The biological function of cartilage, including the transformation to bone, must depend at least in part on the activity of specific intra- and extracellular enzymes. Although not yet completely established, the conversion of ossifying cartilage into bone appears to be mediated by striking changes in enzymatic activities. Thus, more detailed knowledge of the enzymatic spectrum of various kinds of cartilage appears to be important.

The techniques of separation of fractions enriched in various subcellular particles from disrupted cells, developed during recent years (1), have permitted the assessment of the distribution of various enzymes and, therefore, the localization of the various catabolic and anabolic processes within the cell. These studies have strengthened the concept of the cell as a highly organized functional unit. Studies on the intracellular distribution of various enzymatic activities have been carried out for a number of tissues, both normal (2–6) and pathological (7–9), in many species. Cartilage has thus far defied such an analysis, primarily since it has not been possible to homogenize this tissue under conditions which preserve the integrity of the different subcellular particles because of the large amount of connective tissue surrounding its cells.

Lytic enzymes have been used for the dispersion of tissues and the subsequent separation of cells into homogeneous populations (10, 11). The development of such a method, employing sequential digestion with trypsin and collagenase, for the separation of a large number of viable chondrocytes has been described in the accompanying publication (12). We here describe the fractionation of chondrocytes isolated by this method and

the distribution of various enzymes characterizing the different cell particulates. The distribution patterns for mitochondrial, lysosomal, microsomal, and soluble enzymes obtained are basically similar to those found in a prototype tissue, mammalian liver, although some qualitative and quantitative differences were observed. While this work was in progress, two reports on the enzymatic composition of cartilage appeared (13, 14). The present report deals with some enzymes which have not been examined in detail thus far in their intracellular localization.

EXPERIMENTAL PROCEDURE

Materials

Calf thymus deoxyribonucleic acid (type V), yeast-soluble ribonucleic acid (type XI), *p*-nitrophenylphosphate disodium salt, *p*-nitrocatecholsulfate dipotassium salt, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (grade III), *p*-nitrophenyl-β-D-galactopyranoside, glucose-6-phosphate disodium salt, horse heart cytochrome *c* (type III), bovine hemoglobin (type II), nicotinamide adenine dinucleotide phosphate (NADPH) tetrasodium salt (type I), yeast nicotinamide adenine dinucleotide (NAD) (grade III), yeast uridine diphosphate glucose sodium salt, phenolphthalein glucuronide, tetrasodium pyrophosphate, egg white lysozyme, and hexosamine were all purchased from Sigma Chemical Co., St. Louis, Mo. *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide was obtained from Pierce Chemical Co., Rockford, Ill. Lyophilized *Micrococcus lysodeikticus* cells were purchased from Miles Chemical Company, Clifton, N.J.. α-naphthyl acetate was a product of Eastman Chemical Products, Inc., Kingsport, Tenn.

Methods

ISOLATION OF CHONDROCYTES: Isolation of chondrocytes was conducted as described in the accompanying paper (12). The cells so prepared were 99% viable as estimated by staining with trypan blue and subsequent culture of cell samples. For subcellular fractionation the cells were packed by centrifuging for 10 min at 800 *g*, suspended in 0.25 M sucrose (1:3 ratio v/v), and sonicated for 4 sec with a Branson sonifier (Branson Sonic Power, Co., Plainview, L. I., N. Y.) at a current strength of 3.2–3.5 amp. Since such short sonication does not break all of the cells, the nuclear debris obtained after centrifugation at 800 *g* for 10 min was resuspended in sucrose and the suspension was again sonicated. This was repeated and the supernatant solutions were combined and fractionated essentially as described by de Duve et al. (15). The nuclear pellet, and mitochondrial, lysosomal, and microsomal fractions were suspended in 0.25 M sucrose (15) and were assayed along with the final high speed supernatant.

ASSAYS: Acid deoxyribonuclease and acid ribonuclease were assayed in a total volume of 1.0 ml with 400 μg of nucleic acid as substrate. Determination of the breakdown products was done by the method of de Duve et al. (15). Acid phosphatase activity with *p*-nitrophenylphosphate as substrate was determined from the β-nitrophenolate released (16). Aryl sulfatase activity was assayed as described by Roy (17). Hemoglobin was used as the substrate for cathepsin activity (18), and α-naphthyl acetate as substrate for esterase activity by a modification of Weissmann's method (19). *N*-acetyl-β-D-glucosaminidase, *N*-acetyl-β-D-galactosaminidase and β-D-galactosidase activities were measured by the *p*-nitrophenolate ion released (20, 21). Phenolphthalein glucuronide was used as substrate for β-glucuronidase (22). Alkaline and acid pyrophosphatases were measured at pH 7.4 and 5.0 (23). Cytochrome *c* succinate reductase activity was estimated according to a method adapted from Green et al. (24). After preincubation of the mixture at room temperature for 5 min, the reaction was started by addition of succinate and the rate of cytochrome *c* reduction was followed on a Gilford recording spectrophotometer at 550 mm (Gilford Instrument Company, Oberlin, Ohio). Cytochrome oxidase was assayed by the spectrophotometric method of Cooperstein and Lazarow by the rate of oxidation of reduced cytochrome *c* (25). NADPH cytochrome *c* reductase was assayed by following the reduction of cytochrome *c* by NADPH (26). Alkaline

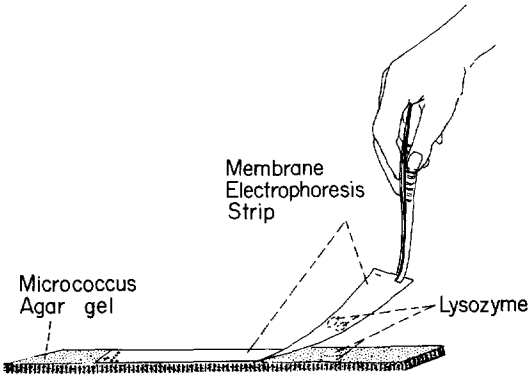

FIGURE 1 Detection of lysozyme activity by microzone electrophoresis. (The apparatus manufactured by Beckman Instruments was used). Electrophoretograms are obtained after electrophoretic separation by placing the membranes upside-down on the surface of an agar gel containing *M. lysodeikticus* in suspension. Lytic activity can be seen by the area of lysis in the agar.

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 (27). After trichloroacetic acid precipitation of protein, inorganic phosphate produced from glucose-6-phosphate (0.02 M) at pH 6.6 in the presence of 0.005 M ethylenediaminetetraacetate (EDTA) in a total volume of 1.0 ml was measured into the supernatant solution. To 0.1 ml of supernatant solution, 0.2 ml of 1% (w/v) ammonium molybdate and 2.0 ml of a 0.05% (w/v) solution of *N*-methyl-*p*-phenylenediamine dihydrochloride in 1% (w/v) sodium bisulfite were added. The absorbance at 770 nm was proportional to the amount of inorganic phosphate present. Lysozyme activity was determined by measuring the decrease in the absorbance at 650 nm in a Gilford recording spectrophotometer of an incubation mixture containing lyophilized *Micrococcus lysodeikticus* cell walls (28). When lysozyme was present in small amounts or was masked by other tissue constituents it was assayed by the method of Schumacher (29). Uridine diphosphate glucose dehydrogenase activity was measured by following the formation of reduced pyridine nucleotide at 340 nm (30). Deoxyribonucleic (DNA) and ribonucleic (RNA) acids were measured by a modification of Schneider's method (31). Hexosamine released after acid hydrolysis was determined by the method of Ross (32), and protein by the method of Lowry et al. (33), with bovine albumin as a standard.

TABLE I

Weight of Wet Tissue and Duration of Incubation for Valid Assays on Chick Embryonic Cartilage

The weight of the tissue and the time of incubation shown represent the minimum values which had to be used for reliable assays.

Assay	Weight of wet cartilage	Time of incubation
	mg	*min*
Acid deoxyribonuclease	5.8	60
Acid ribonuclease	5.8	60
Acid phosphatase	11.7	15
Aryl sulfatase	117.0	60
Cathepsin	58.0	60
Esterase	11.7	15
N-Acetyl-β-D-glucosaminidase	17.5	60
N-Acetyl-β-D-galactosaminidase	25.0	60
β-galactosidase	58.0	60
β-glucuronidase	11.7	30
Alkaline pyrophosphatase	5.8	30
Acid pyrophosphatase	11.7	60
Succinate cytochrome *c* reductase	6.0	4
Cytochrome oxidase	0.6	4
NADPH cytochrome *c* reductase	5.8	4
Alkaline phosphatase	2.8	4
Glucose-6-phosphatase	58.0	4
Lysozyme	1.4	4
Deoxyribonucleic acid	11.7	—
Ribonucleic acid	11.7	—
Hexosamine	5.8	—
Protein	0.6	—

DETECTION OF LYSOZYME ACTIVITY BY MICROZONE ELECTROPHORESIS: Microzone electrophoresis (pH 8.4 for 20 min, Beckman Instruments Inc., Fullerton, Calif.) was carried out on 2.5 μl of a 1/8 cartilage homogenate in water, a 1/3 homogenate of the cell suspension and the subcellular fractions, as well as 2 μl of a 5 mg% egg white lysozyme solution. Electrophoretograms were obtainedrf a the electrophoretic separation by placing the membranes upside-down on the surface of an agar gel containing *Micrococcus lysodeikticus* in suspension (Fig. 1). Lytic activity of the cationic component was clearly shown by an area of lysis in the agar and was photographically documented (29).

UNITS AND PRESENTATION OF RESULTS: For simplicity in the construction of figures presenting the distribution of enzyme activities, the following symbols are used to designate the various subcellular fractions: N, nuclear fraction; M, mitochondrial fraction; L, lysosomal fraction; P, polysomal (microsomal) fraction; S, final supernatant solution. One unit of activity refers to the amount of enzyme which can either decompose or form one micromole of substrate or product per minute under the conditions of the assay. Lysozyme activity was expressed in terms of egg white lysozyme equivalents (34). Nucleic acid breakdown products were expressed as liberated mononucleotides assuming an average extinction coefficient at 260 nm of 8.5×10^3 cm²/mole. The extinction coefficient of cytochrome *c* was taken as 1.85×10^4 cm²/mole at 550 nm.

The distribution patterns of various enzymatic activities and nucleic acids among the different subcellular fractions are shown in the manner proposed by de Duve et al. (15). The mean relative specific activity of components of the fraction is plotted against the mean relative soluble protein content of that fraction. In this way the area of each block is proportional to the percentage of activity recovered in the corresponding fraction, and its height to the degree of purification attained over the homogenate suspension made from whole tissue.

RESULTS

Tissue Concentration and Time of Incubation for Enzyme Assays

In enzyme assays it is necessary that the activities measured are proportional to the amount of enzyme in the tissue and the time of incubation. The weight of wet cartilage and the time of incubation used for each assay performed in this study are shown in Table I. The weights represent the maximum amount of wet tissue which, if incubated for the specific period, yielded linear results when tissue weight was plotted against enzymatic activity, e.g., when one-half as much tissue is used the activity is reduced by one-half. For those assays for which the recording spectrophotometer was used, the time shown is recommended since in longer incubations there was no linear response between activity and incubation time.

TABLE II

Enzyme Activities, Nucleic Acids, Hexosamine, and Soluble Protein in Chick Embryonic Cartilage

Activity and range of activity are given in units per gram of wet tissue while lysozyme, nucleic acids, hexosamine, and protein are expressed as milligrams per gram of wet tissue.

Assay	Activity	Range	Number of experiments
	units/g	*units/g*	
Acid deoxyribonuclease	0.1	0.08–0.119	4
Acid ribonuclease	0.37	0.32–0.41	4
Acid phosphatase	1.29	0.81–1.85	4
Aryl sulfatase	0.32	0.30–0.32	4
Cathepsin	0.236	0.188–0.283	4
Esterase	0.96	0.785–1.12	4
N-Acetyl-β-D-glucosaminidase	0.5	0.44–0.56	3
N-Acetyl-β-D-galactosaminidase	0.18	0.12–0.22	2
β-galactosidase	0.072	0.058–0.084	4
β-glucuronidase	0.64	0.62–0.66	4
Alkaline pyrophosphatase	1.62	0.55–2.8	4
Acid pyrophosphatase	0.70	0.55–0.86	4
Succinate cytochrome *c* reductase	0.088	0.085–0.092	6
Cytochrome oxidase	2.3	2.1–2.4	8
NADPH cytochrome *c* reductase	0.085	0.082–0.089	6
Alkaline hosphatase	1.6	1.2–2.0	4
Glucose-6-phosphatase	0.28	0.22–0.34	4
	mg/g	*mg/g*	
Lysozyme	0.52	0.46–0.56	4
Deoxyribonucleic acid	2.09	1.9–2.3	6
Ribonucleic acid	2.6	1.1–3.2	6
Hexosamine	6.5	5.7–7.4	8
Protein (soluble)	75.2	68.6–81.8	6

Enzyme Activities, Nucleic Acid, Hexosamine, and Protein

Some characteristic enzymatic activities from whole chick embryonic cartilage, along with nucleic acids, hexosamine, and soluble protein, are shown in Table II. Chick embryonic cartilage has the hydrolytic activities present in most tissues. Acid ribonuclease, esterase, alkaline and acid pyrophosphatase, and alkaline phosphatase activities are particularly high. The most striking result, however, is the high lysozyme activity, similar to that observed in mammalian cartilage (34, 41). The activity per gram wet weight range and number of measurements for each assay are shown in Table II. Because of the large amounts of water and insoluble protein in fresh cartilage matrix the activities expressed in terms of wet weight are low. They are, nevertheless, significant when considered in the light of the proportion of cells in this tissue.

A comparison of the various activities for chick embryonic cartilage, bone, and liver is shown in Table III. The values for bone are taken from the studies of Vaes and Jacques (3), and those for liver from the data of de Duve et al. (15, 20, 35). These published data were confirmed, recalculated, and expressed as activity per milligram of DNA.

Distribution of Enzyme Activities and Nucleic Acids in Various Subcellular Fractions of Isolated Chick Embryo Chondrocytes

The distribution of the various enzymatic activities among the various fractions shown in Fig. 2 resembles those recorded for rat liver (15).

TABLE III

Specific Activity of Various Enzymes in Chick Embryonic Cartilage, Rat Liver, and Calvarium

Specific activities of various enzymes are expressed as units per milligram of soluble protein or as units per milligram of DNA. The value for liver and calvarium are calculated from similar studies by de Duve et al. (13), Sellinger et al. (32), and Vaes and Jacques (3).

Assay	Specific activity			Specific activity		
	Cartilage	Liver	Calvarium	Cartilage	Liver	Calvarium
	units × 10^3*/mg protein*			*units/mg DNA*		
Acid deoxyribonuclease	1.3	6.4	14.0	0.04	0.54	0.17
Acid ribonuclease	4.9	13.4	27.3	0.17	1.12	0.34
Acid phosphatase	17.2	—	—	0.61	—	—
Aryl sulfatase	4.3	—	—	0.15	—	—
Cathepsin	3.1	7.4	7.2	0.11	0.62	0.090
Esterase	12.8	—	—	0.46	—	—
N-acetyl-β-D-glucosaminidase	6.6	34.2	68.0	0.24	2.88	0.86
N-acetyl-β-D-galactosaminidase	2.4	—	—	0.086	—	—
β-galactosidase	0.9	1.7	13.1	0.03	0.14	0.16
β-glucuronidase	8.5	3.8	7.1	0.30	0.32	0.09
Alkaline pyrophosphatase	21.5	—	—	0.77	—	—
Acid pyrophosphatase	9.3	—	—	0.34	—	—
Succinate cytochrome *c* reductase	1.2	—	—	0.04	—	—
Cytochrome oxidase	30.7	—	—	1.1	—	—
NADPH cytochrome *c* reductase	1.1	—	—	0.04	—	—
Alkaline phosphatase	21.3	—	—	0.77	—	—
Glucose-6-phosphatase	3.7	80.0	—	0.13	6.65	—
Lysozyme	6.9	—	—	0.25	—	—
Deoxyribonucleic acid	27.9	11.9	81.0	(1.0)	(1.0)	(1.0)
Ribonucleic acid	34.6	51.0	108.0	1.24	4.3	1.34
Hexosamine	86.4	—	—	3.11	—	—
Protein (soluble)	(1000)	(1000)	(1000)	36.0	84	12.6

Electron microscopy was done on glutaraldehyde-fixed, Epon-embedded sections of intact cells as well as the various subcellular fractions. Examination of cells isolated after trypsin digestion (12) revealed that cells looking like chondrocytes and cells looking like fibroblasts were both present. However, the subsequent step in the isolation procedure, which is digestion with collagenase, yielded a homogeneous population of intact, well-preserved cells which had the morphology of chondrocytes. Subcellular fractionation and assays were therefore done only on chondrocytes isolated following the collagenase digestion. Cells released from the tissue during the initial trypsin digestion were discarded.

The subcellular fractions had morphologies comparable to these of analogous fractions isolated by others from tissues such as liver. The degree of morphologic overlap of the different fractions was consistent with the degree of biochemical overlap illustrated in Fig. 2. Current effort is directed toward further characterization of subcellular fractions from larger animals and will be the subject of later communications.

Recovery values based on the sum of the nuclear and cytoplasmic fractions were satisfactory in all

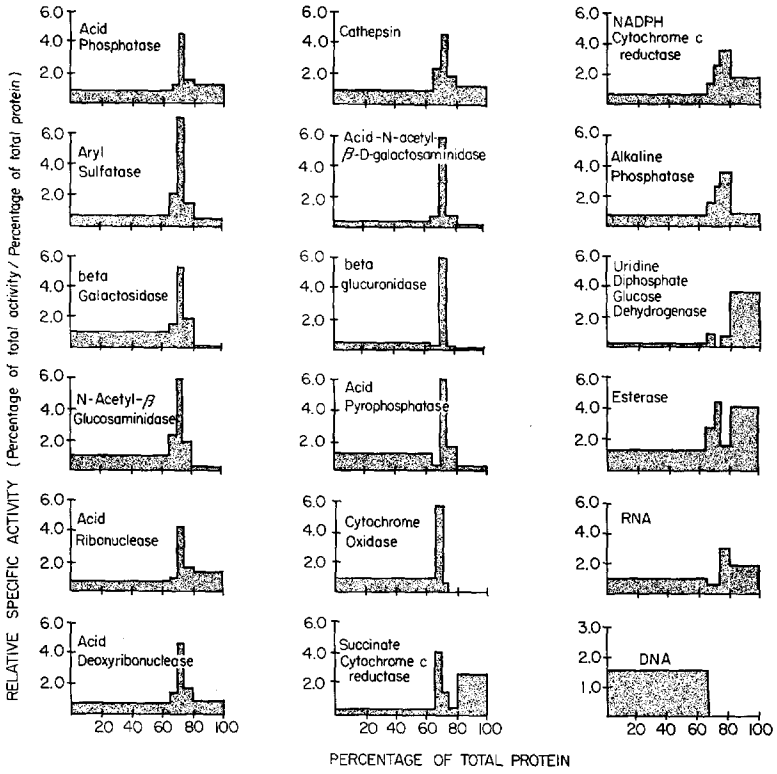

FIGURE 2 Enzyme distribution patterns in subcellular fractions from chick embryonic chondrocytes. Fractions are prepared and represented by their protein content cumulatively from left to right in the order of their isolation: N, M, L, P, S in the manner described by de Duve et al. (15).

cases. The amounts, however, found in the nuclear fraction are higher than for liver. As in liver and bone all acid hydrolases (left column of Fig. 2) show similar distribution patterns, with the "light mitochondrial" fraction having the highest degree of purification.

Cytochrome oxidase, a typical mitochondrial marker, shows the same distribution pattern when chondrocytes are fractionated as described for liver by de Duve et al. (15). Succinate cytochrome *c* reductase is another example of activity associated with the same subcellular particle in liver (15). The distribution pattern shown in Fig. 2, however, indicates that a large part of the total activity is found in the final supernatant solution when chondrocytes from the chick embryonic cartilage were disrupted by a brief sonication, indicating that the enzyme was probably freed from mitochondria during the fractionation procedure.

Alkaline phosphatase is particularly concentrated in the microsomal fraction, with significant amounts of activity in the other fractions as well. The same distribution pattern was obtained for NADPH cytochrome *c* reductase, an apparent microsomal marker in liver (15). Uridine diphosphate glucose dehydrogenase is mainly found in the final supernatant solution, while RNA is distributed between the microsomal fraction and the supernatant solution. The activity found in the nuclear fraction could be attributed to unbroken cells, while the small amounts of RNA found in the lysosomal fraction may be due to microsomal contamination. Under the conditions of the assay, DNA was found only in the nuclear fraction.

Distribution of Lysozyme Activity Assayed by Microzone Electrophoresis

Fig. 3 shows the electrophoretogram of all five subcellular fractions from isolated chondrocytes, as well as of the whole cartilage and cell homogenates. The egg white lysozyme controls show significant areas of lysis of bacterial cell wall in the

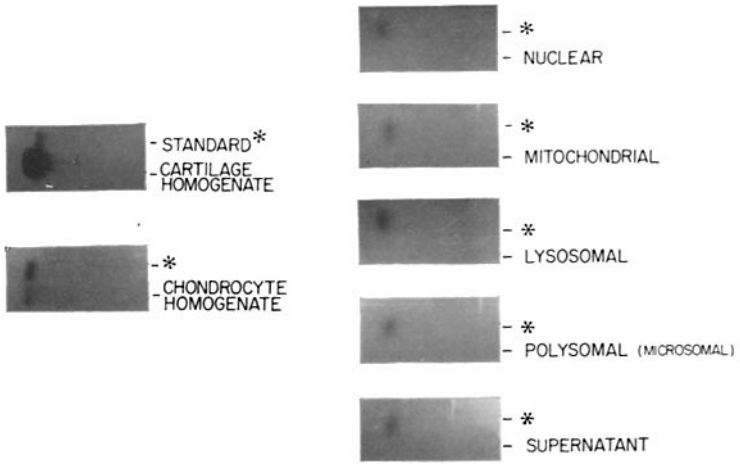

FIGURE 3 Detection of lysozyme activity by microzone electrophoresis. Electrophoresis carried out on cartilage, on chondrocyte homogenates, on each subcellular fraction, and on egg white lysozyme as standard as described in the text. Activity was only seen in whole cartilage and chondrocyte homogenates, while all subcellular fractions were inactive.

agar. The whole cartilage homogenate is obviously rich in lysozyme, and the chondrocyte homogenate contains only traces. None of the subcellular fractions contained enough lysozyme to be detected by this sensitive technique.

DISCUSSION

The studies described here confirm and extend data indicating that cartilage contains significant acid hydrolase activity capable of breaking down both cell constituents and extracellular matrix, an important step in the transformation of cartilage into bone (35, 36). Structure-linked latency studies have been carried out for only two acid hydrolases, acid phosphatase and cathepsin. However, the intracellular distribution and latency studies carried out in other tissues (2–7) strongly suggest that these enzymatic activities belong to the same group of particles, i.e., lysosomes (2). As with lysosomal enzymes in other tissues, the distributions found for the acid hydrolases are not identical for all of the enzymes of this group (15, 37). There are a number of possible interpretations for this phenomenon, one of which is related to the heterogeneity of cell populations found in intact tissue. Since the subcellular fractionation data presented here are derived from an isolated homogeneous cell population, such an explanation cannot be invoked.

In two out of seven runs of this experiment, traces of lysozyme activity were found in the nuclear and microsomal fractions. The observation that lysozyme was occasionally detectable in the nuclear fraction suggests that the association of some enzymes with some cell particulate fractions may be at least in part artifactual. Thus nonspecific binding of lysozyme, which is highly cationic, to nuclear fractions rich in anionic DNA might be expected. Lysozyme associated with the nuclear fraction was easily displaced by adding histones or sodium chloride to the nuclear pellet, thus indicating an easily exchangeable binding which could well have occurred during the fractionation procedure. Other types of affinities of cell fractions for specific enzymes may, in part, account for the nonprecise distribution of enzymes observed in cell fractionation procedures.

Aside from the amount present in body fluids, mammalian lysozymes studied thus far are intracellular and included in lysosomes or lysosome-like particles (6, 38–40). In cartilage, which is a particularly rich source (41), most of the lysozyme is extracellular (12). Disrupted chondrocytes from chick embryonic cartilage contain traces of lysozyme activity masked by other cell constituents. Such low activities cannot be accurately determined by the spectrophotometric assay (28). Electrophoretograms have previously been shown to be highly sensitive methods of demonstrating lysozyme activity in cartilaginous tissues (12, 34). The data clearly demonstrate that the lysosomal fraction of the isolated chondrocytes does not

contain lysozyme activity. The presence of small amounts of lysozyme in the microsomal fraction which was found in a few experiments may suggest that lysozyme is synthesized at this site, since biosynthesis of lysozyme by chick embryonic cartilage is known to occur (42). The fact that, in a tissue which actively synthesizes lysozyme, this protein is almost exclusively extracellular (12), indicates an extremely rapid secretion of lysozyme into the extracellular space. Lysozyme is not the only cartilage enzyme which has its greatest activity extracellularly. Alkaline phosphatase which, like lysozyme, is present in greatest amount near the cartilage-bone junction has also been shown to be concentrated in extracellular space (43). There is, however, no evidence that lysozyme is part of a cell-derived vesicle, as is alkaline phosphatase. The extracellular locus of these two substances particularly near the cartilage-bone junction suggests that they are involved in biochemical events occurring in cartilage matrix in these areas, included among which is calcification.

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