Sodium Chondroitin Sulfate-Protein Complexes of Cartilage

II. METABOLISM*

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It has been long established that cartilage contains acid mucopolysaccharides and protein (1, 2). Whereas the mucopolysaccharide of bovine hyaline cartilage has been identified as chondroitin sulfate A, there have been few investigations until recently concerning the nature of the protein. In 1954 Shatton and Schubert (3) isolated from bovine nasal septa a substance containing a noncollagenous protein associated with chondroitin sulfate, which has been variously designated as chondromucoprotein, mucoprotein, or protein-polysaccharide complex. In this communication the latter term, or simply complex, will be used.

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It has been demonstrated that after the administration of S35O4-, chondroitin sulfate is labeled in the sulfate ester group. The extent of uptake and the rate of disappearance of this label may be used to measure metabolic turnover. Schiller et al. (5) found that turnover rates utilizing C14 precursors were similar to those obtained with S35S and concluded therefrom that turnover studies with S35 measure the metabolism of the entire chondroitin sulfate molecule. On the basis of the disappearance of S35 from the complex, Böstrom (6) studied the turnover of the mucopolysaccharide moiety of the complex. The maximal uptake occurred 24 hours after administration of tracer and the radioactivity declined to about half the maximal value by the 17th day. No information regarding the protein moiety of the complex was obtained.

If the protein-polysaccharide complex is formed as a unit by the chondroblast, the protein and polysaccharide portions of the complex may be metabolized at the same rate. The rate of metabolism of two moieties can be studied simultaneously if each can be labeled specifically. The present study was undertaken to accomplish this end.

EXPERIMENTAL PROCEDURE

Materials—DL-Lysine-1-C14 was obtained from Volk Chemical Corporation. NaS35O4 was obtained from the Atomic Energy Commission. L-Lysine-HCl was obtained from Nutritional Biochemicals Corporation. 1,4-bis-(5-Phenylazo)benzene and aluminum stearate were obtained from Fisher Scientific Company. Male albino rats were obtained from Sprague-Dawley, Inc. The culture of Bacillus cadreus was a gift of Dr. M. Hanke, Department of Biochemistry, University of Chicago.

Methods—Glucuronic acid was determined by the method of Dische (7). Hexosamine was determined by a modification of the method of Boss (8). Nitrogen was determined by a micro-Kjeldahl method. Hydroxyproline was determined by the method of Martin and Axelrod (9). Amino acids were analyzed by paper chromatography. Amino sugars were identified by the method of Kirk and Dyrbe (11). L-Lysine-1-C14 was used to label specifically the protein. This precursor was chosen since lysine is an essential amino acid for the rat and therefore the administered compound is not diluted by endogenously synthesized amino acid. Utilization of the Gale and Epps (12) technique of specific decarboxylation permitted the recovery of the isotope from a protein hydrolysate at the micromolar level. For the metabolic experiments 5 groups of rats, with 10 animals in each group, were utilized. Age at the beginning of the experiment was 99 days; this age was selected in order to minimize growth changes during the experiment. Since no group of rats had an average weight change greater than 10% during the experiment, and the average weight at the outset was the same in all groups, no correction for weight was made in the metabolic data. A total of 20 μc of DL-lysine-1-C14 and 60 μc of NaS35O4 was injected subcutaneously into each animal in 3 divided doses at 4-hour intervals. The first group of animals was killed 24 hours after the initial injection and subsequent groups were killed 3, 5, 0, and 17 days after administration of isotope.

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Preparation of Protein-Mucopolysaccharide Complex—The rats were killed by ether anesthesia and decapitation. The entire chest cage was removed immediately and frozen with dry ice. All subsequent work was carried out at 2-3°C unless otherwise specified. The ribs were dissected, extraneous muscle and fibrous tissue were removed, and the perichondrium was stripped away from the costal cartilage. The cleaned cartilage was cut into pieces 2 to 5 mm long, and crushed by use of a Hughes block. The yield of wet cartilage varied from 2.9 to 3.4 g per group of rats. Extraction of the cartilage followed the procedure of Mathews and Lozaityte (4) with the exception that the initial water extraction of 48 hours was repeated; the extracts were combined and treated together. The yield of complex ranged from 80 to 95 mg.

Since previous studies had shown that further water extraction yields only small amounts of complex, the residue was extracted with 2% NaOH in order to obtain the remaining chondroitin sulfate. For this purpose 4.0 ml of 2% NaOH per g of cartilage were used. After centrifugation and neutralization, saturated (NH₄)₂SO₄ was added. The filtrate was dialyzed until the NH₄⁺ nitrogen was less than 0.02 mg per ml. Chondroitin sulfate was precipitated by the addition of sodium acetate and 95% ethanol. The use of alkali for extractions results in the isolation of chondroitin sulfate almost free of protein. Yields ranged from 40 to 55 mg for preparations which, upon analysis, showed an average molar ratio of hexosamine to 1.25 for nitrogen, 0.99 for uronic acid, and 0.97 for ester sulfate. Paper electrophoresis revealed only a single spot with the mobility of chondroitin sulfate. Chromatography demonstrated the presence of galactosamine and the absence of glucosamine.

Isolation of Radioactive Isotopes and Preparation of Samples for Counting—The protein-mucopolysaccharide complex was hydrolyzed with 6 N HCl for 14 hours in a sealed flask at 100°C, after 28.6 amoles of L-lysine-HCl were added as carrier. The samples were evaporated twice to dryness in a vacuum, clarified by centrifugation, and BaSO₄ precipitated by addition of excess BaCl₂. The clear supernatant was transferred to a Warburg vessel after the pH was adjusted to 6.6. The lysine decarboxylase, suspended in buffer, was placed in the side arm and tipped into the reaction chamber after CO₂ was removed by N₂. The enzymatic reaction was permitted to proceed at room temperature for 90 minutes. The evolved CO₂ was trapped in carbonatofree Ba(OH)₂. Ba₃CO₃ and BaSO₄ were washed in methanol, dried, weighed in counting vials, and suspended in a scintillating go according to the method of Funt and Hetherington (13). The samples were counted in the Packard Tri-Carb liquid scintillation counter. All sulfate-containing samples were counted on the same day so that no correction for isotope decay was needed.

RESULTS

Table I lists the analyses of a representative preparation of protein-mucopolysaccharide complex. The nitrogen analyses indicate a protein content of approximately 25% and a chondroitin sulfate content of approximately 75%. In other preparations the protein content varied from 20 to 25%. Appreciable amounts of collagen were absent since the hydroxyproline content of various preparations was 0.1% or less.

Amino acid analyses of the protein moiety are presented in Table II. The data indicate that the amino acid composition of rat costal cartilage is similar to that of bovine nasal septa complex (4).

Fig. 1 compares the infrared spectra of a known sample of chondroitin sulfate A (14) and the mucopolysaccharides isolated from the complex and from the alkali extract. No significant differences in the spectra are noted, establishing the polysaccharide as either chondroitin sulfate A or chondroitin sulfate B. The infrared spectrum of chondroitin sulfate C is different (14). The polysaccharides from rat cartilage are completely hydrolyzed by testicular hyaluronidase, identifying the polysaccharides as chondroitin sulfate A, since the testicular hyaluronidase does not hydrolyze chondroitin sulfate B. Since hyaluronidase does not hydrolyze keratosulfate, heparin, or the heparin sulfates, these compounds are likewise excluded.

A preliminary experiment was conducted. Since the results were identical with a second, more complete, experiment the presentation is limited to the data derived from the latter experiment.

Fig. 2, which is a semilog plot, compares the rate of disappearance of C¹⁴ with that of S³⁵. The ratio of specific activity at time t to that of time t₀ (t₀ = 1 day after administration of isotope, which is the time of maximal labeling) is plotted against time. There is no apparent difference between the rate of disappearance of C¹⁴ and of S³⁵. Since under these conditions C¹⁴ specifically labels the protein and S³⁵ specifically labels the polysaccharide, it is apparent that both moieties turn over at the same rate.

Additional information regarding the metabolism of the complex may be obtained by inspection of the shape of the curves...
Previous studies have indicated that the disappearance of labeled components from a metabolic pool usually follows first order kinetics, i.e. a plot of the log of radioactivity against time is linear. Such results were found in the studies of both chondroitin sulfate and hyaluronic acid in rat skin by Schiller et al. (5). Unlike those experiments, linearity is not observed in Fig. 2. The nonlinearity suggests the possible presence of more than one metabolic pool of complex in cartilage.

This question was approached in another manner by comparing the rate of disappearance of $^{35}$S from the chondroitin sulfate of the water-extracted complex with that obtained by alkali extraction of the residues (Fig. 3). It should be pointed out that alkali-extracted chondroitin sulfate is probably also protein bound in cartilage since recent work indicates that almost all chondroitin sulfate of cartilage is bound to protein (15). Of note is the finding that the maximal labeling at $t_0$ is lower for the alkali-extracted material, suggesting that the rate of synthesis of this fraction is less than that of the chondroitin sulfate of the water-extracted complex. Furthermore, the rate of disappearance of $^{35}$S from these two fractions is different. At $t_0$, the complex has a specific activity equal to twice that of the alkali extracted chondroitin sulfate, whereas at 16 days this ratio has dropped to approximately 1.3. These findings further substantiate the concept that the chondroitin sulfate of cartilage is not metabolically homogenous.

### DISCUSSION

The existence of a mucopolysaccharide-protein complex raises the question of the mechanism of biosynthesis. The data presented in this paper suggest that the complex is metabolized as a unit. The rate of turnover of any component is a function of both synthesis and breakdown. In the case of chondroitin sulfate of cartilage it seems evident that this compound is manufactured by the chondroblast (16). Since the protein portion turns over at the same rate as the polysaccharide, it seems most
likely that the entire complex is synthesized as a unit and extruded into the matrix.

The problem of mechanism of synthesis of complex proteins is of considerable biological interest. The question as to whether prosthetic groups are synthesized at the same rate as the protein portions of such molecules has not been adequately studied. In the case of synthesis of hemoglobin by rabbit reticulocytes Kruh and Borsook (17) found a close temporal parallelism of heme and globin synthesis. However, London et al. (18) found that such a parallelism does not occur in duck erythrocytes. Richmond et al. (19) reported that irradiation in rabbits results in a greater inhibition of heme synthesis than of globin synthesis.

These considerations are important with respect to the biosynthesis of chondroitin sulfate. Although several studies have indicated that a soluble enzyme which brings about sulfation may be obtained from epiphyses, net synthesis of chondroitin sulfate has not yet been demonstrated. The finding by Salmon and Daughaday (20) that amino acids stimulate sulfate uptake in costal cartilage may indicate that the chondroitin sulfate synthesis requires concomitant synthesis of the protein moiety.

The mechanism of breakdown of matrix is unknown. It seems unlikely that the complex returns to the cells and is degraded by a reversal of the synthetic process. Thus far, enzymes degrading chondroitin sulfate have not been found in cartilage. A possible mechanism of degradation of chondroitin sulfate in tissue is afforded by the observation of French and Benditt (21), showing that chondroitin sulfate may be released from cartilage by the action of proteolytic enzymes. Lack and Rogers (22) have found that plasmin has a similar effect. That this reaction may occur in vivo is suggested by the observation of Thomas (23) that intravenous administration of papain is followed in the rabbit by reversible collapse of cartilage structures. The increase of urinary excretion of inorganic acid concomitant with a loss of metachromasia in the cartilage suggests that chondroitin sulfate may be released from the matrix.

The metabolism of a mucopolysaccharide-protein complex from rat costal cartilage has been studied in vivo. Radioactive tracers were injected into several groups of rats which were killed at various time intervals. Mucopolysaccharide was labeled with $^{35}$S whereas simultaneously the protein moiety was labeled with $^1$H. Recovery of the tracers from cartilage was accomplished by water extraction to obtain the complex, followed by an alkali extraction to yield residual mucopolysaccharide. The results of the radioactive experiments indicate there are no differences in the rates of turnover of mucopolysaccharide or protein moiety, i.e., the complex is metabolized as a unit. Since the log rates of turnover are not linear with time, it appears that the metabolic pool of complex is not homogeneous.

This concept is substantiated when the turnover of mucopolysaccharide from the complex is compared with the turnover of alkali-extracted mucopolysaccharide. Possible mechanisms of synthesis and breakdown of cartilage matrix are discussed.

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