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METABOLISM *IN VIVO* OF CONNECTIVE-TISSUE MUCOPOLYSACCHARIDES

III. CHONDROITIN SULFATE AND KERATOSULFATE OF CARTILAGE

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SUMMARY

The incorporation of glucose into the hexosamine moieties of cartilage mucopolysaccharides has been studied. Isotope appears in the galactosamine moiety of the light mucoprotein fraction first and rapidly disappears from this fraction with an accompanying appearance of isotope in the insoluble residue. Chondroitin sulfate accounts for about 80% of the total polysaccharide and appears to be relatively inert metabolically. Appearance of label in chondroitin sulfate prior to keratosulfate suggests that these polysaccharides do not represent part of the same macromolecular structure. This latter point is not established. The effect of several hormones on the incorporation and turnover of isotope in this tissue was also reported.

INTRODUCTION

Cartilage has been one of the most extensively studied of all connective tissues. It has been used as a source of chondroitin sulfate since the 19th century and although the composition of the polysaccharide fraction was originally thought to be exclusively chondroitin 4-sulfate, keratosulfate and chondroitin 6-sulfate have also been detected[1]. Studies in several laboratories on human material have revealed age-dependent composition changes[2–5]. In particular, there is a continuous increase in the keratosulfate content through the second decade in life and the chondroitin 4-sulfate is progressively replaced by chondroitin 6-sulfate. Qualitative studies suggest that keratosulfate also increases in shark and squid cartilage[6].

Cartilage has been used in studies of radio-sulfate fixation and the effect of hormones on such processes has been reported[7–16].

The physical state of the heteropolysaccharides in this tissue has been the subject of considerable investigation. The chondroitin sulfate may be extracted from cartilage in the form of a protein complex whose properties suggest something more than simple electrostatic attraction[11–14]. The homogeneity of this complex has not been

established but isotope data suggest concomitant formation of at least part of the protein moiety and the polysaccharide chain.

The present study reports the composition of protein complexes isolated from rabbit rib cartilage, the incorporation of carbohydrate precursors into the hexosamine components of the polysaccharides and the effect of various hormonal agents on this process. Preliminary reports have been presented[13,36].

The general experimental design, age distribution of the animals, analytical and counting techniques have been previously described[17]. Information relative to specific experiments is described in the pertinent figure legend.

Cartilage mucoprotein was prepared from rabbit rib cartilage by the method of SCHUBERT *et al.*[18]. Material insoluble in water was extracted by shaking with 10 vol. of 2 % KOH in the cold for 24 h. The KOH extract was repeated twice and after neutralization the soluble portion was dialyzed to remove excess salt. Hexosamines were liberated from polymeric material by hydrolysis in a sealed vessel with 4 N HCl for 16 h. After removal of the acid, the hydrolysate was dissolved in water and the glucosamine and galactosamine resolved by chromatography on Dowex-50, H^+ resin[19]. Quantitation of hexosamine content and radioactivity was carried out as previously described[17]. Neutral sugars were estimated after hydrolysis for 4 h with 1 N H_2SO_4. The hydrolysate was neutralized with $Ba(OH)_2$ and further deionized by treatment with Dowex-50, H^+ and Dowex-1, HCO_3^- resins. After concentration, suitable aliquots were chromatographed in the following solvents: butanol–pyridine–water (6:4:3), ethyl acetate–pyridine–water (2:1:2) upper phase, butanol–acetic acid–water (50:15:35), isopropanol–water, (9:1). Reducing compounds were visualized with a silver nitrate reagent[20].

RESULTS AND DISCUSSION

The composition of the heavy and light mucoprotein fractions and the residue is shown in Table I. In addition to the hexosamine components, both galactose and fucose were demonstrable in all fractions by paper chromatography as described above. Quantitation was not attempted.

TABLE I

HEXOSAMINE CONTENT OF RABBIT RIB-CARTILAGE MUCOPROTEIN AND RESIDUE FRACTIONS

Values are expressed as μmoles/g dry wt. Figures in parentheses are per cent of total glucosamine or galactosamine. Each figure is the average of at least three animals, 15 months of age.

	Control		*Cortisone*		*Growth hormone*	
	Glucos-amine	*Galactos-amine*	*Glucos-amine*	*Galactos-amine*	*Glucos-amine*	*Galactos-amine*
Light mucoprotein fraction	1.4 (6)	12.6 (11)	1.9 (7)	22.4 (14)	6.6* (5)	12.0 (8)
Heavy mucoprotein fraction	0.6 (2.5)	1.9 (2)	0.8 (3)	4.9 (3.9)	6.6 (5)	5.6 (4)
Residue	24.5 (92)	117 (88)	26 (90)	138 (83)	31** (90)	149 (88)

* Decreases with time.
** Increases with time.

The appearance of radioactivity in the galactosamine component of the mucoprotein and residue fractions is illustrated in Fig. 1. The effect of various hormones on the rate of appearance of radioactivity from glucose in the galactosamine and glucosamine components of the light mucoprotein fraction is illustrated in Figs. 3 and 4. The different rates of incorporation of glucose into glucosamine and galactosamine respectively of the light mucoprotein of control animals is illustrated in Fig. 2.

The occurrence of glucosamine and galactosamine in both mucoprotein fractions as well as the residue confirms results reported by GREGORY AND RODEN[21] and by PARTRIDGE and coworkers[13]. Although these fractions are qualitatively homogeneous, satisfactory evidence has not been presented to establish this point.

Studies in cell-free systems have shown that uridine diphospho-*N*-acetyl glucosamine serves as the immediate precursor of the corresponding galactosamine nucleotide[22,23]. The appearance of the glucose carbon chain in galactosamine prior to glucosamine in this system suggests that there is not a linked synthesis of the keratosulfate

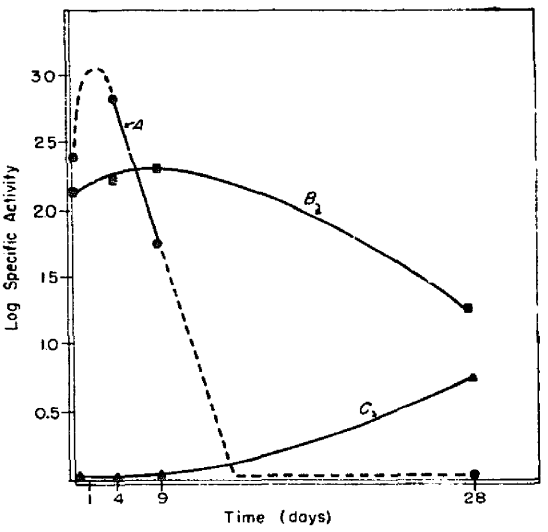

Fig. 1

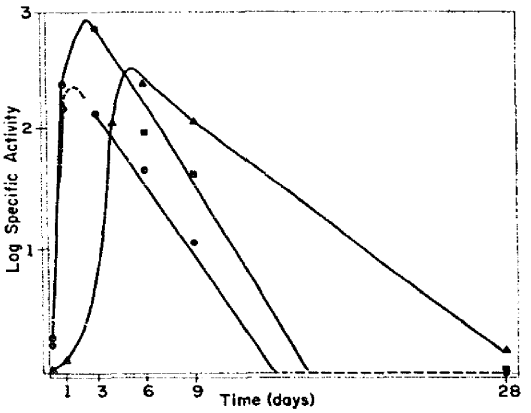

Fig. 3

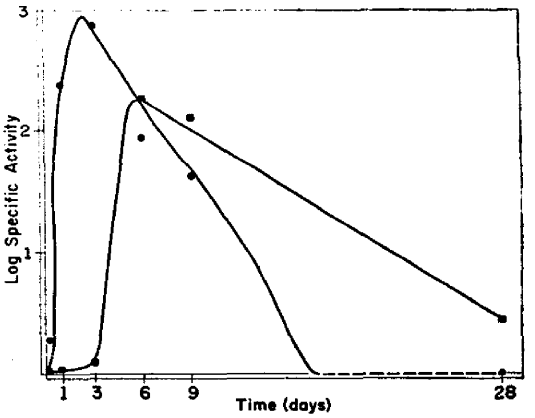

Fig. 2

Fig. 1. Appearance of radioactivity in the galactosamine moiety of rabbit mucoprotein and residue fractions following an injection of uniformly labeled [^{14}C]glucose[17]. Each point is the average of at least two animals, 15 months of age. A, ●, light mucoprotein fraction; B, ■, heavy mucoprotein fraction; C, ▲, redidue.

Fig. 2. Appearance of radioactivity in galactosamine and glucosamine of the light mucoprotein fraction. Each point is the average of at least two animals, 15 months of age. ●—●, galactosamine, light mucoprotein fraction; ■—■, glucosamine, light mucoprotein fraction.

Fig. 3. Effect of growth hormone and cortisone on appearance of radioactivity in the galactosamine moiety of the light mucoprotein fraction. Isotope and hormone administered as previously described[17]. Each point is the average of at least two animals, 15 months of age. ●—●, growth hormone; ■—■, control; ▲—▲, cortisone.

and chondroitin sulfate moieties present in the light mucoprotein fraction. It is possible that, as with certain bacterial lipopolysaccharides, the acceptor site for the keratosulfate must be present before its synthesis takes place[24]. Thus, one may envision that the chondroitin sulfate forms the immediate covalent linkage to protein and until this is synthesized, no appreciable synthesis of keratosulfate may take place. The active metabolic behavior of this fraction coupled with the considerable delay in appearance of isotope in keratosulfate makes this possibility extremely far fetched.

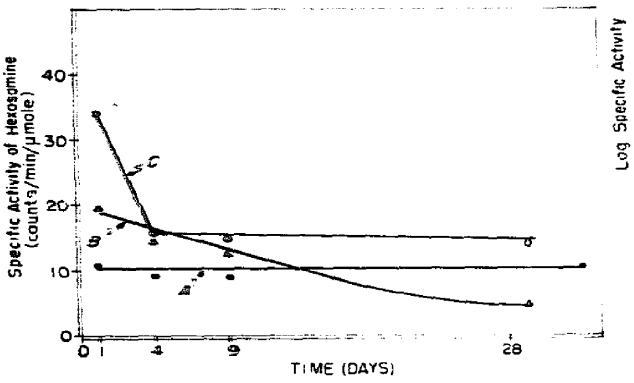

Fig. 4. Effect of growth hormone and TSH on appearance of radioactivity in cartilage galactosamine following a single injection of uniformly labeled ^{14}C glucose. Hexosamine isolated after direct alkali extraction of tissue Note how mixing "pools" distorts data. Each point average of four animals, 15 months of age. A, control group; B, TSH-treated group; C, growth hormone-treated group.

The slow appearance of isotope in the residue fraction and the low specific activities make an accurate determination of polysaccharide "half-life" difficult. Preliminary experiments have been carried out and suggest a turnover time for this fraction in excess of 4 months. In this tissue, as in skin, the polysaccharide "pool" is not homogeneous and apparently undergoes some change either in physical state or linkage to protein following synthesis from simpler precursors. It would appear that the light mucoprotein fraction, operationally defined as being more readily extractable, represents the first appearance of newly synthesized polysaccharide. Since the protein moiety of the mucoprotein complex does not yield a homogeneous protein fraction after removal of the majority of the polysaccharide by exhaustive hyaluronidase digestion, it is extremely difficult to assign any coherent structure to the mucoprotein itself.

The effects of cortisone are in keeping with previous reports of the ability of this hormone to inhibit sulfate fixation and also in agreement with the general effect of cortisone on other heteropolysaccharide systems[25,26]. It would appear that the observed phenomena are not due to a direct influence on sulfate fixation or chain synthesis but rather to an influence on the availability of simple precursors for the formation of the polysaccharide chain; this may be related to capillary permeability, diffusion of nutrients to the cells responsible for synthesis, etc.

The failure of the SCHUBERT technique to solubilize more than 20 % of the total mucopolysaccharide hexosamine present in cartilage is somewhat discrepant from reported results on bovine nasal septa[27]. The above data are for rabbit rib cartilage of the specified age and generalizations to other animals are not valid. The relatively constant composition of the soluble *versus* the insoluble portions is somewhat affected by growth hormone and by the age of the animal. However, sufficient studies have not been carried out to define any consistent trend.

The phenomenon in human cartilage of the replacement of chondroitin 4-sulfate by chondroitin 6-sulfate with the passage of time is of considerable interest. This conversion involves nothing more complex than a transfer of sulfate from the 4 position of the galactosamine moiety to the 6 position. This may require breakdown and resynthesis of the polysaccharide chain, *de novo* synthesis of the chondroitin sulfate C from glucose, or a simple sulfo transferase mechanism. Since similar phenomena have not been established for other animals, it is quite impossible to test these hypotheses experimentally.

Considerable evidence has been adduced to strengthen the original proposal by DAVIDSON AND MEYER[28] that heteropolysaccharide sulfation takes place subsequent to chain synthesis[29–31]. Nevertheless, definitive evidence at this point is missing. It is especially to be noted that all of the sulfo transferase data reported leave 90 % or more of the theoretical hydroxyl acceptors unesterified. In this instance, although the sulfate group qualitatively mirrors the metabolic behavior of the hexose carbons, quantitative studies have not been carried out. The turnover of the sulfate group in this tissue will be the subject of a subsequent report.

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REFERENCES

1 K. MEYER, P. HOFFMAN AND A. LINKER, *Science*, 128 (1958) 896.
2 D. KAPLAN AND K. MEYER, *Nature*, 183 (1959) 1267.
3 R. KUHN AND H. J. LEPPELMAN, *Ann. Chem.*, 611 (1958) 254.
4 M. R. SHETLAR AND Y. F. MASTERS, *Proc. Soc. Exptl. Biol. Med.*, 90 (1955) 31.
5 J. W. LASH AND M. W. WHITEHOUSE, *Biochem. J.*, 74 (1960) 351.
6 J. W. LASH AND M. W. WHITEHOUSE, *Arch. Biochem. Biophys.*, 90 (1960) 159.
7 D. W. DZIEWIATKOWSKI, *J. Biol. Chem.*, 189 (1951) 187.
8 H. BOSTROM AND B. MANSSON, *Arkiv Kemi*, 6 (1953) 23.
9 H. BOSTROM AND E. ODEBLAD, *Arkiv Kemi*, 6 (1953) 39.
10 L. L. LAYTON, *Proc. Soc. Exptl. Biol. Med.*, 76 (1951) 596.
11 J. SHATTON AND M. SCHUBERT, *J. Biol. Chem.*, 211 (1954) 565.
12 H. MUIR, *Biochem. J.*, 69 (1958) 195.
13 S. M. PARTRIDGE, H. F. DAVIS AND G. ADAIR, *Biochem. J.*, 79 (1961) 15
14 G. BERNADI, *Biochim. Biophys. Acta*, 26 (1957) 47.
15 E. A. DAVIDSON, W. SMALL, P. PERCHEMLIDES AND W. BAXLEY, *Biochim. Biophys. Acta*, 46 (1961) 189.
16 E. A. DAVIDSON, W. SMALL AND P. PERCHEMLIDES, *Federation Proc.*, 20 (1961) 162.
17 E. A. DAVIDSON AND W. SMALL, *Biochim. Biophys. Acta*, 69 (1963) 445.
18 B. R. GERBER, E. C. FRANKLIN AND M. SCHUBERT, *J. Biol. Chem.*, 235 (1960) 2870.
19 S. GARDELL, *Acta Chem. Scand.*, 7 (1953) 207.
20 W. TREVELYAN AND S. HARRISON, *Biochem. J.*, 50 (1952) 298.
21 J. GREGORY AND L. RODEN, *Biochem. Biophys. Res. Commun.*, 5 (1961) 430.
22 L. GLASER, *J. Biol. Chem.*, 234 (1959) 2801.
23 B. JACOBSON AND E. A. DAVIDSON, *Biochim. Biophys. Acta*, in the press.
24 H. NIKAIDO, *Biochim. Biophys. Acta*, 48 (1961) 460.
25 S. SCHILLER AND A. DORFMAN, *Endrocrinology*, 60 (1957) 376.
26 E. A. DAVIDSON AND W. SMALL, *Biochim. Biophys. Acta*, 69 (1963) 453.
27 I. MALAWISTA AND M. SCHUBERT, *J. Biol. Chem.*, 230 (1958) 535.
28 E. A. DAVIDSON AND K. MEYER, *J. Biol. Chem.*, 211 (1954) 605.
29 J. B. ADAMS, *Nature*, 186 (1960) 555.
30 E. A. DAVIDSON AND J. G. RILEY, *J. Biol. Chem.*, 235 (1960) 3367.
31 S. SUZUKI AND J. L. STROMINGER, *J. Biol. Chem.*, 235 (1960) 267.