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Desulfation and Depolymerization of Chondroitin 4-Sulfate and Its Degradation Products by Rat Stomach, Liver and Small Intestine[1] (38242)

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Desulfation of chondroitin sulfates in mammals has received increasing attention as evidenced by reports of chondrosulfatases in liver (1, 2), stomach (3, 4), arterial wall (5), cartilage (6) and kidney (7).

Substrate specificities of various chondrosulfatases appear to differ since Held and Buddeke (5) and Suzuki *et al.* (8) observed preferential desulfation of the intact polymer, whereas Tudball and Davidson (1) found desulfation only of high molecular weight oligosaccharides. We found that stomach tissue exhibited not only sulfatase activity but also depolymerase activity resulting in the liberation of inorganic sulfate and organic sulfates (3, 4). We believed it to be of interest to determine the substrate specificities of various alimentary tract tissues and to determine whether the tissues differ in pattern of cleavage, i.e., desulfation of polymer versus depolymerization of sulfated polymer.

We report here the relative desulfation of chondroitin 4-sulfate and of its low mol wt degradation products by rat stomach, liver and small intestine. We also provide here information which bears on the question: "Does desulfation precede depolymerization of chondroitin sulfate?" Sulfatase activity in tissue of young and old rats and in other species also is reported.

Materials and Methods. Preparation of substrate. (a) *^{35}S-Chondroitin sulfate.* ^{35}S-Labeled chondroitin sulfate was prepared from rib cartilage as described previously (4). ^{14}C-Labeled chondroitin sulfate was obtained in the same way by incubation with ^{14}C-glucose (0.8 mCi uniformly labeled ^{14}C-glucose [New England Nuclear, Boston, Mass.] in 30 ml of incubation mixture) instead of $Na_2{}^{35}SO_4$. The sp act for the ^{35}S-chondroitin sulfate was 4.2×10^5 cpm/μmole of disaccharide and for the ^{14}C-labeled chondroitin sulfate was 2.9×10^4 cpm/μmole of disaccharide. When they were digested with chondroitinase ABC (Miles Laboratories, Kankakee, IL) (9), the former gave 84% converted into ΔDi-4S, 8% into ΔDi-6S[2] and 8% as unidentified product, the latter gave 81%, 7%, and 12% for each component. (b) *Degradation products from gastric-enzyme digestion.* ^{35}S-Labeled chondroitin sulfate (50 μl, 0.36 μM as ΔDi-4S) and gastric extract (50 μl containing 1 mg of lyophilized stomach extract powder) were placed in a dialysis bag with a nominal 12,000–14,000 mol wt cutoff (Spectrapor TM2, National Scientific Co., Cleveland, OH), immersed in 25 ml of 0.1 N acetate buffer, pH 4.0, and the mixture was incubated at 37° overnight. More than 90% of the radioactive materials was rendered dialyzable. This procedure was performed with five batches,

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[2] The abbreviations ΔDi-4S and ΔDi-6S are for 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose and 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose, respectively.

and the dialyzates were pooled and concentrated to less than 5 ml in a flash evaporator. Differential dialysis was performed on this concentrate with the Spectrapor TM3 (nominal 3,500 mol wt cutoff) membranes[3] at 2° to afford fractions of nominal mole wt >12,000, 3,500–12,000 and <3,500.

Degradation products obtained by digestion with hyaluronidase. ^{35}S-Chondroitin sulfate (7.2 μmoles in 1 ml of distilled water) was mixed with acetate buffer (1 ml of 0.1 *N*, pH 5.0) and digested at 37° with 50 units of testicular hyaluronidase (Worthington Biochemical Corp., Freehold, NJ) in the presence of toluene. The mixture was incubated for 10 days, and fresh enzyme and toluene were added daily. The incubation mixture was subjected to differential dialysis as described earlier. The fractions of nominal mol wt <3,500 and 3,500–12,000 were used as substrates.

Preparation of tissue extracts (sources of crude enzymes). Tissues were removed from 1-mo and 4-mo old male, Sprague–Dawley rats, sacrificed by ether anesthesia. Mucosa was extracted as described previously (4), except that liver was cut and minced prior to extraction. Hog and dog stomachs were purchased from Pel-Freeze Biologicals, Rogers, AR. Insoluble residues were subjected to 5 cycles of homogenization, freezing and thawing. Supernates were combined and the supernates and residues were dialyzed against several changes of 100 volumes of distilled H_2O for 2 days and then were lyophilized. Protein content was determined colorimetrically (10).

Enzyme assays. Assays were performed by incubation of substrate, and appropriate enzyme source, toluene and antibiotics (dissolved in 0.05 *N* acetate buffer, pH 4.0) in a small tube at 37°. Enzyme action was stopped by boiling for 1 min. Radioelectrophoresis was used for the estimation of sulfatase activity. The incubation mixture was placed on a strip of Whatman 3MM paper, and electrophoresis was performed using 0.3 molar pyridinium-formate, pH 3, at 15mA per cell for 3 hr. The strips were dried at 85° and then were cut into 1-cm wide segments. The segments were each dipped into glass vials containing a scintillation mixture (0.5% of 2,5-diphenyloxazole (PPO) and 0.03% of *p*-bis-[2-(5-phenyloxazolyl)] benzene (POPOP) in toluene), and counted in an Isocap 300 liquid scintillation spectrometer (Nuclear Chicago).

Depolymerase activity was estimated by analysis of radioelectrophoregrams. Incubation mixtures were dialyzed thru a membrane of 12,000–14,000 mol wt cutoff against 100 ml of dist. H_2O for 2 days. The dialyzates were concentrated in a flash evaporator and were electrophoresed. The dialyzable radioactivity which was distributed in the 0–5 cm range (representing depolymerized products) was counted, and the amount of dialyzable organic sulfate was expressed in terms of μmoles sulfate as calculated from the sp act of sulfate in the substrate employed.

Results. Age comparison. Tube incubation assays under conditions of substrate excess were performed to determine the liberation of inorganic sulfate from chondroitin 4-sulfate by extracts from the glandular stomach, liver and small intestine of 1-mo and 4-mo old rats. Assays also were performed with suspensions of the insoluble residues (as enzyme source) remaining after repeated freezing–thawing and homogenization of the tissues. The results of these determinations are given in Table I. Addition of Triton X-100 (5%) to the sediments did not alter the amount of sulfate cleaved. The ratios of lyophilized wt of extracts to insoluble residues were: stomach, 0.14; liver, 0.17 and small intestine, 0.18.

Extracts (supernate fraction) of mucosal scrapings from the whole stomach of dog (age unknown, stomach wet wt 60 and 120 g, respectively) and of hog (adult, stomach wet wt 550 g) were assayed for liberation of sulfate from chondroitin 4-sulfate. The dog stomach extracts cleaved 0.3×10^{-2} μmoles $SO_4^=$/mg protein/hr ±

[3] When tested by us, Spectrapor TM2 retained 95% and 96%, respectively, of aqueous 0.1% solutions of ribonuclease D and cytochrome C (Gallard–Schlesinger, New York, NY) and Spectrapor TM3 95% and 12% of aqueous 10% solutions of polyethylene glycol (Gallard–Schlesinger, New York, NY) of mol wt range 3,300–4,000 and 1,430–1,570, respectively.

TABLE I. Liberation of Sulfate[a] from Chondroitin 4-sulfate by Extracts[b] of Rat Stomach, Liver and Intestine.[c]

	Stomach		Liver		Small intestine	
Age of rat	Supernate	Sediment	Supernate	Sediment	Supernate	Sediment
1 mo	9.6	0.20	9.0	0.22	9.4	0.36
4 mo	0.24	0.21	0.36	0.11	0.22	0.14

[a] Expressed as 10^{-2} μmoles sulfate/mg protein/hr.

[b] Extracts and sediments were obtained from tissues pooled from 10 rats.

[c] Soluble or insoluble residue (5 μg of supernate from tissue of 1-mo old rats or 200 μg of all other preparations) was incubated in a small tube for 1 hr at 37° with ^{35}S-chondroitin 4-sulfate (0.072 μmoles, 2×10^4 cpm) in 0.05 N sodium acetate buffer, pH 4.0 in a final volume of 25 μl. Triton X-100 (5%) was added to the suspension of the insoluble residue. After incubation, the mixture was analyzed by radioelectrophoresis.

5% and the hog cleaved 0.11×10^{-2} μmoles $SO_4^=$/mg protein/hr.

Desulfation of chondroitin 4-sulfate and its degradation products. ^{35}S-Chondroitin 4-sulfate, high (nominal mol wt > 12,000) and low (nominal mol wt 3,500–12,000) mol wt sulfated polysaccharides obtained by selective dialysis of the products of reaction of stomach enzymes with chondroitin 4-sulfate, sulfated polysaccharides (nominal mol wt 3,500–12,000 and <3,500) obtained by selective dialysis of the products of hydrolysis of chondroitin 4-sulfate by testicular hyaluronidase and the unsaturated disaccharide 4-sulfate (ΔDi-4S)[2] obtained by the action of chondroitinase ABC on chondroitin 4-sulfate were used as substrates for the tube assay of extracts of rat (1-mo old) stomach, liver and small intestine (first 15 cm). The results of these assays are given in Table II. In contrast to assays of chondroitin 4-sulfate, assays of the various degradation products with 5 μg of crude enzyme and 1 hr incubation gave counts only slightly higher than background. Accordingly, 200 μg of crude enzyme and 4 hr incubation were employed for the degradation products (still under conditions of substrate excess) to afford accumulation of a sufficient number of counts approx. 6,000.

Comparison of various regions of stomach and small intestine. Extracts of mucosa from the forestomach, body plus antrum of stomach and small intestine (0–15, 15–45

TABLE II. Desulfation[a] of Degradation Products of Chondroitin 4-sulfate by Extracts[b] of Rat Stomach, Liver, and Small Intestine.[c]

Substrate	Stomach	Liver	Small intestine
Chondroitin 4-sulfate	9.1	9.6	10.0
Fraction > 12,000	0.12	0.29	0.024
Fraction 3,500–12,000	0.07	0.29	0.096
H'ase[d] fraction 3,500–12,000	0.03	0.08	0.018
H'ase fraction < 3,500	0.014	0.02	0
ΔDi-4S	<0.004	0	0

[a] Expressed as 10^{-2} μmoles $SO_4^=$/mg protein/hr.

[b] Extracts from scrapings from a pool of 100 1-mo old rats.

[c] Extracts (5 μg for chondroitin 4-sulfate and 200 μg for others) were incubated under conditions of substrate excess with the various substrates (at same specific activity) and 0.05 N sodium acetate buffer, pH 4.0, in a final volume of 25 μl containing toluene and antibiotics after incubation for 1 hr for chondroitin 4-sulfate, or 4 hr (for others). The mixture was analyzed for liberation of sulfate.

[d] H'ase = hyaluronidase.

and 45–100 cm) of 20 1-mo old rats were assayed for sulfatase activity against chondroitin 4-sulfate. After 48 hr incubation at 37°, the following order of activity was found in terms of percent of substrate sulfate liberated as inorganic sulfate: small intestine (15–45 cm) 23% > body stomach 18% > forestomach 12% > small intestine (45–100 cm) 9% > small intestine (0–15 cm) 7%.

Chondroitin sulfates in liver, stomach, and small intestine. Extracts from mucosal scrapings or minced liver obtained from 1-mo old rats were assayed for content of chondroitin sulfates by a method utilizing chondroitinase ABC (9). These results were sought to provide information on cold substrate already present in the tissue extracts. Chondroitin sulfates constituted for liver 3.8%, for stomach 3.8%, and for small intestine (0–15 cm) 0.9% of the wt of dialyzed, lyophilized soluble extract. The ratio of chondroitin sulfate A + B to C was 6 to 1 for liver, 2.2 to 1 for stomach and 3.6 to 1 for intestine.

Tissue and extract wt. The wet wt of liver, stomach and small intestine from a pool of the respective tissues from 20 1-mo old rats and the wt of lyophilized powder of the soluble extracts from these tissues was determined.

The wet wt and lyophilized wt (in parenthesis) were: liver, 130 g (1.8 g); stomach, 34 g (110 mg); small intestine, 0–15 cm, 40 g (140 mg), 15–45 cm, 53 g (250 mg); and 45–100 cm, 72 g (400 mg). The protein content of the lyophilized powder of liver extract was 76%, stomach 72% and small intestine 68%.

Time course of desulfation and depolymerization. Extracts of gastric mucosa were incubated with chondroitin 4-sulfate and at various times the reaction was stopped by heating, and inorganic sulfate and organic sulfate were determined. The results of this kinetic study are shown in Fig. 1. During the early stage of incubation, $^{35}SO_4^=$ released exceeded organic sulfate released; this was reversed at 2–4 hr of incubation, and at 17 hr (not shown in Fig. 1) 21% of substrate ^{35}S was released as organic sulfate and 13% as $^{35}SO_4^=$. Extracts of small intestine gave a similar kinetic pattern. Liver

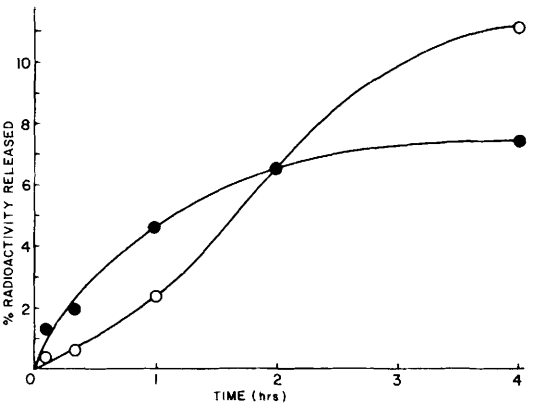

FIG. 1. Liberation of inorganic ●—●—● and dialyzable organic sulfate ○—○—○ from chondroitin 4-sulfate by extract of rat gastric mucosa. Conditions as stated for Table I.

extract, however, gave a different pattern in that liberation of inorganic sulfate exceeded liberation of organic sulfate throughout the course of the experiment, as shown in Fig. 2. Paper electrophoretic analysis was performed of the reaction mixtures of chondroitin 4-sulfate and the three tissue extracts. A large fraction (a maximum of up to 72%) of the radioactivity remained at the origin during the early stages of incubation in the case of liver extracts, but much smaller amounts of radioactive material, 4 and 7%, respectively, remained at the origin for stomach and small intestine extract reaction mixtures. The fraction of radioactivity found at the origin diminished with time. The course of disappearance of the fraction found at the origin and the liberation of inorganic sulfate following the incubation of chondroitin 4-sulfate with liver extract is shown in Fig. 3. Fresh chondroitin sulfate was added to the incubation mixture after 48 hr to determine if the constituents in the liver extract retained the capacity after prolonged incubation to complex with chondroitin sulfate. The same pattern as shown in Fig. 3 was obtained with chondroitin 4-sulfate added to the 48 hr aged liver extract.

After incubation of chondroitin sulfate with the gastric enzyme preparation, the fractions of mol wt ≥ 12,000 and 3,500–12,000 were tested with chondroitinase ABC. Fifty-four percent of the higher

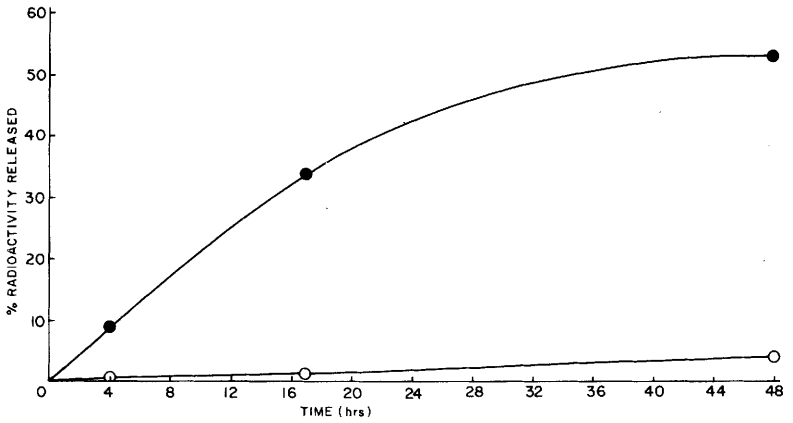

FIG. 2. Liberation of inorganic ●—●—● and dialyzable organic sulfate ○—○—○ from chondroitin 4-sulfate by rat liver extract. Conditions as stated for Table I.

molecular weight fraction and 32% of the lower molecular weight fraction were liberated as ΔDi-4S.

The dialyzable products of 17 hr incubation of ^{14}C- or ^{35}S-chondroitin sulfate with gastric enzymes were examined by radioelectrophoresis (0.1 *N* HCl, 50 mA, 6 hr) to learn whether extensive release of grossly undersulfated or nonsulfated, depolymerized products occurred. No increase in radioactivity was found at or near the origin (region expected for under- or nonsulfated products) with the ^{35}S-substrate and only a 2.2% increase was observed with the ^{14}C-substrate as compared to the control ^{14}C-substrate. Similarly, only a 4.3% increase in radioactivity near the origin was found liberated from the ^{14}C-substrate after incubation with the liver enzyme. This indicates that only a very minor amount of un-

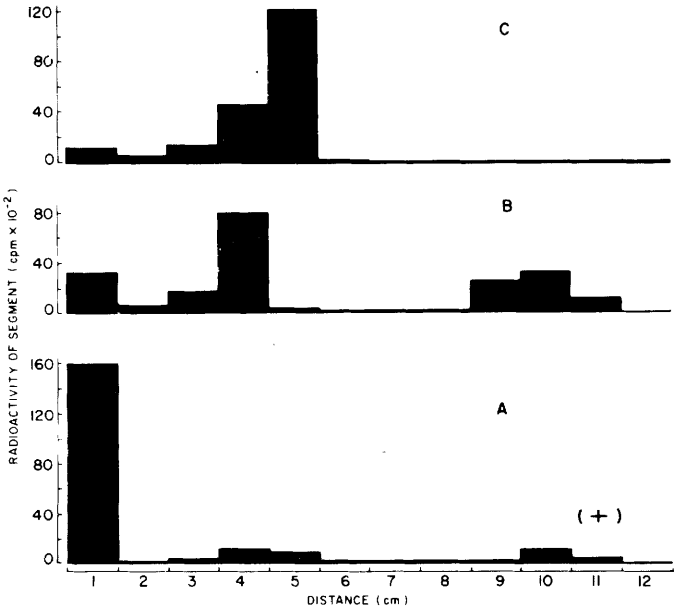

FIG. 3. Paper radioelectrophoresis of: (A) ^{35}S-Chondroitin 4-sulfate incubated with liver extract at pH 4.0 for 1 hr. (B) Above mixture, after 17 hr incubation. (C) Chondroitin 4-sulfate alone. Inorganic sulfate is at 9–11 cm, chondroitin 4-sulfate at 4–5 cm, partially degraded chondroitin 4-sulfate at 3–5 cm and binder-chondroitin 4-sulfate complex at 0–1 cm.

sulfated, or undersulfated, depolymerized product was liberated.

Inhibition by sulfate ion. Sodium sulfate was added to the reaction mixture containing chondroitin 4-sulfate, tissue extract, preservative and acetate buffer, and the amounts of inorganic and organic sulfate liberated were compared to the amounts liberated in the usual incubation mixture, i.e., devoid of added sulfate. The results are given in Table III.

Discussion. Chondrosulfatases were found in stomach, liver and small intestine. The activity of these enzymes were much greater in tissue of younger than of older rats. Interestingly, the younger rats had most of the sulfatase activity in the readily solubilized form, but the older rats had the activity equally distributed between the soluble and insoluble forms.

Chondrosulfatases are apparently distributed in a wide variety of species (8). Chondrosulfatase activity is reported here for the rat liver, forestomach, body and antrum of stomach, and small intestine. The wide distribution of the enzyme regardless of the anatomy and function of the organ in which it is found may be a consequence of its possible lysosomal origin. The lysosomal origin of chondrosulfatases in liver has already been demonstrated (1, 2).

Stomach and small intestine liberate considerable amounts of organic sulfate in addition to inorganic sulfate from chondroitin 4-sulfate. It is of interest to inquire whether desulfation requires prior depolymerization of the substrate or does depolymerization require prior desulfation? Ideally, purified chondroitin 4-sulfatase from these sources would facilitate answering these questions, but the enzyme has not yet been sufficiently purified for such studies. The following information was obtained, however, which suggests that it is likely that desulfation precedes depolymerization. Chondroitin 4-sulfate was preferentially desulfated as compared to the lower mol wt sulfated polysaccharides or ΔDi-4S derived from it. Moreover, studies of the time course of sulfation and depolymerization indicated that the rate of desulfation by gastric and intestinal enzymes was higher at the outset and then subsided, whereas the rate of depolymerization was low initially, and increased with time. It also was observed that sulfate ion not only inhibited desulfation but also inhibited depolymerization. These observations not only suggest that desulfation may precede depolymerization, but they (inhibition data) also suggest that a certain amount of desulfation may have to occur to facilitate depolymerization.

TABLE III. Effect of Added Sulfate on Liberation of Inorganic and Organic Sulfate from Chondroitin 4-sulfate by Extracts of Gastric Mucosa.[a]

	Percent of substrate $^{35}SO_4^=$ liberated as	
	Inorganic $^{35}SO_4^=$	Organic $^{35}SO_4^=$
Without addition of sulfate	17	29
Addition of sulfate	1.0	2.6

[a] Incubation mixture: 10 μl of substrate (0.072 μmoles ^{35}S-chondroitin 4-sulfate containing 40,000 cpm), 10 μl gastric mucosa extract (200 μg), 5 μl antibiotic mixture plus toluene; 0.05 *N* sodium acetate buffer, pH 4.0 was the solvent. Five microliters of distilled H_2O and 5 μl of 0.04 *N* Na_2SO_4 were added to control and experimental tubes, respectively. Tubes were incubated at 37° for 2 days.

The results presented here do not exclude the possibility that high molecular weight (>12,000) polysaccharides formed by very limited depolymerization may be readily desulfated. Unfortunately, such compounds were not available to us for trial. Tudball and Davidson (1) observed that a liver sulfatase acted preferentially on an oligosaccharide believed to be an octasaccharide or larger. Interestingly, liver exhibited greater sulfatase activity toward the relatively high mol wt products of depolymerization than did the stomach or small intestine. Despite the uncertainty remaining about the presence and susceptibility to desulfation of very high mol wt degraded mucopolysaccharide, it is, nevertheless, clear that the products of extensive depolymerization of chondroitin 4-sulfate are poor substrates for the sulfatases from stomach, liver or small intestine.

Liver extract exhibited approximately the same titre of sulfatase activity toward chondroitin 4-sulfate per milligram protein as

stomach or small intestine during the first 15 min, but over a longer period, i.e., 17 hr, liver cleaved at least 2.5 times more sulfate than stomach or intestine.

A possible explanation for the observed differences in extent of desulfation by these tissue extracts may lie in the greater amount of organic sulfates liberated by the stomach and intestine than by liver. Since these sulfatases apparently do not attack lower mol wt organic sulfates or do so only weakly, the liberation in stomach and intestine incubation mixtures of appreciable quantities of organic sulfates, in effect, reduces the amount of substrate for desulfation.

Aside from the greater extent of desulfation exhibited per mg of protein in liver extract, the liver affords about twice as much soluble protein (and hence enzyme) than do extracts of stomach and small intestine, and, accordingly, the liver would appear to have the capacity to desulfate more chondroitin 4-sulfate than stomach and small intestine. If superimposed on this consideration is the preferred localization in the liver of circulating chondroitin 4-sulfate as shown by Wood *et al.* (2), then it is not surprising that liver is considered to be the principal organ for desulfation of circulating chondroitin 4-sulfate. Despite the preeminent position of the liver in desulfation of injected and of circulating chondroitin 4-sulfate (2), it is nevertheless quite possible that various tissues such as stomach and small intestine participate in the desulfation and degradation of chondroitin sulfates and of other polyanions formed or present in these tissues.

Liver extract also differs from stomach and intestine extracts in that liver contains in much greater concentration a stable constituent or constituents which complex with chondroitin 4-sulfate. The resulting complex did not migrate from the origin on electrophoresis under the conditions employed here. The nature of the complexing substance is unknown but may represent basic proteins or possibly enzyme-substrate complexes. It is also conceivable that such binding substances may represent cell membrane receptors or constituents which participate in the sequestering of chondroitin 4-sulfate by liver.

Summary. Stomach, small intestine, and liver contain sulfatases which liberate sulfate from chondroitin 4-sulfate. Sulfatase activity is greater in 1-mo than in 4-mo old rats. For each of the 3 tissue extracts, desulfation occurs more rapidly and extensively with chondroitin 4-sulfate as substrate than with the sulfated products obtained by degradation of chondroitin sulfate with gastric enzymes, hyaluronidase or chondroitinase ABC. Stomach and small intestine liberate organic sulfate in addition to inorganic sulfate, and the ratio of the former to the latter may exceed 1 (in terms of sulfate). The rate of liberation of inorganic sulfate exceeds that of organic sulfate during the early stages of incubation and the reverse occurs in the later stages. Sulfate ion inhibits the liberation of inorganic and to a lesser extent organic sulfate. These results suggest, but do not conclusively prove, that desulfation precedes depolymerization.

Liver liberates chiefly inorganic sulfate and little organic sulfate. Electrophoretic analysis indicates that liver extracts form a transient complex with chondroitin 4-sulfate during the early stages of incubation.

1. Tudball, N., and Davidson, E. A., Biochim. Biophys. Acta **171**, 113 (1969).

2. Wood, K. M., Wusteman, F. S., and Curtis, C. G., Biochem. J. **134**, 1009 (1973).

3. Liau, Y. H., and Horowitz, M. I., Fed. Proc. **32**, abst. 2612 (1972).

4. Liau, Y. H., and Horowitz, M. I., Proc. Soc. Exp. Biol. Med. **144**, 13 (1973).

5. Held, E., and Buddecke, E., Z. Physiol. Chem. **348**, 1047 (1967).

6. Wasteson, A., Lindahl, U., and Hallén, A., Biochem. J. **130**, 729 (1972).

7. Greiling, H., Stichlsatz, H. W., and Kesters, R. W., *in* "Chemistry and Molecular Biology of Intracellular Matrix" (E. A. Balazs, ed.). Academic Press, London and New York (1970).

8. Suzuki, S., *in* "Methods in Enzymology" (V. Ginsburg, ed.), Vol. XXVIII, Part B, p. 921. Academic Press, London and New York (1972).

9. Saito, H., Yamagata, T., and Suzuki, S., J. Biol. Chem. **243**, 1536 (1968).

10. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

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