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Note

Release of inorganic sulfate ion under mild alkaline conditions from sulfated, unsaturated disaccharides obtained from chondroitin sulfates by chondroitinase digestion

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Chondroitin sulfate lyases (EC 4.2.2.4/5; trivial names, chondroitinase ABC, chondroitinase AC, and chondroitinase AC II), isolated from bacteria[1,2], have been used frequently to estimate the proportions of disaccharide repeating units of different sulfation pattern in chondroitin sulfates and dermatan sulfates. These enzymes have also been used for the determination of nonreducing terminal sugars of chondroitin sulfate oligosaccharides on the basis that degradation by these enzymes gives sulfated unsaturated disaccharides, *i.e.*, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose 4- (ΔDi-4*S*, **1**), 6- (ΔDi-6*S*, **2**), and 4,6-di-sulfate (ΔDi-4,6S_2, **3**) and releases the nonreducing terminal monosaccharide or disaccharide groups. When the terminal group is a uronic acid, saturated disaccharides are formed, whereas if sulfated *N*-acetylgalactosamine is the terminal group, it is recovered as such[3]. We previously applied this procedure to the analysis of chondroitin sulfates from chick embryo, epiphyseal cartilage, and rat xiphisternal cartilage that had been metabolically labeled with either $^{35}SO_4^{2-}$ or D-[^{3}H]glucose[4]. Although the analysis revealed the presence of a high proportion of 4,6-bis-sulfated 2-acetamido-2-deoxy-D-galactose units as a common nonreducing terminal group of the newly synthesized chondroitin sulfate chains, the conditions required for quantitative recovery of sulfated mono- and di-saccharides from the enzyme digests were far more rigorous than those for the recovery from digests of oligosaccharide samples. Thus, many of the preliminary tests with the radiolabeled chondroitin sulfates suffered from an erroneously high recovery of inorganic sulfate and sulfated monosaccharide, particularly when the chondroitinase digests were exposed to relatively high temperature under mild alkaline conditions. This observation prompted an investigation of the conditions

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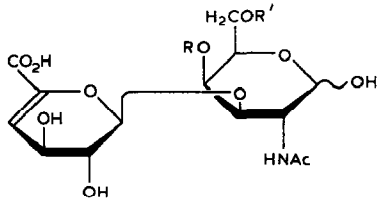
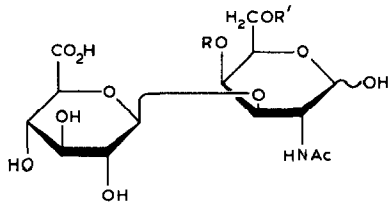
and mechanism for the degradation of the sulfated unsaturated disaccharides **1–3**. The results of this study would eliminate a source of error in the enzymic analysis of terminal sugar groups, as well as of minor disaccharide units in the interior portion. The alkaline lability of the glycosyl linkage to O-3 of the 2-acetamido-2-deoxyhexosamine unit[5–7], and the conversion of **1** and **2** into 2-acetamido-2-deoxy-D-galactose 4- and 6-sulfate, respectively, at 100° under acidic conditions[8,9] have been previously described.





1 R = SO_3^-, R′ = H
2 R = H, R′ = SO_3^-
3 R = R′ = SO_3^-

4 R = $^{35}SO_3^-$, R′ = H
5 R = H R′ = $^{35}SO_3^-$

RESULTS AND DISCUSSION

Fig. 1 shows the time course of degradation of ^{35}S-**1** incubated in 50mM Tris hydrochloride buffer, pH 8.0, at various temperatures. No detectable destruction

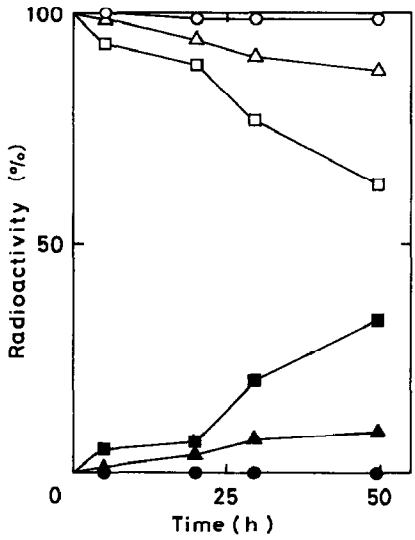
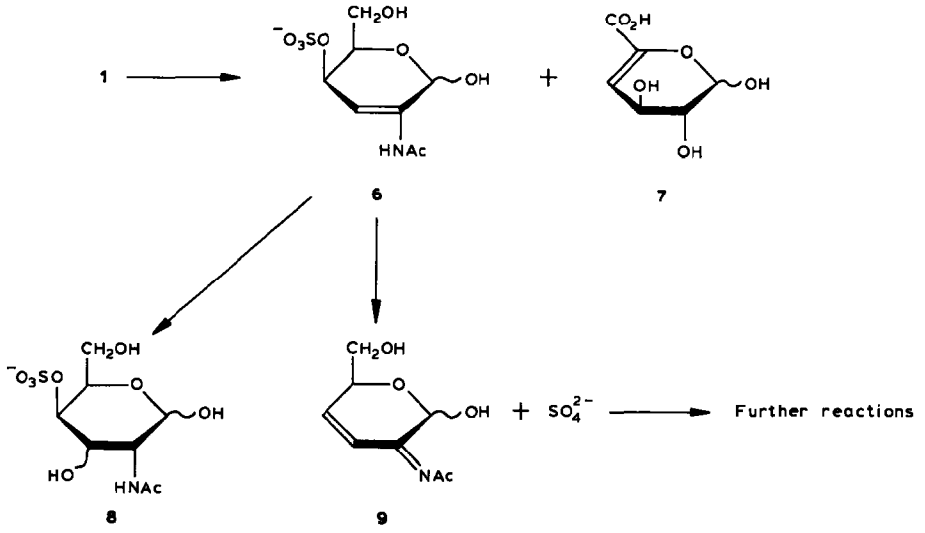


Fig. 1. Time course of degradation of **1** at pH 8. ^{35}S-**1** was incubated in 50mM Tris·HCl (pH 8.0) at 4° (○, ●), 37°, (△, ▲), or 50° (□, ■) for up to 50 h. At the indicated times, an aliquot of the mixture was analyzed by paper electrophoresis. The radioactivity of the zones corresponding to the disaccharide (○, △, □) and SO_4^{2-} ion (●, ▲, ■) on paper electrophoretograms was determined. The total radioactivity of the starting ^{35}S-**1** was set as 100%.



Scheme 1. Proposed pathways of the degradation of **1**. Compound **1** is converted into **6** with release of the unsaturated uronic acid **7**. The double bond of **6** is hydrated to give **8**. Alternatively, **6** is converted into **9** with a concomitant release of SO_4^{2-}. Compounds **2** and **3** may be degraded in a similar fashion, but at somewhat different rates. In view of the presence of resonance double bonds in **9**, it is possible that the compound is further decomposed.

occurred at 4° for up to 50 h, but there was a marked reduction in the radioactivity of the disaccharide at 37° (by 13% after 50 h) and 50° (by 37% after 50 h). The degradation of the disaccharide was accompanied by the release of inorganic [^{35}S]sulfate ion, which accounted for 90% of the radioactivity of the degradation products. Paper chromatography and paper electrophoresis of the reaction mixtures showed, in addition to inorganic [^{35}S]sulfate ion, a radioactively labeled compound having the mobility of 2-acetamido-2-deoxyhexose monosulfate. As 2-acetamido-2-deoxy-D-galactose 4-sulfate was shown to be stable under the same conditions, the inorganic sulfate ion may not be derived directly from the sulfate ester but from a possible 2,3-unsaturated 4-sulfate **6** intermediate formed by alkaline β-elimination of the glycosidic bond (Scheme 1). The sulfate group at C-4 might function as a strong leaving group to promote this reaction.

When ^{35}S-**1** was incubated for 50 h in 50mM acetate buffer, pH 5.0, or in 50mM Tris hydrochloride, pH 7.0, at either 4 or 37°, no destruction of the disaccharide was observed. At 50°, however, about 4% of the total radioactivity was recovered as equimolar amounts of 2-acetamido-2-deoxyhexose [^{35}S]sulfate and inorganic [^{35}S]sulfate ion at the end of incubation (Table I).

When ^{35}S-**2** was treated under similar conditions, it was apparent that the compound was much more stable than the 4-isomer (Table I). Thus, incubation of **2** in 50mM Tris hydrochloride, pH 8.0, at 50° for 50 h resulted in the formation of only 2% of the total radioactivity as inorganic [^{35}S]sulfate ion, as compared to the value of 34% for **1**. The major product from the treatment of **2** was a monosulfated

TABLE I

DEGRADATION OF SULFATED UNSATURATED DISACCHARIDES AFTER INCUBATION FOR 50 h AT pH 5, 7, AND 8 AT VARIOUS TEMPERATURES[a]

Conditions		*Unsaturated disaccharide recovered (%) from*		
pH	*Temp. (%)*	**1**	**2**	**3**
5	4	100	100	99
	37	100	99	100
	50	98	97	99
7	4	100	100	100
	37	100	100	96
	50	96	96	83
8	4	99	99	100
	37	87	93	79
	50	63 (34)[b]	77 (2)[b]	48 (19)[b]

[a]The [^{35}S]sulfate-labeled disaccharide samples were treated under the indicated conditions, and analyzed by paper electrophoresis and paper chromatography. The radioactivity of the corresponding disaccharides recovered was determined. The total radioactivity of each disaccharide added to the reaction mixture is set as 100% radioactivity. [b]The values in parentheses express the percentage of $^{35}SO_4$ ion released.

sugar having the chromatographic and electrophoretic mobility of 2-acetamido-2-deoxyhexose 6-sulfate which accounted for 21% of the total radioactivity of treated ^{35}S-**2**. Apparently, the 4-sulfated disaccharide was more sensitive to alkali than the 6-sulfated isomer, perhaps owing to the presence of the 4-sulfate group as a good leaving group.

Treatment of ^{35}S-**3** under the same conditions also resulted to a large extent, in the degradation of the compound (Table I); at pH 8.0 for 50 h, 21 and 52% of **3** was degraded at 37 and 50°, respectively. As the radioactivity of the substrate is located exclusively at the 6-sulfate residue, the conversion of the 4-sulfate residue into inorganic sulfate ion, if any, would not be detected by the radioactivity measurement. Nevertheless, 19% of the total radioactivity was detected as inorganic [^{35}S]sulfate ion after a 50-h incubation at pH 8.0 and 50°, suggesting that the existence of a sulfate group at C-4 may render the 6-sulfate group more susceptible to degradation under the given conditions. The rest of the labeled products (33%) were accounted for by compounds having the chromatographic and electrophoretic mobility of mono- and bis-sulfated monosaccharides.

These results indicated that the sulfated unsaturated disaccharides **1–3** derived from chondroitin sulfates by chondroitinase digestion were unstable at neutral to alkaline pH. When the sulfated saturated disaccharides **4** and **5** were likewise examined for their stability, both the kinetics and mode of degradation were the same as those observed with the unsaturated homologs, suggesting that the 4,5-unsaturation of the uronic acid group was not required for the degradation.

In view of the properties of the products of chondroitinase digestion, a prolonged incubation at a pH range higher than 7 and at a temperature range higher than 37° should be avoided during chondroitinase analysis. Also, it is noteworthy that up to 10% of ^{35}S-**1** in water was degraded during lyophilization or concentration *in vacuo* in the presence of phosphorus pentaoxide at room temperature.

EXPERIMENTAL

General methods. — Paper electrophoresis was carried out on 60-cm strips of Toyo No. 51A filter paper at room temperature in the apparatus described by Markham and Smith[10], at a potential gradient of 25 V/cm for 60 min. The buffer used was 0.05M ammonium acetate–acetic acid, pH 5.0. Paper chromatography was carried out on 45-cm strips of Toyo No. 50 filter paper at room temperature for 48 h by the descending method with 5:3 (v/v) butyric acid–0.5M NH_4OH. Unsaturated disaccharides on paper strips were located by viewing under u.v. light, reducing sugars and inorganic sulfate ion by staining with the aniline hydrogen phthalate reagent and with the rhodizonate reagent, respectively, and radioactive materials by autoradiography with X-ray film. Radioactivity was measured with an Aloka liquid-scintillation spectrometer. D-Glucuronic acid was determined by the method of Bitter and Muir[11] using D-glucuronolactone as a standard.

Materials. — [^{35}S]Sulfate-labeled **1**, **2**, **4**, and **5** (ammonium salts) were prepared from chondroitin [^{35}S]sulfate (4×10^5 c.p.m./μmol of glucuronic acid) of chick embryo epiphyseal cartilage[2] by sequential digestion with testicular hyaluronidase[13] and chondroitinase ABC. [^{35}S]Sulfate-labeled **3** was isolated from the chondroitinase digest of a chondroitin [^{35}S]sulfate (1.5×10^6 c.p.m./μmol of glucuronic acid) preparation enzymically synthesized from chondroitin 4-sulfate (Seikagaku Kogyo Co., Tokyo) and (adenylyl 3′-phosphate) sulfate (^{35}S-PAPS) with squid cartilage E_6-sulfotransferase[14].

Determination of [^{35}S]*disaccharide degradation.* — The [^{35}S]sulfate labeled disaccharide (2000–5000 c.p.m.) sample was placed in 50mM sodium acetate–acetic acid (pH 5.0), 50mM Tris·HCl (pH 7.0), or 50mM Tris·HCl (pH 8.0) (200 μL each), and the mixture was incubated at either 4, 37, or 50° for up to 50 h. At the indicated times, an aliquot (15 μL) of the mixture was analyzed by either paper electrophoresis (for the isolation of monosulfated disaccharides) or paper chromatography (for the isolation of bis-sulfated disaccharides). As reference compounds for the identification of the separated compounds, authentic samples of inorganic sulfate ion, **1**, **2**, **3**, and 4-mono-, 6-mono-, and 4,6-bis-sulfated derivatives of 2-acetamido-2-deoxy-D-galactose[15] were run on the same chromatographic and electrophoretic strips.

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