Biosynthesis of the Acetylgalactosamine 4,6-Disulfate Unit of Squid Chondroitin Sulfate by Transsulfation from 3'-Phosphoadenosine 5'-Phosphosulfate*

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SUMMARY

A mucopolysaccharide sulfotransferase utilizing 3'-phosphoadenosine 5'-phosphosulfate (sulfate donor) and endogenous proteinpolysaccharides (sulfate acceptor) was characterized from squid cartilage. This enzyme had the unique property of catalyzing the introduction of sulfate into position 6 of an acetylgalactosamine moiety already bearing a sulfate residue at position 4.

The sulfotransferase was separated from endogenous proteinpolysaccharides and purified some 9-fold on DEAESephadex A-50. The enzyme thus purified was effective only when exogenous acceptors were added. Although various mono-, di-, and polysaccharides were active as sulfate acceptors, the presence of a sulfate at position 4 of their acetylgalactosamine residue was essential for the acceptor activity.

These observations suggest that an interpretation of the known change in type of chondroitin sulfates with phylogenetic and ontogenic development must take into consideration the development of a specific family of sulfotransferases with respect to the site of sulfation.

In studies concerned with the fine structure of mucopolysaccharides from various origins, Kawai, Sato, and Anno (6) and Suzuki et al. (2) demonstrated that the squid cartilage is unique in its producing a novel chondroitin sulfate containing acetylgalactosamine 4,6-disulfate residues. This observation prompted us to study the biosynthesis of the disulfated unit in the hope that either unusual sulfotransferase or unusual sulfate-acceptor might be characterized from the tissue. The results of the study presented here clearly indicate that the tissue contains a specific sulfotransferase, thereby producing the over-sulfated chondroitin sulfate.

Before going into the details, it may be appropriate to show in Fig. 1 the structure of the major repeating unit of squid chondroitin sulfate (termed "type E"). The two sulfates are situated on the galactosamine moiety. The difference between type E unit and the well known A and C units is in the number and position of the sulfate residues. In both A and C, only one sulfate is situated on the galactosamine moiety; in A, the sulfate is at position 4, while in C it is at position 6. As will become evident, the squid cartilage carries a variety of polysaccharide chains with different proportions of disulfated (type E), monosulfated (type A and C), and nonsulfated repeating units. The background for determining the distribution of these units in a

Chondroitin sulfate-protein complexes are macromolecules found in the extracellular matrix of human and animal tissues. In the polysaccharide chains, there have been observed multiple variations (for example, see References 1–3) such as the degrees of sulfation, the locations of sulfate ester groups, and the existence of a hybrid chain consisting of N-galactosaminic and iduronic acid. In view of the existence of various different chondroitin sulfate chains, we are quite interested in exploring their differential synthesis.

It is a reasonable assumption that the enzyme system necessary for chondroitin sulfate synthesis exerts a primary control over the differential synthesis. It seems possible, for example, that there is a family of sulfotransferases with more or less sharply developed specificity and they are responsible for determining the site of sulfate incorporation. Considerable support was given to this hypothesis when Suzuki, Theum, and Strominger (4) obtained partial separation of sulfotransferase activities, specific for ChS-A, ChS-C, and heparin sulfate, from an enzyme preparation solubilized from hen oviduct. However, an alternative hypothesis has been proposed by Meicer and Davidson (5). The authors have reported that a soluble sulfotransferase preparation from chick embryo cartilage synthesized ChS-A in the presence of endogenous acceptor whereas in the presence of added protein-free acceptor it synthesized ChS-C, a result which led to their proposal that the site of sulfation depends on whether the acceptor polysaccharide exists as a protein complex or a protein-free polysaccharide.

The abbreviations used are: ChS-A, ChS-B, and ChS-C; chondroitin sulfates A, B, and C; ADi-OS, 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyl)N-galactosamine; ADi-4S, ADi-6S, and ADi diS, derivatives of ADi OS bearing a sulfate at position 4, a sulfate at position 6, and two sulfates at position 4 and position 6 on the hexosamine moiety, respectively (see Fig. 1).

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EXPERIMENTAL PROCEDURE

Material—The following materials were prepared by previously described methods: [35S]3'-phosphoadenosine 5'-phosphosulfate with specific activity of about 5 × 10⁶ cpm per µmole (9); chondroitinase-ABC and chondro 6-sulfatase from Proteus vulgaris (7); ΔDi-OS, ΔDi-4S, ΔDi-6S, and ΔDi-diSn from chondroitin sulfates (2, 7); chondroitin (chemically desulfated product) from ChS-A (10); keratosulfate from bovine cornen (11); charonin sulfate (gluean sulfate) from mollusc (12); acetylgalactosamine 4-sulfate and acetylgalactosamine 6-sulfate (13).

Generous gifts of the following materials are acknowledged: whale cartilage ChS-A and shark cartilage ChS-C from Seikagaku Kogyo Company, Tokyo; 3'-phosphoadenosine 5'-phosphosulfate (unlabeled) from Takeda Chemical Industry, Osaka; ChS-B (dermatan sulfate) of hog intestinal mucosa from Dr. J. A. Cifonelli and Dr. M. B. Mathews, University of Chicago, Chicago; and Pronase-P (45,000 units per g) from Kaken Kagaku Company, Tokyo.

The following commercial materials were used: acetylgalactosamine and hyaluronic acid from Seikagaku Kogyo Company, Tokyo; heparin from Calbiochem; DEAE-Sephadex A-50 from Pharmacia.

Crude Extract—Fresh squid, Ommastrephes sloani pacificus, was purchased locally. For the enzyme preparation, the head cartilage (found in the cephalic region behind the tentacles) was dissected free of soft tissues, cut into slices with a razor, placed in four volumes of ice-cold 0.02 M Tris-HCl, pH 7.0, containing 0.5% Triton X-100, 10% glycerol, and 0.01 M mercaptoethanol, and ground with sea sand in a chilled mortar. The homogenate was centrifuged at 12,000 × g for 20 min. The clear supernatant fluid was collected, dialyzed for 24 hours against three 10-volume changes of 0.02 M Tris-HCl, pH 7.0, containing 10% glycerol and 0.01 M mercaptoethanol, and stored at −20° ("crude extract").

Enzyme Assay—Previous studies of Suzuki et al. (2) revealed the presence of four types of sulfate residues in chondroitin sulfate prepared from squid cartilage, i.e. sulfate linked to position 4 of acetylgalactosamine moiety (type A sulfate), sulfate linked to Position 6 of acetylgalactosamine moiety (type C sulfate), and sulfates located at position 4 and position 6, respectively, of acetylgalactosamine 4,6-dissulfate moiety (hereinafter, the two sulfates will be referred to as type E₄ sulfate and type E₅ sulfate, respectively). The assay described below is so designed as to permit the quantitative measurement of the incorporation of [35S]sulfate into the four different sites and involves (a) precipitation of labeled polysaccharides with ethanol, (b) degradation of the polysaccharides to disaccharides (ΔDi-4S, ΔDi-6S, and ΔDi-diSn) with chondroitinase-ABC, (c) determination of radioactivity in each of the disaccharide fractions after paper-chromatographic separation, (d) further hydrolysis of the ΔDi-diSn product to ΔDi-4S and inorganic sulfate with chondro 6-sulfatase, and (e) determination of radioactivity in each of the sulfatase products after paper electrophoretic separation. The reaction used for the enzymatic determination are summarized in Fig. 1. The radioactivities released as ΔDi-4S and ΔDi-6S by the chondroitinase digestion represent the incorporation of [35S]sulfate into type A sulfate and type C sulfate, respectively, and those released as an inorganic sulfate and ΔDi 4S by the sulfatase digestion represent the incorporation into type E₄ sulfate and type E₅ sulfate, respectively.

Routine incubation mixtures contained, in a final volume of
Incorporation of sulfate into four different sites of chondroitin sulfate by crude and partially purified enzyme preparations

Table I

| Enzyme preparation | Acceptor added | Total activity | Specific activity | Distribution of $^{35}$S (cpm/mg) |
|--------------------|----------------|---------------|------------------|-------------------------------|
|                    |                |               |                  | Type A | Type C | Type E<sub>S</sub> | Type E<sub>G</sub> | Resistant |
| Crude extract      | None           | 1610          | 2.55             | 0.50   | 0.24   | 0.50              | 0.85          | 0.07      |
|                    | None           | 1             | 0.08             | trace  | trace  | trace            | trace         | 1.64      |
|                    | Boiled crude extract | 440          | 13.4             | 0.87   | 0.30   | 0.83             | 7.52          | 1.64      |
| DEAE-Sephadex (Fraction II) |                |               |                  |        |        |                  |               |           |

* * * Total radioactivity (cpm × 10<sup>-4</sup>) incorporated into an ethanol-precipitable polysaccharide fraction (see "Enzyme Assay," Step a/630 mg of the crude extract or 43 mg of these enzyme preparations.

<sup>4</sup> Total radioactivity (see above)/total protein (mg) of enzyme preparation.

<sup>5</sup> Total radioactivity (cpm × 10<sup>-4</sup>) incorporated into the indicated sites total protein (mg) of enzyme preparation.

<sup>6</sup> Total radioactivity (cpm × 10<sup>-4</sup>) incorporated into the indicated sites total protein (mg) of enzyme preparation.

100 μl, Tris-HCl, pH 8.0, 5 μmole; CaCl<sub>2</sub>, 2 μmole; glutathione, 2 μmole; [35S]3'-phosphoadenosine 5'-phosphosulfate, 5 × 10<sup>6</sup> cpm about 0.1 nmole; and the enzyme and acceptor as indicated in the individual experiments. Controls contained heat-inactivated enzyme. After incubation at 25°, the reaction was stopped by immersing the reaction tubes in a boiling water bath for 1 min. Step a: Pronase-P, 0.4 mg, was added to each solution and the mixture was incubated for 1 hour at 37°. To the solution were added 200 μl of water and 700 μl of a solution of 1 g of potassium acetate and 19 mg of EDTA in 100 ml of 95% ethanol to precipitate mucopolysaccharides. The mixture was left for 30 min. Step b: Pronase-P, 0.4 mg, was added to each solution and the mixture was incubated for 1 hour at 37°. To the solution were added 200 μl of water and 700 μl of a solution of 1 g of potassium acetate and 19 mg of EDTA in 100 ml of 95% ethanol to precipitate mucopolysaccharides. The mixture was left for 30 min. Step c: a 20-μl portion of the resulting mixture was subjected to paper electrophoresis (30 min) to separate inorganic sulfate from ADi-4S. After drying, inorganic sulfate and ADi-4S were visualized, respectively, by spraying with BaC<sub>4</sub>(S<sub>2</sub>O<sub>7</sub>)<sub>2</sub> (14) and by viewing under ultraviolet light, cut out, and eluted with water. The eluate was dried in a vacuum over P<sub>2</sub>O<sub>5</sub> and was then dissolved in 40 μl of a solution containing 2 μmole of Tris-acetate, pH 8.0, 40 μg of bovine serum albumin, and 0.012 unit of chondrodi-6-sulfatase. The mixture was incubated at 37° for 20 min. Step d, the resulting mixture was subjected to paper electrophoresis (30 min) to separate inorganic sulfate from ADi-4S. After drying, inorganic sulfate and ADi-4S were visualized, respectively, by spraying with BaC<sub>4</sub>(S<sub>2</sub>O<sub>7</sub>)<sub>2</sub> (14) and by viewing under ultraviolet light, cut out, and counted.

In some experiments (e.g., purification of sulfotransferase by DEAE-Sephadex chromatography, see below), the supernatant solution from boiled (100°, 10 min), centrifuged crude extract (for the preparation, see above) was used as sulfate acceptor.

In the experiments with mono- and disaccharides as acceptor, the reaction products were located, after separation by paper chromatography in Solvent A, by placing the chromatogram on x-ray film for 2 days. The radioactive spots were cut out and counted.

Analytical Methods—The following compounds were determined by the indicated methods: protein by the method of Lowry et al. (15); uronic acid by the method of Dische (16); hexosamine by the modified Elson-Morgan method (17); and sulfate by the method of Dodgson (18) as modified by Kawai, Sono, and Anno (19).

Paper Chromatography and Paper Electrophoresis—The paper chromatographic solvent systems used for descending paper chromatography at about 20°, with Toyo No. 51 A filter paper (60-cm long), were Solvent A, butyric acid-0.5 ammonia, 5:3 (v/v) and Solvent B, 1-butanol-ethanol-water, 13:8:4 (v/v/v). Paper electrophoresis was carried out on 60-cm strips of Toyo No. 51 A paper in the apparatus described by Markham and Smith (20) at a potential gradient of 30 volts per cm. The buffer system used was 0.05 mM ammonium acetate-acetic acid, pH 5.0.

RESULTS

Sulfate Transfer Catalyzed by Crude Extracts—When the crude extract of squid cartilage was incubated with [35S]3'-phosphoadenosine 5'-phosphosulfate under the conditions indicated in Table I, about 7% of the radioactivity of [35S]3'-phosphoadenosine 5'-phosphosulfate was transferred to the acceptor, with ADi-4S, ADi-GS, and ADi-diSn as internal markers.2 Chro-
Fig. 2. Effect of pH on the incorporation of sulfate into four different sites by the crude extract. The conditions of the experiment were those described under "Enzyme Assay," except for the pH of the buffers. The buffers used were 5 mmol of Tris-HCl (pH 7.25 to 9.0) and Tris-maleate (pH 5.75 to 8.6) per incubation mixture. Incubation was carried out for 1 hour with 0.2 mg (as protein) of crude extract. No exogenous acceptor was added.

Sulfate incorporation (cpm x 10^-6/mg)

| pH | Type A | Type C | Type E_d | Type E_s |
|----|--------|--------|----------|----------|
| 5  | 0.8    | 1.2    | 1.5      | 2.0      |
| 6  | 1.0    | 1.5    | 1.8      | 2.5      |
| 7  | 1.2    | 2.0    | 2.2      | 2.8      |
| 8  | 1.5    | 2.5    | 3.0      | 3.5      |

Fig. 3. Effects of NaCl (upper part of figure) and KCl (lower part of figure) concentrations on the incorporation of sulfate into four different sites by the crude extract. The conditions of the experiment were those described under "Enzyme Assay," except that CaCl_2 was omitted and KCl or NaCl was added to give the indicated concentrations. Incubation was carried out for 1 hour with 0.18 mg (as protein) of crude extract. No exogenous acceptor was added.

Sulfate incorporation (cpm x 10^-6/mg)

| Concentration (M) | Type A | Type C | Type E_d | Type E_s |
|-------------------|--------|--------|----------|----------|
| 0.1               | 1.0    | 1.5    | 1.8      | 2.5      |
| 0.2               | 1.2    | 2.0    | 2.2      | 2.8      |
| 0.4               | 1.5    | 2.5    | 3.0      | 3.5      |

The radioactive sulfate remaining at the origin of the chromatogram of chondroitinase digest. It is not clear at present whether this sulfate ester is derived from the same polysaccharide acceptor as that yielding the chondroitin sulfate products or derived from a different acceptor.

In a typical experiment, the H-3-labeled products obtained with the crude extract gave, upon digestion with chondroitinase-ABC, radioactive ΔDi-4S, ΔDi-6S, and ΔDi-diSε in the radioactive sulfate remaining at the origin of paper chromatogram of chondroitinase digest. It is not clear at present whether this sulfate ester is derived from the same polysaccharide acceptor as that yielding the chondroitin sulfate products or derived from a different acceptor.

A study was made to determine if certain cations elicited an activation of some of the activities. As shown in Fig. 3, KCl and NaCl were found to activate the rate of introduction of type E_d sulfate without any significant activation of the other three reactions. From the fact that MgCl_2 and MnCl_2 did not produce any measurable effect on this reaction as well as on the other three reactions, it can be surmised that the chloride ion of itself does not account for the observed activations with sodium and potassium salts. At the optimal concentration of K⁺ ion (0.2 M), the activity ratio of 9:16:1, respectively. A minor product which was depolymerized by Bacillus keratanus "keratanase" (a hydrolase acting on corneal keratosulfate) was also obtained (K. Kimata and S. Suzuki, unpublished observation).
FIG. 4. Effects of CaCl₂ (upper part of figure) and BaCl₂ (lower part of figure) on the incorporation of sulfate into four different sites by the crude extract. The conditions of the experiment were those described under “Enzyme Assay,” except for the variation in the concentrations of CaCl₂ or the replacement of CaCl₂ by varying concentrations of BaCl₂. Incubation was carried out for 1 hour with 0.18 mg (as protein) of crude extract. No exogenous acceptor was added.

The introduction of type E₄ sulfate proceeded approximately 18 times faster than the other reactions. CaCl₂ and BaCl₂, on the other hand, activated all the four reactions, although the concentration at which maximum activation occurred and the extent of activation varied considerably (Fig. 4). Again, the activity for type E₄ sulfate was highest at the optimal Ca²⁺ or Ba²⁺ concentration. At present it is not clear whether these activations by K⁺, Na⁺, Ca²⁺, and Ba²⁺ are effects on the enzymes or on the endogenous mucopolysaccharide acceptors.

A selective activation was also observed when mercaptoethanol or glutathione was added to the reaction mixture (Table II). In this experiment, the crude extract which had been dialyzed against 0.02 M Tris-HCl, pH 7.0, was used. These sulphydryl agents increased most of the reaction velocities, but the activation of type E₄ and type A sulfate incorporation was relatively higher compared with type E₄ and type C sulfate incorporation.

When heat labilities of the activities for type E₄ and E₅ sulfate were compared at 47°C (Fig. 5), the inactivation of the activity for type E₄ sulfate proceeded much more slowly than the activity for type E₅ sulfate. After incubation for 30 min, the former activity had decreased by 40%, while the latter activity had decreased by 90%. These inactivation phenomena might reflect a direct effect on the enzyme proteins or, alternatively, an effect on the stability of endogenous acceptors. The experiments described below indicate that the latter situation is unlikely.

Purification of E₄-Sulfotransferase—The experiments with the crude extract demonstrated the formation of at least four types of sulfate residues from 3'-phosphoadenosine 5'-phosphosulfate and endogenous acceptors. The information of Fig. 2 to 5 and Table II further suggested that the crude extract might contain several mucopolysaccharide sulfotransferases. This inference has been validated in part, at least, by preparation of a specific sulfotransferase capable of catalyzing the transfer of sulfate from 3'-phosphoadenosine 5'-phosphosulfate to Position 6 of acetyl-galactosamine 4-sulfate moieties of acceptor. This enzyme will be referred to as E₄-sulfotransferase.

The crude extract (200 ml) was applied to a DEAE-Sephadex A-50 column (5 x 45 cm) in the cold and washed with 1.5 liters of 0.02 M Tris-HCl, pH 7.0, containing 10% glycerol and 0.01 M
mercaptoethanol. The adsorbent was eluted stepwise with 2 liters each of 0.2 M NaCl and 0.5 M NaCl, and 1.6 liters each of 0.8 M NaCl, 1.0 M NaCl, 1.5 M NaCl, and 2 M NaCl in the same Tris buffer. The flow rate was 60 ml per hour, and 20-ml fractions were collected. Each fraction was checked for the sulfate-incorporating ability, with the boiled crude extract (see "Enzyme Assay") as exogenous sulfate acceptor.

The elution pattern is shown in Fig. 6, together with the patterns of ultraviolet (280 mp)-absorbing material and uronic acid-reacting material. It can be seen that incorporation of sulfate was found in only one peak (Fraction II). The other fractions had appreciable quantities of protein (as measured by absorption at 280 mp), but showed no sulfotransferase activity. Fractions III through VI contained proteoglycan, with the protein content progressively decreasing and the uronic acid content increasing (for more detailed analysis, see Table III).

Fraction II was pooled and concentrated to 20 ml by pressure dialysis against 2 liters of the Tris-glycerol-mercaptoethanol buffer. If the preparation was tested for sulfate incorporation into the individual sites, it was apparent that about 9-fold purification of S-sulfotransferase was realized and a large proportion of the other activities was eliminated (Table I). Since the three activities were not recovered from any of the other fractions of chromatography, the apparent separation of S-sulfotransferase may be interpreted as reflecting the difference in stabilities of sulfotransferase, i.e. the enzymes responsible for incorporation of type A, type C, and type E sulfate seem to be more labile than S sulfotransferase and they are inactivated during the purification.

Properties of E Sulfotransferase The formation of type E sulfate by the purified preparation had an absolute dependence on added acceptor. In addition to the endogenous acceptors in the crude extract, several authentic mucopolysaccharide preparations served as acceptors in the reaction. The relationship

![Graph](http://www.jbc.org/)

**TABLE III**

*Analytical data and acceptor abilities of endogenous proteoglycan fractions*

| Fraction | Ratio to glucuronic acid | Chondroitinase-digestable component | Disaccharides produced by chondroitinase-ABC | \( V_{max} \) | \( K_m \) for PAPS |
|----------|--------------------------|-----------------------------------|---------------------------------------------|---------|---------------|
|          | Galactosamine | Sulfate | Protein | ADi-OS | ADi-S | ADi-6S | ADi-di-S |         |
| III      |             |         |         | %      | %     | %     | %       | 0.19    | 0.33       |
| IV       | 1.86        | 0.72    | 2.93    | 37.3   | 41.8  | 40.9  | 8.1     | 0.23    | 0.22       |
| V        | 1.26        | 0.18    | 61.1    | 7.6    | 46.5  | 17.1  | 28.1    | 0.22    | 1.37       |
| VI       | 1.05        | 0.04    | 73.0    | 3.7    | 39.6  | 20.0  | 36.7    | 0.20    | 0.20       |
|          | 1.02        | 0.02    | 81.0    | 2.1    | 29.5  | 20.1  | 48.3    | 0.19    | 0.33       |

* See Fig. 6.

1 Sum of the disaccharide products (umole)/total glucuronic acid of proteoglycan sample (umole) × 100.

2 The sum of the four disaccharides (umole) is assigned an arbitrary value of 100%.

3 The acceptor concentrations used were 0.1 mM (as glucuronic acid) for Fraction IV, V, and VI, and 0.5 mM for Fraction III; PAPS, 3'-phosphoadenosine 5'-phosphosulfate.
between mucopolysaccharide concentration and reaction velocity is shown in Fig. 7. In these experiments, an [35S]3'-phosphoadenosine 5'-phosphosulfate preparation with relatively low specific activity (about 3.7 × 10^6 cpm per μmole) was added to give a final concentration of 1.33 × 10^{-4} M. It is evident that only mucopolysaccharides containing type A sulfate, i.e. ChS-A and ChS-B, can serve as acceptors. Chondroitin, hyaluronic acid, heparin, corneal keratosulfate, and charonin sulfate (glucan sulfate) did not stimulate the reaction. A commercial preparation of ChS-C exhibited a significant activity. However, this activity is of questionable significance because the ChS-C preparation has been shown to contain about 10% ChS-A. Fig. 7 also shows that analogous reactions (i.e. the introduction of a second sulfate resulting in formation of an acetylgalactosamine 4,6-disulfate moiety) occurred when acetylgalactosamine 4-sulfate and ΔDi-4S were used as acceptors. The products, isolated from the reaction mixtures containing acetylgalactosamine 4-sulfate and ΔDi-4S, were acetylgalactosamine 4,6-disulfate and ΔDi-4S, respectively, as judged by paper chromatography and paper electrophoresis (for the mobilities of authentic samples, see Reference 2). Previously, Suzuki and Strominger (21) reported that 35S could be transferred from [35S]3'-phosphoadenosine 5'-phosphosulfate preparation to mucopolysaccharides containing oligosaccharides and monosaccharides in the presence of crude extracts from hen oviduct. The Es-sulfotransferase preparation was catalyzed at almost maximum rates (see below). The K_m values for acetylgalactosamine 4-sulfate and ΔDi-4S were 0.04 mM and 0.14 mM, respectively.

The reaction with ΔDi-4S was inhibited by one of its products, ΔDi-diS_6. The inhibition by ΔDi-diS_6 was strictly competitive with respect to ΔDi-4S (Fig. 8). The K_i value calculated from Fig. 8 is 0.46 mM for ΔDi-diS_6. One would speculate, therefore, that there is a regulatory interaction of precursor and product on the sulfotransferase.

Properties of Endogenous Acceptors—As shown in Fig. 6, the uronic acid-containing material in the crude cartilage extract could be separated into several subfractions on DEAE-Sephadex. Experiments were therefore designed to examine these subfractions for their chemical compositions and their behaviors toward Es-sulfotransferase.

The uronic acid-reacting fractions from the DEAE-Sephadex column corresponding to Fractions III through VI were separately pooled and concentrated by pressure dialysis. The apparent yields of the fractions from 670 μmoles (as glucuronic acid) of the crude extract were 25.2 μmoles (Fraction III), 25.4 μmoles (Fraction IV), 99.5 μmoles (Fraction V), and 190 μmoles (Fraction VI). The chemical analyses (Table III) indicated that Fraction III through VI contain uronic acid, galactosamine, sulfate, and protein, with the protein content progressively decreasing and the ratio of sulfate to uranic acid or galactosamine increasing. The difference in polysaccharide structure among these fractions was further illustrated by the comparison of disaccharides produced by chondroitinase digestion. As can be seen in Table III the extents to which the fractions were degraded by chondroitinase-ABC are progressively increasing from about 37% (as glucuronic acid) of Fraction III to about 81% of Fraction VI. The analysis of the chondroitinase digests indi-
cated that, from Fraction III to Fraction VI, the proportion of disulfated unit increases from 8.4% to 48% with a compensatory decrease in the proportion of nonsulfated and 4-sulfated units.

All the four fractions served as acceptors in the reaction with E₄-sulfotransferase. The results of a typical assay with varying amounts of each acceptor showed that maximum velocities do not differ among the four fractions (Table III). In the assay conditions used here, an excess of 3'-phosphoadenosine 5'-phosphosulfate (0.133 mM) was used so that the incorporation of ³⁵S-sulfate was enzyme and acceptor-dependent.

The relationship between 3'-phosphoadenosine 5'-phosphosulfate concentration and reaction velocity was also measured under the conditions saturated with the acceptors. The Kₘ values for 3'-phosphoadenosine 5'-phosphosulfate thus obtained are presented in Table III and show that the lower the sulfate content of the acceptor, the greater the affinity of enzyme and 3'-phosphoadenosine 5'-phosphosulfate.

Since the acceptor preparations used in these experiments appear to be mixtures of highly hybridized molecules (see "Discussion") the final interpretation of the data in Table III will have to await further work in the purification of endogenous acceptors.

**DISCUSSION**

The E₄-sulfotransferase system described in this work shows a strict requirement for acceptors, which is typical of many sulfotransferases. There is a correlation between the variation of Vₘₐₓ and Kₘ for 3'-phosphoadenosine 5'-phosphosulfate and the differences in the molecular form of acceptors, as pointed out in the experiments with the four fractions of endogenous acceptors (Table III). However, the site of sulfation on hexosamine residues never changes when different acceptors are used. The purified enzyme specifically catalyzes the sulfation of acetylgalactosamine 4-sulfate residues, leading to acetylgalactosamine 4,6-disulfate formation, whether acetylgalactosamine 4-sulfate in a proteinpolysaccharide form or in a protein-free form is the acceptor. It is remarkable that, even when acetylgalactosamine 4-sulfate in a disaccharide form (ΔD=48) or in the mono-saccharide form is the acceptor, the enzyme introduces an "extra" sulfate to position 6 of the 4-sulfate residue. It is most likely therefore that the specificity of this enzyme does not involve recognition of a particular size or a particular monosaccharide sequence of acceptor molecule.

It should be noted that, although the present study has been restricted to the soluble preparation from the cartilage, a large proportion of the squid sulfotransferases appears to be present in the cell in a membrane-bound form. This is indicated by our control experiments in which a disruption of the squid cartilage without the addition of Triton X-100 has resulted in solubilizing only 50% or less of the sulfotransferase activities solubilized with the aid of the detergent.

At any rate, the detection of such an enzyme as E₄-sulfotransferase in an organism that makes an oversulfated chondroitin sulfate appears to be present in the cell in a membrane-bound form. This is indicated by our control experiments in which a disruption of the squid cartilage without the addition of Triton X-100 has resulted in solubilizing only 50% or less of the sulfotransferase activities solubilized with the aid of the detergent.

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It has been difficult with these fractions to determine the Kₘ values for acceptors, because the plots of velocity against acceptor concentration showed sigmoidal curvature. A comparison of the sulfotransferase patterns found for squid cartilage and 12-day-old chick embryo cartilage, it is apparent that a pattern of sulfotransferases in a given tissue is reflected in the sulfation pattern of chondroitin sulfates occurring in the tissue. Thus, in the chick cartilage the extremely low E₄-sulfotransferase activity is paralleled by the low concentration of acetylgalactosamine 4,6-disulfate residue in the chondroitin sulfates of the tissue. These findings therefore tend to rule out acceptor polymers as a factor determining the site of sulfate incorporation, although conclusive evidence will not be available until we are able to separate all the sulfotransferase activities to define their specificity.
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Biosynthesis of the Acetylgalactosamine 4,6-Disulfate Unit of Squid Chondroitin Sulfate by Transsulfation from 3'-Phosphoadenosine 5'-Phosphosulfate
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