The Sulfation of Chondroitin Sulfate in Embryonic Chick Cartilage Epiphyses

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SUMMARY

With the use of purified chondroitinase of Proteus vulgaris, a method has been devised for the determination of relative amounts of chondroitin, chondroitin-4-SO4, and chondroitin-6-SO4 in cartilage. This method was applied to the analysis of embryonic chick epiphyseal cartilage. With 35S04 and 14C-acetate it was possible to study the relationship of embryo age to rate of formation of chondroitin, chondroitin-4-SO4, and chondroitin-6-SO4. Between 10 and 19 days there is a progressive decrease in synthesis of chondroitin and chondroitin-4-SO4 and a progressive increase in synthesis of chondroitin-6-SO4.

Epiphyseal cartilage of chicken embryos is an active site of biosynthesis of chondroitin sulfate and collagen (1). In tissues, CS4 sulfate is invariably bound to protein (2); however, the extent and position of sulfate esterification vary with both species and age. Embryonic chick cartilage contains both Ch-4-SO4 and Ch-6-SO4. It is not known whether these two polysaccharides exist in the tissue as separate entities or whether both isomers of sulfated galactosamine occur within the same polymer chain.

Previous work has shown that a cell-free preparation from embryonic cartilage catalyzes the formation of CS from UDP-GlcUA and UDP-GalNAc (3). Studies with this preparation and with minced cartilage (4, 5) have partially elucidated the pathway of biosynthesis of the entire complex (6), but the mechanism of sulfation is still not clear. Inorganic sulfate is initially activated to yield adenosine 3'-phospho-5'-phospho-sulfate and the sulfate moiety is then transferred to polysaccharide by a sulfotransferase (7). The exact nature of the polysaccharide acceptor is unknown, but it seems likely that sulfation occurs when the chains have reached a moderately large size (3).

No polysaccharide sulfotransferases have been highly purified. Cell-free experiments have been limited to the study of crude tissue extracts and the effect of added acceptors on the incorporation of sulfate into polysaccharide (7-9). Suzuki, Trenn, and Strominger (10) have reported a partial separation of three sulfotransferase activities, active for heparitin, Ch-4-SO4, and Ch-6-SO4, which occur in crude extracts of hen oviduct. These results provide evidence for the existence of separate enzymes for each acceptor. More recently, Meezan and Davidson (11) have studied the sulfation in cartilage extracts and have concluded that sulfotransferases are probably nonspecific. They suggested that 4-sulfation occurs preferentially when the acceptor is a protein complex while 6-sulfation occurs when free CS chains are present. They also concluded that sulfation of nonsulfated chains does not take place.

In view of changes in the extent of sulfation and proportions of Ch-4-SO4 and Ch-6-SO4 in the course of development of the chick as well as in other species it seems of importance to elucidate further the factors responsible for specificity of site of esterification. It is the purpose of this communication to report the development of techniques for such studies together with their application to the study of embryonic chick cartilage.

EXPERIMENTAL PROCEDURE

Materials

Carrier CS was prepared from epiphyses of tibias and femurs of 13-day-old chick embryos (4). The potassium salt of Ch-4-SO4 was obtained after papain digestion of bovine nasal cartilage (13) and the sodium salt of Ch-6-SO4 was obtained from the Kaken Yaku Kako Company, Ltd., Tokyo, Japan. Carrier-free H35SO4 was purchased from the Union Carbide Corporation.
Suspensions of minced embryonic cartilage (4) were incubated with shaking in sulfate-free Krebs-Ringer-bicarbonate buffer at 37°. The buffer was equilibrated with 95% O₂-5% CO₂ and contained 0.3% glucose.

Preparation of Proteus vulgaris Chondroitinase—P. vulgaris NCTC 4636 was grown at 23° for 48 hours on a medium which contained 1.0% peptone (Oxoid), 0.3% bovine extract (Oxoid Lab-Lemco), 0.1% NaCl, and 0.1% Ch₄-SO₄ and was adjusted to pH 7.0 with NaOH. The cells were collected by centrifugation and the chondroitinase was isolated and purified by a method essentially the same as that of Nakada and Wolfe (14). Separation from sulfatase activity was accomplished by chromatography on DEAE-Sephadex A-50 in 0.005 M potassium phosphate buffer, pH 8.1. The enzyme was stable when stored at -15°. Activity was assayed by measurement of the change in absorbance at 230 mp. One unit of enzyme activity produced a change of absorbance of 1.0 per min with a 10-mm light path when incubated at 25° in 1.0 ml of 0.0625 M Tris-HCl buffer, pH 8.0, containing 0.5 mg per ml of Ch₆-SO₄. Approximately 1000 units were obtained per liter of growth medium. The purified enzyme contained in excess of 200 units per mg of protein.

After purification, the chondroitinase was shown to be free of sulfatase activity by incubation with CS labeled with sulfate-³⁵S. Paper electrophoresis and paper chromatography of the digest indicated that, under the conditions used, less than 3% of the total radioactivity was released as inorganic sulfate, yet the CS was completely hydrolyzed to disaccharide units.

Preparation of Standard Disaccharides—ADi-Ch₄-SO₄ was prepared by digestion of 1.55 g of potassium Ch₄-SO₄ with 25 units of chondroitinase in 250 ml of 0.05 M Tris-HCl buffer, pH 8.0, for 24 hours at room temperature. The entire digest was adjusted to pH 6.0 with HCl and applied to a column (2 x 30 cm) of Dowex 1-X₄ (Cl⁻ form, 200 to 400 mesh) and eluted with a linear gradient of LiCl in 0.01 M HCl. The gradient was made with 500 ml of 0.01 M HCl and 500 ml of 2.0 M LiCl in 0.01 M HCl. The disaccharide peak was detected in the eluate by its absorption at 230 mp. The pooled fractions were neutralized with KOH, concentrated to 10 ml, and applied to a column (1.8 x 85 cm) of Sephadex G-25 (fine grade) equilibrated with water. The disaccharide was eluted just ahead of the Cl⁻ peak. The fractions free of salt were pooled, passed over a column (1.0 x 20 cm) of Dowex 50-X₂ (H⁺ form, 200 to 400 mesh), and finally neutralized to pH 7.0 with KOH. The potassium salt (0.43 g) was obtained as a white powder by lyophilization. Paper chromatography showed that this material contained a small proportion of ADi-Ch₆-SO₄.

ADi-Ch₆-SO₄ was prepared in the same way as ADi-Ch₄-SO₄. The yield from 1.25 g of polysaccharide was 0.27 g of disaccharide.

The ADi-Ch was prepared from Ch₄-SO₄ desulfated by the method of Kantor and Schubert (15). The methyl ester so obtained was treated with 0.1 M KOH at 0-4° for 24 hours and neutralized with HCl, and the polysaccharide was precipitated by the addition of 3 volumes of alcohol. The desulfated CS (4.0 g) was digested with 25 units of chondroitinase in 200 ml of 0.05 M Tris-HCl buffer, pH 8.0, for 24 hours, adjusted to pH 6.0, and applied to a column (2 x 20 cm) of Dowex 1-X₄ (formate form, 200 to 400 mesh). The column was washed with water and eluted with a linear gradient of formic acid prepared with 500 ml of water and 500 ml of 1.0 N formic acid. The disaccharide was detected by its absorption at 230 mp and the fractions containing the peak were pooled and lyophilized. The yield of the free acid of the disaccharide was 1.0 g.

Isolation of Labeled CS—At the end of incubation, tubes were heated in a boiling water bath for 2 min and the sodium salt of CS was isolated after papain digestion (5). The dry material was dissolved in water to yield a solution with 1 to 5 mg of CS per ml and aliquots were then taken for assay of radioactivity and uronic acid and digestion with purified chondroitinase.

CS was digested overnight at room temperature with sulfatase-free chondroitinase (0.1 unit per ml) in 0.05 M Tris-HCl buffer, pH 8.0. The reactions were stopped by heating the tubes in a boiling water bath for 2 min and samples were removed for analyses by paper chromatography in Solvent A. The disaccharides were located on the paper with ultraviolet light and each spot was cut out and assayed for radioactivity.

Analytical Methods—Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer by methods previously described (4). Uronic acid was determined by the carbazole method of Dische (16) and reducing terminal N-acetylgalactosamine by the method of Reissig, Strominger, and Leloir (17) with N-acetylglucosamine as a standard. With this method, N-acetylgalactosamine substituted in position 3 is known to yield an extinction coefficient approximately equal to that of N-acetylglucosamine, whereas that of N-acetylgalactosamine substituted in position 4 is markedly reduced. Hexosamine was determined, after acid hydrolyses, by the method of Johansen, Marshall, and Neuberger (18). Disaccharides were hydrolyzed in sealed tubes with 4.0 M HCl for 1 hour in a boiling water bath, conditions which gave the maximum yield of hexosamine. CS was hydrolyzed in the same way for 8 hours. Ester sulfate was assayed after hydrolysis in 1.0 M HCl at 105° for 17 hours by the method of Dodgson (19).

Paper chromatography was performed on Whatman No. 3MM paper with isobutyric acid-2.0 N ammonia (20), 5:3 (Solvent A), or with 1-butyl alcohol-acetic acid-pyridine-water, 15:3:10:12 (Solvent B).

RESULTS

When CS from 13-day chick embryo cartilage was digested with chondroitinase from P. vulgaris, unsaturated disaccharides were obtained as the products. These were separated and identified by paper chromatography in Solvent A. Three disaccharides were obtained: ADi-Ch₄, ADi-Ch₄-SO₄, and ADi-Ch₆-SO₄.

Each disaccharide was prepared on a larger scale as outlined under "Methods." The results of chemical analysis shown in Table I are in reasonable agreement with the assigned structures, except that the values obtained for the glucuronic acid content are variable and high. The uronic acid content was determined by the reaction with carbazole (16). This method was tested with menthol glucuronic (§igm) which was found to have a glucuronic yield of 48%. This sample was previously analyzed by Prestan, Davies, and Ogston (21) with a similar result by the carbazole method but gave almost exactly theoretical uronic acid content (52.6%) by the CO₂ method. The carbazole value is lower than the theoretical value of 53% and it seems
**Table I**

Analyses of unsaturated disaccharides obtained by chondroitinase digestion of CS

<table>
<thead>
<tr>
<th>Compound</th>
<th>Analysisa</th>
<th>Analysip Rglucuronsioaoidf</th>
<th>Hexosamine content</th>
<th>Uronic acid content</th>
<th>N-Acetyl hexosamine content</th>
<th>230μg x 10^-2</th>
<th>Rgluouronia acidf</th>
<th>Solvent A</th>
<th>Solvent B</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔDi-Ch</td>
<td>42.7</td>
<td>2.82 (0.70)</td>
<td>&lt;0.3</td>
<td>51.9</td>
<td>66.8</td>
<td>63.0</td>
<td>5.5</td>
<td>1.08</td>
<td>1.12</td>
</tr>
<tr>
<td>Expected for C4H10O4N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔDi-Ch-4-SO₄</td>
<td>30.1</td>
<td>2.37 (0.84)</td>
<td>5.79</td>
<td>36.3</td>
<td>59.5</td>
<td>7.8</td>
<td>5.6</td>
<td>0.85</td>
<td>0.99</td>
</tr>
<tr>
<td>ΔDi-Ch-6-SO₄</td>
<td>28.9</td>
<td>2.44 (0.89)</td>
<td>5.36</td>
<td>35.3</td>
<td>46.4</td>
<td>68.1</td>
<td>6.0</td>
<td>0.60</td>
<td>1.05</td>
</tr>
<tr>
<td>Expected for C4H12O4NSK₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* Percentage composition. Molar ratios to hexosamine are in parentheses.

b Determination by microanalytical combustion.

c Determined with free glucuronic acid as standard (16).

d Determined as the N-acetylglucosamine equivalent (17).

e This extinction coefficient is calculated from the hexosamine content.

f Rgluouronia acid is the chromatographic mobility relative to glucuronic acid.

Potassium salt.

**Table II**

CS composition of chick embryo cartilage

Carrier CS from 13-day chick embryos was digested with chondroitinase as outlined under "Methods." The disaccharides were separated by paper chromatography in Solvent A and separately assayed for total uronic acid content. The results are shown in Column A. Columns B and C give values obtained by Thorp and Dorfman (1) for intracellular and extracellular CS, respectively, and Column D gives values obtained by Mathews (22) for cartilage from 14-day embryos.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondroitin</td>
<td>20</td>
<td>20</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>Ch-4-SO₄</td>
<td>29</td>
<td>30</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Ch-6-SO₄</td>
<td>51</td>
<td>51</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

likely, therefore, that the high results with the disaccharides may be due to the unsaturation in the uronic acid moiety.

Reduction of the disaccharides with sodium borohydride at pH 8.0 lowered the hexosamine content by more than 90% in each case but had little effect on the uronic acid content. Unsaturation was further established by the characteristic absorption at 230 nm and by the complete disappearance of the color reaction with carbazole after treatment with aqueous bromine. Under identical conditions, bromine has no effect on the carbazole reaction of glucuronic acid.

The three disaccharides from a digest of CS prepared from 13-day chick embryo cartilage were separated by paper chromatography and separately eluted with water. The amount of each disaccharide was determined with the carbazole reaction. Table II shows these values together with those obtained by Thorp and Dorfman by a different method (1). Good agreement is evident.

The same procedure was applied to CS labeled with sulfate-35S and acetate-14C. Minced cartilage was incubated with the tracer for periods up to 5 hours and the incorporation of radioactivity into CS was measured. Table III shows that the incorporation of each isotope increased throughout the incubation period.

The CS isolated at the different times was digested with chondroitinase and analyzed by paper chromatography. Fig. 1 shows an autoradiograph of one chromatogram and the separation of the three disaccharides. A number of minor spots
are observed and some radioactivity remains at the origin, but the greatest portion of the radioactivity migrates as do the disaccharides. The distribution of the radioactivity is shown in Table III. It is evident that the time of incubation has no effect on the proportion of nonsulfated, 4-sulfated, and 6-sulfated disaccharides. Subsequently, incubation times of 2 or 4 hours were used. It is of interest to note that the distribution of disaccharide in Table III differs from that in Table II. The results in Table III reflect synthesis by cells of epiphyses of 13-day-old chicks while those in Table II reflect content of such epiphyses.

This technique was then applied to a study of the changes in polysaccharide patterns that occur during embryonic development. The effect of age of embryo on the proportion of radioactivity incorporated into each disaccharide was examined and the results are shown in Table IV. Condyles of femurs and tibias were obtained from embryos aged between 11 and 19 days and incubated with sulfate-35S and acetate-14C as before. In addition, whole tibias and femurs from 10-day embryos and entire hind limbs from 9-day embryos were minced and treated in the same way.

The results shown in Table IV indicate a shift of pattern with age. Most consistent and striking is the progressive increase in the proportion of Ch-4-SO4 with a concomitant decrease of Ch-6-SO4. The proportion of unsulfated material seems to show a striking change only after 16 days. The data for days 9 and 10 may be less reliable because well defined epiphyses have not yet developed. This change agrees with the data for days 9 and 10 may be less reliable because well defined epiphyses have not yet developed. This change agrees with the pattern observed by Mathews (22) by chemical analysis of chick cartilage. During the development period studied, the nature of the tissue undergoes considerable change—from a soft plastic lump of epiphyseal cartilage at 11 days to a hard condyle comprised of epiphysial plate, diaphysis, articular cartilage, and a zone of proliferative cells (23) by 19 days. Since there is evidence that these different zones have different activ-

**Table IV**

<table>
<thead>
<tr>
<th>Age of embryo</th>
<th>No. of embryos</th>
<th>% (C)4</th>
<th>% (C)14</th>
<th>% (SO4)4</th>
<th>% (SO4)14</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>43</td>
<td>18</td>
<td>24</td>
<td>23</td>
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<td>10</td>
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<td>11</td>
<td>51</td>
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<tr>
<td>12</td>
<td>40</td>
<td>19</td>
<td>32</td>
<td>32</td>
<td>38</td>
</tr>
<tr>
<td>13 (17-20)</td>
<td>20</td>
<td>41</td>
<td>43</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>26</td>
<td>20</td>
<td>41</td>
<td>42</td>
<td>39</td>
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<td>15</td>
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<td>13</td>
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<tr>
<td>17</td>
<td>9</td>
<td>16</td>
<td>55</td>
<td>55</td>
<td>20</td>
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<tr>
<td>18</td>
<td>7</td>
<td>14</td>
<td>62</td>
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<td>19</td>
<td>8</td>
<td>10</td>
<td>59</td>
<td>57</td>
<td>33</td>
</tr>
</tbody>
</table>

* Calculated as 14C of ΔDi-CH/14C of ΔDi-CH + ΔDi-Ch-4-SO4 + ΔDi-Ch-6-SO4.

**Discussion**

Chondroitinase from *P. vulgaris* degrades CS to a mixture of three unsaturated disaccharides. One of these, ADi-Ch, arises from nonsulfated CS and another, ΔDi-Ch-6-SO4, arises from CS-6-SO4. The remaining disaccharide, ΔDi-Ch-4-SO4, could arise from either Ch-4-SO4 or from dermatan sulfate because the asymmetry at carbon 5 of the uronic acid moiety is lost with the introduction of the double bond (25). In the present work it has been assumed that ΔDi-Ch-4-SO4 arises solely from Ch-4-SO4.

The manner in which these polysaccharides are associated with each other in the tissue is poorly understood. Previous studies on the mucopolysaccharides of cornea (26, 27), chick cartilage, and tadpole cartilage (22) indicate that CS may occur with low levels of sulfation. The ΔDi-Ch observed in this work, therefore, probably arises from undersulfated CS rather than chondroitin itself. However, since the *P. vulgaris* chondroitinase can also degrade hyaluronic acid to disaccharides, it is possible that the ΔDi-Ch arises in part from this polysaccharide. The product would then contain glucosamine instead of galactosamine. However, hyaluronic acid does not occur in epiphyses in appreciable quantities.

The two sulfated polysaccharides, Ch-4-SO4 and Ch-6-SO4, may occur as separate entities, produced perhaps by different
cell types, or the CS may contain both 4- and 6-linked ester sulfate in the same polysaccharide chain. Since there is no evidence for disulfated disaccharides in the digests, 4- and 6-linked ester sulfate do not occur on the same galactosamine residues in the tissue examined.

Mathews (22) has shown that the proportion of Ch-4-SO₄ present in chick cartilage increases during embryo growth and a similar change occurs in tadpole cartilage during metamorphosis. The technique outlined in this paper provides a useful tool for the study of these changes. Because the results obtained with acetate-¹⁴C and sulfate-³⁵S are identical, it is almost certain that the incorporation represents synthesis de novo of CS.

Davidson and Small have shown that the composition of rabbit nucleus pulposus changes as a function of age (28). The ratio of keratan sulfate to CS was found to increase with age, perhaps reflecting different half-lives for the polysaccharides. The results presented indicate that the progressive change with age is manifested not only by change in composition but by change in synthetic capacity as shown by the data in Tables III and IV. The suggestion of Meezan and Davidson (11) that the position of sulfation depends on whether the polysaccharide exists as a protein complex or free polysaccharide chains does not seem reasonable. Available data strongly suggest that CS biosynthesis occurs by way of the stepwise addition of monosaccharide units to a protein acceptor (4-6). It seems unlikely that the fractions isolated by Meezan and Davidson are the physiological acceptors since the material studied must have been derived overwhelmingly from extracellular matrix while sulfation of chondroitin sulfate undoubtedly occurs primarily intracellularly.

The biological significance of Ch-4-SO₄ and Ch-6-SO₄ is not yet clear. The predominance of Ch-6-SO₄ in species which do not form bone is striking, as is the presence of this polysaccharide in embryonic tissues. In humans, however, with maturation of costal cartilage, Ch-6-SO₄ is again the predominant form of chondroitin sulfate.

REFERENCES

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