Alterations of chondroitin sulfate synthesized by chick embryo cartilage cultured in the presence of 6-aminonicotinamide

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SUMMARY

Treatment of day-4 chick embryos with 6-aminonicotinamide (6-AN) impairs limb chondrogenesis and produces micromelia. Interference with limb cartilage development may be related to decreased NAD-dependent synthesis of ATP due to the fact that chondrogenesis is dependent upon anaerobic metabolism. To better understand the effect of 6-AN on chondrogenesis, isolated cartilage epiphyses from day-11 chick embryos were treated in vitro. Sulfate incorporation into total glycosaminoglycans of treated epiphyses was 30% of control. Incorporation of [3H]glucosamine was normal. Fractionation by gel chromatography showed that 40% of the glycosaminoglycans synthesized by treated cells had a molecular weight of less than 15000 compared with 5% of that of the control. A decrease in amount of chondroitin 6-sulfate, an increase of chondroitin 4-sulfate and no change in amount of unsulfated polysaccharide were observed. These results suggest that, upon exposure to 6-AN, chondrocytes produce shorter than normal chondroitin sulfate chains that are preferentially sulfated in the 4 position. Since endochondral bone formation plays an integral role in growth and development of the limb, a defect in production of chondroitin sulfate, a major constituent of cartilage matrix, appears to be involved in 6-AN-induced micromelia.

INTRODUCTION

The nicotinamide analogue, 6-aminonicotinamide (6-AN), is a teratogen that produces major effects in developing cartilage resulting in micromelia in the chick embryo (Seegmiller, Overman & Runner, 1972; Overman, Seegmiller & Runner, 1972). Within the cell, 6-AN forms 6-amino NAD, a competitive analogue of nicotinamide adenine dinucleotide. The dinucleotide analogue inhibits dehydrogenase reactions (e.g. glyceraldehyde-3-phosphate dehydro-

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3 The following abbreviations are used: 6-AN, 6-aminonicotinamide; GAG, glycosaminoglycan; CPC, cetylpyridinium chloride; ΔDi-6,4,-0, 6, 4 and non-sulfated disaccharides; PAPS, adenosine 3’-phosphoadenosyl-5’-phosphosulfate; NAD, nicotinamide adenine dinucleotide; NIC, nicotinamide.
genase) that are associated with synthesis of ATP, an essential molecule in developmental processes (Dietrich, Friedland & Kaplan, 1958; Coper & Neubert, 1964; Ritter, Scott & Wilson, 1975; Sheffield & Seegmiller, 1980). The analogue also inhibits utilization of sulfate to form sulfated proteoglycans by chondrocytes (Caplan, 1972; Overman et al. 1972; Seegmiller & Runner, 1974; Seegmiller, 1977), but the mechanism is not understood. It has not been determined, for example, whether inhibition of sulfate utilization results in undersulfation of an otherwise normal product, or decreased production of chondroitin sulfate. Therefore, experiments were undertaken to determine the specific molecular effects of 6-AN on synthesis of chondroitin sulfate. Evidence is presented in this report that 6-AN alters the molecular weight and position of sulfation of chondroitin sulfate, and possibly affects UDP-N-acetylhexosamine pools in chick limb chondrocytes.

MATERIALS AND METHODS

Tissue preparation and culture conditions

Epiphyses from tibias and femurs of day-11 chick embryos were incubated in groups of 14 at 37 °C and in 10 % CO₂ in air in 3 ml Waymouth's medium (Grand Island Biological). During initial incubation 6-AN was added at a final concentration of 72-9 μM. An equal number of control tissues were incubated in Waymouth's medium or in medium containing 6-AN plus 819 μM nicotinamide. After 14 h of treatment, 25 μCi/ml of [1-³H]D-glucosamine hydrochloride (3-2 Ci/mmmole, Radiochemical Center, Amersham), and 4 μCi/ml H₂³⁵SO₄ (600 mCi/mmmole, New England Nuclear) were added to the cultures and incubation was continued for 3 h. The tissues were quickly rinsed in 0-9 % saline, lyophilized, weighed, digested overnight with papain and treated for an additional 12 h in 0-5 M-NaOH at 4 °C. The solution was neutralized with HCl and the protein was precipitated with 5 % TCA at 4 °C. Unincorporated label was removed by dialysis against 25 mM-Na₂SO₄ followed by distilled water. Glycosaminoglycans (GAG) were precipitated with cetylpyridinium chloride (CPC) in 30 mM-NaCl. The precipitate was dissolved in 2 mM-NaCl, reprecipitated with ethanol and dissolved in water. Aliquots of 100 μl were removed for counting, determination of uronic acid (Bitter & Muir, 1962) and estimation of molecular weight and content of chondroitin sulfate isomers. Carrier chondroitin sulfate, prepared by papain digestion and CPC precipitation of chondroitin sulfate from day-13 chick embryo epiphyses, was added to the sample prior to chondroitinase treatment.

Determination of molecular weight of GAG by gel chromatography

Samples were separately applied to a 90 × 0-9 cm column of Sephacryl S-200 (Pharmacia Fine Chemicals). The column was eluted with 0-2 m-NaCl and
calibrated with chondroitin sulfate extracted from lamprey (5000 daltons) and sturgeon (30000 daltons) cartilage and with blue dextran, phenol red and the radioisotopes used as precursors. (Chondrotin sulfate standards were obtained from Dr M. B. Mathews.)

Analysis of chondroitinase digests of chondroitin sulfate

An aliquot containing carrier chondroitin sulfate was digested with chondroitinase ABC (Miles Research Products) in Tris buffer, pH 8 (Saito, Yamagata & Suzuki, 1968). The disaccharides were separated by descending paper chromatography (Whatmann 3MM) with isobutyric acid: 1 \( \text{N} \) ammonium hydroxide (5:3) for 48 h. The chromatographs were cut into 2 x 3 cm pieces; each piece was eluted with 1 ml of water and counted in Aquasol (New England Nuclear) in a Packard scintillation counter.

Determination of adenosine 3'-phosphoadenosyl-5'-phosphosulfate (PAPS)

Control and treated epiphyses were labeled with \( ^{35}\text{SO}_4^{2-} \) for 45 min, rinsed with saline, frozen and homogenized in 80 % ethanol. Labeling for 90 min did not alter the number of counts recovered in the ethanol extract (unpublished observations). After centrifugation at 12000 \( g \) an aliquot of the supernatant solution was applied to Whatmann 3MM chromatography paper and electrophoresis was performed in 30 mm pyridinium acetate at pH 5-3 for isolation of radioactive PAPS. Standard \( ^{35}\text{S}\)PAPS (New England Nuclear) was added to unlabeled homogenates, ethanol extracted and applied to the paper as a reference standard. The chromatographs were first scanned with a radiochromatograph scanner and the areas co-migrating with the standard were eluted with water and counted with a liquid scintillation system.

Determination of UDP-N-acetylhexosamine

Control and treated tissues were labeled with \(^{3}\text{H}\)glucosamine, homogenized in ethanol, and centrifuged as described above. An aliquot of the supernatant solution was applied to Whatmann 1 chromatography paper and allowed to develop 20 h in 1-butanol:glacial acetic acid:1 \( \text{N} \) ammonium hydroxide (2:3:1) for isolation of radioactive UDP-N-acetylglucosamine (Kim & Conrad, 1976). Standard \(^{3}\text{H}\)UDP-N-acetylglucosamine (New England Nuclear) was added to unlabeled homogenates and an aliquot of the ethanol soluble fraction was applied to the paper as a reference standard. The chromatographs were counted according to the method described above.

RESULTS

Synthesis and characterization of GAG

The activity of \(^{3}\text{H}\)glucosamine and \( ^{35}\text{SO}_4^{2-} \) incorporated into the CPC precipitate of controls was 3437 and 1463 d.p.m./\( \mu \)g uronic acid, respectively
Table 1. Effect of 6-AN on incorporation into the CPC-precipitate by day-11 chick limb cartilage

<table>
<thead>
<tr>
<th>Precursor</th>
<th>N</th>
<th>Control</th>
<th>6-AN + NIC</th>
<th>6-AN treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>^3H^Glucosamine</td>
<td>4</td>
<td>3437 ± 238</td>
<td>3152 ± 340</td>
<td>3106 ± 202</td>
</tr>
<tr>
<td>^35SO_4^-</td>
<td>4</td>
<td>1463 ± 78</td>
<td>1561 ± 181</td>
<td>433 ± 77*</td>
</tr>
</tbody>
</table>

* Significantly different from control, \( P < 0.001 \).

Rates of incorporation of both isotopes were linear over a 12-hour period. For both precursors greater than 90% of the total counts incorporated were digestible with chondroitinase ABC, yielding characteristic disaccharides.

Treatment with 6-AN did not significantly alter the rate of incorporation of \(^3H^\)glucosamine, but inhibited incorporation of sulfate by 70% (Table 1). Addition of nicotinamide to the treatment medium prevented the inhibition of sulfate incorporation.

The molecular weight of chondroitin sulfate was estimated by Sephacryl S-200 chromatography (Hopwood & Robinson, 1973). The radioactivity for the untreated control appeared at the void volume (Fig. 1), with an estimated molecular weight of 30000 daltons or greater. Approximately 5% of the total sample was smaller than 15000 daltons.

Although a major peak of radioactivity for treated cartilage was at the void volume (30000 daltons or greater), approximately 40% of the chondroitin sulfate was smaller than 15000 daltons (Fig. 1). These data suggest that although treated chondrocytes synthesize normal-size chondroitin sulfate chains, a significantly greater proportion of the newly synthesized chains were small when compared with control.

Chondroitinase digestion of \(^3H^\)glucosamine-labeled chondroitin sulfate followed by chromatography yielded 70% of the material in the 6-sulfated disaccharide (ΔDi-6) form (Fig. 2a). Thus day-11 chick cartilage produced predominantly chondroitin-6-sulfate and smaller quantities of the 4- and non-sulfated types as described by Robinson and Dorfman (1969). With \(^35SO_4^-\) labeled material (Fig. 2b), the relative proportions of the 6- and 4-sulfated forms were similar to those shown in Fig. 2a. Essentially no counts appeared in the ΔDi-0 form, and we did not identify the material appearing in the peak to the right of ΔDi-0 (Fig. 2b).

Relative to control, treatment with 6-AN decreased the amount of ΔDi-6, increased the relative amount of ΔDi-4 and produced no changes in proportion of unsulfated disaccharide (Fig. 2a). With the \(^35SO_4^-\)-labeled samples (Fig. 2b), both ΔDi-6 and ΔDi-4 were decreased by 6-AN. However the relative treatment effect was greater for ΔDi-6. Thus in addition to differing in size of newly synthesized chains, control and treated tissues differed according to
Alteration of chondroitin sulfate by 6-AN

Fig. 1. Molecular weight distribution of chondroitin sulfate as determined by Sephacryl S-200 gel filtration of \([3H]\)glucosamine-labeled CPC extracts. The labeled material was extracted from 14 chick epiphyses that were incubated 17 h in medium with or without 6-AN. The isotope was present in the culture medium during the last three h of incubation. The column was eluted with 0.2 m-NaCl and approximately 4 ml fractions were collected. The column was calibrated with standards of known molecular weight. Approximately 40 % of the treated (△—△) sample had a molecular weight less than 15 000 compared with 5 % for the control (○—○).

the position of sulfation, yet there was no increase in the proportion of unsulfated disaccharide in treated tissues.

**Incorporation of \(^{35}\text{SO}_4^=\) into PAPS**

To determine if the change in sulfation produced by 6-AN was associated with an effect on the production of the sulfate donor, PAPS, incorporation of \(^{35}\text{SO}_4^=\) into PAPS was monitored. The amount of \(^{35}\text{SO}_4^=\) incorporated into PAPS was standardized to the amount of cartilage on the basis uronic acid content. Control tissues incorporated 35 d.p.m./μg uronic acid into PAPS (Table 2). Negligible counts were recovered as APS. A standard amount of PAPS carried through the same procedure was recovered from the paper in 90 % yield as PAPS.

In two experiments, epiphyses treated with 6-AN incorporated \(^{35}\text{SO}_4^=\) into PAPS to give 40 and 34 d.p.m./μg uronic acid (Table 2). These values were 114 and 97 % of control, respectively. The amount of sulfate incorporated into APS was negligible. The results suggest that 6-AN does not affect the synthesis of PAPS.

**Incorporation of \([3H]\)glucosamine into UDP-N-acetylhexosamine**

Analysis of the tritium label in the ethanol extract was done to determine if the lack of inhibition of \([3H]\)glucosamine incorporation by 6-AN (Table 1)
Fig. 2(A). Paper chromatography of purified, chondroitinase-digested chondroitin sulfate synthesized in the presence or absence of 6-AN. The isotope used was [3H]glucosamine. Treatment and labeling conditions were similar to those described in Fig. 1. The solvent system for separating the disaccharides consisted of isobutyric acid and 1 N ammonium hydroxide (5:3). The migration of ΔDi-6, -4, and -0 disaccharide standards was measured under ultraviolet light. Relative to control (○—○), treatment with 6-AN (△——△) decreased the amount of ΔDi-6, increased the amount of ΔDi-4, and produced no change in amount of ΔDi-0.

Fig. 2(B). Chromatography of $^{35}$SO$_4$$^{2-}$-labeled, chondroitinase-digested chondroitin sulfate. Relative to control, treatment decreased the amounts of ΔDi-6 and ΔDi-4. Conditions were similar to those described in Fig. 2(A).

was due to a change in activity of the UDP-N-acetylhexosamine pool. After exposure to the isotope for 45 min, tritium counts in the ethanol extract co-chromatographed with the non-radioactive UDP-N-acetylglucosamine. Total counts recovered from control tissues were 491 and 728 d.p.m./μg uronic acid. Approximately 80% of the UDP-N-acetylglucosamine standard applied to the paper was recovered. From 6-AN-treated tissues, total counts co-chromato-
Alteration of chondroitin sulfate by 6-AN

Table 2. Effect of 6-AN on incorporation of $^{35}$SO$_4^{2-}$ into PAPS

<table>
<thead>
<tr>
<th>D.p.m./µg Uronic acid</th>
<th>Control</th>
<th>6-AN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>35</td>
<td>40</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>35</td>
<td>34</td>
</tr>
</tbody>
</table>

Table 3. Effect of 6-AN on incorporation of [3H]glucosamine into UDP-N-acetylatedhexosamine

<table>
<thead>
<tr>
<th>D.p.m./µg Uronic acid</th>
<th>Control</th>
<th>6-AN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>491</td>
<td>287</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>728</td>
<td>557</td>
</tr>
</tbody>
</table>

graphing with UDP-N-acetylglucosamine in two separate experiments were 287 and 557 d.p.m./µg uronic acid (Table 3). The method used did not distinguish UDP-N-acetylglucosamine from UDP-N-acetylgalactosamine. Although there was variation between experiments 1 and 2 in terms of total incorporation of [3H]glucosamine, the lower values for the treatment group (58 and 77 % of control, respectively) suggest that following 6-AN treatment either less added glucosamine was converted to UDP-N-acetylatedhexosamine or the pool size of UDP-N-acetylatedhexosamine was much decreased. The significance of these results awaits measurement of the UDP-N-acetylatedhexosamine pool.

DISCUSSION

Synthesis of chondroitin sulfate involves the stepwise addition of galactosamine and glucuronic acid residues to core protein (Roden & Schwartz, 1975) and concomitant sulfation of galactosamine in the 4- or 6-position (DeLuca, Richmond & Silbert, 1973).

The present study provides two lines of evidence that the inhibition of sulfate utilization by 6-AN (Table 1) represents decreased synthesis of GAG and not undersulfation of the product. First, the proportion of low molecular weight GAG chains produced by treated tissues was greater than that produced by control tissues (Fig. 1). Second, the amount of unsulfated disaccharide (ΔDi-0) was not increased by 6-AN treatment (Fig. 2). If 6-AN treatment had decreased the rate of sulfation without producing an effect on rate of chain elongation, then the treatment should have increased the amount of ΔDi-0 and should not have increased the proportion of shorter GAG chains.
The data are consistent with the hypothesis that 6-AN inhibits chain elongation through decreased rate of chain formation or initiation and/or premature termination of the chains. Presumably the availability of PAPS is unaffected by 6-AN (Table 2). If extensive sulfation in the 4-position occurs, as indicated by the relative increase in ΔDi-4 (Fig. 2), then those hexosamines, sulfated in the 4-position and appearing at the nonreducing ends of GAG chains, presumably would not serve as acceptors for UDP-glucuronic acid (Telser, Robinson & Dorfman, 1965). Hence, chain elongation might terminate prematurely if the sulfation process continued unabated as the chain forming process was inhibited.

The nearly normal levels of glucosamine incorporated into GAG (Table 1) could have several explanations, including changes in activity of the UDP-N-acetylhexosamine pool. Kim and Conrad (1976) provided evidence that the specific activity of the UDP-N-acetylhexosamine pool in chondrocytes depends on the relative contributions of fructose-6-P, derived from glucose, and exogenous glucosamine. They showed that the specific activity of intracellular UDP-N-acetylhexosamine increases with decreasing concentration of glucose in the culture medium. Apparently exogenous glucosamine enters the UDP-N-acetylhexosamine pool whether or not glucose is available.

Although the specific reactions inhibited are not known, there are several sites in the endogenous pathway for synthesis of UDP-N-acetylhexosamine that may be blocked directly or indirectly by 6-AN. For example, decreased levels of ATP in the chondrocyte (Sheffield and Seegmiller, 1980) could block the conversion of glucose to glucose-6-phosphate and thus decrease the amount of UDP-N-acetylhexosamine produced. In other words the size of the UDP-N-acetylhexosamine pool may be decreased while incorporation of [3H]glucosamine by the exogenous pathway is unaffected by 6-AN. The specific activity of label in the UDP-N-acetylhexosamine pool, relative to control, might then be increased due to a relative increase in utilization of exogenous [3H]glucosamine. The net effect of increased specific activity would be an increase in the relative rate of incorporation of [3H]glucosamine into chondroitin. Thus the rate of GAG synthesis could be decreased despite a constant level of [3H]glucosamine incorporation.

Although the present study does not resolve the question concerning the normal incorporation rate for [3H]glucosamine following treatment (Table 1), the fact that radioactivity in UDP-N-acetylhexosamine was decreased by 6-AN (Table 3) suggests that synthesis of UDP-N-acetylhexosamine may have been altered. Because [35S] in PAPS was not decreased (Table 2), reactions for making PAPS (D'Abramo & Lippmann, 1957) may not have been inhibited, despite the low levels of ATP (Sheffield & Seegmiller, 1980).

Schubert & Lacorbiere (1976) reported that in myogenic cell cultures, 6-AN increases glucosamine incorporation and decreases sulfate incorporation. They interpreted the increased glucosamine utilization as an increase in synthesis of GAG. They proposed that 6-AN had induced a 'phenotypic transformation of
myogenic cells to cells with the characteristics expected of chondrocytes. If 6-AN increases the specific activity of the UDP-N-acetylhexosamine pool, then their results would be due, not to a transformation of cell type, but to increased availability of exogenous glucosamine.

Further determinations of pool size will be necessary to resolve the question of effect of 6-AN on cartilage matrix synthesis. Nevertheless, the alterations in synthesis of chondroitin sulfate observed in the present study appear to be involved in the production of 6-AN induced micromelia.

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REFERENCES


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