The Effects of 6-Diazo-5-oxo-L-norleucine, a Glutamine Analogue, on the Structure of the Major Cartilage Proteoglycan Synthesized by Cultured Chondrocytes*

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The glutamine analogue, 6-diazo-5-oxo-L-norleucine (DON), has been shown to inhibit irreversibly the synthesis of both purines and hexosamines by blocking the glutamine-dependent steps in their respective metabolic pathways (1). The effects of DON on hexosamine production are caused by the specific inhibition of a transamidase which catalyzes the transfer of the amide nitrogen from L-glutamine to fructose 6-phosphate to form glucosamine 6-phosphate (2). Consequently, DON has been shown to impair the normal formation of cartilage matrix and to inhibit the synthesis of glycosaminoglycans in avian and mammalian tissues (3–8). However, in spite of its extensive usage, the mechanisms by which DON interferes with chondrogenesis are poorly understood. For example, it has not been determined whether the inhibition of [35S]sulfate incorporation observed in the above studies is a result of undersulfation of an otherwise normal proteoglycan or due to the production of an abnormal molecule.

In this study, we used suspension cultures of chick embryo sternal cells to analyze the effects of DON on the structure of the major cartilage proteoglycan. Under normal conditions, this molecule (Mw ~ 2.5 106) consists of a core protein (Mw ~ 370,000) to which numerous chondroitin sulfate, keratan sulfate, and N- and O-linked oligosaccharides are attached (for review, see Ref. 9). In contrast, we found that incubation in the presence of DON in the synthesis and secretion of a structurally altered proteoglycan (Mw ~ 400,000) that was capable of interacting with hyaluronic acid and link proteins, but which contained considerably fewer and shorter chondroitin sulfate chains and virtually no keratan sulfate chains as compared to proteoglycan from control cultures.

EXPERIMENTAL PROCEDURES

Materials—DON (Lot 104F-0486) was purchased from Sigma, Protein A-Sepharose and prepacked PD-10 columns from Pharmacia Fine Chemicals, carrier-free Na235SO4 (>40 Ci/mg) from ICN Radiochemicals, L-[^3H]proline (>250 mCi/mmol), L-[35S]sulfate (10–30 Ci/mmol), and D-[6-3H]glucosamine HCl (33 Ci/mmol) from Amersham Corp., and keratanase from Miles Scientific. The source of all other materials was the same as described previously (10, 11).

Isolation and Incubation of Cartilage Cells—Cells were isolated and incubated as described previously (10) with the exception that the Krebs medium was supplemented to 0.3 mM with Na2SO4 to minimize the production of undersulfated glycosaminoglycans (12). In a typical experiment, 4–6 106 cells were obtained from 100 17-day chick embryo sternae, and they were incubated at a concentration of 106 cells/ml. In every experiment, a portion of the cells was preincubated in the absence and a portion in the presence of DON for 30 min prior to the addition of either [3H]proline (8 mCi/ml), [35S]sulfate, or [3H]glucosamine (40 mCi/ml each). In one experiment, cells were double-labeled with [3H]serine (20 mCi/ml) and [35S]sulfate (5 mCi/ml). Incubations were continued for 2 h, and labeling was terminated by the addition of excess unlabeled precursor (l-proline, l-serine, and d-glucosamine HCl, 0.5 mg/ml each; Na2SO4, 14 mg/ml). After a 1-h chase, protease inhibitors were added to a final concentration of 10 μM each Na2EDTA and N-ethylmaleimide and 1 mM phenylmeth-
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**Extraction of Newly Synthesized Proteoglycans**—Briefly, the medium was made 4 M in guanidine HCl by the addition of solid (330 mg/ml) and chromatographed on columns of Sephadex G-50 or PD-10. The cell pellet was resuspended in 2 ml of 4% CHAPS in 50 mM sodium acetate, pH 5.8, containing protease inhibitors as above. After stirring for 1 h at 4 °C, an equal volume of 8 M guanidine HCl in the same buffer was added, and extraction was continued overnight. The cell extract was centrifuged (10,000 × g for 20 min), and the supernatant (representing all of the labeled material) was chromatographed as above. The macromolecules from the medium and cell extracts which eluted in the void volume were used for all subsequent experiments.

**Analytical Procedures and Enzyme Digestions**—The following procedures were performed as described previously: gel filtration on Sephacryl columns, chondroitinase ABC and AC II digestions, DEAE-Sepharose chromatography, alkaline borohydride digestion and CsCl density gradient ultracentrifugation (14); SDS-PAGE and bacterial collagenase digestion (10); proteoglycan aggregation (15); analysis of chondroitinase ABC-digested glycosaminoglycan chains (16); keratanase digestion (17) and papain digestion (18). Further details for individual experiments are given under "Results" and in the figure legends and table headings.

**Immunoprecipitation**—The A2 antiserum raised against the major chick cartilage proteoglycan core protein was used; the preparation and specificity of this antiserum have been previously described (17). This antiserum recognizes native and chondroitinase ABC-digested proteoglycan as well as the protein core (17). For the present experiments, duplicate medium samples (1 ml) were dialyzed against RIPA buffer (50 mM Tris, 0.15 M NaCl, 1% sodium deoxycholate, 0.05% SDS, pH 7.2) at 4 °C before addition of 35S sulfate to either the presence or absence of DON (Fig. 1A). Samples were centrifuged for 90 min at 4 °C on a shaker and then mixed with 100 μl of Protein A-Sepharose (prepared by dissolving 0.5 M sodium acetate, pH 8.0, in 0.01 pg/ml; 0.01 pg/ml; 0.01 pg/ml saline) and incubated for an additional 40 min. Samples were centrifuged at 13,000 × g for 3 min; the supernatants were decanted and pellets were rinsed five times with buffer, once with phosphate-buffered saline, and then boiled for 5 min in 200 μl of SDS sample buffer (10) containing 5% 1,4-dithiothreitol. Samples were centrifuged again, and an aliquot of the supernatant was analyzed by SDS-PAGE. Under these conditions, 5–10% of the total [35S] sulfate was precipitated; no attempt was made to achieve quantitative precipitation.

**RESULTS**

**Effects of DON on the Synthesis of Cartilage Macromolecules**—In initial experiments using DON at various concentrations, the incorporation of [35S] sulfate into total (cell plus medium) macromolecules decreased in a dose-dependent manner (Fig. 1A). This inhibitory effect became evident at concentrations of DON > 0.01 pg/ml (0.06 μM) and reached a level of 45–50% of control at 1 μg/ml under these conditions, the ED50 was ~0.03 pg/ml (0.2 μM). Interestingly, no further inhibition occurred up to 100 μg/ml DON. In other experiments, however, incorporation of [35S] sulfate fell as low as 35–40% of control. Similar variations have been previously reported (5). In contrast, incorporation of [3,5]H]proline, [3H]serine, and [3H]glucosamine increased to 134, 130, and 128% of control, respectively. There was no significant effect on secretion, however, since 75–85% of the macromolecular [35S] sulfate and [3H]glucosamine was found in the medium in either the presence or absence of DON (Fig. 1A, inset). Moreover, in an experiment using 5 μg/ml (0.03 mM) DON, the presence of 500 μg/ml (3.4 mM) L-glutamine partially restored [35S] sulfate incorporation (67% of control) while 500 μg/ml D-glucosamine HCl (2.3 mM) nearly completely restored it (93% of control).

Analysis of the DON-treated samples by SDS-PAGE (Fig. 1B) showed that the decrease in [35S] sulfate incorporation was accompanied by the appearance in both cell and medium samples of a component of Mr = ~400,000 which was not present in control samples. This component was most prevalent.

*The molecular weight estimate is based on the mobility of a laminin subunit which has not been completely characterized. Therefore, the Mr ascribed to the altered proteoglycan molecule should not be taken as a definitive value.*

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**FIG. 1.** Dose-dependent effect of DON on the synthesis and secretion of [35S] sulfate-labeled macromolecules. Duplicate suspension cultures of sternal chondrocytes containing 3.4 × 10^5 cells were labeled in the absence or presence of DON at the designated concentrations as described under "Experimental Procedures." A, the total [35S]-labeled macromolecules in cells and medium were measured as the amount of radioactivity eluting in the void volume of a PD-10 column, and the sum was plotted as the percent of the radioactivity in the control sample (shown on the ordinate) versus the DON concentration. The inset shows the relative distribution of [35S]-labeled macromolecules in the cell and medium plotted as a percent of their sum versus the DON concentration. The control values are shown on the ordinate. The values are the mean of at least duplicate analyses.

B, fluorescence autoradiographic analysis of medium and cell samples at various DON concentrations: lanes 1 and 7, 0 μg/ml; lanes 2 and 8, 0.01 μg/ml; lanes 3 and 9, 0.1 μg/ml; lanes 4 and 10, 1 μg/ml; lanes 5 and 11, 10 μg/ml; lanes 6 and 12, 100 μg/ml. Aliquots containing 10,000 cpm (representing 0.3–1% of medium and 3–5% of cell samples) were precipitated with 0.1 volume of 100% trichloroacetic acid or with 3 volumes of 95% ethanol, 1.3% potassium acetate and analyzed by SDS-PAGE on a 6.5% slab gel under reducing conditions.

The migration positions of [35S]methionine-labeled laminin subunits (Mr = 400,000 and 220,000) and entactin (Mr = 150,000) from the rat embryo parietal yolk sac are indicated on the left.
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Effects of DON on Proteoglycan Charge, Size, and Buoyant Density—To examine further the molecular changes in the major cartilage proteoglycan, [35S]sulfate-labeled samples were chromatographed on DEAE-Sepharose. In the control sample (Fig. 3A), a single symmetrical peak representing the majority of the radioactivity eluted between 0.45 and 0.55 M NaCl. In the presence of DON (Fig. 3B), however, the peak eluting in this position (DON-B) now contained only two-thirds of the recovered radioactivity; a second peak (DON-A), representing one-third of the recovered radioactivity, was clearly resolved eluting between 0.3 and 0.4 M NaCl.

In one experiment, individual fractions from each of these peaks (cf. Fig. 3, open circles) were analyzed by gel filtration and SDS-PAGE. In the control sample, over 90% of the total 35S-labeled proteoglycan was excluded from a Sepharose CL-4B column (Fig. 4A) and did not penetrate a 6.5% polyacryl-
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Sepharose CL-4B column with a $K_v = 0.32$ (Fig. 4B, closed squares) and migrated into a 6.5% polyacrylamide gel (Fig. 4B, inset A) to the approximate position of the large subunit of laminin (cf. Fig. 1). This component eluted from a Sepharose CL-2B column with a $K_v = 0.55$ (not shown).

After pooling DEAE-Sephascel fractions (cf. Fig. 3), aliquots were analyzed by isopycnic CsCl density gradient ultracentrifugation. In the control (Fig. 5A) and DON-B samples (Fig. 5B, closed circles), the majority (~75% and 68%, respectively) sedimented to the bottom one-quarter of the gradient, whereas only ~27% of the macromolecules in DON-A sedimented at this density (Fig. 5B, closed squares), suggesting a lower glycosaminoglycan to protein ratio.

In a separate experiment, aliquots of each DEAE-Sephascel pool were dialyzed under associative conditions with 3 mg of bovine nasal cartilage proteoglycan aggregate and subsequently analyzed by Sepharose CL-2B chromatography. In all samples, >85% of the $^{35}$S-labeled macromolecules eluted in the void volume of the column (not shown) showing that DON did not interfere with the ability of the newly synthesized proteoglycans to interact with hyaluronic acid and link protein.

Taken together, the results indicate that incubation with DON led to a significant alteration in the charge, size, and buoyant density of a substantial portion of the newly synthesized major cartilage proteoglycan (DON-A) but did not affect its ability to be secreted or to interact with hyaluronic acid.
Effects of DON on Glycosaminoglycan Size and Charge—To determine whether the decreased hydrodynamic size and density of the DON-treated proteoglycan was due to a decrease in the average size of the glycosaminoglycan chains, pooled peaks from DEAE-Sephacel columns (cf. Fig. 3) were subjected to alkaline borohydride treatment, and the released products were analyzed by analytical Sepharose CL-6B chromatography. In the control sample (Fig. 6A), the 35S-labeled glycosaminoglycans eluted as a polydisperse peak with a \( K_{av} \approx 0.5 \), corresponding to an average \( M_r \approx 20,000 \) (20). In contrast, DON-B and DON-A samples (Fig. 6B) contained a population of smaller glycosaminoglycans (\( K_{av} = 0.7-0.95, M_r = 1,000-5,000 \)) representing about 20 and 80%, respectively, of the total radioactivity. Taken together, these results indicate that the reduction in the hydrodynamic size and buoyant density of the major cartilage proteoglycan synthesized in the presence of DON was due, at least in part, to the presence of relatively short glycosaminoglycan chains.

To investigate further the nature of the glycosaminoglycan chains, pooled samples from DEAE-Sephacel columns were incubated in the absence or presence of chondroitinase ABC, cleaved with alkaline borohydride, and the products analyzed by Sephadex G-50 chromatography. In control samples, 90% of the undigested sample was excluded from the column (Fig. 7A, closed circles), whereas most of the chondroitinase ABC-digested material eluted as disaccharides (Fig. 7A, open circles); only a small proportion, accounting for ~6% of the total digest, eluted as tetrasaccharides or larger. In DON-B, ~80% of the undigested sample was excluded, and the remainder eluted in a broad range from \( K_{av} = 0.15-0.7 \) (Fig. 7B, closed circles). After digestion, ~11% of the sample eluted as tetrasaccharides or larger, and the rest eluted as disaccharides (Fig. 7B, open circles). In contrast, only ~10% of undigested DON-A was excluded. The majority of the glycosaminoglycan chains penetrated the column, with 75% eluting with \( K_{av} = 0.3-0.65 \) (\( M_r = 2,000-5,000 \)) and 15% eluting with \( K_{av} = 0.65-0.95 \) (\( M_r \leq 2,000 \)) (Fig. 7C, closed circles). Some of this material was completely degraded to disaccharides by chondroitinase ABC, but a significant proportion (~45%) now eluted as tetrasaccharides or larger (Fig. 7C, open circles). The latter peak may contain the chondroitinase ABC-resistant linkage region oligosaccharide of chondroitin sulfate (cf. Fig. 9, D-F). Note also that a small amount of chondroitinase ABC-resistant radioactivity in control and DON-B samples, but not in DON-A, eluted in the void volume (Fig. 7, open circles). This material may represent keratan sulfate (cf. Fig. 9, D-F, and Table II).

To determine whether DON also caused a decrease in the degree of sulfation, proteoglycan samples labeled with [3H] glucosamine and purified by DEAE-Sephacel were digested with chondroitinase ABC, and the products were analyzed by paper chromatography (16). The data presented in Table I show that whereas the ratio of 4- to 6-sulfated disaccharides

![Fig. 6. Sepharose CL-6B elution profiles of glycosaminoglycans from cultures incubated in either the absence (A) or presence (B) of DON. Pooled material from DEAE-Sephacel columns (cf. Fig. 3) was dialyzed against deionized water and lyophilized. After treatment with alkaline borohydride, samples were chromatographed on an analytical 0.6 x 90-cm column of Sepharose CL-6B and eluted with 0.5 M sodium acetate, pH 7.0 (14).](https://example.com/fig6)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Origin</th>
<th>6-Sulfated</th>
<th>4-Sulfated</th>
<th>0-Sulfated</th>
<th>3H cpm x 10^-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.6 (7)</td>
<td>5.5 (16)</td>
<td>25.0 (70)</td>
<td>2.5 (7)</td>
<td></td>
</tr>
<tr>
<td>DON</td>
<td>11.0 (30)</td>
<td>4.0 (11)</td>
<td>21.0 (58)</td>
<td>0.3 (1)</td>
<td></td>
</tr>
</tbody>
</table>

*Number in parentheses represents percent of total recovered radioactivity.

![Table 1](https://example.com/table1)
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was similar (4.5 in control versus 5.2 in DON), the DON-treated sample actually contained significantly less unsulfated disaccharide than control. This result confirmed the fact that the inhibition of \[^{35}S\]sulfate incorporation is not due to undersulfation of chondroitin sulfate chains. Note that a relatively large portion of the DON-treated sample remained at the origin. This material contained the chondroitinase ABC-resistant glycosaminoglycans and any oligosaccharides still attached to the protein core (not shown).

Characterization of the Chondroitinase ABC-resistant Material—In order to characterize better the nature of the glycosaminoglycan chains synthesized in the presence of DON, purified \[^{35}S\]sulfate-labeled proteoglycans from control, DON-B, and DON-A samples were sequentially digested with chondroitinase ABC, chondroitinase AC II, and keratanase according to the protocol outlined in Fig. 8. The products of each digestion were subsequently monitored by chromatography on an analytical Sephadex G-50 column (Fig. 9).

The purified intact proteoglycans were initially digested with chondroitinase ABC in the presence of protease inhibitors (21). In the control and DON-B samples, only 15 and 19%, respectively, of the label was excluded from the column, whereas 57% of the label in DON-A was excluded (Fig. 9, A–C). This observation corroborated the paper chromatographic results and supports the concept that the chondroitin sulfate chains in DON-A were shorter than those in either DON-B or control, i.e. the shorter the chondroitin sulfate chains, the larger the percentage of the total protein core-linked \[^{35}S\]sulfate or \[^{3}H\]glucosamine which will be resistant to chondroitinase ABC. Analysis of the excluded material by SDS-PAGE showed the presence in all samples of a component migrating in the molecular weight range of 400 kDa (not shown; cf. Fig. 10).

The nature of the chondroitinase ABC-resistant material was examined by a variety of procedures. The void volume

![Fig. 7. Sephadex G-50 elution profiles of glycosaminoglycans from cultures incubated in either the absence (A) or presence (B and C) of DON before (●) and after (○) chondroitinase ABC digestion. Pooled samples were prepared as described in the legend to Fig. 6. After incubation in the absence or presence of chondroitinase ABC (0.5 unit/ml) in enriched Tris-HCl (16) containing 250 μg of shark cartilage chondroitin sulfate for 1 h at 37 °C, samples were boiled for 2 min and subjected to alkaline borohydride treatment for 30 h at 45 °C. Samples were neutralized with glacial acetic acid, applied to an analytical 0.6 × 90-cm column of Sephadex G-50, and eluted with the buffer described in the legend to Fig. 4. The elution position of disaccharides (Di) and tetrasaccharides (Tetra) generated from chondroitinase ABC or testicular hyaluronidase digestion of chondroitin sulfate and hyaluronic acid, respectively, is indicated in A.](image)

![Fig. 8. Scheme for the sequential digestion of purified \[^{35}S\]sulfate-labeled proteoglycans by chondroitinase ABC, chondroitinase AC II, and keratanase. The products after each digestion were monitored by analytical Sephadex G-50 chromatography (cf. Fig. 9), and the radioactivity eluting in the void volume (V₀) and the included elution volume (Vₑ) was quantitated (cf. Table II). The identity of these products is shown in the boxes.](image)
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Fig. 9. Sephadex G-50 elution profiles of sequential chondroitinase digests of intact \(^{35}S\) sulfate-labeled proteoglycans from cultures incubated in either the absence (A, D) or presence (B, C, E, F) of DON. The digestion scheme is shown in Fig. 8. Pooled DEAE-Sephacel samples were dialyzed against deionized water and lyophilized. After incubation with chondroitinase ABC in the presence of protease inhibitors (21), samples were chromatographed on Sephadex G-50 (A−C). Material eluting the void volume of the Sephadex G-50 column was pooled, dialyzed against deionized water, and lyophilized. Samples were resuspended in buffer, incubated in the absence (O) or presence (C) of chondroitinase AC II (0.5 unit/ml), and subsequently treated with alkaline borohydride as described in the legend to Fig. 7 (D−F).

Material (Fig. 9, A−C) was divided; half was digested with chondroitinase AC II, and then both halves were treated with alkaline borohydride to release the products from the protein core (cf. Fig. 8). The results show that after alkaline borohydride treatment alone, a portion of the radioactivity eluted with a \(K_v\) ~ 0.7 (Fig. 9, D−F, closed circles). In a manner identical to that shown previously (cf. Fig. 7), this peak represented a significantly greater proportion of the radioactivity in the DON samples than in the control. After digestion with chondroitinase AC II (Fig. 9, D−F, open circles), the radioactivity in the included volume now eluted with \(K_v\) ~ 0.85 indicating that it was completely susceptible to the enzyme. Since it is known that chondroitinase ABC digestion can leave attached to the linkage region a modified sulfated disaccharide which is susceptible to chondroitinase AC II (22), it is reasonable to conclude that the material eluting with \(K_v\) ~ 0.7 represents the chondroitin sulfate linkage region oligosaccharide plus at least one sulfated disaccharide (\(K_v\) ~ 0.85) (cf. "Discussion").

A portion of the radioactivity in each sample still eluted in the void volume after sequential chondroitinase ABC and chondroitinase AC II digestions (Fig. 9, D−F, open circles). In the case of the control and DON-B samples, this material was extensively degraded by subsequent keratanase digestion (not shown); there was insufficient radioactivity in the DON-A sample to digest. These results confirmed the identity of the chondroitinase-resistant material eluting in the excluded volume as keratan sulfate.

The quantitation of the \(^{35}S\) sulfate-labeled glycosaminoglycans synthesized in the absence or presence of DON is summarized in Table II. It can be seen that essentially all of the radioactivity can be accounted for as either chondroitin sulfate or keratan sulfate. In every sample, the majority of the radioactivity was sensitive to chondroitinase digestion. In the control and DON-B samples, 7 and 5%, respectively, of the radioactivity was sensitive to keratanase, a value similar to that reported previously for 17-day chick sternal proteogly-
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TABLE II
Quantitation of [35S]sulfate-labeled glycosaminoglycan chains

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>DON-B</th>
<th>DON-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondroitinase ABC sensitive</td>
<td>85</td>
<td>81</td>
<td>43</td>
</tr>
<tr>
<td>Chondroitinase AC II sensitive</td>
<td>7</td>
<td>13</td>
<td>54</td>
</tr>
<tr>
<td>Keratanase sensitive</td>
<td>5</td>
<td>7</td>
<td>&lt;1†</td>
</tr>
</tbody>
</table>

* Percent of total cpm recovered in the included volume of Sephadex G-50 column.
† Estimated. Keratanase digestion not done because of insufficient remaining radioactivity.

Control sample, chondroitinase increased the mobility of the intact proteoglycan from the top of the gel (lane 1) to a migration position slightly more retarded than that of the 400-kDa component (compare lanes 2 and 3). In contrast, chondroitinase ABC digestion had a minimal effect on the mobility of the 400-kDa component (compare lanes 3 and 4). The observed difference in mobility of the proteoglycan between lanes 2 and 4 could represent the presence in the former of keratan sulfate and/or oligosaccharide units which are chondroitinase ABC-resistant.

TABLE III
Substitution of core protein by sulfated glycosaminoglycan chains

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total radioactivity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[35S]Sulfate</td>
</tr>
<tr>
<td>1. Sephadex G-50, V0</td>
<td>Control</td>
</tr>
<tr>
<td>2. DEAE-Sephacel (0.3-0.55 mol NaCl)</td>
<td>Control</td>
</tr>
<tr>
<td>3. Digest, DEAE-Sephacel</td>
<td>Control</td>
</tr>
</tbody>
</table>

* Corrected for total recovery from the various procedures.
† Number in parentheses is percent of [3H] bound to DEAE-Sephacel after papain digestion. This number represents the percent of the total [3H]serine in proteoglycan which is linked to glycosaminoglycan.

Effects of DON on the Substitution of Protein Core by Sulfated Glycosaminoglycan Chains—Since it was possible that the observed effects of DON on proteoglycan mass, charge, and density could be due to a decrease in the number of glycosaminoglycan chains in addition to the observed effect on their size, an estimation of the degree of substitution of protein core serine residues by side chains was made using a protocol similar to that described by Schwartz (18). Proteoglycans double-labeled with [3H]serine and [3S]sulfate in the absence or presence of DON were purified by DEAE-Sephacel chromatography (not shown). Interestingly, in the presence of DON the [3H] label in the proteoglycan region eluted as a single peak in the same position as DON-A, whereas the [3S] label eluted in a bimodal fashion as shown previously (cf. Fig. 3B). The significance of this observation will be discussed later (cf. “Discussion”). One-third of the total [3H]serine eluted with the [3S]sulfate, and the [3S]/[H] ratio in the pooled
material was 0.26 for the control and 0.08 for the DON-treated sample (Table III), suggesting that the latter contained only ~30% of the sulfated glycosaminoglycan/protein as the former.

To estimate the fraction of serine linked to glycosaminoglycan, the purified proteoglycan was digested with papain (18). Inasmuch as it has been previously shown that the chondroitin sulfate chains synthesized in the presence of DON were significantly shorter than control chains (cf. Figs. 6 and 7), the papain digests were chromatographed on DEAE-Sephacel rather than on a gel filtration column. The bound \(^3H\) label was taken to represent serine substituted with glycosaminoglycan chains, and the unbound \(^3H\) label was taken to represent unsubstituted serine. The results (Table III) showed that 34% of the \(^3H\)serine bound to DON-B in the control sample compared to 28% in the DON-treated sample. Thus, under control conditions more than one-third of the labeled serine residues in control proteoglycans was substituted with glycosaminoglycan chains, a value similar to that observed previously in chick cartilage proteoglycans (24, 25).

In the presence of DON, however, the protein core contained only about half as many serine-linked glycosaminoglycans. These results indicate that a decrease in the number of glycosaminoglycan chains significantly contributed to the observed changes in proteoglycan size, charge, and buoyant density.

**DISCUSSION**

The results of this investigation demonstrate for the first time that perturbation of glutamine metabolism induced by DON caused a dramatic and rapid alteration in the structure of the major cartilage proteoglycan synthesized by freshly isolated chick chondrocytes. This alteration was manifested by the production of a molecule with significantly lower apparent M, (~400,000 versus ~2,500,000), buoyant density, and anionic charge. Specifically, the modified proteoglycan contained only ~50% of the normal complement of chondroitin sulfate chains, the attached chains were markedly smaller than normal (M, = 1,000–5,000 versus 20,000), and the number and/or size of the keratan sulfate chains was dramatically reduced. However, our results suggest that the protein core was not significantly affected since the altered proteoglycan could be immunoprecipitated by an antiserum directed against this domain and because the hyaluronic acid binding region remained functional.

The evidence presented here indicates that the inhibitory effect of DON on \(^35S\)sulfate incorporation was mediated by a rapid decrease in glycosaminoglycan synthesis and elongation leading to the production of chondroitin sulfate chains which were, on the average, only about one-eighth the size of normal chains. Indeed, about one-sixth of the affected chains was as small as a hexasaccharide, having the structure GlcA-GalNAc(SO_4)_2-GlcA-Gal-Gal-Xyl. The mechanisms which govern the length of glycosaminoglycan chains have not yet been elucidated. However, it has been suggested that chondroitin sulfate chain length is dependent upon the ratio between available acceptor (protein core) and the metabolic precursors or the chain-elongating transferase enzymes (26). Therefore, it is reasonable to conclude from our data that the occurrence of short chondroitin sulfate chains in DON-A is ultimately due to the relative lack of endogenous hexosamine, specifically UDP-N-acetylhexosamine, caused by DON treatment.

This is confirmed by our observation that addition of exogenous \(^3H\)glucosamine and, to a lesser extent, \(^1H\)-glutamine was capable of reversing the effects of DON. Moreover, the relative lack of endogenous hexosamine would explain the apparent increase in \(^3H\)glucosamine incorporation in the DON-treated chondrocytes as merely reflecting an increase in its intracellular specific activity and not representing an increase in glycosaminoglycan or oligosaccharide synthesis.

It was surprising to find that incubation of chondrocytes with DON under the reported conditions resulted in the synthesis of two distinct proteoglycan subpopulations: altered (DON-A) and largely normal (DON-B). One might have expected a much more polydisperse distribution of proteoglycan monomers containing progressively shorter glycosaminoglycan chains. The fact that we found many small chondroitin sulfate chains on the affected proteoglycan rather than just a few normal-sized chains is consistent with the postulate that at a given time point, many chains are being polymerized concomitantly on a single protein core (27). Our results would indicate, therefore, that at some point during DON treatment there is a rapid inhibition of chondroitin sulfate elongation due to the depletion of endogenous hexosamine. Thus, DON-B was most likely synthesized early in the incubation period when the effects of DON on the hexosamine pool were minimal. Conversely, DON-A was most likely synthesized at times when the effects of DON were maximal. This postulate is supported by the fact that samples labeled with \(^3H\)serine or \(^3H\)proline showed little DON-B by DEAE-Sephacel chromatography or SDS-PAGE, respectively, unlike those labeled with \(^35S\)sulfate, i.e. \(^35S\)sulfate would label nearly completed molecules whereas amino acids would only label molecules in the earlier stages of synthesis. Moreover, preliminary experiments (to be presented elsewhere) show that increasing the time of preincubation in the presence of DON led to a further inhibition of \(^35S\)sulfate incorporation and to a marked increase in the ratio of \(^35S\)sulfate-labeled DON-A/(DON-A + DON-B). Thus, we conclude that most of the de novo synthesized major cartilage proteoglycan was modified in the presence of DON.

Despite its significantly altered structure, proteoglycan synthesized in the presence of DON appeared to be secreted normally. This observation differs from previous studies which report that underglycosylated (18) or undersulfated
production of a proteoglycan containing the same number of chains that are shorter than normal, but the structure of the uronic acid before glycosaminoglycan chains have been added cartilage protein core precursor is able to interact with hyal-precursors (for review, see Ref. 9). These results are thus incubation of chondrocytes with P-D-xyloside results in the resultant proteoglycan is unknown (30). On the other hand, three times larger than that synthesized in the presence of these drugs (6-8,25,31,32) probably results directly from the elaboration of an abnormal cartilage matrix characterized by interference with the availability of substrate for the chain synthesis of UDP-N-acetylhexosamine elongation system; 6-aminonicotinamide inhibits the synthe-

It is useful to compare the structural alterations in cartilage proteoglycan reported here to those produced by 6-amonicotinamide (a nicotinamide analogue) or β-D-xyloside (a chondroitin sulfate acceptor analogue). Exposure of chondrocytes to the former leads to the production of chondroitin sulfate chains that are shorter than normal, but the structure of the resulted proteoglycan is unknown (30). On the other hand, incubation of chondrocytes with β-D-xyloside results in the production of a proteoglycan containing the same number of keratan sulfate chains, but only ~40% as many chondroitin sulfate chains (18, 25); the sizes of both chain types are 25% shorter than normal (25). The estimated M, of the proteoglycan synthesized in the presence of xyloside is 1.2 × 10^6 (25), three times larger than that synthesized in the presence of DON. With both of these analogues, as with DON, there is interference with the availability of substrate for the chain elongation system; 6-amionicotinamide inhibits the synthesis of UDP-N-acetyhexosamine (30) whereas β-D-xyloside competes with protein core as an acceptor for all elongation precursors (for review, see Ref. 9). These results are thus consistent with the hypothesis that chain elongation can be influenced by the availability of substrate (26).

DON, β-D-xyloside, and 6-amionicotinamide cause the elaboration of an abnormal cartilage matrix characterized by reduced metachromatic staining with toluidine blue (6, 31), obviously due to the deficiency of polyanions. Furthermore, the relative lack of matrix exhibited in cultures treated with these drugs (6–8, 25, 31, 32) probably results directly from the presence of relatively fewer and shorter glycosaminoglycan chains which would be expected to occupy a smaller than normal hydrodynamic volume, in addition to exerting less osmotic pressure and resulting in a decrease in interstitial fluid. Thus, the alterations in cartilage proteoglycan structure described here can account not only for the morphologic changes observed in cartilage matrix from DON-treated tissues (3, 6–8) but also permit a better understanding of the role abnormalities in proteoglycan synthesis and assembly may have in contributing to micromelia or other cartilage diseases. It is also likely that DON would be useful in studying proteoglycan metabolism in other cell systems.

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REFERENCES

Effects of DON on Cartilage Proteoglycan Structure