Regulation of Chondroitin Sulfate Synthesis

EFFECT OF β-XYLOSIDES ON SYNTHESIS OF CHONDROITIN SULFATE PROTEOGLYCAN, CHONDROITIN SULFATE CHAINS, AND CORE PROTEIN*

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Chondrocytes synthesize and secrete chondroitin sulfate proteoglycan and cartilage-specific collagen, the major macromolecules of extracellular matrix. Previous studies have shown that chondrocytes (and other cell types) are also capable of synthesizing free polylaccharide chains initiated with β-D-xyloside (1-3). Most likely, the β-xylosides act as initiators of chondroitin sulfate chains at the second glycosyltransferase step, thus eliminating the need for core protein and xylosyltransferase. Studies have been initiated to examine the effect of the artificial production of high levels of soluble chondroitin sulfate chains on the normal synthesis of chondroitin sulfate proteoglycan and collagen (4-8). Preliminary evidence indicates that a reduction in 35S0 incorporation into chondroitin sulfate proteoglycan occurs concomitant with the production of free chondroitin sulfate chains.

Since chondroitin sulfate proteoglycan is the end product of a complex biosynthetic pathway (9), a number of sites exist at which its synthesis may be regulated in the intact cell. In order to identify those sites which serve as loci of control, the various steps of biosynthesis may be assessed and compared under conditions in which normal synthesis is perturbed. In the present study, the regulation of chondroitin sulfate synthesis was examined in chondrocyte cultures producing large amounts of β-xyloside-induced chondroitin sulfate chains, in terms of the capacity of the cultures to produce core protein, chondroitin sulfate chains, and chondroitin sulfate proteoglycan. The observed decrease in chondroitin sulfate proteoglycan synthesis appears to be the result of an inhibition of galactosyltransferase activity by the β-xylosides in the presence of normal production and xylosylation of core protein.

EXPERIMENTAL PROCEDURES

Materials

UDP-[14C]Galactose† (252 μCi/μmol), UDP-[14C]xylose (174 to 198 μCi/μmol), H35SO4, carrier-free (45 Ci/mg), sodium [U-14C]acetate (10 μCi/μmol), and Na2B4H8 (200 μCi/μmol) were obtained from New England Nuclear. UDP-Galactose and UDP-xylose were obtained from Calbiochem. O-β-D-Xylosyl-L-serine was a generous gift from Dr. L. B. Nguyen, University of Alabama in Birmingham, and had been synthesized as previously described (10). Smith-degraded cartilage proteoglycan was prepared by the method of Baker and coworkers (11, 12).

Modified Eagle’s medium (13), fetal calf serum, horse serum, trypsin (95% lyophilized), trypsin/EDTA solution (0.25%), and Hank’s balanced salt solution, calcium- and magnesium-free, were purchased from Grand Island Biological Co. P:12 powdered medium was obtained from North American Biologicals, Inc. Testicular hyaluronidase, purchased from Sigma Chemical Co., was used as provided.

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† The radioimmunoassay which is employed in the present study measures total levels of embryonic chick cartilage core protein, but does not distinguish between unglycosylated or partially or fully glycosylated core protein molecules. The quantitation of core protein is described, all of these species may be included. This is considered in greater detail under “Discussion.”

All sugars discussed in this paper are of the β configuration.
luronidase (20,000 units/mg) was purchased from Leo Holzinger Laboratories, Sweden; cysteinylpyridine chloride, from K & K Laboratories; p-xylene and methyl-p-xylene, from Pfanzelt; p-nitrophenyl-a-D-xlyopyranoside and p-nitrophenyl-p-D-xlyopyranoside, from Koch-Light Laboratories; p-nitrophenyl-a-D-galactopyranoside, p-nitrophenyl-p-D-galactopyranoside, and p-nitrophenyl-N-acetyl-a-D-galactosaminide, from Sigma Chemical Co. Carrier chondroitin sulfate was kindly supplied by Dr. J. A. Cifonelli (University of Chicago).

Methods

Cell Culture—Cultures of chondrocytes were established from sternal cartilage of 15-day-old chick embryos according to procedures described by Cahn et al. (14). Cells were plated at an initial density of 2 to 5 x 10^4 cells/60-mm Falcon tissue culture dish in F-12 medium supplemented with 7% horse serum and 10% fetal calf serum (15). For routine metabolic studies, the cells were permitted to attach to the dishes (2 to 3 days) and subsequent growth was maintained by a complete change of the medium every 2 days with modified Eagle's medium containing 7% horse serum and 10% fetal calf serum. When applicable, multiple sets of plates were incubated in the presence or absence of 1 mM p-nitrophenyl-p-xylene from 6th to 10th day of culture.

Glycosaminoglycan Synthesis—For assay of sulfated glycosaminoglycan synthesis, cells were exposed to 5 micromoles of H_2SO_4 in fresh medium for 48 h on the last day of culture. Media and cells were collected and the amount of radioactive glycosaminoglycan was determined following addition of 12 mg of carrier chondroitin sulfate, papain digestion, cetylpyridinium chloride and ethanol precipitation as described (16). Glycosaminoglycans were also separated and quantitated following extraction of cell and media fractions with 5 M LiCl for 24 h at 4°C. Insoluble material was pelleted at 10,000 x g, and the supernatant fluid was applied to a column (2.5 x 130 cm) of Sephadex G-200 which was eluted with 0.05 M Tris/acetate buffer, pH 5.5, containing 0.25 M KCl and 2.5 M LiCl.

Enzyme Preparation—Cells were scraped from culture dishes, washed twice with Hank's balanced salt solution and suspended in 0.05 M Mes buffer, pH 6.5, containing 0.05 M KCl. Cells were disrupted with a Branson sonifier at 70 watts, followed by three rounds of freezing and thawing. Enzyme assays were performed directly on the crude cell homogenate fractions or on 10,000 x g pellet and supernatant fractions of the cell homogenates.

The concentration of protein in various enzyme fractions was measured by the method of Lowry et al. (17). In order to remove manganous ions which interfere with these determinations, protein was first precipitated from a solution of the enzyme (0.1 to 0.5 ml) with an equal volume of cold 50% trichloroacetic acid. The precipitate was collected by centrifugation, dissolved in 0.1 M NaOH, and assayed as usual.

Xylosyltransferase Assay—This assay was essentially according to Method 9a (11) in 0.05 M Mes buffer, pH 8.5, containing 0.05 M KCl, 0.005 M MnCl_2, and 0.012 M MgCl_2. Incubation mixtures contained the following components in a final volume of 0.075 ml: 0.2 mg of Smith-degraded proteoglycan (0.02 ml), 0.75 micromoles of KF and 1.5 micromoles of UDP-[14C]xylose (specific activity, approximately 30 pCi/micromole; 0.005 ml) and varying amounts of enzyme protein in 0.05 ml of Mes buffer. After incubation for 60 min at 37°C, 0.05 ml of 1% bovine serum albumin and 0.20 ml of 10% trichloroacetic acid, 4% phosphotungstic acid were added. Precipitated protein was recovered by centrifugation, washed twice with 5% trichloroacetic acid, and dissolved in 0.1 ml of 1 M NaOH for liquid scintillation counting.

Galactosyltransferase Assay—This enzyme, which transfers galactose to xylose, was assayed in a total volume of 0.075 ml by incubating 2 micromoles of xylose (0.010 ml), 1.5 micromoles of UDP-[14C]galactose (34.5 micromoles; 0.010 ml), 1.0 micromole of MnCl_2 (0.005 ml), and varying amounts of enzyme protein in 0.05 ml of 0.05 M Tris/acetate, pH 5.5, containing 0.05 M KCl and 0.001 M EDTA. After 60 min at 37°C, the reaction was terminated by heating the tubes at 100°C for 2 min. The cetylpyridinium and galactosylglycosamine standards were spotted on Whatman No. 3MM paper and desalted by high voltage electrophoresis as described (11, 18). Chromatography in ethyl acetate:water (5:1) was carried out for 24 h and the radioactive product was quantitated by liquid scintillation counting of the galactosylxylose area.

Alternatively, galactosyltransferase was assayed with 0.4 micromoles of xylosylserine as substrate instead of xylose (18, 19). After incubation and heat inactivation, the samples were mixed with 0.05 ml of 0.01 M HCl and centrifuged; the supernatant fractions were applied to columns (0.5 x 2.5 cm) of Dowex 50-X2-H+ (200 to 400 mesh) which were washed with 0.05 ml of 0.01 M HCl. The product was eluted with 2 ml of 2 M NH_4OH and evaporated to dryness, and the radioactivity was measured.

Preparation of Core Protein Antigens—The procedures for preparation of cartilage-specific core protein and antisera to core protein have recently been described (20). Briefly, chondroitin sulfate proteoglycan from epiphyses of 12-day-old chick embryos or sternum of adult chickens was extracted and purified in the presence of protease inhibitors (21). Following digestion with testicular hyaluronidase (20), the protein portion containing short chains of chondroitin sulfate was isolated by gel filtration on Sephadex G-200 (1 x 10 cm) in 0.15 M NaCl. The excluded peak was concentrated and used to elicit antibodies to epiphasial cartilage core protein in rabbits as previously described (20). An antigenic fragment of proteoglycan was obtained by further digested with partially purified epiphasial cartilage proteases (20). The antigen fragments were precipitated with 25% [14C] as sodium [U-14C]acetate (100 pCi/micromole) for 6 h in complete medium. Chondroitin sulfate proteoglycan was isolated, treated with hyaluronidase, and separated on Sephadex G-200 as described above.

Radioimmunoassay—A radioimmunoassay system uses the ammonium sulfate precipitation technique of Farr (22). The method was linear (percentage of precipitation versus log antibody concentration) between serum dilutions of 1:12 and 1:96, depending on the particular serum used (20). The percentage of antibody-bound radioactivity (%P) was calculated as follows:

%P = 100 - (cpm Ag) - (cpm Exp-Ppt) x 100

where %P is percent of antibody-bound radioactivity in tubes without unlabeled competitor and %P-exp is percentage of antibody-bound radioactivity in tubes with unlabeled competitor antigen (23).

Sugar Analysis—Monosaccharides were separated and quantitated by the Technicon sugar chromatography system (model SG-1). Samples were prepared for chromatography by hydrolysis in 1 M HCl for 3 h at 100°C, followed by deacidification as described (12). Xylose was also determined as [3H]xylose following treatment of core protein with alkalai NaOH. Core protein was isolated from 0.5 ml of cell extract (0.5 to 0.6 mg of protein) by precipitation with cartilage-specific antisera as previously described (20) or by precipitation with 10% trichloroacetic acid, 4% phosphotungstic acid in the presence of carrier albumin. After drying with ethanol and ether, the precipitates plus either albumin or normal rabbit serum as controls were suspended in 1.0 ml of alkaline borohydride, prepared from 20 mg of NaBH_4 (2 ml of 0.1 M NaOH containing 10 mg/ml of NaBH_4, 2 ml of 0.1 M NaOH containing 10 mg/ml of NaBH_4, and 2 ml of 0.1 M NaOH containing 10 mg/ml of NaBH_4), and left overnight at 25°C. Protein was precipitated and washed three times with 3 ml each of 10% trichloroacetic acid, and the combined supernatant fluids were extracted twice with 5 volumes of ether. The solution was acidified to pH 2 and passed through a column (0.5 x 3 cm) of Dowex 50-X2-H+ (200 to 400 mesh) which was rinsed with 0.01 M HCl. The effluent was evaporated four times with methanol and the desalted sample was subjected to high voltage electrophoresis (Savant Flat Plate, model FP-30A) on Whatman No. 3MM paper in 0.2 M borate buffer, pH 10.0 (19).

RESULTS

Synthesis of Chondroitin Sulfate Proteoglycan by p-D-Xylene-Treated Cultures—Previous studies have shown that derivatives of p-D-xylene stimulate 35S0_4^- incorporation into...
material characterized as protein-free chondroitin sulfate chains (1, 2). In the present study, the effect of the artificial production of high levels of soluble chondroitin sulfate chains, by treatment of cells with β-xylosides for several days, on the normal synthesis of chondroitin sulfate proteoglycan was examined. Synthesis of chondroitin sulfate proteoglycan (CSPG) by chondrocytes grown in the presence and absence of 1 mM p-nitrophenyl-β-D-xyloside for 2 days was assessed by separating and quantitating the 35SO42-labeled large molecular weight CSPG and free chondroitin sulfate chains by gel filtration on Sephadex G-200. The results of a typical experiment are presented in Fig. 1 and indicate that included material, identified as free chondroitin sulfate chains (9), is synthesized by, and found only in, the media of the stimulated cultures (Fig. 1D). The synthesis of free polysaccharide chains is accompanied by an apparent decrease in the production of CSPG as measured by 35SO42 incorporation in both the cell and media fractions from treated cultures (Fig. 1, C and D).

The appropriate fractions from these samples were pooled and quantitated, thereby allowing an estimation of the total synthetic capacity of the cultures. β-Xyloside-stimulated cultures were applied to a column (2.5 × 130 cm) of Sephadex G-200 equilibrated and eluted with 0.05 M Tris/acetate, pH 5.5, containing 0.25 M KCl, and 2.5 M LiCl, in fractions of approximately 8 ml. Aliquots (50 μl) of the labeled material from: A, control cells; B, control media; C, β-xyloside cells; D, β-xyloside media, were assayed for radioactivity.

![Fig. 1. Distribution of 35SO42-labeled polysaccharide in control and β-xyloside-treated cells. Monolayer cultures of chondrocytes were grown as described previously (10). From the 8th to 10th day of culture, plates of cells were labeled with 5 μCi of 35SO42 in the presence or absence (four each) of 1 mM p-nitrophenyl-β-D-xyloside in complete media containing 9.6 mM ascorbate. Media, plus two washes with Hank's balanced salt solution, and cells were separately harvested and extracted with 5 μl LiCl at 0.05 M Tris/acetate, pH 5.5, containing 0.25 M KCl, for 2 days at 4°. Samples from control and stimulated cultures were applied to a column (2.5 × 130 cm) of Sephadex G-200 equilibrated and eluted with 0.05 M Tris/acetate, pH 5.5, containing 0.25 M KCl and 2.5 M LiCl, in fractions of approximately 8 ml. Aliquots (50 μl) of the labeled material from: A, control cells; B, control media; C, β-xyloside cells; D, β-xyloside media, were assayed for radioactivity.](http://www.jbc.org/content/226/17/6318)

![Fig. 2. Inhibition assay using 35SO42-labeled core protein antigen. Unlabeled antigens compared were obtained from β-xyloside-treated or untreated cell and media fractions from embryonic chick sternal chondrocyte cultures. Antigen concentration for the cell fraction is arbitrarily expressed as log of dilution of material derived from six plates of cells (approximately 12 × 10⁶ cells/plate) in a total volume of 2 ml where each milliliter of control media (●) contains 1.25 mg of protein and that from β-xyloside cells (▲) contains 1.06 mg of protein. Antigen concentration in media fraction is expressed as log of dilution of material derived from the media of six plates of cells in a total volume of 6 ml where each milliliter of control media (●) contains 9.9 mg of protein and that of β-xyloside media (▲) contains 10.1 mg of protein. Conditions for the radioimmunooassay have been described under "Methods."](http://www.jbc.org/content/226/17/6318)

### Table 1

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Cells</th>
<th>Media</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-excluded</td>
<td>3.8</td>
<td>4.8</td>
<td>8.6</td>
</tr>
<tr>
<td>(CSPG)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Xyloside-excluded (CSPG)</td>
<td>1.0 (27%)</td>
<td>2.9 (45%)</td>
<td>3.9 (37%)</td>
</tr>
<tr>
<td>β-Xyloside-retarded (chondroitin sulfate chains)</td>
<td>17.4</td>
<td>20.6</td>
<td></td>
</tr>
</tbody>
</table>

**Effect of β-xylosides on chondroitin sulfate synthesis in cultured chondrocytes**

Experimental conditions are identical to those described in the legend to Fig. 1. The appropriate fractions from each radioactive peak eluted from Sephadex G-200 were pooled and concentrated under vacuum. Results are presented as total 35SO42-labeled polysaccharide per four plates. The percentage of CSPG produced in the presence of the β-xyloside is indicated in parentheses.

Levels of core protein in the cell extracts and media fractions from control and β-xyloside-treated cultures were determined by immunoprecipitation with antisera to core protein in an inhibition assay with labeled core protein of known specific activity. As shown in Fig. 2, antigens derived from cells or media of β-xyloside-treated and untreated cultures gave similar levels of inhibition as indicated by the nearly coincident precipitation curves.
the cell layer. Likewise core protein levels in the media fraction from either type of culture were similar but accounted for only 0.1% of the total protein. These results indicate that neither decreased synthesis nor increased solubility of chondroitin sulfate-specific core protein are responsible for the observed decrease in overall CSPG production.

Effect of β-Xylosides on Glycosyltransferase Activity—As previously mentioned, an inhibition of polysaccharide chain initiation or elongation in the presence of normal production of core protein may occur. In order to determine whether the reduced levels of \(^{35}S\)OSG-"-labeled CSPG are caused by an inhibition of enzyme activity or by decreased synthesis of the two specific chain-initiating glycosyltransferases, the following kinds of experiments were performed. Activity levels of xylosyltransferase and galactosyltransferase were measured (25) and found to be similar in cell extracts from equivalent numbers of β-xyloside-treated and untreated cells. In additional cell-free studies using purified preparations of xylosyltransferase (26), p-nitrophenyl-β-D-xyloside, in concentrations exceeding those used to induce chondroitin sulfate chain formation in cell cultures (10 mM) was found not to be inhibitory to the xylosyltransferase reaction.

To further illustrate that chain initiation proceeds unimpeded, the level of xylosylation in both types of cultures was determined directly. Monosaccharides in the hyaluronidase-digested total cell extract, before and after precipitation with core protein antiserum, were separated and quantitated with the Technicon sugar chromatography system. The results (Table II) indicate that β-xyloside-treated cells synthesize core protein which is xylosylated to the same extent as the control cells. Similar results were obtained by reductively labeling the hyaluronidase-digested cell extract, with NaB\(_3\)H\(_4\), in the presence of alkali (24) before and after precipitation with antiserum. One radioactive spot was observed in the treated extract which coincided with authentic xylitol and contained approximately the same radioactivity as the spot from the control extract, although absolute quantitation by this method was not possible.

β-Xyloside Inhibition of Galactosyltransferase—In contrast to the results obtained with xylosyltransferase, the β-xylosides are effective inhibitors of the galactosyltransferase reaction. Inhibition is observed at concentrations of p-nitrophenyl-β-D-xyloside of 1 mM; however, the maximum inhibition is observed at a 10-fold higher concentration (Fig. 3). Furthermore the inhibition is specific for the anomeric linkage and the glycon (Table III).

Previously it was observed (1, 27) that p-nitrophenyl-β-D-xyloside served as an exogenous substrate for the cell-free particulate galactosyltransferase system. Thus as might be expected, the kinetic analysis (Fig. 4) indicates that the β-xylosides are competitive inhibitors with xylose or xylosylserine as galactose acceptors. The effect of p-nitrophenyl-β-D-xyloside on the rate of condensation of xylosylserine and UDP-galactose, when the concentration of the latter was held constant and the xylosylserine concentration was varied over the range 1.5 to 12.5 mM, was examined. As shown (Fig. 4) no change in V\(_{max}\) occurred in the presence of the β-xyloside; however, the slope of the reciprocal plots increased with increasing p-nitrophenyl-β-D-xyloside concentration. These results are consistent with strictly competitive inhibition. When kinetic data are replotted as slope versus inhibitor concentration (28), a plot linear with respect to slope was obtained (Fig.

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

**TABLE II**

<table>
<thead>
<tr>
<th>Component</th>
<th>Before precipitation</th>
<th>After precipitation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>µg/mg protein</td>
<td>µg/mg protein</td>
</tr>
<tr>
<td>Control</td>
<td>β-Xyloside</td>
<td>Control β-Xyloside</td>
</tr>
<tr>
<td>Fucose</td>
<td>3.8</td>
<td>3.8</td>
</tr>
<tr>
<td>Galactose</td>
<td>15.6</td>
<td>13.6</td>
</tr>
<tr>
<td>Mannose</td>
<td>4.8</td>
<td>5.0</td>
</tr>
<tr>
<td>Xylose</td>
<td>3.8</td>
<td>6.8</td>
</tr>
<tr>
<td>Glucose</td>
<td>94.6</td>
<td>73.2</td>
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**TABLE III**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Galactosyltransferase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm x 10(^{-4})</td>
</tr>
<tr>
<td>None</td>
<td>27.0</td>
</tr>
<tr>
<td>p-Xylose</td>
<td>25.3 (93)</td>
</tr>
<tr>
<td>Methyl-β-D-xyloside</td>
<td>22.8 (85)</td>
</tr>
<tr>
<td>p-Nitrophenyl-β-D-xyloside</td>
<td>23.6 (60)</td>
</tr>
<tr>
<td>p-Nitrophenyl-β-D-xyloside</td>
<td>10.4 (38)</td>
</tr>
<tr>
<td>p-Nitrophenyl-β-D-galactoside</td>
<td>27.0 (100)</td>
</tr>
<tr>
<td>p-Nitrophenyl-β-D-galactoside</td>
<td>27.3 (100)</td>
</tr>
<tr>
<td>p-Nitrophenyl-β-D-N-acetylglactosaminide</td>
<td>25.7 (99)</td>
</tr>
<tr>
<td>4-Methylumbelliferyl-β-D-xyloside</td>
<td>11.5 (41)</td>
</tr>
</tbody>
</table>
galactosylxylosylserine formed per h; S, nanomolar xylosylserine. Assays of xylosylserine as indicated in the absence (0) and presence of 2 mM (●) or 4 mM (■) p-nitrophenyl-β-D-xyloside. V, nanomoles of galactosylyxosylserine formed per h; S, nanomolar xylosylserine.

**Table IV**

*Effect of p-nitrophenyl-β-D-xyloside on galactosyltransferase activity*  
A solution of partially purified galactosyltransferase was incubated under standard conditions with 0.5 mg of xylosylated core protein (29) as acceptor instead of xylose, in the absence or presence of the indicated concentrations of p-nitrophenyl-β-D-xyloside. After incubation at 37°C for 60 min, the reaction product was precipitated with trichloroacetic acid/phosphotungstic acid as described for the assay of xylosyltransferase. Results are expressed as counts per min of enzyme activity per 50 μl of galactosyltransferase solution. Percentage of control activity is indicated in parentheses.

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>cpm/50 μl (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4440</td>
</tr>
<tr>
<td>0.2</td>
<td>3400(76)</td>
</tr>
<tr>
<td>0.4</td>
<td>2870(64)</td>
</tr>
<tr>
<td>2.0</td>
<td>1160(26)</td>
</tr>
</tbody>
</table>

4, inset), emphasizing the competitive nature of the inhibition. In additional studies, transfer of galactose to an enzymatically synthesized acceptor, xylosylated-core protein (29), which is the closest approximation available to the natural galactose acceptor, is also inhibited by β-D-xylosides. Concentrations of p-nitrophenyl-β-D-xyloside near or below those used to stimulate chondroitin sulfate chain synthesis in cell culture studies (i.e. 1 mM) inhibited the galactosyltransferase reaction with xylosylated-core protein as acceptor (Table IV).

**DISCUSSION**

Many aspects of the synthesis and secretion of glycosaminoglycans are unknown at both the biochemical and cellular levels. Particularly intriguing is the manner by which the cell coordinates the numerous synthetic reactions required for complex a molecule as chondroitin sulfate proteoglycan. Presumably the biosynthetic sequence of events involves synthesis of the protein moiety by routes that are well established for simple proteins, followed by the stepwise addition of various carbohydrate units from the corresponding nucleotide sugars. At present, it is not known how the rates of core protein synthesis and glycosylation are regulated and coordinated, whether glycosylation occurs before or after completion of the protein core, or how polysaccharide chain synthesis affects core protein synthesis.

The availability of a well controlled and characterized cell culture system provided an opportunity to investigate some of the mechanisms which regulate the synthesis of CSPG. In this system, β-xyloside treatment caused a stimulation of the synthesis of free chondroitin sulfate and was accompanied by an apparent decrease in the production of CSPG, as determined by 35SO42− incorporation into Sephadex G-200-excluded material. Measurement of core protein levels by a radioimmunoassay, specific for the protein portion of cartilage proteoglycan, indicated that the observed decrease in 35SO42−-labeled CSPG was not due to a reduction in synthesis of core protein since comparable amounts were found in both treated and control cultures. Furthermore, the distribution of core protein in the cell and media fractions from both types of cultures was comparable, indicating that overproduction and secretion of an unglycosylated core protein does not occur.

It is recognized that pool sizes of core protein, rather than synthesis per se, are measured by the radioimmunoassay procedure. However, the levels of core protein most likely are a reflection of the synthetic process since previous studies have shown that treatment with puromycin or 5-bromo-2'-deoxyuridine significantly reduces these pools (20). Furthermore, it has been shown that 35SO42− incorporation into CSPG is inhibited greater than 95% in 5 h by treatment with puromycin (25). Thus, core protein must be turning over rapidly since puromycin treatment prevents further de novo synthesis and allows only use or destruction of existing protein pools. It is postulated, therefore, that core protein is continuously synthesized during treatment with β-xylosides.

The core protein that is being measured may actually be of several molecular species, including the following: (a) unglycosylated core protein; (b) core protein attached to xylose; (c) core protein attached to chondroitin sulfate chains. These chains may be present in normal or fewer than normal numbers per core protein, as well as being of normal or shorter than normal length. Such possibilities cannot be distinguished by the radioimmunoassay since the material is digested with hyaluronidase before precipitation. However, some of the molecular species described above under (c) might be expected to yield a more heterogeneous gel filtration pattern caused by molecules intermediate in size between the protein-bound and free chondroitin sulfate chains. Additional information is needed to resolve this question.

As previously shown, production of CSPG is dependent upon the prior synthesis of core protein (16, 30). However, the failure of β-xyloside-treated cells to incorporate 35SO42− into CSPG in the presence of normal amounts of core protein suggests that the synthesis of core protein alone does not mandate the synthesis of complete CSPG. Furthermore, synthesis of core protein is not influenced by later steps of CSPG production since neither increased synthesis of chondroitin sulfate chains nor decreased synthesis of CSPG altered the level of core protein production. These results indicate that there may be several levels of control for CSPG synthesis in the intact cell; at least one which regulates production of core
protein and another which controls the sequence of glycosylation reactions.

The studies of the reactions responsible for conjugation of carbohydrate and protein demonstrate that polysaccharide chain initiation occurred normally in β-xyloside-treated cells. In contrast, the inhibition of galactosyl transfer to a xylosylated core protein acceptor by p-xylosides in concentrations similar to those used in the cell culture studies suggests a possible interruption of the normal biosynthetic scheme at this site. It is proposed, therefore, that the competitive inhibition of the exogenous β-xylosides with the endogenous acceptor in the first galactosyltransferase reaction may be responsible for the observed decrease in 35S incorporation into CSPG by preventing addition of chondroitin sulfate chains to certain core protein molecules.

It is of interest that an artificial substrate should be used preferentially by the intracellular enzyme system rather than the native acceptor. That β-xylosides effectively compete with the xylosylated core protein may result from an intracellular concentration of β-xylosides in excess of the native acceptor. At present, the intracellular concentrations of either compound cannot be adequately determined. Alternatively, the β-xylosides may be more efficiently utilized by the membrane-bound galactosyltransferase system due to their relatively smaller size and hydrophobicity. It should also be mentioned that in cell-free studies it has been shown, that of all the glycosyltransferases, only the first galactosyltransferase will transfer galactose to a monosaccharide acceptor β-xylose, free or in glycosidic linkage, although a substrate such as O-β-d-xylosyl-L-serine, which bears greater resemblance to the natural acceptor, is a slightly better acceptor than the free monosaccharide (11, 18). It is not surprising, therefore, that only β-xylose or derivatives of P-xyloside initiate the synthesis of chondroitin sulfate chains and that analogues with different anomers or sugars are ineffective (1, 2, 27).

Moreover, it may be suggested that a critical control mechanism may function at this site in the biosynthetic scheme since an exogenous substrate can be inserted at this site only. These findings further support the idea that the interaction between xylosyltransferase and galactosyltransferase is complex (31, 32) may be physiologically significant. Such an interaction may regulate the rate of chondroitin sulfate proteoglycan synthesis at this particular locus by promoting a selective association-dissociation of soluble xylosyltransferase (or a xylosyltransferase-core protein complex) with the membrane-bound galactosyltransferase and the rest of the multienzyme system (25, 29). In the presence of high intracellular levels of β-xylosides, the xylosylated core protein complex may then be prevented from binding to the galactosyltransferase acceptor region.

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