Stimulation of Synthesis of Free Chondroitin Sulfate Chains by β-D-Xylosides in Cultured Cells*

(Received for publication, January 23, 1975)

LEONARDO GALLIGANI, JOHN HOPWOOD,† NANCY B. SCHWARTZ,§ AND ALBERT DORFMAN

From the Departments of Pediatrics and Biochemistry, and Joseph P. Kennedy, Jr. Mental Retardation Research Center, University of Chicago, Chicago, Illinois 60637

SUMMARY

β-Xylosides stimulate 2- to 6-fold the synthesis of glycosaminoglycans by three types of nonconnective tissue cells (RG-C6, NB41A, and rat hepatoma cells, and normal and simian virus 40 (SV40)-transformed normal human skin fibroblasts. The effect, which is specific for the anameric linkage and the glycone, is observed in the presence and absence of puromycin. β-Xylosides may substitute for xylosylated core protein as initiators of synthesis of chondroitin sulfate chains. No stimulation of synthesis of heparan sulfate was observed. With the use of a fluorescent xyloside, 4-methylumbelliferyl-β-D-xyloside, it was demonstrated that the free chondroitin sulfate chains secreted into the medium bear the xyloside at the reducing end, and have an average molecular weight of 16,500.

Most connective tissue glycosaminoglycans are linked to serine residues of a protein core by a xylosylserine bond, which is part of a galactosylgalactosylxylosylserine protein-polysaccharide linkage region (1). The biosynthesis of this linkage region tri-saccharide proceeds by the sequential transfer of appropriate monosaccharides by specific glycosyltransferases from uridine nucleotide sugars (2, 3). It has been suggested that the transfer of xylose from UDP-xylose to the protein core (4) regulates the initiation of new polysaccharide chains (5).

Telser et al. (6) showed that puromycin inhibits the biosynthesis of sulfated glycosaminoglycans in embryonic chick epicardial cartilage, presumably by preventing synthesis of core protein. In the same system, Brett and Robinson (7) demonstrated that puromycin inhibition is reversed by the addition of high concentrations of D-xylose. They suggested that xylose substitutes for core protein as an initiator of chondroitin sulfate synthesis of sulfated glycosaminoglycans by three types of nonconnective tissue cells and by normal and SV40-transformed normal human skin fibroblasts.

In this paper, a detailed report of the effect of β-xylosides on glycosaminoglycan synthesis by three different types of nonconnective tissue cells and by normal and SV40-transformed normal human skin fibroblasts is presented.

EXPERIMENTAL PROCEDURE

Materials—Modified Eagle’s medium (13) was obtained from Grand Island Biological Co., and modified Swim’s S-77 medium (14) was prepared in this laboratory. Other materials were obtained from the following sources: fetal calf serum, trypan blue (0.25% solution), and Hanks’ BSS from Grand Island Biological Co.; p-nitrophenyl-α-D-xylopyranoside, p-nitrophenyl-β-D-xylopyranoside, 4-methylumbelliferyl-β-D-xylopyranoside from Koch Light Laboratories; 4-methylumbelliferyl-β-D-galactopyranoside from Pierce Chemical Co.; p-nitrophenyl-β-D-galactopyranoside from Sigma Chemical Co.; puromycin dihydrochloride from Nutritional Biochemicals Co.; gentamycin from Schering Corp.

The radioactive precursors H235SO4, carrier-free, (43 Ci/mg) and sodium [U-14C]acetate (100 Ci/mmol) were purchased from New England Nuclear Corp. Variadase was used as a source of streptococcal hyaluronidase, since the preparation is rich in contaminating hyaluronidase. Commerical Variadase (20,000 units of streptokinase/8 mg) was obtained from Lederle Laboratories. Testicular hyaluronidase (20,000 units/mg) was purchased from Leo-Helsinki Laboratories, Sweden. Chondroitinase ABC, prepared by Seikagaku Kogyo Co., Ltd., was purchased from Miles Laboratories, Inc. Carrier chondroitin 4-sulfate was a gift of Dr. J. A. Cifonelli. Twice crystallized papain, prepared from crude enzyme (type II Sigma) was purchased from Sigma Chemical Co.; papain from Schering Corp.

Cell Culture—Rat glial cells (clonal strain RG-C6) (16) and mouse neuroblastoma cells (C-1300, clonal strain NB41A) (17) were a gift of Dr. G. Sato. Cultures were carried out on the 7th day, when RG-C6 cultures reached a density of 2.5 × 10⁴/100-mm dish, and were fed twice during the following 7 days with 7 ml of modified Eagle’s medium supplemented with 10% fetal calf serum and 5 µg/ml of gentamycin. Experiments were usually carried out on the 7th day, when RG-C6 cultures reached a density of 2.5 to 3.5 × 10⁵ cells/dish and NB41A cultures a density of 2 to 2.5 × 10⁶ cells/dish.

Rat hepatoma cells (HTC), obtained from Drs. D. Haggerty and G. Popjak, were plated at a density of 5 × 10⁴/100 mm dish with 8 ml of modified Swim’s S-77 medium supplemented with 5% fetal calf serum. The cultures usually reached a density of 5 to 6 × 10⁵ cells/dish.

Telser et al. (6) showed that puromycin inhibits the biosynthesis of sulfated glycosaminoglycans in embryonic chick epigeal cartilage, presumably by preventing synthesis of core protein. In the same system, Brett and Robinson (7) demonstrated that puromycin inhibition is reversed by the addition of high concentrations of D-xylose. They suggested that xylose substitutes for core protein as an initiator of chondroitin sulfate synthesis of sulfated glycosaminoglycans by three types of nonconnective tissue cells and by normal and SV40-transformed normal human skin fibroblasts.

In this paper, a detailed report of the effect of β-xylosides on glycosaminoglycan synthesis by three different types of nonconnective tissue cells and by normal and SV40-transformed normal human skin fibroblasts is presented.
fetal calf serum and 5% calf serum. HTC cells were fed every other day, and reached a density of 1.2 to 1.4 x 10^6 cells/dish after approximately 1 week in culture.

Human skin fibroblasts, which had undergone eight transfers, were cultured as described by Matalon and Dorfman (13) in modified Eagle's medium supplemented with 10% fetal calf serum and 10% calf serum. Cells were plated at a density of 2 x 10^4/90-mm dish with 0.5 ml of medium, and were fed two to three times/week. A density of 1.9 x 10^6 cells/plate was obtained in 4 weeks. SV40-transformed normal human skin fibroblasts2 were plated in 100-mm Falcon Petri dishes at the initial density of 2 x 10^5 cells/plate, and grown under the same conditions as normal fibroblasts, to yield a final density of 2.5 x 10^6 cells/plate after 4 weeks.

All cell types were grown at 37° in a humidified atmosphere of 10% CO₂ and 90% air.

**Glycosaminoglycan Synthesis**—On the last day of culture, cells were incubated for 6 hours at 37° under the conditions described in the legends to tables and figures. Cells and media were collected, and the labeled glycosaminoglycans were isolated after addition of 1 mg of carrier chondroitin sulfate as described by Dorfman and Ho (18). For separate determination of media, cell-associated, and intracellular glycosaminoglycans, media were removed and pooled, and the cells were washed twice with Hank's BSS. The cell layers were incubated for 15 min with 3 ml of 0.25% trypsin and centrifuged. The resulting supernatant solution was designated as “extracellular fraction” and pellets were used as “cell fraction.” From each fraction, the labeled glycosaminoglycans were isolated by the procedure of Dorfman and Ho (18).

**Analysis of Glycosaminoglycans**—Glycosaminoglycans were characterized by sequential enzymatic and chemical treatments followed by separation of the products on columns of Sephadex G-50, which were eluted with 0.2 M NaCl or 0.2 M pyridine acetate buffer, pH 5.0.

Streptococcal hyaluronidase digestions were performed in 0.01 M phosphate buffer, pH 6.0, containing 0.15 M NaCl (19). Labeled glycosaminoglycans from RG-C6 and NB41A cultures were incubated at 37° for 18 hours with 2 mg of Varidase in a total volume of 1.1 to 1.3 ml. Labeled material isolated from normal and SV40-transformed normal fibroblasts was incubated at 37° for 4 hours with 0.72 mg of Varidase in a total volume of 5 ml.

Chondroitinase ABC digestion were performed as described by Saito et al. (20) in 0.05 M Tris-acetate buffer, pH 8, using 0.2 to 0.5 units of enzyme. Labeled material isolated from RG-C6, NB41A, and HTC cultures was incubated at 37° for 21 hours in a total volume of 0.7 to 1.0 ml. Glycosaminoglycans isolated from normal and SV40-transformed normal fibroblasts were incubated at 37° for 4 hours in a total volume of 2 ml.

Testicular hyaluronidase digestions were carried out in a total volume of 5 ml of 0.1 M sodium acetate buffer, pH 5.0, containing 0.15 M NaCl using 70,000 units of enzyme (21). Samples were incubated for 4 hours at 37°.

Treatment of glycosaminoglycans with nitrous acid was carried out according to the procedure of Lagunoff and Warren (22), by adding 1 volume of 5% sodium nitrite and 1 volume of 33% acetic acid to the samples. After treatment for 2 hours at room temperature with occasional stirring, samples were lyophilized and redissolved in distilled water.

**Fluorescence Measurements**—Fluorescence was measured with an Aminco-Bowman spectrophotometer following hydrolysis with 0.5 N HCl in boiling water for 30 min. The pH was subsequently adjusted to 10 to 11 with 4 N NaOH. The excitation and emission wavelengths were 350 nm and 448 nm, respectively. 4-Methylumbelliferyl-β-D-glucuronide was used as standard in quantitative determinations. Unhydrolyzed samples were used as blanks.

**Analytical Methods**—Gel chromatography was conducted using columns of Sephadex G-50 and G-200, as described in the legends to figures and tables. Blue dextran and H₂O₂ were used for the calibration of the columns.

Glucuronic acid was assayed, after trichloroacetic acid precipitation of proteins, by the carbazole method of Bitter and Muir (23).

Alkali treatment of proteoglycans was performed at 4° for 4 days in 0.5 N NaOH (24). Samples were neutralized before gel chromatography.

Descending paper chromatography was performed on Whatman No. 3MM paper in (A) butyric acid/0.5 N NaOH (5/3), and (B) 1 M ammonium acetate, pH 5.0/ethanol (3/7) for 40 hours and 18 hours, respectively.

**RESULTS**

**Effect of Xylosides on Glycosaminoglycan Synthesis by RG-C6 and NB41A Cultures**—The effect of the addition of various xylosides and galactosides on the synthesis of sulfated glycosaminoglycans by rat glial and mouse neuroblastoma cells is shown in Table I. The results indicate that of the compounds tested, only β-xylosides stimulate sulfate incorporation into glycosaminoglycans. α-Xylosides and β-galactosides have no significant effect, indicating specificity for anomeric linkage and glycone.

When β-xylosides are added to puromycin-treated cultures, an 11- to 12-fold stimulation of sulfate incorporation in RG-C6 cultures and a 17- to 20-fold stimulation in NB41A cultures is observed. A similar effect of β-xylosides was reported in two strains of hepatoma cells (HTC and H₂) by Schwartz et al. (10). The results obtained with non-cartilage cells are similar to those reported for cartilage cells in which the inhibition of chondroitin sulfate biosynthesis by treatment with puromycin or BrdUrd was overcome by addition of xylose (7–9) or β-xylosides (10–13).

Fig. 1 shows the dose response to p-nitrophenyl-β-D-xyloside of rat glial cells. An effect is obtained at concentrations as low as 0.01 mM with a maximal effect at 0.1 mM. This concentration is much lower than that required for n xylose to overcome the inhibitory action of puromycin in intact chicken cartilage (7) and of BrdUrd in limb bud cells and chondrocytes (8–10). The effect of β-xylosides is rapid, being observed within 1 to 2 hours (10), and its magnitude is not affected by cell density.

**Characterization of Labeled Glycosaminoglycans from RG-C6 and NB41A Cultures**—Rat glial and mouse neuroblastoma cells have been reported to produce low levels of three different types of glycosaminoglycans: hyaluronic acid, chondroitin sulfate, and heparan sulfate (18, 25, 26). Since the addition of β-xylosides...
The synthesis of heparan sulfate, a compound which has been reported to be linked to protein in a manner identical to chondroitin sulfates and dermatan sulfate (28, 29), is minimally increased. The formation of hyaluronic acid shows little elevation in either RG-C6 or NB41A cultures.

Characterization of Sulfated Glycosaminoglycans from HTC Cultures—$^{35}$S-Labeled glycosaminoglycans were isolated from rat hepatoma cultures, following incubation as described in the legend to Table III, and characterized by chondroitinase ABC digestion and nitrous acid degradation. As shown in Table III, the stimulation occurs mainly in the synthesis of chondroitinase ABC-sensitive material, while little stimulation is observed in the synthesis of nitrous acid-degradable material, which may contain heparin or heparan sulfate. (No evidence for the presence of heparin in such cells has been found.)

Effect of 4-Methylumbelliferyl-β-D-xyloside on Glycosaminoglycan Synthesis by Normal and SV40-Transformed Normal Fibroblasts—In order to determine whether β-xylosides stimulate the synthesis of dermatan sulfate, normal human skin fibroblasts and SV40-transformed normal fibroblasts were incubated in the presence or absence of 4-methylumbelliferyl-β-D-xyloside. Total
Characterization of glycosaminoglycans from HTC cultures

Five plates of rat glial cells (4.7 x 10^6 cells/plate) and six plates of mouse neuroblastoma cells (2.9 x 10^6 cells/plate) were labeled for 6 hours at 37°C with 5 μCi of [U-3H]acetate/ml of serum-free medium in the presence or absence of 0.1 mM 4-methylumbelliferyl-β-D-xyloside. After isolation of the labeled glycosaminoglycans from pooled cells and media, the individual products were identified by sequential treatment with streptococcal hyaluronidase, chondroitinase ABC, and nitrous acid, and by separation of the products on Sephadex G-50 as indicated in Table II. Following streptococcal hyaluronidase digestion, the hyaluronic acid and chondroitin disaccharides were identified by paper chromatography in Solvent B. Following hyaluronidase digestion, the hyaluronic acid and chondroitin disaccharides were identified by paper chromatography in Solvent B. After isolation of the total glycosaminoglycans, the hyaluronic acid and chondroitin disaccharides were identified by paper chromatography in Solvent B.

Data are reported as [U-3H]acetate incorporation after subtraction of the residue which is resistant to any treatment.

<table>
<thead>
<tr>
<th>Component</th>
<th>RG-C6</th>
<th>NB41A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td>Total glycosaminoglycans</td>
<td>1,706</td>
<td>11,052</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>583</td>
<td>605</td>
</tr>
<tr>
<td>Chondroitin</td>
<td>194</td>
<td>2,416</td>
</tr>
<tr>
<td>Chondroitin 4/6-sulfate +</td>
<td>613</td>
<td>7,818</td>
</tr>
<tr>
<td>derman sulfate</td>
<td>316</td>
<td>215</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Characterization of sulfated glycosaminoglycans from HTC cultures

Rat hepatoma cells (1.4 x 10^6 cells/plate) were incubated for 6 hours with 10 μCi of [U-3H]acetate/ml of serum-free medium (50 μCi/plate) in the presence or absence of 0.1 mM 4-methylumbelliferyl-β-D-xyloside. After isolation of the total glycosaminoglycans, the individual products were identified by digestion with chondroitinase ABC and degradation with nitrous acid, followed by separation of the products on Sephadex G-50.

<table>
<thead>
<tr>
<th>Component</th>
<th>Control</th>
<th>4-Methylumbelliferyl-β-D-xyloside</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total glycosaminoglycans</td>
<td>3371</td>
<td>8475</td>
</tr>
<tr>
<td>Chondroitin 4/6-sulfate +</td>
<td>1359</td>
<td>5805</td>
</tr>
<tr>
<td>derman sulfate</td>
<td>2012</td>
<td>2670</td>
</tr>
</tbody>
</table>

Characterization of sulfated glycosaminoglycans from normal skin fibroblasts and SV40-transformed normal fibroblasts

Normal skin fibroblasts (1.2 x 10^6 cells/plate) and SV40-transformed normal fibroblasts (2.4 x 10^6 cells/plate) were incubated for 6 hours at 37°C with 5 ml and 10 ml of medium, respectively, in the presence or absence of 0.5 mM 4-methylumbelliferyl-β-D-xyloside. Cells were labeled with 20 μCi of [U-3H]acetate/ml of medium. Labeled glycosaminoglycans were isolated from pooled cells and media by the procedure of Dorfman and Ho (18). The individual components were identified by sequential treatment of the isolated material with streptococcal hyaluronidase, testicular hyaluronidase, chondroitinase ABC, and nitrous acid, followed by separation of the products on a column of Sephadex G-50 (1 x 200 cm) in 0.2 M pyridine-acetate buffer, pH 5.0. The supernatant fractions, obtained after cetylpyridinium chloride precipitation of glycosaminoglycans, were chromatographed on the above column of Sephadex G-50, and the void volume material was degraded with nitrous acid.

Data are reported as [U-3H]acetate incorporation. The values for heparan sulfate are totals of nitrous acid-degradable polysaccharide in cetylpyridinium chloride-precipitable and soluble material.

<table>
<thead>
<tr>
<th>Component</th>
<th>Normal fibroblasts</th>
<th>SV40-transformed normal fibroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td>Total glycosaminoglycans</td>
<td>56,700</td>
<td>120,400</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>40,000</td>
<td>46,500</td>
</tr>
<tr>
<td>Chondroitin 4/6-sulfate</td>
<td>6,200</td>
<td>41,000</td>
</tr>
<tr>
<td>Dermatan sulfate</td>
<td>1,100</td>
<td>14,600</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>9,400</td>
<td>18,300</td>
</tr>
</tbody>
</table>

G-50 columns were treated with testicular hyaluronidase, and rechromatographed on the same columns. The material eluted in the void volume was considered to be dermatan sulfate, while the included material was designated as the chondroitin 4/6-sulfate fraction. The testicular hyaluronidase-resistant material was then subjected to digestion with chondroitinase ABC to which dermatan sulfate is sensitive (20).

Only traces of heparan sulfate were found in the labeled material isolated from cells plus media by cetylpyridinium chloride precipitation (18). Almost all of the heparan sulfate, identified by nitrous acid degradation, was found in the cetylpyridinium chloride-soluble fraction, presumably because of a low degree of sulfation or low molecular weight.

Table IV summarizes the results of the analysis of labeled glycosaminoglycans from cultures of normal and SV40-transformed fibroblasts in the presence or absence of 4-methylumbelliferyl β-D-xyloside. In both strains, the β-xyloside stimulated the incorporation of [U-3H]acetate into glycosaminoglycans 2- to 3-fold. Upon analysis, it is found that the synthesis of the chondroitin 4/6-sulfates fraction is stimulated 6- to 7-fold in normal fibroblasts and 23-fold in SV40-transformed fibroblasts. Furthermore, dermatan sulfate synthesis is stimulated about 13-fold in normal fibroblasts, while less stimulation is found in the transformed cells.

As expected, hyaluronic acid synthesis is not stimulated in either normal or SV40-transformed fibroblasts. Only a 2-fold stimulation is observed in the heparan sulfate fraction of normal
fibroblasts, while no stimulation is found in SV40-transformed cells. This result is similar to what was reported above for RG-C6, NB41A, and HTC cultures.

**Effect of 4-Methylumbelliferyl-β-D-xyloside on Distribution of Glycosaminoglycans in RG-C6 Cultures**—Okayama *et al.* (11) demonstrated that chondroitin sulfate chains synthesized by cartilage in the presence of 4-methylumbelliferyl-β-D-xyloside bear the xyloside at the reducing end of the chains. A similar result was obtained by Levitt (32) utilizing radioactive xylose.

Table V shows the distribution of radioactivity and of 4-methylumbelliferol (determined after hydrolysis) in fractions of RG-C6 cultures. The fluorescent material was found associated with sulfated glycosaminoglycans present predominantly in the medium.

When dialyzed media from control and 4-methylumbelliferyl-β-D-xyloside-treated RG-C6 cultures were chromatographed on a column of Sephadex G-200, the patterns illustrated in Fig. 3 were obtained. While the labeled material from control cultures was eluted mostly in the void volume of the column (Fig. 3A), the material from stimulated cells was eluted in a broad peak in the included volume (Fig. 3B). This peak was coincident with the peak of fluorescence obtained after hydrolysis of the samples.

When the labeled material from control cultures was treated with alkali (24) and rechromatographed on Sephadex G-200, the radioactivity was eluted in an included position (Fig. 3A). Based on the elution volume, an average molecular weight of about 20,000 was calculated for this material (33). Similar calculations gave an average molecular weight of about 12,500 for the material from the stimulated cultures. For this material, it was also possible to calculate the average number of disaccharide units from the molar ratio of glucuronic acid (0.125 μmol/ml) to 4-methylumbelliferol (0.0037 μmol/ml). This ratio of 33 corresponds to an average molecular weight of 16,600.

### Table V

**Effect of 4-methylumbelliferyl-β-D-xyloside on glycosaminoglycan synthesis in RG-C6 cultures**

Eight plates of rat glial cells (3.5 × 10⁷ cells/plate) were incubated for 6 hours in the presence or absence of 0.5 mM 4-methylumbelliferyl-β-D-xyloside. To five plates in each group, 5 μCi of H₂³⁵SO₄/ml of medium were added (25 μCi/plate). Culture fractions were separated as described under “Experimental Procedure.” Glycosaminoglycans were isolated from each fraction as described by Dorfman and Ho (18). Fluorescence measurements were performed on the isolated products before and after hydrolysis as indicated under “Experimental Procedure.”

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Addition</th>
<th>Total glycosaminoglycans</th>
<th>4-Methylumbelliferyl-β-D-xyloside</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>None</td>
<td>32,804</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td>4-Methylumbelliferyl-β-D-xyloside</td>
<td>37,882</td>
<td>0.023</td>
</tr>
<tr>
<td>Tryptic fraction</td>
<td>None</td>
<td>98,332</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td>4-Methylumbelliferyl-β-D-xyloside</td>
<td>96,000</td>
<td>0.023</td>
</tr>
<tr>
<td>Medium</td>
<td>None</td>
<td>145,173</td>
<td>4.10</td>
</tr>
<tr>
<td></td>
<td>4-Methylumbelliferyl-β-D-xylose</td>
<td>1,520,146</td>
<td>10.26</td>
</tr>
</tbody>
</table>

*Figure 3. Gel chromatography of media from RG-C6 cultures incubated in presence or absence of 4-methylumbelliferyl-β-D-xyloside. Fourteen plates of rat glial cells (3.7 × 10⁷ cells/plate) were incubated for 6 hours at 37°C in the presence (B) or absence (A) of 0.1 mM 4-methylumbelliferyl-β-D-xyloside. Media in each group were combined, dialyzed for 8 hours against Na₂SO₄, and then for 5 days against water, concentrated to 8 ml, and applied to a column (2 × 130 cm) of Sephadex G-200 in 0.05 M Tris-acetate, pH 5.5, containing 0.25 M KCl. Fractions (5.5 ml) were collected at 10-minute intervals, and 0.5-ml aliquots of every second fraction were assayed for radioactivity. One-milliliter aliquots of every fourth fraction were used for fluorescence measurements performed as indicated under “Experimental Procedure.” Labeled material from control cultures (A) (●) was treated with alkali (24), and rechromatographed on the same column of Sephadex G-200 after neutralization (A) (○). Blue dextran (BD) and radioactive sulfate were used for the calibration of the column.*
Recent studies have shown that many nonconnective tissue cells synthesize small amounts of glycosaminoglycans (18, 25, 26, 34–37). The results presented here indicate that the addition of \( \beta \)-xylosides to three types of nonconnective tissue cells results in a 2- to 6-fold stimulation in the production of total sulfated glycosaminoglycans with a specific increase in the chondroitin sulfate fraction. Previously, it was demonstrated that \( \beta \)-xylosides overcome the inhibition by puromycin or BrdUrd of chondroitin sulfate synthesis in cartilage cells (10-12). Schwartz et al. (10) also observed that 1-day cultures of limb bud mesenchyme, which are undifferentiated and produce low levels of glycosaminoglycans, can be stimulated by \( \beta \)-xylosides to much higher levels of synthesis. The present results suggest that non-cartilage cells (as well as pre differentiated mesenchyme cells) have a greater potential for synthesizing sulfated glycosaminoglycans than is normally expressed in culture. The low levels of glycosaminoglycans produced by these cells in the untreated state may be due to limited synthesis of specific core protein or xylosyl transferase.

Similar conclusions were reached in the previous study (10), where it was found that stimulation of glycosaminoglycan production occurred when protein synthesis was inhibited greater than 95%, indicating that formation of new transferase enzymes was not involved in the increased glycosaminoglycan synthesis induced by the \( \beta \)-xylosides. Furthermore, since puromycin and BrdUrd-treated chondrocytes and limb bud cells were capable of being rapidly stimulated (1 to 2 hours), the cells must have contained the necessary precursors and enzymic machinery required for synthesis of chondroitin sulfate chains.

In cells of connective tissue (10) and nonconnective tissue origin, only \( \beta \)-xylosides can act as initiators of synthesis of free chondroitin sulfate chains, \( \alpha \)-xylosides being ineffective, presumably because of the specificity of the first galactosyltransferase for the anomeric linkage. In addition, other sugar derivatives do not initiate chondroitin sulfate chain synthesis further along in the sequence, i.e. \( \beta \)-D-galactosidase and \( \beta \)-D-N-acetyl-galactosamine (10). These findings are consistent with previous studies on the substrate specificities of the various chondroitin sulfate glycosyltransferases which showed that only the first galactosyltransferase can use a monosaccharide, \( \beta \)-xylene, as an acceptor.

It is of interest that an apparent lack of specificity for the natural substrate (i.e. xylosylated core protein) should occur only at this reaction site, since recently, an interaction between xylosyltransferase and galactosyltransferase was demonstrated (38-40), which may be of physiological significance. When synthesizing the entire chondroitin sulfate proteoglycan molecule, the chain-initiating xylosyltransferase may serve to transfer core protein from the point of release from the polysomes to the exact membrane location of galactosyltransferase, allowing the remaining steps of chain assembly to proceed on the membrane surface (40). Alternatively, in the absence of core protein or xylosyltransferase, the galactosyltransferase is also capable of initiating the synthesis of free polysaccharide chains with \( \beta \)-xylose or \( \beta \)-N-xylosides.

Analysis of the product formed in the presence of the \( \beta \)-xyloside shows that following initiation, chain assembly also occurs. Thus, the \( \beta \)-xylosides are used not only as substrates for the first galactosyltransferase reaction, but induce formation of complete chondroitin sulfate chains. That the chondroitin sulfate chains produced under the influence of 4-methylumbelliferone-\( \beta \)-D-xylene bear the added substrate at the reducing end is confirmed by obtaining coincident peaks of radioactivity and fluorescence after separation of labeled polysaccharide chains by gel chromatography (Fig. 3B). However, the heterogeneity in size distribution of the xyloside-containing chains is also revealed by these chromatographic patterns.

In similar experiments, Okayama and Lowther (12) reported recently that \( \beta \)-xylosides have a variable effect on the chain length of the chondroitin sulfate, so that \( \beta \)-xylosides with increasingly aliphatic aglycone cause the formation of poly saccharides of progressively shorter chain length. Brett and Robinson (7) showed by comparison of elution profiles that free chondroitin sulfate chains formed in the presence of puromycin and xylose did not markedly differ in size from free chondroitin sulfate chains isolated from cartilage by treatment with alkali. We found, on the basis of the ratio of glucuronic acid to fluorescence of material isolated from the media of stimulated cultures, that the average molecular weight of the chains produced is 16,500.

If calculations are made on the basis of the elution volumes (23), the average molecular weight of the same material is approximately 12,500, while the average molecular weight of the alkali-digested material from control cells is approximately 20,000. All of these calculations are subject to error due to the polydisperse nature of the product studied. However, the observed difference in size between chondroitin sulfate chains produced by the cells in the normal state and the chains produced in the presence of the \( \beta \)-xyloside may, in fact, reflect a different behavior of the polymerizing system, depending on the presence or absence of protein core. Perhaps the mechanisms controlling glycosaminoglycan chain termination may be affected so that free chains are more readily secreted by the cells and, therefore, do not undergo complete polymerization, while the presence of the protein core has the effect of prolonging the time available for polymerization within the endoplasmic reticulum and the Golgi apparatus.

Since rat glial (18, 26), mouse neuroblastoma (25, 26), and hepatoma cells produce more than one type of glycosaminoglycan, the effect of \( \beta \)-xylosides on the individual components was investigated. It was also of interest to establish whether \( \beta \)-xylosides could initiate synthesis of chondroitin 4- and 6-sulfates, dermatan sulfate, heparan sulfate and heparin, which differ in the chemical structure, but are all linked to a protein core via the same linkage region (28, 29, 41–43). The results we obtained with RG-C6, NB41A, and HTC cultures indicate that \( \beta \)-xylosides act as initiators only of free chondroitin sulfate chains and not of heparan sulfate chains. It is possible that heparan sulfate is synthesized at a maximum level in these cells and, therefore, its synthesis is not stimulated by the addition of \( \beta \)-xylosides. Alternatively, while chondroitin sulfate synthesis requires only polymerization and sulfation, these other glycosaminoglycans require additional modifications following, or concomitant with, polymerization, and therefore, their rate of synthesis may be regulated by different mechanisms.

Dermatan sulfate differs from chondroitin sulfate only by the presence of 1-iduronic acid, which is formed by epimerization at C-5 of already polymerized L-gluconic acid residues of chondroitin sulfate chains (44). This problem was examined in normal and SV40-transformed normal fibroblasts. \( \beta \)-Xylosides caused a stimulation of chondroitin 4- and 6-sulfate production in both cell types, while the stimulation in the dermatan sulfate fraction (13-fold) was found only in normal fibroblasts (Table IV). The lack of \( \beta \)-xyloside stimulation of dermatan sulfate synthesis in transformed normal fibroblasts may reflect a limitation of the rate of epimerization, thus preventing conversion of chondroitin
sulfate to dermatan sulfate in these cells, while permitting a
greater accumulation of chondroitin sulfate chains than in normal
cells. Alternatively, these findings may be a result of the relative
distribution and content of L-iduronic acid and D-glucuronic
acid residues in dermatan sulfate produced by SV40-transformed
cells in the presence of β-xylosides, which could make the mole-
cule sensitive to testicular hyaluronidase and result in spuriously
high chondroitin sulfate values.

The results of analyses of the heparan sulfate fraction in both
normal and SV40-transformed fibroblasts are in accord with the
failure of xylosides to stimulate the synthesis of this polysac-
charide in other cell types. All cultures examined produce large
amounts of hyaluronic acid, but the addition of β-xylosides does
not affect this component, which might be expected, in view of
the fact that a xyloside linkage to a protein core has never been
reported for this glycosaminoglycan.

β-Xylosides, with their property of stimulating synthesis of
glycosaminoglycan chains, may be used as specific probes to
assay the potential of a particular cell to produce chondroitin
sulfate chains, and therefore, provide a method for separating
the cellular capacity to produce polysaccharide chains from
xylosylated core protein. Labeling the reducing end of chondroitin
sulfate chains with a fluorogenic xyloside, 4-methylumbelliferyl-
H$_3$PO$_4$ and [U-3H]acetate, may soon provide some answers
xylosylated core protein. Labeling the reducing end of chondroitin
sulfate chains, and therefore, provide a method for separating
assay the potential of a particular cell to produce chondroitin
sulfate values.

Science 172, 1263-1265
U. S. A. 66, 485-499
Chem. 243, 1536-1542
Publishers, Inc., New York
Biophys. 99, 395-400
133, 457-470
Tissue Culture of the Nervous System (Sato, G., ed) pp.
247-250, Plenum Press, New York
Biochem. 242, 462-466
25. Fransson, L.-A. (1970) in Chemistry and Molecular Biology of
823-842, Academic Press, New York
242, 4170-4175
136, 631-637
Acta 237, 214-226
Biophys. Acta 220, 240-243
34. Schwartz, N. B. (1975) FEBS Lett. 49, 342-345
International Santa Catalina Colloquium on Extracellular
Matrix Influences on Gene Expression (Slavkin, H. C., and
York
241, 65-70
240, 2821-2826
(1973) J. Biol. Chem. 475, 7234-7241
Stimulation of synthesis of free chondroitin sulfate chains by beta-D-xylosides in cultured cells.
L Galligani, J Hopwood, N B Schwartz and A Dorfman


Access the most updated version of this article at http://www.jbc.org/content/250/14/5400

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/250/14/5400.full.html#ref-list-1