Proteoglycan and Glycosaminoglycan Synthesis in Embryonic Mouse Salivary Glands: Effects of β-D-Xyloside, an Inhibitor of Branching Morphogenesis

HOLLY A. THOMPSON and BRIAN S. SPOONER
Division of Biology, Kansas State University, Manhattan, Kansas 66506. Dr. Thompson's present address is the Mineralized Tissue Research Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20205.

ABSTRACT   The proteoglycans and glycosaminoglycans synthesized by embryonic mouse salivary glands during normal morphogenesis and in the presence of β-xyloside, an inhibitor of branching morphogenesis, have been partially characterized. Control and p-nitrophenyl-β-D-xyloside-treated salivary rudiments synthesize proteoglycans that are qualitatively similar, based on mobility on Sepharose CL-4B under dissociative conditions and glycosaminoglycan composition. However, β-xyloside inhibits total proteoglycan-associated glycosaminoglycan synthesis by 50%, and also stimulates synthesis of large amounts of free chondroitin (dermatan) sulfate. This free glycosaminoglycan accounts for the threefold stimulation of total glycosaminoglycan synthesis in β-xyloside-treated cultures. Several observations suggest that the disruption of proteoglycan synthesis rather than the presence of large amounts of free glycosaminoglycan is responsible for the inhibition of branching morphogenesis. (a) We have been unable to inhibit branching activity by adding large amounts of chondroitin (dermatan) sulfate, extracted from β-xyloside-treated cultures, to the medium of salivary rudiments undergoing morphogenesis. (b) In the range of 0.1–0.4 mM β-xyloside, the dose-dependent inhibition of branching morphogenesis is directly correlated with the inhibition of proteoglycan synthesis. The stimulation of free glycosaminoglycan synthesis is independent of dose in this range, since stimulation is maximal even at the lowest concentration used, 0.1 mM. The data strongly suggest that the inhibition of branching morphogenesis is caused by the disruption of proteoglycan synthesis in β-xyloside-treated salivary glands.

Epithelial-mesenchymal interactions are required for the development of many diverse organ systems. The tissues are separated by an extracellular matrix, suggesting that developmental events might be mediated by such extracellular macromolecules as collagens (7, 28), proteoglycans (12, 33), and glycoproteins (7, 32). Recently, attention has focused on the role of the extracellular matrix and the basal lamina, a zone of extracellular material having a characteristic ultrastructural organization, in tissue interactions.

Development of the embryonic salivary gland provides a useful model system for studying the role of proteoglycans in epithelial-mesenchymal interactions. The multilobular morphology of salivary glands is established by branching morphogenesis, repetitive budding, and folding of the epithelium. Although these shape changes are generated by forces within the epithelial cells themselves (29), interaction with the mesenchyme is required for branching morphogenesis. Indeed, the pattern of epithelial morphogenesis appears to be regulated by the mesenchyme (21). The two tissues are separated by a basal lamina that contains abundant glycosaminoglycan (5), presumably as proteoglycan. Integrity of the basal lamina is required for maintenance of lobular morphology of the salivary rudiments (1). Furthermore, turnover of basal laminar glycosaminoglycan correlates with branching activity of the epithelium (2). These observations strongly implicate proteoglycans in the process of branching morphogenesis.

One approach to studying the role of proteoglycans in branching morphogenesis is to perturb normal biosynthesis of the molecules. Synthesis of the xylose-linked classes of glycosaminoglycans, which include chondroitin sulfates, dermatan

sulfate, heparin, and heparan sulfate, is initiated by xylosylation of certain serine residues in the proteoglycan core proteins. Two galactose residues are sequentially transferred to each xylosylated serine, and glycosaminoglycan chains are then formed on these linkage regions by addition of the appropriate monosaccharides. Exogenously added β-D-xylosides can disrupt proteoglycan biosynthesis by competing with the endogenous substrate, xylosylated core protein, at the level of the first galactosyltransferase, thereby initiating the synthesis of free glycosaminoglycan chains. Both the β-anomer and the xylose configuration are required, reflecting specificity of the enzyme (9). Beta-xylosides stimulate total glycosaminoglycan synthesis in chondrocytes (19); glial, neuroblastoma, and hepatoma cells (26); and normal and transformed fibroblasts (9), suggesting that synthesis is normally limited by core protein in these systems. A concomitant decrease in proteoglycan synthesis has been reported for β-xyloside-treated chondrocytes (18, 25). We have exploited these characteristics of β-xylosides to investigate the role of proteoglycan synthesis in branching morphogenesis.

Branching morphogenesis of embryonic mouse salivary glands is inhibited severely, but reversibly, by p-nitrophenyl-β-D-xylopyranoside (33). The degree of inhibition is dose-dependent, and similar concentrations of α-xyloside do not alter branching activity of the epithelium. Synthesis of total sulfated glycosaminoglycan is stimulated threefold in β-xyloside-treated glands. These observations suggest that the inhibition of branching morphogenesis results from the effects of competition of β-xyloside with endogenous substrate, core protein, at the level of the first galactosyltransferase in the biosynthetic pathway. The present study demonstrates that synthesis of proteoglycan-associated glycosaminoglycan is inhibited, and free glycosaminoglycan synthesis is dramatically stimulated in β-xyloside-treated glands. Dose-response studies suggest that inhibition of branching morphogenesis results from inhibition of proteoglycan-associated glycosaminoglycan synthesis, rather than from stimulation of free glycosaminoglycan synthesis.

MATERIALS AND METHODS

Materials: The xylose derivative p-nitrophenyl-β-D-xylopyranoside, a product of Koch-Light Laboratories, was purchased from Research Products International Corp. (Mt. Prospect, IL). Grade I guanidine hydrochloride (Sigma Chemical Co., St. Louis, MO) was used in the extraction and elution buffers. Practical grade guanidine hydrochloride (Sigma Chemical Co.) was purified with activated charcoal (3) and used in the dialysis buffer. Benzamidine hydrochloride, 6-aminohexanoic acid, sodium EDTA, whale and shark cartilage chondroitin sulfate, heparin, Sephadex G-50, and Sepharose CL-4B were purchased from Sigma Chemical Co. Butyl nitrite was purchased from Eastman Laboratory and Specialty Chemicals, Eastman Kodak Co., Rochester, NY. Carrier-free [^{35}S]-sulfuric acid was obtained from ICN Chemical and Isotope Division (ICN K & K Laboratories Inc., Plainview, NY). Pronase (45,000 proteolytic U/g) was purchased from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA), and chondroitinase ABC from Miles Laboratories Inc. (Research Products Div., Elkhart, IN).

Organ Culture: Pregnant CD-1 outbred albino mice (Charles River Breeding Laboratories, Inc., Wilmington, MA) were sacrificed by cervical dislocation at 13 d of gestation, counted from day 0 at discovery of the vaginal plug. Embryos were removed and transferred to Hanks' balanced salt solution. Under sterile conditions, salivary glands were dissected from the embryos. Organs were cultured in a modified Ham's F12 medium (27) containing 2X amino acids and pyruvate, antibiotics, and 10% fetal calf serum (Gibco Laboratories, Gibco Div., Grand Island, NY). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO$_2$ in air. For morphogenetic studies, organ rudiments were cultured on Grobstein organ culture assemblies (10) as described previously (33), and the living rudiments were photographed daily.

Labeling and Extraction of Proteoglycan: Pieces of tissue consisting of the submandibular and sublingual glands together with the associated condensed mesenchyme (as in Fig. 1) were grown in submerged culture in Falcon plastic culture dishes (Becton, Dickinson & Co., Oxnard, CA). The salivary rudiments were cultured for 48 h in control medium or in medium containing β-xyloside. Media were then replaced with serum-free F12 containing the appropriate concentration of β-xyloside and 50 μCi [^{35}S]-sulfuric acid/ml medium. Serum-free medium enhances incorporation of labeled precursor but has no discernible effects on branching morphogenesis during the labeling period. After 10 h of incubation with label; tissue, medium, and one Hanks' balanced salt solution rinse of the culture dishes were pooled in siliconized tubes. Samples were stored at −20°C.

Proteoglycan was extracted by incubating the samples for 24 h at 4°C in buffer A (4 M guanidine·HCl, 0.1 M 6-aminohexanoic acid, 10 mM sodium EDTA, 5 mM benzamidine·HCl, and 0.5 M sodium acetate, pH 5.8 [11]). Insoluble material was removed by centrifugation at 1,000 g for 10 min. The pellet was reextracted for 24 h, and centrifuged again. The two supernatants were combined, vortexed, and divided into two equal aliquots. One aliquot was chromatographed on Sepharose CL-4B immediately, whereas the other was digested with pronase before chromatography.

Column Chromatography: Samples were concentrated by ultrafiltration with an Amicon PM10 filter (Amicon Corp. Scientific Sys. Div., Danvers, MA) and dialyzed against buffer A containing 0.1 M sodium sulfate. Samples were applied to a Sepharose CL-4B column (120 cm × 1.5 cm) equilibrated with buffer A containing 0.1 M sodium sulfate and adjusted to pH 7.0. Aliquots of each fraction were dissolved in an aqueous cocktail and radioactivity was measured with a Packard Tricarb liquid scintillation spectrometer (Packard Instrument Co., Inc., United Technologies, Downers Grove, IL).

Pronase Digestion: Aliquots of the guanidine·HCl extract for pronase digestion were concentrated by ultrafiltration as above, then dialyzed against 0.2 M Tris·HCl, pH 8.0. Samples were digested with pronase as described previously (33). Trichloroacetic acid was added (to 5%) to the digested samples and, following incubation at 0°C for 1 h, they were centrifuged at 2,000 g for 10 min. The supernatant was concentrated in a Speed Vac Concentrator (Savant Instruments, Inc., Hicksville, NY), dialyzed against buffer A containing 0.1 M sodium sulfate, and chromatographed as above.

Chondroitinase ABC and Nitrous Acid Digestion: Sepharose CL-4B peaks were treated with chondroitinase ABC, which selectively degrades chondroitin-4-sulfate, chondroitin-6-sulfate, and dermatan sulfate. These glycosaminoglycan classes are referred to collectively as chondroitin (dermatan) sulfate throughout this paper. Fractions in each peak were pooled, lyophilized, resuspended, and dialyzed against 0.01 M Tris·HCl, pH 8.0, and digested with chondroitinase ABC (final concentration 0.15 U/ml) for 24 h at 25°C (23). Commercially obtained chondroitin sulfate controls were digested simultaneously with labeled samples to confirm enzyme activity. Digestion products were separated from chondroitinase ABC-insensitive material by chromatography on Sephadex G-50 columns. Lyophilized samples were dissolved in elution buffer (0.1 M ammonium acetate in 20% ethanol) and applied to the columns (6). Aliquots of the Sephadex G-50 fractions were dried at 50°C and radioactivity was determined.

The chondroitinase ABC-insensitive material was then treated with nitrous acid to selectively degrade the N-sulfated glycosaminoglycans, heparin, and heparan sulfate (4, 6). The Sephadex G-50 excluded fractions were pooled, lyophilized, redissolved in water, and digested as described previously (33). Commercially obtained heparin samples were treated simultaneously with labeled samples. Digested samples were lyophilized, dissolved in elution buffer, and rechromatographed on Sephadex G-50 columns to separate digestion products from nitrous acid-insensitive material.

Preparation of Glycosaminoglycan Fractions: Free glycosaminoglycan synthesized by salivary glands treated with 0.5 mM β-xyloside was prepared for addition to the culture medium of living rudiments. Sepharose CL-4B fractions from the glycosaminoglycan peak were pooled and dialyzed against four changes of 10% F12 over 3 d. Likewise, an equal volume of the 4 M guanidine·HCl elution buffer was dialyzed against 10% F12 to provide a buffer control. The dialyzates were lyophilized and weighed, before being added to culture medium.

Electron Microscopy: Day 13 salivary glands were cultured on Millipore filters (Millipore/Continental Water Systems, Bedford, MA) in Grobstein dishes or on Falcon plastic petri dishes. Rudiments were cultured for 48 h in control medium or medium containing 0.5 mM α- or β-xyloside; fixed with 2.5% glutaraldehyde in 75 mM phosphate buffer, pH 7.2; postfixed with 1% OsO$_4$ in 75 mM phosphate buffer containing 180 mM sucrose, pH 7.2; dehydrated; and embedded in Epon-Araldite. Sections were stained with uranyl acetate and lead citrate, and examined with a Philips 201 electron microscope.

RESULTS

Branching Morphogenesis of Salivary Glands In Vitro

Salivary rudiments dissected from 13-d mouse embryos increase substantially in size during a 48-h culture period (Fig. 1 A and B). New lobes are formed by repetitive branching and folding of the epithelium. Like control rudiments, salivary glands cultured in the presence of 0.5–1.0 mM β-xyloside continue to grow, accumulating total protein per rudiment that is 85–100% of the protein accumulation in control glands. The ultrastructural organization of the epithelium and the surrounding mesenchyme appears normal in the treated rudiments (Fig. 2). However, few new lobes are formed (Fig. 1 C and D). Thus, branching morphogenesis is severely inhibited by 0.5 mM β-xyloside.

Sepharose CL-4B Profiles of Proteoglycans and Glycosaminoglycans Synthesized by Salivary Glands In Vitro

From both control and β-xyloside-treated cultures, three major size classes of sulfated macromolecules are resolved by Sepharose CL-4B chromatography under dissociative conditions (Fig. 3). Although the peaks contain different proportions of radioactivity in the two sample types, the comparable peaks from control and β-xyloside-treated cultures have similar elution volumes. Both the largest size class (PG-1), which elutes at the void volume, and the intermediate size class (PG-2) contain sulfated proteoglycan. All of the radioactivity in PG-1 and PG-2, whether from control or β-xyloside-treated cultures, is shifted to a peak with much larger elution volume in pronase-digested samples (not shown). Because the smallest size class of sulfated macromolecules from control cultures, peak-3, has an elution volume similar to that of the pronase-liberated glycosaminoglycan chains, comparison of the CL-4B profiles of untreated and pronase-digested samples is not useful for distinguishing whether peak-3 contains protein-associated gly-
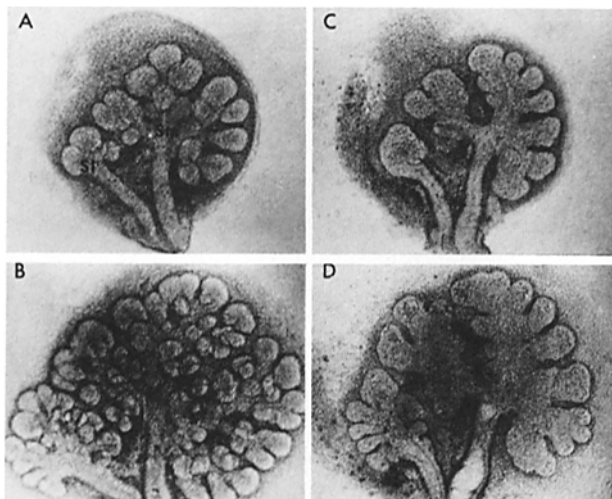
FIGURE 2 Transmission electron micrograph of an epithelial-mesenchymal interface region of a rudiment cultured in 0.5 mM β-xyloside for 48 h (X 7,700). The basal lamina (BL) is continuous over the epithelial (E) surface. Abundant fibrillar collagen (C) is seen in the extracellular matrix surrounding mesenchymal cells (M).

cosaminoglycan. Therefore, fractions containing control peak-3 were pooled, digested with pronase, and rechromatographed on CL-4B. The elution volume is not altered by pronase digestion, suggesting that peak-3 contains either free glycosaminoglycan or small proteoglycan molecules with only a few glycosaminoglycan chains per molecule. Likewise, the elution volume of peak-3 from β-xyloside-treated glands is identical for both untreated and pronase-digested samples (not shown), suggesting that peak-3 contains predominantly glycosaminoglycan chains that have been initiated on the β-xyloside molecules.

The Sepharose CL-4B profiles in Fig. 3 also demonstrate the quantitative effects of 0.5 mM β-xyloside on synthesis of glycosaminoglycan in salivary rudiments. Synthesis of total proteoglycan-associated glycosaminoglycan, PG-1 plus PG-2, is inhibited 50% in treated cultures. However, such cultures accumulate large amounts of newly synthesized free glycosaminoglycan, which presumably is initiated on the β-xyloside molecules. The large amounts of free glycosaminoglycan account for a threefold stimulation of total sulfated glycosaminoglycan synthesis. This value is in agreement with the threefold stimulation measured by other procedures and reported previously (33).

Beta-xylosides have been reported to decrease the number and average chain length of glycosaminoglycans associated with core protein in several systems (15, 17, 18, 20). Beta-xyloside does not appreciably alter the elution volume on CL-4B of the major size classes of sulfated macromolecules: PG-1 is not removed from the excluded to included fractions and the broad PG-2 peak, although overlapping with the free glycosaminoglycan peak, has an elution volume similar to that of

FIGURE 1 Branching morphogenesis of control and β-xyloside-treated salivary glands. A pair of 13-d salivary glands (Sm, submandibular; Sl, sublingual) cultured in control medium is shown at 24 h (A) and 48 h (B) of culture. Rudiments cultured in 0.5 mM β-xyloside are shown at 24 h (C) and 48 h (D) of culture. Although both pairs of glands undergo substantial increase in size, branching activity of the β-xyloside-treated rudiments is inhibited. X 26.
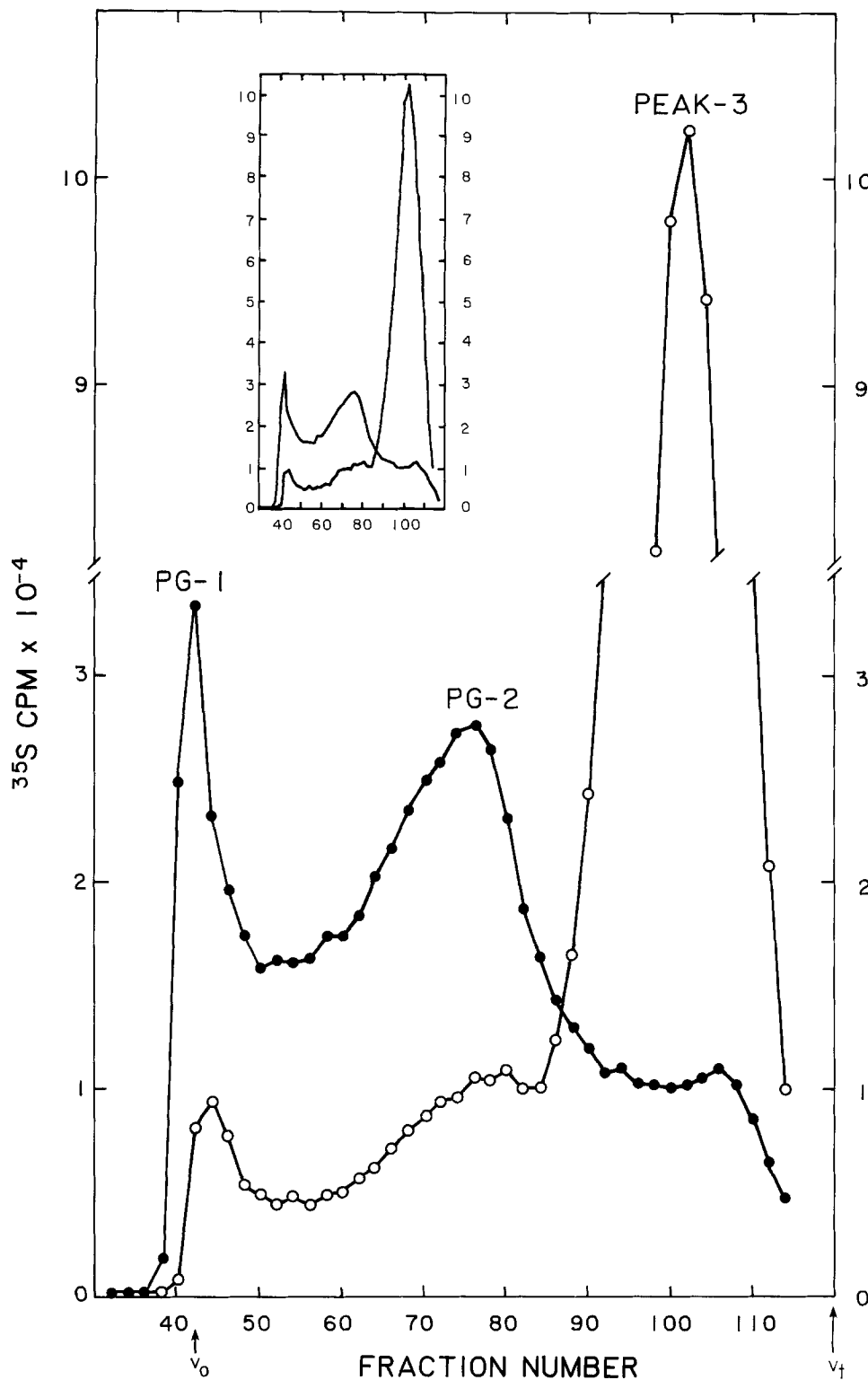
FIGURE 3 Sepharose CL-4B profiles of newly synthesized proteoglycan and glycosaminoglycan. Control (●) and β-xyloside-treated (○) salivary cultures were labeled with [35S]-sulfate, extracted with 4 M guanidine·HCl, and chromatographed. *Inset* shows the entire profile to indicate, for β-xyloside-treated cultures, the large size of *PEAK-3* relative to *PG-1* and *PG-2*. Peaks PG-1 and PG-2 are proteoglycans, and peak-3 appears to be free glycosaminoglycan (see text).

PG-2 from control cultures. However, the possibility that treated rudiments synthesize proteoglycans with fewer or shorter glycosaminoglycan chains cannot be ruled out without direct comparison of the glycosaminoglycan moieties of these proteoglycans.

Chondroitinase ABC and Nitrous Acid Sensitivity of Glycosaminoglycans Synthesized by Cultured Salivary Glands

In order to further characterize the sulfated glycosaminogly-

cans produced by embryonic salivary glands in culture, pooled fractions from each of the Sepharose CL-4B peaks were assayed for sensitivity to chondroitinase ABC and nitrous acid degradation. Chondroitinase ABC is specific for the chondroitin sulfates (chondroitin-4-sulfate, chondroitin-6-sulfate, and dermatan sulfate), whereas nitrous acid degrades heparan sulfate and heparin. Chondroitin (dermatan) sulfate accounts for most of the labeled glycosaminoglycan in PG-1. 88% of the sulfated material, either from control or β-xyloside-treated cultures, is sensitive to chondroitinase ABC digestion (Table I). Peak PG-2 contains both chondroitin (dermatan) sulfate and heparan

TABLE I

Sensitivity of Sepharose CL-4B Proteoglycan Peaks to
Chondroitinase ABC and Nitrous Acid Digestion

	PG-1		PG-2	
	Control	β-Xyloside	Control	β-Xyloside
	%		%	
Chondroitinase-ABC-sensitive	88*	88	74	67
Nitrous-acid-sensitive	2	ND	24	22
Chondroitinase-ABC- and nitrous-acid-insensitive	10	ND	2	11

* Numbers represent percent of total counts per min ^{35}S in sample.
ND, not done.

TABLE II

Sensitivity of Sepharose CL-4B Peak 3 to Chondroitinase ABC
and Nitrous Acid Digestion

	Control		β-Xyloside	
	cpm/rudiment	%	cpm/rudiment	%
Chondroitinase-ABC-sensitive	30	14*	4713	97
Nitrous-acid-sensitive	186	84	139	3
Chondroitinase-ABC- and nitrous-acid-insensitive	6	3	11	<1

* Numbers represent percent of total counts per min ^{35}S in sample.

sulfate, suggesting that this size class includes more than one species of proteoglycan. Chondroitinase ABC degrades 74% of the labeled glycosaminoglycan in PG-2 from control cultures and 67% from β-xyloside-treated cultures. Sensitivity of the sulfated glycosaminoglycan in PG-2 to nitrous acid is also similar: 24% (control cultures) and 22% (β-xyloside-treated cultures). Thus, based on sensitivity to chondroitinase ABC and nitrous acid degradation, β-xyloside does not alter the glycosaminoglycan composition of PG-1 and PG-2.

The sulfated glycosaminoglycan in peak-3 from control cultures is predominantly heparan sulfate. While 84% of the labeled material is sensitive to degradation with nitrous acid, only 14% is digested by chondroitinase ABC (Table II). It is not clear whether this material, which appears to be either free glycosaminoglycan or proteoglycan with only a few glycosaminoglycan chains per molecule, occurs normally in embryonic salivary glands in vivo, occurs only in cultured rudiments, or results from the extraction procedures. However, heparan sulfate with little or no associated protein has been reported in other systems, such as embryonic lung fibroblasts (34), 3T3 cells (14), and PYS-2 cells (22).

In contrast to control peak-3, only 3% of the sulfated glycosaminoglycan in peak-3 from β-xyloside-treated cultures is sensitive to nitrous acid degradation (Table II). However, 3% of the large peak represents 139 cpm [^{35}S]-heparan sulfate/rudiment, a value similar to the 186 cpm [^{35}S]-heparan sulfate/rudiment in peak-3 from control cultures (Table II). The data suggest that this free heparan sulfate is neither dramatically stimulated nor inhibited in the presence of β-xyloside.

The remainder of peak-3 in β-xyloside-treated cultures, 97%, is sensitive to digestion with chondroitinase ABC (Table II). This huge pool of free chondroitin (dermatan) sulfate accounts for 82% of the total sulfated glycosaminoglycan accumulated during the labeling period (data averaged from three experiments). The observations that the elution volume on CL-4B of peak-3 is not altered by pronase digestion and that this large

chondroitin (dermatan) sulfate peak is unique to β-xyloside-treated cultures strongly suggest that its synthesis is initiated on the exogenously added β-xyloside, rather than on the endogenous substrate, core protein.

Effect of Free Glycosaminoglycan on Branching Morphogenesis of Cultured Salivary Glands

Since β-xyloside inhibits synthesis of proteoglycan-associated glycosaminoglycan as well as initiating synthesis of large amounts of free glycosaminoglycan, it is of interest to determine whether either of these effects is related to the inhibition of branching morphogenesis. The effect of free glycosaminoglycan on branching morphogenesis was tested by culturing salivary rudiments in the presence of exogenously added glycosaminoglycans, obtained either from β-xyloside-treated cultures or as commercially available chondroitin sulfate. Chondroitin (dermatan) sulfate synthesized by salivary glands treated with 0.5 mM β-xyloside was prepared as described in Materials and Methods. An identical volume of the guanidine buffer was prepared in the same way, to provide a buffer control. Day 13 salivary glands were cultured in control medium, or medium containing various concentrations of the dialyzed buffer or glycosaminoglycan or commercially obtained chondroitin sulfate. Media were changed daily.

The extent of branching morphogenesis after 48 h of culture is shown in Fig. 4. In the presence of 0.5 mM β-xyloside, branching activity is severely inhibited. We have been unable to significantly alter branching activity by adding 0.51-1.27 mg of the prepared free glycosaminoglycan fractions per ml of medium, although this represents approximately two to five times the amount of free chondroitin (dermatan) sulfate accumulated over 10 h in cultures chronically treated with β-xyloside. The appearance of such glands is indistinguishable

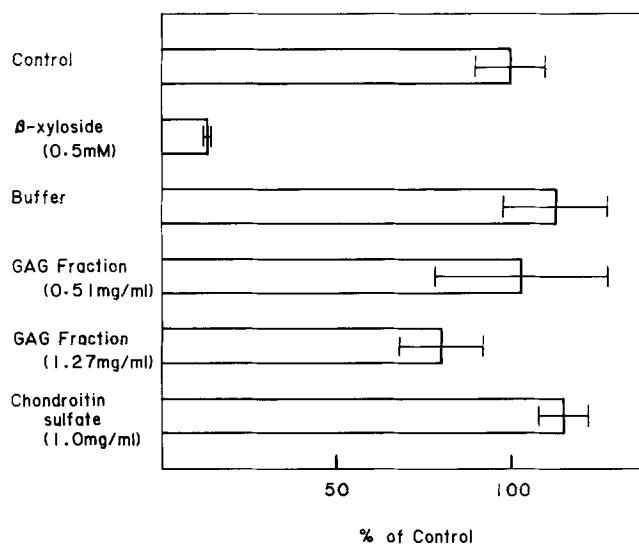
FIGURE 4 The effects of β-xyloside and free glycosaminoglycan on branching morphogenesis of salivary glands. Salivary rudiments were cultured in control medium or medium containing β-xyloside (0.5 mM); free glycosaminoglycan isolated from β-xyloside-treated cultures (0.51 or 1.27 mg/ml); buffer alone, prepared identically to the free glycosaminoglycan; or commercially obtained chondroitin sulfate from cartilage (1.0 mg/ml). The extent of branching morphogenesis after 48 h in culture, i.e., the number of lobes per submandibular gland, is expressed as percent of control. Data is averaged from two experiments, with five to nine rudiments per experimental condition. Error bars indicate standard error of the mean.

from control rudiments. It should be pointed out that this free glycosaminoglycan has been prepared from the CL-4B peak and therefore contains other components of similar molecular size, such as many proteins. The final concentration of glycos-
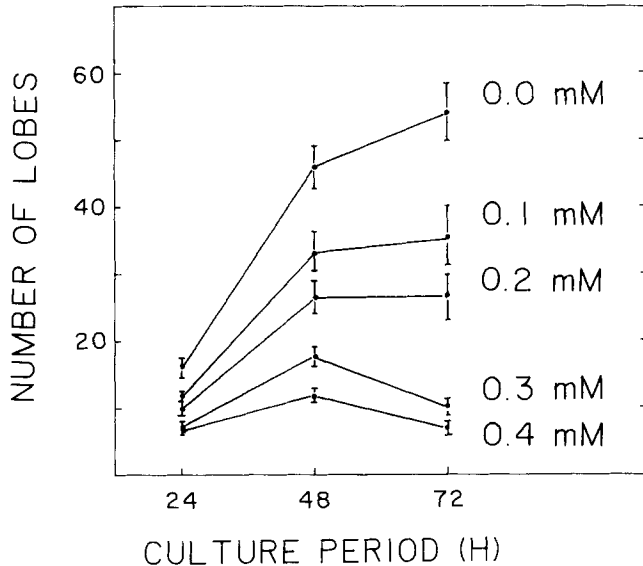

FIGURE 5 Correlation of the inhibition of branching morphogenesis with concentration of β-xyloside. Extent of branching morphogenesis is indicated by the number of lobes per submandibular gland at 24, 48, and 72 h of culture. Error bars indicate standard error of the mean (n = 23-26).

aminoglycan in the medium is thus <0.51-1.27 mg/ml. However, even 1.0 mg/ml of cartilage chondroitin sulfate does not alter branching activity. Together, these data suggest that the inhibition of branching morphogenesis in β-xyloside-treated salivary glands is not caused by the large amounts of free chondroitin (dermatan) sulfate that accumulate in these cultures.

Effects of Increasing Doses of β-Xyloside on Proteoglycan Synthesis, Free Glycosaminoglycan Synthesis, and Branching Morphogenesis

In order to determine whether the inhibition of branching morphogenesis is linked to the inhibition of proteoglycan synthesis, the responses to varying doses of β-xyloside were examined. Salivary glands cultured in control medium for 3 d undergo extensive branching activity, resulting in the formation of many new lobes (Fig. 5). When cultured in 0.1-0.4 mM β-xyloside, a dose-dependent inhibition of morphogenesis is observed. The effects of the same concentrations on glycosaminoglycan synthesis were determined by culturing the rudiments for 48 h in control medium or medium containing 0.1-0.4 mM β-xyloside and labeling with [^{35}S]-sulfate for 10 h. Sepharose CL-4B profiles of the sulfated macromolecules are shown in Fig. 6. The inhibition of proteoglycan-associated glycosaminoglycan synthesis is directly related to the concentration of β-xyloside. However, stimulation of free glycosaminoglycan synthesis is not dose-dependent over this range of β-xyloside concentrations. Rather, free chondroitin (dermatan) sulfate
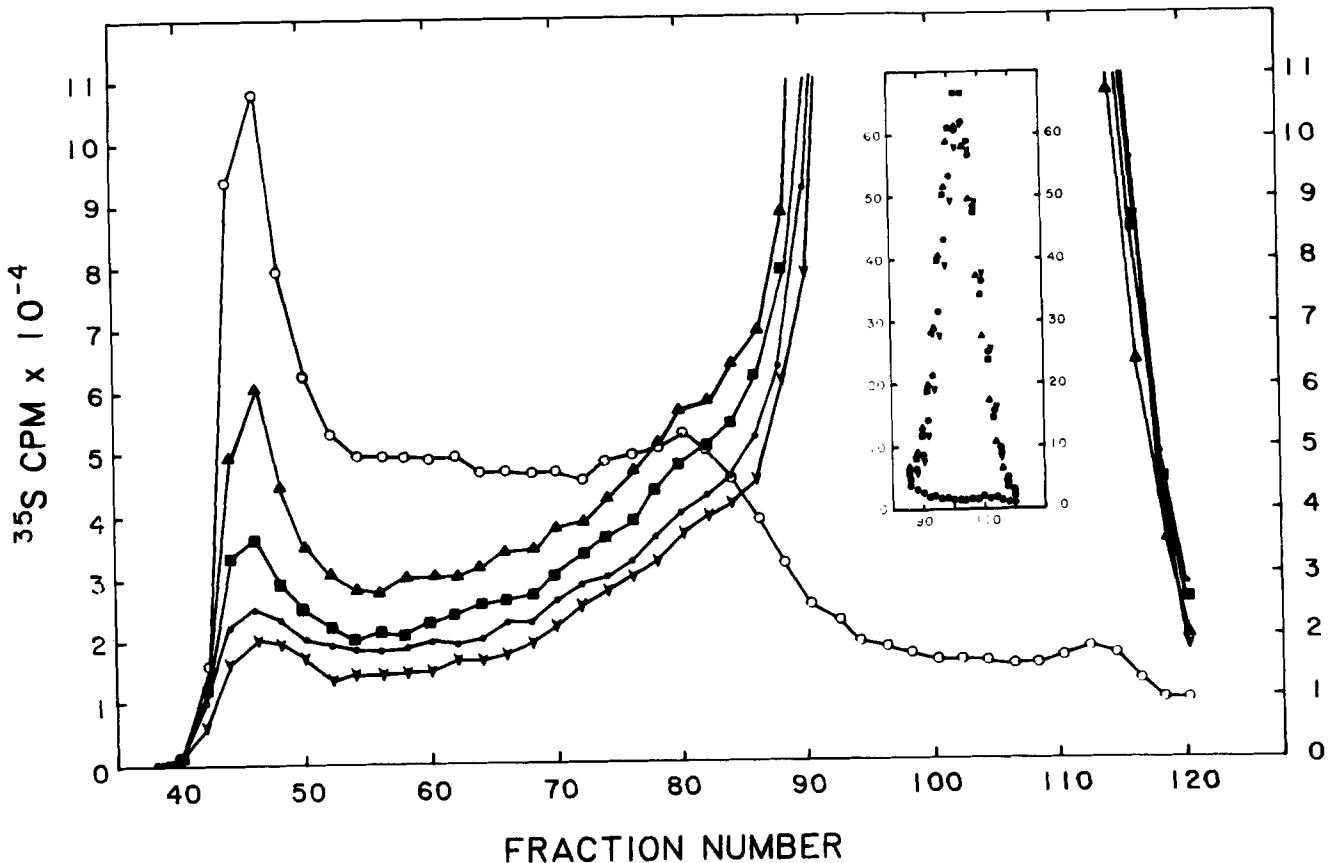

FIGURE 6 Sepharose CL-4B profiles of proteoglycan and glycosaminoglycan extracted from control and β-xyloside-treated cultures. Guanidine·HCl extracts from control cultures (O) elute as three major size classes: PG-1, PG-2, and a very small peak-3. Profiles of extracts from cultures treated with β-xyloside at concentrations of 0.1 mM (▲), 0.2 mM (■), 0.3 mM (●), and 0.4 mM (closed arrowheads) are shown. The inhibition of proteoglycan associated glycosaminoglycan synthesis (PG-1 and PG-2) is directly correlated with concentration of β-xyloside. Inset shows the entire free glycosaminoglycan peak (peak-3). Size of peak-3 is independent of β-xyloside concentration between 0.1 and 0.4 mM.

synthesis is maximally stimulated at the lowest concentration tested, 0.1 mM β-xyloside.

DISCUSSION

Previously we had demonstrated that branching morphogenesis of embryonic salivary glands is inhibited by β-xyloside (33). The inhibition is dose-dependent, specific for the β-anomer, and reversible, and is correlated with a stimulation of total glycosaminoglycan synthesis. In the present study, the effects of β-xyloside on proteoglycan and glycosaminoglycan synthesis, and the relationship between these effects and inhibition of branching morphogenesis have been determined.

Partial characterization, on the basis of mobility on Sepharose CL-4B and sensitivity to degradation with chondroitinase ABC and nitrous acid, does not resolve qualitative differences in the proteoglycans synthesized by control and β-xyloside-treated rudiments. However, quantitatively, synthesis of total proteoglycan-associated glycosaminoglycan is inhibited ~50% in salivary glands treated chronically with 0.5 mM β-xyloside.

In addition to inhibiting proteoglycan-associated glycosaminoglycan synthesis, β-xyloside stimulates synthesis of large amounts of free chondroitin (dermatan) sulfate. It is interesting that heparan sulfate synthesis is not stimulated in the presence of β-xyloside. The galactosyl-galactosyl-xylosyl-serine linkage region is common to heparin and heparan sulfate and chondroitin and dermatan sulfates (16, 24), suggesting that β-xylosides should serve as initiators for synthesis of all of these classes of glycosaminoglycans. Indeed, in SV-40-transformed 3T3 cells, β-xyloside stimulates synthesis of both heparan and chondroitin sulfates (13). However, response to the xylose derivatives varies greatly. In rat hepatocytes, β-xyloside initiates synthesis of heparan sulfate, but only if core protein synthesis is inhibited by cycloheximide (31). In rat glial and mouse neuroblastoma cells, synthesis of chondroitin sulfates is dramatically stimulated; however, as in salivary glands, heparan sulfate synthesis is not stimulated (9). Finally, rat serosal mast cells, which normally produce only heparin proteoglycan, synthesize free glycosaminoglycan chains that are predominantly chondroitin sulfates in response to β-xyloside (30). The significance of the variable response to β-xyloside in terms of regulation of glycosaminoglycan synthesis in these systems is not clear, but poses some intriguing questions.

That the inhibition of branching morphogenesis is not caused by the presence of large amounts of free chondroitin (dermatan) sulfate in the β-xyloside-treated cultures is supported by several observations: (a) We have been unable to inhibit branching morphogenesis of salivary rudiments by adding to the medium large amounts of free chondroitin (dermatan) sulfate extracted from β-xyloside-treated cultures. (b) Similarly, addition of 1.0 mg/ml commercially obtained cartilage chondroitin sulfate fails to inhibit branching activity. (c) Over the range of 0.1–0.4 mM β-xyloside, there is no correlation between the inhibition of branching morphogenesis and the synthesis of free chondroitin (dermatan) sulfate. That is, synthesis is already maximally stimulated at the lowest dose, 0.1 mM, where morphogenesis is only minimally affected.

The present study instead suggests that β-xyloside inhibits morphogenesis by inhibiting synthesis of proteoglycan-associated glycosaminoglycan. The data demonstrate that the inhibition of proteoglycan-associated glycosaminoglycan synthesis by 0.1–0.4 mM β-xyloside *is* dose-dependent, and correlates directly with a dose-dependent inhibition of branching morphogenesis. The biosynthetic inhibition could be accounted for by synthesis of decreased numbers of proteoglycan molecules

or molecules with fewer or shorter glycosaminoglycan chains. Such effects have been reported for β-xyloside-treated chondrocytes, which synthesize proteoglycan with both fewer and shorter glycosaminoglycan chains (17). Further characterization of the proteoglycans synthesized by salivary rudiments in the presence of β-xyloside will be necessary to distinguish between these possibilities. Nevertheless, the present study does establish that disruption of proteoglycan synthesis results in inhibition of branching morphogenesis.

The dynamic nature of glycosaminoglycan in the basal laminae of embryonic salivary glands has been demonstrated by Bernfield and Banerjee (2). Rates of turnover of basal laminar glycosaminoglycan, presumably as proteoglycan, correlate with actively branching and morphogenetically quiescent regions of the epithelium. Both synthesis of basal laminar glycosaminoglycan by the epithelium and degradation by the mesenchyme contribute to turnover rates. The same investigators have shown that the integrity of the chondroitin sulfate-rich basal lamina is required for maintenance of lobular morphology in salivary rudiments (1, 5). They suggest that accumulation of the epithelially produced basal laminar glycosaminoglycan in the interlobular clefts, where rates of synthesis exceed rates of degradation, might contribute to stabilization of lobular morphology, whereas the relatively rapid degradation at the distal regions of lobes is necessary to accommodate growth of the epithelium (2). Both stabilization of interlobular clefts and growth of the epithelial lobes are required for ongoing branching activity. It is therefore reasonable to ask whether β-xyloside, at concentrations that inhibit synthesis of proteoglycan-associated glycosaminoglycan, also affect the ultrastructural organization of the basal lamina. Using standard glutaraldehyde/osmium fixation, we have been unable to detect any obvious differences in the basal laminae of control and β-xyloside-treated rudiments (note the normal appearance of the basal lamina in Fig. 2). However, it should be emphasized that normal ultrastructural appearance does not imply normal function. It is probable that classes of macromolecules other than proteoglycans, such as laminin and/or type IV collagen, are integral components of the basal lamina. Thus, in the presence of 0.5 mM β-xyloside, sufficient proteoglycan might be inserted into the basal lamina to maintain the normal ultrastructural organization, but not to support branching morphogenesis. The organization of anionic sites in basal laminae has been studied in tissues fixed in the presence of ruthenium red (5) or polyethyleneimine (8). Such approaches may be useful for distinguishing changes in the organization of proteoglycan in β-xyloside-treated basal laminae.

Although the studies linking basal laminar glycosaminoglycan with branching morphogenesis suggest that β-xyloside may disrupt basal laminar function, it is also possible that an abnormal extracellular matrix is laid down by the mesenchymal cells. Spooner and Faubion (28) have demonstrated that inhibition of collagen secretion in the presence of L-azetidine-2-carboxylic acid is correlated with both loss of interstitial collagen fibrils and inhibition of branching morphogenesis, suggesting that branching activity is dependent on composition and/or organization of the mesenchymally produced matrix. It is also possible that the inhibition of branching morphogenesis in the presence of β-xyloside results from disruption of proteoglycan synthesis in both epithelial and mesenchymal cells.

The observation that large amounts of free glycosaminoglycan do not substitute for the proteoglycan-associated glycosaminoglycan that mediates branching morphogenesis has important implications for the role of proteoglycans in tissue interactions. The organization of the glycosaminoglycan chains

on core protein may be required for proper insertion into the basal lamina or mesenchymal extracellular matrix. The protein core itself may contribute to interactions of the proteoglycan with other matrix components. Further characterization of the proteoglycans synthesized by β-xyloside-inhibited salivary rudiments will provide insight into the structural features of proteoglycans required for branching morphogenesis.

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