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BIOSYNTHESIS OF CHONDROITIN SULFATE. PURIFICATION OF UDP-D-XYLOSE:CORE PROTEIN β-D-XYLOSYLTRANSFERASE BY AFFINITY CHROMATOGRAPHY*†

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ABSTRACT

The chondroitin sulfate chain-initiating enzyme, UDP-D-xylose:core protein β-D-xylosyltransferase, has been purified from a high-speed supernatant fraction of a homogenate of embryonic chick cartilage by a procedure involving ammonium sulfate fractionation, gel chromatography on Sephadex G-200 resin, and affinity chromatography on a matrix consisting of core protein bound to Sepharose resin. The purified enzyme was homogeneous by electrophoretic and immunological criteria, had a molecular weight of 95,000–100,000 daltons, and was composed of two pairs of dissimilar subunits with molecular weights of ~23,000 and 27,000 daltons, respectively.

INTRODUCTION

Investigations of the structure and properties of glycosyltransferases have been hampered by the difficulties encountered in the isolation of these enzymes, which are often firmly bound to cellular membranes. Of the six glycosyltransferases involved in the biosynthesis of chondroitin sulfate, five are tightly associated with the membranes of the endoplasmic reticulum, and only recently have effective procedures been developed for their solubilization[3,4]. In contrast, the polysaccharide chain-initiating xylosyltransferase (UDP-D-xylose:core protein β-D-xylosyltransferase) is largely soluble and is found in the high-speed supernatant fraction of a cartilage homogenate. Partial fractionation has been achieved by precipitation with ammonium sulfate and gel chromatography on Sephadex G-200 resin or by density-gradient centrifugation[5]. Although the specific activity of the xylosyltransferase was increased 40- to 50-fold over that of the cell homogenate by these procedures, the gel-electrophoretic patterns of such preparations are still rather complex. In the present study, an

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affinity matrix consisting of core protein from cartilage proteoglycan bound to Sepharose resin was used to further purify the xylosyltransferase from embryonic chick cartilage.

EXPERIMENTAL

Materials. — The substrate [Smith degraded chondroitin sulfate proteoglycan from bovine nasal cartilage (PGSD)] for xylosyltransferase was prepared by Smith degradation (periodate oxidation followed by reduction with borohydride and mild acid cleavage) of chondroitin sulfate proteoglycan essentially as described by Baker *et al.*[6].

UDP-D-[^{14}C]xylose (174–198 mCi per mmole) was purchased from New England Nuclear (Boston, Mass. 02118), UDP-D-xylose from Calbiochem (La Jolla, Calif. 92037), 2-(*N*-morpholino)ethanesulfonic acid (MES) from Calbiochem, ammonium sulfate (enzyme grade) from Schwarz Mann (Orangeburg, N.Y. 10962), L-seryl-glycylglycine hemihydrate (Ser-Gly-Gly) from Fox Chemical Company (Los Angeles, Calif. 90021), Sepharose 4B, and Sephadex G-200 and G-100 resins from Pharmacia Fine Chemicals, Inc. (Piscataway, N.J. 08854), cyanogen bromide from Aldrich Chemical Co., Inc. (Milwaukee, Wis. 53233), electrophoresis-grade reagents for polyacrylamide gel electrophoresis from Bio-Rad Laboratories (Richmond, Calif. 94804), and special agar-Noble used for immunochemical procedures from Difco Laboratories (Detroit, Mich. 48232).

The following protein standards were obtained from the sources listed: glucose oxidase and pepsin from Sigma Chemical Co. (St. Louis, Mo. 63178); ribonuclease B and FDP-aldolase from Pharmacia Fine Chemicals, Inc.; trypsin, chymotrypsin, carboxypeptidase, tyrosinase, and catalase from Worthington Biochemical Corp. (Freehold, N.J. 07728); bovine serum albumin from Armour Labs. (Kankakee, Ill. 60901); and hemoglobin from Calbiochem.

Xylosyltransferase assay. — The transfer of D-xylose from UDP-D-xylose to exogenous acceptor was measured as previously described (Method 2a)[5–7]. Briefly, incubation mixtures contained, in a total volume of 0.075 ml, UDP-D-[^{14}C]xylose (0.05 μCi), PGSD (0.2 mg), and varying concentrations of enzyme in standard MES buffer* containing 0.05M potassium chloride. After incubation for 60 min, the reactions were terminated by addition of 1% serum albumin (0.05 ml) and cold 10% trichloroacetic acid–4% phosphotungstic acid (0.2 ml). The resulting precipitate was centrifuged off and washed three times with 5% trichloroacetic acid (0.2 ml). The residue was dissolved in M sodium hydroxide (0.1 ml), quantitatively transferred to a scintillation vial, and the radioactivity was measured. For greater convenience in expressing enzyme activity, a unit of activity is defined in the present work as the

*The following standard buffer solution was used throughout these experiments for preparation of xylosyltransferase and enzyme assays: 0.05M MES, pH 6.5, containing 0.012M magnesium chloride, 0.003M manganous chloride, and either 0.05M or 0.25M potassium chloride, as indicated in the text.

amount of enzyme that catalyzes the incorporation of 1,000 c.p.m. of xylose into PGSD under standard assay conditions. This unit is to be regarded as provisional, since the substrate (PGSD) is not well defined and shows some variation in acceptor activity from one preparation to another.

Enzyme preparation. — Epiphyses from femurs and tibias of 13-day old chick embryos were dissected and homogenized in standard MES buffer containing 0.05M potassium chloride, as previously described[5]. The crude cell homogenate was centrifuged at 10,000 *g* for 20 min at 4°, and the supernatant fluid at 105,000 *g* for 60 min. The resulting supernatant liquid was partially fractionated and concentrated by precipitation with ammonium sulfate between 25% and 70% of saturation[5]. Aliquots of the ammonium sulfate-fractionated enzyme (10–15 ml, ~5 mg of protein per ml) were applied to a column (5.5 × 48 cm) of Sephadex G-200 resin[5], equilibrated and eluted with standard buffer containing 0.25M potassium chloride. The included, eluted fractions containing xylosyltransferase activity were pooled and concentrated by ultrafiltration through an Amicon XM-50 membrane. This Sephadex fraction was stored frozen until purified further.

Additionally, xylosyltransferase was isolated from the 100,000 *g* particulate fraction of a cartilage homogenate by the detergent-salt solubilization procedure described in detail elsewhere[4]. The particulate enzyme was suspended in buffer, Nonidet P-40 and solid potassium chloride were added to final concentrations of 0.5% and 0.5M, respectively, and the mixture was incubated for 30 min at 4°. After centrifugation at 100,000 *g* for 60 min, the supernatant fraction contained more than 85% of the xylosyltransferase activity of the original 100,000 *g* pellet and substantial amounts of four of the other chondroitin sulfate glycosyltransferases[4]. This supernatant fraction was applied directly to a column (2.5 × 143 cm) of Sephadex G-200 resin and eluted with 0.05M tris(hydroxymethyl)aminomethane (Tris) acetate buffer, pH 5.5, containing 0.25M potassium chloride and mM dihydrogen disodium ethylenediaminetetraacetate (EDTA). The included, eluted peak fractions of xylosyltransferase, which were well separated from those containing UDP-D-galactose:D-xylose galactosyltransferase and detergent, were pooled and concentrated, as previously described. Before applying this preparation to an affinity column, it was dialyzed overnight at 4° against standard MES buffer containing 0.05M potassium chloride. Preparations obtained by this procedure had specific activities within the same range as those isolated from the high-speed supernatant fraction of the cartilage homogenate, but an extensive comparison of the two types of products has not been undertaken. The experiments described in the following paragraphs have all been performed with the enzyme obtained from the soluble fraction.

Preparation of PGSD-Sepharose resin. — Sepharose resin was activated by treatment with cyanogen bromide, essentially as described by Cuatrecasas[8]. Cyanogen bromide (10 g) was added to Sepharose 4B resin (50 ml) (Pharmacia) suspended in 0.2M sodium borate buffer (50 ml), pH 9.5. The slurry was adjusted to, and maintained at, pH 11 with 2M sodium hydroxide for 20 min at 20°. The activated Sepharose resin was transferred to a Büchner funnel, washed repeatedly with large volumes of cold

0.2M borate buffer, pH 9.5, and finally suspended in borate buffer (25 ml) containing PGSD (100–200 mg). This mixture was stirred in the cold for 18–20 h, washed with water, and finally equilibrated with standard buffer containing 0.05M potassium chloride.

Amino acid analysis of the PGSD-substituted Sepharose resin after hydrolysis with 6M hydrochloric acid for 21 h at 100° showed the presence of 0.10–0.12 μmoles of serine per ml of packed gel.

Polyacrylamide-gel electrophoresis. — Polyacrylamide-gel electrophoresis was performed in glass tubes (96 × 5 mm) in a Buchler apparatus. A procedure based on the methods of Shapiro *et al.*[9] and Steck *et al.*[10] was developed with 0.05M phosphate buffer, pH 7.1, 0.1% sodium dodecyl sulfate, 0.1% 2-hydroxy-1-ethanethiol and a final acrylamide concentration of 7.5%.

For subunit analysis, enzyme preparations were denatured and reduced in a solution of 4% sodium dodecyl sulfate and 1% 2-hydroxy-1-ethanethiol for 2 h at 37°, and then dialyzed overnight against 0.01M phosphate buffer, pH 7.1, containing 0.1% sodium dodecyl sulfate and 0.1% 2-hydroxy-1-ethanethiol. Samples were mixed with an equal volume of 50% sucrose containing Bromophenol Blue as a tracking dye and layered on top of the gels. All gels were subjected to a current of 5 mA/tube for 5 h. The gels were stained overnight with 0.025% Coomassie Brilliant Blue in 5:2:13 (v/v) 2-propanol–acetic acid–water, destained by diffusion for 8 h in 1:1:8 (v/v) 2-propanol–acetic acid–water, and further destained and rehydrated in 10% acetic acid overnight.

The molecular weight of xylosyltransferase was estimated by the method of Shapiro *et al.*[9] with the use of several standard proteins, as indicated in Fig. 8.

Determination of molecular weight by gel chromatography on Sephadex G-100 resin. — Molecular-weight determination by gel chromatography was performed essentially as described by Andrews[11], on a column (1.5 × 53 cm) of Sephadex G-100 resin, equilibrated with standard MES buffer containing 0.25M potassium chloride. Fractions of 1 ml were collected from the moment of sample application. The effluent volume corresponding to the maximum concentration of protein was estimated, from an elution diagram, by extrapolation of both sides of the protein peak to an apex. The elution volume was plotted against the logarithm of the molecular weight for the proteins listed in Fig. 7.

Ultracentrifugation. — Aliquots (0.2 ml) of a solution of xylosyltransferase, purified by gel chromatography on Sephadex G-200 resin or by affinity chromatography, were layered in 6.5-ml cellulose nitrate tubes on preformed linear gradients of 5 to 20% sucrose prepared[5] in standard MES buffer and containing 0.25M potassium chloride and 0.02M Ser-Gly-Gly. After centrifugation in a Beckman SW 41 rotor at 293,000 *g* for 20 h, 0.5-ml fractions were collected from the top of the tube. Aliquots were assayed for xylosyltransferase activity, and refractive indices were measured with a Zeiss refractometer.

Immunochemical procedures. — Antisera to affinity-purified chick xylosyltransferase were prepared in rabbits[12]. Immunodiffusion was performed as described

by Ouchterlony[13]. Immunoelectrophoresis was performed in a 1.5% agar-Noble matrix[14] prepared on microscope slides in 0.05M sodium barbital buffer, pH 8.6. The voltage was adjusted to 9 volts per cm, and the current was applied for 60 min.

Protein estimation. — The concentration of protein was measured by the method of Lowry *et al.*[15]. In order to remove manganous ions, which sometimes interfered with these determinations, the protein was precipitated from an aliquot (0.2–0.5 ml) of the enzyme solution with an equal volume of 10% trichloroacetic acid–4% phosphotungstic acid. The precipitate was collected by centrifugation, dissolved in 0.1M sodium hydroxide (0.1–0.2 ml), and assayed as usual.

RESULTS

Purification by affinity chromatography. — In preliminary experiments, it was found that xylosyltransferase was adsorbed to the affinity matrix from a crude-enzyme solution (high-speed supernatant fraction of a homogenate) or from a preparation that had been partially purified by gel chromatography on Sephadex G-200 resin. As seen in Fig. 1, a column of PGSD-Sepharose resin retained most of the activity of a partially purified xylosyltransferase preparation, and elution with PGSD yielded a quantitative recovery of the bound enzyme. Nearly all of the applied protein emerged unretarded on washing with the equilibrating buffer, as estimated from absorbance at 280 nm; however, the amount of adsorbed protein that was subsequently eluted by PGSD could not be quantitatively determined, since this substance is itself a protein

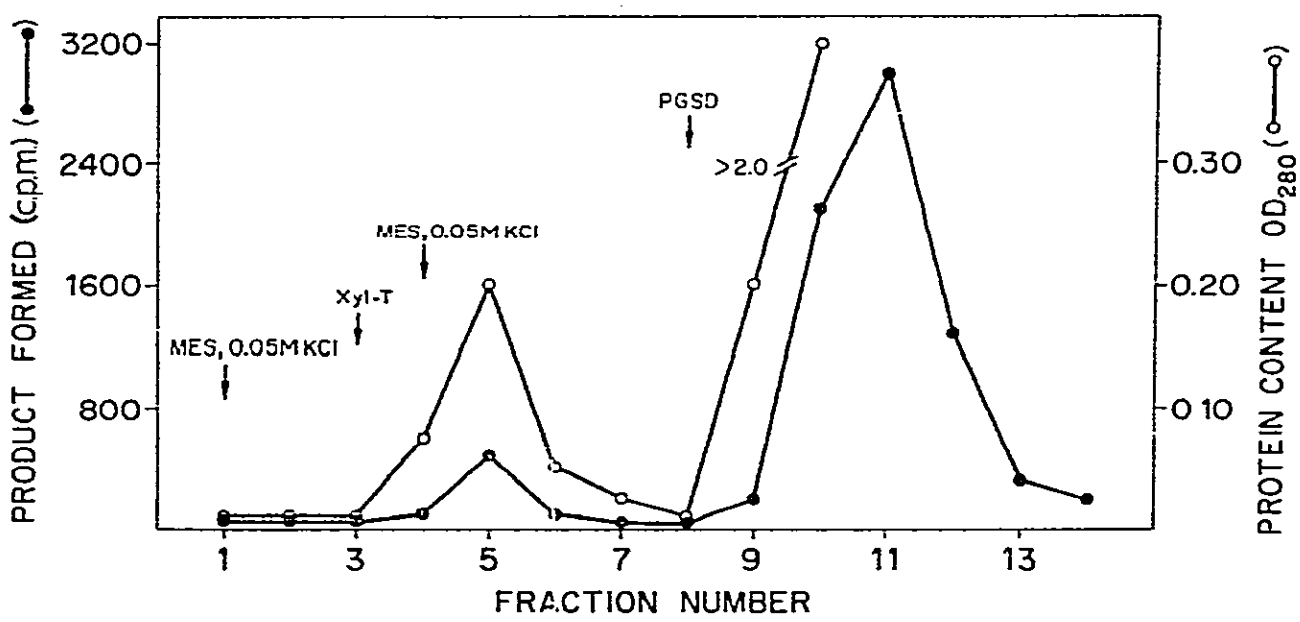


Fig. 1. Chromatography of partially purified xylosyltransferase on a column of PGSD-Sepharose resin. The column (0.5 × 4 cm) was equilibrated with standard MES buffer containing 0.05M potassium chloride. A sample (1.0 ml) containing 130 units of concentrated, partially purified xylosyltransferase (Sephadex G-200 fraction) was applied at the point marked "Xyl-T" in the figure. After being washed with the equilibrating buffer (5 ml), the column was eluted with a solution of PGSD (10 mg/ml) in the same buffer (fraction volume, 1.0 ml). Absorbance at 280 nm (○—○) was measured, and 50-μl aliquots of each fraction were assayed for xylosyltransferase activity (●—●). Approximately 120 units of enzyme were recovered in Fractions 10 to 12.

and was present in large excess (see Fig. 1). There was no adsorption of xylosyltransferase activity on a control column of untreated Sepharose resin, under conditions identical to those described in Fig. 1.

Since the degree of purification could not readily be estimated without removal of PGSD, alternative eluents were sought. It was found that xylosyltransferase activity was also eluted, in lower yield (30–40%), by standard buffer containing 0.25M potassium chloride. Once a column had been saturated with the enzyme, eluted with buffer containing 0.25M potassium chloride, and reequilibrated with buffer of low ionic-strength (0.05M potassium chloride), further batches of xylosyltransferase could be purified on the same column with almost complete recovery of activity on elution with salt-containing buffer alone (*i.e.*, standard buffer containing 0.25M potassium chloride). However, the binding capacity of such a preconditioned column was only approximately 1/3 of the original capacity, corresponding to the proportion of the enzyme that had been removed by the initial elution with buffer of high ionic-strength*.

In a large-scale purification (Fig. 2), a dilute enzyme solution, which had been partially fractionated by gel chromatography, was loaded on a preconditioned column until the affinity matrix became saturated. After the column had been washed until the absorbance at 280 nm had dropped to zero, the enzyme was eluted with standard buffer containing 0.25M potassium chloride. The fraction containing the recovered xylosyltransferase activity represented ~90% of the adsorbed activity and less than 0.5% of the protein applied.

The specific activity of the salt-eluted xylosyltransferase was approximately 4000-fold higher than that of the crude homogenate from embryonic chick cartilage (Table I). In the single affinity-chromatography step, the enzyme was purified, on a previously used column, ~160-fold with a recovery of 90 to 95%. Some variation of the activity was observed from one preparation to another, depending in part on the concentration and total protein content of the enzyme sample applied, and also on the quality of the PGSD-Sepharose resin. It was found preferable to apply a large volume of a dilute solution (0.2–0.4 mg protein per ml) rather than a smaller volume of concentrated material. The average specific activity of 13 consecutive preparations was 9.2×10^3 units/mg, with a range of $6–15 \times 10^3$ units/mg. On repeated affinity chromatography of three preparations of comparatively low activity, which had been obtained from a different batch of PGSD-Sepharose resin, the specific activity increased from $3–5 \times 10^3$ units/mg to $13–19 \times 10^3$ units/mg, corresponding to an overall purification ranging from 6100-fold to 8950-fold.

The highly purified preparations lost most of their activity over a period of one week when stored frozen, but they were stable at 4° for at least 4 months. Concentra-

*For example, 10,400 units of activity were required to completely saturate a particular column (1.2 × 8 cm) of PGSD-Sepharose resin; 4,200 units (40%) were eluted with standard buffer containing 0.25M potassium chloride. Subsequently, 3,500 units were sufficient to saturate the same column, and elution with salt-containing buffer alone gave a recovery of 83% of the activity (2,900 units).

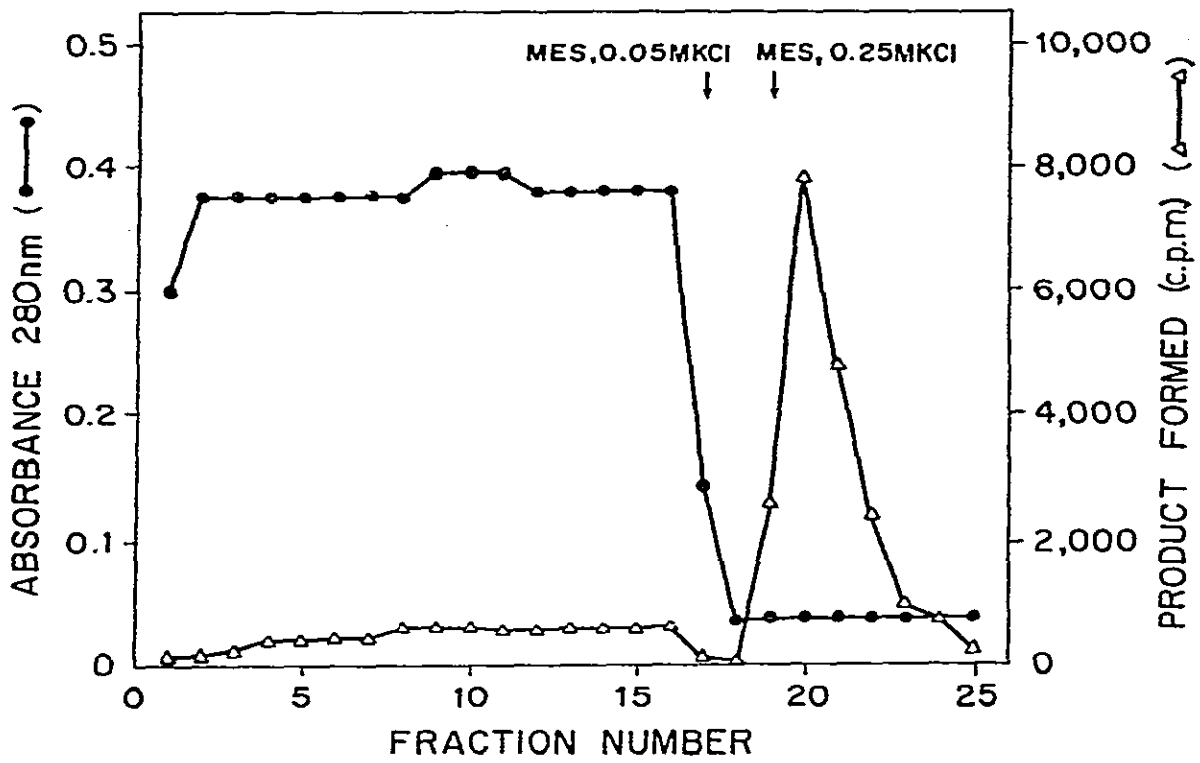


Fig. 2. Elution of xylosyltransferase with potassium chloride from a preconditioned affinity column (previously saturated with enzyme, eluted with standard buffer containing 0.25M potassium chloride, and re-equilibrated with buffer containing 0.05M potassium chloride). Dilute, partially purified enzyme (400 ml; 0.35 mg protein per ml; 6,104 units) was applied to a column (1.2×8 cm) of PGSD-Sepharose resin, resulting in binding of approximately 65% (3,925 units) of the activity, while essentially all of the protein content passed through the column. After being washed with standard buffer containing 0.05M potassium chloride (50 ml), the column was eluted with buffer containing 0.25M potassium chloride (60 ml), to give a recovery of 3,526 units (0.44 mg protein), corresponding to 89% of the bound activity; (●—●) absorbance at 280 nm; (△—△) enzyme activity, expressed as c.p.m. incorporated into product per 50-μl aliquot.

TABLE I

PURIFICATION OF XYLOSYLTRANSFERASE FROM CHICK CARTILAGE

Fraction	*Specific activity (units/mg of protein)*	*Purification (-fold)*
Homogenate	2.12	
25–70% Ammonium sulfate precipitation	22.05	10
Gel filtration on Sephadex G-200 resin	55.60	26
Chromatography on PGSD-Sepharose resin	8,700	4100

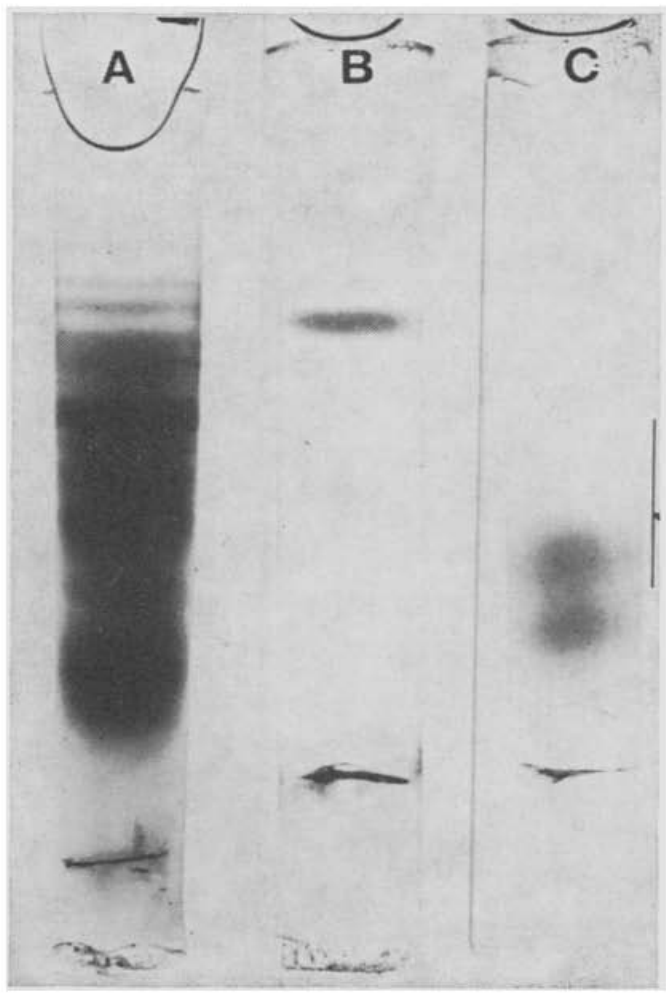
tion by ultrafiltration resulted in visible aggregation with concomitant loss of activity, if the protein concentration exceeded ~0.5 mg/ml.

Criteria of purity. — Xylosyltransferase that had been purified on PGSD-Sepharose resin appeared homogeneous when analyzed by polyacrylamide-gel electrophoresis and by immunochemical procedures. As shown in Fig. 3, the purified enzyme gave a single protein band on electrophoresis in buffer containing 0.1% sodium dodecyl sulfate and 0.1% 2-hydroxy-1-ethanethiol. By comparison, the crude Sephadex fraction contained a large number of components. After pretreatment

with higher concentrations of sodium dodecyl sulfate and 2-hydroxy-1-ethanethiol, two faster-migrating, more diffuse bands were observed, presumably representing the basic monomer units of xylosyltransferase.

The results of immunoelectrophoresis and double diffusion in agar by the Ouchterlony technique are shown in Figs. 4 and 5. In both cases, the antibody against purified chick xylosyltransferase gave a single precipitin line with a number of different enzyme preparations that had been purified by affinity chromatography. (Two of the preparations shown in Fig. 5 had been prepared from a rat chondrosarcoma and will be described in detail elsewhere).

Previous observations have shown that xylosyltransferase purified through the ammonium sulfate step has a low buoyant density[5], perhaps reflecting an association of the enzyme with lipid. Fig. 6 illustrates the distribution of xylosyltransferase activity, after centrifugation for 20 h, of a preparation purified through the Sephadex G-200 resin (A) and affinity chromatography steps (B). Approximately 70% of the activity



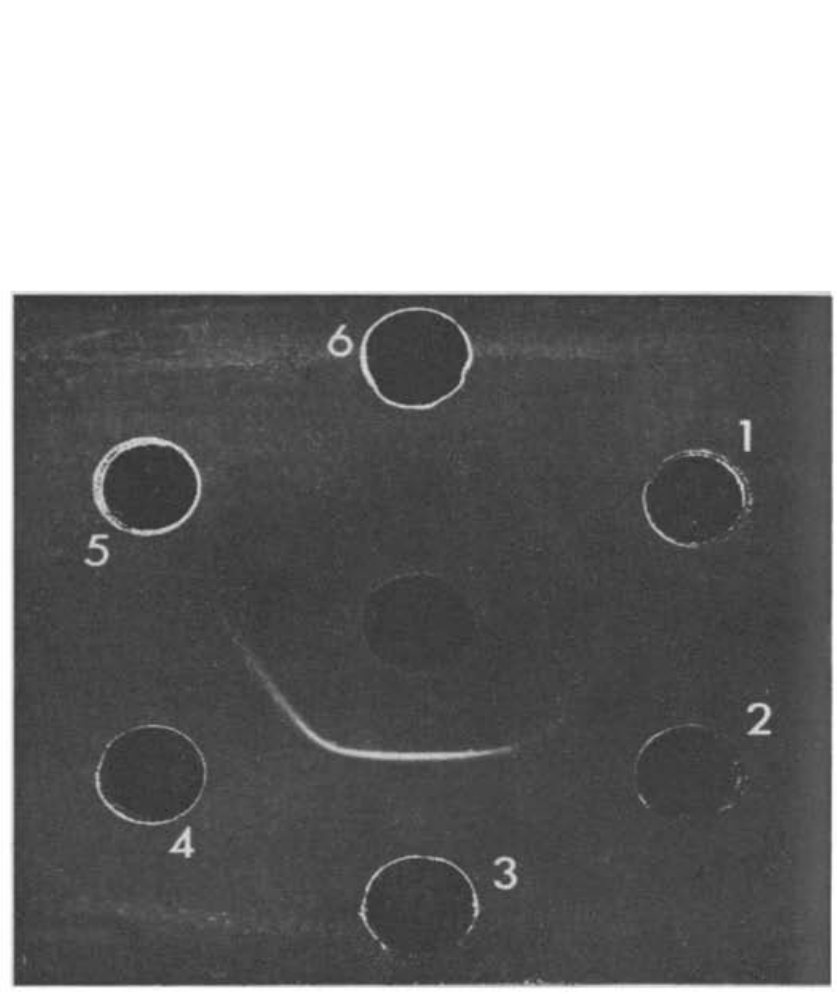


Fig. 3. Polyacrylamide-gel electrophoresis of crude (Sephadex G-200 fraction) and purified preparations of xylosyltransferase. Electrical current was applied at 5 mA/tube (6 volts/cm) for about 5 h. The samples were: (A) crude xylosyltransferase (82 μg); (B) purified enzyme (9 μg; spec. act.: 7,500 units per mg protein; and (C) purified enzyme (9 μg), pretreated with sodium dodecyl sulfate and 1-hydroxy-2-ethanethiol. The gels were stained for protein with Coomassie Brilliant Blue.

Fig. 4. Immunoprecipitation of xylosyltransferase by the Ouchterlony technique. Antiserum to purified xylosyltransferase was applied to the center well and four different purified enzyme preparations (spec. act.: 7,500–9,100 units/mg; amount of protein: 5–25 μg) were applied to wells 1–4. (Wells 5 and 6 contained preparations of xylosyltransferase from a rat chondrosarcoma; these did not precipitate with the antibody to chick enzyme and will be described elsewhere).

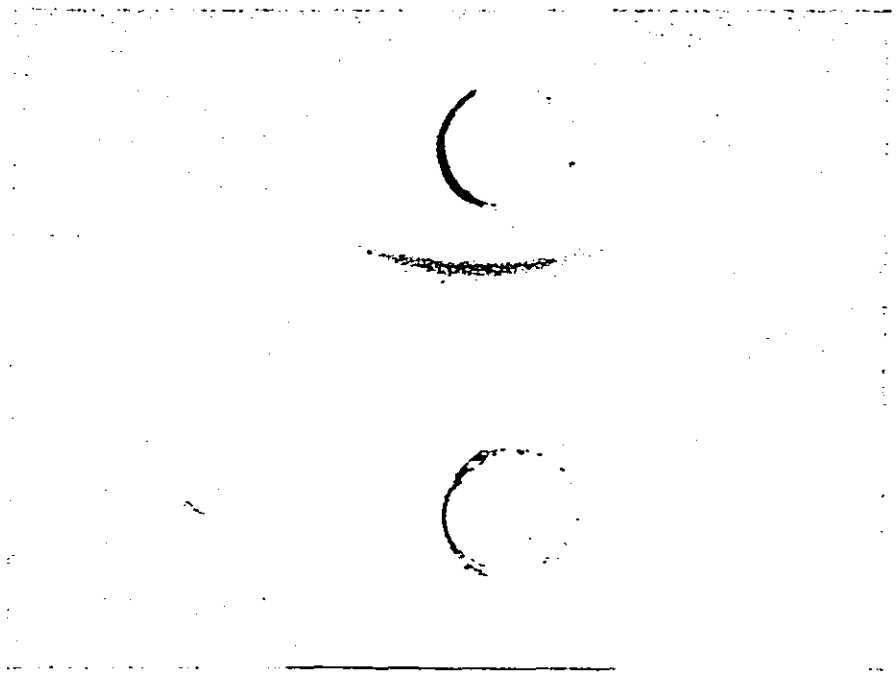


Fig. 5. Immunoelectrophoresis of purified xylosyltransferase. Antiserum (120 μl) to purified enzyme was applied to 10 μg of xylosyltransferase (upper well) that had been subjected to electrophoresis, as described in the Experimental section. The lower well contained only buffer.

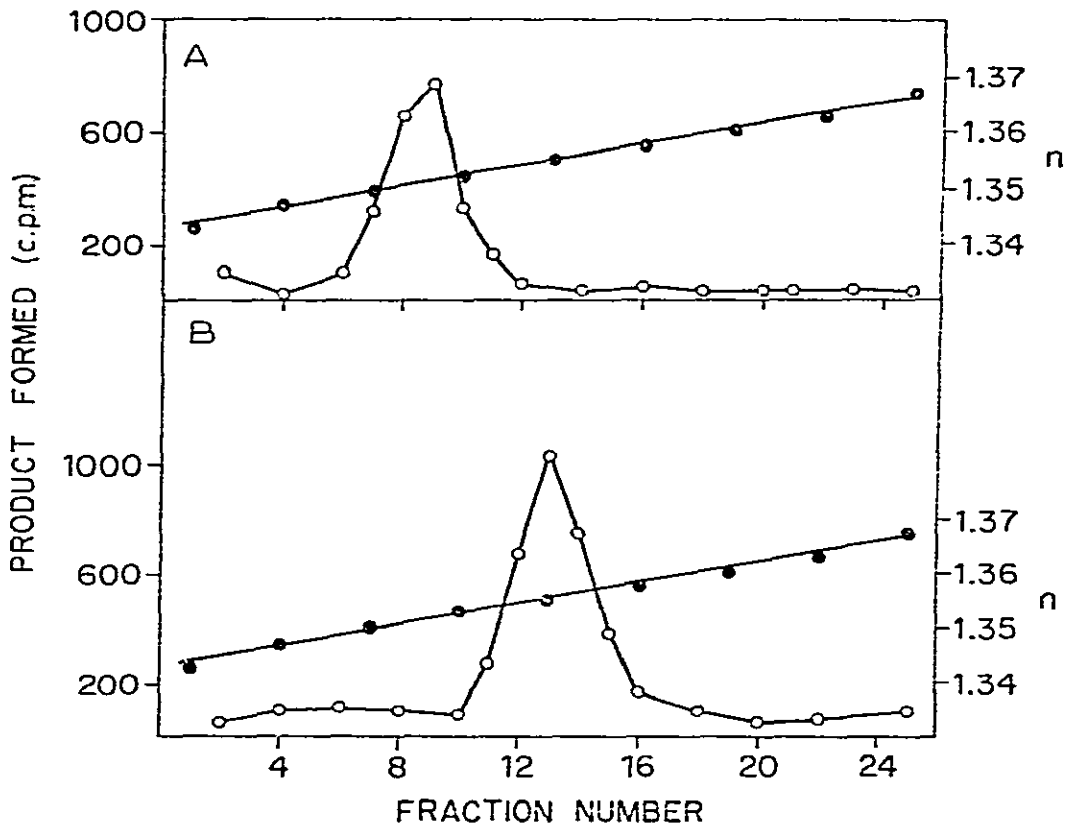


Fig. 6. Density gradient centrifugation of crude and purified xylosyltransferase. A 0.2-ml sample of enzyme was layered on a linear gradient of 5–20% (w/w) sucrose, prepared in standard MES buffer, pH 6.5, containing 0.25M potassium chioride and 0.02M Ser-Gly-Gly, and centrifuged at 41,000 r.p.m. for 20 h at 4° in the SW 41 rotor of a Spinco Model L2-65B ultracentrifuge. Fractions of 0.5 ml were collected, and the refractive index (●—●) and xylosyltransferase activity (○—○) were measured. (A) Crude enzyme (Sephadex G-200 fraction); and (B) purified enzyme. Activity applied: (A) 30 units; and (B) 144 units. Recovery: (A) 21 units (70%); and (B) 34 units (24%). Position of activity: (A) fractions 6–11; (B) fractions 11–16. The fractions were numbered starting from the top of the gradient.

of the Sephadex-purified enzyme was recovered in a band corresponding to a density of 1.045 to 1.047 g per ml, which is similar to the buoyant density previously reported by Stoolmiller *et al.*[5] for a preparation fractionated with ammonium sulfate. In contrast, xylosyltransferase purified by affinity chromatography sedimented toward a higher density (1.059–1.060 g per ml). The recovery of enzyme activity of this preparation after centrifugation was only 24%, suggesting that the chromatography on PGSD-Sepharose resin may result in dissociation from a lipid which stabilizes the enzyme but is not essential for activity.

Molecular-weight determination. — The molecular-weight of xylosyltransferase was determined by chromatography on Sephadex G-100 resin (Fig. 7). The purified enzyme was compared with several standard proteins, and calculation of the molecular weight by Andrews' equation[11] indicated a value of 95,000–100,000 daltons.

As stated previously, polyacrylamide-gel electrophoresis of the purified enzyme after pretreatment with sodium dodecyl sulfate and 2-hydroxy-1-ethanethiol yielded two diffuse bands, presumably subunits of the enzyme. The molecular weights of

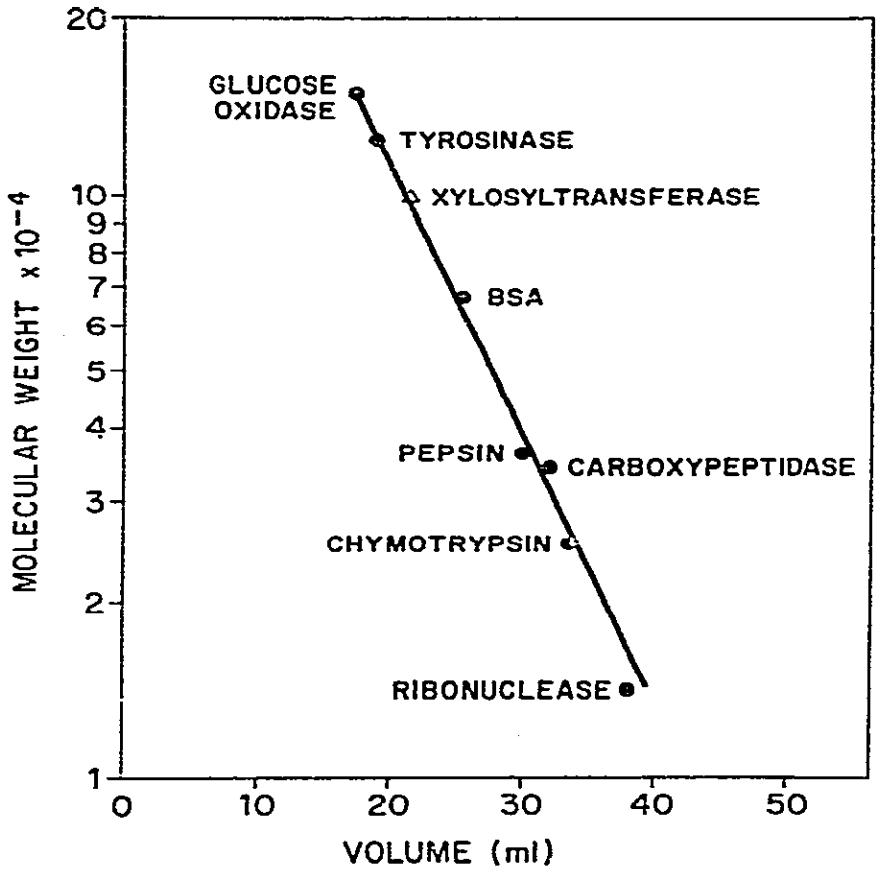


Fig. 7. Determination of the molecular weight of xylosyltransferase by gel chromatography. A column (1.5 × 53 cm) of Sephadex G-100 resin was calibrated with glucose oxidase (150,000 daltons), tyrosinase (125,000 daltons), bovine serum albumin (BSA) (68,000 daltons), pepsin (36,000 daltons), carboxypeptidase (34,000 daltons), chymotrypsin (25,000 daltons), and ribonuclease B (13,700 daltons). Samples (10–15 mg) of each protein, in 1.0 ml of standard buffer containing 0.25M potassium chloride, were chromatographed separately and eluted with the same buffer (fraction volume, 1.0 ml). The positions of the protein peaks for the standards were determined by measurement of the absorbance at 280 nm. Purified xylosyltransferase (400 units) was chromatographed in the same fashion, and its elution volume was determined by enzymic assay of 50-μl aliquots of each fraction. Recovery of activity was greater than 95%. The molecular weight was calculated, as described in the text.

these subunits are ~23,000 and 27,000 daltons, respectively, as determined according to Shapiro *et al.*[9] by comparison with standard proteins (Fig. 8). These results suggest that the native enzyme is composed of two nonidentical sets of subunits.

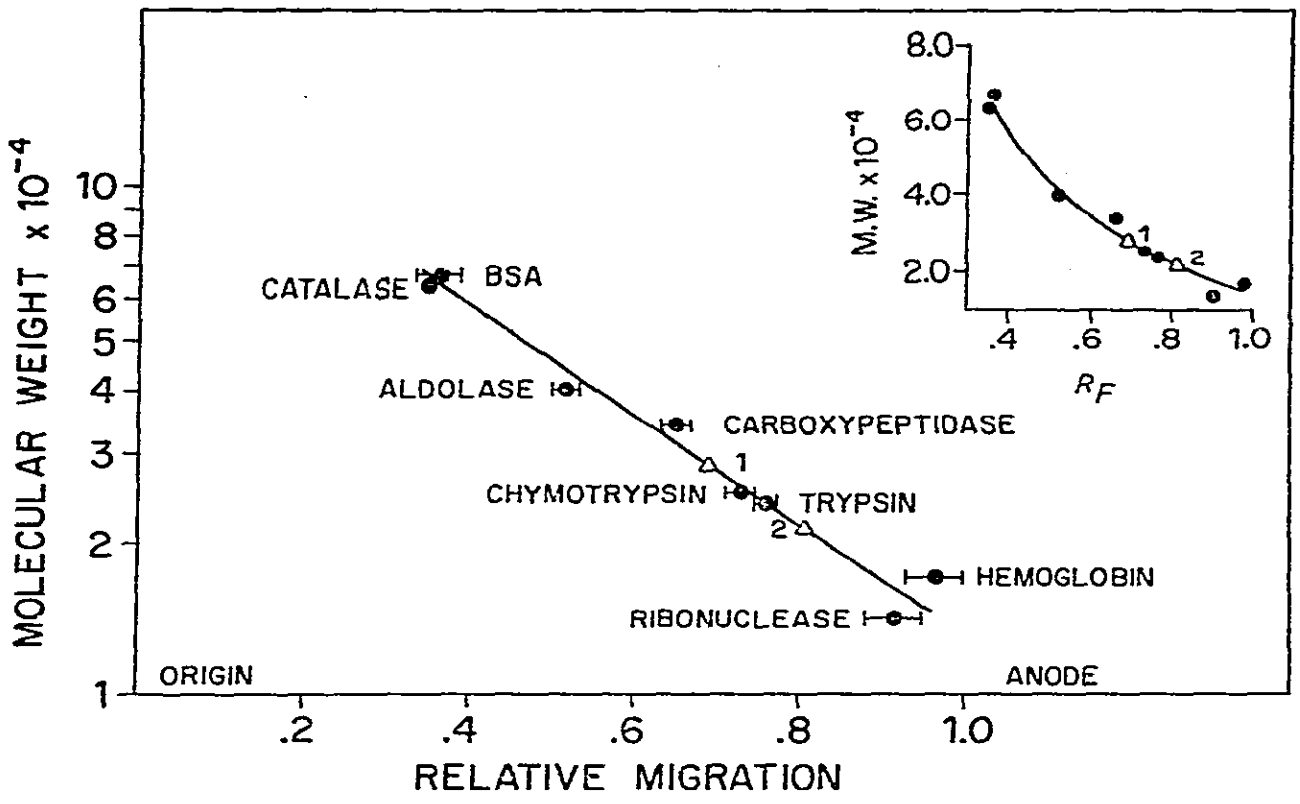


Fig. 8. Determination of the molecular weight of xylosyltransferase subunits by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate. All of the proteins were dissociated by pretreatment with sodium dodecyl sulfate and 2-hydroxy-1-ethanethiol, as described in the text. Approximately 25 μg of each protein were applied to a gel along with Bromophenol Blue as tracking dye. The distance of migration from the origin to the center of the protein band, relative to the distance from the origin to the tracking dye, was plotted against the logarithm of the molecular weight, in each case. The relative positions of the diffuse protein bands obtained after dissociation of xylosyltransferase with sodium dodecyl sulfate are marked by the numerals 1 and 2.

DISCUSSION

The biosynthesis of the chondroitin sulfate chains of cartilage proteoglycans is initiated by transfer of a D-xylosyl group to some hydroxyl groups of the serine residues of the core protein[16]. The xylosyltransferase catalyzing this reaction has previously been purified 47-fold by ammonium sulfate precipitation, gel chromatography, and density gradient centrifugation[5]. The substrate used in these studies (PGSD) had been prepared from bovine nasal cartilage proteoglycan by Smith degradation, which removes the chondroitin sulfate chains and exposes the hydroxyl groups of serine available for xylosylation[6]. In comparison with substrates of lower molecular weight, such as the tripeptide Ser-Gly-Gly, or peptides obtained by partial acid hydrolysis of trypsin-treated chondroitin sulfate proteoglycan, PGSD was a far better D-xylose acceptor and was therefore the substrate of choice in selecting a suitable ligand for affinity chromatography. However, in view of the presence of keratan sulfate in PGSD, there was some doubt whether a PGSD-matrix would be specific for xylosyltransferase or whether glycosyltransferases involved in keratan

sulfate synthesis would also be adsorbed to such a matrix. Since assays for keratan sulfate glycosyltransferases have not yet been developed, this question could not be resolved in the present study, nor was it possible to determine whether any of these enzymes occurred in the partially purified xylosyltransferase preparation used for affinity chromatography. However, since the affinity-purified xylosyltransferase was homogeneous by several criteria, it appears unlikely that keratan sulfate glycosyltransferases were present, and it is further concluded that proteins other than xylosyltransferase were not adsorbed to the PGSD matrix or were not eluted under the conditions used.

It is sometimes difficult or impossible to elute proteins that are strongly adsorbed to affinity columns without resorting to the use of extreme conditions of pH or denaturants, which may destroy biological activity. However, when the same substance is used as affinity ligand and eluent, good recovery of the adsorbed material is usually obtained, and, as could thus be expected, elution with PGSD yielded quantitative recovery of xylosyltransferase activity. Nevertheless, the use of PGSD for elution purposes has certain disadvantages, primarily the necessity of removing it from the eluate by further fractionation procedures, but also the need for relatively large amounts of PGSD which are not readily available. In the search for alternative elution procedures, it was observed that buffer containing 0.25M potassium chloride eluted approximately one third of the activity and, interestingly, total recovery was obtained from a previously used column that had been eluted with salt alone. Elution of preconditioned columns with salt was therefore adopted as a standard purification procedure, and some columns have been in use over one year without significant change in the specific activity of the isolated enzyme, although the retention capacity of the columns has decreased materially over this time period.

The behavior of the enzyme in the presence of the two eluents, PGSD and salt, suggests that two types of interaction are involved in the binding to the affinity column: (a) the enzyme–substrate interaction, which can be dissociated only by the substrate itself; and (b) an interaction between individual xylosyltransferase molecules, which can be dissociated by salt. This assumption would explain the observation that only a fraction of the enzyme applied to a column of fresh PGSD-Sepharose resin is eluted by salt (approximately one third under standard experimental conditions), whereas quantitative recovery is obtained on elution with PGSD. Furthermore, the existence of two types of interaction also provides a ready explanation for the difference between fresh and preconditioned PGSD-Sepharose resin in regard to enzyme recovery on elution with salt. Provided that the substrate sites have initially been saturated with enzyme and the column eluted with salt, retention of additional enzyme on repeated use would then occur exclusively by salt-labile association with the remaining substrate-bound enzyme. If this interpretation is correct, it may be further concluded that the binding of the xylosyltransferase molecules to each other is specific enough to permit complete removal of other proteins in the course of affinity chromatography.

Insofar as purification of xylosyltransferase was the major goal of the present

study and was successfully accomplished by the use of the PGSD matrix, it was immaterial whether purification had occurred by enzyme–substrate or enzyme–enzyme interaction. However, the evidence obtained for specific binding between individual xylosyltransferase molecules spotlights a characteristic feature of many glycosyltransferases, *i.e.*, their tendency to aggregate at low ionic-strength[5,17] and at a protein concentration exceeding approximately 1 mg/ml. It is significant that the interaction is not limited to molecules of a single enzyme species, but may occur between two different glycosyltransferases. As has recently been reported[4,18], PGSD-Sepharose presaturated with xylosyltransferase is capable of adsorbing specifically galactosyltransferase I (UDP-D-galactose:D-xylose galactosyltransferase), which catalyzes the second step of chondroitin sulfate-chain synthesis. This observation substantiates in part a previous suggestion[5,17] that the chondroitin sulfate glycosyltransferases are organized in the membranes of the endoplasmic reticulum in an orderly fashion, and that specific interaction between the individual enzymes could form the basis for juxtaposition of enzymes catalyzing consecutive steps in the chain assembly. Thus, the affinity matrix used in the present study may serve a two-fold purpose, *i.e.*, as a tool for enzyme purification, and perhaps more importantly, as a simplified model for membrane assembly. A study of the interactions between chondroitin sulfate glycosyltransferases with the PGSD matrix as a model membrane is only in its initial stages, and it will be of great interest to extend these investigations to all six glycosyltransferases involved in chondroitin sulfate biosynthesis.

Some properties of the purified xylosyltransferase deserve further comment. The enzyme seems to be a tetrameric structure, composed of two pairs of nonidentical subunits, since the molecular weight of the native enzyme is 95,000–100,000 daltons, whereas the molecular weights of the subunits are 23,000 and 27,000 daltons, respectively. Purification by affinity chromatography appears to have removed some component, possibly lipid, that affected the buoyant density of xylosyltransferase in sucrose, since the purified enzyme was less buoyant than crude-enzyme preparations. This may also explain the higher molecular-weight value (120,000 daltons) obtained for a crude xylosyltransferase preparation[5]. Although the purified xylosyltransferase preparation described here showed only a single component on polyacrylamide-gel electrophoresis and on analysis by immunochemical procedures, it may not yet be concluded with certainty that the enzyme is homogeneous and, *e.g.*, the apparent association with lipids at the stage preceding affinity chromatography makes it desirable to establish whether some lipid is still present in the purified material. Further characterization is presently under way, and it will be of particular interest to compare the properties of xylosyltransferase with those of glycogen synthetase[19] and lactose synthetase[20,21], which are the only glycosyltransferases previously purified to homogeneity or near homogeneity. The latter enzyme is known to be a glycoprotein, containing 12–13% of carbohydrate, and preliminary analyses have shown that xylosyltransferase is similar in this regard, inasmuch as it contains a substantial amount of carbohydrate.

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