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Biosynthesis of Keratan Sulfate: Purification and Properties of a Galactosyltransferase from Bovine Cornea[1]

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A soluble galactosyltransferase was purified 22,000-fold from bovine cornea. The enzyme catalyzes the transfer of galactose from UDP-galactose to *N*-acetyl-D-glucosamine, α- and β-glucosaminides, bovine cornea and nasal septum agalactokeratan, and to glycoproteins containing terminal nonreducing *N*-acetylglucosaminyl units. When *N*-acetyl-D-glucosamine served as acceptor, the product formed by the cornea transferase contained galactose glycosidically linked to carbon atom 4 of *N*-acetyl-D-glucosamine; the same glycosidic linkage was found in [^{14}C]keratan preparations isolated from reaction mixtures where keratan containing terminal nonreducing *N*-acetylglucosaminyl units served as acceptor. The cornea enzyme exhibited a markedly lower K_m with keratan than with *N*-acetyl-D-glucosamine. The physical and kinetic properties of the cornea galactosyltransferase and of the milk A-protein (A-protein + α-lactalbumin = lactose synthase), including modulations of acceptor specificity by α-lactalbumin, were compared. The results of these studies strongly suggest that the two glycosyltransferases are similar, if not identical. Efforts to demonstrate the presence of other soluble galactosyltransferases in cornea were unsuccessful; no change in the ratios of products formed with several acceptors was observed at any stage of purification. It is suggested that in bovine tissues a single galactosyltransferase participates in the synthesis of both high and low molecular weight galactosides including the assembly of the repeating disaccharide [*O*-β-galactopyranosyl-(1 → 4)-*N*-acetylglucosamine] of cornea keratan sulfate.

Keratan sulfate is composed of 8 to 15 repeating disaccharide units of *O*-β-galactosyl-(1 → 4)-*N*-acetylglucosamine polymerized through β,1 → 3 glycosidic linkages and is classified as a glycosaminoglycan (1, 2). Approximately 50% of the galactose residues and 60% of the *N*-acetylglucosamine residues are randomly substituted with ester sulfate groups at carbon atom 6 (3). In cornea, the keratan sulfate chains are linked to protein through *N*-glycosidic bonds to asparagine residues (4), while in cartilage the linkage is through *O*-glycosidic bonds to serine and/or threonine residues (3, 5–7). Keratan sulfate comprises about 7% of the total glycosaminoglycan content of cartilage and over 70% in cornea tissue; hence, most studies dealing with the biosynthesis of keratan sulfate have been carried out with cornea tissue (8, 9). In contrast to other glycosaminoglycans, little is known concerning the specific steps in the biosynthesis of keratan sulfate.

In this article, we report the isolation and extensive purification of a soluble galactosyltransferase from bovine cornea that catalyzes the transfer of galactose to *N*-acetylglucosamine, keratan, and glycoproteins containing terminal nonreducing *N*-acetylglucosamine residues. The physical and

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kinetic properties of cornea galactosyltransferase are presented and compared to those of the A-protein of milk. A preliminary communication reporting some aspects of these studies has been published (10).

MATERIALS AND METHODS

UDP-[*U*-^{14}C]galactose (274 μCi/μmol) was obtained from the New England Nuclear Co., Boston, Massachusetts, and nonradioactive UDP-Gal was from Sigma Chemical Co., St. Louis, Missouri. Dowex 1-X2 (Cl^-) and Dowex 50-X8 (H^+), each 200 to 400 mesh, were purchased from Bio-Rad Laboratories, Richmond, California. Galactose dehydrogenase was obtained from Boehringer–Mannheim, New York, New York; Sephadex and Sepharose 4B were from Pharmacia, Piscataway, New Jersey; α-lactalbumin (crystallized 3×, Lot No. #C8437) was from Gallard Schlesinger Chemical Manufacturing Co., Carle Place, New York; and frozen bovine eyes were from Standard Beef, Detroit, Michigan. 2-Acetamido-*N*-(L-aspart-4-oyl)-1,2-dideoxy-β-D-glucose[3] was purchased from Cyclo Chemical Co., Los Angeles, California. Bacterial chondroitinases and chondrosulfatases were obtained from Miles Laboratory, Elkhart, Indiana. Acylneuraminyl hydrolase (EC 3.2.1.18) was isolated from *Clostridium perfringens* as described by Cassidy *et al.* (11) and bovine testicular β-galactosidase was prepared as described by Distler and Jourdian (12). Crystalline papain was prepared by an affinity chromatography procedure (13). α-Lactalbumin-substituted agarose (4.3 mg of protein/ml of settled Sepharose) was prepared by the method of Trayer and Hill (14).

The following compounds were generous gifts: the $1 \rightarrow 4$, $1 \rightarrow 3$, and $1 \rightarrow 6$ isomers of galactosyl *N*-acetylglucosamine, Dr. Adeline Gauhe, Max-Planck Institute, Heidelberg; methyl 2-acetamido-2-deoxy-α-glucoside, methyl 2-acetamido-2-deoxy-β-glucoside, methyl 2-bromo-acetamido-2-deoxy-β-glucoside, methyl 2-deoxy-2-(*p*-nitrobenzamido)-β-glucoside, di-*N*-acetylchitobiose, and tri-*N*-acetylchitotriose, Dr. Irwin Goldstein, The University of Michigan; 2-acetamido-*N*-(L-aspart-4-oyl)-1,2-dideoxy-β-galactose, Dr. I. Yamashina, Kyoto University, Tokyo; orosomucoid (α_1-glycoprotein) and asialo-orosomucoid, Dr. Karl Schmid, Boston University; and crude heparinase, Dr. A. Linker, University of Utah.

Analytical techniques. Protein concentrations were measured by ultraviolet light absorption (15) or by the method of Lowry *et al.* (16). The concentrations of the purified enzyme solutions were determined using the extinction coefficient $E_{280\,nm}^{0.1\%} = 1.61$ (14). Carbohydrates and carbohydrate derivatives were determined by the following procedures: bound galactose after resin-mediated acid hydrolysis by gas–liquid chromatography of the corresponding *O*-acetylglycitol derivative (17) or with an anthrone reagent (18); free galactose by galactose dehydrogenase (12); hexosamines by a modified Elson–Morgan procedure (19); sulfate by a barium chloroanilate procedure (20); and glucuronic acid by the method of Dische (21). Uridine 5′-diphosphate was quantitated by ultraviolet absorption at 262 nm.

Disc gel electrophoresis was performed in the presence of sodium dodecyl sulfate as described by Weber and Osborn (22); protein bands were detected by Coomassie brilliant blue stain. Chymotrypsinogen, ovalbumin, bovine serum albumin (Schwartz/Mann, Orangeburg, New York), and lactate dehydrogenase (Boehringer–Mannheim Corp.) served as molecular weight standards.

Paper chromatograms were irrigated in a descending fashion on Whatman No. 3MM paper with *Solvent A*, *n*-butyl alcohol:ethyl alcohol:water (10:1:2), for 120 h or on Whatman No. 1 paper developed in multiple descending fashion with *Solvent B*, *n*-butyl alcohol: pyridine:0.1 N hydrochloric acid (5:3:2), for 4, 9, and 12 h. Reducing sugars were detected on chromatograms with an aniline–trichloroacetic acid spray reagent (23) and *N*-acetylhexosamines with an alkaline spray reagent (24).

High-voltage electrophoresis was performed on a Gilson high-voltage electrophorator on Whatman No. 3MM paper saturated with one of the following buffers: *Buffer C*, 1% sodium tetraborate, pH 9.0 (55 V/cm for 40 min); *Buffer D*, 1.25 M pyridine in 0.064 M acetic acid, pH 6.4 (75 V/cm for 30 min); or *Buffer E*, 1.6 M acetic acid in 0.15 M formic acid, pH 2.1 (74 V/cm for 70 min).

Preparation of agalactokeratan sulfate and agalactokeratan. Keratan sulfate was prepared from bovine nasal septum cartilage and bovine cornea by the procedure described by Rodén *et al.* (25). Bovine nasal septum cartilage keratan sulfate contained 21.6% galactose; the galactose to hexosamine to sulfate ratio was 1.00:0.90:1.45. Cornea keratan sulfate contained 34% galactose and the galactose to hexosamine to sulfate ratio was 1.00:0.80:1.19.

Keratan sulfate was desulfated by hydrolysis with Dowex 50-X8 (H^+) in anhydrous methyl alcohol as described by Bhavanandan and Meyer (3). Ninety-four percent of the ester sulfate was removed from the nasal septum preparations and 82% from cornea preparations.

[3] All glycoses were of the D configuration and were pyranosides unless otherwise specified. The accepted nomenclature for disaccharides such as *N*-acetyllactosamine and lactose is 4-*O*-β-D-galactopyranosyl-2-acetamido-2-deoxy-D-glucose and 4-*O*-β-D-galactopyranosyl-D-glucose, respectively. For purposes of simplicity, these compounds are designated galactosyl-(β,1 $\rightarrow$ 4)-*N*-acetylglucosamine and galactosyl-(β,1 $\rightarrow$ 4)-glucose. Similar nomenclature is used for other disaccharides. The prefix "agalacto" refers to prior treatment of compounds such as keratan with β-galactosidase to remove galactose.

Each preparation yielded two fractions: a soluble (62%) and an insoluble (38%) fraction. The soluble keratan was treated with bovine testicular β-galactosidase to remove terminal nonreducing galactosyl residues as described by Distler and Jourdian (12). Maximum release of galactose was achieved after 43 h of incubation; 22% of the total galactose was released from the nasal preparation and 8.2% from the cornea preparation. To separate the agalactokeratan from the enzyme and the galactose, the reaction mixture was placed on a 1.2 × 57-cm column of Sephadex G-25 and the column was eluted with distilled water. The product was detected with anthrone reagent. The content of terminal *N*-acetylglucosaminyl residues of the keratan was determined by the amount of galactose released on treatment with β-galactosidase.

Assay of galactosyltransferase activity. Typical reaction mixtures contained the following components (in micromoles) in final volumes of 0.05 ml: sodium cacodylate–HCl buffer, pH 7.2, 10; *N*-acetylglucosamine, 2.5; manganous chloride, 0.2; bovine serum albumin, 50 μg; UDP-[U-^{14}C]Gal (specific activity) approx. 7×10^5 cpm/mol), 0.045; and 0.1 to 0.5 U of galactosyltransferase in 0.1% bovine serum albumin. Reaction mixtures were initiated by the addition of UDP-[U-^{14}C]Gal and incubated at 37°C for 10 to 30 min, and the reaction was terminated by the addition of 10 μl of 0.25 M sodium EDTA in 1% sodium tetraborate. Aliquots (25 μl) were subjected to high-voltage paper electrophoresis in Buffer C. The areas of the electrophoretograms that corresponded to [^{14}C]acetyllactosamine were cut out and the radioactivity was quantitated in a Packard liquid scintillation spectrometer as recommended by the manufacturer. Reaction rates were linear with respect to time and enzyme concentration provided that less than 10% of the UDP-[U-^{14}C]Gal was consumed. One unit of enzyme is defined as that quantity that transfers 1 nmol of galactose to *N*-acetylglucosamine per minute at 37°C under the conditions described above.

Purification of galactosyltransferase from cornea. Unless otherwise indicated, all steps were performed at 0 to 4°C.

Step 1. Crude extract: Bovine eyes were allowed to thaw partially and the corneas were excised and washed in ice water. The corneas were homogenized in 100-g batches in a Waring Blendor for 1 min with 400 ml of 0.05 M Tris–HCl buffer, pH 7.5, containing 0.1 M sodium chloride, 5 mM magnesium chloride, and 1 mM 2-mercaptoethanol. The homogenates from 925 g of tissue (1160 eyes) were combined, stirred for 6 h, and centrifuged at 18,000g for 15 min. The resulting precipitate was resuspended in an additional 400 ml of buffer–salts mixture per 100 g of tissue and the suspension was stirred for an additional 16 h. The mixture was centrifuged and the supernatant solutions were combined.

Step 2. Ammonium sulfate precipitation: The crude extract from Step 1 was brought to 35% ammonium sulfate saturation by the addition of 19.4 g of ammonium sulfate for each 100 ml of crude extract. The mixture was stirred for 20 min and centrifuged at 18,000g for 15 min, and the precipitate was discarded. The supernatant was brought to 60% saturation with ammonium sulfate (15.1 g/100 ml) and the precipitate was collected by centrifugation. The precipitate was dissolved in a minimum volume of the buffer–salts mixture used for extraction and the solution was dialyzed for a total of 24 h against two changes of 10 volumes each of 0.05 M Tris–HCl, pH 7.5, containing 5 mM magnesium chloride, 5 mM *N*-acetylglucosamine, and 1 mM 2-mercaptoethanol.

Step 3. Affinity chromatography I: A 2 × 16-cm column of the α-lactalbumin-substituted agarose prepared as described by Trayer and Hill (14) was equilibrated with 0.05 M Tris–HCl, pH 7.5, containing 5 mM magnesium chloride, 1 mM 2-mercaptoethanol, and 5 mM *N*-acetylglucosamine. The dialysate from Step 2 was passed through the column (flow rate, 18 ml/h) and the column was washed with the same buffer until the optical density of the eluate at 280 nm was less than 0.1. The galactosyltransferase was eluted from the column with the same buffer–salts mixture lacking *N*-acetylglucosamine. Fractions of 10 ml were collected into tubes containing 0.5 ml of 1 M *N*-acetylglucosamine, a procedure found to stabilize the enzyme. Fractions that contained galactosyltransferase activity were combined and concentrated in an ultrafiltration cell over a Diaflo UM-10 membrane (Amicon Corp., Lexington, Massachusetts).

Affinity chromatography II: Galactosyltransferase from affinity column I was applied to a 1 × 10-cm column of α-lactalbumin-substituted agarose at a flow rate of 10 ml/h. The column was washed and eluted in the same manner as described above for the first affinity chromatography column.

Purification of lactose synthase A-protein from milk. Bovine milk galactosyltransferase (A-protein) was isolated by a procedure similar to that described for cornea galactosyltransferase except that prior to ammonium sulfate fractionation casein was removed by isoelectric precipitation (26) and α-lactalbumin was removed by gel filtration (27).

Preparation of [U-^{14}C]Gal–Keratan. Agalactokeratan from bovine nasal septum from which an equivalent of 0.25 μmol of galactose had been cleaved was added to 0.5 ml of imidazole–HCl buffer, pH 8.0 and ionic strength 0.5, containing 2 μmol of manganous chloride, 0.06 μmol of UDP-[U-^{14}C]Gal (1.3×10^6 cpm), and 0.43 μg of purified bovine cornea galactosyltransferase. The mixture was incubated at 37°C for 22 h and the entire mixture was placed on a 1.2 × 57-cm column of Sephadex G-25. The column was eluted with distilled water. [U-^{14}C]Gal–keratan eluted in the void volume and was concentrated to dryness in a rotary evaporator at 30°C under reduced pressure. The ^{14}C-labeled product contained 3.7×10^5 cpm of bound [U-^{14}C]Gal (0.017 μmol). No attempt was made to saturate the acceptor with [U-^{14}C]Gal.

Isolation and culture of stromal fibroblasts. Bovine cornea stromal preparations were prepared essentially as described by Conrad (28) and the cornea fibroblasts were isolated from stroma in the manner described by Smith (29). The cells were maintained in Eagle's minimum essential medium supplemented with Earle's salts and 10% fetal calf serum (Grand Island Biological Co., Grand Island, New York).

RESULTS

Purification of Bovine Galactosyltransferase and Milk A-Protein

The results of the purification of bovine cornea galactosyltransferase are summarrized in Table I. The extraction procedure yielded a soluble enzyme preparation and less than 10% of the total enzyme activity was detected in the particulate residue after extraction. However, an extended period (16 h) was required for the diffusion of the enzyme from the gelatinous residue into the extracting buffer. Exhaustively extracted cornea residue contained less than 2% of the galactosyltransferase activity found in the soluble fraction. The residue also contained small amounts of a second galactosyltransferase that catalyzed transfer of galactose residues to 3-*O*-β-xylosyl-L-serine; the latter activity is believed to be a galactosyltransferase associated with the biosynthesis of the linkage region common to several glycosaminoglycans (30).

After ammonium sulfate precipitation, the crude cornea enzyme was placed directly onto an α-lactalbumin-substituted agarose affinity column. The capacity of the affinity column to bind the galactosyltransferase was not affected by the presence of high concentrations of extraneous proteins. For example, the application of 10 g of protein did not affect binding of the enzyme. Maximal enzyme recovery was achieved when saturating levels of enzyme (approximately 1000 U of enzyme/ml of packed α-lactalbumin agarose) were used. The cornea galactosyltransferase was purified 22,000-fold (yield, 17%) and had a specific activity of 10,700 U/mg of protein.

The ratios of transferase activity (in the presence and absence of α-lactalbumin) with *N*-acetylglucosamine, glucose, 2-acetamido-*N*-(L-aspart-4-oyl)-1,2-dideoxy-β-glucose, and agalactokeratan as acceptors remained constant at each step of the purification.

Milk A-protein was purified 560-fold from milk that was pretreated to remove the casein fraction. The purified enzyme had a specific activity of 17,184 U/mg of protein; this specific activity compares favorably with values found by others: 14,100 (14), 5,740 (31), and 14,900 U/mg of protein (32).

Homogeneity and Stability of Cornea and Milk Galactosyltransferases

The cornea enzyme yielded a single protein-staining band on disc gel electrophoresis columns run in the presence of sodium dodecyl sulfate (Fig. 1); the estimated molecular weight of the enzyme was 42,200 ± 400. Milk A-protein exhibited two protein

TABLE I

PURIFICATION OF GALACTOSYLTRANSFERASE FROM BOVINE CORNEA[a]

Procedure	Volume (ml)	Total protein (mg)	Total units (nmol/min)	Specific activity (U/mg)	Recovery (%)
Step 1					
Combined 12,000*g* supernatant	5,560	29,000	14,100	0.49	100
Step 2					
Ammonium sulfate	447	9,670	12,400	1.28	88
Step 3					
Affinity chromatography I	53	—	5,020	—	36
Affinity chromatography II	1.25[b]	0.222	2,370	10,700	17

[a] Purification based upon extraction of 1160 corneas.
[b] Concentrated by ultrafiltration.

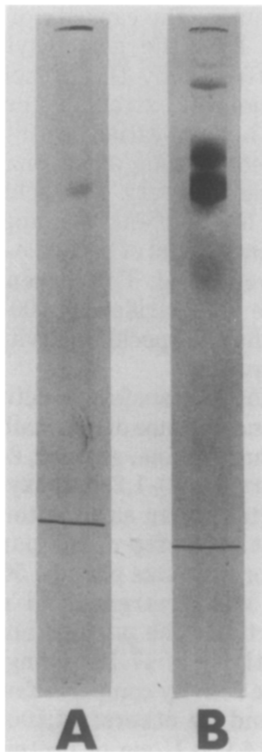

FIG 1. Polyacrylamide disc gel electrophoresis of cornea galactosyltransferase and milk A-protein in the presence of sodium dodecyl sulfate (22). Gel A, 96 U of cornea enzyme (9 μg); gel B, 275 U of milk enzyme (16 μg).

bands with estimated molecular weights of 53,900 ± 900 and 43,000 ± 700, values that agree closely with those reported by Barker *et al.* (32) and by Magee *et al.* (33). Gel columns were not loaded with high concentrations of the cornea enzyme since only small amounts of purified enzyme were available; therefore, it is uncertain if the cornea enzyme contains the high molecular weight component present in the milk A-protein. In addition, when similar protein loads of cornea and milk enzymes were applied to the gel columns (9 and 16 μg, respectively), the cornea enzyme consistently stained much less intensely than the milk enzyme.

Trayer and Hill (14) found that the milk A-protein was stable at a concentration of 1.0 mg/ml for at least 3 months when stored at 4°C in the presence of a solution containing 5 mM manganese chloride, 1 mM 2-mercaptoethanol, and 5 mM *N*-acetylglucosamine. However, the cornea galactosyltransferase at a concentration of 0.18 mg/ml lost about 50% of its activity in 1 month under these conditions. The cornea enzyme could be stored for up to 3 months without loss of activity in 0.1% bovine serum albumin.

Effect of pH and Metal Ions

The effect of pH on bovine cornea and milk galactosyltransferase activities is shown in Fig. 2. The pH profile for both enzymes is remarkably similar with a broad pH optimum ranging from 7.5 to 10.5. As indicated in the figure, enzyme activity is affected by the type of buffer used; this is not attributable to differences in ionic strength of the buffers employed. The pH profile of bovine milk A-protein is similar to that previously reported (34).

Cornea galactosyltransferase activity was completely dependent upon the presence of a divalent cation. Optimal product formation was achieved in reaction mixtures that contained Mn^{2+} ions at final concentrations of

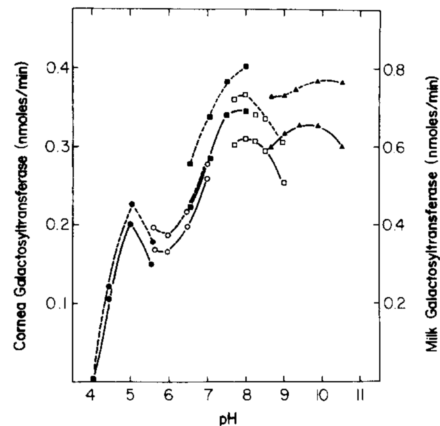

FIG. 2. Effect of pH on cornea and milk A-protein galactosyltransferase activity with *N*-acetylglucosamine as acceptor. Reaction mixtures were similar to those described under Assay of Galactosyltransferases under Materials and Methods except that the following buffers were used: ●, sodium acetate; ○, sodium cacodylate; ■, imidazole–HCl; ▲, glycine; and □, glycylglycine. Each buffer was adjusted to an ionic strength of 0.25 M with sodium chloride. Reaction mixtures contained 0.25 U of cornea enzyme of 0.56 U of milk enzyme and were incubated for 15 min. The solid line represents cornea enzyme and the dashed line, milk enzyme.

4 mM. At the same concentration Co^{2+} and Zn^{2+} showed considerably lower activity (14.9 and 9.2%, respectively), and Mg^{2+}, Ca^{2+}, Fe^{2+}, and Na^{+} gave less than 3% of the activity observed with Mn^{2+} (Table II). No measurable change in product formation was observed when the final concentration of Mn^{2+} was varied between 2.5 and 1000 mM. The cofactor requirements for milk A-protein are similar to those found for the cornea enzyme.

Acceptor Specificity Studies

The cornea and milk galactosyltransferase preparations exhibited almost identical specificities toward a number of low molecular weight acceptor compounds (Table III). The addition of α-lactalbumin to reaction mixtures containing *N*-acetylglucosamine as acceptor inhibited product formation approximately 80%, but α-lactalbumin was required to demonstrate transfer of galactosyl residues to glucose. Both α- and β-methyl glycosides of acetylglucosamine served as acceptors; α- and β-methyl glycosides of glucose did not serve as acceptors even in the presence of α-lactalbumin. The configurations about carbons 2 and 4 of the acceptor compound is of importance as evidenced by the inability of *N*-acetylmannosamine, *N*-acetylgalactosamine, and mannose to serve as acceptor compounds for either the milk or the cornea galactosyltransferases. The di- and trisaccharides of *N*-acetylglucosamine, di-*N*-acetylchitobiose and tri-*N*-acetylchitotriose, serve nearly as well as acceptors as other glycosides of *N*-acetylglucosamine.

The acceptor specificity of the cornea and milk galactosyltransferases for several high molecular weight compounds is also presented in Table III. Agalacto-ovomucoid and asialo-agalacto-α_1-glycoprotein exhibited similar acceptor activities with each enzyme preparation. The surprisingly high activity with agalacto-ovomucoid as acceptor is attributed to the observation that it may have as many as five terminal nonreducing *N*-acetylglucosaminyl residues per mole (35). At least a portion of the *N*-acetylglucosaminyl residues in ovomucoid as well as α_1-glycoprotein are linked in β-configuration to mannose; in keratan *N*-acetylglucosaminyl residues are linked to galactose, and in the chitin derivatives to *N*-acetylglucosamine. The demonstrated transfer of galactosyl residues to the terminal nonreducing *N*-acetylglucosaminyl units of each of these compounds suggests that the galactosyltransferase exhibits little specificity for the penultimate sugar unit or glycosidic bond.

The results of a comparative study of the potential of keratan sulfate and its derivatives to serve as acceptors for the transfer of galactosyl units from UDP-Gal by purified corneal galactosyltransferase are presented in Fig. 3. While the rate of galactosyl transfer to agalactokeratan sulfate was slower than that observed with agalactokeratan, about 75% of the potential acceptor sites of each compound were glycosylated. As anticipated, keratan and keratan sulfate, which contain terminal galactosyl units, were poor acceptors of galactosyl units; that is, the ^{14}C contents of the reaction products were less than 10 and 1%, respectively, the value obtained with agalactokeratan as acceptor.

Kinetic Studies

The K_m values for UDP-Gal, α-lactalbumin, Mn^{2+}, and glycosyl acceptors are presented in Table IV and are strikingly similar for

TABLE II

METAL REQUIREMENTS FOR BOVINE CORNEA AND MILK A-PROTEIN GALACTOSYLTRANSFERASES[a]

Metal ion	Relative activity (%)	
	Cornea galactosyltransferase	Milk A-protein
Mn^{2+}	100	100
Co^{2+}	14.9	14.9
Zn^{2+}	6.1	9.2
Fe^{2+}	4.3	3.5
Mg^{2+}	0.8	1.63
Ni^{2+}	<0.02	<0.02
Ca^{2+}	<0.02	<0.02
None	<0.02	1.2

[a] The incubation conditions were those described under Assay of Galactosyltransferases under Materials and Methods, except that imidazole-HCl buffer, pH 8.0 (ionic strength, 0.5 M), was used. All metal ions were added as the chloride salts to a final concentration of 4 mM.

TABLE III

ACCEPTOR SPECIFICITY OF CORNEA AND MILK GALACTOSYLTRANSFERASES FOR LOW AND HIGH MOLECULAR WEIGHT CARBOHYDRATES AND CARBOHYDRATE DERIVATIVES[a]

Acceptors[b]	Cornea galactosyltransferase (nmol/min/U of enzyme)	Milk A-protein
2-Acetamido-2-deoxyglucose	1.00 (0.21)[c]	1.00 (0.16)[c]
Methyl 2-acetamido-2-deoxy-β-glucoside	0.76	0.80
Methyl 2-acetamido-2-deoxy-β-glucoside	0.76	0.59
Methyl 2-bromo-acetamido-2-deoxy-β-glucoside	0.75	0.54
p-Nitrophenyl 2-acetamido-2-deoxy-β-glucoside	0.67 (0.64)[c]	0.43 (0.40)[c]
2-Acetamido-*N*-(L-aspart-4-oyl)-1,2-dideoxy-β-glucoside	0.65 (0.55)[c]	0.63 (0.58)[c]
Methyl 2-deoxy-2-(*p*-benzamido)-β-glucoside	0.16	0.08
Glucose	0.02 (1.07)[c]	0.01 (1.09)[c]
Tri-*N*-acetylchitotriose	0.64	0.67
Di-*N*-acetylchitobiose	0.54	—
Agalacto-ovomucoid	0.65	0.64
Asialo-agalacto-α_1-glycoprotein	0.42	0.29
Agalactokeratan		
Cornea	0.05	0.09
Nasal septum	0.13	0.12

[a] The incubation conditions were those described under Assay of Galactosyltransferases except that imidazole–HCl buffer, pH 8.0 and ionic strength 0.5, was used. The final concentration of each compound tested was 50 mM except for ovomucoid (0.31 mM), asialo-agalacto-α_1-glycoprotein (0.32 mM), corneal agalactokeratan (0.38 mM), and nasal agalactokeratan (0.33 mM); the concentration of the latter high molecular weight substrates is presented as the concentration of terminal *N*-acetylglucosaminyl groups available to the transferase.

[b] The following compounds were inactive in the presence or absence of α-lactalbumin: 2-amino-2-deoxyglucose, 2-acetamido-2-deoxyglucose-1- and -6-phosphate, glucose-1- and -6-phosphate, α- and β-methyl glucosides, 3-*O*-β-xylosyl-L-serine, 3-*O*-β-L-arabinosyl-L-serine, 2-acetamido-*N*-(L-aspart-4-oyl)-1,2-dideoxy-β-galactoside, 2-acetamido-2-deoxygalactose, and mannose.

[c] Values in parentheses were obtained from incubation mixtures containing 15.5 μg of α-lactalbumin in place of serum albumin.

the cornea and milk galactosyltransferases. Maximal velocities obtained using glucose as acceptor (and in the presence of α-lactalbumin) are also similar for the two enzymes. It is of interest that the K_m of the cornea enzyme for β-galactosidase-treated keratan is less than one-tenth that obtained with *N*-acetylglucosamine as acceptor and approximates the values found for the A-protein by other investigators using glycoprotein acceptors such as ovalbumin (37). The V and K_m values found for the A-protein of milk lactose synthase approximate closely those previously reported (38).

Identification of ^{14}C-Labeled Products

The stoichiometry of the bovine cornea galactosyltransferase-catalyzed reaction using *N*-acetylglucosamine as the acceptor was determined. The ratio of UDP-[^{14}C]Gal consumed to UDP and [^{14}C]galactosyl-(β,1 → 4)-*N*-acetylglucosamine formed was 1.10: 1.04:1.10. The ^{14}C-labeled product was identified by cochromatography with the authentic galactosyl-(β,1 → 4)-*N*-acetylglucosamine in Solvent A, a solvent known to separate the three known position isomers of acetylglucosamine (39), and by release of [^{14}C]galactose on treatment with bovine testicular β-galactosidase. Galactose was identified by paper chromatography in Systems A and B (Table V) as well as by electrophoresis in Buffer C. The demonstration of the formation of a β,1 → 4 glycosidic linkage with *N*-acetylglucosamine as acceptor by the cornea enzyme is in accord

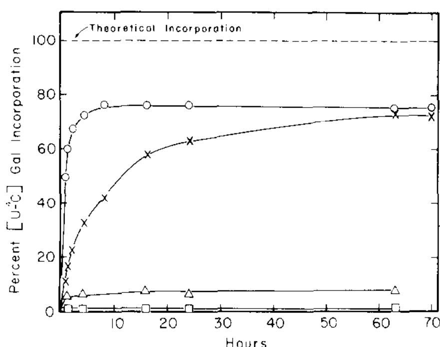

FIG. 3. Transfer of [U-^{14}C]galactose to corneal keratan sulfate and keratan sulfate derivatives with time. The assay conditions described under Materials and Methods were scaled up 10-fold. Each of the potential acceptors, agalactokeratan (○), agalactokeratan sulfate (×), keratan (△), and keratan sulfate (□), was present in the reaction mixtures at a final N-acetylglucosamine concentration of 2.0 μmol; the added agalactokeratan and agalactokeratan sulfate each contained 0.177 μmol of potential galactosyl acceptor sites available to the galactosyltransferase, i.e., the amount of galactose removed from each compound. The specific activity of the UDP-[U-^{14}C]Gal was 0.5 μCi/μmol. Incubations were conducted for the periods of time indicated.

with the results obtained with the milk A-protein (40).

Treatment of the ^{14}C-labeled product obtained when agalactokeratan was incubated with UDP-[U-^{14}C]Gal and cornea galactosyltransferase with β-galactosidase resulted in the quantitative release of the radioactive label as [^{14}C]Gal. After partial acid hydrolysis of [^{14}C]Gal–keratan, 10% of the ^{14}C content was identified by paper chromatography as galactosyl-(β,1 → 4)-N-acetylglucosamine (Table V).

Biosynthesis of Keratan Sulfate-like Polysaccharide by Bovine Cornea Fibroblasts

Fibroblasts isolated from bovine cornea stroma were grown in culture medium supplemented with 10% fetal calf serum. Cells (3.8 × 10^6) were lysed by freezing and thawing and examined for galactosyltransferase activity. Persistently low levels (approximately 0.2–1.0 enzyme unit/mg of protein) of galactosyltransferase were detected even after six cell passages; the transfer of galactose from UDP-galactose to glucose was enhanced by the addition of α-lactalbumin. No galactosyltransferase activity (<0.01 enzyme unit/mg of protein) was detected in fetal calf serum.

DISCUSSION

The purification and characterization of a UDP-galactose:keratan sulfate galactosyltransferase prepared from bovine cornea have been described. The soluble transferase, 22,000-fold purified, transfers galactose to N-acetylglucosaminyl units terminating

TABLE IV

K_m AND V VALUES OF CORNEAL AND MILK GALACTOSYLTRANSFERASES[a]

Compound	Cornea galactosyltransferase		Milk A-protein	
	V (nmol/min/U)	K_m (mM)	V (nmol/min/U)	K_m (mM)
N-Acetylglucosamine	1.00	40.0	1.00	22.0
Glucose[b]	0.62	21.0	0.74	12.0
Agalactokeratan (nasal septum)[c]	0.78	2.8	0.63	4.2
α-Lactalbumin[d]		0.012		0.011
UDP-galactose		0.055		0.066
$MnCl_2$		0.34		0.54

[a] Reaction mixtures were similar to those described under Assay of Galactosyltransferases under Materials and Methods except that imidazole–HCl buffer, pH 8.0 and ionic strength 0.5, replaced the cacodylate buffer. V and K_m values were determined by the procedure of Lineweaver and Burk (36).

[b] Reaction mixtures contained α-lactalbumin (15.5 μg) in place of serum albumin.

[c] Expressed as the concentration of terminal N-acetylglucosaminyl units available to the transferase.

[d] Reaction mixtures contained glucose (2.5 μmol) in place of N-acetylglucosamine.

TABLE V

IDENTIFICATION OF ^{14}C-LABELED PRODUCTS OF CORNEA GALACTOSYLTRANSFERASE[a]

^{14}C-labeled compound obtained	Solvent A ($R_{Gal-(\beta,1\rightarrow 4)-Glc}$)	Solvent B (R_{Glc})	Buffer C (cm)
[^{14}C]Gal-GlcNAc	1.92		2.6
After treatment with β-galactosidase	4.30	0.87	10.6
Partial acid hydrolysate of [^{14}C]Gal–keratan	1.92, 4.35		2.6, 10.6
After treatment with β-galactosidase	4.30	0.89	10.6
Reference compounds			
Galactosyl-(β,1 → 6)-N-acetylglucosamine	1.44		
Galactosyl-(β,1 → 4)-N-acetylglucosamine	1.90		
Galactosyl-(β,1 → 3)-N-acetylglucosamine	2.28		2.6
Galactosyl-(β,1 → 4)-glucose	1.00		
Galactose	4.35	0.89	10.6
Glucose		1.00	
Mannose		1.13	

[a] The incubation conditions and conditions for the resolution of the ^{14}C-labeled products by paper chromatography and paper electrophoresis and their detection are described under Materials and Methods. [U-^{14}C]Gal–keratan (300,000 cpm) was partially acid hydrolyzed at 100°C for 2 h in 1 ml of 0.05 N sulfuric acid (38) and treated with an excess of an equal amount of Dowex 50 (H^+) and Dowex 2 (HCO_3^-); the neutral fraction was reduced in volume and aliquots were used for identification by paper chromatography and paper electrophoresis.

keratan. The β,1 → 4 glycosidic linkage formed is in accord with the established structure of the repeating disaccharide comprising keratan sulfate. Enzyme activity was assayed with low molecular weight and polymeric acceptors at each stage of purification. No changes in the ratios of transferase activity were found, suggesting the absence of other soluble galactosyltransferases in bovine cornea. Small amounts of a second galactosyltransferase that catalyzed the transfer of galactosyl residues to 3-O-xylosyl serine were detected in the particulate fraction. 3-O-β-Xylosyl serine did not serve as a galactosyl acceptor for the purified corneal enzyme. This particulate galactosyltransferase is believed to be associated with the biosynthesis of the linkage region common to several glycosaminoglycans (30).

While the V values for N-acetylglucosamine and agalactokeratan as acceptors were similar, a markedly lower K_m was found with agalactokeratan than with N-acetyl-D-glucosamine as galactosyl acceptor (Table V). Indeed, agalactokeratan may be even a better substrate than is suggested by these results. The values calculated for aglactokeratan are based on the concentration of potential sites (β-N-acetylglucosamine end groups exposed) and are not based on the actual molar concentrations of the glycosaminoglycan. Since only a limited number of terminal N-acetylglucosamine residues in the glycosaminoglycan are available to the enzyme, expressing concentration in terms of glycosaminoglycan rather than total N-acetylglucosamine end groups may provide a more accurate reflection of the relative activity of this acceptor. Nevertheless, we chose the latter method, which yields a maximal K_m value. The lower K_m exhibited by agalactokeratan as compared to N-acetylglucosamine suggests that the chief function of the enzyme is to transfer galactose units to terminal N-acetylglucosamine residues of glycoproteins and/or keratan and not to free N-acetylglucosamine. The fact that low concentrations of agalacto-ovomucoid and asialo-agalacto-α_1-glycoprotein displayed relatively high galactose acceptor activities (Table III) supports this conclusion.

The observed modulation of acceptor specificity of the cornea enzyme by α-lactalbumin prompted a comparison of the physical and chemical properties of the milk

A-protein with bovine cornea galactosyltransferase. With the exception of the aberrant staining behavior of the cornea transferase on sodium dodecyl sulfate polyacrylamide disc gel columns, the milk and cornea enzyme preparations appear nearly identical with respect to their physical characteristics, acceptor specificity, and kinetic properties.

To date, a limited number of studies have been concerned with the biosynthesis of keratan sulfate. Keratan sulfate I is only synthesized in the cornea and by stromal fibroblasts (6, 8). Present studies suggest that both cornea fibroblasts alone and even whole corneas lose their ability to synthesize keratan sulfate when incubated *in vitro* (28, 41–43). However, the fibroblasts synthesize other glycosaminoglycans, including dermatan sulfate, heparan sulfate, and chondroitin-6-sulfate, which are not thought to be found in the normal cornea (42, 43). The inability of the fibroblasts to continue to synthesize keratan sulfate *in vitro* suggests the possibility that galactosyltransferase isolated from bovine cornea may not be synthesized by cornea cells but rather is obtained from adjacent cells or tissues and is subsequently lost during culture; high molecular weight compounds found in serum such as albumin are known to enter the cornea by diffusion (44). In an attempt to answer this question, fibroblasts isolated from bovine cornea stroma were grown in culture medium for extended periods of time and assayed for galactosyltransferase activity. Low but persistent levels (approximately 0.2 U/mg of protein) of galactosyltransferase were detected even after six cell passages. The transfer of galactose from UDP-galactose to glucose in the cultured cells was enhanced by the addition of α-lactalbumin. These results suggest that cornea fibroblasts contain a constitutive galactosyltransferase capable of the addition of galactosyl units to enlongating keratan sulfate chains. The observed loss of keratan sulfate synthesis by cultured cells is therefore probably not solely attributable to the loss of cornea galactosyltransferase.

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