Biosynthesis of Chondroitin Sulfate

ROLE OF PHOSPHOLIPIDS IN THE ACTIVITY OF UDP-D-GALACTOSE-D-XYLOSE GALACTOSYLTRANSFERASE*

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The role of phospholipids in the activity of UDP-D-galactose:D-xylose galactosyltransferase (galactosyltransferase I) from embryonic chick cartilage was investigated. Phospholipase C treatment of particulate galactosyltransferase I caused inactivation of this enzyme to the extent of 60 to 70% as well as hydrolysis of 75 to 80% of the membrane phospholipids. Addition of phospholipid restored activity to nearly control levels. The order of effectiveness of various phospholipids in reactivating phospholipase C-treated galactosyltransferase I was as follows: lysophosphatidylcholine, lysophosphatidylethanolamine, phosphatidylethanolamine, phosphatidylcholine. The effect of phospholipase A on galactosyltransferase I activity was also examined and was found to be concentration-dependent. At concentrations less than 10 μg/mg of pellet protein, phospholipase A slightly activated galactosyltransferase I, whereas at higher concentrations it inhibited the activity in a manner similar to phospholipase C. Galactosyltransferase I was activated moderately and also solubilized by treatment with Nonidet P-40 in the presence of 0.5 M KCl. Following solubilization and purification by gel filtration and affinity chromatography, galactosyltransferase I could be inactivated by detergent removal by dialysis and subsequently reactivated by addition of detergent.

Neither phospholipase C treatment nor exogenous phospholipid had any significant effect on three of the other chondroitin sulfate glycosyltransferases (UDP-D-xylose:core protein xylosyltransferase, UDP-D-glucuronic acid:3-O-B-D-galactosyl-D-galactose glucuronosyltransferase, and UDP-N-acetyl-D-galactosamine:(GlcUA-GalNAc-4-sulfate), N-acetylgalactosaminyltransferase).

On lipid analysis by thin layer chromatography, phosphatidylcholine and phosphatidylethanolamine were found to be the major phospholipids of particulate and solubilized glycosyltransferase preparations from embryonic chick cartilage, while lysophosphatidylcholine and lysophosphatidylethanolamine were barely detectable components. The concentration of these specific phospholipids was diminished greatly following phospholipase C treatment.

Enzymes that catalyze sequential reactions are often obtained either as multienzyme complexes or as components of the same membrane fraction in the course of isolation (2). The glycosyltransferase system which catalyzes the biosynthesis of chondroitin sulfate is similar in this respect. With the exception of a portion of the chain-initiating xylosyltransferase, all of the glycosyltransferases are bound firmly to membranes of the endoplasmic reticulum and are found primarily in the 100,000 x g pellet fraction after high speed centrifugation of a cartilage homogenate (3).

In order to better understand the nature of this multienzyme system and its role in the regulation of chondroitin sulfate synthesis, the composition, structure, and organization of the various components are being investigated. Some of the individual enzymes have been isolated and their properties in aqueous solution are being examined (4, 5). Furthermore, it is important to determine how the enzymes function in the native state, since the glycosyltransferases are attached to or embedded in the membranes of the endoplasmic reticulum, and thus their behavior conceivably might be affected by other membrane components. Since membranes in general contain large amounts of lipid, the effect of perturbation of the lipid environment on the chondroitin sulfate glycosyltransferases was investigated. Of the various means that can be used to alter the phospholipid environment, phospholipase treatments were chosen because of the generally mild conditions under which they selectively modify membrane phospholipids. In the present study one of the chondroitin sulfate glycosyltransferases, UDP-D-galactose:D-xylose galactosyltransferase1 was

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1 All sugars discussed in this paper are of the D configuration.
found to be inactivated by hydrolysis of the membrane phospholipids by phospholipase C, and subsequently reactivated by addition of phospholipids.

**MATERIALS AND METHODS**

UDP-[14C]Galactose (252 μCi/μmol), UDP-[14C]xylose (174 to 198 μCi/μmol), UDP-[14C]glucuronic acid (125 μCi/μmol), and UDP-N-[14C]acetylglucosamine (43 μCi/μmol) were purchased from New England Nuclear. UDP-Galactose, UDP-xylose, and UDP-glucuronic acid were obtained from Calbiochem. Nonidet P-40 was obtained from Shell Oil Co. and bovine serum albumin from Armour Laboratories. Sepharose 4B was purchased from Pharma and cetylpyridinium chloride from K and K Laboratories. 4-O-β-D-galactosyl-β-xylose and 0-β-D-xylo-β-l-serine were generous gifts from Doctors T. Helting and A. C. Stoolmiller, respectively, and had been synthesized as previously described (6–8). 3-O-β-D-Galactosyl-β-galactose and 3-O-β-D-galactosyl-4-O-β-D-galactosyl-β-xylose were prepared as described by 1. Berg et al. (6) and Roden et al. (8) and kindly supplied by Dr. I. Roden. Chondroitin 4-sulfate octasaccharide was nonreducing terminal glucuronic acid (GlueUA=GlcNA-C=Galactose), was isolated from chondroitin 4-sulfate by digestion with testicular hyaluronidase and gel chromatography as described (8). Smith-degraded cartilage proteoglycan was prepared by the method of Baker et al. (9). Silica-coated glass plates (type QT1) were obtained from Quantum Industries. Phospholipase C (Cotidium perfringens) was purchased from Worthington and phosphatase A (Viperus russellii) from Sigma. Phospholipids were obtained from the following sources: phosphatidylincholine (egg) and lysophosphatidylincholine (egg) from Sigma; phosphatidylethanolamine, lysophosphatidylethanolamine, phosphatidylserine, and lysophosphatidylserine from Applied Science Laboratories.

**Enzyme Assays**

**Xylosyltransferase**—This enzyme was assayed essentially according to Method 2a (8), following dialysis of enzyme samples against 0.05 M Mes4 buffer, pH 6.5, containing 0.05 M KCl, 0.003 M MnCl2, and 0.012 M MgCl2. Incubation mixtures contained the following components in a final volume of 0.075 ml; 0.2 μg of Smith-degraded proteoglycan (0.05 ml), 0.75 μmol of KF, and 1.5 nmol of UDP-[3H] xylose (sp. act. approximately 30 μCi/μmol), and varying amounts of enzyme protein in 0.050 ml of Mes buffer. After incubating the reaction mixture for 60 min at 37°, 0.05 ml of 1% bovine serum albumin and 0.20 ml of 10% trichloroacetic acid were added. Precipitated protein was recovered by centrifugation, washed twice in 5% trichloroacetic acid, and dissolved in 0.1 ml of 1 M NaOH for liquid scintillation counting.

**Galactosyltransferase I**—This enzyme, which transfers galactose to 4-O-β-D-galactosyl-β-xylose, was assayed in a volume of 0.075 ml by incubating 2 μmol of xylose (0.010 ml), 1.3 nmol of UDP-[14C] galactose (34.5 μCi/μmol; 0.010 ml), 1.0 nmol of MnCl2 (0.005 ml), and varying amounts of enzyme protein in 0.050 ml of standard buffer. After 60 min incubation, the reaction was terminated by heating the tubes at 100° for 2 min. The entire mixtures and galactosylxylose standards were spotted on Whatman No. 3MM paper and desalted by high voltage electrophoresis as described (8, 10). Chromatography in ethyl acetate/acetic acid/water (5:1:1) was carried out for 24 hours, and the radioactive product was quantitated by liquid scintillation counting of the galactosylotruxyllose area.

Alternatively, galactosyltransferase I was assayed with 0.4 μmol of xylosylserine as substrate instead of xylose (3, 10). After incubation and heat inactivation, the samples were mixed with 0.6 ml of 0.01 M HCl, centrifuged, the supernatant fraction was applied to columns (0.5 × 2.5 cm) of Dowex 50-X2 (H+ form, 200 to 400 mesh) followed by a wash with 6 ml of 0.01 M HCl. The product was eluted with 2 ml of 0.2 M NH4OH, the eluate was evaporated to dryness, and the radioactivity was measured.

**Galactosyltransferase II**—The galactosyltransferase which transfers galactose to 0-β-D-galactosyl-β-xylose was assayed under conditions identical with those for galactosyltransferase I; except that 2 μmol of galactosylxylose were substituted for xylose. The trisaccharide product (galactosylgalactosylxylose) was isolated by paper electrophoresis and paper chromatography and quantitated essentially as described for the assay of galactosyltransferase I (6, 10).

**Glucuronosyltransferase I**—Glucuronosyltransferase I transfers glucuronic acid to a chondroitin sulfate octasaccharide acceptor was measured as previously described (8, 12, 13). Aliquots of enzyme were dialyzed overnight against 0.05 M Hepes buffer, pH 7.0, containing 0.05 M KCl. Reaction mixtures of 0.075 ml contained 30 μg of chondroitin 4-sulfate octasaccharide (0.010 ml), 10 nmol of UDP-N-[14C]galactosamine (43 μCi/μmol; 0.010 ml), 1.0 μmol of MnCl2 (0.005 ml), and varying amounts of enzyme protein in Hepes buffer. After incubation at 37° for 60 min, the reaction product was isolated by cetylpyridinium chloride cellulose column chromatography.

**Enzyme Preparation**

Epipolyphomes from femurs and ribs of 13-day-old chick embryos were dissected and homogenized in standard buffer as previously described (3). The crude cell homogenate was centrifuged at 10,000 × g for 20 min at 4°; subsequently, the 10,000 × g supernatant fluid was centrifuged at 100,000 × g for 60 min.

The concentration of protein in various enzyme fractions was measured by the method of Lowry et al. (14). In order to remove manganese ions which interfere with these determinations, protein was precipitated first from a solution of the enzyme (0.1 to 0.5 ml) with an equal volume of cold 50% trichloroacetic acid. The precipitate was collected by centrifugation, dissolved in 0.1 to 0.2 ml of 0.1 M NaOH, and assayed as usual.

**Solubilization of Glycosyltransferases by Detergent-Salt Treatment**—The 100,000 × g pellet from a cartilage homogenate was suspended in standard buffer, incubated in the presence of 0.5% Nonidet P-40 at 4° for 30 min, and centrifuged at 100,000 × g for 60 min.

The resulting pellet was resuspended by sonication in buffer containing 0.5% Nonidet P-40, and solid potassium chloride was added to a final concentration of 0.5 M. After incubation at 4° for 15 min, the mixture was centrifuged at 100,000 × g for 60 min, and the supernatant and pellet fractions were assayed. In some instances, the enzyme solution in the presence of detergent and salt was kept frozen overnight before extensive centrifugation (5).

**Purification of Galactosyltransferase I**—Solubilized galactosyltransferase I was partially purified by gel filtration on Sephadex G-200 (5). A portion of the enzyme emerged with the void volume, while a substantial amount (60%) appeared in a retarded position and was purified approximately 50-fold. After ultrafiltration to yield a protein concentration of 1 mg/ml, further purification of the retarded fraction of galactosyltransferase I was achieved by affinity chromatography on a matrix of xylosyltransferase-Sepharose (5). Galactosyltransferase I was eluted quantitatively with 0.05 M Mes buffer, pH 6.5, containing 0.25 M KCl, 0.012 M MgCl2, 0.003 M MnCl2, and 1% Nonidet P-40, yielding an enzyme of specific activity approximately 1100-fold higher than that of the crude homogenate.
Phospholipase Analysis

Extraction of phospholipids was carried out according to the procedure of Scanna and Edelstein (15). Briefly, the particulate fraction from a cartilage homogenate (approximately 20 mg of protein in 1.0 ml) was extracted in a separatory funnel by shaking with 95% ethanol/ethyl ether (9/1, v/v) for 2 hours at 0°C. The ethanol/ethyl ether ratio was then changed to 3:5 and the extraction continued overnight at -10°C. The precipitate was removed by centrifugation at 5000 x g, and the organic phase was evaporated to dryness at 0°C under a stream of N2. The lipid extract was dissolved in 1 ml of chloroform/methanol (9/1, v/v).

Phospholipids were visualized by exposure to iodine vapor and identified by comparison with known standards.

Phospholipase Treatment

Phospholipase A or C treatment was carried out in 0.02 M Tris-HCl, pH 7.5, containing 0.005 M CaCl2 at 37°C in the concentration of enzyme to cartilage protein indicated in the text. The incubations were stopped by addition of EDTA to a final concentration of 0.01 M, followed by centrifugation at 100,000 x g for 1 hour. The pellet fractions were resuspended by sonication in the appropriate buffer and the supernatant fractions were dialyzed against the appropriate buffer for at least 2 hours for assay of glycosyltransferase activity.

In the preparation of phospholipids for addition to the enzyme system, the organic solvent was removed under a stream of N2, and the phospholipid was resuspended in buffer by sonic disruption with a Branson ultrasonic apparatus for 1 to 2 min at 70 watts.

RESULTS

Effect of Phospholipase C on Some Chondroitin Sulfate Glycosyltransferases—Treatment of the 100,000 x g particulate fraction from an embryonic chick cartilage homogenate with phospholipase C affected the chondroitin sulfate glycosyltransferases in different ways. As shown in Table I, there was a moderate inactivation of xylosyltransferase, which was not reversed by subsequent addition of phospholipid. Glucurono- syltransferase I and N-acetylgalactosaminyltransferase were activated slightly by phospholipase C treatment alone, and not further affected by addition of exogenous lipid. In contrast, galactosyltransferase I was inactivated substantially by treatment with phospholipase C and reactivated subsequently by addition of phospholipids. Addition of lipid alone had minimal effects on the activities of the four glycosyltransferases examined.

Table I

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Xylosyltransferase</th>
<th>Galactosyltransferase I</th>
<th>Glucuronosyltransferase I</th>
<th>N-Acetylglactosaminyltransferase</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>800</td>
<td>9060</td>
<td>316</td>
<td>158</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>890 (110)</td>
<td>9000 (100)</td>
<td>283 (90)</td>
<td>150 (95)</td>
</tr>
<tr>
<td>Phospholipase C</td>
<td>520 (65)</td>
<td>3780 (41)</td>
<td>352 (110)</td>
<td>204 (133)</td>
</tr>
<tr>
<td>Phospholipase C; phosphatidylcholine</td>
<td>540 (68)</td>
<td>9280 (102)</td>
<td>343 (108)</td>
<td>207 (135)</td>
</tr>
</tbody>
</table>

Effect of Phospholipase C on Particulate Galactosyltransferase I Activity—In a more detailed study (Fig. 1) the effect of phospholipase C on galactosyltransferase I inactivation as a function of time was examined at two concentrations of phospholipase C (0.2 and 2.0 mg/10 mg of pellet protein). At the latter concentration, phospholipase C in the presence of Ca2+ caused a 79% loss of galactosyltransferase I activity. Most of the inactivation (72%) occurred during the first 30 min of incubation. In the presence of phospholipase C alone or Ca2+ alone, only 5 and 7% inactivation, respectively, were observed.

Although the inactivation of galactosyltransferase I by phospholipase C appears to be due to depletion of phospholipids, the possibility was also considered that the inactivation was a consequence of the liberation of inhibitory fatty acids by possible phospholipase A contamination. This alternative could be ruled out since identical results were obtained in the presence of 1% albumin, which is known to bind fatty acids. Furthermore, no lysophosphatides were found by thin layer chromatography in phospholipase C-treated cartilage pellets (Fig. 2). It should also be mentioned that routine preparation of enzyme samples for glycosyltransferase assay following phospholipase C treatment involved centrifugation at 100,000 x g which would remove soluble products of the hydrolysis reaction.

Effect of Phospholipase C on Phospholipid Content of 100,000 x g Particulate Fraction from Cartilage—In the course of phospholipase C treatment, extensive hydrolysis of the membrane-bound phospholipids occurred, concomitantly with the loss of galactosyltransferase I activity. In a typical experiment, inactivation of galactosyltransferase I essentially reached a maximum of approximately 64% in 60 min, at which time 78% of phospholipid phosphorus (1.2 μmol/mg of pellet protein) had been lost.

The effectiveness of phospholipase C treatment was also evident from a qualitative analysis of lipid extracts by thin layer chromatography. In the untreated particulate fraction phosphatidylcholine and phosphatidylethanolamine were the predominant components (Fig. 2) and, appeared to be present in nearly the same proportions as judged from the intensity of staining. After phospholipase C treatment, the content of these two phospholipids was greatly diminished (Fig. 2). (Also see Ref. 17 for a recent report on the quantitation of lipids in cartilage.)
Biosynthesis of Chondroitin Sulfate

The biosynthesis of chondroitin sulfate was investigated using chick cartilage homogenate (10.2 mg of protein) in Tris-HCl, pH 7.5, containing 0.003 M CaCl₂ and 0.2 mg of phospholipase C in buffer without CaCl₂ (O---O) or complete buffer in the absence of phospholipase C (●●●●). Control samples contained 0.2 mg of phospholipase C in buffer without CaCl₂ (O---O) or complete buffer suffusion of the particulate fractions from an embryonic chick cartilage homogenate (10.2 mg of protein) in 0.02 M Tris-HCl, pH 7.5, containing 0.003 M CaCl₂ were treated at 37° with 0.2 mg (●-●) or 2 mg of phospholipase C (●●●●). Control samples contained 0.2 mg of phospholipase C in buffer without CaCl₂ (O---O) or complete buffer without CaCl₂ (O---O) or complete buffer suffusion of the particulate fractions from an embryonic chick cartilage homogenate (10.2 mg of protein) in 0.02 M Tris-HCl, pH 7.5, containing 0.003 M CaCl₂ and 0.001 M EDTA (standard buffer) whereas the pellets were resuspended in 1 ml of the same buffer. Aliquots (200 µl) from each tube were assayed for galactosyltransferase I activity as described under "Materials and Methods." No appreciable activity was found in the supernatant fractions; results for the pellet samples are presented as counts per min of galactosylxyllose formed per 200 µl of enzyme per hour.

Reactivation of Galactosyltransferase I by Addition of Phospholipids—Addition of sonicated dispersions of synthetic phospholipids to phospholipase C-treated galactosyltransferase I led to nearly complete restoration of activity (Fig. 3). Lysophosphatidylcholine was the most effective and stimulated the enzyme activity 2- to 3-fold when added in concentrations of 80 to 160 µg of phospholipid/mg of pellet protein. Qualitatively similar results were obtained with lysophosphatidylethanolamine, although complete reactivation was not achieved at any concentration of this phospholipid. The phosphatides were also effective, but at much higher concentration than the lysophosphatides; for example, 700 µg of phosphatidylcholine/mg of pellet protein were required for complete reactivation of phospholipase C-treated galactosyltransferase I. This concentration of the phospholipid caused minimal stimulation of galactosyltransferase I activity in untreated controls (Table I).

The inactivation and reactivation of galactosyltransferase I as a function of time was followed at the optimum concentration of lysophosphatidylcholine (100 µg/mg of pellet protein). At any point during the course of the incubation with phospholipase C, reactivation to control levels could be achieved by addition of exogenous phospholipid (Fig. 4). As in the previous experiment, addition of phospholipid to untreated controls gave only a moderate activation.

Effect of Phospholipase A on Galactosyltransferase I Activity—Phospholipase A affected galactosyltransferase I activity, but the nature of the effect was concentration-dependent. At concentrations greater than 10 µg of enzyme/mg of pellet protein, inactivation was observed. Whereas at much lower concentrations, phospholipase A stimulated activity (Fig. 5), possibly as a result of slight disruption of the membrane structure and concomitant formation of small amounts of lysophosphatides in the immediate vicinity of the enzyme.

Effect of Detergent on Galactosyltransferase I—As has been shown previously, treatment of the 100,000 × g particulate fraction from embryonic cartilage with 0.50 M KCl and 0.5% Nonidet P-40, results in solubilization of greater than 70% of all six chondroitin sulfate glycosyltransferases (5). Rapid removal of the detergent from such solubilized enzyme preparations by dialysis yielded visible precipitation and after centrifugation of the cloudy solution at 100,000 × g, substantial amounts of each of the chondroitin sulfate glycosyltransferases were again found in the pellet fraction (Table II). Reaggregation of the enzymes was not accompanied by loss of activity, since greater than 90% of the original activity of each enzyme was recovered.

As shown in Fig. 2, the detergent-salt solubilization procedure released membrane phospholipids along with the chondroitin sulfate glycosyltransferases. Most likely, the reaggregated preparations also contained a full complement of lipid since phospholipase C treatment caused substantial loss (approximately 65%) of galactosyltransferase I activity.

Partial separation of the glycosyltransferases from each other and from the bulk of the detergent has been achieved by Sephadex gel filtration (5). In this procedure, galactosyltransferase I was eluted in two distinct peaks. One portion
the reaction was stopped by addition of EDTA to a final concentration of 0.003 M CaCl₂ and treated with 1.5 mg of phospholipase C at 37° C for 1 hour. After centrifugation at 100,000 x g for 1 hour, the pellet fraction was resuspended in 0.05 M Tris-acetate, pH 5.5, containing 0.012 M MgCl₂ and 0.003 M MnCl₂; and 0.001 M EDTA. Aliquots (50 μl) were mixed by sonication with phospholipid to give the final concentration indicated per mg of pellet protein and assayed for galactosyltransferase I activity by the standard procedure. The activity of the control taken through the same procedure without phospholipase C is indicated by the dotted line. Phospholipids used were: lysophosphatidylcholine, O—O; phosphatidylethanolamine, O—O; phosphatidylcholine, O—O; and phosphatidylserine, O—O. Results are expressed as per cent activity of the untreated control, obtained by addition of phospholipid to the phospholipase C-treated preparation.

Fig. 4. Reactivation of galactosyltransferase I activity by lysophosphatidylcholine at various times during phospholipase C treatment. Tubes containing 100,000 x g particulate fraction (10 mg of protein) suspended in 1.0 ml of 0.02 M Tris-HCl, pH 7.5, containing 0.003 M CaCl₂ were incubated with 0.25 mg of phospholipase C. Controls were incubated in the absence of phospholipase C. At the times indicated, the reaction was stopped by addition of EDTA to a final concentration of 0.01 M, and the samples were centrifuged at 100,000 x g for 1 hour; the resulting pellets were resuspended in 1 ml of 0.05 M Tris-acetate, pH 5.5, containing 0.05 M KCl and 0.001 M EDTA. Aliquots (50 μl) were assayed for galactosyltransferase I activity in the presence (O—O) and absence (●—●) of 0.5 μg of lysophosphatidylcholine; control samples (50 μl) likewise were assayed in the presence (O—O) and absence (●—●) of 50 μg of the phospholipid. Results are presented as counts per min of product formed per 50 μl of sample.

emerged with the void volume along with the high molecular weight membrane components and could be inactivated approximately 50% by treatment with phospholipase C. The major portion was found in an included position and was not sensitive to the action of phospholipase C, but probably contained substantial amounts of detergent which may be substituting for lipid.

The included fraction of galactosyltransferase I has been purified further by affinity chromatography (5). Elution of the affinity matrices with 0.25 M KCl/1.0% Nonidet P-40 yielded preparations of the enzyme with specific activities approximately 1100-fold greater than that of the crude homogenate. However, these preparations were only active in the presence of detergent. As summarized in Table III, dialysis of the affinity chromatography-purified galactosyltransferase I against buffer without detergent (in the presence or absence of 0.25 M KCl) resulted in rapid inactivation of the enzyme. Reactivation was achieved, much more slowly, by subsequent dialysis against buffer containing 1% Nonidet P-40. Dialysis against buffer containing only 0.25 M KCl, did not promote reactivation.

**Table III**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Pellet</th>
<th>Supernatant</th>
<th>% total</th>
</tr>
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<tr>
<td>Xylosyltransferase</td>
<td>818</td>
<td>1,290</td>
<td>(61)</td>
</tr>
<tr>
<td>Galactosyltransferase I</td>
<td>11,102</td>
<td>4,785</td>
<td>(30)</td>
</tr>
<tr>
<td>Galactosyltransferase II</td>
<td>1,515</td>
<td>856</td>
<td>(36)</td>
</tr>
<tr>
<td>Glucuronylsytransferase I</td>
<td>1,859</td>
<td>1,931</td>
<td>(51)</td>
</tr>
<tr>
<td>N-Acetylgalactosamintransferase I</td>
<td>1,489</td>
<td>264</td>
<td>(15)</td>
</tr>
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</table>
of the enzyme. However, reactivation in the presence of detergent appeared to be facilitated by addition of a higher concentration of salt (Table III), since dialysis for 24 hours against detergent in the presence of 0.05 M KCl resulted in only 8% reactivation, whereas further dialysis for 24 hours against detergent in buffer containing 0.25 M KCl resulted in an additional 50% increase in activity.

The purified galactosyltransferase I was apparently stable for a relatively long period after removal of detergent, since it could be reactivated to nearly control levels by dialysis against detergent-containing buffer after 18 hours in the absence of detergent. Whereas dialysis against detergent-containing buffer led to complete reactivation, direct addition of Nonidet P-40 to a final concentration of 1.0% in the presence of 0.05 M KCl, 1% Nonidet P-40, 18 hr; 0.25 M KCl, 1% Nonidet P-40, 1 hr; or 1% Nonidet P-40 in the presence of 0.05 M KCl, 1 hr; or 1% Nonidet P-40 in the presence of 0.05 M KCl, 18 hr; or 0.05 M KCl, 1% Nonidet P-40, 1 hr; 0.25 M KCl, 1% Nonidet P-40, 18 hr; 0.05 M KCl, 1% Nonidet P-40, 1 hr; 0.05 M KCl, 1% Nonidet P-40, 18 hr; 0.25 M KCl, 1% Nonidet P-40, 48 hr. reactivated subsequently by the addition of phospholipid. Such indirect means of showing a phospholipid dependence have often been necessary because of the difficulties encountered in the isolation of most membrane-bound enzymes. However, it is recognized that purification of an enzyme to the extent that activation by added phospholipid can be demonstrated directly is the most conclusive evidence for a phospholipid requirement.

In view of the specificity of action of the phospholipases and the generally mild conditions under which treatment can be carried out, these enzymes are ideal agents for the selective modification of membrane structure (18). However, conclusions as to a phospholipid dependence based on the effects of phospholipases should be viewed with caution since phospholipase A produces fatty acids and lysophosphatides whose surfactant properties may inhibit enzymes nonspecifically (23). In contrast, inactivation by phospholipase C may be a more reliable indicator of a phospholipid requirement since the hydrolysis products lack surfactant properties.

In the present study evidence was obtained that degradation by phospholipase C of the phospholipids in the particulate fraction of embryonic chick cartilage results in the inactivation of one of the chondroitin sulfate glycosyltransferases, galactosyltransferase I. Although inactivation was nearly completed within 30 min of incubation, some residual activity remained stable to continued digestion even at a 10-fold higher phospholipase C concentration which may indicate that a portion of the enzyme is located in a position that is less accessible.

Phospholipase C-treated galactosyltransferase I was reactivated by the addition of phospholipid. The effect was not specific for a particular phospholipid, since several phospholipids as well as the nonionic detergent, Nonidet P-40, restored activity. Lysophosphatidylcholine, a minor lipid component of embryonic chick cartilage, was more effective than any other phospholipid. This behavior may be related to the hydrophilic nature of lysophosphatidylcholine which, at the concentration used, is present in a molecular dispersion (18). The higher concentration of phosphatidylcholine (one of the major phospholipids of cartilage) required for optimal activation may be due to its vesicular bilayer nature.

In contrast to phospholipase C, phospholipase A at low concentrations stimulated the particulate galactosyltransferase I. The activating effect may be due to the generation of small amounts of lysophosphatides which remain in the immediate vicinity of the glycosyltransferase. However, addition of lysophosphatidylcholine and lysophosphatidylethanolamine did not activate particulate galactosyltransferase I as much as did phospholipase A treatment. In a study of membrane-bound UDP-galactose-glycoprotein galactosyltransferase (lactose synthetase) in liver microsomes, lysophosphatidylcholine stimulated the activity of this enzyme 13 to 16-fold (22). While preincubation with phospholipase A also stimulated this enzyme, the effect of phospholipase A was enhanced further in the presence of lysophosphatidylcholine. Based on these findings, it was suggested that fluctuation in lysophosphatidylcholine concentration in the immediate environment of the enzyme, caused by endogenous phospholipase A and acyltransferase activities, may influence the activity of this microsomal galactosyltransferase in vivo (22).

The response to phospholipase C of the chondroitin sulfate glycosyltransferases other than galactosyltransferase I indicates that these enzymes vary with respect to their dependence upon the presence of intact membrane phospholipids. Under

### Table III

**Reversible inactivation of galactosyltransferase I**

A preparation of galactosyltransferase I obtained by affinity chromatography on a column of xylosyltransferase-Sepharose (specific activity 6.7 × 10⁶ cpm of product formed/mg of protein) in 0.05 M Mes buffer, pH 6.5, containing 0.25 M KCl, 0.012 M MgCl₂, 0.000 M MnCl₂, and 1% Nonidet P-40 was treated as follows: the enzyme solution (3 ml) was dialyzed against 100 ml of 0.05 M Mes buffer, pH 6.5, containing 0.05 M KCl for 1 (Treatment 2) or 18 (Treatment 4) hours; 1-ml fractions were dialyzed subsequently against the same buffer containing 1% Nonidet P-40 in the presence (Treatments 3, 6, or 7) or absence (Treatment 5) of 0.25 M KCl. At the times indicated aliquots (50 μl) were assayed for galactosyltransferase I activity under standard conditions. Results are presented as counts per min of product formed per 50 μl of enzyme sample and per cent of original activity recovered after each treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Galactosyltransferase activity</th>
<th>Recovery</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>cpm</td>
<td>%</td>
</tr>
<tr>
<td>1 None</td>
<td>1175</td>
<td>100</td>
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<tr>
<td>Dialysis against buffer containing:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. 0.05 M KCl, 1 hr</td>
<td>448</td>
<td>38</td>
</tr>
<tr>
<td>3. 0.05 M KCl, 1 hr; 0.25 M KCl, 1% Nonidet P-40, 18 hr</td>
<td>1281</td>
<td>109</td>
</tr>
<tr>
<td>4. 0.05 M KCl, 18 hr; 0.05 M KCl, 1% Nonidet P-40, 24 hr</td>
<td>185</td>
<td>16</td>
</tr>
<tr>
<td>5. 0.05 M KCl, 18 hr; 0.25 M KCl, 1% Nonidet P-40, 24 hr</td>
<td>296</td>
<td>24</td>
</tr>
<tr>
<td>6. 0.05 M KCl, 18 hr; 0.05 M KCl, 1% Nonidet P-40, 24 hr</td>
<td>875</td>
<td>74</td>
</tr>
<tr>
<td>7. 0.05 M KCl, 18 hr; 0.25 M KCl, 1% Nonidet P-40, 48 hr</td>
<td>1014</td>
<td>86</td>
</tr>
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</table>
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conditions where galactosyltransferase I was inactivated substantially by phospholipase C treatment, xylosyltransferase was inactivated moderately but was not reactivated by the addition of phospholipid. Two other glycosyltransferases were relatively unaffected by treatment with phospholipase C, indicating that they are largely insensitive to hydrolysis of the phospholipase C-accessible membrane phospholipids. Variability in the response to phospholipase C treatment of several enzymes which are all part of the cytoplasmic membrane of Escherichia coli has been reported (23). However, the enzymes measured in this study catalyzed diverse reactions and, therefore, might not be expected to be in close proximity to each other on the membrane. In contrast, the chondroitin sulfate glycosyltransferases catalyze a sequential series of reactions, and, for kinetic efficiency, may be expected to be arranged on the membrane in apposition to each other (3, 5, 24, 25), yet a heterogeneity in their response to phospholipid digestion can still be shown.

As mentioned previously, purification of an enzyme to the extent that activation by phospholipids can be shown, is the most definitive evidence for a phospholipid requirement. Therefore, the dependence of the activity of galactosyltransferase I on a phospholipid environment was shown both by sensitivity of the enzyme to perturbation of its phospholipid environment in the membrane-bound state as well as a requirement for detergent after solubilization and partial purification (5). The highly purified preparations of galactosyltransferase I appear to have a requirement for detergent after solubilization and partial purification (5). The highly purified preparations of galactosyltransferase I appear to have a requirement for detergent after solubilization and partial purification (5). The highly purified preparations of galactosyltransferase I appear to have a requirement for detergent after solubilization and partial purification (5). The highly purified preparations of galactosyltransferase I appear to have a requirement for detergent after solubilization and partial purification (5). The highly purified preparations of galactosyltransferase I appear to have a requirement for detergent after solubilization and partial purification (5). The highly purified preparations of galactosyltransferase I appear to have a requirement for detergent after solubilization and partial purification (5). The highly purified preparations of galactosyltransferase I appear to have a requirement for detergent after solubilization and partial purification (5). The highly purified preparations of galactosyltransferase I appear to have a requirement for detergent after solubilization and partial purification (5). The highly purified preparations of galactosyltransferase I appear to have a requirement for detergent after solubilization and partial purification (5).

In addition to a dependence of this particular glycosyltransferase on a hydrophobic environment for maintenance of its activity, specific interactions between galactosyltransferase I and neighboring proteins or lipids may also alter its mode of action in situ in the intact membrane and thus contribute to the regulation of chondroitin sulfate biosynthesis. In this respect, a specific interaction between galactosyltransferase I and xylosyltransferase has been shown which is prevented in the presence of detergent. If allowed to occur, the complex is dissociated by detergent at elevated ionic strength (24, 25) indicating that hydrophobic, as well as ionic forces participate in this interaction.

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