Initiation of chondroitin sulphate synthesis by β -D-galactosides

Substrates for galactosyltransferase II

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(Received 3 October 1984/17 December 1984; accepted 16 January 1985)

β -Galactosides were found to initiate chondroitin sulphate chain synthesis in chick-embryo cartilage *in vitro* and thereby relieve inhibition by cycloheximide of [3 H]-acetate incorporation into chondroitin sulphate. β -Galactosides with an apolar aglycan group such as phenyl *O*- β -galactoside were active, whereas those with a charged or polar aglycan group such as pyridine 3-*O*- β -galactoside or those with sulphur instead of oxygen in the glycosidic linkage (phenyl β -thiogalactoside) were not. β -Galactosides also serve as substrates for microsomal galactosyltransferase activity from chick-embryo cartilage. Phenyl *O*- β -galactoside and pyridine 3-*O*- β -galactoside were effective substrates for this enzyme, but phenyl *S*- β -thiogalactoside and pyridine 2-*S*- β -thiogalactoside were only slightly active. This galactosyltransferase was shown to be a separate enzyme from galactosyltransferase I, which catalyses transfer of galactose from UDP-galactose to β -xylosides. It is proposed that the enzyme catalysing this reaction is galactosyltransferase II, responsible for transfer of the second galactose residue of the chondroitin sulphate linkage oligosaccharide. No transfer of glucuronic acid from UDP-glucuronic acid to β -galactosides, catalysed by the microsomal preparation could be detected.

β -Xylosides initiate chondroitin sulphate chain synthesis in chondrocytes (Robinson *et al.*, 1975) by serving as substrates for galactosyltransferase I (Rodén, 1980), the second glycosyltransferase involved in chondroitin sulphate chain synthesis (Robinson & Robinson, 1981). The β -xyloside is thus incorporated into the reducing end of the free chondroitin sulphate chain (Fukunaga *et al.*, 1975; Sandy *et al.*, 1980).

β -Galactosides also initiate chondroitin sulphate synthesis in chondrocytes (Robinson *et al.*, 1975), but are less effective than β -xylosides, and the mechanism of their action is unknown. β -Galactosides do not inhibit galactose transfer from UDP-galactose to β -xyloside (Robinson & Robinson, 1981), so that they are unlikely to be substrates for galactosyltransferase I. They might, however, serve as substrates for galactosyltransferase II or for glucuronosyltransferase I, respectively the third and fourth glycosyltransferases involved in chondroitin sulphate biosynthesis (Rodén, 1980). Both of these enzymes are known to catalyse transfer of a monosaccharide residue from the appropriate nucleotide sugar to an oligosaccharide

acceptor with a non-reducing terminal galactose residue (Helting & Rodén, 1969*a,b*; Brandt *et al.*, 1975).

The experiments described in the present paper indicate that the β -galactosides can serve as acceptors for galactosyltransferase II. A preliminary report of these findings has been published (Robinson & Robinson, 1980).

Experimental

Materials

Penta-acetylgalactose (1,2,3,4,6-penta-*O*-aceto- β -galactose) and acetobromogalactose (2,3,4,6-tetra-*O*-aceto- α -galactopyranosyl bromide) were synthesized as described by Westerfield (1966). Pyridine 3-*O*- β -galactoside was prepared by allowing 3.0 g of 3-hydroxypyridine, dissolved in 30 ml of 1.3 M-NaOH, to react with 8.22 g of acetobromogalactose, dissolved in 50 ml of acetone, at room temperature for 5 h (Conchie & Levvy, 1963). The reaction mixture was evaporated to dryness and treated with sodium methoxide in dry methanol as described by Thompson *et al.* (1963). The product (1.08 g) was isolated by ion-exchange chromato-

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graphy on a column (50cm $\times$ 2.0cm) of Dowex 50 (X4; 200–400 mesh; pyridinium form) eluted with a gradient formed from 250ml of 0.1M-formic acid and 250ml of 2.0M-pyridine in 3.0M-formic acid. Tyrosine *O*- β -galactoside was prepared in the same way by reaction of tyrosine (9.05g) with acetobromogalactose (14g) (Conchie & Levvy, 1963). The product (5.0g) was isolated on a column (25cm $\times$ 1.0cm) of Dowex 1 (X4; 200–400 mesh; formate form) eluted with a gradient of formic acid (0–1.0M).

Pyridine 2-*S*- β -thiogalactoside was prepared from 2-mercaptopyridine and acetobromogalactose by the method of Purves (1929). Pyridine 2- β -thiolactoside was prepared by a four-step procedure. Lactose octa-acetate was synthesized from lactose and acetic anhydride in anhydrous pyridine (Hudson & Kunz, 1925), and hepta-acetyl-lactosyl bromide was then formed by the method of Purves (1929). Pyridine 2-*S*-hepta-acetyl-lactoside was prepared by allowing 15g of hepta-acetyl-lactosyl bromide in 30ml of chloroform to react with a mixture of 2-thiopyridine (3.0g) and KOH (1.12g) dissolved in 30ml of methanol. After 16h at 37°C, the product was extracted into chloroform, evaporated to a small volume and crystallized from diethyl ether. Pyridine 2- β -thiolactoside (6.6g) was isolated after deacetylation (Thompson *et al.*, 1963). Benzyl *O*- β -xyloside and pyridine 2-*S*- β -thioxyloside were prepared as described previously (Robinson & Robinson, 1981).

o-Aminophenyl *O*- β -galactoside and *p*-aminophenyl *O*- β -galactoside were gifts from Dr. M. Jermyn (C.S.I.R.O. Division of Protein Chemistry, Melbourne, Vic., Australia). Galactosyl- β (1–4)-xylosyl- β (1–)*O*-serine (Gal-Xyl-Ser) and galactosyl- β (1–3)-galactosyl- β (1–4)-xylosyl- β (1–)*O*-serine (Gal-Gal-Xyl-Ser) were gifts from Dr. L. Rodén (University of Alabama, Birmingham, AL, U.S.A.).

Phenyl *O*- β -[6- 3 H]galactoside and pyridine 3-*O*- β -[6- 3 H]galactoside were prepared from the unlabelled galactosides by oxidation with galactose oxidase and reduction with NaB 3 H $_4$ (Nelsestuen & Kirkwood, 1971). Each galactoside (0.5mmol) was incubated at room temperature for 24h in 2.0ml of 0.05M-glycine/NaOH buffer, pH 8.4, containing galactose oxidase (45 units) and catalase (8.0mg). Then 50mCi of NaB 3 H $_4$ (6.25 μ mol) in 0.2ml of 0.1M-NaOH was added to each reaction mixture, which was left at room temperature for 2h. Finally, 0.2ml of 1.0M-NaBH $_4$ in 0.1M-NaOH was added, and after 1h at room temperature each reaction mixture was neutralized by the addition of 0.05ml of acetic acid. Phenyl *O*- β -[3 H]galactoside was purified on a column (36cm $\times$ 1.7cm) of Dowex 1 (X4; 200–400 mesh; borate form) eluted with a linear gradient formed from 250ml of 0.1M-boric

acid/NaOH buffer, pH 7.0, and 250ml of 0.2M-Na $_2$ SO $_4$ in 0.4M-boric acid/NaOH buffer, pH 7.0; elution then continued with the latter buffer. A large hexose-positive peak, which coincided with a peak of 3 H label, was pooled and desalted by passage through short columns of Dowex 50 (H $^+$ form) and Dowex 1 (acetate form). The material was then evaporated to dryness from methanol solution several times to remove boric acid, and finally dissolved in 10ml of water. The yield of phenyl β -[3 H]galactoside was 0.32mmol with a specific radioactivity of 82mCi/mmol. Analysis by paper chromatography in solvent *D* showed that 10% of the radioactivity in the final preparation was present as free [3 H]galactose.

Pyridine 3-*O*- β -[3 H]galactoside was purified on a column (60cm $\times$ 2.2cm) of Dowex 50W (X2; 100–200 mesh; pyridinium form) eluted with a linear gradient formed from 250ml of 0.2M-acetic acid and 250ml of 2.0M-pyridine in 0.25M-acetic acid. The main 3 H-labelled and hexose-positive peak was pooled, evaporated to dryness and dissolved in distilled water to give 40mM-pyridine β -[3 H]galactoside with a specific radioactivity of 96.5mCi/mmol; paper chromatography in solvent *A* showed a negligible content of free [3 H]-galactose.

D-Galactose, phenyl *O*- β -galactoside, phenethyl *O*- β -galactoside, phenyl β -thiogalactoside, *p*-nitrophenyl *O*- β -galactoside, cycloheximide, Nonidet P-40, *N*-acetylglucosamine, Mes, UDP-galactose, UDP-glucuronic acid, galactose oxidase, and Amberlite CG-120 were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. β -Galactosidase (*Escherichia coli*) and crystalline catalase were from Boehringer Mannheim (Mannheim, Germany).

Sodium [3 H]acetate, NaB 3 H $_4$, UDP-[6- 3 H]-galactose, UDP-[U- 14 C]galactose and UDP-[U- 14 C]glucuronic acid were purchased from Amersham (Australia), Sydney, N.S.W., Australia. Dowex resins and Bio-Gel P-2 and P-4 were from Bio-Rad Laboratories, Richmond, CA, U.S.A., and DEAE-cellulose (DE 52) was from Whatman Biochemicals, Maidstone, Kent, U.K.

General methods

Hexuronate was determined by the carbazole method (Bitter & Muir, 1962), and hexose by the phenol/H $_2$ SO $_4$ method of Dubois *et al.* (1956). Descending paper chromatography was carried out on Whatman 3MM paper in the following solvents: *A*, propan-2-ol/water (4:1, v/v); *B*, ethyl acetate/acetic acid/water (3:1:1, by vol.); *C*, ethyl acetate/pyridine/water (12:3:5, by vol.); *D*, butan-1-ol/acetic acid/water (12:3:5, by vol.); *E*, butan-1-ol/pyridine/water (1:1:1, by vol.). Sugars and glycosides were detected with alkaline AgNO $_3$.

Autoradiography was with Kodirex RP-54 X-ray film for 1–2 weeks' exposure.

Chondroitin sulphate synthesis in intact cartilage

Chick-embryo bones were incubated for 2 h with [^3H]acetate and radioactively labelled chondroitin sulphate was isolated by the procedures described previously (Robinson & Robinson, 1981). Cycloheximide and β -galactosides were added to appropriate incubations 20 min before [^3H]acetate.

Preparation of microsomal enzyme

Femurs and tibiae dissected from 30–40 chick embryos (14 days old) were homogenized in an ice-cold solution composed (final concentrations) of 62.5 mM-Mes, 0.5 M-KCl, 15 mM-MgCl₂ and 1.25 mM-EDTA, all adjusted to pH 6.0 with NaOH. The microsomal fraction was isolated as described previously (Robinson & Robinson, 1981) and resuspended in the same buffered solution at a concentration of 1.0–3.0 mg of protein/ml.

Assay of glycosyltransferase activity

(i) UDP-galactose:D-xyloside galactosyltransferase activity was assayed by incubation of the microsomal preparation (50 μg of protein) for 4 h at 37°C with (final concentrations) 20 mM-pyridine 2- β -thioxyloside, 50 mM-Mes/NaOH buffer, pH 6.0, 0.4 M-KCl, 12 mM-MgCl₂, 10 mM-MnCl₂, 1.0 mM-EDTA and 1.25 mM-UDP-[^3H]galactose (1.98 mCi/mmol) in a final volume of 0.05 ml. The reaction was stopped by addition of 0.5 ml of 6% (w/v) trichloroacetic acid, and the radioactively labelled products were isolated on Amberlite CG-120 (100–200 mesh; H⁺ form) as described previously (Robinson & Robinson, 1981).

(ii) UDP-galactose:galactoside galactosyltransferase activity was assayed by incubation of the microsomal enzyme (50–150 μg of protein) for 6–8 h at 37°C with (final concentrations) β -galactoside (0–50 mM), 50 mM-Mes/NaOH buffer, pH 6.0, 0.4 M-KCl, 12 mM-MgCl₂, 10 mM-MnCl₂, 1.0 mM-EDTA and 1.25 mM-UDP-[^3H]galactose (1.98 mCi/mmol) in a final volume of 0.05 ml. The reaction was stopped by addition of 0.5 ml of 6% trichloroacetic acid, and the radioactively labelled products were isolated on Amberlite CG-120. When phenyl β -galactoside was the substrate, 0.46 mM-UDP-[^{14}C]galactose (4.93 mCi/mmol) was used and the reaction was stopped by addition of 0.05 ml of propan-2-ol; the ^{14}C -labelled products were separated by paper chromatography in solvent B, detected by autoradiography (Robinson & Robinson, 1981) and assayed for radioactivity.

(iii) UDP-galactose:N-acetylglucosamine galactosyltransferase activity (Helting, 1971) was assayed by incubation of the microsomal enzyme for 6 h at 37°C with (final concentrations) 20 mM-N-

acetylglucosamine, 50 mM-Mes/NaOH buffer, pH 6.0, 0.4 mM-KCl, 12 mM-MgCl₂, 10 mM-MnCl₂, 1.0 mM-EDTA and 5.0 mM-UDP-[^3H]galactose (1 mCi/mmol) in a total volume of 0.05 ml. The reaction was stopped by addition of 0.05 ml of propan-2-ol, and the radioactively labelled products were separated by paper chromatography in solvent A. The paper sheet was cut into 1 cm sections, which were analysed for ^3H radioactivity.

(iv) Glucuronosyltransferase activity was assayed by incubation of the microsomal preparation (100 μg of protein) for 6–8 h at 37°C with (final concentrations) acceptor glycoside (0–50 mM), 50 mM-Mes/NaOH buffer, pH 6.0, 0.4 M-KCl, 12 mM-MgCl₂, 20 mM-MnCl₂, 15 mM-KF, 1.0 mM-EDTA, 5 mM-UDP-galactose (in some experiments) and 0.25 mM-UDP-[^{14}C]glucuronic acid (19.5 mCi/mmol) in a total volume of 0.05 ml. These conditions were based on the method of Rodén *et al.* (1972). Reaction products were isolated on Amberlite CG-120 or, when phenyl β -D-galactoside was the substrate, by paper electrophoresis in 0.5 M-pyridine/0.046 M-acetic acid buffer, pH 6.3.

Mixed-substrate experiments were carried out by adding 0–30 mM-phenyl O- β -galactoside or 0–30 mM-benzyl O- β -xyloside to standard incubations with pyridine 3-O- β -galactoside. Transfer of [^3H]galactose to the pyridine derivative alone was measured by using Amberlite CG-120 (Robinson & Robinson, 1981). In a separate series of mixed-substrate experiments, 0–30 mM-pyridine 3-O- β -galactoside was incubated with UDP-[^3H]galactose under the standard conditions in the absence and in the presence of 5 mM- or 10 mM-Gal-Xyl-Ser. Total transfer of galactose to both substrates was determined by adsorption of the products on Amberlite CG-50 and elution with 2 M-ammonia. Partial separation of the products was achieved by passage over small columns of Dowex 1 (X4; CO₃²⁻ form) in 0.4 M-ammonia. The labelled product formed with pyridine 3-O- β -galactoside passed through this resin and was recovered in 90% yield, whereas the labelled serine derivative was absorbed and could be recovered in 50–60% yield by elution with 1.0 M-ammonium formate. Transfer of [^3H]galactose to each acceptor could thus be measured in the one incubation. The two products were also separated by paper chromatography in solvent E; the labelled product formed from pyridine 3-O- β -galactoside ($R_{\text{G:al}} = 1.31$) was well separated from Gal-Gal-Xyl-Ser ($R_{\text{G:al}} = 0.69$).

Characterization of the product of galactose transfer to pyridine β -galactoside

Microsomal enzyme (315 μg) was incubated for 24 h at 37°C with 11.3 mM-pyridine β -[^3H]-

galactoside (96.5 mCi/mmol) and 2 mM-UDP-[^{14}C]galactose (4.93 mCi/mmol) in a total volume of 0.2 ml. The incubation was stopped by addition of 1.0 ml of 6% trichloroacetic acid, and after centrifugation the reaction products and unchanged pyridine β -galactoside were isolated from the supernatant on Amberlite CG-120 and applied to a column (60 cm $\times$ 2 cm) of Dowex 50 (X8; 200–400 mesh; pyridinium form) in 0.1 M-acetic acid. The column was eluted with a linear gradient generated from 250 ml of 0.1 M-acetic acid and 250 ml of 2.0 M-pyridine in 2.5 M-acetic acid. Each peak of ^3H radioactivity was subjected to paper chromatography in solvent A together with pyridine 3-*O*- β -galactoside, pyridine 2- β -thiolactoside and D-galactose standards. The ^3H - and ^{14}C -labelled product of galactose transfer to pyridine 3-*O*- β -galactoside was eluted in fractions 22–24 together with another ^3H -labelled compound. These were separated by preparative paper chromatography in solvent A or by gel filtration on a column (95 cm $\times$ 13 cm) of Bio-Gel P-2 in aq. 10% (v/v) ethanol.

A sample of the galactose transfer product was heated at 100°C for 2 h in 1.0 M-HCl, and the ^{14}C -labelled compounds in the hydrolysate were subjected to paper chromatography in solvent A. Another sample of the product was treated with β -galactosidase in 0.01 M-Tris/HCl buffer, pH 7.2, for 5 h, and the digest mixture was analysed by paper chromatography in solvent C.

Separation of galactosyltransferase activities by ion-exchange chromatography on DEAE-cellulose

A microsomal fraction from chick-embryo epiphyseal cartilage was suspended in 5.0 ml of 0.05 M-Mes/NaOH buffer, pH 6.0, containing 1.0 mM-EDTA and 0.5% (w/v) Nonidet P-40. This was applied to a column (20 cm $\times$ 1.3 cm) of DEAE-cellulose equilibrated with the same solution. The column was eluted with a linear gradient of KCl generated from 250 ml of the Mes buffer and 250 ml of 0.05 M-KCl in the Mes buffer at 4°C. Fractions (10 ml) were collected and assayed for galactosyltransferase activity with 20 mM-pyridine 2- β -thioxyloside, 20 mM-pyridine 3-*O*- β -galactoside and 20 mM-*N*-acetylglucosamine as described above.

Results and discussion

Chondroitin sulphate synthesis initiated by β -galactosides

A number of β -galactosides were tested for their ability to initiate chondroitin sulphate chain synthesis on chondrocytes. They were phenyl *O*- β -galactoside, phenyl *S*- β -thiogalactoside, *p*-nitrophenyl *O*- β -galactoside, phenethyl *O*- β -galactoside, pyridine 3-*O*- β -galactoside, tyrosine *O*- β -

galactoside, *o*-aminophenyl *O*- β -galactoside, *p*-aminophenyl *O*- β -galactoside, pyridine 2-*S*- β -thiogalactoside and pyridine 2-*S*- β -thiolactoside. Chick-embryo epiphyseal cartilage was incubated with 0.1 mM-cycloheximide, and the ability of the β -galactosides to relieve the inhibition of [^3H]acetate incorporation into chondroitin sulphate caused by cycloheximide was measured (Robinson & Robinson, 1981). Only phenyl β -galactoside, phenethyl β -galactoside and *p*-nitrophenyl β -galactoside (Fig. 1) were able to initiate chondroitin sulphate chain synthesis; all the other were inactive.

The concentration of β -galactoside necessary to restore [^3H]acetate incorporation into chondroitin sulphate to the extent measured in the absence of cycloheximide (phenyl β -galactoside, 15 mM; phenethyl β -galactoside and *p*-nitrophenyl β -galactoside, >20 mM) were all much higher than the corresponding values for β -xylosides (benzyl β -xyloside, 0.05 mM), thus confirming previous reports that β -galactosides are poor initiators of chondroitin sulphate synthesis (Okayama *et al.*, 1973; Galligani *et al.*, 1975).

Fig. 1. Effect of β -galactoside on [^3H]acetate incorporation into chondroitin sulphate

Chick-embryo cartilage was incubated for 2 h with [^3H]acetate, 0.2 mM-cycloheximide and with phenyl *O*- β -galactoside (●), *p*-nitrophenyl *O*- β -galactoside (■) or phenethyl *O*- β -galactoside (○). ^3H incorporation is expressed as a percentage of that in an incubation without cycloheximide or β -galactoside (equivalent to 10.2 nmol of acetate/h per mg of tissue hexuronate).

There are several possible reasons why D-galactose (Robinson *et al.*, 1975) and some β -galactosides fail to initiate chondroitin sulphate synthesis in intact chondrocytes. A polar or charged aglycan residue might be a crucial factor, since β -xylosides with charged aglycan groups are less effective than those with non-polar aglycan groups (Robinson & Robinson, 1981). Replacement of oxygen by sulphur in phenyl β -S-thiogalactoside also resulted in complete loss of activity. It seems likely that factors such as a low rate of transport of the β -galactoside into the chondrocyte, a poor affinity of the β -galactosides for the appropriate glycosyltransferases and possibly a high rate of intracellular hydrolysis by β -galactosidases could all serve to make some β -galactoside derivatives ineffective in intact cells.

Glucuronic acid transfer to β -galactosides

Pyridine 3-*O*- β -galactoside, pyridine 2-*S*- β -thiolactoside and phenyl *O*- β -galactoside were all tested for glucuronosyl-acceptor activity by incubation with UDP-[14 C]glucuronic acid and microsomal enzyme. Incubation mixtures contained either UDP-[14 C]glucuronic acid alone or UDP-galactose and UDP-[14 C]glucuronic acid together. No incorporation of radioactivity into product could be detected with any of the substrate combinations tested, even with an acceptor concentration as high as 50 mM.

Previous studies, however, have demonstrated glucuronosyl transfer to D-galactose and β -galactosides by cell-free preparations (Helting & Rodén, 1969b; Brandt *et al.*, 1975), but discrepancies in substrate specificity have been reported for preparations from different tissues such as chick cartilage, chick brain and mouse mastocytoma (Rodén *et al.*, 1972). The chick cartilage enzyme is reported to catalyse glucuronosyl transfer to D-galactose and to lactose, but at rates far slower than to the appropriate substrate 3-*O*- β -D-galactosyl-D-galactose. It is therefore conceivable that the galactosides tested in the present study were substrates, but poor ones, for glucuronosyltransferase. The conditions of assay used were those published by Rodén *et al.* (1972), except that the concentration of KCl was 0.4 M instead of 0.15 M, a difference unlikely to prevent glucuronosyl transfer. Nevertheless, the failure to detect glucuronosyl transfer under the conditions used in the present study cannot eliminate the possibility that phenyl β -galactoside may initiate chondroitin sulphate chains by serving as a substrate for glucuronosyltransferase I.

Galactose transfer to β -galactosides

Transfer of galactose from UDP-galactose to a number of β -galactosides was catalysed by a

microsomal preparation from chick-embryo cartilage. Each galactoside was incubated with microsomal enzyme and UDP-[3 H]galactose for 2–8 h, and the radioactive products were isolated and assayed for radioactivity. Table 1 shows that the rate of galactose transfer to the various β -galactosides was much lower than the rate of transfer to β -xylosides and that pyridine 3-*O*- β -galactoside was nevertheless an active and convenient substrate for galactose transfer. The corresponding thio derivative, pyridine 2-*S*- β -thiogalactoside, was far less active (Fig. 2), suggesting that, in contrast with galactosyl transfer to β -xylosides, the enzyme catalysing galactosyl transfer to β -galactosides is sensitive to a change in the atom involved in the β -glycosidic bond of the acceptor. No transferase activity could be detected when D-galactose was used as acceptor.

Galactosyl transfer to the disaccharide derivative Gal β 1-4Xylp1-*O*-serine (Gal-Xyl-Ser) is also shown in Table 1 and Fig. 2. This acceptor, which is a characteristic substrate for galactosyltransferase II and is not a substrate for galactosyltransferase I (Helting & Rodén, 1969a), was not as effective an acceptor as pyridine 3-*O*- β -galactoside, but was considerably better than pyridine 2-*S*- β -thiolactoside (Gal β 1-4Glcpl-2-*S*-pyridine).

Characterization of the product of galactose transfer to pyridine β -galactoside

Microsomal enzyme was incubated with

Table 1. Galactose transfer from UDP-galactose to β -glycosides catalysed by a microsomal preparation from chicken-embryo cartilage

Each substrate (20 mM) was incubated for 6 h at 37°C with 1.25 mM-UDP-[3 H]galactose or UDP-[14 C]-galactose under standard conditions, and the labelled products of galactose transfer were isolated as outlined in the Experimental section. Values for pyridine 2-*S*- β -thioxyloside and pyridine 3-*O*- β -galactoside are means $\pm$ s.d. with the numbers of separate determinations in parentheses.

Substrate	Galactose transfer (nmol/h per mg of protein)
Benzyl <i>O</i> - β -xyloside	9.5
Pyridine 2- <i>S</i> - β -thioxyloside	7.0 $\pm$ 1.4 (7)
Phenyl <i>O</i> - β -galactoside	1.5
Phenyl <i>S</i> - β -thiogalactoside	<0.2
<i>p</i> -Aminophenyl <i>O</i> - β -galactoside	0.56
<i>o</i> -Aminophenyl <i>O</i> - β -galactoside	0.26
Tyrosine <i>O</i> - β -galactoside	1.1
Pyridine 3- <i>O</i> - β -galactoside	2.2 $\pm$ 0.8 (8)
Pyridine 2- <i>S</i> - β -thiogalactoside	0.34
Pyridine 2- <i>S</i> - β -thiolactoside	0.52
Gal-Xyl-Ser (Gal β 1-4Xylp1- <i>O</i> -Ser)	1.5

Fig. 2. Effect of substrate concentration on galactosyl-transferase activity

[^3H]Galactose transfer to pyridine 2-S- β -thioxyloside (●), to pyridine 3-O- β -galactoside (○), to pyridine 2-S- β -thiogalactoside (■) and to Gal-Xyl-Ser (□) was catalysed by a microsomal preparation from chick-embryo cartilage as outlined in the Experimental section. Incubation time was 6 h.

11.3 mM-pyridine 3-O- β -[6- ^3H]galactoside and 2.0 mM-UDP-[^{14}C]galactose for 24 h. Reaction products were isolated by using Amberlite CG-120 and then separated from unchanged pyridine 3-O- β -[6- ^3H]galactoside by ion-exchange chromatography on Dowex 50. Two main peaks of ^3H radioactivity were eluted from this Dowex 50 column (Fig. 3), each of which was analysed further by paper chromatography in solvent A. The second peak, D-2, contained only unchanged pyridine 3-O- β -[6- ^3H]galactoside (R_{Gal} 1.40) and was not labelled with ^{14}C , whereas the first peak, D-1, was resolved into two ^3H -labelled components. The major component (D1-2; R_{Gal} 0.65) accounted for 21% of the total ^3H label in the incubation and contained no ^{14}C , whereas the minor component (D1-1; R_{Gal} 0.87), which accounted for just 3% of the total ^3H label, also contained ^{14}C . The latter component, D1-1, which was eluted during gel

Fig. 3. Ion-exchange chromatography of products of galactose transfer to pyridine 3-O- β -galactoside. Microsomal enzyme was incubated with pyridine 3-O- β -[6- ^3H]galactoside and UDP-[^{14}C]galactose for 24 h. Cationic products, isolated on Amberlite CG-120, were fractionated on Dowex 50 as outlined in the Experimental section. Fractions (10 ml) were assayed for ^3H radioactivity.

filtration on Bio-Gel P-2 ahead of and separate from component D1-2, had a calculated molar ratio of [^{14}C]galactose to [^3H]galactose of 1.11:1.00, and migrated during paper chromatography in solvent A just behind standard pyridine 2-S- β -thiolactoside (R_{Gal} 0.94). Exhaustive digestion of component D1-1 with β -galactosidase yielded galactose (labelled with ^3H and ^{14}C) as the major radioactive product; it behaved, therefore, as the expected product of transfer of a single residue of galactose from UDP-galactose to the acceptor. The nature of the β -galactosidic linkage was not established; it is, however, presumed to be β (1-3), since in an analogous incubation the labelled product of galactose transfer to Gal-Xyl-Ser migrated during paper chromatography in solvent E to the same position (R_{Gal} 0.67) as standard Gal-Gal-Xyl-Ser (Gal β 1-3Gal β 1-4Xyl β 1-O-serine). The precise structure of the linkage, however, must remain uncertain, since paper chromatography might not separate the different structural isomers of the product.

The major component D1-2 from peak D-1 was formed in similar amounts when pyridine 3-O- β -[6- ^3H]galactoside was incubated with microsomal enzyme in the absence of UDP-galactose. Its rate of synthesis was linearly proportional to incubation time for 8 h and was independent of Mn^{2+}

concentration. In contrast, Mn^{2+} was found to be essential for maximal galactosyltransferase activity. The compound D1-2 was degraded with β -galactosidase to yield free [3H]galactose and a fluorescent compound, which was not 3-hydroxypyridine. We conclude from these findings that compound D1-2 arose not from galactosyl transfer but from enzymic modification of the pyridine ring in the substrate, possibly by microsomal oxygenase (Hayaishi, 1969). A similar unidentified product was formed, in addition to the expected product of galactose transfer, when phenyl *O*- β -[6- 3H]galactoside was incubated with the microsomal preparation and UDP-[^{14}C]galactose in the same way.

Properties of the galactosyltransferase activity

Pyridine 3-*O*- β -galactoside was used for routine assay of galactosyltransferase activity since the product could be isolated by a simple ion-exchange technique. The transfer of [3H]galactose to this substrate was linear with time for 8 h and continued, but at a lower rate, for up to 24 h. Under the standard conditions of incubation, less than 5% of the UDP-[3H]galactose was converted into product after 24 h. An incubation time of 6 h was selected for routine experiments, since it was within the linear part of the time course. The rate of reaction was also directly proportional to the amount of enzyme used over the range 0–3.0 mg of protein/ml. The effects of pyridine 3-*O*- β -galactoside concentration and of UDP-[3H]galactose concentration on [3H]galactose transfer are illustrated in Figs. 2 and 4; the apparent K_m value for the β -galactoside acceptor, determined from a double-reciprocal plot, was >100 mM, whereas the apparent K_m value for UDP-galactose was 1.05 mM, a value 3-fold higher than that previously reported for galactosyltransferase II (Helting & Rodén, 1969a; Rodén *et al.*, 1972; Brandt *et al.*, 1975). An apparent K_m value of 3.75 mM for UDP-galactose was also calculated from the data shown in Fig. 4 for galactose transfer to pyridine 2-*S*-thioxyloside, and this was almost 40-fold higher than values previously reported for galactosyltransferase I (Helting & Rodén, 1969a).

The effect of pH on galactosyl transfer to phenyl *O*- β -galactoside and to pyridine 3-*O*- β -galactoside was measured in 50 mM-Mes/NaOH buffer over the range pH 5–8. The optimum pH with each acceptor was 6.0, but the enzyme activity did not change markedly over the entire range. The enzymic activity was shown to be insensitive to changes in KCl concentration between 0.05 M and 1.0 M and was not affected by addition of Nonidet P-40 (0.4%) to the incubation mixture. In this respect it differs from galactosyltransferase I

Fig. 4. Effect of UDP-galactose concentration on galactose transfer to pyridine 3-*O*- β -galactoside and pyridine 2-*S*- β -thioxyloside

Pyridine 3-*O*- β -galactoside (20 mM) (○) and pyridine 2-*S*- β -thioxyloside (20 mM) (●) were incubated for 6 h at 37°C with microsomal galactosyltransferase (2.0 mg/ml) under standard incubation conditions. Products were isolated on Amberlite CG-120 as outlined in the Experimental section.

(Rodén *et al.*, 1972; Robinson & Robinson, 1981). Assays were routinely carried out in a solution comprising (final concentrations) 0.4 M-KCl, 50 mM-Mes/NaOH buffer, pH 6.0, 12 mM-MgCl₂, 10 mM-MnCl₂ and 1.0 mM-EDTA. The enzymic activity in the microsomal preparation was stable to storage at -20°C for at least 12 months.

During the course of these experiments, it was noticed that the microsomal preparation contained considerable β -galactosidase activity. When the microsomal preparation was incubated with 0.64 mM-pyridine 3-*O*- β -[6- 3H]galactoside (96.5 mCi/mmol), [3H]galactose was released at a rate (about 50 nmol/h per mg of protein) much greater than the measured rate of galactose transfer from UDP-galactose to pyridine 3-*O*- β -galactoside (2.2 nmol/h per mg of protein). If the β -galactoside substrate and the product of galactose transfer were equally susceptible to hydrolysis by the β -galactosidase activity, it can be calculated that about 5–10% of the product might be destroyed during a 6 h incubation with 20 mM-pyridine 3-*O*- β -galactoside; it is conceivable, however, that the loss could be much greater than this. Nevertheless, the linear time course of the reaction and the linear proportionality between acceptor concentration and galactose transfer (Fig. 2), particularly at low acceptor concentration, suggest that hydrolysis of the product is not a serious source of error.

The effect of heat treatment on the galactosyltransferase activity is shown in Fig. 5. The microsomal preparation was heated at 50°C and at 60°C for periods up to 60 min and then cooled and assayed for activity in the standard way. Transferase activity to 20 mM-pyridine 3-*O*- β -galactoside was rather more stable to heat than that to 20 mM-pyridine 2-*S*- β -xyloside, suggesting that different

Fig. 5. Effect of heat treatment on galactosyltransferase. Microsomal enzyme was heated at 50°C (○ and □) or 60°C (● and ■) for 50–60 min, and the activity of galactose transfer to 20 mM-pyridine-3-*O*- β -galactoside (● and ○) and to 20 mM-pyridine 2-*S*- β -thioxyloside (■ and □) was assayed under standard conditions for 6 h as outlined in the Experimental section.

enzymes were involved. Enzymic transfer of galactose to 20 mM-*N*-acetylglucosamine, which is also catalysed by the chick microsomal preparation, was also investigated; heating of the preparation for 5 min at 50°C completely destroyed such activity, as reported by Helting (1971).

Competition between acceptors for galactose transfer

Galactose transfer to pyridine 3-*O*- β -galactoside was measured in the presence of phenyl *O*- β -galactoside and of benzyl *O*- β -xyloside in an attempt to demonstrate that a separate enzyme was involved in galactosyl transfer to the latter substrate. In all of the competition experiments an incubation period of 6 h was used, which lay well within the period of linear incorporation kinetics for pyridine 3-*O*- β -galactoside and benzyl *O*- β -xyloside (Robinson & Robinson, 1981). Surprisingly, no difference in the rate of transfer could be detected on addition of 30 mM-phenyl β -galactoside, though a difference was observed with 30 mM-benzyl β -xyloside (Table 2). This unexpected result, which suggested that the same enzyme might catalyse transfer to β -xylosides and to β -galactosides, was not consistent with previous results that phenyl β -galactoside did not compete with pyridine 2-*S*- β -thioxyloside as substrate for galactosyltransferase I (Robinson & Robinson, 1981). One possible explanation for the lack of competition between phenyl *O*- β -galactoside and pyridine 3-*O*- β -galactoside is that galactosyltransferase II has such low affinity for these aryl glycosides that the enzyme never approaches saturation with substrate. This would mean that, at

Table 2. Effect of benzyl *O*- β -xyloside and phenyl-*O*- β -galactoside on [3 H]galactose transfer to pyridine 3-*O*- β -galactoside. Microsomal enzyme was incubated for 8 h at 37°C with UDP-[3 H]galactose and the substrates shown in the Table. [3 H]Galactose transfer to pyridine 3-*O*- β -galactoside was measured after adsorption of the product on Amberlite CG-120.

[UDP- 3 H]galactose] (mM)	[Pyridine 3- <i>O</i> - β - galactoside] (mM)	[Phenyl <i>O</i> - β - galactoside] (mM)	[Benzyl <i>O</i> - β - xyloside] (mM)	$10^{-4} \times$ [3 H]Galactose transfer to pyridine 3- <i>O</i> - β -galactoside (d.p.m./mg of protein)
1.25	20	0	0	9.4
1.25	20	30	0	9.4
1.25	20	0	10	7.2
1.25	20	0	20	6.6
1.25	20	0	40	5.4
1.25	50	0	0	18.2
1.25	50	30	0	18.2
1.25	50	0	10	13.8
1.25	50	0	20	11.6
1.25	50	0	30	9.1
12.3	20	0	0	16.8
12.3	20	0	10	14.4
12.3	20	0	20	14.9
12.3	20	0	40	12.9

the concentrations used, no competition could be observed, a conclusion supported by the high K_m value (125mM) measured for pyridine 3-*O*- β -galactoside. The competition observed between benzyl *O*- β -xyloside and pyridine 3-*O*- β -galactoside was more difficult to reconcile with two separate galactosyltransferase activities, but further experiments indicated this could nevertheless be so. When the UDP-[3 H]galactose concentration was raised from 1.25mM to 12.3mM, the observed competition was diminished (Table 2), suggesting that part of the competition was for a limited supply of UDP-galactose. Furthermore, when 20mM-pyridine 2-*S*- β -thioxyloside was incubated, in a separate experiment, with 9.2mM-UDP-[14 C]galactose at 37°C for 24h under the assay conditions, the expected product Galp β 1-4Xylp β 1-2-*S*-pyridine was formed (equivalent to a final concentration of 2.8mM) together with an appreciable quantity of another labelled compound, which was tentatively identified as the product of a second galactose transfer, Galp β 1-3Galp β 1-4Xylp β 1-2-*S*-pyridine. The final concentration of the later product was estimated to be 0.15mM. These results suggest that, during the competition experiments with benzyl *O*- β -xyloside and pyridine 3-*O*- β -galactoside, an appreciable concentration of the product Galp β 1-4Xylp β 1-*O*-benzyl may have been formed, which could then have served as a competitive substrate for galactosyltransferase II. If this were so, then such disaccharide substrates must have a much higher affinity for the galactosyltransferase than have the other β -galactosides tested.

In a separate series of experiments, pyridine 3-*O*- β -galactoside was incubated with 5.0mM-UDP-

[3 H]galactose and microsomal enzyme in the presence and absence of Gal-Xyl-Ser. The products were isolated on Amberlite CG-120 and separated on Dowex 1 as outlined in the Experimental section. The results in Table 3 indicate that reciprocal competition did occur between these substrates, although the results were not conclusive, because recovery and separation of the products was not complete.

Although these competition experiments failed to establish that two separate enzymes were involved in galactose transfer to β -xylosides and β -galactosides, more direct evidence was obtained by ion-exchange chromatography on DEAE-cellulose of microsomal enzyme solubilized with 0.5% Nonidet P-40 as described in the Experimental section. Although complete separation of the activity transferring galactose to pyridine 2-*S*- β -thioxyloside from that to pyridine 3-*O*- β -galactoside was not achieved, the peaks of activity were partially resolved and were separate from a peak of activity transferring galactose to *N*-acetylglucosamine (Fig. 6). This result, together with the different heat-lability (Fig. 5) of the two activities and the different response to addition of Nonidet P-40, indicated that galactose transfer to β -galactoside is not catalysed by galactosyltransferase I, but probably by galactosyltransferase II, the third glycosyltransferase of chondroitin sulphate chain synthesis. We propose that this activity enables β -galactosides to function as initiators of chondroitin sulphate synthesis in intact cells. It is probable, however, that within the chondrocyte there are additional galactosyltransferases capable of transferring galactose to β -galactosides; these could be involved, perhaps, in

Table 3. Effect of pyridine 3-*O*- β -galactoside on galactose transfer to Gal-Xyl-Ser
Microsomal enzyme was incubated for 6h at 37°C with 50mM-UDP-[3 H]galactose (1.0mCi/mmol) and the other substrates shown in the Table under the standard conditions outlined in the Experimental section. Transfer of [3 H]galactose to pyridine 3-*O*- β -galactoside and to Gal-Xyl-Ser were determined by ion-exchange chromatography as described in the Experimental section.

Substrates added		Transfer of [3 H]galactose to	
Pyridine 3- <i>O</i> - β -galactoside (mM)	Gal-Xyl-Ser (mM)	Pyridine 3- <i>O</i> - β -galactoside (d.p.m.)	Gal-Xyl-Ser (d.p.m.)
0	0	0	0
0	5	0	4650
5	0	5270	188
5	5	3550	4010
10	0	8460	396
10	5	7290	3330
20	0	15300	251
20	5	12700	2520
30	0	18800	396
30	5	16700	3280

Fig. 6. Ion-exchange chromatography of galactosyltransferase on DEAE-cellulose

Microsomal enzyme was dissolved in a solution containing 0.5% Nonidet P-40 and 1.0mM-EDTA in 0.05M-Mes/NaOH buffer, pH6.0, and fractionated on a column (20cm $\times$ 1.3cm) of DEAE-cellulose as outlined in the Experimental section. Fractions (10ml) were assayed for galactose transfer to 20mM-pyridine 2-S- β -thioxyloside (○), to 20mM-pyridine 3-O- β -galactoside (●) and to N-acetylglucosamine (■).

glycoprotein or glycolipid biosynthesis, so that β -galactosides may serve as substrates for more than one such galactosyltransferase.

We thank Professor D. A. Lowther for his support and encouragement during this work. The work was supported by a grant from the Australian Research Grants Committee (D280/16025). J. A. R. thanks Monash University for a Monash Postgraduate Research Scholarship.

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