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Enzymatic Synthesis and Carbon-13 Nuclear Magnetic Resonance Conformational Studies of Disaccharides Containing β-D-Galactopyranosyl and β-D-[1-^{13}C]Galactopyranosyl Residues†

H. A. Nunez and R. Barker*

ABSTRACT: Partially purified UDPgalactosyltransferase (EC 2.4.1.22) from bovine milk has been used to synthesize millimolar amounts of compounds such as Galβ(1→4)Glc, Galβ(1→4)GlcNAc-β-hexanolamine, and Galβ(1→4)-GlcNAcβ(1→4)GlcNAc. The same method has been used to prepare similar compounds containing ^{13}C-enriched galactopyranosyl moieties. Galβ(1→4)GlcNAc-β-hexanolamine was also synthesized in a solid-phase system in which the GlcNAc-β-hexanolamine glycoside was covalently linked to agarose beads. At pH 7.0 and at 1–5 mM Mn^{2+} the yields of the galactosyl saccharides are greater than 90% by using 10% excess of UDPGal donor. The use of a 90% enriched [1-^{13}C]galactosyl residue allowed the determination of the most abundant conformer about the galactopyranosyl–glycoside linkage by analysis of the carbon–carbon coupling constants from C1 of Gal to the C3′, C4′, and C5′ of GlcNAc or Glc.

The carbohydrate structures of the cell-surface glycoproteins and glycolipids are involved in cell–cell recognition, the immune response, hormone receptor functions, and internalization of various macromolecular materials. All are highly specific phenomena, and the specificity seems to be due, at least in part, to the structure of the oligosaccharide moieties involved. The nature of the individual sugar components and their sequence, linkage position, anomeric configuration, and overall configuration all appear to be important (Hughes, 1975). A well-documented case is the relationship of carbohydrate structure to the immune response elicited by the ABO and other antigenic groups of animal cell surfaces. However, even in this case very little is known about the conformation of the monosaccharide moieties about the glycosidic linkages of the oligosaccharide and the role that this may play in the interaction betwen antigen and antibody.

In general, the glycosubstances can be obtained in small amounts from biological sources, making studies of their physical, chemical, and biochemical properties difficult. Synthetic approaches are complex, exemplified by the elegant works in the laboratories of Lemieux (Lemieux & Driguez, 1975), Jeanloz (N-U-Din et al., 1974), Schuerch (1972) and their co-workers. In this paper we describe an approach using a partially purified UDPgalactosyltransferase for the preparation in high yield of millimolar amounts of a number of disaccharides containing D-Gal[1] in β(1→4) linkage. The ap-

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[1] Abbreviations used: ^{13}C NMR, ^{13}C nuclear magnetic resonance spectroscopy; Gal, α- or β-D-galactopyranose; Galβ(1→4)Glc (lactose), 4-*O*-(β-D-galactopyranosyl)-D-glucopyranose; Galβ(1→4)GlcNAc (*N*-acetyllactosamine), 4-*O*-(β-D-galactopyranosyl)-2-acetamido-2-deoxy-β-D-glucopyranose; Galβ(1→4)GlcNAcβ(1→4)GlcNAc, 4-*O*-(β-D-galactopyranosyl)-2-acetamido-2-deoxy-4-*O*-(β-D-glucopyranosyl)-2-acetamido-2-deoxy-D-glucopyranose; Galβ(1→4)GlcNAc-β-hexanolamine, 6-amino-1-hexyl 4-*O*-(β-D-galactopyranosyl)-2-acetamido-2-deoxy-β-D-glucopyranoside; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; GlcNAc-β-hexanolamine, 6-amino-1-hexyl 2-acetamido-2-deoxy-β-D-glucopyranoside; GlcNAcβ(1→4)GlcNAc (chitobiose), 2-acetamido-2-deoxy-4-*O*-(β-D-glucopyranosyl)-2-acetamido-2-deoxy-β-D-glucopyranose.

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proach has also permitted the preparation of ^{13}C-enriched disaccharides to facilitate studies of the solution conformation about the glycosidic bond. The product disaccharides are also substrates for GDPfucosyltransferases, leading to the possibility of extending this synthetic approach to more complex oligosaccharides. In addition, the feasibility of an enzymatic solid-phase synthesis of oligosaccharide is demonstrated by the transfer of the galactosyl moiety to a GlcNAc residue linked through a six-carbon spacer arm to agarose beads.

The reaction catalyzed by UDPgalactosyltransferase requires substrate amounts of UDPGal and of an acceptor such as GlcNAc. The enzyme is activated by Mn^{2+}. The modifying protein α-lactalbumin alters the specificity of the enzyme to

$$\text{UDPGal} + \text{acceptor} \xrightarrow[\substack{(\alpha\text{-lactalbumin})\\ \text{galactosyl-}\\ \text{transferase}}]{Mn^{2+}} \text{Gal}\beta(1\rightarrow4)\text{-acceptor} + \text{UDP}$$

permit glucose to serve as an acceptor (Brew et al., 1968). The reaction is essentially irreversible and the product UDP is a potent inhibitor.

Materials and Methods

GlcNAc-β-hexanolamine was synthesized by the method of Chiang et al. (1979). Chitobiose was a gift of Dr. J. C. Speck, Jr., Department of Biochemistry, Michigan State University. UDPGal was synthesized by the methods of Moffat (1966). [1-^{13}C]UDPGal was synthesized in the same manner by using D-[1-^{13}C]Gal synthesized as described previously (Serianni et al., 1979). [U-^{14}C]Gal was obtained from New England Nuclear. The UDPgalactosyltransferase was partially purified as described previously be affinity chromatography on UDP hexanolamine agarose (Barker et al., 1972).

Synthesis of 4-O-(β-D-Galactopyranosyl) Oligosaccharides. In a typical synthesis, 0.1 mmol of the acceptor and 0.11 mmol of UDPGal were dissolved in 15 mL of 25 mM cacodylate buffer, pH 7.0, containing 10–20 units of UDPgalactosyltransferase, 3 mM $MnCl_2$, and 0.1% mercaptoethanol. When Glc was the acceptor, the reaction mixture contained 1 mg/mL α-lactalbumin. The reaction mixture was kept at 37 °C until reaction was completed (see Figure 1), as estimated by LC, thin-layer chromatography, electrophoresis, or radioactivity measurements. The reaction mixture was then transferred to a column containing 30 mL of Dowex 1 (Cl^- form) and eluted with 200 mL of water. The eluate was concentrated to 5 mL and then transferred to a 4 × 100 cm column of Bio-Rad P-2 (400 mesh) and eluted with water. The elution was monitored with a refractive index detector (Pharmacia). The saccharide product emerges first and is well separated from contaminating salts. Alternatively, in the case of products containing an amino group, the reaction mixture was transferred to a 2 × 35 cm column of Dowex 50 (H^+) and eluted with 1 M pyridinium acetate. Repeated evaporation from water in vacuo yielded the disaccharide glycoside as the acetate salt. Prior to NMR analysis products were passed through a small column (1 mL) of Chelex 100 to remove traces of Mn^{2+}.

The following compounds have been prepared by this method: Galβ(1→4)GlcNAc; Galβ(1→4)GlcNAc-β-hexanolamine; Galβ(1→4)GlcNAcβ(1→4)GlcNAc; [1-^{13}C]Galβ-(1→4)GlcNAc; [1-^{13}C]Galβ(1→4)GlcNAc-β-hexanolamine; [1-^{13}C]Galβ(1→4)Glc.

Solid-Phase Synthesis. GlcNAc-β-hexanolamine was coupled to Sepharose 4B (Pharmacia) by the method of March et al. (1974) to give 6–10 μmol of ligand per mL of packed, wet gel. The gel was equilibrated with 25 mM cacodylate buffer, pH 7.0, containing 5 mM $MnCl_2$ and 0.1% mercaptoethanol. To approximately 5 mL of settled gel in a total volume of 7 mL, UDPGal was added (a twofold excess over the amount of 2-acetamido-2-deoxyglucose attached to the gel) and 10 units of UDPgalactosyltransferase was added to give a final volume of 10 mL. The reaction mixture was agitated gently in a closed vial on a reciprocating shaker at 35 °C. The transfer of [U-^{14}C]Gal was measured on 20-μL samples of the supernatant phase. After 18 h, approximately 85% of the theoretical transfer had taken place. Neither addition of fresh enzyme nor addition of UDPGal increased the extent of substitution. The gel was transferred to a small column and washed exhaustively with water. When no further radioactivity could be detected in the eluant, an aliquot was incubated with 2 M NaOH at 40 °C for 3 h to release 80% of the ligand (Naoi & Lee, 1974) which could be recovered by chromatography on Dowex 50 as described above.

High-Pressure Liquid Chromatography. A Whatman Partisil PXS 10/25 SAX column equilibrated with 0.2 M, pH 3.4, potassium phosphate buffer was used to estimate the amount of UDPGal, UDP, and UMP. Aliquots of 5–10 μL of the reaction mixture were diluted to 100 μL, and 20 μL of the resulting solution was injected into the system and eluted with the equilibration buffer. The UV absorbant material was monitored by using a LKB Uvicord II, Type 8303A UV detector.

^{13}C NMR. Proton-decoupled spectra were obtained with Bruker WP-60 (15.08-MHz) and NT-360 (90.5-MHz) spectrometers, equipped for Fourier transform operation. Samples were dissolved in ^2H_2O at 0.05–0.2 M concentration. Chemical shifts are given in parts per million as calculated by the computer after a sweep offset has been entered to obtain the literature value of 97.4 ppm for the resonance of Cl for the β-glucopyranose anomer in a water solution of D-glucose (Walker et al., 1976). This method produces relative chemical shifts with errors within ±0.1 ppm with respect to internal Me_4Si.

Electrophoresis. The progress of reactions of compounds containing a hexanolamine moiety was followed by using horizontal paper electrophoresis in triethanolamine bicarbonate buffer, 0.05 M, pH 7.5. After electrophoresis for 90 min at 100 V/cm, the paper was dried and analyzed for ninhydrin-positive material. Under these conditions, the disaccharide has R = 0.8 relative to the monosaccharide derivative. UDPGal, UDP, and UMP present in the reaction mixture are well separated in 60 min under the same conditions.

Thin-Layer Chromatography. Thin-layer chromatography was carried out by using (20 × 20 cm) plates coated with silica gel G (250 μm) and 5-μL samples of the reaction mixture. Plates were developed with 1-propanol–acetic acid–water (85:12:3 v/v) for 60–90 min. Under these conditions, R_f values for the compounds of interest are 0.39 for GlcNAc, 0.20 for Galβ(1→4)GlcNAc, 0.20 for Galβ(1→4)Glc, and 0.1 for Galβ(1→4)GlcNAcβ(1→4)GlcNAc. Spots were visualized by spraying the plates with 2 N sulfuric acid and charring.

Radioactivity Assay. As an alternative method to follow the progress of the reaction or to assay the UDPgalactosyltransferase activity, the method of Brew et al. (1968) was used. An aliquot (10–100 μL) of the reaction mixture was placed into a 0.6 × 5 cm Dowex 1 (Cl^- form), eluted with 2 mL of water into a scintillation vial, and counted. Under these conditions, only the neutral and positively charged species pass through the column.

Results and Discussion

Purification and Storage of UDPgalactosyltransferase. The enzyme was purified through the first steps of the method

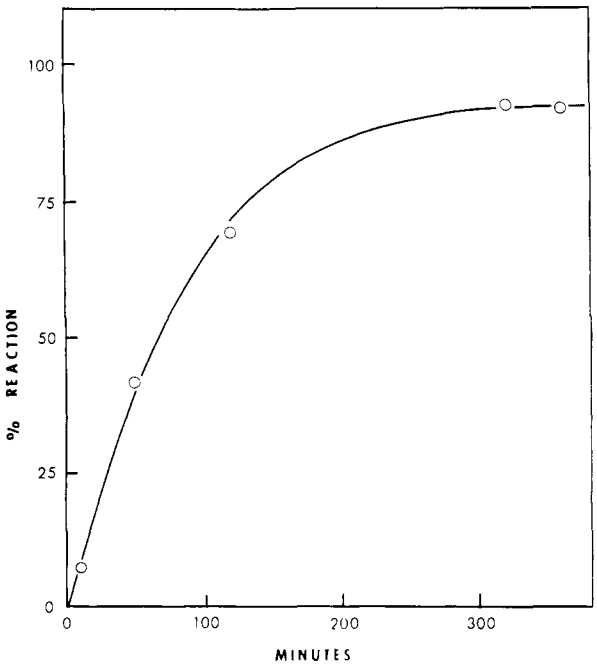


FIGURE 1: Enzymatic synthesis of Galβ(1→4)GlcNAc-β-hexanolamine, followed by high-pressure liquid chromatographic estimation of UDP formation. Aliquots of 20 μL from the reaction mixture (see Materials and Methods) were diluted with 30 μL of 0.2 M, pH 3.4, potassium phosphate buffer, 20 μL of the mixture was injected onto a Whatman Partisil PXS 10/25 SAX column which was developed with the same buffer, and the eluant was monitored with a UV detector.

described by Barker et al. (1972). Partial purification by adsorption and elution from UDP–Sepharose gives a preparation that can be kept at 4 °C for an indefinite time in 25 mM GlcNAc and 5–20 mM Mn^{2+} at pH 7.4 in 25 mM cacodylate buffer. Cacodylate buffer inhibits bacterial growth and also permits long reaction times in synthetic steps. For use with acceptors other than GlcNAc, the enzyme can be dialyzed against and stored in 25 mM cacodylate buffer, pH 7.4, and 1% mercaptoethanol at 4 °C for several weeks with little loss of activity.

Galactosyltransferase Reaction. The result of a typical experiment using 100 μmol of GlcNAc-β-hexanolamine is shown in Figure 1. Maximum UDPgalactosyltransferase activity is obtained at pH 7.4 and Mn^{2+} concentration up to 50 mM (Powell & Brew, 1974; Tsopanakis & Herries, 1978). For maximization of the yields of product, however, pH 7.0 ± 0.2 and Mn^{2+} concentration of 1–5 mM are preferable, since these conditions minimize the nonenzymatic degradation of UDPGal catalyzed by Mn^{2+} (Nunez & Barker, 1976). The disappearance of UDPGal and the appearance of UDP can be followed in all the cases by LC or electrophoresis. The simultaneous nonenzymatic degradation of UDPGal to galactose 1,2-phosphate and UMP can also be monitored by these methods. Depending on the characteristics of the acceptor, formation of oligosaccharide can be followed by TLC, electrophoresis, or transfer of radioactivity, as described under Materials and Methods.

The concentration of UDPgalactosyltransferase is not critical, although it is best used at high concentrations to reduce the reaction times and the effect of nonenzymatic side reactions. The enzyme becomes more labile as it is purified, and it is difficult to keep concentrated solutions.

UDPgalactosyltransferase is subjected to both product inhibition and substrate inhibition (Brew et al., 1968). For these reasons, UDPGal concentrations were kept below 10 mM. When UDPgalactosyltransferase at an activity of 4 units/mL of reaction mixture is used, more than 95% of the galactose is transferred in 6 h at 37 °C (Figure 1). With less concentrated enzyme solutions, the reaction times increase significantly. The acceptor substrate usually was equimolar with respect to UDPGal. Use of a 10% excess of the UDPgalactose, however, assures complete conversion of the acceptor substrate and facilitates product purification.

Solid-Phase Synthesis. To facilitate purification, it would be convenient to carry out the enzymatic synthesis of oligosaccharides covalently linked to a solid matrix. To permit this, the acceptor should be attached to the solid matrix through a covalent linkage which can be cleaved after formation of the desired glycosidic bond. The hexanolamine glycosides provide such a linkage to Sepharose since the bond formed with CNBr-activated Sepharose is base labile (Naoi & Lee, 1974). With 6–10 μmol of GlcNAc-β-hexanolamine per mL of Sepharose and a twofold excess of UDPGal at pH 6.8–7.1 in 1.5 mM Mn^{2+} and 1% mercaptoethanol, UDPgalactosyltransferase converts approximately 85% of the acceptor into Galβ(1→4)GlcNAc-β-hexanolamine in a few hours at 37 °C. In separate experiments, both Galβ(1→4)GlcNAc-β-hexanolamine and GlcNAc-β-hexanolamine could be cleaved from the Sepharose matrix to the extent of 80% in 2 M NaOH at 37 °C for 3 h. Longer treatment did not increase the amount of liberated saccharide. These results seem to corroborate the studies that show a diversity of linkages formed when a primary amine group is coupled to CNBr-activated Sepharose (Porath, 1974). The liberated glycoside was identical in all respects to the product formed by the enzyme acting on GlcNAc-β-hexanolamine in solution, indicating that the matrix did not affect the specificity of the enzyme. It appears that UDPgalactosyltransferase from bovine milk has a high degree of specificity for the GlcNAc residue but that the remainder of the acceptor molecule is much less critical. Galβ(1→4)-GlcNAc-β-hexanolamine–Sepharose is a useful affinity adsorbent for GDPfucosyltransferases. The suitability of the ligand as a substrate for the same enzyme is now being investigated both in solution and in the solid-phase system (Rosevear, Nunez, and Barker, unpublished experiments).

Product Purification. An anion-exchange resin, Dowex 1 in the Cl^- form, was used as the first step in the purification process since the acceptors and the products of the enzymatic reaction are neutral or posiviely charged at pH 7. The excess of UDPGal and the UDP and UMP formed which are retained by the resin can be recovered by a gradient elution (Moffat, 1966) and reused. The concentrated oligosaccharide products were purified by gel filtration on Bio-Rad P-2 or, in the case of those compounds containing a free amino group, selectively eluted with pyridinium acetate from Dowex 50.

Characterization of Products. The product of the action of the UDPgalactosyltransferase prepared from bovine whey on GlcNAc and UDPGal has been well characterized by chemical and enzymatic means as Galβ(1→4)GlcNAc. This characterization is fully supported by the ^{13}C NMR data obtained in this study. Thus, the resonances in the anomeric region at 104.3, 96.3, and 92.0 ppm can be assigned to C1 of a β-D-Gal residue and the C1′α and C1′β of GlcNAc, respectively. The C1 of an α-D-Gal residue would resonate approximately 4 ppm upfield. The appearance of two resonances at 80.2 and 79.7 ppm corresponding to the C4′α and C4′β, respectively, and the disappearance of the resonances at 71.5 and 71.2 ppm relative to the spectrum of unsubstituted GlcNAc (Nunez et al., 1977) support the presence of a 1→4 glycosidic linkage.

Table I: Carbon-13 Chemical Shift Assignments[a]

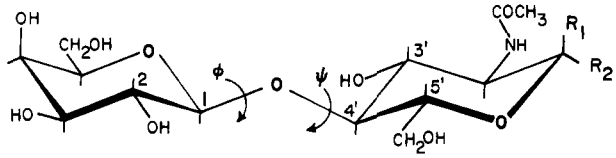
	chemical shifts (ppm)							
	GlcNAc	Gal[b]	GlcNAc-β-hexanolamine	Galβ(1→4)-GlcNAc-β-hexanolamine	Galβ(1→4)-GlcNAc	Galβ(1→4)-Glc[c]	GlcNAcβ-(1→4)-GlcNAc[d]	Galβ(1→4)-GlcNAcβ-(1→4)GlcNAc
carbon 1								
Rα[e]	92.2	93.8			91.6	93.4	91.8	92.0
Rβ	96.4	98.0			96.2	97.3	96.2	96.3
GlcNAc			102.7				102.9	
GlcNAc'				102.4				102.8
Gal				104.3	104.1	104.3		104.3
carbon 2								
Rα	55.5	70.0			55.1	72.9	55.0	55.0
Rβ	58.1	73.6			57.6	75.3	57.5	57.6
GlcNAc			57.2				57.0	
GlcNAc'				56.5				56.6
Gal				72.5	72.3	72.5		72.4
carbon 3								
Rα	72.1	70.8			70.6	72.7	70.7	70.7
Rβ	75.3	74.4			74.9	75.9	73.6	73.6
GlcNAc			75.2				74.9	
GlcNAc'				73.9				74.0
Gal				74.0	73.9	74.1		74.0
carbon 4								
Rα	71.5	70.9			80.1	80.0	81.3	81.2
Rβ	71.2	70.4			79.7	79.9	80.9	80.8
GlcNAc			71.6				71.1	
GlcNAc'				79.9				79.7
Gal				70.1	69.9	70.1		70.0
carbon 5								
Rα	73.0	72.0			71.5	71.6	71.4	71.4
Rβ	77.3	76.6			76.1	76.3	75.9	76.0
GlcNAc			77.4				77.3	
GlcNAc'				76.5				76.8
Gal				76.9	76.6	76.9		76.2
carbon 6								
Rα	62.0	62.8			61.3	61.5	61.5	61.5
Rβ	62.2	62.6			61.3	61.6	61.5	61.5
GlcNAc			62.4				62.0	
GlcNAc'				61.5				62.0
Gal				62.5	62.3	62.6		62.3
carbonyl								
Rα	175.9				176.0		176.0	176.1
Rβ	176.2				176.0		176.0	176.1
GlcNAc			175.9				176.1	
GlcNAc'				175.7				176.1
methyl								
Rα	23.3				23.3		23.3	23.3
Rβ	23.5				23.5		23.5	23.6
GlcNAc			23.8					
GlcNAc'				23.6			23.5	23.6

[a] Chemical shifts are given in ppm as calculated by the computer after a sweep offset has been entered to obtain a chemical shift value of 97.4 ppm for C1 of the β-glucopyranose anomer in a water solution of D-glucose (Walker et al., 1976). They are accurate to ±0.1 ppm. [b] Chemical shifts taken from Walker et al. (1976). [c] Several reports for lactose chemical shifts (Dorman & Roberts, 1971; Johnson & Jankowski, 1972; Voelter et al., 1973) differ by 1.3 ppm. The chemical shifts for Glc C3β and Glc C5β have recently been reassigned (Sillerud et al., 1978, footnote). [d] Chemical shifts differ up to 0.2 ppm from the values reported by Colson & King (1976). [e] Rα and Rβ specify the chemical shifts of the designated carbon in the reducing moiety of the saccharides.

When GlcNAc-β-hexanolamine is used as an acceptor, the disaccharide product is also readily characterized by ^{13}C NMR. A resonance at 104.3 ppm is characteristic of C1 of a β-D-Gal residue. The resonance of the other carbons of Gal can be assigned (Table I) in comparison to those of other β-D-galactopyranosides (Colson & King, 1976) with the advantage that C2 and C3 can unequivocally be assigned by using their coupling constant to C1 in the enriched compounds (Walker et al., 1976). Glycosidation of the GlcNAc at C4 is confirmed by the substantial downfield shift (from 71.6 to 79.9 ppm) of the resonance for the C4 carbon. There are smaller effects on C3 and C5, and the resonances of other carbons in the acceptor are only slightly affected (Table I). The resonance at 102.4 ppm can be assigned to C1 of the β-GlcNAc residue (Colson & King, 1976). The presence of a β-D-galactopyranosyl residue in the product is confirmed by the quantitative release of glactose on treatment with β-galactosidase (Craven et al., 1965).

When 2-acetamido-2-deoxy-4-*O*-(α-D-glucopyranosyl)-2-acetamido-2-deoxy-D-glucopyranose (chitobiose) or the homologous trisaccharides are used as acceptors, similar high yields of the corresponding β(1→4)-linked-D-galactopyranosyl derivatives are obtained. In these cases, the resonance of C1 of the β-D-galactopyranosyl moiety occurs at 104.3 ppm, and the resonance of C4 at which substitution has occurred is moved downfield from 71.1 to 79.7 ppm. The remaining

Scheme I



α-**1a**, R_1 = OH; R_2 = H
β-**1a**, R_1 = H; R_2 = OH
1b, R_1 = H; R_2 = $O(CH_2)_6NH_2$

carbons have been assigned the chemical shifts listed in Table I.

Glucose can be used as an acceptor when α-lactalbumin is included in the reaction mixture. Similar yields are obtained and similar isolation procedures can be utilized. NMR parameters of the product Galβ(1→4)Glc (lactose) are listed in Table I.

Conformation about the Galactosyl Linkage. The description of the conformation of the β-D-galactopyranosyl–glycosidic linkage in **1a** and **1b** requires the specification of two torsion angles, ϕ and ψ, as indicated in Scheme I. Although the following discussion deals with **1b** specifically, similar data were obtained and similar conclusions can be drawn with respect to all of the Galβ(1→4)-linked saccharides listed under Materials and Methods. NMR spectrometry is useful for the study of glycosidic conformations in solution, since the three-bond C–C and C–H coupling constants are expected to show an angular dependence similar to that described by Karplus (1963) for vicinal protons (Hamer et al., 1978). Also, there is evidence that the two-bond homo- and heteronuclear coupling constants between ^{13}C and ^{1}H depend strongly on the configuration of the C, H, and O substituents along the coupling pathway (Schwarcz et al., 1975; Walker et al., 1976; Bock & Pedersen, 1977). Carbon–carbon coupling cannot be observed in natural-abundance ^{13}C spectra, and some C–H couplings are difficult to observe. Specific ^{13}C enrichment, however, generally allows coupling to the enriched atom to be observed. In **1a** and **1b**, 90% enriched with ^{13}C at C1 of the galactosyl residue, ψ can be evaluated from the three-bond C1–C3′ and C1–C5′ coupling constants and ϕ can be evaluated from the two-bond C1–C4′ coupling constant. An important feature of these compounds is that, at 90.5 MHz, the proton-decoupled ^{13}C NMR spectrum is completely resolved, and resonances for the carbons involved in the galactopyranosyl–glycosidic linkage are easily observable. A partial spectrum of the β-hexanolamine glycoside, **1b**, at 90.5 MHz is shown in Figure 2. The chemical shifts of the galactopyranosyl residue are those expected for a β-D-galactopyranoside (Perlin, 1970). The coupling constants $^{1}J_{C1-C2}$, $^{2}J_{C1-C3}$, and $^{2}J_{C1-C5}$ have the same magnitude (46, 3.7, and 0 Hz, respectively) as those observed in the reducing sugar for the β anomer (Walker et al., 1976). Thus, there appears to be no distortion of the galactopyranosyl ring in the disaccharide. The resonance due to C4′ is approximately 1 Hz broader than that of C4′ in the nonenriched saccharide and the resonances of the doublet of C2 in the enriched compound which should be among the narrowest peaks in the spectrum. In addition, C5′ is broadened by approximately 1 Hz with respect to C3′ (Figure 2), indicating that, if present, $^{3}J_{C1-C5'} \leq 1.5$ Hz and $^{3}J_{C1-C3'} \simeq 0$ Hz.

Interpretation of the three-bond $^{3}J_{C1-C3'}$ and $^{3}J_{C1-C5'}$ coupling constant values which define ψ requires that relationships between dihedral angles and coupling constant values be established for closely related model structures. Since two- and three-bond C–C coupling constants through a glycosidic

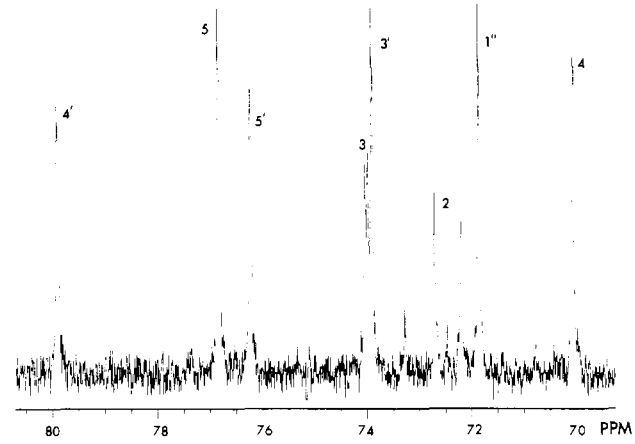


FIGURE 2: Partial 90.5-MHz ^{13}C NMR spectrum of [1-^{13}C]Galβ-(1→4)GlcNAc-β-hexanolamine in D_2O. The proton-decoupled spectrum was obtained with a NT-360 spectrometer at 20 °C using a 0.2 M solution in a 5-mm tube. Transients (3000) were accumulated at a sweep width of 5434 Hz at 0.16 Hz/computer point and 45° pulse. Chemical shifts are expressed in ppm downfield from tetramethylsilane (see the text). The peak numbers correspond to those of **1b** in Figure 3.

linkage have not been described previously, we have examined several 1-^{13}C-enriched ethyl glycosides (Nunez & Barker, 1979). In all cases (i.e., 1-^{13}C-enriched ethyl α- and β-pento- and hexofuranosides and pento- and hexopyranosides), the three-bond coupling constant $^{3}J_{C1-C2'} \simeq 3$ Hz. The values can be related to those observed for coupling through the ring oxygen in 1-^{13}C-enriched methyl glycosides and reducing sugars (Walker et al., 1976) or pentofuranoses, pentofuranosides, and 1,4-aldonolactones (unpublished observations), in which the dihedral angle can be assigned. In these cases, $^{3}J_{C1-C6} = 3.7 \pm 0.3$ Hz (180°) in hexopyranoses and lies in a range of 0–2.6 Hz for pentofuranoses, pentofuranosides, and 1,4-aldonolactones (110–140°). Thus, the observation (Figure 2) that $^{3}J_{C1-C5'}$ is less than 1.5 Hz and that $^{3}J_{C1-C3'}$ is approximately 0 Hz implies that the favored conformer has C3′ and C5′ at approximately 120° with respect to C1 when viewed along the O1–C4′ bond.

We do not have an appropriate model compound to establish the values of coupling through a glycosidic oxygen for angles of less than 120°; nevertheless, it is expected that there is a minimum at 90° and another maximum at 0°, as is the case for other homo- or heteronuclear coupling constants between nuclei of $I = 1/2$ (Inch, 1972).

These observations indicate that the most stable conformer in aqueous solution has the C3′ at an angle slightly smaller than 120° and C5′ at an angle slightly larger than 120° with respect to C1, since any further decrease in absolute value for the C3′ angle will concomitantly increase the angle for C5′ at which point a resolvable $^{3}J_{C1-C5'}$ coupling constant should appear. A different conformer with C3′ and C5′ turned through 180° could also account for these results. In the second rotamer, both C3′ and C5′ lie at approximately 60° with respect to C1. In this latter rotamer strong steric interactions occur between the two pyranosyl rings. Although ^{13}C NMR cannot distinguish between these two possible rotamers for **1**, we favor the former in accord with the conclusions drawn from model building and theoretical calculations (Rees & Smith 1975), optical rotation (Rees, 1970), and X-ray crystallography (Marchessault & Sundararajan, 1975; Cook & Bugg, 1973) for lactose and cellobiose, in which the stereorelationships around the glycosidic linkage are similar to that of **1**. The same ambiguities with respect to assignment of ϕ would exist for $^{3}J_{C1-H4'}$ measurements since an angle near 180°

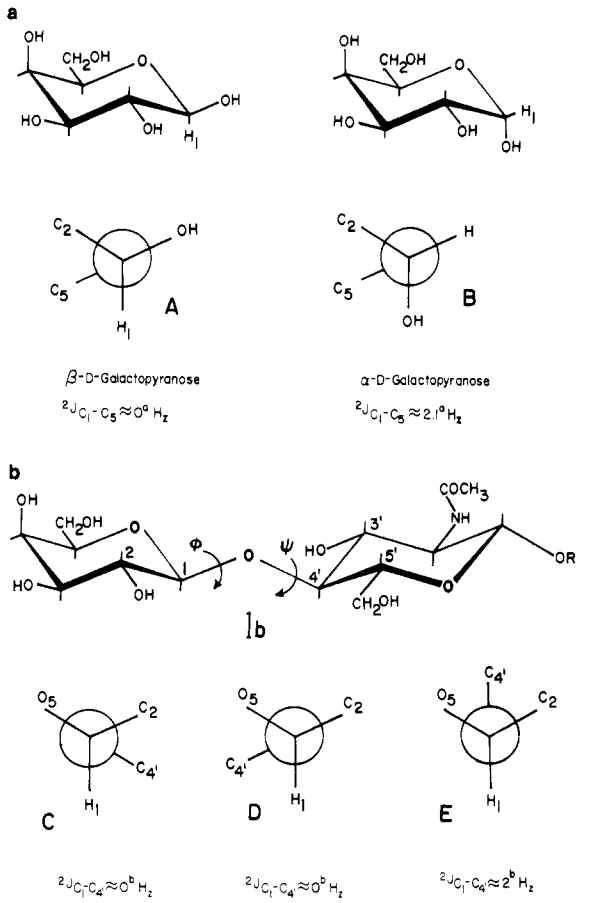


FIGURE 3: Rotamer projections along the indicated bonds for the $^4C_1$ conformation of α- and β-D-galactopyranose and the galactosyl–glycosidic linkage of [1-^{13}C]Galβ(1→4)GlcNAc-β-hexanolamine. (a) Experimentally observed coupling constants; (b) expected coupling constants.

or 0° would give a similar coupling constant value, as has been shown by Hamer et al. (1978) for methyl-β-cellobiose and methyl-β-cellobiose heptaacetate and by Excoffier et al. (1977) for cellobiose octaacetate.

In hexopyranoses and hexopyranosides the values of the two-bond, through the ring oxygen, C–C coupling constants are strongly influenced by the configuration of the C, O, and H substituents on the C originating the coupling. The results of Bock & Pedersen (1977) allowed a generalization, similar to that found previously by Perlin (Schwarcz et al., 1975), that for the two-bond C–H coupling constant, an O or a C *anti* to the coupled C makes a positive contribution to the coupling, whereas an O or a C *gauche* makes a negative contribution. Thus, in the specific case of [1-^{13}C]Gal, for which a projection through the C1–O5 bond is shown in Figure 3, the $^2J_{C1-C5}$ is 2.1 Hz for the α anomer, whereas coupling is small and not resolved for the β anomer. In the spectrum of **1b** (Figure 2), resonances due to C4′ and C5 show no coupling, although the line width for C5 is greater. These results would be expected if the position of C4′ (through the glycosidic bond) is similar to that of C5 (through the ring oxygen) with respect to the substituents on C1. As can be seen in the projection through C1–O1 in Figure 3, in only two (C and D) of the three most stable rotamers that define ϕ, the coupling contributions would cancel each other to give small or zero coupling. Both rotamers are sterically possible and both would fulfill the condition that C3′ and C5′ are at about the same angle with respect to C1. However, from model building, from studies on cellobiose and lactose as mentioned before, from ab initio molecular orbital calculations in related structures (Jeffrey et al., 1978), and from an extension of the exoanomeric effect put forward by Lemieux et al. (1979), conformer D or one close to it in which C4′ is near eclipsed with H1 seems to be most probable since it would permit hydrogen bonding between the 3′-OH and the ring oxygen of the galactosyl moiety and would account for the observed line broadening. Our studies (Nunez & Barker, 1979) with simple glycosides of 1-^{13}C- and 2-^{13}C-enriched monosaccharides also indicate that in these compounds the aglycon C1′ (equivalent to C4′) is generally trans to C2, as inferred from $^2J_{C1-C1'} \geq 1$ Hz and a $^3J_{C2-C1'} \simeq 3$ Hz.

It is noteworthy that, in the reducing disaccharides, the conversion from the α to the β form in the reducing moiety has no effect on the coupling constants observed in the region of the glycosidic bond, indicating that the conformation about the glycosidic linkage is unaffected by this relatively remote change. Similarly, the substitution of Glc for GlcNAc has little effect on the conformation about the glycosidic bond.

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Biosynthesis of Heparin. Hydrogen Exchange at Carbon 5 of the Glycuronosyl Residues†

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ABSTRACT: O-Desulfated heparin is known to incorporate tritium into its glycosyluronic acid moieties when incubated in ^3H_2O with heparosan-*N*-sulfate D-glucuronosyl 5-epimerase. We have now established the location of the incorporated tritium as follows. L-[^{3}H]Idosan and a mixture of D-[^{3}H]-glucose and L-[^{3}H]idose were isolated from enzymatically tritiated O-desulfated heparin by esterification of the carboxyl groups with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, reduction with sodium borohydride, acid hydrolysis, and deaminative cleavage. When the mixture of the two hexoses was converted to methyl [^{3}H]glycosides and subsequently degraded by successive periodate oxidation, hypobromite oxidation, and acid hydrolysis, the only ^{3}H-labeled fragment was glyceric acid. The same result was obtained upon degradation of the L-[^{3}H]idosan, indicating that in both the D-glucuronic acid and L-iduronic acid residues, the tritium was located at C-5. In another approach, D-[^{3}H]glucuronic acid was isolated from the ^{3}H-labeled polysaccharide and was then converted to methyl (methyl D-[^{3}H]glucopyranoside)uronate. On reduction with sodium borohydride in anhydrous methanol, this compound lost its radioactivity and yielded unlabeled methyl α-D-glucopyranoside. This finding indicates that the label was located at C-5, since it was previously shown that the hydrogen in this position is completely exchanged under the reaction conditions used and that hydrogen atoms in other positions are not affected. It is concluded that incubation of O-desulfated heparin in ^3H_2O with heparosan-*N*-sulfate D-glucuronosyl 5-epimerase introduces tritium at C-5 of the glycuronosyl moieties of the substrate and that no exchange of hydrogen atoms at C-2, C-3, or C-4 with protons of the medium or with the C-5 hydrogen occurs during the reaction.

Heparin biosynthesis is initiated by the assembly of monosaccharide units into a polymer composed of alternating D-glucuronosyl and 2-acetamido-2-deoxy-D-glucosyl moieties. This polysaccharide subsequently is modified by successive N-deacetylation, N-sulfation, C-5 D-glucuronosyl epimerization, and finally O-sulfation of most of the L-iduronosyl and 2-deoxy-2-sulfamido-D-glucosyl moieties. The epimerization, catalyzed by heparosan-*N*-sulfate D-glucuronosyl 5-epimerase, is accompanied by an exchange of the C-5 hydrogen atoms with protons of the aqueous medium, as indicated by the loss of radioactivity from heparosan *N*-sulfate specifically labeled with tritium in the C-5 position of the D-glucuronosyl moieties (Lindahl et al., 1976, 1977; Jacobsson et al., 1979). Some direct evidence is available for the reversibility of this process, inasmuch as the enzyme is known to catalyze incorporation of tritium from ^3H_2O into the uronosyl residues of heparan sulfate and a heparin derivative from which the *O*-sulfate groups have been removed (Jensen et al., 1979; I. Jacobsson, U. Lindahl, J. Jensen, L. Rodén, D. S. Feingold, and H. Prihar, unpublished results). However, the exact location of the incorporated tritium has not been established.

In the present investigation, a procedure has been developed for the determination of the position of incorporated tritium, in D-glucuronosyl as well as in L-iduronosyl units. Application of this methodology to enzymatically tritiated O-desulfated heparin shows that radioactivity is present in both D-glucuronosyl and L-iduronosyl groups and, furthermore, that the label is associated with the C-5 position only.

Materials and Methods

Materials. L-[6-^{14}C]Idose, a product of Centre d'études nucléaires de Saclay, France, was converted to L-[6-^{14}C]idosan by the method of Sorkin & Reichstein (1945). D-[U-^{14}C]-Glucose and D-[5-^{3}H]glucose were purchased from the Radiochemical Center, Amersham/Searle, Chicago, IL. Methyl α-D-[U-^{14}C]glucopyranoside and methyl α-D-[5-^{3}H]glucopyranoside were prepared from these compounds by the method of Fischer (1893, 1895). The position of the label in the

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