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METHYL β-LACTOSIDE: 600-MHz ^{1}H- AND 75-MHz ^{13}C-N.M.R. STUDIES OF ^{2}H- AND ^{13}C-ENRICHED COMPOUNDS

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ABSTRACT

Using UDP-D-galactose: 2-acetamido-2-deoxy-D-glucose 4-β-D-galactosyltransferase (EC 2.4.1.22), several methyl β-lactosides have been prepared with ^{2}H- and/or ^{13}C-enrichment at specific sites to facilitate study by ^{13}C (75 MHz) and ^{1}H (600 MHz) n.m.r. spectroscopy. ^{13}C-Chemical shift assignments were verified and the ^{1}H-spectrum of β-lactoside was fully assigned. Sites of enrichment were selected to permit all of the potential three-bond C-C and C-H couplings through the glycosidic bond to be obtained. Replacement of H-3 of the D-glucose residue of methyl β-lactoside with ^{2}H allowed resolution of C-1–H-4′ coupling in the 600-MHz ^{1}H-spectrum. Single or multiple ^{13}C-enrichment at C-1, C-2, C-3, C-1′, C-3′, and/or C-4′ in the disaccharide allowed observation of intra- and inter-residue couplings. ^{13}C-Spin–lattice relaxation-times (T_1) are interpreted in terms of molecular motion in solution. The data suggest that methyl β-lactoside has an extended conformation with little rotation about the glycosidic bond. Inter-residue couplings are best explained by torsion angles of $\phi \sim 40°$ and $\psi \sim 15°$, indicating that the conformations of β-lactoside in solution and in the crystal are similar.

INTRODUCTION

Many biochemical processes involve highly specific binding of proteins to ligands that may be simple molecules or components of complex structures. Such processes are responsible for cell–cell recognition, enzyme–substrate complex formation, antibody–antigen interactions, and the many other "recognition" events that are responsible for biological structure and function. During the past fifty years, studies of the blood-group substances and of bacterial and tissue antigens have demonstrated that complex oligosaccharides are the ligands for many essential recognition events and account for the high degree of specificity displayed in them. These complex oligosaccharides are rich in biological information. Information is contained in the composition of the oligosaccharide, in the sequence of its com-

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ponent monosaccharides, in their anomeric form, and in the position of glycosidic linkages. In addition, the conformations of the monosaccharides and of the glycosidic bonds determine the range of structures to be recognized and refine the information contained in other levels of structure. To fully appreciate the biochemical roles of oligosaccharides, it is necessary to establish the conformations they assume in solution and the ways in which their conformations are affected when "recognition" occurs.

N.m.r. spectroscopy gives four kinds of data reflecting the solution conformations of oligosaccharides, namely, chemical shifts, coupling constants, nuclear relaxation rates, and nuclear Overhauser enhancements (n.O.e.). Chemical shifts are sensitive to those structural variations, including conformations, that affect nuclear shielding, such as the spatial proximity of nuclei to electronegative groups. Coupling constants vary with the geometry of bonds linking the coupled nuclei. ^{13}C-Spin–lattice relaxation-rates ($1/T_1$) vary with the orientation and relative rates of rotation of C-H dipoles in the structure, and ^{1}H-relaxation rates and n.O.e. measurements can be interpreted in terms of internuclear ^{1}H–^{1}H distances.

The application of n.m.r. spectroscopy to studies of solution conformations of oligosaccharides is complicated by several factors. Complex oligosaccharides are difficult to prepare or isolate; ^{13}C–^{1}H couplings can be difficult to observe in ^{1}H-coupled ^{13}C-spectra, and ^{13}C–^{13}C couplings are virtually impossible to observe in compounds with ^{13}C at natural abundance levels (1.1%); and ^{1}H-n.m.r. spectra of oligosaccharides are very complex, even at 300 MHz.

To circumvent these difficulties, we have developed methods for the synthesis of 0.1–1.0 mmol of oligosaccharides of the Type 2, human blood-group[1,2]. To facilitate observation of ^{13}C–^{13}C and ^{13}C–^{1}H coupling, procedures were developed to incorporate isotopes of C, H, and/or O at specific sites in monosaccharides[3,4] which could then be used to prepare specifically enriched oligosaccharides[1,2]. With specific ^{13}C-enrichment, accurate ^{13}C–^{1}H-coupling constants (±0.2 Hz) can be obtained from ^{1}H-spectra, provided the spectra are interpretable. Spectrometers operating at very high fields (500–600 MHz) and ^{2}H-substitution for ^{1}H can be used to simplify otherwise complex spectra. ^{13}C–^{13}C Couplings (>1 Hz) generally are observable in the unenriched portion of the ^{13}C-spectrum or, in difficult cases, can be obtained from doubly enriched compounds[2].

We now report on the preparation of a series of methyl β-lactosides (methyl 4-*O*-β-D-galactopyranosyl-β-D-glucopyranosides) enriched at various positions with ^{13}C and/or ^{2}H. All couplings about the glycosidic bond and the ^{13}C-relaxation times (T_1 values) have been measured and interpreted in terms of the most probable conformation about the glycosidic bond and the motional properties of the molecule in solution.

EXPERIMENTAL

Materials. — Glycolaldehyde, D-arabinose, D-lyxose, ATP, and glycolytic enzymes were purchased from the Sigma Chemical Co. [U-^{14}C]Gal and UDP-[U-^{14}C]Gal were obtained from New England Nuclear Corp. Potassium [^{13}C]cyanide (K^{13}CN), 99.7% pure and 90.7 atom% ^{13}C, was supplied by the Los Alamos Scientific Laboratory, University of California, Los Alamos, NM. D-Glyceraldehyde[5] and D-threose[6] were prepared from D-fructose and 4,6-*O*-ethylidene-D-galactose, respectively. The bovine galactosyltransferase was purified by the method of Barker *et al.*[7].

^{13}C- and ^{2}H-enriched hexoses. — D-[1-^{13}C]Gal and D-[1-^{13}C]Glc were synthesized as described previously[3]. D-[2-^{13}C]Gal was synthesized by the addition of cyanide to D-[1-^{13}C]lyxose[3], which was prepared from D-threose as described previously[3]. D-[1,3-^{13}C; 3-^{2}H]Gal and D-[1,3-^{13}C; 3-^{2}H]Glc were similarly prepared by the addition of K^{13}CN to D-glyceraldehyde in $^{2}H_2O$ and reduction with Pd/$^{2}H_2$, followed by separation of the [1-^{13}C,^{2}H]-enriched tetroses[4,8]. Two additional cycles of cyanide addition, reduction with $^{1}H_2$, and separation, using first K^{12}CN and then K^{13}CN, gave the desired products. D-[4-^{13}C]Glc was prepared enzymically from D-[4-^{13}C]fructose by the sequential action of hexokinase (EC 2.7.1.1), phosphoglucose isomerase (EC 5.3.1.9), and potato acid phosphatase (EC 3.1.3.2). D-[4-^{13}C]Fructose was prepared from DL-[1-^{13}C]glyceraldehyde and dihydroxyacetone phosphate by the action of muscle aldolase (EC 4.1.2.13) and potato acid phosphatase. DL-[1-^{13}C]Glyceraldehyde was produced as a racemic mixture from glycolaldehyde as described previously[8]. Dihydroxyacetone phosphate was prepared from dihydroxyacetone (1,3-dihydroxypropanone) and Mg^{2+}-ATP by the action of glycerolkinase (EC 2.7.1.30). Details of the method will be described elsewhere.

Methyl β-D-galacto- and -gluco-pyranosides. — A solution of the ^{13}C- and/or ^{2}H-enriched hexose (1 mmol) in 10 mL of anhydrous methanol was made 25mM in H_2SO_4 and boiled under reflux until the amount of β-pyranoside reached a maximum, as assessed by g.l.c. of the trimethylsilyl ethers. The β-pyranoside was isolated by chromatography on Dowex 1-X2 (HO$^-$) resin, as described by Austin *et al.*[9].

α-D-Galactose 1-phosphate. — Yeast galactokinase (EC 2.7.1.6), partially purified through the DEAE-cellulose chromatography step of the procedure of Schell and Wilson[10], was used with Mg^{2+}-ATP to phosphorylate ^{13}C- and/or ^{2}H-enriched D-galactose. An aqueous solution of the crude galactokinase (40 mL) from 4 g of dried yeast (galactose-adapted *Kluyveromyces fragilis*, Sigma) was stored in 2-mL aliquots at —20°. The enzyme preparation contains adenylyl kinase, so that the conversion can be accomplished by using slightly more than 50% of the stoichiometric amount of ATP.

The reaction mixture contained 1.0 mmol of enriched D-Gal, 0.6 mmol of ATP, 0.17 mmol of MgCl$_2$, 33 μmol of dithiothreitol, 0.1 mmol of NaF, and 1.65 mmol of triethanolamine–acetate (pH 8.0), and 2.0 mL (~7 units) of galactokinase in a total volume of 33 mL. After 12 h at 33°, the mixture was diluted to 150 mL and applied rapidly to a column (2.5 × 10 cm) of Dowex 1-X2 (HCO$_3^-$) resin (200–400 mesh) equilibrated with 0.02M TEAB (pH 7.5). A 1-L linear gradient of 0.02→0.4M TEAB (pH 7.5) was started immediately, and 8-mL fractions were collected at a flow rate of 2 mL/min. α-D-Gal 1-phosphate was eluted in fractions 81–93, and was obtained as its triethylammonium salt by repeated concentration *in vacuo* from H_2O; yield, 80%.

^{13}C- and ^{2}H-enriched UDP-Gal. — The phosphoromorpholidate procedure of Moffatt[11], as modified by Kochetkov *et al.*[12], was used to prepare ^{13}C- and/or ^{2}H-enriched sugar nucleotides.

Methyl β-lactosides. — These were prepared and isolated as described previously[1], with the following modifications. The initial concentration of UDPGal was increased to 20mM and that of the acceptor (methyl β-D-glucopyranoside) to 100mM. The following methyl β-disaccharides were prepared in 0.1–0.2 mmol amounts: βD-Gal(1→4)βD-Glc, βD-Gal(1→4)βD-[4-^{13}C]Glc, βD-[2-^{13}C]Gal(1→4)βD-Glc, βD-[2-^{13}C]Gal(1→4)βD-[4-^{13}C]Glc, and βD-[1,3-^{13}C;3-^{2}H]Gal(1→4)βD-[1,3-^{13}C;3-^{2}H]Glc.

Instrumentation. — Carbon-13 n.m.r. spectra were obtained at 75 MHz with a Bruker WM-300 superconducting FT spectrometer equipped with quadrature detection. Spectra were obtained at ~35° with 16 K real spectral points, and the spectrometer was locked to the resonance of $^{2}H_2O$ in the sample. Exponential multiplication employed in the Fourier transformation was selected to yield line-broadenings of 0.2 Hz. Chemical shifts (±0.15 p.p.m.) are given relative to internal Me_4Si by setting spectral parameters to give anomeric resonances of α- and β-D-[1-^{13}C]glucopyranose at 93.6 and 97.4 p.p.m. respectively[13].

Proton n.m.r. spectra were obtained at 600 MHz with the MPC-HF superconducting spectrometer at the NMR Facility for Biomedical Studies in the Department of Chemistry at Carnegie–Mellon University, Pittsburgh, PA. Correlation spectra were obtained with a homonuclear lock (H_2O) and were enhanced to improve resolution. Chemical shifts (±0.01 p.p.m.) are given relative to internal sodium 3-(trimethylsilyl)-1-propanesulfonate.

Samples (0.2M) were treated with Chelex 100 and flushed with nitrogen. For ^{13}C spin-lattice relaxation-rates, samples were made 50mM in acetate buffer (pH 5.0) and 3mM in ethylenediaminetetra-acetate in 50% $^{2}H_2O$, and flushed with nitrogen.

RESULTS AND DISCUSSION

Methyl β-lactoside was selected for study of solution conformation because of its intrinsic value as a model for other glycosides, and because it resembles *N*-acetyl-lactosamine, the core structure of the ABO blood-group system. The same enzyme (UDP-D-galactose:2-acetamido-2-deoxy-D-glucose 4-β-D-galactosyltransferase) can be used to make both disaccharides, and both can serve as substrates for the 2-α-L-fucosyltransferase which forms the αL-Fuc(1→2)βD-Gal-linked trisaccharides of the ABO Type 2 group. Studies, therefore, can be extended to the trisaccharide and higher oligosaccharides in the series.

Crystalline methyl β-D-lactoside has[16] an extended conformation with torsion angles $\phi \simeq 45°$ and $\psi \simeq 15°$. A similar conformation has been proposed for cellobiose[17] and for *N*-acetyl-lactosamine[1,14] in aqueous solution. In an earlier study[1], inter-residue coupling was used to infer the solution conformation of the glycosidic bond. The torsion angle ϕ was defined by $^2J_{C-1,C-4'}$ and consideration of steric factors, and ψ was defined by $^3J_{C-1,C-3'}$ and $^3J_{C-1,C-5'}$. The conformation deduced was in good agreement with that predicted by hard-sphere calculations of non-bonded interaction energies[14]. The present study was undertaken to obtain all of the potentially useful couplings about the glycosidic bond and to measure ^{13}C-spin–lattice relaxation-rates which can be interpreted in terms of molecular motion[15].

The questions addressed, concerning methyl β-D-lactoside in solution, are: (*1*) are the conformations of the monosaccharide units the same in the disaccharide as in simple glycosides? (*2*) does the disaccharide exist in a stable conformation or is rotation about the glycosidic bond rapid? (*3*) if the conformation about the glycosidic bond is relatively stable, what is it?

Much of what is known about the conformations of sugars in solution comes from the evaluation of ^{1}H–^{1}H, three-bond coupling-constants. In oligosaccharides, the evaluation of these couplings can be difficult due to resonance overlap. Further, ^{1}H–^{1}H coupling across glycosidic bonds involves pathways with at least four bonds between the coupled nuclei. For these reasons, ^{13}C–^{13}C and ^{13}C–^{1}H couplings become important indicators of both intra- and inter-residue conformations. Although ^{13}C–^{1}H coupling can be obtained from ^{1}H-coupled ^{13}C-spectra, they can be obtained more accurately and more readily from ^{1}H-spectra of specifically ^{13}C-enriched compounds. ^{13}C–^{13}C Coupling generally is not observable in natural-abundance compounds, but can be obtained from the natural abundance portion of the spectrum of specifically ^{13}C-enriched compounds. In fact, if two sites are enriched, coupling between them can be observed with concentrations in the range needed for studies of protein–ligand interactions.

Based on these considerations, our approach has been to prepare specifically ^{2}H- and ^{13}C-enriched compounds, and to use high-field n.m.r. spectrometry so that critical intra- and inter-residue coupling constants can be measured.

The assignment of chemical shifts is an essential prerequisite to other applications of n.m.r. spectroscopy. Previous ^{13}C-chemical shift assignments of the compounds used in this study[1,13,14,18] are confirmed here by observations with ^{13}C-enriched compounds[13]. ^{1}H-Chemical shifts are listed in Table I; these assignments can be made with certainty, because of ^{1}H–^{13}C couplings in specifically enriched

TABLE I

^{1}H-CHEMICAL SHIFTS OF METHYL β-D-GLYCOPYRANOSIDES IN ^2H_2O

Compound	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6a*	*H-6b*	*CH*$_3$
Gal	4.30	3.48	3.63	3.90	3.68	3.78	3.73	3.56
Glc	4.36	3.24	3.48	3.36	3.44	3.91	3.70	3.55
Lactose–Gal	4.43	3.52	3.64	3.91	3.70	3.77	3.73	—
Lactose–Glc	4.39	3.29	3.63	3.63	3.58	3.97	3.79	3.56

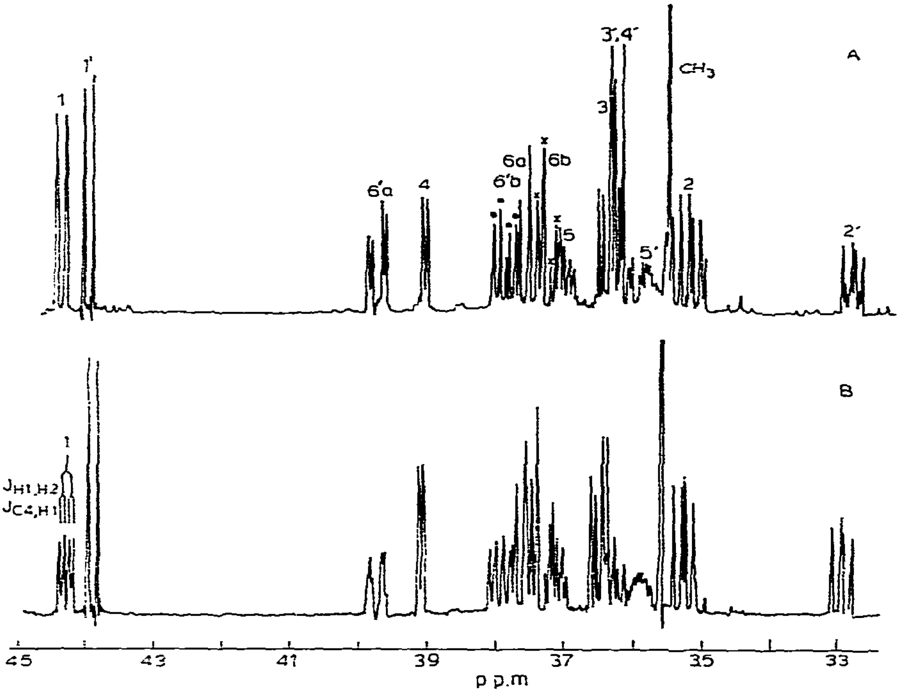


Fig. 1. 600-MHz, ^{1}H-N.m.r. spectra of A, methyl β-lactoside (each ^{1}H multiplet was assigned as indicated, and the resonances of H-6'b and H-6b are labeled (●) and (X), respectively); and B, βD-Gal(1→4)βD-[4-^{13}C]Glc1Me (^{13}C-enrichment at C-4 of Glc permits measurement of the three-bond, inter-residue, C-4'-H-1 coupling-constant). Each spectrum was obtained after 50 transients with a spectral width of 770 Hz at 0.124 Hz/data point.

compounds. The ^{1}H-chemical shifts for methyl β-D-glucopyranoside agree closely with those reported by Perkins *et al.*[18], and those for methyl β-D-galactopyranoside with the values reported by Lemieux *et al.*[14]. For methyl β-lactoside, all ^{1}H-resonances are moved downfield (deshielded), with H-4 being most strongly affected (0.27 p.p.m.) and H-1 least (0.03 p.p.m.). For the Gal residue of the disaccharide, only H-1 is strongly deshielded (0.13 p.p.m.) relative to methyl β-D-galactopyranoside, probably due to interaction with O-3 and O-6 of Glc.

For methyl β-lactoside, the resonance for H-1 of Gal is downfield of H-1 of Glc, whereas, in the monosaccharide methyl glycosides, the reverse is the case. Without specific ^{13}C-enrichment, the opposite assignment might have been made. This observation points again to the great care that must be exercised in making assignments based on spectral similarities alone.

Intra-residue conformations. — The conformations of the Gal and Glc components of methyl β-lactoside can be compared with those of the parent methyl glycosides on the basis of three-bond ^{1}H–^{1}H and two- and three-bond ^{13}C–^{1}H coupling-constants. All of the intra-residue $^3J_{H\text{-}H}$ values could be obtained from the 600-MHz ^{1}H-spectra (Fig. 1). In addition, ^{13}C–^{1}H and ^{13}C–^{13}C couplings involving C-1, C-2,

TABLE II

INTRA-RESIDUE ^{1}H–^{1}H COUPLING CONSTANTS[a]

Coupled nuclei	*Methyl β-D-glycopyranoside*		*Methyl β-lactoside*	
	Gal	*Glc*	*Gal*	*Glc*
1–2	7.9	8.0	7.8	8.0
2–3	9.9	9.4	10.0	9.5
3–4	3.5	9.1	3.5	[b]
4–5	1.1	10.0	1.0	9.9
5–6a	7.9	2.3	8.2	2.2
5–6b	4.3	6.1	3.8	5.1
6a–6b	−11.7	−12.4	−11.8	−12.3

[a]Accurate to ±0.2 Hz. [b]Resonance overlap precludes analysis.

TABLE III

INTRA-RESIDUE ^{13}C–^{13}C COUPLING CONSTANTS[a]

Coupled nuclei	*Methyl β-D-glycopyranoside*		*Methyl β-lactoside*	
	Gal	*Glc*	*Gal*	*Glc*
One bond				
1–2	46.7	46.8	46.7	[b]
2–3	39.8	38.9	39.8	[b]
3–4	38.5	39.3	38.8	39.0
4–5	—	41.0	—	41.8
Two bond				
1–3	4.6	4.4	4.8	4.7
1–5	0	0	0	0
2–4	0	2.8	0	2.2
3–5	1.4	2.5	1.5	
4–6	—	0	—	0
1–Me	1.9	2.0	—	2.2
Three bond				
1–4	0	0	0	0
1–6	4.3	4.1	4.3	4.0
2–5	0	—	0	—
3–6	4.2	4.1	4.3	4.0
2–Me	3.0	—	—	—

[a]Accurate to ±0.2 Hz. [b]Resonance overlap precludes analysis.

and C-3 of the Gal residue and C-1′, C-3′, and C-4′ of the Glc residue were obtained (Tables II–IV). Only a small number of differences in coupling constants exist between the methyl glycopyranosides and the disaccharide, and these are unlikely to reflect substantial changes in intra-residue conformations.

TABLE IV

INTRA-RESIDUE, ^{13}C–^{1}H COUPLING-CONSTANTS[a]

Coupled nuclei	*Methyl β-D-glycopyranoside*		*Methyl β-lactoside*	
	Gal	*Glc*	*Gal*	*Glc*
C-1–H-1	160.7	160.7	161.9	161.0
C-2–H-2	148.6	—	148.6	—
C-4–H-4	—	146.4	—	147.5
C-1–H-2	6.1	6.3	6.2	6.4
C-2–H-1	broad	—	0.5	—
C-2–H-3	[b]	—	[b]	—
C-3–H-2	5.1	4.9	5.0	5.2
C-3–H-4	4.5	4.5	4.6	4.5[c]
C-4–H-3	—	[b]	—	5.0
C-4–H-5	—	[b]	—	
C-1–H-3	1.3	1.2	—	—
C-1–H-5	2.5	2.4	[d]	[d]
C-1–Me	4.5	4.5	—	4.6
C-2–H-4	5.5	—	5.0 ±0.3	—
C-3–H-1	1.2	1.2	1.1	1.1
C-3–H-5	[d]	2.5	[d]	[d]
C-4–H-2	—	0	—	0.5
C-4–H-6[a]	—	2.3	—	2.8
C-4–H-6[b]	—	1.1	—	0.5

[a]Accurate to ±0.2 Hz unless otherwise indicated. [b]Resonance overlap precludes analysis. [c]Tentative assignment, may be 4.9 Hz (assigned to C-1–H-4′). [d]Envelope of more than 12 lines obscures couplings.

The differences in $^{3}J_{H,H}$, all involving H-6a and H-6b in the Gal and Glc residues of the disaccharide (Table II), are probably due to changes in the rotational freedom of the hydroxymethyl groups (see below).

Significant changes occur in $^{2}J_{C\text{-}2,C\text{-}4}$ for Glc, which decreases from 2.8 to 2.2 Hz, and in $^{3}J_{C\text{-}4,H\text{-}2}$ for Glc, which increases from 0 to 0.5 Hz in the disaccharide. A similar change occurs in $^{1}J_{C\text{-}1,H\text{-}1}$ for Gal, which increases from 160.7 to 161.9 Hz in the disaccharide. These small changes probably are due to the substitution of Gal for H at C-4 of Glc, and of Glc for Me at C-1 of Gal.

Molecular motion. — The motion of a molecule in solution can be considered in terms of the overall motion of the molecule and in terms of the relative motion of its parts with respect to each other. The overall motion of a (1→4)-linked disaccharide having an extended conformation can be anisotropic by rotation about the long axis or one of the two short axes of the molecule, or isotropic, with no preferred axis of motion. Within the molecule, relative motion can involve the monosaccharide residues and the aglycon (if any), which can rotate with respect to each other about the glycosidic bond(s). In addition, in hexopyranosides, the hydroxymethyl groups can rotate about the C-5–C-6 bonds. Each type of motion is important to the evaluation

TABLE V

^{13}C-RELAXATION TIMES FOR METHYL β-D-LACTOSIDE[a]

	Gal					*Glc*						
	1	*2*	*3*	*4*	*5*	*6*	*1*	*2*	*3*	*4*	*5*	*6*
T_1 (s)	0.64	0.66	0.67	0.51	0.64	0.44	0.73	0.69	0.70	0.66	0.66	0.33
n.O.e. (±10%)	1.9	2.2	2.1	2.2	2.1	2.0	2.0	2.1	2.1	1.8	2.0	2.1

[a] At 38±1°, times are accurate to ±5%.

of conformation and each can be assessed by measurement of ^{13}C-spin–lattice relaxation-rates.

The ^{13}C-relaxation in the glycosides used in this study occurs through dipolar interactions with directly bonded protons. Each carbon is bonded to at least one proton and each exhibits full n.O.e. (Table V)[15]. For compounds of this type, ^{13}C-spin–lattice relaxation-rates are proportional to the number of directly bonded protons and the correlation time of the C-H vector, and inversely proportional to the sixth power of the C-H bond-length. Molecular motion generates constantly changing angles between the C-H dipoles and the applied field, resulting in fluctuating magnetic fields that are responsible for relaxation. When motion is isotropic, the resultant local fields are the same for each ^{13}C-nucleus and all relax at the same rate if the dipolar mechanism predominates and if all C-H bonds are the same length. Anisotropic motion results in different ^{13}C-nuclei having different relaxation rates. Those whose C-H dipoles align with a preferred axis of rotation relax more rapidly than those with C-H dipoles more nearly normal to that axis. Thus, provided the dipolar mechanism predominates, that non-bonded, dipolar interactions are not significant, and that C-H bond-lengths are the same, differences in ^{13}C-spin–lattice relaxation-rates can be interpreted in terms of anisotropic motion.

In methyl β-lactoside, all C-H bonds except H-4 of Gal are axial and their C-H dipoles make the same angle with respect to the planes of the monosaccharide rings. As the compound rotates in solution, the equatorial C-4–H-4 bond-dipole of Gal should cause C-4 of Gal to relax at a rate different from that of the other ring carbons if rotation is anisotropic. If, on the other hand, rotation is isotropic, C-4 of Gal should relax at the same rate as other ring carbons, provided that the C-4–H-4 bond is the same length as the C-H bonds of other carbons. The data in Table V show that all of the ring carbons of methyl β-lactoside relax at similar rates, except for C-4 of Gal which relaxes faster. As stated by Berry *et al.*[15], this difference suggests that the C-4–H-4 dipole is more nearly aligned with a preferred axis of rotation and that the disaccharide tumbles as a fairly rigid structure, at least in part anisotropically, with a principal axis of rotation roughly parallel to the C-4–H-4 Gal bond. This interpretation is based on the assumption that the C-4–H-4 bond-length is not substantially less than other C-H bond-lengths in the molecule.

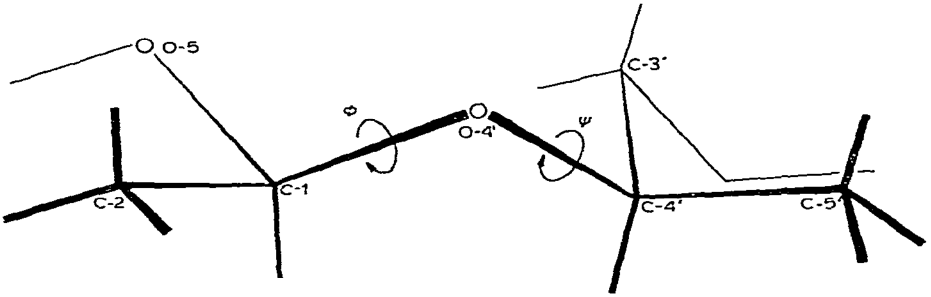
The similarity of T_1 values for the ^{13}C-nuclei of the ring atoms with axial hydrogens suggests that the two rings rotate at the same rate. Although the relaxation rates would be similar if the two rings rotated at the same rate but in opposite directions, this possibility, which involves substantial energy-barriers, seems remote. The data in Table V indicate that the T_1 values for the ring atoms of Gal with axial hydrogens are slightly shorter than those of Glc. The difference is small but reproducible. It may be due to greater motion in the Glc residue, differences in C-H bond-lengths, or, as is more likely, to a difference in the C-H dipole orientations of the two residues with respect to the principal axis of rotation. If the last possibility applies, the C-H dipoles in the Glc residue must be more nearly normal to the axis of rotation than those in the Gal residue.

The magnitudes of coupling constants between nuclei linked through the glycosidic bond also provide information on the dynamics of rotation about that bond. If rotation occurs at a rapid and constant rate, couplings between C-1 and C-3′, C-1 and C-5′, and C-1 and H-4′ would all have values that are the average of the appropriate Karplus curve. If rotation is rapid, with discrete energy minima, and not of constant rate, coupling values would be the time-weighted average of contributing values. In the case of slow rotation, with long residence times at one or more preferred conformations, the molecule can be thought of as existing in one or more stable conformations that may be observable in the n.m.r. spectrum. The values observed are ${}^3J_{\text{C-1,C-3}'}$ 0 Hz, ${}^3J_{\text{C-1,C-5}'}$ 1.3 Hz, and ${}^3J_{\text{C-1,H-4}'}$ 4.9 Hz. A coupling of 0 Hz can be achieved only if the majority of contributing conformations involve a dihedral angle close to ±90°. If other conformers contributed significantly, ${}^3J_{\text{C-1,C-3}'}$ values greater than 0 would be expected. A similar argument can be made with respect to ${}^3J_{\text{C-1,H-4}'}$, which has a value close to the maximum[20], indicating that conformers other than those with dihedral angles close to 0 or 180° make minor contributions. Thus, the observation of coupling constants that differ greatly from the average expected in a freely rotating system implies either rapid rotation with long dwell-times in one (or a few) stable conformations, or a relatively stable conformation with vibrational motion about the most stable arrangement.

In methyl β-lactoside, two conformations can give approximately the same couplings between C-1 and C-3′, C-5′, and H-4′. One of these creates strong, steric interactions between the Gal ring-oxygen and the hydroxymethyl group of Glc. In this case, then, the observation of distinctly non-average coupling-constants implies the existence of a single, relatively stable conformation about the C-4′–O-4′ bond.

Unfortunately, a similar assessment cannot be made of the conformation about the C-1–O-4′ bond. Only two couplings can be observed (${}^3J_{\text{C-2,C-4}'}$ and ${}^3J_{\text{C-4}',\text{H-1}}$) and both have values that could arise from conformational mixing. However, rotation about this bond would increase T_1 values for Gal relative to Glc. The opposite effect is observed (Table V). Thus, the T_1 values and coupling data both support the conclusion that methyl β-lactoside has a fairly stable conformation about the glycosidic bond.

Conformation of the glycosidic bond. — Two angles (ϕ and ψ) determine the conformation about glycosidic bonds, as shown in Scheme 1. Estimates of these angles can be made from coupling constants between nuclei linked by the bond. The three-bond couplings that relate to ϕ and ψ for a β-(1→4) linkage are listed in Scheme 1 with the values obtained in this study.



ϕ = 40°: $^3J_{C\text{-}4',H\text{-}1}$ = 38Hz and $^3J_{C\text{-}2,C\text{-}4'}$ = 3.1Hz
ψ = 15°: $^3J_{C\text{-}1,H\text{-}4'}$ = 49Hz, $^3J_{C\text{-}1,C\text{-}3'}$ = 0 Hz, and $^3J_{C\text{-}1,C\text{-}5'}$ = 16 Hz

Scheme 1

Interpretation of ^3J values requires measurement of couplings in standard compounds of fixed geometry in order to establish the relationship of dihedral angle to ^3J. For glycosides, the coupling pathways (either COCC or COCH) involve oxygen, and examples with fixed geometry are few. Satisfactory models are provided by the [1-^{13}C]-enriched hexopyranoses and their simple glycosides[13]. Probable maximum values of $^3J_{COCC}$ can be obtained from coupling between C-1 and C-6, which are antiperiplanar. Values of $^3J_{COCH}$ at 60° can be obtained from C-1–H-5 couplings in hexopyranosides[13,21,22] and $^3J_{COCH}$ at 120° from C-1–H-5 couplings in pentopyranosides. In addition, C-1–H-4 and C-1–C-5 couplings for angles in the range 90–150° can be observed in the furanosides[23]. The values obtained in this study, taken with those reported previously, show that the COCC and COCH couplings both follow the Karplus relationship[24] with ^3J values ranging from 4.4 to 0 Hz and 6.0 to 0 Hz, respectively. It is possible, therefore, to assign dihedral angles (±10°) from observed ^3J values.

The torsion angle ϕ, which specifies the conformation about the C-1–O-4' bond, is reflected in two coupling constants, $^3J_{C\text{-}4',H\text{-}1}$ 3.8 Hz and $^3J_{C\text{-}2,C\text{-}4'}$ 3.1 Hz. The first of these is not easily obtained with the natural-abundance compound. Although the C-4' resonance is well separated from those of other carbons, the ^{1}H-coupled resonance is split by H-3', H-4', H-5', H-6'a, and H-6'b, as well as by H-1. In the [4'-^{13}C]-enriched compound, however, the H-1 resonance is easily observed, as is coupling to C-4' (*cf.* Figs. 1A and 1B). The value of $^3J_{C\text{-}4',H\text{-}1}$ corresponds to a C-4'–H-1 dihedral angle of 40 ±10°.

Coupling between C-2 and C-4' was observable in the 75-MHz, ^{13}C-n.m.r. spectra of the [2-^{13}C]-, [4'-^{13}C]-, and [2,4'-^{13}C]-enriched lactosides. Values of $^3J_{C\text{-}2,C\text{-}4'}$ were 3.0, 3.1, and 3.1 Hz, respectively. These couplings correspond to a C-2–C-4' dihedral angle of 150 ±10°.

The difference between the C-4'–H-1 and C-2–C-4' dihedral angles should

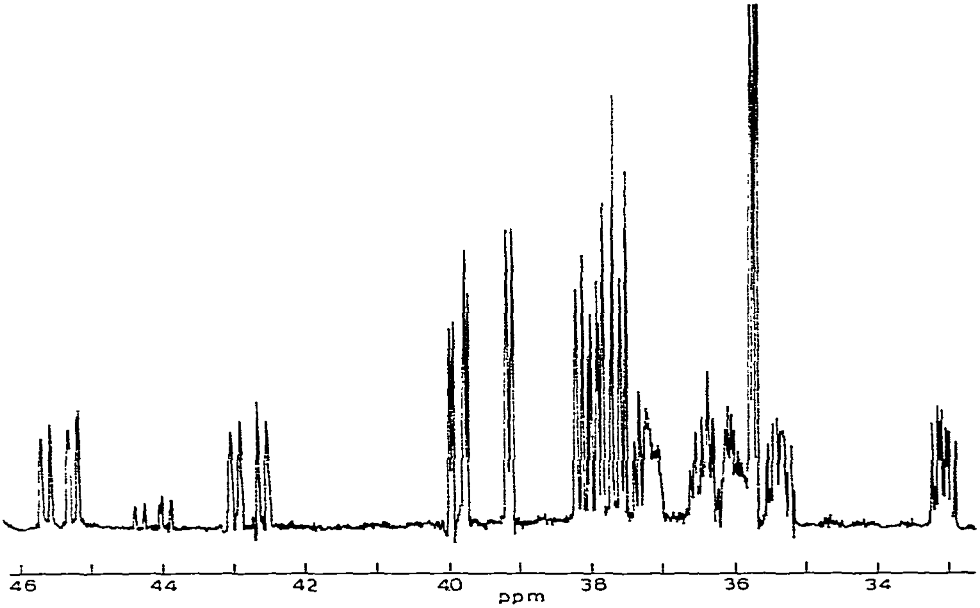


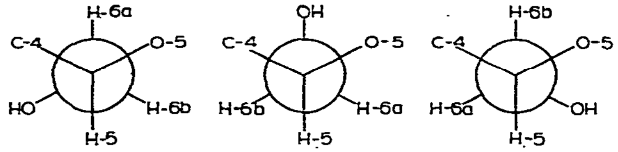
Fig. 2. 600-MHz, ^{1}H-N.m.r. spectrum of βD-[3-^{2}H;1,3-^{13}C]Gal(1→4)βD-[3-^{2}H;1,3-^{13}C]Glc1Me. Deuteration at C-3′ simplifies the spectrum, so that resonances due to H-4′, centered at 3.63 p.p.m., are observed. This allows measurement of the three-bond, inter-residue, C-1–H-4′ coupling-constant.

be 120°, and the experimental observations are in good agreement with this expectation. The alternative conformation, in which the dihedral angles are C-4′–H-1 ~140° and C-2–C-4′ ~20°, is strongly sterically hindered. The couplings obtained in this study indicate that the torsion angle ϕ is ~40°, in good agreement with conclusions drawn earlier that ϕ is ~50° for analogous disaccharides[1,14,17].

Three three-bond coupling-constants define the torsion angle ψ. They arise from coupling between C-1 and H-4′, C-3′ and C-5′. Previously, it was reported[1] that $^3J_{\text{C-1,C-3}'} = 0$ Hz and $^3J_{\text{C-1,C-5}'} \simeq 1.5$ Hz. In this study, we find $^3J_{\text{C-1,C-3}'} = 0$ Hz and $^3J_{\text{C-1,C-5}'} = 1.6$ Hz in spectra in which couplings of 1 Hz are resolvable. The third constant, $^3J_{\text{C-1,H-4}'} = 4.9$ Hz, was obtained from the 600-MHz ^{1}H-spectrum of a multiply enriched lactoside, βD-[3-^{2}H;1,3-^{13}C]Gal(1→4)βD-[3-^{2}H;1,3-^{13}C]-Glc1Me (Fig. 2). This compound was prepared to uncover the H-4′ resonance which is overlapped by that of H-3′ in the unenriched sample. The H-4′ resonance occurs as a well-resolved multiplet due to splitting by ^{13}C-3′ (4.5 Hz) and H-5′ (9.9 Hz), as well as by C-1. Couplings were assigned on the basis of those observed in methyl βD-[3-^{2}H;1,3-^{13}C]glucopyranoside, where $^2J_{\text{C-3,H-4}} = 4.5$ Hz and $^3J_{\text{H-4,H-5}} = 10.0$ Hz.

The observed couplings fit best with dihedral angles between C-1–C-3′ of 105°, C-1–C-5′ of 135°, and C-1–H-4′ of 15°. These values give $\psi = 15°$, in good agreement with conclusions drawn earlier[1,14,17].

Conformation of hydroxymethyl groups. — The hydroxymethyl group of gluco-



	A	B	C	$\frac{B+C}{2}$	Obs.
$J_{H-5,H-6a}$	106[a]	22[a]	22	22	23
$J_{C-4,H-6a}$	10	46	.10	28	23
$J_{H-5,H-6b}$	3.0	2.2	106	64	6.1
$J_{C-4,H-6b}$	4.6	10	1.1	1.1	1.1

[a] Values taken from ref. 24.

Fig. 3. Rotamers about the C-5–C-6 bond of the hydroxymethyl groups in β-D-hexopyranosides.

pyranose and simple glucopyranosides has been proposed to alternate between two relatively stable conformations[25,26] (Fig. 3, B and C). The 600-MHz spectra give $^3J_{H-5,H-6}$ values of 2.3 and 6.1 Hz for methyl β-D-glucopyranoside and 2.2 and 5.1 Hz for the Glc residue in the lactoside. We have also obtained $^3J_{C-4,H-6}$ couplings of 2.3 and 1.1 Hz from the ^{1}H-spectrum of the [4-^{13}C]-enriched compounds. In methyl β-D-glucopyranoside, H-6a, the downfield C-6 proton, is coupled to both H-5 and C-4 by ~2.3 Hz, whereas H-6b, the upfield C-6 proton, is coupled to H-5 and C-4 by 6.1 and 1.1 Hz, respectively. The observation of these paired couplings allows the H-6 protons to be identified. Thus, H-6a is the "equatorial" proton when the C-6–O-6 bond bisects the O-5–H-5 angle (Fig. 3C). The values of the four coupling constants indicate that rotamers B and C contribute approximately equally to the observed coupling in both methyl β-D-glucopyranoside and methyl β-lactoside (Fig. 3).

For the Gal residue, only $^3J_{H-5,H-6a}$ and $^3J_{H-5,H-6b}$ are available (Table I). The values observed (~8.0 and ~4.0 Hz) indicate that either rotamer A or C of Fig. 3 predominates.

In A, H-6a would be strongly coupled to H-5, whereas H-6b would be strongly coupled in C. If both rotamers contribute equally, both couplings should be 6.4 Hz. Although the data indicate that one rotamer predominates, there is little basis for selecting one rotamer over the other, since neither exhibits strong steric repulsions between HO-6 and HO-4. Usually, resonances of protons *syn* to a substituent are shifted upfield[26]. In rotamer A, H-6a is *syn* to C-4 and O-5, whereas H-6b is *syn* only to O-5. In rotamer C, the reverse is true. Thus, if rotamer A predominates, the resonance at higher field should have the larger H-5–H-6 coupling; it does not. DeBruyn and Anteunis[26] have proposed that this anomaly is due to deshielding by HO-4, which is quasi-*syn*-axial to H-6a in rotamer A and to H-6b in rotamer B. They proposed that rotamer A is favored on the basis of an unfavorable interaction of the dipoles of the C-6–O-6 and C-1–O-1 bonds in C. This interaction would be weak and perhaps less significant than the *gauche* interaction between the C-6–O-6 and C-5–O-5 bonds in rotamer C. It appears that either rotamer A or C is possible, although A may be favored. Simultaneous observation of ${}^{3}J_{\text{H-5,H-6ab}}$ and ${}^{3}J_{\text{C-4,H-6ab}}$ would resolve the matter.

Couplings of H-6a and H-6b are best interpreted as indicating that the hydroxymethyl group of Glc is oscillating rapidly about the C-5–C-6 bond between rotamers B and C, whereas, in Gal, the average conformation more closely resembles rotamer A. This conclusion implies that there is more motion in the Glc hydroxymethyl group than in that of Gal. On this basis, T_1 for C-6 of Gal should be shorter than T_1 for C-6 of Glc. The reverse is observed (Table V). However, as pointed out above, ^{13}C-relaxation rates are determined in part by the angular motion of C-H dipoles and bond lengths, so that differences of this kind are difficult to interpret. It is also possible that the Gal hydroxymethyl group oscillates more rapidly than that of Glc, but with a smaller amplitude, causing it to relax more slowly.

In this study, we have drawn conclusions about the conformation and motional properties of methyl β-lactoside in solution from coupling constants and ^{13}C-T_1 values. Proton n.O.e. and T_1 values, from which distances between ^{1}H nuclei can be derived[14], would provide additional probes of conformation. Such studies may only be possible at 600 MHz since, at 300 MHz, the ^{1}H-spectrum of methyl β-D-lactoside is complex; H-3′, H-4′, H-5′, H-3, and H-5 resonate in the range 3.57–3.70 p.p.m. Although ^{1}H-T_1 values in complex spectra can be obtained using the INEPT pulse sequence, satisfactory assessment of n.O.e. and T_1 values may require compounds specifically enriched with ^{2}H and ^{13}C to facilitate observation.

In summary, methyl β-lactoside in aqueous solution has a conformation similar to that in the crystalline state. It is a relatively rigid molecule that rotates to some degree anisotropically about its long axis. Within the time scale of n.m.r. observations, it has restricted rotation about the glycosidic bond which has torsion angles $\phi \simeq 40°$ and $\psi \simeq 15°$, as shown in Scheme 1.

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