Synthesis and Solution Conformation of the Type 2 Blood Group Oligosaccharide αLFuc(1→2)βDGal(1→4)βDGlcNAc†

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ABSTRACT: Partially purified glycosyltransferases and chemically synthesized sugar nucleotides have been used to prepare a number of oligosaccharides related to the type 2 (human) blood group (H) substance. Specific ^{13}C enrichment of the Gal and Fuc residues of the trisaccharide αLFuc(1→2)-βDGal(1→4)βDGlcNAc-hexanolamine has been used to evaluate both intra- and interresidue conformations in solution. Bovine *N*-acetylglucosamine β(1→4) galactosyltransferase and porcine β-galactoside α(1→2) fucosyltransferase were partially purified by affinity chromatography on UDP-agarose and GDP-agarose, respectively, to give preparations that could be stored for months. UDP-[1-^{13}C]Gal and GDP-[1-^{13}C]Fuc were chemically synthesized. The following oligosaccharides were prepared in 20–50-mg amounts and purified by ion-exchange and gel-filtration chromatography: αLFuc(1→2)-βDGal(1→4)βDGlcNAc-hexanolamine, αLFuc(1→2)βD[1-^{13}C]Gal(1→4)βDGlcNAc-hexanolamine, αL[1-^{13}C]Fuc(1→2)βD[1-^{13}C]Gal(1→4)βDGlcNAc-hexanolamine, αL[1-^{13}C]-Fuc(1→2)βD[1-^{13}C]Gal(1→4)βDGlcNAc, αLFuc(1→2)βD-[1-^{13}C]Gal(1→4)βDGlcNAc, αL[1-^{13}C]Fuc(1→2)βD[1-^{13}C]-Gal(1→4)βDGlc, αLFuc(1→2)βD[1-^{13}C]Gal-hexanolamine, αL[1-^{13}C]Fuc(1→2)βD[1-^{13}C]Gal-ethanol, αLFuc(1→2)βD-[1-^{13}C]Gal-ethanol, αL[1-^{13}C]Fuc(1→2)βDGal-ethanol, and αLFuc(1→2)βD[2-^{13}C]Gal-ethanol. βDGal(1→4)DGlcNAc disaccharides were prepared essentially as described previously [Nunez, H. A., & Barker, R. (1980) *Biochemistry 19*, 489]. Fucosylated oligosaccharides were prepared with a 2-fold excess of acceptor over GDPFuc and partially purified β-galactoside α(1→2) fucosyltransferase in sodium cacodylate buffer, pH 6.0, with 2 mM $MnCl_2$ at 33 °C. After 24 h of incubation, yields of the fucosylated compounds were typically 80%. The remaining GDPFuc was hydrolyzed to fucose during the incubation. Purification of the product trisaccharide was achieved by deproteinization and ion-exchange and gel-filtration chromatography. Specific ^{13}C enrichment and comparison with ^{13}C-enriched model compounds allowed unambiguous assignment of ^{13}C resonances. Fucosylation at O2 of βDGal(1→4)βDGlcNAc-hexanolamine caused a 5.6-ppm downfield shift of the C2 resonance of Gal. The resonance of C1 was shifted upfield by 2.5 ppm, while those of C3 and C4 were shifted downfield by 1.0 and 0.5 ppm, respectively. The C4 resonance of GlcNAc is also shifted downfield by 2.4 ppm. Fucosylation of the disaccharide βDGal(1→4)DGlcNAc resulted in a similar pattern of chemical shift changes. Interresidue coupling constants ($^3J_{C1-C1'} \simeq 1.5$ Hz observed as line broadening, $^3J_{H1-C2'} \simeq 3.2$ Hz, $^3J_{C1'-C3''} \simeq 0$ Hz, $^3J_{C1'-C5''} \simeq 1.0$ Hz observed as line broadening, and $^2J_{C1'-C4''} \simeq 1.5$ Hz) in the enriched oligosaccharides allowed estimation of the most abundant conformer for the ϕ and ψ torsion angles in the βDGal(1→4)GlcNAc ($\phi' \simeq 60°$ and $\psi' \simeq 15°$) and αLFuc-(1→2)DGal ($\phi \simeq 55°$ and $\psi \simeq 0°$) glycosidic linkages of the trisaccharide.

The oligosaccharide components of glycoproteins and glycolipids are of interest because of their demonstrated roles in several recognition events. Knowledge of the solution conformations of these oligosaccharides is limited yet is essential for the complete understanding of their specificities. The most promising approach to establishing solution conformations of such complex oligosaccharides appears to be NMR[1] spectroscopy, which, in certain systems, can give data interpretable in terms of conformations of component monosaccharide rings and conformations about the glycosidic bonds (Lemieux et al., 1980; Nunez & Barker, 1980). Application of NMR spectroscopy requires the isolation or synthesis of substantial amounts of oligosaccharides (>20 μmol). Data acquisition is facilitated by larger amounts of material, and certain interactions can be assessed only if compounds enriched with ^{13}C at specific sites are prepared (Nunez & Barker, 1980).

An important class of immunologically active oligosaccharides with well-defined structures is the ABO blood group of antigens. ABO substances can be isolated from biological sources, although generally in small quantities, and chemical syntheses are very complex (Jacquinet & Sinay, 1976, 1977; Lemieux & Driquez, 1975). An alternative approach, capable of providing sufficient amounts of oligosaccharides for NMR spectroscopy, that proceeds in high yield and permits the incorporation of ^{13}C-enriched monosaccharides was applied by Nunez & Barker (1980) to the synthesis of type 2 disaccharides. The required sugar nucleotide (UDPGal) was synthesized chemically and then used with the appropriate glycosyltransferase to give the disaccharide βDGal(1→4)-βDGlcNAc-hexanolamine in 80% yield. Several ^{13}C-enriched compounds were prepared and solution conformations proposed. The extension of this approach to the synthesis of fucosylated di- and trisaccharides of the ABO type 2 human

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[1] Abbreviations: NMR, nuclear magnetic resonance; UDP, uridine 5′-diphosphate; GDP, guanosine 5′-diphosphate; GMP, guanosine 5′-monophosphate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(5-phenyloxazol-2-yl)benzene; BSA, bovine serum albumin; SP-Sephadex, sulfopropyl-Sephadex.

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blood group system is described in this paper.

Materials and Methods

Raw bovine milk was obtained from the Michigan State University dairy. Porcine submaxillary glands were purchased from Bio-Resources, Dallas, TX. L-[^{14}C(U)]Fucose, GDP-L-[^{14}C(U)]fucose, UDP-D-[^{14}C(U)]galactose, and D-[^{14}C-(U)]galactose were purchased from New England Nuclear. UDP-galactose, Dowex 50-X8 (100–200 mesh), Dowex 1-X8 (100–200 mesh), Chelex-100, Bio-Gel P2 (−400 mesh), sulfopropyl-Sephadex, Sephadex G50, Sepharose 4B, sodium cacodylate, and GMP were purchased from Sigma Chemical Co. Dichlorodimethylsilane was purchased from Aldrich. α-L-Fucosidase from beef kidney was purchased from Boehringer Mannheim. All other chemicals were of the highest purity commerically available.

βDGlcNAc-hexanolamine was synthesized by the method of Barker et al. (1972). D-[1-^{13}C]Gal was synthesized as described previously (Serianni et al., 1979a). D-[2-^{13}C]Gal was synthesized by the sequential addition of ^{13}CN and CN to D-threose (Serianni et al., 1979b). UDP-[1-^{13}C]Gal was synthesized by the method of Nunez et al. (1980) with the following modification. β-D-Galactopyranose tetraacetate, prepared by hydrolysis of the corresponding α-bromo compound, was anomerized in 75% aqueous pyridine at room temperature to an equilibrium mixture of α- and β-D-galactopyranose tetraacetates, with equilibrium favoring the α anomer. After phosphorylation, the anomers were separated as previously described by Dowex 1 (C1) chromatography. L-[1-^{13}C]Fuc (P. R. Rosevear and R. Barker, unpublished method) was used in the synthesis of GDP-[1-^{13}C]Fuc (Nunez et al., 1981). Ethyl and hexanolamine β-D-galactosides were synthesized by the procedure of Chiang et al. (1979).

Synthesis of GDP-Sepharose. GDP-hexanolamine was synthesized from GMP-morpholidate and 6-[*N*-(trifluoroacetyl)amino]-1-hexanol by the procedure of Moffatt (1966). Coupling of GDP-hexanolamine to Sepharose 4B was accomplished with a procedure modified from that of Cuatrecases (1970). Sepharose 4B (55 g, 110 mL) was suspended in 110 mL of 2.5 M phosphate buffer, pH 12.0, and placed in an ice bath with efficient stirring. Cyanogen bromide, 22 g, dissolved in 25 mL of acetonitrile was injected into the Sepharose 4B suspension, and the pH was maintained at 12.0 for 9 min with 8 N NaOH. The activated gel was filtered quickly, washed with 2 L of ice water, and added to 0.7 mmol of GDP-hexanolamine in 70 mL of water. After adjustment to pH 10.2 with 1 N HCl, the mixture was shaken for 15 h at 4 °C. The gel was filtered and washed with 2 L of water. The absorbance (280 nm) of the washings indicated that 5 μmol of GDP-hexanolamine was bound per mL of Sepharose. Before use, the affinity gel was washed as described by Sadler et al. (1979).

High-pressure liquid chromatography (HPLC) was performed on a Whatman Partisil PXS 10/25 SAX column at 1000 psi with 0.2 M potassium phosphate buffer, pH 3.4. Compounds were detected by monitoring the column eluate at 260 nm with an Altex dual-wavelength UV detector.

Radioactivity was measured with a Beckman LS-7000 scintillation counter with aqueous samples (0.2–1.0 mL) mixed with 2–5 mL of scintillation fluid (8 g of PPO, 0.2 g of dimethyl-POPOP, 1 L of Triton X-100, and 2 L of toluene).

^{13}C NMR spectra were obtained with Bruker WP-60 and WH-180 and Nicolet 360 (15.08, 45, and 90 M Hz, respectively) Fourier-transform spectrometers. All spectra were obtained with proton decoupling.

In the Nicolet spectrometer, a two-level decoupling program was used to minimize sample heating. A spectral window of 1800 Hz was used when the entire spectrum of the compound was required, and a spectral window of 1000 Hz was used for doubly enriched compounds. All spectra were obtained with 16 384 spectral points at 24 °C. Chemical shifts, given in parts per million, are referenced to the C1 resonance of β-D-glucopyranose in a water solution and are accurate to ±0.1 ppm with respect to internal tetramethylsilane (Walker et al., 1976).

Assays of hexanolamine-containing compounds having a primary amino group were performed with fluorescamine (Naoi & Lee, 1974). Hexanolamine, 0–80 μmol, was used as a standard. Fluorescence was determined by excitation at 390 nm and emission at 475 nm on a Aminco-Bowman spectrophotofluorometer with a xenon lamp power supply.

Protein concentrations were measured with a fluorescamine assay (Bohlen et al., 1973) with bovine serum albumin as a standard.

Bovine N-acetylglucosaminide $\beta(1\rightarrow4)$ galactosyltransferase was purified by the method of Barker et al. (1972). The enzyme can be stored for at least 2 years in 25 mM sodium cacodylate, pH 7.4, 25 mM $MnCl_2$, 25 mM GlcNAc, and 1 mM mercaptoethanol. The rate of formation of βDGal(1→4)DGlcNAc, lactose, or βDGal(1→4)βDGlcNAc-hexanolamine was followed by measuring the rate of transfer of ^{14}C from UDP-[^{14}C(U)]galactose into disaccharide during a 10-min incubation at 37 °C as described by Bell et al. (1976).

Porcine β-galactoside $\alpha(1\rightarrow2)$ fucosyltransferase was partially purified by using the procedure of Beyer et al. (1980) with modifications. All glass apparatus and glass wool bed supports were carefully cleaned and treated before use with dichlorodimethylsilane. Two kilograms of porcine submaxillary glands was processed at a time, and all procedures were performed at 5 °C with 25 mM sodium cacodylate, pH 6.0 (buffer A). Initial processing followed a procedure used to solubilize a porcine CMP-*N*-acetylneuraminate:β-D-galactoside $\alpha(2\rightarrow3)$ sialyltransferase (Sadler et al., 1979). Complete solubilization of the fucosyltransferase from the membrane fragments could be achieved with only two Triton X-100 extractions. Fucosyltransferase, from the Triton extract, was adsorbed to SP-Sephadex and eluted with buffer containing 0.3 M NaCl as described by Sadler et al. (1979). The filtrates were treated again with SP-Sephadex as described above and discarded. Fractions containing fucosyltransferase activity eluting from the SP-Sephadex columns were combined, mixed with an equal volume of glycerol, and stored at −20 °C (Beyer et al., 1980).

The SP-Sephadex purified enzyme from 4 kg of tissue was further purified on a 5 × 10 cm column of GDP-Sepharose with the procedure of Beyer et al. (1980). The enzyme was eluted from the affinity resin with 2 M NaCl. Fractions containing fucosyltransferase were pooled and stored at 4 °C under which conditions the enzyme is stable for at least 3 months. Fucosyltransferase from the GDP-Sepharose column (200 mL) was desalted on 5 × 100 cm columns of Sephadex G-50 (fine) equilibrated with 25 mM sodium cacodylate, pH 6.0. Fractions (20 mL) were collected at a flow rate of 200 mL/h. Fucosyltransferase activity was pooled and quickly applied at a flow rate of 50 mL/h to a 1-mL GDP-Sepharose column equilibrated with 25 mM sodium cacodylate, pH 6.0. The column was washed with 20 mL of buffer and the fucosyltransferase eluted with 2 M NaCl in buffer. Fractions (1 mL) were collected. The enzyme elutes in 5–8 mL and can be stored without loss of activity for 2 months at 4 °C. Longer periods of storage require dilution to 50% with glycerol and temperatures of −20 °C. Syntheses of fucosylated compounds

were carried out with this enzyme preparation. The enzyme had a specific activity of 0.88 μmol of αLFuc(1→2)βDGal-(1→4)DGlc formed min^{-1} (mg of protein)$^{-1}$ at saturating concentrations of GDP-fucose and lactose at 33 °C.

Further purification can be achieved with a βDGal(1→4)-βDGlcNAc-hexanolamine-Sepharose column, having 4 μmol of ligand/mL of Sepharose, equilibrated in 25 mM sodium cacodylate, pH 6.0, containing 0.5 mM GMP. The enzyme from the Sephadex G50 column is made 0.5 mM in GMP and applied at a flow rate of 10 mL/h to the βDGal(1→4)-βDGlcNAc-hexanolamine-Sepharose column. Elution is easily achieved by removal of GMP from the eluting buffer. Fucosyltransferase prepared in this manner is near 100000-fold purified but is less stable in the glycoside synthesis reaction.

Porcine β-galactoside α(1→2) fucosyltransferase activity was determined as described by Beyer et al. (1980) with 100-μL reaction mixtures containing 0.5 μmol of lactose, βDGal(1→4)DGlcNAc, or βDGal(1→4)βDGlcNAc-hexanolamine, 5 μmol of sodium cacodylate, pH 6.0, 2 μmol of $MnCl_2$, 0.2 mg of BSA, 2 nmol of GDPFuc containing approximately 5000 cpm GDP-[^{14}C(U)]Fuc, and enzyme solution at 33 °C for 15–60 min. Appropriate controls were included to correct for GDP-fucose hydrolysis. Specific activities are reported in terms of the acceptor substrate lactose at 33 °C and are in good agreement with those reported by Beyer et al. (1980). (One unit = 1 μmol of Fuc transferred to lactose per min at 33 °C.)

βDGal(1→4)DGlcNAc Disaccharides. Galactosylated compounds were prepared on a 140-μmol scale at 33 °C with procedures similar to Nunez & Barker (1980). Reaction mixtures contained 16 mL of *N*-acetylglucosaminide β(1→4) galactosyltransferase (43 units/mL) in 25 mM sodium cacodylate, pH 7.4, 25 mM $MnCl_2$, 2 mM β-mercaptoethanol, 60 μL of 0.01 mCi/mL UDP-[^{14}C(U)]Gal, 140 μmol of UDPGal, disodium salt, 150 μmol of acceptor, and 4 mg of BSA in a total volume of 18 mL. After 6 h, another 6 mL of *N*-acetylglucosaminide β(1→4) galactosyltransferase was added and the incubation continued for another 12 h.

Protein was removed from the reaction mixture by repeated concentration and dilution in a 10-mL Amicon cell with an Amicon PM10 membrane. The protein-free filtrates were passed over a 1 × 4 cm column of Dowex 1-X8 (100–200 mesh) and eluted with water. Alternatively, hexanolamine glycosides were purified by adsorption to a 1 × 5 cm column of Dowex 50-X8 (H^+) (100–200 mesh) and elution with 1 M pyridinium acetate. Pyridinium acetate is removed by repeated evaporation in vacuo at 40 °C. The galactosyl disaccharide was separated from the acceptor at a flow rate of 5 mL/h on a 4 × 90 cm column of Bio-Gel P2 (−400 mesh) equilibrated in 0.1 M triethylammonium bicarbonate, pH 7.4. Triethylammonium bicarbonate in the final product was removed by repeated evaporation in vacuo at 40 °C. Nonreducing disaccharides were treated with 0.5 mL of Chelex-100 (Na^+), and reducing disaccharides were treated with 0.5 mL of Chelex-100 (H^+) before NMR analysis. Hexanolamine glycosides were completely eluted from the Chelex-100 (Na^+) resin with 1 M pyridinium acetate, which can be removed by repeated evaporation from water.

Preparation of Fucosylated Oligosaccharides. Most syntheses were performed by using 50 μmol of the limiting reactant with 4 mL of β-galactoside α(1→2) fucosyltransferase (0.62–0.88 unit/mL), 30 μL of 0.01 mCi/mL GDP-[^{14}C-(U)]Fuc, 0.04 mmol of sodium cacodylate, pH 6.0, 0.15 mmol of $MnCl_2$, 7.5 mg of BSA, 50–100 μmol of acceptor oligosaccharide, and 50 μmol of GDPFuc in a total volume of 7.4 mL. After incubation for 6 h at 33 °C an additional 2 mL of β-galactoside α(1→2) fucosyltransferase was added and incubation continued for 24 h.

Incorporation of radioactivity from GDP-[^{14}C(U)]Fuc into oligosaccharide was measured in a 20-μL aliquot of the reaction, which was diluted with 0.5 mL of water, and transferred to a column (Pasteur pipet) containing 1.0 mL of Dowex 1-X8 (Cl^-; 100–200 mesh). The column was rinsed with 1.5 mL of water and the eluant collected in a scintillation vial for counting (12 mL of scintillation fluid). Hydrolysis of GDP-fucose to fucose was measured in a reaction with a hexanolamine glycoside as an acceptor. A 20-μL sample was diluted with 0.5 mL of water and applied to a Pasteur pipet column containing equal proportions of Dowex 1-X8 (Cl^-; 100–200 mesh) and Dowex 50-X8 (H^+; 100–200 mesh). The column was washed with 1.5 mL of water and the eluate collected in a scintillation vial and counted for radioactivity. Only fucose from the hydrolysis of GDP-fucose elutes from the column.

High-pressure liquid chromatography on a Partisil PXS 10/25 SAX column was also used to estimate the proportions of GMP, GDP, and GDP-fucose in the reaction mixture and provides a useful measure of the progress of the reaction. Retention times for GMP, GDP-fucose, and GDP are 7, 12, and 24 min, respectively.

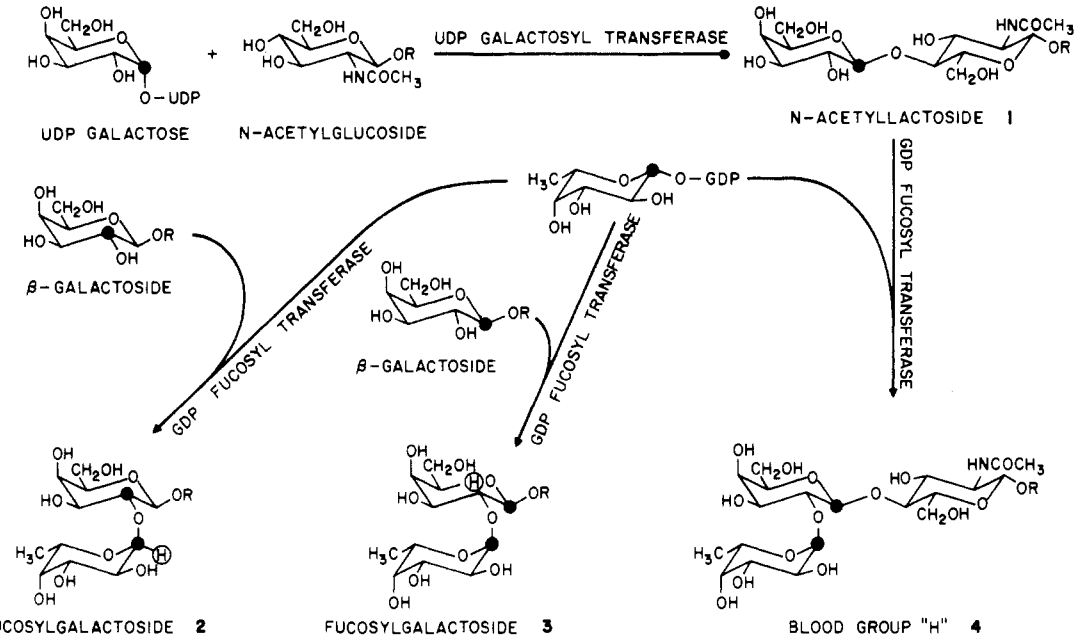
Purification of Fucosylated Oligosaccharides. Protein was removed from the reaction mixtures by repeated concentration and dilution in an Amicon cell with an Amicon PM10 membrane. The protein-free filtrates were applied to a 1 × 4 cm column of Dowex 1-X8 (Cl^-; 100–200 mesh) and 0.5 mL of Chelex-100 (Na^+), which was eluted with water. The eluate was concentrated to ~4 mL and applied to a 4 × 90 cm Bio-Gel P2 (−400 mesh) column equilibrated with 0.1 M triethylammonium bicarbonate, pH 7.4, and eluted at a flow rate of 5 mL/h with the same buffer. The fucosylated product is well separated from the acceptor. Occasionally, some salt eluted with the fucosylated product and was removed by rechromatography on a 1 × 40 cm Bio-Gel P2 (−400 mesh) column equilibrated in water at a flow of 5 mL/h. Nonreducing compounds were treated with 0.5 mL of Chelex-100 (Na^+) and reducing compounds with 0.5 mL of Chelex-100 (H^+) before NMR analysis. Hexanolamine glycosides were eluted completely from the Chelex-100 (Na^+) with 1 M pyridinium acetate, which then was removed by repeated evaporation from water.

Results and Discussion

As shown in Scheme I, the chemical synthesis of UDP-[1-^{13}C]Gal, GDP-[1-^{13}C]Fuc, and simple glycosides of βD[1-^{13}C]- and [2-^{13}C]Gal together with the purification of galactosyl and fucosyltransferases permits the preparation of blood group H substance and a number of model disaccharides with ^{13}C enrichment at specific sites. The synthesis of UDP-[1-^{13}C]Gal and galactosyltransferase has been described previously (Nunez & Barker, 1980; Barker et al., 1972), and the synthesis of GDP-[1-^{13}C]Fuc is described elsewhere (Nunez et al., 1981). Fucosyltransferase from porcine submaxillary glands has been purified by Beyer et al. (1980). The procedure depends on affinity chromatography with GDP-hexanolamine-Sepharose. Two affinity chromatography steps give fucosyltransferase suitable for the synthesis of fucosyl glycosides (11 000-fold purification, specific activity 0.88 unit mg^{-1}) that is stable for months in 50% glycerol at −20 °C.

Purification of the porcine β-galactoside α(1→2) fucosyltransferase 100 000-fold can be achieved with the affinity column βDGal(1→4)βDGlcNAc-hexanolamine-Sepharose containing 3 μmol of ligand/mL of resin. Adsorption of the

Scheme I



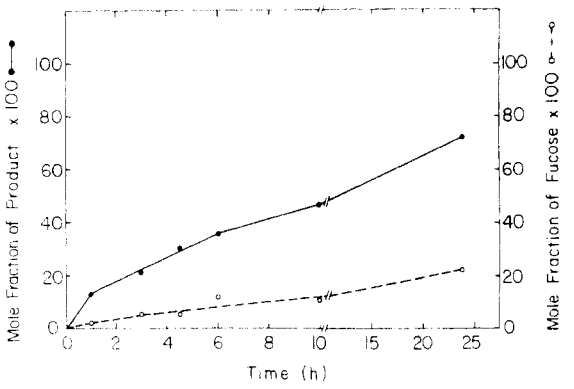


FIGURE 1: The kinetics of fucosylation of βDGal(1→4)βDGlcNAc-hexanolamine with porcine β-galactoside α(1→2) fucosyltransferase. (●) Fuc transferred to βDGal(1→4)βDGlcNAc-hexanolamine plus (○) GDPFuc hydrolyzed.

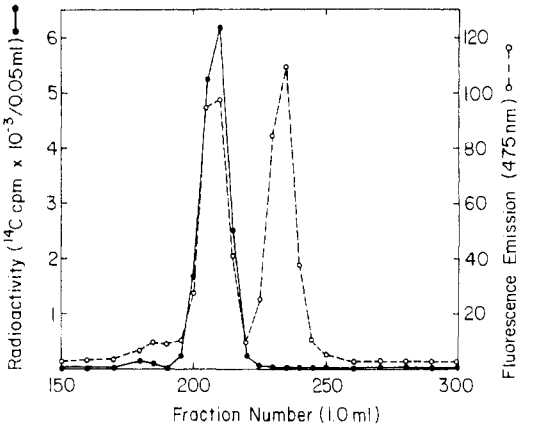


FIGURE 2: Separation of αLFuc(1→2)βDGal-hexanolamine and βDGal-hexanolamine by gel filtration on a Bio-Gel P2 (−400 mesh) column equilibrated with 0.1 M triethylammonium bicarbonate, pH 7.5. (○--○) The primary amino group in the hexanolamine aglycon as measured with fluorescamine; (●–●) ^{14}C transferred from GDP-[^{14}C(U)]Fuc.

enzyme requires 5 mM GMP in the buffer, and elution is achieved by removal of the GMP. The high specificity of this affinity resin most probably results from the lack of negative charge on the adsorbant and the inability of most nucleotide-binding enzymes to interact with it. The highly purified fucosyltransferase is less stable than the 11 000-fold purified enzyme both in storage and in the fucosylation reaction mixture. Fortunately, the less highly purified enzyme is suitable for synthesis of fucosylated oligosaccharides. Although the cruder mixture contains proteins that bind to GDP, either because of its negative charge or because of their affinity for all or part of the GDP moiety, the mixture appears to contain no enzymes that use the galactosyl receptors used in this study to form other than the desired product. Indeed, the products all appear to be single compounds.

Synthesis and Purification of Fucosylated Oligosaccharides. Fucosylations were performed at 33 °C because the GDP-Sepharose-purified enzyme is more stable at this temperature than at 37 °C. Initially, reactions with 10 μmol of GDPFuc were performed with a 2-fold excess of acceptor over the GDPFuc. With the acceptor, βDGal(1→4)βDGlcNAc-hexanolamine, both the kinetics of the reaction and the extent of GDPFuc hydrolysis can be followed (Figure 1). In this experiment, 90% utilization of GDPFuc is achieved with 72% incorporation into product and 22% hydrolysis to Fuc. Larger scale reactions with ^{13}C-enriched acceptors were performed with a 1:1.2 ratio of GDPFuc to acceptor. The progress of the reaction for the synthesis of αLFuc(1→2)βD[1-^{13}C]Gal-(1→4)βDGlcNAc followed by HPLC shows that no GDPFuc remains after 20 h. The only products observable are GMP and GDP. The yield of trisaccharide is 83%, based on starting GDPFuc. Hydrolysis of GDPFuc to Fuc plus GDP ranged from 10 to 20% in these preparations. Beyer et al. (1980) noted that a GDPFuc hydrolase activity copurified with porcine β-galactoside α(1→2) fucosyltransferase, which may be intrinsic to the fucosyltransferase. Nonenzymatic hydrolysis of GDPFuc under the reaction conditions is less than 10% after 30 h of incubation at 33 °C (Nunez & Barker, 1976).

Purification of the fucosylation reactions, after incubation for 12–24 h, was achieved by deproteinization, deionization, and gel filtration. Protein was removed by pressure dialysis in an Amicon cell with an Amicon PM10 membrane. The filtrate contained over 98% of the radioactivity initially present in GDPFuc and less than 2% of the initial protein. Deionization and removal of GMP, GDP, and any unreacted GDPFuc were achieved with ion-exchange resins, and the products were separated by gel filtration. Figure 2 shows a

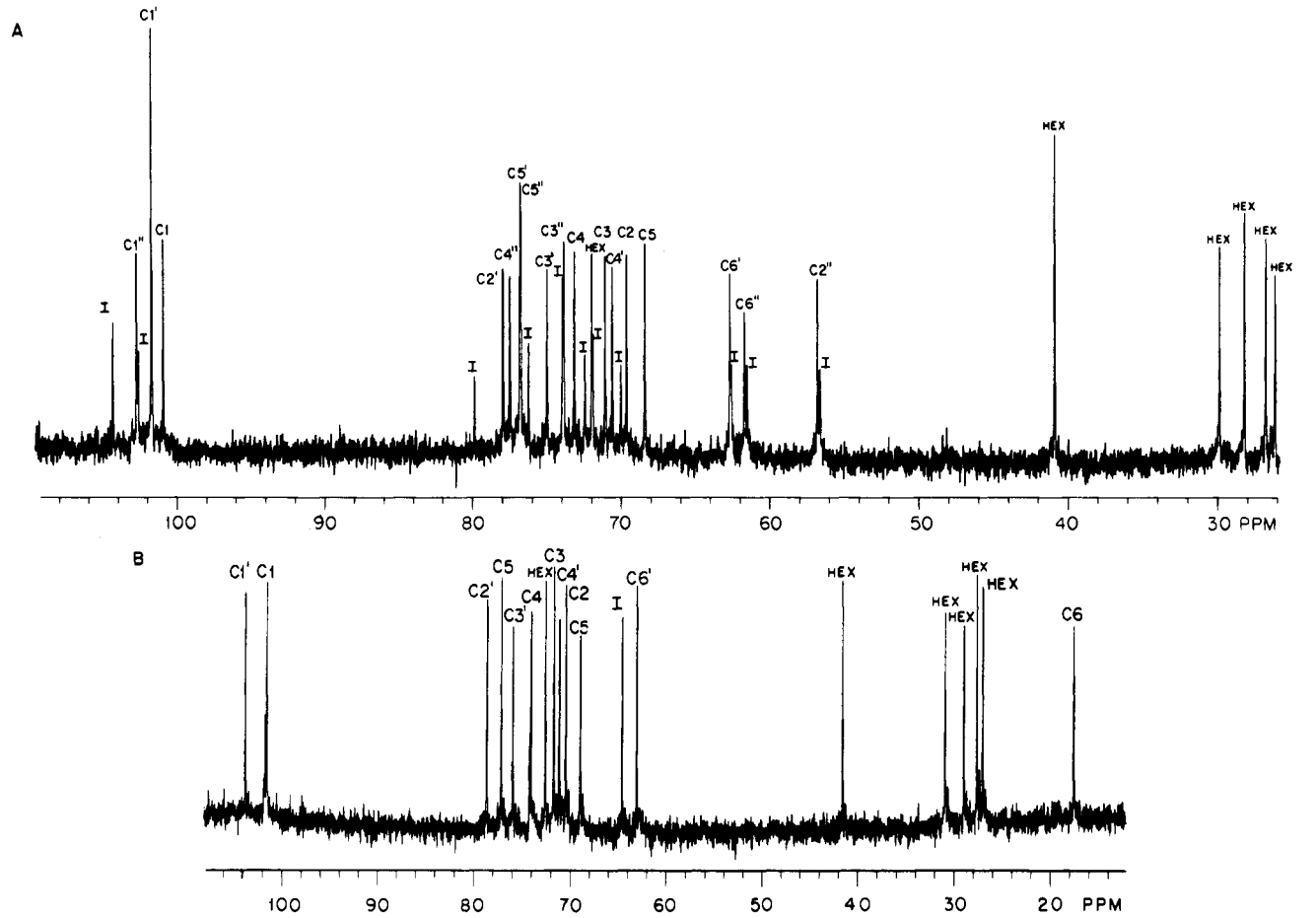


FIGURE 3: (A) The 90-MHz natural abundance ^{13}C NMR spectrum of αLFuc(1→2)βDGal(1→4)βDGlcNAc-hexanolamine. The proton-decoupled spectrum was obtained at 20 °C with a 0.05 M solution in a 10-mm tube. Transients (8000) were accumulated at a sweep width of 5000 Hz with 16K data points. Chemical shifts are in ppm downfield from tetramethylsilane. Hex = hexanolamine; C = Fuc; C′ = Gal; C″ = GlcNAc; I = disaccharide. (B) The 90-Hz natural abundance spectrum of αLFuc(1→2)βDGal-hexanolamine.

typical separation for αLFuc(1→2)βDGal-hexanolamine. A 2-fold excess of acceptor was used in this reaction. Removal of the triethylammonium bicarbonate buffer in vacuo gave the fucosylated product ready for NMR analysis. Fucosylated compounds synthesized in this fashion were αLFuc(1→2)-βDGal(1→4)βDGlcNAc-hexanolamine, αLFuc(1→2)βD[1-^{13}C]Gal(1→4)βDGlcNAc-hexanolamine, αL[1-^{13}C]Fuc(1→-2)βD[1-^{13}C]Gal(1→4)βDGlcNAc-hexanolamine, αLFuc(1→-2)βD[1-^{13}C]Gal(1→4)βDGlcNAc, αL[1-^{13}C]Fuc(1→2)βD[1-^{13}C]Gal(1→4)βDGlcNAc, αL[1-^{13}C]Fuc(1→2)βD[1-^{13}C]-Gal(1→4)βDGlc, αLFuc(1→2)βDGal-hexanolamine, αLFuc-(1→2)βD[1-^{13}C]Gal-hexanolamine, αL[1-^{13}C]Fuc(1→2)βD-[1-^{13}C]Gal-hexanolamine, αL[1-^{13}C]Fuc(1→2)βD[1-^{13}C]-Gal-ethanol, αLFuc(1→2)βD[1-^{13}C]Gal-ethanol, αL[1-^{13}C]-Fuc(1→2)βDGal-ethanol, and αLFuc(1→2)βD[2-^{13}C]Gal-ethanol.

The use of mono- and disaccharide-hexanolamine derivatives as acceptors for fucosyltransferase generates a number of oligosaccharides suitable for covalent attachment to Sepharose 4B. The affinity chromatography adsorbants so formed can be applied to the purification of antibodies and specific carbohydrate binding proteins such as other glycosyltransferases.

Characterization of Enzymatically Synthesized Products. The product of the bovine *N*-acetylglucosaminide β(1→4) galactosyltransferase reaction has been well characterized by chemical and enzymatic means and, recently, by ^{13}C NMR spectrometry (Nunez & Barker, 1980). The product of the porcine β-galactoside α(1→2) fucosyltransferase reaction has recently been characterized by serologic properties and specific fucosidase digestion, indicating exclusive formation of the Fuc α(1→2)Gal glycosidic linkage (Beyer et al., 1980).

Carbon-13 NMR spectroscopy offers an alternative approach for characterization of complex oligosaccharides (Excoffier et al., 1977; Gagnaire et al., 1977; Lemieux et al., 1980; Nunez & Barker, 1980). The ^{13}C NMR of the enzymatically synthesized dissacharides βDGal(1→4)βDGlcNAc-hexanolamine, βD[1-^{13}C]Gal(1→4)βDGlcNAc-hexanolamine, and βD[1-^{13}C]Gal(1→4)βDGlcNAc was assigned by Nunez & Barker (1980). Lemieux et al. (1980) also interpreted the ^{13}C and ^{1}H NMR spectra of an extensive series of chemically synthesized oligosaccharides of the ABO type 1 blood group.

Enzymatic fucosylation of the disaccharide βDGal(1→4)-βDGlcNAc-hexanolamine gives the trisaccharide αLFuc(1→-2)βDGal(1→4)βDGlcNAc-hexanolamine (Scheme I), the most complex oligosaccharide prepared in this study. Specific ^{13}C enrichment and comparison with ^{13}C-enriched model compounds facilitated complete assignment of its ^{13}C NMR spectrum (Figure 3). The 90-MHz proton-decoupled ^{13}C NMR spectrum of αLFuc(1→2)βDGal(1→4)βDGlcNAc-hexanolamine can be divided into three regions: the anomeric region from 105 to 95 ppm, a region from 85 to 55 ppm containing C2–C6 of the carbohydrates and C1 of hexanolamine, and a third region from 50 to 15 ppm containing C2–C6 of the hexanolamine, C6 of fucose, and the methyl carbon of the *N*-acetyl group of GlcNAc. Assignments of specific resonances are given in Table I.

The resonances in the anomeric region at 100.9, 101.7, and 102.7 ppm are due to C1 of Fuc, Gal, and GlcNAc, respectively. Resonances in Figure 3 are labeled in accordance with IUPAC nomenclature with Fuc, Gal, and GlcNAc being

Table I: Carbon-13 Chemical Shifts of αLFuc(1→2)βDGal(1→4)βDGlcNAc and Related Compounds[a]

	R[b]									GalGlcNAc[c]		FucGalGlcNAc	
	α	β	ethyl-Gal	methyl-Fuc	GlcNAc-hex[c,d]	GalGlcNAc-hex[c,d]	FucGal-GlcNAc-hex[d]	Gal-hex[d]	FucGal-hex[d]	α	β	α	β
C1													
Glc	93.6	97.4											
GlcNAc	92.2	96.4			102.7	102.4	102.7			91.6	96.2	92.1	96.5
Gal	93.8	98.0	104.0			104.2	101.7	104.3	103.5	104.1		102.3	
Fuc	93.8	97.8		101.2			100.9		101.3			101.4	
C2													
Glc	73.2	75.9											
GlcNAc	55.5	58.1			57.2	56.5	56.8			55.1	57.6	55.5	57.9
Gal	70.0	73.6	72.3			72.5	77.9	72.4	78.4	72.5		77.9	
Fuc	69.8	73.4		69.6			69.6		70.2			69.8	
C3													
Glc	74.5	77.5											
GlcNAc	72.1	75.3			75.2	73.9	73.8			70.6	74.9	70.8	75.1
Gal	70.8	74.4	74.4			74.0	75.0	74.6	75.8	73.9		72.0	
Fuc	71.0	74.6		71.3			71.0		71.5			71.2	
C4													
Glc	71.4	71.3											
GlcNAc	71.5	71.2			71.6	79.9	77.5			80.1	79.7	77.7	77.5
Gal	70.9	70.4	70.2			70.1	70.6	70.4	70.9	69.9		70.8	
Fuc	73.5	73.1		73.5			73.1		73.9			73.3	
C5													
Glc	73.0	77.4											
GlcNAc	73.0	77.3			77.4	76.5	76.8			71.5	76.1	70.8	76.7
Gal	72.0	76.6	76.6			76.9	76.9	76.7	77.0	76.6		76.8	
Fuc	67.8	72.3		67.9			68.4		68.7			68.5	
C6													
Glc	62.3	62.5											
GlcNAc	62.0	62.2			62.4	61.5	61.5			61.3	61.3	61.6	61.6
Gal	62.8	62.6	62.5			62.5	62.6	62.6	62.9	62.3		62.7	
Fuc	17.7	17.2		16.9			16.3		17.4			16.8	
carboxyl of *N*-acetyl	175.9	176.2			175.9	175.7				176			
methyl of *N*-acetyl	23.3	23.5			23.8	23.6				23.3	23.5		
C1 of hexanolamine					71.6	71.6	71.7	72.0	71.8				

[a] Chemical shifts are in ppm relative to C1 of β-glucopyranose in water at 25 °C at 97.4 ppm. [b] R specifies the chemical shift of the reducing moiety taken from Walker et al. (1976). [c] Chemical shifts taken from Nunez & Barker (1980). [d] hex = β-D-hexanolamine glycoside.

Table II: ^{13}C NMR Chemical Shift Differences between αLFuc(1→2)βDGal(1→4)βDGlcNAc-hexanolamine and Related Glycosides[a,b]

	GlcNAc						Gal						Fuc					
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
GGlhex-FGGlhex[c]	−0.3	−0.3	0.1	2.4	−0.3	0.0	2.5	−5.6	−1.0	−0.5		0.9						
FGhex-FGGlhex							1.8	0.5	0.8	0.3	0.1	0.3	0.4	0.6	0.5	0.7	0.3	1.1
GGl-FGGl α	−0.5	−0.4	−0.2	2.4	0.7	0.3	1.8	−5.4	1.9	−0.9	−0.2	−0.4						
β	−0.3	−0.3	−0.2	2.2	−0.6	−0.3												
Fmet-FGGlhex													0.3	0.0	0.3	0.4	−0.5	0.6
Fmet-FGGl													−0:2	−0.2	0.1	0.2	−0.6	0.1

[a] Chemical shift differences are in ppm and are accurate to ±0.5 ppm. [b] Negative signs indicate downfield chemical shifts. [c] Abbreviations used: GGlhex, βDGal(1→4)βDGlcNAc-hexanolamine; FGGlhex, αLFuc(1→2)βDGal(1→4)βDGlcNAc-hexanolamine; FGhex, αLFuc(1→2)βDGal-hexanolamine; GGl, βDGal(1→4)DGlcNAc; FGGl, αLFuc(1→2)βDGal(1→4)DGlcNAc; Fmet, methyl-αLFuc.

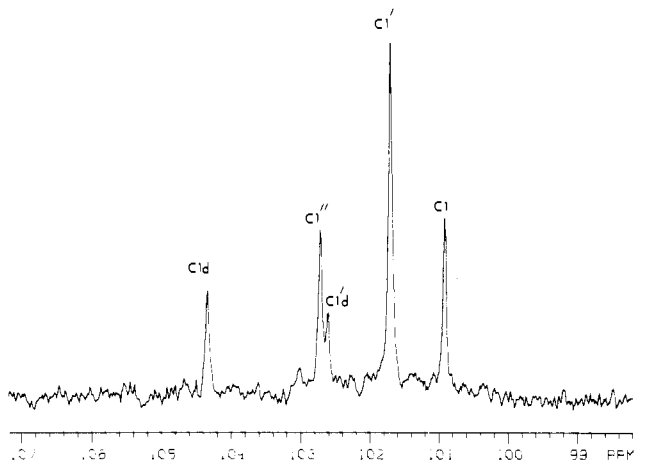


FIGURE 4: Partial 90-MHz proton-decoupled ^{13}C NMR spectrum of the anomeric region of αLFuc(1→2)βDGal(1→4)βDGlcNAc-hexanolamine. The spectrum was obtained at 20 °C with a 0.05 M solution in a 10-mm tube. Transients (8000) were accumulated at a sweep width of 5000 Hz with 0.3 Hz/data point. Resonances labeled C, C′, and C″ represent Fuc, Gal, and GlcNAc in the trisaccharide, respectively. Carbons C1*d* and C1′*d* correspond to Gal and GlcNAc of the disaccharide βDGal(1→4)βDGlcNAc-hexanolamine.

represented by C, C′, and C″, respectively. Derivatization of Gal with Fuc produces shifts of all C1 resonances. The shifts are apparent in the 90-MHz proton-decoupled ^{13}C NMR spectrum of a sample containing both αLFuc(1→2)βDGal-(1→4)βDGlcNAc-hexanolamine and βDGal(1→4)-βDGlcNAc-hexanolamine (Figure 4). Carbons C1*d* and C1*d*′ (Figure 4) represent C1 of Gal and GlcNAc, respectively, in the dissacharide. Derivatation of C2 of βDGal shifts its C1 resonance upfield (from 104.2 to 101.7 ppm) while C1 of GlcNAc shifts downfield by 0.11 ppm. C1 of Fuc in the trisaccharide is shifted downfield when compared to C1 of Fuc in αL[1-^{13}C]Fuc-methanol. The presence of an α-L-fucopyranosyl linkage was confirmed by comparison to the methyl glycosides and by specific hydrolysis with an α-L-fucosidase from beef kidney.

Chemical shift differences resulting from derivatization of βDGal(1→4)βDGlcNAc-hexanolamine with Fuc are apparent in all carbons except C6″ and C5′ (Table II). As expected, large chemical shift changes in C1′, C2′, and C3′ result from glycosidation of C2′ Gal with Fuc (Dorman & Roberts, 1970; Lemieux et al., 1980). Large upfield shifts of C4″ GlcNAc (2.4 ppm) and C6′ Gal (0.9 ppm) also result from fucosylation at C2′ Gal. All of the Fuc resonances (except C2) are slightly shifted relative to those of αLFuc-methanol. These long-range effects on chemical shifts as a consequence of fucosylation also indicate the need for caution in assigning resonances (or structures) in complex oligosaccharides based on chemical shifts of the constituent sugars and simple glycosides. Lemieux et al. (1980) have proposed that chemical shift differences resulting from glycosylation may reflect small changes in nonbonded interactions, valence angles, and/or hybridization of the affected nuclei.

Unequivocal assignment of most chemical shifts is possible through the use of ^{13}C enrichment at specific sites. The 45-MHz proton-decoupled ^{13}C NMR spectrum of αLFuc(1→2)βD[1-^{13}C]Gal(1→4)βDGlcNAc-hexanolamine (Figure 5) allows assignment of C1′, C2′, C3′, and C6′ of Gal since C1′ Gal is enriched with ^{13}C to the 90% level (Walker et al., 1976). The anomeric region shows two ^{13}C-enriched resonances, that at 104.2 ppm is the C1 of Gal in the contaminating disaccharide βD[1-^{13}C]Gal(1→4)βDGlcNAc-hexanolamine. Natural abundance carbons of the disaccharide are not observed. The ^{13}C-enriched resonance at 101.7 ppm is the C1′ of Gal in the trisaccharide. Derivatization of Gal at C2 with Fuc resulted in a downfield shift of C2′ (from 72.3 to 77.9 ppm); C2′ can be assigned unequivocally on the basis of a large coupling (46 Hz) to C1 of [1-^{13}C]Gal (Figure 5B). C3′ of Gal (75.0 ppm) is assigned on the basis of $^2J_{C1'-C3'}$ = 3.5 Hz and a broadening indicating a small $^3J_{C1'-C6'}$ coupling was used to assign C6′ Gal at 62.6 ppm (Walker et al., 1976).

In the reducing trisaccharide αLFuc(1→2)βD[1-^{13}C]Gal-(1→4)DGlcNAc, the chemical shift of Gal C1′ differs slightly (102.32–102.28) depending on the anomeric configuration at C1″ of GlcNAc. Comparison of the trisaccharide chemical shifts to those of βDGal(1→4)GlcNAc shows the characteristic shifts of carbons α and β to the site of substitution. C4″ of GlcNAc is shifted upfield (2.4 ppm).

The pattern of chemical shifts in αLFuc(1→2)βDGal-hexanolamine (Table I) is similar to that in the αLFuc(1→2)-βDGal residue of the trisaccharide except that all of the resonances in the disaccharide are downfield of those in the trisaccharide (Figure 3B, Tables I and II).

Solution Conformations. A complete description of the conformation of an oligosaccharide includes the conformations of component monosaccharides and specification of the ϕ and ψ angles of its glycosidic bonds. There is little evidence that intraresidue conformations differ from those of the parent monosaccharides or their simple glycosides (Lemieux & Koto, 1974; Nunez & Barker, 1980; Lemieux et al., 1980). Several studies have been made of the conformation about the glycosidic linkage using $^3J_{C-H}$ and $^3J_{C-C}$ coupling (Lemieux & Koto, 1974; Excoffier et al., 1977; Gagnaire et al., 1977; Nunez & Barker, 1980; Lemieux et al., 1980). Lemieux et al. (1980) have used hard-sphere calculations, including consideration of the exoanomeric effects, to predict conformations of several type 1 ABO and Lewis blood group oligosaccharides.

To evaluate ψ for the αLFuc glycosidic bond, the trisaccharide αLFuc(1→2)βD[1-^{13}C]Gal(1→4)βDGlcNAc-hexanolamine was prepared to determine whether coupling of C1 Fuc to the enriched C1′ Gal is observable. The line width of C1 Fuc was broadened in comparison with C1 GlcNAc or any

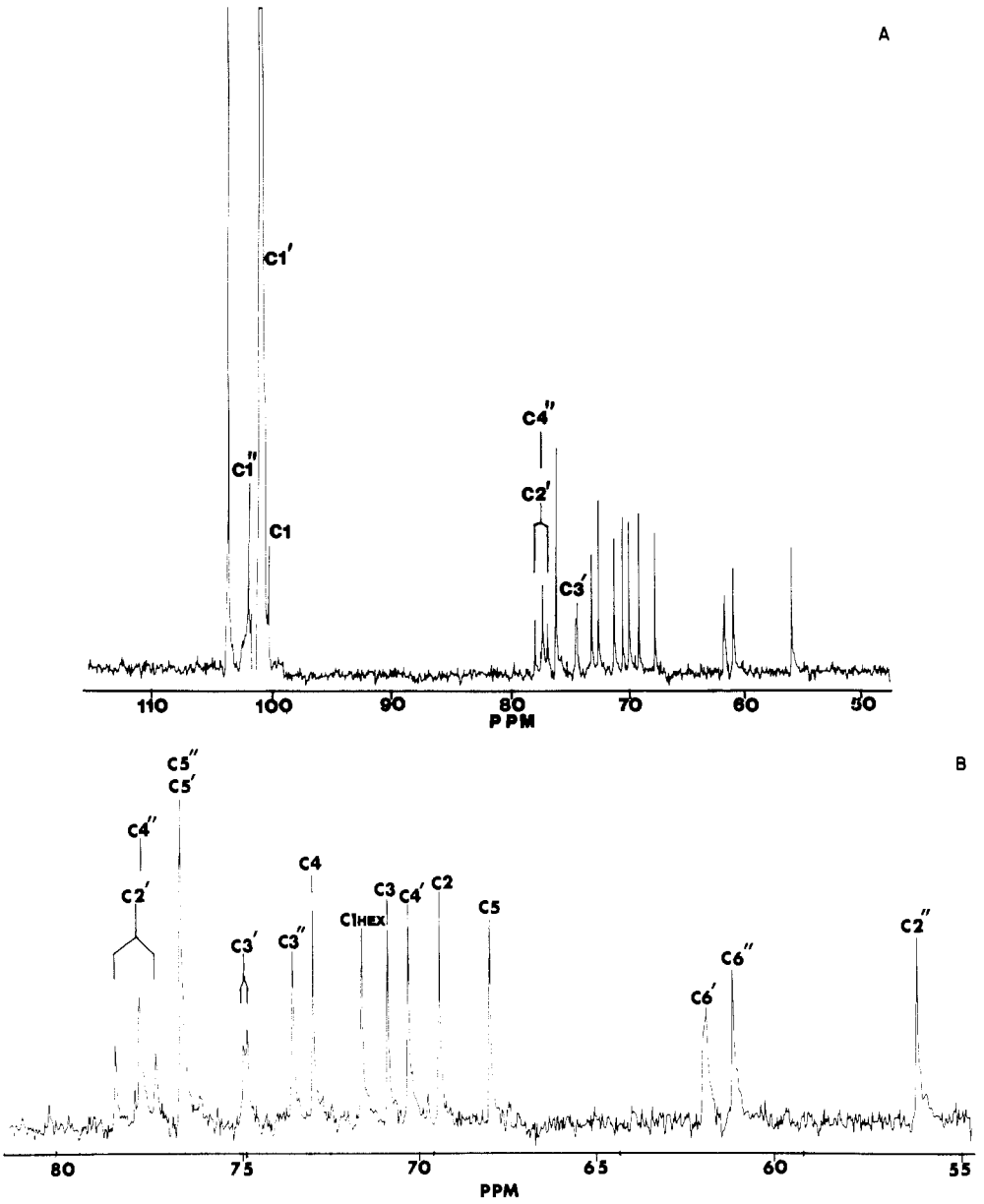


FIGURE 5: (A) The 45-MHz proton-decoupled ^{13}C NMR of the trisaccharide αLFuc(1→2)βD[1-^{13}C]Gal(1→4)βDGlcNAc-hexanolamine. The spectrum was obtained at 25 °C with a 17 mM solution in a 10-mm tube. Transients (36000) were accumulated at a sweep width of 8000 Hz at 0.9 Hz/computer point. (B) The C2–C6 region of αLFuc(1→2)βD[1-^{13}C]Gal(1→4)βDGlcNAc-hexanolamine.

other single resonance. Repeated attempts to resolve a coupling failed. To increase the sensitivity in the measurement, we synthesized the trisaccharide with 90% ^{13}C enrichment at both C1 Gal and C1 Fuc. Again, no splitting could be detected although line broadening was apparent in both ^{13}C-enriched resonances (Figure 6). Line broadening was measured by comparing the line widths of ^{13}C-enriched resonances in the doubly enriched trisaccharide with those of the singly enriched trisaccharide and parent disaccharide as well as with dioxane as an internal standard. The line broadening in both enriched-carbon resonances can be accounted for by an unresolved coupling of 1–1.5 Hz.

Several doubly enriched di- and trisaccharides [αL[1-^{13}C]Fuc(1→2)βD[1-^{13}C]Gal-ethanol, αL[1-^{13}C]Fuc(1→2)-βD[1-^{13}C]Gal(1→4)DGlcNAc, αL[1-^{13}C]Fuc(1→2)βD[1-^{13}C]Gal(1→4)DGlc, and αL[1-^{13}C]Fuc(1→2)βD[1-^{13}C]Gal-hexanolamine] were prepared for evaluation of the ψ torsion angle in the αLFuc(1→2)Gal glycosidic linkage. In no case was $^3J_{C1-C1'}$ coupling observed although line broadening of approximately 2.5 Hz was observed in the doubly enriched saccharides compared to the singly enriched compounds. Line

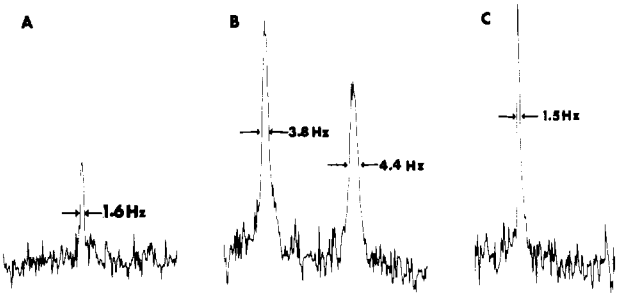


FIGURE 6: Carbon-13 NMR line-width measurements of the ^{13}C-enriched resonances in (A) αLFuc(1→2)βD[1-^{13}C]Gal(1→4)-βDGlcNAc-hexanolamine, (B) αL[1-^{13}C]Fuc(1→2)βD[1-^{13}C]Gal-(1→4)βDGlcNAc-hexanolamine, and (C) the natural abundance ^{13}C resonance of dioxane. Transients (1000) were accumulated at a sweep width of 1000 Hz at 0.36 Hz/data point.

broadening is interpreted as indicating unresolved coupling (1–1.5 Hz) in these cases also.

Interpretation of $^3J_{C-C}$ couplings requires that the dependence of $^3J_{C-C}$ on dihedral angles be established. Although an appropriate series of rigid model compounds is not available,

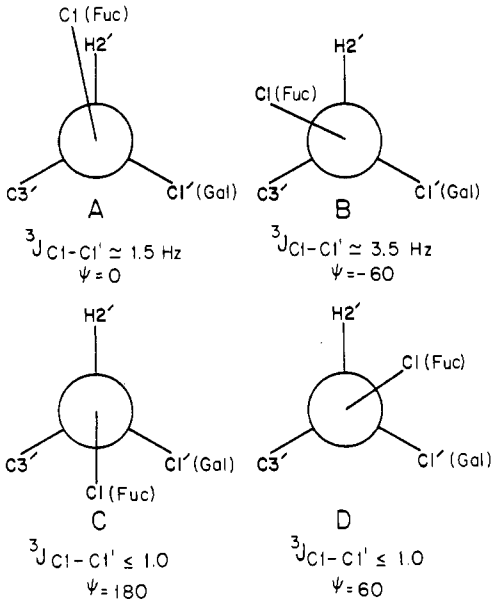


FIGURE 7: Possible ψ torsional angles about the αLFuc(1→2)βDGal glycosidic linkage.

it is probable that a Karplus relationship holds and that $^3J_{C-C}$ values have a minimum near 90° and maxima near 180 and 0° (Inch, 1972). In the following discussion, for convenience, specific values will be assigned to various dihedral angles. This specificity is not meant to imply that rigid conformations exist, rather the values are approximate and should be considered to be the "weighted average" of the rotameric structures present in solution.

Three-bond coupling through O, between C1 and C6 in hexoses and hexopyranosides where the dihedral angle is 180°, falls in the range 3–4 Hz (Walker et al., 1976). These values may represent a near maximum value for $^3J_{C-C}$ coupling through oxygen in carbohydrates. Coupling between C1 and C4 (dihedral angle 60°) in the 1-^{13}C-enriched carbohydrates was not observed (Walker et al., 1976). The vicinal relationship between C1 and C4 in the pyranose ring is gauche and therefore only a small coupling constant would be predicted. However, within the cyclic system there are two pathways through which coupling can occur. The pathway through C2 and C3 is the virtual mirror image of the pathway through O5 and C5, and, if couplings have different signs, they would tend to cancel. Vicinal coupling constants in pentofuranosides, pentofuranoses, and 1,4-aldonolactones (dihedral angle = 110–140°) are in the range of 0–2.5 Hz (H. A. Nunez et al., unpublished observations). In addition, coupling from C1 to C2′ in ethyl glycopyranosides is approximately 3 Hz (Nunez & Barker, 1979). Here the rotameric population probably favors the conformation in which C2′ is trans to C1. If this rotamer accounts for 70% of the population, the maximum value for $^3J_{C1-C2'}$ would be ~4 Hz.

These data and the inter-ring $^3J_{C1-C1'}$ coupling of approximately 1.5 Hz, estimated from the 2.5-Hz line broadening, were used to predict the most probable rotamer for ψ in the αLFuc(1→2)βDGal glycosidic linkage. Projections in Figure 7 show the expected variation in coupling constant with dihedral angle ψ for the αLFuc(1→2)βDGal glycosidic linkage. Rotamers with C1 Fuc eclipsed (or near eclipsed) by either C1′ or C3′ Gal are omitted because of unfavorable nonbonded interactions. Rotamer B with C1 anti to C1′ can be eliminated also since a readily observable coupling constant (3.5 Hz) is expected for this arrangement. Rotamers C and D would have $^3J_{C1-C1'} \simeq$ 0–1.0 Hz. These and other conformations with C1

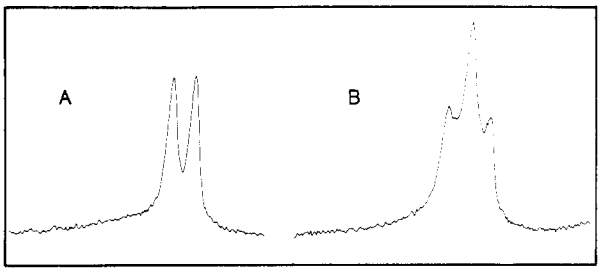


FIGURE 8: Partial 180-MHz ^{1}H NMR showing the H1 Fuc resonance in (A) (A) αLFuc(1→2)βDGal-ethanol and (B) αLFuc(1→2)βD[2-^{13}C]Gal-ethanol. Transients (512) were accumulated at a sweep width of 2500 Hz at 0.15 Hz/data point.

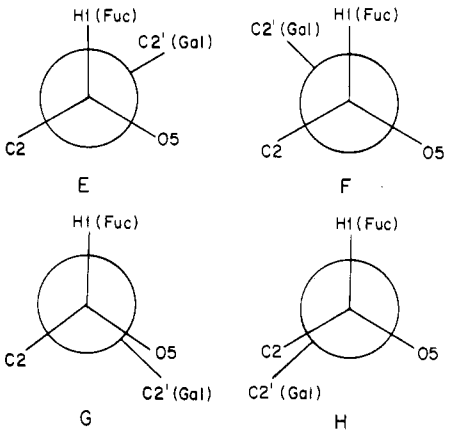
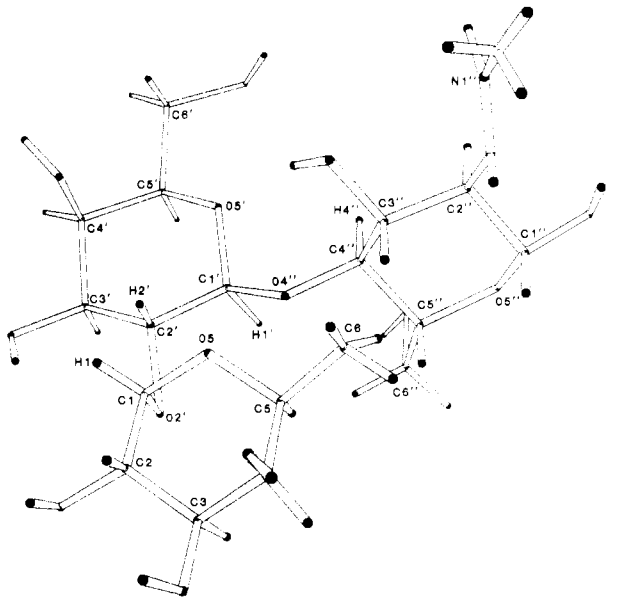


FIGURE 9: Torsion angles ϕ = 55 and 125° of αLFuc(1→2)βDGal glycosidic linkage; each would have $^3J_{H1-C2'} \simeq$ 3.2 Hz.

Fuc <120° to C1′ Gal are not expected to show line broadening of 2.5 Hz since, in the disaccharide βD[1-^{13}C]Gal(1→4)βDGlcNAc-hexanolamine, C3′ of GlcNAc is ~120° to C1 Gal and no line broadening is observed whereas the C5′ resonance is slightly broadened and it was assumed that this carbon is at an angle slightly larger than 120° with respect to C1 Gal (Nunez & Barker, 1980). Similar line broadening (or coupling) would be expected for a rotamer with C1 Fuc at an angle slightly greater than 120° to C1′ Gal (Figure 7, rotamer A) and for conformers with C1 at an angle slightly less than 60°. Models do not completely resolve this ambiguity although steric interactions are greater in the conformers with C1 <60° with respect to C1′. Thus, the observed coupling, together with considerations of steric interactions, suggests that the most stable conformer is rotamer A which has $\psi \simeq 0°$.

The torison angle ϕ about the αLFuc(1→2)βDGal glycosidic linkage was evaluated by using the model disaccharide αLFuc(1→2)βD[2-^{13}C]Gal-ethanol. In this compound, $^3J_{C2'-H1}$ should obey a Karplus relationship (Hamer et al., 1978). The 180-MHz ^{1}H NMR spectra of H1 of αLFuc(1→2)βDGal-ethanol and αLFuc(1→2)βD[2-^{13}C]Gal-ethanol are shown in Figure 8. The assignment of these resonances to H1 Fuc was made from the spectrum of αL[1-^{13}C]Fuc(1→2)βDGal-ethanol in which H1 Fuc is split by ^{13}C1 ($^1J_{C-H}$ = 170 Hz). The anomeric proton in the unenriched compound (Figure 8A) appears as a doublet ($^3J_{H1-H2}$ = 3.5 Hz); no long-range coupling is observed. In the [2-^{13}C]Gal disaccharide (Figure 8B), H1 is a triplet due to coupling with C2′ as well as H2. The coupling constants are estimated as $^3J_{H1-H2}$ = 3.4 Hz and $^3J_{H1-C2'}$ = 3.2 Hz. The latter interresidue coupling corresponds to a dihedral angle between the coupled atoms of approximately 55° (or 125°) (Hamer et al., 1978; Lemieux & Koto, 1974). Four conformations satisfy this requirement (Figure 9). Of these, F, G, and H have significant steric interactions between the Fuc and Gal rings. Thus, rotamer E is the most

Chart I



probable for the dihedral angle ϕ.

Lemieux & Koto (1974) described the exoanomeric effect that stabilizes certain conformations of ϕ. The exoanomeric effect produces maximal stabilization when a pair of electrons of the aglycon oxygen is antiperiplanar to those of the ring oxygen (Lemieux et al., 1979), a situation that occurs when $\phi = \pm 60°$ (Lemieux et al., 1980). Lemieux & Koto (1974) observed $^3J_{C1'-H1} = 3.8$ and 4.2 Hz for alkyl α- and β-glycopyranosides, respectively, and assigned ϕ values of $\sim 60°$. Based on $^3J_{C2'-H1} = 3.2$ Hz, the value of ϕ for the αLFuc-(1→2)βDGal glycosidic linkage appears to be $\sim 55°$. It may well be determined by the exoanomeric effect.

The values for ϕ and ψ (55 and 0°, respectively) deduced from the coupling observed in this study are in excellent agreement with those estimated by Lemieux et al. (1980) ($\phi = 40°$ and $\psi = 20°$) on the basis of hard-sphere calculations and NMR parameters. Note that Lemieux et al. (1980) were evaluating conformations in type 1 blood group oligosaccharides in which the Gal moiety is linked β(1→3) to GlcNAc rather than β(1→4) as in the trisaccharide described here.

The elegant study of Lemieux et al. (1980) clearly demonstrates that the most stable conformation about a single glycosidic bond in an oligosaccharide is influenced by changes in the conformation about other glycosidic bonds in the molecule. The difference between the estimates of ϕ and ψ for the αLFuc(1→2)βDGal linkage made by Lemieux et al. (1980) and those made in this study are therefore to be expected. Nunez & Barker (1980) evaluated ϕ and ψ for the disaccharide βD[1-^{13}C]Gal(1→4)βDGlcNAc-hexanolamine and assigned values of 60 and 15°, respectively. In this conformation, C1 Gal lies at an angle slightly greater than 120° with respect to C5′ GlcNAc and slightly less than 120° with respect to C3′ GlcNAc, causing broadening of the C5′ resonance. It is important to determine whether fucosylation alters the conformation about the glycosidic linkage in the disaccharide βDGal(1→4)βDGlcNAc-hexanolamine. Unfortunately, evaluation of this conformation in αLFuc(1→2)βD[1-^{13}C]Gal(1→4)βDGlcNAc-hexanolamine was hindered by overlap of the C5″ GlcNAc and C5′ Gal resonances (Figure 5B). The resonance of C3″ GlcNAc is well separated from other resonances and is not noticeably broadened in comparison to other single carbon resonances in the spectrum, indicating $^3J_{C1'-C3''} \simeq 120°$. Since an appreciable change in coupling to C5″ GlcNAc would stimultaneously cause a change in coupling to C3″ GlcNAc, the value of ψ of the glycosidic bond in the βDGal(1→4)GlcNAc moiety of the trisaccharide is proposed to be similar to that in the disaccharide ($\simeq 15°$). This conformation permits hydrogen bonding between OH3″ of GlcNAc and O5′ Gal as proposed for lactose (Hirotsu & Shimada, 1974).

The ϕ torsional angle in the βDGal(1→4)GlcNAc glycosidic linkage in the trisaccharide αLFuc(1→2)βD[1-^{13}C]Gal(1→4)βDGlcNAc-hexanolamine was evaluated with geminal coupling constants $^2J_{C1'-C4''}$ as described by Nunez & Barker (1980) for the parent disaccharide. The presence of substantial broadening in the resonance of C4″ (Figure 5B) indicates that the most stable conformer has C4″ bisecting the O5′–H1′ angle ($\phi \simeq 60°$) (Nunez & Barker, 1980). This conformation maximizes stabilization due to the exoanomeric effect (Lemieux et al., 1979) and is similar to that obtained by X-ray diffraction studies on lactose (Hirotsu & Shimada, 1974).

Studies with 1-^{13}C- and 2-^{13}C-enriched aldopyranosides indicate that in these compounds, also, the aglycon carbon equivalent to C4″ GlcNAc is anti to C2 (Nunez & Barker, 1979). Ethyl α-D-[2-^{13}C]galactopyranoside was found to have a $^3J_{C2-C1'} = 3.5$ Hz, indicating an anti relationship between C2 and C1′. Two-bond, $^2J_{^{13}C1-C1'}$, coupling in this glycoside appeared as broadening (1.0–1.5 Hz) of the aglycon. Since C4″ GlcNAc is equivalent to the C1′ aglycon in the 1-^{13}C- and 2-^{13}C-enriched aldopyranosides, the findings support the conclusion that C4″ GlcNAc is anti to C2′ Gal in the trisaccharide. A computer simulation of the proposed structure for the trisaccharide is shown in Chart I.

Although fucosylation at C2 of the disaccharide does not affect the ϕ or ψ torsion angles of the βDGal(1→4)DGlcNAc glycosidic linkage, it does cause a substantial shift in the resonance of C4″ GlcNAc (2.4 ppm upfield). This change may reflect changes in nonbonded interactions, torsional angles, or hybridization of the affected nucleus.

In summary, we have shown that the trisaccharide of the type 2 blood group H substance αLFuc(1→2)βDGal(1→4)-βDGlcNAc-hexanolamine can be synthesized readily in 20–50-mg amounts using the galactosyltransferase from bovine milk and the fucosyltransferase from porcine submaxillary glands. The synthesis of the trisaccharide and smaller amounts of a number of fucosyl disaccharides with ^{13}C enrichment in the Fuc and Gal moieties allows inter- and intraresidue C–C and C–H couplings to be measured and the conformation of the oligosaccharides in solution to be evaluated. Conformations of the component monosaccharide residues are similar to those of the unsubstituted monosaccharides or their simple glycosides. The conformation of the βDGal(1→4)βDGlcNAc glycosidic bond in the trisaccharide is similar to that in the disaccharide ($\phi \simeq 60°$ and $\psi \simeq 15°$). The αLFuc(1→2)βDGal glycosidic bond has $\phi \simeq 55°$ and $\psi \simeq 0°$ in the trisaccharide and in related disaccharides.

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Single-Crystal Electron Paramagnetic Resonance Studies of Photolyzed Oxy- and Nitric Oxide–Cobalt Myoglobins†

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ABSTRACT: Low-temperature photodissociation of oxygen from oxy–cobalt myoglobin was studied by single-crystal electron paramagnetic resonance (EPR) spectroscopy at 5 K. The photolyzed oxy–cobalt myoglobin exhibited an EPR spectrum consisting of two nonequivalent sets (species I and II) of the principal values and eigenvectors of the g tensors: $g_1^{I} = 3.55$, $g_2^{I} = 3.47$, and $g_3^{I} = 2.26$ for species I, and $g_1^{II} = 2.04$, $g_2^{II} = 1.93$, and $g_3^{II} = 1.86$ for species II, which resembled neither the deoxy nor the oxy form. Possible models of the photodissociated state of oxy–cobalt myoglobin are proposed by comparison with cobalt porphyrin complexes. The photolyzed product of nitric oxide–cobalt myoglobin exhibited new EPR signals at $g = 4.3$ and a very broad signal at around $g = 2$. The principal g values have been determined from the single-crystal EPR measurements: $g_1 = 4.39$, $g_2 = 4.27$, and $g_3 = 4.00$. Analysis of another EPR signal around $g = 2$ was difficult due to its broadness. Magnetic interactions were observed. An isotropic EPR signal at $g = 4.3$ suggested a weakly spin-coupled system between cobaltous spin ($S = {}^1/_2$ or ${}^3/_2$) and nitric oxide spin ($S = {}^1/_2$).

The light-induced dissociations of ligands such as oxygen (O_2), carbon monoxide (CO), and nitric oxide (NO) from myoglobin (Mb),[1] hemoglobin (Hb), and other hemoproteins are well-known. The elucidation of the electronic and stereochemical natures of these photolyzed products has an important implication in our understanding of the molecular mechanisms of ligand binding and photolysis.

Artificial myoglobins and hemoglobins substituted with cobaltous protoporphyrin IX for protoheme are capable of reversible oxygenation (Hoffman & Petering, 1970; Yonetani et al., 1974a). As previously demonstrated, oxy–CoMb and oxy–CoHb are readily photolyzed at liquid helium temperature (4.2 K). The optical absorption spectra of the photolyzed products are indistinguishable from those of the corresponding deoxy compounds. However, the photolyzed product exhibits new EPR extrema at $g = 3.87$ and around $g = 1.9$, which resemble neither the deoxy nor the oxy form, suggesting the possibility that the photolyzed product may be in a new

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[1] Abbreviations: Mb, myoglobin; Hb, hemoglobin; EPR, electron paramagnetic resonance; Co^{II}TpivPP, cobalt(II) *meso*-tetrakis($\alpha,\alpha,\alpha,\alpha$-*o*-pivalamidophenyl)porphyrin.

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