Glycolipid Membrane Surface Structure: Orientation, Conformation, and Motion of a Disaccharide Headgroup†

J.-P. Renou,‡ J. B. Giziewicz, Ian C. P. Smith, and Harold C. Jarrell*
Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6
Received August 4, 1988; Revised Manuscript Received October 24, 1988

ABSTRACT: The orientation of the disaccharide headgroup of a lactose-containing lipid, 3-*O*-(4-*O*-β-D-galactopyranosyl-β-D-glucopyranosyl)-1,2-di-*O*-tetradecyl-*sn*-glycerol (DTLL), relative to the surface of bilayer membranes has been determined via ^{2}H NMR. The lactosyl headgroup is extended away from the membrane surface into the aqueous phase. The headgroup motion has axial symmetry as evidenced by the spectral line shape and order parameter tensor. ^{2}H NMR of oriented multibilayers of DTLL confirms that the director of motional averaging is the bilayer normal. The two sugar residues have segmental order parameters S (glucose, 0.53; galactose, 0.51) which indicate that the headgroup fluctuates about the bilayer normal as a rigid unit. ^{2}H spin–lattice relaxation times T_{1z} for deuterons on each of the two sugar rings are similar, indicating further that there is no substantial motion about the disaccharide linkage within the headgroup. The magnitude of the relaxation times (4 ms) suggests that the rigid body motions of the headgroup are approaching the Larmor frequency; however, they increase with increasing temperature, indicating that the motions are rapid enough to be in the fast motional regime ($\omega_0^2\tau_c^2 < 1$). The conformation about the galactose–glucose intersaccharide linkage, calculated from the ^{2}H NMR data, is shown to differ substantially from those found in X-ray diffraction studies of crystalline lactose and high-resolution NMR studies of methyl lactoside in nonviscous solution. The orientations of the hydroxymethyl groups in the headgroup have been calculated from the ^{2}H NMR data. For the galactosyl residue the data are consistent with the presence of more than one rotamer about the C5″–C6″ bond which are in fast exchange on the ^{2}H NMR time scale. The hydroxymethyl group of the glucose residue exists in two rotameric forms about the C5′–C6′ bond which have relative populations of ca. 2:1 and which are in slow exchange on the ^{2}H NMR time scale (10^{-5} s). The two rotamers differ from those deduced from X-ray crystallography of crystalline lactose and ^{13}C NMR studies of methyl lactoside in solution.

Glycolipids in cell membranes may be divided into two distinct classes: glycosphingolipids and glycoglycerolipids. Glycosphingolipids are implicated in cell–cell recognition (Critchly et al., 1979), surface membrane receptors (Fishman & Brady, 1976), and antigenicity (Hakomori, 1984). Glycoglycerolipids which occur in plants (Quinn & Williams, 1978), bacteria (Rogers et al., 1980), and mycoplasma (Wieslander et al., 1978) have only recently received more intensive investigation with regard to their physical properties in model and biomembrane systems (Wieslander et al., 1978; Sen et al., 1982; Endo et al., 1982; Hinz et al., 1985). In view of the important roles played by the glycolipid headgroup, it is clear that an understanding of the structure and dynamics of the carbohydrate moiety can provide valuable insight into the properties of the membrane surface and into the process of cell surface recognition, at the molecular level. To date the headgroup conformation of glycolipids has been explored through the use of energy minimization calculations (Bock et al., 1985; Wynn & Robson, 1986; Wynn et al., 1986) and high-resolution NMR[1] studies (Czarniecki & Thornton, 1976; Sillerud et al., 1978; Scarsdale et al., 1986). In one recent report the two approaches were combined with the results from ^{1}H NMR used as distance constraints in the conformational energy minimization calculations to select the most likely conformation from a set of energetically similar conformers (Scarsdale et al., 1986). Such assessments of glycolipid headgroup structure are valuable. However, they deal only with intramolecular interactions and/or with single molecules in an isotropic environment. Lipids actually occur in the anisotropic molecular environment of the biological membrane, where the intramolecular interactions may differ from those of isolated molecules. More importantly, strong intermolecular interactions at the membrane surface may occur. As a result, a direct measure of the headgroup properties within a bilayer environment is a necessary extension of the approaches mentioned previously.

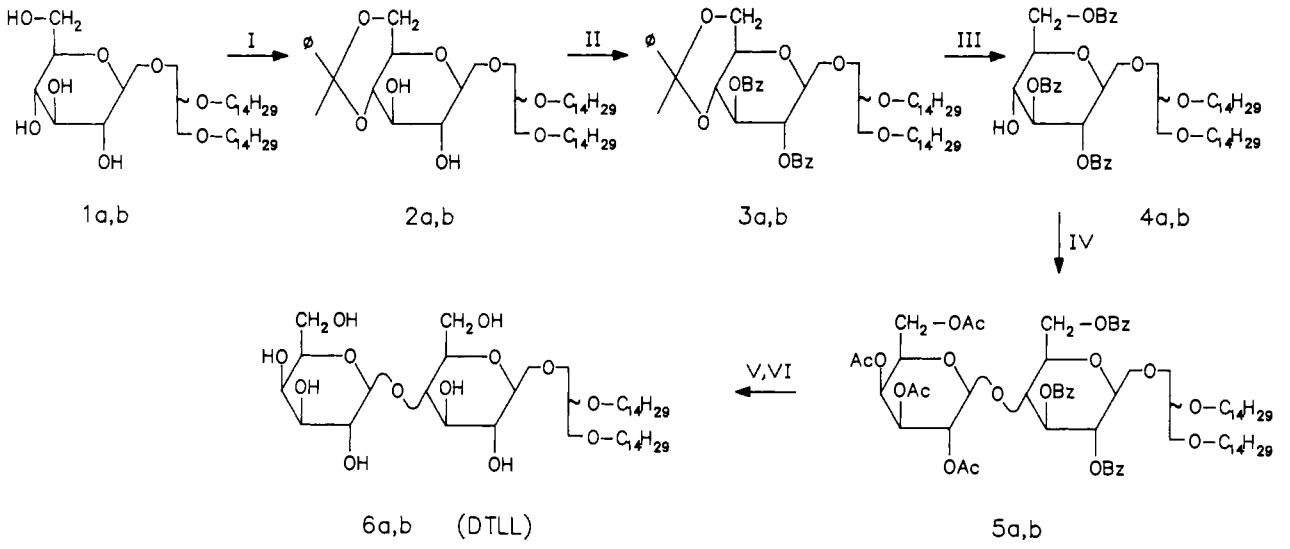
The first ^{2}H NMR studies on glycolipid headgroups involved cerebrosides (Skarjune & Oldfield, 1979, 1982) and demonstrated that valuable information about the nature of the surface of membranes containing this important class of lipids may be obtained. Previous studies from this laboratory have demonstrated that ^{2}H NMR can provide detailed information on the headgroup properties of glycoglycerolipids (Jarrell et al., 1986, 1987a,b). The headgroup orientation of monoglycosylglycerolipids relative to the bilayer surface has been shown to be sensitive to the type of linkage to the glycerol backbone and to the type of carbohydrate which constitutes the headgroup (Jarrell et al., 1987a). In addition, both rates and amplitudes of the headgroup motion are sensitive to the nature of the carbohydrate (Jarrell et al., 1987b). ^{2}H NMR results on aqueous multilamellar dispersions of a gluco-

† Issued as NRCC Publication No. 29635.
‡ On leave from the Institut National de la Recherche Agronomique, Station de Recherches sur la Viande, Thiex 63122 Ceyrat, France.

[1] Abbreviations: NMR, nuclear magnetic resonance; DTLL, 3-*O*-(4-*O*-β-D-galactopyranosyl-β-D-glucopyranosyl)-1,2-di-*O*-tetradecyl-*sn*-glycerol, 3-*O*-β-lactosyl-1,2-di-*O*-tetradecyl-*sn*-glycerol; β-DTGL, 3-*O*-β-D-glucopyranosyl-1,2-di-*O*-tetradecyl-*sn*-glycerol; DMPC, dimyristoylphosphatidylcholine; TLC, thin-layer chromatography; DSC, diffential scanning calorimetry.

0006-2960/89/0428-1804$01.50/0

Scheme I[a]



[a] a: *rac*-glycerol, deuterated galactose moiety. b: *sn*-glycerol, deuterated glucose moiety. (I) PhCHO, $ZnCl_2$; (II) NaH, BzCl/DMF; (III) $BH_3{\cdot}(CH_3)_3N$, $AlCl_3$/THF; (IV) $Hg(CN)_2$, $HgBr_2/CH_3CN$; (V) H_2, 10% Pd/C/AcOH; (VI) CH_3ONa/CH_3OH.

cerebroside (Skarjune & Oldfield, 1982) and its analogous glycoglycerolipid (Jarrell et al., 1986) reveal that the headgroups have similar orientational and motional properties. The latter results suggest that information obtained on glycoglycerolipids may be applicable to the corresponding glycosphingolipids.

The present study extends previous studies on monoglycosylglycerolipids to a disaccharide-containing lipid, 3-*O*-(4-*O*-β-D-galactopyranosyl-β-D-glucopyranosyl)-1,2-di-*O*-tetradecyl-*sn*-glycerol (DTLL). The lactosyl residue is of interest since it occurs in lactosylceramide, a precursor for more complex glycosphingolipids (Hakomori, 1984; Feizi, 1985). In addition, the lactosyl group provides a surface moiety with which to probe directly surface interactions such as binding to galactose-specific lectins (Soriano et al., 1983). The present study elucidates the membrane surface characteristics of the lactosyl headgroup with regard to the orientation of the carbohydrate relative to the membrane surface, the degree of motional restriction, as reflected by the molecular order parameter, and the rates of headgroup motion. In addition, the conformation about the intersaccharide linkage is deduced and compared with those reported from high-resolution NMR studies of methyl lactoside and X-ray crystallographic data for lactose.

Materials and Methods

3-O-(4-O-β-D-Galactosyl-β-D-glucopyranosyl)-1,2-di-O-tetradecyl-sn-glycerol (DTLL). Hepta-*O*-acetyl-α-lactosyl bromide (Hudson & Kunz, 1925) (8.85 mmol) was added in one portion to a mixture of 1,2-di-*O*-tetradecyl-*sn*-glycerol (Jarrell et al., 1986) (8.85 mmol, 4.28 g) and mercury(II) cyanide (10 mmol, 2.52 g) in dry acetonitrile–methylene chloride (150 mL, 2:1 v/v), and the resulting mixture was stirred at room temperature overnight with the exclusion of moisture. The reaction mixture was concentrated under vacuum to a residue which was dissolved in chloroform. The solution was washed with 1 M aqueous potassium bromide, dried over sodium sulfate, and concentrated. The product was purified by silica gel chromatography with ethyl acetate–hexane–chloroform (1:4:4 v/v/v) as eluant. Recrystallization from ethanol–water afforded the acetylated lipid (2.92 g, 54.2%), mp 35.5–36.5 °C. Anal. Calcd for $C_{57}H_{98}O_{20}$: C, 62.05; H, 8.95. Found: C, 62.18; H, 9.01.

Deacetylation of the lipid derivative (2.34 g) under standard conditions (Jarrell et al., 1986) afforded DTLL (1.67 g, 97.4%), mp 199–200 °C [lit. (Ogawa & Beppu, 1982) mp 210–213 °C]. Anal. Calcd for $C_{43}H_{84}O_{13}$: C, 63.83; H, 10.46. Found: C, 63.64; H, 10.52. The product had a ^{13}C NMR spectrum which was in agreement with published data for DTLL (Ogawa & Beppu, 1982).

Deuteration of DTLL. DTLL (100 mg, preexchanged by evaporation to dryness of a methanol–O^2H_1 solution) in 1,2-dimethoxyethane–^2H_2O (15:10 v/v) was refluxed for 1 h in the presence of Raney nickel [preexchanged with ^2H_2O (Koch & Stuart, 1977)]. The reaction mixture was filtered and concentrated to a residue which was recrystallized from methanol to give DTLL: homogeneous on TLC (methanol–chloroform, 2:8 v/v; ethyl acetate–methanol–water 12:2:1 v/v/v); mp 193–196 °C. ^{13}C NMR revealed deuteration at C3″ and C4″ of the galactose residue (ca. 60% and 90% ^2H, respectively). Anal. Calcd for $C_{43}H_{82}{}^2H_2O_{13}$: C, 63.67; H, 10.69. Found: C, 63.51; H, 10.52. Exchange for 2 h gave DTLL deuterated at C2″ (80%), C3″ (100%), C4″ (100%), and C6″ (80%) of the galactose moiety and C3′ (50%) of the glucose residue, as determined by ^{13}C NMR. DTLL ^2H-labeled in the glucose residue was prepared as outlined in Scheme I.

3-O-(4,6-O-Benzylidene-β-D-glucopyranosyl)-1,2-di-O-tetradecyl-rac-glycerol (**2a**). 3-*O*-β-D-glucopyranosyl-1,2-di-*O*-tetradecyl-*rac*-glycerol (**1a**) (Jarrell et al., 1986) (1.5 g) was added to a mixture of anhydrous $ZnCl_2$ (1.7 g) and benzaldehyde (30 mL) and the mixture stirred overnight. The reaction mixture was diluted with chloroform and the solution washed with aqueous sodium bicarbonate, dried over anhydrous sodium sulfate, and concentrated. The crude product was chromatographed on silica gel with hexane as eluant to remove benzaldehyde and then with ethyl acetate–hexane (1:4 v/v) to give **2a** (1.44 g, 83%) after crystallization from methanol–water. After being dried for 15 h at 40 °C and 0.04 Torr, compound **2a** had an mp of 62.5–63 °C. Anal. Calcd for $C_{44}H_{70}O_8$: C, 71.89; H, 10.70. Found: C, 71.59; H, 10.76.

3-O-(2,3-Di-O-benzyl-4,6-O-benzylidene-β-D-glucopyranosyl)-1,2-di-O-tetradecyl-rac-glycerol (**3a**). Compound **2a** (1.3 g, 1.77 mmol) was added to a suspension of sodium hydride (256 mg, 10.6 mmol) in dry dimethylformamide (30 mL) and the mixture stirred at ambient temperature for 30

min. Benzyl chloride (1.23 mL, 10.6 mmol) was added and the mixture stirred at room temperature overnight. The reaction was quenched with methanol (5 mL) for 30 min and the resulting mixture diluted with chloroform, washed with water, and concentrated under vacuum. The residue was fractionated on silica gel with a step gradient of 3–45% ethyl acetate in hexane to give crystalline **3a** (990 mg, 60.6%). Recrystallization from ethyl acetate–methanol at 4 °C afforded **3a** (909 mg, 56%) in two crops having an mp of 44–46 °C. Anal. Calcd for $C_{58}H_{90}O_8 - CH_3OH$: C, 74.80; H, 10.00. Found: C, 75.04; H, 10.15.

3-O-(2,3,6-Tri-O-benzyl-β-D-glucopyranosyl)-1,2-di-O-tetradecyl-rac-glycerol (**4a**). Compound **3a** (831 mg, 0.9 mmol) was treated with borane–trimethylamine complex (10 mmol) and anhydrous aluminum chloride (10 mmol) in dry tetrahydrofuran (50 mL) (Garreg et al., 1983). After 3 h additional borane–trimethylamine and aluminum chloride (5 mmol) were added, and the mixture was stirred overnight at ambient temperature. The reaction mixture was concentrated and the residue dissolved in chloroform, washed with dilute aqueous hydrochloric acid, dried over sodium sulfate, and concentrated. The residue was coevaporated several times with methanol and the crude product fractionated on a silica gel column with a 4–6–8–10% step gradient of ethyl acetate–hexane as eluant. The product was crystallized from diethyl ether–methanol at −15 °C in 82% yield and had an mp of 48–49 °C. Anal. Calcd for $C_{58}H_{92}O_8$: C, 75.94; H, 10.11. Found: C, 75.77; H, 10.27.

3-O-(4-O-β-D-[2,3,4,6,6-$^2H_5$]Galactopyranosyl-β-D-glucopyranosyl)-1,2-di-O-tetradecyl-rac-glycerol ([$^2H_5$]-**6a**). Tetra-*O*-acetyl-D-[2,3,4,6,6-$^2H_5$]galactopyranosyl bromide was prepared according to the procedure used for its glucose analogue (Jarrell et al., 1986) and had deuteration levels, as determined by ^{13}C NMR, of C2 (75%), C3 (30%), C4 (100%), and C6 (60%). Compound **4a** (230 mg) in dry acetonitrile (15 mL) was treated at room temperature overnight with the ^2H-labeled acetylated galactosyl bromide (3.7 mmol) in the presence of mercury(II) cyanide (316 mg, 1.25 mmol) and mercury(II) bromide (450 mg, 1.25 mmol). Concentration of the reaction mixture afforded a residue which was dissolved in chloroform, washed with 1 M potassium bromide, dried over sodium sulfate, and concentrated. Fractionation of crude **5a** on a silica gel column with 12% followed by 16% ethyl acetate in hexane afforded **5a** as a colorless syrup.

Deprotection of **5a** by hydrogenolysis with 10% Pd/C in acetic acid followed by Zemplēn deacetylation afforded, after recrystallization from methanol, pure **6a** in 35% yield. The product was homogeneous on TLC (methanol–chloroform, 1:4 v/v) and comigrated with authentic DTLL.

3-O-(4-O-β-D-Galactopyranosyl-β-D-[2,3,4,6,6-$^2H_5$]glucopyranosyl)-1,2-di-O-tetradecyl-sn-glycerol ([$^2H_5$]-**6b**). 3-*O*-β-D-[2,3,4,6,6-$^2H_5$]Glucopyranosyl-1,2-di-*O*-tetradecyl-*sn*-glycerol (Jarrell et al., 1986) was treated as described above for compounds **2a**–**6a**. Compound **6b** had an mp of 191–193 °C. Anal. Calcd for $C_{43}H_{81}{}^2H_3O_{13}$: C, 63.59; H, 10.80. Found: C, 63.73; H, 10.62. ^2H-Labeling in the glucose residue was determined by ^{13}C NMR to be C2 (100%), C3 (30%), C4 (100%), and C6 (60%) (Jarrell et al., 1986).

Methods. Column chromatography was performed with silica gel (230–400 mesh, Aldrich Chemical Co., Milwaukee, WI).

Differential scanning calorimetry (DSC) was performed as described previously (Jarrell et al., 1986).

Samples for ^2H NMR consisted of 30–40 mg of dry lipid hydrated with a 3-fold excess of ^2H-depleted water (Aldrich Chemical Co., Milwaukee, WI) in a 5-mm (o.d.) sample tube. Hydrated samples were cyclically heated to 75 °C with vortex mixing and freeze-thawed to homogeneity (4–5 cycles). Oriented multibilayers were prepared as described elsewhere (Jarrell et al., 1987a).

^2H NMR spectra were obtained at 30.7 MHz as described previously (Jarrell et al., 1986). Typically, spectra were obtained with a $\pi/2$ pulse width of 2.3 μs (5-mm solenoid coil) or 3.8 μs (10-mm solenoid coil), 60-μs delay between the $\pi/2$ pulses of the quadrupolar echo sequence (Davis et al., 1976), and a recycle time of 100 ms. Relaxation times, T_{1z}, were measured with the inversion–recovery procedure in combination with the quadrupolar echo sequence as described elsewhere (Perly et al., 1985). Measurements on oriented multibilayers were performed as described previously (Jarrell et al., 1987b) with angular settings having an estimated accuracy of ±5°.

The orientation of the motional axis relative to each of the carbohydrate rings was computed from the ^2H NMR data as described previously (Jarrell et al., 1986). The atom positions for the calculations were obtained from the X-ray diffraction data of lactose (Hirotsui & Shimada, 1974). Quadrupolar splittings were measured from the 90° oriented sample ("dePaked") spectra which were calculated from the powder spectra as described previously (Bloom et al., 1981). The shapes of the lines in dePaked spectra were approximated, when required, with the curve analysis routine in the Nicolet 1280 NMR software and a Gaussian line-shape function. An alternative analysis of molecular ordering is given in the Appendix which follows the method outlined previously (Dufourc et al., 1983).

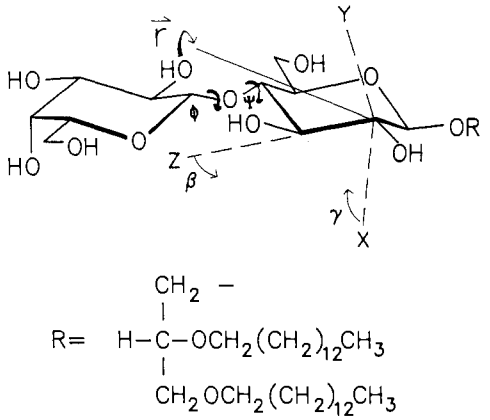
Conformational energies were calculated by the hard-sphere procedure (Venkatachalam & Ramachandran, 1967) using the Kitaigorodsky expression (Kitaigorodsky, 1961):

$$HS = 30000e^{-13(d/d_0)} - 0.14(d/d_0)^{-6} \text{ kcal/mol}$$

where d is the internuclear distance of two given atoms (separated by more than two bonds) and d_0 is 1.11 times the sum of their van der Waals radii. The exoanomeric effect, although frequently included in conformational energy calculations of oligosaccharides in aqueous solutions (Bock, 1983), was not included since for membrane systems the importance of the effect is not known. For aqueous solution, the exoanomeric effect has been suggested to be of little significance (Lipkind et al., 1984). It should also be emphasized that the effects of hydration have also been ignored. In the present study, the energies of conformers which fit the ^2H NMR are used qualitatively to assess whether or not the deduced structures are energetically reasonable.

Results and Discussion

Glycoglycerolipids which contain one sugar moiety as the headgroup are known to exhibit polymorphic phases (Wieslander et al., 1978; Sen et al., 1982; Mannock et al., 1985; Jarrell et al., 1986, 1987b) while lipids having two or more carbohydrate residues in the lipid headgroup form only lamellar structures in water (Wieslander et al., 1978, 1981; Iwamoto et al., 1982; Endo et al., 1983). Previous studies have indicated that the temperature at which the gel to liquid crystal transition occurs is sensitive to the constituent saccharide moieties in the headgroup (Iwamoto et al., 1982; Endo et al., 1983). Differential scanning calorimetry (DSC) reveals that 3-*O*-β-lactosyl-1,2-di-*O*-tetradecyl-*sn*-glycerol (DTLL) has an endothermic transition at 66 °C which is attributed to the gel to liquid crystal transition. ^2H NMR (Figure 2) reveals that at and above 70 °C the spectra are indicative of axially symmetric motion, while at or below 65 °C the spectra are not



$$R= \begin{array}{l} CH_2- \\ | \\ H-C-OCH_2(CH_2)_{12}CH_3 \\ | \\ CH_2OCH_2(CH_2)_{12}CH_3 \end{array}$$

FIGURE 1: Structure of 3-*O*-β-lactopyranosyl-1,2-di-*O*-tetradecyl-*sn*-glycerol (DTLL) and the molecule-fixed axis system. The definitions of the β and γ angles relating the motional axis to the molecule-fixed axis system are shown. The conformational angles φ and ψ are also indicated.

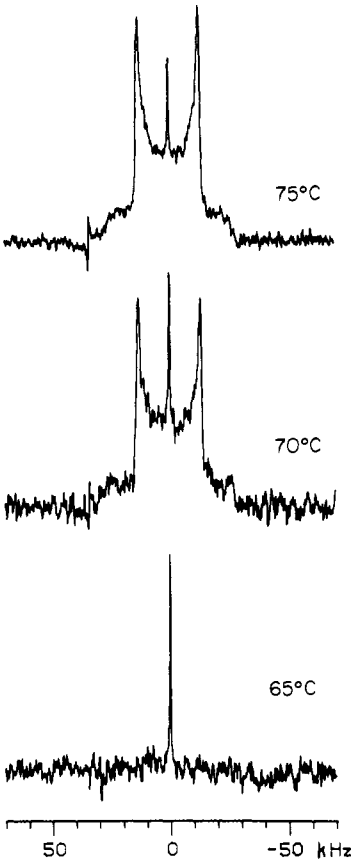


FIGURE 2: Temperature dependence of ^2H NMR spectra (30.7 MHz) of an aqueous dispersion of DTLL ^2H-labeled at C3″ and C4″ of the galactose residue.

representative of lipid in the liquid crystalline phase. Note that at 65 °C (Figure 2) essentially no signal attributable to DTLL labeled in the galactosyl residue is detectable. A similar result was obtaned for glucose-labeled DTLL (data not shown). While this aspect was not investigated further, it is reasonable to attribute the decrease in signal intensity to a significant decrease in the transverse relaxation time T_{2e}. Such a dramatic change in T_{2e} has been reported for dimyristoylphosphatidylcholine (DMPC) ^2H-labeled at C6 of the sn-2 chain where T_{2e} decreased by an order of magnitude on going from the liquid crystalline L_α phase to the intermediate $P_{\beta'}$ phase (Meier et al., 1986). Studies of dipalmitylglycoglycerolipids having the diglucosyl headgroups maltose and cellobiose (Iwamoto et al., 1982) exhibit the corresponding

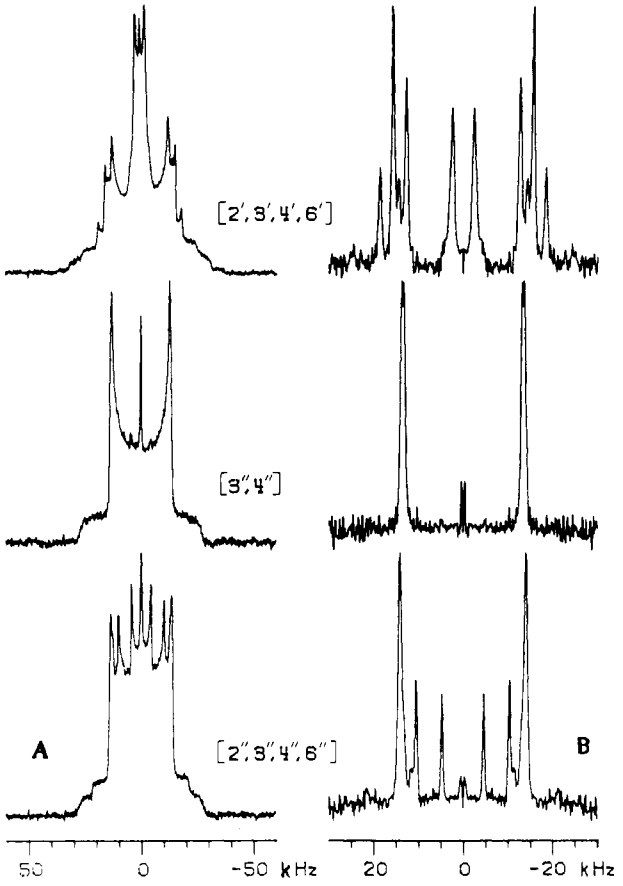


FIGURE 3: ^2H NMR spectra (30.7 MHz) of aqueous dispersions of DTLL ^2H-labeled at the indicated positions at 75 °C: (A) powder spectrum; (B) dePaked spectrum calculated from (A).

phase transition at 52 and 54 °C, respectively, which are substantially lower than that of DTLL. Since DTLL has shorter alkyl chains (C_{14}) than the dipalmitylglycoglycerolipids (C_{16}), the former may be expected to exhibit lower transition temperatures than the latter for the same headgroup (Hinz et al., 1985). Since lactose differs from cellobiose only in that the terminal saccharide moiety is galactose (the C4 epimer of glucose) rather than glucose, the higher T_c of DTLL relative to those of the diglucosyl lipids suggests that interheadgroup interactions within the bilayer plane are stronger for the former relative to the latter lipids. If such headgroup interactions are present, they may be reflected in the rates and amplitudes of the headgroup motion as well as in the conformation of the lactosyl residue.

Headgroup Order and Conformation. ^2H NMR spectra of aqueous multilamellar dispersions of the variously labeled lipids in the liquid crystalline phase are shown in Figure 3. The spectra for each of the ^2H-labeled pyranose rings have axially symmetric shapes reflecting the corresponding effective symmetry of the headgroup motion. Because the spectra are superpositions of powder patterns associated with each of the labeled ring positions, the spectra were "dePaked" to give the 90° oriented sample spectra (Figure 3B). Resonance assignments were made from the integrated intensities of the dePaked spectra and the known level of deuteration at each of the ring positions. The quadrupolar splittings associated with the various ^2H-labeled ring positions are given in Table I.

In order to relate the motion of the headgroup and its average orientation to the bilayer surface, the coincidence of the bilayer normal with the director of motional averaging of the lactosyl moiety must be confirmed. Oriented multibilayers

Table I: Headgroup Orientational Parameters and Experimental and Calculated Quadrupolar Splittings ($\Delta\nu_Q$) for DTLL at 75 °C

	orientation (deg)[a]		$\Delta\nu_Q$ (kHz)[b]						
carbohydrate moiety	β	γ	C2′	C2″	C3′	C3″	C4′	C4″	S
glucose	−19 ± 3	7 ± 5	24.8		28.5		31.2		0.53
			(24.8)		(28.5)		(31.1)		
			15.2[c]		5.6[c]		4.9[c]		
galactose	−80 ± 4	210 ± 4		29.5		26.6		27.8	0.51
				(29.1)		(26.2)		(27.4)	
				31.2[d]		31.2[d]		10.4[d]	

[a] Angles β and γ are defined as in Figure 1. Note that an additional solution representing a 180° rotation about the motional axis is not shown. [b] Values in parentheses are the experimental quadrupolar splittings. [c] Calculated assuming the conformation of crystalline lactose and the orientation of the motional axis relative to the galactose ring as determined from the ^{2}H NMR data. [d] As in footnote *c* but with the orientation of the motional axis relative to the glucose ring as determined from the ^{2}H NMR data.

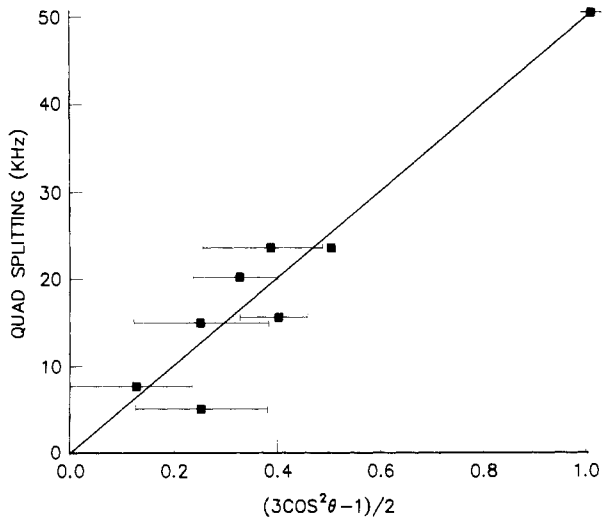


FIGURE 4: Quadrupolar splitting as a function of the orientation (θ) of the bilayer normal relative to the magnetic field direction for oriented multibilayers of DTLL ^{2}H-labeled in the galactosyl residue.

of DTLL were examined for the dependence of the quadrupolar splitting ($\Delta\nu_Q$) on the orientation θ of the bilayer normal (normal to the plane of the glass plates) to the magnetic field direction (Figure 4). The results are consistent with a $P_2(\cos\theta)$ dependence of $\Delta\nu_Q$ on the angle θ and thus with the bilayer normal as the director of motional averaging for DTLL.

On the ^{2}H NMR time scale (10^{-5} s) the pyranose rings of galactose and glucose are rigid so that the quadrupolar splitting, $\Delta\nu_Q$, for a given position is given by (Dufourc et al., 1983; Petersen & Chan, 1977)

$$\Delta\nu_Q = \frac{3}{4}\frac{e^2qQ}{h}S\frac{3\cos^2\theta_i - 1}{2} \quad (1)$$

where e^2qQ/h is the quadrupolar coupling constant (158 kHz for the OC–^{2}H group and 164 kHz for the OC–^{2}H$_2$ group (Jarrell et al., 1986), S is the segmental order parameter describing the anisotropic motion of the sugar ring relative to the bilayer normal, and θ_i is the angle between the ith C–^{2}H bond and the molecular rotation axis. Equation 1 assumes that the ordering is axially symmetric. In the axis system attached to the molecule (Figure 1), the segmental motional axis has the direction cosines ($\cos\gamma\sin\beta$, $\sin\gamma\sin\beta$, $\cos\beta$) so that

$$\cos\theta_i = a_x^i\cos\gamma\sin\beta + a_y^i\sin\gamma\sin\beta + a_z^i\cos\beta$$

where a_x^i, a_y^i, and a_z^i are the direction cosines of the ith C–^{2}H bond in the molecular axis system, as calculated from X-ray crystallographic data of lactose (Hirotsui & Shimada, 1974). The orientation of the motional axis in the molecular frame for each pyranose ring was calculated from the quadrupolar splittings as described previously (Dufourc et al., 1983; Jarrell et al., 1986), Table I. The values of the segmental order parameter S for each pyranose ring (galactose, 0.51; glucose, 0.53) are, within experimental error, the same, indicating that both rings are undergoing motions of the same amplitude relative to the director of motional averaging and that there is no segmental motion (conformational equilibrium) within the disaccharide headgroup. Therefore, on the ^{2}H NMR time scale the lactosyl group is moving as a rigid moiety and has a fixed conformation about the galactose–glucose glycosidic bond. The latter conclusion while interesting is not surprising since ^{13}C NMR studies of methyl lactoside in aqueous solution also concluded that there is little rotation about the glycosidic bond (Hayes et al., 1982). In addition a recent high-resolution ^{1}H NMR study of a complex glycolipid containing the lactosyl moiety as part of the carbohydrate headgroup concluded that in solution there is only one preferred conformation about the galactose–glucose bond (Scarsdale et al., 1986). The present results indicate that the headgroup of DTLL reorients as a rigid unit with a segmental order parameter of ca. 0.52. DTLL corresponds to the attachment of a galactose residue to the 4′ position of the corresponding monoglucosylglycerolipid 3-*O*-β-D-glucopyranosyl-1,2-di-*O*-tetradecyl-*sn*-glycerol (β-DTGL), for which an S value of 0.45 has been reported (Jarrell et al., 1986). The results for DTLL suggest that addition of a second carbohydrate moiety attenuates the amplitude of the overall headgroup motion relative to that of the parent monoglycosyl lipid. If the conformation of the lactosyl residue is fixed on the time scale of ^{2}H NMR measurements (10^{-5} s), it is of considerable interest to compare it with that deduced for this moiety in molecules which are in isotropic solution.

^{13}C NMR of methyl β-D-lactoside (Hayes et al., 1982) suggested that the conformation about the galactose–glucose glycosidic bond closely resembled that observed for lactose in the crystalline state (Hirotsui & Shimada, 1974). In the present study, the orientation of the motional axis of the lactose residue of DTLL relative to each of the constituent pyranose rings and the molecular ordering have been determined (Table I). By use of the orientation of the motional axis relative to the glucose (or galactose) ring and the X-ray-determined conformation of crystalline lactose, quadrupolar splittings for deuterons on the galactose (or glucose) ring may be calculated from eq 1 (Table I). Such calculations show (Table I) that, given the experimentally determined ring orientations, ordering, and the conformation of crystalline lactose, the predicted quadrupolar splittings do not agree with experiment. Hence, the lactosyl conformation in DTLL must differ from that of crystalline lactose and methyl β-D-lactoside in aqueous solution. The conformation about the intersaccharide linkage was deduced as follows. For one of the two possible glucose orientations (Table I) the conformation about the glycosidic bond was varied by coordinate rotations about the C1″–O and

Table II: Orientational Order Parameters[a] for the Lactosyl Residue of DTLL

conformation (deg)		order parameters[b]				
ϕ	ψ	S^*_{xx}	S^*_{yy}	S^*_{zz}	η^*	potential energy (kcal/mol)
−35	−40	−0.274	−0.237	0.511	0.074	3.9
−41	−33[c]	−0.275	−0.217	0.492	0.12	4.2
−27	−55	−0.275	−0.254	0.529	0.04	6.6
35	30	−0.330	−0.237	0.353	0.866	3.2
43	39[c]	−0.333	−0.0718	0.405	0.645	5.1
27	23	−0.326	−0.0033	0.326	0.998	3.0

[a] Order parameters S_{ij} determined in the molecule-fixed axis system were diagonalized to give the order matrix $\bar{S}^*$, where S^*_{zz} corresponds to the segmental order parameter S. [b] Average deviation in the calculated S_{ij} values over five combinations of the six S_{CD} values was <±5%. [c] Range of ϕ and ψ angles considered as possible conformations which fit the ^{2}H NMR data.

C4′–O bonds of the galactose residue, and the direction cosines of the C–^{2}H bonds were calculated. Quadrupolar splittings were calculated from eq 1, the determined S value, and orientation of the motional axis in the molecule-fixed axis system. Rotations about the two bonds were performed in 5° steps. Two conformations reproduced the experimental $\Delta\nu_Q$ values satisfactorily. However, one of the two possibilities could be excluded as improbable since atoms in the galactose ring are in close proximity to those of the glucose ring, in particular the hydroxymethyl residues nearly overlap (C6″–C6′ separation is ca. 1.9 Å which is less than the van der Waals radius for a CH_2 group). The second conformation had ϕ and ψ angles (Figure 1) of 40 ± 2° and −80 ± 10°, respectively, which differ considerably from the corresponding angles (ϕ 45°, ψ −15°) associated with crystalline lactose. It should be emphasized that in these calculations the glucose orientation was held fixed so that the results are limited by the accuracy with which the orientation of the glucose ring relative to the motional axis of the headgroup is determined. While these calculations give conformations which are consistent with the ^{2}H NMR data, they may not be energetically reasonable. Some insight into the conformational energy of the deduced structure may be obtained by potential energy calculations. For oligosaccharide structures, a common potential energy function uses the Kitaigorodsky potential (Kitaigorodsky, 1961) and the exoanomeric effect (Bock, 1983). In the present study, only an estimate of the conformational energy of the ^{2}H NMR deduced structure is desired. It is not obvious what potential energy function should be used to describe the conformation of oligosaccharides associated with a membrane surface. For this reason, the Kitaigorodsky potential was used in energy calculations without the inclusion of any additional energy terms. While admittedly crude, this approach can be useful since in the present study only a qualitative estimate of the relative energy of a given conformation is desired and not a search for the true minimum energy conformation(s). By this procedure, the conformation of crystalline lactose as determined by X-ray diffraction has an energy of 2.5 kcal/mol. For the structure deduced by ^{2}H NMR the energy was estimated to be 17 kcal/mol. This value is significantly higher than that of the minimum energy configurations. Two conclusions are possible. Either the presence of the membrane surface is such that additional interactions make the deduced conformation favorable, or as a result of the inaccurracy in determining the glucose headgroup orientation relative to the membrane plane, the best fit conformation is incorrect. The latter difficulty may be addressed by searching for a conformation and orientation of the entire lactosyl residue which best fit the ^{2}H NMR data.

Since the preceding results are consistent with a rigid headgroup structure, a more general analysis may be used in which the order tensor for the lactose unit is determined from the ^{2}H NMR results as outlined in the Appendix. In this approach the orientation of the most ordered axis of the headgroup is determined relative to the molecular frame, and the asymmetry in the ordering is determined. Conformations of the lactosyl residue were generated by rotations about the C4′–O4′ and C1″–O4′ bonds in 5° steps and the associated order matrices calculated from the C–^{2}H bond order parameters, as outlined in the Appendix. Solutions were sought which have the same order matrix for any combination of five of the six available S_{CD} values. An additional constraint in the calculations was that the potential energy of a given conformer be ≤30 kcal/mol. The latter criterion is quite arbitrary but was chosen to exclude energetically unreasonable possibilities while providing sufficient latitude to allow for consideration of energetically unlikely (based only on intramolecular interactions) conformers. The results are given in Table II and demonstrate that the possible conformations which fit the above criteria fall into two distinct angular ranges. The conformational energies, as calculated in this study, for the two sets of possible conformations are quite similar. The most notable difference lies in the calculated asymmetry parameter η^* in the segmental ordering (Table II). An asymmetry of >0.7 in segmental ordering is concluded to be unlikely for several reasons. Since the ^{2}H NMR line shapes (Figure 3) reflect effective axially symmetric averaging, the combination of internal motion about the glycerol headgroup linkage and overall molecular reorientation must be nearly axially symmetric. Dimyristoylphosphatidylcholine (DMPC) has been shown to approximate a cylindrical molecular shape and as a result exhibits a small asymmetry (≤0.1) in the ordering of the glycerol backbone (Strenk et al., 1985). Thus DTLL may be expected to exhibit similar symmetry in its ordering. Spectral simulations of ^{2}H NMR line shapes associated with ^{2}H-labeled phospholipids also deduced that acyl chain ordering exhibits a small asymmetry (Meier et al., 1986). The consistency of more than one set of order parameters with the NMR data may reflect the fact that for the glucose residue the C–^{2}H bond orientations are nearly parallel, giving rise to similar bond order parameters. As a result, determining the order parameters from the six available order parameters is less definitive than would otherwise be expected.

Inspection of Table II reveals that the S (S^*_{zz}) value lies in the range 0.49–0.53, in excellent agreement with the results obtained explicitly assuming axially symmetric ordering. The C2′–C3′ bond of the glucose residue is related to the most ordered axis of the order tensor (the local motional axis) by β −33 ± 3° and γ 20 ± 2° in good agreement with the results obtained above by considering only the glucose residue. The conformation about the glycosidic linkage gives −35 ± 6° and −40 ± 10° for the ϕ and ψ angles, respectively. This differs considerably from the conformation found for crystalline lactose (45° and −15°) (Hirotsui & Shimada, 1974) and methyl lactoside in aqueous solution (40 ± 10°, 15°) (Hayes et al., 1982). While the orientation of the most ordered axis

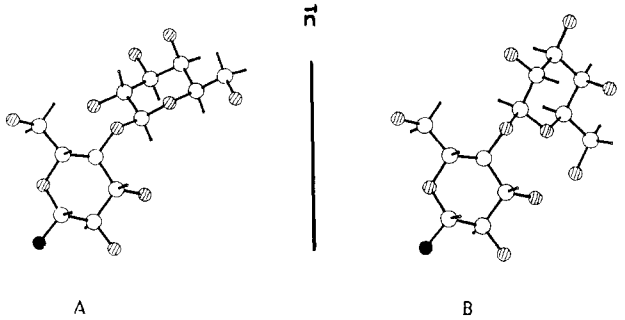


FIGURE 5: Orientation of the lactosyl residue relative to the motional axis $\vec{n}$ as calculated from the quadrupolar splittings. The conformation about the intersaccharide linkage is (A) that calculated from the NMR data in this study and (B) that for crystalline lactose. (○) Hydrogen; (hatched circle) oxygen; (○) carbon; (●) O3 of glycerol backbone.

in the molecular frame is similar to that assuming axially symmetric ordering, the conformation deduced is considerably different. The conformational energy, 4.5 ± 0.5 kcal/mol, is significantly less than that (17 kcal/mol) of the conformation deduced assuming axially symmetric ordering. Interestingly, a recent ^{1}H NMR study on a complex sphingolipid which contained a lactosyl residue attached to a sphingosine residue concluded that the conformation about the glucose–galactose bond differs substantially (ϕ 20°, ψ 0°) from that expected from minimum energy calculations (ϕ 60°, ψ −10°) (Scarsdale et al., 1986). The present results are consistent with a single conformation about the galactose–glucose glycosidic linkage but do not represent a definitive determination of the conformation of the lactosyl residue at the membrane surface. Because conformational analysis by either high-resolution or solid-state NMR requires accurately determined relative atomic positions, the starting point of any analysis is the X-ray or neutron diffraction data for the residues of interest. In the case of proton coordinates only, neutron diffraction data are considered to be useful (Bock, 1983). In the present study only X-ray data are available. In cases where only X-ray data are available, the heavy atom positions are used, and the proton positions maybe calculated in an "optimized structure". The latter approach has been used in the conformational analysis of oligosaccharide in nonviscous solution (Bock, 1983). In order to assess the sensitivity of the deduced conformation of the lactosyl headgroup to the initial structure (X-ray vs calculated), the ^{2}H data were analyzed by use of proton positions of an "optimized" lactosyl residue. From the starting structure an S value of 0.62 ± 0.01 and an asymmetry in ordering of 0.25 ± 0.04 were obtained. The corresponding conformation was found to have ϕ 5 ± 4° and ψ −50 ± 8°. Clearly, there is a large discrepancy between the conformation deduced solely from the X-ray data and that deduced from the optimized structure. However, in both cases the conformation most consistent with the ^{2}H NMR results differs from that of crystalline lactose. A pictorial representation of the orientation and conformation of the lactosyl residue as deduced from the X-ray data for lactose is presented in Figure 5. It is clear that the headgroup is oriented away from the bilayer surface into the aqueous environment. This result is independent of whether the X-ray-defined or optimized structure is used in the analysis of the ^{2}H NMR data. For comparison, the conformation of crystalline lactose is shown in Figure 5B assuming the same orientation of the glucose ring relative to the director of motional averaging as that given in Figure 5A.

Hydroxymethyl Group Orientations. Inspection of Figure 3 reveals that two quadrupolar splittings are observed for the two deuterons of the hydroxymethyl group of glucose (C6′) and galactose (C6″) (Table III). For the C6′-labeled glucose

Table III: Calculated Quadrupolar Splittings for Deuterons at C6′ and C6″

carbohydrate residue	angle[a]	deuteron	$\Delta\nu_Q$ (kHz)[b]
glucose C6′	0	*pro-R*	26.4
	0	*pro-S*	59.4
	203[c]	*pro-R*	33.8 (36.6)
	201[c]	*pro-S*	4.1 (4.2)
	295[d]	*pro-R*	4.5 (4.2)
	294[d]	*pro-S*	3.5 (4.2)
galactose C6″	0	*pro-R*	29.6
	0	*pro-S*	31.3
	104[e]	*pro-R*	20.4 (20.3)
	95[e]	*pro-S*	8.8 (9.1)

[a] Angle in degrees through which the hydroxymethyl group was rotated about the C5′–C6′ or C5″–C6″ bond relative to the group's orientation in crystalline lactose. Rotation is clockwise as viewed from C5′–C6′ (C5″–C6″). Using order and orientational parameters as in Table I. [b] Absolute values of $\Delta\nu_Q$; values in parentheses are the observed values. [c] Figure 6D, major rotamer. [d] Figure 6E, minor rotamer. [e] Figure 6F.

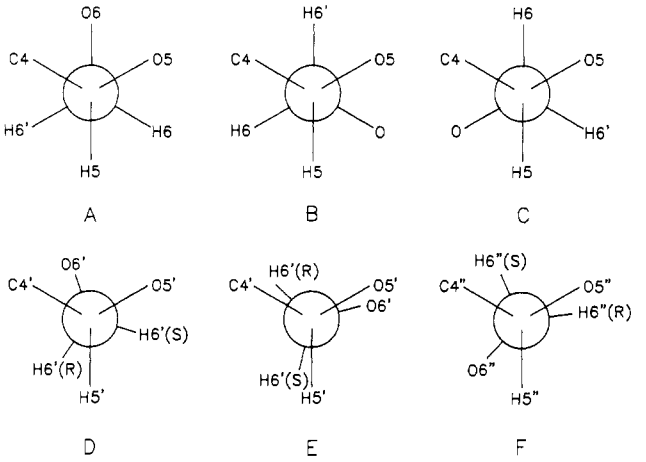


FIGURE 6: Conventional minimum energy rotamers about the C5–C6 bond: (A) gauche (−); (B) gauche (+); (C) trans. Calculated orientations of the hydroxymethyl group of (D and E) the glucosyl residue and (F) the galactosyl moiety of DTLL.

position the two powder patterns have relative intensities of ca. 2:1 while those for the galactose C6″ position are of nearly equal intensity. The hydroxymethyl group of the glycolipids α- and β-DTGL have previously been shown to give rise to two quadrupolar splittings, which was interpreted as due to the presence of two unequally populated rotamers about the C5′–C6′ bond which are in slow exchange on the deuterium NMR time scale (10^{-5} s) (Jarrell et al., 1986, 1987b). ^{2}H NMR studies on other glycolipids concluded that the hydroxymethyl group may exhibit fast rotameric interconversion (Skarjune & Oldfield, 1982) or have one orientation relative to the sugar ring (Skarjune & Oldfield, 1979). The glucose residue of DTLL is interpreted in terms of two rotamers in a ratio of 2:1 which are in slow exchange. In order to elucidate the orientation of the exocyclic group relative to the sugar ring, the hydroxymethyl group was rotated about the C5′–C6′ bond, and the quadrupolar splittings were calculated from the known S value and orientation of the sugar ring relative to the motional axis as described previously (Jarrell et al., 1986). The major conformer has the C6′–O6′ bond gauche (−) to the C5′–O5′ bond (Figure 6D) while in the minor rotamer O6′ nearly eclipses the ring oxygen atom (O5′) (Figure 6E). Similar results have been obtained with other glycoglycerolipids (Jarrell et al., 1986, 1987b). In general the C5–C6 segment of a glycopyranosyl group may exist in the three energy minimum orientations shown in Figure 6A–C. The most populated rotamer of the present study corresponds nearly

to that in Figure 6A. The calculated orientation of the minor rotamer gives the unlikely result that O6′ and O5′ are nearly eclipsed. It is tempting to attribute the latter result to the inaccuracy of the calculations and that the minor rotamer has the C6′–O6′ bond gauche (+) (Figure 6B) to the C5′–O5′ bond, which would be more consistent with the results of high-resolution NMR studies on a variety of glucopyranoside derivatives (Nishida et al., 1984). However, for the orientation shown in Figure 6B quadrupolar splittings of 27 and 54 kHz are expected for the *pro-R* and *pro-S* deuterons at C6′, a result which does not agree with the experiment (Table III). Additionally, in rotamer Figure 6B the *pro-R* deuteron would have an axial orientation relative to the sugar ring and as a result would be expected to exhibit a quadrupolar splitting which is of the same order as those associated with the axial ring deuterons (25–30 kHz) which is clearly not the case since a splitting of 4 kHz is observed. It is possible that, due to the influence of interactions within the bilayer surface, nonstandard conformers are populated.

The hydroxymethyl group of the galactose residue gives rise to only two quadrupolar splittings of equal integrated intensity (Figure 3), suggesting that either there is one rotamer present or there are two or more rotamers which are in fast exchange on the ^{2}H NMR time scale. If there is only one rotamer present, the orientation of the C6″–O6″ bond relative to the sugar ring may be calculated as described above for the corresponding glucose residue. The calculated rotamer (Table III) (Figure 6F) does not conform to any of the expected minimum energy conformers that have been assumed in the analysis of high-resolution NMR studies of galactose derivatives (Nishida et al., 1984) or methyl lactoside (Hayes et al., 1982). As discussed for the glucose residue, this may reflect the limitations of the current calculations which result from the requirement of using calculated conformations about the glycosidic bond as well as the orientation of the headgroup relative to the bilayer normal. If, however, there was rapid exchange between two or more rotamers, the calculated orientation would be expected to differ from the three most likely rotamers. High-resolution ^{1}H NMR results for the galactopyranosyl residue of lactose (Hayes et al., 1982) are consistent with the three possible rotamers (Figure 6A–C) in rapid exchange and having populations of ca. 67/21/12 for gauche (+), trans, and gauche (−), respectively. If a similar equilibrium exists in DTLL, quadrupolar splittings of 12 and 19 kHz are expected for the deuterons at C6″, respectively, which is in surprisingly good agreement with the observed values (Table III). The good agreement between calculated and observed values may be fortuitous and should not be interpreted as confirming the relative populations of the exchanging sites, since relatively minor changes in these ratios lead to large changes in the calculated splittings. Clearly any deviation from the ideal geometries shown in Figure 6A–C, as is observed for the glucose residue, will effect the calculated $\Delta\nu_Q$ values for the *pro-R* and *pro-S* deuterons. ^{2}H longitudinal relaxation times (T_{1z}) for the deuterons at C6″ suggest that such an equilibrium, rather than one stable conformer, exists (vide infra). Interestingly, if an equilibrium does exist between the orientations shown in Figure 6 the gauche (−) rotamer (Figure 6C) must be present in order to account for the observed 20.1-kHz splitting. In addition the gauche (+) (Figure 6B) must be the most populated orientation in order to explain the observed splittings.

Rates of Headgroup Motion. While the residual quadrupolar interactions give information on a molecular unit's orientation and motional amplitude, the rates of molecular motion may be probed through relaxation times. The longitudinal relaxation times, T_{1z}, for deuterons at the various labeled positions of DTLL were measured at several temperatures, Table IV. For the ring positions the relaxation rates, T_{1z}^{-1}, decrease with increasing temperature indicating that the correlation time (τ_c) for headgroup motion is such that $\omega_o^2\tau_c^2 < 1$. The relaxation times for deuterons on both rigid sugar rings have essentially identical values, indicating that the disaccharide headgroup is moving as a rigid unit. The relaxation times for deuterons on the glucose hydroxymethyl group have values similar to those on the rigid ring, indicating that there is little internal motion on this time scale about the C5′–C6′ bond, in agreement with the conclusions from the analysis of quadrupolar splittings presented above. The relaxation times for the corresponding deuterons at C6″ of the galactose residue are significantly longer than those for deuterons attached to the rigid ring, suggesting internal motion about the C5″–C6″ bond. The latter result is consistent with a rapid exchange between two or more sites such as are shown in Figure 6. The present conclusions are consistent with ^{13}C NMR spin–lattice relaxation studies of lactose derivatives (Hayes et al., 1982; Czarniecki & Thornton, 1977) which concluded that little internal motion about the interglycosidic bond was present. In addition, the hydroxymethyl group of the glucose residue did not undergo any fast motion about its C5–C6 bond while that of the galactose residue was found to be undergoing significant motion leading to increased relaxation times for the galactose C6′ carbon.

Table IV: ^{2}H Spin–Lattice Relaxation Times for the Lactosyl Residue of DTLL in the Liquid Crystalline Phase

carbohydrate residue	^{2}H-labeled position	temperature (°C)	T_{1z} (ms)
galactose[a]	C2″–C4″	75	4.7 ± 0.6
		80	5.8 ± 0.5
		90	8.0 ± 1.0
	C6″	75	10.8 ± 0.4, 13.3 ± 0.5
		80	12 ± 1, 15 ± 1
		90	10 ± 2, 13 ± 3
glucose[b]	C2′	75	4.0 ± 0.3
	C4′	75	3.9 ± 0.4
	C6′ (*R*)	75	4.3 ± 0.2
	C6′ (*S*)	75	4.3 ± 0.2

[a] Error estimates are from the fitting of the relaxation data to a single exponential. [b] Precision estimates are from the average of two measurements.

The short relaxation times are similar to those reported in monoglycosylglycerolipids (Jarrell et al., 1987b) and indicate that the correlation time must be approaching the Larmor frequency since at 30.7 MHz the shortest relaxation time is expected to be of the order of 2 ms (Davis, 1983). Since the relaxation time for the C6′ deuterons, which have a quadrupolar splitting of 4 kHz (S_{CD} of 0.03), is the same as that for the ring deuterons (S_{CD} 0.2), the relaxation rates lack the S_{CD}^2 dependence expected if relaxation were dominated by collective order director fluctuations (Brown, 1982), a result which has been concluded recently for another lipid system (Jarrell et al., 1988). More quantitative analysis of the relaxation data requires the choice of a specific model for the molecular motion. The present data are insufficient to discriminate between the possible models for molecular motion. However, the molecular motion(s) must have a correlation time in the nanosecond range or longer to account for the short relaxation times. Inspection of the partially T_{1z}-relaxed spectra of DTLL ^{2}H-labeled in the glucose headgroup (Figure 7) reveals that the relaxation rate is dependent upon the orientation of the bilayer normal relative to the magnetic field direction. Similar

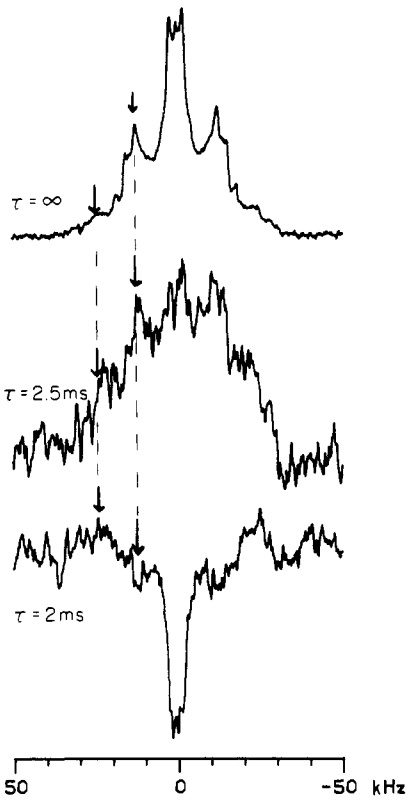


FIGURE 7: Partially T_{1z}-relaxed spectra of DTLL ^{2}H-labeled at C2′, C3′, C4′, and C6′ in the glucosyl group. The spectra (30.7 MHz) were acquired at 75 °C with the inversion–recovery sequence modified to include the quadrupolar echo sequence. The spacings between the 180° pulse and the first 90° pulse are indicated. The $\pi/2$ pulse width (5-mm solenoid coil) was 2.3 μs.

results have been obtained with monoglycosylglycerolipids (Jarrell, unpublished results) and indicate that lateral diffusion of the lipid molecules over the curved surface of the multilamellar liposomes is too slow to lead to complete averaging of the orientation-dependent relaxation rate. Such averaging has been established to occur in the liquid crystalline phase of multilamellar dispersions of phospholipids (Brown & Davis, 1981), but not in the gel phase of lecithins or cerebroside or the liquid gelatin phase of cholesterol–lipid mixtures (Siminovitch et al., 1985). The absence of orientational averaging of T_{1z} suggests that either the glycolipids form liposomes which are substantially larger than those formed by phospholipids or lateral diffusion of glycolipids is significantly slower than that of phospholipids. Wieslander et al. (1981) have shown that the rate of lateral diffusion of diacylglycoglycerolipids is a factor of two to six times greater than that of lecithins (Lindblom et al., 1981). Hence, the present results suggest that liposomes of DTLL are either very large or have less curvature than that which occurs with phospholipids. Monogalactosyldiacylglycerols have been shown to form lamellar structures having only slight curvature (Mannock et al., 1985; Sen et al., 1981) while the corresponding digalactosyl lipid forms large liposomes (Sen et al., 1981). The present results cannot distinguish between the two possibilities.

Conclusions

The present study demonstrates that ^{2}H NMR of glycolipids in a membrane environment can provide detailed information about the membrane surface. Glycoglycerolipids containing a disaccharide headgroup have been proposed to form lamellar structures in which the headgroup is parallel to the bilayer surface (Iwamoto et al., 1982). In contrast, the present study indicates that for DTLL the carbohydrate headgroup extends away from the bilayer surface into the surrounding aqueous environment. The surface of liposomes composed of DTLL is characterized by motion of relatively small amplitude which has a significant motional component in the nanosecond range. The observation of an anisotropy in the relaxation rates may be explained either by a lateral diffusion rate which is slow relative to that of phospholipid systems or, more likely, by a curvature of DTLL liposomes being considerably less than that of phospholipid systems. The headgroup presents a relatively rigid structure in which the only significant internal motion occurs about the C5″–C6″ bond of the galactosyl residue. This motion is most likely a jump between unequally populated sites. The conformation about the lactosyl moiety, as deduced from ^{2}H NMR data, differs from that reported for crystalline lactose and for methyl lactoside in aqueous solution, suggesting that intermolecular interactions within the bilayer plane may be involved. It should be emphasized that while the present study does not yield a definitive conformation for the headgroup, a conformation consistent with the ^{2}H NMR data may be elucidated. It will therefore be possible to probe relative changes in this structure as a result of surface interactions.

It is clear that ^{2}H NMR can provide details relating to cell–surface topology. Applications of NMR to the direct examination of ligand–receptor interactions such as lectin– or antibody–carbohydrate binding in a membrane environment should provide valuable insight into how surface structure affects such interactions.

Acknowledgments

We are grateful to H. Seguin for elemental analyses, to F. Cooper for mass spectroscopic analyses, and to Dr. D. Bundle for providing the atomic coordinates of the "optimized" lactose residue.

Appendix

Order Matrix Analysis of the Lactosyl Residue of DTLL. In the molecule-fixed axis system defined with the origin at O4′ and the Z axis along the O4′-C1″ bond, the electric field gradient of the nth deuteron attached to the nth rigid sugar ring of the headgroup is given by (Dufourc et al., 1983; Strenk et al., 1985)

$$V_{ij}^n = eq\frac{3\cos\theta_i^n\cos\theta_j^n - \delta_{ij}}{2}$$

where $\cos\theta_i^n$ $(i, j = x, y, z)$ are the direction cosines of the nth C–^{2}H bond in the molecular frame and the tensor in the principal frame of the C–^{2}H bond is assumed to be axially symmetric. The component of the average field gradient tensor parallel to the motional axis of the headgroup is given by

$$V = \bar{n}V\bar{n}^*$$

where $\bar{n} = (\cos\gamma_x, \cos\gamma_y, \cos\gamma_z)$ are the direction cosines of the director $\bar{n}$ in the molecular frame. The observed bond order parameter is given by

$$S_{CD}^n = \sum_{i,j}\cos\theta_i^n\cos\theta_j^n\frac{3\cos\gamma_i\cos\gamma_j - \delta_{ij}}{2} = \sum_{i,j}\cos\theta_i^n\cos\theta_j^n S_{ij}$$

The molecular order parameters S_{ij} are the same for all sites in the lactosyl residue while the direction cosines, $\cos\theta_i^n$, depend upon the conformation (defined by the angles ϕ and ψ, Figure 1) and are not time dependent. What is required in this analysis is that for a chosen conformation and any five of the six available S_{CD} values the same S_{ij} values are obtained. In addition, the condition $-0.5 < S_{zz} < 1.0$ must be met. When

a given conformation gave a self-consistent set of S_{ij} values, the order matrix was diagonalized (Dufourc et al., 1983) to give the molecular order parameter S^*_{zz} and the anisotropy of the ordering:

$$\eta^* = \left| \frac{S^*_{xx} - S^*_{yy}}{S^*_{zz}} \right|$$

Conformations were generated by clockwise rotations of the atomic coordinates of the galactosyl residue, starting with the X-ray crystallographic data of lactose (Hirotsui & Shimada, 1974). The residue was rotated about the O4′–C4′ bond (incrementing the ψ angle, Figure 1) by 5° steps followed by rotation about the C1″–O4′ bond by 5° steps through 360° (incrementing the ϕ angle, Figure 1) for each new value of the ψ angle. Direction cosines for the C–^{2}H bonds, and the order parameter S_{ij} for various combinations of five of the six S_{CD} values, were calculated. Since the signs of the bond order parameters are unknown, all possible sign combinations were examined. For a given set of S_{ij} values from each combination of five S_{CD} values the sixth S_{CD} order parameter was calculated and required to agree with experiment to within ±0.01 (corresponding to an error of ±1.2 kHz in the $\Delta\nu_Q$ values which is outside experimental error, estimated to be ±0.4 kHz). An additional constraint imposed upon the solutions was that the conformational energy be less than 30 kcal/mol. In order to relate the results of the calculations, Table III, to those obtained by treating the two sugar residues separately, the conformation having the smallest η^* value was transformed to the coordinate system of Figure 1 having the molecular Z axis along the glucose C2′–C3′ bond, and the diagonal order matrix was calculated. The most ordered axis, Z^*, was found to be related to the molecular frame by the angles β and γ of −33 ± 3° and 21 ± 2°, respectively, which is in good agreement with the results obtained in the analysis of the glucose residue alone (Table I).

Registry No. **1a**, 85648-57-3; **1b**, 118112-33-7; **2a**, 118112-27-9; **2b**, 118142-07-7; **3a**, 118112-28-0; **3b**, 118112-34-8; **4a**, 118112-29-1; **4b**, 118142-08-8; **5a**, 118112-32-6; **5b**, 118112-35-9; **6a**, 118203-77-3; **6a** acetyl derivative, 118142-05-5; [$^2H_2$]-**6a**, 118142-06-6; [$^2H_6$]-**6a**, 118112-26-8; (±)-[$^2H_5$]-**6a**, 118112-30-4; **6b**, 118170-00-6; hepta-*O*-acetyl-α-lactosyl bromide, 118203-78-4; 1,2-di-*O*-tetradecyl-*sn*-glycerol, 36314-51-9; tetra-*O*-acetyl-D-[2,3,4,6,6-$^2H_5$]galactopyranosyl, 118112-31-5.

References

Bloom, M., Davis, J. H., & MacKay, A. I. (1981) *Chem. Phys. Lett. 80*, 198–202.

Bock, K. (1983) *Pure Appl. Chem. 55*, 605–622.

Bock, K., Breimer, M. E., Brignole, A., Hansson, G. C., Karlsson, K. A., Larson, G., Leffler, H., Samuelsson, B. E., Stromberg, N., Eden, C. S., & Thurin, J. (1985) *J. Biol. Chem. 260*, 8545–8551.

Brown, M. F. (1982) *J. Chem. Phys. 77*, 1576–1599.

Brown, M. F., & Davis, J. H. (1981) *Chem. Phys. Lett. 79*, 431–435.

Critchly, D. R., Ansell, S., & Dills, S. (1979) *Biochem. Soc. Trans. 7*, 314–319.

Czarniecki, M. F., & Thornton, E. R. (1976) *J. Am. Chem. Soc. 98*, 1023–1023.

Czarniecki, M. F., & Thornton, E. R. (1977) *J. Am. Chem. Soc. 99*, 8279–8282.

Davis, J. H. (1983) *Biochim. Biophys. Acta 737*, 117–171.

Davis, J. H., Jeffrey, K., Bloom, M., Valic, M. I., & Higgs, T. P. (1976) *Chem. Phys. Lett. 42*, 390–394.

Dufourc, E. J., Smith, I. C. P., & Jarrell, H. C. (1983) *Chem. Phys. Lipids 33*, 153–172.

Endo, T., Inoue, K., & Nojima, S. (1982) *J. Biochem. 92*, 953–960.

Endo, T., Inoue, K., Nojima, S., Sekiya, T., Oki, K., & Nozawa, Y. (1983) *J. Biochem. 93*, 1–6.

Feizi, T. (1985) *Nature 314*, 53–57.

Fishman, P., & Brady, R. O. (1976) *Science 194*, 906–915.

Garreg, E. K. M., Hultberg, P. J., & Oscarson, S. (1983) *J. Carbohydr. Chem. 2*, 305–311.

Hakomori, S.-H. (1984) in *The Cell Membrane* (Haber, E., Ed.) pp 181–201, Plenum Press, New York.

Hayes, M. L., Serianni, A. S., & Barker, R. (1982) *Carbohydr. Res. 100*, 87–101.

Hinz, H.-J., Six, L., Ruess, K.-P., & Lieflander, M. (1985) *Biochemistry 24*, 806–813.

Hirotsui, K., & Shimada, A. (1974) *Bull. Chem. Soc. Jpn. 47*, 1872–1879.

Hudson, C. S., & Kunz, A. (1925) *J. Am. Chem. Soc. 47*, 2052–2055.

Iwamoto, K., Sunamoto, J., Inoue, K., Endo, T., & Nojima, S. (1982) *Biochim. Biophys. Acta 691*, 44–51.

Jarrell, H. C., Giziewicz, J. B., & Smith, I. C. P. (1986) *Biochemistry 25*, 3950–3957.

Jarrell, H. C., Jovall, P. Å., Giziewicz, J. B., Turner, L., & Smith, I. C. P. (1987a) *Biochemistry 26*, 1805–1811.

Jarrell, H. C., Wand, A. J., Giziewicz, J. B., & Smith, I. C. P. (1987b) *Biochim. Biophys. Acta 897*, 69–82.

Jarrell, H. C., Smith, I. C. P., Jovall, P. Å., Mantsch, H. H., & Siminovitch, D. J. (1988) *J. Chem. Phys. 88*, 1260–1263.

Kitaigorodsky, A. Z. (1961) *Tetrahedron 14*, 230–236.

Koch, H. J., & Stuart, R. S. (1977) *Carbohydr. Res. 59*, C1–C6.

Lemieux, R. U., Bock, K., Delbaere, L. T. J., Koto, S., & Rao, V. S. (1980) *Can. J. Chem. 58*, 631–653.

Lindblom, G., Johansson, L. B.-A., & Arvidson, G. (1981) *Biochemistry 20*, 2204–2207.

Lipkind, G. M., Verovsky, V. E., & Kochetkov, N. K. (1984) *Carbohydr. Res. 133*, 1–13.

Mannock, D. A., Brain, A. P. R., & Williams, W. P. (1985) *Biochim. Biophys. Acta 817*, 289–298.

Meier, P., Ohmes, E., & Kothe, G. (1986) *J. Chem. Phys. 85*, 3598–3617.

Nishida, Y., Ohrui, H., & Meguro, H. (1984) *Tetrahedron Lett. 25*, 3251–3254.

Ogawa, T., & Beppu, K. (1982) *Agric. Biol. Chem. 46*, 255–262.

Perly, B., Smith, I. C. P., & Jarrell, H. C. (1985) *Biochemistry 24*, 4659–4665.

Petersen, N. O., & Chan, S. I. (1977) *Biochemistry 16*, 2657–2667.

Quinn, P. J., & Williams, W. P. (1978) *Prog. Biophys. Mol. Biol. 34*, 109–173.

Rogers, H. J., Perkins, H. R., & Ward, J. R. (1980) *Microbial Cell Walls and Membranes*, Chapter 3, Chapman & Hall, New York.

Scarsdale, J. N., Yu, R. K., & Prestegard, J. H. (1986) *J. Am. Chem. Soc. 108*, 6778–6784.

Sen, A., Williams, W. P., & Quinn, P. J. (1981) *Biochim. Biophys. Acta 663*, 380–389.

Sen, A., Williams, W. P., Brain, A. P. R., & Quinn, P. J. (1982) *Biochim. Biophys. Acta 685*, 297–306.

Sillerud, L. O., Prestegard, J. H., Yu, R. K., Schafer, D. E., & Konigsberg, W. (1978) *Biochemistry 17*, 2619–2626.

Siminovitch, D. J., Ruocco, M. J., Olejniczak, E. T., Gupta, S. K., & Griffin, R. G. (1985) *Chem. Phys. Lett. 119*, 251–255.

Skarjune, R., & Oldfield, E. (1979) *Biochim. Biophys. Acta 556*, 208-221.
Skarjune, R., & Oldfield, E. (1982) *Biochemistry 21*, 3154-3160.
Soriano, P., Dijkstra, J., Legrand, A., Spanjer, H., Londos-Gagliardi, D., Roerdink, F., Scherphof, G., & Nicolau, C. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 7128-7131.
Strenk, L. M., Westerman, P. W., & Doane, J. W. (1985) *Biophys. J. 48*, 765-773.
Venkatachalam, C. M., & Ramachandran, G. N. (1967) in *Conformation of Biopolymers* (Ramachandran, G. M., Ed.) Vol. 1, Academic Press, New York.
Wieslander, Å., Ulmius, J., Lindblom, G., & Fontell, K. (1978) *Biochim. Biophys. Acta 512*, 241-253.
Wieslander, Å., Christiansson, A., Rilfors, L., & Lindblom, G. (1980) *Biochemistry 19*, 3650-3655.
Wieslander, Å., Rilfors, L., Johansson, L. B.-A., & Lindblom, G. (1981) *Biochemistry 20*, 730-735.
Wynn, C. H., & Robson, B. (1986) *J. Theor. Biol. 123*, 221-230.
Wynn, C. H., Marsden, A., & Robson, B. (1986) *J. Theor. Biol. 119*, 81-87.

Relationship between Guanosine Tetraphosphate and Accuracy of Translation in *Salmonella typhimurium*†

Didier Nègre,‡ Jean-Claude Cortay,‡ Pierluigi Donini,§ and Alain J. Cozzone*,‡
Laboratoire de Biologie Moléculaire, Université de Lyon, Villeurbanne, France, and Dipartimento di Biologia Cellulare, Università La Sapienza, Roma, Italy

Received May 16, 1988; Revised Manuscript Received September 23, 1988

ABSTRACT: In bacteria a high level of mistranslation is observed in amino acid starved *rel*⁻, but not *rel*⁺, strains, and mistranslation can be studied qualitatively by means of "stuttering" experiments in two-dimensional protein gels. It has been suggested that the low level of mistranslation that occurs in *rel*⁺ strains is assured by guanosine 5′-diphosphate 3′-diphosphate (ppGpp), a nucleotide whose intracellular concentration greatly increases in *rel*⁺ cells under amino acid starvation. In the present study the relationship between level of ppGpp and mistranslation was analyzed by performing stuttering experiments in amino acid starved bacteria that contained either high or low levels of ppGpp. Three strains of *Salmonella typhimurium* were used in these experiments: a *relA*⁺ *hisT*⁺ strain (TA997), a *relA*⁺ *hisT* strain (TA1001), and a *relA hisT* strain (PD2). These strains were first characterized with respect to macromolecular syntheses and ppGpp levels under exponential growth and under amino acid starvation. Both *rel*⁺ strains exhibited stringent control over RNA synthesis. ppGpp accumulated to high levels when TA997 was starved for either of three amino acids. Starvation of TA1001 for histidine did not cause accumulation of ppGpp, whereas starvation for lysine and arginine produced high levels of ppGpp. Extracts from the three strains, obtained either under exponential growth or under amino acid starvation, were then subjected to two-dimensional electrophoretic analysis: mistranslation was observed whenever ppGpp was absent. In particular, starvation of TA1001 for histidine resulted in high mistranslation frequencies, while under lysine and arginine starvation mistranslation was undetectable, regardless of whether the cells were *rel*⁺ or *rel*⁻. The results reported in this study provide strong evidence that ppGpp is indeed the molecule responsible in vivo for maintaining the frequency of mistranslation at a low level in *rel*⁺ bacteria.

Starvation for amino acids in eukaryotes causes incorporation of illegitimate amino acids at the starved-for codons according to a predictable scenario, whereas amino acid starvation of *rel*⁺ bacteria produces only a small increase in mistranslation (Hall & Gallant, 1972; Pollard, 1984). The *relA* gene of *Escherichia coli* and its homologue in *Salmonella typhimurium* preside over a variety of physiological events during amino acid starvation of these bacteria, including protection against translation errors. When mistranslation is studied in *rel*⁻ strains, however, the error frequencies that are produced are higher by several orders of magnitude (Hall & Gallant, 1972; Gallant & Foley, 1979; Cashel & Rudd, 1987). Thus, amino acid starved *relA* bacteria have been very useful in the study of in vivo translational errors.

The events that take place in *rel*⁺ bacteria under amino acid starvation have been collectively termed the stringent response (Gallant, 1979; Cashel & Rudd, 1987). A central element of the response is the production of guanosine 5′-triphosphate 3′-diphosphate (pppGpp) and of guanosine 5′-diphosphate 3′-diphosphate (ppGpp). There is good evidence that the two nucleotides are produced as a result of the interaction between a codon present in an RNA message and a codon-specific uncharged tRNA on ribosomes possessing an active *relA* product (Block & Haseltine, 1974). High levels of these nucleotides are normally produced by amino acid starved *rel*⁺, but not *rel*⁻, bacteria, and it is thought that ppGpp is an effector molecule that mediates many of the other metabolic events that take place during the stringent response (Cashel & Rudd, 1987).

The evidence for a causal role of ppGpp is circumstantial, since it is based on the correlation between production of the

† This work was supported by the Centre National de la Recherche Scientifique (UA 1176), the Fondation pour la Recherche Médicale, and the Italian National Research Council (Ingegneria Genetica Grant 86.00085.51).
* Address correspondence to this author at the Laboratoire de Biologie Moléculaire, 43 Boulevard du Onze Novembre, 69622 Villeurbanne Cedex, France.
‡ Université de Lyon.
§ Università La Sapienza.

0006-2960/89/0428-1814$01.50/0