However, the first two approximants give reasonable error estimates, and higher order moments are not considered.

Supplementary Material Available

One plot of Q_{DA}/Q_D vs. BR surface density for DMPC vesicles with rhodamine B chloride at 25 and 40 °C and C_{18}DiI at 30 °C and one plot of ellipticity (θ) at 590 nm vs. temperature (°C) for BR reconstituted into DMPC vesicles (open circles) or DPPC vesicles (closed squares) (2 pages). Ordering information is given on any current masthead page.

Registry No. DMPC, 13699-48-4; DPPC, 2644-64-6; C_{18}DiI, 41085-99-8; OR, 65603-19-2.

References

Anderson, J. M. (1981) *FEBS Lett. 124*, 1-10.

Baird, B. A., Pick, U., & Hammes, G. G. (1979) *J. Biol. Chem. 254*, 3818-3825.

Becher, B. M., & Cassim, J. Y. (1975) *Prep. Biochem. 5*, 161-178.

Cantley, L. C., Jr., & Hammes, G. G. (1975) *Biochemistry 14*, 2976-2981.

Casadio, R., & Stoeckenius, W. (1980) *Biochemistry 19*, 3374-3381.

Chen, R. (1967) *Anal. Biochem. 19*, 374-387.

Cherry, R. J., Mueller, U., Henderson, R., & Heyn, M. P. (1978) *J. Mol. Biol. 121*, 283-298.

Dewey, T. G., & Hammes, G. G. (1980) *Biophys. J. 32*, 1023-1036.

Ebrey, T. G., Becher, B., Mao, M., Kilbride, P., & Honig, B. (1977) *J. Mol. Biol. 112*, 377-397.

Fleming, P. J., Koppel, D. E., & Strittmatter, P. (1979) *Biochemistry 18*, 5458-5464.

Fung, B. K.-K., & Stryer, L. (1978) *Biochemistry 17*, 5241-5248.

Hammes, G. G. (1981) *Protein-Protein Interaction*, pp 257-287, Wiley, New York.

Henderson, R., & Unwin, P. N. T. (1975) *Nature (London) 257*, 28-32.

Heyn, M. P., Blume, A., Rehorek, M., & Dencher, N. A. (1981a) *Biochemistry 20*, 7109-7115.

Heyn, M. P., Cherry, R. J., & Dencher, N. A. (1981b) *Biochemistry 20*, 840-849.

Ikemoto, N. (1982) *Annu. Rev. Physiol. 44*, 297-318.

King, G. I., & Schoenborn, B. P. (1982) *Methods Enzymol 88*, 241-248.

Kouyama, T., Kinosita, K., & Ikegami, A. (1983) *J. Mol. Biol. 165*, 91-107.

Lanyi, J. K., & MacDonald, R. E. (1979) *Methods Enzymol. 56*, 398-407.

Ovchinnikov, Y. A. (1982) *FEBS Lett. 148*, 179-191.

Racker, E., Violand, B., O'Neal, S., Alfonzo, M., & Telford, J. (1979) *Arch. Biochem. Biophys. 198*, 470-477.

Rehorek, M., Dencher, N. A., & Heyn, M. P. (1983) *Biophys. J. 43*, 39-45.

Snyder, B., & Freire, E. (1982) *Biophys. J. 40*, 137-148.

Stoeckenius, W., & Bogomolni, R. A. (1982) *Annu. Rev. Biochem. 52*, 587-616.

Stryer, L. (1978) *Annu. Rev. Biochem. 47*, 819-846.

Wolber, P. K., & Hudson, B. S. (1979) *Biophys. J. 28*, 197-210.

Cardiolipin Conformation and Dynamics in Bilayer Membranes As Seen by Deuterium Magnetic Resonance†

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ABSTRACT: Deuterium nuclear magnetic resonance was used to investigate the structure and dynamics of the cardiolipin polar region in pure cardiolipin bilayers and in mixed cardiolipin–lecithin systems. The *Escherichia coli* mutant B 131GP defective in both the biosynthesis and degradation of glycerol was used for biosynthetic labeling of cardiolipin. Deuterated glycerol added to the growth medium was specifically incorporated into membrane phospholipids, and deuterated cardiolipin was purified from lipid extracts. Deuterium NMR spectra yielded characteristic quadrupole splittings that could be assigned to the corresponding backbone and head-group deuterons. Both glycerol backbones in cardiolipin were found to adopt identical conformations. As observed in other lipids, the glycerol backbones of cardiolipin are oriented perpendicular to the bilayer surface. The glycerol head group of cardiolipin was distinctly less flexible than the polar groups of other lipids as suggested by T_1 relaxation time measurements. Only small changes in the quadrupole splittings could be detected between a pure cardiolipin bilayer and a mixed cardiolipin lecithin system.

In 1906 Wassermann demonstrated the presence of specific antibodies in the sera of syphilis patients (Wassermann et al., 1906) against an antigen that later turned out to be cardiolipin (Pangborn, 1942). Cardiolipin can be found in the membranes of almost all organisms. When different human organs are compared, the largest percentage of cardiolipin is present in heart and skeletal muscle (Rouser et al., 1969; Simon & Rouser, 1969). Furthermore, it is characteristically associated with subcellular membranous particles displaying high metabolic activity, e.g., mitochondria. The cardiolipin content of a particular membrane can be influenced by a number of different biological processes such as aging (Bruce, 1974), transition from exponential to stationary growth phase [yeast (Jakovcic et al., 1971); *Escherichia coli* (Kanemasa et al., 1967)], or infection of *E. coli* [bacteriophage f1 (Chamberlain & Webster, 1976)]. The chemistry and biochemistry of cardiolipin have been reviewed recently (Ioannou & Golding, 1979).

Studies with a mutant deficient in the synthesis of cardiolipin have suggested that this lipid is a dispensable component of

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the bulk lipid phase of *E. coli* membranes (Pluschke et al., 1978). On the other hand, studies on different membrane systems indicate that cardiolipin may be essential for the activity of some membrane-bound enzymes, notably those of the inner mitochondrial membrane such as cytochrome *c* oxidase (Awasthi et al., 1971; Vik et al., 1981; Robinson, 1982) and the mitochondrial phosphate carrier (Cheneval et al., 1983). Both enzymes are inhibited by the drug adriamycin, which is explained by a complexation of adriamycin with cardiolipin and a concomitant depletion of cardiolipin from the protein (Goormaghtigh et al., 1982a; Chevenal et al., 1983).

A second physiological function of cardiolipin has been suggested by its tendency to form an inverted hexagonal (H_{II}) phase under certain conditions. In model membranes composed of either pure cardiolipin or cardiolipin–phospholipid mixtures, the inverted hexagonal phase can be induced by quite different agents, e.g., divalent metal ions, local anesthetics, or proteins (Cullis et al., 1978; de Kruijff & Cullis, 1980; Vasilenko et al., 1982; de Kruijff et al., 1979, 1982). On the other hand, drugs such as adriamycin and also some integral membrane proteins specifically stabilize the bilayer structure and suppress the bilayer–hexagonal phase transition of cardiolipin (Taraschi et al., 1983; Goormaghtigh et al., 1982b; de Kruijff et al., 1981). It has been suggested that cardiolipin via the formation of an inverted hexagonal phase could act as a carrier for Ca^{2+} facilitating the movement of Ca^{2+} across the cell membrane. However, Ca^{2+} transport studies with artificial black membranes and multilamellar vesicles containing 5–25% cardiolipin showed no Ca^{2+} translocation across the membrane and hence argue against this hypothesis [cf. Sakolove et al. (1983)].

Of primary concern in the present study were the conformation and dynamics of the head-group segments of cardiolipin. By selective deuteration of the individual carbon segments, the three glycerol residues of cardiolipin were made spectroscopically visible via deuterium magnetic resonance (^2H NMR),[1] and the quadrupole splittings as well as the T_1 relaxation times were measured. The same method was employed previously to investigate in detail the head-group properties of synthetic and natural phosphatidylglycerols where selective deuteration was achieved either by chemical synthesis (Wohlgemuth et al., 1980) or by biochemical incorporation of selectively deuterated glycerol into the phospholipids of *E. coli* (Gally et al., 1981; Borle & Seelig, 1983a). The biochemical incorporation of deuterated glycerol in high yield was facilitated by using mutants of *E. coli* that were defective in both the biosynthesis and degradation of glycerol. Exponentially growing *E. coli* cells contain about 15% phosphatidylglycerol and 5% cardiolipin. In the stationary phase and under a variety of suboptimal conditions, the cardiolipin content tends to increase at the expense of phosphatidylglycerol (Cronan & Vagelos, 1972). Since cardiolipin is formed by the reaction of two phosphatidylglycerol molecules (Hostettler et al., 1972; Hirschberg & Kennedy, 1972), the present investigation was a natural extension of our previous experience with phosphatidylglycerol.

Materials and Methods

Synthesis of Deuterated Glycerols. Optically active *sn*-[1,1-$^2H_2$]glycerol and *sn*-[3,3-$^2H_2$]glycerol were synthesized as described by Lok et al. (1976) starting from L- and D-serine, respectively, and using $LiAlD_4$ as reducing agent. [2-^2H]-Glycerol was prepared by reducing dihydroxyacetone with $NaBD_4$ (Renoll & Newman, 1955).

Incorporation of Selectively Deuterated Glycerol into E. coli Membrane Phospholipids. Strain B131GP is a derivative of strain BB20-14 (Bell, 1974), which is defective in both the biosynthesis and degradation of glycerol [genotype *gps* A, *glp* D, *glp* R, Glp K, *pho* A, *fad* E, *ton* A, *rel* 1; for nomenclature, see Bachmann (1983)]. In our hands BB20-14 produced less cardiolipin than most wild-type *E. coli* strains. B131GP was obtained from a tryptophan-auxotrophe derivative of BB20-14 by transduction to prototrophy using *E. coli* K12Y *mel* as the donor. It probably has obtained the wild-type *cls* allele by cotransduction (Pluschke et al., 1978) and produces normal amounts of cardiolipin.

The cells were grown on mineral salt medium (Vogel & Bonner, 1956), 0.4% glucose, 0.3% α-amino acids (casein hydrolysate, Oxoid), and 0.004% deuterated glycerol. At the end of the exponential growth phase, the same amount of deuterated glycerol was added again, and after a further 2 h of growth in the stationary phase the cells were harvested by centrifugation.

Purification of Cardiolipin. The total lipid extract of *E. coli* strain B131 GP contained phosphatidylethanolamine (accounting for ~80 wt % of the membrane lipids), phosphatidylglycerol (~15 wt %), and cardiolipin (~5 wt %). Cardiolipin was purified from *E. coli* cells by two different methods with no significant differences in the final ^2H NMR spectra.

(*a*) *Method 1.* A total phospholipid fraction was prepared from cells by methanol–diethyl ether (3:1 v/v) extraction (Takahashi et al., 1967). After evaporation of the solvent, the phospholipids were dissolved in methylene dichloride, filtered, and further purified by precipitation from anhydrous acetone at −10 °C (Baer, 1951). The precipitate was dissolved in methylene dichloride–methanol (2:1 v/v), and the lipids were separated on a Sephadex LH-20 column (Eichberg & Burnham, 1970). Residual phosphatidylethanolamine was removed by column chromatography of the crude cardiolipin on silica gel (solvent: methylene dichloride–methanol–H_2O, 65:25:4 v/v/v). The purified cardiolipin was washed with a 0.1 M EDTA solution saturated with NaCl (pH 8.5) to yield the sodium salt.

(*b*) *Method 2.* A total phospholipid extract was prepared from cells by methylene chloride–methanol (1:1 v/v) extraction. It was dissolved in methanol–methylene dichloride (2:1 v/v) at a concentration of about 2 mg of lipid/mL, and cardiolipin was precipitated by adding Ba^{2+}–acetate (Vasilenko et al., 1982). The precipitate (cardiolipin with small amounts of phosphatidylethanolamine and phosphatidylglycerol) was dissolved in methylene dichloride–methanol–ammonia (65:25:4 v/v/v). Residual lipids were removed on a Sephadex LH-20 column as described above. Finally, cardiolipin was converted to its sodium salt by washing the lipid with 0.1 M EDTA solution saturated with NaCl (pH 8.5). Residual Ba^{2+} was determined with atomic absorption spectroscopy and found to be less than 0.4×10^{-3} mol of Ba^{2+}/mol of cardiolipin.

Thin-layer chromatography of the purified cardiolipin still revealed trace amounts (<5%) of phosphatidylethanolamine and phosphatidylglycerol but no protein. The second method resulted in a higher yield and was thus the preferred purification procedure.

The fatty acid composition of purified cardiolipin was determined by gas chromatography on a 15% Silar 10 C column

[1] Abbreviations: NMR, nuclear magnetic resonance; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; CL, cardiolipin.

[cf. Gilkes & Weeks (1977)] yielding 5.7 mol % myristic acid, 39.3 mol % palmitic acid, 7 mol % palmitoleic acid, 14.2 mol % stearic acid, and 32.6 mol % vaccenic acid. The fatty acid composition of cardiolipin from strain B131 GP is thus similar to that of wild-type *E. coli* with palmitic acid predominantly in the *sn*-1 position and vaccenic acid in the *sn*-2 position [cf. Overath & Thilo (1978)].

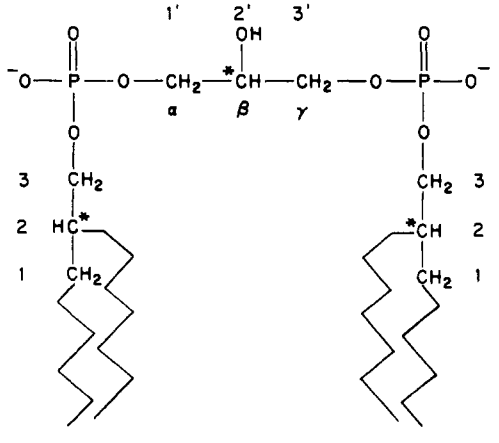
^2H and ^{31}P NMR Measurements. NMR spectra were recorded for pure cardiolipin bilayer dispersions and for mixtures of cardiolipin with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) containing 20 wt % cardiolipin. Approximately 20–70 mg of cardiolipin was dispersed in approximately the same amount of buffer (10 mM PIPES buffer, pH 7.0, containing 1 mM EDTA). In mixtures with phosphatidylcholine again 20–70 mg of cardiolipin was measured. The total amount of lipid in the sample was hence 100–350 mg, and the corresponding amount of buffer was added. Deuterium-depleted water was used to minimize the isotropic ^2H NMR signal arising from the natural abundance of deuterium in water. A few samples were also dispersed in buffer containing in addition 0.1 M NaCl. Within experimental error 0.1 M NaCl had no influence on the line shape and the quadrupole splittings of the deuterium spectra.

All NMR measurements were made with a Bruker Spectrospin CXP-300 spectrometer operating at 46.1 MHz for ^2H and 121.48 MHz for ^{31}P. The experimental conditions are the same as detailed before (Seelig et al., 1981; Tamm & Seelig, 1983).

^2H NMR spectra of random dispersions of lipid bilayers were computer-simulated by assuming an angular-independent intrinsic line width (defined by T_2). The computer program was kindly provided by Dr. K. R. Jeffrey, and the details of the calculations have been described elsewhere (Siminovitch et al., 1984).

Results

Nomenclature and Stereospecificity. Naturally occurring phosphatidylglycerol has the L (backbone)–D (head group) conformation and in the stereospecific numbering nomenclature is called 1,2-diacyl-*sn*-glycero-3-phospho-1-glycerol. In bacteria cardiolipin is made from two molecules of phosphatidylglycerol and altogether contains three optically active glycerols. For comparison with other polar groups we use the stereospecific numbering as well as the nomenclature α, β, and γ for the three glycerol head-group segments (asterisks denote asymmetric carbon atoms):



It should be noted that cardiolipin possesses three asymmetric carbon atoms and the naturally occurring compounds exhibit optical activity. The arrangement of the three optically active glycerol segments is analogous to that of lyxose [cf. Le Coq & Ballou (1964) and Powell & Jacobus (1974)], and the asymmetry of cardiolipin was also confirmed by phospholipase D hydrolysis (Tucker & White, 1971) and ^{31}P NMR spectroscopy. High-resolution ^{31}P NMR spectra of monomerically dissolved cardiolipin showed two signals of equal intensity, demonstrating the physical inequivalence of the two phosphate residues (Henderson et al., 1974).

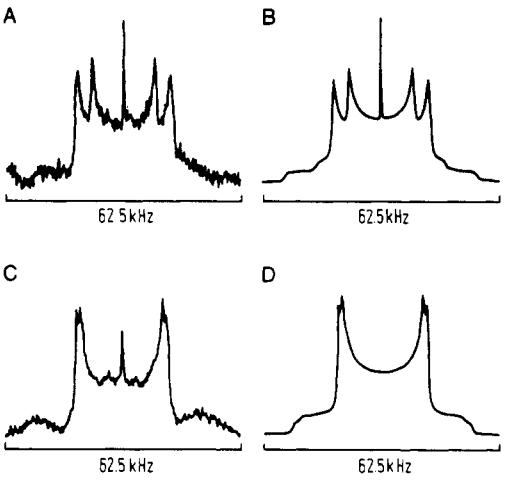


FIGURE 1: ^2H NMR spectra (at 46.1 MHz) of cardiolipin extracted from *E. coli* strain B131GP grown on [2-^2H]glycerol. (A) Cardiolipin (50 mg) dispersed in buffer; 18 000 scans; 15 °C; 62.5-kHz spectral width. (B) Simulated spectrum with an intensity distribution of 2 ($\Delta\nu_Q$ = 25.5 kHz):1 (17.1 kHz):0.035 (isotropic peak) [T_2 = 0.4 ms (25.5 kHz); 0.5 ms (17.1 kHz); 2.0 ms (isotropic peak)]. (C) Cardiolipin–DOPC (20:80 w/w) (66 mg of CL) dispersed in buffer; 50 000 scans; 20 °C, 125-kHz total spectral width. (D) Simulated spectrum with an intensity distribution of 2 (23.8 kHz):1 (21.8 kHz) (T_2 = 0.5 ms for both resonances).

Addition of stereospecifically deuterated glycerol to the growth medium of strain B131GP leads to a stereoselective deuteration of the backbone as well as the head-group segments. Thus, addition of optically active [1,1-$^2H_2$]glycerol will introduce deuterium labels at the *sn*-1 position of both backbones and at the *sn*-1′ (α) position of the head group of cardiolipin.

Cardiolipin Deuterated at sn-2 and sn-2′ Segments. Incorporation of [2-^2H]glycerol leads to a labeling of the two *sn*-2 backbone segments and the β head-group segment of cardiolipin.

Figure 1A shows the corresponding ^2H NMR spectrum of pure cardiolipin bilayers dispersed in buffer (50:50 w/w). Two quadrupole splittings with separations of about 17 and 25 kHz (at 15 °C) can clearly be resolved. The assignment of the resonances follows from a computer simulation of the spectra (Figure 1B) that yields an intensity ratio of 1:2 for the inner and outer signal. Thus, the 17-kHz signal must be assigned to the β head-group segment (one deuteron) whereas the 25-kHz splitting arises from the *sn*-2 backbone segments (two deuterons). If cardiolipin is diluted with 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (cardiolipin–DOPC, 20:80 w/w), the quadrupole splitting of the β-segment is found to increase by 4 kHz, whereas that of the *sn*-2 segments is reduced by 2 kHz (cf. parts C and D of Figure 1). Figure 1C contains an additional weak signal with a quadrupole splitting of about 6 kHz. This spectral contribution is attributed to a trace amount of deuterated phosphatidylglycerol present in this sample. The temperature dependence of the quadrupole splittings of cardiolipin with and without DOPC is shown in Figure 2A.

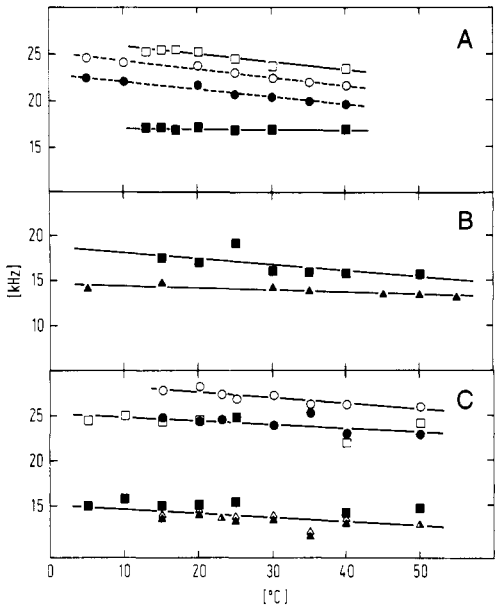


FIGURE 2: Temperature dependence of the quadrupole splittings of cardiolipin. (A) Deuteration of the *sn*-2 and *sn*-2′ (β) segment. (□) Backbone and (■) head group of pure cardiolipin bilayers; (○) backbone and (●) head group of cardiolipin in a CL–DOPC (20:80 w/w) mixture. (B) Quadrupole splittings of the *sn*-1 and *sn*-1′ (α) segments. (■) Cardiolipin; (▲) CL–POPC (20:80 w/w) mixture. The quadrupole splitting in the range of 13–16 kHz (cf. Figure 3) is a superposition of the head-group *sn*-1′ deuterons and the 1*R* deuteron of the backbone. (C) Quadrupole splittings of cardiolipin deuterated in the *sn*-3 and *sn*-3′ (γ) segments. In pure cardiolipin bilayers only one quadrupole splitting can be resolved for the head-group segment (■) and one for the backbone segment (□). In mixtures of cardiolipin with DOPC the intrinsic line width appears to be smaller and two backbone signals can be resolved at all temperatures [(○, ●) difference ~2 kHz]. At some temperatures it is also possible to detect two slightly different head-group splittings (△, ▲).

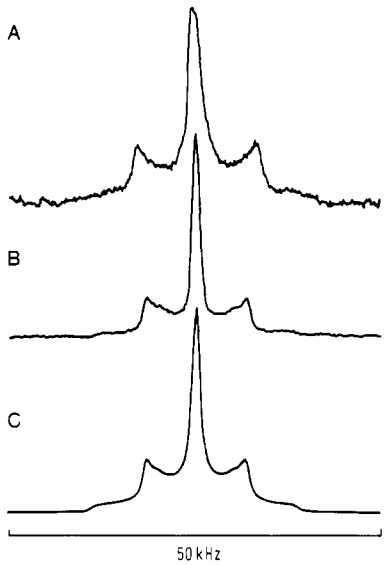


FIGURE 3: ^{2}H NMR spectra of cardiolipin isolated from *E. coli* strain B131GP grown on [1,1-$^2H_2$]glycerol. (A) 47 mg of cardiolipin dispersed in buffer; 14 000 scans; 40 °C; 50-kHz spectral width. (B) Cardiolipin–POPC (20:80 w/w) (41 mg of CL) dispersed in buffer; 22 000 scans; 45 °C; 50-kHz spectral width. (C) Simulated spectrum of (B) with an intensity distribution of 4 ($\Delta\nu_Q$ = 13.4 kHz):2 (isotropic peak):0.35 (10.3 kHz) (T_2 = 0.4 ms for all resonances).

Cardiolipin Deuterated at sn-1 and sn-1′ Segments. Labeling at the C-1 and C-1′ carbon atoms is achieved by growth on [1,1-$^2H_2$]glycerol. Corresponding ^{2}H NMR spectra of pure cardiolipin bilayers and cardiolipin (20 wt %) in mixture with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (80 wt %)

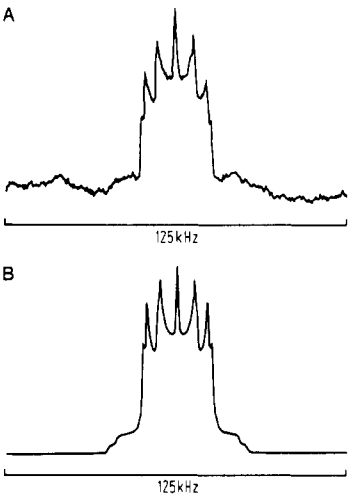


FIGURE 4: ^{2}H NMR spectrum and simulation of cardiolipin extracted from *E. coli* strain B131GP grown on [3,3-$^2H_2$]glycerol. Measuring temperature 35 °C. (A) Cardiolipin–DOPC (20:80 w/w) (75 mg of CL) dispersed in buffer; 54 000 scans; 35 °C; 125-kHz spectral width. (B) Simulation of the spectrum with an intensity distribution of 2 ($\Delta\nu_Q$ = 25.6 kHz):2 (22.6 kHz):1 (14.6 kHz):1 (13.1 kHz):0.15 (isotropic line) (T_2 = 0.5 ms with the exception of the isotropic signal where T_2 = 0.4 ms).

are shown in parts A and B of Figure 3, respectively. The spectra can be explained by a superposition of two components with quadrupole splittings of 0 and 13–16 kHz, respectively, and an intensity ratio of 2 (isotropic component):4 (broad component) (cf. computer simulation, Figure 3C). The ^{31}P NMR spectra of the samples exhibit the characteristic features of a lipid bilayer with no indication of an isotropic phase. The assignment of the resonances is made by analogy with phosphatidylglycerol (Gally et al., 1981; Borle & Seelig, 1983a). The isotropic resonance arises from the 1*S* deuterons of the glycerol backbone (one deuteron for each glycerol backbone), the C–D vectors of which are oriented close to the magic angle. The 1*R* deutrons of the backbone as well as the two α-deuterons of the head group (*sn*-1′ segment) accidentally gave rise to the same quadrupole splitting of 16 kHz. The temperature dependence of this quadrupole splitting is summarized in Figure 2B.

Cardiolipin Deuterated at sn-3 and sn-3′ Segments. Three quadrupole splittings can be discerned for cardiolipin derived from growth medium containing [3,3-$^2H_2$]glycerol (Figure 4A). By comparison with deuterated phosphatidylglycerol, the two largest splittings of 23 and 26 kHz are assigned to the glycerol backbone segments, whereas the 13 kHz is attributed to the γ head-group segment. At some temperatures the head-group splitting can be further resolved into two splittings with slightly different separations. This fact has been taken into account by the computer simulation shown in Figure 4B. The temperature dependence of the *sn*-3 quadrupole splittings of pure cardiolipin and mixtures of cardiolipin (20 wt %) and DOPC (80 wt %) is illustrated in Figure 2C. No significant differences were observed between the two bilayer systems.

The occurrence of two quadrupole splittings is explained by the physical inequivalence of the two deuterons at the *sn*-3 segment; i.e., the two C–D bond vectors are inclined at slightly different angles with respect to the bilayer normal. This conclusion follows from a comparison with lipids containing only one *sn*-3 segment such as phosphatidylcholine (Gally et al., 1975), phosphatidylethanolamine (Gally et al., 1981; Ghosh & Seelig, 1982), and phosphatidylglycerol (Borle & Seelig, 1983a) since in all three bilayers the *sn*-3 segment gives rise to two quadrupole splittings. For bilayers of *E. coli* phos-

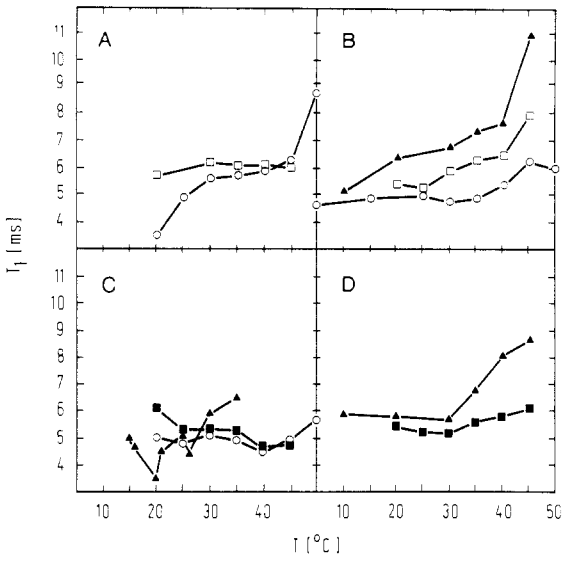


FIGURE 5: Deuterium spin–lattice (T_1) relaxation times of cardiolipin and cardiolipin–phosphatidylcholine mixtures (20:80 w/w) dispersed in buffer. (A) Head group in pure cardiolipin bilayer: (□) 1,1-$^2H_2$ segment (head group and 1*R* backbone deuteron); (○) 2-^2H segment. (B) Head group in cardiolipin–phosphatidylcholine mixed bilayer: (□) 1,1-$^2H_2$ segment (head group and 1*R* backbone deuteron); (○) 2-^2H segment (backbone and head group not resolved); (▲) 3,3-$^2H_2$ segment. (C) Backbone in pure cardiolipin bilayer: (■) 1,1-$^2H_2$ segment 1*S* deuteron); (○) 2-^2H segment; (▲) 3,3-$^2H_2$ segment. (D) Backbone in cardiolipin–phosphatidylcholine mixed bilayer: (■) 1,1-$^2H_2$ segment (1*S* deuteron); (▲) 3,3-$^2H_2$ segment.

phatidylethanolamine, it was further demonstrated by stereospecific monodeuteration that the two quadrupole splittings can be assigned to individual deuterons of the *sn*-3 segment (Gally et al., 1981). By analogy, we conclude that the same situation prevails in cardiolipin.

^2H NMR Spin–Lattice Relaxation Times. Spin–lattice (T_1) relaxation times were measured by inverting the magnetization by a 180° pulse and observing the return to equilibrium by using the quadrupole echo sequence. Figure 5 summarizes the results of these measurements for various glycerol segments as a function of temperature and lipid composition. Inspection of Figure 5 reveals that the T_1 relaxation times of all segments investigated are remarkably constant, independent of temperature, and almost independent of the location of the segment. All relaxation times fall in the range of 5–8 ms, and the addition of 80 wt % phosphatidylcholine does not influence the T_1 behavior. It may be noted that the relaxation rate of the isotropic signal (Figure 3) is identical with that of the well-resolved quadrupole splittings. This provides an additional support for the above assignment since isotropically tumbling water is characterized by a much longer T_1 relaxation time (Borle & Seelig, 1983b).

Discussion

Glycerol Backbone. At the present level of resolution, no spectral differences can be detected between the two backbone glycerols of cardiolipin. Corresponding segments in the two halves of the molecule are characterized by essentially identical quadrupole splittings, suggesting a rather symmetric conformation of the cardiolipin molecule in the bilayer membrane.

A detailed comparison of the quadrupole splittings of cardiolipin with those of the backbone segments of phosphatidylethanolamine (Gally et al., 1981; Ghosh & Seelig, 1982) and phosphatidylglycerol (Borle & Seelig, 1983a) reveals a very close quantitative agreement. Numerical values characteristic of the individual segments are as follows: *sn*-3 segment 23–26 kHz; *sn*-2 segment 25 kHz; *sn*-1 segment 13–17 kHz (1*R* deuteron) and 0 kHz (1*S* deuteron). Since the quadrupole splittings are structural parameters and as such are quite sensitive to even small structural changes, it follows that the glycerol backbone conformation must be virtually identical for the three types of lipids.

The following molecular picture can be derived from a quantitative analysis of the quadrupole splittings [cf. Gally et al. (1975, 1981)]. When the small spectral difference of the *sn*-3 segment is neglected, it follows that the glycerol C(2)–C(3) bond must be oriented essentially parallel to the bilayer normal. Under these conditions the C–D bond vectors of the C(2) and the C(3) segments make the same average angle with the bilayer normal, yielding identical quadrupole splittings for all three deuterons involved. However, if the C(2)–C(3) axis were held exactly parallel to the bilayer normal, the predicted quadrupole splitting would be 42 kHz (assuming tetrahedral bond angles) whereas the experimental values are only 25 kHz. The reduction compared to the theoretical value suggests some wobbling motion of the C(2)–C(3) axis. Quantitatively, the wobbling motion is characterized by an order parameter of $S \sim 0.6$. The same wobbling parameter has been deduced for the glycerol backbone of phosphatidylcholine (Seelig et al., 1977).

The characteristic feature of the C(1) carbon is the inequivalence of the two deuterons with quadrupole splittings of 0 and 13–17 kHz. With the above assumption about the orientation of the C(2)–C(3) bond, the inequivalence of the two deuterons at C(1) can only be explained if the C(1)–C(2) bond rotation angle deviates on the average from the symmetric trans state toward either the gauche$^+$ or the gauche$^-$ state. In the trans state both deuterons of the C(1) segment make the same angle (109.47°) with the C(2)–C(3) axis. Any deviation from the trans state induces an asymmetry in the bond vector orientation. If ψ denotes the bond rotation angle (referenced to the trans state with $\psi = 0°$), then the angle α between the C–D bond vector and the C(2)–C(3) rotation axis is given by

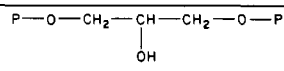
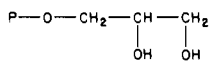
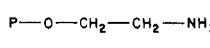
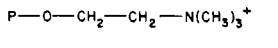
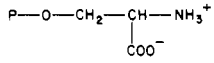
$$\cos \alpha = (1/9)[1 - 4 \cos \psi \pm 4(3^{1/2}) \sin \psi]$$

where the sign differentiates between the two deuterons. No single bond rotation angle ψ can be found that produces the two quadrupole separations of 0 and 17 kHz. Hence, a more complicated dynamic model must be invoked in which the C(1)–C(2) axis assumes different rotational isomeric states. Such a model is also suggested by comparing different single crystal studies of phospholipids where torsion angles close to the trans and gauche$^+$ states and also intermediate angles have been found (Hauser et al., 1981). However, since the torsion angles in solution and the population of the different rotational isomeric states are not known, a more detailed analysis is not possible at present.

The measurement of the T_1 relaxation times provides insight into the motional properties of the deuterated segments. All three glycerol backbone segments of cardiolipin exhibit T_1 relaxation times of 5–8 ms with almost no temperature dependence between 5 and 40 °C. The glycerol backbone is therefore characterized by rather long correlation times of 0.5–5 ns that can be assigned to the rotation of the glycerol backbone around an axis parallel to the bilayer normal [cf. Ghosh & Seelig (1982)]. Off-axis motions are probably even slower, but only the faster motional components determine T_1.

Glycerol Head Group. Each phospholipid head group exhibits a characteristic set of quadrupole splittings that is rather independent of the chemical nature of the attached fatty acyl chains. A comparison of cardiolipin with other polar groups

Table I: Deuterium Quadrupole Splittings and T_1 Relaxation Times of Different Lipid Head Groups

	head-group segment		
	sn-1′ (α)	*sn*-2′ (β)	*sn*-3′ (γ)
cardiolipin[a]			
quadrupole splittings (kHz)	13–17	17–20	13–16
T_1 relaxation time (ms)[d]	5–7	3–9	5–8
phosphatidylglycerol[b]			
quadrupole splittings (kHz)	10–11	4–5	0.5–1
T_1 relaxation time (ms)[d]	8–19	8–19	8–22
phosphatidylethanolamine[c]			
quadrupole splittings (kHz)	10	4	
phosphatidylcholine[c]			
quadrupole splittings (kHz)	6	5	
phosphatidylserine[c]			
quadrupole splittings (kHz)	2, 17[e]	15	

[a] This work. [b] Borle & Seelig (1983a). [c] Cf. Seelig & Seelig (1980). [d] Data refer to the temperature interval of 15–40 °C. [e] Two inequivalent deuterons.

is shown in Table I. The data refer to the bilayer phase at temperatures above the gel-to-liquid-crystal phase transition temperature. Of particular relevance is the comparison between cardiolipin and its parent compound phosphatidylglycerol. For the latter the quadrupole splittings decrease distinctly from the ester linkage (α-segment) toward the free end (γ-segment) of the glycerol residue, indicating increasing motional freedom in the same direction. In contrast, practically identical quadrupole splittings of 13–17 kHz are observed for the α- and γ-segment of cardiolipin while the β-segment with a splitting of 17–20 kHz exhibits the largest splitting measured so far for a head-group segment. This behavior is obviously dictated by the geometry of the cardiolipin molecule. Both ends of the glycerol head group serve as anchor points for phosphatidic acid residues, leading to a distinct restriction of the segmental flexibility.

Compared to the other lipids listed in Table I, the conformational restraints imposed on cardiolipin appear to be quantitatively similar to those of phosphatidylserine, in particular with respect to the large quadrupole splitting of the β-segment. For phosphatidylserine this rigidity could be caused by the terminal carboxylate group, which most likely serves as an additional anchor group for this polar residue at the lipid–water interface.

A further difference between cardiolipin and phosphatidylglycerol shows up in the deuterium T_1 relaxation times. For cardiolipin all head-group segments are characterized by short relaxation times (5–8 ms) with almost no temperature dependence. On the other hand, the head-group segments of phosphatidylglycerol have longer relaxation times (8–22 ms) with a distinct temperature dependence. From the latter it can be concluded that the motions fall into the fast correlation time regime with correlation times of 0.05–0.5 ns. In contrast, the correlation times of cardiolipin are clearly longer, by as much as a factor of 10, reflecting again the rather rigid structure of the cardiolipin head group.

In summary, the following conclusions can be drawn from the present study. (1) By using an *E. coli* mutant defective in the synthesis and degradation of glycerol, it was possible to selectively deuterate individual glycerol segments of cardiolipin. (2) The ^2H NMR spectra yielded characteristic quadrupole splittings for the backbone and head-group deuterons. The α- and β-resonances of the head group overlapped with the corresponding *sn*-1 and *sn*-2 resonances of the glycerol backbone, whereas the γ-resonance could be assigned unambiguously. (3) The glycerol head group of cardiolipin was less flexible than most other phospholipid polar groups. (4) Both glycerol backbones were found to adopt the same conformation and orientation within the cardiolipin bilayers. This conformation appears to be ubiquitous for all phospholipid backbones investigated so far. All glycerol backbones are oriented perpendicular to the surface of the membrane. (5) In mixed bilayers composed of cardiolipin and phosphatidylcholine, only small changes can be detected in the quadrupole splittings. The intensity of the ^2H NMR parameters to the addition of other lipids provides evidence for a rather stable head-group conformation, a phenomenon that is also found with other lipids.

With the availability of deuterated cardiolipin and the assignment of the various resonances, it appears now possible to study the interaction of cardiolipin with metal ions, membrane-bound enzymes, and antibodies.

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References

Awasthi, Y. C., Chuang, T. F., Keenan, T. W., & Crane, F. L. (1971) *Biochim. Biophys. Acta 226*, 42–52.
Bachmann, B. J. (1983) *Microbiol. Rev. 47*, 180–230.
Baer, E. (1951) *J. Biol. Chem. 189*, 235–247.
Bell, R. M. (1974) *J. Bacteriol. 117*, 1065–1076.
Borle, F., & Seelig, J. (1983a) *Biochemistry 22*, 5536–5544.
Borle, F., & Seelig, J. (1983b) *Biochim. Biophys. Acta 735*, 131–136.
Bruce, Å. (1974) *J. Lipid Res. 15*, 103–108.
Chambertain, B. K., & Webster, R. E. (1976) *J. Biol. Chem. 251*, 7739–7745.
Cheneval, D., Müller, M., & Carafoli, E. (1983) *FEBS Lett. 159*, 123–126.
Cronan, J. E., & Vagelos, P. R. (1972) *Biochim. Biophys. Acta 265*, 25–60.
Cullis, P. R., Verkeij, A. J., & Ververgaert, P. H. J. Th. (1978) *Biochim. Biophys. Acta 513*, 11–20.
De Kruijff, B., & Cullis, P. R. (1980) *Biochim. Biophys. Acta 602*, 477–490.
De Kruijff, B., Verkleij, A. J., van Echteld, C. J. A., Gerritsen, W. J., Mombers, C., Noordam, P. C., & de Gier, J. (1979) *Biochim. Biophys. Acta 555*, 200–209.
De Kruijff, B., Verkleij, A. J., van Echteld, C. J. A., Gerritsen, W. J., Noordam, P. C., Mombers, C., Rietveld, A., de Gier, J., Cullis, P. R., Hope, M. J., & Nayar, R. (1981) *International Cell Biology* 1980–1981 (Schweiger, H. G., Ed.) pp 559–571, Springer-Verlag, Berlin and Heidelberg.

De Kruijff, B., Verkleij, A. J., Lennissen-Bijvelt, J., Van Echteld, C. J. A., Hille, J., & Rijnbout, H. (1982) *Biochim. Biophys. Acta 693*, 1–12.

Eichberg, J., & Burnham, J. D. (1970) *J. Lipid Res. 11*, 386–388.

Gally, H. U., Niederberger, W., & Seelig, J. (1975) *Biochemistry 14*, 3647–3652.

Gally, H. U., Pluschke, G., Overath, P., & Seelig, J. (1981) *Biochemistry 20*, 1826–1831.

Ghosh, R., & Seelig, J. (1982) *Biochim. Biophys. Acta 691*, 151–160.

Gilkes, N. R., & Weeks, G. (1977) *Biochim. Biophys. Acta 464*, 142–156.

Goormaghtigh, E., Brasseur, R., & Ruysschaert, J. M. (1982a) *Biochem. Biophys. Res. Commun. 104*, 314–320.

Goormaghtigh, E., Vandenbranden, M., Ruysschaert, J. M., & de Kruijff, B. (1982b) *Biochim. Biophys. Acta 685*, 137–143.

Hauser, H., Pascher, I., Pearson, R. H., & Sundell, S. (1981) *Biochim. Biophys. Acta 650*, 21–51.

Henderson, T. O., Glonek, T., & Myers, T. C. (1974) *Biochemistry 13*, 623–628.

Hirschberg, C. B., & Kennedy, E. P. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 648–651.

Hostettler, K. Y., van den Bosch, H., & van Deenen, L. L. M. (1972) *Biochim. Biophys. Acta 260*, 507–513.

Ioannou, P. V., & Golding, B. T. (1979) *Prog. Lipid Res. 17*, 279–318.

Jakovcic, S., Getz, G. S., Rabinowitz, M., Jakob, H., & Swift, H. (1971) *J. Cell Biol. 48*, 490–502.

Kanemasa, Y., Akamatsu, Y., & Nojima, S. (1967) *Biochim. Biophys. Acta 144*, 382–390.

Lecocq, J., & Ballou, C. E. (1964) *Biochemistry 3*, 976–980.

Lok, C. M., Ward, J. P., & van Dorp, D. A. (1976) *Chem. Phys. Lipids 16*, 115–122.

Overath, P., & Thilo, L. (1978) *Int. Rev. Biochem.* 19.

Pangborn, M. C. (1942) *J. Biol. Chem. 143*, 247–256.

Pluschke, E., Hirota, Y., & Overath, P. (1978) *J. Biol. Chem. 253*, 5048–5055.

Powell, G. L., & Jacobus, J. (1974) *Biochemistry 13*, 4024–4026.

Renoll, M., & Newman, M. S. (1955) *Organic Syntheses*, Collect. Vol. III, pp 502–504, Wiley, New York.

Robinson, N. C. (1982) *Biochemistry 21*, 184–188.

Rouser, G., Simon, G., & Kritchevsky, G. (1969) *Lipids 4*, 599–606.

Sakolove, P. M., Brenza, J. M., & Shamoo, A. E. (1983) *Biochim. Biophys. Acta 732*, 41–47.

Seelig, J., & Seelig, A. (1980) *Q. Rev. Biophys. 13*, 19–61.

Seelig, J., Gally, H., & Wohlgemuth, R. (1977) *Biochim. Biophys. Acta 467*, 109–119.

Seelig, J., Tamm, L. K., Hymel, L., & Fleischer, S. (1981) *Biochemistry 20*, 3922–3932.

Siminovitch, D. J., Rance, M., Jeffrey, K. R., & Brown, M. F. (1984) *J. Magn. Reson. 58*, 62–75.

Simon, G., & Rouser, G. (1969) *Lipids 4*, 607–614.

Takahashi, T., Hashimoto, T., & Hara, I. (1967) *Bull. Soc. Chim. Biol. 49*, 65–70.

Tamm, L. K., & Seelig, J. (1983) *Biochemistry 22*, 1474–1483.

Taraschi, T. F., de Kruijff, B., & Verkleij, A. J. (1983) *Eur. J. Biochem. 129*, 621–625.

Tucker, A. N., & White, D. C. (1971) *J. Bacteriol. 108*, 1058–1064.

Vasilenko, I., de Kruijff, B., & Verkleij, A. J. (1982) *Biochim. Biophys. Acta 684*, 282–286.

Vik, S. B., Georgevich, G., & Capaldi, R. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1456–1460.

Vogel, H. J., & Bonner, D. M. (1966) *J. Biol. Chem. 218*, 97–106.

Wasserman, A., Neissen, A., & Bruck, C. (1906) *Dtsch. Med. Wochenschr. 32*, 745–746.

Wohlgemuth, R., Waespe-Šarčevič, N., & Seelig, J. (1980) *Biochemistry 19*, 3315–3321.