Structure of *Escherichia coli* Membranes. Glycerol Auxotrophs as a Tool for the Analysis of the Phospholipid Head-Group Region by Deuterium Magnetic Resonance†

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ABSTRACT: Glycerol selectively deuterated at various positions was synthesized and supplied to the growth medium of *Escherichia coli* strain T131 GP, which is defective in endogenous glycerol synthesis as well as glycerol degradation and lacks the ability to synthesize cardiolipin. The procedure enables the stereospecific labeling of the membrane phospholipids (~80% phosphatidylethanolamine, ~20% phosphatidylglycerol). Deuterium magnetic resonance spectra were obtained for cell membranes and lipid dispersions either from total lipid extracts or from purified phosphatidylglycerol or -ethanolamine. When glycerol deuterated at various positions was used, all resonances of the phospholipid glycerol backbone and the terminal glycerol moiety in phosphatidylglycerol could be assigned. The results indicate that the molecular conformation of the glycerol backbone is independent of the phospholipid species investigated and is also not altered by the presence of high amounts of membrane proteins. For the quantitative interpretation of the deuterium magnetic resonance splittings, a model is proposed which assumes essentially free rotation around the glycerol C(2)–C(3) bond combined with an asymmetric and restricted jump process around the C(1)–C(2) bond. This model is compatible with known X-ray structures of phospholipid molecules. The two deuterons of both the glycerol backbone C(1) and C(3) segments were found to be magnetically inequivalent. Stereoselective monodeuteration eliminated one set of quadrupole splittings in both cases.

Technical innovations such as the development of new NMR[1] pulse methods and the introduction of high-field NMR spectrometers have made it possible to measure ^2H NMR spectra even at very low concentrations of deuterium, opening a new avenue for the structural analysis of phospholipids in biological membranes. However, there remains the problem of selective deuteration at specific sites. For the bacterium *Escherichia coli*, a promising approach is the use of mutants defective in the synthesis of the precursors of phospholipid biosynthesis which can be supplied in deuterated form to the growth medium and are incorporated into the membrane phospholipids. *E. coli* mutants defective in fatty acid synthesis have already been employed successfully to incorporate deuterated fatty acids into *E. coli* inner and outer membranes (Gally et al., 1979, 1980; Davis et al., 1979; Nichol et al., 1980; Kang et al., 1979), and a detailed picture of the phospholipid conformation in the hydrophobic part of the bilayer membrane has been obtained. In the present study, we have investigated the polar head-group region of *E. coli* membranes by the same method. Mutants defective in both the biosynthesis and degradation of glycerol incorporate externally added glycerol specifically into membrane phospholipids (Cronan et al., 1970; Lin et al., 1971). In addition, introduction of a mutation in the *cls* gene (Pluschke et al., 1978) into such a strain leads to a defect in cardiolipin synthesis and simplifies the glycerol-containing phospholipids of this organism to phosphatidylethanolamine and phosphatidylglycerol. When appropriately deuterated glycerol was incorporated, it was possible to study the motion and structure of the derived phospholipids both in membranes and after isolation. The objectives of this investigation are (i) to provide the assignments of all glycerol backbone and glycerol head-group resonances, (ii) to relate the spectral parameters to a possible model for the glycerol backbone conformation, and (iii) to compare the phospholipid conformation of cell membranes and derived liposomes at the level of the glycerol backbone.

Materials and Methods

Synthesis of Deuterated Glycerols. Perdeuterated glycerol ([$^2H_5$]glycerol) was purchased from Merck, Sharp & Dohme (Canada) and used without further purification. *sn*-[1,1-$^2H_2$]Glycerol and *sn*-[3,3-$^2H_2$]glycerol were obtained by hydrolyzing 2,3-*O*-isopropylidene-*sn*-[1,1-$^2H_2$]glycerol and 1,2-*O*-isopropylidene-*sn*-[3,3-$^2H_2$]glycerol, respectively. The latter compounds were synthesized according to Lok et al. (1976) using $LiAlD_4$ instead of $LiAlH_4$ in the reduction step. [2-^2H]Glycerol was prepared by reducing dihydroxyacetone with $NaBD_4$ (Renoll & Newmann, 1955). By means of horse liver alcohol dehydrogenase, the stereospecific incorporation of a single deuteron into a CH_2 group was achieved (Günther et al., 1973; Wohlgemuth et al., 1980). (3*R*)-*sn*-[3-^2H]Glycerol was prepared by exchange of 1,2-*O*-isopropylidene-*sn*-glycerol in deuterium oxide and (1*S*)-*sn*-[1-^2H]glycerol by exchange of 2,3-*O*-isopropylidene-*sn*-[1,1-$^2H_2$]glycerol in H_2O.

Incorporation of Selectively Deuterated Glycerol into E. coli Membrane Phospholipids. The *E. coli* K12 mutant T131GP [genotype *cls*, *gps*A, *glp*D, *glp*R, *glp*K, *pho*A, *fad*E, *ton*A, *rel*1; for nomenclature see Bachmann et al. (1976)] is a *cls* derivative [cf. Pluschke et al. (1978)] of strain BB20-14 kindly provided by Dr. R. M. Bell (Bell, 1974). The genetics and biochemistry of glycerol and phospholipid metabolism relevant to this study have been reviewed (Lin, 1976; Raetz, 1978). Briefly, cells growing on mineral salts medium (Vogel & Bonner, 1956), 0.4% glucose, and 0.002% deuterated glycerol take up glycerol and convert it to *sn*-glycero-3-phosphate (cf. Figure 1A for the conversion of *sn*-[3,3-^2H]glycerol to

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[1] Abbreviations used: NMR, nuclear magnetic resonance; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-1′-glycerol; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; Pipes, 1,4-piperazinediethanesulfonic acid.

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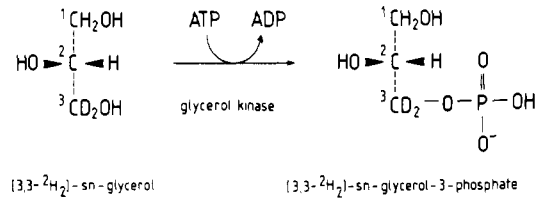
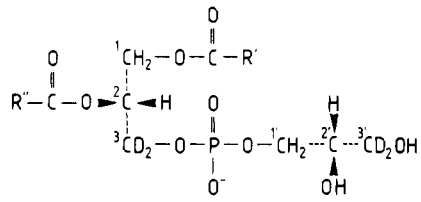
A



[3,3-$^2H_2$]-sn-glycerol [3,3-$^2H_2$]-sn-glycerol-3-phosphate

B



[3,3-$^2H_2$]-3-sn-phosphatidyl-[3',3'-$^2H_2$]-1'-sn-glycerol

FIGURE 1: Stereospecific incorporation of *sn*-[3,3-$^2H_2$]glycerol into *E. coli* phospholipids. (A) Conversion of *sn*-[3,3-$^2H_2$]glycerol to *sn*-[3,3-$^2H_2$]glycerol 3-phosphate. (B) Stereochemistry and stereospecific numbering of the resulting 3-*sn*-phosphatidyl-1'-*sn*-glycerol.

sn-[3,3-$^2H_2$]glycero-3-phosphate). Acylation of *sn*-glycero-3-phosphate gives rise to *sn*-3-phosphatidic acid which is the precursor of both phosphatidylethanolamine (~80%) and phosphatidylglycerol (~20%). The supplementation of the mutant with *sn*-[3,3-$^2H_2$]glycerol leads to the stereospecific labeling of the 3 positions in PE and PG and of the 3' position in PG (cf. Figure 1B). Correspondingly, *sn*-[1,1-$^2H_2$]glycerol and *sn*-[2-^{2}H]glycerol label positions 1 and 1' and 2 and 2', respectively. It should be noted that the *cls* mutation in strain T131GP prohibits the synthesis of cardiolipin, the third typical phospholipid of wild-type *E. coli*. The fatty acid composition of both PE and PG in strain T131GP is that of wild-type *E. coli* with palmitic acid predominantly in the *sn*-1 position and vaccenic and palmitoleic acids in the *sn*-2 position.

A total membrane fraction (inner and outer membranes) was prepared by sonication and high-speed centrifugation. A total phospholipid fraction was prepared from cells by chloroform/methanol extraction (Ames, 1968) and purified by silicic acid chromatography or acetone precipitation. PE and PG were separated on a DEAE-cellulose column (Rouser et al., 1969). The phospholipids were dispersed at 50 °C in 0.01 M Pipes buffer solution of pH 7.0 containing 0.1 M NaCl and 0.001 M EDTA. Deuterium-depleted water was used in all sample preparations to eliminate isotropic signals due to natural abundance of deuterium in water. The other experimental conditions are the same as described earlier (Gally et al., 1979).

Deuterium NMR Measurements. Deuterium NMR spectra were recorded at 61.4 MHz with a Bruker-Spectrospin WH-400-FT spectrometer and at 46.4 MHz with a Bruker-Spectrospin CXP-300 spectrometer. The WH-400 instrument is essentially designed for high-resolution work; the pulse width for a 90° pulse was 16 μs, the spectral width 100 or 50 kHz, and the recycle time 0.15 s. The CXP-300 spectrometer is equipped with high-power transmitters and special pulse-programmer hardware, allowing detection of very broad resonance lines. ^{2}H NMR spectra were obtained by employing the quadrupole echo technique (Davis et al., 1976) with 5-μs, 90° pulse, and pulse separations of 30 μs.

Results

Supplying perdeuterated glycerol to the *E. coli* growth medium leads to deuteration of all three glycerol backbone

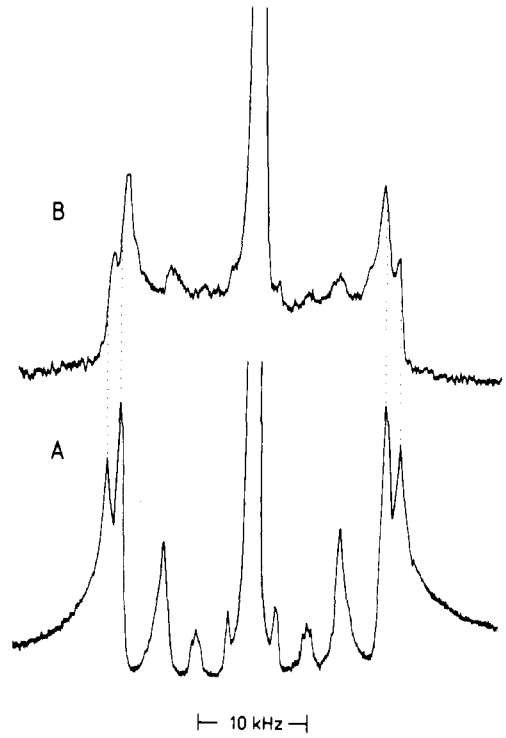


FIGURE 2: ^{2}H NMR spectra of multilamellar liposomes derived from *E. coli* phospholipids. *E. coli* strain T131GP was grown on perdeuterated glycerol (measuring temperature 47 °C; spectral width 100 kHz). (A) ^{2}H NMR frequency 61.4 MHz; WH-400 spectrometer; single-pulse mode. (B) ^{2}H NMR frequency 46.4 MHz; CXP 300 spectrometer; quadrupole-echo mode.

segments of PE and PG and, simultaneously, of the three head-group segments of PG. Accordingly, the ^{2}H NMR spectrum of the total lipid extract is rather complex as evidenced by Figure 2. Figure 2A was recorded under high-resolution conditions (WH-400 spectrometer). Altogether, five quadrupole doublets can be discerned with splittings ranging from 4 to 28 kHz. Since the buffer was made up with deuterium-depleted water and since the phosphorus NMR spectra are characteristic of a purely lamellar structure, the strong central deuterium signal cannot be explained by nonlamellar phases such as micelles. Instead, it must result from lipid segments in which the C–D bond vectors are either aligned close to the magic angle or where the segmental flexibility is such as to completely average the static quadrupole splitting constant (cf. below). Due to insufficient radiofrequency power of the WH-400 spectrometer, the spectral shapes are distorted and Figure 2A does not correspond to the theoretical line shape of a spin-1 powder pattern. Deuterons with small quadrupole splittings are exaggerated in intensity compared to those with large splittings. The same sample was therefore measured with the CXP-300 spectrometer using the quadrupole echo technique (Figure 2B). Under these conditions, the spectral shape conforms to an axially symmetric spin-1 powder pattern, but the inner resonances are less clearly resolved. Nevertheless, within experimental error, both techniques lead to the same quadrupole splittings, which is reassuring in view of the fact that almost all earlier ^{2}H NMR work on membranes has been performed with high-resolution spectrometers.

The assignment of the quadrupole splittings of Figure 2 to the backbone and head-group segments of PE and PG becomes possible by (i) growth of *E. coli* T131GP on selectively deuterated glycerols, (ii) isolation of PE from the lipid mixture, since PE is deuterated in the backbone only, and (iii) comparison of the present results with the head-group data obtained for selectively deuterated 1,2-dipalmitoyl-*sn*-glycero-

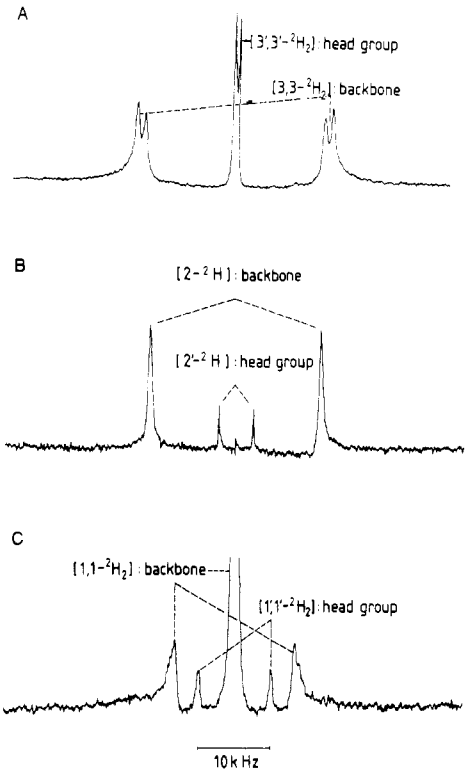


FIGURE 3: ^2H NMR spectra (at 61.4 MHz) of multilamellar liposomes derived from total phospholipid extract. PE ~80 wt %; PG ~20 wt %. (A) *E. coli* T131GP grown on *sn*-[3,3-$^2H_2$]glycerol (measuring temperature 36 °C). (B) Growth medium supplied with *sn*-[2-^{2}H]glycerol (48 °C). (C) Growth medium supplied with *sn*-[1,1-$^2H_2$]glycerol (48 °C).

3-phosphoglycerol (DPPG) (Wohlgemuth et al., 1980).

The first possibility is illustrated in Figure 3 which comprises spectra of the total phospholipid extract of *E. coli* T131GP mutant grown on *sn*-[3,3-$^2H_2$]glycerol (Figure 3A), *sn*-[2-^{2}H]glycerol (Figure 3B), and *sn*-[1,1-$^2H_2$]glycerol (Figure 3C), respectively. The simplest spectral pattern is obtained for *sn*-[2-^{2}H]glycerol, yielding an intense outer and a weak inner signal with spacings of 23.6 and 4.75 kHz, respectively. Because of its intensity, the larger doublet must be assigned to the glycerol backbone C(2) segment of both PE (~80 wt %) and PG (~20 wt %) while the weak signal originates from the C(2′) segment of the PG glycerol head group (~20 wt % only). A quadrupole splitting of 3.1–3.9 kHz has also been found for the same head-group segment in DPPG bilayers above the phase transition temperature Wohlgemuth et al., 1980).

A more complex spectrum with three quadrupole splittings of 26.9-, 24.8-, and 0.67-kHz separation (at 36 °C) is observed for lipids derived from *E. coli* T131GP grown on *sn*-[3,3-$^2H_2$]glycerol (Figure 3A). By comparison with synthetic DPPG, the smallest splitting can be attributed to the terminal C(3′) segment of the PG glycerol head group whereas the two other splittings arise from the two inequivalent deuterons of the C(3) backbone segment. This is proven in more detail in Figure 4 which shows ^2H NMR spectra of pure PE dispersions. Compared to Figure 3A, the central signal is strongly reduced in intensity (a small impurity of PG apparently remains) and only the two C(3) backbone signals are left. If the bacterium is grown on monolabeled (3*R*)-*sn*-[3-^{2}H]glycerol, the ^2H NMR spectrum of the corresponding PE dispersion is further simplified and shows just one quadrupole splitting, the size of which corresponds to the inner splitting of [3,3-$^2H_2$]PE (Figure 4B). This demonstrates unambiguously that the two deuterons of the C(3) segment are motionally inequivalent.

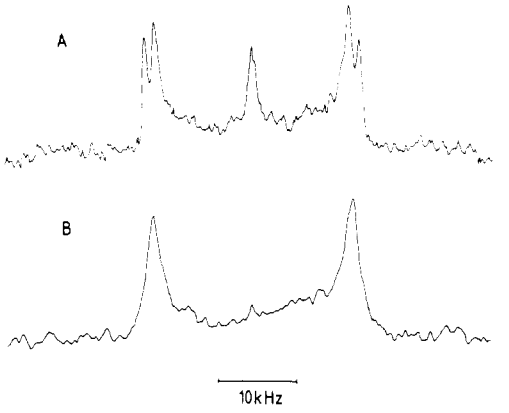


FIGURE 4: ^2H NMR spectra (at 46.1 MHz) of PE liposomes purified from total lipid extract. (A) [3,3-$^2H_2$]PE. Growth medium supplied with *sn*-[3,3-$^2H_2$]glycerol (measuring temperature 47 °C). The sample presumably contains an impurity of PG, giving rise to the central peak. The two quadrupole splittings have seperations of 27.3 and 24.7 kHz. (B) (3*R*)-*sn*-[3-^{2}H]PE. Growth medium supplied with (3*R*)-*sn*-[3-^{2}H]glycerol (measuring temperature 37 °C). The resonances are broader than in (A) due to unresolved proton–deuteron dipolar couplings. The quadrupole splitting is 25.3 kHz.

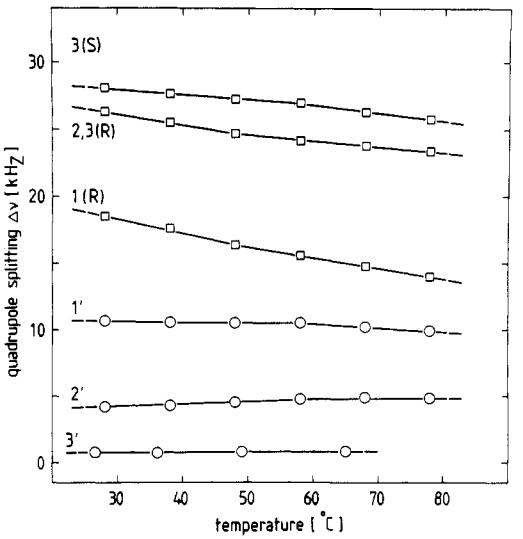


FIGURE 5: Variation of the quadrupole splittings of the glycerol backbone (segments 1*R*, 2, 3*S*, 3*R*) and the glycerol head group (segments 1′, 2′, and 3′) with temperature. Data refer to the total lipid extract.

Three signals with quadrupole splittings of 16.7, 10.1, and ~0 kHz (at 48 °C) are also obtained for phospholipids where *sn*-[1,1-$^2H_2$]glycerol is incorporated into the head group and backbone (Figure 3C). The 10-kHz splitting corresponds in size and appearance to that of 1,2-dipalmitoyl-*sn*-glycero-3-phospho-1′-glycerol labeled at the C(1′) segment (Wohlgemuth et al., 1980). This assignment is also supported by comparison with [1,1-$^2H_2$]PE purified from the total lipid extract where the 10-kHz signal is absent. The origin of the two remaining signals can be understood by labeling with (1*S*)-*sn*-[1-^{2}H]-glycerol. The spectrum of monolabeled PE lacks the 16.7-kHz signal, and only the strong central signal of the 1*S* deuteron is observed whereas the ^{31}P NMR spectrum of this lipid dispersion is typical of a purely lamellar phase. A summary of the compounds investigated in this study together with the assignment of the various quadrupole splittings is provided in Table I. The variation of the quadrupole splittings with temperature is shown in Figure 5. It may be noted that all quadrupole splittings decrease with increasing temperature except for the C(2′) segment of the glycerol head group which has the opposite characteristics. The unusual temperature

Table I: Deuterium Quadrupole Splittings (kHz) of *E. coli* Phospholipids Labeled in the Glycerol Backbone and the Glycerol Head Group at 37 °C

compound	glycerol backbone segments					glycerol head-group segments		
	1*R*	1*S*	2	3*R*	3*S*	1′	2′	3′
[$^2H_5$]PE	~15.0	~0	not resolv.	25.2	27.7			
[1,1-$^2H_2$]PE	17.6	0						
(1*S*)-[1-^{2}H]PE		0						
[2-^{2}H]PE			25.3					
[3,3-$^2H_2$]PE				24.7	27.3			
(3*R*)-[3-^{2}H]PE				25.3				
[$^2H_5$]PG	14.4	0	not resolv	22.0	22.0	10.95	4.17	
[1,1-$^2H_2$]PG	14.6	1.0				12.1		
$^2H_5$-labeled total lipid	17.7	0	not resolv	25.5	27.7	10.5	4.3	0
1,1-$^1H_2$-labeled total lipid	16.8	0				10.4		
2-^{2}H-labeled total lipid			24.6				4.2	
3,3-$^2H_2$-labeled total lipid				24.8	26.9			0.67
representative quadrupole splittings for each segment	17	0	25	25	27	10.5	4.2	0.6
DPPG (46 °C)[a]						10.3	3.3	0.7
DPPC (50 °C)[b,c]				27	29			

[a] Wohlgemuth et al. (1980). [b] Gally et al. (1975). [c] N. Waespe-Šarčević and J. Seelig, unpublished results.

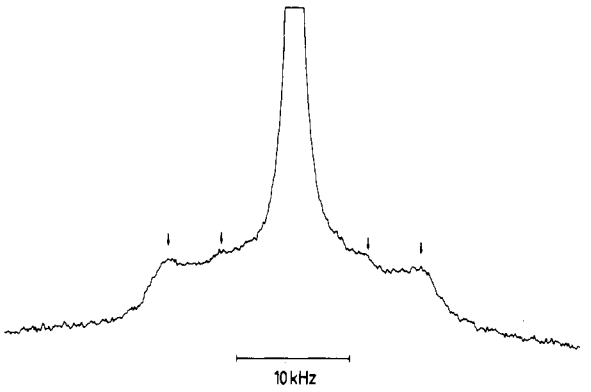


FIGURE 6: ^{2}H NMR spectrum (at 46.1 MHz) of *E. coli* cell membranes derived from *E. coli* T131GP grown on perdeuterated glycerol. Measuring temperature 37 °C. Spectrum was obtained by using the quadrupole echo technique.

behavior of the C(2′) segment has also been observed for the C(2′) segment of synthetic DPPG (Wohlgemuth et al., 1980) and reflects the fact that the quadrupole splittings are determined not only by the randomness of the motion but also by geometric changes (cf. Seelig, 1977).

A ^{2}H NMR spectrum of cell membranes derived from *E. coli* T131GP grown on perdeuterated glycerol is shown in Figure 6. Compared to the corresponding lipid extract (Figure 2B), the intrinsic line width is distinctly larger, eliminating most of the fine structure revealed by the lipid extract. Nevertheless, in addition to a strong central signal, two quadrupole splittings of 24.0 (intense) and 13.2 kHz (weak) can be resolved. ^{2}H NMR spectra of cell membranes obtained from bacteria grown on selectively deuterated glycerol have generally the same appearance as that shown in Figure 6, being also characterized by rather broad lines. Table II provides a summary of the measured quadrupole splittings. In no case was it possible to resolve the PG head-group signals.

Table II: Deuterium Quadrupole Splittings (kHz) of *E. coli* Cell Membranes at 37 °C

substrate supplied	glycerol backbone segments		
	1	2	3
sn-[2-^{2}H]glycerol		24.6	
sn-[3,3-$^2H_2$]glycerol			24.2
perdeuterated glycerol	13.2[a]	24.0	24.0

[a] In addition, a strong central signal is observed.

Discussion

The stereospecific deuteration of the phospholipid glycerol backbone by chemical means is tedious. Chemical deuteration of the glycerol C(3) segment has been achieved for 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) (Gally et al., 1975) and for 1,2-dimyristoyl- and 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (Browning & Seelig, 1980). The present in vivo labeling method is faster and simpler than chemical synthesis. Moreover, it allows for the first time the specific labeling and spectral assignments of all three glycerol backbone segments.

Inspection of Table I reveals that each segment has its own specific ^{2}H NMR spectrum, characterized by (i) the number of splittings and (ii) the size of the quadrupole splittings. For example, the C(3) segment always exhibits two closely spaced splittings of about 25 and 27 kHz, not only for PE and PG purified from *E. coli* lipids but also for synthetic DPPC and PS (Gally et al., 1975; Browning & Seelig, 1980). A corresponding similarity is found for the C(1) segment of PE and PG. Again, one observes two quadrupole splittings, this time, however, with distinctly different separations (17 and 0 kHz). For both segments, stereospecific monodeuteration eliminates one quadrupole splittings and unambiguously proves that the two splittings arise from the motional inequivalence of the individual deuterons. A similar result has recently been obtained for the C(3′) head group segment of 3,3′-DPPG (Wohlgemuth et al., 1980). The C(2) segment of the glycerol backbone carries only one deuteron and consequently produces just one quadrupole splittings, which agrees in size with the smaller splittings of the C(3) group. Again, one observes practically no difference between PE and PG. Though purely empirical in nature, this comparison of deuterium quadrupole splittings nevertheless allows the conclusion that the average conformation of the glycerol backbone is relatively constant and does not change appreciably from phospholipid to phospholipid. Finally, the close spectral similarity of the glycerol head-group segments of *E. coli* PG and synthetic DPPG is also obvious, reinforcing and extending the above conclusion to the phospholipid head-group region.

High-resolution NMR data indicate a limited flexibility of the glycerol backbone (Hauser et al., 1980). On the basis of

this intrinsic flexibility, the following model can be proposed for the quantitative interpretation of the ^2H NMR glycerol backbone data. From the single-crystal structural analysis of 1,2-dilauroyl-*rac*-glycerophosphoethanolamine (Hitchcock et al., 1974) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (Pearson & Pascher, 1979), it is known that the glycerol C(2)–C(3) bond vector is parallel to the bilayer normal. Also, there are no preferred conformations about the C(2)–C(3) bond (cf. Pearson & Pascher, 1979). If we assume that the same average orientation holds true for the liquid-crystalline membrane, it follows that the C–D bond vectors of the C(3) and the C(2) segment make the same average angle with the bilayer normal. Fast rotation around the C(2)-C(3) axis would lead to an axially symmetric pattern with identical quadrupole splittings for all three deuterons involved. If the small difference between the C(3) deuterons is neglected, this is indeed borne out by the experiment. A molecular order parameter of $S \approx 0.65$ can be calculated for the fluctuations of the C(2)–C(3) axis around the bilayer normal (Seelig & Gally, 1976). The single-crystal data further show that the glycerol C(1)–C(2) bond may occur in either the trans (*t*) or the gauche (*g*) conformation. In the trans state, both deuterons make the same angle (109.47°) with the C(2)–C(3) rotation axis; in the gauche state, the two deuterons are inclined at different angles to this axis (0° and 109.47°). A jump between a trans and a gauche state combined with a fast rotation around the C-(2)–C(3) axis would therefore produce two distinctly different quadrupole splittings, as required by the experimental observation. The exact nature of the envisaged two-site jump process is not important in the present context; for example, the change in the torsion angle must not be precisely 120°, but could assume some intermediate value. However, it should be emphasized that the jump must be *asymmetric*. A jump between symmetric, mirror-like conformations as it probably occurs in the ethanolamine and choline head groups (Seelig & Gally, 1976; Seelig et al., 1977) would always lead to identical splittings for a given methylene segment.

From the ^2H NMR spectra of *E. coli* membranes, only the quadrupole splittings of the glycerol backbone segments can be derived. The quadrupole splittings of the membrane (Table II) are almost identical with those of the corresponding pure lipid vesicle. The glycerol backbone conformation is therefore not altered to any appreciable extent by the presence of a high amount of protein in the membrane. A similar conclusion has been reached earlier for the fatty acyl chain region of *E. coli* membranes (Gally et al., 1979). Taken together, both sets of data are supporting the view that glycerophospholipids, independent of their specific chemical structure, are characterized by a unique and ubiquitous average molecular conformation of the glycerol backbone and the fatty acyl chains. The molecular differences are expressed only at the level of the polar groups [cf. Seelig & Seelig (1980)].

The main difference between the ^2H NMR spectra of *E. coli* cell membranes and derived liposomes are the relative broad lines of the intact membranes. This effect has already been noted earlier for *E. coli* fatty acid auxotrophs (Gally et al., 1980) and has simultaneously been observed in lipid–protein recombinants (Seelig & Seelig, 1978; Rice et al., 1979; Seelig et al., 1981). Some of the line broadening must probably be attributed to slow motional effects (cf. Rice et al., 1979). Another possibility in the present case would be a specific binding of the glycerol head groups to some membrane proteins which could also smooth out the fine structure of the ^2H NMR spectra.

The *E. coli* glycerol auxotroph employed in this study is a convenient source of deuterated and nondeuterated PG and PE with natural fatty acid composition. Future applications which can be envisaged are (i) ion binding studies to the phosphoglycerol head group by using a combined approach of NMR spectroscopy and differential scanning calorimetry, (ii) reconstitution of membrane proteins in a lipid matrix containing deuterated PG in order to shed light on possible PG–protein interactions, (iii) preparation of deuterated cardiolipin, and (iv) the investigation of phospholipid–cholesterol interactions at the level of the glycerol backbone. Such studies eventually might lead to a better understanding of the functional differences between the various lipid species and thus provide an explanation for the diversity of lipids in biological membranes.

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Active-Site Topography of Human Coagulant (α) and Noncoagulant (γ) Thrombins†

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ABSTRACT: Human α- and γ-thrombins (clotting vs. nonclotting activities, respectively) were conjugated at their active-site serine with a series of 13 phenylsulfonyl fluoride spin-labeling reagents. One class (*indole-site reagents*), predominantly para-substituted phenylsulfonyl fluorides with an overall linear conformation, was sensitive to indole binding at an apolar site common to both α- and γ-thrombins. A second class (α–γ *difference reagents*), all meta substituted, which had an overall bent conformation, exhibited more immobilization when conjugated to γ- vs. α-thrombin, attributable to either a steric obstruction or increased hydrophobicity where the nitroxide moiety binds. A third class exhibited relatively *mobile* spin-label spectra, indicative of little or no interaction with either the indole site or α–γ difference site mentioned above. When spin-labeled bovine trypsin and α-thrombin were compared, several similarities were found with most of the meta-substituted reagents, but not with para-substituted reagents. The most notable difference, however, between the thrombins and trypsin was the strong immobilization of all spin-labeled α- or γ-thrombins upon exposure to ligands which bind to the basic specificity pocket, while the ESR spectra of the corresponding spin-labeled trypsin derivatives were totally unaffected by ligand binding to its structurally homologous binding pocket. These differences were attributed to both critical substitutions and/or deletions in the sequence lining the thrombin specificity pocket. From analysis of CPK models, a topographical map was derived describing the relationship of the indole and α–γ difference sites to other loci in the catalytic center. These two regions were approximated by oblate ellipsoids of revolution (minor axis 4 Å, major axis 5 Å) which extended out from the phenylsulfonyl ring. The indole site ellipsoid was attached to the para position of the phenyl ring by its minor axis; the α–γ difference site ellipsoid was attached to the meta position of the phenyl ring, also by its minor axis (i.e., at an orientation relative to the indole site by a 60° rotation in the plane of the phenyl ring). This "double ellipsoid" model overlapped in a region approximately 2.8 Å wide. These data provide direct physical evidence for tertiary structural differences in certain active-site regions of γ-thrombin, which may be related to its loss of clotting and other biologically important activities.

Coagulant α-thrombin (EC 3.4.21.5) is a serine protease generated in the final preclotting events of blood coagulation and has several bioregulatory functions in hemostasis [see Fenton et al. (1979)]. Although the human and bovine enzymes have been crystallized (McKay et al., 1977; Tsernoglou & Petsko, 1977), the three-dimensional structure has thus far been unobtainable, and thrombin structural features have been inferred only from amino acid sequence homologies between its larger B chain (Figure 1) and the pancreatic serine proteases (Magnusson et al., 1975; Elion et al., 1977). In the human system, autolytic or limited tryptic fragmentation of the α-thrombin B chain gives rise to two and subsequently three B-chain fragments of β- and γ-thrombins, respectively (Figure 1). These enzyme forms lack fibrinogen clotting activity and certain other biological functions but retain similar estero- and amidolytic and most other proteolytic activities of the parent form. Because the functional residues of the catalytic triad (His-43, Asp-99, and Ser-205) are contributed by the noncovalently bound B-chain fragments in γ-thrombin, the fragments remain in noncovalent association in the functional enzyme form (Fenton et al., 1979). Comparisons between α- and γ-thrombins have shown that the noncovalent form (i.e., γ-thrombin) more readily denatures (Bauer et al., 1980; Chang et al., 1980), yet displays remarkably similar intrinsic fluorescence and other properties as dansyl-, anthraniloyl-, or spin-label-active serine conjugates (Berliner & Shen, 1977a–c).

In this work, the complete fluorosulfonylphenyl spin-label series of Berliner & Wong (1974) has been employed to examine active-site topography and differences between human α- and γ-thrombins. The method involves the isomorphous incorporation of several topographically sensitive spin-labels in both α- and γ-thrombins and subsequent comparisons of nitroxide mobility both between the two thrombin forms and each structural class of spin-label. The nitroxide tumbling

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