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Structure of *Escherichia coli* Membranes. Phospholipid Conformation in Model Membranes and Cells As Studied by Deuterium Magnetic Resonance†

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ABSTRACT: The dynamic conformation of phospholipid molecules in membranes of *Escherichia coli* has been investigated by means of deuterium magnetic resonance. *E. coli* strains which were deficient in the synthesis of cardiolipin were grown in the presence of selectively deuterated elaidic acid, oleic acid, and palmitic acid. A total of 50–85% of the natural fatty acids were replaced by the deuterium-labeled analogues, and well-resolved deuterium magnetic resonance spectra of intact *E. coli* cells could be obtained in less than 1-h measuring time. The spectra reveal a striking similarity between the phospholipid conformation in a biological membrane and that of phospholipid model membranes. If the deuterium label is attached at the C-2 segment of the fatty acyl chains or at the cis double bond, the deuterium magnetic resonance spectra are rather unique and can be considered as spectral fingerprints of the phospholipid conformation in the fluid membrane. Almost identical fingerprints are observed for native *E. coli* membranes, for liposomes formed from extracted *E. coli* lipids, and for synthetic phospholipids. The phospholipid conformation in the fluid membrane as derived from these spectral patterns is closely related to the structural model suggested for phospholipids in the gel phase and in the crystalline state. The orientational fluctuations of the fatty acyl chain segments in the membrane are quantitatively described by the deuterium order parameters. A detailed order profile has been established for *E. coli* membranes by incorporating palmitic acid and oleic acid selectively deuterated at altogether 10 different carbon atoms. The shape of the curve drawn through deuterium order parameters of intact *E. coli* cells closely resembles that of synthetic 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine. It can be concluded that the order profiles characteristic of saturated and cis unsaturated fatty acyl chains are qualitatively not altered by the presence of membrane proteins. Due to instrumental limitations, lipids in the gel state or lipids tightly bound to membranous proteins cannot be resolved in the present experiments.

Studies in the past 10 years have lead to the consensus view that a fluid bilayer of phospholipids forms the basic two-dimensional matrix of most biological membranes into which and around which various proteins are situated. A more detailed molecular description of phospholipid arrangement in biological membranes requires knowledge about the conformation of both the polar and apolar parts of phospholipids in the bulk lipid phase and at the sites of interaction of lipids with intrinsic and peripheral proteins. The purpose of this study is to characterize the hydrocarbon chain conformation of phospholipids in a biological membrane and to establish similarities and differences between the native membrane and protein-free phospholipid bilayers. As a representative membrane system we have chosen *Escherichia coli* in which the hydrocarbon chain composition of the phospholipids can be manipulated by addition of fatty acids to the growth medium

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Table I: Fatty Acid Composition of Membrane Phospholipids in *E. coli* Cells

		fatty acids[a] (mol %)				
strain	supplement	16:0	14:0 plus 12:0	cis unsaturated[b] cyclopropane	*trans*-Δ^9-18:1	*cis*-Δ^9-18:1
T2GP	oleate	25	10			65
T2GP	elaidate	6	9		85	
T106GP		29	19	52		
T106GP	palmitate	71[c]	7	22		

[a] The number before the colon refers to chain length; the number after the colon refers to the number of double bonds. [b] Sum of *cis*-Δ^9-16:1, *cis*-Δ^{11}-18:1, and their cyclopropane derivatives. [c] Since the addition of saturated fatty acids to the medium reduces the in vivo rate of saturated fatty synthesis (Silbert et al., 1973), >50% of the total fatty acids are medium-derived 16:0.

[see Overath & Thilo (1978) for a review]. The method of choice for the analysis of the hydrocarbon chain conformation is deuterium magnetic resonance (^{2}H NMR)[1] which has lead to a quantitative and detailed picture for the organization of phospholipids in pure lipid bilayers (Seelig, 1977; Seelig & Browning, 1978; Büldt et al., 1978). While this study was in progress several ^{2}H NMR studies on lipid organization in membranes of *Acholeplasma laidlawii* and *E. coli* have been published (Stockton et al., 1977; Gent & Ho, 1978; Davis et al., 1979; Kang et al., 1979; Smith et al., 1979).

Materials and Methods

Incorporation of Selectively Deuterated Fatty Acids into E. coli Phospholipids. The strains of *E. coli* used in the present study are derivatives of the well-characterized fatty acid requiring strain K1062 (Overath et al., 1971; Overath & Träuble, 1973). Strain T2GP is defective in cardiolipin synthesis [*cls*; cf. Pluschke et al. (1978)], unsaturated fatty acid synthesis (*fab*B), and fatty acid degradation (*fad*E). Cells of this strain were grown in the presence of either 0.1% oleate at 37 °C or 0.1% elaidate at 40 °C as previously described (Overath & Träuble, 1973). For incorporation of deuterated palmitate, strain T106GP (*cls*, *fad*E, obtained from T2GP by transduction) which has regained the ability to synthesize unsaturated fatty acids was used. Cells of this strain were first grown as described above without fatty acid supplement at 37 °C and subsequently grown for two generations in the presence of 0.1% palmitate and 0.5% Brij 35. Cells were harvested by centrifugation and washed twice with 100 mM NaCl, 10 mM sodium phosphate, pH 7.0 (or 10 mM 1,4-piperazinediethanesulfonic acid, pH 7.0), and 0.5% Brij 35 to remove adsorbed fatty acids. The detergent was then removed by washing the cells twice with the same buffer without Brij 35. Cells were stored at −30 °C and thawed immediately before the NMR measurements.

A total phospholipid fraction was prepared from the cells by $CHCl_3/CH_3OH$ extraction (Ames, 1968) and silicic acid chromatography. The phospholipids were then dispersed at 50 °C in the buffers indicated in the figure legends and centrifuged. After three washings the pellet was transferred to a 10-mm NMR sample tube.

Deuterated fatty acids were prepared as described previously (Seelig & Seelig, 1974; Seelig & Waespe-Šarčevič, 1978).

Deuterium NMR Measurements. In order to obtain sufficient sensitivity, we had to make the deuterium NMR measurements at 61.4 MHz using a superconducting magnet. The pulse width for a 90° pulse was 16 μs, the spectral width was 50 kHz, and the recycle time was 0.15 s. The measuring time for most cell membranes was between 30 and 60 min/spectrum. Due to insufficient radiofrequency power of the NMR spectrometer used, the spectral shapes are slightly distorted and do not correspond to the theoretical line shape of a spin-1 powder pattern. The quadrupole splitting is, however, not affected by this distortion. Lipids in the gel state give rise to rather broad lines and are not detected in the present experiments.

For a quantitative comparison of deuterium NMR spectra of liquid-crystalline bilayers, we use the deuterium order parameter, S_{CD}, which is related to the experimental quadrupole splitting, $\Delta\nu_Q$, according to

$$\Delta\nu_Q = (3/4)(e^2qQ/h)S_{CD} \quad (1)$$

e^2qQ/h is the static deuterium quadrupole coupling constant which is about 170 kHz for aliphatic deuterons (Burnett & Muller, 1971) and 175.3 kHz for olefinic deuterons (Kowalewski et al., 1976). Only the absolute value of the deuterium order parameter can be determined since the sign of the quadrupole splitting is generally unknown. The order parameter is related to the angular fluctuations of the C–D bond vector around the bilayer axis, i.e., the normal to the bilayer surface. If θ denotes the instantaneous angle between the C–D bond vector and the bilayer normal, then S_{CD} is given by

$$S_{CD} = (1/2)(3\,\overline{\cos^2\theta} - 1) \quad (2)$$

where the bar indicates a time average over all motions which are fast compared to the static quadrupole splitting constant. The molecular interpretation of S_{CD} can be complicated at times since it is a tensorial average which not only depends on the randomness of motion but also on geometrical factors, i.e., the angles between the C–D bond vector and the principal axes of the molecular diffusion tensor [cf. Seelig (1977)]. The geometrical factors are of particular importance for the motions of the cis and trans double bond as has been discussed in more detail elsewhere (Seelig & Waespe-Šarčevič, 1978).

Results and Discussion

Incorporation of Selectively Deuterated Fatty Acids into Phospholipids. The *E. coli* mutants used in this study are defective in cardiolipin synthesis and therefore contain essentially only two phospholipid components, phosphatidylethanolamine (80%) and phosphatidylglycerol [20%; cf. Pluschke et al. (1978)]. Deuterated fatty acids added to the growth medium of strains T2GP and T106GP are extensively incorporated into the phospholipids as shown in Table I. Previous studies using related strains have shown that medium-derived elaidate (*trans*-Δ^9-octadecenoate) will be distributed almost equally between the *sn*-1 and *sn*-2 position of the glycerol moiety. Medium-derived oleate (*cis*-Δ^9-octadece-

[1] Abbreviations used: NMR, nuclear magnetic resonance; Pipes, 1,4-piperazinediethanesulfonic acid; Brij 35, poly(oxyethylene) dodecyl ether; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine.

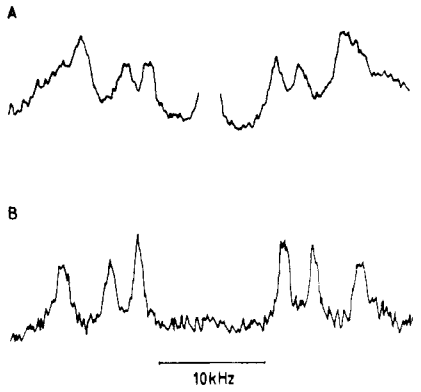

FIGURE 1: Deuterium NMR spectra (61.4 MHz) of [2,2-$^2H_2$]elaidate-enriched strain T2GP cells and derived liposomes. (A) Intact cells suspended in 0.1 M NaCl, 0.01 M $MgCl_2$, and 0.01 M Pipes, pH 7.0; temperature, 36 °C; 20000 free induction decays (~1-h measuring time). The central line arises from the natural abundance of deuterium in water. (B) Liposomes derived from elaidate-enriched cells in 0.1 M NaCl, 0.01 M $NaPO_4$, pH 7.0, and 0.01 M EDTA. Measuring temperature, 41 °C; 15000 free induction decays.

noate) is preferentially incorporated into the *sn*-2 position while palmitate (hexadecanoate) is mainly located at the *sn*-1 position (Silbert, 1970; Silbert et al., 1973; Linden et al., 1973; Nishihara et al., 1975).

Both the outer and inner (cytoplasmic) membranes of *E. coli* contain fluid lipid domains of similar composition which can undergo a fluid ↔ ordered phase transition upon a decrease in temperature. Under the conditions of the NMR experiments described below, essentially all lipid molecules which can take part in the thermal transition are in the fluid state. It should be noted that for the *cls*⁻ strains used here, the absence of cardiolipin and the compensatory increase in phosphatidylglycerol shift the transition by about 6 °C to lower temperatures (G. Pluschke, unpublished experiments) when compared to previous studies with cardiolipid-containing membranes (Overath & Thilo, 1978). As the phospholipid content in the cytoplasmic membrane is about 2.5 times higher than in the outer membrane (Overath et al., 1975), the cytoplasmic membrane is expected to contribute at least 2/3 to the deuterium NMR signal obtained from intact cells. While the lateral arrangement of the phospholipids in the cytoplasmic and outer membranes appears to be similar because the ordered ↔ fluid transition occurs over a similar temperature range, the cytoplasmic membrane most likely contains a lipid bilayer while the outer membrane is believed to exhibit extended phospholipid domains only on the inner half facing the cytoplasm (Smit et al., 1975).

Spectral "Fingerprints" of Phospholipid Conformation. *E. coli* strain T2GP grown in the presence of selectively deuterated elaidic acid ensures a high degree of deuterium labeling (cf. Table I) which facilitates the measurements of the deuterium magnetic resonance spectra. In a first experiment we have used [2,2-$^2H_2$]elaidic acid in order to probe the hydrocarbon chain conformation in the vicinity of the polar head groups. Figure 1 shows representative spectra of [2,2-$^2H_2$]-elaidate-enriched *E. coli* cells and derived liposomes. Figure 1 demonstrates that one obtains well-resolved deuterium NMR spectra of intact cells within a reasonable time (~1 h/spectrum). There is a remarkable similarity between the spectrum of cells (Figure 1A) where the membranes contain 60–70 wt % protein [for details of the membrane composition cf. Overath et al. (1975)] and that of the protein-free lipid bilayers formed from the extracted and purified phospholipids (Figure 1B). Therefore, it can be concluded that the hydrocarbon chain ordering near the glycerol backbone is very similar in both

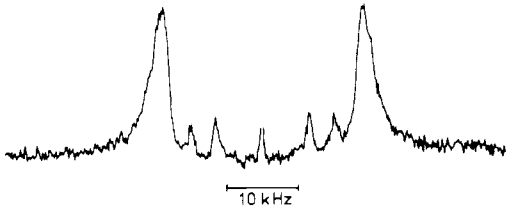

FIGURE 2: Deuterium NMR spectrum (61.4 MHz) of liposomes derived from strain T106GP cells grown in the presence of [2,2-$^2H_2$]palmitic acid; temperature, 41 °C. Buffer: 0.1 M NaCl, 0.01 M $NaPO_4$, and 0.01 M $MgCl_2$, pH 7.0.

systems. The results presented in Figure 1 are not unique to *E. coli* lipids. The same spectral pattern has been observed for bilayers of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine, both with saturated and with unsaturated fatty acyl chains [Seelig & Browning (1978) and references cited therein]. In all these systems the C-2 deuterated phospholipids give rise to three quadrupole splittings, which agree is size and intensity if compared under corresponding conditions.

Figure 1A thus provides unambiguous experimental evidence that this spectral "fingerprint" of the C-2 segments is also typical for phospholipids in intact biological membranes and reflects a general feature of phospholipid organization. On the basis of studies with phospholipids labeled in one chain only, the largest splitting in Figure 1 can be assigned to the deuterons of the *sn*-1 chain, while the two smaller splittings arise from the *sn*-2 chain (Seelig & Seelig, 1975, 1977; Seelig & Waespe-Šarčevič, 1978). The differences in the quadrupole splittings can be explained by a conformational model in which the beginnings of the two fatty acyl chains have different average orientations with respect to the bilayer surface (Seelig & Seelig, 1975; Schindler & Seelig, 1975). On the average, the *sn*-1 chain is extended perpendicular to the bilayer surface at all segments while the *sn*-2 chain begins parallel to the membrane surface and is bent perpendicular to it after the C-2 segment. This model is in close agreement with the only known single crystal X-ray structure of a phospholipid bilayer (Hitchcock et al., 1974) and is also supported by recent neutron diffraction studies on selectively deuterated phosphatidylcholine bilayers in the gel state as well as in the fluid state (Büldt et al., 1978; Zaccai et al., 1979). Taken together, the X-ray, neutron diffraction, and deuterium NMR experiments strongly suggest that very similar conformational constraints are imposed on phospholipid molecules in lipid bilayers independent of the physical state or chemical composition of the particular environment.

The deuterium NMR spectrum of *E. coli* strain T106GP grown in the presence of [2,2-$^2H_2$]palmitic acid exhibits just one pair of rather broad lines with a quadrupole splitting characteristic of an extended *sn*-1 chain (not shown). However, the corresponding spectrum of the extracted lipids (Figure 2) reveals a second spectral component of much lower intensity typical for the bent *sn*-2 chain. The differences in the spectra between elaidic acid and palmitic acid containing lipids can be explained by the different extent of incorporation of these fatty acids into *E. coli* membranes. As mentioned above, elaidic acid will replace about 85% of the natural fatty acids and is therefore distributed approximately equally between the *sn*-1 position and *sn*-2 position of the phospholipids. In contrast, palmitic acid is incorporated to a much lesser extent (~60%) and will be attached preferentially at the *sn*-1 position. As expected, the deuterium NMR spectrum of Figure 2 contains an intense component from [2,2-$^2H_2$]palmitic acid in the *sn*-1 position and only a minor component from the same

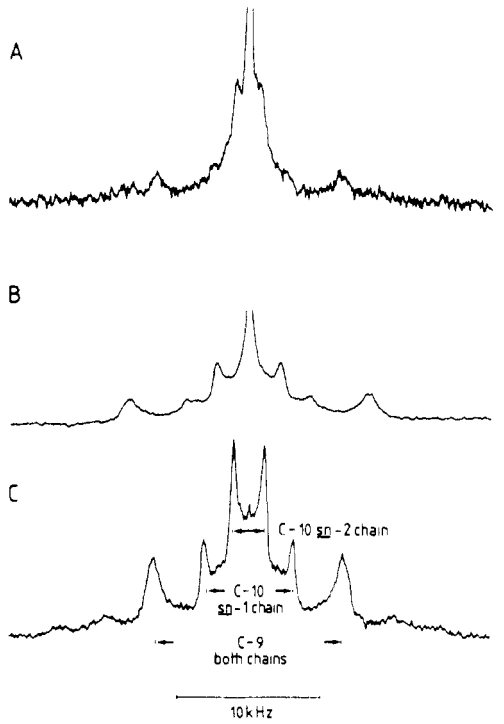

FIGURE 3: Deuterium NMR spectra (61.4 MHz) of [9,10-$^2H_2$]-oleate-enriched strain T2GP cells and related model systems. (A) Intact cells suspended in 0.1 M NaCl, 0.01 M Pipes, and 0.01 M $MgCl_2$, pH 7.0; 40 °C; 28 000 free induction decays (~80-min measuring time). The central peak is due to the natural abundance of deuterium in water. (B) Liposomes derived from *E. coli* T2GP strain grown on [9,10-$^2H_2$]oleic acid containing medium. Buffer composition was as described in (A). 20 °C; 3000 free induction decays (~10-min measuring time). (C) Liposomes prepared from synthetic 1,2-di[9,10-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine (DOPC). 50 wt % DOPC; 50 wt % H_2O. 35 °C; 1200 free induction decays (~7-min measuring time).

fatty acid in the *sn*-2 position. In cells the deuterium NMR signal of the *sn*-2 chain is lost in the noise, and only the signal of the *sn*-1 chain is observable.

In an analogous deuterium NMR study Stockton et al. (1977) incorporated [2,2-$^2H_2$]palmitic acid into membranes of *A. laidlawii*. The unusual shape of the deuterium line suggested to the authors "the presence of possibly three overlapping powder doublets". No resolution of the three doublets was achieved, which in the light of the present experiments seems to be due to the rather heterogeneous lipid composition of the *A. laidlawii* membrane and to the lower extent of enrichment with specifically deuterated palmitate.

The close resemblance of the phospholipid conformation in intact membranes and pure lipid bilayers is not limited to the C-2 position of the fatty acyl chains but can also be demonstrated for the hydrophobic membrane interior. The most striking example is provided by the deuterium NMR spectra of the cis double bond as illustrated in Figure 3. In these experiments strain T2G was grown in the presence of [9,10-$^2H_2$]oleic acid (cf. Table I). Figure 3 compares the deuterium NMR spectra of cells (Figure 3A) and derived liposomes (Figure 3B) with the spectrum of liposomes prepared from synthetic 1,2-di[9,10-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine (DOPC) (Figure 3C). The assignment of the quadrupole splittings to the various deuterons is indicated in Figure 3C and is based on results obtained for DOPC labeled in one chain only (A. Seelig and J. Seelig, unpublished results) and on the analysis of the deuterium NMR spectra of 1-palmitoyl-2-[9,10-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine (POPC) (Seelig & Waespe-Šarčevič, 1978). The C-9 deuterons of the *sn*-1

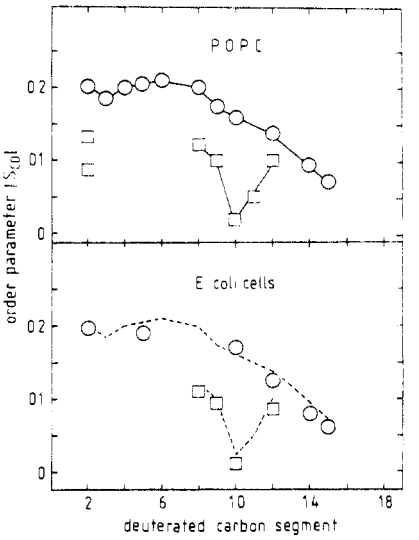

FIGURE 4: Variation of the deuterium order parameter, $|S_{CD}|$, with segment position. POPC: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; 50 wt % lipid; 50 wt % H_2O. Data taken from Seelig & Seelig (1977) and Seelig & Waespe-Šarčevič (1978); measuring temperature, 27 °C. *E. coli* cells: strains T106GP and T2GP grown on media supplemented with selectively deuterated palmitic or oleic acid, respectively. Cells were suspended in 0.1 M NaCl, 0.01 M $MgCl_2$, and 0.01 M Pipes, pH 7.0. Temperature, 40 °C. (○) Deuterium label attached at palmitic acyl chain. (□) Deuterium label attached at oleic acyl chain.

and *sn*-2 chain cannot be resolved at ambient temperatures but appear as individual signals at lower temperatures ($T <$ 0 °C). It is obvious from inspection of Figure 3 that the average orientation of the cis double bond is rather similar in all three systems and is not altered dramatically by the presence of membrane proteins.

The observation of different quadrupole splittings for the C-9 and C-10 deuterons of both double bonds rules out the possibility that the C=C bond vector is *exactly* parallel to the bilayer normal, since this orientation would produce identical splittings for all deuterons. The quantitative analysis of the corresponding problem in POPC bilayers leads to the conclusion that a relatively small average tilt angle of 7–8° between the bilayer normal and the C=C bond vector is sufficient to account for the observed differences between the C-9 and C-10 deuterons (Seelig & Waespe-Šarčevič, 1978). From the same analysis it further follows that even small variations of the tilt angle would produce dramatic changes in the quadrupole splittings. The close similarity of the deuterium NMR spectra shown in Figure 3 thus demonstrates that even minor details in the dynamic chain structure such as the average orientation of the cis double bond are not distinctly modified by the presence of membrane-bound proteins.

Order Profile of Hydrocarbon Chains. Measuring the order parameter $|S_{CD}|$ as a function of the segment position leads to a bilayer profile for the hydrocarbon chain ordering. Such profiles have been reported for a variety of pure phospholipid model systems and have been summarized recently (Seelig & Browning, 1978). In addition, the order profile of *A. laidlawii* enriched to 70% with deuterated palmitic acid has been determined (Stockton et al., 1977). Figure 4 summarizes the results obtained for *E. coli* cells grown in the presence of selectively deuterated palmitic (strain T106GP) and oleic acids (strain T2GP). To a first approximation, the fatty acid composition of the *E. coli* cells can be described as an equimolar mixture of saturated and unsaturated fatty acids. The closest model systems available to date are bilayers composed of synthetic POPC and, therefore, the order profile of this

membrane is also included in Figure 4 (Seelig & Seelig, 1977; Seelig & Waespe-Šarčevič, 1978). Again one notices a striking similarity between the shapes of the order profiles of pure POPC and *E. coli* cells, despite the fact that these cells are surrounded by two different membranes, have a heterogeneous fatty acid composition, contain more than 50 wt % protein in the membranes, and have phosphoethanolamine and phosphoglycerol instead of phosphocholine as polar groups. For palmitate-enriched *E. coli* cells, the order profile resembles that of the *sn*-1 palmitic acyl chain of POPC; for the oleate-enriched cells, it agrees with that of the *sn*-2 oleic acyl chain. The molecular interpretation of the order profiles can therefore proceed in close analogy with that given for POPC bilayers. The *sn*-1 palmitic acyl chain in *E. coli* cells exhibits the same variation of order parameter as all the other saturated phospholipid membranes investigated so far. After a plateau region comprising the first 8–10 chain segments, the order parameter decreases to almost zero at the terminal methyl group. In contrast, the *sn*-2 oleic acyl chain is characterized by a conspicuous decrease of the $|S_{CD}|$ parameter at the cis double bond. The reason for this discontinuity is not a particularly random movement of the cis double bond but is related to the specific geometry of this segment and to its alignment in the bilayer as discussed above. Indeed, after correction for these geometric factors, the statistical order of the *sn*-2 oleic acyl chain of POPC is found to be the same as that of the adjacent *sn*-1 palmitic acyl chain (Seelig & Waespe-Šarčevič, 1978). It is safe to assume that a similar conclusion holds true for the cis unsaturated phospholipid chain in *E. coli* membranes; i.e., the statistical fluctuations are independent of the chemical nature of the fatty acyl chains but are determined mainly by the distance of the deuterated segment from the polar interface. It should be noted that the agreement of the absolute values for the order profile for POPC and *E. coli* cells shown in Figure 4 is fortuitous.

In a related deuterium NMR study Davis et al. (1979) have grown *E. coli* in media supplemented with perdeuterated palmitic acid. Under these conditions most of the deuterium resonances cannot be resolved. Nevertheless, the moments of the spectra may be compared to those of [$^2H_{31}$]dipalmitoyl-*sn*-glycero-3-phosphocholine model membranes. The qualitative and quantitative agreement between the two types of systems is again found to be rather close in the liquid-crystalline state.

Conclusion

Deuterium NMR is an extremely sensitive method to detect conformational changes in membranes. For example, the well-established stiffening and condensing effect of cholesterol is reflected quite clearly in the deuterium NMR spectrum by an increase of the residual quadrupole splitting by as much as 25 kHz (Gally et al., 1976; Stockton & Smith, 1976; Haberkorn et al., 1977; Oldfield et al., 1978). Analogously, the binding of di- and trivalent ions to the polar head groups of phosphatidylcholine and phosphatidylethanolamine induces ion-specific conformational changes which are accompanied by variations of the quadrupole splitting of the order of 5–10 kHz (Brown & Seelig, 1977; H. Akutsu and J. Seelig, unpublished results).

The close similarity between the deuterium NMR spectra of *E. coli* membranes and pure phospholipid bilayers must therefore be considered as strong evidence for the constancy of the phospholipid conformation in these systems. In particular, in both systems the *sn*-1 chain is found to be straight and the *sn*-2 chain is bent, the cis double bond is tilted by a few degrees with respect to the bilayer normal, and the order profiles of corresponding chains are almost superimposable. This confirms our previous conclusion derived from a comparison of various model systems that phospholipids in membranes are subjected to rather similar conformational constraints and that the spectrum of conformations available to the various types of phospholipids is almost identical (Seelig & Browning, 1978). The principal features of this phospholipid structure are the same in single crystals, in the gel state, and in the liquid-crystalline state and, as shown in the present study, are not modified appreciably by the presence of membrane-bound proteins. As far as the influence of proteins is concerned, this conclusion must, however, be qualified in two respects. First, due to instrumental limitations only phospholipids in the liquid-crystalline state are detected in the present experiments. No conclusions can be reached regarding the interaction between membranous proteins and gel-state lipids. We also must leave open the possibility that signals from a number of tightly bound, slowly exchanging lipids were not resolved. ^2H NMR studies on *E. coli* (strain L 51) grown on specifically deuterated palmitic acid suggest that at 37 °C the amount of immobilized lipids is less than 3% of the total phospholipid (C. P. Nichol, J. H. Davis, G. Weeks, and M. Bloom, unpublished results), but the situation may be different for the system described in this study. Second, the present discussion has emphasized the qualitative similarities between phospholipid model membranes and intact *E. coli* cell membranes. However, a closer look will reveal small, but nevertheless significant quantitative differences between pure phospholipid bilayers and protein-containing membranes. This refinement of the present picture will be provided in a subsequent paper.

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Entrapment of Proteins in Phosphatidylcholine Vesicles†

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ABSTRACT: The trapping efficiency of globular proteins in four different types of phosphatidylcholine vesicles was systematically studied. Vesicles were generated in a mixture of ^{125}I-labeled proteins of various molecular weights. The trapped proteins were separated from untrapped proteins by gel filtration and ultrafiltration and subsequently analyzed by gel electrophoresis and autoradiography. Entrapment of proteins was demonstrated by their resistance to trypsin digestion. The relative amount of each entrapped protein species was then compared to that of the original protein solution. In multilamellar vesicles and large unilamellar vesicles, proteins of molecular weight up to 97 000 had the same trapping efficiency as sucrose. In small unilamellar vesicles generated by either sonication or ethanol injection, however, the relative trapping efficiency of protein decreased progressively as the molecular weight of the protein became greater. For example, the trapping efficiency of α-amylase (M_r 97 000) was only half of that for sucrose. The apparent decrease in trapping efficiency with the protein's molecular weight in small unilamellar vesicles can be accounted for by the combination of the bound water layer at the vesicle's internal surface and the steric hindrance when protein is captured during vesicle formation.

In recent years, phospholipid vesicles (liposomes) of various sizes and composition have been used as carriers, both in vitro and in vivo, to introduce biologically active substances into cells [for a recent review, see Pagano & Weinstein (1978)]. In particular, phospholipid vesicles offer an attractive method for the enzyme replacement therapy (Cohen et al., 1976; Roerdink et al., 1976; Tyrrell et al., 1976; Belchetz et al., 1977). In these experiments, enzymes are trapped within the internal aqueous space of the vesicle which is subsequently exposed to cells. It is therefore of crucial importance that the amount of enzyme trapped in the vesicle be quantitative and well controlled. However, it has been the experience of the investigators that trapping of proteins in lipid vesicles, particularly small unilamellar vesicles (SUV),[1] is difficult and sometimes impossible. It is the purpose of this communication to report a systematic study on the trapping of proteins of various molecular weights in four different types of phospholipid vesicles. The results clearly showed a deviation from the ideal trapping of large proteins in SUV but not in larger vesicles.

Materials and Methods

Materials. Total lipids were extracted from hen yolks with $CHCl_3$–MeOH (2:1 v/v) by the method of Folch et al. (1957). Phosphatidylcholine was purified by silicic acid column chromatography (Litman, 1973). [^{3}H]Dipalmitoyl-PC was synthesized by a catalytic hydrogenation with tritium gas on purified dipalmitoleoyl-PC at the New England Nuclear, Inc., and subsequently purified by silicic acid chromatography. All lipids were stored in sealed ampules under N_2 at −70 °C and periodically examined for purity by TLC. Lysozyme (Worthington), chymotrypsinogen and peroxidase (Miles), conalbumin (Sigma), and α-amylase (Calbiochem) were purchased from commercial sources and used without further purification. Proteins were iodinated with Na^{125}I (New England Nuclear) by using chloramine-T (Hunter & Green-

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[1] Abbreviations used: SUV-son, small unilamellar vesicles prepared by sonication; SUV-EtOH, small unilamellar vesicles prepared by the EtOH injection method; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; PC, phosphatidylcholine; $NaDodSO_4$, sodium dodecyl sulfate.

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