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Lateral Diffusion, Protein Mobility, and Phase Transitions in *Escherichia coli* Membranes. A Spin Label Study†

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ABSTRACT: The coefficient of lateral diffusion in *Escherichia coli* membranes is determined as $D_{diff} = 3.25 \times 10^{-8}$ cm²/sec at 40° using a spin label technique developed previously. This value compares well with the rate of lateral diffusion in dipalmitoyllecithin (DPL) membranes at 50°. The rate of rotational and translational diffusion of membrane proteins may be estimated from the hopping frequency ($\nu \approx 10^7$ Hz) of the lipid molecules. For a protein with a radius $R = 25$ Å we obtain a rotational relaxation time τ_r of about $\tau_r = 75$ μsec and a coefficient of lateral diffusion $D_{diff}^{P} \sim 3 \times 10^{-10}$ cm²/sec. The electron spin resonance (esr) spectra of spin labels incorporated in DPL model membranes undergo characteristic changes in the temperature range of the lipid phase transition. The transition temperature, T_t, determined from the changes in spectral intensity and the order parameter S depends on the distance between the paramagnetic center and the membrane surface. If the NO group is deeply buried within the hydrocarbon phase the obtained values of T_t agree well with dilatometric and spectroscopic measurements (90° light scattering, 8-anilino-1-naphthalenesulfonate (ANS) fluorescence). Lower values of T_t are, however, observed if the NO group is near the semipolar region of the membrane. The same spectral changes have been observed in intact membranes of an *E. coli* fatty acid auxotroph (containing predominantly *trans*-Δ⁹-octadecenoic acid, *cis*-Δ⁹-octadecenoic acid, and *trans*-Δ⁹-hexadecenoic acid), indicating a lipid phase transition in these membranes. The transition temperatures T_t obtained with stearic acid labels carrying the NO group near the methyl end of the chain agree well with the previously reported breaks in the temperature dependence of some transport systems of the respective *E. coli* mutant (P. Overath *et al.*, *Nature (London), New Biol., 234*, 264 (1971)). This shows that these breaks are caused by phase transitions of the membrane lipids. The occurrence of a lipid phase transition in *E. coli* membranes and the approximate equality of the coefficient of lateral diffusion in these membranes with the value of D_{diff} in DPL model membranes strongly support the presence of (continuous) lipid layers in the *E. coli* membranes. "Polar" spin labels (Tempo, digitoxigenin) and stearic acid labels with the NO groups near the carboxyl end indicate a "pretransition" some 6–8° below the main transition. This shows that the result of spin label studies with membranes may depend critically on the position of the paramagnetic center within the membrane.

In recent years spin label probes have been used extensively to study the structure and dynamics of lipids in biological membranes (see reviews by Jost *et al.*, 1971 and Mehlhorn and Keith, 1972). Several groups have applied the spin label technique to the crystalline–liquid crystalline phase transition in dispersions of synthetic phospholipids (Barratt *et al.*, 1969; Hubbell and McConnell, 1971; Jost *et al.*, 1971). Discontinuities in the temperature dependence of the rotational correlation time τ (Barratt *et al.*, 1969), the line width ΔH (Sackmann and Träuble, 1972), and the so-called order parameter S (Hubbell and McConnell, 1971) have been taken as evidence for the occurrence of phase transitions. Similar discontinuities in the electron spin resonance (esr) spectra of spin labeled membranes of *Mycoplasma laidlawii* (Tourtelotte *et al.*, 1970), of plant and rat liver mitochondria (Raison *et al.*, 1971), and yeast cells (Eletr and Keith, 1972) suggest that similar phase transitions occur in biological membranes. This interpretation is supported by calorimetric studies (Steim *et al.*, 1969) showing peaks in the specific heat at about the same temperatures. Historically, calorimetry was the first method used to demonstrate lipid phase transitions in model membranes (Chapman *et al.*, 1967) and biological membranes (Steim *et al.*, 1969).

A large body of evidence is now available demonstrating that the crystalline–liquid crystalline phase transition involves a transition from an ordered to a fluid lipid structure. The importance of the membrane fluidity is now gaining full recognition, for example, in the processes of membrane assembly (*cf.* Rothfield and Romeo, 1971; Sumper and Träuble, 1973), membrane fusion (Frye and Edidin, 1970), rotational and translational motion of membrane components in immune response (Taylor *et al.*, 1971), and sensory transduction (Kaissling and Priesner, 1970; Adam and Delbrück, 1968; Brown, 1972; Cone, 1972).

For some purposes it may suffice to characterize the membrane fluidity by the macroscopic viscosity (η). A more direct approach to the problem of membrane fluidity is the study of the molecular motions within the lipid hydrocarbon chains and the translational motion of the lipid molecules. An attempt is made in the present paper to correlate the translational and rotational motion of membrane macromolecules with the mobility of the lipid molecules.

The rate of lateral diffusion in lipid model membranes was measured recently by an analysis of the effect of spin label

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interactions on the line shape of esr spectra (*Static Method;* Träuble and Sackmann, 1972). Devaux and McConnell (1972) studied the spread of an initially concentrated spot of radicals within stacked lipid lamellae (*Kinetic Method*). Both approaches yield the same order of magnitude for the coefficient, D_{diff}, of lateral diffusion within the plane of membranes ($D_{diff} \approx 3 \times 10^{-6}$ cm²/sec). Preliminary measurements indicated the same speed of lateral diffusion in *Escherichia coli* membranes (Träuble and Sackmann, 1972). A value of $D_{diff} \approx 7.5 \times 10^{-8}$ cm²/sec (40°) was measured (*Static Method*) by Scandella *et al.* (1972) for sarcoplasmic reticulum membranes. The main purpose of the present paper is (1) a systematic study of the rate of lateral diffusion in *E. coli* membranes and (2) a comparison of the spin label method with other techniques for the study of lipid phase transitions. Stearic acid spin labels (*N*-oxyl-4′,4′-dimethyloxazolidine derivatives) were used to measure the coefficient of lateral diffusion. The lipid solubility of the spin labels Tempo[1] (2,2,6,6-dimethylpiperidin-1-oxyl) and a *N*-oxyl-4′,4′-dimethyloxazolidine (NODO) derivative of digitoxigenin provide a further possibility of characterizing the membrane fluidity.

In a previous publication (Overath and Träuble, 1973) thermal phase transitions in *E. coli* membranes (cells, membranes, and dispersions of the extracted lipids) were investigated by dilatometry, light scattering, and fluorescence probing. This paper may be consulted for the pertinent literature and for the properties of the *E. coli* system used in the present work.

I. Materials and Methods

Membranes. Three types of *E. coli* membranes from strain K1062 containing either *trans*-Δ⁹-octadecenoic acid (*trans*-18:1), *trans*-Δ⁹-hexadecenoic acid (*trans*-16:1), or *cis*-Δ⁹-octadecenoic acid (*cis*-18:1) as main lipid constituent were investigated in this work. The preparation of the membranes and the analysis of their fatty acid composition was described in a previous publication (Overath and Träuble, 1973). The protein-to-lipid weight ratio of *trans*-18:1, *trans*-16:1, and *cis*-18:1 membranes was 2.16:1, 1.75:1, and 2.26:1, respectively.

Spin Labels. The spin labeled stearic acids of the general form I(*m*,*n*) (*cf.* Figure 1a) and the 2,2,6,6-tetramethylpiperidin-1-oxyl (Tempo) radicals were products from Synva, Palo Alto, Calif. The *N*-oxyldimethyloxazolidine derivative of digitoxigenine was a gift of Mrs. R. Rösen.

Incorporation of the Spin Labels. The spin labeled fatty acids were added to aliquots of the membrane suspensions (1.4 mg of protein) in phosphate buffer (12 g of K_2HPO_4, 3 g of KH_2PO_4, 2 g of $(NH_4)_2SO_4$, and 0.2 g of $MgSO_4 \cdot 7H_2O$ per liter of water) and the volume was adjusted to 0.2 ml with buffer.

The samples were incubated for 1 hr at 40°. After centrifugation (20 min, 30,000*g*) the pellet was suspended in 0.1 ml of buffer. Comparison of the intensities of the esr spectra of the resuspended pellet and the supernatant showed that about 95% of the spin label had been incorporated into the membranes. For the experiments at low label concentration (no label interaction) the weight ratio label-to-lipid was $m \approx 0.0127$ corresponding to a molar ratio $c \approx 0.025$. For the diffusion measurements molar ratios between 0.025 and 0.2 were used.

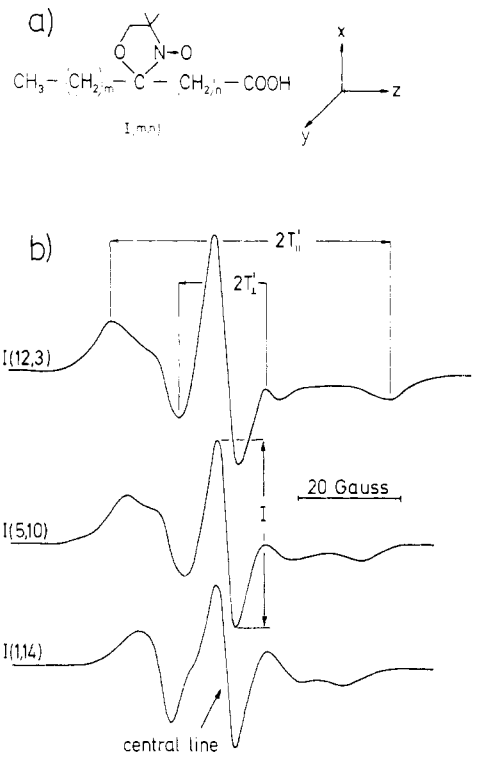

FIGURE 1: (a) Chemical structure of *N*-oxyl-4′,4′-dimethyloxazolidine (NODO) derivatives of stearic acid. The unpaired electron is located at the nitrogen atom in a $2p\pi$ orbital. The NODO ring is parallel to the *xy* plane. The long axis of the $2p\pi$ orbital is directed parallel to the normal of the NODO ring (or parallel to the long axis of the alkyl chain). (b) Esr spectra of the stearic acid labels I(12,3), I(5,10), and I(1,14) in dipalmitoyllecithin membranes at 25°. The spectra are characteristic for radicals performing a rapid anisotropic tumbling in the absence of radical interaction. Note the two pairs of side bands separated by $2T_{\parallel}'$ and $2T_{\perp}'$.

Spin labeled dipalmitoyllecithin dispersions were prepared in 0.1 M NaCl by cosonication for about 10 min at 45° and at 60 W.

Esr Measurements. The esr spectra were recorded using a Varian V4502 X-band spectrometer. The static magnetic field was measured with an AEG gaussmeter. The samples were sealed in capillaries with an inner diameter of 1.1 mm. Spectra were recorded at increasing and decreasing temperature, while the temperature was monitored to an accuracy of 0.2°.

Fluorescence Measurements. 8-Anilino-1-naphthalenesulfonate (ANS) served as fluorescence probe. The temperature dependence of the fluorescence intensity (excitation, 360 nm; emission, 490 nm) was measured using an Aminco-Bowman spectrofluorimeter as described previously (Overath and Träuble, 1973).

Analysis of the Esr Spectra. The esr spectra of fatty acid spin labels in lipid bilayers are determined by the rapid anisotropic motion of the fatty acid molecules (Seelig, 1970; Hubbell and McConnell, 1971) which are anchored with their polar heads in the membrane–water interface. The alkyl chains can rotate about their long axes, which in turn precess about the normal to the membrane surface. Superimposed on this rigid body motions are intrachain motions involving trans–gauche isomerizations about the C–C bonds.

Typical esr spectra of fatty acid labels performing such an anisotropic motion are shown in Figure 1b: an outer pair of

[1] Abbreviations used are: ANS, 8-anilino-1-naphthalenesulfonate; DPL, dipalmitoyllecithin; NODO, *N*-oxyl-4′,4′-dimethyloxazolidine; Tempo, 2,2,6,6-tetramethylpiperidin-1-oxyl; NO, nitroxide; *cis*-18:1, *cis*-Δ⁹-octadecenoic acid; *trans*-18:1, *trans*-Δ⁹-octadecenoic acid; *trans*-16:1, *trans*-Δ⁹-hexadecenoic acid.

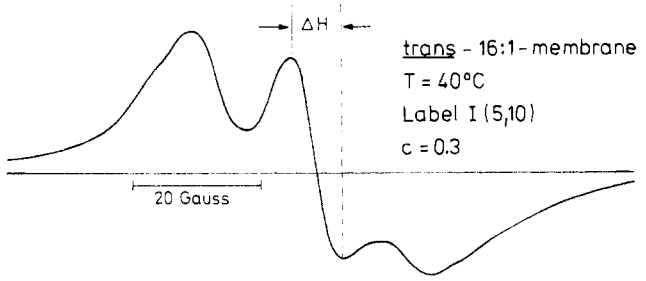

FIGURE 2: Exchange broadened first derivative esr spectrum of the label I(5,10) in *trans*-16:1 *E. coli* membranes. Measured at a temperature (40°) above the lipid phase transition (T_t = 28°). The total width of the central line is denoted by ΔH. c is the label-to-lipid molar ratio.

lines is separated by a magnetic field distance $2T_{\parallel}'$, an inner pair of lines is separated by $2T_{\perp}'$.

The "fluidity" of a membrane can be characterized by the so-called order parameter S (Seelig, 1970; Hubbell and McConnell 1971) defined by

$$S = (T_{\parallel}' - T_{\perp}')/(T_z - T_x) \quad (1)$$

where $T_z - T_x = 25$ gauss (McConnell and McFarland, 1970). S determines the average orientation of the long axis of the $2p\pi$ orbital of the radical (*cf.* Figure 1a) with respect to the normal, z', of the membrane. According to Hubbell and McConnell (1971) the order parameter of fatty acid labels is given by

$$\log S - \log S_0 = C + n \log P_t \quad (2)$$

where n denotes the number of CH_2 groups between the carboxyl group and the paramagnetic NODO ring (*cf.* Figure 1a). P_t is the fraction of CH_2 groups in trans configuration. C is a small correction term which is virtually independent on n (*cf.* Hubbell and McConnell, 1971). S_0 characterizes the (anisotropic) rigid body motion of the alkyl chains and is defined as

$$S_0 = \tfrac{1}{2}\langle 3\cos^2\vartheta - 1\rangle \quad (3)$$

where ϑ denotes the momentary angle between the z' axis and the long axis of the alkyl chain and where $\langle\ \rangle$ symbolizes the time average. In a plot of log S *vs.* n, S_0 is determined by the intercept with the ordinate.

According to eq 2 log S decreases linearly with increasing distance, n, between the NODO ring and the polar group of the label. Deviations from this linear dependence indicate alterations in the chain mobility in a direction from the polar groups to the membrane interior.

Determination of the Coefficient of Lateral Diffusion (D_{diff}). The rate of lateral diffusion is directly related to the number of encounters per second between randomly diffusing particles. The short-range exchange interaction operating upon collisions between spin label molecules provides a convenient parameter to measure the rate of lateral diffusion. This exchange interaction causes a detectable broadening of the esr spectra if the molar ratio label-to-lipid exceeds c = 0.025.

A typical exchange broadened spectrum of a fatty acid label in an *E. coli* membrane is shown in Figure 2. The width, ΔH, of the central line is given by

$$\Delta H = \Delta H_0 + \Delta H_{dip} + \Delta H_{ex} \quad (4)$$

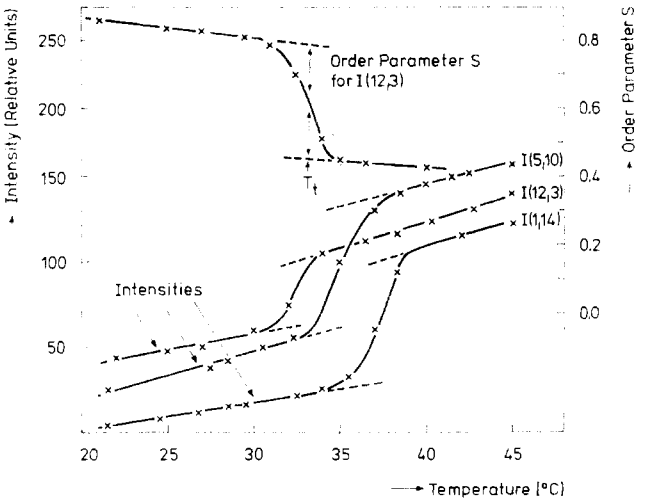

FIGURE 3: Lipid phase transitions in aqueous dispersion of dipalmitoyllecithin (3×10^{-3} M) indicated by changes in the esr spectra of the stearic acid labels I(12,3), I(5,10), and I(1,14). For the definition of the order parameters S *cf.* eq 1. The spectral intensity is defined as the vertical distance between the maximum and the minimum of the central line (Figure 1b). The transition temperature T_t is defined as the temperature where the measured curves (——) bisect the vertical distance between the extrapolated straight (– – –) lines characteristic for the two states $T \ll T_t$ and $T \gg T_t$.

ΔH_0 is the line width at negligible label interaction ($c < 0.02$), ΔH_{dip} is the line broadening due to the dipole–dipole interaction between the radicals, and ΔH_{ex} is the broadening caused by the exchange interaction. ΔH_{dip} can be neglected for label concentrations smaller than $c \approx 0.1$; for concentrations between c = 0.15 and c = 0.4 the value of ΔH_{dip} is approximately 1 G (Sackmann and Träuble, 1972). ΔH_{ex} may be determined as follows. First ΔH_0 is taken from an esr spectrum of the system at a label concentration $c < 0.01$. Then spectra are recorded for at least three different label concentrations larger than $c \approx 0.025$ and the line width ΔH of these spectra is determined. ΔH_{ex} is then obtained using eq 4.

The exchange broadening ΔH_{ex} is a direct measure for the probability of spin exchange per second (the so-called exchange frequency W_{ex}) according to

$$W_{ex} = 1.4 \times 10^6\, \Delta H_{ex}\ [\text{Hz}] \quad (5)$$

For a two-dimensional system W_{ex} is related to the label-to-lipid molar ratio c by the following equation (Träuble and Sackmann, 1972)

$$W_{ex} = \frac{4}{3}\frac{d_c D_{diff}}{F\lambda}\frac{c}{1+c} \quad (6)$$

F denotes the area per lipid molecule ($F \approx 60$ Å^2 for $T > T_t$; Chapman *et al.*, 1967), d_c is the critical distance for the onset of spin exchange ($d_c \approx 20$ Å; Träuble and Sackmann, 1972), and λ is the length of one diffusional jump in the lipid lattice ($\lambda \approx 8$ Å; Träuble and Sackmann, 1972). With these values one obtains

$$W_{ex} = 5.5 \times 10^{14} D_{diff}\frac{c}{1+c} \quad (7)$$

where W_{ex} is measured in Hz and where D_{diff} has the dimension cm^2/sec.

According to eq 7 W_{ex} is expected to increase linearly with

$c/(1 + c)$. D_{diff} is determined by the slope of the straight line in a W_{ex} *vs.* $c/(1 + c)$ plot.

II. Results

(A) Phase Transitions in Dispersions of Dipalmitoyllecithin (DPL). Esr Spectra. Stearic acid spin labels carrying nitroxide groups at carbon atom 5 (I(12,3)), 12 (I(5,10)), and 16 (I(1,14)), respectively, were incorporated into DPL bilayers. Esr spectra were recorded in the temperature range between 20 and 50°. If not stated otherwise, the label-to-lipid molar ratio was $c = 0.01$. The intensity of the esr spectra (*cf.* legend to Figure 3) and the order parameter S are plotted in Figure 3 as a function of the temperature. Apparently the spectral intensity and the order parameter are equally suitable for the indication of lipid phase transitions.

Surprisingly, the observed transition temperatures, T_t, depend on the position of the nitroxide groups in the alkyl chain: labels carrying the nitroxide group near the carboxyl end indicate lower values of T_t than labels with the NO group near the methyl end: $T_t = 33°$ for I(12,3); $T_t = 37.5°$ for I(1,14). The value $T_t = 37.5°$ agrees well with the results of 90° light scattering and ANS fluorescence measurements (*cf.* Table II and Figure 6a).[2]

The question arises whether these differences in T_t are due to an effect of the spin labels on the lipid structure or if the inner and outer (semipolar) region of the lipid bilayer undergo conformational changes at different temperatures.

The three labels might differ in their alkyl chain flexibility and, consequently, in their sensitivity to environmental motions. In order to check this possibility we have plotted in Figure 4 the logarithm of the order parameter S as a function of the number n of CH_2 groups between the carboxyl group and the NODO ring for temperatures below and above the phase transition (20 and 42°). For DPL straight lines are obtained. From the slope of these lines we obtain for the fraction P_t of trans configurations within the hydrocarbon chains of the label the values $P_t = 0.93$ at 20°, $P_t = 0.88$ at 30°, and $P_t = 0.85$ at 42° (*cf.* eq 2). This indicates that the alkyl chain flexibility of the spin labels is virtually the same slightly below (30°) and above (42°) the phase transition. The decrease in S at the phase transition therefore reflects mainly a decrease in S_0 (*cf.* Figure 4) which is equivalent to an increase of the mean angular deviation of the alkyl chains $\langle\vartheta\rangle$, from the normal z' of the membrane. S_0 decreases from $S_0 = 0.95$ at 20° and $S_0 = 0.88$ at 30° ($T < T_t$) to $S_0 = 0.65$ at 42° ($T > T_t$), corresponding to an increase of $\langle\vartheta\rangle$ from $\langle\vartheta\rangle = 12°$ and $\langle\vartheta\rangle = 16°$ to $\langle\vartheta\rangle = 30°$.

It is furthermore conceivable that the three spin labels cause different perturbations of the lipid matrix. In order to clarify this question, we have measured the transition temperatures of membranes containing different concentrations of the label I(12,3) and I(1,14) with the spin label technique. The results are given in Table I. We have also measured the transition temperature of I(12,3)-containing DPL membranes with the ANS fluorescence technique (*cf.* Table I). Table I shows that the transition temperature is not appreciably affected by the spin labels for label concentrations lower than $c \approx 0.04$.

Tempo Solubility. According to McConnell *et al.* (McConnell and McFarland, 1970; McConnell *et al.*, 1972) the hyperfine coupling and the g value of Tempo depend on the environmental polarity. The spectrum of Tempo in lipid dispersions exhibits two separate high field lines, w and m, characteristic for the local polarities of the water and the membrane phase, respectively. The intensity, I_m, of the high field line m may be used as a measure for the relative concentration of Tempo contained in the lipid bilayer. Figure 5 shows I_m as a function of the temperature for DPL dispersions, demonstrating that the Tempo solubility in DPL bilayers increases sharply between 30 and 35° defining $T_t = 32.5°$. In an analogous experiment Hubbell and McConnell (1971) obtained $T_t = 29°$; 90° light scattering and ANS fluorescence measurements (*cf.* Figure 6a) gave $T_t = 38°$ for the same Tempo containing preparation (*cf.* Table II).

ANS Fluorescence Measurements. A conformational change in DPL membranes at a temperature below the main transition has also been observed using the polarity dependent fluorescence label ANS as shown by the lower curve of Figure 6b (increasing temperature). Besides a strong fluorescence enhancement at about 38° (main transition) a smaller pretransitional effect is observed at about 32°. This effect is most

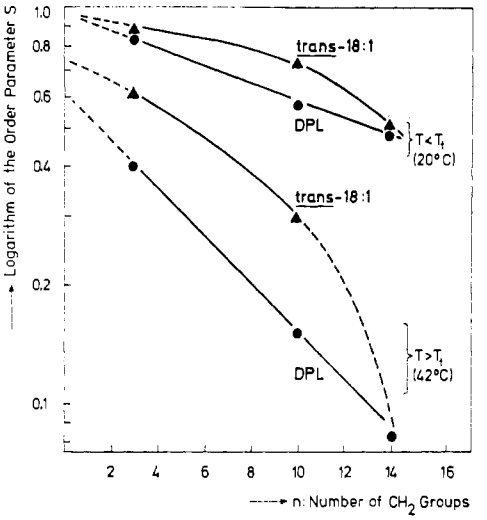

FIGURE 4: Order parameter S (*cf.* eq 1) of stearic acid spin labels I(m,n) (*cf.* Figure 1a) in dipalmitoyllecithin (DPL) bilayers and in *trans*-18:1 *E. coli* membranes. Log S is plotted for a temperature above (42°) and below (20°) the lipid phase transition as a function of the number, n, of CH_2 groups between the carboxyl end and the NODO ring of the label: (▲) *trans*-18:1 containing *E. coli* membranes; (●) DPL model membranes. The intercept of the straight lines (obtained for the DPL system) with the ordinate yields the values $S_0 = 0.65$ at 42° and $S_0 = 0.95$ at 30°. The same plot for 30° (not shown) yields $S_0 = 0.88$.

TABLE I: Transition Temperature T_t [°C] of DPL Dispersions Containing Different Concentrations of the Labels I(12,13) and I(1,14).

Molar Ratios Label-to-Lipid	Esr Spectroscopy I(1,14)	Esr Spectroscopy I(12,13)	ANS Fluorescence[a] I(12,3)
0.01	37.5	33	38
0.04	37	32	37
0.16	35	29	36

[a] ANS fluorescence measurements were performed only with membranes containing I(12,3).

[2] This value of T_t is smaller than the transition temperature $T_t = 41°$ reported in the literature (Barratt *et al.*, 1969). The discrepancy is most probably due to impurities in the dipalmitoyllecithin used in this work.

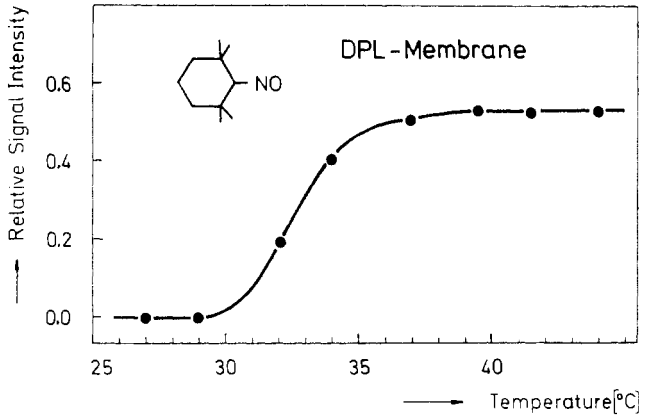

FIGURE 5: Temperature dependence of the solubility of Tempo in DPL lipid lamella. The intensity of the high field line of Tempo adsorbed to the membrane is plotted as a function of the temperature. Lipid and Tempo concentrations were 3×10^{-3} M and 3×10^{-4} M, respectively. At 42° about 10% of the total Tempo content was dissolved in the membrane.

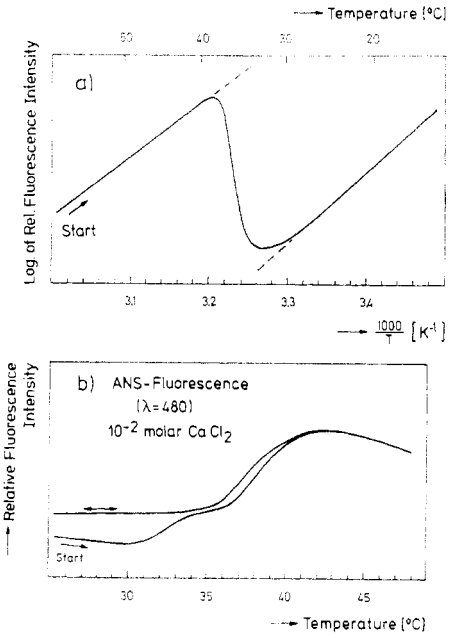

FIGURE 6: (a) Demonstration of phase transitions of DPL dispersions using ANS as fluorescence probe. The lipid dispersion (25 mg of DPL in 20 ml of water) contained 3×10^{-4} M Tempo, 10^{-5} M ANS, and 0.1 M NaCl (excitation, λ_{ex} 360 nm; emission, λ_{em} 480 nm). The sample was equilibrated at $T > T_t$ for about 10 min prior to the measurement. The transition temperature (T_t = 38°) is defined in analogy to Figure 3. By plotting the logarithm of the fluorescence intensity straight lines are obtained above and below T_t. (b) Temperature dependence of the ANS fluorescence intensity of a DPL dispersion containing $CaCl_2$ (10^{-2} M). The dispersion was prepared at 20°. In the first temperature run a clearly visible pretransition is observed at about 32°. The pretransition is absent in subsequent temperature sweeps.

clearly seen in the presence of $CaCl_2$ or $LaCl_3$. The pretransition is observed only if the dispersions are prepared at room temperature and measured at increasing temperature (*cf.* Figure 6b, start) suggesting that an irreversible adsorption of a small amount of ANS takes place in the first temperature run at the pretransition.

The results of this section are summarized in Table II.

(B) Phase Transitions in E. coli Membranes. Esr Spectra. Typical spectra of the labels I(12,3) and I(5,10) incorporated into *trans*-18:1 *E. coli* membranes are presented in Figure 7 for temperatures between 16 and 44°. The label concentrations were sufficiently low ($c \leq 0.02$) to exclude radical interactions. Comparison with Figure 1b shows that the fatty acid molecules in the *E. coli* membrane perform essentially the same anisotropic motion as in the DPL dispersion suggesting that the environment of the labels in the *E. coli* membrane is virtually the same as in the DPL bilayers.

According to Figure 7 the field distance $2T_{||}'$ decreases sharply with increasing temperature at about 30° for I(12,3) and at 37° for I(5,10). These spectral changes are qualitatively the same as those occurring at the phase transition in DPL model membranes suggesting that they are caused by a lipid phase transition in the *E. coli* membrane.

Figure 8 shows the order parameter S and the intensity of the central line as a function of the temperature for the labels I(12,3) and I(5,10) incorporated into *trans*-18:1 membranes. In accordance with the findings for the DPL membrane, the label I(12,3) with the paramagnetic center near the carboxyl group indicates a lower transition temperature, T_t', than the other two labels (*cf.* Table II).

The corresponding results for the *trans*-16:1 membranes are given in Figure 9. Also in this case the label I(12,3) indicates a lower transition temperature. Table III summarizes the results for the *trans*-18:1, the *trans*-16:1, and the *cis*-18:1 membranes.

The Solubility of Tempo and Digitoxigenin in trans-18:1 Membranes. The solubility of Tempo in *E. coli* membranes was determined from the esr intensity as described above. Figure 10a shows the esr spectra of digitoxigenin in suspensions of *trans*-18:1 membranes. This spectrum is a superposition of a sharp triplet spectrum of (free) digitoxigenin in water and a broader three-line spectrum arising from the membrane-bound label (Rösen, 1973). The concentration of the free label has been determined from the intensity of the sharp line at the right side of the spectrum (*cf.* Figure 10a). Figure 10b shows that the solubilities of both labels increase sharply between 28 and 35° defining a transition temperature of about 32°.

Table II summarizes the transition temperatures of the

TABLE II: Comparison of Transition Temperatures (°C) of DPL Dispersions and *trans*-18:1 *E. coli* Membranes Obtained with Different Techniques.

Method	DPL Dispersions	*trans*-18:1 Membrane
Spin label		
I(1,14)	38	36
I(5,10)	35	37
I(12,3)	32[d]	29[d]
Tempo	32[d]	32[d]
Digitoxigenin		31[d]
ANS fluorescence	38	41[a]
90° light scattering	38	38[a]
Dilatometry	40	40[a,b]
Calorimetry	41.75[c] (34)[d]	

[a] Data of Overath and Träuble (1973). [b] Dispersions of extracted lipids. [c] Data of Hinz and Strutevant (1972). [d] Transition temperature T_t' of pretransition.

TABLE III: Comparison of Transition Temperature (°C) of *E. coli* Membranes (Suspensions in Phosphate Buffer) Obtained with Different Techniques.

	Stearic Acid Spin Label								
	I(12,3)		I(5,10)		I(1,14)		90° Light Scattering[b,c]		*In Vivo* NphGal Hydrolysis[b,d]
E. coli Membranes	T_t	ΔT[a]	T_t	ΔT[a]	T_t	ΔT[a]	T_t	ΔT[a]	T_t
cis-18:1	*14*	4	*17*	4			*13*	12	16
trans-16:1	*24*	3	*28*	3	*28*	3	*27*	7	31
trans-18:1	*29*	3	*37*	3	36	3–4	*38*	4	37

[a] ΔT is the width of the transition. [b] Data taken from the previous paper by Overath and Träuble (1973). [c] 90° light scattering measurements were performed with dispersions of the extracted lipids in water. [d] NphGal, *o*-nitrophenyl β-D-galactopyranoside.

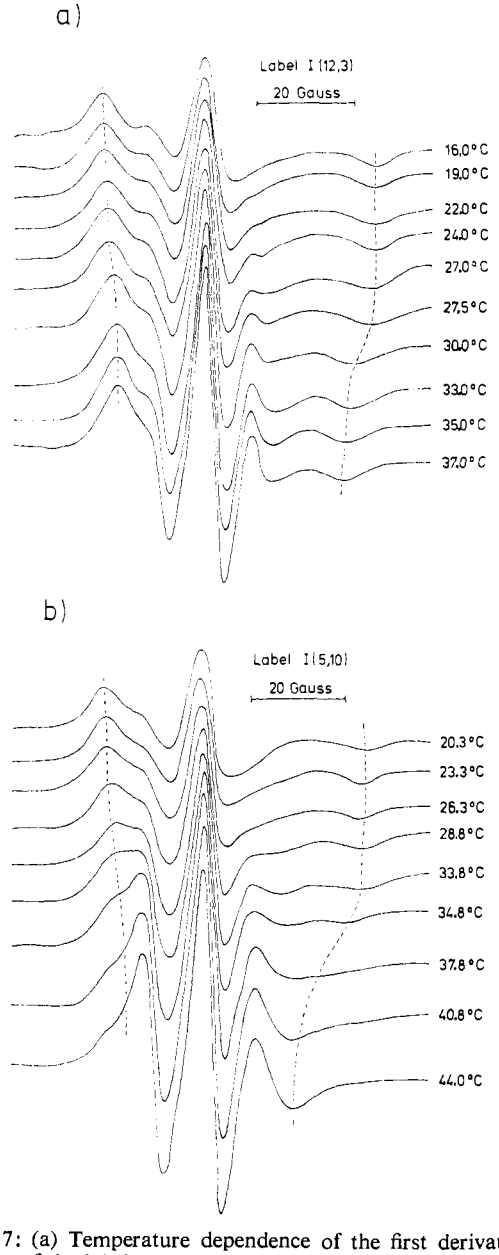

FIGURE 7: (a) Temperature dependence of the first derivative esr spectrum of the label I(12,3) incorporated in *trans*-18:1 containing *E. coli* membranes. A conformational change is indicated at about 30° by an abrupt decrease in the field distance $2T_{||}'$ (Figure 1b) of the two outermost side bands (*cf.* broken lines). (b) Temperature dependence of the spectrum of the label I(5,10) incorporated in *trans*-18:1 membranes. This label indicates a conformational change at a higher temperature (about 37°).

trans-18:1 membranes and the DPL dispersions obtained with different methods.

(*C*) *Lateral Diffusion in E. coli Membranes.* The coefficient of lateral diffusion, D_{diff}, was determined for the *trans*-16:1 membranes (T_t = 28°) at 40° using the label I(5,10). Esr spectra were recorded at 40° for five different label concentrations (*cf.* Table IV). The exchange frequency W_{ex} was determined from these spectra as described in Materials and Methods. By plotting W_{ex} *vs.* the label density, $c/(1 + c)$, we obtain a straight line.

$$W_{ex}\ [\text{MHz}] = 18c/(1 + c)$$

The slope of this line yields $D_{diff} = 3.25 \times 10^{-8}$ cm²/sec by applying eq 7.

For a two-dimensional lipid lattice with a lattice constant λ

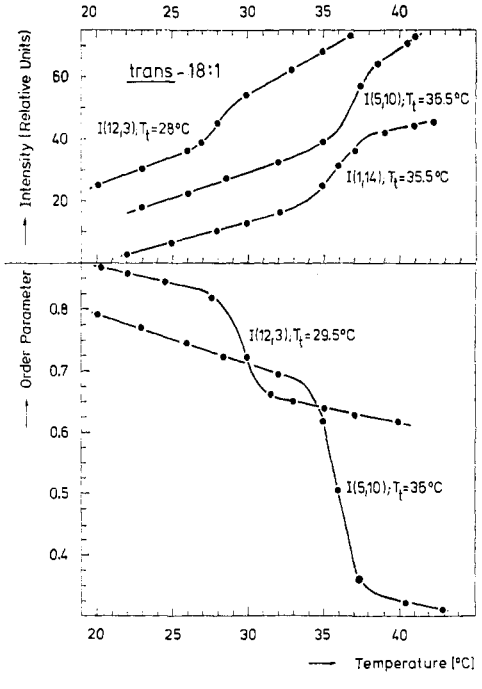

FIGURE 8: Demonstration of the lipid phase transition in *trans*-18:1 containing *E. coli* membranes using the stearic acid labels I(12,3), I(5,10), and I(1,14). The lipid phase transition is indicated by a sharp decrease in the order parameter *S* or by a corresponding increase in the intensity of the central line. As for DPL model membranes the label I(12,3) indicates a lower value of the transition temperature than the labels I(5,10) or I(1,14).

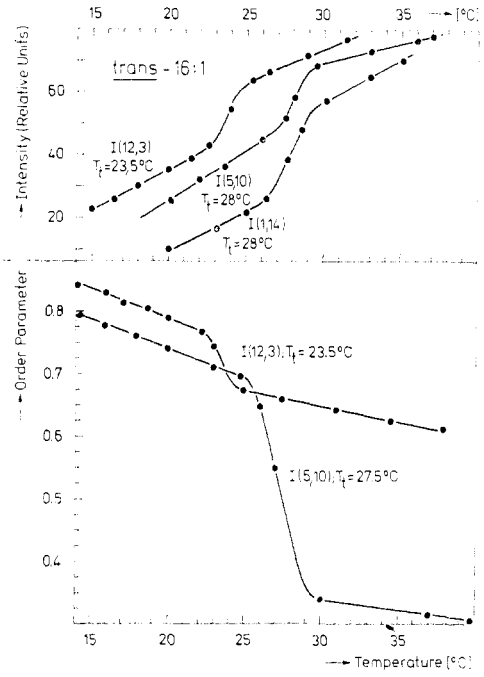

FIGURE 9: Temperature dependence of the order parameter S and the spectral intensities of the three stearic acid labels I(12,3), I(5,10), and I(1,14) in *trans*-16:1 containing *E. coli* membranes.

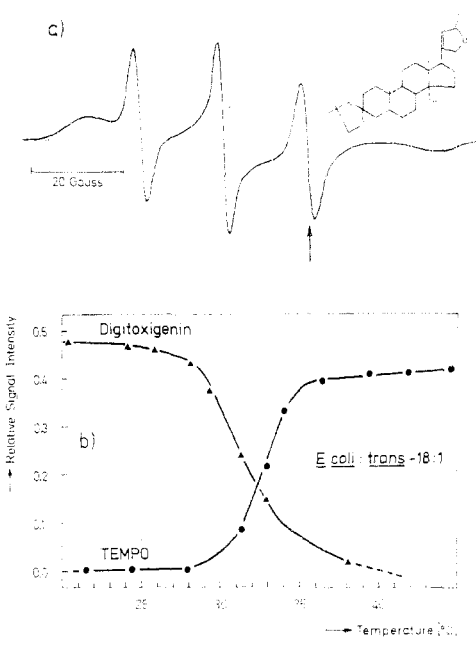

FIGURE 10: Temperature dependence of the solubility of Tempo and digitoxigenin in *trans*-18:1 containing membranes. (a) Esr spectrum of a *N*-oxyl-4′,4′-dimethyloxazolidine derivative of digitoxigenin in a suspension of *trans*-18:1 membranes. The sharp triplet arises from the radicals dissolved in water. The broad three-line spectrum is due to the membrane-bound label. The concentration of the radicals in water can be estimated from the intensity of the sharp line at the right side of the spectrum (arrow). (b) Temperature dependencies of the relative concentrations of Tempo in membranes and digitoxigenin in water. The ordinate gives the intensities of the high-field line of membrane-bound Tempo (*cf.* Figure 5) and of the sharp line at the right side of the digitoxigenin spectrum, respectively. At $T < T_t$ about 77% of digitoxigenin is dissolved in water. At $T > T_t$ this value decreases to about 70%.

(length of one diffusional jump) the hopping frequency ν is related to the diffusion coefficient by

$$\nu = 2D_{\text{diff}}/\lambda^2 \tag{8}$$

The average distance of travel per second $\sqrt{\bar{x}^2}$ may be calculated according to the Einstein relation $D_{\text{diff}} = \bar{x}^2/2\tau$ by putting $\tau = 1$ sec. For $\lambda = 8$ Å the above value of D_{diff} yields $\nu \approx 10^7$ Hz and an average distance of travel per second of $\bar{x} = 27{,}000$ Å/sec. This result shows that the lateral mobility of stearic acid labels in *trans*-16:1 membranes at 40° is about the same as that of androstane in DPL monolayers at 50° ($D_{\text{diff}} = 3 \times 10^{-8}$ cm²/sec, Träuble and Sackmann, 1972). Scandella *et al.* (1972) determined $D_{\text{diff}} = 7.5 \times 10^{-8}$ cm²/sec for the diffusion of phospholipids in sarcoplasmic reticulum at 40°. Since these membranes contain a high fraction of unsaturated lipids they are expected to be more fluid than DPL and *E. coli* membranes above T_t. Apparently, the diffusion rates of androstane and fatty acids are about equal to the self diffusion of phospholipids.

TABLE IV: Exchange Frequency W_{ex} of the Fatty Acid Label I(5,10) in *trans*-16:1 Membranes of *E. coli* at 40°.[a]

Molar Ratio, c, Label-to-Lipid	Label "Density" $c/(1+c)$	Total Line Width ΔH [gauss]	Exchange Frequency W_{ex} [MHz]
0.050	0.0475	4.5	0.7
0.101	0.092	5.1	1.5
0.200	0.167	5.9	2.8
0.300	0.231	8.0	4.2
0.400	0.286	9.0	5.6

[a] The natural line width of the central line has been taken as $\Delta H_0 \approx 4$ G. A value of $\Delta H_{dip} \approx 1$ G has been assumed for the label-to-lipid molar ratios $c > 0.2$. A value of 1 G of the external field corresponds to a precession frequency of about 2.8×10^6 Hz for a g factor of 2.0023.

III. Discussion

The present work shows that the spin label technique is a sensitive method for the detection of conformational changes in artificial and biological membranes. Spin label studies allow the characterization of the membrane fluidity in terms of the lateral mobility, the rotational tumbling, and the chain flexibility of the probes.

The esr spectra of spin labels in DPL and in *E. coli* membranes exhibit similar spectral changes at the characteristic phase transition: the side bands move toward the central line indicating a decrease in the order parameter by 50%. The intensity of the central line increases by about a factor of 2 corresponding to an increase in the rotational motion of the labels. This suggests that a large part of the lipids in *E. coli* membranes is organized similarly to the lipids in a bilayer. This confirms the results of a previous investigation (Träuble and Overath, 1973).

Main Transition. The transition temperatures, T_t, obtained with the label I(1,14) agree well with the results of 90° light scattering, fluorescence, and dilatometric studies. This is valid for both *E. coli* and DPL dispersions.

In a previous paper (Träuble and Haynes, 1971) the structural changes at the phase transition were interpreted in terms of the formation of rotational isomers within the hydrocarbon chains. This model explains (1) the increase in specific volume

($\Delta V/V$ = 1.02% for *trans*-18:1 lipids, $\Delta V/V$ = 1.4% for DPL dispersions), (2) the decrease in bilayer thickness, and (3) the rather large lateral expansion at the phase transition with increasing temperature (Chapman *et al.*, 1967).

The lateral expansion of the lipid lattice at the phase transition is also reflected in an abrupt decrease of the order parameter S_0 with increasing temperature (S_0 = 0.88 at 30°; S_0 = 0.65 at 42°). This decrease in S_0 corresponds to an increase in the mean angular deviation $\langle\vartheta\rangle$ (*cf.* eq 3) from $\langle\vartheta\rangle$ = 16° ($T < T_t$) to $\langle\vartheta\rangle$ = 30° ($T > T_t$).

Consider a lipid monolayer of thickness h with the lipid hydrocarbon chains forming a triangular lattice with a lattice constant a (*cf.* Träuble and Sackmann, 1972; Figure 5). X-Ray experiments by Chapman *et al.* (1967) show $a = 2/\sqrt{3} \times 4.5 = 4.8$ Å and $h = 23$ Å at $T < T_t$. Assuming a fatty acid label of length h occupies one site in the lipid lattice one estimates $\langle\vartheta\rangle$ = 17° for $T < T_t$ and $\langle\vartheta\rangle$ = 23° for $T > T_t$ ($a = 5.3$ Å; $h = 19$ Å). In this estimate the lipid hydrocarbon chains were considered as rigid bodies with their long axes perpendicular to the plane of the membrane. The much greater experimental value of $\langle\vartheta\rangle$ = 30° at $T > T_t$ indicates that the *lipid hydrocarbon chains* undergo a tumbling motion resulting in a mean angular deviation of their long axes by 7° with respect to the normal of the membrane.

Pretransition. Surprisingly the labels Tempo, digitoxigenin, and I(12,3) indicate transition temperatures T_t' about 8° lower than the main transition T_t, both for DPL and *E. coli* membranes. Similar pretransitions located about 8° below T_t have been demonstrated calorimetrically by Chapman *et al.* (1969) and by Hinz and Sturtevant (1972) (*cf.* Table III). Ladbrooke and Chapman (1969) discussed this effect in terms of a conformational change within the lipid polar head groups. The rather small volume change at the pretransition (Overath and Träuble, 1973) confirms this interpretation.

The following arguments support the view that the labels Tempo, digitoxigenin, and I(12,3) sense primarily structural changes in the polar region of the membranes. (1) Hamilton and McConnell (1968, Figure 2) established an empirical relationship between the hyperfine splitting a_N and the environmental polarity of Tempo. Using these results we estimate from the observed ratio $a_N(H_2O)/a_N(DPL)$ = 1.05 that the nitroxide group of the membrane-bound Tempo experiences a rather high polarity (similar to that of ethylene glycol). (2) Digitoxigenin contains several polar substituents and therefore is expected to adsorb to the membrane surface. (3) The label I(12,3) is expected to be sensitive against structural changes within the polar groups due to the close proximity of the NODO ring to the carboxyl end of the label.

Lateral Diffusion. According to section IIC the lateral mobility of lipids in *E. coli* membranes is about the same as in DPL membranes ($D_{diff} = 3.25 \times 10^{-8}$ cm²/sec for *E. coli* at 40°, $D_{diff} = 3 \times 10^{-8}$ cm²/sec for DPL at 50° (Träuble and Sackmann, 1972)). This suggests that the lipids in *E. coli* membranes providing the diffusion medium are organized as bilayers or monolayers. In fact, about 80% of the lipids in *E. coli* and in sarcoplasmic reticulum membranes appear to be free or not immobilized by membrane proteins (*cf.* Träuble and Overath, 1973; McConnell *et al.*, 1972).

Independent evidence for rapid lateral diffusion in intact membranes has been provided by Overath *et al.* (1971) and by Tsugagoshi and Fox (1972) from experiments in which the lipid composition of *E. coli* fatty acid auxotrophs has been altered during the growth period. The possibility of rapid lateral diffusion is critically important for the interpretation of experiments dealing with the question whether membrane proteins and/or lipids are synthesized at distinct sites in organisms like *E. coli* or *B. subtilis* (Lin *et al.*, 1972; Weaver-Green and Schaechter, 1972; Tsugagoshi *et al.*, 1971; Mindich and Dales, 1972; Autissier *et al.*, 1971; Autissier and Kepes, 1971, 1972; Wilson and Fox, 1971). Patches or zones of newly synthesized membrane molecules will not be conserved if rapid lateral diffusion within the plane of the membrane is possible. Therefore the presence of distinct growth zones cannot be excluded even if the newly synthesized lipid molecules are found to be homogeneously distributed among the progeny.

Lateral Mobility of Membrane Proteins. The rapid lateral diffusion of the lipid molecules in a fluid membrane is expected to lead to a considerable mobility of larger membrane components (proteins, glycolipids, glycoproteins that penetrated into the membrane). In the following an attempt is made to correlate the lateral diffusion of such membrane macromolecules with the rate of lateral diffusion of the lipid molecules.

Continuum Model. The membrane is considered as a two-dimensional continuum of thickness d characterized by the viscosity η. The diffusion of membrane components is described by the Stokes–Einstein relation ($D = kT/6\pi\eta R$; where R is the radius of the diffusing particle). Then the diffusion coefficient, D_{diff}^P, of an assumed cylindrical protein molecule (radius R, height d, molecular weight M_P) is related to the diffusion coefficient, D_{diff}^L, of the lipid molecules (molecular weight M_L) by

$$D_{diff}^P = \sqrt{M_L/M_P}\, D_{diff}^L$$

According to this relation the diffusion coefficient of a protein of molecular weight M_P = 100,000 is expected to be only ten times smaller than the value D_{diff}^L of the lipid molecules ($M_L \approx 800$). However, since the protein molecules are not very large compared to the lipid molecules (which provide the diffusion medium), the applicability of a continuum consideration is questionable.

Free Volume Model. In this model the rate-limiting step for the lateral diffusion of a membrane protein is assumed to be the formation of free volume by lipid displacements at the boundary of the protein molecule. A similar concept was developed for the diffusion in polymers by Kumins and Kwei (1968). If the free volume corresponding to a lipid vacancy is thought of as being distributed over half the periphery of the protein we obtain an estimate for the elementary diffusional step λ_P of the protein: obviously $\lambda_P = F_L/\pi R$, where F_L is the area of one lipid vacancy. F_L is approximately equal to the square (λ^2) of the average distance λ between two lipid molecules. For a two-dimensional lattice the diffusion coefficient of a component P is given by

$$D_{diff}^P = \lambda_P^2 \nu_P/2 \qquad (9)$$

Since an upper limit of ν_P is given by the hopping frequency ν ($\approx 10^7$ Hz) of the lipid molecules it follows

$$D_{diff}^P = \frac{1}{2}\frac{F_L^2\lambda^2}{\pi^2R^2}\nu_P \leq \frac{1}{2}\frac{F_L\lambda^2}{\pi^2R^2}\nu = \frac{1}{\pi}\frac{F_L}{F_P}D_{diff}^L$$

F_P denotes the area of the protein molecule and $D_{diff}^L = {}^1/_2\lambda^2\nu_L$ is the diffusion coefficient of the lipid molecules.

According to this relation a cylidrical protein molecule of molecular weight 100,000 (radius R = 25 Å) would diffuse by a factor of 100 more slowly ($D_{diff}^P \approx 3 \times 10^{-10}$ cm²/sec) than

the lipid molecules ($D_{diff}^{L} = 3 \times 10^{-8}$ cm²/sec). This corresponds to an average distance of travel per second of about 3000 Å/sec. This estimate is in good order of magnitude agreement with the rate of lateral diffusion of surface antigens in mouse human cell hybrids ($D_{diff}^{P} \approx 2 \times 10^{-10}$ cm²/sec) reported by Frey and Edidin (1970).

There is evidence (*cf.* Träuble and Overath (1973) or Jost *et al.* (1973)) that membrane bound (integral) proteins are surrounded by firmly attached lipid molecules. Träuble and Overath (1973) estimated that integral protein molecules in *E. coli* membranes are surrounded by about 130 lipid molecules on average.

A protein of $R = 25$ Å would thus be encircled by a belt of two to three layers of lipid molecules. This would result in an increase of the effective radius R_{eff} of the protein by about 16–24 Å. Since $D_{diff}^{P} \propto R_{eff}^{-2}$, the diffusion rate of this coated protein would be smaller by a factor of 3–4 than the value of D_{diff}^{P} of the "naked" protein.

Rotational Mobility of Membrane Protein. The rotational relaxation time, τ_r, of a cylindrical protein M with radius R embedded in the lipid matrix may be estimated from the hopping frequency, ν, of the lipid molecules on the basis of a simple model described recently (Träuble and Sackmann, 1973). The rotational axis of M is assumed perpendicular to the plane of the membrane. Moreover it will be assumed that the cross section of the protein is not exactly circular but exhibits at least one "spike" of a few ångströms in height. This spike may be a local protrusion of the polypeptide chain or it may be a lipid molecule firmly attached to the protein.

Obviously M can rotate only if a vacancy is temporarily created in the neighborhood of the spike. The rotation may thus be visualized as a sequence of rotational steps each involving an angular change θ_λ. The value of θ_λ is related to the average distance, λ, between the lipid molecules by $\theta_\lambda = \lambda/R$ (Träuble and Sackmann, 1973). The coefficient of rotational diffusion, D_r, is then given by

$$D_r \text{ [rad}^2\text{/sec]} = \frac{1}{2}\nu_r\theta_\lambda^2 = \frac{1}{2}\nu_r\frac{\lambda^2}{R^2}$$

where ν_r ($= \nu$) denotes the frequency of rotational steps of the protein molecule. The rotational correlation time, τ_r, is given by $\tau_r = (2\pi)^2/D_r$.

Consider a protein molecule with a radius $R = 25$ Å embedded in a (DPL or a *trans*-16:1 *E. coli*) membrane characterized by a hopping frequency $\nu \sim 10^7$ sec^{-1} and an average distance of $\lambda = 8$–10 Å between the lipid molecules. The above equation predicts a rotational correlation time of $\tau_r = 50$–75 μsec ($R = 25$ Å). Recently, Brown (1972) reported a value of $\tau_r \approx 20$ μsec for the rotational correlation (relaxation) time of rhodopsin ($R \approx 20$ Å; *cf.* Brown, 1972) in the visual receptor membrane. The somewhat smaller value for τ_r appears plausible because the lipid molecules in the visual receptor membrane contain a high fraction of unsaturated hydrocarbon chains. Thus for egg-lecithin at 40° ($D_{diff}^{L} = 15 \times 10^{-8}$ cm²/sec; $\nu \propto D_{diff}$) one obtains $\tau_r = 10$–15 μsec.

The results of the above model remain virtually unchanged if M exhibits two (or more) spikes. The reason is that the relaxation time of free rotation of a protein with $M_P = 50{,}000$ is of the order of 10^{-9} sec at room temperature as estimated from the kinetic energy of rotation. Therefore, two lipid sites at different spikes must be vacant simultaneously only for about 10^{-9} sec in order to allow one rotational step of M. This value is, however, very short compared to the lifetime of a lipid vacancy which is about equal to the inverse of the jump frequency ($\approx 10^{-7}$ sec).

Acknowledgment

The drawing of the figures by Miss I. Otto is gratefully acknowledged.

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Purification and Characterization from *Chromobacterium violaceum* of an Enzyme Catalyzing the Synthesis of γ-Cyano-α-aminobutyric Acid and Thiocyanate†

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ABSTRACT: A cell-free extract of *Chromobacterium violaceum* D 341, a strain that vigorously produces cyanide and converts it into γ-cyanoaminobutyric acid, has been shown to catalyze the synthesis of this cyanoamino acid. An enzyme, γ-cyanoaminobutyric acid (γ-CNabu) synthase, has been purified 270-fold from the extract. It is an acidic protein with a molecular weight near 130,000. It is activated by simple thiols and generally inactivated by disulfides. Pyridoxal 5′-phosphate can serve as its cofactor. In the presence of the enzyme homocystine and cyanide react to give γ-CNabu and thiocyanate. As a possible intermediate for this reaction γ-thiocyanoaminobutyric acid (*S*-cyanohomocysteine), a new sulfur amino acid, has been synthesized. It has been shown to form by a nonenzymatic cyanolysis of homocystine and to serve as a good substrate in place of homocystine for the enzymatic synthesis of γ-CNabu and thiocyanate.

A recent contribution from this laboratory described the isolation and identification of γ-cyano-α-aminobutyric acid as a new product of cyanide assimilation from *Chromobacterium violaceum* strain D 341 (Brysk and Ressler, 1970). This report describes the purification from the same organism of an enzyme that catalyzes the synthesis of γ-CNabu[1] and some of its properties. In the presence of this isolated enzyme, cyanide and a four-carbon chain of homocystine are utilized to form γ-CNabu, and sulfur from homocystine is converted into thiocyanate (reaction 1). Although roles for homocysteine in transmethylation, transsulfuration, and desulfurase reactions are well established, the corresponding disulfide, homocystine, has not previously been implicated in a synthetic aspect of metabolism.

$$\text{homocystine} + 2CN^- \xrightarrow[\text{enzyme}]{} \gamma\text{-cyanoaminobutyric acid} + SCN^- + \text{homocysteine} \quad (1)$$

In a preliminary consideration of a possible route for the enzymatic utilization of homocystine, it has been found that homocystine and cyanide react nonenzymatically to form γ-thiocyano-α-aminobutyric acid and that the enzyme herein described catalyzes the reaction of this amino acid with cyanide to give γ-CNabu and thiocyanate. The enzyme is provisionally named γ-thiocyanoaminobutyric acid thiocyano-lyase (adding CN) (EC 4.4.1) and for convenience is referred to as γ-CNabu-synthase.

Experimental Section

Synthesis of γ-Thiocyano-α-L-aminobutyric Acid. L-Hcy was treated with cyanogen halide as for acetylcysteine and cysteine (Aldridge, 1951; Catsimpoolas and Wood, 1964).

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[1] Abbreviations used are: ME, 2-mercaptoethanol; Hcy, homocysteine; Cm-Hcy, carboxymethylhomocysteine; Cm-HcySH, carboxymethylthiohomocysteine; γ-CNabu, γ-cyano-α-aminobutyric acid; γ-SCNabu, γ-thiocyano-α-aminobutyric acid; β-CNala, β-cyanoalanine; pyridoxal-P, pyridoxal 5′-phosphate.