Studies of the Crystalline–Liquid Crystalline Phase Transition of Lipid Model Membranes. I. Use of Spin Labels and Optical Probes as Indicators of the Phase Transition

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Abstract: The crystalline–liquid crystalline phase transition of dipalmitoyllecithin model membranes with a transition temperature (T_t) of 41° was studied by optical methods and by the spin-labeling technique. Structural sensitive fluorescent and absorption probes were used to indicate changes in the arrangement of the lipid polar head groups of the membranes. The number of binding sites for these probes on the membrane surface increases by a factor of about 3 when the system is heated above the transition temperature. This effect is due to a lateral expansion of the membrane and to a loosening of the polar group structure at the transition temperature. The spin-labeling technique was used to study changes within the hydrocarbon phase of the lipid model membranes. An androstane derivative with a paramagnetic N–O group was used as a steroid spin label. Several steroid–lecithin mixtures with a steroid content ranging from $c = 0.01$ to $c = 0.27$ ($c =$ molar ratio steroid:lecithin) were investigated at temperatures between 18 and 55°. For low label concentrations ($c < 0.01$) clear triplet spectra are observed at all temperatures. The line width of these spectra decreases sharply when the system is heated above the phase transition, indicating a sharp increase in the tumbling motion of the label molecules. With increasing steroid concentration the shape of the esr spectrum is modified due to increasing magnetic interactions (dipolar and exchange interaction) between the label molecules. For $T < T_t$ increasing label concentration produces a gradual broadening of the spectra until for $c > 0.035$ the spectra coalesce into broad one-line spectra. When the system is heated above T_t these broad spectra resolve into sharp triplet spectra. The changes in the esr spectra caused by the magnetic interaction between the steroid labels were used to study (1) the molecular organization of the steroid–lecithin system and (2) the influence of the lipid-phase transition on the structure of the mixed membranes. As a parameter which reflects the membrane structure we have evaluated the exchange frequency W_{ex} denoting the rate of spin exchange between the steroid molecules. With increasing temperature W_{ex} decreases sharply at the phase transition. This characteristic temperature dependence is in complete contrast to the behavior of paramagnetic radicals in organic solvents and suggests a sudden change in the internal structure of the mixed steroid–lecithin system at the lipid-phase transition. The details of these structural changes are discussed in two subsequent papers.

1. Introduction

Phospholipids, proteins, and water are the major building units of cell membranes. Among these the phospholipids are commonly considered as the structure-determining constitutents of biological membranes. This view is supported by the fact that phospholipids in aqueous environment may form a variety of highly organized structures depending upon the water content, the temperature, and other parameters.[1] The most popular of these structures consists of lamellar bilayers separated by layers of water. In these bilayers the lipid hydrocarbon chains are interacting hydrophobically and the lipid polar head groups face the water. Most models of biological membranes postulate a lipid bilayer as an essential building unit of the membrane.[2–5]

Much attention has been given in recent years to functional and structural changes in biological membranes.[6–18] These changes are possibly related to the capability of lipid–water systems to undergo reversible structural changes upon alterations of the water content, the ion concentration, and possibly other parameters. Of particular interest here is the so-called crystalline–liquid crystalline phase transition which produces remarkable changes in the physical state and the organization of the lipid molecules. Extensive calorimetric studies of this phenomenon have been performed by Chapman and coworkers[19–23] using synthetic phos-

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pholipids. It has been shown that the phase transition temperature T_t depends upon the length of the hydrocarbon chains, the chemical nature of the lipid polar head groups, and the water content of the system. A lower limiting value of T_t, the so-called Krafft point, is reached for water contents larger than about 30%. For the dipalmitoylphosphatidylcholine–water system studied in the present paper this value is about 41°.[20,23] The exact nature of the structural changes at the phase transition is not well known. The calorimetric measurements together with X-ray, infrared, and nmr studies[19–23] indicate that below T_t the hydrocarbon chains are in a quasicrystalline arrangement while above T_t they are in a more fluid state with considerable molecular motion. There is also evidence[24–26] that the polar groups have a higher degree of rotational freedom above the phase transition.

The structural changes at the phase transition are expected to affect the properties of lipid membranes in the following way. 1. Changes in the polar head group structure might affect the binding of ligands (ions, dyes, and larger molecules) to the membrane surface.[24] 2. Structural changes in the hydrocarbon chains might affect the interaction between the lipid molecules and other membrane constituents such as cholesterol, steroids, and membrane proteins. 3. From these effects it is expected that the membrane permeability is different below and above the phase transition.[27]

The study of the lipid-phase transition is hampered by the fact that lipids show neither an intrinsic fluorescence nor an optical absorption which could be used for spectroscopic studies. Only a limited number of techniques, for example, X-ray and nmr methods, can be applied directly. Thus a main problem in this field is the search for useful indicators.[28,29]

In the present study fluorescent probes and optically absorbing probes were used as indicators for changes in the polar head group structure. Changes in the hydrocarbon phase were monitored with the spin-labeling technique. The experiments to be described here were performed in an attempt (1) to learn more about the physical nature of the structural changes at the phase transition and (2) to elucidate the influence of the phase transition on the organization of a mixed system containing lecithin and steroid molecules.

The second question is of some importance because biological membranes are multicomponent systems and most probably the membrane properties depend upon the precise organization of the membrane constituents. It is hoped that the study of the two-component system lecithin–steroid would give an idea of how heterogeneous membranes may be organized.

Our knowledge of the arrangement of steroid molecules within a lipid matrix is rather limited and restricted mainly to the case of the lipid–cholesterol system. This system has been studied by several authors using monolayer,[30–35] X-ray,[36] and bilayer techniques. In most cases the lipid material was in a state above the crystalline–liquid crystalline phase transition. Not much is known about this system for $T < T_t$.

In the present study we have chosen as a representative of the steroid class an androstane derivative (*cf.* Figure 1a); this molecule differs from cholesterol mainly in the substitution of the hydrocarbon chain in the 17β position by an OH group. This molecule was chosen because it can be prepared easily as a spin label and because it has a structure similar to many steroid hormones. The interest in this system is enhanced by recent experiments[37,38] which indicate that the activity of steroid hormones is connected with the interaction of these molecules with cell membranes. For example, it has been shown[37,38] that steroid hormones play a role in the interaction of ribosomes with endoplasmic membranes.

As a parameter which is sensitive to the molecular organization of the steroid–lecithin system, we have measured the magnetic interaction between the spin-labeled steroid molecules. The esr spectra are modified by these interactions and depend sensitively upon the arrangement, the mobility, and the orientation of the steroid molecules within the lipid matrix.

The general principles of the spin-labeling technique have been described in recent articles by Hamilton and McConnell,[39] Ohnishi,[40] and Griffith and Waggoner.[41] The reader is referred to these papers. Other articles[42–45] deal with biological applications of this technique. Two research groups have applied the spin-labeling technique to the study of the crystalline–liquid crystalline phase transition. Barratt, *et al.*,[46] observed a sharp increase in the tumbling rate of the spin labels when the lipid material was heated above T_t. Hubbell and McConnell[42,44] reported an abrupt decrease in the order parameter at the transition temperature; from these data they were able to calculate the change in entropy at the transition temperature. These results are in good accord with earlier conclusions drawn from calorimetric X-ray, nmr, and ir studies[20,22,23] which state that the mobility of the hydrocarbon chains increase sharply at the transition point with increasing temperature. In these and earlier spin label studies the

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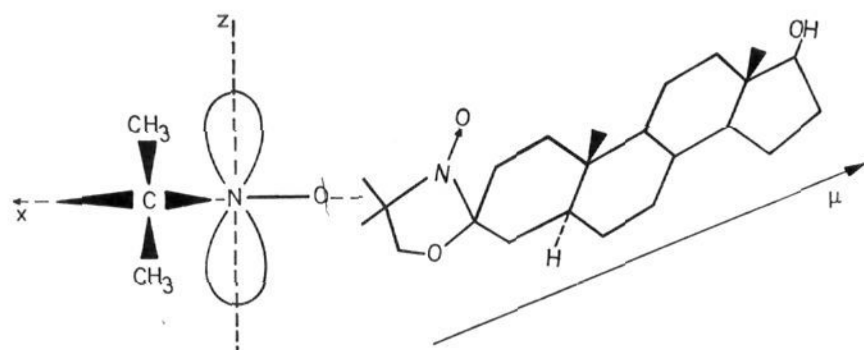
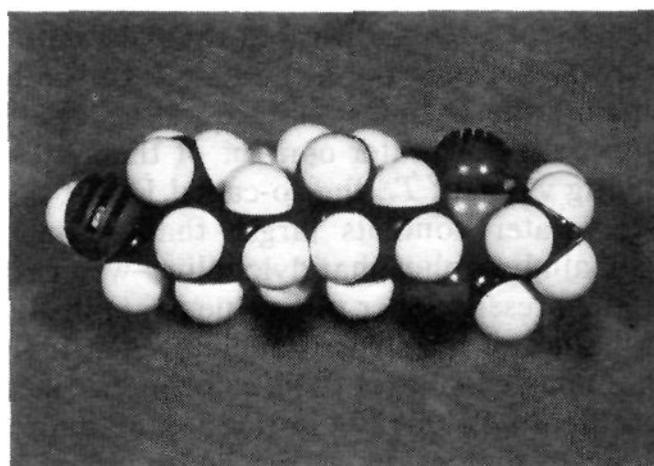


Figure 1a. Structure of the used steroid spin label molecule. Right side: chemical structure of the *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5α-androstan-3-one-17β-ol. The unpaired electron is located at the nitrogen atom of the N–O group in a 2pπ orbital. The more hydrophilic end of the molecule is determined by the OH group. Left side: definition of the principal axes system of the hyperfine coupling tensor **T**, assuming planarity of the oxazolidine ring. The long axis of the 2pπ orbital is perpendicular to the long axis of the steroid molecule (μ axis).



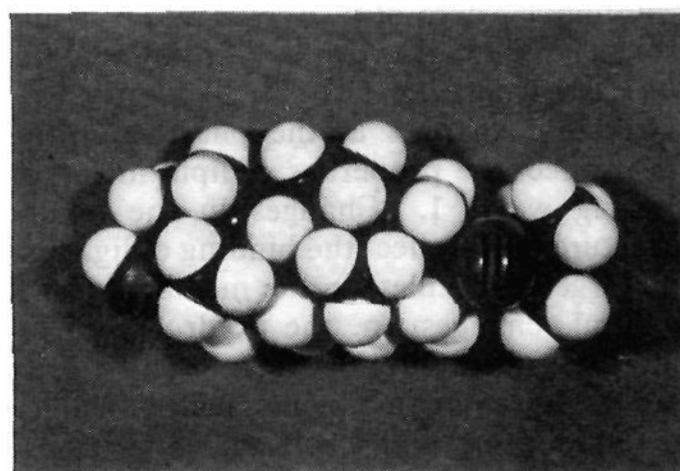


Figure 1b. Space filling model of the label molecules: (top) viewed parallel to the plane of the steroid nucleus (the long axis of the 2pπ orbital is perpendicular to the plane of the figure); (bottom) viewed in a direction normal to the plane of the steroid nucleus.

label concentration was kept as low as possible in order to avoid perturbations of the system and interactions between the label molecules.

In our experiments the spin labels may be considered as part of the system. In order to obtain information about the arrangement of the steroid molecules within the lipid matrix the label concentration was varied from very low values up to fairly high concentrations. With the onset of substantial label interaction the esr spectra, become rather complicated and an analysis of these spectra requires computer simulation of the measured spectra.

In the present paper (I) we report the experimental results of the optical and the esr measurements. The computer analysis of the esr spectra will be given in an accompanying paper (II). In a third paper (III) a theoretical model will be developed to explain the experimental findings.

2. Description of the Probe Molecules and Their Use

2.1. Optical Probes. (a) 8-Anilino-1-naphthalene sulfonate (ANS) was used as a conformational sensitive fluorescent probe.[28,47–49] This molecule has been used in recent years by several workers[48,50–52] as a conformational probe of protein and membrane structure.

As was shown by Stryer[48] and Turner and Brand,[53] the quantum yield Q of ANS is highly sensitive toward the polarity of its local environment and increases with decreasing polarity from $Q = 0.004$ in water to values close to 1 in highly apolar environment. When ANS is added to an aqueous dispersion of phospholipids it binds to the lipid material due to its hydrophobic ring system. Upon binding to dipalmitoyllecithin model membranes the quantum yield increases from $Q = 0.004$ in water to $Q = 0.08 \pm 0.02$.[24,54] The binding is accompanied by a shift of the maximum emission wavelength from $\lambda = 520$ nm in water to $\lambda = 485$ nm in the bound state. The value of $Q = 0.08$ is characteristic of a local environment with a dielectric constant of about $\epsilon \approx 40$ (*cf.* Stryer[48]). This indicates that the ANS molecules are buried within the polar head groups of the phospholipid structure and not within the hydrocarbon phase (ϵ approximately 2–4). Thus changes in the polar head group arrangement are expected to affect the binding of ANS, or, in other words, ANS is expected to be an indicator of changes in the polar head group arrangement.

(b) Bromthymol Blue (BTB), a pH indicator of a p*K* 7.2 was used as a conformational sensitive absorption indicator (*cf.* Chance and Mela[55]). Also this molecule binds to the lipid structure due to its aromatic ring system. In aqueous solution of pH 7 BTB has a molar extinction coefficient of $E_{615} = 1.4 \times 10^4$ at $\lambda = 615$ nm. Upon binding to the lipid structure the extinction coefficient is reduced to $E_{615} = 2.4 \times 10^2$. The same spectral changes are observed when BTB is dissolved in a medium with a dielectric constant of about $\epsilon \approx 30$. This indicates that BTB is buried within the lipid polar head groups and that BTB is a possible probe for changes in the polar head group arrangement.

2.2. Spin Label. An *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5α-androstan-3-one-17-β-ol was used as a spin label. This molecule was chosen because it is chemically and structurally similar to a series of important steroid hormones such as testosterone, androsterone, etc., and because it is incorporated easily into lipid structures. The chemical structure and a space-filling model of this molecule are shown in Figures 1. Figure

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1b demonstrates the approximately cylindrical shape of the molecule with a length of about 15 Å and a cross-sectional area of about 40 Å^2; the latter value is close to the molecular area of dipalmitoyllecithin molecules in the condensed state of monolayers or in bilayer structures.[21,36]

The paramagnetic center is the nitroxide group with an unpaired electron in a $2p\pi$ orbital localized at the nitrogen atom.[39,56] The long axis of the $2p\pi$ orbital is perpendicular to the long axis of the steroid molecule. The fine structure of the paramagnetic resonance spectrum is determined by the hyperfine coupling between the unpaired electron and the spin angular momentum I of the nitrogen nucleus ($I(^{14}N) = 1$). The component of I in the direction of the external field can take on three values, $I_H = +1, 0, -1$, andt herefore the esr spectrum is split into three lines.

In the simplest case of an isolated spin label molecule having a fixed orientation with respect to the applied field, the esr spectrum can be described quantitatively using the spin Hamiltonian[57]

$$\mathcal{H} = |\beta| \boldsymbol{S}\cdot\mathbf{g}\cdot\boldsymbol{H}_0 + h\boldsymbol{S}\cdot\mathbf{T}\cdot\boldsymbol{I} - \beta_N \boldsymbol{I}\cdot\mathbf{g}_N\cdot\boldsymbol{H}_0 \quad (1)$$

The first and the last term in this expression describe the action of the applied field H_0 on the electron spin angular momentum S and the nuclear spin angular momentum I. The second term represents the electron-nuclear hyperfine interaction characterized by the nuclear hyperfine tensor $\mathbf{T}$. $\mathbf{g}$ and $\mathbf{g}_N$ are the $\mathbf{g}$-factor tensors of the electron and of the ^{14}N nucleus; β and β_N denote the electron and the nuclear Bohr magnetons, respectively.

The components of the $\mathbf{g}$ and $\mathbf{T}$ tensors have been measured by Griffith, *et al.*,[58] and by Hubbell (*cf.* Hubbell and McConnell[44]). In the coordinate system of Figure 1a the values[59] are

$$(T_x, T_y, T_z) = (5.8, 5.8, 30.8 \pm 0.5)\ \text{G} \quad (2)$$

$$(g_x, g_y, g_z) = (2.0089, 2.0058, 2.0021 \pm 0.001)\ \text{G} \quad (3)$$

The $\mathbf{g}$ tensor determines the position of the central line (0) of the triplet spectrum whereas the $\mathbf{T}$ tensor determines the splitting of the spectrum. In the following the term "splitting" is used to denote the distance $T(+1/-1)$ in gauss between the $+1$ and -1 lines. Due to the high anisotropy of the $\mathbf{T}$ tensor the splitting is strongly dependent upon the orientation of the $2p\pi$ orbital with respect to the external field. The splitting is greatest (smallest) when the external field is parallel (perpendicular) to the z axis. The maximum (minimum) value of $T(+1/-1)$ is 62 (12) G.

In the next complicated case of a system of immobilized spin labels with an isotropic directional distribution, the resulting spectrum is a superposition of many spectra with different values of the splitting $T(+1/-1)$ and with different field positions of the central line due to the anisotropy of the g value. This leads to an asymmetric spectrum with an apparent splitting of 61 G (*cf.* McConnell and McFarland,[60] Figure 6).

In the presence of molecular motions of the spin labels, the spin Hamiltonian becomes time dependent and in the general case a quantitative evaluation of the spectra is rather complicated. An evaluation is, however, possible in the two limiting cases: (a) of very fast free (isotropic) rotation, and (b) of very fast anisotropic motion of the label molecules.

(a) "Very fast" free rotation is defined by the condition that the inverse of the correlation time τ for the molecular motion is large compared to $|T_z - T_x|$ ($\approx 7.10^8$ c/sec).[60] In this case one observes a sharp triplet spectrum with a reduced effective splitting $T(+1/-1)$ which is given by twice the trace of the $\mathbf{T}$ tensor.

$$a_H = {}^1/_2 T(+1/-1) = {}^1/_3(T_x + T_y + T_z) = 14.1\ \text{G} \quad (4)$$

Thus the observation of a sharp triplet spectrum with a splitting given by $2a_H$ indicates rapid isotropic motion of the labels.

(b) Very fast anisotropic motion. It was shown by McConnell[61] that in this case (characterized by a mean angular deviation of the rotation axes from an average rotation axis) an effective or time average Hamiltonian can be derived to describe the spectra. The observed spectra consist of a central line (0) and two pairs of side lines which are separated by $2T_\parallel$ and $2T_\perp$ where $T_\parallel$ and $T_\perp$ are the time averages of the $\mathbf{T}$ tensor along the directions parallel and perpendicular to the rotation axis. For a detailed discussion of this case the reader is referred to the papers by McConnell[61] and McConnell and McFarland.[60]

Concerning the incorporation and localization of the steroid molecules within the lipid structure, the following remarks are pertinent. (1) The steroid label has a very low water solubility (10^{-5}–10^{-6} mol/l.), whereas it dissolves readily in less polar (organic) solvents such as benzene or chloroform. (2) Amphiphatic steroids from which the label is derived spread as monolayers on air–water or oil–water interfaces with the hydrophobic steroid nucleus in the air (oil) phase and the hydrophilic OH group in the aqueous phase.[35,61] (3) According to Hubbell and McConnell[42] the androstane label, when it is incorporated into oriented lipid membranes, exhibits rotational motion preferentially about its long axis, whereas the same label without the OH group shows little or no anisotropy.

These data suggest that the steroid is intercalated between the hydrocarbon chains of the lipid molecules with the OH group "anchored" in the polar membrane–water interface. Possible conformations of the steroid label within a lipid monolayer are shown in Figure 2. A more detailed discussion of this point will be given in part III.

Since the five-membered nitroxide ring of the steroid label is rigidly linked to the steroid nucleus, a rotational motion of the label indicates that the entire molecule undergoes rotational motion. Thus the results can be correlated directly with the environmental viscosity.

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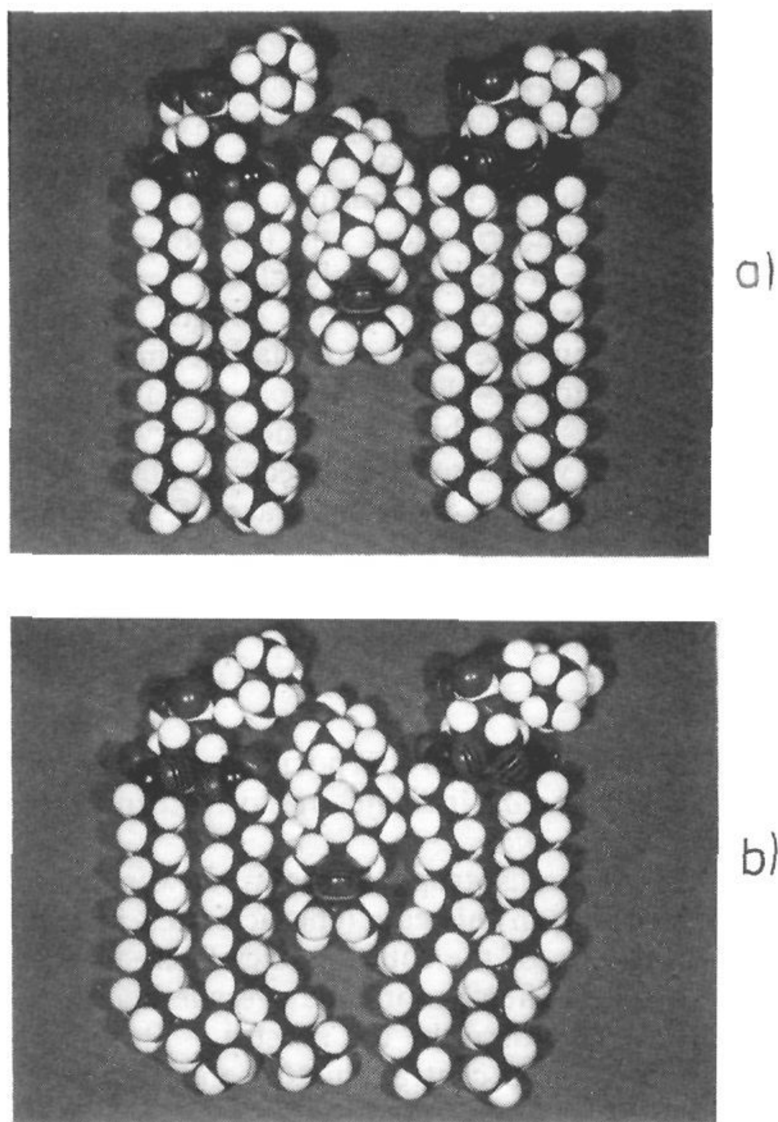

Figure 2. Steroid label molecule sandwiched between two lipid molecules. The polar hydroxide group is assumed to be anchored within the polar interface. (a) The hydrocarbon chains are considered as rigid rods ($T < T_t$); (b) in order to obtain better incorporation of the steroid nucleus (between the lipid molecules) rotational isomers (kinks) of the hydrocarbon chains have been formed[66] ($T > T_t$).

3. Materials and Methods.

3.1. Materials. Lecithin. Synthetic L-dipalmitoylphosphatidylcholine (mol wt 750) was obtained from Fluka and used without further purification. Thin layer chromatography[21] showed that this material contained approximately 97% dipalmitoylphosphatidylcholine. The impurities were lyso derivatives, palmitic acid, phosphatidic acid, and traces of unidentified material. The capillary melting point of this lecithin is 230°. According to calorimetric data by Ladbrooke and Chapman,[20] the transition temperature T_t of the dipalmitoylphosphatidylcholine–water system at excess water is $T_t \approx 41°$. Concerning the influence of cholesterol on the phase transition, Ladbrooke, *et al.*,[62] have shown that upon addition of cholesterol to the lecithin the transition curve becomes broad and the transition temperature T_t decreases and reaches a value of about $T_t \approx 30°$ at a cholesterol concentration of about 30 mol %. For higher concentrations of cholesterol the crystalline–liquid crystalline phase transition disappears. Upon addition of androstanol Ladbrooke and Chapman[20] observed only a small lowering of the transition temperature; androstanol evoked, however, an additional pretransition at a somewhat lower temperature.

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ANS (8-anilino-1-naphthalene sulfonate) was obtained from Pierce Chemical Co. as an ammonium salt and used without further purification.

BTB (Bromthymol Blue) was obtained from Merck. The measurements were performed at pH 7 using phosphate buffered solutions with an ionic strength of $J \approx 0.01$.

The **spin label** (an *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5α-androstan-3-one-17β-ol) was prepared by Dr. W. Kühnle[63] of this laboratory according to the method described by Keana, *et al.*[64]

3.2. Measurements. Esr spectra were recorded using a Varian 4502 spectrometer with a klystron as a source of microwave radiation operating at 9.5 MHz. A microwave power of about 5 mW was used to avoid saturation effects. Resonance absorption occurred at an external field strength of about 3000 G. First derivative spectra were recorded on a X-Y recorder with the *x*-axis driven by the Fieldial at a magnetic field sweep of 20 G/min. The microwave frequency was monitored with a Hewlett-Packard 5245/L 2590-A frequency counter and the static magnetic field H_0 was measured with an AEG gaussmeter. The samples were contained in sealed calibrated capillaries with an internal diameter of 1.1 mm. Sequences of spectra were recorded at increasing and decreasing temperatures. During a run the temperature was monitored by a small copper–constantan thermocouple to an accuracy of 0.2°. The recording of one spectrum took about 5 min. Once the spectrum was recorded the temperature was changed to a new fixpoint about 3° higher (or lower) with a heating (cooling) rate of 0.5°/min.

Optical Measurements. The absorption spectra were measured with a Cary 14 spectrophotometer; the temperature dependence of the optical absorption was recorded continuously using a Zeiss PMQ 2 spectrophotometer. An Aminco-Bowman spectrofluorimeter was used for the fluorescence measurements. Temperature dependencies were recorded continuously using a thermopile dipping into the cuvettes. The temperature was changed with a rate of 1°/min. For the evaluation of the titration measurements the fluorescence signal was corrected for inner filter effects arising from the ANS absorption and the light scattering of the lipid vesicles.

3.3. Preparation of the Lipid–Water Model Systems and Incorporation of the Spin Label Molecules into These Structures. Two methods were used for the preparation of lipid–water model systems. Most measurements were made with aqueous solutions of lipid monolayer vesicles containing an inner organic phase (three-component system). A few comparative measurements were made with aqueous dispersions containing bimolecular lipid lamellae (two-component system).

1. Preparation of Lipid Monolayer Vesicles (*cf.* Träuble and Grell[65]). The lecithin and the spin label molecules were dissolved in a mixture of organic solvents with a density of 1. A droplet of this solution was dispersed ultrasonically (power input 70 W) in 2

(63) We are very much indebted to Dr. W. Kühnle for the preparation of the spin label.

(64) J. F. W. Keana, J. B. Keana, and D. Beetham, *J. Amer. Chem. Soc.*, **89**, 3055 (1967).

(65) H. Träuble and E. Grell in "Neurosciences Research Report on Carriers and Specificity in Membranes," Vol. 9, M. Eigen and L. DeMaeyer, Ed., MIT Press, Cambridge, Mass., 1971, p 373.

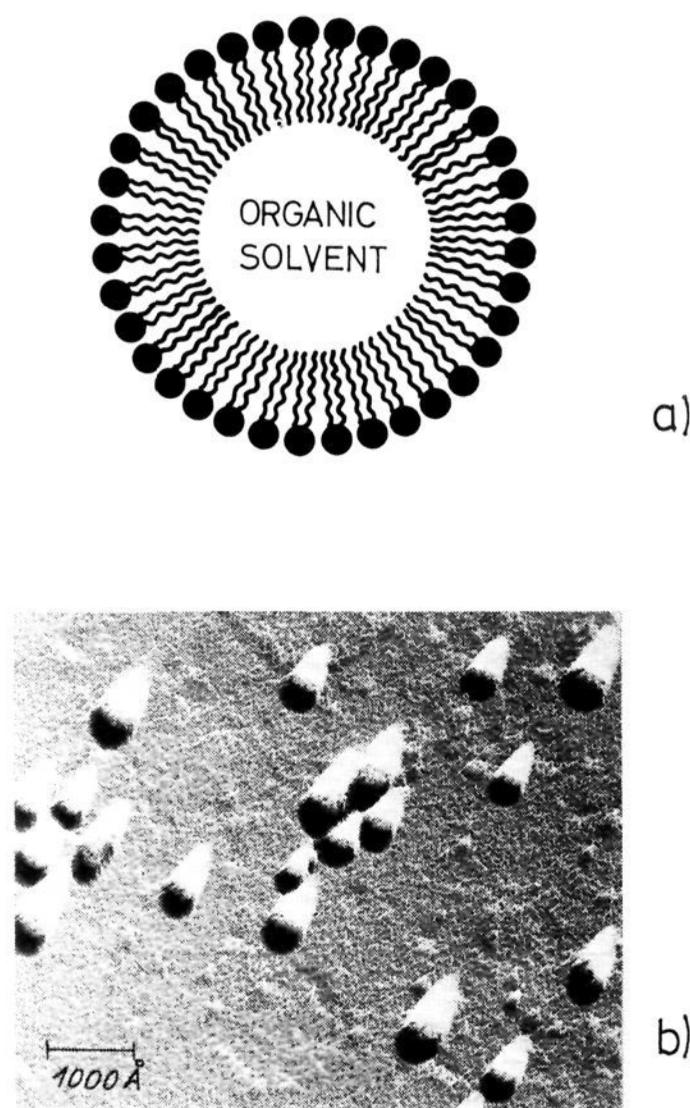

Figure 3. Lipid monolayer vesicles. (a) Schematic drawing. (b) Electron microscope picture; shadow-casting with Ge. The picture demonstrates the presence of spherical vesicles with an average diameter of about 500 Å. The vesicles remain stable during the drying on the EM grid. The high contrast of the vesicles arises from the inner organic phase.

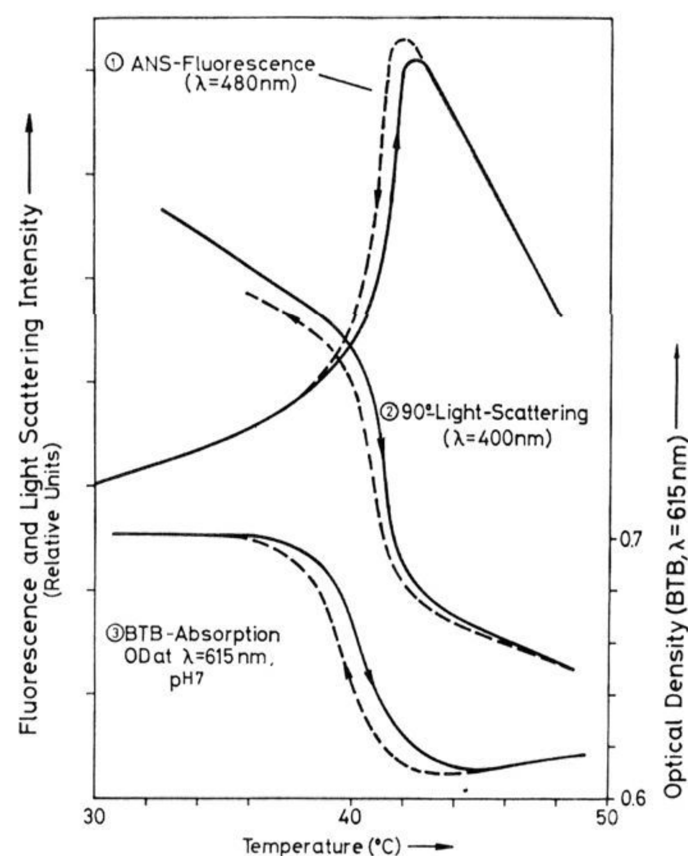

Figure 4. Optical demonstration of the lipid-phase transition using 8-anilino-1-naphthalene sulfonate (ANS) as a fluorescence probe (curve 1), Bromthymol Blue (BTB) as an absorption indicator (curve 3), and 90° light-scattering measurements. The measurements were carried out with sonicated dipalmitoyllecithin dispersions: lecithin content, 2×10^{-4} M, pH 7; ANS concentration, 2×10^{-5} M; BTB concentration, 5×10^{-5} M. A sharp increase in the ANS fluorescence is observed at the phase transition. This effect is mainly due to an increase in the number of bound ANS molecules. The phase transition leads to a sharp decrease in 90° light scattering and in the BTB absorption. The values of the transition temperature T_t deduced from the fluorescence and light-scattering measurements agree exactly with the results of calorimetric studies.[21]

mM CsCl at 45° for about 10 min. In a typical preparation 50 mg of dipalmitoyllecithin and 2 mg of steroid label were dissolved in 0.5 ml of benzene–chloroform; an aliquot of 0.2 ml of this solution was dispersed in a 20-ml aqueous phase. It was shown by electron microscopy that the solution contained spherical vesicles with an average diameter of about 500 Å (*cf.* Figure 3b). With these vesicles an inner organic phase is enclosed by a lipid monolayer with the lipid polar head groups directed toward the aqueous phase (*cf.* Figure 3a). Good preparations are obtained only when the lipid material and the volume of the organic phase are in a correct relation to match the volume and surface requirements of vesicles with a diameter of about 500 Å. The observed esr spectra indicate that with this procedure almost all the label molecules are incorporated into the lipid monolayers (*cf.* Figure 5).

2. Preparation of Aqueous Lipid Dispersions. The lipid and the steroid molecules were dissolved in a benzene–chloroform mixture. The solvent was evaporated in a nitrogen atmosphere to produce a thin film on the wall of a glass tube. Water was added and the sample was dispersed ultrasonically for about 10 min at 45° with an energy supply of 70 W. In a typical preparation 20 mg of lecithin and 3 mg of steroid label were dissolved in 0.4 ml of benzene–chloroform; then 20 ml of 2 mM CsCl was added for the ultrasonic irradiation. As reported by Barratt, *et al.*,[46] with this procedure not all of the label molecules are incorporated into the lipid structure. This is apparent from the observation of an additional narrow spectrum superimposed upon the relatively broad spectrum of the incorporated label molecules. This narrow spectrum is attributed to small aggregates of label molecules tumbling very rapidly in the bulk aqueous phase. Apart from this effect we obtained qualitatively the same esr spectra with lipid dispersions as with monolayer vesicle preparations.

For the esr measurements the solutions were concentrated to a final volume of about 2 ml corresponding to a lipid concentration of about 1.4×10^{-2} M; for the optical measurements much lower lipid concentrations of about 10^{-4} M lipid were used.

4. Results and Discussion.

4.1. Optical Measurements. The optical indicators (ANS and BTB) were added to the lipid model systems (without labels) after the preparation. As shown in Figure 4 the phase transition is revealed by a sharp increase in the ANS fluorescent signal and by a decrease in the BTB absorption. The observed transition temperature of $T_t \approx 40°$ is in good accord with calorimetric data by Ladbrooke and Chapman[20] for the same dipalmitoylphosphatidylcholine–water system at high water content.

Titrations of the lipid–water system with indicator molecules were carried out at 25 ($T < T_t$) and 45° ($T > T_t$). The evaluation of these measurements using Scatchard plots showed that the changes in optical properties observed upon heating the system above

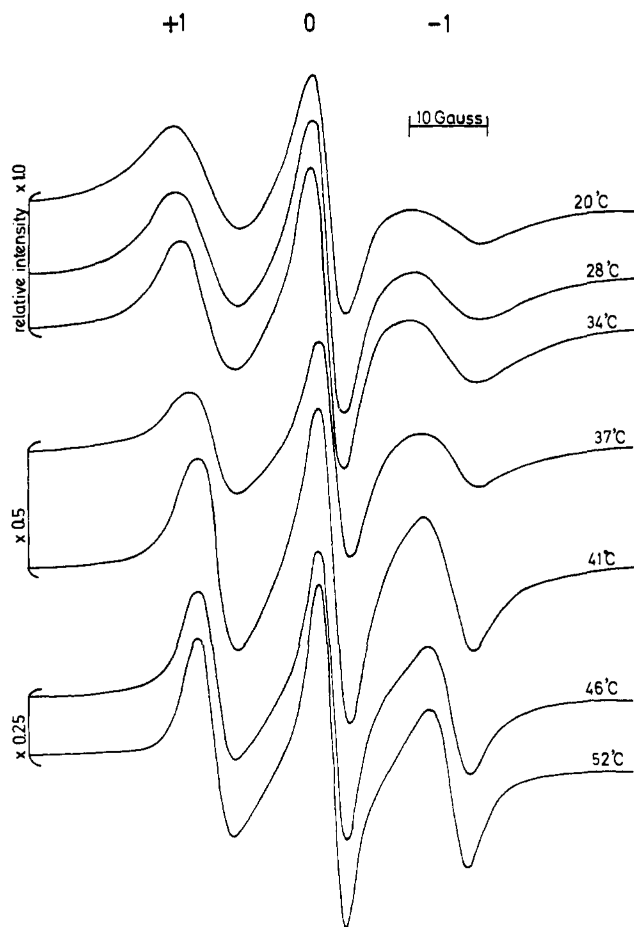

Figure 5. First-derivative esr spectra of the steroid label buried within the lipid phase of monolayer vesicles. The lipid-phase transition occurs between 30 and 40°. The label to lipid molar ratio was c = 0.01 corresponding to an average distance $d_{la} \approx 75$ Å between neighboring label molecules; low label concentration, negligible interaction between the label molecules. Increasing temperature produces a sharpening of the three lines of the spectrum. The three lines are denoted by the corresponding magnetic quantum numbers (+1, 0, −1) of the nitrogen nuclear spin in the magnetic field. The static magnetic field increases from left to right. Note the different ordinate scales.

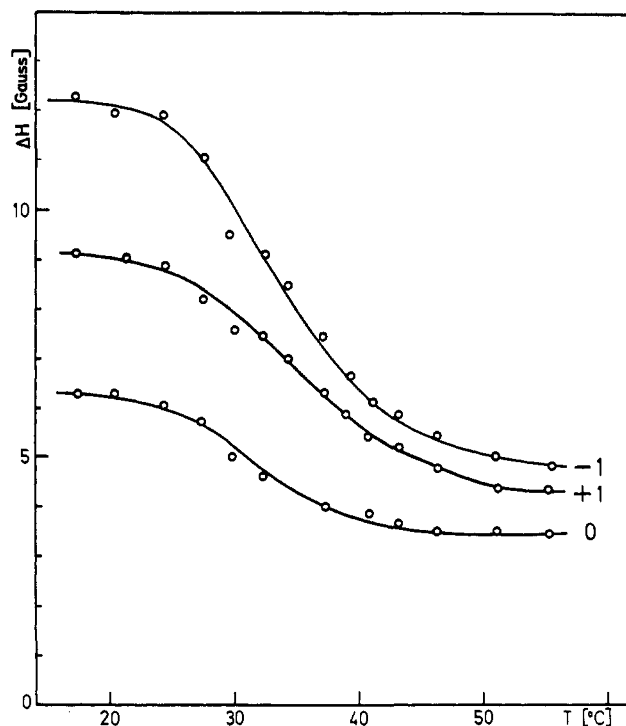

Figure 6. Temperature dependence of the line widths ΔH of the three lines (+1, 0, −1) of the triplet spectra in Figure 5. The distance between the maximum and minimum of each line was taken as a measure of the line width. The lipid-phase transition produces a sharp decrease in the line width ΔH.

T_t are mainly due to an increase in the number of binding places on the membrane surface.[24] The calculated numbers of binding places per hundred lipid molecules are, in the case of BTB, n = 7.7 at 25° and n = 20 at 45°, and, in the case of ANS, n = 2.5 at 25° and n = 6.7 at 45°. This indicates that the polar groups are more loosely packed above the phase transition. This conclusion is in good accord (a) with the previously reported expansion of the membrane surface by about 20% when the system is heated above the phase transition (*cf.* Träuble and Haynes[66]) and (b) with the increase in rotational freedom of the polar head groups at T_t observed in proton resonance measurements of the same system.[25]

4.2. Esr Measurements. Esr spectra were recorded for different label concentrations in the temperature range between 18 and 55° which covers the region of the lipid phase transition. Five samples with a label to lipid molar ratio between c = 0.01 and c = 0.27 were used. Table I summarizes the values of the label to lipid molar ratios of our specimens and the corresponding values of the average next-nearest-neighbor distances d_{la} between the label molecules calculated on the assumption that the steroid molecules are distributed statistically within the lipid matrix. In the case of the lowest label concentration (c = 0.01) the average distance d_{la} is large enough ($d_{la} \approx 75$ Å) to neglect interactions between the label molecules. For the higher label concentrations ($c > 0.03$) interactions between the label molecules become important. The esr spectra for these two cases are shown in Figures 5 and 7, respectively.

(66) H. Träuble and D. H. Haynes, *Chem. Phys. Lipids*, 7, 324 (1971).

Table I. Label to Lipid Molar Ratios c for Five Different Preparations[a]

No. of prepn	Molar ratio c label:lipid	d_{la}, Å[b]
a	0.01	74.8
b	0.035	40.6
c	0.075	28.2
d	0.13	22.0
e	0.27	16.1

[a] The corresponding next-nearest-neighbor distances d_{la} were calculated assuming a statistical distribution of the label molecules within the two-dimensional lattice of the lipid molecules (*cf.* part III, eq 5). According to Pethica (*cf.* part III) the hydrocarbon chains of the lipid matrix form a triangular lattice with a lattice constant a. The molecular area F per lipid molecule is then given by $F = a^2\sqrt{3}$. F was taken equal to 48 Å^2 according to low-angle X-ray studies by Ladbrooke and Chapman.[20] [b] Next-nearest-neighbor distance between label molecules (la subscript = label).

(A) Low Label Concentration (c = 0.01), Negligible Interaction between the Label Molecules. In the whole temperature range between 20 and 52° relatively sharp

triplet spectra are observed (Figure 5). The three lines of these spectra are characterized in the following by the corresponding quantum numbers (+1), (0), (−1) of the nitrogen nucleus. The differences in the shape of the two side bands (+1), (−1) arise from the anisotropy of both the nuclear hyperfine coupling tensor **T** and the **g** tensor of the nitroxide radical.

As demonstrated by Figure 5, raising the temperature produces a narrowing of all three lines. The temperature dependence of the line widths ΔH is plotted in Figure 6. The widths of the three lines (+1), (0), (−1) decrease from H = (9.0), (6.3), (12.0) G at 20° to H = (4.3), (3.4), (4.8) G at 52°. The line widths decrease sharply between about 30 and 40° which is the temperature range of the crystalline–liquid crystalline phase transition. As is seen in Figure 5 in this temperature range the asymmetry of the spectrum is sharply reduced with increasing temperature indicating a sharp increase in the tumbling rate of the label molecules. A similar observation has been reported by Hubbell and McConnell.[44]

The correlation time τ characteristic of the tumbling motion of the steroid molecules[67,68] will be estimated in part II as $\tau \approx 10^{-8}$ sec at 20°. Upon heating the samples above T_t the value of τ decreases by a factor 3–4.

The spectra in Figure 5 may be compared with the two limiting cases (a) of free isotropic rotation of spin labels (label molecules dissolved in an organic solvent), and (b) of fast anisotropic rotation of spin labels (fatty acid labels within lipid bilayer structures: Hubbell and McConnell,[44] Figure 2; McConnell and McFarland,[60] Figures 8a and 11).

It is clear from such a comparison that our spectra are intermediate between these two cases, and at least for $T > T_t$ they are closer to the case of isotropic rotation. This is supported by the fact that the observed splitting $T(+1/-1)$ = 32 G is almost identical with twice the isotropic hyperfine coupling constant a_H = 14.1 G (*cf.* eq 4).

This compares well with studies by Hubbell and McConnell.[42] These workers observed a nearly isotropic motion of the androstane label incorporated in phosphatidylserine vesicles. A highly anisotropic motion of the androstane label has been observed, however, in the case of the Homarus walking-leg nerve fiber. Hubbell and McConnell conclude that the membranes of this system exhibit a rather rigid semipolar surface due to the presence of acidic phospholipids which may be linked together by salt bridges.

Highly anisotropic molecular motions of long labeled fatty acids in lipid structures have been observed by several workers.[43,60,69] This is, however, not surprising because the hydrocarbon chains of the fatty acids can align much better along the hydrocarbon chains of the lipid molecules than the steroid molecules and therefore the fatty acids perform rotational motions preferentially about the long axes of the molecules.

The near isotropy of our spectra may be explained along the following lines.

(1) As demonstrated by Figure 2a fairly large pockets of free volume are created at places where the steroid nucleus is incorporated into the lipid matrix, when it is assumed that the hydrocarbon chains behave as rigid rods ($T \ll T_t$). Even in the case of higher mobility of the hydrocarbon chains ($T > T_t$) small pockets of free volume may be present. Therefore the steroid nucleus is expected to have a high degree of motional freedom and deviations of the steroid axis from the mean direction of the hydrocarbon chains may well occur.

(2) It has been demonstrated by Seelig[43] and Rottem, *et al.*,[70] that the degree of disorder within the hydrocarbon phase increases exponentially with increasing distance from the layer surface. Since the nitroxide group of the steroid molecule is buried relatively deep (≈12 Å) within the hydrocarbon phase of the lipid membrane, we expect a high degree of molecular tumbling for the paramagnetic end of the molecule.

(3) In contrast to labeled fatty acids, the 2pπ orbital of the steroid label is oriented perpendicular to the long axis of the molecule. Therefore a rotation about the long axis of the molecule has a large averaging effect. This shows that the observation of nearly isotropic spectra does not exclude an anisotropic motion of the steroid molecules.

In the accompanying paper II it will be shown that the spectra of Figure 5 can be described very well as a superposition of three Lorentzian lines which are separated by distances given by the isotropic coupling constant a_H.

(B) High Label Concentration (c = 0.035–0.27), Interactions between the Label Molecules Important. Figure 7 shows the esr spectra of the samples with high label concentrations in the temperature range between 18 and 55°. Below the phase transition, at 20°, a gradual broadening of the spectra is observed with increasing label concentration; the triplet spectrum observed at low c values (Figure 7.1) goes over into a broad line with a line width of about 20.5 G for $c \approx$ 0.27 (Figure 7.4).

Heating the samples above the phase transition produces a dramatic change in the shape of the spectra. This effect is seen most clearly in Figure 7.4 for the highest label concentration. In the region of the crystalline–liquid crystalline phase transition between 30 and 40°[71] the broad line spectrum ($T < T_t$) resolves into a triplet spectrum ($T > T_t$). The sharpness of the lines increases with further increasing temperature. The same characteristic behavior is observed for the lower label concentrations, with the difference that these spectra show some fine structure already at low temperature.

Above the phase transition sharp triplet spectra are observed in all cases; however, the width of the central line increases with increasing label concentration ($\Delta H(0)$ = 3.4 G for c = 0.01; $\Delta H(0)$ = 5.5 G for c = 0.27).

The observed broadening of the esr spectra with increasing label concentration will be explained in part II as a result of the interaction between the label molecules. Two interaction mechanisms have been dis-

(67) D. Kivelson, *J. Chem. Phys.*, 33, 1094 (1960).
(68) G. E. Pake, "Paramagnetic Resonance," W. A. Benjamin, New York, N. Y., 1962.
(69) W. L. Hubbell in "Neurosciences Research Report on Carriers and Specificity in Membranes," Vol. 9, M. Eigen and L. DeMaeyer, Ed., MIT Press, Cambridge, Mass., 1971, p 357.
(70) S. Rottem, W. L. Hubbell, L. Hayflick, and H. M. McConnell, *Biochim. Biophys. Acta*, 219, 104 (1970).
(71) The transition temperature T_t decreases somewhat with increasing steroid content. This effect parallels the decrease in T_t when cholesterol is added to the lecithin.[20]

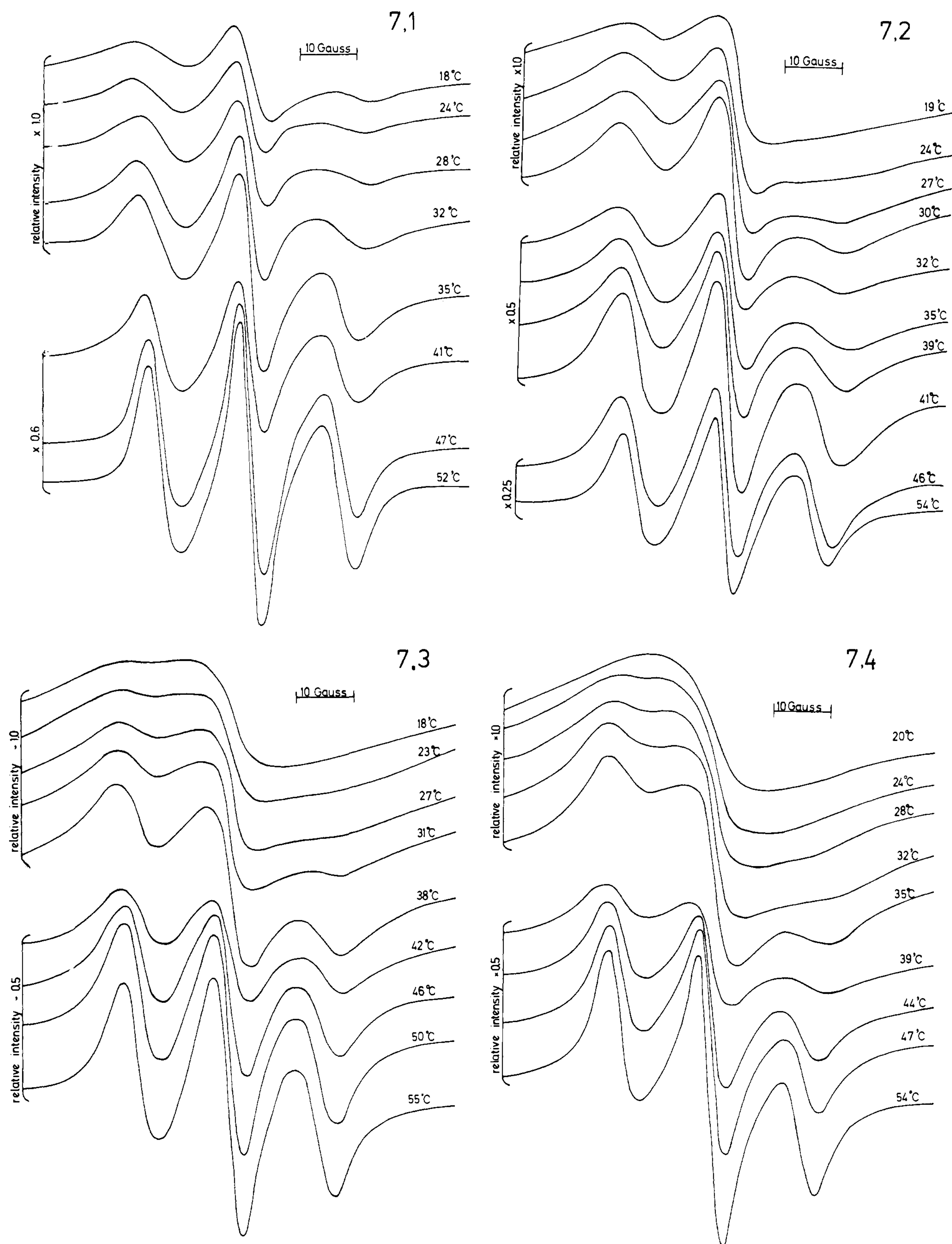

Figure 7. First-derivative esr spectra of the steroid label buried within the lipid phase of monolayer vesicles. Temperature dependence of esr spectra at different label concentrations. Figures 7.1–7.4: increasing label concentration c (c = label:lipid molar ratio) or decreasing average distance d_{la} between the label molecules. Figure 7.1: $c = 0.035, d_{la} = 40.6$ Å; Figure 7.2: $c = 0.075, d_{la} = 28.2$ Å; Figure 7.3: $c = 0.13, d_{la} = 22.0$ Å; Figure 7.4: $c = 0.27, d_{la} = 16.1$ Å. The steroid concentration is high enough that interactions between the label molecules play a role. For $T < T_t$ a gradual broadening of the spectra is observed with increasing label concentration; for $c = 0.13$ the spectrum has collapsed into one broad line. Upon heating the system above the phase transition the broad spectra are resolved into triplet spectra. This effect is reversible. Left to right: increasing strength of the external magnetic field.

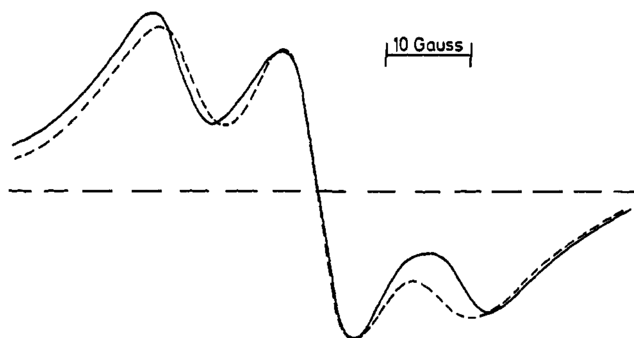

Figure 8. Comparison between an experimental spectrum (—) taken from Figure 7.3 (c = 0.13, T = 38°) and a computer simulated spectrum (---). The theoretical curve was calculated from a Bloch equation modified by a term for the exchange interaction between the label molecules. The general shape and much of the fine structure of the experimental curve are contained in the theoretical curve.

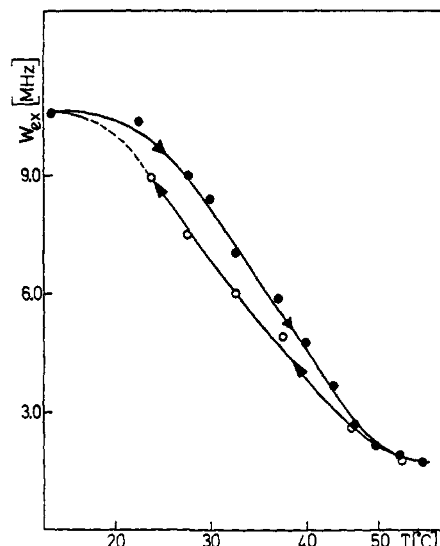

Figure 9. Temperature dependence of the exchange frequency W_{ex} calculated from the spectra in Figure 7.3 (c = 0.13). W_{ex} decreases sharply in the temperature range of the lipid-phase transition. A small hysteresis is observed between the two branches measured at increasing and decreasing temperature.

cussed in the literature as possible reasons for a esr line broadening (*cf.* Pake[68]): (a) the magnetic dipole–dipole interaction and (b) the spin-exchange interaction between neighboring molecules.

A quantitative evaluation of our spectra in terms of these two mechanisms requires a computer simulation of the measured spectra. This analysis, to be described in part II, shows that the general shape of the spectra is determined essentially by the spin exchange mechanism. This is illustrated in Figure 8 which shows a measured spectrum taken from Figure 7.3 (c = 0.13, T = 38°) together with a computer simulated spectrum which was calculated taking into account the exchange mechanism only. The computer simulation and the curve fitting was based on a modified Bloch equation including exchange terms (*cf.* McConnell[67]). As a measure of the exchange interaction we have used the exchange frequency W_{ex} which denotes the probability of spin exchanges between the interacting molecules per second.

The calculated and the measured spectra differ somewhat in the positions of the maxima and minima of the side bands. As will be shown in part II, the fit can be improved by taking into account also the magnetic dipole–dipole interaction. Only small values of the dipole–dipole interaction are necessary to improve the fit. The values of W_{ex} determined under the assumption of exchange interaction only are about 15% too large. Thus, to a first approximation, the spectra of Figure 7 can be evaluated on the basis of exchange interaction only. The values of W_{ex} calculated in this way are plotted in Figure 9. This figure shows the temperature dependence of W_{ex} for a relatively high label concentration (c = 0.13). The most important feature of this curve is the sharp decrease of W_{ex} in the temperature range of the crystalline–liquid crystalline phase transition. To our knowledge a decrease in W_{ex} with increasing temperature has not been observed so far with other systems.

The spin-exchange interaction has been studied by Plachy and Kivelson[72] for solutions of di-*tert*-butyl nitroxide in *n*-pentane and propane. An increase of W_{ex} with increasing temperature and decreasing viscosity was observed. Similar results were reported by Powles and Mosley[73] for solutions of biphenyl ions in diethylene glycol dimethyl ether over the temperature range from −90 to +70°. The increase in W_{ex} with temperature observed in these studies can be understood on the basis of a diffusional theory proposed by Pake and Tuttle[74] (*cf.* part III).

In this theory the frequency of collisions between two radicals leading to exchange interaction is calculated on the basis of a diffusional model. Proportionality is obtained between W_{ex} and T/η where η denotes the solvent viscosity.

Application of this model to our case would mean that the viscosity of the system increases with increasing temperature. This is quite unreasonable. It is more likely to assume that the sharp decrease in W_{ex} in the temperature range of the crystalline–liquid crystalline phase transition is related to a radical change in the organization of the steroid–lecithin system. In part III of this series a theoretical model will be developed for these structural changes.

Acknowledgment. The authors would like to thank Dr. W. Kühnle for his help with chemical problems. We are further indebted to Miss I. Otto for experimental assistance.

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(73) J. G. Powles and M. H. Mosley, *Proc. Phys. Soc., London*, **78**, 370 (1961).
(74) G. E. Pake and T. R. Tuttle, *Phys. Rev. Lett.*, **3**, 423 (1959).