Molecular Architecture of Liquid Crystalline Bilayers[1,2]

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Abstract: The short-range order of liquid crystalline bilayers is investigated with paraffinic spin labels bearing different polar groups. The observed polarity profile of the hydrocarbon layer is attributed to electrostatic interactions between the nitroxide dipole and the dipolar regions of the bilayer. The molecular ordering of the hydrocarbon chains depends significantly on the type of polar group and decreases in the order $COOH > OH > COOCH_3 \approx CH_2OOCCH_3 > CH_3$. The intrinsic flexibility of the hydrocarbon chains is also determined by the polar groups; it can be distinguished between stiff paraffinic chains which are structural elements of the liquid crystalline state and liquid-like chains which are merely dissolved in the hydrocarbon layer. The parallel alignment of the hydrocarbon chains in the bilayer is distorted if the length of the spin-labeled molecule exceeds the thickness of the bilayer. In the case of dicarboxylic acid labels the molecules can occur in an extended or a folded configuration. The ordering of the spin-labeled molecules shows a distinct chain-length dependence. The order parameter increases with increasing chain length, reaching an upper limit at a total chain length of about 10 to 12 carbon atoms. The spin-label data can also be used to determine the thickness of the liquid crystalline bilayer.

Lyotropic mesophases (*e.g.*, soap–water systems[4] or phospholipid–water systems[5]) have received much attention as model systems for biological membranes. It is assumed that the aggregation of the amphiphilic molecules into a variety of different structures is of significance for the function of natural membranes. X-Ray diffraction has provided detailed information about the dimensions and overall geometry of such lyotropic mesophases, but "we have little reliable knowledge of the arrangement of the molecules within the aggregates."[6,7]

McConnell and coworkers and several other groups have shown that the spin-label technique is a suitable means for the investigation of biological membranes.[8] In previous studies, therefore, we have used this method to gain insight into the short-range order of smectic liquid crystals of lamellar structure, which can be regarded as the simplest form of a bilayer membrane. Using spin-labeled fatty acids we have obtained quantitative results on essentially two aspects of the physical state of the bilayer, namely (1) the rotational mobility of the hydrocarbon chains and (2) the preferential orientation and the flexibility of the carbon skeleton.[9,10] We have now synthesized various new spin labels and have also extended our investigation to a series of different lyotropic phases, including phospholipid dispersions, so that we can provide a more sophisticated picture of the bilayer structure. We wish to report experimental results on (1) the polarity profile of liquid crystalline bilayers, (2) the influence of the polar groups, (3) the influence of the bilayer thickness, and (4) the effect of the chain length on the orientation of the lipid molecules. Furthermore, we wish to show how the spin-label technique can yield information about the dimensions of the bilayer.

(1) A preliminary report has been given at the Symposium of Passive Transport through Cell Membranes, Rotterdam, 1971; "Biomembranes," L. A. Manson, Ed., Plenum Press, New York, N. Y., 1972, in press.

(2) Work supported by the Swiss National Science Foundation under Grant No. 3.131.69.

(3) Predoctoral fellow of the Swiss National Science Foundation.

(4) P. Ekwall, L. Mandell, and K. Fontell in "Liquid Crystals 2," Part II, G. H. Brown, Ed., Gordon and Breach, New York, N. Y., 1969, p 325.

(5) V. Luzzati in "Biological Membranes," D. Chapman, Ed., Academic Press, New York, N. Y., 1968, p 71.

(6) Reference 4, p 343.

(7) G. H. Brown, J. W. Doane, and V. D. Neff, "Structure and Physical Properties of Liquid Crystals," Butterworths, London, 1971, p 70.

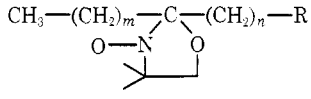
(8) B. G. McFarland and H. M. McConnell, *Proc. Nat. Acad. Sci. U. S.*, 68, 1274 (1971), and references cited therein.

(9) J. Seelig, *J. Amer. Chem. Soc.*, 92, 3881 (1970).

(10) J. Seelig, *ibid.*, 93, 5017 (1971).

I. Spin Labels

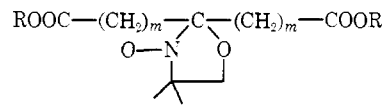
The spin labels used in this investigation are of the following general structure.



The various spin labels are summarized in Table I. Most of the spin labels are derivatives of stearic acid [I(*m*,*n*)] or of the corresponding methyl or ethyl esters [II(*m*,*n*)]. The position of the label group on the hydrocarbon chain is varied systematically in order to probe the different regions of the bilayer. The influence of the polar group has been studied by preparing spin-labeled stearyl alcohols [III(*m*,*n*)], stearyl acetates [IV(*m*,*n*)], and long-chain paraffins [V(*m*,*n*)].

In another set of experiments the effect of the chain length has been investigated. The label group was positioned two carbon–carbon bonds apart from the polar group [I(*m*,2), II(*m*,2)], and the length of the hydrocarbon tail was varied. The total chain length increased from 5 to 18 carbon atoms.

Finally, we have also synthesized spin labels carrying two polar groups with the following symmetrical structure.



VI(*m*,*m*), R = H
VII(*m*,*m*), R = C_2H_5
m = 6, 7

Chemical Synthesis. The starting materials for the synthesis of the labels II(*m*,*n*) are the methyl or ethyl esters of the corresponding keto carboxylic acids. The latter are prepared from commercially available dicar-

Table I

General notation	End group, R	Spin labels (*m*,*n*) Chain length: 18 C atoms	Varying chain length
I(*m*,*n*)	COOH	I(*m*,*n*) + II(*m*,*n*): (13,2),[9] (12,3),[9] (11,4), (10,5), (9,6),[9] (8,7), (7,8), (6,9), (5,10),[12b] (4,11), (3,12)	I(*m*,*n*): (0,2),[12c] (1,2), (2,2), II(*m*,*n*): (4,2), (7,2), (9,2)
II(*m*,*n*)	$COOCH_3$ $COOC_2H_5$		
III(*m*,*n*)	CH_2OH	III(*m*,*n*) + IV(*m*,*n*): (13,2), (11,4), (10,5), (6,9), (3,12)	
IV(*m*,*n*)	CH_2OOCCH_3		
V(*m*,*n*)	CH_3	V(14,1)	Chain length: 10 atoms V(7,0), (6,1), (5,2)

boxylic acids according to the method of Jones[11] and can be purified either by vacuum distillation or by recrystallization from *n*-pentane. The keto group is converted into the spin-label group according to the procedure of Keana.[12] Saponification of II(*m*,*n*) in methanol yields I(*m*,*n*). Labels III(*m*,*n*) and IV(*m*,*n*) are prepared as follows. The starting materials are again the methyl or ethyl esters of the corresponding keto carboxylic acids. The keto group is protected by ketalization with triethyl orthoformate, and the keto esters are reduced with lithium aluminum hydride to keto alcohols. The protecting groups are removed with concentrated HCl at 80°, and the resulting long-chain keto alcohols are esterified with acetic anhydride. Oxazolidine formation and oxidation with *m*-chloroperbenzoic acid lead to IV(*m*,*n*) and saponification to III(*m*,*n*). Labels V(*m*,*n*) are readily prepared from commercially available ketones (Fluka Ltd., Switzerland) according to ref 12. Labels VII(6,6) and VII(7,7) are synthetized from diethyl 1,15-pentadecan-8-onedicarboxylate and diethyl 1,17-heptadecan-9-onedicarboxylate again using the Keana method. The preparation of the keto dicarboxylic acids by the thermal decomposition of lead salts of appropriate dicarboxylic acids has been described by Kenner and Morton.[13] Saponification of VII(*m*,*n*) yields VI(*m*,*n*).

II. Liquid Crystalline Systems

The chemical composition of four different liquid crystalline model systems used in this study is given in Table II. Following the terminology of Brown, Doane, and Neff[7] we have denoted these phases L_1, since the molecules are arranged into a lamellar structure with coherent double layers of amphiphilic molecules separated by layers of water. Phase L_{1a} has already been used in our previous work.[14] The complete phase diagrams of the ternary mixtures L_{1b}, L_{1c}, and L_{1d} have been reported by Ekwall and coworkers.[4,15] The essential difference between phases L_{1a} and L_{1b} is the length of the amphiphilic molecules, which have a chain length of ten carbon atoms in phase L_{1a} and eight carbon atoms in phase L_{1b}. The double layer of phase L_{1b} is therefore a few ångströms shorter than that of phase L_{1a}. The two other systems of Table II have been chosen in order to study the influence of the water content on the molecular architecture of the double layer. The thickness of the interplanar water region increases from approximately 10 Å (L_{1c}) to 30 Å (L_{1d}). Also included in this study are a few results with dispersions of phospholipid bilayers which were prepared by sonicating purified egg-yolk lecithin[16] in 0.1 *N* KCl/0.01 *M* Tris–HCl buffer at pH 8.5 (~30 mg of lecithin/ml of buffer).

Table II

Phase	Water, wt %	Sodium decanoate, wt %	Decanol, wt %
L_{1a}	30	28	42
		Sodium caprylate, wt %	Caprylic acid, wt %
L_{1b}	30	35	35
		Sodium caprylate, wt %	Decanol, wt %
L_{1c}	25	38	37
L_{1d}	50	14	36

III. Experimental Section

The liquid crystalline phases were doped with a small amount of spin label (~0.1 mg of label/g of liquid crystalline material), and the bilayers were then oriented homogeneously in a flat rectangular quartz cell (spacing of the large surfaces 0.03 mm) as described previously.[9,10] Epr spectra were recorded with the orientation of the magnetic field parallel and perpendicular to the normal of the large cell surface. All measurements were performed at room temperature (~22°) on a Varian E-3 spectrometer. The quantities which can be measured directly from the epr spectrum are the hyperfine splittings $T_\parallel$ and $T_\perp$ which are the splittings observed when the magnetic field is parallel and perpendicular, respectively, to the normal of the large cell surface. Since the bilayer planes are oriented parallel to this surface, the direction of the normal is, to a first approximation, identical with the direction of the long molecular axes of the lipid molecules with the bilayers. From $T_\parallel$ and $T_\perp$ the isotropic hyperfine coupling constant a_N and the degree of order S_3 can be calculated.[9] The degree of order S_3 is defined by the following equation

$$S_3 = (1/2)(3\langle\cos^2\theta_3\rangle - 1) \quad (1)$$

where $\langle\cos^2\theta_3\rangle$ is a measure of the mean angular deviation of the nitrogen $2p\pi$ orbital from the reference axis, *i.e.*, the normal of the bilayer plane In a completely rigid hydrocarbon chain the nitrogen $2p\pi$ orbital is extended parallel to the long axis of the labeled molecule. Therefore the order parameter S_3 also yields

(11) R. G. Jones, *J. Amer. Chem. Soc.*, **69**, 2350 (1947).
(12) (a) J. W. Keana, S. B. Keana, and D. Beetham, *ibid.*, **89**, 3055 (1967); (b) A. D. Keith, A. S. Waggoner, and O. H. Griffith, *Proc. Nat. Acad. Sci. U. S.*, **61**, 819 (1968); (c) W. Balthasar, unpublished results.
(13) J. Kenner and F. Morton, *Ber.*, **72**, 456 (1939).
(14) The system sodium decanoate–decanol–water has not been investigated by Mandell, *et al.*, as was stated erroneously in ref 9. These authors have studied the system sodium caprylate–decanol–water. Our experiments show, however, that in both systems the lamellar phases L_I have a similar range of existence.
(15) L. Mandell, K. Fontell, and P. Ekwall, *Advan. Chem. Ser.*, No. 63, 89 (1967).
(16) W. S. Singleton, M. S. Gray, M. L. Brown, and J. L. White, *J. Amer. Oil Chem. Soc.*, **42**, 53 (1965).

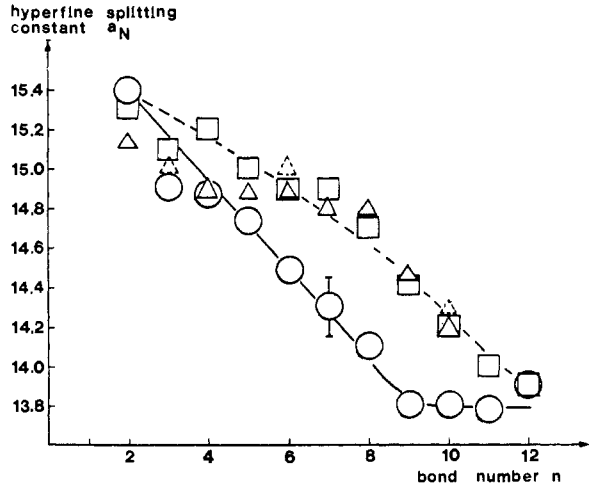


Figure 1. Polarity profile of liquid crystalline bilayers as measured by stearic acid spin labels I(m,n): ○, phase L_{1c}; □, phase L_{1d}; △, phospholipid dispersion; ⧋, phospholipid spin labels (data calculated from ref 8, Table I).

information about the alignment of the molecular axis in the bilayer and about the internal flexibility of the hydrocarbon chain. In the case of a completely rigid chain, the following interpretation of S_3 may be given. For $S_3 = 1$ the long molecular axis of the hydrocarbon chain is oriented parallel to the direction of the bilayer normal; for $S_3 = 0$ the molecule is either tumbling isotropically or the mean angular deviation amounts to half of the tetrahedral angle of 109.4°.

IV. Polarity Profile of Liquid Crystalline Bilayers

From measurements with pure organic solvents it is known that the hyperfine splitting constant a_N is an indicator of the polarity of the immediate spin-label environment, increasing polarity of the solvent leading to increasing values of a_N. For the spin labels of section II, a_N varies between 13.8 G in a pure hydrocarbon solvent and 15.6 G in an aqueous solution, but is independent of the total chain length, the position of the label group on the chain, or the type of polar group.

The latter two factors can no longer be ignored if the same spin labels are dissolved in a liquid crystalline bilayer. In Figure 1 we have selected three bilayers which are typical examples for the variation of a_N with the position of the label group. The essential features of Figure 1 are (1) the gradual decrease of a_N, spread over eight to ten carbon–carbon bonds, from about 15.3 G at the polar interface to 13.8 G in the interior of the double layer; (2) the distinct influence of water: the less water the faster the decrease of a_N. Similar polarity profiles have also been obtained for the lamellar phases L_{1a} and L_{1b} which are not included in Figure 1. Figure 1 is based on measurements with stearic acid labels I(m,n). The stearyl alcohols III(m,n) yield virtually the same results, whereas smaller values of a_N are found for some of the esters II(m,n) and IV(m,n). This is simply the effect of the weak hydrophilicity of the ester group; the ester probes are preferentially dissolved in the hydrocarbon part of the double layer (*cf.* section V).

In order to explain the observed polarity profile, it could be argued that the gradual decrease of a_N is caused by rapid, one-dimensional fluctuations of the spin label in and out of the bilayer, a_N being a time average between a water and a hydrocarbon environment. The mean residence time of the label group in the hydrocarbon region would then depend on the distance of the label group from the water region. This assumption implies that the size of the spin-labeled molecule has a distinct influence on the actual value of a_N, since long-chain fatty acids exhibit a higher affinity to a hydrocarbon environment than short-chain acids.[17] This is not confirmed experimentally as can be seen from Figure 7 in section VII where fatty acids of different chain length (fixed label position $n = 2$) yield practically the same value of a_N. Furthermore, if phospholipid spin labels are incorporated into a phospholipid bilayer, the same polarity profile is found as with the much smaller stearic acid labels. (In Figure 1 the points denoted by dashed triangles represent phospholipid spin labels; data calculated from ref 8, Table I.) We may therefore conclude that fluctuations cannot be responsible for the variation of a_N.

It can also be excluded that the observed polarity differences correspond to real changes of the macroscopic dielectric constant within the bilayer. By averaging the hyperfine splitting constant a_N for the different label positions and comparing the result with organic solvents with a similar a_N value, an average dielectric constant of $\epsilon \sim 10$ is obtained for the hydrocarbon region. This value is not in agreement with dielectric measurements of "black" lipid membranes and theoretical calculations of the dielectric constant of a lipid bilayer ($\epsilon \sim 2$–4).[18]

We therefore wish to propose a model which describes the polarity profile as a long-range electrostatic interaction of the nitroxide dipole with the oriented dipoles and surface charges of the hydrocarbon–water interface. This purely electrostatic model has been adapted from recent theories of electrochromism and solvatochromism[19,20] and is based on an electrostatic explanation of the solvent dependence of a_N which we therefore want to discuss first.

The nitroxide bond has a dipole moment of approximately 4 D.[21] In solution the electric field of this dipole polarizes and orientates the surrounding molecules, creating a reaction field which has the same direction as the inducing dipole.[22] If a certain polarizability of the nitroxide bond is assumed, the effect of the reaction field is to increase the original dipole moment. A quantitative expression for the effective dipole moment has been derived by Böttcher.[23]

$$\mu^* = (\mu/3)(n_D^2 + 2)(2\epsilon + 1)/(2\epsilon + n_D^2) \qquad (2)$$

This equation refers to a pure dipole liquid with the dielectric constant ϵ, the refraction index n_D, and the dipole moment μ of the free molecule. μ^* is the total dipole moment comprising the permanent plus the induced moment. The numerical value of the hyperfine splitting constant is proportional to the unpaired spin density on the nitrogen nucleus. Since the nitroxide bond can be described formally by a resonance between the structures A and B, increasing contributions from

(17) P. Mukerjee, *J. Phys. Chem.*, 69, 2821 (1965).
(18) S. Ohki, *J. Theor. Biol.*, **19**, 97 (1968).
(19) W. Liptay, *Z. Naturforsch. A*, **20**, 1441 (1965).
(20) W. Liptay, *Angew. Chem.*, **81**, 195 (1969).
(21) E. G. Rozantsev, "Free Nitroxyl Radicals," Plenum Press, New York, N. Y., 1970.
(22) L. Onsager, *J. Amer. Chem. Soc.*, **58**, 1486 (1936).
(23) C. J. F. Böttcher, "Theory of Electric Polarisation," Elsevier, Amsterdam, 1952, p 70.

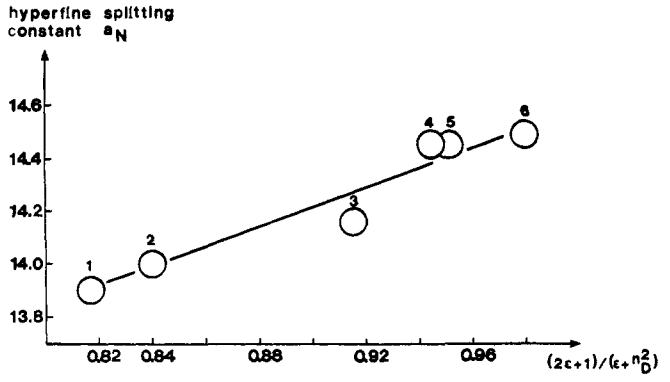


Figure 2. Solvent dependence of the hyperfine splitting constant a_N (label I(13, 2), ~25°): (1) cyclohexane, (2) 1,4-dioxane, (3) chlorobenzene, (4) benzotrifluoride, (5) 1,2-dichloroethane, (6) benzonitrile.

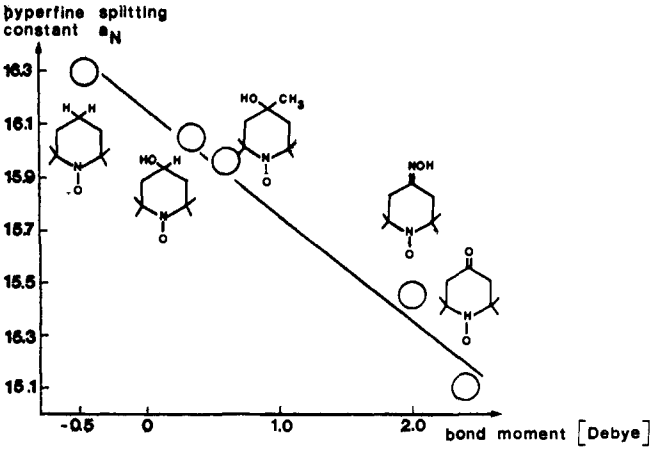
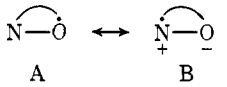


Figure 3. Dependence of the hyperfine splitting constant a_N on the dipole moment of the substituent R in compound VIII (data taken from ref 25).

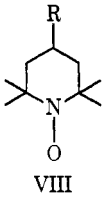
the dipolar form B will lead to increasing values of a_N. Therefore, if the electrostatic model is correct, a linear relationship between a_N and μ^* is to be expected.



A B

In order to test this prediction label I(13,2) was dissolved in organic solvents of different dielectric constant, and the measured hyperfine splitting constant was plotted *vs.* $(2\epsilon + 1)/(2\epsilon + n_D^2)$. ($n_D$ and μ refer to the pure dipole compound; the first two expressions on the right-hand side of eq 2 are therefore constant for all solvents.[24]) The refraction index was approximated with n_D^{25} equal to 1.45, which is an average value measured for esters II(*m*,*n*). Figure 2 shows the experimental result, which confirms the expected linear relationship up to $\epsilon \sim 30$. The failure to account for larger dielectric constants is due to a poor approximation for the reaction field used to derive eq 2 and to specific hydrogen bonding effects. (The same range of validity is also found for the electrostatic theory of solvatochromism.[19,20]) The reaction field induced at the position of the nitroxide dipole is of the order of 10^7 V/cm, and from the slope of Figure 2 it can be estimated that a change of the electric field strength by 10^6 V/cm brings about a change of a_N by 0.4 G.

A more direct proof for the influence of an electrostatic field on a_N has been reported by Brière, *et al.*[25] These authors have synthesized a series of spin labels



VIII

VIII with the substituent R having different dipole moments. As can be seen from Figure 3 a linear relation between a_N and the dipole moment of the substituent R is found. As has been pointed out by Brière, *et al.*, it is unlikely that this effect is propagated through σ bonds, but must be due to direct electrostatic interactions.

Let us now proceed to a discussion of the liquid crystalline bilayer. The L_1 phases are composed of a hydrocarbon part (~18 to 24 Å) sandwiched between two dipole layers (~2 Å) each being in contact with a water phase (~7 to 80 Å). In comparison with the vast amount of literature on the hydrocarbon part of such double layers, almost no mention has been made of the dipolar region, although the latter is an essential factor in originating the double layer. This disregard of the dipolar groups is probably due to the fact that dielectric measurements have been the main source of information to date. Unfortunately the dipolar regions do not contribute significantly to the over-all electrical properties; these are determined almost exclusively by the dielectric constant and the resistance of the hydrocarbon part.[26] However, the dipolar regions cannot be neglected if, instead of the bulk properties, the molecular properties are investigated as is the case with spin labels.

The molecular dipoles of the dipolar region are aligned almost parallel to each other. The electric field of a specific dipole is therefore not averaged out by surrounding dipoles, but the lines of force will penetrate into the hydrocarbon region. Superimposed on the dipole fields are Coulomb fields which arise from dissociated carboxyl groups (L_1 phases) or from the absorption of ions (phospholipid dispersion). Using a crude static model of an oriented dipole layer with interposed surface charges, we have estimated that the electric field strength decreases from about 10^8 V/cm at 2 Å to a few 10^6 V/cm at 10 Å distance from the dipolar region. This change is comparable with the difference of the reaction fields between polar and nonpolar solvents. We therefore assume that the electric field of the dipolar region polarizes the nitroxide dipole in much the same way as does the reaction field of an isotropic liquid, thus simulating different dielectric constants at different points in the hydrocarbon layer. The difference between the two situations is that the reaction field is always oriented parallel to the nitroxide dipole, which does not hold true for the nitroxide bond in the hydrocarbon bilayer. Close to the polar region

(24) Reference 23, p 71.
(25) R. Brière, H. Lemaire, and A. Rassat, *Bull. Soc. Chim. Fr.*, 3273 (1965).
(26) D. A. Haydon, *J. Amer. Oil Chem. Soc.*, 45, 230 (1968).

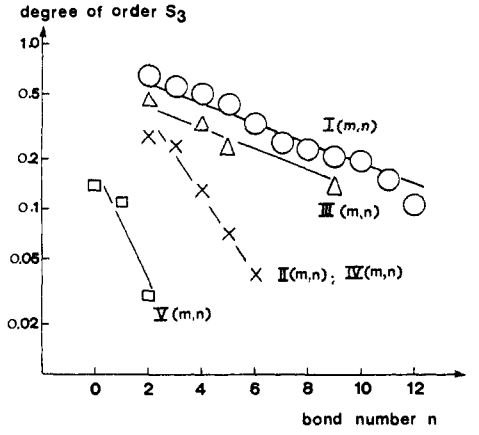


Figure 4. Phase L_{1a}. Order parameter S_3 as a function of bond number n: 18 carbon atoms chain length [V(7,0) and V(5,2) 10 carbon atoms chain length].

the nitroxide dipole is oriented preferentially perpendicular to the bilayer normal, but the motion becomes more and more isotropic with increasing distance from the polar region.[9] In the hydrocarbon interior it is much easier for the nitroxide dipole to align itself in the direction of maximum field intensity. We therefore assume that the observed variation of the hyperfine splitting constant a_N, since it does not show a simple $1/r^2$ or $1/r^3$ dependence, arises from the combined effects of varying average orientation of the nitroxide groups and the superposition of coulombic and dipolar fields.

On this basis we can also give a tentative explanation of the influence of water on the polarity profile (Figure 1). The L_{1d} phase has a higher water content (larger spacing between two adjacent lipid bilayers) and therefore contains more dissociated carboxyl groups.[27] The higher charge density increases the contribution of coulombic fields to the total field intensity, inducing a less rapid decrease of the field intensity in phase L_{1d} compared to phase L_{1c} with the smaller water content and less surface charges.

It should finally be noted that the "polarity" of lipid membranes as determined by the incorporation of fluorescence labels[28] can be interpreted in terms of the same electrostatic model.

V. Significance of the Polar Groups for the Ordering of the Hydrocarbon Chains

A plot of the order parameter S_3 as a function of the label position n for four types of polar groups is presented in Figure 4. The most notable observations are the following. (1) At a given label position the order parameter decreases as a function of the polar groups in the order $COOH > CH_2OH > COOCH_3 > CH_3$. This result can be explained by the well-known differences in the hydrophilicity of the four polar groups. The carboxyl group is a strong polar group anchoring the spin label rigidly in the bilayer surface, whereas the hydrophilic tendencies are less pronounced for the alcohols and are again smaller for the esters and the methyl group. It is also interesting to note that both types of esters II(m,n) and IV(m,n) yield essentially the same order parameter S_3 despite the distinct differences in the ordering of the corresponding acids I(m,n) and alcohols III(m,n). (2) The motion of the methylene groups adjacent to the polar surface is restricted to an anisotropic rotation about the long molecular axis of the alkyl chain, but it becomes more isotropic for methylene groups which are located in the interior of the double layer. When a logarithmic scale is used for S_3 (as in Figure 4), a linear dependence on n is obtained for all four series of spin labels. Such a relationship is to be expected if it is assumed that the hydrocarbon chains possess a certain intrinsic flexibility and that each methylene segment contributes the same increment to the total chain flexibility. Under these conditions the following approximation for S_3 can be derived.[9]

$$\log S_3 = n \log S_\alpha + \text{constant} \qquad (3)$$

S_α is a quantity characteristic of the intrinisic chain flexibility, large values of S_α being typical for a rather stiff alkyl chain. The molecular origins of the chain flexibility are fast internal rotations about carbon–carbon bonds. It is possible to analyze S_α further in terms of a cooperative rotational isomeric model, in which the chain flexibility is determined by two parameters, namely the temperature T and the apparent energy difference E_σ between trans and gauche conformations.[10] Large values of E_σ mean that the trans conformation is energetically more favored than the gauche conformation leading to an extended and more rigid hydrocarbon chain. The numerical analysis of Figure 4 is summarized in Table III. It is obvious from

Table III

	S_α	E_σ, cal mol^{-1}
Stearic acid labels I(m,n)	0.843	1400
Stearyl alcohol labels III(m,n)	0.843	1400
Ester labels II(m,n) and IV(m,n)	0.60	~500–600
Paraffin labels V(m,n)	0.50	

inspection of Table III that the stearic acids and the stearyl alcohols have the same intrinsic flexibility. Since the bilayer is composed of sodium decanoate and decanol, it is safe to assume that the stearic acid and stearyl alcohol labels which carry the same polar groups as the bilayer components are true structural elements of the bilayer. As such they participate in the increased rigidity of the liquid crystalline state characterized by S_α. The only difference in the behavior of the two types of amphiphilic molecules of phase L_{1a} is the larger overall-motion of the alcohols.

The ester labels and the paraffin labels, however, are clearly more flexible than the corresponding acids or alcohols as can be seen from Figure 4 and Table III. Since a value of $E_\sigma \approx 500$–800 cal mol^{-1} is well established for a polymethylene chain in the liquid state,[29] we may conclude that these molecules are more or less dissolved in the hydrocarbon part of the bilayer and that the hydrocarbon chain assumes a configuration which is similar to that in a pure liquid hydrocarbon solvent.

(27) P. Ekwall, I. Danielsson, and L. Mandell, *Kolloid-Z.*, **169**, 113 (1960).

(28) *E.g.*, T. Gulik-Krzywicki, E. Shechter, M. Iwatsubo, J. L. Ranck, and V. Luzzati, *Biochim. Biophys. Acta*, **219**, 1 (1970).

(29) T. M. Birshtein and O. B. Ptitsyn, "Conformation of Macromolecules," Interscience, New York, N. Y., 1966.

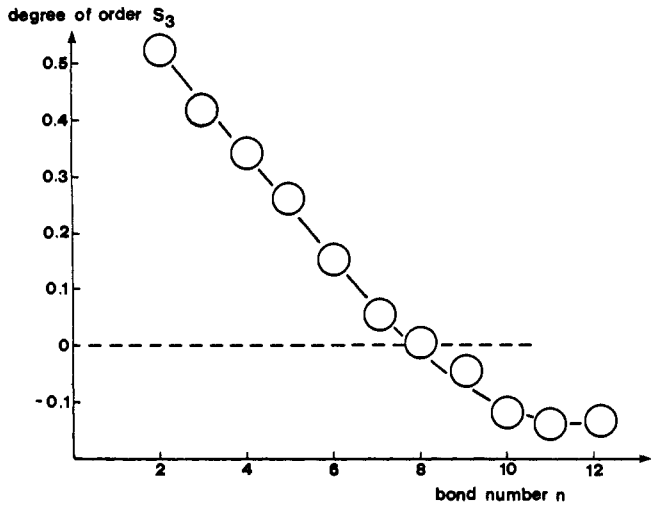


Figure 5. Order parameter S_3 of stearic acid spin labels I(m,n) incorporated into the sodium caprylate–caprylic acid bilayer L_{1b}.

VI. Influence of the Bilayer Thickness

Another topic of interest is the distortion of the parallel alignment of the hydrocarbon chains due to incompatibilities between the length of the spin labels and the dimension of the bilayer. This shall be illustrated by two types of experiments.

Let us first consider the sodium caprylate–caprylic acid phase L_{1b}. The hydrocarbon part of this bilayer is a few ångströms shorter than the stearic acid spin labels if the latter are thought to be in a rather extended configuration. Therefore, in order to avoid contact with the water region, the hydrocarbon chain must either assume a more coiled configuration or the end of the chain must be bent in some way. The experimental results are shown in Figure 5. Again the order parameter S_3 decreases with increasing bond number n, and the intrinsic flexibility of the first five carbon–carbon bonds is almost the same as for the sodium decanoate–decanol bilayer. Approximately in the middle of the bilayer the order parameter approaches a value of $S_3 = 0$, which in this case does not correspond to a completely random tumbling of the respective hydrocarbon segment, but to a mean angular deviation from the bilayer normal of about 54.7°. This can be concluded, because a further increase of the bond number n leads to negative S_3 values, clearly indicating a rather stiff configuration of the hydrocarbon tail with a preferential orientation perpendicular to the bilayer normal. (If the hydrocarbon chain were completely flexible, the order parameter $S_3 = 0$ would represent a totally isotropic motion, and an increase of n would then not bring about any further change of S_3.) The question of whether the hydrocarbon chain finally turns back on itself could be decided with spin labels bearing the label group even further down the methylene chain.

A second alteration of the direction of the hydrocarbon chains is detected by the dicarboxylic acid labels VI(m,m). If label VI(6,6) is dissolved in the sodium caprylate–caprylic acid bilayer L_{1b}, epr spectra as shown in Figure 6 are obtained. These spectra can be interpreted as a superposition of two different configurations of the spin label. Signal A comes from the linear, extended form of label VI(6,6) where the two carboxyl groups are anchored in opposite polar regions of the

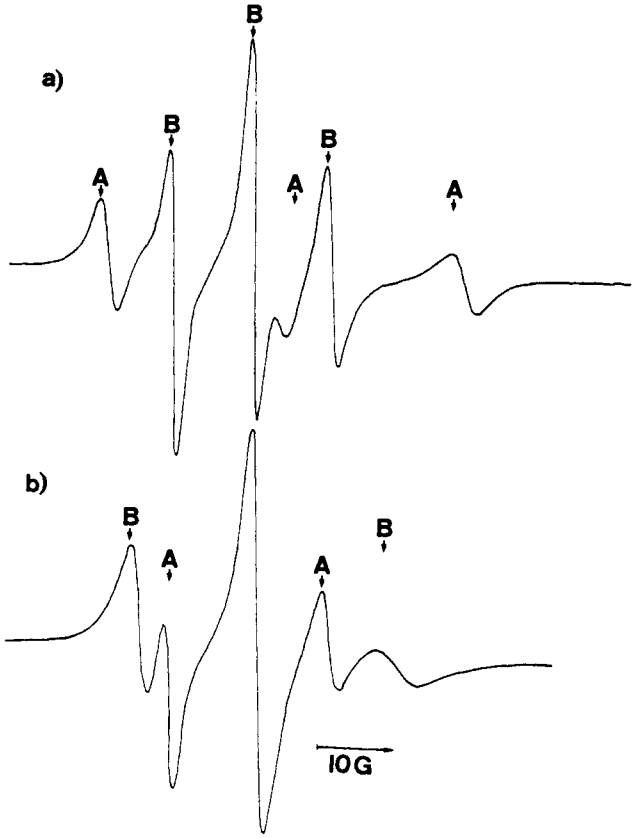


Figure 6. Spin label VI(6,6) in an oriented sample of phase L_{1b}: (a) applied magnetic field parallel to the bilayer normal; (b) applied magnetic field perpendicular to the bilayer normal.

bilayer, whereas signal B is due to a folded configuration of the label with both carboxyl groups lying in the *same* polar region. In both cases the spin-label group itself is located approximately in the middle of the hydrocarbon region ($a_N \sim 14.2$), but the nitrogen $2p\pi$ orbital is aligned parallel to the bilayer normal ($S_3 = 0.51$) in the extended form and perpendicular to this axis in the folded form ($S_3 = -0.24$). From the symmetrical structure of label VI(6,6) and from the inspection of molecular models, it follows that in the folded configuration the hydrocarbon chain must be bent backwards at the position of the label group.

The rate of exchange between the linear and the folded configuration is slow with respect to the time scale of the epr experiment. A detailed investigation of the temperature dependence of this configurational equilibrium is in progress.

If label VI(6,6) is incorporated into the sodium decanoate–decanol bilayer L_{1a}, only signal B of the folded hydrocarbon chain is observed. Such a behavior can easily be explained because label VI(6,6) is *shorter* than the hydrocarbon layer of phase L_{1a}. If the hydrocarbon chain assumes a linear configuration, at least one carboxyl group would be located in a nonpolar environment. Such a situation is energetically highly unfavorable.[30] The molecule is therefore forced into a folded configuration so that both carboxyl groups can be anchored in a hydrophilic environment.

Additional support for this explanation is obtained with label VI(7,7). Using phase L_{1b} as a solvent system,

(30) A. Parsegian, *Nature (London)*, **221**, 844 (1969).

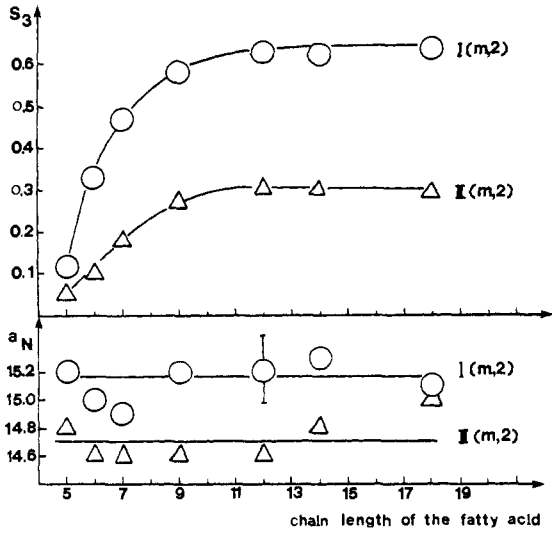


Figure 7. Phase L_{1a}. Chain-length dependence of the order parameter S_3 and the isotropic hyperfine splitting constant a_N.

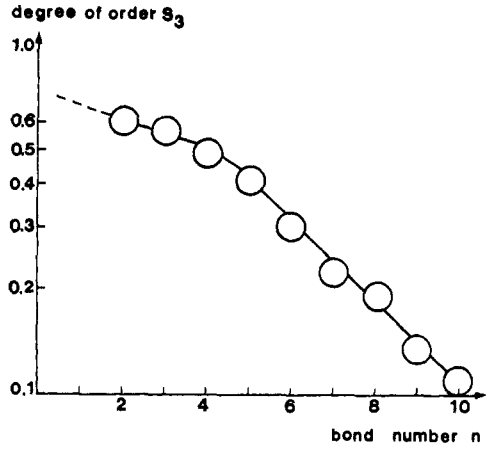


Figure 8. Order parameter S_3 of stearic acid spin labels I(m,n) incorporated into the sodium caprylate–decanol bilayer L_{1c}.

only signal A arising from the linear configuration is observed. In this case the molecule is *longer* than the hydrocarbon layer of the sodium caprylate–caprylic acid system and the carboxyl groups can easily be accommodated into opposite polar regions; *i.e.*, label VI(7,7) is extended throughout the whole hydrocarbon layer from one polar surface to the other. However, if the bilayer thickness is increased (using phase L_{1a} as solvent system), again both the linear and the folded configurations of label VI(7,7) are observed. It can be concluded from these experiments that depending on the components added to a liquid crystalline bilayer the molecular order can be far more subtle than a simple parallel alignment of the molecules.

VII. Effect of the Chain Length

The influence of the chain length on the ordering of the spin labels is described in Figure 7, where the isotropic hyperfine splitting constant a_N and the degree of order S_3 have been plotted *vs.* the total chain length of the spin label. The spin-label group is positioned two methylene units away from the polar head, and the length of the hydrocarbon tail is varied. It can be seen from Figure 7 that the hyperfine splitting constant a_N is approximately 0.5 G smaller for the esters than for the acids but in both cases is almost independent of the chain length. This confirms earlier conclusions, based on the flexibility of the hydrocarbon chain (*cf.* section V), that the ester labels must be dissolved in a rather hydrophobic environment. Figure 7 also shows that the order parameter S_3 is a function of two variables. Firstly, S_3 depends again on the type of head group; the acids are better aligned in the bilayer than the esters, which is in agreement with the results shown in Figure 4. Secondly, the order parameter S_3 increases for both series of spin labels with increasing chain length, reaching an upper limit at a chain length of about 11–12 carbon atoms. A further lengthening of the hydrocarbon tail has no effect on the alignment of the spin labels. At least two different factors are responsible for the initial rise of S_3. First, the cooperative interactions governing the chain configuration are less effective in short chains; here the molecules assume a relatively coiled configuration. If more carbon atoms are added to the chain the cooperative interactions become more significant, leading to a more extended chain. Using the formalism of the rotational isomeric model it can be shown, however, that this argument alone is not sufficient to explain the chain-length dependence of the order parameter.[31] The second important factor is hydrophobic interactions. The increase of the chain length probably induces stronger hydrophobic forces between the hydrocarbon moieties, thus restricting the motional freedom of the molecules. In terms of the rotational isomeric model this means that the intrinsic flexibility S_3 and the energy parameter E_σ must increase with increasing chain length. The synthesis of appropriate spin labels in order to test this prediction experimentally is in progress.

VIII. Determination of the Bilayer Thickness

If the flexibility of all segments of a hydrocarbon chain is known, it should be possible, in principle, to determine the thickness of the bilayer. How this can be accomplished by using the rotational isomeric model shall be outlined briefly for phase L_{1c}. This liquid crystalline system has been chosen because the dimensions of its bilayer are known from X-ray investigations.

The flexibility profile of phase L_{1c} as measured by stearic acid spin labels I(m,n) is presented in Figure 8. Two linear regions of different gradient S_α are observed. The carbon–carbon bonds adjacent to the polar surface are clearly less flexible ($S_\alpha = 0.90$) than the segments in the central region of the bilayer ($S_\alpha = 0.77$). This corresponds to E_σ parameters of $E_\sigma \approx 1700$ cal mol^{-1} for the first five carbon–carbon bonds and $E_\sigma \approx 1150$ cal mol^{-1} for the following two or four, depending on whether the calculation refers to the hydrocarbon chain of a caprylic acid or a decanol molecule.

Knowing the E_σ parameters, the next step is to determine the average length of the vector $\mathbf{r}$ which connects the ends of the carbon skeleton. $\mathbf{r}$ is given by the sum of the skeleton bond vectors $\mathbf{l}_i$ ($|\mathbf{l}| = 1.537$ Å).

$$\mathbf{r} = \sum_{i=1}^{n} \mathbf{l}_i \qquad n = 7 \text{ or } 9 \tag{4}$$

(31) H. Limacher and J. Seelig, manuscript in preparation.

Let us consider for a moment a fictitious molecule of known geometry where the molecular motion of the hydrocarbon chain is completely frozen in. A Cartesian coordinate system is attached to each chain segment. Using appropriate transformation matrices of order 3, the projection of the ith bond vector along the axis of the first coordinate system is then found by stepwise transformations through $i - 1$ foregoing coordinate systems. In the reference frame of the first bond eq 4 can therefore be expressed in the following form

$$\mathbf{r} = \sum_{i=1}^{n} (\mathbf{T}_1\mathbf{T}_2 \ldots \mathbf{T}_{i-1})\mathbf{l}_i \qquad (5)$$

(for $i = 1$, $\mathbf{T}_0$ is the identity matrix of order 3).

Let us now return to the problem of a *flexible* hydrocarbon chain in a lipid bilayer. In this case the average orientation of the vector has been found to be parallel to the bilayer normal.[9] Furthermore, each carbon–carbon bond can assume three conformations (trans, gauche$^+$, gauche$^-$) with rapid interconversions occurring between these conformational states. In order to determine the average length of the vector, the serial product of transformation matrices must be replaced by its statistical mechanical average.[32] Omitting details of this rather lengthy calculation[31] the final result can be expressed as follows. The average length of the carbon skeleton of caprylic acid (decanol) with the chain aligned parallel to the bilayer normal amounts to 7.6 Å (9.1 Å). Addition of appropriate van der Waals radii for the terminal methyl protons and the carboxyl groups (hydroxyl group) increases the chain length to 9.1 Å (11.3 Å). Furthermore, it is known from studies with rigid steroid spin labels that the hydrocarbon chains are subjected to wobbling motions around the bilayer normal, the mean angular deviation being about 30°. Taking into account this overall motion reduces the projection of the chain on the direction of the bilayer normal to 8.5 Å (10.6 Å). The molecular ratio of decanol molecules to caprylic acid molecules in phase L_{1c} is 1.02. The average length of all hydrocarbon chains therefore amounts to 9.52 Å. The total thickness of the bilayer is given by twice this length plus additional 2.4 Å for the van der Waals distance between the terminal methyl groups in the middle of the bilayer. The final result is a thickness of 21.4 Å, which is in fair agreement with a value of 21.7 Å determined by X-ray diffractions.[15] Using the same formalism we have analyzed five more sodium caprylate–decanol–water systems with varying bilayer thickness. In no case was the difference between the spin label data and the X-ray investigation larger than 2%.[31] These results are particularly satisfying because they lend additional support to the semiempirical approach used for describing the chain flexibility and because they give us some confidence to apply the same method to determine the dimension of unknown liquid crystalline bilayers.

(32) P. J. Flory, "Statistical Mechanics of Chain Molecules," Interscience, New York, N. Y., 1969, p 99.

H_2BH_3 as an Intermediate in Tetrahydridoborate Hydrolysis[1]

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Abstract: The rate law corresponding to a simple, rate-determining, proton transfer fails to correlate rates of BH_4^- hydrolysis in basic solutions. A rate law arising from a mechanism involving an intermediate which can react with OH^- to regenerate BH_4^- successfully correlates all the observed rate constants. Under appropriate conditions BH_4^- exchanges hydrogen isotopes with the solvent. The exchange rate appears to be about half the rate of regeneration of BH_4^- from the intermediate. These observations strongly suggest an intermediate of composition BH_5 but with the added proton equivalent to only one of the original four. It is isoelectronic with CH_5^+, and a structure analogous to **1** is suggested.

The purpose of this paper is to firmly establish a number of key facts about the acid-catalyzed aqueous solution hydrolysis of tetrahydridoborate ion (BH_4^-) and to develop a mechanism consistent with all the facts. The overall reaction is shown in eq 1. A number of authors[2–6] have shown that the

$$BH_4^- + 4H_2O \xrightarrow{HA} 4H_2 + B(OH)_4 \qquad (1)$$

release of H_2 is stepwise, and intermediates, $BH_{4-n}OH_n^-$, are involved, but the second and fourth hydrogen molecules are released considerably more rapidly than the first, so that the rate of the first step substantially determines the rate of the whole process. It has been generally,[7] but not universally,[8] supposed that the release of the first mole of H_2 from BH_4^- was a one-step reaction involving a rate-determining proton transfer and having a rate law given by eq 2, which would be appro-

$$-d(BH_4^-)/dt = (BH_4^-)\left\{\sum_{HA} k_{HA}(HA)\right\} \qquad (2)$$

(1) Based on the Ph.D. Thesis of J. E. C. Hutchins, University of Minnesota, 1969; presented, in part, at the 155th National Meeting of the American Chemical Society, San Francisco, Calif., March 31–April 5, 1968.

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(3) J. A. Gardiner and J. W. Collat, *Inorg. Chem.*, **4**, 1208 (1965).

(4) K. N. Mochalov and C. G. Gil'manchin, *Dokl. Akad. Nauk SSSR*, **132**, 134 (1960).

(5) R. E. Davis, E. B. Bromels, and C. L. Kibby, *J. Amer. Chem. Soc.*, **84**, 885 (1962).

(6) L. A. Levine and M. M. Kreevoy, *ibid.*, **94**, 3346 (1972).

(7) R. E. Davis and C. G. Swain, *ibid.*, **82**, 5949 (1960).

(8) R. E. Mesmer and W. L. Jolly, *Inorg. Chem.*, **1**, 608 (1962).