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VALINOMYCIN AS A PROBE FOR THE STUDY OF STRUCTURAL CHANGES OF BLACK LIPID MEMBRANES

G. STARK, R. BENZ, G. W. POHL AND K. JANKO
Fachbereich Biologie, Universität Konstanz, D-7750 Konstanz (Germany)
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SUMMARY

The formation and stability of thin films made from lecithins with saturated fatty acid chains of different length was studied. They show phase transitions similar to those in macroscopic lipid–water systems. Smaller structural changes were observed by examining the temperature dependence of conductivity in the presence of valinomycin and K^+ and by comparing the conductance of membranes formed from lecithins with different length of their fatty acids. The results were interpreted on the basis of a recently published transport model.

INTRODUCTION

Structural changes in lipids and lipid–water systems have been examined with different systems and methods. Differential thermal analysis[1], X-ray diffraction[2] and ESR studies[3] mainly were the tools, which lead to the detection of phase changes in macroscopic lipid and lipid–water phases. During the last years these investigations were also extended to biological and artificial membranes[4–7]. Monolayer studies revealed that phase changes can be observed at an air–water interface[8–10]. The detection here relies on variations in the surface pressure–area curve. Depending on the surface pressure and on the temperature, monolayers may show either a "condensed" or "expanded" behaviour. Structural transitions in lipid vesicle suspensions could be followed by temperature dependent adsorption of fluorescent and absorbing molecules[11]. There is a lack of similar experiments with flat lipid membranes formed by the technique of Müller and Rudin (see refs 16, 18). This system, which seems to be the artificial membrane system best suited for electrochemical studies, offers two other detection possibilities for structural changes, namely the appearance and stability of these black membranes and the electrical current across them, which should very sensitively depend on their structure. The paper contains corresponding studies with flat bilayer membranes formed from lipids with fatty acid chains of different length. As a probe for structural changes the valinomycin induced K^+ current was used and interpreted on the basis of a recently published carrier model[12–14].

MATERIALS AND METHODS

Membranes were formed from the following 1,2-diacyl-3-*sn*-phosphatidylcholines (lecithins):

(1) Lipids with saturated fatty acid chains: (a) distearoylphosphatidylcholine (Supelco), which was used unpurified; (b) dipalmitoylphosphatidylcholine (Fluka AG.), used after purification by column chromatography; (c) dimyristoyl- and dilaurinoylphosphatidylcholine synthesized according to the method of Baer and Buchnea[15] in our own laboratory.

(2) Lipids with unsaturated fatty acids: dierucoyl- and dioleoylphosphatidylcholine synthesized like (1c).

The purity of the lipids was checked by thin-layer chromatography. The membranes were generally formed from a 0.5–1 % (w/v) lipid in *n*-decane solution. As the lipids with saturated hydrocarbon chains were not soluble in pure decane, a small amount of butanol or chloroform (less than 10 % per volume) was added. Valinomycin was obtained from Calbiochem.

The chamber used for bilayer formation was made from Teflon and surrounded by a metal block, which was connected to one of two thermostats brought up to different temperatures. The arrangement of Teflon chamber and metal block had to be carefully adjusted to get the same temperature on either side of the membrane during the heating or cooling cycle. The temperature was measured by a thermocouple (Philips Thermocoax, Type 2ABAC 05) in the immediate neighbourhood of the membrane. Heating or cooling of the device was effected by switching from the high to the low temperature bath and *vice versa*. Though the aqueous solutions were stirred, there was a temperature gradient in the vertical direction. From the upper to the lower edge of the circular hole (diameter 3 mm) this temperature difference, however, was less than 0.5 °C. We used calomel electrodes or platinized platinum electrodes (at lower membrane resistances). The current was measured by a Keithley Microvolt Ammeter 150B. Both current and thermovoltage were plotted by a stripchart recorder (Hewlett Packard 7100 BM). Current–voltage curves were measured using two current and two voltage electrodes. Capacitance measurements were performed by an a.c. bridge method[16]. The membrane area was determined photographically according to the method of White[17].

RESULTS AND DISCUSSION

The formation of black lipid membranes from saturated lecithins

The technique of membrane formation has been described very often in the past (*e.g.* refs 16 and 18) so that the reader who is interested in details should refer to those papers. First a lamella from the lipid solution is spread over an aperture. Under the combined influence of gravity and capillary forces this lamella gradually becomes thinner until small "black" fields appear, which spread over the whole area. At room temperature the transition to the black state has been observed for lipids, which contain only or at least a larger part of unsaturated or branched fatty acids. With lipids containing only straight saturated fatty acids the attempts to get stable black lipid membranes only succeeded at higher temperatures[19]. Studying the temperature dependence of formation of black films with distearoyllecithin, we found that at 25 °C the lamellas were unstable and at the latest broke after 1 min with no indication of transition into the black state. The same was found at 40 °C. At 42 °C the lamellas were stable, appeared very rigid, but showed no black spots within 1 h. Slowly increasing the temperature at 44 °C the membrane suddenly

changed its appearance, looked fluid, got thinner and showed small black areas after some time. At 45 °C the membrane became completely black and showed satisfactory stability. This holds for all temperatures above 45 °C up to at least 60 °C. The thinning process, which is very slow at 45 °C gets faster with increasing temperature. This abrupt transition from a rigid solid-like state to a mobile fluid-like state at 44 °C resembles the melting of liquid crystals. We believe therefore, that the appearance of stable black membranes above a certain temperature indicates a phase transition of the lipids corresponding to the transition of a gel-like structure to a liquid–crystalline structure in macroscopic lipid–water mixtures[1,2]. The thinning of the membrane lamella and the transition to the black state only occur above the "melting point" of the lipid molecules, which leads to a considerable and visible decrease of the viscosity of the lamella.

Table I shows that this melting point (structural transition) depends on the chain length of the fatty acids. This is already known from other membrane systems such as vesicles[11] and lipid–water mixtures[1]. In contrary to these systems black lipid films contain a considerable amount of solvent (*n*-decane). To look for an influence of the solvent on the phase transition, the transition temperatures of the three systems were compared (Table I). While the temperatures for vesicles and lipid–water mixtures show good agreement, those of the black films deviate. Obviously the interaction of the lipid molecules with decane makes the membrane more fluid, so that the melting of the hydrocarbon chains of the lipid molecules already occur at lower temperatures. With decreasing chain length of the lipids the transition temperature of the black films was less well defined and the stability of the films decreased. For dimyristoyllecithin films there is only an upper limit indicated, because the stability at lower temperatures was not sufficient to perform the experiments.

The appearance of the structural transition can be demonstrated also in another way. If black membranes are formed above the melting point and cooled, they break at the latest at the melting point. In no case we were able to undercool a membrane below this temperature and "freeze" it. With other lipids and very small membrane areas (which increases membrane stability) such an undercooling was observed by Krasne *et al.*[20].

The temperature dependence of conductivity

As the current across a membrane should depend very sensitively on its structure, temperature-dependent structural changes should be observable by measuring the temperature dependence of conductivity. The experiments were performed with

TABLE I

TRANSITION TEMPERATURES FOR LECITHINS WITH DIFFERENT CHAIN LENGTHS OF FATTY ACIDS AND FOR DIFFERENT SYSTEMS

Chain lengths	*Transition temperature* (°C)		
	Lipid–water mixtures[1]	*Lipid vesicles*[11]	*Lipid films*
C_{18} (stearoyl)	58	58	44
C_{16} (palmitoyl)	41	39	31
C_{14} (myristoyl)	23	18	<31

valinomycin and K^+ present in the aqueous phases. Valinomycin is known to act as a carrier for K^+ across lipid bilayer membranes. Its transport mechanism has been carefully studied in the past and quantitatively described by appropriate rate constants[12–14].

Fig. 1 shows the dependence of the current on the temperature at a constant voltage. For membranes with unsaturated fatty acids such as dierucoyllecithin membranes the current depends on the temperature in the usual exponential manner with an experimental activation energy of about 17 kcal/mole. For membranes formed from saturated lipids the data may be represented in a semi-logarithmic plot by two lines, which cross at a temperature dependent on the chain length of the fatty acids. This change of the activation energy of the current at a certain temperature can be interpreted as a second structural transition of the membrane. One might argue that this transition is not generated by the membrane itself, but is a peculiarity of the valinomycin system brought about possibly by a combination of different temperature dependent rate constants. However, these transitions occur not only in the presence of valinomycin, but also in the presence of other charge carriers such as trinitrophenol. With this compound the activation energy is altered in the inverse direction at the transition point compared with the valinomycin data. This may result from the completely different conductance mechanism of trinitrophenol. One has to conclude now, that two different "probes" lead to the same result. It seems therefore justified to speak of a second structural change of the membrane.

The data of Fig. 1 were obtained by cooling a membrane preformed at high temperature. For two reasons this cooling process has to be performed within a relatively short period of time. First, the total number of valinomycin molecules within the membrane should remain constant during the cooling cycle, so that the temperature dependence of the current comes only from the rate constants determining the carrier transport (see next sections). The exchange of valinomycin molecules between the membrane and the adjacent water phases is slow. This has been shown previously[13] and can be also demonstrated in another way (Fig. 2).

Heating up a membrane from a temperature T_1 to a temperature T_2 and keeping

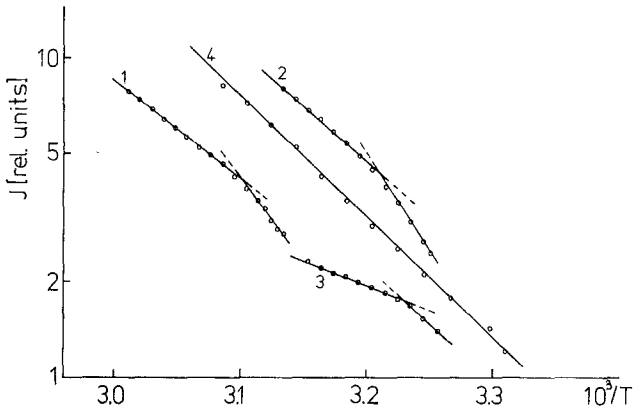

Fig. 1. Semi-logarithmic plot of the temperature dependence of conductivity for membranes formed from lecithins with different fatty acid chains. (1) distearoyllecithin, (2) dipalmitoyllecithin, (3) dimyristoyllecithin, (4) dierucoyllecithin. Aqueous solution: 10^{-8} M valinomycin, 1 M KCl. Different scale for the single curves.

it constant at T_2, the current first rises to a higher value and then decreases to almost the initial value. This first increase, which goes parallel to the temperature, can be assigned to temperature-dependent rate constants, whereas the decrease after approx. 8 min is due to a decrease of the total number of valinomycin molecules within the membrane with increasing temperature (see Eqn 2). Such a negative temperature coefficient of the partition coefficient is not observed for the K^+ carrier monactin. Besides the exchange between membrane and water is faster in this case so that the separation of both temperature-dependent processes appears not so clear. Fig. 2 shows that the net effect of the temperature on the current in the presence of valinomycin is approximately zero. In order to measure temperature dependences like Fig. 1, which are only governed by the rate constants, it is therefore necessary to keep the cooling time shorter than 5 min. A second reason for this time limitation is due to the fact that the membrane structure changes with time. This can be concluded from the observations that at constant temperature the current slowly increases with time. This increase goes parallel with an increasing capacity of the membrane. A reasonable explanation for these effects might be that the solvent decane diffuses out of the membrane so that the membrane thickness decreases. This explanation is supported by the observation that the effect is relatively small at room temperature but well pronounced at elevated temperatures. The second structural transition as discussed above (and indicated by Fig. 1) may also be interpreted as an influence of the solvent decane. Several degrees above the temperature, where the membrane "crystallizes", a phase separation between the lipid molecules and decane might take plase. Such phase separations between lipid molecules of different chain lengths have been observed by H. Träuble using vesicles (personal communication). Another possibility is a small change in the orientation of the lipid molecules (possibly of the polar head groups). With the present instrumental device it was not possible to study the reversibility of the effect, as the heating could not be effected fast enough.

The influence of the fatty acid chain length

In the preceding sections it was shown that the structure of bilayer membranes depends on the temperature and that presumably the solvent decane has a marked influence, which seems to increase with decreasing chain length of the lipids. To

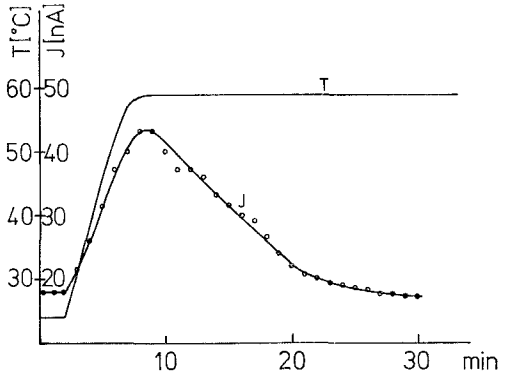

Fig. 2. Time dependence of the current after a temperature jump (dioleoyllecithin/1 M LiCl, 10^{-2} M KCl, 10^{-8} M valinomycin).

answer the question if there are structural differences between membranes formed from lipids with different chain length, conductance experiments in the presence of valinomycin were performed and analyzed with respect to the rate constants describing the K^+ transport induced by those molecules. Besides, the capacitance of the different lipid membranes was measured. Fig. 3 shows that the conductance at $c_K = 1$ M rather strongly depends on the chain length. This is also true for smaller K^+ concentrations (*e.g.* $c_K = 10^{-2}$ *M*). From the capacitance measurements (Fig. 4) we may conclude that the thickness of the membrane does not depend strongly on the fatty acid chain length of the lipids. Increasing the chain length by two methylene groups (that means 10–15 % for a straight chain of 14–18 carbon atoms) corresponds to a decrease of the capacitance mean value of about 5 %. This experimental finding can be explained by the solvent content of the membrane as discussed above. The solvent apparently influences the membrane thickness. This is in agreement with the results of Fettiplace *et al.*[21]. A reduction of the chain length of the lipid molecules obviously has little influence on the "solvent layer". Thus the term "bimolecular lipid membrane" (often used in the literature and sometimes also in this paper) should be understood with some reservations.

In order to find out which of the rate constants is responsible for the variable conductance, it was necessary to compare the current–voltage curves of the membranes[13]. Fig. 5 contains the results for the saturated lipids (as for dioleoyllecithin membranes, see ref. 13).

Interpretation of the results

The mechanism of valinomycin-induced K^+ transport has been studied in a quantitative manner on the basis of an "Eyring model"[12–14]. The model assumes that the carrier molecules are located in the membrane interface. Charge transport from the aqueous phase across the interface proceeds by a chemical reaction between free neutral carrier molecules S from the membrane and ions M^+ from the aqueous

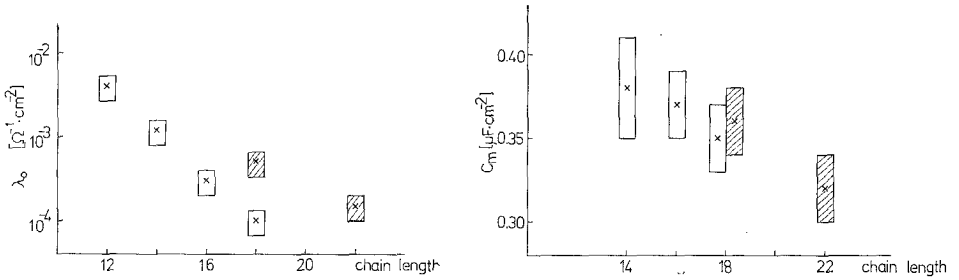

Fig. 3. Conductivity of lecithin membranes as a function of the chain length of the fatty acids of the lipid molecules. Shaded rectangles, unsaturated fatty acids; unshaded rectangles, saturated fatty acids. The height of the rectangles corresponds to the experimental error. The mean value (×) is indicated. The aqueous solution contained 10^{-8} M valinomycin and 1 M KCl. The temperature in case of the saturated lipids was 55 °C, with the unsaturated lipids 25 °C.

Fig. 4. Capacitance of lecithin membranes as a function of the chain length of fatty acids of the lipid molecules. Shaded rectangles, unsaturated fatty acids; unshaded rectangles, saturated fatty acids. The rectangles indicate the maximal deviation from the mean value (×). The capacitances were measured immediately after formation of the black membrane. The aqueous solution contained 0.1 M KCl, pH 5.8. The temperature in the case of saturated lipids was 55 °C, with the unsaturated lipids 25 °C.

phase. This heterogeneous reaction is described by a recombination (or association) rate k_R and a dissociation rate k_D:

$$M^+(a) + S(m) \underset{k_D}{\overset{k_R}{\rightleftharpoons}} MS^+(m) \qquad (1)$$

where a = aqueous phase and m = membrane.

The charged complexes MS^+ (which are assumed to be the only charge carriers within the membrane) may jump with a rate constant k_{MS} over the energy barrier of

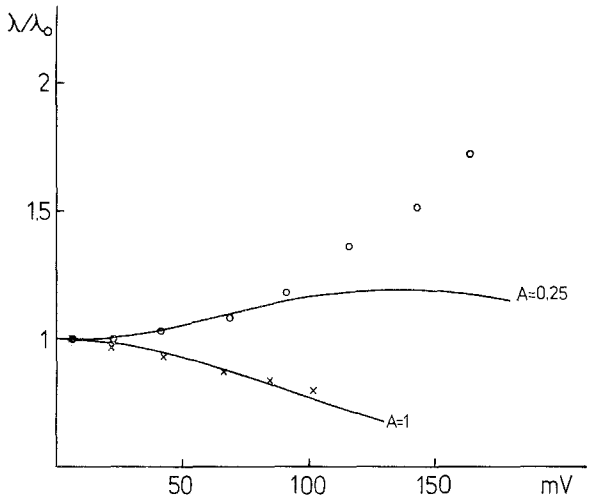

Fig. 5. Voltage dependence of conductivity for saturated lipids at 55 °C. ○, dipalmitoyllecithin/1 M KCl, 10^{-8} M valinomycin; ×, dilaurinoyllecithin/1 M KCl, 10^{-8} M valinomycin. Full lines according to Eqn 4. At 10^{-2} M KCl the data of all lecithins ($C_{18:0}$–$C_{12:0}$) approximately agree with the open circles, the same holds at 1 M KCl for distearoyllecithin. The data for dimyristoyllecithin at 1 M KCl lie between both curves. The unsaturated lipids dioleoyl- and dierucoyllecithin behave at all K^+ concentrations like dipalmitoyl- and distearoyllecithin (see also ref. 13).

the interior of the membrane. The same holds for the free carrier S (rate constant k_S). It was shown that the whole transport model can be described by these four rate constants and by the total number of carrier molecules N_0 (moles/cm^2) in the membrane (or instead of N_0 by the partition coefficients γ_S and γ_{MS} for S and MS^+ and the equilibrium constant K for the complex formation in the aqueous phase).

The formal analysis of the model gives the following expression for the electrical current J across a bilayer membrane (Eqn 5 of ref. 13, somewhat rewritten by using Eqns 15, 16 and 17 of ref. 12).

$$J = FN_0 \frac{k_R c_M k_{MS}}{k_R c_M + k_D} \frac{\sinh u/2}{(1 + A \cosh u/2)}$$

$$= \frac{Fd\gamma_S k_R k_{MS} c_M c_0}{k_D(Kc_M + 1)} \frac{\sinh u/2}{(1 + A \cosh u/2)} \qquad (2)$$

with

$$A = \frac{2k_{MS}}{k_D} + \frac{k_{MS}}{k} \frac{k_R c_M}{{}_S k_D} \qquad \text{(Eqn 11 of ref. 13)}$$

F = Faraday constant. $u = FU/RT$ reduced voltage. U = voltage across the membrane. R = gas constant. T = absolute temperature. d = membrane thickness. c_M = metal ion concentration in the aqueous phase. c_0 = total carrier concentration in the aqueous phase. The other parameters are defined in the text.

From Eqn 2 one derives the following expression for the conductivity in the limit of small voltages λ_0:

$$\lambda_0 = (J/U)_{u\approx 0} = \frac{F^2 d c_M c_0}{2RT(Kc_M + 1)} \frac{\gamma_S k_R k_{MS}}{k_D(1 + A)} \tag{3}$$

The constant A may be got by interpretation of the current–voltage curve[13]. If one defines λ as an integral conductivity ($\lambda = J/U$), one gets (Eqn 12 of ref. 13):

$$\frac{\lambda_s}{\lambda_0} = \frac{2(1 + A)\sinh u/2}{u(1 + A\cosh u/2)} \tag{4}$$

As A is the sum of one metal concentration dependent term and a second independent term, one may derive k_{MS}/k_D and k_R/k_S by studying the concentration dependence of λ/λ_0 (ref. 13).

The marked dependence of conductivity from the chain length of the lipids (Fig. 3) can be analyzed with the help of Eqn 3. From Fig. 4 one can see that the thickness d of the membrane varies less than 10 % between distearoyl- and dimyristoyl lecithin (assuming equal dielectric constant), whereas λ_0 changes by one order of magnitude. Neglecting the small variation of d, the set of parameters of Eqn 3, which depends on membrane properties is given by $\gamma_S k_R k_{MS}/k_D \cdot (1 + A)$. From the analysis of the current–voltage curves (by comparing the λ/λ_0–U curves at $c_K = 10^{-2}$ and 1 M in Fig. 5) one derives 2 $k_{MS}/k_D = 0.25$ independent of the chain length. The parameter A is equal for distearoyl- and dipalmitoyllecithin and only slightly increases for dimyristoyl- and dilaurinoyllecithin. Therefore, mainly the product $\gamma_S k_R$ is responsible for the variable conductance. It can be concluded in another way from Fig. 5 that presumably the recombination rate k_R is the changing quantity. For distearoyl- and dipalmitoyllecithin there follows 2 $k_{MS}/k_D \gg k_{MS}k_R/k_S k_D$, as the λ/λ_0–U curves are independent of the K^+ concentration. For dilaurinoyllecithin the ion concentration dependent term of $A(k_{MS}k_R/k_S k_D = 0.75)$ has increased compared with dipalmitoyllecithin, whereas k_{MS}/k_D remained constant. Obviously k_R/k_S increases with decreasing chain length. One can suppose that the same is true for k_S, as the diffusion across a thinner membrane should be faster. It seems therefore reasonable to conclude that k_R is the predominant quantity of the effect, because it must more than compensate a possible increase of k_S. A similar derivation can be made for the temperature dependence of the current with the aid of the left side of Eqn 2. From a previous study[14] we know that for neutral membranes holds: $k_R c_M < k_D$ ($c_M \leqslant 1$ M). The parameter A turned out as nearly temperature independent for all ion concentrations (therefore also k_{MS}/k_D). As N_0 was kept constant by performing the experiments within a short period of time, again k_R seems to be the responsible rate constant for the observed temperature dependence. Of course the other rate constants will also be temperature dependent, but their influence seems to cancel out.

Having interpreted the observed effects as a dependence of the recombination rate on the structure of the membrane, we may ask for the molecular basis of the structural changes. The complex formation occurs at the interface, but we do not know the actual site. Thus complex formation could occur at the aqueous side of the interface, but also more towards the interior of the membrane. It is therefore impossible to decide, whether a change of k_R reflects a change of the polar headgroups or of the fatty acids or most probably of both. Recently it was shown that the recombination rate k_R for valinomycin/K^+ at a membrane surface is several orders of magnitude smaller than in a methanolic solution[14]. The reason for this could be the strong molecular interaction of the lipid molecules within a membrane which leads to a decrease of fluidity. It has been shown by spin label studies that the flexibility of the fatty acid chains is more restricted near the headgroups compared with the other end[22]. A possible interpretation of our result seems to be that the interaction of the lipid molecules at the interface increases with the chain length of their fatty acids and decreases with increasing temperature.

It was shown previously[13] and can be also seen in Fig. 5, there is a deviation of the experimental data from the theoretical Eqn 4. Eqn 4 was derived from Eqn 2 under the assumption that all the rate constants are independent of the voltage. Stimulated by the interpretation of the preceding results one might speculate, if the deviation could be caused by a structural change of the membrane at high voltages. Such a structural change could result from the Maxwell pressure of the charged membrane capacitance. It was shown by White[17] that this pressure leads to an increase of the membrane capacitance or to a reduced thickness. Taking again k_R as influenced rate constant one can deduce from Eqn 2 that an increasing k_R gives an increasing current and therefore a higher conductance. The effect is more pronounced for small values of k_R compared with k_D, because at high k_R the increase of the nominator is partially cancelled by an increase of the denominator. This agrees with the experimental data, where the deviation of Eqn 4 is more pronounced for a superlinear current–voltage curve, which is equivalent to a small k_R.

From the results of Fig. 2 one can see that with valinomycin as a probe it is possible to separate the influence of partition coefficient and rate constants on the temperature dependence of conductivity. In principle one can therefore measure the activation energy of the partition coefficient. For dioleoyllecithin membranes its absolute value approximately seems to agree with the activation energy for k_R. As those measurements extend over a relatively long period of time, there might, however, be also an influence of a time-dependent membrane structure (decane effect). At very long times the membrane torus also seems to play a role. On addition of valinomycin to the aqueous phase the torus is not in equilibrium with it. There is a continuous flow of valinomycin from the aqueous phase to the torus so that at long times (more than 1 h) the concentration in the water seems to decrease, which leads also to a decreasing conductance of the membrane. These effects render a quantitative evaluation difficult and will be studied more intensively in the future.

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