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TRANSPORT KINETICS OF DIPICRYLAMINE THROUGH LIPID BILAYER MEMBRANES

EFFECTS OF MEMBRANE STRUCTURE

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Summary

Charge-pulse current-relaxation studies have been performed with lipid bilayer membranes in the presence of the hydrophobic ion dipicrylamine. From the analysis of the relaxation times and amplitudes the translocation rate constant k_i of dipicrylamine as well as the partition coefficient β between membrane surface and water could be evaluated. In a first series of experiments membranes made from monoolein or dioleoylphosphatidylcholine in a number of different *n*-alkane solvents were studied, as well as virtually solvent-free bilayer membranes made from monolayers. The thickness d of the hydrocarbon layer of these membranes varied between 5.0 and 2.5 nm. While β was almost insensitive to variations in d, a strong decrease of k_i with increasing membrane thickness was found; the observed dependence of k_i on d approximately agreed with the theoretically expected influence of membrane thickness on the height of the dielectric barrier. No specific differences between Mueller-Rudin films and solvent-free (Montal-Mueller) membranes other than differences in thickness were found. In a further series of experiments the chemical structure of the lipid was systematically varied (number and position of double bonds in the hydrocarbon chain, nature of the polar head group). The translocation rate constant k_i was much larger in phosphatidylethanolamine membranes than in phosphatidylcholine membranes. A strong increase of k_i was found when the number of double bonds in the hydrocarbon chain was increased from one to three. These changes were discussed in terms of membrane fluidity and dielectric barrier height. Much higher values of k_i were observed in lipids with ester linkage between hydrocarbon chain and glycerol backbone, as compared with the corresponding ether analogs. This finding is qualitatively consistent with determinations of dipolar potentials in monolayers of ester and ether lipids. When cholesterol is added to phosphatidylcholine membranes, the translocation rate constant k_i increases up to five-fold, while the partition coefficient β remains virtually constant. The variation of k_i in this case can be largely

accounted for by a decrease in membrane thickness and a concomitant reduction in dielectric barrier height. In membranes made from the negatively charged lipid phosphatidylserine the partition coefficient of dipicrylamine strongly increased with ionic strength, as expected from the Gouy-Chapman theory of the surface potential.

Introduction

Artificial lipid bilayer membranes have been used as models for the investigation of transport systems such as carriers or lipophilic ions [1—5] (for a review see ref. 6). Most of the studies have been performed with membranes obtained by the method of Mueller et al. [7]. These membranes contain a certain amount of solvent and their thickness was found to depend on the chain length of the *n*-alkane used for membrane formation [8,9] and of the lipid [10—12]. Membranes formed from monolayers according to the method of Montal and Mueller [13] have a smaller thickness [11], although these membranes may still contain traces of solvent [14]. The influence of membrane thickness on transport processes has been investigated for the gramidicin A channel [15] and recently for valinomycin-mediated Rb^+ transport [16]. Whereas the lifetime of the gramicidin A channel in membranes from the same lipid was largely dependent on the chain length of the solvent, a comparatively smaller influence on carrier mediated ion transport was found. In particular, the rate constants of translocation of the complexed and the uncomplexed carrier molecule were found to be rather insensitive to the nature of the solvent [16].

Compared with ion carriers, the transport mechanism of lipophilic ions across lipid bilayer membranes is relatively simple. The transport was shown to occur in three distinct steps [5]: adsorption of the ion from the aqueous phase to the membrane-solution interface, translocation to the opposite interface, and desorption into the aqueous solution. By studying the transport kinetics of lipophilic ions information on the structure and dynamics of the lipid membrane may be obtained [17—20]. Kinetic studies with lipophilic ions have been performed using the voltage-jump relaxation method [5,18—20] and more recently, the charge-pulse relaxation technique [21,22]. The latter method was used throughout this study. Its main advantage, apart from a better time resolution, lies in the fact that the pertubation of the membrane can be kept very small.

Besides the influence of solvent, we have also investigated the dependence of transport kinetics on the structure of the lipid. The rate constant k_i for translocation of a lipophilic ion across the membrane interior may depend on the shape of the dielectric barrier, on the dipolar potential in the membrane surface, as well as on the viscosity of the membrane. In order to study the influence of these factors on the transport of a lipophilic ion species (dipicrylamine) we have used phosphatidylcholine with fatty-acid residues differing in the hydrocarbon chain length, as well as in the number and position of the double bonds. In addition, we have varied the polar head-group and the nature of the linkage between hydrocarbon chain and polar residue (ester or ether bonds). In a further series of experiments the influence of different amounts of choles-

terol on the transport properties of a dioleoylphosphatidylcholine membrane has been studied.

A number of recent publications have dealt with effects of surface charge of the membrane on ion transport [23—25]. It has been found that in the presence of negative surface charges the permeability of lipophilic anions is reduced, whereas the permeability of cations is increased [23,25,26]. Furthermore, it has been shown that the transport of charged molecules is strongly dependent on the ionic strength of the aqueous solutions. In these investigations only the stationary membrane conductance has been studied. The charge-pulse relaxation method which we have applied to negatively charged phosphatidylserine membranes yields a more detailed discription and allows to separate the effects of ionic strength on the translocation rate and on the partition coefficient of the lipophilic ion.

Materials and Methods

Lipid bilayer membranes were obtained from various lipids by two different methods [7,13]. Solvent-containing membranes were formed from a 1—3% (w/v) lipid solution in a *n*-alkane (Merck, Darmstadt, G.F.R., standard for gas chromatography), across a circular hole with an area of about 2 mm^2, as described earlier [27]. Bilayer membranes from monolayers ("solvent-free" membranes) were formed in the usual way [11] across a small hole (0.2—0.3 in diameter) in a thin Teflon foil. Most of the phospholipids used in this study were synthesized by K. Janko [28]. The 1,2-diacyl-*sn*-glycerol-3-phosphorylcholines had the following fatty acid residues: palmitoleoyl (Δ^9 - $C_{16:1}$), oleoyl (Δ^9 - $C_{18:1}$), elaidinoyl (*trans*Δ^9 - $C_{18:1}$), petroselinoyl (Δ^6 - $C_{18:1}$), vaccenoyl (Δ^{11} - $C_{18:1}$), linoleoyl ($\Delta^{9,12}$ - $C_{18:2}$), linolenoyl ($\Delta^{9,12,15}$ - $C_{18:3}$) eicosenoyl (Δ^{11} - $C_{20:1}$), erucoyl (Δ^{13} - $C_{22:1}$), and nervonoyl (Δ^{15} - $C_{24:1}$). In addition, a phosphatidylcholine with a mixed chain was used: L-1-oleoyl-2-stearoyl-3-phosphatidylcholine. An ether phosphatidylcholine (DL-1-*O*-oleyl-2-*O*-palmityl-3-phosphatidylcholine) was obtained from Calbiochem. San Diego, California. Egg phosphatidylcholine, egg phosphatidylethanolamine, and phosphatidylserine from ox brain were isolated and purified by standard methods [29,30]. D,L-dioleoylphosphatidylethanolamine and its ether analog were synthesized as described previously [28].

Monoolein as well as most of the fatty acids used for lipid synthesis were obtained from NuCheck Prep, Elysian, Minn. U.S.A. Cholesterol was purchased from Eastman. The purity of all lipids was checked by thin layer chromatography. Dipicrylamine (Fluka, Buchs, Switzerland, Puriss.) was used as a concentrated stock solution in water or in ethanol. Small amounts of the stock solution were added to the aqueous solutions to get a final concentration of 10^{-8} M or $3 \cdot 10^{-8}$ M. The ethanol content in the aqueous phase never exceeded 0.1% (v/v). The aqueous solutions used in the membrane experiments were unbuffered (pH ~ 6) and contained between $3 \cdot 10^{-2}$ and 3 M NaCl (Merck, analytical grade) as an inert electrolyte. It has been checked previously [21] that the transport kinetics of dipicrylamine are pH-independent in the vicinity of pH 6. The measurements were performed 20 to 30 min after blackening of the membrane. The temperature was 25° C throughout.

The charge pulse experiments were performed as described earlier [21]. A voltage source was connected to the membrane by a fast FET switch through silver/silver-chloride electrodes. The membrane capacity was charged up to a voltage between 5 and 10 mV by a charge pulse of 50 ns duration. The impedence of the switch in the "open" position was larger than $10^{12}\Omega$. The voltage decay across the membranes was measured with a high-impedance voltage follower, input impedance $>10^{12}$ Ω and recorded with a storage oscilloscope. With this experimental set-up relaxation processes with time constants down to 1 μs could be resolved. In a number of test experiments with dummy circuits the performance of the whole set was carefully tested. No distortion of the signal caused by the amplifier system or by the electrodes was observed [21,22]. The specific membrane capacity C_m has been determined as described earlier [28], using rectangular voltage pulses of 10 mV.

Evaluation of the relaxation data

The model for the transport of lipophilic ions across lipid bilayer membranes which has been described previously in full detail [5,6,21] is based on the assumption that the transport is carried out in three distinct steps: (i) adsorption from the aqueous phase to the membrane-water interface (rate constant k_{am}), (ii) translocation over the central barrier in the middle of the membrane (rate constant k_i), and (iii) desorption to the aqueous phase (rate constant, k_{ma}).

In this study we slightly simplify the previously given theoretical treatment [21] assuming that of all three rate constants only k_i is voltage dependent. This is not a serious restriction because we have shown that approximately 90% of the voltage drops across the central barrier of the membrane [21]. In addition, the voltage applied to the membranes in our experiments was rather small ($\leqslant$10 mV).

For the theoretical analysis of the charge-pulse experiment we assume that the membrane which separates two identical solutions of the lipophilic ion is in equilibrium at times $t < 0$. At $t = 0$ the membrane capacity is charged up nearly instantaneously to an initial voltage V_m^o. Thereafter the external circuit is switched to virtually infinity resistance; the voltage V_m decays by conduction processes within the membrane. For small voltages ($|V_m^o| << 25$ mV) and in the absence of diffusion polarisation, the time course of V_m is given by [21]:

$$V_m(t) = V_m^0 [a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2)] \quad (1)$$

$$a_1 + a_2 = 1 \quad (2)$$

The relaxation times τ_1 and τ_2 and the relaxation amplitues a_1 and a_2 are known functions of the rate constants k_{ma}, k_{am} and k_i [21]. In the systems studied here, however, the behaviour of V_m at long times is governed by slow diffusion of lipophilic ions in the aqueous layers adjacent to the membrane surfaces [21]. This means that only the short relaxation time τ_1 may be obtained from the experiments. It is found that $V_m(t)$ exhibits a fast, purely exponential decay, followed by a slow and, in general, nonexponential decay process. As the time ranges of both processes are widely separated, the fast relaxation time τ_1 may be evaluated simply by plotting (at short times t) the logarithm of $V_m(t)$ vs. t.

Under these conditions where one decay process is much faster than the other, the fast process is exclusively determined by redistribution of lipophilic ions across the central carrier (rate constant k_i); accordingly, the theoretical expressions for τ_1 and a_1 reduce [21] to:

$$\tau_1 = \frac{1}{2k_i(1 + bN_t)}, \tag{3}$$

$$a_1 = \frac{bN_t}{1 + bN_t}, \tag{4}$$

$$b = \frac{F^2}{4RTC_m}. \tag{5}$$

N_t is the total concentration (mol · cm^{-2}) of lipophilic ions adsorbed to both membrane surfaces at equilibrium; C_m is the specific membrane capacity, F the Faraday constant, R the gas constant and T the absolute temperature. From the experimental value of N_t determined at a given aqueous concentration c of the lipophilic ions, the partion coefficient β may be calculated which is defined in the following way:

$$\beta = \frac{N_t}{2c} = \frac{k_{am}}{k_{ma}}. \tag{6}$$

β is equal to the thickness of an aqueous layer containing the same amount of lipophilic ions as one membrane surface. It is seen from Eqns. 3 and 4 that a measurement of τ_1 and a_1 contains information on k_i and the ratio k_{am}/k_{ma}, whereas the single values of k_{am} and k_{ma} cannot be determined from such an experiment.

Results and Discussion

In all systems studied here the fast voltage-decay was found to be purely exponential. From the observed relaxation time τ_1 and the relaxation amplitude a_1 the two parameters k_i and N_t were calculated according to Eqns. 3 and 4. For each set of experimental conditions between 5 and 15 membranes were used. The standard deviations were usually less than 10—15% for k_i and less than 20% for N_t in the case of Mueller-Rudin membranes. For membranes made from monolayers the scatter of the experimental results was considerably larger, the standard deviations being about 30% for k_i and about 40% for N_t.

The specific membrane capacity C_m which is needed for the calculation of the parameter b (Eqn. 5) and of the dielectric thickness d of the membrane was taken from the literature or, if necessary, was measured in the course of this study. d was obtained from the relation:

$$C_m = \epsilon_0 \epsilon_m / d \tag{7}$$

where $\epsilon_o = 8.85 \cdot 10^{-12}$ C · V^{-1} · m^{-1} is the permittivity of free space and $\epsilon_m \simeq 2.1$ is the dielectric constant of the hydrocarbon layer of the membrane.

(a) Influence of solvent

Membranes were formed from dioleoylphosphatidylcholine and glycerol-

monooleate dissolved in different *n*-alkanes and from monolayers of the same lipids ("solvent-free" membranes). The results on the kinetic behaviour of dipicrylamine in these membranes are summarized in Table I. It is seen that there is a large increase of the translocation rate constant k_i in the series from *n*-octane films to solvent free membranes; in the case of dioleoylphosphatidylcholine membranes k_i changes by a factor of about 18 and in the case of monoolein membranes by a factor of about 13. This increase of k_i is paralleled by a decrease in membrane thickness d, as obtained from the specific membrane capacity C_m [8,9,11,28].

The translocation rate constant k_i is approximately given by

$$k_i = k_{i0}\, e^{-w} \tag{8}$$

where k_{i0} is a constant and w is the height of the energy barrier in the center of the membrane, expressed in units of kT (k is the Boltzmann constant). The main contribution to w is the dielectric interaction of the ion with the membrane and the adjacent aqueous phases [31,33]. For two membranes of dielectric thickness d and d^*, respectively, the difference Δw in the electrostatic energy of an ion located in the central plane of the membrane is given by [33]:

$$\Delta w = w(d) - w(d^*) = h\left(\frac{1}{d^*} - \frac{1}{d}\right) \tag{9}$$

$$h = \frac{e_0^2}{4\pi\epsilon_0\epsilon_m kT} \ln\left(\frac{2\epsilon_w}{\epsilon_w + \epsilon_m}\right) \simeq 17.8 \text{ nm} \tag{10}$$

(T = 298 K)

e_0 is the elementary charge and $\epsilon_w \sim 78.5$ the dielectric constant of water. If k_i

TABLE I

Kinetic parameters of dipicrylamine transport through membranes made from dioleoylphosphatidylcholine and monoolein dissolved in different *n*-alkanes. The aqueous phase contained 0.1 M NaCl; T = 25°C. The values of the specific capacity C_m and of the dielectric thickness d of the membranes from dioleoylphosphatidylcholine and monoolein were taken from refs. 28 and 11, respectively. The results for dioleoylphosphatidylcholine dissolved in *n*-decane were taken from ref. 21. The partition coefficient β of solvent-free membranes has to be considered as a lower limit because of the presence of a large excess of lipid which probably reduces the aqueous dipicrylamine concentration.

Solvent	C_m (nF · cm^{-2})	d (nm)	τ_1 (μs)	a_1	k_i (s^{-1})	N_t (pmol · cm^{-2})	β (× 10^{-2} cm)
	Dioleoylphosphatidylcholine/10^{-8} M dipicrylamine						
n-Octane	377	4.9	350	0.67	460	0.81	4.1
n-Decane	374	5.0	410	0.65	430	0.74	3.7
n-Dodecane	422	4.4	210	0.68	780	0.95	4.8
n-Tetradecane	486	3.8	110	0.59	1830	0.74	3.7
n-Hexadecane	624	3.0	42	0.49	5950	0.65	3.3
Solvent-free	728	2.6	38	0.36	8470	0.43	2.2
	Monoolein/3 · 10^{-8} M dipicrylamine						
n-Octane	394	4.7	550	0.25	680	0.14	0.23
n-Decane	390	4.8	520	0.27	710	0.15	0.25
n-Dodecane	416	4.5	420	0.23	910	0.13	0.22
n-Tetradecane	469	4.0	330	0.18	1240	0.11	0.18
n-Hexadecane	585	3.2	87	0.18	4670	0.14	0.23
Solvent-free	745	2.5	53	0.092	8530	0.08	0.13

and k_i^* are the translocation rate constants in membranes of dielectric thickness d and d^*, respectively, then the ratio k_i/k_i^* should be approximately equal to

$$\frac{k_i}{k_i^*} \simeq e^{-\Delta w} \tag{11}$$

provided that the effect of membrane thickness on k_i is mainly of dielectric origin. In Fig. 1 the ratio k_i/k_i^* has been plotted as a function of dielectric thickness d, where k_i^* is the rate constant measured with n-decane as solvent. In addition to the experimental points, theoretical curves are represented in Fig. 1, which have been calculated from Eqns. 9—11 with $d^* = 5.0$ nm for dioleoylphosphatidylcholine and $d^* = 4.8$ nm for monoolein. Fig. 1 shows that the experimentally observed dependence of k_i on thickness d roughly agrees with Eqn. 11, for phosphatidylcholine. It is also seen from Fig. 1 that the highest experimental value of k_i/k_i^* for phosphatidylcholine, which has been obtained from solvent-free films nearly falls on the theoretical curve. This indicates that, at least for this lipid, there is no particular structural difference between Mueller-Rudin films and solvent-free (Montal-Mueller) membranes other than the difference in thickness. For monoolein films the agreement is less satisfactory, the value of k_i/k_i^* for solvent-free membranes being by a factor of about 2 to 3 smaller than predicted by Eqn. 11.

The observed thickness dependence of k_i for dipicrylamine is in sharp contrast to the results of recent studies on valinomycin-mediated Rb^+ transport [16] in monoolein membranes made from a series of n-alkane solvents (n-decane to n-hexadecane) and solvent-free monoolein membranes. In these experiments the translocation rate constant k_{MS} of the positively charged ion-carrier complex was found to be almost insensitive to a variation in membrane thick-

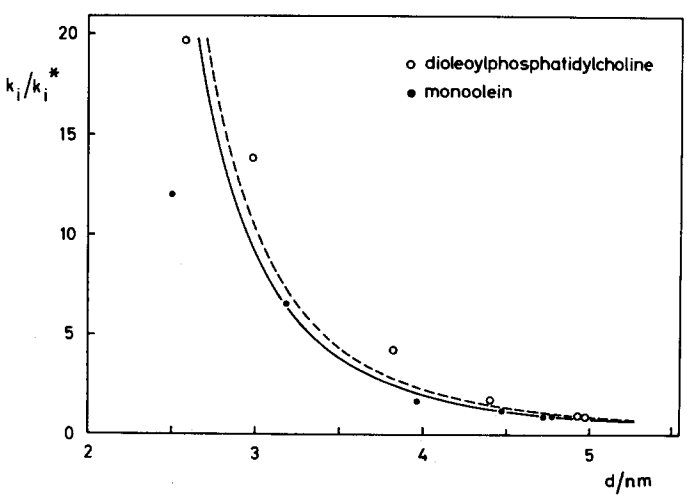

Fig. 1. Ratio k_i/k_i^* for dipicrylamine transport across membranes made from dioleoylphosphatidylcholine and monoolein as a function of membrane thickness d. k_i is the translocation rate constant in a membrane of thickness d; k_i^* is the value of k_i for n-decane as solvent. Dotted line: theoretical curve for dioleoylphospahtidylcholine. Full line: theoretical curve for monoolein. For further explanations see text.

ness. The reason for this different behavior is not clear so far. Possibly the translocation rate of the valinomycin · Rb^+ complex which has a larger size than the dipicrylamine ion is more sensitive to the fluidity of the membrane; it is conceivable that the fluidity increases with increasing *n*-alkane content of the membrane. This could then lead to a mutual compensation of the effects of fluidity and dielectric barrier height on k_{MS}. Another possibility would be that the adsorption plane of the ion-carrier complex shifts more toward the interior of the membrane as the membrane becomes thicker. In contrast to the large variation of k_i for dipicrylamine, there is only an insignificant change of the partition coefficient β with membrane thickness (Table I). This result is expected for a lipophilic ion which is mainly adsorbed to the membrane surface, if the structure of the surface does not appreciably depend on the presence of solvent in the membrane.

Indeed, Haydon [32] has presented arguments suggesting that the density of the polar head-groups is nearly the same in a solvent-less bimolecular leaflet and in a hydrocarbon-containing film made from the same lipid. The conclusion that the *n*-alkane present in the film has essentially the effect of increasing the thickness of the hydrophobic layer without changing the structure of the surface, is also consistent with the thickness dependence of k_i as discussed above.

It is interesting to note that a similar strong influence of the membrane thickness on k_i was found for the transport of tetraphenylborate across lipid bilayer membranes (Benz, R., unpublished results). For membranes from monoolein k_i increase from 11 s^{-1} (*n*-decane as solvent) to 130 s^{-1} (*n*-hexadecane as solvent).

(b) Influence of lipid structure

The results obtained with a variety of different phosphatidylcholines and phosphatidylethanolamines are given in Tables II, III and IV. The effects of the following variations in the structure of the hydrocarbon chains have been studied: position of the double bond, cis-trans isomerism, number of the double bonds, and nature of the linkage between hydrocarbon chain and glycerol moiety (ester or ether bond). For comparison, previous data [21] on the chain-length dependence of the kinetic parameters have also been included in Table II.

In contrast to the large variation of k_i with chain length (by a factor of more than 20 between C_{16} and C_{24}), the dependence of k_i on the position and cis-trans configuration of the double-bond is relatively small. Also the partition coefficient β was found to be rather insensitive to these structural variations.

A strong variation of the translocation rate constant k_i (by a factor of more than ten) was observed when the number of double bonds in the C_{18} fatty-acid chain was increased from one to three (Table III). A similar behaviour has been reported previously for the translocation rate constant of the valinomycin · Rb^+ complex in a series of monoglyceride membranes [16]. A possible reason for the observed increase in k_i could be a change in fluidity of the membrane with increasing unsaturation of the fatty acid chains. A detailed interpretation of the variation of k_i is difficult, however, because the specific membrane capacity C_m increases with unsaturation of the lipid (Table III) and this increase of C_m may result from a decrease in membrane thickness and/or from an increase of

TABLE II

Kinetic parameters of dipicrylamine transport through membranes made from phosphatidylcholines and phosphatidylethanolamines differing in the chain length and in the position of the double bond. The solvent was *n*-decane. The aqueous phase contained 10^{-8} M dipicrylamine and 0.1 M NaCl; T = 25°C. The values of the specific membrane capacity were taken from ref. 28 and the experimental data for the systems denoted by an asterisk from ref. 21.

Fatty acid residues	C_m (nF . cm^{-2})	τ_1 (ms)	a_1	k_i (s^{-1})	N_t (pmol · cm^{-2})	β (× 10^{-2} cm)
	Phosphatidylcholines					
Dipalmitoleoyl (Δ^9-$C_{16:1}$) *	387	0.32	0.45	850	0.36	1.8
Dipetroselinoyl (Δ^6-$C_{18:1}$)	399	0.27	0.53	880	0.46	2.3
Dioleoyl (Δ^9-$C_{18:1}$) *	374	0.41	0.65	430	0.74	3.7
Divaccenoyl (Δ^{11}-$C_{18:1}$)	390	0.72	0.45	370	0.35	1.8
Dielaidinoyl (*trans*-Δ^9-$C_{18:1}$)	335	0.44	0.65	430	0.62	3.1
1-Oleoyl-2-stearoyl	370	0.51	0.44	550	0.32	1.6
Dieicosenoyl (Δ^{11}-$C_{20:1}$) *	358	0.84	0.71	170	0.93	4.7
Dierucoyl (Δ^{13}-$C_{22:1}$) *	327	1.42	0.74	90	0.99	5.0
Dinervonoyl (Δ^{15}-$C_{24:1}$) *	287	2.85	0.79	37	1.15	5.8
Egg	339	0.47	0.62	420	0.57	2.9
	Phosphatidylethanolamines					
Dioleoyl (Δ^9-$C_{18:1}$)	372	0.13	0.37	2510	0.22	1.1
Egg	328	0.16	0.25	2310	0.12	0.6

dielectric constant ϵ_m. Both changes would lead to a reduction of dielectric barrier height and to an increase of k_i. It is also interesting to note that the partition coefficient β greatly decreases (by a factor of eight) when the number of double bonds of the fatty-acid chain is varied from one to three (Table III). So far, we have no clear explanation for this finding. Table IV summarizes experiments with lipids differing in the nature of the linkage between hydro-

TABLE III

Kinetic parameters of dipicrylamine transport through membranes from phosphatidylcholines with C_{18} fatty acid residues of different degree of unsaturation. The lipids were dissolved in *n*-decane. The aqueous phase contained 0.1 M NaCl and 10^{-8} M dipicrylamine, 25°C. The values of the specific capacity C_m were taken from ref. 28 and the experimental data for dioleoylphosphatidylcholine from ref. 21.

Fatty acid residues	C_m (nF : cm^{-2})	τ_1 (μs)	a_1	k_i (s^{-1})	N_t (pmol · cm^{-2})	β (× 10^{-2}, cm)
Dioleoyl (Δ^9-$C_{18:1}$)	374	410	0.65	430	0.74	3.7
Dilinoleoyl ($\Delta^{9,12}$-$C_{18:2}$)	416	210	0.38	1500	0.27	1.4
Dilinolenoyl ($\Delta^{9,12,15}$-$C_{18:3}$)	582	86	0.12	4810	0.09	0.45

TABLE IV

Kinetic parameters of dipicrylamine transport through membranes from lipids with ester or ether linkage of the hydrocarbon tail. The lipids were dissolved in *n*-decane. The aqueous phase contained 0.1 NaCl and 10^{-8} M dipicrylamine. The values of the specific capacity C_m were taken from ref. 28. T = 25°C.

Lipid	C_m (nF · cm^{-2})	τ_1 (ms)	a_1	k_i (s^{-1})	N_t (pmol · cm^{-2})	β (× 10^{-2} cm)
1-Oleoyl-2-stearoyl-3-phosphatidylcholine	370	0.51	0.44	550	0.32	1.6
1-*O*-oleoyl-2-*O*-palmityl 3-phosphatidylcholine	352	7.5	0.63	24	0.62	3.1
Deoleoyl-phosphatidyl-ethanolamine	372	0.13	0.37	2510	0.22	1.1
Di-*O*-oleoyl-phosphatidylethanolamine	357	0.79	0.46	350	0.32	1.6

carbon chain and glycerol backbone. Such a comparison between ester and ether analogs of phosphatidylcholine and phosphatidylethanolamine is particularly interesting because it is known that monolayers from ester and ether lipids differ in the magnitude of the surface potential [34]. It is therefore likely that also in a bilayer membrane the dipolar potential drop in the membrane-water interface is different for ester and ether lipids. Variations in the dipolar potential in turn may influence the ion permeability of the membrane [17,20,35]; see also ref. 37 for a clearly written review on surface potentials of lipid bilayers. There is strong evidence that as a result of the existence of dipolar layers, the interior of a phosphatidylcholine membrane is positive by several hundred millivolts with respect to the aqueous phases [35]. When the ester linkage in phosphatidylcholine is replaced by an ether bond the absolute magnitude of this potential difference is considerably reduced; for instance, at a packing density of one lipid molecule per 0.6 nm^2 (60 Å^2) the surface potentials of distearoylphosphatidylcholine (ester) and of distearylphosphatidylcholine (ether) monolayers differ by about 120 mV; for dioleoylphosphatidylcholine and dioleylphosphatidylcholine the difference is about 90 mV [34].

The existence of dipolar layers in the membrane surface affects in general, both the partion coefficient β and the translocation rate constant k_i of the hydrophobic ion. The relative magnitude of the effects on β and on k_i depends on the location of the adsorption plane of the hydrophobic ion relative to the dipolar layer [20]. If a fraction δV_D of the total dipolar potential V_D drops between the adsorption plane and the membrane surface, then the dipolar potential may be expected to change the energy of an adsorbed ion by the amount $z\delta FV_D$ per mol (z is the valency of the ion); accordingly, the partition coefficient β is modified by the factor exp $(-z\delta FV_D/RT)$. On the other hand, the activation energy for the translocation of the ion should then increase by the amount $z(1-\delta)\,FV_D$ which would change k_i by the factor exp$[-z(1-\delta)$-$FV_D/RT]$. If the adsorption plane is located outside (on the aqueous side) of the dipolar layer than V_D acts only on k_i leaving β unaffected ($\delta = 0$).

Unfortunately, such theoretical concepts cannot be rigorously tested in membrane experiments, because any modification of lipid structure also changes other parameters besides the dipolar potential. Table IV shows that the

partition coefficient is not much affected if ester bonds in phosphatidylcholine or phosphatidylethanolamine are replaced by ether bonds. One may therefore expect that a large fraction of ΔV_D should act on the translocation rate constant k_i. Indeed, it is seen that k_i is much smaller in the lipids with ether bonds (by a factor of 23 in the case of phosphatidylcholine and by a factor of 7 in the case of phosphatidylethanolamine). This is in qualitative agreement with expectation, as replacing ester by ether bonds makes the potential in the membrane interior less positive. The quantitative agreement, however, is poor; if the difference in the dipolar potential of ester and ether analogs is of the order of 100 mV, as suggested by the monolayer experiments, the translocation rate constants should differ by a factor of the order of one hundred, which is much larger than the observed ratio of the k_i values. This discrepancy may result from changes of membrane properties other than the dipolar potential. Furthermore, as the size of the hydrophobic ion is not negligible, the ion creates a perturbation of the dipolar layer which tends to diminish the influence of V_D.

Table II also contains a comparison between phosphatidylcholines and phosphatidylethanolamines. Although the membrane thickness (estimated from C_m) is almost the same in both cases, the translocation rate constant k_i is about six times larger in the case of phosphatidylethanolamine. The partition coefficient β on the other hand is significantly smaller in phosphatidylethanolamine. These differences cannot be explained by changes in the dipolar potential, as V_D is virtually the same for both lipids [32]. The larger value of β and the smaller value of k_i in phosphatidylcholine membranes possibly result from a stronger interaction of dipicrylamine with the zwitterionic head group of this lipid.

(c) Membranes containing cholesterol

Membranes were formed from dioleoylphosphatidylcholine-cholesterol mixtures (in *n*-decane) with cholesterol : phospholipid molar ratios ranging between 1 : 5 and 4 : 1. It is seen from Table V that the partition coefficient β is almost insensitive to the composition of the membrane; on the other hand, the translocation rate constant k_i is found to increase with increasing cholesterol content. An even stronger increase of k_i has been observed with membranes from monoolein containing cholesterol [17,20]. For the monoolein-cholesterol membranes the variation of k_i has been interpreted in terms of dipolar potential changes resulting from the incorporation of cholesterol. This explanation seems unlikely in this case since the dipolar potential of phosphatidylcholine membranes is apparently not much changed by the presence of cholesterol [32,35]. On the other hand, it is seen from Table V that the specific membrane capacity increases with the molar ratio cholesterol : phosphatidylcholine, indicating that membranes with a high cholesterol content have a reduced thickness. This change in membrane thickness seems to account to a large extent for the increase in translocation rate constant k_i.

Kinetic studies with valinomycin at membranes from dioleoylphosphatidylcholine/cholesterol mixtures support this hypothesis. Even at high molar ratios of cholesterols only a small influence on valinomycin mediated Rb^+ transport was observed. (Benz, R., unpublished results).

On the other hand it is not clear if the molar ratio lipid/cholesterol is the

TABLE V

Kinetic parameters of dipicrylamine transport through membranes made from dioleoylphosphatidylcholine/cholesterol mixtures dissolved in *n*-decane. The mole fraction x of cholesterol (referred to total lipid) is given in the first column. Conditions: 25°C; 0.1 M NaCl and 10^{-8} M dipicrylamine. The results for pure dioleoylphosphatidylcholine and the specific capacity C_m for the same lipid were taken from refs. 21 and 28, respectively.

x	C_m (nF cm^{-2})	τ_1 (μs)	a_1	k_i (s^{-1})	N_t (pmol · cm^{-2})	β ($\times 10^{-2}$ cm)
0	374	410	0.65	430	0.74	3.7
0.17	369	430	0.65	410	0.72	3.6
0.33	379	370	0.66	450	0.80	4.0
0.50	392	320	0.62	590	0.68	3.4
0.67	418	270	0.58	760	0.62	3.1
0.80	510	90	0.59	2270	0.77	3.9

same in the bulk phase and in the black part of the membrane. Pagano et al. [36] have shown that this is not given in certain cases. Because of this finding a detailed discussion and interpretation of the effects of cholesterol on the properties of the membranes may be difficult.

Membranes from monoolein/cholesterol mixtures dissolved in *n*-decane show an increasing specific capacity with increasing mole fraction of cholesterol [16]. From the results given in this study it is possible to conclude that the strong influence of cholesterol on the transport of lipophilic ions as reported by G. Szabo [17,20] is only partly caused by the change of the dipolar potential of the membranes and partly by the thinning of the membranes with increasing cholesterol content. Membrane experiments with monoolein/cholesterol mixtures dissolved in *n*-hexadecane, where no thickness decrease is observed, show a much smaller influence of cholesterol on the kinetics of dipicrylamine or tetraphenylborate (Cros, D. and Benz, R., in preparation).

(d) Effects of surface charge

Some experiments were performed with the negatively-charged lipid phosphatidylserine. The results are given in Table VI. The ionic strengths of the aqueous solutions were varied between $3 \cdot 10^{-2}$ and 3 M at a constant dipicrylamine concentration of 10^{-8} M.

TABLE VI

Kinetic parameters of dipicrylamine transport through membranes made from phosphatidylserine in aqueous NaCl solutions of different ionic strength J (T = 25°C). The lipid was dissolved in *n*-decane. For the calculation of N_t a value of C_m = 355 nF · cm^{-2} for the membrane capacity was used. The aqueous phase contained 10^{-8} M dipicrylamine.

J/M	τ_1 (ms)	a_1	k_i (s^{-1})	N_t (pmol · cm^{-2})	β ($\times 10^{-2}$ cm)
0.03	0.63	0.016	790	0.006	0.030
0.1	0.73	0.033	660	0.013	0.065
0.3	0.78	0.10	570	0.043	0.22
1	0.81	0.33	420	0.18	0.90
3	0.59	0.55	380	0.46	2.3

From the data represented in Table VI it is seen that there is a strong increase of the relaxation amplitude a_1 of the fast relaxation process with increasing ionic strength. This corresponds to the increase of the partition coefficient β which is presumably caused by a decrease of surface potential. We have tried to fit the observed dependence of β on ionic strength with the Gouy-Chapman theory, neglecting any ionic-strength dependence of the activity coefficient of dipicrylamine in aqueous solution. This has been confirmed for neutral dioleoylphosphatidylcholine membranes in the presence of 1 M NaCl and 10^{-8} dipicrylamine [21]. If the only effect of ionic strength on β occurs via a change in surface potential ψ_o, then the following relations hold [37,38]:

$$\beta = \beta_0 \exp(-zF\Delta\psi/RT) \tag{12}$$

$$\Delta\psi = 2\frac{RT}{F} \ln\left[\frac{\sigma}{\sigma_0} + \sqrt{\left(\frac{\sigma}{\sigma_0}\right)^2 + 1}\right] \tag{13}$$

$$\sigma_0 = \sqrt{8\epsilon_0\epsilon RTJ} \tag{14}$$

β_0 is the limiting value of β at infinite ionic strength ($\psi_o = 0$), z the valency of the hydrophobic ion, σ the surface charge per unit area, and J the ionic strength of the inert 1 : 1 electrolyte added to the solution. The results obtained from Eqns. 12—14 are shown in Fig. 2. The full circles represent the values of $\Delta\psi$ calculated from β(c) with $\beta_0 = 0.25$ cm. The theoretical curve has been drawn using $\beta_0 = 0.25$ cm and a charge density of one elementary charge per 0.52 nm^2 ($\sigma \sim 0.31$ C $\cdot$ m^{-2}). This charge density is close to the expected value of about one elementary charge per 0.60 nm^2 in a densely packed monolayer of phosphatidylserine. It is seen from Fig. 2 that the Gouy-Chapman theory gives a reasonable fit to the experimental data. The value of the partition coefficient β_0 at infinite ionic strength, which is needed for an optimal fit

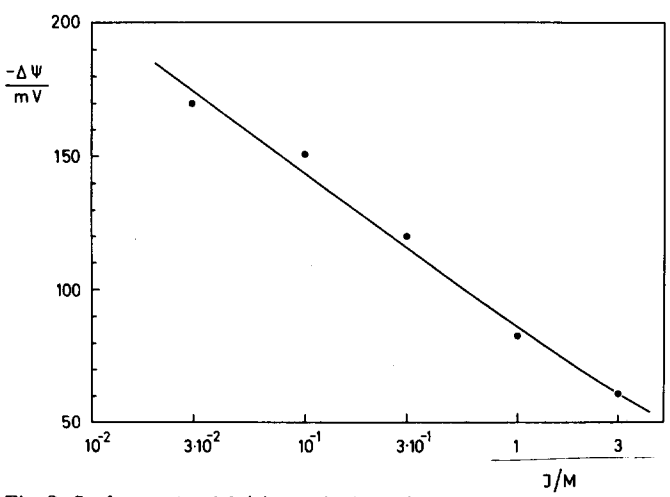

Fig. 2. Surface potential $\Delta\psi$ as calculated from the ionic strength dependence of partition coefficient β of dipicrylamine in membranes from phosphatidylserine. J is the ionic strength of the inert electrolyte (NaCl) added to the solutions. The values of $\Delta\psi$ were calculated from Eqn. 12 using a value of $\beta_0 = 0.25$ cm. The theoretical line corresponds to a surface charge density of 1 elementary charge per 0.52 nm^2.

is higher than the β values observed with uncharged membranes; this could mean that the results are influenced to some extent by variations in the activity coefficient of dipicrylamine with ionic strength.

The effect of ionic strength on the translocation rate constant k_i is relatively small (Table VI). Between J = 0.03 M and J = 3 M k_i decreases by a factor of about two. This change could result from a small variation in membrane thickness and/or a change in the fluidity of the lipid.

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References

1 Mueller, P. and Rudin, D.O. (1967) Biochim. Biophys. Res. Commun. 26, 398—404
2 Eisenman, G., Ciani, S.M. and Szabo, G. (1968) Fed. Proc. 27, 1289—1304
3 Tosteson, D.C. (1968) Fed. Proc. 27, 1269—1277
4 Le Blanc, Jr., O.H. (1969) Biochim. Biophys. Acta 193, 350—360
5 Ketterer, B., Neumcke, B. and Läuger, P. (1971) J. Membrane Biol. 5, 225—245
6 Haydon, D.A. and Hladky, S.B. (1972) Q. Rev. Biophys. 5, 187—282
7 Mueller, P., Rudin, D.O., Tien, H.T. and Wescott, W.C. (1962) Nature 194, 979—980
8 Fettiplace, R., Andrews, D.M. and Haydon, D.A. (1971) J. Membrane Biol. 5, 277—296
9 White, S.H. (1975) Biophys. J. 15, 95—117
10 Taylor, J. and Haydon, D.A. (1966) Disc. Far. Soc. 42, 51—59
11 Benz, R., Fröhlich, O., Läuger, P. and Montal, M. (1975) Biochim. Biophys. Acta 394, 323—334
12 Requena, J. and Haydon, D.A. (1975) Proc. R. Soc. A, 347, 161—177
13 Montal, M. and Mueller, P. (1972) Proc. Natl. Acad. Sci. U.S. 69, 3561—3566
14 White, S.H., Petersen, D.C., Simon, S. and Jafuso, M. (1976) Biophys. J. 16, 481—488
15 Hladky, S.B. and Haydon, D.A. (1972) Biochim. Biophys. Acta 274, 294—312
16 Benz, R., Fröhlich, O. and Läuger, P. (1976) Biochim. Biophys. Acta, 464, 465—481
17 Szabo, G. (1974) Nature 252, 47—49
18 Bruner, L.J. (1975) J. Membrane Biol. 22, 125—141
19 Andersen, O.S. and Fuchs, M. (1975) Biophys. J. 15, 795—830
20 Szabo, G. (1976) In Extreme Environment; Mechanism of Microbial Adaption (Heinrich, M.R., ed.), Academic Press, New York, in the press
21 Benz, R., Läuger, P. and Janko, K. (1976) Biochim. Biophys. Acta 455, 701—720
22 Benz, R., Gisin, B.F., Ting-Beall, H.P., Tosteson, D.C. and Läuger, P. (1976) Biochim. Biophys. Acta 455, 665—684
23 McLaughlin, S.G.A., Szabo, G., Eisenman, G. and Ciani, S.M. (1970) Proc. Natl. Acad. Sci. U.S. 67, 1268—1275
24 Stark, G., Benz, R., (1971) J. Membrane Biol. 5, 133—153
25 McLaughlin, S.G.A. and Harary, H. (1976) Biochemistry 15, 1941—1948
26 Haydon, D.A. and Myers, V.B. (1973) Biochim. Biophys. Acta 307, 429—443
27 Benz, R., Stark, G., Janko, K. and Läuger, P. (1973) J. Membrane Biol. 14, 339—364
28 Benz, R. and Janko, K. (1976) Biochim. Biophys. Acta 455, 721—738
29 Singleton, W.S., Gray, M.S., Brown, M.L. and White, J.L. (1965) J. Am. Oil Chem. Soc. 42, 53—56
30 Sanders, H. (1967) Biochim. Biophys. Acta 144, 485—487
31 Neumcke, B. and Läuger, P. (1969) Biophys. J. 9, 1160—1170
32 Haydon, D.A. (1975) Ann. New York Acad. Sci. 264, 2—16
33 Parsegian, A. (1969) Nature 221, 844—846
34 Paltauf, F., Hauser, H. and Phillips, M.C. (1971) Biochim. Biophys. Acta 249, 539—547
35 Hladky, S.B. and Haydon, D.A. (1973) Biochim. Biophys. Acta 318, 464—468
36 Pagano, R.E., Ruysschaert, J.M. and Miller, J.R. (1972) J. Membrane Biol. 10, 11—30
37 McLaughlin, S.G.A., in F. Bronner and A. Kleinzeller (eds.), Current Topics in Membranes and Transport, Vol. 9, in the press
38 Läuger, P. and Neumcke, B. (1973) in Membranes, A Series of Advances (Eisenmann, G., ed.), Vol. II, 1—59, Marcel Dekker, New York