Biochimica et Biophysica Acta 813 (1985) 221–229
Elsevier

BBA 72478

Ca^{2+}-induced phase separation in black lipid membranes and its effect on the transport of a hydrophobic ion

Alfred Miller [a], Günther Schmidt [a], Hansjörg Eibl [b] and Wolfgang Knoll [a]

[a] *Physik Department E22, Technische Universität München, James-Franck-Strasse, D-8046 Garching and* [b] *Max-Planck-Institut für Biophysikalische Chemie, Am Fassberg, D-3400 Göttingen (F.R.G.)*

(Received October 22nd, 1984)

Key words: Ion transport; Hydrophobic ion; Phase separation; Ca^{2+}; Voltage jump–current relaxation; (Lipid bilayer)

Voltage jump-current relaxation studies have been performed with dipicrylamine-doped black membranes of binary lipid mixtures. As in the case of the carrier-mediated ion transport (Schmidt, G., Eibl, H. and Knoll, W. (1982) J. Membrane Biol. 70, 147–155) no evidence was found that the neutral lipid phosphatidylcholine (DPMPC) and the charged phosphatidic acid (DPMPA) are heterogeneously distributed in the membrane over the whole range of composition. However, besides a continuous dilution of the surface charges of DPMPA by the addition of DPMPC molecules, different structural properties of mixed membranes influence the kinetics of the dipicrylamine transport. The addition of Ca^{2+} to the electrolyte induces a lipid phase separation within the membrane into two fluid phases of distinctly different characteristics of the translocation of hydrophobic ions. Thus, it is possible to determine a preliminary composition phase diagram for the DPMPA/DPMPC mixtures as a function of the Ca^{2+} concentration.

Introduction

The induction of phase separation in artificial membranes of lipid mixtures is a well-established phenomenon [1–7] although its physiological significance for biological membranes is still debated [8–13]. Particularly interesting are isothermally triggered lateral or transversal redistributions of charged lipids by ionic changes in the aqueous phase like variations of the pH and/or concentrations of mono- and divalent ions or by the addition of charged polypeptides [2,3,5,6,14,15].

In order to address the question of how a membrane function like the transport of ions across the hydrophobic barrier is influenced by the structure and the order of the lipid that surrounds the functional unit we have performed electrical measurements with model ionophores incorporated into bilayer membranes of binary lipid mixtures [16]. We could show that these so-called black films are well-suited to study the coupling of model transport systems to the lateral organisation of the two lipid components. By the analysis of the kinetic parameters of the carrier-mediated ion transport it was demonstrated that Ca^{2+} also in these model membranes can induce a lateral phase separation which modifies the transport characteristics considerably.

If the concept of the lipid-controlled function of an integral protein is more generally valid, transport systems other than carriers should also be sensitive to changes in the lateral distribution of the lipids in a mixed membrane. We have extended therefore our voltage jump-current relaxation studies to the transport of hydrophobic

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DPMPA, 1,2-dipentadecylmethylidene phosphatidic acid; DPMPC, 1,2-dipentadecylmethylidenephosphatidylcholine.

ions. As in the case of the carrier valinomycin, the molecular details of the translocation of dipicrylamine across lipid membranes are well established [17–19]. Moreover, the analysis of the kinetic data requires the determination of only one exponential decay. A major advantage, however, is the fact that the transport of dipicrylamine across a charged membrane is distinctly different from the translocation across a neutral one. It is therefore possible to observe even small changes of the membrane composition and determine rather accurately the phase boundaries in the Ca^{2+}-induced demixing region of the phase diagram.

Materials and Methods

The synthesis of the lipids used in this study – 1,2-dipentadecylmethylidenephosphatidylcholine (DPMPC) and 1,2-dipentadecylmethylidene phosphatidic acid (DPMPA) – is described elsewhere [20]. All measurements were performed with black lipid membranes which were formed by the method of Mueller et al. [21] from 1% (w/v) lipid solutions in *n*-decane (Fluka, purum). Membrane areas were typically $10^{-2} \cdot cm^2$. The experiments were started 20–30 min after the complete blackening of the membranes. The temperature was 36°C where all lipids are fluid [22,23]. Dipicrylamine (Fluka, puriss) was added to the buffered electrolyte solutions (1 M NaCl/10 mM Hepes (pH 6, adjusted by NaOH and HCl)) to give final concentrations between $1 \cdot 10^{-9}$ and $4 \cdot 10^{-5}$ M. At that pH, DPMPA has one negative charge [23]. For the studies of the calcium effect, various amounts of $CaCl_2$ salt were added to the aqueous solutions. The relaxation behavior was independent of the way Ca^{2+} was added: in some cases, it was applied after the membrane had been formed in Ca^{2+}-free buffer, in others (mostly for the high Ca^{2+} concentrations), the Ca^{2+} was already in the electrolyte before the membrane was formed.

The set-up for the voltage jump-current relaxation studies was similar to those described previously [24,25] with some modifications: a commercial pulse generator (HP 8013 B) triggered via opto-coupler a self-designed pulsformer [26] which reduced the slow voltage increase during the pulse to less than 0.1% of the pulse height with only a slight increase of the noise level.

A storage oscilloscope (Tectronix 5115, with plug-in amplifier 5 A 22 N and time-base 5 B 12 N) was used as a fast amplifier. Its output was fed into a waveform recorder (Gould Biomation 2805) whose memory could be transferred to a microcomputer by a self-designed interface. Single-shot relaxation measurements were averaged (up to 512 times) and the data finally stored on a floppy disk for later analysis.

Results and Discussion

Analysis of the current relaxation

The transport of hydrophobic ions like dipicrylamine through a lipid bilayer membrane is assumed to occur in three consecutive steps as first suggested by Ketterer et al. [17]: (i) adsorption from the aqueous phase into the potential energy minimum at the membrane interface; (ii) translocation across the central barrier to the opposite potential minimum; (iii) desorption into the aqueous phase. These kinetic processes are assumed to be first-order and have been described by rate constants: K_{ma} for the adsorption and desorption, K_i for the translocation step. It has been established that for many cases the inequality $K_{ma} \ll K_i$ is well-satisfied for dipicrylamine [17,27]. Then, the electric current transient, $J(t)$, following the application of a voltage jump mirrors the voltage-dependent redistribution of the lipophilic ions between the two potential minima at the membrane interfaces. For certain membranes, several additional effects have been reported, some of which modify the time dependence of the transient current considerably. A limited number of 'binding sites' for dipicrylamine ions at the membrane interface are responsible for a saturation behavior of the current at high dipicrylamine concentrations [17,27–30]. Electrostatic interaction between the hydrophobic ions in the membrane [30,31] and membrane fluidity changes [27] have been proposed as a reason for the observed dipicrylamine concentration dependence of the translocation rate constant K_i and hence the current transients. Most seriously affected is the experimental determination of the kinetic parameters of the ion relaxation across the membrane if adsorption and desorption and diffusive processes in

the aqueous boundary layer can no longer be ignored. As has been shown by Jordan and Stark [19] such processes can modify substantially the time dependence of the current relaxation and in some cases even prevent a determination of the translocation rate constant K_i. It turned out, however, that for the lipids used in this study, even when working with the charged DPMPA it always was possible to determine a relaxation time, τ, from the initial slope of the current relaxation after a voltage jump according to Ref. 32:

$$\frac{1}{\tau} = -\frac{d}{dt}(\ln J(t))_{t=0} \quad (1)$$

The initial conductivity, $\lambda_0 = \lambda(t=0)$, which is given by $\lambda_0 = \frac{J_0}{U \cdot A}$ with J_0 = current at zero time, A = membrane area, is a second parameter which is highly dependent on the lipid composition of the membrane.

Three examples of current relaxations after a voltage jump of $U = 50$ mV are shown in Fig. 1 for a DPMPC membrane, a DPMPA membrane and a 1 : 1 mixture of both lipids. All measuring curves are obtained by averaging 100 times. For better comparison, the curves are all scaled to unity by dividing each data point by the appropriate J_0. It can be seen that indeed with sufficient accuracy for our purposes each current relaxation can be characterized by two parameters, i.e., the relaxation time and the initial conductivity.

Kinetics of dipicrylamine translocation across mixed membranes

In order to find the best experimental conditions that allow us to analyse the transport kinetics of dipicrylamine over the whole composition range of binary lipid mixtures as membranes we first performed a set of measurements at different dipicrylamine concentrations in the aqueous phase. On the basis of these results, we choose for the following experiments a bulk dipicrylamine concentration of $1 \cdot 10^{-6}$ M. At that concentration, the conductivities are sufficiently high and saturation effects that come into play for DPMPC-rich membranes [27,30] do not disturb yet too much. In a first series of measurements, we investigated in detail the kinetics of the dipicrylamine translocation across membranes composed of binary mix-

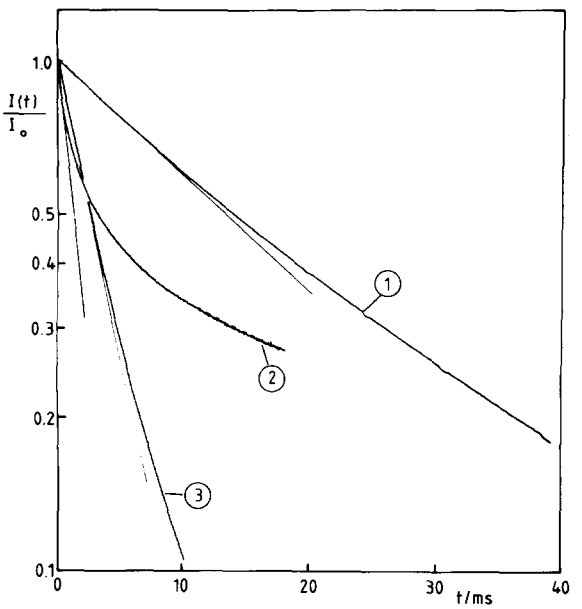

Fig. 1. Semilogarithmic plots of current relaxations after the application of a voltage jump of $U = 50$ mV. Shown are the averaged curves (scaled to unity by dividing each data point $J(t)$ by the current at zero time, J_0) of a pure DPMPC membrane (1), pure DPMPA (2) and a 1:1 mixture of both lipids (3). From the analysis of the straight lines, the relaxation times, τ, and the initial conductivities, λ_0, are deduced: (1) $\tau = 20.3$ ms, $\lambda_0 = 5.6 \cdot 10^{-4}\ \Omega^{-1} \cdot cm^{-2}$. (2) $\tau = 2.5$ ms, $\lambda_0 = 9 \cdot 10^{-5}\ \Omega^{-1} \cdot cm^{-2}$. (3) $\tau = 3.7$ ms, $\lambda_0 = 7.5 \cdot 10^{-4}\ \Omega^{-1} \cdot cm^{-2}$. $T = 36°C$; pH 6; 10 mM Hepes; 1 M NaCl; dipicrylamine concentration $= 1 \cdot 10^{-6}$ M.

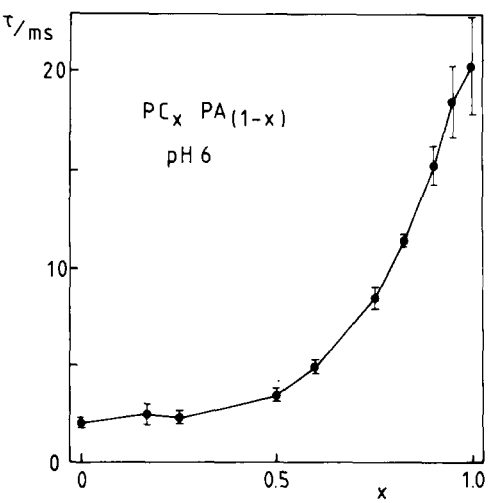

Fig. 2. Dependence of the relaxation time, τ, on the DPMPC content, x, in mixed membranes. Mean values ± S.D.

tures of DPMPC and DPMPA molecules. In Fig. 2 is shown how the relaxation time, τ, increases as the mole fraction x of DPMPC in the mixture is increased. Fig. 3 demonstrates the corresponding dependence of the initial conductivity λ_0 on the DPMPC fraction x. It can be seen that already small amounts of a second lipid component modify considerably the transport parameters of dipicrylamine incorporated into the bimolecular film. As in the studies with an ion carrier as a structural probe [16], we conclude from that finding that no major difference exists between the composition of the torus and the membrane. From the initial conductivity, λ_0, and the relaxation time, τ, the interfacial dipicrylamine concentration N_t can be calculated [32]. The result is shown in Fig. 4. The observed behavior is determined by at least three different contributions: (i) an increasing dilution of the surface charges of DPMPA by the addition of DPMPC, (ii) an increasing saturation behavior with increasing DPMPC content, and (iii) modifications due to variations in the structural properties of lipid mixtures. The first contribution would cause a monotonous decrease of N_t with increasing surface charge density σ (i.e., increasing DPMPA content) as predicted by the Gouy-Chapman theory for charged membranes [31,33]. The dashed line has been calculated with the assumption of a homogeneous mixture and a constant mean molecular area of 0.50 nm^2. The two other contributions are less easy to quantify. For the increasing saturation of N_t with increasing DPMPC content, a smooth variation might be assumed. From monolayer experiments with DPMPA/DPMPC mixtures, on the other hand, it is well known that at intermediate compositions peculiar structural properties can be expected [34]. It is not clear, however, how these properties influence the partition coefficient β which is given by [35]:

$$\beta = \frac{N_t}{2c_s} \tag{2}$$

with c_s = interfacial dipicrylamine concentration.

In view of our phase separation studies it is important to note that the variations of kinetic parameters of the dipicrylamine transport between membranes of different composition are by far more pronounced than those found in the analysis of a carrier-mediated ion transport [16]. Especially, the large variations of the conductivity λ_0 over a relatively narrow range of compositional variation

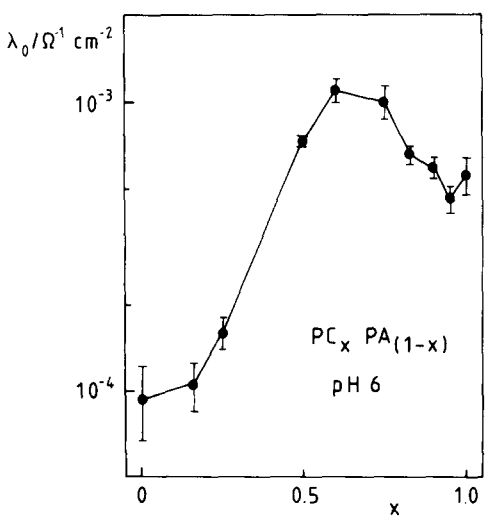

Fig. 3. Dependence of the initial conductivity, λ_0, on the DPMPC content, x, in membranes of binary lipid mixtures. Mean values ± S.D.

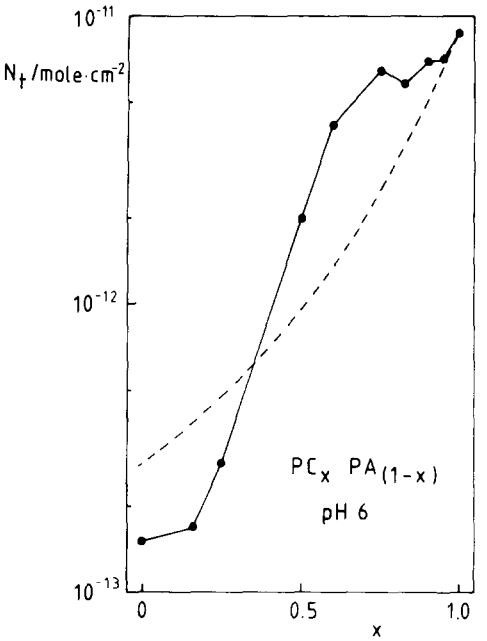

Fig. 4. Interfacial dipicrylamine concentration, N_t, calculated from the experimental data of Figs. 2 and 3 as a function of the DPMPC mole fraction, x, in mixed membranes.

is very helpful, as it allows one in many cases to neglect the current contributions by the DPMPA-rich segregated domains.

Ca^{2+}-induced phase separation in mixed membranes

The main aim of the present study was to test whether the results and interpretations concerning the coupling of membrane structure and function obtained by the analysis of the kinetics of the carrier-mediated ion transport are valid also for the translocation of a hydrophobic ion and might therefore be generally extended to other transport systems. For that purpose, voltage jump-current relaxation studies were performed with membranes of different binary lipid mixtures with increasing Ca^{2+} concentrations in the bulk aqueous phase. Typical examples of averaged current relaxation curves are shown in Fig. 5 for different Ca^{2+} concentrations. The membrane-forming solution contained a 1 : 1 mixture of DPMPA and DPMPC. All measuriang curves could be analysed in terms of an initial slope $-1/\tau = d\,(\ln I(t))/dt$ and an iniatial conductivity $\lambda_0 = J_0/(U \cdot A)$. The results for all investigated mixtures are summarized in Fig. 6 for the relaxation times and in Fig. 7 for the conductivities, respectively.

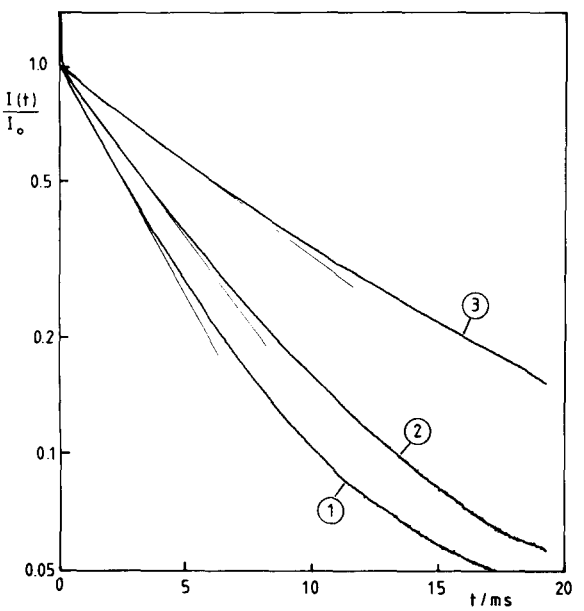

Fig. 5. Semilogarithmic plots of current relaxations after the application of a voltage jump of $U = 50$ mV. Shown are the averaged curves (scaled to unity by dividing each data point $J(t)$ by the current value at zero time, J_0) of a 1 : 1 mixture of DPMPA and DPMPC with no Ca^{2+} in the aqueous phase (1); with $c_{Ca^{2+}} = 1 \cdot 10^{-3}$ M (2); and $c_{Ca^{2+}} = 1 \cdot 10^{-2}$ M (3). The relaxation times, τ, and initial conductivities, λ_0, are: $\tau = 3.5$ ms and $\lambda_0 = 7.5 \cdot 10^{-4}\ \Omega^{-1} \cdot cm^{-2}$ for (1); $\tau = 5.1$ ms and $\lambda_0 = 8.8 \cdot 10^{-4}\ \Omega^{-1} \cdot cm^{-2}$ for (2); $\tau = 8.6$ ms and $\lambda_0 = 8 \cdot 10^{-4}\ \Omega^{-1} \cdot cm^{-2}$ for (3), respectively. The aqueous phase contained 1 M NaCl/10 mM Hepes (pH 6); $T = 36°C$; dipicrylamine concentration $= 1 \cdot 10^{-6}$ M.

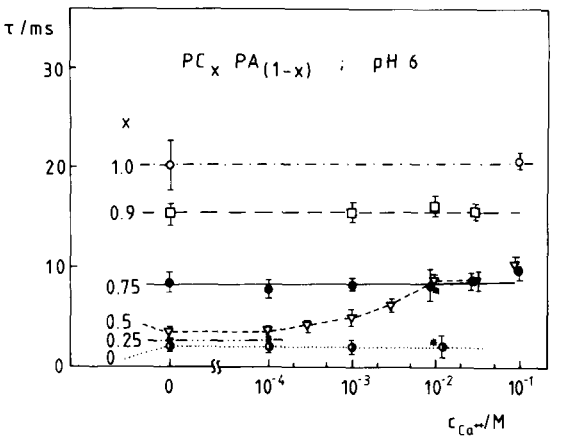

Fig. 6. Relaxation times, τ, as a function of Ca^{2+} concentration in the bulk aqueous phase for membranes of different lipid composition: (○-·-○) PC, $x = 1$; (□——□) $x = 0.9$; (●——●) $x = 0.75$; (▽- - - - -▽) $x = 0.5$; (*-···-*) $x = 0.25$; (◑·····◑) $x = 0$. Data points are mean values ± S.D. of at least five membranes.

For a pure DPMPC membrane ($x = 1$), no in-

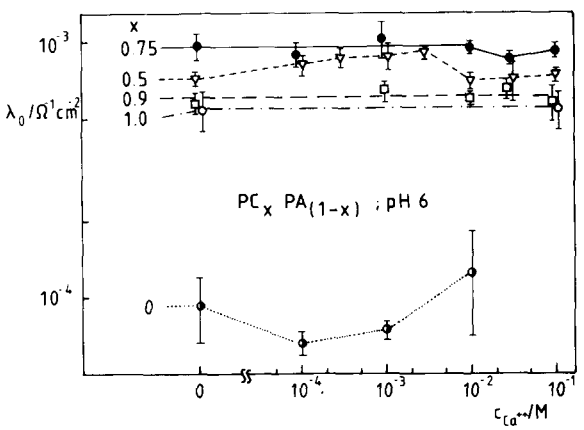

Fig. 7. Initial conductivities, λ_0, as a function of the Ca^{2+} concentration in the bulk aqueous phase for membranes of different lipid composition. The symbols are as in Fig. 6. Data points are mean values ± S.D.

fluence of the Ca^{2+} level on the kinetic parameters of the dipicrylamine transport was found, in agreement with results obtained with valinomycin [16].

The same behavior is seen for membranes that contain also some DPMPA as long as its fraction does not exceed 25% (i.e., $x \geqslant 0.75$). Presented are our data for $x = 0.9$ and 0.75. Again, no variation of τ with increasing Ca^{2+} concentration ($c_{Ca^{2+}}$) is found (up to 0.03 M). λ_0, which increases with increasing DPMPA content (see also Fig. 3), remains also constant, independent of the Ca^{2+} concentration in the aqueous phase.

Measurements at the other end of the phase diagram, i.e., with DPMPA-rich membranes were much more difficult due to a drastically reduced membrane stability. For a mixture with $x = 0.16$, measurements only up to $c_{Ca^{2+}} = 1 \cdot 10^{-3}$ M were possible and showed no variations of the transport characteristic compared to the Ca^{2+}-free electrolyte. Only for $x = 0$, i.e., pure DPMPA membranes, data could be obtained up to $c_{Ca^{2+}} = 1 \cdot 10^{-2}$ M. For all these conditions, the relaxation times remain constant. The same holds true for the conductivities although for these data a larger experimental error might hide some variations. Quite a different behavior is found for a black lipid membrane with a starting composition of $x = 0.5$, i.e., equimolar in DPMPC and DPMPA. Beginning already at about $c_{Ca^{2+}} = 1 \cdot 10^{-4}$ M, τ increases slightly until it reaches at $c_{Ca^{2+}} = 1 \cdot 10^{-2}$ M the same value as the mixture with $x = 0.75$. Upon a further increase of $c_{Ca^{2+}}$ both relaxation times remain constant (up to $c_{Ca^{2+}} = 3 \cdot 10^{-2}$ M). The conductivity, λ_0, seems to increase slightly from $7.2 \cdot 10^{-4}\ \Omega^{-1} \cdot cm^{-2}$ at $c_{Ca^{2+}} = 0$ M to $9.0 \cdot 10^{-4}\ \Omega^{-1} \cdot cm^{-2}$ at $c_{Ca^{2+}} = 3 \cdot 10^{-3}$ M but drops then down again to $\lambda_0 = 7 \cdot 10^{-4}\ \Omega^{-1} \cdot cm^{-2}$ at $c_{Ca^{2+}} = 10^{-2}$–10^{-1} M. It never exceeds, however, the values of the membranes with $x = 0.75$.

It is quite instructive to compare this behavior with results obtained with the carrier incorporated into a 1:1 mixed membrane. There, too, Ca^{2+} effects could be observed starting at $c_{Ca^{2+}} = 1 \cdot 10^{-4}$ M and ending at $c_{Ca^{2+}} = 1 \cdot 10^{-2}$ M. At that Ca^{2+} level, however, kinetic parameters determined the ion transport which were within experimental error not distinguishable from those of a pure DPMPC membrane. This had led us to the conclusion that Ca^{2+}-bound DPMPA molecules aggregate to clusters of negligable conductivity until finally at $c_{Ca^{2+}} = 1 \cdot 10^{-2}$ M, the current-carrying membrane areas contain only DPMPC molecules. Obviously, the kinetic parameters of the dipicrylamine translocation under equivalent experimental conditions do not indicate a pure DPMPC environment but are instead representative of a mixed membrane with $x = 0.75$. Although in the case of the carrier-mediated transport, too, the current-carrying DPMPC-rich membrane areas could have contained some 10% DPMPA molecules, we have to address the question of whether we do see with dipicrylamine a different phase behavior upon the addition of Ca^{2+} or whether only the current relaxation misleads to such a conclusion due to, e.g., the fact that the current through the DPMPA-rich membrane areas may not be neglected in the case of the dipicrylamine translocation. Suppose a mixed membrane with an initial mole fraction x of DPMPC molecules separates into two phases with mole fractions x_1 and x_2, respectively. If the overall membrane area remains constant upon a phase separation, the following equation holds:

$$x_1 P_1 + x_2 P_2 = x \tag{3}$$

Here P_1 and P_2 are the fractions of membrane areas of phase 1 and phase 2, respectively, hence:

$$P_1 + P_2 = 1 \tag{4}$$

The current through each of the two different membrane regions is given by:

$$J_i = \lambda_i(x_i) U A P_i \tag{5}$$

where i stands for phase 1 or phase 2.

By a combination of Eqns. 3, 4 and 5, one obtains for the ratio of the two current contributions:

$$\frac{J_2}{J_1} = \frac{\lambda_2(x_2)(x_1 - x)}{\lambda_1(x_1)(x - x_2)} \tag{6}$$

Now, one of the phase boundaries at $c_{Ca^{2+}} = 1 \cdot 10^{-2}$ M is located at $x_2 = 0.75$ and we know its corresponding conductivity $\lambda_2 = 9.6 \cdot 10^{-4}\ \Omega^{-1} \cdot cm^{-2}$. We know also from Fig. 3 that the conductivity λ_1 of the second, DPMPA-rich phase is

about one order of magnitude lower. A calculation on the basis of Eqn. 6 for a mixture with $x = 0.5$ shows that as long as x_1 remains below about 0.25 the current through the demixed membrane is dominated, as in the case of the carrier transport, by the DPMPC-rich membrane areas, i.e., $J_2/J_1 \geqslant 10$. It is therefore reasonable that for this equimolar mixture an increasing relaxation time is found upon the addition of Ca^{2+} (see Fig. 6). The current through the separated DPMPA-rich domains with the faster kinetic is simply too low to be detected. The increase of τ corresponds to a gradual change of composition of the DPMPC-rich domains from $x = 0.5$ to $x = 0.75$. By comparing these relaxation times obtained for different Ca^{2+} concentrations with those found for different mixtures (see Fig. 2), it is therefore possible to quantify the DPMPC mole fraction of these domains, thereby obtaining one phase boundary of the coexistence region in detail.

On the other hand, Eqn. 6 also tells us that wherever the second phase boundary x_1 is located it is possible to choose the initial mole fraction, x, in such a way that the current contributions of the two membrane regions corresponding to phase 1 and 2, respectively, are comparable. One experimental example is given in Fig. 8. Here, the initial mole fraction was choosen at $x = 0.25$. (Up to a Ca^{2+} concentration of $1 \cdot 10^{-4}$ M, no change in the kinetic parameters of the dipicrylamine translocation was found (see also Fig. 6).) At $1 \cdot 10^{-2}$ M Ca^{2+}, the relaxation process could be well-fitted by the sum of two exponentials, one with a relaxation time $\tau_1 = 9.6$ ms and the second with $\tau_2 = 3.4$ ms. While the longer relaxation time is nearly identical to those values found for a mixed membrane with $x = 0.75$, the faster process has a time constant similar to those found for the dipicrylamine translocation through a membrane rich in DPMPA (see (Fig. 2). The conductivity of a pure DPMPA membrane did not change much if Ca^{2+} was added (see Fig. 7). If we assume that the same holds true for membranes with only little DPMPC, i.e., that we can approximate the conductivity of a DPMPA-rich membrane by its value in a Ca^{2+}-free environment, we can correlate x_1 and $\lambda(x_1)$ according to the experimental data presented in Fig. 3. As can be seen from Eqn. 6, the knowledge of J_2/J_1 allows one then to derive the

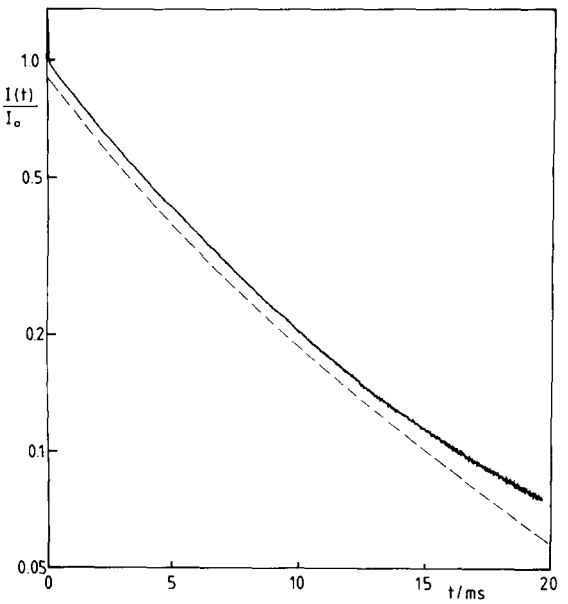

Fig. 8. Semilogarithmic plot of the current relaxation after the application of a voltage jump of $U = 50$ mV. The membrane was composed of a binary mixture of DPMPA and DPMPC with $x = 0.25$. The aqueous phase contained $1 \cdot 10^{-2}$ M $CaCl_2$ in addition to 1 M NaCl and 10 mM Hepes (pH 6; $T = 36$°C); dipicrylamine concentration $= 1 \cdot 10^{-6}$ M. The full line is the averaged experimental relaxation curve. The broken curve is calculated according to $J(t) = J_{0,1} \exp(-t/\tau_1) + J_{0,2} \exp(-t/\tau_2)$ with $J_{0,1} = J_{0,2} = 0.5$ and $\tau_1 = 9.6$ ms, $\tau = 3.4$ ms. For better clarity, this fit curve is somewhat shifted relative to the experimental data.

desired second phase boundary which is given by the mole fraction of DPMPC, x_1. This is illustrated in Fig. 9 for $x = 0.25$. Even if one admits large experimental errors in the determination of J_2/J_1, the range of possible values of x_1 is rather limited. For the example of Fig. 8 we estimate the range for J_2/J_1 to be between 0.5 and 3 and obtain therefore a possible range for $x_1 = 0.1$–0.2. Pure DPMPA domains seem to be unlikely because $J_2/J_1 = 5.2$ is certainly outside the experimental error.

All these results are summarized in Fig. 10. For very low Ca^{2+} concentrations, a homogeneous mixture of DPMPC and DPMPA over the whole range of composition is suggested. This region extends for mixtures rich in DPMPC ($x \geqslant 0.75$) and possibly also for mixtures with only very little DPMPC ($x \leqslant 0.1$) up to high Ca^{2+} levels ($c_{Ca^{2+}} = 3 \cdot 10^{-2}$ M). At intermediate compositions, the

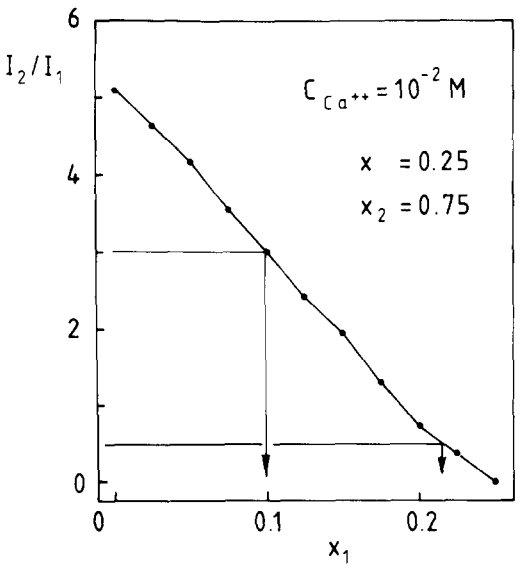

Fig. 9. Ratio of the current contributions I_2/I_1 through the two membrane areas of different composition, $x_{1,2}$, in a demixed membrane. For details see text, in particular Eqns. 3–6.

mixtures are destabilized upon the addition of Ca^{2+} and separate into two coexisting fluid phases. For the upper phase boundary, rather accurate values could be deduced. For the lower one, only estimates were obtained. Completely unknown, so far, are the molecular details of the interactions that lead to that instability.

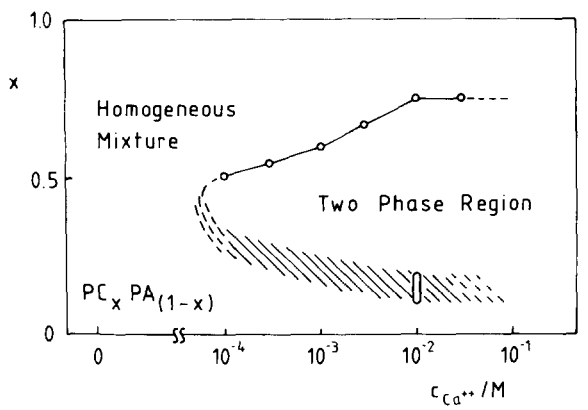

Fig. 10. Tentative phase diagram of binary mixture of DPMPA and DPMPC as a function of the Ca^{2+} concentration in the bulk aqueous phase.

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