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Permeability of vesicular phospholipid bilayer membranes to thallium and its facilitation

Israel R. Miller

Department of Membrane Research, Weizmann Institute of Science, Rehovot 76100 (Israel)

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The rate of release of Tl^+ from phospholipid vesicles of different composition was measured by pulse polarography as a function of temperature or in the presence of valinomycin, tetraphenylboron (TPB^-) or dipicrylamine (DPA^-) as transport facilitators. The release from pure dipalmitoylphosphatidylcholine (DPPC) vesicles increased abruptly around the pretransition temperature. The release from lipid mixtures with broad transition temperature region increased continuously with temperature. The steepness of the increase decreased with the width of the transition peak. Valinomycin, TPB^- (tetraphenylboron) and DPA^- (dipicrylamine) facilitate release of Tl^+ from unilamellar vesicles above their phase transition temperature with a first-order release rate constant. They do not facilitate release below the phase transition. Bursts of release were observed upon their addition to the vesicles but after annealing, which was completed within less than a minute, the vesicles were resealed. No facilitated release from multilamellar vesicles could be discerned. The entrapped volume into the multilamellar vesicles is determined from the difference between the maximal facilitated release and the total release after lysis of the liposomes by Triton X-100. The volume entrapped in the multilamellar vesicles determined this way amounted to 10–20% of the total entrapped volume.

Introduction

Unilamellar lipid bilayer vesicles are a convenient tool for investigation of lipid membrane permeability under different conditions and under the influence of different agents penetrating the membrane, modifying its structure and eventually facilitating the ion transport. In a previous paper we have measured polarographically the rate of release of Cd^{2+} from dipalmitoylphosphatidylcholine (DPPC) vesicles [1]. The polarographic method or the voltammetric methods in general, using different types of electrodes (e.g., rotating electrodes) besides the dropping mercury electrode are suitable for measuring the rate of release from vesicles. They all measure very accurately the concentration in the external solution and are not affected by the substance entrapped within the vesicles. Similarly to some fluorescence methods [2,3] the voltammetric method permits measurement of outflow kinetics in real time. Its advantage is the wide choice of ionic and non ionic permeants, amenable for investigation.

The alkali ions are reduced only at very negative potentials (approx. -2 V relative to normal calomel electrode) and can be measured only in the presence of quaternary ammonium salts as supporting electrolyte. Even so the measurements are obscured by H^+ reduction. We have chosen therefore for our investigation Tl^+, which shows strong resemblance to K^+ with respect to ionic

Abbreviations: TPB^-, tetraphenylboron anion; DPA^-, dipicrylamine anion.

radius, hydration and binding [4,5] and is reduced around 0.65 V relative to the normal calomel electrode. We measured the release rate as a function of temperaturer and of surface charge with particular consideration of events occurring during phase transition. The effectiveness of transport facilitators like valinomycin or hydrophobic anions depends also very strongly if the lipid is in the liquid-crystalline or in the rigid-crystalline phase. The phase transition temperatures and the surface charge were adjusted by the lipid composition.

Experimental

Materials

The lipids used were dipalmitoylphosphatidylcholine DPPC purchased from Dr. Berchtold's (CH-3007 Bern, Switzerland) and egg phosphatidylcholine and bovine brain phosphatidylserine purchased from Lipid Products, Nutfield, U.K. The salts K_2SO_4, Na_2SO_4, Tl_2SO_4 were all pro analysis grade and used without further purification. Valinomycin, tetraphenylboron (TPB^-) and dipicrylamine (DPA^-) were purchased from Sigma.

Vesicle formation

The vesicles were formed by the Deamer and Bangham procedure [6]. Briefly: 3 ml of phospholipid solution 1.5–2 mg/ml in diethylether/methanol (4 : 1, v/v) were injected into 4 ml of an aqueous solution of 0.1 M Tl_2SO_4 + 0.1 M (K_2SO_4 or Na_2SO_4) at 60°C and at a rate of 0.2 ml/min. A slow stream of N_2 was then bubbled through the solution, at this temperature, for additional ten minutes to remove most of the organic solvent. The rest of the organic solvent was removed together with the untrapped Tl^+ by consecutive dialysis against 0.5 l of 0.1 M K_2SO_4 or Na_2SO_4, respectively, changed every 1 h for 5 h or every 0.5 h for 3 h at 4°C. The concentration of Tl^+ in the extravesicular solution was reduced to less than $2 \cdot 10^{-5}$ M or $5 \cdot 10^{-5}$ M, respectively, and after dilution the initial concentration in the polarographic cell was below $4 \cdot 10^{-7}$ M or $1 \cdot 10^{-6}$ M. The exception were egg PC vesicles which had even at 4°C an outflow half-time of about 50 h.

The average radius r_i of the vesicles was derived from the entrapped volume V_i(l) by the relation:

$$V_i = v_p R_i / 3d$$

where v_p is the molar volume of the phospholipid molecule and d is the thickness of the bilayer. The total entrapped volume V_i was determined polarographically after release of the vesicular content with 0.1%. Triton X-100 or by sonication. The entrapped volume of the different preparations varied between 6 and 81 per mole lipid and the radius varied between 1100 Å and 1500 Å. This is an underestimate since 10–20% of the entrapped volume was in multilamellar vesicles. The entrapped volume in the unilamellar vesicles was derived from the maximal facilitated release by valinomycin or the organic anions.

Polarographic determination

We used pulse polarography in the measurements employing PAR 170 with a drop timer. The sensitivity of this method is somewhat lower than the derivative pulse or the cyclic voltammetry after preformation of the respective amalgam on the hanging mercury drop. It had, however, the advantage that time dependence could be recorded directly as follows: After completing the polarogram, the recorder was transferred to the time base and thus the sampled pulse current at the final set potential was measured as a function of time. Then either the temperature was scanned at a certain rate or different additives like valinomycin, organic anions etc. were injected and the current was measured isothermically, as a function of time. Concentration/current relation was obtained by running the polarograms at different Tl^+ concentrations and by changing the Tl^+ concentration when the recorded was on time base at constant potential as demonstrated in Fig. 1. At room temperature, increment of 10^{-7} M in concentration of Tl^+ produced an increase of 1.8 nA in pulse polarographic current at 2 s drop time at a potential of 0.9 V relative to the normal Ag | AgCl electrode.

The rate of release is considered to be a first-order reaction and the reverse reaction could be neglected since the outer concentration of the depolarizer was always by at least two orders of

magnitude lower than its intravesicular concentration.

Results

The temperature dependence of the release rate

The rate of release at 4°C was extremely slow if the lipids were below the phase transition temperature like in the case of DPPC or mixtures of DPPC with egg PC at ratios 70 : 30 and 50 : 50 or even a mixture of 50% DPPC, 30% egg PC and 20% PS (all the mixtures had broad phase transition between 10°C and 37°C) [1,8]. Vesicles formed from DPPC or from DPPC with 30% PC released less then 2% of their content per day at 4°C. The release from vesicles of the (DPPC, egg PC, PS) mixture was up to 5% per day, while the release from egg PC vesicles reached 20–25% per day at this temperature. The rate of release increases with temperature. In Fig. 2 the time dependence of the pulse polarographic current is presented. The temperature which is gradually increased is marked along the scan. The corrected current for a constant temperature (20°C) which is proportional to the Tl^+ concentration in the extravesicular solution, can be derived from the presented curves with the aid of the measured variation of the current with temperature for any fixed concentration of Tl^+.

It is evident from the curve a in Fig. 2 that there is an abrupt release from the vesicles of DPPC around 35°C which is the temperature region of the pre-transition. This differes from the reported maximal leakiness at the transition temperature which was obtained after abrupt increase

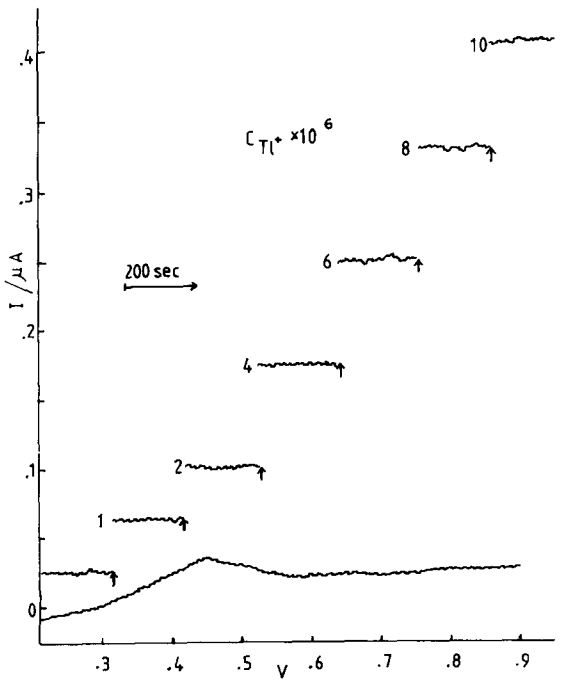

Fig. 1. Calibration of pulse polarographic current of Tl^+. After potential scan in absence of Tl^+ till 0.9 V, the recorder is changed to time base. Subsequent amounts of Tl^+ added as indicated by arrows. The concentration of Tl^+ is noted near each current line.

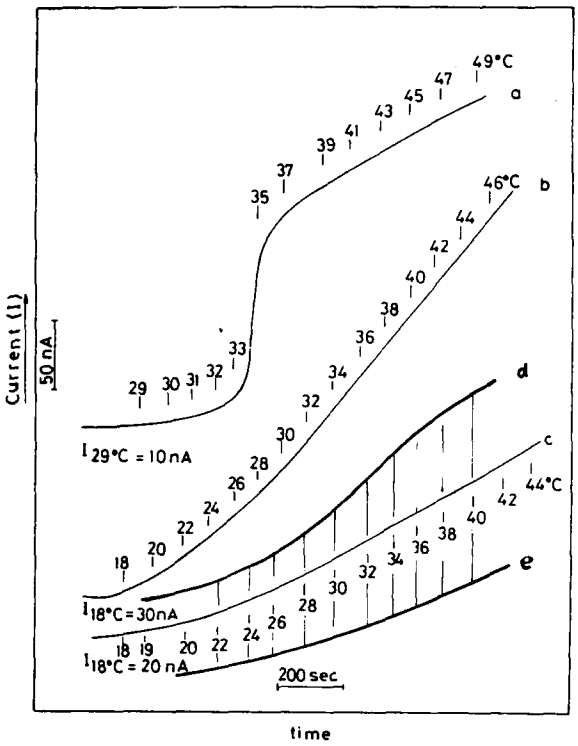

Fig. 2. Temperature scan. After completing the pulse polarogram between −0.2 V and −0.9 V relative to the normal N(Ag| AgCl) electrode at 29°C for DPPC vesicles loaded with 0.05 M K_2SO_4 and 0.05 M Tl_2SO_4, and at 18°C for vesicles from the lipid mixtures the temperature was increased at nearly constant rate, as indicated. The currents above the background at the initial temperature are presented on each scan. Scans: (a) DPPC, supporting electrolyte K_2SO_4; (b) DPPC + PC + PS, supporting electrolyte K_2SO_4; (c) DPPC + PC + PS, supporting electrolyte Na_2SO_4; (d) 70 : 30 DPPC/PC; (e) 50 : 50 DPPC/PC.

of temperature from below the phase transition to different temperatures around and above the phase transition [1,7,9]. Indeed if the temperature is changed abruptly from 4°C to 37°C only about 15% of the inner content is released in the first instant and the release continues very slowly after annealing of the vesicles at this temperature.

No abrupt increase in release at any particular temperature is observed from vesicles made of mixtures of DPPC and egg PC at ratios 70:30 (and 50:50 and of 20% PS, 30%PC and 50% DPPC having broad phase transitions. Curves b and c corresponding to release from negatively charged vesicles show that a distinct lowering of the rate of release increase with temperature if K_2SO_4 is replaced by Na_2SO_4 as the supporting electrolyte in the outer solution.

A more efficient exchange (antiport) mechanism mediated by the negatively changed lipids can be excluded since facilitated release of Tl^+ even by valinomycin which is specific to K^+ is at room temperature (see next paragraph) not any more efficient to the presence of K_2SO_4 than in the presence of Na_2SO_4 as supporting electrolyte. The influence of the composition of the bathing solution on Tl^+ release during the temperature raise can not be therefore attributed to the permeability difference of the cationic species. It may be related to the permeability of the neutral complexes [10] and to the higher structural stability of the lipid layer in the presence of Na^+ during the temperature increase. The behaviour of the facilitated transport as it will be shown in the next paragraph speaks in favour of the increased structural stability in the presence of Na^+.

Facilitated transport of Tl^+

The transport of Tl^+ could be facilitated by valinomycin and by the organic anions tetraphenylboron (TPB^-) or dipicrylamine (DPA^-). TPB^- and DPA^- are not known as carriers but the organic anions and their neutral complexes with cations are more permeable than inorganic ions [11]. Neither of these agents were active way below the phase transition, e.g., the release of Tl^+ from DPPC vesicles was not enhanced by them at room temperature, or even at 30°C. However, as seen in Fig. 3 the increase in release during the heating scan is much more pronounced in the presence of TPB^- added at 30°C. At 37°C which is below the main transition of DPPC (41.6°C), TPB^- does enhance release of Tl^+. Its effect is, however, very irregular and irreproducible; it depends very strongly on the rate of addition of TPB^-. It seems that TPB^- as well as the other additives effects the structure and even the local phase transition temperatures of the surrounding lipids. This effect has been observed when using optical probes and is particularly pronounced close to phase transition [12–14].

Very reproducible results could be obtained above the phase transition as in the case of egg PC

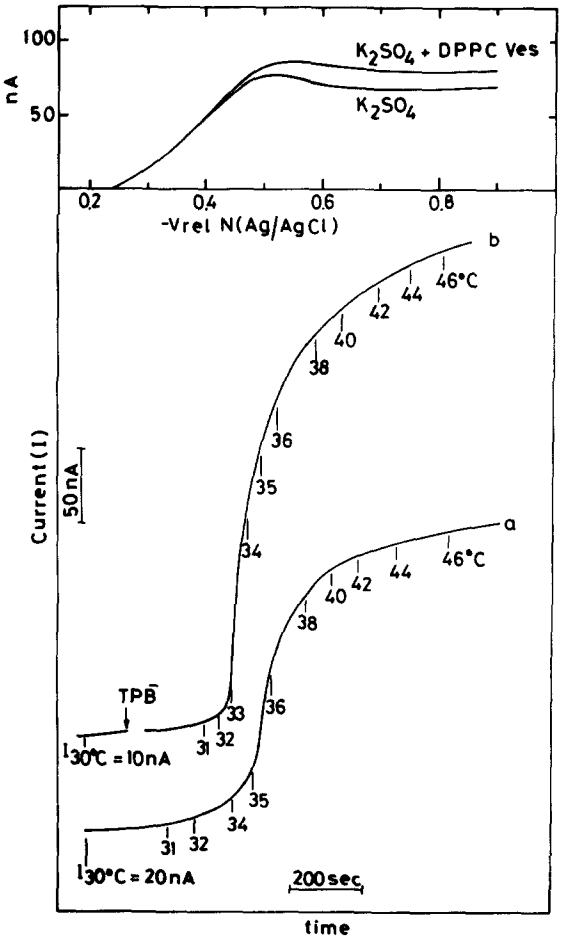

Fig. 3. Temperature scan. Procedure as in Fig. 1, initial temperature 30°C. (a) Tl^+ in DPPC without any carrier. (b) $5 \cdot 10^{-7}$ M TPB^- added as indicated. Inset: Pulse polarograms of supporting electrolyte and after addition of DPPC (32 μg/ml). Vesicles loaded with 0.05 M K_2SO_4 and 0.05 M Tl_2SO_4.

or even within the broad phase transition region as in the case of the lipid mixture (DPPC, PC, PS) at 25°C and of DPPC/PC 70:30 and 50:50 at 30°C. Unfortunately in the case of the PC vesicles only about 50–60% of the Tl^+ expected to be in the vesicles remained entrapped. The rest leaked out during the dialysis and the incubation at 4°C. The release of Tl^+ was enhanced when the vesicles were inserted into the 0.1 M K_2SO_4 at 24°C (Fig. 4). The rate of release is further augmented by addition of valinomycin, dipicrylamine (DPA^-) or tetraphenylboron (TPB^-) at concentrations of the order of 10^{-7} M. In all the cases the rate of release increases with concentration of the consecutively added facilitators.

Similar results were obtained with the lipid mixture (DPPC, PC, PS) in the presence of K_2SO_4 as supporting electrolyte. When K_2SO_4 was replaced by Na_2SO_4 the behaviour of valinomycin still remained the same but TPB^- and particularly DPA^- behaved differently (Fig. 5). Added DPA^- and also TPB^- at its lowest concentration caused a burst of release during the first few seconds after addition. The membranes seem to become leaky upon the addition of the lipid soluble anions but anneal very rapidly and the rate of release decreases to a value slightly above the initial rate. This behaviour is in keeping with the stabilising and possibly rigidifying effect of Na^+ on the membrane which upon impact of the interacting additive undergoes a local structural perturbation with transient performation and leakiness. The annealing of the membrane is accomplished by rearrangement of the lipid molecules around the

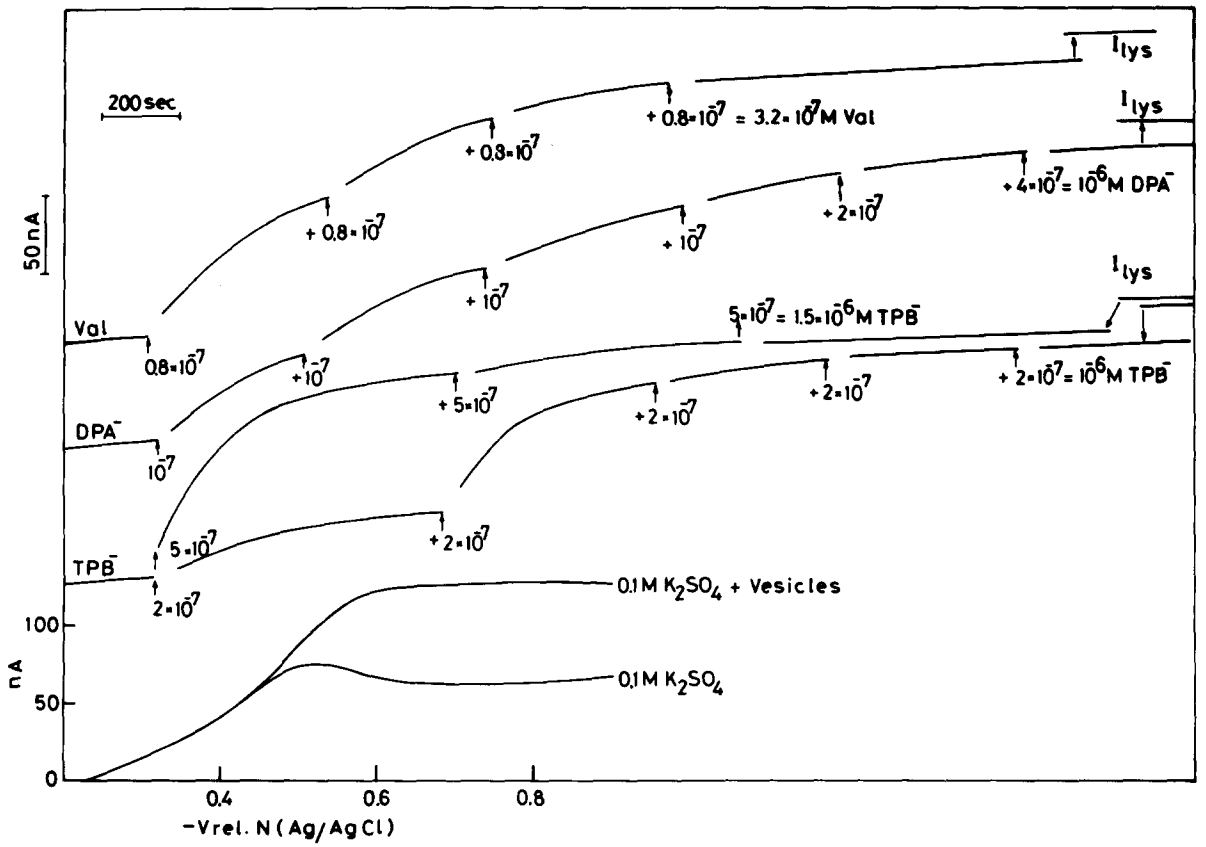

Fig. 4. Kinetics of facilitated release of Tl^+ from egg PC vesicles. Supporting electrolyte 0.1 M K_2SO_4. The charging current background at the start of the time scan varied between 60 and 70 nA. The name of the facilitator is given at the start and at the end of each run. Their addition times and concentrations are indicated. Current after lysis with Triton X-100 (I^{lys}) is given for each run. Low inset: Pulse polarograms of supporting electrolyte alone and after addition of Tl^+ vesicles loaded with Tl_2SO_4 and K_2SO_4.

interacting additive resealing the perforation.

To carry out accurate kinetic analysis only one concentration of facilitator was added to each lipid vesicle sample and the release was measured for a longer time. In Figs. 6 and 7 the measured current is plotted as a function of time for different concentrations of facilitators (valinomycin, DPA^- or TPB^-) added to vesicles produced from the phosphatidylcholine mixtures DPPC/egg PC 70:30 (Fig. 7) and 50:50 (Fig. 6). Here more diluted solution of facilitators were added under vigorous stirring of the vesicle suspension to assure their even distribution. It is evident from these figures that the rate of increase in polarographic current which represents the rate of release is proportional within experimental accuracy to the concentration of the facilitator added. The difference between the different facilitators was not very large. For 50:50 DPPC/PC their relative efficiencies were as follows:
$TPB^- >$ valinomycin $> DPA^-$.

Discussion

The kinetics of carrier facilitated transport has been discussed in detail and extensively reviewed by many authors [11,15–18]. The treatment dwelled mainly on transport through planar lipid bilayers with potential control. The release kinetics from vesicles [1,10] can be described as a homogeneous first-order reaction:

$$-\frac{dc_i}{dt} = kc_i \qquad (1)$$

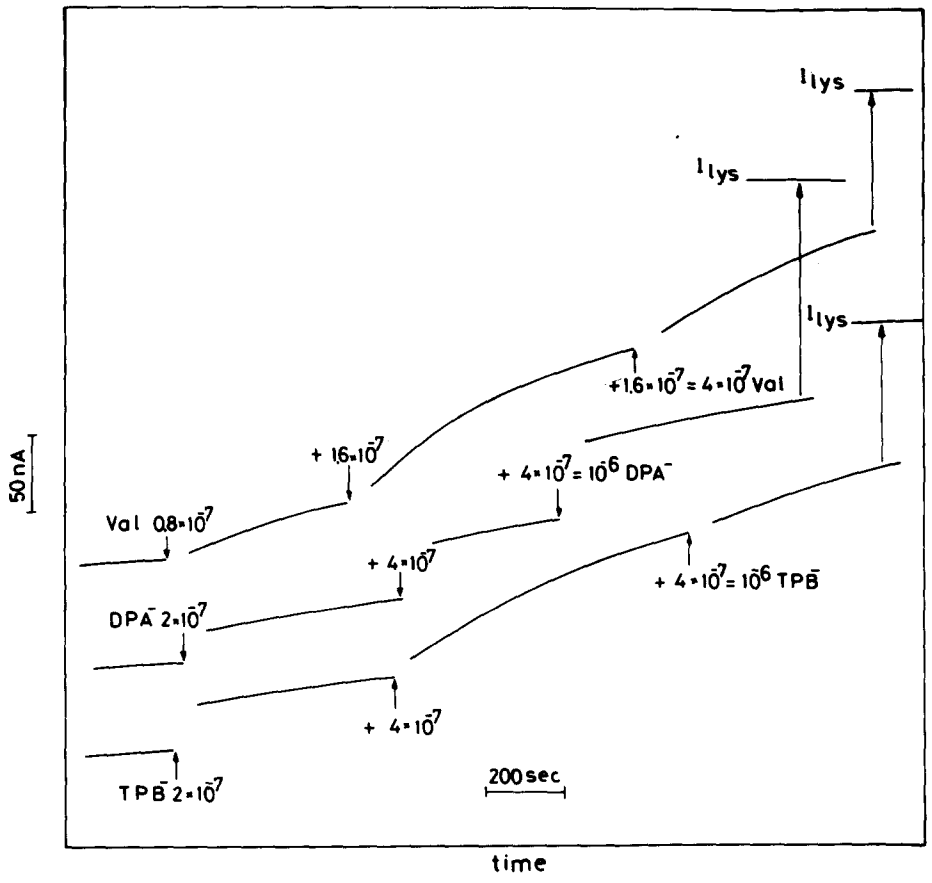

Fig. 5. Kinetics of facilitated release of Tl^+ from mixed lipid vesicles (50:30:20 DPPC/PC/PS) at 24°C. Initial net current was around 70 nA. Supporting electrolyte 0.1 M Na_2SO_4. The facilitators, the addition and concentration are indicated.

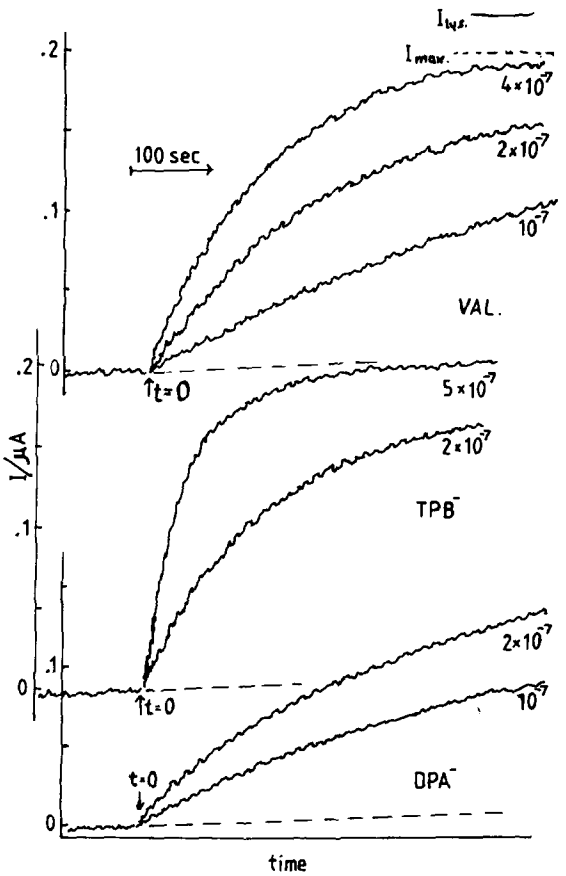

Fig. 6. Facilitated release of Tl^+ from 50:50 DPPC/egg PC. The name of the facilitator is noted below the group of current vs. time curves it generated; its concentration is noted on each curve.

where c_i is the intravesicular concentration. The back reaction is neglected since the outer concentration is by orders of magnitude lower. It can also be described as a heterogeneous reaction.

$$\frac{dn}{dt} = -V_i \frac{dc_i}{d_t} = Ak_p c_i \tag{2}$$

with a permeability rate constant k_p which is the product of the complexation constant and the complex translocation rate constant as well as of the surface concentration of the carrier. V_i being the entrapped volume and A the area of the vesicles. Thus:

$$k_p = k\frac{V_i}{A} = \frac{kR}{3} \tag{3}$$

R is the mean radius of the vesicles. Solution of

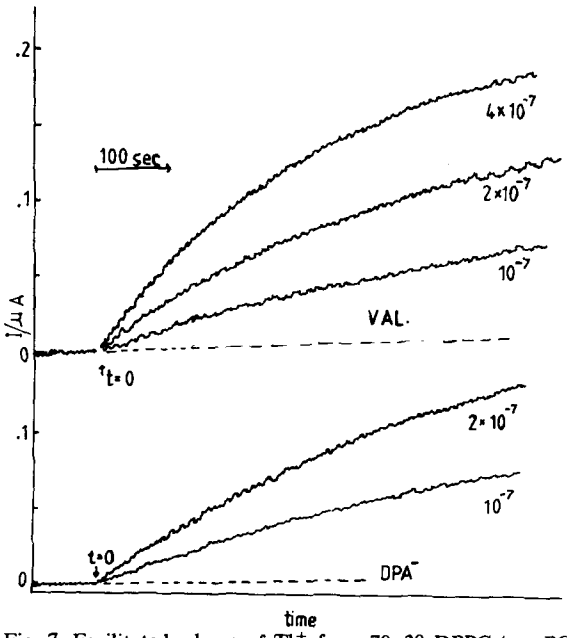

Fig. 7. Facilitated release of Tl^+ from 70:30 DPPC/egg PC. Facilitators and their concentrations are indicated.

Eqn. 1 in the absence of complexation or at very fast complex dissociation when the internal concentration of the active permeant is proportional to its concentration after its out flow renders:

$$\ln\frac{I_\infty - I_0}{I_\infty - I_t} = kt \tag{4}$$

where I_∞ is the current measured at infinite time after the facilitators have been added. This is usually lower by 10–20% than the current measured after lysis of the vesicles by Triton X-100. I_0 and I_t are the currents at the moment of injection of the carrier and at the time t.

Since Tl^+ forms an ion pair complex $TlSO_4^-$ with a stability constant $K_S = 23$ [19], the concentration of free Tl^+ which can interact with the carrier or the hydrophobic anion is in the presence of excess of SO_4^{2-}, approximately:

$$C_{Tl^+}^{free} = C_{Tl^+}^{total}/K_S\left(C_{SO_4^{2-}} + (1/K_s) - C_{Tl^+}^{total}\right) \tag{5}$$

and the solution of Eqn. 2 becomes

$$kt = \ln\frac{(I_\infty - I_0)(B - I_\infty + I_t)}{(I_\infty - I_t)(B - I_\infty + I_0)} \tag{6}$$

$B = \left(C_{SO_4^{2-}} + (1/K_s) \times \text{current per } C_{Tl^+}\right)$.

The choice of Eqn. 6 or 4 for the description of the kinetics of facilitated release of Tl^+ from the vesicles will depend on the rate of dissociation of the ion pair $TlSO_4^-$. If the dissociation is a very fast process and the binding of Tl^+ to the facilitators and their translocation across the membrane is rate controlling, then Eqn. 4 is valid. In the opposite case it will be Eqn. 6. Applying the two equations to the data presented in Figs. 4, 5, 6 and 7 shows that Eqn. 4 represents faithfully the unimolecular kinetics indicating that the dissociation of $TlSO_4^-$ is, as expected a much faster process that the Tl^+ translocation through the membrane.

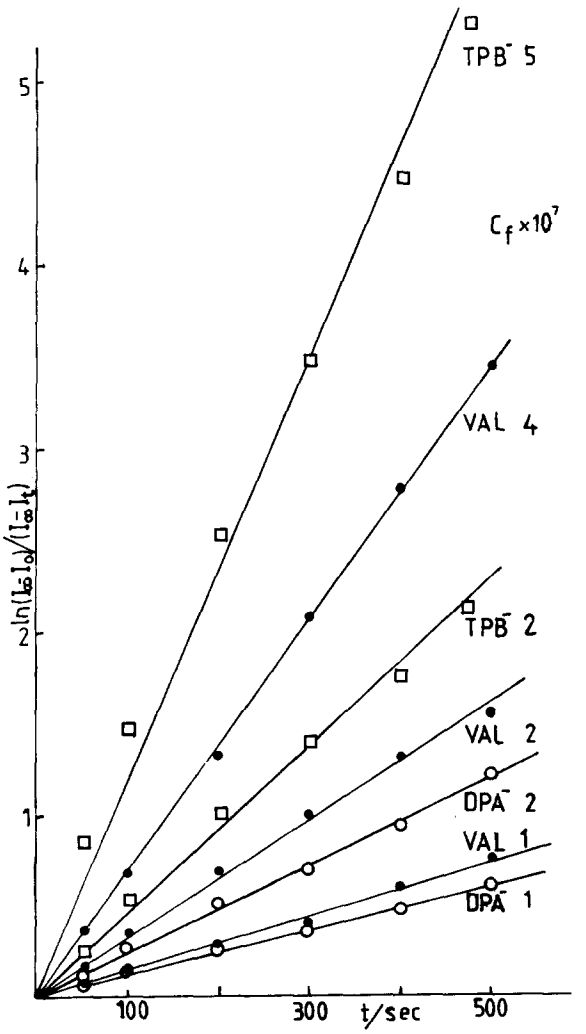

Fig. 8. First-order kinetic analysis of Tl^+ release from 50:50 DPPC/PC vesicles $\ln(I_\infty - I_0)/(I_\infty - I_t)$ plotted against time in the presence of valinomycin (●——●), DPA^- (○——○), TPB^- (□——□). The concentrations of the facilitators are indicated.

In Fig. 8 we plotted $\ln(I_\infty - I_0)/(i_\infty - i_t)$ against time according to Eqn. 4, for the release of Tl^+ from 50:50 DPPC/egg PC vesicles facilitated by valinomycin, DPA^- and by TPB^-. The release rate constant is nearly proportional to the concentration of the facilitator. At 30°C, it is about $1.2 \cdot 10^{-3}$ s^{-1} for 10^{-7} M DPA^- about $1.6 \cdot 10^{-3}$ s^{-1} for 10^{-7} M valinomycin and $2.3 \cdot 10^{-3}$ s^{-1} for 10^{-7} M TPB^-. These values correspond to the respective heterogeneous rate constants k_p of $4 \cdot 10^{-9}$, $5.3 \cdot 10^{-9}$ and $7.7 \cdot 10^{-9}$ $cm \cdot s^{-1}$, respectively, for a vesicle radius of 100 nm. The rate constants in vesicle made of the DPPC/egg PC mixtures at a ratio of 7:3 are at the same temperature slightly above half of these in the 1:1 DPPC/egg PC vesicles. The efficiency of the different facilitators is a function of the distribution coefficient of the facilitator between the aqueous solution and the membrane and of their binding, translocation and dissociation rates.

We measured by the same pulse polarographic method the uptake of DPA^- from aqueous solutions at concentrations between $2 \cdot 10^{-6}$ and $4 \cdot 10^{-6}$ M by egg PC (Fig. 9). Under these conditions about one DPA^- molecule per an area of about 750 $Å^2$ or $2.2 \cdot 10^{-11}$ $mol \cdot cm^{-2}$ has been adsorbed which corresponds to $2.2 \cdot 10^{-6}$ $\mu C \cdot cm^{-2}$. This value is in good agreement with the DPA^- adsorption on a dierucoylphosphatidylcholine membrane determined by measuring the total of transferred adsorbed charges, applying large amplitude potential pulses [20,21]. The absorbance measurements [22] of the DPA^- adsorbed on a single bilayer membrane gave in this concentration region about four times higher adsorption values. It seems that the adsorbed DPA^- molecules which do not contribute to the electrical conductance are only weakly bound and diffuse fast on the surface of the vesicles thus contributing fully to the polarographic currents. The adsorption of TPB^- is about half of that of DPA^-. The K^+ complex of a valinomycin analogue is almost eighty times weaker [11]. This makes TPB^- about three times more efficient as Tl^+ translocator than DPA^-. One can not draw direct conclusions with respect to valinomycin but it seems that it translocates by

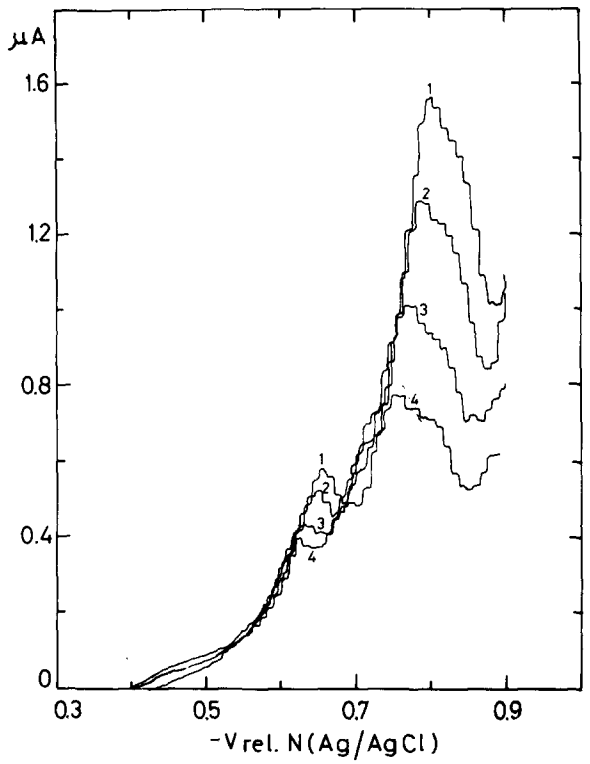

Fig. 9. Polarograms of $4 \cdot 10^{-6}$ M DPA^- in the presence of increasing concentrations of PC vesicles (without Tl^+) 1, No egg PC; 2, $8 \cdot 10^{-6}$ M egg PC; 3, $1.6 \cdot 10^{-5}$ M egg PC; 4, $2.4 \cdot 10^{-6}$ M egg PC. The DPA^- adsorbed on the vesicles acquires a diffusion coefficient which is a factor of 200 lower than that opf the free DPA^-. If all the DPA^- would be adsorbed the current would decrease to less then 8% of the value measured in the presence of free DPA^-. Thus $8 \cdot 10^{-6}$ M egg PC vesicles absorb between (6 and 7)$\cdot 10^{-7}$ M DPA^- when the concentration of free DPA^- is about $3.3 \cdot 10^{-6}$ M.

about an order of magnitude more efficiently.

The rate constant of the release from egg PC vesicles at 24°C without any added facilitator was below $2 \cdot 10^{-4}$ s^{-1} and from vesicles made of 50:50 DPPC/egg PC mixtures approx. $6 \cdot 10^{-5}$ s^{-1}. This corresponds to the respective heterogeneous rate constants of $6 \cdot 10^{-10}$ and $2 \cdot 10^{-10}$ $cm \cdot s^{-1}$. This is by more than three orders of magnitude smaller than the permeability of TlCl and by more than an order of magnitude than that of $CdCl_2$ [19] through planar bilayer membranes formed from decane solution. This difference may result from slower formation of the neutral permeant salt, and from its larger size and polarity.

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