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MECHANISM OF ION TRANSPORT THROUGH LIPID BILAYER-MEMBRANES MEDIATED BY PEPTIDE *CYCLO*-(D-Val-L-Pro-L-Val-D-Pro)$_3$

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SUMMARY

The cyclic dodecapeptide PV, *cyclo*-(D-Val-L-Pro-L-Val-D-Pro)$_3$, a structural analogue of the ion-carrier valinomycin, increases the cation permeability of lipid bilayer membranes. This paper reports the results of two types of relaxation experiments, namely relaxation of the membrane current after a voltage jump and decay of the membrane voltage after a charge pulse in lipid bilayer membranes exposed to PV. From the relaxation data, the rate constant for the translocation of the ion carrier complex across the membrane, as well as the partition coefficient of the complex between water and membrane solution interface were computed and found to be about one order of magnitude less than the comparable values for valinomycin (Val). Furthermore, the dependence of the initial membrane conductivity on ion concentration was used to evaluate the equilibrium constant, K, of complexation between PV and some monovalent cations in water. The values of K yield the following selectivity sequence of PV: $Na^+ < NH_4^+ < K^+ < Cs^+ < Rb^+$. These and earlier results are consistent with the idea that PV promotes cation movement across membranes by the solution complexation mechanism which involves complexation between ion and carrier in the aqueous phase and transport of the carrier across the membrane. In the particular form of the solution complexation mechanism operating here, the PV present in the PV-cation complex carrying charge across the membrane derives from the side from which the current is flowing (cis-mechanism). As shown previously, valinomycin, in contrast to PV, acts by an interfacial complexation mechanism in which the Val in the Val-cation complex derives from the side toward which current is flowing (trans-mechanism). The comparison of the kinetic properties of these two closely related compounds yields interesting insights into the relationship between chemical structure and function of ion carriers.

INTRODUCTION

The effects of ionophores on the ionic permeability of lipid bilayer membranes have been studied extensively in the past, in most cases by analysing the steady-state

Abbreviation: cyclic dodecapeptide PV, *cyclo*-(D-Val-L-Pro-L-Val-D-Pro)$_3$.

electric properties of the membranes [1, 2]. These studies have yielded valuable information on the molecular basis of ion transport. Nevertheless, many questions concerning the mechanism of carrier-mediated ion transport cannot be approached by stationary methods alone. As carrier-induced conductance is the result of a series of discrete transport steps of an ion, the use of kinetic techniques becomes indispensable [3–5].

Techniques that yield quantitative information on the rate of the single transport steps can be applied in two ways. On the one hand, carriers can be used as probes to describe the nature of the barrier a lipid membrane presents to permeating ions. On the other hand, a well-characterized membrane can be used to probe the carrier property of a molecule. In this way, information is gained that will facilitate the design of artificial carriers with predetermined characteristics. While the former approach has been applied to study the properties of lipid membranes of varying chemical composition [6, 7] we shall attempt in this contribution to make use of the latter possibility in the evaluation of the artificial ionophore PV.

PV, *cyclo*-(-L-Val-D-Pro-D-Val-L-Pro-)$_3$, is a neutral homodetic cyclopeptide that was designed to display the properties of an ion carrier such as valinomycin. The peptide, which was synthesized by the solid-phase method [8], was found to complex with the alkali ions (refs. 8–11 and Grell, E., unpublished), the binding constants being in general three orders of magnitude greater than for valinomycin. NMR spectroscopy revealed [10] that the cation complexes of PV have S_6 symmetry and are essentially isostructural with the K^+ complex of valinomycin. The PV-cation complexes have dissociation rate constants that are several orders of magnitude smaller than the corresponding valinomycin complexes. Uncomplexed PV exists in two conformational states that interconvert only at very slow rates. Such slow molecular movements have not been observed with valinomycin. The observation of Grell, E. (unpublished) that the affinity of PV for K^+ is sufficiently strong that it can be quantitatively determined even in water revealed another property in which PV differs markedly from valinomycin.

PV was the first synthetic peptide to exhibit ion carrier activity on lipid bilayer membranes [12]. Steady-state measurements showed that the membrane conductance induced by PV in solutions of either Na^+ or K^+ was proportional to the aqueous PV concentration. However, the conductance with K^+ was four orders of magnitude less than in the case of valinomycin. The current-voltage curves were of the saturating type even at low salt concentrations. A further peculiarity was observed when PV was added only to one side of a bilayer separating otherwise identical solutions of either K^+ or Na^+ salts. Under these conditions, a zero-current potential of up to $\approx$ 100 mV was observed, the PV-containing side being negative.

These and other observations on the stationary state of membranes exposed to PV were recognized to be consistent with two different hypotheses or a combination of both [12, 34]. These hypotheses can be stated in terms of the model shown in Fig. 1. The first possibility is that the rate coefficient for translocation of free PV (k_S) is small compared with the permeability of the unstirred layer to PV and the rate coefficient for desorption of free PV(S) to the membrane (k_S^{ma}). The second possibility is that the rate of dissociation of PV cation complexes both on the membrane (k_D) and in the aqueous solutions (k_D^a) is slow compared to the permeability of the unstirred layers to PV and the rate coefficient for desorption of the PV-cation complex (MS) to the

membrane (k_{MS}^{ma}). In both of these situations, the S present in MS complexes crossing the membrane during the stationary state of current flow derives from S and/or MS present in the aqueous solution on the side of the membrane from which current is flowing (cis) rather than from S diffusing back through the membrane from the side toward which current is flowing (trans). Two kinds of information support the second hypothesis, i.e., that k_D and k_D^a for PV are very low. First, estimates of k_D in methanol from proton NMR spectra of K^+-PV complexes indicate values of about 1 s^{-1}, several orders of magnitude lower than k_D for K^+ valinomycin complexes [10]. Furthermore, the dependence of stationary state conductance on salt concentration is consistent with the second but not the first hypothesis [12].

This paper reports observations of the kinetic properties of membranes exposed to PV. These observations and related studies, (Anderson, O., Lev, A. A., Beall, P. T., Gisin, B. F. and Tosteson, D. C., unpublished) permit quantitative estimates of the partition coefficient of PV-metal complexes between water and membranes (γ_{MS}) and of the association constant of PV for various metal cations in water. The observations also allow estimates of the rate coefficient for translocation of PV-metal in complexes across the membrane interior (k_{MS}) and, less reliably, across the interface between membrane and adjacent aqueous solutions (k_{MS}^{ma}).

MATERIALS AND METHODS

Black lipid membranes were formed in the usual way [13] from a 1–2 %(w/v) lipid solution in *n*-decane. As lipids we used dioleoyl-L-α-phosphatidylcholine (dioleoyllecithin) and glycerylmonooleate (monoolein). The lecithin was synthesized by Janko, K. [6], while the monoolein was obtained from NuCheck Prep (Elysian, Minn., U.S.A.). The purity of both lipids was checked by thin-layer chromatography.

The cell used for bilayer formation was made from Teflon and was inserted into a thermostated metal block. The temperature was kept at 25 °C in all experiments. The membranes had an area of either $2 \cdot 10^{-2} \cdot cm^2$ or $8 \cdot 10^{-2} \cdot cm^2$. The diameter of the membrane had no influence on the specific conductivity and on the relaxation times of the membrane. The specific conductivity, however, slightly depended on the age of the membrane. For this reason, all measurements were carried out at a fixed time (15–20 min) after the membrane turned completely black. PV [8] was used as a concentrated solution in chloroform (10^{-3} M). Small amounts of this stock solution were added directly to the aqueous phase to get a final PV concentration of 10^{-7} M or 10^{-6} M. 10^{-5} M PV solutions were obtained by evaporating the chloroform and subsequently adding the aqueous salt solution. The unbuffered salt solutions (chlorides of various cations) had a pH of about 6. In some cases LiCl (which is not transported by PV to any appreciable extent) was added to keep the ionic strength constant at 1 M, but the results were found to be independent of the presence of LiCl.

The potential measurements were carried out in the following way. Membranes from dioleoyllecithin were formed in a solution containing 10^{-6} M PV and 1 M salt. 10 min after blackening of the membrane the PV concentration in one aqueous compartment was raised by adding a known amount of PV dissolved in ethanol under stirring. The potential was measured through silver-silver chloride electrodes with a Keithley 602 electrometer. After about 5 min the membrane potential V_m reached a stationary value. V_m was corrected for small asymmetry potentials of the electrodes

($\leqslant 2$ mV) by subtracting the residual potential that remained after destroying the membrane at the end of the experiment.

Stationary-conductance measurements and voltage-jump experiments were carried out as described previously [6]. A battery-operated pulse generator with a rise time of about 200 ns was used together with a storage oscilloscope (Tektronix 7633/7A22). The current was measured as a voltage drop across a series resistor.

The charge-pulse experiments [14–16] were performed in the following way. The membrane capacitance was charged by a brief current pulse to a voltage smaller than 10 mV. For this purpose a variable voltage-source was connected to the membrane during about 50 ns via a fast FET switch. After the pulse the switch had an impedance larger than $10^{12}\ \Omega$ in the "open" position. The subsequent decay of the membrane voltage was measured using a high, impedance voltage follower with a band width of 1 MHz (Analog Devices 42 K, input impedance $> 10^{12}\ \Omega$). The output signal of the voltage follower was recorded with a storage oscilloscope. The time resolution of the whole set up was close to 1 μs.

THEORY

A detailed model for carrier-mediated ion transport in thin-lipid membranes has been described previously [17, 18]. This model contains two parallel reaction sequences by which ions may be transported through the membrane (Fig. 1). The first sequence (interfacial complexation mechanism) is the classical carrier mechanism which involves complexation of an aqueous ion M^+ with a carrier molecule S in the membrane-solution interface. The complex MS^+ is translocated to the opposite interface where the ion is released by dissociation. In the second mechanism (solution complexation) the complex MS^+ forms in the aqueous phase, adsorbs to the membrane-solution interface, and is then translocated to the other side. In this limiting case it is assumed that the rate coefficients for the reaction between S and M^+ in the

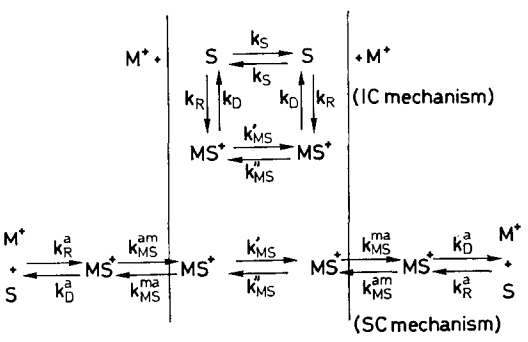

Fig. 1. Reaction scheme for the transport of an ion M^+ mediated by the carrier S. In the interfacial-complexation (IC) mechanism the complex MS^+ is formed in the membrane-solution interface. In the solution-complexation (SC) mechanism complexation takes place in the aqueous phase, and the complex MS^+ is subsequently adsorbed to the interface. k_R, k_D, $k_S{}^a$, and $k_D{}^a$ are the rate constants of association and dissociation of MS^+ in the interface and in the aqueous solution (superscript a). k_{MS} and k_S are the rate constants of translocation of MS^+ and S across the membrane, $k_{MS}{}^{am}$ and $k_{MS}{}^{ma}$ are the rate constants of exchange of MS^+ between aqueous solution and membrane interface.

aqueous solutions (k_R^a and k_D^a) are low compared to the diffusion coefficients for S and MS^+ in water. Thus S and MS^+ are assumed to behave as completely independent species in the unstirred layers. This assumption is not, of course, always justified [35, 36].

(a) Voltage-jump experiments

We consider the case that the system is at equilibrium at times $t < 0$ so that the concentration of the complex MS^+ in the membrane-solution interface, N_{MS}, is the same on both sides (N_{MS} is expressed in mol/cm^2). If at time $t = 0$ a voltage is applied to the membrane, an initial current $J(0)$ is observed which relaxes to a stationary current $J(\infty)$. $J(0)$ depends on the rate constants k'_{MS} and k''_{MS} for the translocation of MS^+ from left to right and from right to left (Fig. 1), respectively, and is given by:

$$J(0) = FN_{MS}(k'_{MS} - k''_{MS}) \tag{1}$$

$J(0)$ is referred to unit area of the membrane. F is the Faraday constant. The dependence of k'_{MS} and k''_{MS} on the applied voltage V_m is approximately given by [17]:

$$k'_{MS} = k_{MS}e^{u/2} \tag{2}$$

$$k''_{MS} = k_{MS}e^{-u/2} \tag{3}$$

$$u = \frac{V_m}{RT/F} = \frac{\Psi' - \Psi''}{RT/F} \tag{4}$$

R is the gas constant, T the absolute temperature, and Ψ', Ψ'' are the electrical potentials in the left- and right-hand aqueous phases. The interfacial concentration N_{MS} is related to the aqueous concentration c_{MS} of MS^+ by the dimensionless partition coefficient γ_{MS} [17]:

$$\gamma_{MS} = \frac{2}{d}\frac{N_{MS}}{c_{MS}} = \frac{2}{d}\frac{k_{MS}^{am}}{k_{MS}^{ma}} \tag{5}$$

d is the membrane thickness, k_{MS}^{am} and k_{MS}^{ma} are the rate constants of exchange of MS^+ between aqueous phase and membrane-solution interface (Fig. 1). Introducing the equilibrium constant K of complex formation in the aqueous phase, c_{MS} may be expressed by the concentrations c_M and c_S of M^+ and S in water:

$$K = \frac{c_{MS}}{c_M c_S} = \frac{c_{MS}}{c_M(c_0 - c_{MS})} \tag{6}$$

$c_0 = c_S + c_{MS}$ is the total concentration of the carrier in water. Eqns. 1–6 may be combined to give the specific membrane conductance $\lambda = J/V_m$ at time zero:

$$\lambda(t = 0) = \frac{2F^2 N_{MS} k_{MS}}{uRT} \sinh(u/2) \tag{7}$$

$$N_{MS} = \frac{\gamma_{MS} c_0 d}{2} \cdot \frac{c_M K}{1 + c_M K} \tag{8}$$

Eqn. 7 holds for any combination of the interfacial- and solution-complexation mechanisms. The conductance λ_0 in the ohmic limit ($|u| \ll 1$) is given by

$$\lambda_0(t = 0) = \frac{F^2 N_{MS} k_{MS}}{RT} \tag{9}$$

so that

$$\left(\frac{\lambda}{\lambda_0}\right)_{t=0} = \frac{\sinh(u/2)}{u/2} \tag{10}$$

This relation is strictly valid if the barrier in the center of the membrane is sharp and if the full voltage drops across the barrier [17]. In order to account for the real shape of the barrier [11, 19, 21], the empirical expression

$$\left(\frac{\lambda}{\lambda_0}\right)_{t=0} = \frac{\sin(nu)}{nu} \tag{11}$$

may be used, where n is an adjustable parameter.

In the case of a carrier which acts by the interfacial-complexation mechanism or by a combination of interfacial- and solution-complexation mechanisms, the time course, $J(t)$, of the current after a voltage jump is governed by at least two relaxation times [3]. The experimental results presented below, however, give evidence that peptide PV acts almost exclusively by the solution-complexation mechanism. In that case the charge transport through the membrane may be described in the same way as the transport of hydrophobic ions. Provided that changes in the concentration of MS^+ in the unstirred aqueous layers near the membrane may be neglected, $J(t)$ is then given by [20]:

$$J(t) = J(\infty) + [J(0) - J(\infty)]e^{-t/\tau} \tag{12}$$

$$\frac{1}{\tau} = k_{MS}^{ma} + 2k_{MS}\cosh(u/2) \tag{13}$$

Implicit in the derivation of Eqns. 12 and 13 is the assumption that the rate constant k_{MS}^{ma} for the exchange between membrane and water is independent of voltage and that the barrier in the center of the membrane is sufficiently narrow. In order to account for the real shape of the barrier, Eqn. 13 may be replaced by (compare Eqn. 11):

$$\frac{1}{\tau} = k_{MS}^{ma} + 2k_{MS}\cosh(nu) \tag{14}$$

(b) Charge-pulse experiments

If the membrane capacitance is charged up at $t = 0$ to an initial voltage V_m^0, the voltage decays to zero by redistribution of ions within the membrane and by transport of ions through the membrane. Under the assumptions (a) that only k_{MS} is voltage dependent, but not k_{MS}^{ma}, and (b) that V_m is small ($|V_m| \ll RT/F$), the decay of $V_m(t)$ is described by two exponential relaxation processes [16]:

$$V_m(t) = V_m^0(a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2}) \tag{15}$$

The relaxation times τ_1 and τ_2 and the relaxation amplitudes a_1 and a_2 may be obtained by straightforward calculation [16]. In the case of PV where the relation $k_{MS}^{ma} \ll k_{MS}$ holds (see below), the theoretical expressions reduce to the following approximate relations [16]:

$$\tau_1 \approx \frac{1}{2k_{MS}(1+sN_{MS})} \tag{16}$$

$$\tau_2 \approx \frac{1+sN_{MS}}{k_{MS}^{ma} sN_{MS}} \tag{17}$$

$$a_1 \approx \frac{sN_{MS}}{1+sN_{MS}} \tag{18}$$

$$a_2 = 1-a_1 \tag{19}$$

$$s \equiv \frac{F^2}{2RTC_m}$$

C_m is the specific membrane capacity. Using Eqns. 16–18 the unknown quantities k_{MS}, k_{MS}^{ma} and N_{MS} may be determined from the experimental parameters τ_1, τ_2 and a_1.

RESULTS

(a) *Voltage-jump experiments*

If a voltage V_m is suddenly applied to the membrane in the presence of PV and K^+, a large initial current is observed which declines within about 1 ms to a much lower value (Figs. 2 and 3). In 1 M K^+ and 1 μM PV the initial current (at $V_m =$ 10 mV) is about 33 $\mu A/cm^2$, about 4000 times larger than the steady-state current (at the same voltage). The early decay of the current J is purely exponential, as shown by Fig. 4 where $J(t)$ is plotted on a logarithmic scale. The final approach of $J(t)$ to the steady state (Fig. 3), however, is much slower than predicted by an exponential function with a single time constant. $J(t)$ cannot be represented by the sum of two exponentials, either. A clue to the origin of the slow phase of $J(t)$ is given by the observation that the steady-state current $J(\infty)$ can be increased by about 50 % by stirring of the aqueous solutions. This suggests that the slow decay of the current is associated with concentration changes in the solution layers adjacent to the membrane surfaces. The occurrence of diffusion polarization due to unstirred layers has already been observed with hydrophobic ions [20, 28] and has also been reported from studies with ion carriers [29, 30]. The conclusion that the transport of MS^+ is affected by the diffusional resistance of unstirred layers is consistent with the following observation. If the voltage is switched off after the steady state of J is reached (about 10 s after the initial voltage-jump), a current in the opposite direction is observed which decays to zero within about 10 s (lower trace of Fig. 3). This slow backtransport of charge is easily explained by the assumption that a concentration difference of MS^+ is built up across the membrane during current flow and that after switching-off the voltage this concentration difference is equalized partly by transport of MS^+ through the membrane, and partly by diffusion in the aqueous phases.

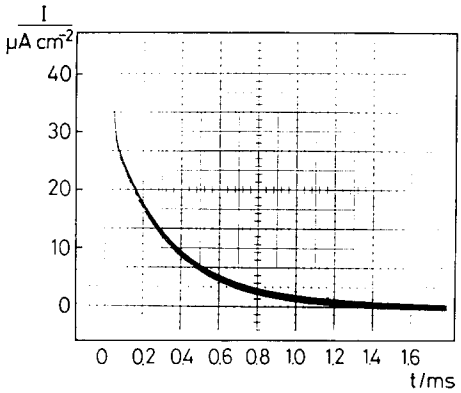

Fig. 2. Membrane current $J(t)$ after a voltage jump ($V_m = 10$ mV) at time $t = 0$. Dioleoyllecithin membrane in the presence of 1 M KCl and 1 μM PV, 25 °C. The RC time-constant of the circuit, which is determined by the product of the external series resistance ($R_S = 1$ kΩ) times the membrane capacity ($c_M = 6$ nF), was 6 μs.

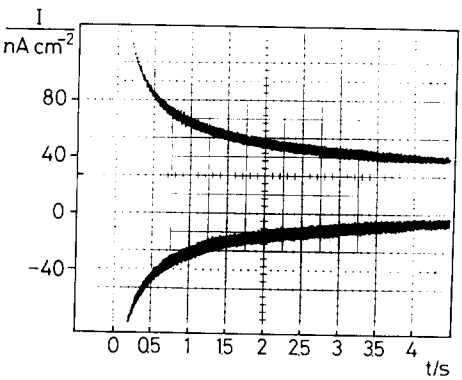

Fig. 3. Same conditions as in Fig. 2, but $J(t)$ recorded at longer times and with increased sensitivity at $V_m = 50$ mV. $R_S = 50$ kΩ (RC time-constant 0.3 ms). Upper trace: decay of the membrane current after the voltage jump. Lower trace: backflow of the current at the end of the experiment; after the steady-state was reached (about 10 s after the voltage-jump) the voltage was switched to zero and the resulting current was recorded.

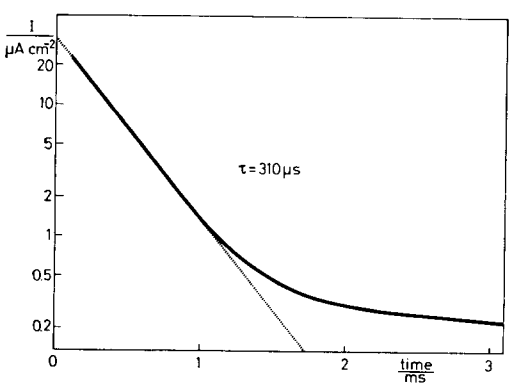

Fig. 4. Plot of $J(t)$ from the experiment of Figs. 2 and 3 on a logarithmic scale.

A striking result of the voltage-jump experiments is the large difference between the initial and the steady-state membrane current. A likely explanation of this finding is that the translocation of the PV-cation complex MS^+ across the interior of the membrane is fast, but that at least one of the other transport steps (e.g. the dissociation of MS^+ or the translocation of S) is very slow. In this case a voltage-jump is followed by a large initial current which results from the redistribution of MS^+ between the two potential minima in the membrane-solution interfaces and which declines to a much smaller steady-state current.

As indicated above, in previous work [13] it was suspected that dissociation of PV-cation complexes both on the membrane-water interface and in the aqueous solutions may be very slow. Under these circumstances, the steady-state current is limited by the rate at which PV-cation complexes can diffuse across the unstirred layers and be transported across the membrane-water interface. The dependence of the second relaxation time, τ_2, in the charge pulse experiments (see below) on salt concentration is also consistent with this hypothesis.

The experimental results of the voltage-jump experiments are summarized in Table I. The relaxation time τ has been obtained from the linear part of the plot of log(current) versus time (compare Fig. 4). The evaluation of τ from $J(t)$ is only meaningful if it is guaranteed that the early part of the current decay is not affected by diffusion polarization. The notion that diffusion polarization is indeed negligible during the initial phase of $J(t)$ is supported by the following argument [21]. The maximum charge that may be transported during time t^* through the membrane-solution interface by diffusional flow of ions in the aqueous phase is given by [31]

$$Q_{\text{diff}} = 2Fc\sqrt{\frac{Dt^*}{\pi}} \tag{20}$$

TABLE I

KINETIC DATA OF PV-MEDIATED ION TRANSPORT THROUGH DIOLEOYLLECITHIN MEMBRANES, AS OBTAINED FROM VOLTAGE-JUMP EXPERIMENTS

$T = 25$ °C. n is the number of membranes used for each set of experimental conditions. The standard deviations are given together with the mean values. $\lambda_0(t = 0)$ is the initial ohmic conductivity ($1\ S = 1$ Siemens $= 1\ \Omega^{-1}$), λ_S is the steady-state conductivity. The relaxation time τ has been obtained from the linear part of the plot of log (current) versus time. λ_S has been determined at $V_m = 50$ mV, the other data at $V_m = 10$ mV.

Ion	n	c_M/M	c_0/μM	$\lambda_0(t=0)$ / mS · cm^{-2}	λ_S / μS · cm^{-2}	τ / μs	k_{MS} / s^{-1}	N_{MS} / pmol · cm^{-2}
Na^+	5	1	1	0.022±0.006	0.5±0.2	417±42	1200±220	0.005±0.001
K^+	6	1	0.1	0.32 ±0.06	0.2±0.1	352±60	1420±300	0.06 ±0.01
	5	1	1	3.2 ±0.8	0.6±0.3	321±80	1560±510	0.54 ±0.08
	5	1	10	32.3 ±9.0	5.8±2	338±60	1480±350	5.78 ±0.5
	6	0.3	1	2.3 ±0.5	1.0±0.4	397±85	1260±280	0.48 ±0.06
	5	0.1	1	1.4 ±0.2	0.9±0.3	352±60	1420±290	0.26 ±0.04
	5	0.03	1	0.68 ±0.2	0.9±0.4	315±95	1590±490	0.12 ±0.03
	7	0.01	1	0.25 ±0.05	0.4±0.2	385±85	1300±320	0.05 ±0.01
Rb^+	7	1	1	2.9 ±0.9	0.4±0.2	344±70	1450±390	0.53 ±0.06
Cs^+	6	1	1	3.4 ±0.8	0.5±0.3	320±72	1560±420	0.58 ±0.07
NH_4^+	5	1	1	1.3 ±0.3	0.8±0.3	370±70	1350±330	0.26 ±0.04

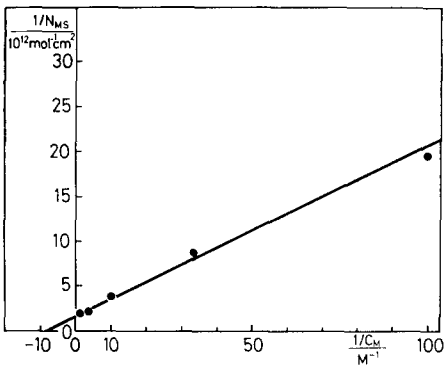

Fig. 5. Interfacial concentration N_{MS} as a function of the K^+ concentration c_M in water (double-reciprocal plot). 1 μM PV, 25 °C, dioleoyllecithin membrane.

Q_{diff} is referred to unit area of the membrane; c is the concentration and D the diffusion coefficient of the charge carrier MS^+ in water. With $t^* = 1$ ms, $c = 1$ μM and $D = 2 \cdot 10^{-6} \cdot cm^2 \cdot s^{-1}$ (calculated from the Stokes radius of the PV-cation complex), Q_{diff} becomes equal to $5 \cdot 10^{-9}$ $C \cdot cm^{-2}$. In the presence of 1 M K^+ and 1 μM PV, however, the charge transport during the first ms is about $5 \cdot 10^{-8}$ $C \cdot cm^2$ ($V_m = 50$ mV 10-times higher than Q_{diff}). The relaxation time τ may be used to calculate the translocation rate constant k_{MS} according to

$$\tau \approx \frac{1}{2k_{MS}} \tag{21}$$

This relation follows from Eqn. 13 in the ohmic limit $|\cosh(u/2) \approx 1|$ under the assumption that the translocation across the central barrier is much faster than the subsequent transport steps ($k_{MS} \gg k_{MS}^{ma}$). Eqn. 21 also holds at arbitrary k_{MS}^{ma}, provided that the translocation is faster than diffusion in the aqueous phase. The values of k_{MS} are given in Table I; it is seen that k_{MS} is independent of the complexed ion (within the limits of experimental error) and is about $1 \cdot 10^3 \cdot s^{-1}$.

From λ_0 ($t = 0$) and k_{MS}, the equilibrium concentration of MS^+ in the interface, N_{MS}, may be obtained using Eqn. 9. The calculated values of N_{MS} are also represented in Table I. According to Eqn. 8, N_{MS} depends on the equilibrium constant K of complexation in water:

$$\frac{1}{N_{MS}} = A\left(\frac{1}{c_M} + K\right)$$

($A = 2/K\gamma_{MS}c_0 d$). K may, therefore, be obtained by plotting $1/N_{MS}$ versus $1/c_M$. This has been done in Fig. 5 for the potassium complex with the result $K_{K^+} \simeq 8$ M^{-1}. Values of K for other ions have been determined from charge-pulse data.

The voltage dependence of the initial conductance $\lambda(t = 0)$ and of the relaxation time τ are represented in Figs. 6 and 7. It is seen that $\lambda(t = 0)$ increases with voltage (corresponding to a superlinear current-voltage characteristic), whereas τ is a decreasing function of voltage. Both findings are consistent with the notion that

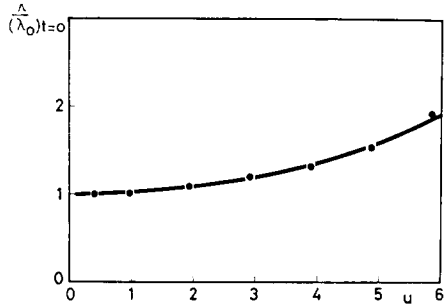

Fig. 6. Dependence of the initial conductance $\lambda(t = 0)$ on the reduced voltage $u = V_m F/RT$. λ_0 is the ohmic limit of λ. Dioleoyllecithin membrane. $T = 25$ °C. The aqueous phases contained 1 M KCl and 1 μM PV. The experimental points are mean values from five membranes. The theoretical line has been drawn according to Eqn. 11 with $n = 0.35$.

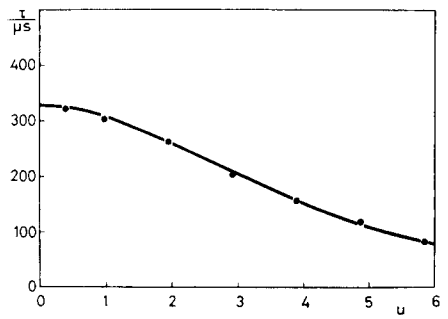

Fig. 7. Relaxation time τ of the membrane current as a function of the reduced voltage $u = V_m F/RT$. Dioleoyllecithin membrane, $T = 25$ °C. The aqueous phases contained 1 M KCl and 1 μM PV. The experimental points are mean values from five membranes. The theoretical line has been drawn according to Eqn. 14 with $k_{MS}^{ma} \approx 0$ and $n = 0.35$.

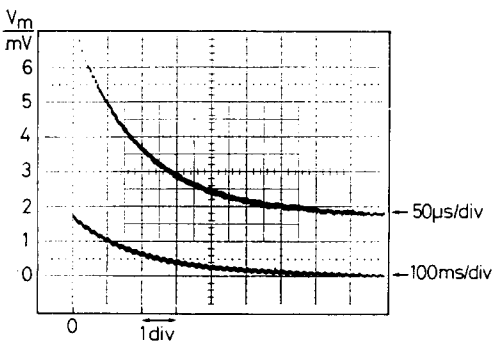

Fig. 8. Oscillogram of a charge-pulse experiment with a dioleoyllecithin membrane in the presence of 1 μM PV and 1 M KCl at 25 °C. The membrane capacitance has been charged up at time zero to a voltage $V_m^0 \approx 7.15$ mV by a current pulse of 50 ns duration. The decay of the voltage has been recorded at two different time-scales (in the lower trace the fast initial decay is not resolved).

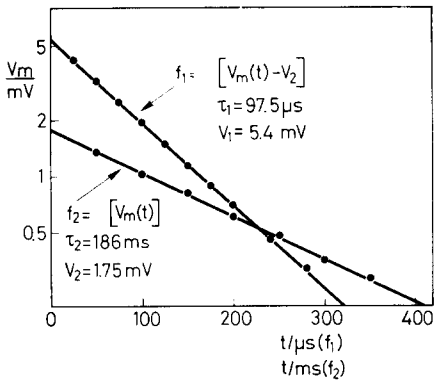

Fig. 9. Analysis of $V_m(t)$ from Fig. 8. $V_2 \approx 1.75$ mV is the amplitude of the slower process (Eqn. 17). Note the different time-scales of the abscissa.

the transport of MS^+ through the interior of the membrane is limited by an energy barrier (compare Eqns. 11 and 14). The superlinear behaviour of the initial current may be compared with the saturating characteristics of the steady-state current J_S [12]. According to the findings presented in this paper, the saturation of J_S which has been observed previously mainly originates from diffusion polarization in the aqueous phases.

(b) Charge-pulse experiments

The oscillogram of a typical charge-pulse experiment is represented in Fig. 8. The decay of the voltage V_m is recorded at two different time-scales; Fig. 8 shows that $V_m(t)$ contains two processes with widely separated time constants. In order to check whether $V_m(t)$ obeys the theoretically predicted relation

$$V_m(t) = V_1 e^{-t/\tau_1} + V_2 e^{-t/\tau_2} \tag{22}$$

(compare Eqn. 15), the experimental values of $V_m(t)$ have been plotted on a logarithmic scale in Fig. 9. It is seen that both processes are purely exponential, in agreement with Eqn. 22. The finding that $V_m(t)$ is described by Eqn. 22 even at times of the order of 1 s indicates that diffusion polarisation does not seriously affect the charge-pulse experiment under the given conditions. Nevertheless, the interpretation of the second decay process (time constant τ_2) has to be considered as tentative.

At smaller ion concentrations ($c_M \simeq 0.1$ M) an additional slow process of low amplitude is observed in the time range of seconds which is not accounted for by Eqn. 22. Whether this additional decay process originates from diffusion polarisation or from residual charge-transport by the interfacial-complexation mechanism is not clear.

The results of charge-pulse experiments with different monovalent cations are represented in Table II. From the relaxation times τ_1, τ_2 and the relaxation amplitude a_1 the kinetic parameters k_{MS}, k_{MS}^{ma} and N_{MS} have been calculated according to Eqns. 16–18. It is seen that the translocation rate constant k_{MS} is almost the same for the different ions; furthermore, the values of k_{MS} of Table II agree fairly well with

TABLE II

KINETIC ANALYSIS OF PV-MEDIATED ION TRANSPORT THROUGH DIOLEOYLLECITHIN AND MONOOLEIN MEMBRANES BY THE CHARGE-PULSE METHOD

$T = 25$ °C. n is the number of membranes used for each set of experimental conditions. The standard deviations are given together with the mean values. For the evaluation of N_{MS} from Eqn. 18 a value of $s = 4.7 \cdot 10^{12} \cdot cm^2/mol$ ($C_m = 0.4\,\mu F/cm^2$) has been used. The partition coefficient γ_{MS} has been calculated according to Eqn. 5 from N_{MS} and c_{MS} with $d = 5.0$ nm).

Ion	n	c_M / M	c_0 / μM	τ_1 / μs	τ_2 / ms	a_1	k_{MS} / s^{-1}	k_{MS}^{ma}*) / s^{-1}	N_{MS} / pmol · cm^{-2}	γ_{MS}
Dioleoyllecithin										
Na^+	6	1	1	580±100	3000±1000	0.023±0.01	840±200	15±4	0.0046±0.002	—
K^+	16	1	10	11± 5	115± 20	0.97 ±0.05	1400±600	8±3	6.2 ±1.5	2500
	15	1	1	85± 30	190± 40	0.75 ±0.02	1500±500	7±2	0.58 ±0.05	2300
	6	1	0.1	320± 80	750± 100	0.22 ±0.02	1200±400	6±2	0.054 ±0.02	2100
	10	0.1	1	160± 40	205± 50	0.39 ±0.02	1200±400	8±3	0.30 ±0.05	—
Rb^+	12	1	1	100± 50	160± 30	0.74 ±0.03	1300±400	8±2	0.55 ±0.05	2100
	8	0.1	1	135± 40	170± 40	0.72 ±0.02	1050±350	8±3	0.48 ±0.05	—
Cs^+	8	1	1	110± 40	200± 50	0.74 ±0.03	1100±300	7±2	0.56 ±0.04	2200
	8	0.1	1	225± 60	170± 60	0.66 ±0.02	800±500	9±3	0.37 ±0.03	—
NH_4^+	6	1	1	180± 30	165± 60	0.61 ±0.03	1100±200	10±3	0.30 ±0.05	—
	7	0.1	1	400± 80	525± 90	0.24 ±0.03	950±300	8±3	0.061 ±0.02	—
Monoolein										
K^+	7	1	1	12± 5	300± 50	0.88 ±0.10	5000±1000	4±2	1.52 ±0.30	6000

* The values of k_{MS}^{ma} have to be considered as tentative (see text).

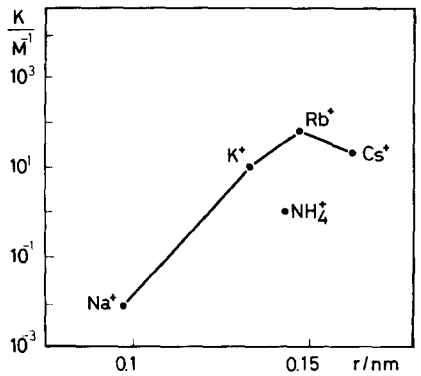

Fig. 10. Equilibrium constants K of complexation for different ions, as obtained from charge-pulse experiments. r is the cation radius. The values of K for K^+, Rb^+, Cs^+ and NH_4^+ have been determined according to Eqn. 23 from measurements at $c_M^a = 0.1$ M and $c_M^b = 1$ M. In the case of Na^+ Eqn. 24 has been used.

k_{MS} as obtained by the voltage-jump method (Table I). The interpretation of the slow decay process (time constant τ_2) in terms of the desorption rate constant k_{MS}^{ma} (Eqn. 17) has to be considered as tentative. As mentioned above, τ_2 may be influenced by the limited diffusion rate in the unstirred aqueous layers adjacent to the membrane and, besides, we cannot exclude that other conduction processes contribute to the membrane current at long times.

As seen from Table II (experiments with 1 M K^+, the concentration N_{MS} of complexes in the interface is proportional to the aqueous complex concentration $c_{MS} \approx c_0$ over at least three orders of magnitude up to $c_{MS} = 10^{-5}$ M (at 1 M K^+ the total carrier concentration c_0 is nearly equal to c_{MS}). From the values of N_{MS} the equilibrium constant K of complexation may be determined. If the interfacial concentrations which are obtained at two different cation concentrations c_M^a and c_M^b are N_{MS}^a and N_{MS}^b, then

$$K = \frac{(N_{MS}^a/c_M^a) - (N_{MS}^b/c_M^b)}{N_{MS}^b - N_{MS}^a} \tag{23}$$

In this way, the complexation constants of K^+, Rb^+, Cs^+ and NH_4^+ have been determined (Fig. 10). The complexation constant of K^+ which has been calculated from Eqn. 23 ($K = 10$ M^{-1}) is nearly the same as determined from the voltage-jump experiments (Fig. 5, $K = 8$ M^{-1}). In the case of Na^+ where K is rather small, the experiments at $c_M = 0.1$ M are too inaccurate and therefore the following method has been used. If it is assumed that the partition coefficient γ_{MS} does not appreciably depend on the nature of the complexed ion, then the ratio of the complexation constants of two different ion species i and j is given by (compare Eqn. 8):

$$\frac{K_i}{K_j} \approx \frac{N_{MS}^i/N_{MS}^j}{1 + c_M K_j (1 - N_{MS}^i/N_{MS}^j)} \tag{24}$$

N_{MS}^i and N_{MS}^j are determined at identical concentrations $c_M^i = c_M^j = c_M$ and identical c_0. A test of the assumption $\gamma_{MS}^i \approx \gamma_{MS}^j$ has been made with NH_4^+; it was

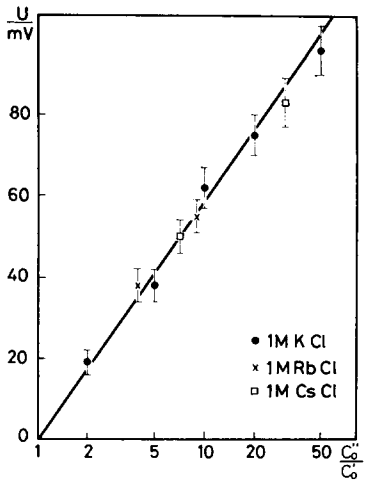

Fig. 11. Zero-current potential V_m in the presence of unequal concentrations c'_0, c''_0 of PV on both sides of the membrane. $T = 25$ °C, dioleoyllecithin membrane. c'_0 has been held constant at 1 μM. The salt concentration is 1 M on both sides. The potential is positive on the side with the smaller PV concentration. The slope of the line corresponds to the theoretical value of $(RT/F) \ln 10 = 59.2$ mV per decade.

found that the values of K, as determined by the two different methods (Eqns. 23 and 24), agreed within a factor of two.

The sequence of the complexation constants in water, as obtained from the charge-pulse experiments (Fig. 10) is $Na^+ < NH_4^+ < K^+ < Cs^+ < Rb^+$. This selectivity sequence agrees with the sequence determined by spectrophotometric titration in water (Grell, E., unpublished); the complexation constants obtained by the latter method are consistently higher by a factor of 3–5, however. The reason for this discrepancy is not clear at the moment; possibly the difference arises from the different PV concentrations (10^{-4} M and 10^{-6} M) used in the two types of experiments. The selectivity sequence for PV is almost identical with the sequence for valinomycin ($Na^+ < NH_4^+ < Cs^+ \lesssim K^+ < Rb^+$), except for the $K^+ : Cs^+$ ratio [32]. If the complexation constants of PV for the different cations are arranged according to the ion radius (Fig. 10) the value for NH_4^+ falls off the sequence of the other ions. The same behaviour is found for valinomycin, whereas in the case of the macrotetrolides the position of NH_4^+ in the selectivity sequence is normal [32].

(c) Zero-current membrane potential

The occurrence and quantitative analysis of a zero-current membrane potential when PV is added to only one side of an otherwise symmetrical system has been described previously [12]. We have measured therefore the zero-current potential in the presence of known but unequal concentrations of PV on each side and with identical K^+, Rb^+ or Cs^+ solutions on either side of the membrane ($c_M = 1$ M). The results are represented in Fig. 11. It is seen from Fig. 11 that the zero-current potential

V_m is represented to a high degree of accuracy by

$$V_m = \frac{RT}{F} \ln \frac{c''_0}{c'_0} = (59.2 \text{ m}V) \log \frac{c''_0}{c'_0} \tag{25}$$

V_m was always positive on the side with the smaller PV concentration. These results differ somewhat from those reported earlier from similar experiments [12]. In that case, significant deviations from Eqn. 25 were observed when the ratio c''_0/c'_0 was greater than 10. The origin of this difference in the results is not clear. In the earlier experiments, the bilayers were formed from sheep red cell lipids containing cholesterol while in the experiments reported here the bilayers were formed from dioleoyllecithin. The salt concentration was 0.1 M in the previous and 1 M in the present experiments. The deviation from Eqn. 25 in the earlier work was interpreted in terms of the development of small (approx. 3 %) differences in the concentrations of PV and K^+PV across the unstirred layers. Both sets of observations confirm that K^+PV is the major charge carrier in this system (i.e. that the transference number for K^+PV approximates 1 under these conditions). The presence of a steady-state electrical potential difference when the PV concentration is different on the two sides of a membrane separating otherwise identical solutions proves that charge is transported mainly by the solution complexation mechanism in this system. However, it leaves unresolved the issue of whether failure of PV to act as an effective carrier is due to a low membrane permeability for free PV (low k_S) or to a slow rate of dissociation of metal-PV complexes (k_D and $k_D{}^a$).

The finding that the measured values of V_m closely agree with the theoretical limit of 59.2 mV per decade (at 25 °C) indicates that there are no appreciable concentration changes of MS^+ in the unstirred layers. In principle, such concentration changes can occur if there is a finite flux of free carrier S across the membrane and if the reaction $MS^+ \rightleftarrows M^+ + S$ in water is fast enough.

DISCUSSION

Despite their structural similarity, PV and valinomycin behave differently when exposed to lipid bilayers in the presence of alkali ions. The results of our kinetic experiments support the idea that PV acts almost exclusively by the solution-complexation mechanism in which the ion-carrier complex is formed in the aqueous phase and then translocated through the membrane. On the other hand, valinomycin has been shown to function mainly by the interfacial complexation mechanism in which an ion from the aqueous solution combines with a membrane-bound carrier molecule in the membrane-solution interface [18].

In general, a carrier which partitions between membrane and aqueous phase may be expected to act by a combination of both transport mechanisms. It is, therefore, useful to define a quantitative measure for the relative contribution of each transport mechanism to the overall transport. For this purpose we consider the stationary state of a symmetrical membrane-solution system with ideally stirred aqueous phases ($c'_M = c''_M = c_M$, $c'_S = c''_S = c_S$, $c'_{MS} = c''_{MS} = c_{MS}$, $V_m \neq 0$). As the principal difference between interfacial- and solution-complexation mechanism is independent of the presence or absence of unstirred layers, and in order to simplify

the discussion, we neglect diffusion polarisation in the following. Accordingly, we also omit the possibility that a concentration difference of MS^+ between the two unstirred layers creates (by the reaction $MS^+ \rightleftarrows M^+ + S$) a concentration difference of S and a back-flow of S across the membrane. Under these circumstances the fluxes Φ'_{IC} and Φ''_{IC} of the ion M^+ due to the interfacial-complexation (IC) mechanism at the left-hand (′) and right-hand (″) interface are given by

$$\Phi'_{IC} = k_R c_M N'_S - k_D N'_{MS} \tag{26}$$

$$\Phi''_{IC} = -k_R c_M N''_S + k_D N''_{MS} \tag{27}$$

Similarly, the transport of M^+ (in the form MS^+) due to the solution-complexation (SC) mechanism is described by

$$\Phi'_{SC} = k^{am}_{MS} c_{MS} - k^{ma}_{MS} N'_{MS} \tag{28}$$

$$\Phi''_{SC} = -k^{am}_{MS} c_{MS} + k^{ma}_{MS} N''_{MS} \tag{29}$$

It may be shown that $\Phi'_{IC} = \Phi''_{IC} \equiv \Phi_{IC}$ and $\Phi'_{SC} = \Phi''_{SC} \equiv \Phi_{SC}$. As a measure for the contribution of the solution-complexation mechanism we may therefore define the ratio Φ_{SC}/Φ_{IC} for which straightforward calculation yields the relation

$$\rho \equiv \frac{\Phi_{SC}}{\Phi_{IC}} = \frac{k^{ma}_{MS}}{k_D}\left(1 + \frac{c_M k_R}{2k_S + k^{ma}_S}\right) \tag{30}$$

(k_S^{ma} is rate constant of desorption of free carrier S). In the limit of small ion concentrations ($c_M k_R \ll 2k_S + k_S^{ma}$) Eqn. 30 simply states that the relative contribution of the solution-complexation mechanism is given by the ratio of the rate constants of desorption and dissociation of MS^+. The solution-complexation mechanism tends to dominate whenever $k_D < k_{MS}^{ma}$.

The presence of unstirred layers near the membrane surface seriously limits the efficiency of the solution-complexation mechanism [18]. As the total carrier concentration in the aqueous phase is usually very low (of the order of 1 μM or less), ion transport through the membrane leads to large changes in the MS^+ concentration in the aqueous solution adjacent to the membrane surface. In the extreme case the transport is entirely limited by the diffusion of the complex through the unstirred layers which are much thicker ($\delta \simeq 10^{-2}$ cm) than the membrane. With an aqueous diffusion coefficient of MS^+ of $D = 2 \cdot 10^{-6} \cdot cm^2 \cdot s^{-1}$ and a concentration $c_{MS} = 1$ μM the maximum possible current density compatible with the solution-complexation mechanism becomes $J = FDc_{MS}/\delta \simeq 2 \cdot 10^{-8}$ A $\cdot$ cm^{-2}. On the other hand, with a carrier of the interfacial-complexation type, such as valinomycin, current densities of the order of 10^{-3}–10^{-2} A $\cdot$ cm^{-2} may be easily obtained [18]. For a given value of k_{MS}, the IC mechanism permits much higher currents than the solution-complexation mechanism because the ratio of the membrane to the unstirred layer permeability can be as high as 10^6.

According to Eqn. 30 the finding that $\Phi_{SC} \gg \Phi_{IC}$ can be explained by the assumption that the dissociation rate constant k_D of PV is much smaller than the desorption rate constant k_{MS}^{ma}. From the relaxation studies reported here, only the latter could be approximately determined ($k_{MS}^{ma} \leqslant 10\ s^{-1}$). NMR experiments indicate that in methanol the dissociation rate constant of PV is many orders of mag-

nitude smaller than that of valinomycin [10]. This is consistent with the finding that PV forms a relatively stable potassium complex even in water (stability constant $K \simeq 8\ M^{-1}$), whereas in the case of valinomycin complexation is negligible under the same conditions [18]. For valinomycin which acts as an interfacial-complexation carrier the relation $k_D \gg k_{MS}^{ma}$ may be inferred from Eqn. 30. From studies with lipid bilayers k_D is found to be of the order of $10^5\ s^{-1}$ at 25 °C [3, 15]; k_{MS}^{ma} could not be determined for valinomycin so far. It appears therefore that the poor efficiency of PV as a carrier (as compared with valinomycin) is mainly due to the fact that PV is a much stronger complexating agent than valinomycin. The conclusion that very strong complexing agents are poor carriers is general. Consider a hypothetical carrier whose strength of complexation may be varied by some kind of chemical modification, while the values of k_S, k_{MS}, γ_S and γ_{MS} remain fixed. At low values of the complexation constant $K_h = k_R/k_D$, where k_D is large, the carrier functions according to the interfacial-complexation mechanism. The efficiency of the carrier then increases with increasing K_h. At high values of K_h, however, k_D becomes small since k_R is limited by the diffusional access of M^+. In the extreme case the dissociation of MS^+ in the membrane becomes completely blocked and the carrier switches to the less efficient solution-complexation mechanism.

It is interesting to note that valinomycin seems to have an almost ideal value of k_D. This may be shown in the following way. The specific conductance of a membrane containing Nmol of carrier per unit area is given by [3]

$$\lambda_0 = \frac{F^2 N}{2RT} \cdot \frac{c_M k_R}{k_D + c_M k_R} \cdot \frac{k_D k_{MS}}{k_D + 2k_{MS} + c_M k_R k_{MS}/k_S} \tag{31}$$

(pure interfacial-complexation mechanism). If k_D is varied under otherwise fixed conditions, λ_0 goes through a maximum which is reached at

$$k_D^* = \sqrt{c_M k_R k_{MS}(2 + c_M k_R/k_S)} \tag{32}$$

From the values of k_R, k_{MS} and k_S which have been obtained with a phosphatidylinositol membrane in the presence of valinomycin and K^+ [3] one calculates $k_D^* = 2 \cdot 10^4 \cdot s^{-1}$ at $[K^+] = 0.1$ M, which is near the experimentally determined value $k_D = 5 \cdot 10^4 \cdot s^{-1}$. A similar estimate is obtained from experiments with glycerol-monooleate membranes [15]: $k_D^* = 1 \cdot 10^5 \cdot s^{-1}$, $k_D = 3 \cdot 10^5 \cdot s^{-1}$. It is also pertinent to mention that all depsipeptide analogues of valinomycin studied so far turned out to be less effective carriers than valinomycin itself [2]. It is possible, of course, that still more effective carriers exist in which all the rate constants are larger than those of valinomycin.

For the rate constant of desorption of the PV complex only a tentative value of $k_{MS}^{ma} \simeq 10 \cdot s^{-1}$ could be given, which is about 100-times smaller than k_{MS}. This means that the barrier for the jump of MS^+ from the interfacial energy minimum into the water phase is higher by about 6 kJ/mol than the barrier for the translocation across the membrane. The partition coefficient of the PV complex between membrane and water, $\gamma_{MS} \simeq 10^3 - 10^4$, is considerably lower than the partition coefficients of other carrier complexes and hydrophobic ions ($\gamma_{MS} \simeq 1.6 \cdot 10^4$ for the valinomycin/Rb^+ complex in monoolein membranes [7], $\gamma \simeq 10^5$ for tetraphenylborate and dipicrylamine in dioleoyllecithin membranes).

One kinetic parameter is common to the solution- and interfacial-complexation mechanism, the translocation rate constant k_{MS} of the complex. The finding that k_{MS} is practically the same for Na^+, K^+, Rb^+, Cs^+ and NH_4^+ (Tables I and II) is consistent with the close structural similarity of the PV complexes of these ions, as inferred from NMR studies [10]. Compared with valinomycin/K^+ complex where k_{MS} is about $2 \cdot 10^5 \cdot s^{-1}$ in monoolein membranes [15], the translocation rate constant of the PV/K^+ complex is surprisingly small $5 \cdot 10^3 \cdot s^{-1}$ in monoolein membranes and $1 \cdot 10^3 \cdot s^{-1}$ in dioleoyl phosphatidylcholine membranes).

This large difference in the k_{MS} values of valinomycin and PV cannot be explained simply on the basis of the size of the complexes. Both complexes are pumpkin-shaped with a height of ≈ 1.2 nm and a diameter of ≈ 1.6 nm. Differences, however, exist in the nature of the side chains. In PV, a proline ring replaces the methyl group of the lactyl residues and the isopropyl group of the hydroxiisovaleryl residues of valinomycin. This gives the outer hydrocarbon coat of the PV complex a more rigid structure and possibly less hydrophobic properties as compared with the valinomycin complex. The value of k_{MS} is directly related to the energy difference between the equilibrium position of the complex in the interface and the top of the barrier in the center of the membrane. The shape of the energy barrier is determined by the hydrophobic interaction of the complex with the membrane and by the image force [24], as well as by the interfacial potential due to orientation of dipoles in the polar layers of the membrane [25, 26]. All these interactions exhibit a very strong dependence on position of the interfacial energy minimum of MS^+ which may result from minor differences in the shape, flexibility and hydrophobicity of the complex and may, therefore, result in appreciable changes in the height of the energy barrier (a change by a factor of one hundred in k_{MS} corresponds to an energy difference of 5.7 kJ/mol which is small compared with the estimated total barrier height of the order of 100 kJ/mol). Both the low value of k_{MS} and the low value of γ_{MS} (as compared with valinomycin) may be understood if it is assumed that the PV-cation complex is located nearer to the aqueous side of the membrane-solution interface.

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