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Kinetics of Ion Transport in Lipid Membranes Induced by Lysine-Valinomycin and Derivatives

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Summary. Lysine-valinomycin and two N_ϵ-acyl derivatives are compared with respect to their potency to transport Rb^+ ions across thin lipid membranes. Lysine-valinomycin acts as a neutral ion carrier only above a pH of about 7 of the aqueous solutions, while at lower pH the molecules seem to be positively charged due to a protonation of the ε-NH_2 group of the lysine residue.

A kinetic analysis based on voltage jump relaxation experiments and on the nonlinearity of the current-voltage characteristics showed that the conductance increment Λ per carrier molecule for uncharged lysine-valinomycin is similar to that of natural valinomycin. The attachment of a rather bulky side group such as the dansyl or para-nitrobenzyloxycarbonyl group reduced Λ by approximately one order of magnitude.

Some of the relaxation data of the valinomycin analogues were influenced by an unspecific relaxation of the pure lipid membrane. This structural relaxation represents a limitation to the possibility of analyzing specific transport systems in thin lipid membranes by the voltage jump or charge pulse techniques. It is shown that the time dependence of this structural relaxation – which was first published by Sargent (1975) – is at variance with a three capacitor equivalent circuit of the membrane, which was suggested by Coster and Smith (1974) on the basis of a.c. measurements. A modified equivalent circuit has been found to represent a satisfactory analogue for the current relaxation in the presence of valinomycin. It turned out, however, that such an equivalent circuit provides little insight into the molecular mechanism of transport.

Key words: Lipid membranes – Valinomycin – Ion transport – Fast kinetics.

Introduction

The depsipeptide valinomycin is one of the most extensively used ionophores in cell biology. It was first isolated by Brockman and Schmidt-Kastner (1955) from cul-

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tures of Streptomyces fulvissimus. Moore and Pressman (1964) reported a strong increase of the potassium permeability of mitochondrial membranes in the presence of this compound, an effect which was subsequently found for a variety of different biological membranes. The mechanism of its action has been mainly studied by using artificial lipid membranes which mimic the hydrophobic barrier of biomembranes. An essential result of this analysis was the finding that valinomycin behaves as an ion carrier for alkali ions in contrast to the pore formers gramicidin A or alamethicin (for a review see Eisenman et al., 1973; Stark et al., 1974). The unique complexing properties of valinomycin and especially its large preference of potassium over sodium caused an increasing interest into its molecular structure. Approximately one hundred different analogues have been synthesized up to now in order to elucidate the molecular basis of its activities (Ovchinnikov et al., 1974; Gisin et al., 1969; Gisin and Merrifield, 1972; Ovchinnikov and Ivanov, 1977). While most of them have been studied with respect to their binding properties of alkali ions and their antimicrobial activity, little is known about the details of their transport behaviour in membranes. Only valinomycin (Stark et al., 1971; Laprade et al., 1975; Knoll and Stark, 1975, 1977; Pohl et al., 1976) and the peptide analogue PV (proline-valinomycin) (Benz et al., 1976) have been analyzed in thin lipid membranes by fast kinetic experiments. This approach in favourable cases allows the quantitative determination of the single transport parameters of a given transport model. It can therefore contribute to characterize the "transport capacity" of different analogues on the basis of single transport steps. In this way a relationship between the molecular structure of an ionophore and its permeability properties in lipid membranes in terms of partition coefficient, translocation rate across the membrane and interfacial reaction rates may be established. A first attempt to compare the transport properties of three valinomycin analogues with a variable degree of methylation was presented by Eisenman et al. (1975). Their study was based on steady state measurements and showed that the effect of an increasing methylation is principally to decrease the dissociation rate of the complex. A detailed kinetic analysis was, however, not performed.

This paper reports a comparative kinetic study of three other valinomycin analogues. Lysine-valinomycin (Lys-VAL) differs from natural valinomycin (VAL) only by replacement of one L-valine residue by an L-lysine. This substitution allows the investigation of the effect of an additional charge which is due to protonation of the ε-NH_2-group at low pH. The two other analogues are derivatives of Lys-VAL in which the dansyl (Dns) or the paranitrobenzyloxycarbonyl (PNZ) groups have been attached to the lysyl-residue. Their behaviour will indicate, if and how the transport properties of carrier molecules are influenced by a modification of their shape and structure. The fluorescence emitted by dansyllysine-valinomycin has already been used to estimate its concentration inside the membrane (Pohl et al., 1976).

The results presented below will also show that the possibility of performing a kinetic analysis of ion transport – which is induced by specific ionophores – is limited by the existence of unspecific structural relaxation processes of the unmodified membrane.

Materials and Methods

A. Synthesis of Valinomycin Analogues

The structure of the valinomycin analogues used are shown in Figure 1. Paranitrobenzyloxycarbonyl-lysine-valinomycin (PNZ-Lys-VAL) was prepared using the stepwise segment condensation scheme devised for the solid phase synthesis of VAL (Gisin et al., 1969). The lysine residue was introduced in the last segment as N_α-t-butyloxycarbonyl-N_ϵ-paranitrobenzyloxycarbonyl-L-lysyl-D-α-hydroxyisovaleric acid. Cleavage from the solid support followed by cyclization gave PNZ-Lys-VAL. Lysine valinomycin (Lys-VAL) was obtained from this compound by catalytic hydrogenation and dansyl-lysine-valinomycin (Dns-Lys-VAL) (Pohl et al., 1976) by dansylation of Lys-VAL. For a detailed account of these syntheses see Gisin and Dhundale (1979). In the present study Lys-VAL was used in its hydrochloride form.

B. Membrane Experiments

Black lipid membranes were formed from commercially available glycerylmonooleate (Nu Check) and from dioleoyllecithin (prepared in our laboratory by K. Janko). The technique of membrane formation, the steady state electrical measurements as well as the voltage jump-current relaxation method have been described repeatedly (Stark et al., 1971, 1974; Knoll and Stark, 1975; Pohl et al., 1976). Since the amplitude of the observed current relaxations was occasionally very small, signal averaging was used as described previously (Pohl et al., 1976). To improve the time resolution of the setup to about 1 μs a transient recorder (Biomation, model 805) in combination with the signal averager (Nicolet, model 1072) was used. The application of signal averaging techniques improved the current sensitivity by at least a factor of 20.

The relaxation of the unmodified membrane (Fig. 5), which extended over at least four orders of magnitude in time, was obtained by measuring the response of

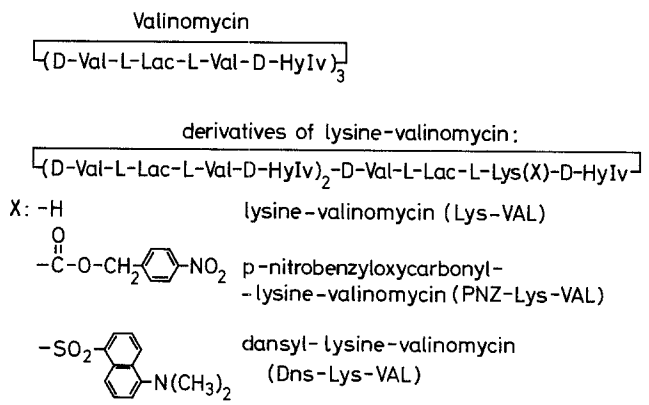

Fig. 1. Structure of the valinomycin analogues

the same membrane with different sample intervals of the transient recorder and by using different external resistors to improve the current sensitivity at long times after a voltage jump. An increase in the external resistor is accompanied by a simultaneous increase of the time constant of the initial current spike. A comparison of two relaxation curves obtained with two different external resistors, at long times compared to the initial current spikes, gave a satisfactory agreement. This indicates that the relaxation measurements were not influenced by the choice of the external resistor introduced for the current measurement. The relaxations shown in Figure 5 are composed of three different measurements using the resistors 10^2 Ω, 10^3 Ω, and 10^4 Ω. Usually 512 signals were averaged. The time interval between two successive voltage pulses usually was ten times larger than the voltage pulse length. Only those membranes were accepted, which showed constant electrical properties throughout the testing time of about 10–15 min.

Results and Discussion

A. Steady state experiments

Addition of lysine-valinomycin to the membrane forming solution induced a pH-dependent conductance increase reminiscent of a titration curve (Fig. 2). Above pH 8 and below pH 6 the conductance seems to assume constant values (though a further increase at high pH cannot be excluded within the experimental error). The same experiment performed with natural VAL instead of Lys-VAL gave equal conductance values at pH 8 and pH 4. This indicates that the effect observed in the presence of Lys-VAL is due to this compound and is not caused by a change of the state of protonation of the lipid molecules. A possible interpretation of this result would be to assume neutral Lys-VAL molecules inside the membrane above a pH of

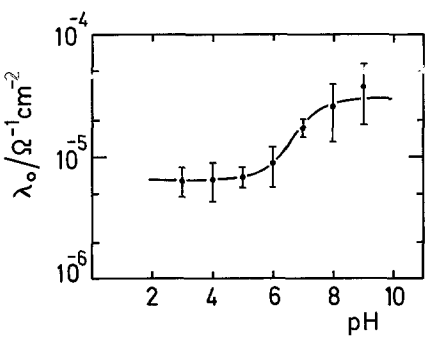

Fig. 2. Membrane conductance as a function of pH. Experimental conditions: Dioleoyllecithin membranes with 10^{-3} M Lys-VAL in the membrane forming solution, 1 M RbCl in unbuffered water, 25° C. The ground level conductance without Lys-VAL was between 10^{-7}–10^{-6} Ω^{-1} cm^{-2} except at pH 9, where slightly higher values were obtained. The pH of the unbuffered aqueous solutions was carefully controlled before and after a measurement. Only those membranes were accepted where the deviation from the indicated value was less than 0.5 pH units. Above pH 9 the membrane stability was very poor. The solid line was drawn assuming a pK of 7 (see text)

about 8 which become progressively protonated at lower pH. The carrier-ion complexes, if formed with 1 : 1 stoichiometry, would then bear a single charge at high pH and possibly two positive charges at low pH. The smaller conductance found at low pH would then be expected from a lower concentration and/or a lower translocation rate across the membrane diffusion barrier of the twofold charged complexes. It was tried to describe the pH-dependence of the conductance tentatively on the basis of simplifying assumptions which are outlined in Appendix A. The resulting Eq. (A4) agrees fairly well with the experimental data, if an apparent pK of about 7 is assumed. This result is consistent with extraction experiments performed with Lys-VAL by Ovchinnikov and Ivanov (1977), who reported a protonation of the ε-NH_2 group of lysine below pH 7, a value considerably lower than the aqueous pK of this group (10.5). A drop in pK is to be expected when an amino group is transferred from water into an unpolar medium (Cohn and Edsall, 1965).

The interpretation of the results of Figure 2 implies that the chloride anion dissociates into the aqueous solution as the Lys-VAL-HCl complex is incorporated into the membrane, leaving the molecule positively charged at low pH. At high pH neutral Lys-VAL molecules are obtained by dissociation of H^+ from the lysyl-residue. The assumption of a complete dissociation of Cl^- from the complex is supported by the observation that the transport efficiency of Lys-VAL does not depend on the external chloride concentration. Similar conductance values were found at high and low pH, if 1 M RbCl in the aqueous phase was replaced by 0.5 M Rb_2SO_4. Further experiments have shown that the conductance is almost completely determined by a movement of Rb^+-ions across the membrane independent of the pH of the aqueous solutions. If RbC'l was replaced by LiCl, the same conductance value of about $2 \cdot 10^{-7}\ \Omega^{-1}\ cm^{-2}$ was found in the absence and presence (10^{-3} M) of Lys-VAL in the membrane forming solution. Since these measurements were performed at pH 4, where the carrier molecules are supposed to be protonated, they indicate that there is only a negligible contribution of electrogenic proton transport even at low pH.

In conclusion, the conductance of lipid membranes in the presence of Lys-VAL is presumably determined by singly charged ion carrier complexes at pH > 7 and by twofold charged complexes at pH < 7. For reasons explained later in this paper, a kinetic analysis was possible only at high pH. The following results for Lys-VAL and its derivatives were all obtained in a pH range where virtually all molecules are neutral. The results may therefore be directly compared with those of natural VAL.

The assumption of a 1 : 1 stoichiometry for Lys-VAL induced Rb^+-transport may in principle be verified by measuring the conductance as a function of the involved species. In view of the relatively small conductance values observed even at high Lys-VAL concentrations, such measurements are, however, not of great significance. Larger conductance values were found for the two derivatives Dns-Lys-VAL and PNZ-Lys-VAL. In these two analogues the ε-NH_2 group of lysine is blocked by large hydrophobic groups. A linear relationship between the conductance and the concentration of Dns-Lys-VAL in the membrane forming solution was already reported (Pohl et al., 1976). Figure 3 shows the dependence of the conductance on the Rb^+-concentration in water for the different analogues. In all cases, a linear relationship is found at low Rb^+-concentrations. At high Rb^+-concentrations a saturation

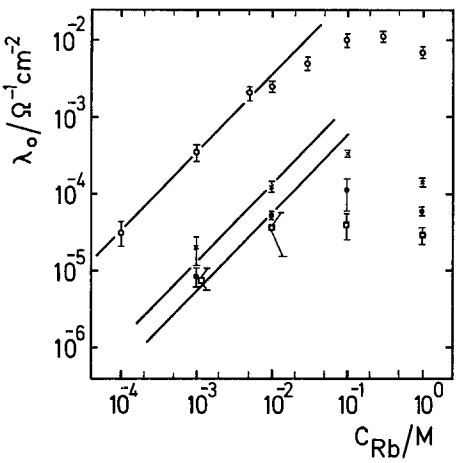

Fig. 3. Conductance as a function of Rb^+-concentration in water for the different analogues. The data were obtained with glycerylmonooleate membranes at 10° C using the following concentrations of the valinomycin species in the membrane forming solution:
○ $5 \cdot 10^{-4}$ M VAL (taken from Knoll and Stark, 1975); × 10^{-4} M Dns-Lys-VAL; ● 10^{-4} M PNZ-Lys-VAL; □ 10^{-3} M Lys-VAL. The pH of the aqueous solutions was about six except at the experiments with Lys-VAL (pH 8). The solid lines were drawn with slope 1

behaviour is apparent with a slight maximum between 0.1 M and 1 M. In case of normal VAL, the saturation effect was in fair agreement with predictions from a kinetic analysis performed at different Rb^+-concentrations (Knoll and Stark, 1975). For the analogues studied in the present paper there are larger discrepancies, as will be discussed later. The qualitative behaviour is, however, the same for all valinomycin species. This analogy refers also to other experimental findings which are not presented in detail: The saturation in the relationship between conductance and Rb^+-concentration almost disappears if dioleoyllecithin is used instead of glycerylmonooleate. The ion selectivity sequence of conductance (in the linear range of conductance versus ion concentration) is the same for VAL and PNZ-Lys-VAL, i.e.,

$$\lambda_0(\mathrm{RbCl}) \geq \lambda_0(\mathrm{KCl}) > \lambda_0(\mathrm{CsCl}) \geq \lambda_0(\mathrm{NaCl}),\ \lambda_0(\mathrm{LiCl})\,.$$

Differences between VAL and Lys-VAL and its derivatives were, however, observed with respect to the reproducibility of the conductance data. While the VAL data are usually reproducible within a factor of 2, the values for Lys-VAL may differ up to a factor of 10 for membrane solutions prepared at different days. The data presented in Figure 3 were obtained with single preparations. This relatively large scatter might be caused by the rather complex equilibration process of the carrier molecules between torus and membrane. Similar to Dns-Lys-VAL where aggregation formation has been observed in aqueous solutions (Pohl et al., 1976), Lys-VAL might exists in the membrane forming solution not in monomeric form.

The close correspondence with respect to the transport mechanism for the different valinomycin species is also apparent from their current-voltage characteristics (Fig. 4). The change from a superlinear curve at low concentrations of the transported ion to a sublinear curve at high concentrations is a characteristic for carrier mediated ion transport (Stark and Benz, 1971). Therefore, a kinetic analysis on the basis of the voltage jump method and on the nonlinearities of the current-voltage characteristics has been performed, as described in previous publications (Stark et al., 1971; Knoll and Stark, 1975).

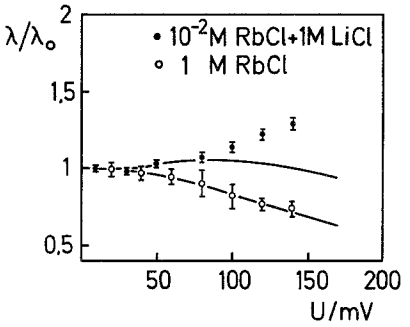

Fig. 4. Current voltage curve at two different ion concentrations for 10^{-4} M Dns-Lys-VAL in the membrane forming solution. The ratio λ/λ_0 of the conductance λ observed at a given voltage U over the conductance λ_0 in the ohmic region at low voltages is plotted. The solid lines were calculated from the voltage jump data (Table 2)

B. Relaxation Experiments

I. The Unmodified Membrane. The electric current across lipid membranes doped with valinomycin after a voltage jump shows a characteristic relaxation, which has been analysed on the basis of a carrier transport model (Stark et al., 1971). The current amplitude of these relaxations usually is large enough so that relaxations which are due to the unmodified lipid membrane can be neglected. This is no longer permissible when substances are studied which induce relaxations of considerably smaller current amplitude than natural VAL. In such a case, the behaviour of the pure lipid membrane has to be taken into account. Current relaxations which cannot be assigned to a specific transport species will be called "unspecific" in the following. Such unspecific current relaxations have been first published by Sargent (1975) for membranes formed from oxidized cholesterol. They could be clearly separated from the electrostrictive phenomena, which have been often described as a result of decreasing membrane thickness (i.e., increasing membrane capacitance) at high membrane voltages. Since part of the specific relaxations described below are of rather small current amplitude, the unspecific relaxations had to be measured under the same experimental conditions.

In Figure 5, the current spike from the initial loading of the membrane capacitance is omitted. A continuous decrease of the current over at least three orders of magnitude in time is observed which extends beyond the applied voltage pulse length of 10 ms. Similar results were obtained for lecithin membranes. Though not studied in great detail, the current relaxation appears to be identical for voltage jumps from 0 to 30 mV and from −15 mV to +15 mV. This indicates – in agreement with Sargent (1975) – that electrostrictive effects or changes of the area of the bimolecular film (i.e., influences on torus and lenses) are not responsible for the observed phenomenon, since they depend on the square of the voltage (i.e., are not present at a voltage jump from −V to +V). A formal description of the current relaxation through a series of exponentials requires the assumption of at least four different relaxation times. This is of consequence for a phenomenological treatment of the membrane in terms of an equivalent electrical circuit. The most simple circuit, consisting of a parallel arrangement of a single membrane capacitance and a membrane resistance, shows an exponential decay of the current after a voltage jump from an initial value, which is determined by the external resistor of the circuit, to the steady state value determined by the membrane resistance. A more sophisticated version uses three of these elements, two for the polar regions of the membrane and one for

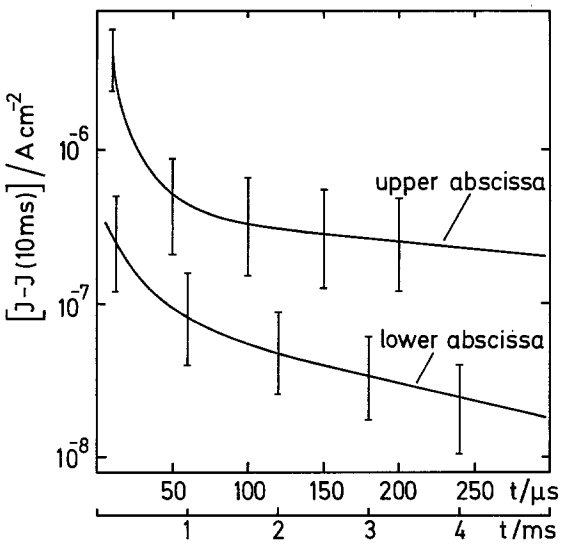

Fig. 5. Unspecific relaxation of the current across glycerylmonooleate membranes following a voltage jump from 0 to 30 mV. The membranes were formed in 1 M LiCl (T = 10° C). The bars indicate the approximate scatter of the data obtained from five different membranes. The current spike which is due to the initial loading of the membrane capacitance is not shown

the hydrocarbonlike interior. Coster and Smith (1974) determined the values of the capacitances and resistancies by very sensitive a.c. measurements. The band width of their setup was, however, restricted to 100 Hz. The current relaxation reported in this paper could be detected with high sensitivity by using signal averaging techniques for frequencies ranging from less than 10 Hz up to 1 MHz. For a three element equivalent circuit the current relaxation should be composed of two exponentials, as is analysed in detail in Appendix B. The necessity of at least five exponentials (including the initial current spike not shown in Fig. 5) for a quantitative description of the current relaxation indicates that the three element equivalent circuit is inadequate. It can be used as an approximation only in case of a small frequency range.

The mechanistic basis of the observed unspecific relaxation remains largely obscure. As is illustrated in Figure 6, both charge transfer and/or (as was suggested by Sargent, 1975) a voltage dependent orientation of dipoles might contribute to the observed effects, which could take place either within the polar region or the hydrocarbonlike interior of the membrane. The concentration of charged species inside the membrane is usually assumed to be extremely small, since we consider the bare membrane surrounded by a salt solution. Though it is difficult to exclude the presence of hydrophobic impurities completely, the observed effects seem to be rather associated with the polar region of the membrane. Little is known about the rotational mobility of the polar head groups, the concentration and mobility of "free" ions inside the polar region and the magnitude of the electric field across it. The rather complex time course of the relaxation represents another interesting problem. It might be caused by some cooperative action of adjacent head groups. Further studies – such as the dependence of the current relaxation on the kind and concentration of the ions present in water and on the amplitude of the voltage jump – are necessary to distinguish between the different possibilities outlined above. They might contribute to elucidate the structure and flexibility of the polar region.

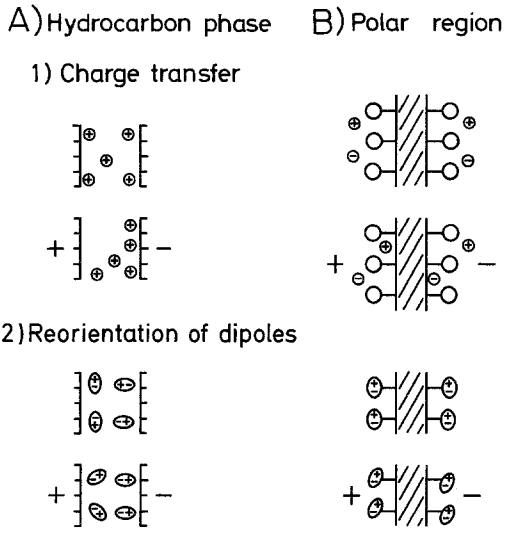

Fig. 6. Possible origin of the current relaxation observed with unmodified lipid membranes after application of a voltage jump (see text for details)

II. Carrier Kinetics. The specific current relaxation induced by valinomycin in the presence of Rb^+ has been found to consists of two exponential processes (Laprade et al., 1975; Knoll and Stark, 1975), i.e., it may be fitted by the equation

$$J = J_\infty(1 + \alpha_1 e^{-t/\tau_1} + \alpha_2 e^{-t/\tau_2}) . \tag{1}$$

A relaxation of the type of Eq. (1) was predicted from a simple carrier model, which describes ion transport on the basis of four rate constants (Stark et al., 1971). The amplitude of a third exponential is zero, if it is assumed that only the rate constant of the translocation of the charged carrier-ion complexes is voltage dependent, while the three other rate constants are not affected by a voltage across the membrane. Using this assumption the two rate constants of the interfacial reaction (k_R, k_D) as well as the translocation rate constants of the free and complexed carrier molecules (k_S, k_{MS}) may be obtained from the four experimental quantities α_1, α_2, τ_1, and τ_2.

An additional source of information about the rate constants is the nonlinearity of steady state current-voltage curves (Stark and Benz, 1971). Their shape according to the simple carrier model depends only on one parameter A, which is a function of the rate constants:

$$A = 2\,z + v c_M \tag{2}$$

with $z = k_{MS}/k_D$, $v = k_{MS}k_R/k_S k_D$ and c_M = ion concentration in water. z and v may be obtained by measuring the concentration dependence of A.

If for technical reasons only one of the two relaxation times can be resolved, the rate constants in favourable cases may be determined from z, v, τ, and α (Stark et al., 1971). The knowledge of the rate constants enables one to characterize the

efficiency of a given carrier species either in terms of the turnover number f per carrier molecule and per second (Stark et al., 1974)

$$f = \left(\frac{1}{k_S} + \frac{1}{k_{MS}} + \frac{2}{k_D}\right)^{-1} \tag{3}$$

or in terms of the conductance increment Λ per carrier molecule (Pohl et al., 1976)

$$\Lambda = \frac{\lambda_0}{N_0} = \frac{F^2}{2\,RT}\,\frac{k_R c_M}{(k_R c_M + k_D)}\,\frac{k_{MS}}{(1 + 2\,z + v c_M)} \tag{4}$$

(λ_0 = steady state membrane conductance at low voltages, N_0 = total number of carrier molecules per unit area of membrane, F = Faraday constant, T = temperature, R = gas constant).

A kinetic analysis of valinomycin induced Rb^+-transport through monoglyceride membranes was performed previously (Knoll and Stark, 1975). A comparison between these results and the unspecific relaxation of the bare membrane (Fig. 5) shows that the contribution of the latter does not exceed a few per cent (usually less than one per cent) of the total relaxation observed in the presence of valinomycin in the time range where the relaxation times and amplitudes according to Eq. (1) were determined. This is no longer true in case of some of the analogues considered in this paper. In Figure 7 the specific relaxation of PNZ-Lys-VAL induced Rb^+-transport is compared with an unspecific relaxation observed in 1 M LiCl. The data obtained in 1 M RbCl need more than one exponential term for an adequate fitting. If, however, the unspecific relaxation is subtracted from the total relaxation, a single straight line is obtained in the semilogarithmic plot. Therefore, only a single specific relaxation time may be obtained within the experimental error given by the presence of the unspecific relaxation.

The subtraction procedure used in Figure 7 raises some questions. Firstly, its application means to assume a linear superposition of specific and unspecific relaxation

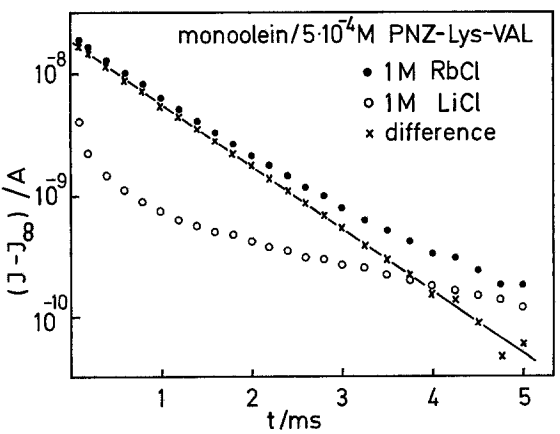

Fig. 7. Current relaxation following a voltage jump of 30 mV (membrane area $5 \cdot 10^{-3}$ cm^2)

phenomena. Secondly, the relatively large deviations between different membranes of the unspecific relaxation (see Fig. 5) results in the uncertainty as to which membrane should be used. Therefore, the subtraction method was only used to make sure about the significance of the presence of two different specific relaxation times. Otherwise the unspecific relaxation was neglected in the analysis of the data summarized in Table 1. The values for PNZ-Lys-VAL and Dns-Lys-VAL are affected only to a minor extent by the unspecific relaxation, if the analysis is restricted to a limited time range. In Figure 7 the relaxation time and its amplitude differ only slightly, if obtained by the subtraction method or by taking the unmodified data between 100 μs and 3 ms, which may be reasonably fitted by a single straight line. In case of Dns-Lys-VAL two relaxation times have been detected in this way, which differ by a factor of 10. In contrast to its derivatives a substantial influence of the structural relaxation can, however, not be excluded for Lys-VAL. The analysis of the Lys-VAL data on the basis of the carrier model, as discussed above, is therefore tentative. The rate constants given in Table 2 represent only rough approximations. The approximative nature of the values for Lys-VAL may also be concluded from the following test of consistency: For the relaxation amplitudes α_1 and α_2 and the quantities z and ν (obtained from the concentration dependence of the current-voltage characteristic) the relation

$$\alpha_1 + \alpha_2 = (2\,z + \nu c_M)\cosh(u/2) \tag{5}$$

should hold (Stark et al., 1971). For the Lys-VAL data there is a difference of a factor 8 between both sides of the Equation, while for Dns-Lys-VAL a good agreement is obtained (see solid lines of Fig. 4). For Lys-VAL and Dns-Lys-VAL the relaxation data (i.e., τ_1, τ_2, α_1, and α_2) were sufficient for the calculation of the rate constants in Table 2. In case of PNZ-Lys-VAL, where only one relaxation time could be resolved within the experimental error, kinetic and steady state data together (i.e., τ, α, z, and ν) had to be used, as described above. Table 2 contains also values of the partition coefficient γ_S^{mb} of the free carrier species S between the membrane forming solution (concentration c_s^b) and the membrane, defined as the concentration ratio $\gamma_S^{mb} = c_S^m/c_S^b$. It is an equilibrium property of the system which is obtained from the steady state conductance if the rate constants are known (Benz et al., 1973). In view of the rather bad reproducibility of the conductance data for Lys-VAL and its derivatives, the values are, however, subject to a larger error. They were calculated from the conductance values at 1 M RbCl found throughout the relaxation experiments (Table 1).

A comparison of the transport behaviour of valinomycin and its lysine-analogues on the basis of the data summarized in Table 2 yields the following essential characteristics: The substitution of one L-valine by an L-lysine in natural VAL has only a relatively small effect on the kinetic parameters of the transport, though this statement is weakened by the approximate nature of the Lys-VAL data. The comparatively low conductance increase induced by Lys-VAL in artificial lipid membranes is mainly caused by a smaller partition coefficient. The conductance increment per carrier molecule (or the turnover number) is reduced by about one order of magnitude, if rather bulky side groups, such as the nitrobenzyloxycarbonyl or the dansyl group, are attached to Lys-VAL. This is mainly caused by a reduction of the translocation rate constants across the membrane interior and to a minor extent by a

Table 1. Steady state current-voltage and voltage jump relaxation data (mean value of 5 membranes and standard error) of Rb^+-transport across glycerylmonooleate membranes mediated by valinomycin and analogues. The experiments were performed with $5 \cdot 10^{-4}$ M VAL, PNZ-Lys-VAL or Dns-Lys-VAL or with 10^{-3} M Lys-VAL in the membrane forming solution. The aqueous solution contained 1 M RbCl. The pH was about six except in the experiments with Lys-VAL, where it was kept between pH 7 and 8. The temperature was 10° C. In the case of Lys-VAL the data are influenced by the unspecific relaxation of the membrane (see text). The voltage jump was from 0 to 30 mV

	$\tau_1/\mu s$	$\tau_2/\mu s$	α_1	α_2	z	v	$\lambda_0/\Omega^{-1}\,cm^{-2}$
VAL	18.7 ± 2	7.2 ± 0.9	13.4 ± 2.8	13.9 ± 4.9	–	–	$(3 \pm 0.5)\ 10^{-3}$
Lys-VAL	66 ± 12	13.5 ± 4.7	1.5 ± 0.3	6.6 ± 5.4	0.2	0.4	$(3 \pm 1.6)\ 10^{-4}$
PNZ-Lys-VAL	830 ± 62	–	0.5 ± 0.06	–	0.15	0.4	$(3 \pm 0.4)\ 10^{-4}$
Dns-Lys-VAL	345 ± 22	35.3 ± 4	0.51 ± 0.07	0.22 ± 0.06	0.175	0.35	$(7 \pm 1)\ 10^{-4}$

Table 2. Analysis of the voltage jump data of Table 1. The conductance increment Λ and the partition coefficient γ_s^{mb} refer to experiments performed with 1 M RbCl in water. The data for Lys-VAL represent only rough approximations. The error estimates do not include systematic errors, such as a contribution of the unspecific relaxation of the membrane, but refer to the statistical variations observed for five different membranes (standard error)

	$k_R c_M/s^{-1}$	k_D/s^{-1}	k_{MS}/s^{-1}	k_S/s^{-1}	f/s^{-1}	Λ/Ω^{-1}	γ_s^{mb}
VAL	$(6.7 \pm 1.8)\ 10^4$	$(2.2 \pm 0.6)\ 10^4$	$(3.9 \pm 0.1)\ 10^4$	$(6.2 \pm 1)\ 10^3$	$3.6 \cdot 10^3$	$4.1 \cdot 10^{-15}$	1.2
Lys-VAL	$(9.8 \pm 0.2)\ 10^3$	$(1.8 \pm 0.3)\ 10^4$	$(2.5 \pm 1.3)\ 10^4$	$(4.6 \pm 2.2)\ 10^3$	$2.7 \cdot 10^3$	$4.3 \cdot 10^{-15}$	0.15
PNZ-Lys-VAL	$(1.4 \pm 0.2)\ 10^3$	$(5.6 \pm 2.2)\ 10^3$	$(8.4 \pm 3.2)\ 10^2$	$(5.3 \pm 0.5)\ 10^2$	$2.9 \cdot 10^2$	$3.3 \cdot 10^{-16}$	4.8
Dns-Lys-VAL	$(5.3 \pm 0.5)\ 10^3$	$(1.9 \pm 0.3)\ 10^4$	$(1.9 \pm 0.4)\ 10^3$	$(1.3 \pm 0.2)\ 10^3$	$7.1 \cdot 10^2$	$7.9 \cdot 10^{-16}$	4.6

reduction in the association rate, while the dissociation rate constant remains almost unaffected. The reduction in the conductance increment per carrier molecule is more than compensated by the increase of the partition coefficient induced by the side groups, so that the membrane conductance produced by Dns-Lys-VAL and PNZ-Lys-VAL is larger than that produced by Lys-VAL at identical concentrations in the membrane forming solution.

The association rate of Lys-VAL and its derivatives is too small compared to the dissociation rate to explain the saturation (and the maximum) of the conductance observed at high Rb^+-concentrations (Fig. 3). A reason for this discrepancy may consist in the rather complex equilibrium behaviour between membrane and torus, which might depend on the Rb^+-concentration in water. Unfortunately, the existence of the unspecific relaxation does not permit to resolve the specific relaxations of the Lys-VAL derivatives at small Rb^+-concentrations. A possible dependence of γ_S^{mb} on the Rb^+-concentration can, therefore, not be tested experimentally. For the same reason, the specific relaxation induced by Lys-VAL could only be resolved at high pH of the aqueous phases (where the uncomplexed carrier molecules are supposed to be neutral) and even here only within a substantial error. This clearly shows the limitations of a kinetic analysis of specific transport systems based on voltage jump or charge pulse experiments, which stem from the presence of the unspecific relaxation of the pure lipid membrane.

These limitations are also present in alternating current studies, as may be concluded from experiments reported by Pickar and Amos (1976). They studied the effect of pentachlorophenol in lecithin membranes and they also had to correct their data for effects of the undoped membrane. Their analysis as well as that of other authors (Ashcroft et al., 1977; Zimmermann et al., 1977) was based on a three element equivalent circuit for the membrane (Fig. 8 without R_{ext}). In this kind of approach the variation of the elements on incorporation of an additive into the membrane is measured. From a change of C_p or R_p an "interfacial activity" is usually inferred, while a change of C_h or R_h is assumed to reflect a modification of the properties of the hydrocarbon core of the membrane. This procedure appears of questionable validity in view of the following arguments:

Firstly, as has been shown in the last section, the three element circuit is insufficient to explain the high frequency behaviour of the undoped membrane. Secondly, we will now show that one has to extend this circuit in order to explain the specific effect of valinomycin and its analogues in lipid membranes. The current relaxation after a voltage jump, which is applied to a three element circuit, consists of only two exponential terms, as is shown in Appendix B [Eq. (B5)]. The relaxation of the current across a lipid membrane, which is doped with valinomycin, has been found, however, to consist of three exponential terms (if the unspecific relaxation of the bare membrane shown in Fig. 5 is neglected). Two of these exponentials are generated through the presence of valinomycin, while the third one, the "initial current spike", is present also in the absence of this compound. The different number of exponentials indicates that the three element circuit is insufficient to describe the behaviour of lipid membranes doped with valinomycin.

We will now try to suggest an extended equivalent circuit of this membrane system and we will study the question whether it is possible to distinguish between interfacial and inner membrane phenomena on the basis of this circuit. A better

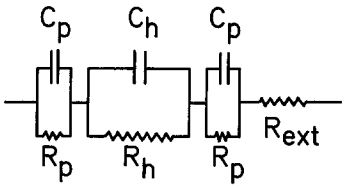

Fig. 8. Equivalent electrical circuit of the membrane assuming independent capacitances and resistances for the polar region (index p) and hydrocarbon region (index h)

phenomenological description of the relaxation of the membrane current may be obtained by assuming that the specific effect of valinomycin is "in parallel" to the effect of the untreated membrane. The latter may be approximated by a single capacitance, which is responsible for the initial current spike. The two remaining exponentials of the current relaxation which are specifically induced by valinomycin, may then be interpreted on the basis of Figure 8, i.e., with a "specific" three element circuit "plus" an additional series resistance, which, however, is now part of the membrane. A comparison of Eq. (1) and (5) shows that the same current relaxation is expected for the valinomycin effect in the membrane and for this circuit. In other words, the five parameters of the carrier model N_0, k_R, k_D, k_{MS}, and k_S can be "translated" into the five circuit parameters C_h, R_h, C_p, R_p, and R_{ext}. The following data [obtained from the relaxation data of Table 1 by using Eq. (B5)] refer to glycerylmonooleate membranes doped with $5 \cdot 10^{-4}$ M valinomycin in the membrane forming solution. The current relaxation observed in this case can be simulated by

$$
\begin{aligned}
C_p &= 2.3\ \mu\text{F/cm}^2, \\
C_h &= 4.3\ \mu\text{F/cm}^2, \\
R_p &= 160\ \Omega/\text{cm}^2, \\
R_h &= 2.4\ \Omega/\text{cm}^2, \\
R_{\text{ext}} &= 11.8\ \Omega/\text{cm}^2.
\end{aligned}
$$

R_{ext} comprises also the resistance of the aqueous solution and of the electrical testing circuit (together about 10% of R_{ext} under the experimental conditions used here). Parallel to the circuit of Figure 8 a capacitance of about 0.4 μF/cm^2 (simulating the capacitance of the undoped membrane) has to be added. This complete equivalent circuit of the membrane was checked by an experiment. The same current relaxation was found as with the membrane. The validity of this equivalence between membrane and circuit extends, however, only to small voltages. At high voltages the carrier system becomes nonlinear (i.e., the relaxation times and amplitudes become voltage dependent), while the equivalent circuit contains only linear elements. A further limitation of this circuit concerns the interpretation of its elements in terms of membrane properties. C_h, C_p, R_p, R_h, and R_{ext} depend on all rate constants of the carrier model, as may be seen from a detailed inspection of the corresponding equations. There is no direct correlation between individual elements of the circuit and individual rate constants. This means that a direct assignment of circuit elements either to the membrane interface or to the membrane interior is not possible. In view of this fact we consider the application of equivalent circuits as a substitute for mechanistic models of very limited usefulness.

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Appendix A

pH-Dependence of Lysine-Valinomycin Induced Conductance

The derivation is based on the following simplifying assumptions:
a) Lys-VAL inside the membrane (index m) is neutral at high pH (species A) and is positively charged at low pH (species HA^+) according

$$\frac{c_A^m \, c_{H^+}}{c_{HA^+}^m} = K \,. \tag{A1}$$

b) The membrane conductance λ_0 is proportional to the concentration of the species A and HA^+ with different proportionality constants a and b:

$$\lambda_0 = a \, c_A^m + b \, c_{HA^+}^m \,. \tag{A2}$$

From Eqs. (A1) and (A2) one obtains with the notation $c_t^m = c_A^m + c_{HA}^m+$:

$$\lambda_0 = c_t^m \frac{a \, K + b c_{H^+}}{K + c_{H^+}} \,. \tag{A3}$$

Eq. (A3) may be rewritten by introducing the pH-independent conductances $\lambda_0^1 = bc_t$ and $\lambda_0^2 = ac_t$ obtained at sufficiently low and high pH:

$$\lambda_0 = \lambda_0^1 \frac{(\lambda_0^2/\lambda_0^1) \, K + c_{H^+}}{K + c_{H^+}} \,. \tag{A4}$$

Here we have implicitly assumed that the total carrier concentration c_t^m in the membrane does not depend on the pH of the aqueous solutions. Since the experiments of Figure 2 were performed by adding Lys-VAL to the membrane forming solution, c_m^t is assumed to be buffered from the torus surrounding the membrane. Eq. (A4) was fitted to the experimental data of Figure 2 by using the values

$$\lambda_0^1 = 6.6 \cdot 10^{-6} \, \Lambda^{-1} \, \mathrm{cm}^{-2}, \; \lambda_0^2 = 3 \cdot 10^{-5} \, \Omega^{-1} \, \mathrm{cm}^{-2}, \text{ and } K = 10^{-7} \, \mathrm{M} \,.$$

The value of the equilibrium constant K may, however, vary to a certain extent depending on the correctness of the above mentioned simplifying assumptions.

Appendix B

An Equivalent Electrical Circuit of a Lipid Membrane as Studied by Voltage Jump Current Relaxation Experiments

A circuit often assumed to simulate the electrical behaviour of an unmodified lipid membrane is shown in Figure 8. It consist of three elements, each of which is built by a parallel arrangement of a resistance and a capacitance. Two of the elements are usually assigned to the polar regions (index p), while the

third one is believed to represent the hydrocarbonlike interior of the membrane (index h). The external resistor R_{ext} comprises the resistance of the aqueous solution as well as that of the electrical testing circuit. The application of Kirchhoff's laws yields

$$J = \frac{U_p}{R_p} + C_p \frac{dU_p}{dt} = \frac{U_h}{R_h} + C_H \frac{dU_h}{dt}, \tag{B1}$$

$$U = 2\,U_p + U_h + JR_{ext}\,. \tag{B2}$$

U is total voltage applied to the circuit and U_p, U_H represent the voltages across the corresponding elements p and h. Eqs. (B1) and (B2) may be rearranged to the following second order differential equation:

$$\frac{d^2U_h}{dt^2} + \alpha \frac{dU_h}{dt} \times \beta U_h = \gamma \tag{B3}$$

with

$$\alpha = \left(\frac{1}{R_{ext}C_h} + \frac{1}{R_hC_h} + \frac{1}{R_pC_p} + \frac{2}{R_{ext}C_p} \right),$$

$$\beta = \frac{R_{ext} + R_h + 2\,R_p}{R_{ext}R_hC_hR_pC_p}$$

$$\gamma = \frac{U}{R_{ext}R_pC_pC_h}\,.$$

Eq. (B3) has to be solved considering the conditions of a voltage jump experiment, i.e., a sudden increase of the voltage from 0 to U at time $t = 0$:

$$U_h(t = 0) = U_p(t = 0) = 0\,,$$

$$J(t = 0) = \frac{U}{R_{ext}} = C_h \frac{dU_h}{dt} = C_p \frac{dU_p}{dt} \tag{B4}$$

The solution of the problem is obtained by standard techniques and reads:

$$J = J_\infty(1 + \alpha_1 e^{b_1 t} + \alpha_2 e^{b_2 t}) \tag{B5}$$

with

$$J_\infty = \frac{U}{R_{ext} + 2\,R_p + R_h},$$

$$\alpha_1 = -\frac{[R_h + 2\,R_p + R_{ext}(1 + b_2R_hC_h)]}{(b_2 - b_1)R_{ext}R_hC_h}(1 + b_1R_hC_h)\,,$$

$$\alpha_2 = \frac{[R_h + 2\,R_p + R_{ext}(1 + b_1R_hC_h)]}{(b_2 - b_1)R_{ext}R_hC_h}(1 + b_2R_hC_h)\,,$$

$$b_{1,2} = \varepsilon \pm \sqrt{\delta}$$

$$\varepsilon = -\frac{1}{2}\left(\frac{1}{R_{ext}C_h} + \frac{1}{R_hC_h} + \frac{1}{R_pC_p} + \frac{2}{R_{ext}C_p}\right),$$

$$\delta = \frac{1}{4}\left(\frac{1}{R_{ext}C_h} + \frac{1}{R_hC_h} - \frac{1}{R_pC_p} - \frac{2}{R_{ext}C_p}\right)^2 + \frac{2}{R_{ext}^2C_hC_p}.$$

Eq. (B5) are largely simplified, if the following special case is considered:

$$C_p \gg C_h; \quad R_h \gg R_p \gg R_{ext}. \tag{B6}$$

Then,

$$J_\infty = \frac{U}{R_h},$$

$$b_1 = -\frac{1}{R_pC_p}; \quad b_2 = -\frac{1}{R_{ext}C_h}; \quad (-b_1 \ll -b_2); \tag{B7}$$

$$\alpha_1 \ll \alpha_2; \quad \alpha_2 = \frac{R_h}{R_{ext}}.$$

The conditions (B6) were fullfilled in case of the a.c. measurements reported by Coster and Smith (1974). The current relaxation observed after a voltage jump should then consist mainly of a single exponential (index 2) which corresponds to the case where the membrane is simulated only by a single element instead of three (as is easily shown). The addition of the two elements, which specially consider the polar regions of the membrane (index *p*), contribute a second exponential term of very small amplitude [with the restrictions of Eq. (B6)]. Its relaxation time $\tau_1 = R_pC_p$ is much larger than the relaxation time $\tau_2 = R_{ext}C_h$ (which represents the initial current spike usually observed in case of voltage jump experiments).

In contrast to the statement of Eq. (B5) one needs, however, at least five exponentials to fit the current decrease (including the initial current decrease), as was first shown by Sargent (1975) for membranes formed from oxidized cholesterol and is reported in this paper for glycerylmonooleate and lecithin membranes. This indicates that the equivalent circuit of Figure 8 is inadequate. It may, however, be shown that the results obtained from the voltage jump experiments agree qualitatively with the a.c. measurements of Coster and Smith (1974) if the band width of the voltage jump experiments is correspondingly reduced. A phenomenological description of the electrical behaviour of unmodified lipid membranes which includes the high frequency range has to consist of considerably more elements than assumed in Figure 8.

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