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Alkali Ion Transport through Lipid Bilayer Membranes Mediated by Enniatin A and B and Beauvericin

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Summary. Stationary conductance measurements with lipid bilayer membranes in the presence of enniatin A and B and beauvericin were performed. For comparison, some valinomycin systems were investigated. It was found that the conductance in the case of enniatin A and B is caused by a carrier ion complex with a 1:1 stoichiometry, whereas for beauvericin, a 3:1 carrier ion complex has to be assumed to explain the dependence of the conductance on carrier and ion concentration in the aqueous phase. The current-voltage curves measured with dioleoyl phosphatidylcholine membranes show a superlinear behavior for the three carriers in the presence of potassium. On the other hand, supralinear current-voltage curves were observed with membranes from different monoglycerides, except for beauvericin. The results obtained with enniatin A and B are in a satisfactory agreement with an earlier proposed carrier model assuming a complexation between carrier and ion at the membrane water interface.

The discrimination between potassium and sodium ions is much smaller for the enniatins than for valinomycin. This smaller selectivity as well as the fact that potassium ions cause the highest conductance with lipid bilayer membranes may be due to the smaller size of the cyclic enniatin molecules, which contain 6 residues in the ring *vs.* 12 in the case of valinomycin. Charge-pulse relaxation studies were performed with enniatin A and B, beauvericin, and valinomycin. For monoolein membranes only in the case of valinomycin, all three relaxations predicted by the model could be resolved. In the case of the probably more fluid membranes from monolinolein ($\Delta^{9,12}$-$C_{18:2}$) and monolinolenin ($\Delta^{9,12,15}$-$C_{18:3}$) for all carrier systems except for beauvericin, three relaxations were observed.

The association rate constant k_R, the dissociation rate constant k_D, and the two translocation rate constants k_{MS} and k_s for complexed and free carrier, respectively, could be calculated from the relaxation data. The carrier concentration in the aqueous phase had no influence on the rate constants in all cases, whereas a strong saturation of the association rate constant k_R with increasing ion concentration was found for the enniatins. Because of the saturation, k_R did not exceed a value of $4 \times 10^5\ \text{M}^{-1}\,\text{sec}^{-1}$ with 1 M salt irrespective of carrier, ion, or membrane-forming lipid.

A similar but less pronounced saturation behavior was also observed for the translocation rate constant k_S of the free carrier. The other two rate constants were independent of the ion concentration in the aqueous phase. In the case of the enniatins, the translocation rate constant k_{MS} was not independent from the kind of the transported ion. In the series K^+, Rb^+ and Cs^+, k_{MS} increases about threefold. The turnover numbers for the carriers as calculated from the rate constants range between $10^4\,\text{sec}^{-1}$ and

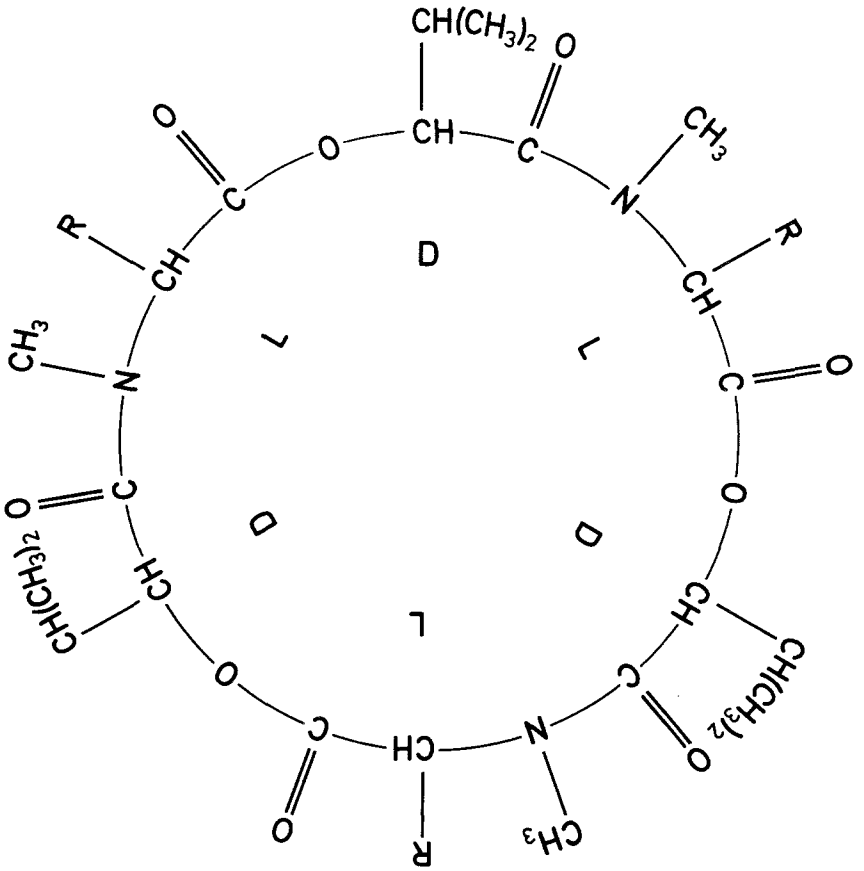
0022-2631/78/0043-0367 $05.60

$10^5 sec^{-1}$ and do not show a strong difference between the individual carriers. The conductance difference in the systems investigated here is therefore mainly caused by the partition coefficients, which are smaller for the enniatins than for valinomycin.

Certain macrocyclic compounds, such as valinomycin, the macrotetrolides, and the enniatins, have been shown to increase the permeability of natural and artificial membranes for alkali ions [9, 18, 27, 36, 37]. Valinomycin and the macrotetrolides actin antibiotics act as mobile carriers within the membrane [20]. In bulk organic phases like ethanol and methanol they form complexes with alkali ions with high stability constants [10, 15, 32, 38]. (For a review *see* ref. [9]). For carrier-mediated ion transport simple models have been developed [8, 23, 24]. The transport properties of valinomycin and the macrotetrolides observed in stationary conductance measurements as well as in kinetic experiments are well explained by a model which has been described in full detail in previous publications [23, 34]. It has been shown that a 1:1 ion-carrier complex is responsible for charge transfer across lipid bilayer membranes and that the interfacial complexation between carrier molecules and ions at the membrane-water interface is needed to explain the high current densities [23, 33]. An alternative theory has been developed for carrier molecules which act mainly by solution complexation (SC-mechanism) [2]. In this case the membrane conductivity under stationary conditions is many orders of magnitude lower than for the interfacial complexation mechanism (IC-mechanism) [2, 35].

An increase of the K^+ transport across biological and artificial membranes has also been reported for enniatin B [18, 37], a member of a family of four antibiotics (Fig. 1) which are cylohexa-depsipeptides containing three N-methyl-L-amino acids and three D-α-hydroxyisovaleric acid residues [16, 28]. Enniatin A (cyclo [N-methyl-L-isoleucin D-α-hydroxyisovaleric acid]$_3$), enniatin B (cyclo [N-methyl-L-valine D-α-hydroxyisovaleric acid]$_3$), and enniatin C (cyclo [N-methyl-L-leucin-D-α-hydroxyisovaleric acid]$_3$) have been found in certain strains of *Fusarium* [29], whereas beauvericin (cyclo [N-methyl-L-phenylalanin-D-α-hydroxyisovaleric acid]$_3$) was produced by the fungus *Beauveria bassiana* [16].

The increase of cation permeability of lipid bilayer membranes in the presence of enniatin B and beauvericin has been explained on the basis of carrier-ion complexes with 2:1 and 3:2 stoichiometry [18]. These sandwich complexes have been proposed in order to explain the second or third power dependence of conductivity *vs.* total carrier concentration c_0 in the aqueous phase. NMR studies with enniatin B-K^+ complexes in



	R
enniatin A	$-CH(CH_3)C_2H_5$
enniatin B	$-CH(CH_3)_2$
enniatin C	$-CH_2CH(CH_3)_2$
beauvericin	$-CH_2C_6H_5$

Fig. 1. Structure of the enniatins and beauvericin

organic solvents support this hypothesis, although the stability constant of the 2:1 complex in ethanol is two orders of magnitude lower than that of the 1:1 complex in the same solvent [18].

So far, detailed data on conductance behavior of lipid bilayer membranes in the presence of enniatin A and B and of beauvericin are lacking. In order to test whether the ion transport mediated by these compounds can be described by the kinetic models previously developed for valinomycin and the macrotetrolides, stationary conductance

measurements as well as electrical relaxation experiments have been carried out. Electrical relaxation methods, either by the voltage-jump [5, 6, 17, 19, 34] or charge-pulse technique [1, 4, 13, 14] have already been applied to the kinetic analysis of carrier mediated ion transport. The latter technique has been used throughout this study. Its main advantage, besides a minimal pertubation of the membrane ($V_m \leqq 10$ mV), lies in the increased time resolution (≈ 200 nsec).

Materials and Methods

Optically black lipid bilayer membranes were obtained in the usual way [6] from a 1–3% (wt/vol) lipid solution in *n*-decane (Merck, Darmstadt, G.F.R., standard for gas chromatography). The cell used for bilayer formation was made from Teflon. The circular hole in the wall between the two compartments had a diameter of about 2 mm (membrane area 2×10^{-2} cm^2). The temperature was kept at 25° throughout. The aqueous phase contained alkali ion chlorides (Merck, analytical grade) in different concentrations (10^{-3}–3 M) dissolved in twice distilled water. Synthetic enniatin A and B [30, 31] (generously supplied by Dr. O. Studer, Hoffmann-LaRoche, Basel, Switzerland, and Dr. B. Pressman, Miami, Fla.), as well as beauvericin (Bachem, Marina Del Rey, Calif.) and valinomycin (Calbiochem, San Diego, Calif.) were used as concentrated stock solutions in ethanol. Small amounts of the stock solutions were added to the unbuffered salt solutions (pH $\approx$ 6) to get a final concentration of the antibiotics between 10^{-8} and 5×10^{-5} M. The ethanol concentration in the aqueous phases never exceeded 0.5% (v/v), a concentration which did not affect the electrical properties of the membranes to any appreciable extent.

Membranes were formed from the following lipids: L-1,2-dioleoyl-3-phosphatidylcholine, synthesized in our own laboratory according to ref. [3] and monoglycerides with different fatty acid residues: oleoyl ($\Delta^9 - C_{18:1}$), linoleoyl ($\Delta^{9,12} - C_{18:2}$), and linolenoyl ($\Delta^{9,12,15} - C_{18:3}$). The purity of the lipids was checked by thin-layer chromatography and was found to be greater than 99%.

The stationary conductance measurements were performed using silver-silver chloride electrodes in series with a voltage source and a Keithley 150 B or 610 C electrometer. The experiments were carried out under steady-state conditions, which were reached in the case of valinomycin about 20 min after blackening of the membrane. In the case of the other carriers, steady state was obtained faster. All data were recorded 20–30 min after the membranes were in the black state.

The charge pulse experiments were carried out as described in previous publications [1, 4]. The membrane capacitance was charged up to a voltage of about 10 mV by a brief current pulse (10 nsec to 50 nsec duration) through platinized platinum electrodes. The voltage transients across the membrane were recorded with a Tektronix 7633/7A13 storage oscilloscope. The evaluation of the data from the oscillographic records was performed as described earlier [4].

Results

Stationary Conductance Data

The description and the mathematical treatment of the transport model which assumes a 1:1 stoichiometry between carrier molecule and

ion has been given in detail in previous publications [23, 33]. Therefore, only the main equations are presented here, which will be used later for the analysis of the results. The model for carrier-mediated ion transport is based on the assumption that the association between carrier S (total aqueous concentration c_0) and ion M^+ (aqueous concentration c_M) takes place at the membrane-solution interface with the rate constants of association and dissociation being k_R and k_D, respectively. Complex MS^+ and free carrier S cross the membrane with rate constants k_{MS} and k_S [23]. The translocation rate constant of the complex is assumed to be the only voltage-dependent rate constant:

$$k'_{MS} = k_{MS}\, e^{u/2}$$
$$k''_{MS} = k_{MS}\, e^{-u/2}. \tag{1}$$

k'_{MS} and k''_{MS} are the rate constants of translocation of MS^+ from left to right and from right to left, respectively; $u = FV/RT$ is the reduced voltage (F is the Faraday constant, V the voltage, R the gas constant and T the absolute temperature). The partition coefficients γ_S and γ_{MS} for the free and the complexed carrier are defined as the ratio of the average membrane concentration divided by the bulk aqueous concentration. The membrane conductance λ_0 in the limit of small voltages is given by the following equation [33]:

$$\lambda_0 = \frac{F^2 d}{2RT} \frac{z\,\gamma_s k_R\, c_M\, c_0}{(K c_M + 1)(1 + 2 \cdot z + v\, c_M)}. \tag{2}$$

d is the membrane thickness, K the equilibrium constant of complex in the aqueous phase, and $z = k_{MS}/k_D$ as well as $v = k_{MS} k_R / k_S k_D$ are combinations of the four rate constants. The voltage dependence of the conductance may be expressed by the following relation [33]:

$$\frac{\lambda}{\lambda_0} = \frac{2(1+A)\sin h(u/2)}{u[1 + A\cos h(u/2)]} \tag{3}$$

where $A = 2z + v\, c_M$. It is possible, in principle, to derive from the current voltage relationship (λ/λ_0) and the membrane conductance λ_0 as a function of c_M the quantities z, v and K, provided that these quantities are sufficiently large.

However, relaxation studies with the voltage jump [19] or with the charge pulse technique [4] have shown that k_R is not independent of the ion concentration c_M in the aqueous phase and that the product $k_R c_M$ saturates at high ion concentrations [4, 19]. Therefore, the evaluation of

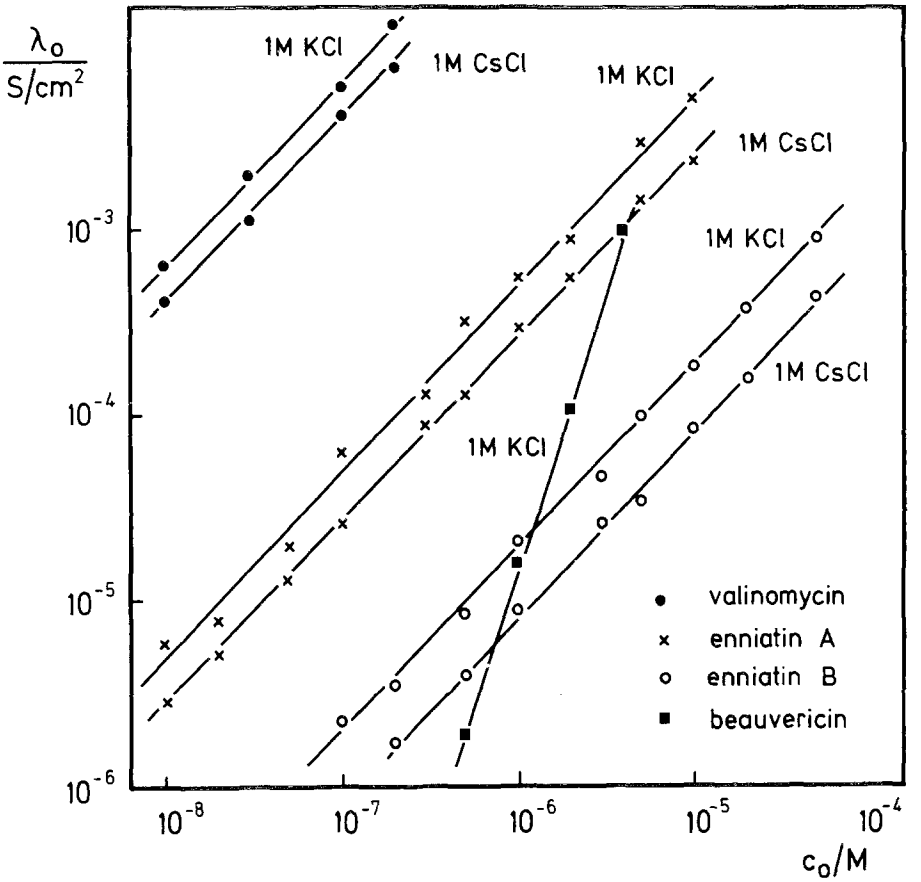


Fig. 2. Conductance of dioleoyl phosphatidylcholine membranes as a function of the carrier concentration c_0 with 1 M KCl or 1 M CsCl in the aqueous phase. The conductance in the absence of carriers is about 2×10^{-7} S cm^{-2}; $T = 25$ °C

the aqueous equilibrium constant K on the basis of Eqs. (2) and (3), assuming a concentration independent k_R may lead to erroneous results. Furthermore, it is known from studies with valinomycin and trinactin that only part (50–70%) of the applied voltage acts on the carrier [5, 19, 21].

Fig. 2 shows the membrane conductance at small voltages ($|u| \ll 1$, $|V| \ll 25$ mV) for dioleoyl phosphatidylcholine membranes. In the case of valinomycin as well as of enniatin A and B in the presence of K^+ or Cs^+, the experimental points may be fitted well with lines of slope one. Similar results were found for other alkali ions like Rb^+ in the case of valinomycin and Li^+, Na^+, and Rb^+ in the case of enniatin A and B. For beauvericin a strikingly different result was found (Fig. 2). The data

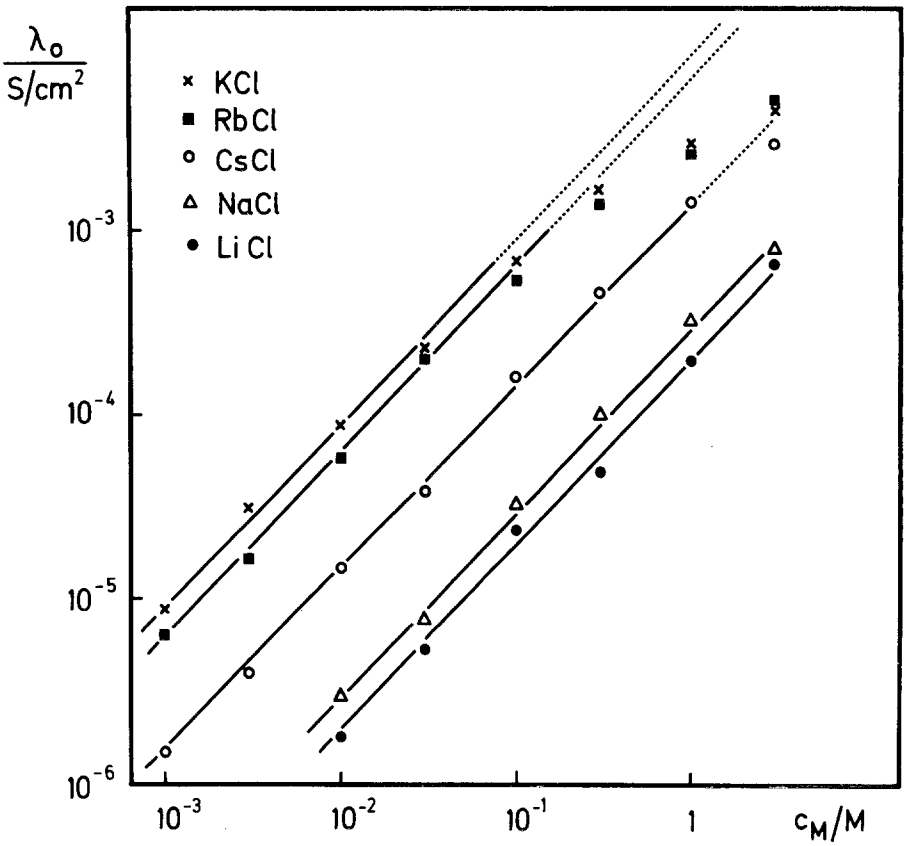


Fig. 3. Conductance of dioleoyl phosphatidylcholine membranes as a function of the concentration c_M of different salts in the aqueous phase, enniatin A ($c_0 = 5 \times 10^{-6}$ M); $T = 25$ °C

obtained for 1 M KCl and various concentrations of beauvericin are best fitted with a line of slope three. The same is true for other alkali ions (Na^+, Rb^+, and Cs^+). The reason for this difference between the enniatins and beauvericin is not clear, but it is interesting to note that the slope of three has also been reported in a study of beauvericin and enniatin B in membranes made from brain lipids [18]. It is also seen from Fig. 2 that the efficiency of the four carrier systems increases from enniatin B to enniatin A to valinomycin, whereas the conductivity in the presence of beauvericin ranges approximately between enniatin B and enniatin A.

Fig. 3 contains the results obtained with dioleoyl phosphatidylcholine membranes with 5×10^{-6} M enniatin A and different concentrations of alkali ions. There is a linear relationship between ion concentration and conductivity for LiCl and NaCl and at lower concentrations also for K^+,

Rb^+, and Cs^+. At higher concentrations of these ions saturation is observed quite similar to saturation phenomena which have been found in the presence of valinomycin [6, 19]. In the simplest case this may be caused by a non-negligible value for v. However, other reasons also, as previously discussed, like saturation of the carrier in the aqueous phase or a saturation of the product $k_R c_M$, may explain the deviation from linearity. On the basis of Eqs. (2) and (3) a value of $K = 2\,\text{M}^{-1}$ would be obtained for the enniatin A/K^+ complex in the aqueous phase from the data given in Fig. 3 and the current-voltage relationships in dioleoyl phosphatidylcholine membranes (Fig. 5). However, with glycerolmonooleate membranes, a value of $K = 5\,\text{M}^{-1}$ would be obtained from a similar approach. This deviation shows clearly that some of the assumption implicit in the use of Eqs. (2) and (3) are not fullfilled (*see* section *Charge-Pulse Relaxation Studies*).

The deviation from the linearity in the λ_0 *vs.* c_M plot is smaller in the case of enniatin B, as seen from Fig. 4; but also in this case, discrepancies between different types of membranes were observed, which lead to a higher apparent value of K ($K \approx 1.5\,\text{M}^{-1}$) in experiments with glycerolmonooleate membranes than with dioleoyl phosphatidylcholine membranes ($K \leqq 0.2\,\text{M}^{-1}$) in the presence of K^+ as transported ion. For enniatin B the deviations for the different lipids could result from saturation of $k_R c_M$ with increasing KCl-concentration in the case of glycerolmonooleate membranes. This explanation is consistent with the finding that the association constant k_R for the valinomycin/Rb^+ complex is larger for monoglyceride membranes than for phosphatidyl choline membranes [4]. A direct proportionality between ion concentration and conductivity without any deviation at high salt concentrations was found in the presence of beauvericin. For enniatin A and B the results given in Figs. 2, 3, and 4 may be explained by the assumption of a 1:1 carrier ion complex. For beauvericin, on the other hand, a 3:1 stoichiometry between carrier and ion seems likely. However, other explanations which are less simple, such as a concentration dependent partitition coefficient, may also explain the slope of three in Fig. 2. As already mentioned, a similar slope has been found for beauvericin/Cs^+ and for enniatin B/K^+ with membranes from brain lipids [18]. In the case of beauvericin, our results are in agreement with the previous study. The reason for the descrepancy in the case of enniatin B is not clear. It may be caused by the use of different lipids for membrane formation, which may be more or less favorable for the formation of 1:1 and 3:1 complexes.

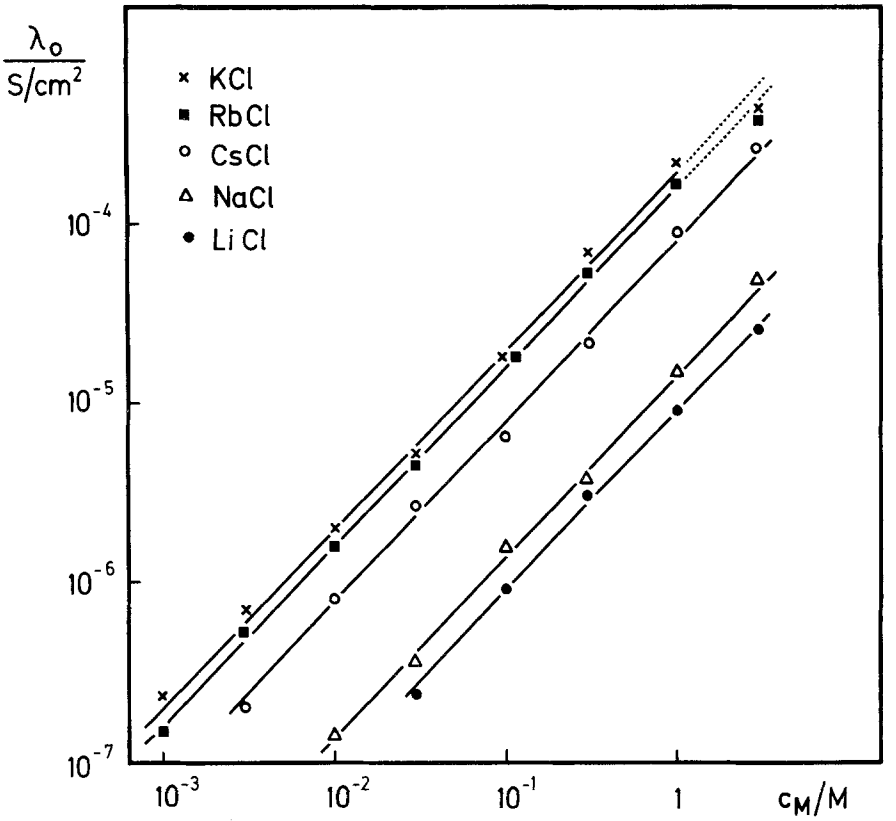


Fig. 4. Conductance of dioleoyl phosphatidylcholine membranes as a function of the concentration c_M of different salts in the aqueous phase; enniatin B ($c_0 = 10^{-5}$ M); $T = 25\,°C$

For membranes formed from monolinolein and monolinolenin the specific conductance in the presence of the enniatins may reach extremely high values ($\simeq 10^{-2}\,S\,cm^{-2}$). Therefore, a similar estimate as given in the Appendix of ref. [33] shows that, if the enniatins act by the solution-complexation (SC) mechanism, diffusion polarization should occur in these experiments. However, diffusion polarization was never observed in the stationary experiments with the enniatins. It may be concluded from this finding that these carriers act mainly by the interfacial-complexation (IC) mechanism.

Current-Voltage Relationships

Figures 5 and 6 contain the current-voltage relationships for the different carriers obtained with membranes from two different neutral lipids, dioleoyl phosphatidylcholine (Fig. 5) and monoolein (Fig. 6). The

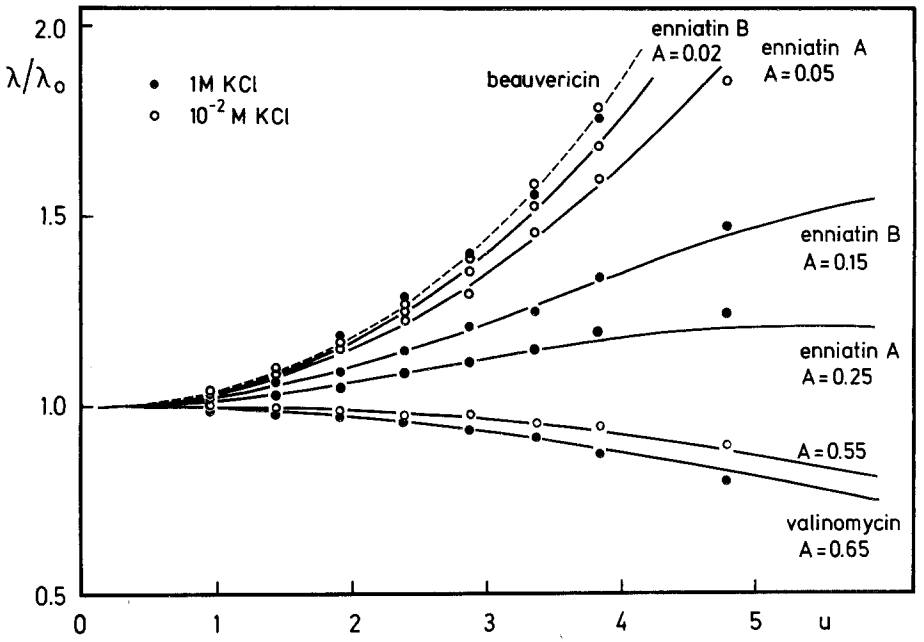


Fig. 5. Conductance ratio λ/λ_0 as a function of voltage for membranes from dioleoyl phosphatidylcholine. The aqueous phase contained, besides the different carriers, either 1 M KCl or 10^{-2} M KCl; $T=25\,°C$. The concentration of the carriers was (in M): 10^{-7} valinomycin, 5×10^{-6} enniatin A, 10^{-5} enniatin B and 2×10^{-6} beauvericin. The full lines were calculated from Eq. (3) with the values of A given in the text. For beauvericin (dotted line) *see* text

data of the two figures were derived using two different KCl concentrations, 1 and 10^{-2} M. In the case of valinomycin the ionic strength was kept constant at 1 M by using corresponding concentrations of LiCl. For the other carriers this was not possible because of the relatively poor selectivity of these carriers between Li^+ and K^+. For dioleoyl phosphatidylcholine membranes (Fig. 5) all carriers except valinomycin produce a superlinear current-voltage relationship. This suggests that the rate-limiting step in these cases is the migration of the complexes across the potential barrier in the middle of the membrane, whereas the interfacial reaction is in equilibrium ("equilibrium domaine" [12]). The differences in the shape of the current-voltage relationships for the same carrier but different ion concentration is an additional argument for the existence of an interfacial reaction (IC-mechanism [23]) rather than a SC-mechanism, where the complexation in the solutions is in equilibrium. A reasonable fit of the data given in Fig. 5 may be achieved with Eq. (3) using the following values for A for the different systems:

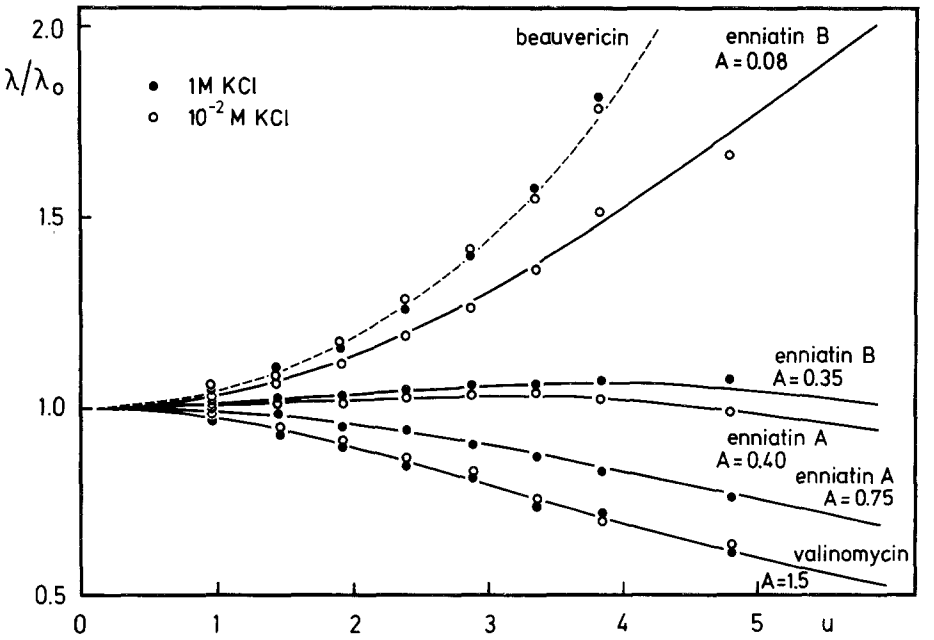


Fig. 6. Conductance ratio λ/λ_0 as a function of voltage for membranes from monoolein. The aqueous phase contained, besides the different carriers, either 1 M KCl or 10^{-2} M KCl; $T=25\,°C$. The carriers had the following concentrations (in M): 10^{-7} valinomycin, 1.2×10^{-6} enniatin A, 5×10^{-6} enniatin B, and 2×10^{-6} beauvericin. The lines were calculated from Eq. (3) with the values of A given in the text. For beauvericin (dotted line) *see* text

Valinomycin, 1 M KCl: $A=0.65$, 10^{-2} M KCl: $A=0.55$

Enniatin A, 1 M KCl: $A=0.25$, 10^{-2} M KCl: $A=0.05$

Enniatin B, 1 M KCl: $A=0.15$, 10^{-2} M KCl: $A=0.02$.

In the case of beauvericin, where Eq. (3) is not appropriate for curve fitting, the current-voltage relationship can be represented by the function $2\cdot\sinh(u/2)/u$, irrespective of the KCl concentration.

In kinetic studies it has been found that the translocation rate constant k_{MS} is larger for a monoglyceride membrane than for a membrane from the corresponding phosphatidylcholine, possibly indicating a larger fluidity of the membrane from monoglycerides [4]. This is, in principle, also reflected in Fig. 6. The current-voltage relationships for all carriers except beauvericin are shifted to a more sublinear behavior. This means that the interfacial reaction becomes more and more rate limiting, i.e., the system is the "kinetic domain" [12]. This is also reflected by

larger values for A in case of monoolein compared with dioleoyl phosphatidylcholine membranes.

The current voltage curves of the different systems given in Fig. 6 may be fitted using Eq. (3) with the following values for A:

Valinomycin, 1 M KCl: $A=1.5$, 10^{-2} M KCl: $A=1.5$

Enniatin A, 1 M KCl: $A=0.75$, 10^{-2} M KCl: $A=0.40$

Enniatin B, 1 M KCl: $A=0.35$, 10^{-2} M KCl: $A=0.08$.

In the case of beauvericin the shape of the current voltage curves does not differ for 1 and 10^{-2} M KCl. They may be represented in a way similar to that for membranes from dioleoyl phosphatidylcholine.

The kinetic constants of valinomycin-mediated potassium transport across monoolein membranes have been measured by charge-pulse experiments [4]. The value for A as calculated from the single rate constants ($A=7.5$ for $c_M=1$ M) and the value for A obtained from fitting the λ/λ_0 curves of Fig. 5 with Eq. (3) ($A=1.5$ for $c_M=1$ M) show a large difference. This may be caused by the rather incomplete description of the current-voltage curves by Eq. (3). A better description is achieved by the assumption that only the fraction α of the applied voltage acts on the charged complex [17, 19]. This leads to the following modification of Eq. (3):

$$\frac{\lambda}{\lambda_0}=\frac{2(1+A)\sin h(\alpha u/2)}{\alpha u[1+A\cos h(\alpha u/2)]}. \tag{4}$$

Equation (4) gives a much better fit of the current-voltage curve presented in Fig. 6 for valinomycin and 1 M KCl, using the parameters $A=7.5$ and $\alpha=0.6$. Similar deviations have been observed for the valinomycin-Rb^+ system at monoolein membranes [19].

The relatively small difference between the current-voltage curves measured with monoolein membranes for 1 M KCl and 10^{-2} M KCl in the presence of valinomycin may be caused by the saturation effect which has been observed for k_R [4, 19]. In addition, for $\alpha=0.5$ the variation of the current-voltage curves for values of A ranging between 1 and 100 is rather small [19].

Figure 7 contains the current-voltage curves obtained for 1 M KCl in the presence of enniatin A and B with membranes from monolinolein/*n*-decane. As with valinomycin, it is not possible to fit the curves with $\alpha=1$ and the values of A which can be calculated from the rate constants (given in Table 3, dotted lines in Fig. 7).

A much better fit is achieved if the same values of A are used together with $\alpha=0.75$ in Eq. (4). This is a hint that also in the case of the enniatins

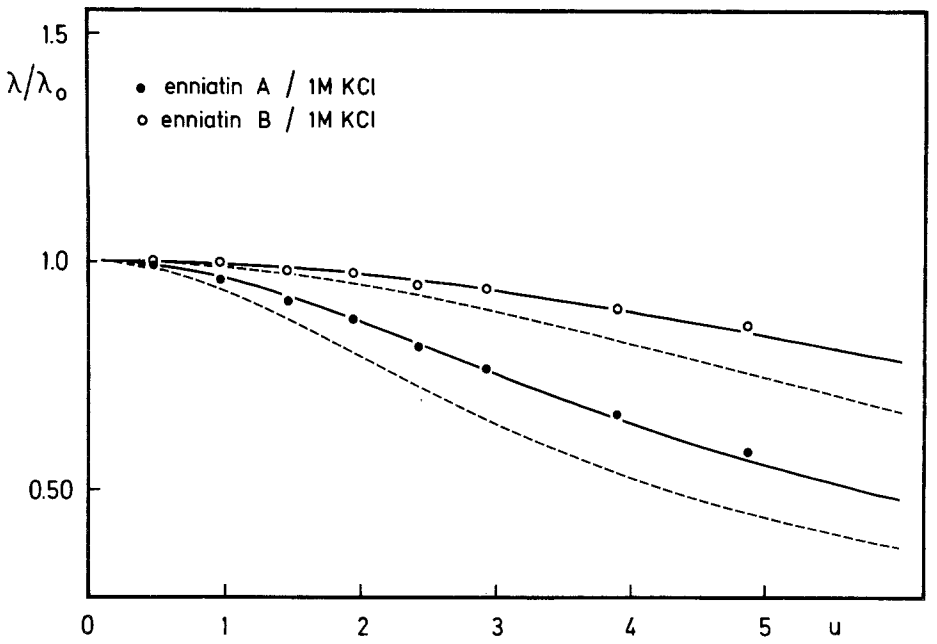


Fig. 7. Conductance ratio λ/λ_0 as a function of voltage for membranes from monolinolein. The aqueous phase contained, besides 1 M KCl, 1.2×10^{-6} M enniatin A or 5×10^{-6} M enniatin B; $T = 25$ °C. The dotted lines were calculated from Eq. (3) with $A = 10$ (enniatin A) and $A = 0.8$ (enniatin B) and the full lines from Eq. (4) with $\alpha = 0.75$ and the same values for A

only a portion of the total voltage applied to the membrane acts on the complexes.

However, the deviations with enniatin B are lower than with enniatin A and with valinomycin, possibly because of the less pronounced saturation of λ/λ_0 observed with this carrier. It is therefore likely that the error in A (Figs. 5 and 6) introduced by the incomplete description of λ/λ_0 by Eq. (3) is lower if the kinetics of the carrier is shifted more towards the equilibrium domaine.

Ion Selectivity

The selectivity of carriers like valinomycin and the macrotetrolides has extensively been studied by Eisenman and coworkers using zero current potential measurements with different cations on both sides of a bilayer membrane [11]. In these studies a strong selectivity for K^+ over Na^+ has been found for these carriers, whereas the discrimination between the larger alkali ions is comparatively poor. For the enniatins

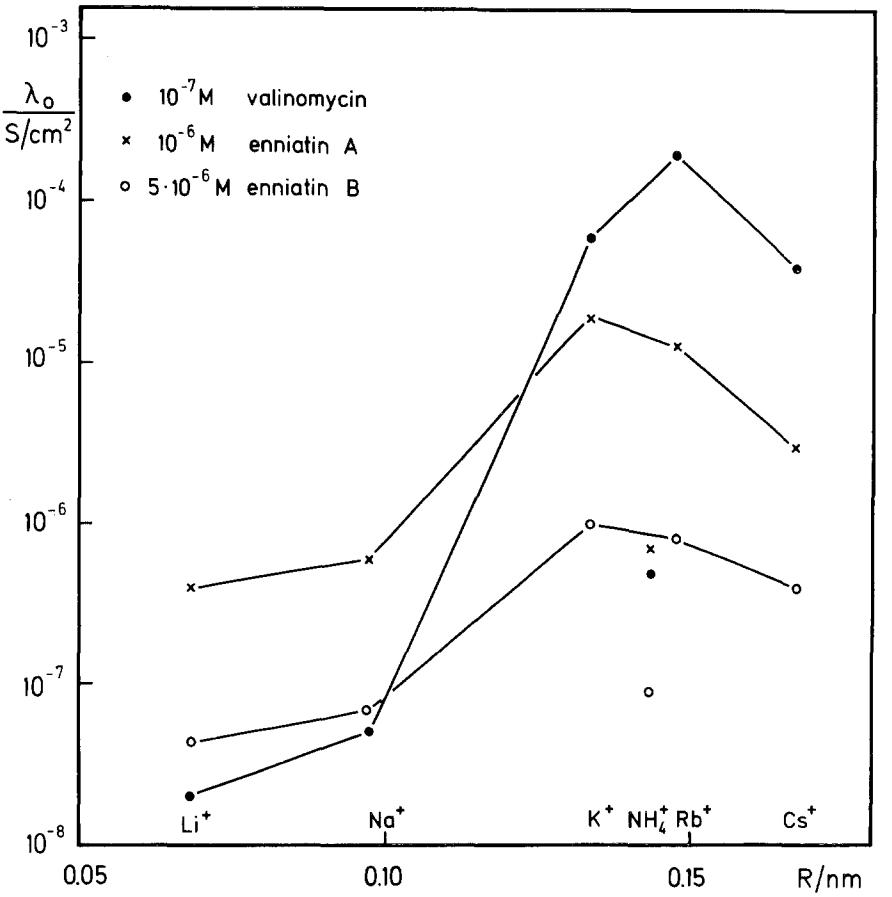


Fig. 8. Conductance of dioleoyl phosphatidylcholine membranes in the presence of valinomycin, enniatin A, enniatin B, and different 10^{-2} M salts as a function of the cation radius R; $T=25\,°C$

only limited selectivity data are available, showing that the selectivity between Na^+ and K^+ is much smaller [27].

For an analysis of ion selectivity, Eqn. 2 is written in the form

$$\lambda_0=\frac{F^2 d}{2RT}\,\frac{k_{MS}K\gamma_{MS}c_M c_0}{(K_{c_M}+1)(1+A)}. \tag{5}$$

This equation may be used to discuss the conductance at constant carrier concentration c_0 in the presence of different ion species with the same concentration c_M. Figure 8 shows the results obtained with dioleoyl phosphatidylcholine membranes and different carriers.

As can be seen from Fig. 8, the discrimination between K^+ and Na^+ is very strong for valinomycin, (approximately 1000-fold), whereas for

enniatin A (about 30-fold), and for enniatin B (about 15-fold) the selectivity between these two ions is much smaller. In addition, there is also a change in the selectivity sequence between the different carriers; it is possibly caused by the size of the ring. For valinomycin with 12 acid residues in the ring, Rb^+ is transported best, whereas for the enniatins and beauvericin with only 6 residues, the conductance is the largest for K^+.

According to Eq. (5) the differences in the conductances observed with the different ion species may result mainly from a change in the aqueous equilibrium constant K rather than from a change in k_{MS} or γ_{MS}, although some variation seems also to be possible in these constants (*see* next section). However, these considerations may not be valid for beauvericin, because a different ion transport mechanism is likely to be effective for this carrier. The selectivity for beauvericin is very poor. In the presence of 2×10^{-6} M beauvericin and 10^{-2} M KCl a conductance λ_0 of 1.2×10^{-6} S cm^{-2} is observed with dioleoyl phosphatidylcholine membranes. For 10^{-2} M NaCl or 10^{-2} M LiCl, λ_0 is only about four to five times lower.

Charge-Pulse Relaxation Studies

The analysis of the charge-pulse experiment in terms of the carrier model has been described extensively in a previous publication [4]. Therefore, only the main equations, from which the rate constants can be calculated from the experimental data, are given here. After a brief charge pulse of about 10 to 50 nsec duration the decay of the voltage across the membrane with time in the presence of a carrier system is given by the following equation:

$$V_m(t) = V_m^0(a_1 e^{-\lambda_1 t} + a_2 e^{-\lambda_2 t} + a_3 e^{\lambda_3 t} \tag{6}$$

$$a_1 + a_2 + a_3 = 1. \tag{7}$$

If all three relaxation processes with the relaxation times $\tau_i = 1/\lambda_i (\tau_1 < \tau_2 < \tau_3)$ and the relative relaxation amplitudes a_i are resolved, the four rate constants and N_0 may be obtained in the following way. Defining the quantities

$$P_1 = \lambda_1 + \lambda_2 + \lambda_3 \tag{8}$$

$$P_2 = \lambda_1 \lambda_2 + \lambda_1 \lambda_3 + \lambda_2 \lambda_3 \tag{9}$$

$$P_3 = \lambda_1 \lambda_2 \lambda_3 \tag{10}$$

$$P_4 = a_1 \lambda_1 + a_2 \lambda_2 + a_3 \lambda_3 \tag{11}$$

$$P_5 = a_1 \lambda_1^2 + a_2 \lambda_2^2 + a_3 \lambda_3^2 \tag{12}$$

the rate constants and N_0 are given by the following equations [4]:

$$k_{MS} = \frac{1}{2}\left(\frac{P_5}{P_4} - P_4\right) \tag{13}$$

$$k_D = \frac{1}{2k_{MS}}\left[\frac{P_1 P_5}{P_4} - P_2 + \frac{P_3}{P_4} - \left(\frac{P_5}{P_4}\right)^2\right] \tag{14}$$

$$k_S = \frac{1}{2k_D}\left(\frac{P_3}{P_4}\right) \tag{15}$$

$$k_R = \frac{1}{c_M}(P_1 - P_4 - 2k_S - 2k_{MS} - k_D) \tag{16}$$

$$N_0 = \frac{2RTC_m}{F^2}\left(\frac{P_4}{k_{MS}}\right)\left(1 + \frac{k_D}{k_R c_M}\right). \tag{17}$$

A typical charge pulse experiment is given in Fig. 9. As the decay of the voltage across the membrane V_m extends over a large time range, V_m was recorded with different sweep times. The analysis of the curve was performed by digitizing the data and using a computer fit program [4]. Otherwise, the three relaxations could not be resolved with sufficient accuracy.

In a first set of experiments, charge pulse relaxation studies were performed with different carriers and 1 M KCl on membranes from monoolein/*n*-decane. The results are given in Table 1. Experimental data and rate constants for valinomycin were taken from ref. [4]. In contrast to valinomycin where all three relaxation processes predicted by the theory could be resolved, with the enniatins only two relaxations were observed and in the case of beauvericin only one relaxation process was visible. The appearance of only two out of three relaxation processes may have different causes. One reason may be that two relaxation processes have very similar time constants, so that they are seen as one single exponential. This is possible but not very probable because time constants and relative amplitudes of the relaxations are dependent on the carrier concentration [4], whereas in experiments with largely different enniatin A and B concentrations only two relaxations could be resolved. Therefore, the second explanation, that the amplitude of one relaxation process (possibly of the fast one) is too small to be detected, is more likely. This situation arises, for instance, if the stability of the complex is very small.

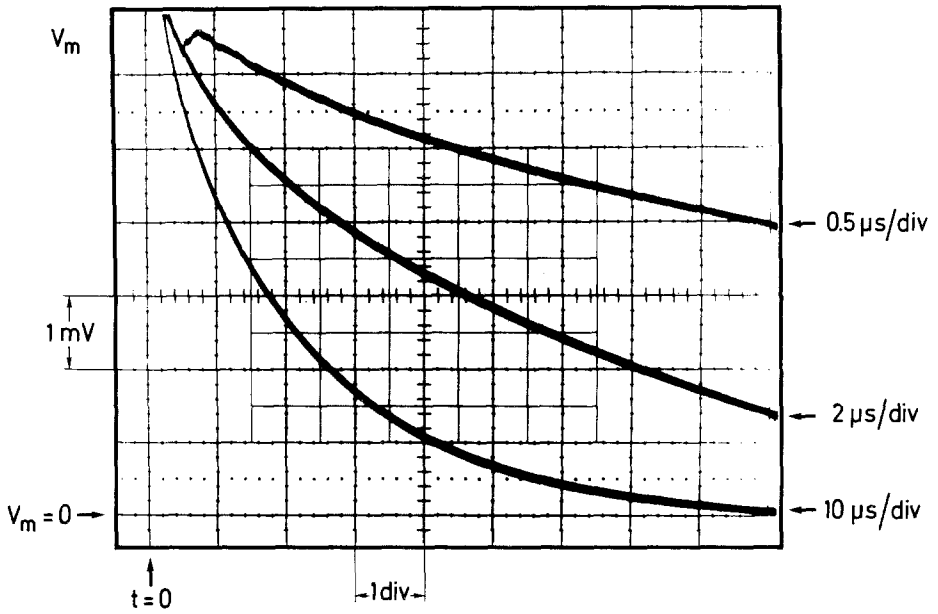


Fig. 9. Typical charge pulse experiment with 1.2×10^{-6} M enniatin A and 1 M CsCl at a membrane from monolinolein/*n*-decane; $T = 25\,°C$. At time $t = 0$ the membrane capacitance was charged up to a voltage of $V_m^0 = 8.84$ mV by a current pulse of about 20 nsec. A repetitive pulse sequence was used with waiting times of 500 µsec between the pulses. The decay of V_m was recorded with different sweep times, as indicated on the right side of the oscillogram. The base lines at 2 µsec/*div.* and 0.5 µsec/*div.* were shifted by arbitrary amounts. The base line for 2 µsec/*div.* is at -1.42 mV and for 0.5 µsec/*div.* at -1.82 mV. The curve was fitted according to Eq. (6) with the following values for τ_i and V_i: $\tau_1 = 0.62$ µsec, $V_1 = 0.57$ mV; $\tau_2 = 3.22$ µsec, $V_2 = 1.74$ mV; and $\tau_3 = 22.1$ µsec, $V_3 = 6.53$ mV. From this data the following values for the rate constants and for N_0 were calculated from Eqs. (8)–(17): $k_R = 1.89 \times 10^5\ \text{M}^{-1}\,\text{sec}^{-1}$; $k_D = 6.62 \times 10^5\ \text{sec}^{-1}$; $k_{MS} = 3.75 \times 10^5\ \text{sec}^{-1}$; $k_S = 8.67 \times 10^4\ \text{sec}^{-1}$; and $N_0 = 5.87 \times 10^{-13}$ mol cm^{-2}

As has been shown in a recent paper [1], the stability of the valinomycin/Rb^+ complex as well as its translocation rate constant increase with increasing numbers of double bonds in the fatty acid chain of the membrane forming lipid. In order to test the hypothesis that the relaxation amplitude of the fastest process was too small to be detected in the case of monoolein membranes, experiments with monoglycerides with a C_{18}-chain and more than one double bond (monolinolein and monolinolenin) were performed. With membranes from these lipids the three relaxations predicted by the theory for valinomycin and enniatin A and B (but not for beauvericin) could be resolved. The experimental results for membranes from monolinolein are given in Table 2. As can be

Table 1. Relaxation times τ_i and relative relaxation amplitudes a_i from charge pulse relaxation experiments with monoolein/*n*-decane membrane[a]

τ_1 μsec	τ_2 μsec	τ_3 μsec	a_1	a_2	a_3
		10^{-7} M valinomycin			
0.87	2.6	52	0.29	0.30	0.41
		1.2×10^{-6} M enniatin A			
–	9.8	59	–	0.63	0.37
		2×10^{-5} M enniatin B			
–	6.5	25	–	0.042	0.96
		2.10^{-6} M beauvericin			
–	–	1100	–	–	1

[a] The aqueous phase contained, besides the carrier, 1 M KCl; $T = 25\,°C$. The data for valinomycin were taken from ref. [4]; for the rate constants of this system the following values have been calculated [4]: $k_R = 2.9 \times 10^5\,M^{-1}\,sec^{-1}$, $k_D = 2.7 \times 10^5\,sec^{-1}$; $k_{MS} = 2.1 \times 10^5\,sec^{-1}$; $k_S = 3.8 \times 10^4\,sec^{-1}$; $N_0 = 7.8 \times 10^{-13}$ mol cm^{-2} and $\gamma_0 = 1.6 \times 10^4$. The assignment of the observed relaxation processes in the case of enniatin A and B and of beauvericin is tentative.

seen from the data, the relaxation amplitude of the fastest process decreases in the series valinomycin, enniatin A, and enniatin B.

This is also reflected in the values of the rate constants (Table 3). The stability of the ion carrier complex and the translocation rate constant k_{MS} is highest for valinomycin and much lower for the enniatins. Another interesting result is the strong increase of the recombination rate constant k_R with decreasing KCl or RbCl concentration in the aqueous phase, whereas it is independent of the carrier concentration c_0 as are the other rate constants. A similar behavior of k_R has already been observed with valinomycin and Rb^+ as transported ion [4, 19]. Wheras in the case of valinomycin only a relatively small effect on k_S has been observed [4], a much stronger influence on this rate constant is visible in the case of enniatin A. With decreasing KCl concentration from 3 M to 0.03 M, k_S increases about sevenfold.

The other two rate constants k_D and k_{MS} seem to be independent of ion concentration for a given set of experimental conditions. However, in contrast to the findings with valinomycin [4], the rate constant of translocation, k_{MS}, is not independent of the kind of the ion. In the series

Table 2. Relaxation times τ_i and relative relaxation amplitudes a_i from charge pulse experiments with monolinolein/*n*-decane membranes at different ion concentrations c_M and different carrier concentrations c_0 in the aqueous phase[a]

c_0 μM	c_M M	τ_1 μsec	τ_2 μsec	τ_3 μsec	a_1	a_2	a_3
			Valinomycin/KCl				
0.1	1	0.27	1.9	31	0.35	0.076	0.58
			Enniatin A/KCl				
3.6	1	0.61	2.1	68	0.74	0.11	0.15
1.2	1	1.2	3.2	86	0.22	0.39	0.39
0.4	1	1.4	3.8	150	0.057	0.32	0.62
1.2	3	1.7	4.3	220	0.093	0.53	0.37
1.2	0.3	1.5	3.1	63	0.22	0.26	0.52
1.2	0.1	1.4	2.9	48	0.23	0.19	0.57
1.2	0.03	1.4	3.5	35	0.18	0.25	0.57
			Enniatin A/RbCl				
1.2	1	1.0	3.0	46	0.22	0.38	0.40
1.2	0.3	1.0	3.2	26	0.17	0.29	0.55
			Enniatin A/CsCl				
1.2	1	0.61	2.9	20	0.084	0.28	0.63
			Enniatin B/KCl				
10	1	1.9	4.4	29	0.090	0.75	0.16
			Enniatin B/RbCl				
10	1	1.5	4.2	14	0.042	0.59	0.37
			Enniatin B/CsCl				
10	1	–	4.9	18	–	0.079	0.92
			Beauvericin/KCl				
2	1	–	–	180	–	–	1

[a] The ionic strength in the enniatin A experiments (except for 3 M) was kept constant at 1 or 0.5 M (at 0.03 M KCl because of the low selectivity); $T = 25\,^{\circ}C$.

K^+, Rb^+ and Cs^+, k_{MS} increases about twofold. A similar effect occurs also in the case of enniatin B.

The partition coefficient $\gamma_0 = N_0/dc_0$ describes the total partitioning of the carrier molecules in the membranes. γ_0 is used here because it is very difficult to estimate the extent of complex formation in the aqueous phase.

Table 4 contains the experimental results obtained with membranes

Table 3. Rate constants k_R, k_D, k_{MS}, and k_S of carrier-mediated ion transport across monolinolein/*n*-decane membranes as calculated from the data of Table 2[a]

c_0 μM	c_M M	k_R $10^4\,M^{-1}\,sec^{-1}$	k_D $10^4\,sec^{-1}$	k_{MS} $10^4\,sec^{-1}$	k_S $10^4\,sec^{-1}$	N_0 pmol cm^{-2}	γ_0 10^3
			Valinomycin/KCl				
0.1	1	27	21	110	11	0.53	13
			Enniatin A/KCl				
3.6	1	31	18	16	2.5	3.1	2.1
1.2	1	27	22	15	2.2	0.92	1.9
0.4	1	35	17	13	2.8	0.36	2.2
1.2	3	9.6	18	8	0.95	0.90	1.9
1.2	0.3	81	12	14	6.0	0.62	1.3
1.2	0.1	210	13	17	8.0	0.58	1.2
1.2	0.03	580	19	15	7.0	0.73	1.5
			Enniatin A/RbCl				
1.2	1	25	29	19	3.5	1.0	2.1
1.2	0.3	65	31	21	6.9	0.84	1.7
			Enniatin A/CsCl				
1.2	1	23	67	36	7.8	0.72	1.5
			Enniatin B/KCl				
10	1	17	26	3.1	3.6	4.5	1.1
			Enniatin B/RbCl				
10	1	19	35	4.3	8.3	3.2	0.8

[a] The total partition coefficient γ_0 was determined from N_0 according to $N_0/c_0 \cdot d$. For the membrane capacity C_m [*Eq.* (17)] a value of 0.464 μF cm^{-2} [1] was used.

from monolinolenin ($C_{18:3}$). Besides beauvericin, in all systems investigated with membranes from this lipid all three relaxations predicted by the theory could be resolved. The time constant of the fastest relaxation process is in all systems smaller than observed with membranes from monolinolein or from monoolein. In addition, its relative relaxation amplitude is larger with monolinolenin membranes. In the case of beauvericin, only two relaxations were observed. As can be seen from Table 4, the three relaxation amplitudes of the enniatin B/K^+ systems and also the longest relaxation time τ_3 strongly depend on the concentrations c_0 and c_M. Despite these large variations in the relaxation parameters, the rate constants k_R, k_D, k_{MS}, and k_S (Table 5) calculated according Eqs. (8)–(17) are independent of the enniatin B concentration. The association rate constant k_R shows a similar strong dependence on

Table 4. Relaxation times τ_i and relative relaxation amplitudes a_i from charge pulse experiments with monolinolenin ($\Delta^{9,12,15}-C_{18:3}$)/*n*-decane membranes at different ion concentration C_M and different carrier concentrations c_0 in the aqueous phase[a]

c_0 μM	c_M M	τ_1 μsec	τ_2 μsec	τ_3 μsec	a_1	a_2	a_3
			Valinomycin/KCl				
0.1	1	0.27	1.9	31	0.35	0.076	0.58
			Enniatin A/KCl				
1.2	1	1.2	3.2	86	0.22	0.39	0.39
			Enniatin A/RbCl				
1.2	1	1.0	3.0	46	0.22	0.38	0.40
			Enniatin A/CsCl				
1.2	1	0.61	2.9	20	0.084	0.28	0.63
			Enniatin B/KCl				
1	1	0.86	2.6	54	0.066	0.23	0.71
3	1	0.72	2.1	40	0.22	0.24	0.53
10	1	0.43	1.4	27	0.63	0.16	0.20
10	3	0.74	2.2	69	0.31	0.37	0.32
10	0.3	0.39	1.6	14	0.70	0.092	0.21
10	0.1	0.47	1.5	11	0.62	0.13	0.26
			Enniatin B/RbCl				
10	1	0.36	1.3	15	0.52	0.18	0.30
10	0.3	0.43	1.5	8.2	0.45	0.19	0.36
			Enniatin B/CsCl				
10	1	0.25	1.4	7.0	0.11	0.38	0.52
			Beauvericin/KCl				
2	1	–	1.2	63	–	0.21	0.79

[a] The ionic strength in the enniatin B experiments (except for 3 M) was kept constant at 1 M by adding LiCl; $T=25\,°C$.

the KCl-concentration as in the case of enniatin A and valinomycin [4, 19], whereas the influence of c_M on k_S is similarly small, as in the case of enniatin A. The other two rate constants k_D and k_{MS} are independent of c_M.

As can be seen from Table 5 also in the case of monolinolenin, the rate constant k_{MS} is dependent on the kind of the transported ion for the two carriers enniatin A and B. In the series K^+, Rb^+, and Cs^+, k_{MS} increases about twofold in the case of enniatin A and about threefold in the case of enniatin B. A similar effect on k_{MS} for the different valino-

Table 5. Rate constants k_R, k_D, k_{MS}, and k_S of carrier mediated ion transport across membranes from monolinolenin ($\Delta^{9,12,15}-C_{18:3}$)/*n*-decane as calculated from the data of Table 4[a]

c_0 μM	c_M M	k_R 10^4 M^{-1} sec^{-1}	k_D 10^4 sec^{-1}	k_{MS} 10^4 sec^{-1}	k_S 10^4 sec^{-1}	N_0 pmol cm^{-2}	γ_0 10^3
			Valinomycin/KCl				
0.1	1	40	10	260	19	0.42	13
			Enniatin A/KCl				
1.2	1	35	8.1	72	7.8	0.52	1.3
			Enniatin A/RbCl				
1.2	1	58	19	100	10	0.99	2.6
			Enniatin A/CsCl				
1.2	1	37	32	150	21	0.99	2.6
			Enniatin B/KCl				
1	1	39	34	26	6.9	0.39	1.2
3	1	37	29	33	6.5	0.73	0.75
10	1	42	33	30	5.8	2.9	0.90
10	3	11	35	25	2.1	1.5	0.46
10	0.3	98	26	31	12	3.5	1.1
10	0.1	290	31	29	15	3.1	0.96
			Enniatin B/RbCl				
10	1	41	40	48	11	2.0	0.62
10	0.3	85	47	42	17	2.5	0.77
			Enniatin B/CsCl				
10	1	39	170	84	16	1.5	0.46

[a] The total partition coefficient γ_0 was determined from $N_0/c_0 d$. For the membrane capacity C_m [Eq. (17)] a value of 576 μF cm^{-2} [1] was used.

mycin systems at monolinolenin membranes has not been found ([1] and this study).

The translocation rate constants k_{MS} and k_S for the different carrier systems increase with the number of double bonds in the fatty acid chain in the membrane-forming lipid. This effect which is less pronounced for k_S has been previously discussed in terms of membrane fluidity [1].

Discussion

In this study carrier-mediated ion transport by the enniatins was investigated. The results given here make it very likely that these two

carriers act in a way similar to the well known ion carrier valinomycin, although the size of the enniatins is much smaller. In particular, evidence has been obtained that enniatin A and B form a 1:1 complex with the transported metal ion and that this complexation reaction takes place at the membrane water interface (IC-mechanism). No evidence was found for the proposed 2:1 or 3:2 carrier ion complexes in the case of enniatin A and B [18]; this is probably not surprising since the stability constant of the 2:1 complex of enniatin B in organic phases is at least two orders of magnitude lower as the 1:1 complex [18]. So far, no explanation besides the use of different lipids may be given for the discrepancy between our findings and the 2:1 enniatin B/K^+ reported in the literature [18].

In the case of beauvericin, the 3:1 stoichiometry between carrier and ion which has been reported from bilayer experiments [18] is consistent with the results presented here. Unfortunately it could not be proven by kinetic experiments that a 3:1 complex is actually responsible for the observed concentration dependence of conductance. Possibly there are also other explanations, for example a concentration-dependent partition coefficient. On the other hand, the beauvericin molecule with its three N-methyl phenylalanins is considerably different from the enniatins which contain N-methyl isoleucin (enniatin A) and N-methyl valin (enniatin B). This difference in structure may lead to altered complex formation properties. Such differences are also seen in the current voltage curves. In all systems investigated here a strongly superlinear $I(V)$ curve was found for beauvericin, independent of salt or carrier concentration. Therefore, it cannot be excluded that complex formation in the case of beauvericin occurs mainly in the aqueous phase.

It is interesting to note that in the case of cyclic polyethers also a cubic dependence of the conductance on carrier concentration has been observed [25]. In this case no variation of the partition coefficient with carrier concentration has been found [25].

The selectivity of the enniatins is much smaller than the selectivity of valinomycin or the macrotetrolids. Especially the discrimination between K^+ and Na^+ is very low. This may be caused by the smaller size of the ring which contain only six acid residues *vs.* twelve for valinomycin. The same reason may also be responsible for the modified selectivity sequence. Whereas valinomycin transports Rb^+ best, the stability constant is highest for K^+ in the case of the enniatins [15]. The selectivity of beauvericin is very poor. The conductance for K^+ is only about fivefold higher than for the other ions under otherwise identical

conditions. This fact may tentatively be explained by the formation of sandwich complexes. If several carrier molecules form a complex with an ion, the selectivity for different ions may be poor because of the possibility that the carrier molecules can arrange differently for different ions. The current-voltage curves measured in two types of membranes also reveal considerable differences between enniatin A and B and beauvericin. In the series valinomycin, enniatin A, enniatin B, and beauvericin the $I(V)$ curves become more and more superlinear, irrespective of the membrane-forming lipid. Whereas in the case of valinomycin and the enniatins the $I(V)$ curve may be approximately fitted with a theoretical expression derived on the basis of the earlier proposed carrier model [23], such a fit is not possible for beauvericin. However, in previously studied systems modifications of the carrier model are needed for a more accurate fitting of the current voltage curves [4, 17, 19]. Especially the voltage-dependence of the translocation rate constant of the complex is better described if it is assumed that only part of the applied voltage acts on the complex [17, 19, 21]. This seems also to be true for the enniatins as Fig. 7 shows. The ion-concentration dependence of the association rate constants k_R and of the translocation rate constant k_S are not easily understood on the basis of the simple carrier model. For valinomycin-mediated Rb^+ transport the decrease of k_R with increasing ion concentration c_M has been explained by a finite number of sites for the complexation reaction which become saturated at high c_M [19]. Possibly other explanations also have to be considered for the observed concentration dependence of k_R.

It is interesting to note that, although there is some increase in k_R with increasing fluidity of the lipid (enniatin B) k_R did not exceed, irrespective of the nature of carrier and of the transported ion, a value of about $4 \times 10^5\,\mathrm{M}^{-1}\,\mathrm{sec}^{-1}$ at 1 M salt. The reason for this finding is not clear, but it may explain the relatively small change of k_R with the structure of the monoglyceride, as found previously [1]. In another study with different phosphatidylcholines, where $k_R c_M$ possibly does not saturate, a pronounced chain-length dependence of k_R has been found [6].

The increase of the translocation rate constant k_{MS} in the series enniatin B, enniatin A, and valinomycin is an interesting result. It may be caused by the different size of the complexes. In all cases the complexes have cylindrical shape with approximately the same diameter of 1.5 nm [26] but a height of 1.3 nm (valinomycin) and of 0.7 nm (enniatin B), respectively. The enniatin B-K^+ complex has approximately the form of a disc. Models of the different carrier complexes show that in

the case of valinomycin the ion is perfectly shielded, whereas for the enniatins the central ion is exposed to both sides. Therefore it may well be that some water molecules are associated with the complex and are transported with it (E. Grell, *personal communication*). In addition, a larger fraction ($\sim 75\,\%$) of the applied voltage (as compared with valinomycin) seems to act on the ion complexes of enniatin A and B, and therefore it may well be that these complexes are more strongly associated with the polar headgroups, thus reducing the translocation rate constant k_{MS}.

The smaller conductance of the enniatins in lipid bilayer membranes is mainly caused by smaller partition coefficients of these molecules in contrast to valinomycin. In membranes of lower fluidity also the complex stability and the translocation rate constants k_{MS} and k_S are comparatively smaller. A characteristic parameter for the efficiency of the ion transport rate of carrier molecules is the turnover number in the limit of high c_M [22]:

$$f = \left(\frac{2}{k_D} + \frac{1}{k_S} + \frac{1}{k_{MS}} \right)^{-1} . \tag{18}$$

Table 6 contains the turnover numbers of the three carrier systems

Table 6. Turnover numbers for the different carrier molecules calculated according to Eq. (18) from the rate constants given in Tables 1, 3, and 5 for 1 M salt

Fatty and residue of the monoglyceride	Salt	Turnover number $10^4\,\mathrm{sec}^{-1}$
	Valinomycin	
Oeoyl $\Delta^9-C_{18:1}$	KCl	2.6
Linoleoyl $\Delta^{9,12}-C_{18:2}$	KCl	5.1
Linolenoyl $\Delta^{9,12,15}-C_{18:3}$	KCl	3.9
	Enniatin A	
Linoleoyl $\Delta^{9,12}-C_{18:2}$	KCl	1.7
	RbCl	2.5
	CsCl	5.4
Linolenoyl $\Delta^{9,12,15}-C_{18:3}$	KCl	2.6
	RbCl	4.6
	CsCl	8.6
	Enniatin B	
Linoleoyl $\Delta^{9,12}-C_{18:2}$	KCl	1.5
	RbCl	2.4
Linolenoyl $\Delta^{9,12,15}-C_{18:3}$	KCl	4.1
	RbCl	6.2
	CsCl	12

calculated from the rate constants given in Tables 1, 3, and 5 for 1 M salt. The turnover numbers range between $10^4\,sec^{-1}$ and $10^5\,sec^{-1}$ for the three carriers valinomycin, enniatin A, and enniatin B. Judged by the value of f the efficiency for the different carriers is approximately the same and the large variation observed in the conductance is mainly caused by the difference in the partition coefficients. Because of the decreasing dissociation rate constant k_D for valinomycin in the series monoolein, monolinolein, and monolinolenin, the turnover number shows a maximum, whereas it increases in this series for the other carriers.

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