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An Extended Kinetic Analysis of Valinomycin-Induced Rb-Transport through Monoglyceride Membranes

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Summary. The time course of the current following a voltage jump, which is applied to monoglyceride bilayers in the presence of valinomycin, shows two relaxation times. This is basically in agreement with a simple carrier model which has been described in full detail formerly. Relaxation times and amplitudes allow a calculation of the rate constants of the transport model. The presented data supplement an analysis which was hitherto based only on the slower relaxation process and on information derived from the nonlinearity of current-voltage characteristics. The additional resolution of the faster relaxation time allowed an approximate determination of the voltage dependence of the translocation rate constant for the carrier-ion-complex and provided evidence for a small voltage dependence of the interfacial reaction. The dependence of the relaxation parameters on the ion concentration in the aqueous phase was interpreted assuming a saturation of the ion concentration at the reaction plane at high bulk concentrations.

The cyclodepsipeptide valinomycin strongly and selectively increases the cation permeability and the electric conductance of thin lipid membranes. Studies performed in different laboratories are consistent with the view that the charge carrying species is a 1:1 complex between a valinomycin molecule and a monovalent cation (Liberman & Topaly, 1968; Tosteson, Andreoli, Tieffenberg & Cook, 1968; Stark & Benz, 1971). These complexes are preferably formed at the membrane interface, where there is an energy minimum for valinomycin (Hsu & Chan, 1973), and are able to move freely across the interior of the membrane (Krasne, Eisenman & Szabo, 1971). The formation and dissociation of the complexes as well as their movement from one interface to the other may be described by rate constants. These rate constants may be quantitatively determined from the nonlinearity of current-voltage curves and from voltage jump relaxation experiments (Stark, Ketterer, Benz & Läuger, 1971). According to the outlined simple carrier transport model, the time course of the current following a voltage jump should be composed of two relaxation processes. Within an experimental time resolution of about 2 μsec the current, however, followed a single exponential decay for phosphatidylinositol and

lecithin membranes of varying chain length of the lipid molecules, while the faster process could not be resolved (Stark *et al.*, 1971; Benz, Stark, Janko & Läuger, 1973). Recently, Laprade, Ciani, Eisenman and Szabo (1974) found for membranes formed from diglycerides, in addition, a second, faster process, which, however, they described as being at variance with the original formulation of the model.

The present paper contains an extensive study on monoglyceride membranes also exhibiting a two-exponential current decay. It was found, that both relaxation processes are in agreement with the main features of the original model. A detailed analysis, however, showed that some refinements of the transport mechanism had to be introduced in order to explain the results quantitatively. It is suggested that the ion concentration at the reaction plane saturates. Besides, further evidence for a voltage dependence of the interfacial reaction was obtained.

Materials and Methods

The monoglycerides used for the formation of black lipid membranes were obtained from Nu Check Prep and Sigma. They were pure as checked by thin-layer chromatography. Most experiments were done with monoolein (18:1-monoglyceride) and monoeicosenoin (20:1-monoglyceride), some also with 16:1-, 22:1-, and 24:1-monoglycerides. A 0.5–1 %(w/v) lipid solution in *n*-decane (Fluka, purum) was used. Valinomycin (Calbiochem) in most cases was added to the lipid phase to give a concentration between 5×10^{-5} and 5×10^{-4} M. The aqueous RbCl-solutions were unbuffered (pH about 6). Their ionic strength was held constant at 1 M by adding LiCl, which is transported by valinomycin only to a very small extent.

The cell for bilayer formation as well as the set-up used for voltage jump relaxation experiments has been described in previous publications (Benz *et al.*, 1973). A pulse generator with a rise-time of 4 nsec was used (Philips PM 5712). To improve the time resolution of the method, care was taken to minimize the voltage drop in the aqueous solutions surrounding the membrane. Using a manipulator and specially shaped platinized platinum electrodes the distance between membrane and electrodes usually was kept less than 1 mm. With this method it was possible to hold the resistance R_S between both electrodes (without membrane) below 30 Ω. At sufficient high differences between initial and stationary current the external resistor R_A (introduced for current measurement) could be kept as small as 30 Ω. Then at a membrane capacity C_m of about 2 nF the time constant $\tau_c = (R_A + R_S) C_m$, which determines the initial charging process and limits the time resolution of the method, was about 0.1 μsec. To obtain a satisfactory amplitude resolution over the whole time range of the current decay a measurement was repeated with different external resistors corresponding to different time ranges of the relaxation process. The resolution of the relaxation amplitude depends on the time resolution (i.e. on R_A) and on the membrane conductance. With a time resolution of better than 2 μsec ($\tau_c \approx 0.1$ μsec) and an applied voltage of 100 mV, the minimum relaxation current, which could be resolved was about 2×10^{-7} A (half of the noise width of the circuit). Since most of the experiments, however, were performed at 10 °C, where the relaxation times are longer than at 25 °C, this limiting time resolution was not necessary ($R_A > 30$ Ω) (Fig. 1). Thereby the amplitude resolution was improved. The measurements were not performed until the experimental data assumed time-independent values. This was reached 5–10 min after the blackening process.

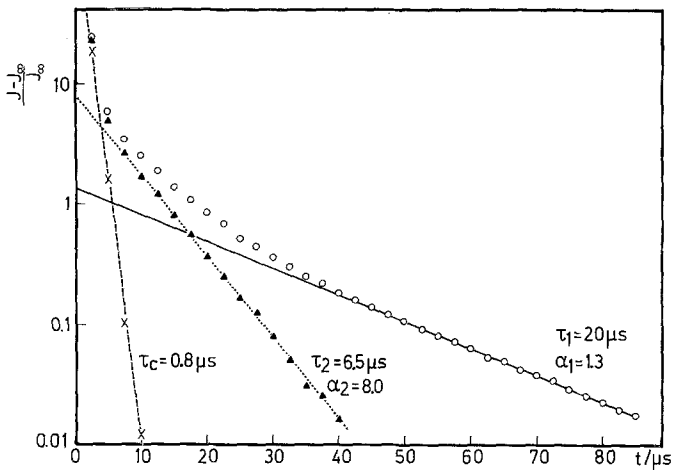

Fig. 1. Typical current relaxation following a voltage jump of 90 mV across a monoolein membrane ($T = 10\,°C$, $c_{Rb} = 0.1$ M). The experimental data (o) at times longer than 40 μsec follow a straight line, which is assumed to represent the slower relaxation process. At times shorter than 40 μsec a faster process becomes visible. It is easily separated from the time constant τ_c of the membrane capacity. ($J_\infty = 1.5 \times 10^{-6}$ A, $R_A = 300\,\Omega$, noise 5×10^{-8} A)

Analysis of the Current Relaxation

An attempt will be made to interpret the experimental data on the basis of a carrier model, which has been described in full detail elsewhere (Läuger & Stark, 1970; Stark *et al.*, 1971; Benz *et al.*, 1973). It assumes that the carrier molecules are preferentially located at the membrane interfaces, which is supported by NMR studies (Hsu & Chan, 1973). There they may react with ions M^+ (concentration c_M) from the aqueous phase (formation rate constant k_R, dissociation rate constant k_D). The mean diffusion time across the membrane interior for the free carrier molecule S is $1/k_S$, for the complex MS^+ $1/k_{MS}$ (k_S, k_{MS} translocation rate constants). If a voltage is applied to the membrane, k_{MS} has to be replaced by k'_{MS} and k''_{MS}, which takes into account that the movement of the positively charged complexes is hindered or favored by the electric field.

According to this model the time course of the current J following a voltage jump is (Stark *et al.*, 1971):

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

with J_∞ = stationary current, α_1, α_2 = relaxation amplitudes and τ_1, τ_2 = relaxation times. Relaxation times and amplitudes are functions of the

rate constants:

$$\tau_1 = (a-b)^{-1} \tag{2}$$

$$\tau_2 = (a+b)^{-1} \tag{3}$$

$$\alpha_1 = e+f \tag{4}$$

$$\alpha_2 = e-f \tag{5}$$

where

$$a = \tfrac{1}{2}\left[k_R\, c_M + k_D + 2k_S + (k'_{MS} + k''_{MS})\right] \tag{6}$$

$$b = \tfrac{1}{2}\left[(k_R\, c_M - k_D + k_S - (k'_{MS} + k''_{MS}))^2 + 4k_R\, c_M\, k_D\right]^{\frac{1}{2}} \tag{7}$$

$$e = \frac{(2k_S + k_R\, c_M)(k'_{MS} + k''_{MS})}{4k_D\, k_S} \tag{8}$$

$$f = \frac{1}{2b}\left[e(k_R\, c_M + k_D + 2k_S - (k'_{MS} + k''_{MS})) - (k'_{MS} + k''_{MS})\right]. \tag{9}$$

A plot of $\log[(J-J_\infty)/J_\infty]$ versus t employing Eq. (1) should be represented by the sum of two straight lines, if τ_1 and τ_2 are sufficiently different. From these lines τ_1, τ_2 and α_1, α_2 may be determined. Fig. 1 shows an example. Two relaxation times may be resolved and separated from the time constant τ_c of the initial charging process of the membrane capacity. Therefore, the time decay of the current allows to determine four experimental quantities, which, using Eqs. (2)–(9), allow to calculate the four rate constants of the model. While the dissociation rate constant k_D and the translocation rate constant k_S may be obtained directly, the formation rate constant k_R in all equations appears in the combination $k_R\, c_M$. The translocation rate constant k_{MS}, which appears as the sum $(k'_{MS} + k''_{MS})$ is obtained by an extrapolation to small voltages U:

$$(k'_{MS} + k''_{MS})_{U \to 0} = 2k_{MS}. \tag{10}$$

For membranes formed from lecithins or phosphatidylinositol the fast relaxation time could not be resolved within the time resolution of our present set-up. In these cases an analysis is possible using information derived from the nonlinearity of current-voltage curves. Then, however, an a priori assumption about the voltage dependence of k_{MS} is necessary, which had been introduced via an Eyring expression. With monoglyceride membranes this assumption may now be the subject of a critical test, since the additional knowledge of the faster relaxation time allows an extended analysis (*see* later section). Eqs. (2)–(9) may be easily converted into Eqs. (35)–(43) of Stark *et al.* (1971), setting $(k'_{MS} + k''_{MS}) = 2k_{MS} \cosh u/2$ (with $u = FU/RT$ reduced voltage).

In conclusion, the resolution of the two relaxation processes both allows a simplified evaluation of the rate constants (analysis of a single current relaxation) using less assumptions and supplies more information about the transport mechanism.

One also has to consider the possibility that one or both relaxation processes are not generated by the carrier itself but only result from voltage-dependent structural changes of the membrane. E.g., small changes of the orientation of the polar head groups of the lipid molecule could change the membrane capacity and simulate a relaxation. Then, however, the relaxation should also be observable without specific "conductance probes", which could not be found. A similar study with the macrotetrolide trinactin also showed two relaxation processes. They were, however, clearly different from the valinomycin system concerning relaxation times and especially relaxation amplitudes, which were considerably smaller. These observations provide sufficient evidence as to a direct involvement of valinomycin.

At high voltages (above 150 mV) a third relaxation with a comparatively slow time constant $\tau_3 (\tau_3 > 100\ \mu\text{sec})$ and very small relaxation amplitudes $(\alpha_3 < 0.05)$ became visible. It seems possible that this relaxation results from structural changes of the membrane at high voltages.

Concentration Dependence of Current Relaxation

Fig. 2 shows how the relaxation times and amplitudes depend on the Rb-concentration in the aqueous phases. α_1 approaches zero at small concentrations, while α_2 attains a constant value. Such a behavior is consistent with Eqs. (4) and (5). At 10^{-3} M only one process was present, which was assigned to τ_2 and α_2.

The evaluation of the rate constants may be performed at all concentrations where both relaxation times could be resolved. In the frame of the transport model used hitherto, all four rate constants should not depend on the Rb-concentration. This requirement is met by k_D, k_{MS} and k_S within the experimental error (deviation from the mean values less than factor 2). On the contrary, the formation rate constant k_R decreases from $10^6\ \text{M}^{-1}\ \text{sec}^{-1}$ at 10^{-2} M to less than $10^5\ \text{M}^{-1}\ \text{sec}^{-1}$ at 1 M (Fig. 3).

Looking for an explanation of this discrepancy one realizes that k_R was determined as the combination $k_R \cdot c_M$, where c_M is the ion concentration at the place of the complex formation. Calculating k_R in Fig. 3, c_M was set equal to the bulk aqueous concentration. This may be approxi-

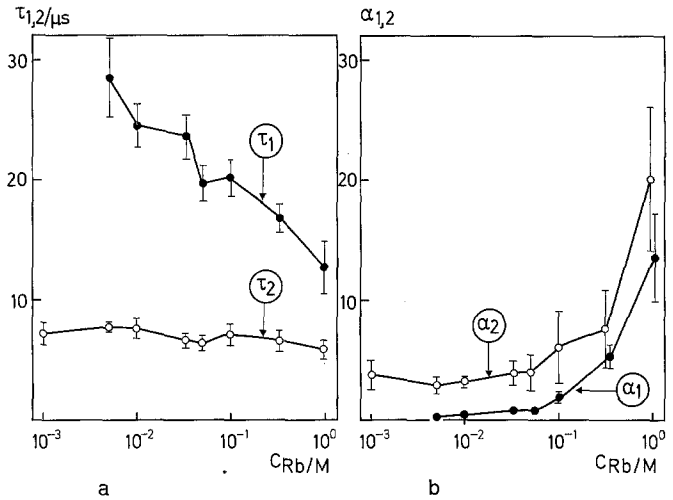

Fig. 2. Dependence of the relaxation data on the Rb^+ concentration (*a*) relaxation times, (*b*) relaxation amplitudes. Each point represents the mean value of at least five membranes. Bars indicate the standard error. The measurements were performed with monoolein membranes ($c_{val} = 5 \times 10^{-4}$ M in lipid, $T = 10$ °C, $U = 30$ mV, $c_{Rb} + c_{Li} = 1$ M)

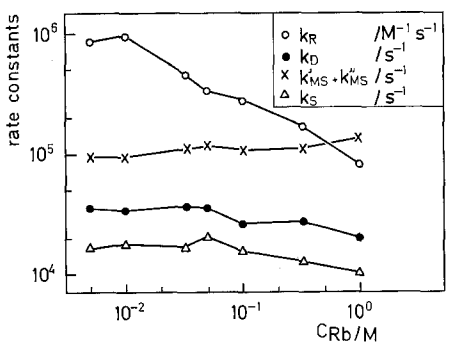

Fig. 3. Concentration dependence of the rate constants according the data of Fig. 2

mately correct, if the carrier molecules are located "on" the membrane interface and "see" the full bulk concentration. If, however, their energy profile has a minimum "in" the interface, which is equivalent with a position between the polar head groups (possibly extending even towards the hydrophobic part of the membrane), they could experience a strongly reduced ion concentration c_M^*. If the product $k_R\, c_M^*$ (which is the measured quantity) is plotted versus the aqueous concentration c_M (Fig. 4*a*), a saturation at high c_M is found. In the line of the present argumentation this finding supports the interpretation that the local concentration c_M^* saturates at high bulk concentrations c_M. One could imagine that the

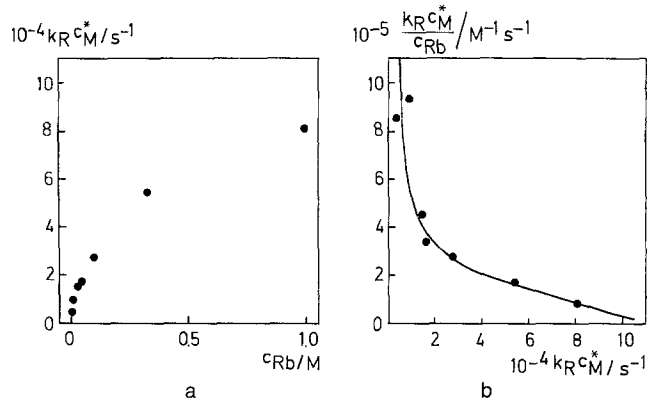

Fig. 4. Concentration dependence of the rate of complex formation. (*a*) Linear plot; (*b*) Scatchard plot: Full line was calculated assuming two classes of "binding sites" with $K_1 = 3.5 \times 10^{-3}$ M, $K_2 = 0.39$ M, $C_2/C_1 = 17.3$, $k_R c_M^*(\max) = 1.1 \times 10^5 \sec^{-1}$ [*see* Eq. (13)]

penetration of ions through the polar headgroups to the place of complex formation is only possible at certain structural defects within the liquid crystalline lattice of the lipid molecules (which might even be created or increased by the carrier molecules themselves). This limited number of defects might be occupied already at a relatively small concentration c_M. According to this conception Fig. 4*b* contains a Scatchard plot of the data of Fig. 4*a*. It shows that at least two classes of "binding sites" must be postulated to explain the data. Additionally these "binding sites" must favor Rb over Li, since the experiments were performed at constant total concentration ($c_{Rb} + c_{Li}$). For identical behavior of Rb and Li the degree of occupation would be the same at all Rb-concentrations.

Other possibilities of explaining the observed discrepancies have been envisaged also. The concentration dependence of the rate constants (Fig. 3) indicates that the description of the interfacial reaction is inadequate. For the sake of simplicity it had been formulated as a simple bimolecular reaction. Grell & Funck (1973) interpreted their kinetic study of complex formation of valinomycin with alkali ions in methanol on the basis of a two-step model:

$$M^+ + S \underset{k_{21}}{\overset{k_{12}}{\rightleftarrows}} M^+ - S \underset{k_{32}}{\overset{k_{23}}{\rightleftarrows}} MS^+. \tag{11}$$

They found that the rate constant k_{12} of formation of the intermediate complex $M^+ - S$ is almost diffusion controlled (at least for K^+ and NH_4^+), while its conformational rearrangement into the final complex MS^+ (rate constant k_{23}) represents the rate-limiting step of the complex formation.

Besides, they found k_{21} to be considerably larger than k_{23}. The two-step mechanism [Eq. (11)] on a kinetic basis behaves equivalent to a one-step bimolecular reaction, if the concentration of the intermediate complex $M^+\text{–}S$ is much smaller compared with the concentration of the final complex MS^+. In methanol, the condition $c_{M-S} \ll c_{MS}$ has been established for valinomycin and K^+ (Grell & Funck, 1973). It should hold even better for Rb^+, which is favored against K^+ by valinomycin. Therefore, transforming the conditions in methanol to a membrane interface, the approximation of reaction (11) by a simple bimolecular reaction appears justified. Nevertheless, one might argue that through the special situation of a membrane interface (possibly through the strongly reduced fluidity) the ratio $c_{M-S}/c_{MS} = k_{32}/k_{23}$ might be enhanced. Then the approximation is no longer valid. Therefore an alternative model has been analyzed taking into account the supplemented reaction (11), but, using the restricting condition that the first step occurs much faster than the second one. This condition is given in methanol and considerably facilitates a mathematical treatment (*see* Appendix). Indeed, a saturation of the formation rate results [Eq. (A.5)]. It is, however, accompanied by the same concentration dependence of the translocation rate constant k_S, which is contradictory to the data of Fig. 3. Therefore, the interpretation on the basis of a reduced and limited ion concentration at the place of the complex formation is favored at present, though in view of the simplified treatment, an influence of the supplemented reaction (11) cannot be excluded completely.

In previous publications a strong reduction of the formation rate constant k_R for valinomycin and the macrotetrolides with increasing chain length of the lipid molecules has been reported (Benz *et al.*, 1973; Benz & Stark, 1975). In the frame of the present argumentation this would imply that the concentration c_M^* depends on the chain length of the lipids. It was assumed that c_M^* is determined by the number of structural defects. Since an increase in the chain length should enhance the degree of order in a bilayer, a reduction of the number of defects and, as a consequence, a decrease of c_M^* appears understandable. The number of defects may be very small. From Fig. 4 a limiting value of about 10^5 sec^{-1} for $k_R c_M^*$ is estimated. If the data of Grell and Funck (1973) in methanol are accepted as an upper limit of k_R ($k_R \leqq 10^7$ M^{-1} sec^{-1}), a lower limit of $c_M^* \approx 10^{-2}$ M follows. Assuming a width of the reaction layer of 5 Å we find a lower limit of 3×10^{11} binding sites/cm^2, which has to be compared with a number of about 2×10^{14} lipid molecules/cm^2. For lipids with longer chain lengths this number may even decrease by several orders of magnitude. Though

such numbers of structural defects appear reasonable, further experiments are necessary to confirm their existence.

The saturation of $k_R c_M^*$ at high c_M should also have an influence on the membrane conductance λ_0 as a function of c_M. If valinomycin is added to the lipid phase, λ_0 is given by (Benz *et al.*, 1973):

$$\lambda_0 = \frac{F^2 d}{2RT} \frac{c_S^b \gamma_S^{mb} k_{MS} k_R c_M^*/k_D}{(1+2k_{MS}/k_D + k_{MS} k_R c_M^*/k_S k_D)} \tag{12}$$

where c_S^b = valinomycin concentration in the bulk lipid phase, γ_S^{mb} = partition coefficient of S between the bulk phase and the membrane, F = Faraday constant, R = gas constant, T = absolute temperature, and d = membrane thickness.

For reasons which have been discussed before, Eq. (12) contains the concentration c_M^* instead of the bulk concentration c_M. If $k_R c_M^*$ were a linear function of c_M, Eq. (12) would predict a linear relationship between λ_0 and c_M at small c_M and a saturation behavior at high c_M (Fig. 5, dotted line). In contrast, the experimental data show a flat maximum (*see also* Laprade *et al.*, 1974). If, however, the concentration dependence of $k_R c_M^*$ (Fig. 3) is taken into account in Eq. (12), a fairly good agreement is obtained (full line of Fig. 5). It demonstrates the internal consistency of stationary and relaxation experiments. A full agreement, however, is not observed, since again the curve only shows a saturation and not a maximum. This may be easily verified from Eq. (12) in combination with Eq. (13), which is formulated on the basis of two classes of "binding sites"; i.e.,

$$c_M^* = \frac{c_1 c_M}{K_1 + c_M} + \frac{c_2 c_M}{K_2 + c_M} \tag{13}$$

where

c_1, c_2 = concentration of binding sites 1, 2

K_1, K_2 = dissociation constants.

Therefore additional effects, though of minor importance, must play some role.

If valinomycin is added to the water, the denominator of λ_0 must contain an additional concentration-dependent factor $(K c_M + 1)$, which considers the complex formation of valinomycin in water (K = association constant in water). Then, the applied model also predicts a maximum, which was found in the form of a more pronounced one (Fig. 5). The differences between both kinds of addition of the carrier molecules allow an evaluation of the equilibrium constant K in water (Benz *et al.*, 1973). For

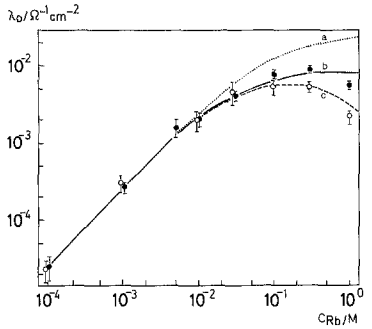

Fig. 5. Dependence of the stationary conductance λ_0 on the Rb^+-concentration for monoolein membranes ($T=10\,°C$, $c_{Li}+c_{Rb}=1\,M$). Each point represents the mean value of at least five membranes. Bars indicate standard error. ●, 5×10^{-4} M valinomycin in the membrane-forming solution (membrane area $5\times10^{-3}\,cm^2$). ○, 10^{-7} M valinomycin in water (membrane area $2.5\times10^{-2}\,cm^2$); data multiplied by the constant factor 0.79. Lines according Eq. (12): (*a*) with mean values of the rate constants $\overline{k_{MS}/k_D}=1.5$, $\overline{k_{MS}k_R/k_Dk_S}=43$; (*b*) using the concentration-dependent data of Fig. 3 and $\gamma_S^{mb}=2$; (*c*) considering, in addition, complex formation in the aqueous phase ($K=1.5$)

valinomycin and Rb^+ at 5 °C, $K=1\,M^{-1}$ had been found using lecithin membranes. The data of Fig. 5 may be explained assuming $K=1.5\,M^{-1}$.

Fig. 5 demonstrates that for monoolein membranes an adequate description of λ_0 as a function of c_M is only possible taking into account the saturation of $k_R c_M^*$. For lecithin membranes (Benz *et al.*, 1973) this was not necessary. This could imply that the half saturation of "binding sites" occurs at higher concentrations c_M compared to monoolein membranes. Therefore, the adequate description of the concentration dependence of λ_0 – which is an independent check of the model – also indicates, whether saturation effects have to be considered or not. In contrast to lecithin membranes, they seem to be present at negatively charged membranes. The discrepancies observed earlier (Stark & Benz, 1971; Stark *et al.*, 1971) may have the same basis. A study is under way to clear this question.

At our previous kinetic analysis – where the fast relaxation could not be resolved – all experimental parameters containing k_R were obtained at 1 M salt concentration. Therefore, in the frame of the present extended argumentation, these data for k_R simply correspond to the product $k_R c_M^*$ at $c_M=1\,M$.

The data have been interpreted applying a transport model which assumes an independent action of all valinomycin molecules inside the membrane. This lack of mutual interaction is mainly based on the observation of a strict proportionality between the stationary conductance λ_0 and the valinomycin concentration (Tosteson *et al.*, 1968; Stark & Benz,

Table 1. Dependence of relaxation data from the valinomycin concentration in the lipid phase at 25 °C

$c_{val/M}$	$\tau_1/\mu sec$	$\tau_2/\mu sec$	α_1	α_2
10^{-4}	5.8	2.3	8.9	27.7
5×10^{-4}	8.3	2.9	5	6.7

1971) and has also been found for the macrotetrolides (Szabo, Eisenman & Ciani, 1969; Stark & Benz, 1971). For monoolein membranes, such a proportionality was observed up to valinomycin concentrations of at least 5×10^{-4} M in the membrane-forming solution. There was, however, some influence on the relaxation data. Table 1 shows that the relaxation amplitudes decrease and the times increase at higher valinomycin concentrations. The variation of the corresponding rate constants, however, was rather small (less than a factor of 2). The effect could be caused by small structural changes (or fluidity changes) induced by the high valinomycin concentration inside the membrane (about 10^{11} molecules/cm^2 at 5×10^{-4} M). Similar, but more pronounced effects have been observed at high concentrations of hydrophobic ions (L. J. Bruner, *personal communications*).

Voltage Dependence of Current Relaxation

In the frame of the carrier model used up to now it was assumed that a voltage applied across the membrane only acts on the translocation rate constant k_{MS}. This is consistent with the picture that the carrier molecules and their ion complexes are situated "on" the membrane interface, thus not experiencing the electric field, which only exists inside the membrane (at least at high ionic strength). In the last section some evidence was presented that ions have to penetrate the hydrophilic head groups of the lipid molecules in order to react with a carrier molecule. From this should follow a certain voltage dependence of the rate constants describing the interfacial reaction. A similar conclusion was obtained earlier from large discrepancies at high voltages observed at the mathematical description of stationary current-voltage curves (Stark & Benz, 1971), and was also suggested by Hladky (1974) and Laprade *et al.* (1974). The voltage dependence of k_{MS} had been introduced via the exponential expressions (Zwolinsky, Eyring & Reese, 1949; Läuger & Stark, 1970)

$$k'_{MS} = k_{MS}(0)\, e^{-0.5\,u}$$
$$k''_{MS} = k_{MS}(0)\, e^{0.5\,u} \qquad (14)$$

with $u = FU/RT$ reduced voltage and U = applied voltage. They are strictly valid only, if the energy barrier separating both interfaces has a very steep maximum in the middle of the membrane and if the interfacial reaction does not depend on the voltage. Therefore, the inadequate description of the current-voltage curves at high voltages could arise from a broader shape of the energy barrier and/or from an additional voltage dependence of complex formation and dissociation. Hall, Mead and Szabo (1973) tried to calculate the shape of the barrier using a special fitting procedure to current-voltage curves (assuming the interfacial reaction as voltage independent). Hladky (1974) also took into account a voltage dependence of the interfacial reaction. But since he introduced three additional free parameters, without having further experimental information, the good agreement between data and theory appears almost self-evident. Therefore, the question is open, whether only the voltage dependence of k_{MS} is inadequate, and (or) if also a certain voltage dependence of k_R and k_D has to be introduced. The resolution of the faster relaxation time provides an experimental approach to this special problem.

Eqs. (1)–(9) were derived assuming that only k_{MS} is voltage dependent. The kind of voltage dependence, i.e. the shape of the barrier, however, is largely arbitrary. Moreover, these equations only contain the sum $(k'_{MS} + k''_{MS})$, which therefore is directly obtained from the kinetic analysis. As will be shown below, the difference $(k'_{MS} - k''_{MS})$ may be evaluated from the current-voltage characteristic. Therefore, both observations together allow an approximative determination of k'_{MS} and k''_{MS}. For reasons based on thermodynamic considerations (*see* Appendix B) for the ratio k'_{MS}/k''_{MS} (in the case of negligible voltage dependence of the other rate constants) the following must hold (Eq. B.3):

$$\frac{k'_{MS}}{k''_{MS}} = e^{-u}. \tag{15}$$

From a violation of Eq. (15) it immediately follows that k_R and k_D must be voltage dependent.

Figs. 6 and 7 show the voltage dependence of the relaxation parameters for 18:1- and 20:1-monoglyceride membranes. The main effect is associated with the amplitude α_2 of the faster relaxation process. Figs. 8 and 9 contain the corresponding voltage dependence of the rate constants analyzed according to Eqs. (1)–(9). The sum $k'_{MS} + k''_{MS}$ increases much less than expected on the basis of Eq. (14). The other rate constants proved to be approximately constant within the experimental error, although the decrease of the dissociation rate constant k_D at high voltages observed with

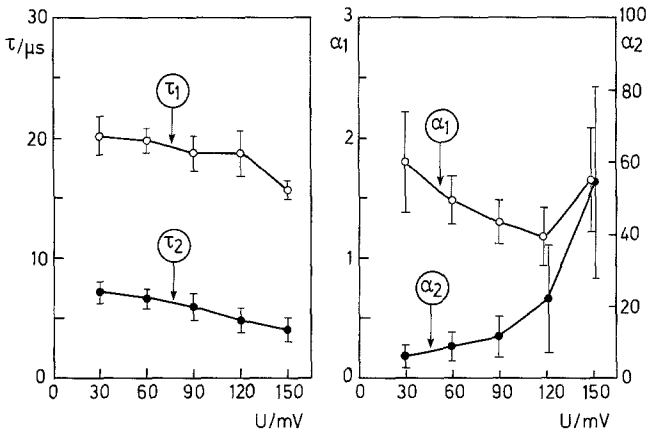

Fig. 6. Dependence of the relaxation data on the applied voltage for 18:1-monoglyceride membranes. Mean values of five membranes, bars indicate standard error ($c_{val}=5\times10^{-4}$ M in lipid, $T=10$ °C, $c_{Rb}=0.1$ M, $c_{Li}=0.9$ M)

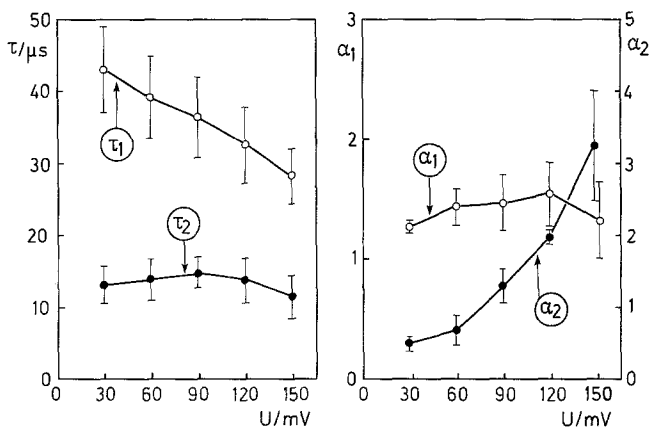

Fig. 7. Same as Fig. 6, but for 20:1-monoglyceride membranes

monoolein membranes should be significant. As may be already expected from the preceding discussion, the approximative character of Eq. (14) also appears in the presentation of stationary current-voltage curves (Fig. 10). The shape of these curves have been found to be largely independent on the Rb-concentration for $c_{Rb}\geqq10^{-2}$ M. Following the treatment given earlier (Läuger & Stark, 1970; Stark & Benz, 1971) the integral conductance λ for an arbitrary voltage dependence of k_{MS} is expressed as

$$\lambda=\frac{J}{U}=\frac{h(k'_{MS}-k''_{MS})}{[g(k'_{MS}+k''_{MS})+1]\,u} \tag{16}$$

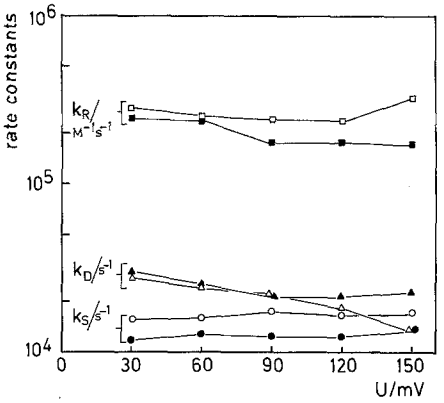

Fig. 8. Voltage dependence of the rate constants k_R, k_D and k_S. The values were calculated at each voltage according to the data of Figs. 6 and 7 using Eqs. (2)–(9). Open symbols: 18:1-monoglyceride membranes; full symbols: 20:1-monoglyceride membranes

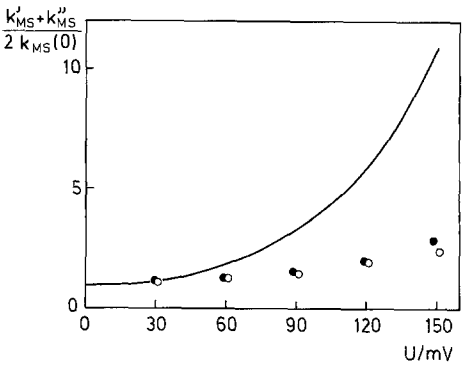

Fig. 9. Voltage dependence of $(k'_{MS}+k''_{MS})$ according to the data of Figs. 6 and 7 using Eqs. (2)–(9). $2\,k_{MS}(0)$ was obtained by extrapolation to zero voltage. Open circles: 18:1-monoglyceride membranes ($k_{MS}(0)=5\times10^4\,\mathrm{sec}^{-1}$); full circles: 20:1-monoglyceride membranes ($k_{MS}(0)=1.1\times10^4\,\mathrm{sec}^{-1}$); full line calculated according Eq. (14)

with

$$g=(k_R\,c_M+2k_S)/2k_S\,k_D$$
$$h=F^2\,N_0\,k_R\,c_M/2RT(k_R\,c_M+k_D)$$
$$N_0=\text{total concentration of carrier molecules/cm}^2$$
$$u=FU/RT \text{ reduced voltage.}$$

k'_{MS} and k''_{MS} represent the voltage dependence of k_{MS}; i.e., instead of Eq. (14):

$$k'_{MS}=k_{MS}(-u)$$
$$k''_{MS}=k_{MS}(u) \qquad (17)$$

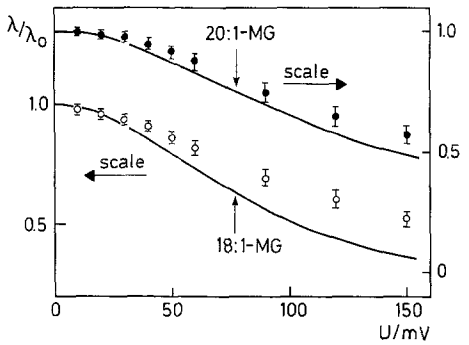

Fig. 10. Reduced stationary conductance λ/λ_0 as a function of voltage ($c_{val}=5\times10^{-4}$ M in lipid, $c_{Rb}=0.1$ M, $c_{Li}=0.9$ M, $T=10$ °C). Open circles: 18:1-monoglyceride membranes; full circles: 20:1-monoglyceride membranes. Mean values of five membranes, the bars indicate maximum and minimum observed value. Full lines calculated according Eq. (21) with the special voltage dependence of Eq. (14); data for $k_{MS}(0)$ have been taken from Fig. 9, for k_R, k_D, k_S from Fig. 8 (mean values)

$k_{MS}(u)$ should be a steady and differentiable function, which may be developed into a Taylor series at $u=0$:

$$k_{MS}(u)=k_{MS}(0)+\dot{k}_{MS}(0)\,u+\tfrac{1}{2}\ddot{k}_{MS}(0)\,u^2+\cdots. \tag{18}$$

From Eqs. (17) and (18) it follows with the notation $\dot{k}_{MS}(0)=\beta k_{MS}(0)$: (19)

$$(k'_{MS}-k''_{MS})_{u\to0}=-2\beta u k_{MS}(0). \tag{20}$$

The physical meaning of β is illustrated for $\beta=0.5$. In this case, as may be shown combining Eqs. (18) and (B.3), the rate constants k_R, k_D and k_S are voltage independent for $u\to0$. For $\beta<0.5$, however, a certain voltage dependence of these rate constants must exist.

Using Eqs. (16) and (20), the reduced conductance λ/λ_0 may be written as

$$\frac{\lambda}{\lambda_0}=\frac{(J/U)}{(J/U)_{U\to0}}=\left|\frac{(k'_{MS}-k''_{MS})}{2\beta u k_{MS}(0)}\right|\frac{[g\cdot2k_{MS}(0)+1]}{[g(k'_{MS}+k''_{MS})+1]}. \tag{21}$$

Eqs. (1)–(9) as well as Eq. (21) are strictly valid only for $\beta=0.5$. In Eq. (21) the ratio λ/λ_0 as well as the quantities g, $k_{MS}(0)$ and $(k'_{MS}+k''_{MS})$ are known from Figs. 8, 9 and 10. Therefore $(k'_{MS}-k''_{MS})$ as a function of U may be determined if $\beta=0.5$. This difference is alternatively also obtained from the voltage dependence of the initial current J_0 following a voltage jump, since $J_0=FN_{MS}(k'_{MS}-k''_{MS})$ ($N_{MS}=$ interfacial concentration of MS^+ at zero voltage). Therefore, with the knowledge of $(k'_{MS}+k''_{MS})$ and $(k'_{MS}-k''_{MS})$ the voltage dependence of k_{MS} (i.e. of k'_{MS} and k''_{MS}) can be calculated. Fig. 11 shows the result. $k_{MS}(+|U|)$ and $k_{MS}(-|U|)$ indicate how the movement

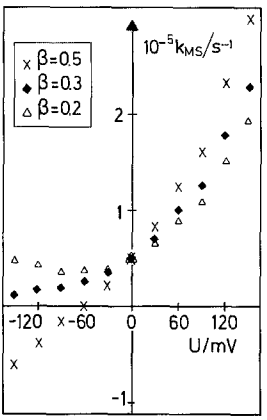

Fig. 11. Approximate voltage dependence of k_{MS} according to the data of Figs. 8, 9 and 10 and Eqs. (2)–(9) and (21) for 18:1-monoglyceride membranes, and different values of the parameter β. Only $\beta=0.3$ is physically reasonable *(see text)*

of the complex in the direction and opposite the direction of an electric field is influenced. Therefore, for physical reasons $k_{MS}(+|U|)$ should be a monotonic increasing and $k_{MS}(-|U|)$ a monotonic decreasing function of U. In addition, only positive values are reasonable. Fig. 11 shows that $\beta=0.5$ yields negative values for k''_{MS}, if $U>60$ mV. Only for $\beta\approx0.3$ ($\beta\approx0.36$ for 20:1-monoglyceride membranes) the just-mentioned conditions are fulfilled over the whole voltage range. Since Eqs. (1)–(9) and (21), which have been used for the calculation, are strictly valid only for $\beta=0.5$, the voltage dependence of k_{MS} given in Fig. 11 ($\beta=0.3$) is only an approximation. It may be considered, however, as an evidence for $\beta<0.5$. This is equivalent with the statement that in addition to the translocation rate constant k_{MS}, other processes must be voltage dependent, too. It means also that Eq. (15) is not obeyed, but that the generalized Eq. (B.3) has to be used, which includes additional voltage dependences. As has been already mentioned above, the rate constants of the interfacial reactions k_R and k_D should be involved. But even an influence on the translocation rate constant k_S of the neutral free carrier molecules S cannot be completely excluded. The Maxwell pressure of the loaded membrane capacity could influence structural properties of the membrane (e.g. fluidity) and thus even influence the movement of neutral species. Therefore, the assumption that only k_{MS} is voltage dependent, which underlies also Eqs. (1)–(9), and (21) and hence the data of Figs. 8, 9 and 11, is inadequate. Nevertheless, it should be a good approximation at least at not too high voltages, since k_{MS} experiences the main part of the voltage drop across the membrane.

From the voltage dependences of k_{MS} and from the observation that the parameter β increases with the chain length of the lipid molecules, conclusions may be drawn about the shape of the energy barrier. In view of the only approximative character of $k_{MS}(U)$ (Fig. 11) this discussion, however, appears premature at present. But the obvious voltage dependence of the rate constants k_R and k_D supports the conclusion already obtained in the last section, that the free rubidium ions experience a part of the voltage drop and therefore have to penetrate the polar head groups of the lipid molecules in order to react with free carrier molecules[1]. Consequences of this idea on the interpretation of stationary current-voltage curves are considered in Appendix B.

Conclusion

The results presented in the last sections have been interpreted on the basis of a simple carrier model widely used at present. The limitation of this model especially with respect to the description of the interfacial reaction and the voltage dependence of the rate constants are clearly visible. Through introduction of suitable modifications the model was supplemented. These modifications are partly only approximative and therefore preliminary. The concentration c_M^* at the place of complex formation has been assumed to be in equilibrium with the bulk concentration c_M also at high voltages. This assumption is supported by the absence of a suitable relaxation. An improved quantitative description of valinomycin-induced ion transport should omit such simplifying assumptions and should allow also for an extended voltage dependence of the transport parameters. Such an analysis, however, will inevitably require a higher degree of mathematical effort and also additional experimental information about the transport system.

This information may be obtained by using different relaxation methods, such as the charge pulse method (Feldberg & Kissel, 1975) or the temperature jump method (Knoll & Stark, *in preparation*). Besides, the analysis outlined in this paper may now be applied to other lipids, such as phospholipids and other carrier molecules, for which the resolution of the faster relaxation process has recently been possible, too.

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1 A similar conclusion has been obtained by D. Eisenman, S. Krasne and S. Ciani, who suggested a small voltage dependence of k_R and k_D (*Ann. N.Y.Acad. Sci., in press*). (*See also* Hladky, 1974.)

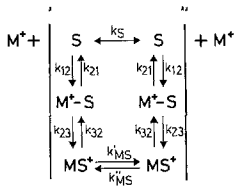

Fig. 12. Carrier model with a nontransported "precomplex"

Appendix A

Carrier Model with Supplemented Interfacial Reaction

Fig. 12 shows an extended carrier model considering the two-step interfacial reaction (11). It will be analyzed with the special assumption that the equilibration of S and $M^{\pm}-S$ following a voltage jump occurs much faster than the relaxation of the rest of the reaction scheme in which we at present are only interested. Then, the interfacial concentrations of S and $M^{\pm}-S$ may be assumed in equilibrium, i.e.

$$\frac{N'_S}{N'_{M-S}}=\frac{N''_S}{N''_{M-S}}=\frac{k_{21}}{k_{12}\,c_M}. \tag{A.1}$$

If we denote $N'_S+N'_{M-S}=N'$ and $N''_S+N''_{M-S}=N''$, we obtain with Eq. (A.1):

$$N'_S=\frac{N'\,k_{21}}{k_{12}\,c_M+k_{21}} \quad \text{and} \quad N'_{M-S}=\frac{N'\,k_{12}\,c_M}{k_{12}\,c_M+k_{21}}. \tag{A.2}$$

Similar relations hold for N''_S and N''_{M-S}.

Using Eq. (A.2) the time derivative of the interfacial concentrations N', N'', N'_{MS} and N''_{MS} are expressed by

$$\begin{aligned}\frac{dN'}{dt}=&-\frac{k_{23}\cdot k_{12}\,c_M}{k_{12}\,c_M+k_{21}}\,N'+k_{32}\,N'_{MS}\\&-\frac{k_S\,k_{21}}{k_{12}\,c_M+k_{21}}\,N'+\frac{k_S\,k_{21}}{k_{12}\,c_M+k_{21}}\,N'',\end{aligned} \tag{A.3}$$

$$\frac{dN'_{MS}}{dt}=\frac{k_{23}\cdot k_{12}\,c_M}{k_{12}\,c_M+k_{21}}\,N'-k_{32}\,N'_{MS}-k'_{MS}\,N'_{MS}+k''_{MS}\,N''_{MS}. \tag{A.4}$$

Analogous equations hold for dN''/dt and dN''_{MS}/dt. This system of linear differential equations is equivalent to the system describing the original carrier model (Stark *et al.*, 1971), if the following notations are

made:

$$\begin{aligned} N &\hat{=} N_S & N_{MS} &\hat{=} N_{MS} \\ k_R &\hat{=} \frac{k_{12}\, k_{23}}{k_{12}\, c_M + k_{21}} & k_S &\hat{=} \frac{k_S \cdot k_{12}}{k_{12}\, c_M + k_{21}} \\ k_D &\hat{=} k_{32} & k_{MS} &\hat{=} k_{MS}. \end{aligned} \tag{A.5}$$

Using the same transformations, the initial conditions of our problem (equilibrium at time $t=0$) may be translated into those of the original model, too. Therefore, the relaxation of the current according to the modified model under the specified conditions obeys Eqs. (1)–(9), provided the transformations (A.5) are introduced. These transformations show the same concentration dependence of k_R and k_S.

Appendix B

A Carrier Model with an Extended Voltage Dependence of the Rate Constants

We consider a generalized carrier model with all rate constants being voltage dependent. Then at voltages U different from zero the rate constants k'_R and k'_D of the left side interfacial reaction generally will be different from those of the right membrane interface k''_R and k''_D. We also permit k'_S different from k''_S. This problem is formally identical to that of an asymmetrical membrane, which has been treated previously (Stark, 1973). The stationary current J was obtained as

$$J = \frac{FN_0(k'_R\, c'_M\, k'_{MS}\, k''_D\, k''_S - k''_R\, c''_M\, k''_{MS}\, k'_D\, k'_S)}{D} \tag{B.1}$$

with D being a rather bulky function of the rate constants, which is different from zero. In contrast to the previous treatment, however, the rate constants are now assumed to be functions of the applied voltage.

We now assume to have different concentrations c'_M and c''_M on both sides of the membrane and consider the equilibrium potential at $J=0$. For thermodynamic reasons the Nernst potential must be obtained, which is for the transport of a monovalent cation M^+:

$$\frac{c'_M}{c''_M} = e^{+u} \quad \text{with } u = FU/RT. \tag{B.2}$$

Therefore, combining Eq. (B.1) ($J=0$) and Eq. (B.2) results in

$$\frac{k'_R\, k'_{MS}\, k''_D\, k''_S}{k''_R\, k''_{MS}\, k'_D\, k'_S} = e^{-u}. \tag{B.3}$$

Now we consider the case $c'_M = c''_M$, but externally apply the same voltage u. Then, the current will be different from zero. The shape of current-voltage curves is independent from the level of the membrane conductance, i.e. from the valinomycin concentration (apart from very high concentrations). Therefore, the voltage dependence of the rate constants does not depend on the presence of a current, from which it follows that Eq. (B.3) again must be obeyed. This equation shows that the voltage dependences of the rate constants may not be arbitrarily chosen.

Now we proceed to the assumption of special voltage dependences of the rate constants. This paper presents some evidence that the ion concentration c^*_M at the place of complex formation is considerably smaller than the bulk concentration c_M. In accordance with Eq. (13) one may write $c^*_M = \gamma_M c_M$. Since c^*_M experiences a part of the voltage drop across the membrane γ_M will depend on the voltage. In the following we use the simple relations

$$\begin{aligned} c^{*\prime}_M &= \gamma^0_M e^{-\delta u} c_M \\ c^{*\prime\prime}_M &= \gamma^0_M e^{\delta u} c_M . \end{aligned} \tag{B.4}$$

They include an increase of the left interfacial concentration $c^{*\prime}_M$ and a corresponding decrease of $c^{*\prime\prime}_M$ with $(-|u|)$. One may imagine that either the dissociation constants $K_{1,2}$ and/or the number of binding sites are influenced [*see* Eq. (13)]. The "real" rate constants k_R and k_D of the bimolecular interfacial reaction and the translocation rate constant k_S are considered as being voltage independent. The voltage dependence of k_{MS} [Eq. (14)] also is modified:

$$\begin{aligned} k'_{MS} &= k_{MS}(0)\, e^{-\beta u} f(u) \\ k''_{MS} &= k_{MS}(0)\, e^{\beta u} f(u) \qquad \text{with } f(0) = 1. \end{aligned} \tag{B.5}$$

The function $f(u)$ allows for energy barriers with a broader maximum compared to the "Eyring barrier".

Then, the voltage dependence of the current J is obtained from Eq. (B.1) using $k'_D = k''_D$, $k'_S = k''_S$, $k'_R c'_M = k_R \gamma^0_M e^{-\delta u} c'_M$ and $k''_R c''_M = k_R \gamma^0_M e^{\delta u} c''_M$. From Eq. (B.3) follows $\delta = 0.5 - \beta$. A simple calculation yields for the reduced conductance λ/λ_0 in the limit of small concentrations $c_M (c'_M = c''_M)$:

$$\lambda/\lambda_0 = \frac{2[1 + 2k_{MS}(0)/k_D]\, f(u) \cdot \sinh u/2}{u[1 + (2k_{MS}(0)/k_D)\, f(u) \cdot \cosh \beta u]}. \tag{B.6}$$

For $\beta = 0.5$ and $f(u) = 1$, Eq. (B.6) is equivalent to the simple carrier model used up to now. An identical result (though with another meaning of β) is obtained, if exponential voltage dependences of k_R and k_D are introduced, as has been done by Hladky (1974).

The ratio $k_{MS}(0)/k_D$ may be obtained applying Eq. (B.6) to stationary current voltage curves at small concentrations c_M. The question arises, how the determination of $k_{MS}(0)/k_D$ is influenced by the assumption of a voltage dependence of the interfacial reaction (i.e. $\beta < 0.5$) and by a broader shape of the energy barrier ($f(u) < 1$). As may be concluded from Eq. (B.6), at a given ratio λ/λ_0 the value of $k_{MS}(0)/k_D$ has to increase with decreasing β and to decrease with decreasing $f(u)$ (if $f(u) \cosh \beta u > 1$). This means that a shift of the reaction plane towards the interior of the membrane will enlarge k_{MS}/k_D, while a broader energy barrier will reduce it. Hladky (1975) tried to reinterpret the valinomycin data of Benz *et al.* (1973) using much smaller values for k_{MS}/k_D. Then, with his voltage dependence of $k'_{MS} + k''_{MS}$ a thermodynamically correct fit of the current-voltage curves is only possible with $\beta = 0.5$. This disagrees with the results of the present paper. For smaller values of β (e.g. $\beta = 0.4$), $k_{MS}/k_D \ll 1$ (as used by Hladky) is in contrast to the experimental data of Benz *et al.* (1973).

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