J. Membrane Biol. 26, 385–403 (1976)

Modification of Valinomycin-Mediated Bilayer Membrane Conductance by 4,5,6,7-Tetrachloro-2-methylbenzimidazole

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Received 3 November 1975

Summary. The compound, 4,5,6,7-tetrachloro-2-methylbenzimidazole (TMB), has been found to markedly modify the steady-state valinomycin-mediated conductance of potassium (K^+) ions through lipid bilayer membranes. TMB alone does not contribute significantly to membrane conductance, being electrically neutral in solution. In one of two classes of experiments (I), valinomycin is first added to the aqueous phases, then changes of membrane conductance accompanying stepwise addition of TMB to the water are measured. In a second class of experiments (II), valinomycin is added to the membrane-forming solution, followed by TMB additions to the surrounding water. In both cases membrane conductance shows an initial increase with increasing TMB concentration which is more pronounced at lower K^+ ion concentration. At TMB concentrations in excess of 10^{-5} M, membrane conductance becomes independent of K^+ ion concentration, in contrast to the linear dependence observed at TMB concentrations below 10^{-7} M. This transition is accompanied by a change of high field current-voltage characteristics from superlinear (or weakly sublinear) to a strongly sublinear form. All of these observations may be correlated by the kinetic model for carrier-mediated transport proposed by Läuger and Stark (*Biochim. Biophys. Acta* **211**:458, 1970) from which it may be concluded that valinomycin-mediated ion transport is limited by back diffusion of the uncomplexed carrier at high TMB concentrations. Experiments of class I reveal a sharp drop of conductance at high ($> 10^{-5}$ M) TMB concentration, not seen in class II experiments, which is attributed to blocked entry of uncomplexed carrier from the aqueous phases. Valinomycin initially in the membrane is removed by lateral diffusion to the surrounding torus. The time dependence of this removal has been studied in a separate series of experiments, leading to a measured coefficient of lateral diffusion for valinomycin of 5×10^{-6} cm^2/sec at 25 °C. This value is about two orders of magnitude larger than the corresponding coefficient for transmembrane carrier diffusion, and provides further evidence for localization of valinomycin in the membrane/solution interfaces.

Valinomycin is a cyclic antibiotic known to strongly increase the conductance of lipid bilayer membranes in the presence of potassium

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(K^+) and rubidium (Rb^+) ions (Andreoli, Tieffenberg & Tosteson, 1967; Lev & Buzhinsky, 1967; Mueller & Rudin, 1967). X-ray analysis (Pinkerton, Steinrauf & Dawkins, 1969), spectroscopic studies of complexation in organic solvents (Shemyakin *et al.*, 1969), and further studies of bilayer membrane conductance (Liberman & Topaly, 1968; Stark & Benz, 1971) have established that valinomycin selectively transports alkali cations by formation and translocation of charged, lipid-soluble 1 : 1 complexes. Theoretical descriptions of membrane conductance by this mechanism have been based upon: (a) the assumption of appropriate chemical equilibria between aqueous and membrane phases (Ciani, Eisenman & Szabo, 1969), or (b) a kinetic treatment of complex formation, translocation, and dissociation as discrete rate processes (Läuger & Stark, 1970). Though the two approaches are equivalent in the ohmic or low field limit (Ciani, Eisenman, Laprade & Szabo, 1972), the latter is more appropriate to the description of nonlinearities of current-voltage characteristics at high applied electric field. The kinetic approach to carrier-mediated transport has been applied to other ionophorous antibiotics as well as valinomycin, and has been extended steadily in recent publications (Laprade, Ciani, Eisenman & Szabo, 1974; Benz & Stark, 1975; Eisenman, Krasne & Ciani, 1975; Hladky, 1975; Knoll & Stark, 1975).

In previous publications we have reported the blocking by 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB) of valinomycin-mediated K^+ ion conductance of lipid bilayer membranes (Kuo & Bruner, 1973), and have correlated blocking efficiency with lipophilicity for TTFB and three additional substituted benzimidazoles (Kuo, Fukuto, Miller & Bruner, 1975). One of the latter compunds, 4,5,6,7-tetrachloro-2-methylbenzimidazole (TMB), shows a significant blocking effect although, in contrast to TTFB, it is not ionized in the pH range (5–8) of our experiments. Electrically neutral TMB would be expected to introduce minimal modification of carrier-mediated membrane conductance due to surface potential effects, in contrast to the situation illustrated by the effect of anionic dinitrophenol upon nonactin-K^+ conductance (McLaughlin, 1972). Furthermore, electrical neutrality insures that TMB does not itself contribute directly to membrane conductance, in contrast to TTFB (Kuo & Bruner, 1973). Thus modification of valinomycin-mediated K^+ ion conductance of bilayer membranes by TMB should represent a particularly favorable case for interpretation in terms of a corresponding modification of one or more of the rate processes postulated by Läuger and Stark (1970). This is the objective of the present paper.

Theoretical Considerations

To facilitate the presentation and interpretation of our results we summarize here the essential features of the Läuger-Stark (1970) kinetic model for carrier-mediated ion transport in the steady-state. We simplify their development by neglecting complexation in the aqueous phases between valinomycin and alkali cations. This can be justified, at least for K^+ ion, by the complexation studies of Shemyakin *et al.* (1969), and by studies of the dependence of bilayer membrane conductance upon aqueous phase cation concentration (Stark & Benz, 1971).

With this simplification three sets of rate processes are considered:

(a) *The Exchange of Uncomplexed Carrier between the Aqueous Phases and the Membrane/Solution Interfaces.* The interfacial surface density of uncomplexed carrier N_S is given by,

$$N_S = \tfrac{1}{2}\, d\, \gamma_S\, c_0 \tag{1}$$

where d is the membrane thickness, γ_S is a dimensionless partition coefficient, and c_0 is the concentration of carrier in the aqueous phases.

(b) *The Formation and Dissociation of Carrier-Ion Complexes in the Membrane/Solution Interfaces.* This is a heterogeneous reaction between aqueous phase cations and interfacial free carriers, having formation and dissociation rate constants k_R and k_D, respectively. The surface density of complexed carrier N_{MS} is related to other parameters by

$$K_h = \frac{k_R}{k_D} = \frac{N_{MS}}{c_M\, N_S} \tag{2}$$

where c_M is the aqueous phase concentration of transported alkali cation, and K_h is the equilibrium constant of the heterogeneous reaction.

(c) *The Translocation of Both Complexed and Uncomplexed Carriers across the Membrane.* These processes are characterized by rate constants k_{MS} and k_S, respectively. A key assumption of the model is that only the rate constant k_{MS} is modified by an applied electric field. Eyring expressions assuming a barrier potential with a sharp central maximum are introduced to describe this modification. Perturbation of k_{MS} by an applied field, and the associated current flow, gives rise to perturbation of N_{MS} and of N_S as well. Throughout this section values of k_{MS}, N_{MS}, and N_S appropriate to the limit of zero electric field are implied.

The expression for the current density J, developed by Läuger and Stark, simplified by neglect of carrier complexation in the aqueous phases,

is presented below with minor notational changes.

$$J=\frac{\beta c_M J^s \sinh(u/2)}{1+(\alpha+\beta c_M)\cosh(u/2)}. \tag{3}$$

Here $u=F(\Delta\Psi)/RT$ is the reduced voltage, where F is the Faraday constant, $\Delta\Psi$ is the applied transmembrane potential difference, R is the gas constant, and T is the absolute temperature. In addition,

$$J^s=Fd\gamma_S c_0 k_S \tag{4}$$

$$\alpha=2k_{MS}/k_D \tag{5}$$

$$\beta=(k_{MS}/k_S)K_h. \tag{6}$$

It is useful to consider Eq. (3) in three limiting cases:

I. $(\alpha \text{ and } \beta c_M \ll 1)$

$$\begin{aligned} J &\approx \beta c_M J^s \sinh(u/2) \\ &= 2FN_{MS} k_{MS} \sinh(u/2) \end{aligned} \tag{7}$$

by use of Eqs. (1), (2), (4), and (6). In this case the rate-limiting step is translocation of the charged complex across the potential barrier provided by the hydrocarbon core of the membrane. The current-voltage characteristic is superlinear.

II. $(\beta c_M \gg \alpha \text{ and } 1)$

$$\begin{aligned} J &\approx J^s \tanh(u/2) \\ &= 2FN_S k_S \tanh(u/2) \end{aligned} \tag{8}$$

using Eqs. (1) and (4). Here the rate-limiting step is back-diffusion of the neutral carrier across the membrane interior. The current-voltage characteristic is sublinear, saturating at high applied potential.

III. $(\alpha \gg \beta c_M \text{ and } 1)$

$$\begin{aligned} J &\approx (\beta c_M/\alpha) J^s \tanh(u/2) \\ &= FN_S k_R c_M \tanh(u/2) \\ &= FN_{MS} k_D \tanh(u/2) \end{aligned} \tag{9}$$

using Eqs. (7), (5), and (2). In this case the rate-limiting step is the formation or dissociation of ion-carrier complexes, the net rates for these processes always being equal in steady state. Again the current-voltage characteristic is sublinear.

Steady-state measurements alone cannot determine all five of the constants, γ_S, k_S, k_{MS}, k_R and k_D, but can fix three combinations of these. Relaxation methods are required to determine all five constants (Stark, Ketterer, Benz & Läuger, 1971). We have chosen to designate the three combinations by α, β, and J^s (which fixes the product $\gamma_S k_S$), and will determine how they are modified upon introduction of TMB. This procedure should – in the context of the three limiting cases discussed above – shed light on the manner in which TMB modifies valinomycin-mediated K^+ ion conductance in bilayer membranes.

Experimental results are normally presented in terms of ohmic conductance,

$$\lambda_0 = \lim_{\Delta\Psi \to 0} \left(\frac{J}{\Delta\Psi} \right) \tag{10}$$

and in terms of the ratio λ/λ_0 of chord conductance at higher fields to ohmic conductance. From preceding results,

$$\frac{\lambda}{\lambda_0} = \frac{2}{u} \frac{[1+(\alpha+\beta c_M)] \sinh(u/2)}{[1+(\alpha+\beta c_M)\cosh(u/2)]} \tag{11}$$

and

$$\lambda_0 = \frac{\beta c_M}{1+(\alpha+\beta c_M)} \left(\frac{FJ^s}{2RT} \right). \tag{12}$$

Thus α and β may be determined from measured nonlinearity of current-voltage characteristics for various values of K^+ ion concentration c_M. Then J^s may be evaluated by reference to λ_0.

Materials and Methods

Bilayer membranes were formed by the brush technique from bacterial phosphatidylethanolamine (Supelco) in *n*-decane. Conductance cells of teflon and quartz construction were employed. Membranes were formed on a circular aperture of 1 mm diameter, except where otherwise noted. All measurements were carried out at 25 °C. Steady-state electrical measurements in both the ohmic and nonohmic range employed apparatus previously described (Huebner & Bruner, 1972).

Valinomycin (Calbiochem) was added either to the aqueous phases or to the membrane-forming solution. In either case membranes were permitted to thin fully and to achieve a stable conductance level before stepwise additions of TMB to the aqueous phases were made. At least five minutes were allowed after each addition of TMB to permit attainment of a stable conductance level before measurement, except in the case of studies of certain time-dependent effects to be described below. The aqueous phases were stirred continuously, except for one experiment to be described below. Further experimental details have been published elsewhere (Kuo *et al.*, 1975).

Results

In the first series of experiments ohmic membrane conductance has been measured in the presence of 10^{-7} M valinomycin in the aqueous phases, at various fixed concentrations of KCl, with total ionic strength held constant by appropriate additions of LiCl. The variation of ohmic conductance with concentration of added TMB is illustrated in Fig. 1.

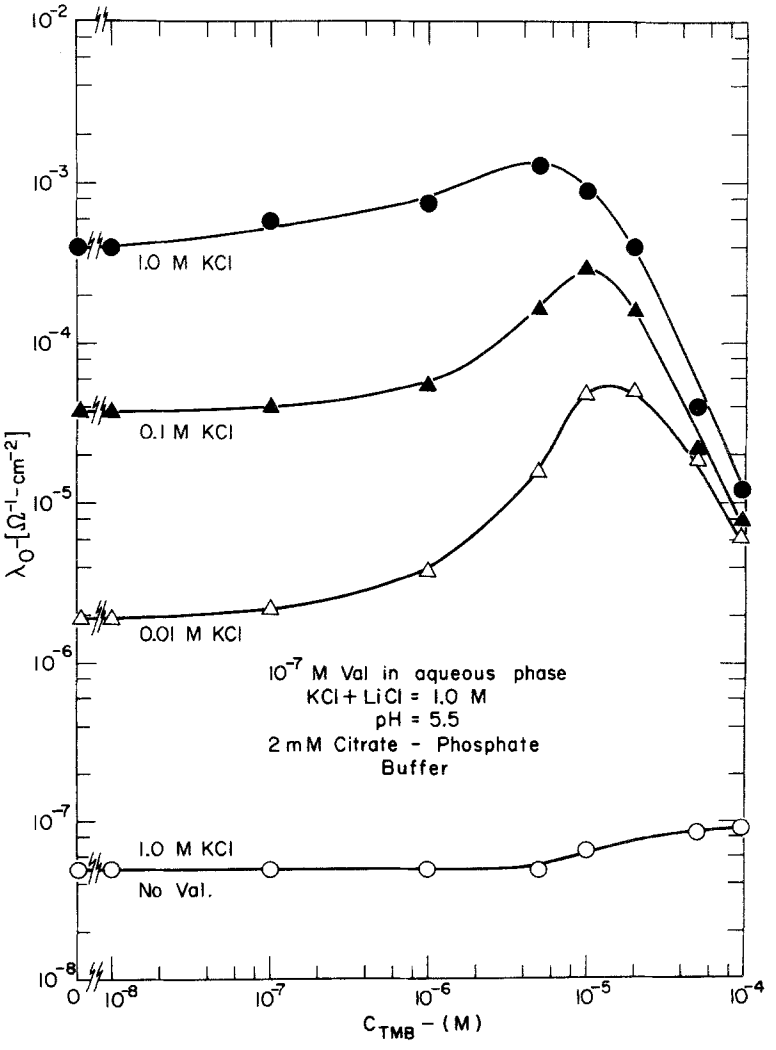

Fig. 1. Steady-state ohmic membrane conductance due to valinomycin-mediated K^+ ion transport is plotted versus the concentration of added TMB. Both valinomycin (10^{-7} M) and TMB have been added to the aqueous phases. The lowest-lying curve illustrates membrane conductance in the presence of TMB, but with valinomycin absent

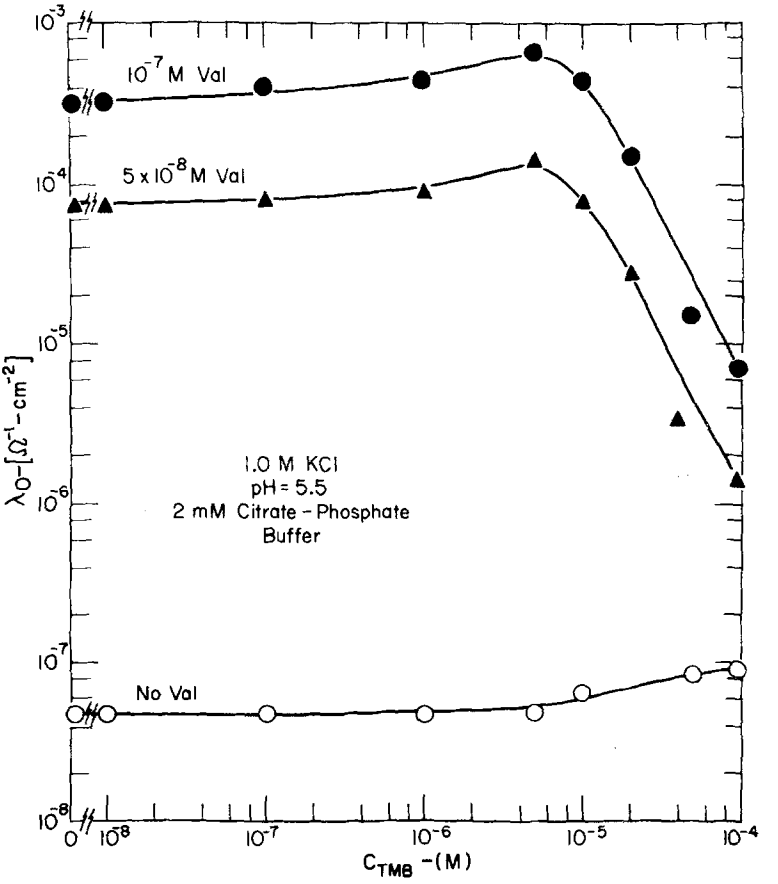

Fig. 2. The proportionality of the steady-state ohmic membrane conductance to the aqueous phase concentration of valinomycin, at all concentrations of added TMB, is illustrated. This provides evidence for the operation of the valinomycin-K^+ ion carrier mechanism throughout the full range of TMB concentration

Essential features of the results are: (a) valinomycin-K^+ conductance is unmodified by TMB concentrations lower than 10^{-7} M, and is proportional to K^+ ion concentration in that range; (b) carrier-mediated conductance shows an initial increase with increasing TMB concentration, which is more pronounced at lower K^+ ion concentration; and (c) further increase of TMB concentration, above 5×10^{-6} M, leads to a sharp decrease of conductance, with conductance becoming virtually independent of K^+ ion concentration.

The data of Fig. 2 establish that membrane conductance scales with aqueous phase valinomycin concentration throughout the full range of TMB concentration. This indicates that carrier-mediated transport is

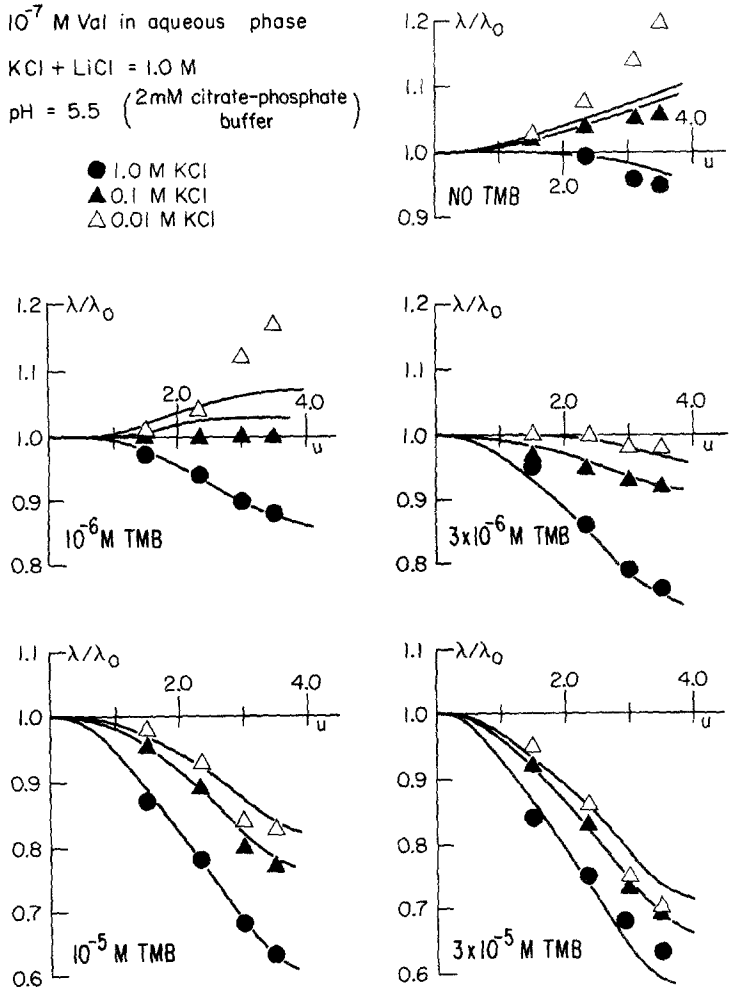

Fig. 3. The ratio of steady-state high field chord conductance to ohmic conductance λ/λ_0 is plotted versus reduced voltage u, defined immediately following Eq. (3). In each case illustrated, 10^{-7} M valinomycin has been added to the aqueous phases, as well as TMB at the concentration indicated. Increasing sublinearity of current-voltage characteristics with increasing TMB concentration is evident

operative throughout the range, and that the observed conductance variations must be associated with rate changes induced by added TMB.

Observed nonlinearities of current-voltage characteristics are plotted in Fig. 3 for various concentrations of TMB. At each concentration of TMB three plots of λ/λ_0 versus u are shown, for differing concentrations of K^+ ion. It is seen that, at any concentration of TMB, a decrease of superlinearity or an increase of sublinearity accompanies an increase of K^+ ion concentration. In addition, at any fixed K^+ ion concentration, a

Table 1. Variation with TMB concentration of the parameters of carrier-mediated membrane conductance

	c_{TMB} (M)	α	β (M^{-1})	J^s [a] (amp/cm^2)
A. 10^{-7} M valinomycin added to aqueous phases	None	0.3	0.2	1.7×10^{-4}
	10^{-6}	0.3	0.4	1.7×10^{-4}
	3×10^{-6}	0.5	0.9	1.5×10^{-4}
	10^{-5}	0.8	2.7	7.7×10^{-5}
	3×10^{-5}	1.4	4.9	1.1×10^{-5}
	10^{-4}	— [b]	— [b]	6.2×10^{-7} [c]
B. 2×10^{-4} M valinomycin added to membrane-forming solution	None	0.3	0.3	2.5×10^{-5}
	10^{-6}	0.4	0.6	1.7×10^{-5}
	3×10^{-6}	0.6	1.1	1.5×10^{-5}
	10^{-5}	0.6	3.6	1.2×10^{-5}
	3×10^{-5}	1.0	7.5	5.2×10^{-6}
	10^{-4}	— [b]	— [b]	2.3×10^{-6} [c]

[a] All values of J^s, except those given for a TMB concentration of 10^{-4} M, are calculated using tabulated values of α and β, Eq. (12), and ohmic conductance data at 1 M K^+ ion concentration.
[b] Nonlinear current-voltage data could not be obtained at 10^{-4} M TMB concentration because of reduced membrane stability in high applied fields.
[c] These values are calculated using Eq. (12), ohmic conductance data at 1 M K^+ ion concentration, and the assumption that $\beta c_M \gg 1 + \alpha$.

transition toward increasingly sublinear current-voltage characteristics accompanies an increase of TMB concentration.

The data of Fig. 3 may be used, as discussed in the theoretical section above, to determine the parameters α and β at each concentration of TMB. The results of this analysis are presented in Part A of Table 1. The smooth curves through the data points of Fig. 3 have been drawn using Eq. (11) and the tabulated values of α and β. In addition, using Eq. (12) and ohmic conductance data, we have calculated J^s at each concentration of TMB and listed the results in Part A of Table 1. As the concentration of TMB increases, α is seen to increase by about a factor of five, while β increases by roughly a factor of 25, and J^s decreases by more than two orders of magnitude.

In a second series of experiments, 2×10^{-4} M valinomycin has been added to the membrane-forming solution rather than to the aqueous phases. Stepwise addition of TMB to the aqueous phases after formation of black membranes with stable conductance then yields the results shown in Fig. 4. Again there is an initial increase of conductance with increasing TMB concentration, which is more pronounced at lower K^+ ion con-

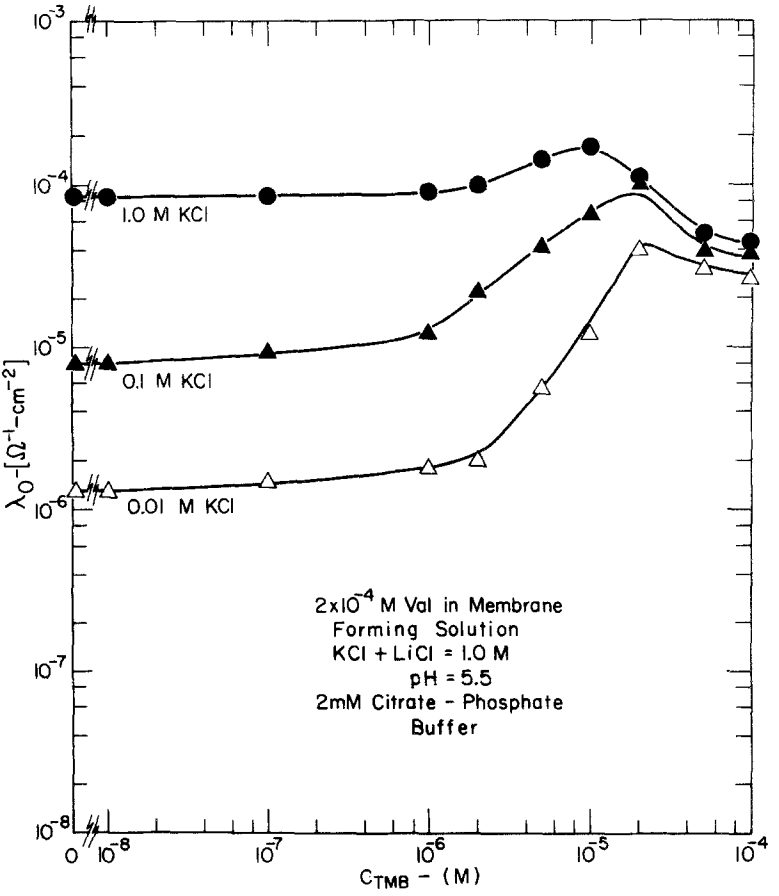

Fig. 4. Steady-state ohmic membrane conductance due to valinomycin-mediated K^+ ion transport is plotted versus the concentration of added TMB. Valinomycin (2×10^{-4} M) has been added to the membrane-forming solution in this case, while TMB has been added to the aqueous phases

centration. Now, however, the subsequent decrease of carrier-mediated conductance at high TMB concentration is very much reduced, being nearly eliminated at the lowest K^+ ion concentration.

Plots of λ/λ_0 versus u for the second series of experiments are presented in Fig. 5. Values of α, β, and J^s determined for this series are listed in Part B of Table 1. In this case we again see that, as the concentration of TMB increases, α changes relatively little while β increases markedly. In this case, however, the decrease of J^s is much smaller.

The data presented above may be interrelated to a remarkable degree by the Läuger-Stark model, by reference to Eqs. (11) and (12) in particular.

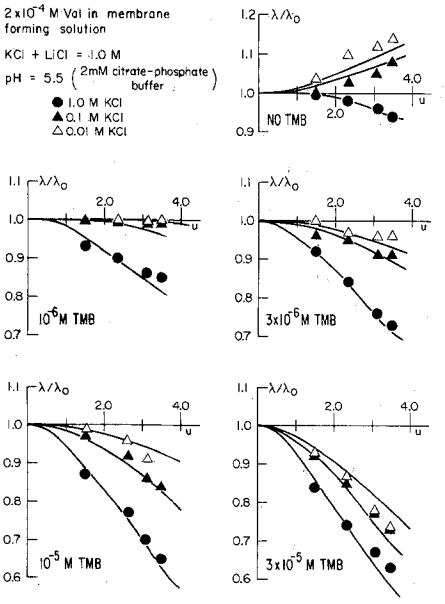

Fig. 5. The conductance ratio λ/λ_0 is again plotted versus reduced voltage u. In each case illustrated 2×10^{-4} M valinomycin has been added to the membrane-forming solution, while TMB at the concentrations indicated has been added to the aqueous phases. Increasing sublinearity of current-voltage characteristic with increasing TMB concentration is again evident

An increase of β with increasing TMB concentration correlates the following experimental observations:

(a) The ohmic membrane conductance due to valinomycin-mediated K^+ ion transport shows an initial increase with increasing TMB concentration.

(b) This initial increase is more pronounced at lower concentration, c_M, of transported ion.

(c) The ohmic conductance becomes nearly independent of c_M at high TMB concentration. This observation is consistent with an approach to the second of the three limits discussed in the theoretical section above.

(d) A transition from superlinear (or weakly sublinear) to strongly sublinear current-voltage characteristics takes place as the TMB concentration is raised from zero to 10^{-4} M. This observation is consistent with a transition – with increasing β – from the first to the second of the three limits described above.

The preceding remarks apply with equal validity to the results obtained when valinomycin is added either to the aqueous phases or to the membrane-forming solution. The most significant point of difference between

the two sets of experiments is the much larger decrease of J^s observed in the former case at high concentrations of added TMB. We recall from Eq. (8) that J^s is proportional to the product of N_S, the interfacial concentration of uncomplexed carrier, and k_S, its rate constant for membrane translocation. When valinomycin is added to the aqueous phases, N_S is governed by partition of the carrier between the water and the membrane, as described by Eq. (1). When valinomycin is added to the membrane-forming solution, on the other hand, the Läuger-Stark model need only be modified to the extent of recognizing that in this case the interfacial concentration of uncomplexed carrier is governed by its partition between the torus and the membrane (Stark & Benz, 1971; Benz, Stark, Janko & Läuger, 1973). Since the rate constant k_S must be independent of the mode of addition of valinomycin to the system, we immediately conclude that the sharper decrease of J^s observed when valinomycin is in the aqueous phases must be associated with a more pronounced decrease of interfacial free-carrier concentration in this case. Thus we conclude that the blocking of carrier-mediated conductance observed in this case at high TMB concentration results from a TMB-induced decrease of γ_S, the coefficient of partition of valinomycin between water and the membrane interface.

The blocking by TMB of carrier-mediated membrane conductance, when valinomycin is in the water, will occur even though a high level of valinomycin-K^+ ion conductance is established prior to the addition of TMB. This is so because valinomycin already in the membrane can diffuse to the torus, and will not be replenished from the aqueous phases because of blocking by TMB. To check this point experimentally we have performed a series of measurements of the time course of loss of valinomycin-K^+ ion conductance following the addition of TMB. A square wave of 20 mV amplitude and 2.5 sec period was applied to the membrane, and the current response was monitored continuously on a recorder. The experiments were performed using membrane diameters of 1, 2 and 3 mm. The results are shown in Fig. 6.

For each membrane diameter it is possible, at sufficiently long times, to resolve a clearly linear segment on the semi-log plot of membrane conductance versus time. The interpretation of these results is facilitated by reference to the solution of the classical problem of radial diffusion out of an infinitely long cylinder, or a right cylinder with sealed end surfaces. For our purposes the approximate solution presented by Jost, 1960, may be adapted as follows;

$$\frac{\lambda_0(t)}{\lambda_0(0)} = \frac{\bar{c}_m(t)}{c_m(0)} \approx \frac{4}{(2.405)^2} \exp\left[-t/\tau\right] \tag{13}$$

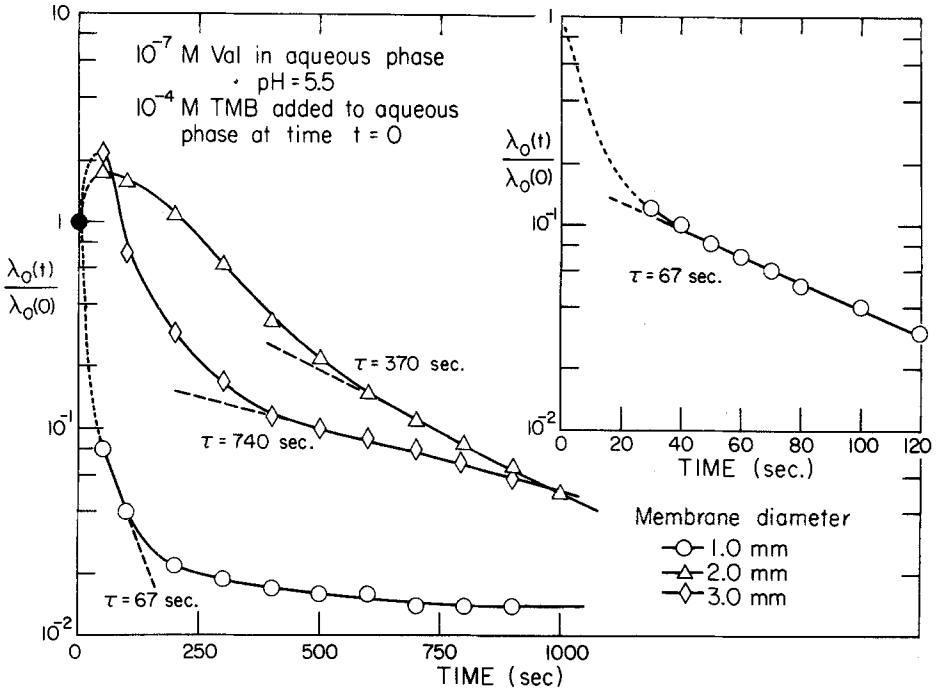

Fig. 6. The variation of ohmic membrane conductance with time $\lambda_0(t)$ after addition of 10^{-4} M TMB to the aqueous phases, is illustrated on a semi-log plot. Addition of TMB takes place at $t=0$, at which time the membrane conductance is $\lambda_0(0)$, resulting from prior addition of 10^{-7} M valinomycin to the aqueous phases. The ratio $\lambda_0(t)/\lambda_0(0)$ has been plotted on the logarithmic ordinate scale. Results are shown for three different membrane diameters, with linear ranges of conductance decay and corresponding relaxation times being illustrated. Only for the 1 mm diameter membrane is a steady-state low conductance reached, corresponding to fully blocked conductance. The conductance decay for this case is replotted on an expanded time scale in the inset at the upper right

where

$$\tau = \frac{r_0^2}{(2.405)^2 D}. \tag{14}$$

Here $\bar{c}_m(t)$ is the average concentration of carrier remaining on the membrane at time t, $c_m(0)$ is the initial (assumed uniform) membrane concentration of carrier, and τ is a characteristic relaxation time involving r_0, the membrane radius, and D, the coefficient for lateral diffusion of the carrier in the membrane/solution interface. In Fig. 7 we present a log-log plot of measured values of τ versus membrane diameter. A close proportionality between τ and the square of the membrane diameter, as predicted by Eq. (14), is evident. Use of Eq. (14) and the three data points of Fig. 7 yields an average value of the interfacial diffusion coefficient of $D = 5 \times 10^{-6}$ cm^2/sec for valinomycin.

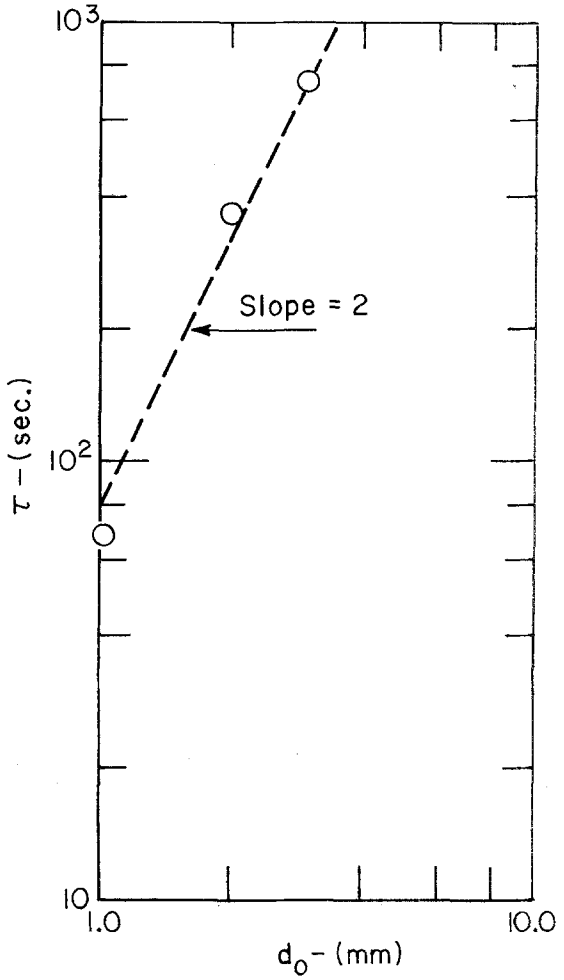

Fig. 7. The relaxation time τ for conductance decay, from Fig. 6, is plotted versus membrane diameter d_0 using log-log scales. The variation of τ with $(d_0)^2$, predicted by Eq. (14), is illustrated

Several additional points should be made regarding the data of Fig. 6. First, because of the finite time required to add TMB, to disperse it by stirring through the aqueous phases, and to permit it to penetrate the aqueous unstirred layers adjacent to the membrane, the recording of meaningful conductance data could not begin for about 30 sec after the beginning of TMB addition. This time range is indicated by dotted line segments on the curves of Fig. 6. A second point is that, for both the 2 and 3 mm diameter membranes, an initial increase of conductance is clearly resolved. We associate this with the increase of stationary-state conductance seen in Fig. 1 at concentrations of TMB below the range where

blocking begins. This increase in turn reflects an increase of the parameter β, as discussed above. A third point is that the exact solution of the problem of diffusion out of a cylinder, also presented by Jost (1960), consists of an infinite series of decaying exponential terms, of which only the leading term appears on the right of Eq. (13). This term, having the longest relaxation time, should dominate at all times $t \gtrsim \tau$, but the predicted presence of faster relaxations undoubtedly contributes to the regions of positive curvature in the curves of Fig. 6, which precede the linear segments.

Finally, it should be emphasized that the blocking of valinomycin-mediated conductance is never "complete", i.e., the conductance is never reduced to that of an unmodified membrane. Blocking by TMB simply means that entry of the uncomplexed carrier from the water into the membrane is impeded to a sufficient extent that loss of valinomycin to the torus, which must occur even in the absence of TMB, is able to shift the equilibrium membrane concentration of the carrier to a much lower value. A similar dynamic balance between entry from the aqueous phase and loss to the torus undoubtedly fixes the membrane concentration of TMB as well. In one experiment such as those of Fig. 6, using a 2-mm diameter membrane, stirring was stopped after 300 sec (data not shown). The decrease of conductance was immediately interrupted; it in fact rose slightly over the next several hundred seconds before resuming a slow decrease. An immediate interpretation is that entry of TMB from the water was impeded by a widened diffusion barrier resulting from cessation of stirring. Continuing loss of TMB to the torus then shifted its equilibrium interfacial membrane concentration to a lower value, thereby partially reversing the blocking effect. This observation also suggests that the interfacial diffusion coefficient of TMB is significantly higher than that of valinomycin, a conclusion which is also supported by the fact that the aqueous phase TMB concentration required for blocking is one to two orders of magnitude greater than the corresponding valinomycin concentration. This conclusion is of particular importance because our interpretation of the linear segments of the curves of Fig. 6 implicitly assumes that the concentration of TMB on the membrane interfaces has reached a steady-state value prior to the time range covered by these segments.

Observations reported by Benz *et al.* (1973) and by Hladky (1973) are pertinent to the discussion of the preceding paragraph. In both reports an increase with membrane area of the membrane conductance per unit area is described. The implication is that the membrane concentration of carrier near the border is depleted by diffusion of carrier into the torus, the effect being proportionately larger for smaller membranes. The carrier

is added to the aqueous phase in these studies. In addition, Hladky reports a two- to sevenfold increase of carrier-mediated conductance at the onset of stirring, which is reversed upon cessation of stirring. The interpretation is that stirring narrows the aqueous phase diffusion barrier to the entry of carrier from the water into the membrane.

Discussion

It was pointed out in the theoretical section above that the individual rate constants introduced by the Läuger-Stark model could not be determined by stationary-state conductance measurements alone. We can, however, make some inferences about how the individual rate constants describing valinomycin transport are modified by the addition of TMB, provided we introduce one assumption. We assume that k_S and k_{MS}, the rate constants describing translocation of uncomplexed and of complexed carriers, respectively, will be similarly modified upon addition of TMB; i.e., the ratio k_{MS}/k_S is assumed to remain constant. With this assumption it follows from Eq. (6) that the observed increases of β must be attributed to an increase of the heterogeneous rate constant K_h, defined by Eq. (2). This in turn implies either an increase of the recombination rate constant k_R, or a decrease of the dissociation rate constant k_D, upon addition of TMB. An increase of k_R could account for the fact that β is observed to increase much more than α, but this appears unlikely; there is no obvious explanation for enhancement of carrier complexation at moderate (10^{-6} – 10^{-5} M) TMB concentrations, followed by blocked entry of the uncomplexed carrier from the aqueous phases at higher TMB concentrations. A decrease of k_D, as well as blocked carrier entry, might, however, be plausibly attributed to steric hindrance by TMB adsorbed to the membrane/solution interfaces. In this case there would have to be a concomitant decrease of k_{MS}, and hence by our initial assumption a decrease of k_S as well, to account for our experimental observation that β increases much more than α. Such a decrease of k_S might very well account for the decrease of J^s which is observed at high aqueous phase TMB concentrations when valinomycin is added to the membrane-forming solution. For the reasons cited we conclude that our observations on the changes of α and β with increasing TMB concentration are most likely to reflect associated decreases of k_D, k_{MS} and k_S. This conclusion must be considered tentative, however, pending availability of results of relaxation studies which are planned.

Refinements of the original Läuger-Stark model of carrier-mediated transport which have been put forward (Knoll & Stark, 1975) include: (a) modification of the Eyring expressions for the voltage dependence of the translocation rate constants for the complexed carrier; (b) replacement of the bulk aqueous phase concentration of transported ion c_M by an effective concentration c_M^* to take account of interfacial saturation effects; and (c) introduction of voltage-dependent rate constants, k_R and k_D, to describe the interfacial reactions. Some of these questions have been addressed by previous investigators as well (Hladky, 1972; Benz *et al.*, 1973; Hall, Mead & Szabo, 1973; Hladky, 1974). We find nevertheless that the unmodified Läuger-Stark model is able to correlate in a very satisfactory way the essential features of our experimental results on the modification by TMB of valinomycin-mediated K^+ ion conductance. Model parameters (α and β) derived from analysis of nonlinear current-voltage characteristics, evaluated as a function of TMB concentration, may be used to explain the major features of our data for ohmic carrier-mediated conductance, the latter also being measured as a function of TMB concentration. These major features include: (a) an initial increase of carrier-mediated conductance with increasing concentration of TMB, this increase being more pronounced at lower aqueous phase concentrations of the transported ion; and (b) a nearly complete loss of dependence of ohmic conductance upon concentration of the transported ion, which occurs at high TMB concentrations. Finally, a comparison of results from experiments involving introduction of valinomycin into either the aqueous phase or into the membrane-forming solution establishes that, in the former case, the entry of valinomycin from water into the membrane is impeded by an elevated concentration of TMB in the aqueous phases. This may be interpreted as a TMB-induced reduction of the partition coefficient governing the distribution of the carrier between the aqueous phases and the membrane interfaces. This partition coefficient γ_S is a key parameter of the original Läuger-Stark model. The results cited above are all consistent with the conclusion that, at high TMB concentration, the steady-state membrane current is limited by back-flux of the uncomplexed carrier.

Our result for the lateral diffusion coefficient of valinomycin at 25 °C ($D = 5 \times 10^{-6}$ cm^2/sec) implies that the environment of the carrier is quite fluid so far as lateral movement is concerned. By way of contrast we note that the rate constant for translocation of valinomycin through the interior of bilayer membranes is $k_S \approx 10^4$ sec^{-1} (Stark *et al.*, 1971; Benz *et al.*, 1973; Knoll & Stark, 1975). An equivalent coefficient for transverse

diffusion, of order $k_S d^2$ where d is the membrane thickness, is then approximately 10^{-8} cm^2/sec. The coefficient for lateral diffusion is thus seen to exceed that for transverse diffusion of the carrier by at least two orders of magnitude. The coefficient for transverse diffusion determined from the studies of conductance relaxation cited above is of the same order as that which can be deduced from microviscosity studies of nonpolar fluorescent probes in the hydrocarbon region of phospholipid dispersions (Cogan, Shinitzky, Weber & Nishida, 1973). The diffusional anisotropy revealed by our measurements provides further convincing evidence for the localization of valinomycin in energy minima at the membrane/solution interfaces, a conclusion supported experimentally by the NMR studies of Hsu and Chan (1973) as well.

It is a pleasure to acknowledge many stimulating discussions with Dr. G. Stark, which took place during the course of a visit to our laboratory. This work has been supported by a grant from the United States Army Research Office.

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