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Kinetic Analysis of Carrier-Mediated Ion Transport by the Charge-Pulse Technique

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Summary. The charge-pulse technique has been used previously for the study of quasi-stationary processes in membranes which required only a moderate time resolution. It is shown here that a time resolution of about 400 nsec may be achieved with this technique and that it may be applied to the kinetic analysis of carrier-mediated ion transport. By this method we have studied the transport of alkali ions through optically black monoolein membranes in the presence of the ion carrier valinomycin. All three relaxation processes that are predicted by theory have been resolved. From the relaxation times and the relaxation amplitudes the rate constants for the association (k_R) and the dissociation (k_D) of the ion-carrier complex, as well as the translocation rate constants of the complex (k_{MS}) and the free carrier (k_S) could be obtained. For 1 M Rb^+ at 25 °C the values are $k_R = 3 \times 10^5\ M^{-1}\ sec^{-1}$, $k_D = 2 \times 10^5\ sec^{-1}$, $k_{MS} = 3 \times 10^5\ sec^{-1}$, $k_S = 4 \times 10^4\ sec^{-1}$. The activation energies of the single rate constants which have been estimated from experiments at two different temperatures range between 50 and 90 kJ/mol.

The kinetics of carrier-mediated ion transport through lipid bilayer membranes has been studied by electrical relaxation experiments (Stark, Benz, Ketterer & Läuger, 1971; Benz, Stark, Janko & Läuger, 1973; Gambale, Gliozzi & Robello, 1973; Benz & Stark, 1975; Hladky, 1975*b*; Knoll & Stark, 1975; Laprade, Ciani, Eisenman & Szabo, 1975; Sandblom, Hägglund & Eriksson, 1975). In these studies the voltage-jump (or voltage-clamp) method has been used which consists in measuring the time course of the membrane current after a sudden displacement of the voltage. The time resolution of the voltage-jump technique is often limited by an RC time constant which is given by the product of the membrane capacitance times the resistance of the external circuit. (If the current is controlled by a feedback circuit, the time resolution is usually limited by the bandwidth of the feedback system.) An alternative method with a potentially better time resolution is the so-called charge pulse technique. The membrane capacitance is charged to an initial voltage by a brief current pulse of 10–100 nsec duration; at the end of the pulse the external charging circuit is switched to virtually infinite resistance. The membrane voltage V_m then

decays by conduction processes across the membrane as well as by redistribution of charges within the membrane. From a measurement of V_m as a function of time, information on the mechanism of ion transport in the membrane may be obtained. This method, which has occasionally been used in neurophysiology (e.g., Hodgkin, Huxley & Katz, 1952), has been worked out in considerable detail in the course of studies of fast electrode reactions (Delahay, 1962; Reinmuth & Wilson, 1962; Weir & Enke, 1967; Kudirka, Daum & Enke, 1972; *see* Weir & Enke for a more complete survey of the literature). Recently, the charge-pulse method has been applied to the study of electrical properties of lipid bilayer membranes in the presence of hydrophobic ions (Borisova, Ermishkin, Liberman, Silberstein & Trofimov, 1974; Gavach & Sandeaux, 1975) and of ion carriers (Feldberg & Kissel, 1975). In these studies the slow phase of the voltage decay has been recorded, i.e., that part of the voltage relaxation which is mainly determined by the steady-state conductance of the membrane. In the following, we show that with increased time resolution of the measuring system also the early phases of the voltage relaxation may be analyzed which are governed by fast concentration changes of the ion-carrier complex in the membrane-solution interfaces. Under favorable conditions the rate constants of the single transport steps of the carrier system may be obtained from such an analysis.

Theory

In the following we base the analysis of the charge-pulse experiment on a model of carrier-mediated ion transport that has already been used for the treatment of steady-state and voltage-jump relaxation data (Läuger & Stark, 1970; Stark *et al.*, 1971). Specifically, this model assumes that complex formation between an ion M^+ and a carrier molecule S takes place in the membrane-solution interface and may be described by overall rate constants k_R (association) and k_D (dissociation). The translocation of the complex MS^+ from one interface to the other is treated, to a first approximation, as a simple first-order reaction with a rate constant k_{MS}. Similarly, the translocation of the free carrier S is described by a rate constant k_S. Of all the rate constants only k_{MS} is assumed to be voltage dependent. This is a rather crude approximation since both S and MS^+ are probably located some distance away from the membrane-solution interface toward the interior of the membrane and experience part of the voltage drop across the membrane (Stark & Benz, 1971; Eisenman,

Krasne & Ciani, 1975; Hladky, 1975*a*). The resulting voltage dependence of k_R and k_D is neglected here but may later be introduced into the model when more experimental data will become available. The dependence of k_{MS} on the externally applied voltage V_m is calculated on the basis of a single barrier of the Eyring type (Läuger & Stark, 1970):

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

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

$$u = \frac{V_m}{RT/F} = \frac{\psi' - \psi''}{RT/F}. \tag{3}$$

k'_{MS} and k''_{MS} are the rate constants of translocation from the left to the right and from the right to the left interface, respectively; ψ' and ψ'' are the electrical potentials in the left-hand and right-hand aqueous solution; R is the gas constant, T the absolute temperature and F is Faraday's constant. Again, Eqs. (1) and (2) have to be considered as approximations which may be replaced in a more refined analysis by expressions taking into account the shape of the energy barrier (Hall, Mead & Szabo, 1973) as well as the fact that only part of the voltage drops across the central barrier.

We assume that the membrane separates identical solutions of the transported ion M^+ (concentration c_M) and that the system is at equilibrium at times $t \leqq 0$. At time $t = 0$ the membrane capacitance is charged up virtually instantaneously to a voltage V_m^0. Denoting the concentrations of S and MS^+ in the left interface by N'_S and N'_{MS} and the concentrations in the right interface by N''_S and N''_{MS} (expressed in moles/cm^2) then the rate of change of these quantities after the charge pulse is given by

$$\frac{dN'_S}{dt} = -k_R c_M N'_S + k_D N'_{MS} - k_S (N'_S - N''_S) \tag{4}$$

$$\frac{dN''_S}{dt} = -k_R c_M N''_S + k_D N''_{MS} - k_S (N''_S - N'_S) \tag{5}$$

$$\frac{dN'_{MS}}{dt} = k_R c_M N'_S - k_D N'_{MS} - k'_{MS} N'_{MS} + k''_{MS} N''_{MS} \tag{6}$$

$$\frac{dN''_{MS}}{dt} = k_R c_M N''_S - k_D N''_{MS} - k''_{MS} N''_{MS} + k'_{MS} N'_{MS}. \tag{7}$$

Implicit in these equations is the assumption that the exchange of S and MS^+ between interface and water is slow with respect to the time

scale of the relaxation experiment (Stark & Benz, 1971). It is seen that the sum of the right-hand sides of Eqs. (4) and (5) is zero; this means that the total concentration N_0 of the carrier per unit area is a constant:

$$N_0 = N_S' + N_S'' + N_{MS}' + N_{MS}''. \tag{8}$$

The rate of decay of the voltage V_m after the initial charge pulse is determined by the specific membrane capacitance C_m and by the current density J in the membrane:

$$\frac{dV_m}{dt} = -\frac{J}{C_m} = -\frac{F}{C_m}(k_{MS}' N_{MS}' - k_{MS}'' N_{MS}''). \tag{9}$$

According to Eqs. (1) and (2) the translocation rate constants k_{MS}' and k_{MS}'' depend on voltage and therefore are functions of time. In the following we restrict the analysis to small voltages ($|u| \ll 1$ or $|V_m| \ll 25$ mV) where the approximations

$$k_{MS}' \approx k_{MS}\left(1 + \frac{u}{2}\right) \tag{10}$$

$$k_{MS}'' \approx k_{MS}\left(1 - \frac{u}{2}\right) \tag{11}$$

hold. This is not a serious restriction, as the sensitivity of the method is usually sufficient to obtain all the information required for the evaluation of the rate constants from an experiment with V_m^0 of the order of a few millivolts.

Eqs. (4)–(7) together with Eq. (9) represent a system of five linear differential equations for the five unknown functions: $N_S'(t)$, $N_S''(t)$, $N_{MS}'(t)$, $N_{MS}''(t)$, and $V_m(t)$. The solution which may be obtained by standard methods (compare Appendix A) yields $V_m(t)$ in the form

$$V_m(t) = V_m^0(a_1 e^{-\lambda_1 t} + a_2 e^{-\lambda_2 t} + a_3 e^{-\lambda_3 t}) \tag{12}$$

$$a_1 + a_2 + a_3 = 1. \tag{13}$$

In principle, the relaxation times $\tau_i = 1/\lambda_i$ and the relaxation amplitudes a_i ($i = 1, 2, 3$) may be expressed as functions of the rate constants and of N_0, but the resulting equations are rather cumbersome. We therefore use an alternative method here which requires that all three relaxation times τ_i and amplitudes a_i are measurable. Defining the quantities

$$P_1 = \lambda_1 + \lambda_2 + \lambda_3 \tag{14}$$

$$P_2 = \lambda_1 \lambda_2 + \lambda_1 \lambda_3 + \lambda_2 \lambda_3 \tag{15}$$

$$P_3 = \lambda_1 \lambda_2 \lambda_3 \tag{16}$$

$$P_4 = a_1 \lambda_1 + a_2 \lambda_2 + a_3 \lambda_3 \tag{17}$$

$$P_5 = a_1 \lambda_1^2 + a_2 \lambda_2^2 + a_3 \lambda_3^2 \tag{18}$$

it may be shown by straightforward analysis (*see* Appendix A) that

$$k_{MS} = \frac{1}{2}\left(\frac{P_5}{P_4} - P_4\right) \tag{19}$$

$$k_D = \frac{1}{2k_{MS}}\left[\frac{P_1 P_5}{P_4} - P_2 + \frac{P_3}{P_4} - \left(\frac{P_5}{P_4}\right)^2\right] \tag{20}$$

$$k_S = \frac{1}{2k_D}\frac{P_3}{P_4} \tag{21}$$

$$k_R = \frac{1}{c_M}(P_1 - P_4 - 2k_S - 2k_{MS} - k_D) \tag{22}$$

$$N_0 = \frac{2RTC_m}{F^2}\frac{P_4}{k_{MS}}\left(1 + \frac{k_D}{c_M k_R}\right). \tag{23}$$

In this way the four rate constants k_R, k_D, k_S, k_{MS}, as well as the carrier concentration N_0 may be calculated from the five experimental quantities P_1, P_2, P_3, P_4 and P_5. The consistency of the analysis may be controlled by measuring the τ_i and the a_i at different ion concentrations c_M and different aqueous carrier concentrations c_0 and checking whether the calculated values of the rate constants are independent of c_M and c_0.

The Case $c_M k_R, k_D \gg k_S, k_{MS}$

A useful specialization of the above model concerns the case that the complexation reaction is always at equilibrium, i.e., that $c_M k_R$ and k_D are much larger than k_S and k_{MS}. Under these circumstances the explicit expressions for the τ_i and a_i become relatively simple. Since a discussion of this special case gives valuable insight into the mechanism of the relaxation process, we quote in the following the results of the calculation. If $c_M k_R$ and k_D are much larger than the other rate constants, then any perturbation of the system leads to a fast relaxation of the interfacial reaction, followed by slower relaxation processes that are governed by jumps across the central barrier. The shortest relaxation time is therefore given by $\tau_1 = 1/(c_M k_R + k_D)$. The amplitude a_1 [Eq. (12)] of the fast process, however, is zero, because a voltage change does not perturb the interfacial

equilibrium as long as $c_M k_R$ and k_D are voltage independent. The time constants τ_2, τ_3 and the amplitudes a_2, a_3 of the two slow relaxation processes may then be calculated from Eqs. (4)–(7), as shown in Appendix B. For the following we introduce the abbreviations

$$p=\frac{c_M k_R}{k_D}; \qquad b=\frac{F^2}{4RTC_m}.$$

p is the concentration ratio of complexed and uncomplexed carrier in the membrane. $1/b$ is the surface density of elementary charges that is needed to charge the membrane capacitance C_m to a voltage of $4RT/F$ ($1/b \simeq 4\times 10^{-13}$ mol cm$^{-2} \simeq 2\times 10^{11}$ cm^{-2}).

The result then reads

$$\lambda_1=\frac{1}{\tau_1}=c_M k_R+k_D \tag{24}$$

$$\lambda_2=\frac{1}{\tau_2}=\frac{k_S+pk_{MS}(1+bN_0)+|k_S+pk_{MS}(1-bN_0)|\sqrt{1+\vartheta}}{1+p} \tag{25}$$

$$\lambda_3=\frac{1}{\tau_3}=\frac{k_S+pk_{MS}(1+bN_0)-|k_S+pK_{MS}(1-bN_0)|\sqrt{1+\vartheta}}{1+p} \tag{26}$$

$$a_2=\begin{cases}\frac{1}{2}\left(1-\frac{1}{\sqrt{1+\vartheta}}\right) & \left(bN_0<1+\frac{k_S}{pk_{MS}}\right)\\ \frac{1}{2}\left(1+\frac{1}{\sqrt{1+\vartheta}}\right) & \left(bN_0>1+\frac{k_S}{pk_{MS}}\right)\end{cases} \tag{27}$$

$$a_3=1-a_2 \tag{28}$$

$$\vartheta=4bN_0\left[\frac{pk_{MS}}{k_S+pk_{MS}(1-bN_0)}\right]^2. \tag{29}$$

It is seen from Eqs. (27)–(29) that a_2 and a_3 are always positive (or zero); this means that $V(t)$ monotonically decreases with time after the charge pulse. (It should be noted, however, that the concentrations N_S', N_S'', N_{MS}', N_{MS}'' are nonmonotonous functions of time.)

For a discussion of Eqs. (25) and (26) it is useful to introduce the steady-state time-constant τ_m of the membrane, which is defined as the product of the specific ohmic membrane resistance R_m^0 in the steady state, times the specific membrane capacitance C_m. Introducing R_m^0 from Stark *et al.* (1971), τ_m is found to be (in the limit $c_M k_R, k_D \gg k_S, k_{MS}$):

$$\tau_m=R_m^0 C_m=\frac{p+1}{2bN_0}\left(\frac{1}{pk_{MS}}+\frac{1}{k_S}\right). \tag{30}$$

Depending on the amount of carrier present in the membrane, the value of the dimensionless quantity bN_0 varies within wide limits. To give a specific example: for a glyceryl-monooleate membrane in an aqueous solution of 10^{-7} M trinactin and 1 M KCl, a value of $bN_0 \simeq 3$ is calculated from the data of Benz and Stark (1975); but considerably smaller and larger values are also possible. It is therefore interesting to discuss the voltage relaxation in the limit of small and of large bN_0.

At small carrier concentrations ($bN_0 \ll 1$) Eqs. (25)–(28) reduce to

$$\tau_2 \approx \frac{p+1}{2(k_S + p k_{MS})} \tag{31}$$

$$\tau_3 \approx \tau_m \gg \tau_2 \tag{32}$$

$$a_2 \approx \frac{\vartheta}{4} \approx 0, \qquad a_3 \approx 1 - \frac{\vartheta}{4} \approx 1. \tag{33}$$

In this case one amplitude (a_2) vanishes so that only one relaxation process is observed which corresponds to the steady-state decay of a voltage across a parallel combination of C_m and R_m^0. This means that at low carrier concentration the information obtained from a charge-pulse experiment is already contained in a steady-state conductance measurement.

Feldberg and Kissel (1975) used the charge-pulse method to study the steady-state conductance of bilayer membranes in the presence of ion carriers by measuring the slowest relaxation process (corresponding to τ_3). For the analysis of their data they assumed that the membrane is in a true quasistationary state during the slow phase of the voltage decay. This seems justified in the limit $bN_0 \ll 1$; in the general case, however, even the slowest relaxation process is influenced by a redistribution of MS^+ between the two interfaces. Indeed, under the assumptions of our calculations (small voltage, Eyring-barrier, fast complexation reaction) Eqs. (11) and (12) of Feldberg and Kissel (1975) reduce to $V_m/dt = V_m/\tau_m$ which corresponds to the case $bN_0 \ll 1$ [Eqs. (30)–(33)], whereas for arbitrary bN_0, τ_3 is different from τ_m [Eq. (26)]. In the analysis of Feldberg and Kissel the redistribution of MS^+ may be allowed for by a suitable correction (S. W. Feldberg, *personal communication*).

If the carrier concentration is large ($bN_0 \gg 1$, $bN_0 \gg k_S/pk_{MS}$) Eqs. (25)–(28) assume the form

$$\tau_2 \approx \frac{p+1}{2pk_{MS}bN_0} \tag{34}$$

$$\tau_3 \approx \frac{p+1}{2k_s} \gg \tau_2 \tag{35}$$

$$a_2 \approx 1 - \frac{1}{bN_0} \approx 1, \quad a_3 \approx \frac{1}{bN_0} \approx 0. \tag{36}$$

In this case the amplitude of the slowest relaxation process (a_3) vanishes.

Materials and Methods

Monoolein was obtained from Nu Check Prep (Elysian, Minnesota). The sample contained about 98% of the α-isomer and gave a single spot in a thin-layer chromatogram. *n*-decane was from Merck (standard for gas chromatography). Valinomycin was obtained from Calibiochem (San Diego, California) and was dissolved in ethanol. Identical amounts of the concentrated ethanolic stock solution were added to the aqueous compartments of the membrane cell to get a final concentration between 5×10^{-9} and 2×10^{-7} M. The ethanol concentration in the aqueous phases never exceeded 0.1% (v/v). The pH of the unbuffered aqueous solutions was about 6; the ionic strength was held constant by addition of LiCl as an inert electrolyte.

Optically black lipid membranes were formed in the usual way (Läuger, Lesslauer, Marti & Richter, 1967) from a 1–2% (w/v) solution of monoolein in *n*-decane. The circular membranes had a diameter of either 2 or 3 mm.

The charge-pulse experiment was performed in the following way. The membrane was charged up to a voltage of about 10 mV (or less) by a brief current pulse. For this purpose a variable voltage source (output voltage 10 mV – 5 V) was connected to the membrane cell during 20–50 nsec by means of a fast FET-switch (2N5653, Pan Elektronik, Taufkirchen, G.F.R.). The impedance of the switch in the "open" position was larger than $10^{12}\,\Omega$. The switch was triggered repetitively by a separate, battery-operated impulse generator with waiting periods between the pulses of at least 20 times the longest relaxation time of the membrane. The voltage transient across the membrane was amplified with an Analog Devices Mod. 46 K preamplifier (bandwith 10 MHz) and recorded with a storage oscilloscope (Tectronix 7633 with amplifier 7A 13). The original photographs of the oscillographic records were subsequently enlarged to 15 × 20 cm in order to match the resolution of the digitizer (Hewlett Packard 9864 A). The digitized data from the voltage versus time curves were further analyzed (*see below*) with a Hewlett Packard 9820 A calculator (with plotter 9862 A).

The current was applied to the membrane through a pair of platinized platinum electrodes of large area (about 1 cm^2 each electrode). Pairs of electrodes were selected such that the asymmetry voltage at zero current was less than 0.5 mV. In some experiments the voltage was measured with a separate pair of electrodes.

The performance of the whole set-up was carefully tested with dummy circuits. In one set of experiments the cell was replaced by a resistor (representing the resistance of the solutions and the electrodes) in series with a parallel combination of a resistor and a capacitor of the appropriate values (the "membrane"). In a second set of test experiments the arrangement of cell, membranes and electrodes was the same as used later in the valinomycin experiments, but the membrane was formed in a 1 M KCl solution without carrier and the carrier-induced conductance was simulated by introducing a resistor in parallel to the membrane. In all these cases a purely exponential decay of the voltage with the expected time constant was observed after the charging pulse; examples are given in Figs. 1 and 2.

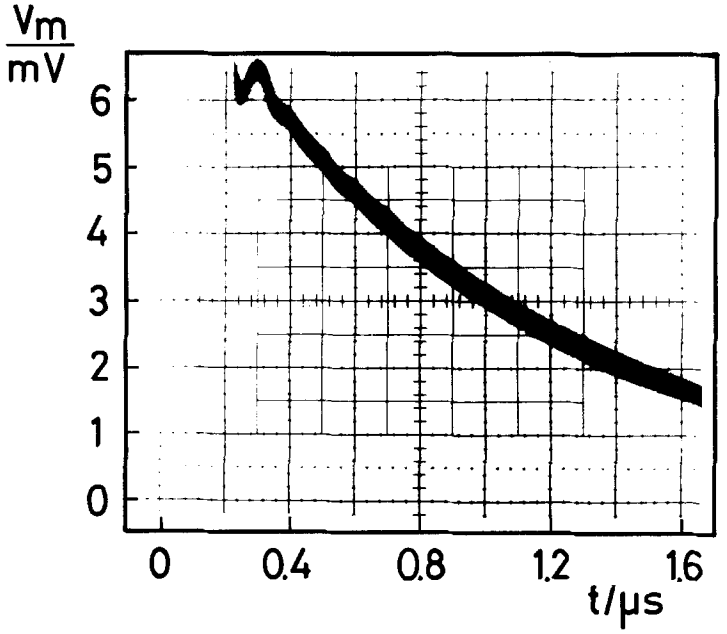

Fig. 1. Oscillographic record of a test experiment with a monoolein/*n*-decane membrane in 1 M KCl (solution resistance 30 Ω). The membrane capacitance was $C_M \simeq 10$ nF (membrane area $A \simeq 2.6\,\text{mm}^2$), the membrane resistance was of the order of $10^8\,\Omega$; an external resistance $R_e = 100\,\Omega$ was introduced parallel to C_M. At time $t=0$ a charging pulse of about 50 nsec duration was applied to the membrane. For times $t > 400$ nsec the decay of the membrane voltage V_m is purely exponential with a time constant of 0.99 μsec which agrees with the calculated time constant $\tau = R_e C_M = 1.0$ μsec. The distortion of the signal at $t < 400$ nsec is due to oscillations of the FET switch in the charging circuit

In this way it has been established that the electrodes themselves do not introduce voltage transients in the time range considered here and that the distortion of the signal by the amplifier system is negligible. The time resolution of the set-up was limited by oscillations of the FET switch at the termination of the charging pulse and was about 400 nsec.

As discussed in the previous section, the decay of the membrane voltage V_m is governed by three relaxation times $\tau_1 < \tau_2 < \tau_3$:

$$V_m(t) = V_1 \exp(-t/\tau_1) + V_2 \exp(-t/\tau_2) + V_3 \exp(-t/\tau_3). \tag{37}$$

The relaxation times τ_i and the voltage amplitudes V_i were evaluated from the record of $V_m(t)$ in the following way. For long times ($t \gg \tau_2$) the relation $V_m(t) \approx V_3 \exp(-t/\tau_3)$ holds; accordingly, a plot of $ln[V_m(t)]$ at long times t gives a straight line from which τ_3 and V_3 are obtained. If then the logarithm of the difference $V_m(t) - V_3 \exp(-t/\tau_3)$ is plotted as function of t, again a straight line results at sufficiently long times which gives τ_2 and V_2. Repetition of this procedure with $V_m(t) - [V_2 \exp(-t/\tau_2) + V_3 \exp(-t/\tau_3)]$ finally yields τ_1 and V_1. In each case the regression line is calculated using a least-squares fit program. An example is given in Fig. 4. The accuracy of this method is highest if the relaxation times τ_i are widely separated and if the voltage amplitudes V_i are of comparable magnitudes.

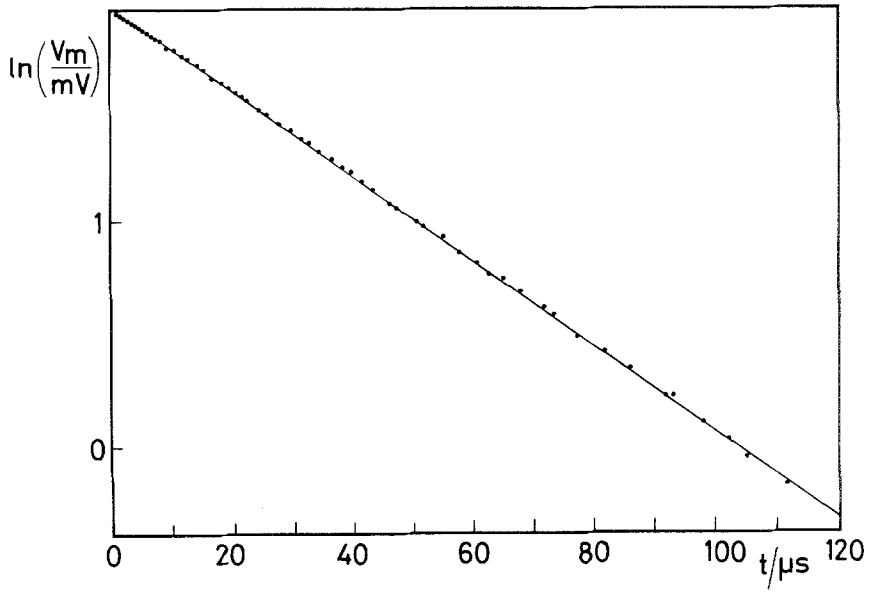

Fig. 2. Computer plot of the logarithm of the membrane voltage V_m as a function of time t. The conditions of the experiment were the same as in Fig. 1 except that an external resistance of $R_e = 5\,\mathrm{k\Omega}$ was used. The regression line corresponds to a time constant of 52.9 μsec (correlation coefficient 0.99991)

Results

Oscillographic records of a charge-pulse experiment in the presence of valinomycin and Rb^+ are represented in Fig. 3. As the decay of the membrane voltage V_m extends over a wide time range, V_m was recorded with different sweep times. The values of $V_m(t)$ from the different records were plotted on a logarithmic scale and were analyzed as described in the previous section. This is shown in Fig. 4 for the data of Fig. 3. From three successive plots (A, B, and C) the three relaxation times τ_i and the voltage amplitudes V_i [Eq. (37)] were evaluated by calculating the regression line that gave the best fit to the plotted points in the limit of long times.

In this way the values of the parameters $\tau_i = 1/\lambda_i$ and $a_i = V_i/V_m^0$ were determined for each set of experimental conditions (Table 1). By inserting the λ_i and a_i into Eqs. (14)–(23), the rate constants k_R, k_D, k_{MS}, k_S and the total carrier concentration N_0 in the membrane could be calculated. The results are summarized in Table 2 which also contains the distribution coefficient γ_S of the uncomplexed carrier S:

$$\gamma_S = \frac{2N_S}{c_S d}. \tag{38}$$

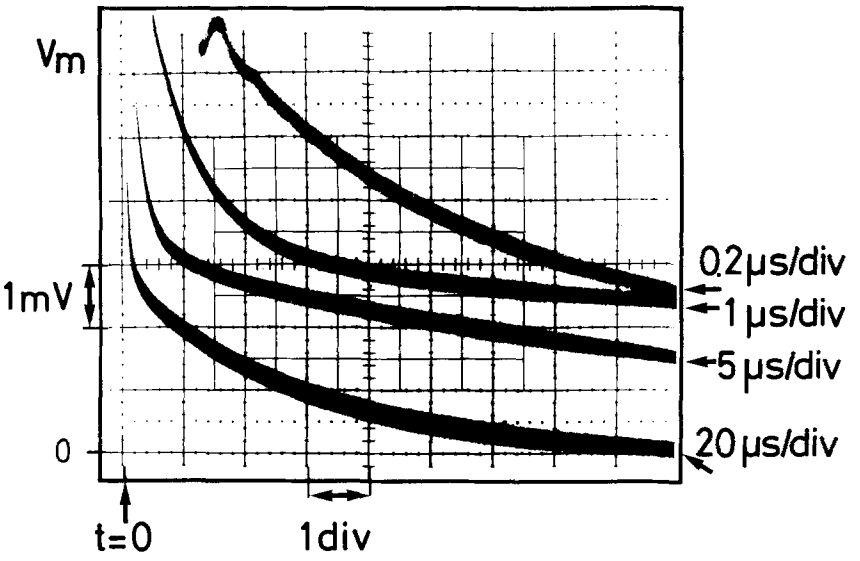

Fig. 3. Decay of the membrane voltage V_m after a charge pulse. Monoolein/*n*-decane membrane in 2×10^{-7} M valinomycin and 1 M RbCl; $T=25$ °C; membrane area $A=2$ mm^2. At time $t=0$ the membrane capacitance was charged up by a current pulse of about 50 nsec duration to a voltage of $V_m^0=10.2$ mV. A repetitive pulse sequence was used with waiting times of 1 msec between pulses. The decay of V_m was recorded with different sweep times, as indicated on the right side of the oscillogram. The base lines of the records at 5 μsec/div, 1 μsec/div and 0.2 μsec/div were shifted by arbitrary amounts. The base line for 5 μsec/div is at 0.32 mV, for 1 μsec/div at −0.05 mV and for 0.2 μsec/div at −1.35 mV

N_S is the interfacial concentration of S at equilibrium ($N_S'=N_S''=N_S$):

$$N_S=\frac{N_0}{2}\left(1+\frac{c_M k_R}{k_D}\right). \tag{39}$$

$d\simeq5$ nm is the membrane thickness and c_S the concentration of S in the aqueous phase. At 25 °C, where complexation between valinomycin and alkali ions in water is negligible up to $c_M=1$ M (Stark and Benz, 1971), c_S could be set equal to the total carrier concentration c_0 in water. Only in the experiments with 1 M rubidium chloride at 10 °C the complexation in water had to be taken into account; from the data of Knoll and Stark (1975) the complexation constant was estimated to be ~1.5 M^{-1}.

It is seen from Table 1 that the three relaxation amplitudes a_1, a_2, a_3 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 values of the rate constants k_D, k_{MS}, and k_S which are calculated from Eqs. (19)–(23) are fairly constant over the whole range of experimental conditions (the agreement at $c_0=5$ nM is likely to be

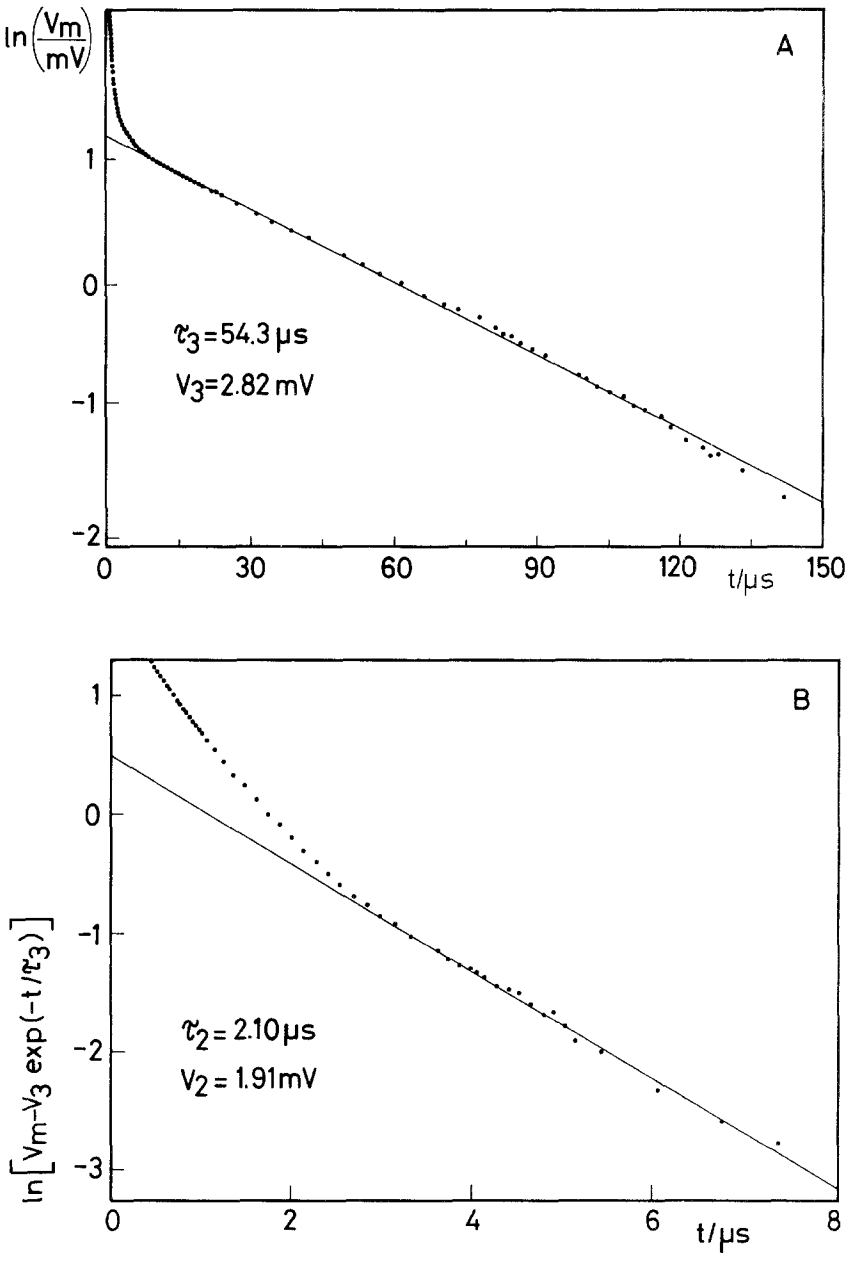

Fig. 4. Analysis of the data taken from Fig. 3. As described in Materials and Methods, the relaxation times τ_i and the voltage amplitudes V_i [Eq. (37)] were evaluated from the three successive plots *A*, *B*, and *C*. In each case the regression line which was drawn gave the best fit to the plotted points in the limit of long times t. The correlation coefficients were calculated to be $r=0.9995$ (plot *A*, $t>15$ μsec), $r=0.9969$ (plot *B*, $t>3$ μsec), $r=0.9997$ (plot *C*, $t>0.5$ μsec)

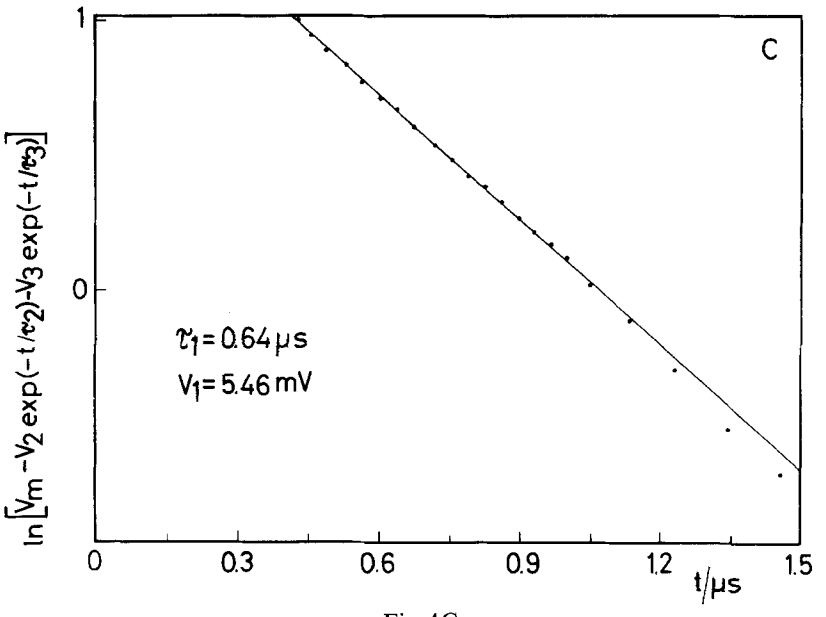

Fig. 4C

Table 1. Relaxation times τ_i and relative relaxation amplitudes a_i from charge-pulse experiments with monolein/*n*-decane membranes at different ion concentrations c_M and different valinomycin concentrations c_0 in the aqueous phase[a]

c_0 (nM)	c_M (M)	τ_1 (µsec)	τ_2 (µsec)	τ_3 (µsec)	a_1	a_2	a_3
			KCl, 25 °C				
100	1.0	0.865	2.59	51.9	0.295	0.300	0.405
			RbCl, 25 °C				
5	1.0	0.868	3.14	675	0.013	0.042	0.950
10	1.0	0.854	3.16	299	0.035	0.069	0.896
50	1.0	0.823	2.75	83.0	0.109	0.219	0.672
100	1.0	0.696	2.04	63.1	0.357	0.236	0.407
200	1.0	0.599	2.01	44.4	0.518	0.202	0.281
100	0.30	0.939	3.10	47.6	0.293	0.267	0.440
100	0.10	0.711	3.29	36.2	0.182	0.307	0.510
100	0.03	0.878	4.26	30.2	0.093	0.190	0.718
100	0.01	0.989	4.70	30.5	0.102	0.097	0.801
			RbCl, 10 °C				
100	1.0	3.35	7.52	414	0.374	0.195	0.431
			CsCl, 25 °C				
100	1.0	0.821	3.66	33.1	0.102	0.407	0.491

[a] The ionic strength has been held constant at 1 M by addition of LiCl. The values of τ_i and a_i are mean values that have been determined from five to nine membranes for each set of experimental conditions.

Table 2. Rate constants k_R, k_D, k_{MS}, k_S of valinomycin-mediated cation transport through monoolein/*n*-decane membranes as calculated from the data of Table 1[a]

c_0 (nM)	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 (10^{-14} mol cm^{-2})	γ_S (10^3)	n
			KCl, 25 °C					
100	1.0	29± 4	27± 6	21± 4	3.8±0.8	78 ±40	7.5	9
			RbCl, 25 °C					
5	1.0	42± 20	25±19	32±15	2.8±1.6	3.2± 2.5	4.8	5
10	1.0	29± 7	25±14	35±15	3.9±1.6	5 ± 3	4.6	6
50	1.0	30± 6	23±14	27± 5	4.4±0.8	30 ±15	5.2	6
100	1.0	37± 12	24± 6	27± 3	3.5±0.4	68 ±30	5.3	7
200	1.0	32± 3	23± 4	29±10	4.6±0.9	130 ±40	5.4	9
100	0.30	66± 17	21±10	25± 7	4.0±0.6	80 ±26	8.2	8
100	0.10	190± 40	38±15	35±13	4.5±1.0	61 ±20	8.1	8
100	0.03	430±120	43±14	30± 8	5.6±2.0	65 ±30	10.0	6
100	0.01	630±300	31± 3	29± 5	7.8±0.5	52 ±25	8.6	7
			CsCl, 25 °C					
100	1.0	22± 4	56±10	25± 8	4.1±0.5	72 ±30	10.3	8
			RbCl, 10 °C					
100	1.0	11±2	3.7±2	6.3±0.7	0.98±0.3	65 ±20	8.2	9
			RbCl, 10 °C (Knoll & Stark, 1975)					
[b]	1.0	8.1	1.7	5.5	1.0	–	7.9	

[a] The partition coefficient γ_S of the free carrier has been determined from N_0 according to Eqs. (38) and (39). For the membrane capacity C_m [Eq. (23)] a value of 0.390 μF/cm^2 has been used (Benz, Fröhlich, Läuger & Montal, 1975). In addition to the standard deviations, the number n of membranes used for each set of experimental conditions is indicated.

[b] Valinomycin added to the membrane-forming solution (5×10^{-4} M).

fortuitous, because the small value of a_1 leads to relatively large errors). This finding is consistent with the carrier model used for the analysis if it is assumed that the single carrier molecules act independently of each other. A somewhat larger variation (by about a factor of two) is observed in the case of the partition coefficient γ_S. This variation presumably originates from the difficulty to establish partition equilibrium between the membrane and the aqueous phase. The association rate constant k_R is also found to be independent of the carrier concentration c_0, but it shows a very pronounced variation with ion concentration c_M. A decrease of k_R with increasing c_M has already been observed by Knoll and Stark (1975) for valinomycin-mediated rubidium transport in monoolein mem-

Table 3. Activation energies E of the rate constants k_R, k_D, k_{MS} and k_S (100 nM valinomycin, 1 M Rb^+)

$E(k_R)$ (kJ mol^{-1})	$E(k_D)$ (kJ mol^{-1})	$E(k_{MS})$ (kJ mol^{-1})	$E(k_S)$ (kJ mol^{-1})
57	87	68	60

branes; as a possible explanation they proposed that a finite number of sites for the complexation reaction exists which become saturated at high c_M.

Knoll and Stark (1975) determined the rate constants of the valinomycin/Rb^+ system at 10 °C using the voltage-jump method. For the purpose of comparison, we have also carried out a few experiments at 10 °C with the charge-pulse technique. The values of the rate constants, as obtained by the two different methods, are in satisfactory agreement, as Table 2 shows.

From the two sets of measurements at 10 and 25 °C the activation energies E of the rate constants may be estimated (Table 3). In view of the uncertainties in the determination of γ_S, the difference in the γ_S values between 10 and 25 °C should be regarded as insignificant. It may be concluded that the enthalpy change ΔH associated with the transfer of valinomycin from water to the monoolein membrane is likely to be small ($|\Delta H| \lesssim 20$ kJ/mol); a larger value ($\Delta H = -100$ kJ/mol) has been observed with dipolmitoleoyllecithin membranes (Benz *et al.*, 1973).

Discussion

In this study we have tested the possibility to apply the charge-pulse method to the analysis of fast transport processes in lipid bilayer membranes. The charge-pulse technique has been used previously for the investigation of quasistationary processes in membranes which required only a moderate time resolution. We have demonstrated here that using relatively simple instrumentation a time resolution of about 400 nsec may be achieved and that the charge-pulse technique may be applied to the kinetic analysis of carrier-mediated ion transport in thin lipid membranes.

The method which we have used for the analysis of the experimental data requires that all three relaxation processes predicted from the model can be resolved. This presents difficulties in cases where one or two of the relaxation amplitudes a_i become too small or where the relaxation times

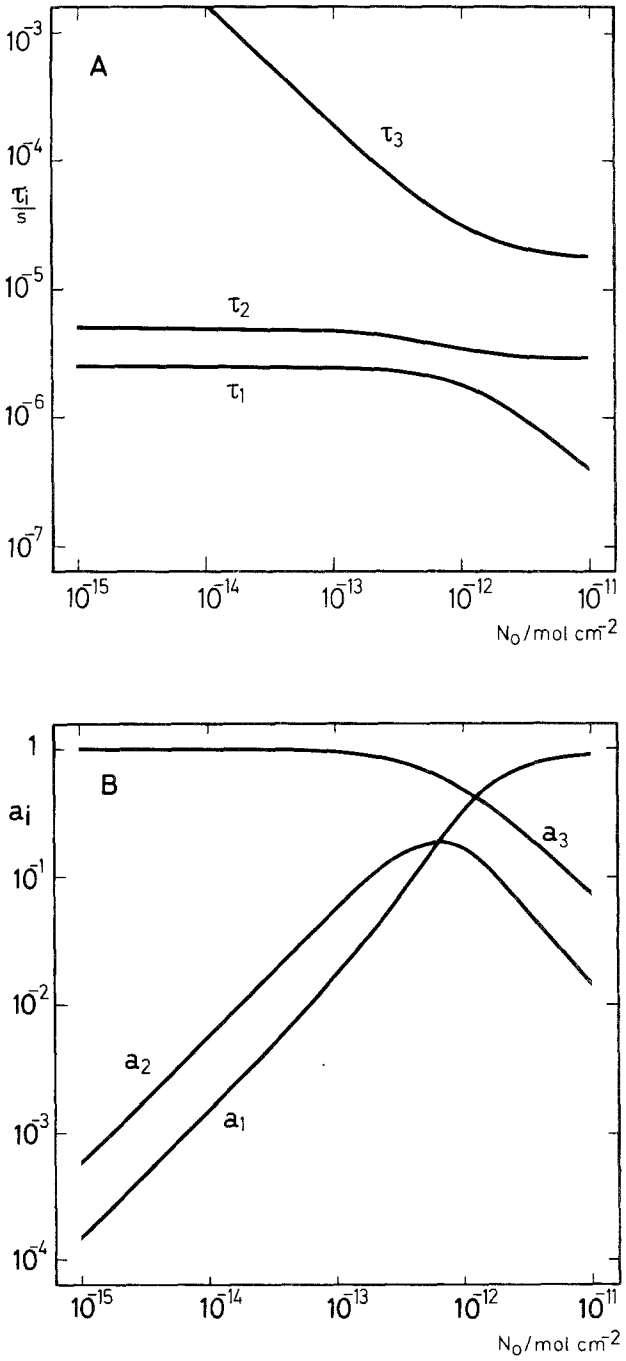

Fig. 5. Relaxation times $\tau_i(A)$ and relaxation amplitudes $a_i(B)$ as a function of the total carrier concentration N_0 in the membrane. The τ_i and a_i have been calculated from Eqs. (A 8)–(A 11), (A 16) and (A 19) for a given set of rate constants ($c_M k_R = k_D = k_{MS} = k_S = 10^5$ sec^{-1}). For the membrane capacity a value of $C_m = 0.40\,\mu$F/cm^2 has been used. $T = 298$ °K

τ_i are not sufficiently different. Since the τ_i and the a_i depend on the experimental conditions, this problem may usually be overcome by a suitable choice of the carrier concentration in the membrane. To illustrate this point we have plotted in Fig. 5 the relaxation times and amplitudes as functions of the total carrier concentration N_0 in the membrane for an arbitrarily chosen set of rate constants. It is seen that the longest relaxation time τ_3 and the three amplitudes a_1, a_2, a_3 strongly depend on N_0 (at small N_0, τ_3 is proportional to the membrane resistance and therefore inversely proportional to N_0). For a reasonable accuracy of the analysis, the a_i values should be larger than 0.02, provided that the τ_i differ by at least a factor of three.

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Appendix A

Derivation of Eqs. (19)–(23)

Introducing the variables:

$$r = N'_S + N''_S, \qquad s = N'_{MS} + N''_{MS}$$

we obtain from Eqs. (4)–(8):

$$\frac{dr}{dt} = -(c_M k_R + k_D) r + N_0 k_D \tag{A1}$$

or (with $1/\tau = c_M k_R + k_D$):

$$r(t) = r(\infty) + [r(0) - r(\infty)]\, e^{-t/\tau}. \tag{A2}$$

For the stationary state ($dr/dt = 0$) Eq. (A1) yields

$$r = N_0 \frac{k_D}{c_M k_R + k_D}. \tag{A3}$$

In the charge-pulse experiment $r(\infty)$ is identical with $r(0)$. According to Eq. (A2), this means that r is a constant and is given by Eq. (A3). In a similar way the relation

$$s(t) \equiv s = N_0 \frac{c_M k_R}{c_M k_R + k_D} \tag{A4}$$

is obtained. With

$$y_1 = N_S' - N_S'', \quad y_2 = N_{MS}' - N_{MS}'', \quad y_3 = u,$$

Eqs. (4)–(11) yield the following system of differential equations:

$$\frac{dy_i}{dt} = \sum_{j=1}^{3} A_{ij} y_j \quad (i=1, 2, 3) \tag{A5}$$

where

$$\begin{aligned} &A_{11} = -(c_M k_R + 2k_S); \quad && A_{12} = k_D; && A_{13} = 0 \\ &A_{21} = c_M k_R; && A_{22} = -(k_D + 2k_{MS}); && A_{23} = -sk_{MS} \\ &A_{31} = 0; && A_{32} = -4bk_{MS}; && A_{33} = -2bsk_{MS} \end{aligned}$$

$$b = \frac{F^2}{4RTC_m}.$$

The solution of Eqs. (A5) which fulfills the boundary condition $y_i(\infty)=0$ has the form

$$y_i(t) = \sum_{j=1}^{3} B_{ij} e^{-\lambda_j t} \quad (i=1, 2, 3) \tag{A6}$$

where the λ_j are the roots of the characteristic equation

$$\mathrm{Det}\,(A_{ij} + \delta_{ij}\lambda) = 0. \tag{A7}$$

δ_{ij} is Kronecker's delta ($\delta_{ij}=0$ for $i \neq j$ and $\delta_{ij}=1$ for $i=j$). Eq. (A7) may be written as

$$\lambda^3 - P_1\lambda^2 + P_2\lambda - P_3 = 0 \tag{A8}$$

$$P_1 = c_M k_R + k_D + 2k_S + 2k_{MS} + 2bsk_{MS} \tag{A9}$$

$$P_2 = 2k_{MS}(c_M k_R + 2k_S)(bs+1) + 2k_D(k_S + bsk_{MS}) \tag{A10}$$

$$P_3 = 4bsk_D k_S k_{MS}. \tag{A11}$$

According to Vieta's theorem, P_1, P_2, and P_3 may be expressed by the roots λ_j:

$$P_1 = \lambda_1 + \lambda_2 + \lambda_3 \tag{A12}$$

$$P_2 = \lambda_1\lambda_2 + \lambda_1\lambda_3 + \lambda_2\lambda_3 \tag{A13}$$

$$P_3 = \lambda_1\lambda_2\lambda_3 \tag{A14}$$

[compare Eqs. (14)–(16)].

With the initial conditions $y_1(0)=y_2(0)=0$, $y_3(0)=u_0=V_m^0 F/RT$, one finds from Eqs. (A5) and (A6):

$$-\left(\frac{dy_3}{dt}\right)_{t=0}=2bsk_{MS}u_0=\lambda_1 B_{31}+\lambda_2 B_{32}+\lambda_3 B_{33}. \tag{A15}$$

As $B_{3j}/u_0=a_j$ $(j=1,2,3)$ [Eq. (12)], one obtains

$$P_4=a_1\lambda_1+a_2\lambda_2+a_3\lambda_3=2bsk_{MS} \tag{A16}$$

[compare Eq. (17)]. Finally, an expression for P_5 may be derived in the following way. In analogy to Eq. (A15) one obtains from the initial conditions:

$$-\left(\frac{dy_2}{dt}\right)_{t=0}=sk_{MS}u_0=\lambda_1 B_{21}+\lambda_2 B_{22}+\lambda_3 B_{23}. \tag{A17}$$

Furthermore, introduction of the solutions (A6) into $dy_3/dt=A_{31}y_1+A_{32}y_2+A_{33}y_3$ yields the following relations:

$$B_{2j}=B_{3j}\frac{\lambda_j-2bsk_{MS}}{4bk_{MS}} \quad (j=1,2,3). \tag{A18}$$

Multiplication of both sides with λ_j and summation over j gives, according to Eqs. (A15) and (A17):

$$4bsk_{MS}^2(bs+1)=a_1\lambda_1^2+a_2\lambda_2^2+a_3\lambda_3^2=P_5 \tag{A19}$$

[compare Eq. (18)]. The relations (19)–(23) are then obtained by consecutive elimination of k_{MS}, k_D, k_S, k_R, and N_0 from Eqs. (A12)–(A14), (A16), and (A19).

Appendix B

Derivation of Eqs. (25)–(29)

If the rates of association and dissociation of the complex are much larger than the translocation rates, then during the whole relaxation process the relation

$$\frac{N'_{MS}}{N'_S}=\frac{N''_{MS}}{N''_S}=\frac{c_M k_R}{k_D}=p \tag{B1}$$

holds. Introducing new variables

$$v=\frac{N_S'+N_{MS}'}{N_0}-\tfrac{1}{2},$$

$$z=2bN_0\frac{pk_{MS}}{p+1}t,$$

Eqs. (4)–(11) reduce to

$$\frac{du}{dz}=-u-4v \tag{B2}$$

$$\frac{dv}{dz}=-\frac{1}{4bN_0}u-\frac{k_S+pk_{MS}}{bN_0pk_{MS}}v. \tag{B3}$$

The functions $u(z)$ and $v(z)$ have to fulfill the boundary conditions

$$u(0)=\frac{V_m^0F}{RT}, \qquad u(\infty)=0$$

$$v(0)=v(\infty)=0.$$

The solution of Eqs. (B2) and (B3) which is obtained by standard methods may be written in the form ($V_m=uRT/F$):

$$V_m(t)=V_m^0(a_2e^{-\lambda_2t}+a_3e^{-\lambda_3t}) \tag{B4}$$

where λ_2, λ_3, a_2, and a_3 are given by Eqs. (25–(29).

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