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A LASER-TEMPERATURE-JUMP METHOD FOR THE STUDY OF THE RATE OF TRANSFER OF HYDROPHOBIC IONS AND CARRIERS ACROSS THE INTERFACE OF THIN LIPID MEMBRANES

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The first application of a laser-temperature-jump apparatus for the study of ion transport through planar (artificial) lipid membranes is described. The relaxation of the electric current is detected, either continuously at a constant applied voltage or discontinuously by a series of short voltage pulses. The second technique, a combined voltage- and temperature-jump method, is especially appropriate to investigate the kinetics of the adsorption/desorption process of hydrophobic ions and neutral carriers of cations at the membrane interface and to separate this phenomenon from the diffusion process through the unstirred aqueous layers adjacent to the membrane. The aim is to determine the rate-limiting step of transport. The permeation rate of the hydrophobic anion 2,4,6-trinitrophenolate is limited by the inner membrane barrier. For tetraphenylberate the rate constant of translocation across the inner barrier and that of desorption from the membrane into water are found to be of comparable magnitude. The membrane permeability of the neutral macrocyclic ion carrier enniatin B is strongly interface limited by its comparatively small rate of desorption into water. These results show that the frequently used a priori assumption of partition equilibrium at the membrane interfaces during transport is not justified.

1. Introduction

The permeation of hydrophobic substances through cell membranes is frequently interpreted on the basis of two physical quantities only, the partition coefficient between membrane and water (i.e. the membrane solubility of the substance) and the diffusion coefficient inside the membrane. As a consequence, the permeability coefficient P is proportional to the product of both quantities. A measurement of P permits determination of the diffusion coefficient if assumptions are made concerning the partition coefficient (e.g. by identifying it with the partition coefficient of a suitable macroscopic two-phase system, such as oil/water) [1–5].

The simple approach to a physical interpretation of membrane permeability is, however, only correct if the partition equilibrium at the membrane/water interface is maintained during transport. This means that the adsorption/desorption process at the interface must be fast compared to the diffusion process across the membrane interior [6]. The validity of this assumption is difficult to verify in practice, as may be concluded from the lack of appropriate data in the literature. Indirect evidence for a limitation of membrane permeability through the interfacial adsorption/desorption process has been obtained for some substances and membranes [5,6]. A rigorous determination of the rate-limiting step in transport requires the application of fast kinetic methods. We are studying the permeation of hydrophobic ions and neutral ion carriers through artificial lipid membranes. We have recently shown [7] that a detailed analysis of the current relaxation following a voltage jump – at least in principle – allows the determination of both the rate of translocation of hydrophobic ions across the membrane interior and also their rate of desorption from the membrane. In practice, however, this analysis provides fairly accurate data for the rate of translocation,

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while it is more difficult to separate the adsorption/desorption process from the diffusion process in the aqueous phases adjacent to the membrane.

In the present paper we analyse the adsorption/desorption process on the basis of temperature-jump relaxation experiments. It has been shown previously that this technique, well known from the study of fast reactions in homogeneous solutions, may also be applied to investigate ion transport through planar lipid membranes [8]. In that study the temperature jump was produced by absorption of an intense light flash. The original setup has been improved by using a laser instead of a flash lamp. In addition, a new technique — a combined voltage- and temperature-jump method — is introduced which allows to distinguish membrane phenomena from diffusion in the unstirred layers.

We find that the a priori assumption of a fast partition equilibrium between membrane and water is not justified for hydrophobic substances. The identification of the rate-limiting step in transport has to be established separately for each species.

2. Materials and methods

2.1. Methods

The temperature-jump method has become an important tool for the study of the mechanism of fast reactions [9,10]. In spite of the numerous applications to homogeneous solutions this method has been rarely used to investigate the molecular basis of ion transport through biological membranes [11,12]. For temperature-jump experiments with planar (artificial) lipid membranes we have so far applied two different versions of this technique: a slow version using two (three) thermostats held at different temperatures [13] and a fast one employing light-induced T-jumps [8]. As light source a flash-lamp was used with an emission spectrum extending over the complete UV and visible region. The T-jumps obtained were, however, relatively small (0.1–0.5°C), although a dye was added to the aqueous solutions to improve light absorption. Furthermore T-jumps >0.1°C were only achieved after a relatively difficult procedure of light focusing.

We have therefore tried to improve the method by installing a high-intensity Nd-glass laser (JK Lasers Ltd., GB). It is used in all experiments described here. The laser operates in the near infrared (emission wavelength 1.06 μm). It can emit two types of pulses: in the fixed Q mode the pulses are 400 μs long and have a maximum energy of 20 J, corresponding to an intensity of 2×10^4 W/cm^2. Alternatively, in the Q switch mode 40 ns pulses are emitted with a maximum energy of 1.5 J per pulse ($I \approx 4 \times 10^7$ W/cm^2). Higher intensities are obtained by focusing the laser beam.

As has been pointed out in the literature, the temperature jumps achieved with Q-switched Nd-radiation are only a few tenths of a degree [14,15]. Therefore, those authors used the stimulated Raman effect to shift the wavelength of the radiation to regions where water absorbs more strongly. However in the fixed Q mode, the intrinsic absorption of water at the unshifted wavelength is large enough to yield sufficiently high T-jumps [16]. The intensity is well below the level at which photochemical artifacts have to be considered; however, in the Q-switch mode photodissociation and other photoeffects may occur [17–19]. Since there are further artifacts produced by the high intensity of light in the Q-switch mode (see below), only the fixed Q mode is employed in the present study. A drawback, of course, is the relatively poor maximum time resolution of 400 μs given by the duration of the light pulse.

The relaxation of the membrane conductance following a temperature jump is studied using the apparatus illustrated in fig. 1. Either a constant voltage or a series of short voltage pulses can be applied to the

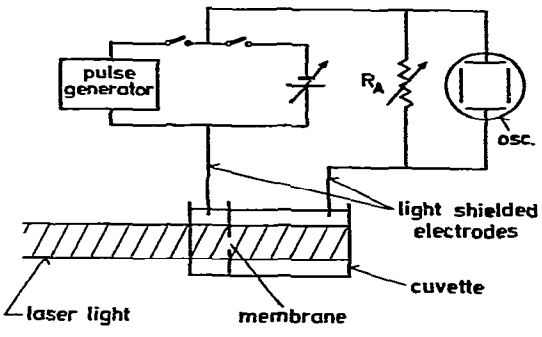

Fig. 1. Schematic diagram of the experimental arrangement for laser-temperature-jump measurements on planar lipid membranes. For higher intensities the laser beam may be focused.

membrane via two Ag—AgCl electrodes. The time dependence of the current is measured with a storage oscilloscope (Tektronix 549) via the voltage drop across an external resistor R_A. To avoid photoartifacts at the electrodes, these are light shielded and placed in separate vessels connected to the cuvette by salt bridges (not shown in fig. 1).

Fig. 2 illustrates the basic features of the voltage

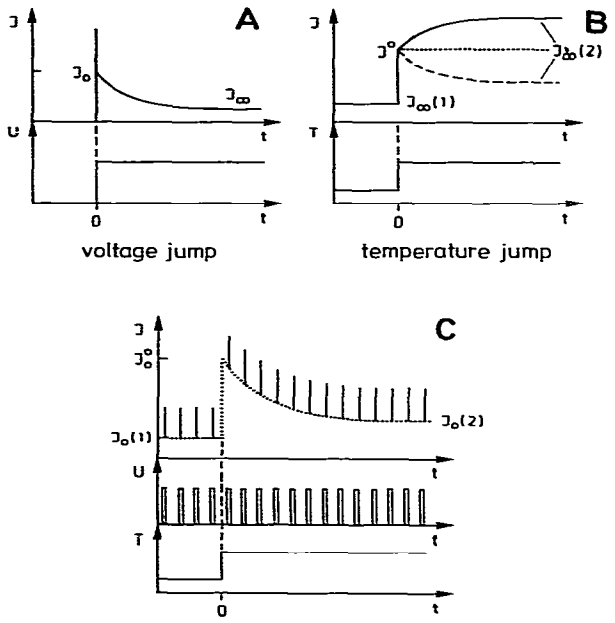

Fig. 2. Principle of the kinetic methods. (A) Typical current relaxation following a voltage jump as obtained with hydrophobic ions and ion carriers (see refs. [20,7,26]). The initial current spike is due to the charging of the membrane capacitance. The actual relaxation of the transport system starts at J_0. (B) Temperature-jump current-relaxation method at constant voltage. The T-jump at $t = 0$ generates a transition from the steady-state current $J_\infty(1)$ to the new current level $J_\infty(2)$. (C) Combined voltage- and temperature-jump method. Here the T-jump is superimposed on a series of short voltage pulses. The method permits monitoring the T-jump-induced relaxation of the initial current J_0 after a voltage jump (see text for details). The ground level and the spikes from the discharge of the membrane capacitance at the end of the pulses are suppressed. Only one of the two possible relaxation curves from J_0^0 to J_0 (2) is shown (cf. fig. 2B).

jump, the temperature jump and a combined version of these methods. In all cases the relaxation of the electric current is observed. The relaxations shown are typical for hydrophobic ions and neutral ion carriers.

The voltage-jump method (fig. 2A) has been described in detail in previous publications [20,21,7]. The "normal" temperature-jump method (fig. 2B) can only be applied if a steady-state current exists at fixed applied voltage. This condition is satisfied for systems where diffusion through the unstirred aqueous layers adjacent to the membrane is fast enough not to influence the ion transport through the membrane.

If unstirred layer effects (diffusion polarization) cannot be neglected, a steady-state current will not be reached [20]. For this case, a combined voltage- and temperature-jump method has been developed (fig. 2C). The time course of the membrane conductance following a temperature jump is monitored by a series of voltage jumps. The duration of the pulses is chosen so that the initial current spike (arising from the loading of the membrane capacity) is cut off directly below J_0 (see fig. 2A). At J_0 the current spike is completed, but the actual relaxation of the current, which is due to the combined action of membrane processes and unstirred layer diffusion, has not yet started (compare fig. 2A). In this way, the initial current J_0 is obtained, a quantity unaffected by the unstirred layers. J_0 is proportional to the ion concentration inside the membrane [see section 3.1.2, eq. (18)]. Thus, the relaxation of J_0 is measured, reflecting the changes in the membrane concentration of the substance under study.

A detailed discussion of the current response after a temperature jump for both the T-jump at constant voltage and the combined V- and T-jump is given in section 3. Qualitatively, the initial current increase from $J_\infty(1)$ to J^0 [and from $J_0(1)$ to J_0^0] is caused by an instantaneous increase of the ion mobility inside the membrane; the subsequent relaxation from J^0 to $J_\infty(2)$ [or from J_0^0 to $J_0(2)$] reflects the temperature-dependent decrease (or increase) of the concentration inside the membrane. Thus, the relaxation contains information on the adsorption/desorption process at the interface. In our present study it is this special aspect of transport which primarily interests us.

The magnitude of the temperature jumps normally used ranges from 0.3 (unfocused laser beam with 17 J/pulse) to 1.3°C (focused laser beam). In principle

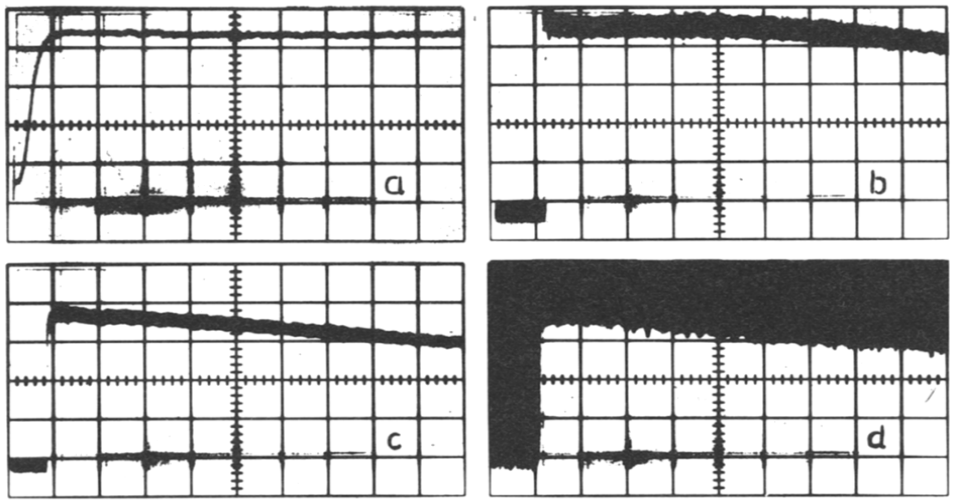

Fig. 3. Oscilloscope records of the current relaxation following a T-jump for different systems ($\Delta T = 0.3-0.4°C$). (a) T-jump at constant voltage for dioleoyllecithin membranes with 10^{-6} M nonactin and 1 M KCl in water. Oscilloscope sensitivity: 1 ms and 10 nA per division. (b) T-jump at constant voltage for diphytanoyllecithin membranes with 10^{-6} M nonactin and 1 M KCl in water. Oscilloscope sensitivity: 1 s and 1.5 nA per division. (c) T-jump at constant voltage for membranes formed from dioleoyllecithin with 10^{-3} M valinomycin in the mmebrane forming solution and 1 M KCl in water. Oscilloscope sensitivity: 1 s and 15 nA per division. (d) T-jump with the combined temperature- and voltage-jump method for dioleoyllecithin membranes with 10^{-5} M dipicrylamine and 0.1 M NaCl in water. Pulse length 2 ms, interval between pulses 15 ms. Oscilloscope sensitivity: 2 s and 1.5 nA per division.

higher temperature jumps are possible, but the membrane stability is considerably impaired if the laser beam is focused more strongly (see below).

At the wavelength of 1.06 μm the intrinsic absorption of water alone is sufficient to obtain reasonable temperature jumps. On the other hand, it is small enough to keep the temperature gradient over the membrane (and even over the cuvette) small. The decadic absorption coefficient A for 1.06 μm and water (or an aqueous solution of a colourless salt) at room temperature is 0.063 cm^{-1} [19]. By Lambert–Beer's law $\approx$40% of the laser intensity is absorbed by the aqueous solution in the cuvette (length of the cuvette: 4 cm). The temperature increase $\Delta T(x)$ at a distance x from the front surface of the cuvette — again calculated from Lambert–Beer's law — is

$$\Delta T(x) = (2.303 A I_0/C) \exp(-2.303 A x) , \qquad (1)$$

with I_0 being the intensity of the laser beam and C the heat capacity of the medium. With the parameter values $x = 0.8$ cm (membrane distance from the window of the cuvette) and $I_0 = 10$ J/cm^2, one obtains from eq. (1) $\Delta T = 0.31°C$. This value agrees well with the mean temperature increase found experimentally: $\Delta T_{exp} = 0.35 \pm 0.10°C$. It was obtained from the initial current increase of valinomycin-induced cation conductance following a temperature jump [compare fig. 3c and eq. (16)]. The activation energies of the single transport steps of this carrier system have been determined previously by a voltage-jump analysis [8].

The application of this method implies that the temperature of the membrane is the same as the temperature of the surrounding aqueous solutions. This assumption is supported by a simple estimate which shows that thermal equilibration between a 50 Å thick membrane and the adjacent aqueous phases is completed in less than 1 ns. The limitations of the two temperature-jump methods are the following: The time resolution of the T-jump method at constant voltage is limited by the length of the laser pulse (400 μs) and/or by the time resolution of the detection system. The latter is given by the characteristic charging time τ_C of the membrane capacity C_M [8]: $1/\tau_C = (1/R_M + 1/R_{ext})/C_M$. R_{ext} is the sum of the external measurement resistance R_A and the solution resistance between the electrodes.

Fig. 3a shows a T-jump experiment with a membrane doped with the macrocyclic ion carrier nonactin. For this special transport system (as well as for the other systems shown in fig. 3) a specific current relaxation could not be resolved within the time limitations of the method. The rise time of the current increase ($\approx$500 μs) following a T-jump is of the same order as the duration of the laser pulse ($\approx$400 μs) and the charging time τ_C of the detection system (200 μs).

For the combined V- and T-jump method the time resolution is usually limited by the interval between two pulses. The minimum interval length is determined by the condition that the initial state of the system has to be re-established at any new pulse. First, the membrane capacitance must be discharged again; secondly, the perturbation of the (originally symmetrical) ion concentration at the membrane interfaces, caused by the preceding voltage pulse, must be given time to decay. The minimum interval length has been determined experimentally by varying the interval length and observing the corresponding changes of J_0.

For long observation times the temperature stability of the system is important. Calculations have shown that, after a T-jump with the unfocused laser beam (diameter of the heated cylindrical volume 1.8 cm), the temperature should remain constant for at least 15 s, if only loss of heat by diffusion is taken into account [22]. In practice, the temperature stability is impaired by convection. Figs. 3b–3d show the current behaviour at long times for membranes doped with three different compounds. The current remains fairly constant for at least 4 s. This may serve as evidence for a constant temperature over this time range. Whether the slow decrease of the current at times $t >$ 4 s reflects decreasing temperature or is due to specific system relaxations cannot be ascertained. It was found, however, that the time range of constant current was reduced considerably when the laser beam was focused (i.e. the heated volume was smaller).

Further limitations of the T-jump method concern the magnitude of the relaxation amplitude. The sensitivity of our present detection system is sufficient to resolve a relaxation if, at a T-jump of 1°C, the activation energy of the membrane conductance is larger than $\approx$1.3 kJ/mole. For larger T-jumps the limit would be even lower. In principle, this could be achieved by a stronger focusing of the laser light. Then, however, the available time range of the method would be reduced due to heat conduction (see above). Moreover, the presence of the following artifacts makes further increase of ΔT rather unfavourable: The fast heating is accompanied by a shock wave in the aqueous phase [14], which may lead to mechanical distortions of the membrane and even membrane rupture. Besides, the shock wave may cause electrical oscillations in the measuring circuit which are superimposed on the current relaxation. With the unfocused laser beam, the oscillations do not represent a major obstacle and the stability of the membranes is good. At temperature jumps of more than 1°C, however, oscillations become a severe problem. In addition, the probability of membrane rupture increases strongly.

Summarizing, the use of a Nd-glass laser instead of a flash lamp represents an improvement in several aspects. First, no dye has to be added to the solution, since the intrinsic absorption of water alone is sufficient. Secondly, higher temperature jumps are achieved in the fixed Q mode, enabling better resolution of relaxation amplitudes. Last but not least the experiments are considerably easier ot perform. Finally, all numerical calculations are performed using a PDP 11/40 computer.

2.2. Materials

Optically black lipid membranes were formed from a 0.5–1% (wt/vol) solution of lipids in n-decane (Merck, Darmstadt, GFR, standard for gas chromatography). The lipids were 1,2-dioleoyl-3-sn-phosphatidylcholine (dioleoyllecithin), rac-1,2-diphytanoylphosphatidylcholine (diphytanoyllecithin) and phosphatidylserine, which was extracted from ox brain. The area of the hole across which the membranes were formed was $\approx$0.1 cm^2.

The electrolyte solutions were made with alkali-ion chlorides (Merck, analytical grade). The pH of the unbuffered solutions was $\approx$ 6. Trace concentrations of the various hydrophobic ions and carriers were added to the aqueous phases in the form of ethanolic solutions. The ethanol content never exceeded 1%.

Hydrophobic ions: sodium tetraphenylborate (TPB) and 2,4,6-trinitrophenol (TNP) from Merck, dipicrylamine (DPA) from Fluka.

Carriers: valinomycin and nonactin from Böhringer; cyclo-(D-VAL-L-Pro-L-VAL-D-Pro)$_3$ (PV), synthesized

by Gisin [23]; enniatin B (cyclo [N-methyl-L-valine-D-α-hydroxyisovaleric acid]$_3$) from Hoffmann–LaRoche.

The temperature was kept at 25°C, unless stated otherwise.

3. Theory

3.1. Hydrophobic ions

The basis for a theoretical treatment of temperature-jump experiments with lipid-soluble ions is the model originally proposed by Ketterer et al. [20]. According to this model, transport is assumed to proceed by the following steps: the hydrophobic ions reach the membrane by diffusion through the aqueous phase, adsorb at the interface, translocate across the membrane interior, desorb from the membrane interface and diffuse through the aqueous solution. The model is illustrated in fig. 4. The hydrophobic ion of valency z is assumed to be the only charge carrier within the membrane. Its concentration in the bulk solution is C. The translocation rate constants across the inner membrane barrier are k_i' and k_i'', the rate constant for desorption is k and the rate constant of adsorption is βk. Accordingly, β represents a partition coefficient defined by

$$\beta = N/C \,, \tag{2}$$

where N is the equilibrium ion surface density at one interface. The reader should consult Ketterer et al. [20] for a more detailed discussion of the model, and Jordan and Stark [7] for its rigorous analysis on the basis of voltage-jump relaxation experiments. Here we shall concentrate on the description of T-jump experiments and

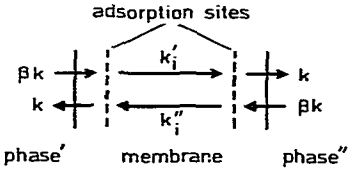

Fig. 4. Kinetic model for the transport of lipid soluble ions through membranes [20]. The diffusion processes in the two aqueous phases are not indicated.

we shall study the question whether this method provides new information about the model.

3.1.1. T-jump at constant voltage

The method may be applied to any system which shows a steady-state current at constant voltage (fig. 2B). This condition excludes those cases where the current is influenced by diffusion in the unstirred aqueous phases. In the following we assume rapid diffusion in water, i.e. absence of diffusion polarization. Then, only the membrane processes must be analysed and the ion concentrations in water may be considered time independent. The equations describing the kinetic scheme of fig. 4 are

$$\mathrm{d}N'/\mathrm{d}t = \beta k C - (k + k_i')N' + k_i''N'' \,, \tag{3}$$

$$\mathrm{d}N''/\mathrm{d}t = \beta k C + k_i'N' - (k + k_i'')N'' \,. \tag{4}$$

$N'(N'')$ denotes the ion surface density at interface $'$ (interface $''$). The steady-state interfacial concentration, obtained by equating eqs. (3) and (4) to zero, is,

$$\bar{N}' = \beta C(k + 2k_i'')/(k + k_i' + k_i'') \,. \tag{5}$$

An analogous expression holds for $\bar{N}''$ (the superscripts $'$ and $''$ are exchanged). We assume that the voltage affects only the translocation process across the membrane interior, which may be represented – in a simplified manner – by a single energy barrier [20];

$$k_i' = k_i e^{zu/2} \,, \quad k_i'' = k_i e^{-zu/2} \,, \quad u = VF/RT \tag{6}$$

(k_i is the rate constant at zero voltage, V the voltage, F Faraday's constant, T the absolute temperature, R the gas constant). It is further assumed that the T-jump, taking place at time $t = 0$, gives rise to an instantaneous change of the model parameters:

$$k_i - \Delta k_i \to k_i \,, \quad k - \Delta k \to k \,, \quad \beta - \Delta\beta \to \beta \,.$$

While Δk_i and Δk are always positive (positive temperature coefficient of rate constants), $\Delta\beta$ may be positive or negative depending on the sign of the enthalpy change associated with the partition coefficient. The change of the model parameters results in a shift of the steady-state interfacial concentrations $\bar{N}'(1) \to \bar{N}'(2)$ and $\bar{N}''(1) \to \bar{N}''(2)$; the numbers 1 and 2 refer to the situation before and after the temperature jump. The interfacial concentrations are calculated from eq. (5). The time course of this process is obtained by standard methods from eqs. (3) and (4):

$$N'(t) = \bar{N}'(2) + a\mathrm{e}^{-t/\tau_1} + b\mathrm{e}^{-t/\tau_2} , \quad (7)$$

$$N''(t) = \bar{N}''(2) + a(k_i'/k_i'')\mathrm{e}^{-t/\tau_1} - b\mathrm{e}^{-t/\tau_2} , \quad (8)$$

$$1/\tau_1 = k , \quad 1/\tau_2 = k + k_i' + k_i'' . \quad (9)$$

The constants a and b are long expressions and will not be explicitly given. They are determined from the initial conditions $N'(0) = \bar{N}'(1)$ and $N''(0) = \bar{N}''(1)$.

The quantity of practical interest is the current density $J(t)$. As we assume a single steep energy barrier, the current density is [20]

$$J(t) = zF[N'(t)k_i' - N''(t)k_i''] . \quad (10)$$

Eq. (10) in combination with eqs. (7)–(9) and eq. (6) gives the final result

$$J(t) = J_\infty(2)(1 + \alpha_T \mathrm{e}^{-t/\tau}) , \quad (11)$$

with

$$J_\infty(2) = 2zF\beta C\tau k k_i \sinh(zu/2) , \quad (12)$$

$$1/\tau = 1/\tau_2 = k + 2k_i \cosh(zu/2) , \quad (13)$$

$$\begin{aligned}\alpha_T = \{&2\beta C \cosh(zu/2)(k\Delta k_i - k_i\Delta k) \\ &- \Delta\beta C[k + 2k_i \cosh(zu/2)](k - \Delta k)\} \\ &\times \{\beta C k[k - \Delta k + 2\cosh(zu/2)(k_i - \Delta k_i)]\}^{-1} .\end{aligned} \quad (14)$$

For $|\Delta k_i| \ll k_i$ and $|\Delta k| \ll k$ one obtains

$$\alpha_T \approx \left[\frac{\alpha_V}{1 + \alpha_V}\left(\frac{\Delta k_i}{k_i} - \frac{\Delta k}{k}\right) - \frac{\Delta\beta}{\beta}\right] . \quad (14a)$$

$$\alpha_V = 2k_i \cosh(zu/2)/k , \quad (15)$$

is the amplitude of the current relaxation following a voltage jump [20,7]. The time course of the voltage-jump relaxation corresponds exactly to that of the T-jump [eq. (11)]. The relaxation time is identical, too. Differences are confined to the relaxation amplitude: The voltage-jump relaxation describes the voltage-dependent redistribution of the ions between the two interfaces. The amplitude α_V of this process approaches zero when $k_i/k \to 0$. The amplitude α_T of the T-jump-induced relaxation consists of two terms. The first one is proportional to α_V and may be understood as a temperature-dependent redistribution of ions between the interfaces. Even in the case $\alpha_V \to 0$ (i.e. when the voltage-jump relaxation disappears), a T-jump relaxation may be resolved, if $\alpha_T = -\Delta\beta/\beta$ is sufficiently large. This second term of α_T is induced by the temperature-dependent increase (or decrease) of the charge-carrier concentration at the membrane interfaces. Therefore, the T-jump method can provide information on the kinetics of hydrophobic ions in membranes even in cases where the voltage-jump method fails. This is true for picrate, as will be shown in section 4.

The current relaxation given by eq. (11) only reflects the time-dependent change of the interfacial concentrations N' and N''. In addition, there is an instantaneous increase $\Delta J = J^0 - J_\infty(1)$ of the current at time $t = 0$ (see fig. 2B), produced by an abrupt change of the translocation rate constant $k_i - \Delta k_i \to k_i$. From eq. (10) in combination with eq. (6) one finds the relation

$$J^0/J_\infty(1) = k_i/(k_i - \Delta k_i) . \quad (16)$$

J^0 corresponds to $J(0)$ of eq. (11). For the evaluation of the experimental data the following form of eq. (11) was used:

$$F(t) = [J(t) - J_\infty(2)]/[J(0) - J_\infty(2)] = \mathrm{e}^{-t/\tau} . \quad (17)$$

3.1.2. Combined temperature and voltage jump

The method has been applied in those cases where a steady-state current at constant voltage was not observed due to long lasting diffusion processes in the unstirred aqueous layers adjacent to the membrane. The initial current density $J(0)$ following a voltage jump does depend, however, only on membrane processes. This may easily be seen from eq. (10). For a voltage jump starting from symmetrical conditions ($N' = N'' = N$), we find [in combination with eq. (6)]

$$J_0 = J(0) = 2zFNk_i \sinh(zu/2) . \quad (18)$$

The proportionality between J_0 and N may be used to monitor the time dependence of the interfacial concentration N after a T-jump by a sequence of short voltage jumps (see fig. 2C). The changes in N, induced by an increase (or decrease) of the partition coefficient β with temperature, give rise to a diminution (or accumulation) of charge carriers in the aqueous phases. The ion concentration C in the neighbourhood of the membrane will therefore be a function of time and will also depend on the distance from the interface. The situation is illustrated in fig. 5.

The function $N(t)$ depends on the diffusion velocity in water and on the rate of the adsorption/desorption process at the membrane interface. Therefore, the time

Fig. 5. Schematic concentration profile for hydrophobic anions near the membrane interface after a T-jump (no voltage applied). The charge-carrier concentration N at the membrane interfaces decreases in case of TPB^- and TNP^-; it increases for the neutral ion carrier enniatin B (see figs. 7–9).

dependence of the voltage-jump-induced initial current J_0, measured after a T-jump has been applied contains information about the kinetics of the adsorption/desorption process. An outline of the theory, which strongly parallels the treatment of the voltage-jump problem (7) is given in appendix A. The result is

$$J_0(t) = J_0(2)\,[1 - (\Delta\beta/\beta)F(t)]\ , \tag{19}$$

with

$$J_0(2) = 2zF\beta C k_i \sinh(zu/2)\ , \tag{20}$$

reflecting the steady-state concentration $\bar{N}(2) = \beta C$, which the system approaches after the T-jump.

The function $F(t)$ is

$$F(t) = \frac{4\xi}{\pi}\int_0^\infty \frac{du\,\exp(-u^2 kt)}{(u^2-1)^2 + 4\xi^2 u^2}\ , \tag{21}$$

with $\xi = \frac{1}{2}\beta(k/D)^{1/2}$, where D is the diffusion coefficient of the hydrophobic ion in water. $F(t)$ is determined by the current observed in a T-jump experiment: Since $F(0) = 1$ and $F(t \to \infty) = 0$ [eq. (A.4)] one finds from eq. (19)

$$F(t) = [J_0(t) - J_0(2)]/[J_0(0) - J_0(2)]\ . \tag{22}$$

It is formally identical to $F(t)$ as defined in the constant-voltage variant of the method [left side of eq. (17)]. $F(t)$ depends on three parameters: the desorption rate constant k, the partition coefficient β, and the diffusion coefficient D [see eq. (21)]. In case of rapid diffusion in water, $F(t)$ converges to $\exp(-kt)$ [eq. (A.5)]. Again, the formal similarity to eq. (17) is evident in this special case. The relaxation time is, however, different in both cases. In contrast to the constant-voltage version [eq. (13)], only the desorption rate constant k is important. This reflects the different initial conditions. In the combined temperature- and voltage-jump method, the system remains symmetrical throughout the evolution of the relaxation. In the constant-voltage version, the voltage creates an asymmetry in the system leading to a dependence on the translocation rate constants k_i' and k_i''. The "instantaneous" current increase $\Delta J = J_0^0 - J_0(1)$ at $t = 0$ (see fig. 2C) is identical in both versions of the method [eq. (16)].

The diffusion coefficient D of hydrophobic ions in water is either known or may be calculated from Stokes' law. The other two parameters important for $F(t)$, k and β, may be obtained by numerical integration of eq. (21) and by fitting the result to the experimental data evaluated according to eq. (22). At short times $F(t)$ is mainly determined by k. This may easily be seen from eq. (23), obtained by combining eqs. (18), (22) and (A.2).

$$\mathrm{d}\ln F(t)/\mathrm{d}t|_{t=0} = -k\ . \tag{23}$$

In case of rapid diffusion [i.e. exponential decrease of $F(t)$] eq. (23) is also valid for times $t > 0$. If diffusion in water has to be taken into account, the shape of $F(t)$ at times $t > 0$ is influenced by k and β. The general behaviour of $F(t)$ for various values of the two parameters is shown in fig. 6. The most rapid decay of $F(t)$ corresponds to the situation where diffusion in water is relatively fast, that is when $\xi \to 0$ ($D \gg k\beta^2/4$) and exponential behaviour is found [see eq. (A.5)]. In this case the concentration profile in the aqueous solution is uniform. If $k \to 0$ the time scale of adsorption/desorption is long in comparison to the time required for diffusion in the aqueous phase to adjust the ionic concentrations near the interface; thus equilibration in the unstirred layers is effectively instantaneous. When $\beta \to 0$ few ions are adsorbed onto the membrane. As a result small changes in the ionic partitioning between water and membrane cannot significantly alter the aqueous concentration profile. If diffusion is relatively slow a different limit is reached, as indicated in eq. (A.6). Here the function $F(t)$ is independent of k and is determined solely by the rate of diffusion in water and the partition coefficient be-

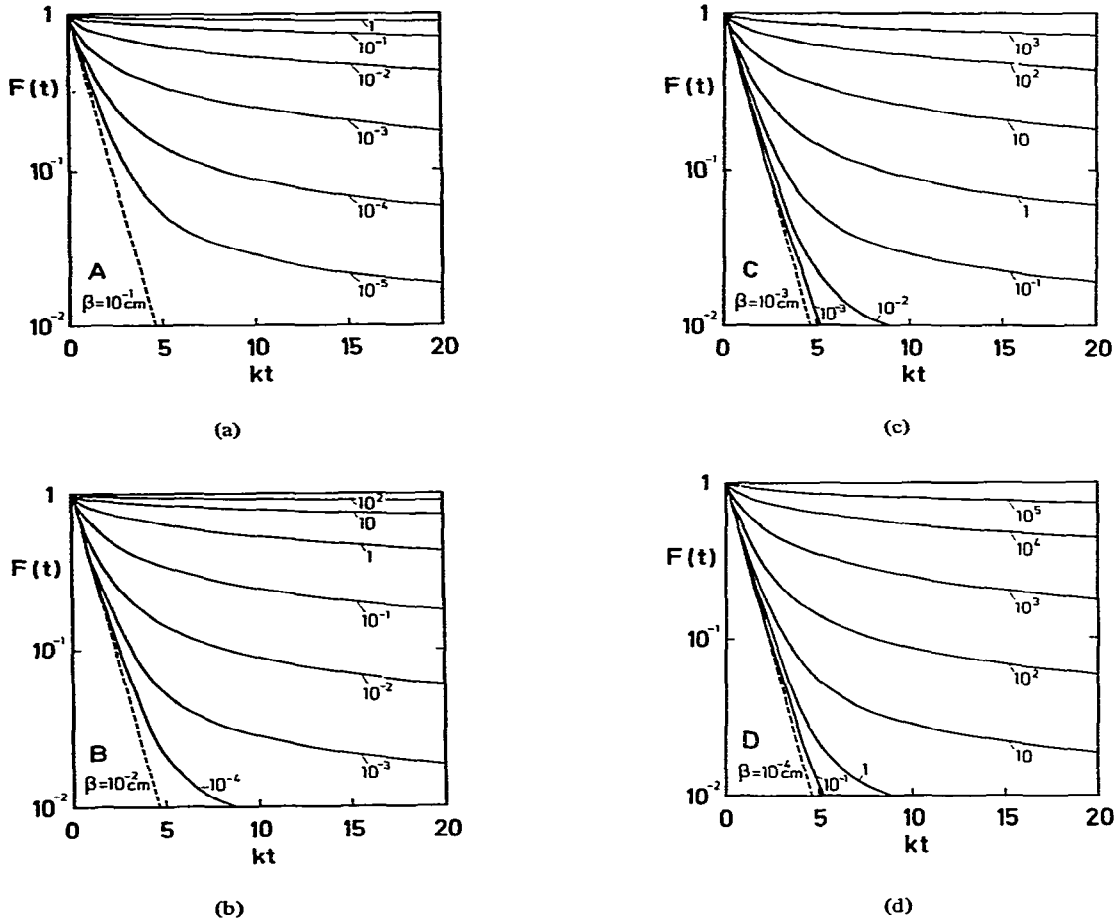

Fig. 6. T-jump-induced relaxation of $F(t) = [J(t) - J_\infty]/(J_0 - J_\infty)$ as a function of the reduced time kt for various values of β and k. (A) $\beta = 10^{-1}$ cm; (B) $\beta = 10^{-2}$ cm; (C) $\beta = 10^{-3}$ cm; (D) $\beta = 10^{-4}$ cm. The values of k ($10^{-5} \leqslant k \leqslant 10^{5}$ s^{-1}) are indicated in each graph. The dashed line represents the limiting case $F(t) = \exp(-kt)$ obtained for rapid diffusion in the aqueous phase. For all graphs the diffusion coefficient D was chosen to be 5.0×10^{-6} cm^2 s^{-1}, which is approximately the value for TPB and DPA.

tween membrane and water.

Only certain combinations of the parameters D, β, and k are experimentally accessible as illustrated in fig. 6. The curves have been calculated with $D = 5 \times 10^{-6}$ cm^2 s^{-1}, approximately the value for DPA and TPB [38]. For this value of D the function $F(t)$ is sensitive to the choice of k only if $\beta^2 k \leqslant 10^{-3}$ cm^2 s^{-1}, since the experimental uncertainty in the determination of $F(t)$ is ≈ 0.1. Larger values of $\beta^2 k$ correspond, in practice, to the $\xi \to \infty$ limit, i.e. the conditions under which $F(t)$ is independent of k. Further restrictions on the range of k-values are imposed by the limited time resolution of the method (see section 2). Assuming a lower limit of 1 ms and an upper limit of 4 s, we find that for $\beta \geqslant 10^{-2}$ cm a determination of k is hardly possible. For smaller values of β, the range of k accessible by our method is:

$\beta = 10^{-3}$ cm: $0.1 \leqslant k \leqslant 10^{2}$ s^{-1} ,

$\beta = 10^{-4}$ cm: $0.1 \leqslant k \leqslant 10^{3}$ s^{-1} .

We shall now compare the advantages of the voltage- and the temperature-jump methods for the study of the adsorption/desorption process. The voltage-jump method permits a relatively simple determination of the translocation rate constant k_i and the partition coefficient β from the initial values of the current and its slope. The desorption rate constant k is found from a rather complex analysis of the shape of the current relaxation [7]. The T-jump method, on the other hand, permits direct determination of k from the initial slope of $\ln F(t)$, while establishment of β requires computer analysis of the time course of the current relaxation. Therefore, the two methods are complementary with respect to a simple and straightforward evaluation of the desorption rate constant k and the adsorption rate constant βk. The T-jump method extends the range of experimentally accessible k-values considerably. The V-jump method fails if either $k_i/k \gg 1$ or $k_i/k \ll 1$ [7]. In these cases the T-jump technique provides the only source of information on k.

3.2. Neutral carriers of monovalent cations

The application of the T-jump method to the study of the adsorption/desorption process at lipid membranes is not confined to ionic substances, but may in principle be used for any substance which modifies the conductance of the membrane. This will be demonstrated for neutral ion carriers such as valinomycin, enniatin and the macrotetrolides. The action of these so-called ionophores has been intensively studied in the past (for a review see e.g. refs. [24,25]). It is usually assumed that the energy profile for these substances inside the membrane is similar to that for hydrophobic ions, i.e. they concentrate at the membrane interfaces. Here, they can bind cations from the adjacent aqueous phase. The cations are transported across the membrane interior as carrier-ion complexes and may dissociate at the opposite interface. The kinetics of these processes have been investigated by the voltage-jump method [26–29,21], by the charge pulse technique [30–32] and by noise analysis [33]. In all cases the interpretation was based on the assumption that the substances behave as membrane-bound carriers, i.e. the exchange between membrane and water was largely neglected. This was justified by the finding that the exchange of carrier molecules between membrane and water — including unstirred layer effects — is slow compared to the velocity of ion permeation through the membrane [34–36]. The available evidence for a relatively slow equilibration process between membrane and water is, however, only qualitative, since a clear separation of unstirred layer diffusion and adsorption/desorption processes has not been achieved. We will study the problem on the basis of T-jump experiments and show that its mathematical treatment can be reduced to the formalism developed for hydrophobic ions.

We denote the interfacial concentrations of carriers and carrier-ion complexes by N'_S (N''_S) and N'_{MS} (N''_{MS}), the corresponding rate constants of translocation across the membrane interior by k_S and k_{MS}, the rate constants of desorption from the interface into water by k_S^{ma} and k_{MS}^{ma}, and the rate constants of adsorption by $\beta_S k_S^{ma}$ and $\beta_{MS} k_{MS}^{ma}$ (see fig. 10 in appendix B). Complex formation is described as a bimolecular reaction. It can take place either in water (rate constants $\bar{k}_R, \bar{k}_D$) or at the interface (rate constants k_R, k_D). The reader should consult ref. [36] for a more detailed discussion of the carrier model.

The formal equivalence of the carrier problem to that of hydrophobic ions has been established under the following assumptions:

(1) The interfacial concentrations on both sides of the membrane are approximately equal, i.e. $N'_S \approx N''_S \equiv N_S$ and $N'_{MS} \approx N''_{MS} \equiv N_{MS}$.

(2) A T-jump changes the equilibrium constants K and K_h of the complex formation in water and at the interfaces. The corresponding relaxation processes are assumed to be fast compared to the equilibration of the carrier species between membrane and water.

(3) The diffusion coefficients of carrier and carrier-ion complex are set equal, i.e. $D_S \approx D_{MS} = D$.

(4) The concentration c_M of cations M^+ in water is sufficiently high so that changes in the unstirred layers due to current flow and complex formation with the carrier molecules can be neglected. As a result, c_M is time independent.

(5) The time range of the phenomena under study is short enough not to be influenced by the exchange process of the carrier molecules between the membrane and its surrounding torus of bulk lipid material.

The validity of these assumptions is discussed in section 4. Condition (1) allows the initial current to be expressed in the same form as in eq. (18), if k_i is

replaced by k_{MS}, N by N_{MS} and if $z = 1$ [26]. The kinetic equations of the carrier problem can be transformed into those for hydrophobic ions by means of the following substitutions (see appendix B):

$$k \rightarrow (k_S^{ma} + k_{MS}^{ma} K_h c_M)/(1 + K_h c_M) , \quad (24)$$

$$\beta \rightarrow \beta_s (1 + K_h c_M)/(1 + K c_M) , \quad (25)$$

with the equilibrium constants $K = \bar{k}_R/\bar{k}_D$ and $K_h = k_R/k_D$. As a result, the relaxation of the current is again given by eqs. (19)–(23).

4. Results and discussion

4.1. Hydrophobic ions

Temperature-jump experiments were performed in the presence of the hydrophobic anions tetraphenylborate (TPB), dipicrylamine (DPA) and 2,4,6-trinitrophenol (TNP) with membranes formed from the neutral lipids dioleoyllecithin, diphytanoyllecithin and the negatively charged lipid phosphatidylserine. The systems were also studied by voltage-jump experiments. In the presence of TPB and DPA current relaxations of large amplitude were found and strong evidence for the influence of diffusion in the unstirred layers was obtained [7,20,37]. For TNP, on the other hand, a voltage-jump relaxation could not be resolved. This indicates that unstirred layer diffusion is comparatively fast in this case and that ion permeation is limited by the membrane. Therefore, the constant-voltage version of the T-jump technique can be applied to TNP, while the combined V- and T-jump method has to be used for TPB and DPA. The method allows a determination of the rate constant k of desorption only if $\beta \leqslant 10^{-3}$ cm (see section 3.1.2). This condition is not satisfied for neutral dioleoyllecithin membranes in the presence of TPB and DPA, where values of $\beta > 10^{-2}$ cm have been reported [7,20]. The inadequacy of the T-jump method for the system DPA/dioleoyllecithin may be also concluded from fig. 3d. There, the current does not show a significant change up to 4 s after the T-jump (apart from the initial increase produced by the temperature dependence of k_i). Therefore, we used negatively charged membranes, where β is considerably smaller due to electrostatic repulsion of the anions. We concentrated on the anion TPB, which we have studied recently by voltage-jump analysis [7], and compared the results obtained by the two kinetic methods.

Fig. 7 shows an original record of a TPB-induced T-jump relaxation and its analysis according to section 3.1.2. The instantaneous rise of J at $t = 0$ (from $J_0(1)$ to J_0^0, compare with fig. 2) is not shown. The decrease of the current from J_0^0 to $J_0(2)$ reflects a reduced membrane concentration of TPB with increasing temperature, i.e. a negative enthalpy of the partition coefficient β (see section 4.3). The error bars for $F(t)$ result largely from the uncertainty in J_0^0 and in $J_0(2)$, which have to be obtained by extrapolation [for $J_0(2)$ usually a second record was taken with a larger time scale]. $F(t)$ is clearly nonexponential indicating the influence of unstirred layer diffusion. The solid line represents the best fit of eq. (21) to the experimental data. It was found that the shape of $F(t)$

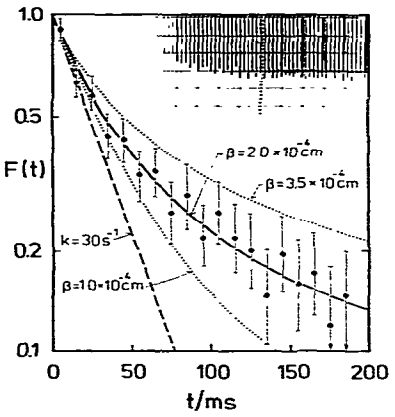

Fig. 7. Semilogarithmic plot of $F(t)$ versus t for the current relaxation of the system tetraphenylborate/phosphatidylserine following a temperature jump of 0.6°C (combined V- and T-jump). The membrane was formed from a 0.4% solution of phosphatidylserine in decane in the presence of 0.1 M NaCl and 10^{-5} M TPB in the aqueous phase (25°C, 100 mV pulses of 2 ms duration, interval between pulses 10 ms, measurement resistance $R_A = 33$ kΩ, $I_\infty(1) = 0.7$ μA, charging time $\tau_C = 0.9$ ms). The inset shows the oscilloscope record with 50 ms and 50 μV per division. The steady-state level $I_0(1)$ is suppressed. $F(t)$ was calculated from the experimental data according to eq. (22); the bars indicate the measurement uncertainty. The dashed line with slope $-30\,s^{-1}$ is the tangent to $F(t)$ for $t \rightarrow 0$ [see eq. (23)]. The curves were calculated from eq. (21) with $D = 5.2 \times 10^{-6}$ cm^2 s^{-1} [38] and $k = 30$ s^{-1} for the indicated values of β.

Table 1
Comparison of the T-jump and V-jump analysis of tetraphenylborate permeation through phosphatidylserine membranes[a)]

System	Method	k_i (s^{-1})	k (s^{-1})	β (cm)
10^{-5} M TPB in 0.1 M NaCl	T-jump	–	56 ± 16	$(2.0 \pm 0.8) \times 10^{-4}$
10^{-6} M TPB in 0.1 M NaCl	V-jump	41 ± 16	61 ± 15	$(2.3 \pm 0.7) \times 10^{-4}$

a) The data represent mean values of 5 membranes and the standard deviation.

– though being very sensitive to β (see dotted lines) and k [initial slope of $F(t)$] – is less sensitive to a variation of the diffusion coefficient D. For the latter a literature value was taken [38].

Table 1 shows that the T-jump data agree well with those obtained previously from a V-jump analysis [7]. The T-jump experiments were performed at higher concentrations of TPB in water. This was done in order to increase the membrane conductance and, as a consequence, to improve the time resolution of the method. The applied concentration of 10^{-5} M is, however, still in the linear region of the conductance–concentration relationship for negatively charged phosphatidylserine membranes. The good agreement obtained for both independent kinetic methods represents a further support for the simple kinetic model (fig. 4) used to describe the permeation of hydrophobic ions through lipid membranes. In addition, the results of both methods show that for TPB the partition equilibrium at the interface is not maintained during current flow, since the value of the rate constant k_i of translocation across the membrane interior is very similar to that of the rate constant k of desorption from the membrane into water.

The second hydrophobic ion studied in detail is the picrate anion (TNP). The conductance increase induced in lipid membranes by picrate alone is relatively small (1–2 orders of magnitude). A further strong increase in the conductance (up to 3 orders of magnitude) is observed, however, if neutral macrocyclic cation carriers such as valinomycin are added [39]. It was found that the high conductance in this case is not due to a high cation permeability (the experiments were performed in the absence of cations normally transported by valinomycin), but is induced by a considerably enhanced picrate permeability [40]. Ginsburg and Stark [40] suggested that the translocation rate of the relatively small TNP molecules across the membrane is strongly increased in the neighbourhood of the comparatively large, but highly mobile valinomycin molecule.

Because of the small membrane conductance, the time resolution of the current measurement at a T-jump experiment in the presence of TNP alone was not sufficient to resolve the relaxation at room temperature. At 3°C, however, this could be achieved, since the molecular process underlying the relaxation is slow enough. In the presence of valinomycin, the T-jump-induced current relaxation was found to be identical to the pure TNP system at 3°C. Due to the considerably higher membrane conductance (i.e. improved time resolution) the relaxation could now be resolved at higher temperatures.

Fig. 8 illustrates typical experiments. As in case of TPB, the current shows a decrease after the "initial jump". But the function $F(t)$ now decays exponentially within the experimental error limits. The result is identical for the T-jump at constant voltage and for the combined V- and T-jump method. This indicates that the slow V-jump-induced current relaxation of very small amplitude observed previously for this system [40] is of no consequence for the present experiments. The identity of the results obtained with both T-jump methods does not exclude the existence of a very fast V-jump relaxation not resolvable by our present methods. The existence of such a relaxation (within the framework of our kinetic model) is, however, unlikely in view of the following arguments: The exponential decay of $F(t)$ shows that unstirred layer diffusion is relatively fast. Thus, interpretation of the experiment performed at constant voltage yields [see eqs. (17) and (13)]

$$1/\tau_{exp} = k + 2k_i \cosh(zu/2) , \qquad (26)$$

while the combined V- and T-jump experiment indicates [see eq. (A.5)]

$$1/\tau_{exp} = k . \qquad (27)$$

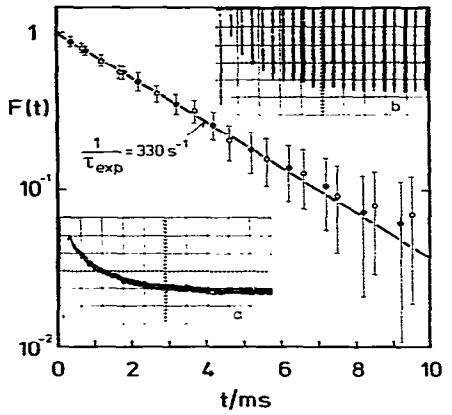

Fig. 8. Semilogarithmic plot of $F(t)$ versus t for the current relaxation of the system picrate/valinomycin/diphytanoyllecithin following a T-jump of 0.3°C; (a) at constant voltage (•), (b) with voltage pulses (○). The membrane was formed from a 0.5% solution of diphytanoyllecithin in decane containing 10^{-3} M valinomycin. The aqueous solutions contained 10^{-2} M picrate (pH 6 with LiOH) and 1 M LiCl (25°C). Inset (a) shows the oscilloscope record of the T-jump at an applied constant voltage of 100 mV (measurement resistance R_A = 3.3 kΩ, $I_\infty(1)$ = 15.5 μA, charging time τ_C = 40 μs). The oscilloscope sensitivity was 50 μV and 2 ms per division. The base line is suppressed. Inset (b) shows the record of the current relaxation with the combined T- and V-jump method at the same membrane as in (a). 100 mV pulses of 0.2 ms duration were applied. The pauses between pulses were 0.8 ms long. All other data are the same as in (a). The first spike appearing in the record occurred before the T-jump; it defines $I_\infty(1)$. The bars in the graph indicate the measurement uncertainty. The solid line was drawn with slope $-330\ s^{-1}$ according to eq. (17).

Both equations can only be satisfied if $k_i \ll k$. This condition is equivalent to a small voltage-jump amplitude α_V [compare eq. (15)]. The relative magnitude of the rate constants k and k_i may be assessed according to eq. (26) by measuring the voltage dependence of the experimental time constant τ_{exp}. Experiments indicate that $1/\tau_{exp}$ increases by only ≈50% if the voltage across the membrane is varied from 15 to 200 mV. Hence, on the basis of eq. (26) we obtain $k_i/k \approx 3 \times 10^{-2}$. This result accounts for both observations, namely the identical T-jump relaxation curves at constant voltage and with voltage pulses, and the small unresolvable voltage-jump amplitude α_V. Nevertheless, the question arises whether the valinomycin facilitated permeation of TNP can be described by the simple model of fig. 4, which was developed for the unfacilitated permeation of hydrophobic ions. Though this problem cannot be decided on the basis of the present experimental data, there are two arguments which show that our interpretation represents at least a good approximation. The very weak voltage dependence of τ_{exp} may indicate that the observed current relaxation originates mainly from the adsorption/desorption process at the interface, since charge transfer across the membrane interior, irrespective of the detailed mechanism, is expected to be voltage dependent. Secondly, at 3°C the same relaxation time τ_{exp} was found in the absence and presence of valinomycin (see table 2). This justifies the use of eq. (27) to interprete our data.

Table 2
Temperature-jump relaxation data for 2,4,6-trinitrophenol[a)]

System	Lipid	T(°C)	$1/\tau_{exp}(s^{-1})$
TNP + 10^{-3} M VAL	18:1 PC	25	130 ± 14
	16:4 CH_3-PC	25	381 ± 58
		14	94 ± 11
		3	19 ± 5
TNP	16:4 CH_3-PC	3	23 ± 5

a) The experiments were performed in 10^{-2} M solutions of TNP in 1 M LiCl (pH 6) in the presence and absence of valinomycin in the membrane-forming solution for the lipids dioleoyllecithin (18:1 PC) and diphytanoyllecithin (16:4 CH_3-PC). The mean values (with standard error) given for $1/\tau_{exp}$ correspond to the rate constant k of desorption [eq. (27)].

Table 2 summarizes the results obtained with TNP. A pronounced temperature dependence of the desorption rate constant k was found (activation energy E_A = 97 kJ/mole). k is larger by a factor of 3 for diphytanoyllecithin — which has branched fatty-acid residues — than for dioleoyllecithin. If one accepts our interpretation on the basis of the model shown in fig. 4, then the relation $k_i/k \ll 1$ indicates that, in contrast to the anion TPB, the rate-limiting step of transport is now the translocation across the membrane interior, i.e. the adsorption/desorption process is in equilibrium.

4.2. Neutral carriers of monovalent cations

Measurements were made on dioleoyllecithin membranes doped with one of the following substances: the depsipeptides valinomycin and enniatin B, the macrotetrolide nonactin and proline-valinomycin (PV), a peptide analogue of valinomycin. For valinomycin and non-

actin a relaxation could not be resolved. The current was fairly constant up to at least 4 s after the T-jump (see fig. 3). This is in agreement with results obtained earlier with a "slow" T-jump method [13]. There, it was found that the exchange of valinomycin between membrane and the unstirred aqueous phases took place within a time range of 30 min. This may partly reflect the relatively high partition coefficient β_S of the order of 10^{-2} cm, which was reported for valinomycin and also for the macrotetrolides [28,29,36]. At high values of β_S, the relatively slow diffusion process through the unstirred layers may play a dominant role. In this case the fast T-jump method described in the present paper is less suitable (compare section 2). For the valinomycin analogue PV smaller values of β_S have been reported [23], which should be more favourable for a fast T-jump analysis. The result was, however, more or less identical to that of normal valinomycin, i.e. a relaxation could not be resolved.

The rise time of the "initial jump", reflecting the temperature-dependent change of the translocation rate constant k_{MS} [analogous to eq. (16)], was within the limitations of the apparatus (see fig. 3a). The same was found for diphytanoyllecithin membranes doped with valinomycin. For this system we have reported a delayed increase of k_{MS} after a T-jump [8]. This finding, which was obtained with the flash-lamp apparatus could not be confirmed with our present laser device. We do not know the origin of this discrepancy; it might be caused by the presence of a dye in case of the flash-lamp method or by an impurity in the lipid preparation.

So far the only neutral ion carrier for which a T-jump relaxation could be resolved is enniatin B. The molecular characteristics of this substance and also its transport behaviour in membranes are very similar to valinomycin [41,32]. But its partition coefficient β_S is considerably smaller due to the more polar nature of this compound [32]. This favours a T-jump analysis.

Fig. 9 shows a current relaxation with positive amplitude. This differs from the results obtained with the hydrophobic ions TPB and TNP, and also with the depsipeptide valinomycin, where a decrease of the concentration with increasing temperature was found (see figs. 7 and 8 for TPB and TNP, and ref. [13] for valinomycin). The data agree well with the solid curve calculated according to eqs. (21)–(23). The curve is

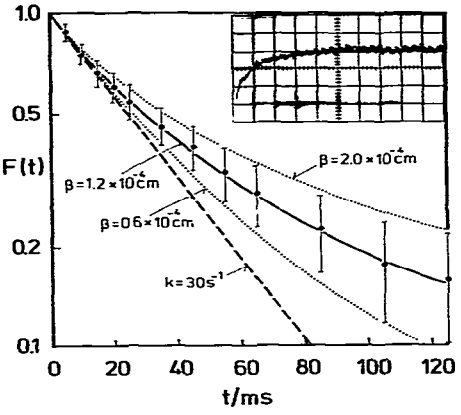

Fig. 9. Semilogarithmic plot of $F(t)$ versus t for the current relaxation of the system enniatin B/dioleoyllecithin following a T-jump of 0.6°C. The membrane was formed from a 1% solution of dioleoyllecithin in decane in the presence of 1 M KCl and 10^{-5} M enniatin B in the aqueous phase (25°C, applied voltage 100 mV, measurement resistance 33 kΩ, $I_\infty(1) = 0.73$ μA, charging time $\tau_C = 0.8$ ms). The inset shows the oscilloscope record with a sensitivity of 0.1 mV and 50 ms per division. The steady-state level $I_\infty(1)$ is suppressed, also most of the initial rise from $I_\infty(1)$ to I^0 (cf. fig. 2B). The theoretical curves were calculated from eqs. (21)–(23) with $D = 3.4 \times 10^{-6}$ cm^2 s^{-1} and $k = 30$ s^{-1}.

clearly nonexponential, showing the influence of unstirred layer diffusion. An interpretation on the basis of eqs. (21)–(23) is only correct, if the assumptions summarized in section 3.2 are fulfilled. These are discussed in the following:

(1) The left-hand interfacial concentrations (N'_S, N'_{MS}) are approximately equal to those of the right membrane interface (N''_S, N''_{MS}), if the condition of symmetry underlying eq. (18) is met, e.g. by applying the combined V- and T-jump method. At a T-jump with constant applied voltage, the condition of symmetry is fulfilled, if

$$(2z + \upsilon c_M) \cosh(u/2) \ll 1 , \qquad (28)$$

with $z = k_{MS}/k_D$ and $\upsilon = (k_{MS}/k_S)\, k_R/k_D$ (cf. fig. 10); eq. (28) guarantees that, at a V-jump, the initial current J_0 is identical to the steady-state current density J_∞ (see eqs. (5)–(7) of ref. [36]). z and υ may be obtained by an analysis of the steady-state current–voltage characteristic [34,36]. For the experimental conditions of fig. 9, Benz [32] has found: $2z + \upsilon c_M = 0.15$.

(2) Exact data for the relaxation times $1/\tau_h = k_R c_M + k_D$ and $1/\bar{\tau} = \bar{k}_R(c_S + c_M) + \bar{k}_D$ describing the process of complex formation at the interface and in water are not available for the experimental situation considered here. A rough approximation of their order, however, may be obtained from experiments with similar systems. Benz [32] analysed enniatin B in membranes formed from highly unsaturated monoglycerides by the charge pulse technique. Taking his data for k_R and k_D, one obtains $\tau_h \approx 2\,\mu s$. This is more than four orders of magnitude faster than the process of fig. 9. Though the rate constants of carrier-mediated ion transport have been found to depend on the structure of the lipid used for membrane formation [36], we believe that, in view of the large difference in the time scale to the process under study, the condition of relatively fast complex formation at the interface is easily met. Kinetic data for the complex formation of enniatin B in water have not been reported. Grell and Oberbäumer [41] analysed the structural analogue valinomycin; on the basis of their data one obtains $\bar{\tau} \approx 2\,\mu s$. If we assume that enniatin B behaves not too differently, we can expect the free and the complexed species of this compound to equilibrate relatively fast in comparison to the time course of the relaxation shown in fig. 9.

(3) The size of free enniatin B and its ion complex are approximately equal, since the ion is "buried" inside the ring structure of this ligand system [42]. The value $D = 3.4 \times 10^{-6}$ cm^2/s was calculated according to Stokes' and Einstein's laws on the basis of the structural data of ref. [42].

(4) The constancy of c_M may be inferred from the absence of diffusion polarization in a voltage-jump experiment.

(5) The diameter of the membranes used in this study was ≈3 mm. The mean diffusion time τ_d across the membrane is many orders of magnitude larger than the time range considered in fig. 9. For $D = 10^{-7}$ cm^2/s — the upper limit of the lateral diffusion coefficient of lipid molecules — one finds $\tau_d \approx 5 \times 10^4$ s. Though the exchange between membrane and torus may be somewhat accelerated by convection, this process is by far too slow to be of relevance for our experiment.

Summarizing, we conclude that the conditions required for applying the theory seem to be fairly well fulfilled. From analysis of data collected using five different membranes we found

$$k = 45 \pm 15\ s^{-1}\ , \quad \beta = (1.0 \pm 0.5) \times 10^{-4}\ cm\ .$$

Eqs. (24) and (25) indicate how k and β have to be interpreted in terms of the rate constants of the carrier model. k is determined by the rate constants of desorption k_S^{ma} and k_{MS}^{ma} of the free carrier S and its ion complex MS^+. The interfacial association constant $K_h = k_R/k_D$ appears as a weighting factor determining their relative importance. The exact value of K_h is not known for dioleoyllecithin membranes. In principle, it may be obtained by a measurement of k as a function of the ion concentration c_M. These experiments could not be performed, since at low concentrations c_M the conductance increase induced by enniatin B is too small for a successful application of the T-jump method. An estimate of K_h is obtained as follows.

For unsaturated monoglycerides Benz [32] found $K_h \approx 1$ and $1 \leqslant k_{MS}/k_S \leqslant 10$. Since for dioleoyllecithin membranes $\upsilon = 0.13$ (see above), we get $K_h = k_R/k_D \leqslant 0.13\ M^{-1}$, if we assume that the ratio k_{MS}/k_S is similar for monoglyceride and lecithin membranes[‡]. As a consequence we find

$$k_S^{ma} + 0.13\, k_{MS}^{ma} \leqslant 45\ s^{-1}\ , \quad \text{i.e.}$$

$$k_S^{ma} < 45\ s^{-1} \quad \text{and} \quad k_{MS}^{ma} < 345\ s^{-1}\ . \qquad (29)$$

The translocation rate constants k_S and k_{MS} of enniatin B across the interior of monoglyceride membranes were in the range of 10^5–10^6 s^{-1} [32]. For the depsipeptide valinomycin k_S and k_{MS} were less than one order of magnitude smaller in lecithin membranes compared to monoglyceride membranes [43]. If we assume a similar behaviour for the structural analogue enniatin B, we find from eq. (29) that the rate constants of desorption are at least two orders of magnitude smaller than the rate constants of translocation for both the free and the complexed forms of enniatin B. This means that the membrane transport of enniatin B is limited by the interface.

Eq. (25) allows calculation of the partition coefficient β_S from the measured parameter β, if K_h and K

[‡] The argument would have been simpler, if we had performed our experiments with membranes made from highly unsaturated monoglycerides. The relatively poor stability of this kind of membranes, however, makes them less appropriate for T-jump experiments.

are known. The association constant K of complex formation in water was estimated to $K \leqslant 0.2\ M^{-1}$ [32]. Assuming the same limit for K_h as above ($K_h \leqslant 0.13\ M^{-1}$), we find

$$\beta_S \approx \beta = 1.0 \times 10^{-4}\ cm.$$

This value may be compared to those obtained by Benz [32], who found $\beta_S \approx 0.4 \times 10^{-4}$ cm–1.1×10^{-4} cm from his charge pulse studies on monolinolein and monolinolenin membranes.

4.3. Activation energies of transport

We analysed our data on the basis of models which describe ion transport as a passage across a series of activation barriers (compare figs. 4 and 10). The free energies of activation may be calculated from the corresponding experimentally determined rate constants by applying Eyring's theory of absolute rate processes [6]. For the transport of hydrophobic ions we obtain (see fig. 9 of ref. [20])

$$k_i = f_i \exp(-\Delta F_i/RT)\ , \tag{30}$$

$$k = f \exp(-\Delta F/RT)\ , \tag{31}$$

$$\beta k = l f_a \exp(-\Delta F_a/RT)\ , \tag{32}$$

ΔF_i, ΔF and ΔF_a represent the free energies of activation of the symmetrical inner membrane barrier and of the asymmetrical interfacial barrier. f_i, f and f_a are frequency factors, which are of the order of $RT/hL \approx 6 \times 10^{12}\ s^{-1}$ (h is Planck's constant, L is Avogadro's constant). l is the jump length for a jump from the aqueous solution into the energy minimum at the interface. The change in free energy $\Delta F^0(\beta)$ under standard conditions associated with the partition coefficient β is obtained from eqs. (31) and (32) as

$$\Delta F^0(\beta) = \Delta F_a - \Delta F\ . \tag{33}$$

The corresponding change in the enthalpy $\Delta H^0(\beta)$ is obtained from the temperature dependence of β:

$$\mathrm{d} \ln \beta / \mathrm{d}(1/T) = -\Delta H^0(\beta)/R\ . \tag{34}$$

It was calculated from the amplitude ΔT of the T-jump and the amplitude of the measured current relaxation. Since $J \propto \beta$ [compare eq. (12) or (18)] one finds for small ΔT

$$\Delta\lambda/\lambda = [\Delta H^0(\beta)/RT]\,\Delta T/T\ , \tag{35}$$

where $\Delta\lambda$ represents the change in the membrane conductance induced by ΔT. From an equation analogous to eq. (35) the activation energy ΔE_i of the rate constant k_i is obtained. Here, $\Delta\lambda$ represents the initial conductance increase induced by the temperature dependence of k_i [compare eq. (16)]. ΔE_i is related to the enthalpy of activation ΔH_i by $\Delta E_i = \Delta H_i + RT$ [6].

Table 3 summarizes the results obtained for the different systems under study. In the case of enniatin B ΔE_i refers to the rate constant k_{MS} and $\Delta H^0(\beta)$ to the partition coefficient β_S of the neutral species.

For the picrate system, $\Delta H^0(\beta)$ and ΔE_i were measured at three different temperatures. While the data at 25 and 14°C agree fairly well, those at 3°C show a clear deviation. We do not know the origin of this discrepancy; it might indicate a change in certain structural properties of the membrane between 14 and 3°C.

Enniatin B shows a positive value of $\Delta H^0(\beta)$ in contrast to the other substances under study. In this respect it also differs from valinomycin, for which a negative $\Delta H^0(\beta)$ was found with the slow T-jump method [13]. In the case of enniatin B the two temperature-dependent processes [characterized by ΔE_i and $\Delta H^0(\beta)$] cannot be separated by the slow method in contrast to valinomycin, where the adsorption/desorption process including unstirred layer diffusion is comparatively slow (see section 4.2). For enniatin B the slow method responds to the sum of ΔE_i and $\Delta H^0(\beta)$. Experiments performed under the same conditions as the fast T-jump measurements gave a value of 121.5 ± 9 kJ/mole, which is in reasonable agreement with $\Delta E_i + \Delta H^0(\beta) = 98.5$ ± 17 kJ/mole.

We shall analyse the behaviour of tetraphenylborate in more detail, since all model parameters could be determined in this case. The data refer to negatively charged phosphatidylserine membranes. Hence, β contains an electrostatic contribution reflecting the repulsion of the anions. The actual anion concentration at the interface may be calculated by applying the theory of Gouy–Chapman [44]. Assuming an interfacial charge density of one charge per 60 Å^2 and an ionic strength of 0.1 M one can infer the partition coefficient β^0 of a neutral membrane from the measured values for a negatively charged one (table 1). The result is $\beta^0 = 4.1 \times 10^{-2}$ cm, which compares well with the experimental value of $\beta = 3 \times 10^{-2}$ cm reported for neutral dioleoyllecithin membranes [20]. Assuming identical desorption rate constants k for neutral and negatively charged

Table 3
Activation energies of the processes analysed by T-jump methods [a]

System	Lipid	Electrolyte	T(°C)	$\Delta H^0(\beta)$ (kJ mole^{-1})	ΔE_i (kJ mole^{-1})
10^{-5} M TPB	PS	0.1 M NaCl	25	−14.7 ± 2.9	33.1 ± 6.3
10^{-2} M TNP, 10^{-3} M VAL [b]	18:1 PC	1 M LiCl	25	−37.7 ± 11.3	41.5 ± 13.0
	16:4 CH_3-PC	1 M LiCl	25	−37.3 ± 7.5	44.8 ± 4.6
			14	−30.2 ± 3.4	44.4 ± 2.5
			3	−40.6 ± 12.2	95.5 ± 18.4
10^{-2} M TNP	16:4 CH_3-PC	1 M LiCl	3	−48.6 ± 17.6	107.3 ± 27.7
10^{-5} M enniatin B	18:1 PC	1 M KCl	25	+16.8 ± 4.2	81.7 ± 13.0

a) $\Delta H^0(\beta)$ is the enthalpy change associated with the partition coefficient β of hydrophobic ions or neutral carrier molecules between membrane and water. ΔE_i is the activation energy for the translocation of the ions (or ion complexes) across the central membrane barrier. The data are obtained from at least 4 membranes for each set of experimental conditions. Mean values are given together with the standard deviations. (18:1 PC ≙ dioleoyllecithin, 16:4 CH_3-PC ≙ diphytanoyllecithin).
b) In the membrane forming solution.

membranes, one finds $k\beta^0 = 2.3$ cm/s and from eq. (32) $\Delta F_a = 31$ kJ/mole (for a jump length $l = 10^{-7}$ cm). Similarly $\Delta F = 63$ kJ/mole and $\Delta F_i = 64$ kJ/mole are obtained by making use of eqs. (30) and (31). Hence, from eq. (33) $\Delta F^0(\beta) = -32$ kJ/mole for a neutral membrane. An ion having its position at one interfacial energy minimum is separated from the opposite interface on one side and the aqueous solution on the other side by almost identical barrier heights ($\Delta F_i \approx \Delta F$).

An ion being adsorbed from the aqueous phase has to overcome a barrier of $\Delta F_a = 31$ kJ/mole. This value for ΔF_a means that the adsorption process is influenced by unstirred layer diffusion and simultaneously by the adsorption barrier at the interface (at high values of ΔF_a corresponding to small values of βk unstirred layer diffusion is relatively fast). This may be concluded qualitatively from the fact that, on the one hand, the relaxation curve shown in fig. 7 is clearly nonexponential; i.e. eq. (A.5), the condition for rapid unstirred layer diffusion is violated. On the other hand, the time dependence of the relaxation is also influenced by βk, as may be seen from the theoretical curves in fig. 7. The relative contributions of unstirred layer diffusion and interfacial barrier ΔF_a may be assessed as follows: According to Zwolinsky et al. [6], diffusion through the unstirred layers may be described as a passage of the ion across an infinite series of activation barriers. The rate constant k_{diff} of the transport across a single barrier is related to the diffusion coefficient D by

$$D = k_{diff} l^2 , \qquad (36)$$

where l is the distance between two energy minima. Assuming $D = 5 \times 10^{-6}$ cm^2/s and $l = 10^{-7}$ cm, we obtain $k_{diff} = 5 \times 10^8$ s^{-1}. For a similar treatment of the adsorption process we have to replace $k\beta^0$ by $k^{am} = k\beta^0/l$ (k^{am} is the rate constant of adsorption for molecules within an aqueous layer of thickness l adjacent to the membrane interface). From the data given above we find $k^{am} = 2.3 \times 10^7$ s^{-1}. This is more than one order of magnitude smaller than k_{diff}. Smaller values of l increase the difference between k_{diff} and k^{am} (the value $l = 10^{-7}$ cm probably represents an upper limit).

For enniatin B, the difference between k_{diff} and k^{am} is considerably larger. An analogous calculation gives $k_{diff} = 3.4 \times 10^8$ s^{-1} and $k^{am} < 4.5 \times 10^4$ s^{-1} for the neutral species S. This shows clearly that the interface represents a considerable barrier for the membrane diffusion of this compound. The relatively small adsorption rate constant k^{am} for the neutral compound enniatin B as compared to the hydrophobic ion tetraphenylborate might indicate that the adsorption plane is positioned more towards the hydrophobic side of the membrane interface in the case of enniatin B; i.e. molecules moving from water to the interfacial energy minimum will experience a relatively strong hindrance through the polar head groups of the lipid molecules.

While our results represent the first data on the kinetics of adsorption of hydrophobic substances at planar

lipid membranes there have been several investigations at spherical lipid vesicles and micelles [45–50]. Suspensions of such microscopic particles may be studied in a conventional T-jump setup developed for homogeneous solutions. The rate constant of association of the fluorescent dye N-phenyl naphthylamine was found to be diffusion controlled [47]. Similar high values were reported for the adsorption of the fluorescent molecule ANS (1-anilino-8-naphthalenesulfonate) [45] and for the calcium ionophore X537A [46]. Values close to diffusion controlled were also obtained for the insertion of tenside molecules into micelles [49,50]. On the other hand, the rate of recombination of monomeric lipid molecules from water with lipid vesicles was found to be many orders of magnitude smaller [48]. In this case the incorporation of lipid molecules is limited by the vesicle surface.

The available data do not appear sufficient for a detailed discussion of the differences observed for the various substances and interfaces. The activation barrier ΔF_a may be expected to depend on factors such as the size of the adsorbing molecule, the exact location of the plane of adsorption ("onto" or "within" the polar head groups) and the nature, packing density and steric arrangement of the lipid molecules. It would be interesting to see whether ΔF_a is different for lipid vesicles and planar lipid films, since the extremely small radius of curvature of a vesicle leads to a wedge-shaped arrangement of the outer lipid monolayer. The lower packing density of the polar region might thus facilitate the penetration of molecules into the lipid matrix, i.e. reduce the barrier of adsorption as compared to planar lipid membranes. The question whether diffusion across the membrane interior or the interfacial adsorption/desorption process limits the membrane permeability of a given substance, might thus depend not only on the substance under study but also on structural characteristics of the membrane.

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Appendix A. Temperature-jump theory for hydrophobic ions including diffusion in the aqueous phase

The combined temperature- and voltage-jump method can be treated as a symmetrical problem. The concentrations C' and C'' on both sides of the membrane are identical, since the duration of the voltage pulses is short enough not to influence the system. For the same reason we a sume $k_i' = k_i''$. The initial conditions are

$$C' = C'' = C_B\,, \qquad N' = N'' = (\beta - \Delta\beta)C_B\,,$$

where C_B is the bulk concentration of hydrophobic ions in the aqueous phase. The temperature jump changes the partition coefficient from $\beta - \Delta\beta$ to β. The concentration of hydrophobic ions near the interfaces is a function of their distance x from the membrane. The system is described by the diffusion equation in water (A.1), the equation for the adsorption/desorption process at the interface (A.2) and an appropriate boundary condition (A.3):

$$\partial C/\partial t = D\,\partial^2 C/\partial x^2\,, \tag{A.1}$$

$$\mathrm{d}N/\mathrm{d}t = \beta k C_0 - kN\,, \tag{A.2}$$

$$D\,\partial C/\partial x|_{x=0} = \mathrm{d}N/\mathrm{d}t\,, \tag{A.3}$$

C_0 is the concentration at $x = 0$. Substituting $n(t) = N(t) - (\beta - \Delta\beta)C_B$ and $c(x,t) = C(x,b) - C_B$, one obtains

$$\partial c/\partial t = D\,\partial^2 c/\partial x^2\,,$$

$$\mathrm{d}n/\mathrm{d}t = \beta k c_0 - kn + k\Delta\beta C_B\,,$$

$$D\,\partial c/\partial x|_{x=0} = \mathrm{d}n/\mathrm{d}t\,,$$

and the initial conditions $n(0) = c(x,0) = 0$. The treatment of this system by Laplace transformation is completely analogous to the voltage-jump theory in ref. [7] and yields relations (19)–(21).

The function $F(t)$ has the following properties:

$$\text{(a)} \quad F(0) = 1 \quad \text{and} \quad F(t \to \infty) = 0\,, \tag{A.4}$$

(b) rapid diffusion: $\xi \to 0$ $(D \gg k\beta^2/4)$

$$F(t) = \mathrm{e}^{-kt}\,, \tag{A.5}$$

(c) slow diffusion: $\xi \to \infty$ $(D \ll k\beta^2/4)$

$$F(t) = \mathrm{e}^{at}\,\mathrm{erfc}\,[(at)^{1/2}]\,, \tag{A.6}$$

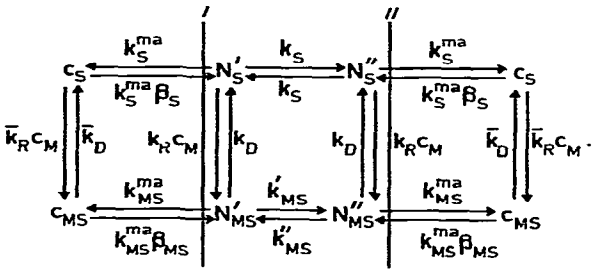

Fig. 10. Reaction scheme of ion transport mediated by a neutral carrier. See text for explanation of symbols.

with $a = D/\beta^2$.

(d) $t \to \infty$:

$$F(t) \to [\beta/(\pi D t)^{1/2}][1 + (1 - 2\xi^2)/kt + \ldots]. \qquad (A.7)$$

Appendix B. Temperature-jump theory for neutral carriers of monovalent cations

The model used to interpret carrier mediated transport is illustrated in fig. 10. The diffusion processes within the unstirred aqueous layers have been omitted for clarity. The model will be analysed under the conditions of a T-jump experiment using assumptions (1)–(5) summarized in section 3.2.

Since the system is symmetrical (assumption 1), we have to consider the kinetic processes only at one interface. The time-dependent diffusion processes of the free carrier S and the complexes MS^+ in the unstirred layers are influenced by the chemical reaction of the complex formation. Therefore,

$$\partial c_S/\partial t = D_S\, \partial^2 c_S/\partial x^2 - \bar{k}_R c_S c_M + \bar{k}_D c_{MS}\,, \qquad (B.1)$$

$$\partial c_{MS}/\partial t = D_{MS}\partial^2 c_{MS}/\partial x^2 + \bar{k}_R c_S c_M - \bar{k}_D c_{MS}\,. \qquad (B.2)$$

The concentration c_M of cations is time independent and identical on both sides of the membrane (assumption 4). The kinetic equations for the interfacial concentrations are

$$dN_S/dt = \beta_S k_S^{ma} c_S^0 - (k_S^{ma} + k_R c_M)N_S + k_D N_{MS}\,, \qquad (B.3)$$

$$dN_{MS}/dt = \beta_{MS} k_{MS}^{ma} c_{MS}^0 + k_R c_M N_S - (k_{MS}^{ma} + k_D)N_{MS}\,. \qquad (B.4)$$

c_S^0 and c_{MS}^0 are the concentrations of S and MS^+ directly at the interface ($x = 0$). The boundary conditions are

$$D_S\, \partial c_S/\partial x|_{x=0} = \beta_S k_S^{ma} c_S^0 - k_S^{ma} N_S\,, \qquad (B.5)$$

$$D_{MS}\, \partial c_{MS}/\partial x|_{x=0} = \beta_{MS} k_{MS}^{ma} c_{MS}^0 - k_{MS}^{ma} N_{MS}\,. \qquad (B.6)$$

The time derivative of the total carrier concentration $c = c_S + c_{MS}$ in water is the sum of eqs. (B.1) and (B.2),

$$\partial c/\partial t = D\, \partial^2 c/\partial x^2\,. \qquad (B.7)$$

Here, assumption (3), that the diffusion coefficient of carrier and carrier-ion complex are equal, was used. Because of assumption (2), the chemical reaction between carrier S and ion M^+ may be considered to be in equilibrium. Hence,

$$c_S = c/(Kc_M + 1)\,, \quad c_{MS} = Kc_M c/(Kc_M + 1), \qquad (B.8)$$

$$N_S = N/(K_h c_M + 1)\,, \quad N_{MS} = K_h c_M N/(K_h c_M + 1), \qquad (B.9)$$

where $N = N_S + N_{MS}$ represents the total carrier concentration at one interface. Using eqs. (B.3), (B.4), (B.8), (B.9) and relation (36)

$$\beta_S K_h = \beta_{MS} K\,, \qquad (B.10)$$

one obtains for the time derivative of N:

$$\frac{dN}{dt} = \beta_S \frac{k_S^{ma} + k_{MS}^{ma} K_h c_M}{Kc_M + 1} c^0 - \frac{k_S^{ma} + k_{MS}^{ma} K_h c_M}{K_h c_M + 1} N\,. \qquad (B.11)$$

Taking the sum of eqs. (B.3), (B.4) and (B.5), (B.6), one finds (using assumption 3)

$$D\, \partial c/\partial x|_{x=0} = dN/dt\,. \qquad (B.12)$$

Eqs. (B.7), (B.11) and (B.12) are transformed into eqs. (A.1)–(A.3) of the corresponding problem for hydrophobic ions by making the identifications stated in eqs. (24) and (25) (see section 3.2).

References

[1] R. Collander and H. Bärlund, Acta Botan. Fenn. 11 (1933) 1.
[2] A. Finkelstein, J. Gen. Physiol. 68 (1976) 127.
[3] J.M. Wolosin, H. Ginsburg, W.R. Lieb and W.D. Stein, J. Gen. Physiol. 71 (1978) 93.

[4] E. Galluci, S. Micelli and C. Lippe, Arch. Intern. Physiol. Biochim. 78 (1971) 881.
[5] M. Poznansky, S. Tong, P.C. White, J.M. Milgram and A.K. Solomon, J. Gen. Physiol. 67 (1976) 45.
[6] B.J. Zwolinsky, H. Eyring and C. Reese, J. Phys. Colloid. Chem. 53 (1949) 1426.
[7] P.C. Jordan and G. Stark, Biophys. Chem. 10 (1979) 273.
[8] W. Knoll and G. Stark, J. Membr. Biol. 37 (1977) 13.
[9] M. Eigen and L. De Maeyer, in: Techniques of organic chemistry, Vol. 8, Pt. 2, ed. A. Weissberger (Interscience, New York, 1963) p. 895.
[10] M. Eigen, Quart. Rev. Biophys. 1 (1968) 3.
[11] L.E. Moore, J.P. Holt Jr. and B.D. Lindley, Biophys. J. 12 (1972) 157.
[12] L.E. Moore, Biochim. Biophys. Acta 375 (1975) 115.
[13] G. Stark, R. Benz, G.W. Pohl and K. Janko, Biochim. Biophys. Acta 266 (1972) 603.
[14] D.H. Turner, G.W. Flynn, N. Sutin and J.V. Beitz, J. Am. Chem. Soc. 94 (1972) 1554.
[15] S. Ameen, Rev. Sci. Instr. 46 (1975) 1209.
[16] H. Hoffmann, E. Yeager and J. Stuehr, Rev. Sci. Instr. 39 (1968) 649.
[17] R.V. Ambartzumian and V.S. Letokhov, in: Chemical and biochemical applications of lasers, Vol. 3, ed. C.B. Moore (Academic Press, New York, 1977) p. 167.
[18] J.F. Holzwarth, in: Technique and application of fast reactions in solution, eds. W.J. Gettins and E. Wyn-Jones (Reidel, Dordrecht, 1979) p. 47.
[19] D.M. Goodall, R.C. Greenhow and B. Knight, J.F. Holzwarth and W. Frisch, in: Technique and application of fast reactions in solution, eds. W.J. Gettins and E. Wyn-Jones (Reidel, Dordrecht, 1979) p. 561.
[20] B. Ketterer, B. Neumcke and P. Läuger, J. Membr. Biol. 5 (1971) 225.
[21] W. Knoll and G. Stark, J. Membr. Biol. 25 (1975) 249.
[22] W. Brock, Ph.D. Thesis, University of Konstanz, in preparation.
[23] R. Benz, B.F. Gisin, H.P. Ting-Beall, D.C. Tosteson and P. Läuger, Biochim. Biophys. Acta 455 (1976) 665.
[24] G. Eisenman, G. Szabo, S. Krasne, S. McLaughlin and S. Krasne, in: Progress in surface and membrane science, Vol. 6, eds. J. Danielli, M. Rosenberg and D. Cadenhead (Academic Press, New York, 1973) p. 139.
[25] G. Stark, in: Membrane transport in biology, Vol. 1, ed. D.C. Tosteson (Springer, Berlin, 1978) p. 447.
[26] G. Stark, B. Ketterer, R. Benz and P. Läuger, Biophys. J. 11 (1971) 981.
[27] R. Laprade, S. Ciani, G. Eisenman and G. Szabo, in: Membranes, Vol. 3, ed. G. Eisenman (Dekker, New York, 1975) p. 127.
[28] S.B. Hladky, Biochim. Biophys. Acta 375 (1975) 327.
[29] R. Benz and G. Stark, Biochim. Biophys. Acta 382 (1975) 27.
[30] S.W. Feldberg and G. Kissel, J. Membr. Biol. 20 (1975) 269.
[31] R. Benz and P. Läuger, J. Membr. Biol. 27 (1976) 171.
[32] R. Benz, J. Membr. Biol. 43 (1978) 367.
[33] H.-A. Kolb and P. Läuger, J. Membr. Biol. 41 (1978) 167.
[34] G. Stark and R. Benz, J. Membr. Biol. 5 (1971) 133.
[35] S.B. Hladky, Biochim. Biophys. Acta 307 (1973) 261.
[36] R. Benz, G. Stark, K. Janko and P. Läuger, J. Membr. Biol. 14 (1973) 339.
[37] O.S. Andersen and M. Fuchs, Biophys. J. 15 (1975) 795.
[38] J.F. Skinner and R.M. Fuoss, J. Phys. Chem. 68 (1964) 1882.
[39] D.C. Tosteson, in: Perspectives in membrane biophysics, ed. D.P. Agin (Gordon and Breach, New York, 1972) p. 129.
[40] H. Ginsburg and G. Stark, Biochim. Biophys. Acta 455 (1976) 685.
[41] E. Grell and I. Oberbäumer, in: Molecular biology, biochemistry and biophysics, Vol. 24, eds. I. Pecht and R. Rigler (Springer, Berlin, 1977) p. 371.
[42] W. Simon and W.E. Morf, in: Membranes, Vol. 2, ed. G. Eisenman (Dekker, New York, 1973) p. 329.
[43] R. Benz, O. Fröhlich and P. Läuger, Biochim. Biophys. Acta 464 (1977) 465.
[44] S. McLaughlin, in: Current topics in membrane transport, Vol. 9, eds. F. Bronner and A. Kleinzeller (Academic Press, New York, 1977) p. 71.
[45] D.H. Haynes and P. Simkowitz, J. Membr. Biol. 33 (1977) 63.
[46] D.H. Haynes, V.C.K. Chin and B. Watson, Arch. Biochem. Biophys. 203 (1980) 73.
[47] P. Woolley and H. Diebler, Biophys. Chem. 10 (1979) 305.
[48] L. Thilo, Biochim. Biophys. Acta 469 (1977) 326.
[49] R. Folger, H. Hoffmann and W. Ulbricht, Ber. Bunsenges. Physik. Chem. 78 (1974) 986.
[50] H. Hoffmann, I. Kielmann, W. Ulbricht, E.A.G. Anianson, S.N. Wall, M. Almgren, R. Zana, J. Lang and C. Tondre, J. Phys. Chem. 80 (1976) 905.