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A laser-T-jump study of the adsorption of dipolar molecules to planar lipid membranes

I. 2,4-dichlorophenoxyacetic acid

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Abstract. The adsorption of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) as well as of other dipolar molecules to the interface of artificial lipid membranes gives rise to a change of the dipole potential between the membrane interior and water. As a consequence of the adsorption of the neutral species, the conductance of planar membranes, observed in the presence of the macrocyclic ion carriers nonactin or valinomycin, may change by many orders of magnitude. Using this effect in combination with a laser-T-jump technique, the kinetics of the adsorption process were measured and were interpreted on the basis of a Langmuir-isotherm. A partition coefficient (at small concentrations) of $\beta_{HA} = 4.7 \cdot 10^{-4}$ cm, a rate constant of desorption $k_{HA} \geqq 100\,s^{-1}$ and a maximum surface density $N_D = 7.7 \cdot 10^{13}/cm^2$ were found. The concentration at half saturation is $K_{HA} = 2.7 \cdot 10^{-4}\,M$.

Using these values the membrane conductance induced by the ion carrier nonactin and the shape of the current-voltage relationship as a function of the ligand concentration in water was analyzed. A maximum dipole potential of $V_D^{max} = -239$ mV and a contribution of $b = 3.1 \cdot 10^{-15}\,V\,cm^2$ per single adsorbed 2,4-D molecule was found. 74% of the dipole potential acts on the inner membrane barrier separating the two interfacial adsorption planes of nonactin. The remainder (26%) favours interfacial complex formation between nonactin and K^+ from the aqueous phase. The data hold for membranes formed from dioleoyllecithin in *n*-decane.

Key words: Adsorption, lipid membranes, laser-T-jump, Langmuir isotherm, 2,4-D

Introduction

The adsorption of lipophilic molecules to biological membranes is of great importance for their permeability properties. The measurement of the concentration of adsorbed molecules as a function of the free concentration in water has, however, turned out to be difficult. Therefore, starting with the pioneering work of Collander and Bärlund (1933) the membrane concentration on nonelectrolytes is usually estimated via the oil/water partition coefficient, thus avoiding a direct determination.

The present study deals with adsorption to an artificial membrane system. Planar lipid membranes have proven to be an excellent in vitro-system for the investigation of mechanisms of ion transport across biological membranes. It was found, however, that these membranes can also be used to study the behaviour of nonelectrolytes which, as a consequence of their adsorption to the membrane interface, modify the electrical properties of the membrane. The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) (Smejtek and Paulis-Illangasekare 1979 a, b) and phloretin, the inhibitor of certain routes of transport across biological membranes (Andersen et al. 1976; Melnik et al. 1977; Reyes et al. 1983) as well as other dipolar molecules (McLaughlin 1973; Szabo 1974) were found to modify the dipole potential at the membrane/water interface. As a result, the membrane permeability for hydrophobic ions and for alkali ions in the presence of macrocyclic ion carriers may be changed by many orders of magnitude.

We use this effect to study the kinetics of adsorption of dipolar molecules using a laser-T-jump method. The data are analyzed on the basis of Langmuir's equation. The maximum number of available sites at the interface, the aqueous concentration at half-saturation and the contribution of a single adsorbed molecule to the dipole potential were obtained. In favourable cases the method also allows one to determine the rate constants of adsorption and desorption.

The present paper concentrates on the methodological aspects, illustrating the procedure with the herbicide 2,4-D. In a second paper the adsorption of

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phloretin will be considered and compared with a series of analogues which show only small structural differences (Reyes et al. 1983). The method allows one to study the effect of substituents on membrane adsorption in a more detailed way than has so far been possible.

Experimental

The experiments were performed on optically black lipid membranes made from a 1% solution of 1,2-dioleoyllecithin (Avanti Polar Lipids) in *n*-decane (Fluka, standard for gas chromatography). The aqueous solutions normally contained 0.1 *M* KCl, 0.9 *M* LiCl, 10 m*M* buffer and 10^{-7} *M* nonactin (Boehringer). In some experiments valinomycin (Boehringer) was used instead of nonactin. It was added to the membrane forming solution (10^{-3} *M*). The solutions also contained appropriate concentrations of either 2,4-dichlorophenoxyacetic acid (2,4-D) (Aldrich) or phloretin (Sigma) added from stock solutions in ethanol. Most of the experiments in the presence of 2,4-D (pK $\approx$ 2.7) were performed at pH 2 (phosphate buffer). In the case of phloretin (pK = 7.35) the pH was adjusted to 5 (acetate buffer). The pH was selected to ensure that the substances were largely in the protonated (neutral) form. The PTFE-cuvette used for bilayer formation was cleaned after every experiment (ethanol in case of phloretin, chromic acid in the case of 2,4-D).

The laser-T-jump method as applied to planar lipid membranes was described in previous publications (Brock et al. 1981; Stark et al. 1986). In brief, the temperature of the membrane and of the surrounding aqueous phase is increased by a few tenths of a degree (typically 0.2°–0.3 °C) by absorption of a high intensity infrared flash (wavelength 1.06 μm), which is produced by a Nd-glass laser (JK Lasers Ltd. GB). The response of the electric current was observed at constant applied voltage. The current transient after amplification was stored in a digital oscilloscope (Tektronix 7603/7D20, 1024 words memory size, 8 bit resolution). The time range of the signal extended over more than two orders of magnitude in time. To allow for simultaneous detection of the fast and slow components of the signal the time base of the oscilloscope was controlled by an external clock. The clock emits signals at a constant rate, which after a preselected number of points can be changed to another value. In this way the time per oscilloscope division can be changed up to four times.

The digital data were transferred to a microcomputer (Victor, Sirius I) and fitted to the equations outlined below. The criterion for the selection of the values of the parameters given in the legends to the figures and tables was to minimize chi-square. This was achieved by using procedures such as that proposed by Marquardt (Bevington 1969). The uncertainty of the values is about 20%. This refers to those cases, where a good agreement between theory and experiment was observed (as for all 2,4-D data).

The time range of the method is limited by the width of the laser flash (400 μs in the fixed *Q* mode of the laser) and at long times by the temperature stability of the membrane and its environment (limited by heat conduction and convection to about 5 s). The heating time can be reduced to 40 ns by using the *Q*-switch mode of the laser. The problems of signal distortion associated with this technique (due to the presence of shock waves) have been solved recently. For 2,4-D and phloretin the comparatively low time resolution of the fixed *Q*-mode was sufficient. For some of the analogues of phloretin the high time resolution of the *Q*-switch-technique was necessary (cf. the subsequent paper). Details of the fast technique will be published elsewhere (R. Awiszus, G. Stark, in preparation).

The measurement of the current-voltage characteristics was carried out as described previously (Stark and Benz 1971). All experiments were performed at 25 °C.

Theory

The theoretical treatment of adsorption of dipolar lipophilic molecules is based on a model proposed by Ketterer et al. (1971) to explain saturation effects in the presence of lipid soluble ions. The model is illustrated in Fig. 1. The molecules adsorb to the membrane/water-interface, where they find a fixed surface density, N_D, of binding sites. The rate constants of adsorption and desorption are βk and k. The rate of adsorption for a symmetrical problem is given by

$$\frac{dN}{dt} = \beta k C_0 \left(1 - \frac{N}{N_D}\right) - k N. \tag{1}$$

C_0 is the aqueous concentration adjacent to the membrane (bulk aqueous concentration C_B), N is the actual surface density at time t. The factor $(1 - N/N_D)$ accounts for the probability that an adsorbing molecule will find a free site at the interface.
At equilibrium

$$N = \beta C_B/(1 + q), \tag{2}$$

with

$$q = \beta C_B/N_D \ . \tag{3}$$

β is the concentration independent partition coefficient at low surface density

$$\beta = N/C_B, \quad (N \ll N_D). \tag{4}$$

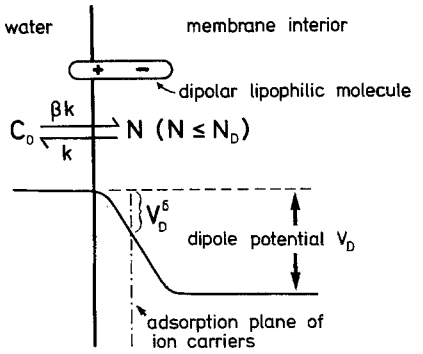

Fig. 1. The dipole potential V_D induced by adsorption of dipolar lipophilic molecules at the membrane/water interface (intrinsic dipole potential of the interface not shown). The part, V_D^δ, of V_D acts at the adsorption plane of the sensor molecules (see text)

The aqueous concentration at half saturation of the binding sites, K_S, obtained from Eqs. (2) and (3) is

$$K_S = N_D/\beta. \tag{5}$$

A T-jump taking place at time $t = 0$ gives rise to an instantaneous change of the model parameters:

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

Consequently the surface density, N, will change its value. The kinetics of this process are influenced by the diffusion in the unstirred aqueous layers adjacent to the membrane. A decrease (increase) of N gives rise to a transient increase (decrease) of the aqueous concentration C near the membrane interface. Therefore, the solution of the problem requires Eq. (1), the diffusion equation, i.e. (D = diffusion coefficient)

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2}, \tag{6}$$

and the boundary condition

$$D\left.\frac{\partial C}{\partial x}\right|_{x=0} = \frac{dN}{dt}. \tag{7}$$

The surface density of binding sites, N_D, is assumed to be (virtually) temperature independent.

We show (for a more general case) in appendix A that Eq. (1) may be reduced to the problem of simple partitioning of molecules between membrane and water, i.e. $N \ll N_D$. This holds, if the amplitude of the T-jump is small enough to ensure a small perturbation of the system. Then, the general problem is formally identical to that obtained in the case of negligible saturation ($N_D \to \infty$, $q = 0$), which was considered previously (Jordan and Stark 1979; Brock et al. 1981; Brock 1981). The result is

$$N(t) = N_\infty[1 - (\Delta\beta/\beta)F(t)], \tag{8}$$

with

$$F(t) = \frac{2\eta}{\pi}\int_0^\infty \frac{\exp(-k^* t v^2)\,dv}{(v^2-1)^2 + \eta^2 v^2}, \tag{9}$$

$$\eta = \beta^*\sqrt{k^*/D} \tag{10}$$

$$\beta^* = \beta/(1+q)^2 \quad \text{and} \tag{11}$$

$$k^* = k(1+q) \tag{12}$$

v is a dimensionless integration variable. N_∞ is the equilibrium interfacial concentration at the temperature $T + \Delta T$.

Since $F(0) = 1$ and $F(t \to \infty) = 0$ one finds from Eq. (8)

$$F(t) = \frac{N(t) - N_\infty}{N_0 - N_\infty} \tag{13}$$

with $N_0 = N(t=0) = N_\infty(1 - \Delta\beta/\beta)$ being the interfacial concentration before the T-jump.

Therefore, measurement of $N(t)$ or $F(t)$ allows one to determine the effective partition coefficient, β^*, and the effective rate constant of desorption, k^*, by fitting Eqs. (9) and (10) to the data.

In the case of comparatively fast aqueous diffusion, i.e. $\eta \to 0$ ($D \gg k^* \beta^{*2}/4$), Eq. (9) is reduced to the simple exponential

$$F(t) = \exp(-k^* t). \tag{14}$$

In general, however, $F(t)$ depends also on the effective partition coefficient, β^*. This is an effect of diffusion through the unstirred aqueous layers. In the case of sufficiently large values of β^* ($D \ll k^* \beta^{*2}/4$), $F(t)$ becomes independent of k^*, i.e.

$$F(t) = e^{\varkappa t}\,\mathrm{erfc}[(\varkappa t)^{1/2}] \tag{15}$$

with $\varkappa = D/\beta^{*2}$.

The measurement of $F(t)$ proceeds as follows: We assume that the membrane – in addition to the neutral lipophilic substance L under study – contains a conductance probe with the following properties:

1) There is no T-jump induced relaxation in the presence of the conductance probe alone, i.e. in the absence of L.

2) The current density J – which is due to the presence of the probe molecules at a constant voltage V – depends on the surface density N of adsorbed molecules L, i.e.

$$J = f(N). \tag{16}$$

3) A change of N is followed by a virtually simultaneous change of J. A possible delay in the adjustment of Eq. (16) is much shorter than the time behaviour of the adsorption process of the ligand L.

4) The presence of the conductance probe has no influence on the adsorption of L, i.e. molecular inter-

actions between L and the conductance probe can be neglected.

A T-jump experiment – at sufficiently small ΔT– represents a minor perturbation of the system. Consequently, since $|N - N_0| \ll N_0$, the function f may be approximated by the first two members of a Taylor expansion, i.e.

$$f(N) \approx f(N_0) + (N - N_0)\left(\frac{df}{dN}\right)_{N_0}. \tag{17}$$

Combining Eqs. (13), (16) and (17) gives

$$F(t) = \frac{J(t) - J_\infty}{J_0 - J_\infty}, \tag{18}$$

J_∞ = steady state current at the temperature $T + \Delta T$. Thus, the kinetics of adsorption are monitored via the relaxation $J(t)$ of the electric current.

In reality, the measurement of the initial current J_0 is influenced by the heating time (width of the laser flash). Similarly the measurement of J_∞ is also complicated by the finite time range of the method (see "Experimental"). Therefore, the experimental data were analyzed in the following way. If t_1 is the minimum time ($t_1 \geqq 400\,\mu s$) and t_2 is the maximum time of measurement ($t_2 \leqq 5$ s), the experimental quantity $F_{exp}(t)$ is defined as

$$F_{exp}(t) = \frac{J(t) - J(t_2)}{J(t_1) - J(t_2)}, \tag{19}$$

i.e. J_0 and J_∞ are replaced by $J(t_1)$ and by $J(t_2)$.

Using Eqs. (18) and (19) one obtains

$$F_{exp}(t) = \frac{F(t) - F(t_2)}{F(t_1) - F(t_2)}. \tag{20}$$

By applying Eq. (20) in combination with Eqs. (9) and (10), $F_{exp}(t)$ was numerically evaluated and it was compared to the experimental data analyzed according to Eq. (19). In this way β^* and k^* were determined. These quantities were further analyzed on the basis of Eqs. (3), (11), and (12) to obtain the model parameters β, k and N_D. This was achieved by measuring the dependence of β^* and k^* on the bulk concentration C_B.

As conductance probes the neutral macrocyclic ion carriers nonactin and valinomycin were used. In the presence of these substances the K^+-conductance of lipid membranes is drastically enhanced. Both compounds meet the requirements for a probe molecule mentioned above (see "Results and discussion"). The conductance may be interpreted on the basis of a carrier mechanism which is well established through the work of many different groups (e.g., Szabo et al. 1969; Markin et al. 1969; Stark and Benz 1971; Hladky 1974; Laprade et al. 1982). The following model was used to interpret the carrier-conductance (Läuger and Stark 1970): Metal ions M^+ (e.g. K^+) from the aqueous phase (concentration C_M) undergo complex formation with neutral carrier molecules S at the membrane/water interface (rate constants k_R and k_D). The velocity of movement of the free carrier S and of the carrier-ion-complex MS^+ across the membrane interior is determined by the rate constants k_S and k_{MS}. The magnitude of these rate constants depends on the height of the energy barriers which separate energy minima at the two membrane/water interfaces for both the species S and MS^+. The application of a voltage V to the membrane leads to an asymmetric energy barrier for the positively charged complex MS^+ (i.e. $k_{MS} \rightarrow k'_{MS}$, k''_{MS}). The latter represents the driving force for a net movement of ions M^+ across the membrane. The voltage dependence of k_{MS} is described as (Knoll and Stark 1975)

$$\begin{aligned} k'_{MS} &= k_{MS} \exp(-\xi u)\, f(u) \\ k''_{MS} &= k_{MS} \exp(\xi u)\, f(u), \end{aligned} \tag{21}$$

with $u = FV/RT$ reduced voltage (F = Faraday constant, R = gas constant, V = voltage, T = temperature). The function $f(u)$ is a correction term which allows for arbitrary barrier shapes. For a steep and narrow energy barrier in the middle of the membrane: $f(u) = 1$. Different authors (e.g., Hladky 1974; Ciani 1976) used trapezoidal- or image force-barriers to fit the model to experimentally observed steady state current-voltage relationships. We used an empirical function $f(u)$ determined as follows: The steady state current-voltage curve, described as the voltage dependence of the ratio λ/λ_0 ($\lambda = J/V$ membrane conductance, λ_0 = voltage independent conductance observed at low voltages) is given by (Stark and Benz 1971; Knoll and Stark 1975):

$$\frac{\lambda}{\lambda_0} = \frac{2(1 + A) f(u) \sinh(u/2)}{u(1 + A f(u) \cosh(u/2))}, \tag{22}$$

with

$$A = k_{MS}/k_D(2 + k_R C_M/k_S) \tag{23}$$

Eq. (22) was obtained using $\xi = 0.5$. This implies that the voltage dependence of $k_R C_M$ is neglected. In reality, ξ is slightly smaller than 0.5, i.e. $k_R C_M$ is weakly voltage dependent [see also discussion in context with Eq. (29)].

Approximate values for the ratios k_{MS}/k_D and k_R/k_S were estimated by fitting Eqs. (22) and (23) to experimentally observed current-voltage curves at different concentrations C_M using the assumption $f(u) = 1$. The results observed for K^+-complexes of valinomycin and of macrotetralides such as nonactin are $(k_{MS}/k_D)_{K^+} \leqq 0.5$ (Stark and Benz 1971; Benz and Stark 1975; Hladky 1974, 1975 a). The stability of the carrier complexes MS^+ formed with Na^+ or Li^+ is smaller than that formed with K^+ by several orders of magnitude (Szabo et al. 1969; Krasne and Eisenman

1976). Therefore, the relation $(k_{MS}/k_D)_{Na^+} \ll (k_{MS}/k_D)_{K^+}$ holds (assuming an identical translocation rate constant k_{MS} for K^+- and Na^+-complexes). Consequently, the assumption $(k_{MS}/k_D)_{Na^+} \approx 0$ appears as a satisfactory approximation to reality. Setting $A = 0$ the function $f(u)$ may be calculated by fitting Eq. (22) to experiments performed in the presence of Na^+ or Li^+. It was found that the data were in good agreement with the empirical function

$$f(u) = \frac{1}{g} \frac{g + hu^2(g-2)}{1 + hu^2} \tag{24}$$

with $g = 8.83$ and $h = 0.1$. The numerical values hold for nonactin in dioleoyllecithin membranes formed from solutions in *n*-decane.

The form of Eq. (24) was chosen to obtain an "inverse sigmoid" behaviour of $f(u)$ suggested by the data. Details of this procedure will be published elsewhere. Using Eq. (24) improved values for the ratios k_{MS}/k_D and k_R/k_S are obtained from the measurement of current-voltage curves in the presence of K^+.

Equation (22) was derived from the current-voltage relationship

$$J = B \frac{(k_R C_M/k_D) k_{MS} f(u) \sinh(u/2)}{1 + A f(u) \cosh(u/2)} \tag{25}$$

with

$$B = \frac{2F\beta_s C_t}{KC_M + 1} .$$

$\beta_S = N_S/C_S$ is the partition coefficient of the neutral carrier S between interface and water, $C_t = C_{MS} + C_S$ is the total carrier concentration in water. $K = C_{MS}/C_M C_S$ is the equilibrium constant of complex formation in water.

The adsorption of dipolar molecules L to the interface gives rise to a dipole potential V_D. We neglect discrete charge effects and assume that V_D is proportional to the surface density N of the molecules, i.e.

$$V_D = -bN \tag{26}$$

$$b = \mu/\varepsilon\varepsilon_0 \tag{27}$$

μ = dipole moment perpendicular to the membrane interface produced through adsorption of a single molecule L, ε_0 = permittivity of free space. ε = dielectric constant within the absorbed layer of molecules.

The presence of the dipole potential modifies the height of the energy barrier of the membrane interior experienced by the positively charged carrier complexes MS^+. In addition a part, V_D^δ, of V_D acts on the interfacial complex formation (cf. Fig. 1). The two effects may be accounted for by considering the following potential dependences for the translocation rate constant, k_{MS}, and for the product $k_R C_M$:

$$k_{MS} = k_{MS}^0 \exp[-F(V_D - V_D^\delta)/RT] \tag{28}$$

$$k_R C_M = (k_R C_M)_0 \exp(-FV_D^\delta/RT) \tag{29}$$

k_{MS}^0 and $(k_R C_M)_0$ represent rate constants in the absence of L. Equation (29) may be understood as an increase of the concentration $C_M^* = \gamma_M C_M$ at the plane of complex formation induced by negative values of V_D, i.e. $\gamma_M = \gamma_M^0 \exp(-FV_D^\delta/RT)$ and $k_R C_M = k_R^0 C_M^*$ (Knoll and Stark 1975). There is also a comparatively weak dependence of $k_R C_M$ on the voltage difference across the membrane (Hladky 1974, 1975 a; Knoll and Stark 1975), which is neglected in the present treatment. This is justified by the fact that V_D acts across a very thin layer (comparable to the dimension of the dipolar molecule), whereas the membrane voltage, V, drops across the complete thickness of the membrane. Therefore, at comparable absolute values of V_D and V, the effect of V_D on the interfacial reaction is considerably larger.

Using Eqs. (26), (28) and (29), the effect of adsorption of dipolar molecules L (surfaces density N) on the current density J caused by macrocyclic ion carriers may be expressed as

$$J = E \frac{\exp(b\alpha N)}{(1 + A(N)\sigma)}, \tag{30}$$

with

$$E = \frac{2F\beta_s C_t}{KC_M + 1} \frac{(k_R C_M)_0}{k_D} k_{MS}^0 f(u) \sinh(u/2), \quad \text{and}$$

$$\alpha = F/RT, \quad \sigma = f(u)\cosh(u/2).$$

$A(N)$ corresponds to Eq. (23), i.e. $A(0) = A$ and

$$A(N) = 2z_0 \exp[b(1-a)\alpha N)] + v_0 \exp(b\alpha N), \tag{31}$$

$z_0 = k_{MS}^0/k_D$, $v_0 = (k_R C_M)_0 k_{MS}^0/k_S k_D$ and $a = V_D^\delta/V_D$.

$A(N)$ may be obtained from an analysis of the current-voltage relationship [Eq. (22)], using Eq. (31) instead of Eq. (23). The experiments were performed at sufficiently small concentrations C_M so that the second term of Eq. (31) could be neglected, i.e. $v_0 \exp(b\alpha N) \ll 2z_0 \exp[b(1-a)\alpha N]$.

The complete analysis may be summarized as follows: The study of the T-jump induced relaxation of the electric current [according to Eq. (20) in combination with Eq. (9)] as a function of the aqueous concentration C_B of a dipolar ligand L allows one to determine the model parameters β, k and N_D. As a result, the surface density N may be calculated as a function of C_B using Eqs. (2) and (3). The further analysis based on the current-voltage relationship and on the conductance as a function of N enabled us to estimate the contribution, b, of a single ligand molecule to the dipole potential V_D. This is achieved by applying Eqs.

(22) and (30) in combination with Eq. (31). The measurement of $A(N)$ via the current-voltage characteristic allows one to estimate the quantities z_0 and $b(1-a)$. By the additional measurement of $J(N)$ a separate determination of b and a is obtained.

The analysis was completed by the following test of consistency. The model parameters may be used to predict the concentration dependence of the relaxation amplitude of the T-jump experiments. Using Eqs. (2), (3), (30), and (31) one finds ($\Delta J/J = (J_0 - J_\infty)/J_\infty$)

$$\frac{\Delta J/J}{\Delta T} = \alpha b\, g(N) \frac{C_B}{(1+\beta C_B/N_D)^2} \frac{\Delta\beta}{\Delta T}, \qquad (32)$$

with

$$g(N) = \frac{1 + 2 z_0 \delta a \exp[b(1-a)\alpha N]}{1 + [2 z_0 \exp(b(1-a)\alpha N) + v_0 \exp(b\alpha N)]\delta}.$$

Equation (32) predicts small amplitudes for low and high concentrations of the ligand L. There is a maximum at intermediate concentrations. For $z_0 = v_0 \to 0$ the maximum is found at $C_B = N_D/\beta$.

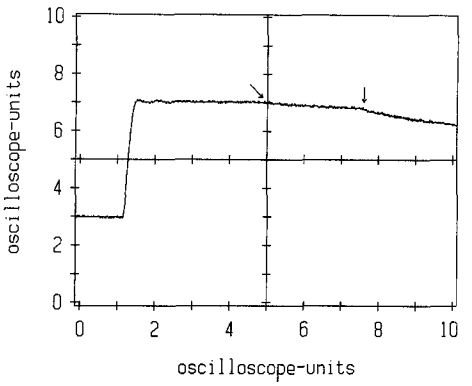

Fig. 2. Record of the current response following a T-jump at a planar lipid membrane in the presence of the sensor molecule valinomycin (control experiment). The membrane (area $7 \cdot 10^{-2}$ cm^2) was formed from a 1% solution of dioleoyllecithin in n-decane in the presence of 10^{-3} M valinomycin. Aqueous solution 1 M KCl, applied voltage 50 mV, membrane conductance $3 \cdot 10^{-3}$ S cm^{-2}. Oscilloscope: Vertical sensitivity 50 nA/division, the time scale was changed at the indicated arrows from 1 ms/division to 100 ms/division and finally to 1 s/division. The stepwise current increase mirrors the rise of the temperature (see text)

Results and discussion

The use of the ion carriers nonactin or valinomycin as probes for the adsorption of dipolar lipophilic substances suggests itself for the following reasons:

The process of adsorption is accompanied by an enormous increase of the electric conductance extending over many orders of magnitude (Andersen et al. 1976; Smejtek and Paulis-Illangasekare 1979 a). The amplitude of a T-jump induced relaxation in the presence of the probe alone is virtually zero within a time range of microseconds up to several seconds. This is illustrated in Fig. 2. Following a T-jump there is an almost instantaneous increase of the current. The effect is caused by the mobility increase (rate constant k_{MS}) of the carrier complexes MS^+, the charge carriers responsible for the membrane conductance. The rise time mirrors the width of the laser pulse (≈ 400 µs). After the initial stepwise increase the current stays constant and shows only a slow decrease in the region of seconds. This indicates that the specific relaxations of the valinomycin system are either faster or slower than the time range considered here. In fact it was found that the characteristic time constants of valinomycin mediated transport are either in the time range of 10–50 µs or in the range of minutes (Brock et al. 1981). Besides, the amplitude of the T-jump induced fast process is too small to be resolved within the present accuracy. The slow process reflects the temperature dependent partitioning of valinomycin between membrane and water, i.e., the process considered here for 2,4-D. Because of the large partition coefficient of valinomycin and/or because of the small exchange rate between membrane and water, the equilibration takes place within about 30 min (Stark et al. 1972). As a result, the current, after the initial step, is virtually constant from the shortest accessible time (time resolution about 10 µs in the Q-switch mode of the laser) up to several seconds. Similar experiments to the one shown in Fig. 2 were performed with nonactin. It was found that the temperature stability in the range of seconds is even better than that observed in the presence of valinomycin (see also Brock et al. 1981).

The intrinsic relaxation processes of the probe system also determine the time range within which the probe may be used as an indicator of the kinetics of the adsorption process (cf. "Theory", assumption 3 for the conductance probe). The carrier kinetics should be fast compared to the adsorption kinetics of the ligand L. The problem is treated in detail in appendix B. The intrinsic relaxation times of the nonactin – or the valinomycin-system depend on the kind of lipid, on the ion concentration in water, and on the membrane voltage. For membranes made from dioleoyllecithin or monoolein in n-decane the relaxation times in the case of valinomycin were found to be < 50 µs (Benz et al. 1973; Knoll and Stark 1975). For nonactin in monoolein membranes in the presence of 0.1 M KCl solutions the relaxation time was $\leqq 10$ µs (Hladky 1975 b; Laprade et al. 1982). This should also hold for membranes formed from dioleoyllecithin. The problem is less severe in the presence of the dipolar ligands, since the kinetics of the carrier systems are accelerated because of an increase of the rate constants. Therefore, signal distortion caused by the finite response of the probe system may be neglected for processes slower

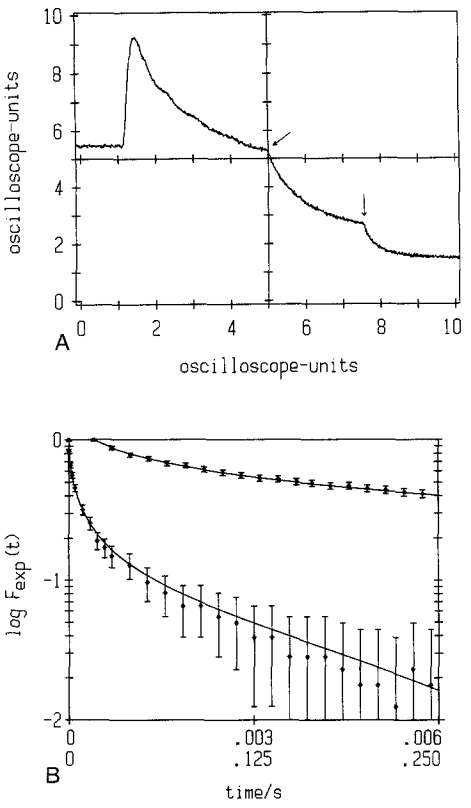

Fig. 3 A and B. T-jump induced current relaxation observed in the presence of 2,4-dichlorophenoxyacetic acid and nonactin. Aqueous solution: 0.1 *M* KCl, 0.9 *M* LiCl, 10 m*M* phosphate buffer (pH 2), 10^{-7} *M* nonactin and $4 \cdot 10^{-4}$ *M* 2,4-D. Membrane area $7 \cdot 10^{-2}$ cm^2, applied voltage 50 mV, membrane conductance $9.4 \cdot 10^{-4}$ S cm^{-2}. **A)** Record of the current transient. Oscilloscope: Vertical sensitivity 20 nA/division, the time scale was changed at the indicated *arrows* from 1 ms/unit to 10 ms/unit and finally to 200 ms/unit. **B)** Analysis of the data according to Eq. (19) using two different time scales. The *solid lines* were calculated from Eqs. (15) and (20) with $t_1 = 400$ µs, $t_2 = 0.4$ s, $D = 5.9 \cdot 10^{-6}$ cm^2/s and $\beta^* = 10^{-4}$ cm. Using these values, $F(t_1) = 0.63$ and $F(t_2) = 0.038$ is obtained. The *error bars* indicate the measurement uncertainty

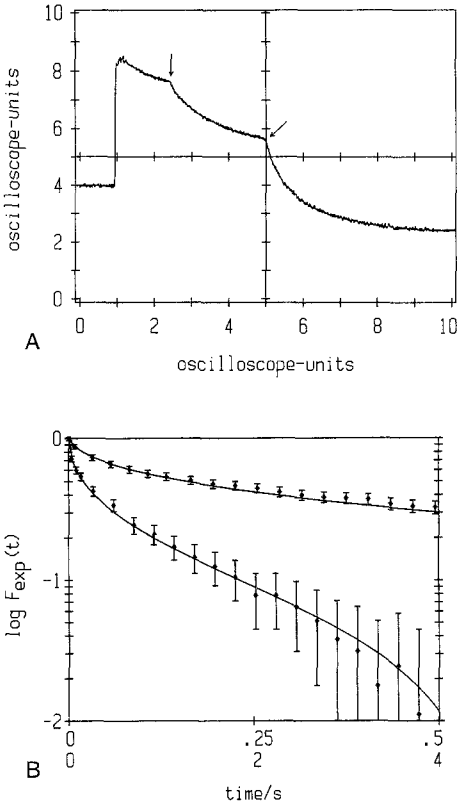

Fig. 4 A and B. T-jump induced relaxation of the electric current in the presence of phloretin and nonactin. Aqueous solution: 0.1 *M* KCl, 0.9 *M* LiCl, 10 m*M* acetate buffer (pH 5), 10^{-7} *M* nonactin and 10^{-5} *M* phloretin. Membrane area $7 \cdot 10^{-2}$ cm^2, applied voltage 50 mV, membrane conductance $4.6 \cdot 10^{-4}$ S cm^{-2}. **A)** Record of the current transient. Oscilloscope: Vertical sensitivity 20 nA/division, the time scale was changed at the indicated *arrows* from 5 ms/division to 50 ms/division and finally to 1 s/division. **B)** Analysis of the data according to Eq. (19) using two different time scales. The *solid lines* were calculated according to Eqs. (15) and (20) with $t_1 = 900$ µs, $t_2 = 5$ s, $D = 5.5 \cdot 10^{-6}$ cm^2/s and $\beta^* = 1.5 \cdot 10^{-3}$ cm. Using these values, $F(t_1) = 0.95$ and $F(t_2) = 0.16$ is obtained. The *bars* indicate the measurement uncertainty

than 10 µs. This is also about the time resolution of our electrical detection system in the T-jump experiments.

Summarizing, the system nonactin/K^+ represents an excellent means to study the kinetics of adsorption of dipolar substances within a time range of 10 µs to at least 4 s. The same holds for the system valinomycin/K^+ though in this case signal distortion cannot be excluded at times < 50 µs.

Applications of the method to 2,4-D and phloretin are shown in Figs. 3 and 4. In both cases a decreasing current is observed. This is interpreted as a reduction of the dipole potential due to a decrease of the surface density N with increasing temperature. The current transient extends over several orders of magnitude in time. The variable time base was changed twice to permit a complete detection of the signal. The data were transformed using Eq. (19) and were found to be in fair agreement with the applied theory [Eqs. (9), (10), and (20)]. In both cases, clearly non-exponential behaviour was observed. This indicates a strong influence of unstirred layer diffusion. In fact a reasonable fit to the data in Figs. 3 and 4 is obtained assuming $k^* \geqq 500$ s^{-1} (2,4-D) and $k^* \geqq 25$ s^{-1} (phloretin). The values for β^* vary only slightly within the range of permissible values for k^* ($\beta^* = (1 \pm 0.2) \cdot 10^{-4}$ cm for 2,4-D and $\beta^* = (1.5 \pm 0.2) \cdot 10^{-3}$ cm for phloretin). Therefore, for both substances only a lower limit could be derived for k^*, while β^* could be determined with reasonable accuracy. For the further analysis it was assumed that the adsorption process is completely unstirred layer controlled, i.e., Eq. (15) was applied.

While the two substances show the same behaviour in principle, they differ with respect to the time

scale of the effect. The kinetics of phloretin are slower by at least one order of magnitude. Therefore, its effective partition coefficient β^* must be larger (i.e., more substance has to diffuse across the unstirred layers). This is confirmed by the values obtained from the analysis.

For 2,4-D, less than 80% of the actual transient are detected within the experimental time resolution of a fixed Q-experiment (under the conditions of Fig. 3 $F(t_1) = 0.63, t_1 = 400$ µs). Using the Q-switch mode of the laser, t_1 may be reduced to 10 µs. $F(t_1) \approx 0.9$ is found in such an experiment. The maximum accessible time t_2 is, however, considerably smaller under Q-switch conditions ($t_2 \approx 50$ ms) because of an impaired temperature stability. Thus, the detection of the fast part of the signal is improved while that of the slow part is worsened ($F(t_2) \geqq 0.1$). A comparison between the two experimental methods will be presented in Awiszus and Stark (1988).

Identical results were obtained in the presence of the chemically different conductance probes nonactin or valinomycin. A variation of the probe concentration by an order of magnitude also had no effect on the shape of the transient. We think, therefore, that any influence of the probe molecules on the adsorption behaviour of the ligand L can be largely neglected.

In the following we concentrate on the behaviour of 2,4-D. The effective partition coefficient β^* decreases with increasing concentration, C_B, in water (Fig. 5). This is predicted by the theory. It is a characteristic of the Langmuir isotherm and means that the change, ΔN, of the surface density decreases towards zero as N increases towards the saturating level N_D. Analysis of the data yields the partition coefficient β and the maximum surface density N_D. The data are less accurate at small and at large concentrations. This is largely due to the fact that the relaxation amplitude has a maximum at intermediate concentrations. Within the given experimental error, the data agree with the assumption of a single set of identical binding sites. The deviations observed at small concentrations might, however, indicate a certain variability in the affinity of the sites. Thus, the applied theory is presumably an idealization.

Summarizing, the analysis of the T-jump data enables us to calculate the surface density, N, of adsorbed ligand molecules as a function of the concentration in water. This in turn allows an extended interpretation of the dipole potential induced by these molecules as is shown below.

Smejtek and Paulis-Illangasekare (1979 a) found that the current-voltage characteristics of membranes formed from egg lecithin/cholesterol, or from monoolein in the presence of nonactin, are continuously changed from superlinear to sublinear behaviour as the concentration of 2,4-D in water is increased. As a result an increase of the parameter A in Eq. (22) was reported.

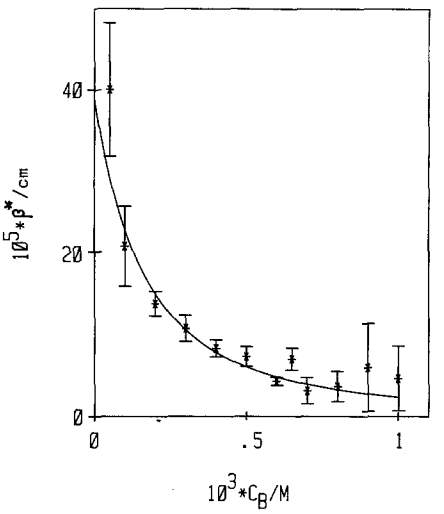

Fig. 5. The effective partition coefficient β^* as a function of the concentration, C_B, of 2,4-D in water. The *data points* are mean values of at least 5 different measurements, the *bars* represent the standard deviation. The *solid line* was calculated according to Eqs. (3) and (11) using $\beta = 3.9 \cdot 10^{-4}$ cm and $N_D = 7.7 \cdot 10^{13}$ cm^{-2}

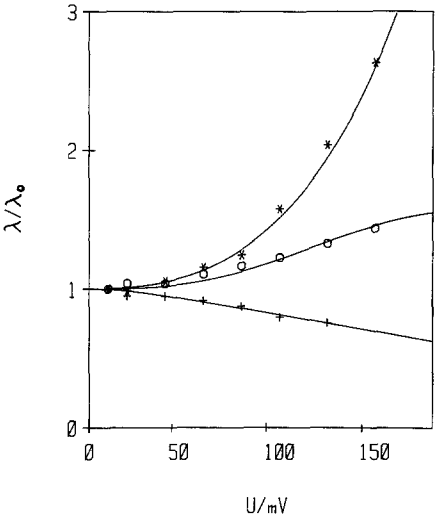

Fig. 6. Current-voltage characteristic of three different dioleoyllecithin membranes in the presence of 10^{-7} M nonactin, 0.1 M KCl, 0.9 M LiCl, 10 mM phosphate buffer (pH 2) and increasing concentrations of 2,4-D. The *solid lines* were fitted to the data using Eq. (22) in combination with Eq. (24). The following values were obtained for the parameter A: $*$:$C_B = 0$, $A = 0$; o: $C_B = 3 \cdot 10^{-4} M$, $A = 0.13$; $+$:$C_B = 10^{-3} M$, $A = 0.7$

The knowledge of N as a function of C_B permits a refined analysis. The measurement of $A(N)$ and of $J(N)$ and their analysis according to Eqs. (30) and (31) yields the quantities b and a, i.e., the contribution of a single adsorbed molecule to the dipole potential, V_D, and the fraction V_D^δ/V_D, acting on the interfacial reac-

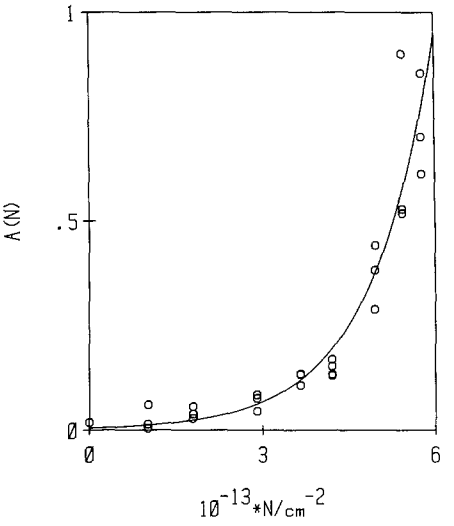

Fig. 7. The parameter A as a function of the surface density N of adsorbed molecules 2,4-D. The data were obtained from single membranes except for $N = 0$ (mean value of 19 membranes). Eq. (31) was fitted to the data. The following values of parameters were obtained (using $v_0 = 0$): $z_0 = 2.4 \cdot 10^{-3}$, $b(1 - a) = 2.3 \cdot 10^{-15}$ V cm^2

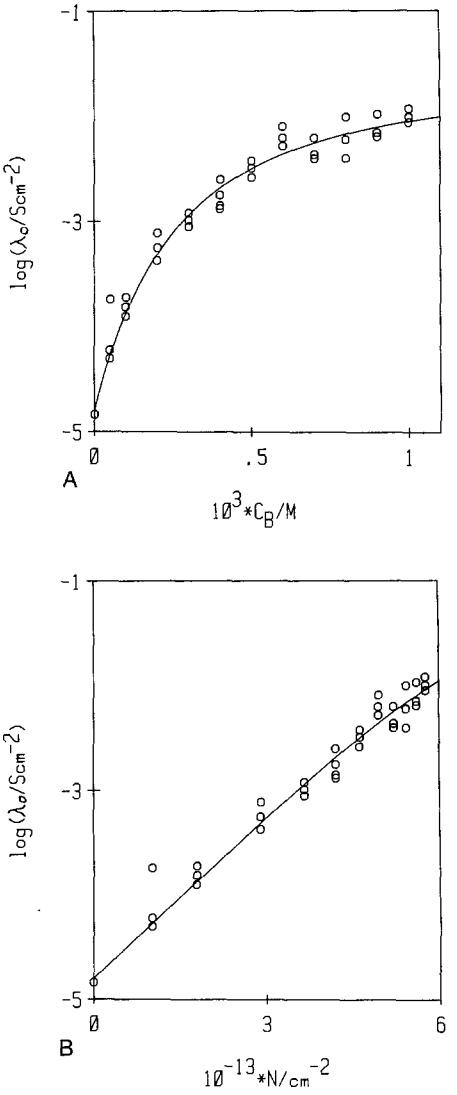

Fig. 8 A and B. Zero voltage conductance, λ_0, as a function of the concentration of 2,4-D. Experimental conditions see legend to Fig. 6. The data represent single membranes except for $N = 0$ (mean value of 25 membranes). **A**)The dependence of λ_0 on the bulk concentration C_B in water. **B**)The dependence of λ_0 on the surface density N at the membrane interface. N was calculated from C_B according to Eqs. (2) and (3) using the values for β and N_D from the T-jump analysis (see legend to Fig. 5). The *solid lines* represent a fit of Eqs. (30) and (31) to the data using values for z_0 and $b(1 - a)$ from the analysis of the current-voltage characteristic (cf. legend to Fig. 7). The free parameters were obtained as $E = 8 \cdot 10^{-7}$ A cm^{-2} and $b = 3.1 \cdot 10^{-15}$ V cm^2.

tion of the carrier. Our experimental results obtained with dioleoyllecithin membranes are summarized in Figs. 6–8. Instead of the current, the reduced conductance λ/λ_0 is plotted as a function of the voltage. There is a steep (exponential) increase of A with increasing surface density N as is predicted by Eq. (31) (neglecting $v_0 \exp(b \alpha N) \leqq 0.1 \cdot 2 z_0 \cdot \exp(b(1 - a) \alpha N)$. Neglecting the second term is justified by the finding that A is only very weakly dependent on the ion concentration C_M. Saturating behaviour is observed, if $\log \lambda_0$ is plotted versus C_B. It is converted to an almost exponential dependence, if λ_0 is plotted as a function of the surface density N (cf. Fig. 8). This indicates that the denominator in Eq. (30) is of minor importance.

The values of the model parameters are summarized in Table 1. Instead of β and k, the pH-corrected values for the neutral species HA (using Eq. (A4) and $K_a = 500\, M^{-1}$) are listed. The value for β_{HA} may be compared with the predictions of a macroscopic octanol/water-system (Hansch and Leo 1979). A concentration ratio of $\gamma = 646$ was reported for the two solvents in equilibrium with each other with respect to 2,4-D. A value of $\beta_{HA} = \gamma d/2 = 1.6 \cdot 10^{-4}$ cm (assuming a membrane thickness $d = 50$ Å) is extrapolated from such a two phase system. It is about three times smaller than that found for the interface of a dioleoyllecithin membrane. The main difference between the partition equilibrium of an oil/water system and the interface of a lipid membrane applies to the saturation behaviour of the latter. The surface density, N_D, of binding sites listed in Table 1 corresponds to an area per binding site of 130 Å^2 or a maximum number one 2,4-D molecule per 2.5 lipid molecules.

Table 1. Table of the model parameters for the adsorption of 2,4-D to the membrane/water interface of dioleoyllecithin membranes

β_{HA}	[cm]	$4.7 \cdot 10^{-4}$
k_{HA}	[s^{-1}]	$\geqq 100$
N_D	[cm^{-2}]	$7.7 \cdot 10^{13}$
K_{HA}	[M]	$2.7 \cdot 10^{-4}$
b	[V cm^2]	$3.1 \cdot 10^{-15}$
a		0.26
V_D^{max}	[mV]	−239
$\Delta H_0(\beta)$	[kJ mol^{-1}]	− 54

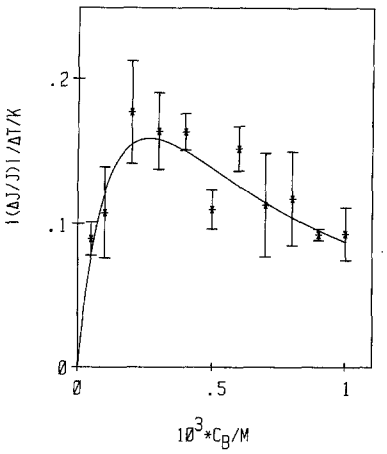

Fig. 9. The reduced relaxation amplitude $|\Delta J/J|/\Delta T$ as a function of the concentration of 2,4-D in water. Experimental conditions compare legend to Fig. 3. The *solid line* was calculated according to Eq. (32) using values for the parameters given in Table 1. The only free parameter, $\Delta\beta/\Delta T$, is obtained as $2.85 \cdot 10^{-5}$ cm/K

With all binding sites occupied a maximum dipole potential of about 240 mV is induced, negative towards the membrane interior. This value, has, however, not been observed experimentally. At the largest concentration $C_B = 10^{-3}\,M$, V_D is about -180 mV. The orientation and the magnitude of the dipole moment induced through adsorption of 2,4-D is sufficient to cancel the intrinsic positive dipole potential of an unmodified (pure) membrane formed from dioleoyllecithin. A value of 224 mV – as a rough estimate – was reported for the latter, using negatively and positively charged lipophilic ions as probes (Pickar and Benz 1978). A fraction $a = 0.26$ of V_D induced by 2,4-D acts between the adsorption plane of nonactin and the aqueous phase. The remainder $(1 - a) = 0.74$ leads to a reduction of the membrane barrier sensed by the translocation rate constant of the nonactin/K^+-complex. This is similar to the hydrophobic ion tetraphenylborate (Smejtek and Paulis-Illangasekare 1979 b).

The main contribution of the present study is the determination of the surface density, N, as a function of the ligand concentration, C_B, in water. As a consequence, the mean contribution, b, of a single molecule to the dipole potential is obtained. It may be further interpreted as follows: Treating the layer of adsorbed dipolar molecules as a plate condenser, the dielectric constant, ε, inside the layer may be estimated using Eq. (27). If the dipole moment, μ, produced through adsorption of a single 2,4-D molecule is equated with the dipole moment of a single 2,4-D molecule (μ_L = 3.33 Debye, McClellan 1974), $\varepsilon = 4$ is obtained. This would indicate that the adsorption plane of 2,4-D is at the lipophilic side of the membrane/water interface. The procedure is, however, a rough approximation only, since the orientation of other dipolar molecules (e.g. water) might be changed during the adsorption process, i.e. there might be further contributions to the dipole moment μ (see Awiszus and Stark 1988, for a further discussion of this problem).

There is an independent test of the analysis performed so far. The relaxation amplitude $|\Delta J/J|/\Delta T$ should show a maximum at $C_B \approx N_D/\beta$. It is found that Eq. (32) adequately describes the experimental results (Fig. 9). The scaling factor $\Delta\beta/\Delta T$ allows one to estimate the change in the enthalpy $\Delta H_0(\beta)$ associated with the binding process (see Table 1).

The applied procedure is based on a series of simplifications. Perhaps the most essential one is the assumption that the consequences of ligand adsorption for the conductance probe may be reduced to a matter of electrostatics only. Thus, a possible change of the microviscosity of the membrane interior and its effect on the translocation rate constants k_{MS} and k_S is neglected. Nevertheless a consistent analysis of the different experimental approaches was achieved. One cannot, however, exclude the possibility that a more refined analysis, based on additional voltage-jump relaxation experiments will lead to a modified dependence of the rate constants on the ligand concentration, i.e., to a modification of Eqs. (28) and (29). This would, however, affect the values of the dipolar quantities b, a, and $V_D^{\max}$ only. The quantities β_{HA}, k_{HA}, N_D and K_{HA}, which describe the adsorption behaviour of the ligand, were obtained without using details of the carrier transport mechanism. The values of these parameters are therefore invariant, if the voltage dependence and/or the dependence on the dipole potential of the rate constants is changed.

A similar study was performed with phloretin. A Langmuir-like adsorption isotherm of this substance to lipid membranes was reported previously (De Levie et al. 1979; Verkman and Solomon 1980; Verkman 1980). A combination of the dipole properties with those of adsorption has, however, not been performed. The behaviour of phloretin and of some structural analogues will be treated in part II (Awiszus and Stark 1988).

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Appendix A

The kinetics of adsorption of a weak acid HA as studied by a T-jump experiment at planar lipid membranes

The dipolar ligands used in the present study are weak acids HA. Only the neutral form HA binds to the

membrane/water interface and modifies the dipole potential, as is discussed in detail in the main text. Here we present a brief sketch of the general problem which, in the actual case, is reduced to the equations used for data analysis.

We assume that the binding sites at the interface may be occupied either by neutral HA or by negatively charged A^- (surface densities N_{HA} and N_A) with different partition coefficients β_{HA} and β_A. The protonation reaction proceeds either homogeneously in the aqueous phase or heterogenously at the membrane/water interface (equilibrium constants K_a and K_h, respectively). Therefore Eq. (1) must be applied separately to both of the two species HA and A^-, i.e., to N_{HA} and to N_A. This is also true for the diffusion Eq. (6), which in addition must be supplemented by the rate of association and dissociation in water. The problem is analogous to that of the adsorption of neutral carrier molecules which was treated in a previous publication (Brock et al. 1981). By applying the same procedure it is found that the general mathematical problem can be reduced to that outlined in the main text, i.e. to the solution of Eqs. (1), (6), and (7), provided the following identities are used:

$$N \to N_{HA} + N_A, \quad C \to C_{HA} + C_A, \tag{A1}$$

$$k \to \left(\frac{k_A}{K_h C_H} + k_{HA}\right) \frac{K_h C_H}{K_h C_H + 1} \tag{A2}$$

$$\beta \to \beta_{HA} \frac{K_a C_H}{K_a C_H + 1} \frac{K_h C_H + 1}{K_h C_H}. \tag{A3}$$

Eqs. (A1)–(A3) are obtained with the assumption of sufficiently well buffered aqueous solutions, i.e. a time independent proton concentration C_H. If the negatively charged species A^- are excluded from the binding sites ($N_A \to 0$, $K_h \to \infty$), Eqs. (A 2) and (A3) are reduced to

$$k \to k_{HA} \quad \text{and} \quad \beta \to \beta_{HA} K_a C_H/(K_a C_H + 1). \tag{A4}$$

Eqs. (A4) were used in the analysis of the experimental data.

The previous treatment of the adsorption kinetics (Brock et al. 1981) was performed neglecting saturation phenomena, i.e., $N \ll N_D$. The general problem (including saturation) is reduced to the previous one, if the solution is limited to a small perturbation of the system. This is shown as follows:

Substituting $n(t) = N_\infty - N(t)$ and $c(x,t) = C_B - C(x,t)$, and recalling $N_\infty = \beta C_B/q$ (Eq. (2)) one obtains from Eqs. (1), (6), and (7)

$$\frac{dn}{dt} = \frac{\beta k c_0}{1+q} - nk\left(1 + q - q\frac{c_0}{C_B}\right), \tag{A5}$$

$$\frac{\partial c}{\partial t} = D\frac{\partial^2 c}{\partial x^2}, \quad \text{and} \tag{A6}$$

$$D\left.\frac{\partial c}{\partial x}\right|_{x=0} = \frac{dn}{dt}. \tag{A7}$$

At a sufficiently small amplitude of the T-jump, i.e., $c_0 = c(0,t) \ll C_B$ and $(1 + q - q\,c_0/C_B) \approx (1+q)$, the problem is identical to that found in the absence of saturation ($q \ll 1$). Using the substitutions $k^* = k(1+q)$ and $\beta^* = \beta/(1+q)^2$, Eqs. (A5)–(A7) become formally independent of the degree of saturation. Therefore, the solution in the form of Eq. (9), which was originally derived for $q \to 0$ (Brock et al. 1981), is valid for arbitrary values of q.

Appendix B

The influence of carrier kinetics on the analysis of the time dependence of the adsorption of the ligand L

A change of the surface density N of ligand molecules is detected by measurement of the electrical conductance induced by carrier mediated transport of K^+-cations. The shape of the adsorption kinetics may be distorted by intrinsic relaxation processes of carrier transport, if the two phenomena proceed within the same time range. To avoid an interference of the two processes, carrier kinetics should be fast compared with the adsorption kinetics of the ligand. The problem may be formulated as follows:

The electric current density, J, caused by the movement of the carrier complexes MS^+ is

$$J = F(N'_{MS} k'_{MS} - N''_{MS} k''_{MS}), \tag{B1}$$

with N'_{MS}, N''_{MS} = surface densities of the complex MS^+ at the left and right membrane interface. Using Eq. (21) (and $\xi = 0.5$) one obtains

$$J = F k_{MS} f(u) m(t), \tag{B2}$$

with

$$m(t) = N'_{MS} \exp(-u/2) - N''_{MS} \exp(u/2). \tag{B3}$$

The change of J following a variation of the surface density N is obtained from Eqs. (B 2) and (B 3) in combination with Eqs. (26)–(29). The dependence $J(N)$ is determined by $k_{MS}(N)$ and by $m(N)$.

Suppose there is a stepwise (instantaneous) change ΔN. The quantity, $k_{MS}(N)$, will respond instantaneously. $m(N)$, however, will be delayed due to the specific relaxation processes of carrier transport. The problem is similar to that of a voltage-jump experiment (Stark et al. 1971). $N'_{MS}(t)$ and $N''_{MS}(t)$ may be written as

$$N^i_{MS}(t) = \bar{N}^i_{MS} + \sum_{k=1}^{3} A^k_i \, e^{-t/\tau_k}, \quad i = ' \text{ or } ''. \tag{B4}$$

For the dependence of the three relaxation times τ_k on the rate constants of the carrier model see Stark et al.

1971 (Eqs. 35–39). τ_k is a function of N (cf. Eqs. 26–29). Equation (30) was derived by neglecting relaxation processes of carrier transport, i.e., by setting $N^i_{MS}(t) = \bar{N}^i_{MS}$ ($\bar{N}^i_{MS}$ = steady state concentration $N^i_{MS}(N)$). This is justified, if the times τ_k are fast compared with the kinetics of ligand adsorption (see main text).

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