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A stopped-flow kinetic study of the interaction of potential-sensitive oxonol dyes with lipid vesicles

R.J. Clarke and H.-J. Apell

Department of Biology, University of Konstanz, D-7750 Konstanz, F.R.G.

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The interaction of the dyes oxonol V and oxonol VI with unilamellar dioleoylphosphatidylcholine vesicles was investigated using a fluorescence stopped-flow technique. On mixing with the vesicles, both dyes exhibit an increase in their fluorescence, which occurs in two phases. According to the dependence of the reciprocal relaxation time on vesicle concentration, the rapid phase appears to be due to a second-order binding of the dye to the lipid membrane, which is very close to being diffusion-controlled. The slow phase is almost independent of vesicle concentration, and it is suggested that this may be due to a change in dye conformation or position within the membrane, possibly diffusion across the membrane to the internal monolayer. The response times of the dyes to a rapid jump in the membrane potential has also been investigated. Oxonol VI was found to respond to the potential change in less than 1 s, whereas oxonol V required several minutes. This has been attributed to lower mobility of oxonol V within the lipid membrane.

1. Introduction

Voltage-sensitive dyes have been widely used for more than 10 years for measuring membrane potentials of cells, cell organelles and membrane vesicles [1–5]. A change in membrane potential results in an alteration in the dye fluorescence or absorption signal. From this dye response the membrane potential can be determined, assuming that the dye response has previously been calibrated at known membrane potentials. A commonly used calibration procedure is that developed by Hoffman and Laris [6], in which a diffusion potential for K^+ is generated in the presence of valinomycin and a K^+ concentration gradient.

The response times of various voltage-sensitive dyes to a change in membrane potential have been found to vary between less than microseconds and seconds, and various mechanisms have been proposed to explain the dye response. In general, in order to produce a change in the dye absorbance or fluorescence spectrum, the change in membrane potential must cause a perturbation of the dye's electronic system or a change in its chemical environment. This may come about in various ways:

(1) a potential dependent partition of the dye between the aqueous and lipid phases [8,11–15, 17–19];

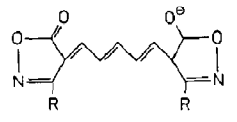
(2) a change in dye orientation or position in the lipid membrane [10,11,17];

(3) a change in the degree of dye aggregation within the lipid membrane or in the aqueous solution [7,8,10,11,16,17];

(4) a direct electrochromic effect, i.e., the electric field created by the potential causes a distortion of the dye's electronic structure [9].

It is also possible that the response of a given dye results from a combination of two or more of these basic mechanisms. The degree to which each mechanism contributes to the magnitude of the

Correspondence (present) address: R.J. Clarke, School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.



oxonol V R= ⬡
oxonol VI R=-$CH_2CH_2CH_3$

Fig. 1. Structures of oxonol V and VI.

overall dye response may also depend on the conditions under which the experiment is performed, e.g., relative concentrations of dye to lipid, temperature, ionic strength, and pH. It is therefore important for accurate measurement of membrane potential that the calibration of dye response be carried out under identical experimental conditions.

Oxonol dyes, such as oxonol V and VI, have been widely used as potential-sensitive dyes in membrane experiments. Oxonol V and VI (see fig. 1) have a pK_a near 4.2 [20] and are thus anionic at physiological pH values. They show relatively large variations in absorbance and fluorescence due to changes in membrane potential, and have been found to be particularly useful for measuring inside-positive potentials [18,21,22,27]. They have been applied in studies with submitochondrial particles [12–14,20,23,26,28], lipid vesicles [13,18, 20–22,24,30–33,35,36], mitochondria [20], bacterial chromatophores [25,35], bacterial spheroblasts [35], chromaffin granules [27], and animal cells [34,37,38]. When the dyes bind to the lipid membrane, red shifts of both the absorbance and fluorescence spectra are observed [14,18,20,24,27]. At low dye/lipid concentration ratios a fluorescence enhancement also occurs [14,18,20,27], which is probably due to the restricted motion of the dye molecule and its protection in the lipid phase from quenching agents. At increasing dye concentrations, however, the fluorescence enhancement diminishes and, at high dye/lipid concentration ratios, fluorescence quenching may be observed [20]. This is probably due to the accumulation of dye within the lipid, leading to quenching by dye aggregation or through an inner filter effect [20,27]. It is generally believed that an inside-positive membrane potential leads to an increase in dye binding to the lipid, which causes a red shift of the absorbance spectrum [12,23,25] and either an increase or decrease in fluorescence [12,18,27], depending upon the relative dye/lipid concentrations. It should be noted that the red shift of the absorbance spectrum could itself lead to a decrease in fluorescence due to a change in the optimum excitation wavelength; furthermore, increased overlap of the absorbance and fluorescence spectra may give rise to increased self-reabsorption. However, if the excitation wavelength is chosen so as to give maximum fluorescence of membrane-bound dye and the dye concentration is kept low, these effects seem not to be important.

Oxonol dyes have also been used in the measurement of transient membrane potentials generated by ion pumps [18,27–30,32,34,36]. In this case it is important that the dye response rate be much faster than the rate of change of the potential. With this in mind, a kinetic investigation of the interaction of oxonol V and VI with lipid vesicles and their response to changes in membrane potential has been carried out. From a comparison of the kinetic properties of oxonol V and VI, information concerning the effect of dye structure on the response rate can be obtained.

2. Materials and methods

2.1. Materials

Dioleoylphosphatidylcholine was obtained from Avanti Polar Lipids (Birmingham, AL); oxonol V (bis(3-phenyl-5-oxoisooxazol-4-yl)pentamethine oxonol) and oxonol VI (bis(3-propyl-5-oxoisooxazol-4-yl)pentamethine oxonol) were from Molecular Probes (Junction City, OR); valinomycin was from Boehringer-Mannheim. The purity of the dyes was checked by thin-layer chromatography and the results indicated only traces of degradation products. Therefore, they were used without further purification. The phospholipid contents of vesicle suspensions were determined by use of the phospholipid B test from Wako (Osaka). All other reagents were obtained from Merck (analytical grade). Dialysis tubing was purchased from Serva (Heidelberg).

2.2. Vesicle preparation

Lipid vesicles were prepared from synthetic dioleoylphosphatidylcholine according to a previously described dialysis method producing unilamellar vesicles with an average outer diameter of 72 nm and a narrow size distribution (half-width about 10 nm) [39,40]. All vesicle suspensions were prepared in buffer H (30 mM imidazole, 1 mM L-cysteine, 1 mM EDTA, 5 mM $MgSO_4$) containing various amounts of Na_2SO_4 and K_2SO_4. The pH of the buffer was adjusted to 7.2 with H_2SO_4.

2.3. Stopped-flow measurements

Stopped-flow experiments were carried out using a stopped-flow unit (ZWS 11) of Sigma Instruments, Berlin, with fluorescence detection at right angles to the incident light beam. By the use of appropriate filters, the excitation wavelength was maintained at 580 (± 5) nm and the emission was observed at wavelengths ≥ 630 nm. The current signal of the photomultiplier was converted to voltage by a current-voltage converter (1 A = 25000 V). The output signal of the converter was amplified 10 times and passed through an active low-pass filter (bandwidth 1 kHz). The voltage signal for each transient was then collected as 3968 12-bit data points using a Nicolet 4094A digital oscilloscope and stored on floppy disk. At the end of an experiment the data could be plotted on an X-Y recorder. The solutions in the drive and reservoir syringes were equilibrated to a temperature of 22°C prior to each experiment. The drive syringes were driven by compressed air. The mixing time of the stopped-flow unit was determined to be approx. 7 ms.

The interaction of the dyes with phosphatidylcholine vesicles in the absence of a membrane potential was investigated by mixing a dye solution with an equal volume of vesicle suspension. Experiments were performed at varying dye/lipid concentration ratios. All solutions contained 75 mM K_2SO_4 to prevent any undesired osmotic effects.

In order to investigate the response time of the dyes to a change in membrane potential, stopped-flow experiments were performed in which a voltage jump is produced. In these experiments a suspension of vesicles equilibrated with dye, valinomycin and buffer containing 2.5 mM K_2SO_4 was mixed with an equal buffer volume containing 75 mM K_2SO_4. In this way a diffusion potential for K^+ of approx. 66 mV (inside positive) was generated within the mixing time. To prevent osmotic effects, the vesicles were prepared in buffer containing 72.5 mM Na_2SO_4, so that the total ($K_2SO_4 + Na_2SO_4$) concentration remained constant at 75 mM. The relaxation times obtained are the average from at least four transients.

2.4. Static fluorescence measurements

Fluorescence measurements were carried out in a Perkin Elmer 650-40 fluorescence spectrophotometer. The thermostatically controlled cuvette holder was equipped with a magnetic stirrer. The excitation wavelength was set to 580 nm (slit width 20 nm) and the emission wavelength to 660 nm (slit width 20 nm). The oxonol V stock solution contained 0.03 mM dye in ethanol. 1 μl of this solution was added to 1 ml of buffer in the cuvette to obtain a final (total) oxonol concentration of 30 nM.

The cuvette was filled with 1 ml of buffer and equilibrated in the thermostatted cuvette holder to 22°C. After measuring the background fluorescence, oxonol V was added. Following the attainment of a constant fluorescence signal, an aliquot of the vesicle suspension was added. Fluorescence changes, ΔF, caused by additions of varying amounts of vesicle suspensions were determined as relative changes with respect to the fluorescence level, F_0, prior to the addition; they were corrected for the small dilution effect which was determined separately by adding a known amount of buffer solution.

3. Theory

Consider a vesicle as being a supramolecule with an undefined (arbitrarily large) number of binding sites for dye. The 'molecular weight' of a vesicle is then given by

$$M_v = L\left[\tfrac{4}{3}\pi\left(r_o^3 - r_i^3\right)\right]/\bar{v} \qquad (1)$$

where r_o and r_i denote the external and internal radii of the vesicle, respectively, L Avogadro's constant, and $\bar{v}$ the partial specific volume of lipid in the vesicle membrane [41]. Assuming a membrane thickness of 4 nm, mean values of r_o and r_i have been found to be 36 and 32 nm, respectively [39,40]. It is known that the vesicle radii follow a Gaussian distribution which causes an uncertainty in the value of M_v and the number of lipid molecules per vesicle [40]. The numbers given are valid for an average vesicle with the mean radius. The value of $\bar{v}$ has been previously determined to be approx. 0.985 $cm^3\ g^{-1}$ [42,43]. Introducing these values into eq. 1 gives $M_v = 3.56 \times 10^7$ g mol^{-1}. The molecular weight of dioleoylphosphatidylcholine, M_l, is 786.14 g mol^{-1}. Thus, the number of lipid molecules per vesicle, n, is (for the mean radius) given by

$$n = \frac{M_v}{M_l} = 45\,200 \tag{2}$$

Accordingly, the molar vesicle concentration can then be directly calculated from the molar lipid concentration by dividing by 45 200.

The binding of dye, D, to vesicles, V, can be represented by the following reaction scheme:

$$D + V \underset{k''}{\overset{k'}{\rightleftarrows}} DV$$

$$D + DV \underset{2k''}{\overset{k'}{\rightleftarrows}} D_2V$$

$$D + D_2V \underset{3k''}{\overset{k'}{\rightleftarrows}} D_3V$$

...

Implicit in these relations is the assumption that there are no interactions between the dye molecules in the lipid membrane, so that the dye association rate constant, k', is identical for each step and the dissociation rate constant increases only by a statistical factor corresponding to the number of dye molecules in the vesicle. The total concentration of dye bound to the lipid, C^*_{DV}, and that of the vesicles, C^*_V, are given by

$$C^*_{DV} = C_{DV} + 2C_{D_2V} + 3C_{D_3V} + \ldots \tag{3}$$

$$C^*_V = C_V + C_{DV} + C_{D_2V} + C_{D_3V} + \ldots \tag{4}$$

The overall rate of change of concentration of bound dye is, according to the reaction scheme above,

$$\frac{dC^*_{DV}}{dt} = k'C_DC_V + k'C_DC_{DV} + k'C_DC_{D_2V} + \ldots - \left(k''C_{DV} + 2k''C_{D_2V} + 3k''C_{D_3V} + \ldots\right) \tag{5}$$

Combination with eqs. 3 and 4 yields,

$$\frac{dC^*_{DV}}{dt} = k'C_DC^*_V - k''C^*_{DV} \tag{6}$$

Now, according to the method of Schwarz [44,45], let us introduce the term

$$r = \frac{C^*_{DV}}{C^*_V} \tag{7}$$

which represents the ratio of bound dye to the total vesicle concentration. Substituting for C^*_{DV} from eq. 7 in eq. 6 gives,

$$\frac{dr}{dt} = k'C_D - k''r \tag{8}$$

We now introduce the total dye concentration C^*_D:

$$C^*_D = C_D + C^*_{DV} \tag{9}$$

as well as the equilibrium constant for dye binding to the vesicle

$$K = \frac{k'}{k''} = \frac{C^*_{DV}}{C_DC^*_V} \tag{10}$$

Combination of eqs. 7–10 then yields

$$\frac{dr}{dt} = -(k'C^*_V + k'')(r - \bar{r}) \tag{11}$$

$$\bar{r} = \frac{\bar{C}^*_{DV}}{C^*_V} = \frac{KC^*_D}{KC^*_V + 1} \tag{12}$$

$\bar{r}$ and $\bar{C}^*_{DV}$ designate the equilibrium values of r and C^*_{DV}, respectively.

In the stopped-flow experiments the binding of dye to the vesicles has been followed by monitoring the change in dye fluorescence. The fluorescence, F, at time, t, can be defined by the following equation,

$$F = n_w f_w + n_l f_l \tag{13}$$

where n_w and n_l are the number of moles of dye in the aqueous and lipid phases, respectively, f_w and f_l being the corresponding values of fluorescence per mole of dye.

Eq. 13 may be rewritten introducing the total number, $n = n_l + n_w$, of dye molecules and expressing n_l by $n_l = VC^*_{DV} = VrC^*_V$ (V, total volume of suspension); this yields:

$$F = rVC^*_V(f_l - f_w) + f_w n \tag{14}$$

According to eq. 14 the rate of change of F and the equilibrium value $\bar{F}$ of F are given by:

$$\frac{dF}{dt} = VC^*_V(f_l - f_w)\frac{dr}{dt} \tag{15}$$

$$F - \bar{F} = VC^*_V(f_l - f_w)(r - \bar{r}) \tag{16}$$

Combining eqs. 11, 15 and 16 then yields

$$\frac{dF}{dt} = -(k'C^*_V + k'')(F - \bar{F}) \tag{17}$$

Thus, it can be seen that the fluorescence signal should follow a single-exponential time function with a relaxation time, τ, given by

$$1/\tau = k'C^*_V + k'' \tag{18}$$

A plot of the reciprocal relaxation time vs. the total vesicle concentration should yield a straight line, from which the rate constants k' and k'' can be determined from the slope and intercept, respectively.

If only one of the rate constants, k' or k'', can be accurately determined, the other can be indirectly calculated by using the value of the dye's membrane-water partition coefficient, γ, determined under equilibrium conditions, which is defined by

$$\gamma = \frac{n_l/V_l}{n_w/V_w} \tag{19}$$

where V_l and V_w denote the respective volumes of the lipid and water phases. On combination of eqs. 19 and 13, it can be shown that the fluorescence of a dye-vesicle mixture is given by

$$F = n\frac{f_w V_w + \gamma f_l V_l}{V_w + \gamma V_l} \tag{20}$$

Titration of a dye solution with a vesicle suspension and fitting the experimental data obtained to eq. 20 enable one to determine γ. If the volume of the lipid phase is much smaller than that of the water phase, which is normally the case, it can be assumed that V_w is approximately equal to the total suspension volume, V. Under these conditions, by comparison of eqs. 10 and 19 it can be shown that:

$$K = \frac{k'}{k''} = \gamma\bar{V} \tag{21}$$

where $\bar{V}$ is the lipid volume per mole of vesicles, and is expressed as:

$$\bar{V} = L\left[\tfrac{4}{3}\pi\left(r_o^3 - r_i^3\right)\right] \tag{22}$$

Since for the vesicles used in this study, r_o and r_i have been found to be 36 and 32 nm, respectively, $\bar{V}$ can be calculated to be 3.5×10^4 dm^3 mol^{-1}. Thus, k', k'' and γ are related by the equation

$$\frac{k'}{k''} = (3.5 \times 10^4 \text{ dm}^3 \text{ mol}^{-1})\gamma \tag{23}$$

4. Results

4.1. Determination of partition coefficients

The membrane-water partition coefficient, γ, of oxonol V has been determined by fluorescence titration of the dye with vesicles (as described in section 2) and fitting of the data to eq. 20. The value of γ obtained from the fit was $(5.9 \pm 0.2) \times 10^5$ (see fig. 2). The identical procedure has previously been carried out for oxonol VI [18], resulting in a value of γ for oxonol VI of $(1.9 \pm 0.1) \times 10^4$. Thus, oxonol V is significantly more lipophilic than oxonol VI, which perhaps reflects the less favourable hydration of the phenyl moieties of oxonol V in comparison to that of the propyl moieties of oxonol VI.

4.2. Oxonol V-vesicle interaction

When dye is rapidly mixed with vesicles in the stopped-flow apparatus, as described in section 2,

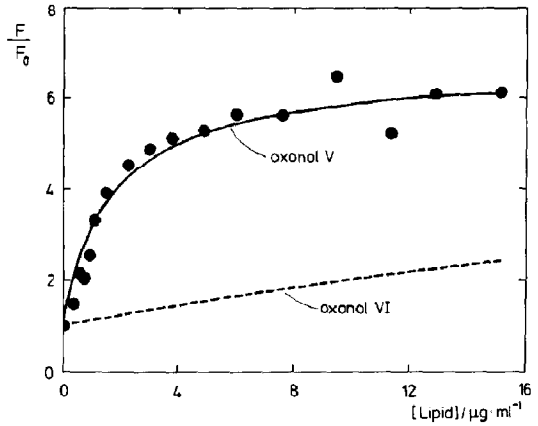


Fig. 2. Fluorescence intensity F of oxonol V at 660 nm (excitation wavelength 580 nm) as a function of lipid concentration at a constant total dye concentration of 30 nM. F is referred to the intensity, F_0, at zero lipid concentration. The continuous line represents a fit of the data to eq. 20. For comparison, the broken line shows the expected variation in fluorescence intensity of oxonol VI over this lipid concentration [18].

two relaxation processes are observed, both of which are characterized by an increase in fluorescence (see fig. 3). The two processes are widely separated in terms of their time scales, the faster process occurring in the range of tens of milliseconds and the slower in the range of tens of seconds. If the reciprocal relaxation time of the faster process is plotted vs. vesicle concentration (see fig. 4A), one can see that a straight line is obtained, suggesting that the process being observed is due to the binding of dye to the vesicle membrane (cf. eq. 18). From the slope of the plot, the second-order rate constant for the binding step can be estimated as:

$$k' = 1.18(\pm 0.05) \times 10^{11}\ \mathrm{M^{-1}\,s^{-1}}$$

which is similar to the rate constant for binding of the fluorescence indicator N-phenylnaphthylamine to lipid vesicles [46]. Theoretically, it should be possible to determine the rate constant, k'', for dissociation of dye from the membrane from the intercept of the plot. However, the value is too small to allow accurate determination from the experimental data. Nevertheless, k'' can be indirectly estimated by using the previously determined value of the partition coefficient, γ. On inspection of eq. 23 (see section 3), it can be seen that k'' is given by

$$k'' = \frac{k'}{3.5 \times 10^4 \times \gamma}$$

Thus, substituting the experimentally determined values of k' and γ into this equation, it can be found that,

$$k'' \approx 5.7\ \mathrm{s^{-1}}$$

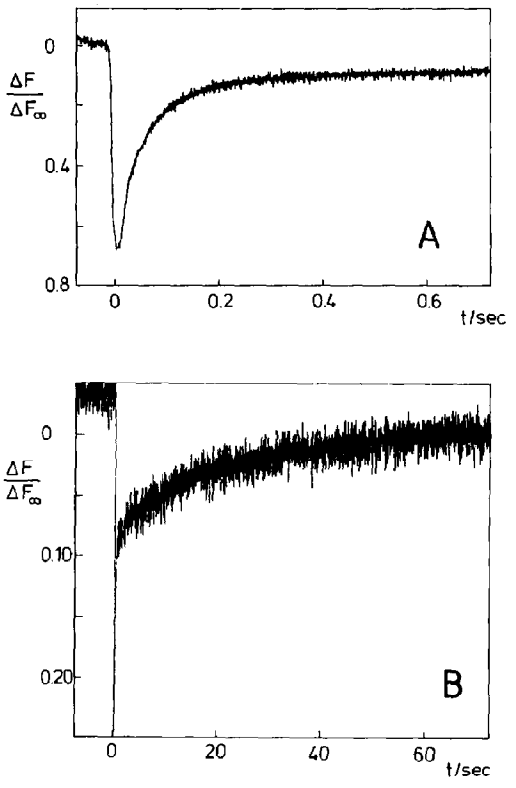


Fig. 3. Stopped-flow traces. (A) Oxonol V in buffer H containing 75 mM K_2SO_4 and vesicles in buffer H containing 75 mM K_2SO_4 were mixed to final concentrations of 75 nM dye and 0.14 nM vesicles (lipid concentration, 6.3 μM); $T = 22\,^\circ$C, pH 7.2. (B) As in panel A, but over a longer time range. The fact that the final value of the fluorescence does not reach that of the pretrigger level is due to a small degree of electrical disturbance which arises from electrical control of the delivery of compressed air. This disturbance, however, is instantaneous and does not affect the time course of the transients.

The dependence of the reciprocal relaxation time of the faster process on dye concentration is depicted in fig. 4B. At low dye concentrations, the reciprocal relaxation time appears to be practically independent of dye concentration, as expected from section 3. At high dye concentrations (i.e., at dye/lipid concentration ratios ≥ 0.05), however, there is a marked increase in the reciprocal relaxation time. Similar behaviour has previously been reported by others for binding of oxonol VI to submitochondrial particles [12], of the dye diS-C_3-(5) to a glycerylmonooleate dispersion [13] and of merocyanine 540 to submitochondrial particles [15]. These authors have suggested that this change in dye dependence is due to a breakdown in pseudo-first order conditions. This can be interpreted on the basis of the reaction scheme shown in section 3. Here, it was assumed that a vesicle possesses an indefinitely large number of dye-binding sites, such that it can

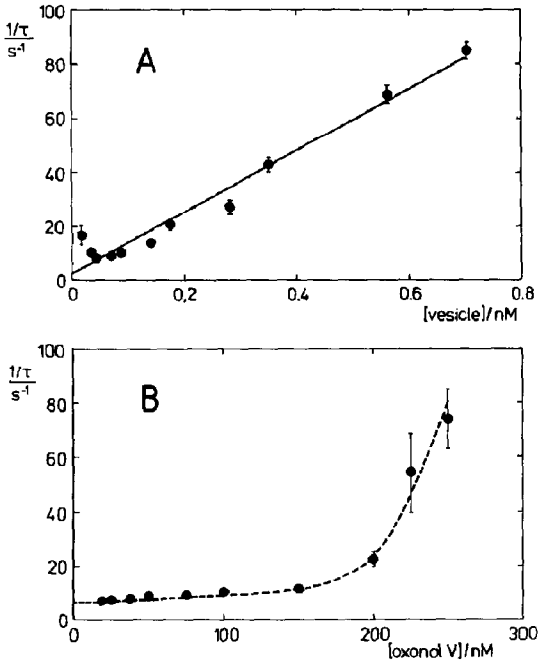


Fig. 4. Kinetic plots for the faster process of oxonol V. (A) Dependence of the reciprocal relaxation time on vesicle concentration. [Oxonol V] = 75 nM. (B) Dependence of the reciprocal relaxation time on oxonol V concentration. [Vesicle] = 0.070 nM.

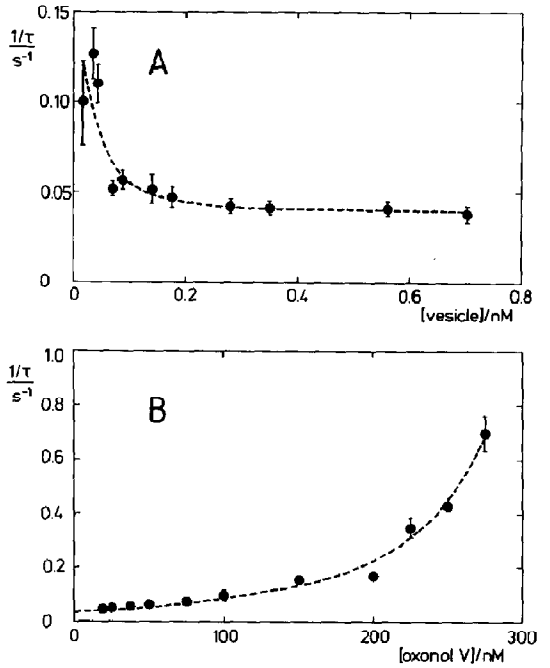


Fig. 5. Kinetic plots for the slower process of oxonol V. (A) Dependence of the reciprocal relaxation time on vesicle concentration. [Oxonol V] = 75 nM. (B) Dependence of the reciprocal relaxation time on oxonol V concentration. [Vesicle] = 0.070 nM.

act as a sink for dye molecules. As one proceeds to higher dye concentrations, however, this assumption becomes invalid, and thus a dependence of the reciprocal relaxation time on dye concentration would be expected.

In the case of the slower relaxation process, the concentration dependences are very different (see fig. 5). It can be seen that at high vesicle concentration and low dye concentrations, the reciprocal relaxation time is relatively insensitive to changes in the concentrations. If one proceeds to high dye/lipid concentration ratios, however (i.e., high dye concentrations at constant vesicle concentration or low vesicle concentrations at constant dye concentration) a dramatic acceleration in the reaction rate is observed. This acceleration begins to manifest itself at dye/lipid concentration ratios greater than approx. 0.015, corresponding to one dye molecule per 66 lipid molecules.

The origin of the slow phase is less clear in comparison. However, some possible mechanisms can be considered. A plausible explanation is that the vesicles possess two classes of binding sites with different association and dissociation rate constants. In this case, the reciprocal relaxation time of the slow phase should show a linear dependence on the vesicle concentration, as has been observed for the fast phase. Since such behaviour has not been found, a simple dye-vesicle binding reaction can be ruled out. A more likely explanation is that the slow phase is due to a change in dye conformation or position within the vesicle membrane. Such a reaction could result in a change in dye fluorescence simply due to the change in chemical environment of the dye or via coupling to the faster binding step. Thus, the following reaction scheme could be envisaged,

$$D + V \overset{K_1}{\rightleftarrows} DV \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} D^{\#}V \qquad (24)$$

If the binding step is assumed to be always at equilibrium on the time scale of the second step, it can be shown that the concentration dependence of the reciprocal relaxation time of the second step is governed by the following equation:

$$\frac{1}{\tau} = k_2\left(\frac{K_1 C_V^*}{1 + K_1 C_V^*}\right) + k_{-2} \qquad (25)$$

At sufficiently high vesicle concentrations, such that $K_1 C_V^* \gg 1$, eq. 25 reduces to

$$\frac{1}{\tau} = k_2 + k_{-2} \qquad (26)$$

Thus, at high vesicle concentrations this mechanism would predict a constant value of $1/\tau$, irrespective of the dye or vesicle concentration, which is in fact in agreement with the experimental observations. At low vesicle concentrations, however, such that $K_1 C_V^* \ll 1$, eq. 25 reduces to:

$$\frac{1}{\tau} = k_2 K_1 C_V^* + k_{-2} \qquad (27)$$

Thus, at low vesicle concentrations, a linear increase of $1/\tau$ with vesicle concentration would be expected. In actual fact, however, there is a decrease in the value of $1/\tau$ to a limiting value as the vesicle concentration is increased (see fig. 5A). The increase in the value of $1/\tau$ at high dye concentrations (see fig. 5B) is also not directly explicable in terms of this mechanism. A possible explanation, however, is that at high dye/lipid concentration ratios, variation in the values of k_2 and k_{-2} occurs due to perturbation of the membrane structure as dye accumulates in the membrane. This is supported by recent measurements made by Fumero et al. [48], which showed effects of both oxonol V and VI on the thermal phase transition of suspensions of dimyristoylphosphatidylcholine. That the slow phase is associated with movement of the dye within the vesicle membrane is suggested by the results of Bashford et al. [24], who found that the rate of the process is greatly diminished on increasing the membrane cholesterol content and that it disappears altogether when measurements are made below the gel-liquid crystalline phase transition temperature. Thus, a possibility is that the slow phase reflects dye diffusion across the membrane to the internal lipid monolayer, a process whose rate would be expected to depend on the structure of the dye and the membrane fluidity.

If one extrapolates from the graphs to zero dye concentration (see fig. 5B) or infinite vesicle concentration (see fig. 5A), where no effects due to dye accumulation would be expected, the apparent rate constant of the slow process, k_s, can be estimated to be

$$k_s = 0.036(\pm 0.004)\ s^{-1}$$

This value is of the same order as membrane permeation rate constants measured for a number of hydrophobic fluorescent dyes [17,24,25,47].

If one proceeds to higher dye concentrations at constant vesicle concentration, at approx. 225 nM oxonol V a third relaxation process appears, which is characterized by a decrease in fluorescence (see fig. 6). With 225 nM oxonol V and 3.2 μM lipid in the buffer a dye/lipid concentration ratio of 0.07 is obtained, corresponding to one dye molecule per 14 lipid molecules. The amplitude of the process increases rapidly as the dye concentration is increased further. The rate of this process is intermediate between those of the other two relaxations previously mentioned. A likely explanation

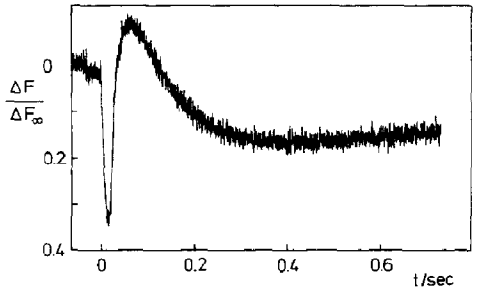


Fig. 6. Stopped-flow experiment in which oxonol V in buffer H containing 75 mM K_2SO_4 and vesicles in buffer H containing 75 mM K_2SO_4 were mixed to final concentrations of 250 nM dye and 0.07 nM vesicles; $T = 22°$ C, pH 7.2.

for this process is that it is due to the formation of dye aggregates of low fluorescence within the lipid membrane. This is consistent with the observation under equilibrium conditions that at high concentrations of oxonol V a quenching of dye fluorescence occurs on binding to the vesicles [20,27].

4.3. Oxonol V response to membrane potential

If a dye-vesicle mixture containing valinomycin in a low K^+ concentration buffer is rapidly mixed in the stopped-flow apparatus with a high K^+ concentration buffer so as to produce an inside-positive membrane potential (as described in section 2), a slow relaxation characterized by an increase in fluorescence is observed (see fig. 7). The reciprocal relaxation time calculated for this process under the conditions shown in fig. 7 is:

$$1/\tau = 4.2(\pm 0.5) \times 10^{-3}\ s^{-1}$$

A control experiment was also performed in which a vesicle-dye-valinomycin mixture was mixed with a buffer of the same K^+ concentration, so that no potential was produced. In this case, no change in dye fluorescence was observed. One might have expected a small decrease in fluorescence due to dilution of the vesicle suspension and the consequent dissociation of some dye molecules from the vesicles. However, the partition coefficient of the dye for the lipid phase is so high that the amount of dye which dissociates is too small to observe.

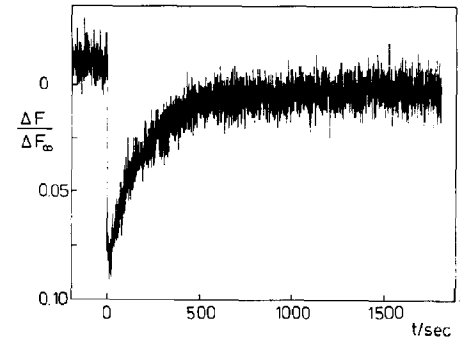


Fig. 7. Stopped-flow experiments in which a suspension of vesicles equilibrated with oxonol V, and valinomycin, and in buffer H containing 2.5 mM K_2SO_4 and 72.5 mM Na_2SO_4 was mixed with buffer H containing 75 mM K_2SO_4. The membrane potential produced was 66 mV. Final concentrations were: [oxonol V] = 75 nM, [vesicle] = 0.14 nM, [valinomycin] = 16.5 nM. $T = 22°$ C, pH 7.2.

4.4. Oxonol VI-vesicle interaction

When oxonol VI is rapidly mixed with vesicles in the stopped-flow apparatus, two relaxation processes, both showing an increase in fluorescence, are evident (see fig. 8), as has previously been found for oxonol V. In the case of oxonol VI, however, over the vesicle concentration range measured the relaxation time for the faster process was too close to the mixing time of the apparatus (which is approx. 7 ms) to permit accurate determination. It should be noted that for the experiments with oxonol VI, it was necessary to

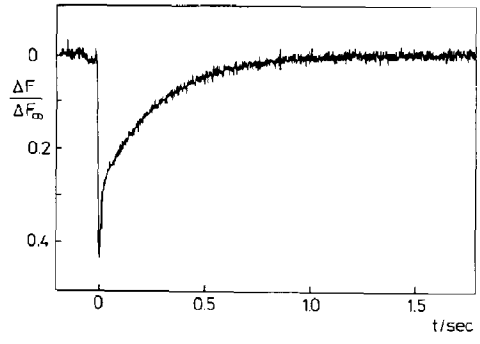


Fig. 8. Stopped-flow experiment in which oxonol VI in buffer H containing 75 mM K_2SO_4 and vesicles in buffer H containing 75 mM K_2SO_4 were mixed to final concentrations of 75 nM dye and 2.11 nM vesicles; $T = 22°$ C, pH 7.2.

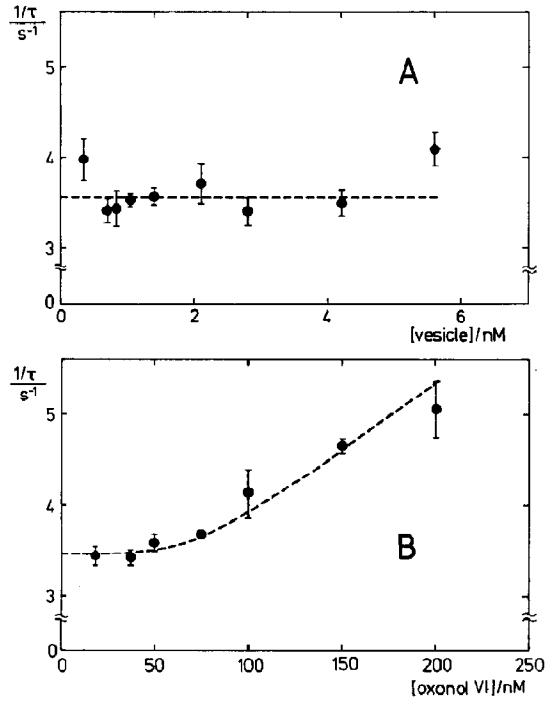


Fig. 9. Kinetic plots for the slower phase of oxonol VI. (A) Dependence of the reciprocal relaxation time on vesicle concentration. [Oxonol VI] = 75 nM. (B) Dependence of the reciprocal relaxation time on oxonol VI concentration. [Vesicle] = 2.11 nM.

use a higher vesicle concentration range than with oxonol V, because of the lower affinity of oxonol VI for the lipid membrane. Nevertheless, limits can be given for the rate constants of the dye association and dissociation reactions. Based on the apparatus mixing time, the dye-vesicle dissociation rate constant is

$$k'' < 140\ s^{-1}.$$

According to the membrane-water partition coefficient for oxonol VI of 1.9×10^4 [18] and eq. 23 (section 3), the association rate constant can be evaluated as

$$k' < 9.3 \times 10^{10}\ M^{-1}\ s^{-1}.$$

The rate of the slower process could be reliably measured as a function of vesicle and dye concentration (see fig. 9). The reciprocal relaxation time was found to be independent of vesicle concentration within the range of vesicle concentrations investigated, and at low dye concentrations the reciprocal relaxation time was also found to be insensitive to dye concentration. Thus, the behaviour of oxonol VI is very similar to that previously observed for oxonol V. Analogously to oxonol V, the slower process could be interpreted as being due to dye diffusion across the membrane. In the same way, the increase in reciprocal relaxation time at high dye concentrations (i.e., at dye/lipid concentration ratios ≥ 0.001) can be interpreted as being due to dye-induced perturbation of the membrane structure. From the plotted data the apparent rate constant of the slow process can be estimated as:

$$k_s = 3.6(\pm 0.3)\ s^{-1}.$$

4.5. Oxonol VI response to membrane potential

If an experiment similar to that previously described for oxonol V is carried out, in which a potential of 66 mV is rapidly generated across the membrane, a single relaxation process characterized by an increase in fluorescence is observed (see fig. 10). The reciprocal relaxation time calculated for this process under the conditions given is:

$$1/\tau = 3.5(\pm 0.1)\ s^{-1}$$

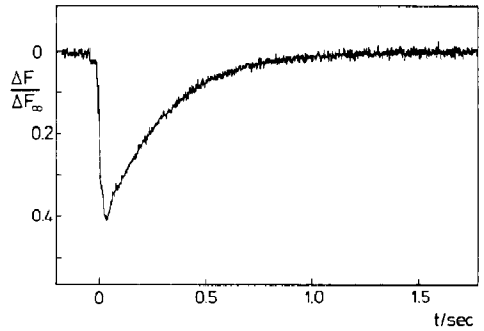


Fig. 10. Stopped-flow experiment in which a suspension of vesicles equilibrated with oxonol VI, and valinomycin, and in buffer H containing 2.5 mM K_2SO_4 and 72.5 mM Na_2SO_4 was mixed with buffer H containing 75 mM K_2SO_4. The membrane potential produced was 66 mV. Final concentrations were: [oxonol VI] = 75 nM, [vesicle] = 0.84 nM, [valinomycin] = 100 nM. $T = 22°C$, pH 7.2.

A control experiment was also performed in which a vesicle-dye-valinomycin mixture was mixed with a buffer of the same K^+ concentration, so that no potential was produced. In this case a small decrease in fluorescence was observed, which is probably due to a small amount of dye leaving the lipid and redissolving in the aqueous medium as a result of the dilution of the dye-vesicle suspension. The reciprocal relaxation time of this process is approximately the same as that observed in the experiment in which a potential is produced, but the amplitude is approx. 8-fold smaller.

5. Discussion

Both dyes have been found to interact with lipid vesicles via a two-step process. Based on the dependence of the reciprocal relaxation times on vesicle concentration, the two steps can be interpreted as being due to rapid binding of the dye to the external lipid monolayer of the vesicle, followed by a slow change in the dye's environment within the membrane, possibly due to diffusion across the membrane to the internal monolayer. The values of the various rate constants and partition coefficients determined are listed in table 1.

The rate constants for the binding step, k', can now be compared to the theoretical diffusion-controlled values of the rate constant, calculated from the Smoluchowski equation [46]. Since the vesicles are much larger than the dye molecules, they can be considered as remaining stationary during the course of the reaction and their diffusion coefficient may therefore be neglected. The radius of collision can also be considered to equal the vesicle radius. Thus, the diffusion-controlled rate is given by

$$k'_{\text{diff}} = 4\pi LDr \text{ [24]} \tag{28}$$

where D denotes the dye diffusion coefficient and r the vesicle radius. According to the calculations of Smith et al. [13], the diffusion coefficients of oxonol V and VI are equal to 6.4×10^{-6} and 6.7×10^{-6} cm^2 s^{-1}, respectively. Using these values and an average vesicle radius of 36 nm, the theoretical diffusion-controlled rate constants k'_{diff} for the two dyes are calculated to be 1.74×10^{11} M^{-1} s^{-1} (oxonol V) and 1.83×10^{11} M^{-1} s^{-1} (oxonol VI). The difference between the values is due to the different diffusion coefficients which could be caused by a slightly smaller degree of friction between the oxonol VI molecule and the solvent compared to oxonol V. The k'_{diff} is comparable with the experimentally determined values of k' (see table 1). The small differences reveal that the dye-binding step for both dyes is very close to being diffusion-controlled. Thus, the lower affinity of oxonol VI for the lipid phase is probably predominantly due to a higher dissociation rate constant.

The observed rate constants for the binding step are also comparable to values previously obtained for similar systems. The interactions of oxonol V with azolectin vesicles [13,24] and chromaffin granules [27] as well as those of oxonol VI with bacterial chromatophores [25] and submitochondrial particles [12,13] have been investigated by others. The values of the association rate constant obtained therein lie within the range 10^6–10^7 M^{-1} s^{-1}, i.e., much lower than the values reported in the present article. However, the method which has been used in order to obtain these values involves measuring the dependence of the observed reaction rate on dye concentration, and the dye concentrations used have been in the micromolar range. At such high dye concentrations, perturbation of the membrane structure and dye aggregation in the membrane are likely to become apparent. Thus, the physical significance of these values is uncertain. The rate constants reported here for the binding step represent the values for the association of a single dye molecule to a single vesicle. Thus, it has been possible to compare the

Table 1

	Oxonol V	Oxonol VI
γ	$5.9\,(\pm 0.2)\times 10^5$	$1.9\,(\pm 0.1)\times 10^4$ [18]
k' (M^{-1} s^{-1})	$1.18\,(\pm 0.05)\times 10^{11}$	$< 9.3\times 10^{10}$
k'' (s^{-1})	5.7	< 140
k_s (s^{-1})	$3.6\,(\pm 0.4)\times 10^{-2}$	$3.6\,(\pm 0.3)$
$1/\tau$ (s^{-1}) (voltage response)	$4.2\,(\pm 0.5)\times 10^{-3}$	$3.5\,(\pm 0.1)$

results obtained directly with the theoretical diffusion-controlled values. This is one advantage of expressing the rate constant in terms of vesicle concentration rather than monomeric phospholipid concentration. In agreement with the results obtained here, binding of the fluorescent dye *N*-phenylnaphthylamine [46] as well as the peptide alamethicin [45] to phospholipid vesicles, has been found to be very close to diffusion-controlled.

In the case of the second step of the dye-vesicle interaction, which has been interpreted as being a membrane-associated change in dye conformation or position, the measured first-order rate constants are in good agreement with values obtained for the slow phase of interaction of oxonol V with azolectin vesicles [24] and of oxonol VI with submitochondrial particles [12]. It has been found that the rate constant for oxonol VI is approx. 100-fold greater than that of oxonol V. Thus, although oxonol V binds to the lipid membrane more strongly than does oxonol VI, it appears to have a lower mobility within the membrane. This may be partly due to the bulkier structure of the oxonol V dye molecule in comparison to oxonol VI. Another possibility is that, because of their differing structures, the two dyes take up different orientations in the lipid membrane, with that of oxonol V being less favourable for membrane permeation.

The experiments in which a potential is produced across the membrane have also shown that oxonol V responds much more slowly to potential changes as compared to oxonol VI. The rates of response are consistent with the previously proposed response mechanism, in which the increase in dye fluorescence is considered to arise from movement of the dye across the lipid membrane to the internal monolayer and because of the small intravesicular volume there is a net increase in the concentration of bound dye [18,22,25,36]. In the case of oxonol VI, there is a good correlation between the values of k_s and $1/\tau$ for the voltage response. For oxonol V, however, the value of $1/\tau$ is almost an order of magnitude smaller than k_s, i.e., the dye appears to move more slowly within the membrane in the presence of an electrical potential difference. Other authors have observed an apparent decrease in the rate of association of oxonol dyes with chromaffin granules [27] and mitochondrial particles [12] in the presence of an electrical potential. Smith and Chance [12] have suggested that a change in membrane surface potential or in intrinsic dye affinity for sites in the membrane might explain a lower dye-binding rate. In order to achieve a lower dye diffusion rate, the electrical potential must somehow alter the membrane structure or cause a change in the dye interaction with the lipid molecules. In the case of the dye merocyanine 540, it has been suggested [11] that an electrical potential causes the production of an electric field which reorients the dye molecules in the membrane. Dye dimerisation within the lipid membrane has also been proposed [7,8,10,11,16,17]. If an electrical potential results in a new dye orientation which is less favourable for membrane permeation or in the production of dye dimers which have a lower mobility in the membrane, then a decrease in diffusion rate might be expected. An alternative explanation is that the electrical potential causes a change in membrane fluidity. Evidence for an increase in lipid microviscosity of vesicles on formation of a membrane potential has been presented by Corda et al. [49], using the method of fluorescence depolarisation. At this stage, however, a definitive description of the underlying cause of the difference in values of k_s and $1/\tau$ for oxonol V cannot be given.

Comparison of the response rates of the two dyes to a change in membrane potential shows that oxonol VI is much more suitable than oxonol V as a probe for monitoring rapid kinetic processes such as potential generation by ion pumps.

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References

1 C.L. Bashford and J.C. Smith, Methods Enzymol. 55 (1978) 569.
2 L.B. Cohen and B.M. Salzberg, Rev. Physiol. Biochem. Pharmacol. 83 (1978) 33.
3 A.S. Waggoner, Annu. Rev. Biophys. Bioeng. 8 (1979) 47.
4 J.C. Freedman and P.C. Laris, Int. Rev. Cytol. Suppl. 12 (1981) 177.
5 A.S. Waggoner, in: The enzymes of biological membranes, 2nd edn., ed. A.N. Martonosi (Plenum, New York, 1985) vol. 3, p. 313.
6 J.F. Hoffman and P.C. Laris, J. Physiol. 239 (1974) 519.
7 P.J. Sims, A.S. Waggoner, C.H. Wang and J.F. Hoffman, Biochemistry 13 (1974) 3315.
8 A.S. Waggoner, C.H. Wang and R.L. Tolles, J. Membrane Biol. 33 (1977) 109.
9 L.M. Loew, G.W. Bonneville and J. Surow, Biochemistry 17 (1978) 4065.
10 W.N. Ross, B.M. Salzberg, L.B. Cohen, A. Grinvald, H.V. Davila, A.S. Waggoner and C.H. Wang, J. Membrane Biol. 33 (1977) 141.
11 P.R. Dragsten and W.W. Webb, Biochemistry 17 (1978) 5228.
12 J.C. Smith and B. Chance, J. Membrane Biol. 46 (1979) 255.
13 J.C. Smith, S.J. Frank, C.L. Bashford, B. Chance and B. Rudkin, J. Membrane Biol. 54 (1980) 127.
14 J.C. Smith, L. Hallidy and M.R. Topp, J. Membrane Biol. 60 (1981) 173.
15 J.C. Smith, J.M. Graves and M. Williamson, Arch. Biochem. Biophys. 231 (1984) 430.
16 A.P. Singh and P. Nicholls, J. Biochem. Biophys. Methods 11 (1985) 95.
17 G. Cabrini and A.S. Verkman, J. Membrane Biol. 92 (1986) 171.
18 H.-J. Apell and B. Bersch, Biochim. Biophys. Acta 903 (1987) 480.
19 E.B. George, P. Nyirjesy, M. Basson, L.A. Ernst, P.R. Pratap, J.C. Freedman and A.S. Waggoner, J. Membrane Biol. 103 (1988) 245.
20 J.C. Smith, P. Russ, B.S. Cooperman and B. Chance, Biochemistry 15 (1976) 5094.
21 H.S. van Walraven, K. Krab, M.J.M. Hagendoorn and R. Kraayenhof, FEBS Lett. 184 (1985) 96.
22 K. Krab, H.S. van Walraven, M.J.C. Scholts and R. Kraayenhof, Biochim. Biophys. Acta 809 (1985) 228.
23 C.L. Bashford and W.S. Thayer, J. Biol. Chem. 23 (1977) 8459.
24 C.L. Bashford, B. Chance, J.C. Smith and T. Yoshida, Biophys. J. 25 (1979) 63.
25 C.L. Bashford, B. Chance and R.C. Prince, Biochim. Biophys. Acta 545 (1979) 46.
26 B. Chance, Y. Nakase and F. Itshak, Arch. Biochim. Biophys. 198 (1979) 360.
27 D. Scherman and J.P. Henry, Biochim. Biophys. Acta 599 (1980) 150.
28 J.B. Hughes, S. Joshi and D.R. Sanadi, J. Biol. Chem. 257 (1982) 6697.
29 A.B. Bennett and R.M. Spanswick, J. Biol. Chem. 71 (1983) 95.
30 J. Navarro and A. Essig, Biophys. J. 46 (1984) 709.
31 T.J. Beeler, L. Dux and A.N. Martonosi, J. Membrane Biol. 78 (1984) 73.
32 Y.P. Loh, W.W.H. Tam and J.T. Russell, J. Biol. Chem. 259 (1984) 8238.
33 H.S. van Walraven, M.J.M. Hagendoorn, K. Krab, N.P. Haak and R. Kraayenhof, Biochim. Biophys. Acta 809 (1985) 236.
34 C.L. Bashford and C.A. Pasternak, Biochim. Biophys. Acta 817 (1985) 174.
35 J.P. Armitage and M.C.W. Evans, FEBS Lett. 126 (1981) 98.
36 T.J. Beeler, R.H. Farmen and A.N. Martonosi, J. Membrane Biol. 62 (1981) 113.
37 C.L. Bashford, G.M. Alder, M.A. Gray, K.J. Micklem, C.C. Taylor, P.J. Turek and C.A. Pasternak, J. Cell. Physiol. 123 (1985) 326.
38 C.L. Bashford and C.A. Pasternak, Trends Biochem. Sci. 11 (1986) 113.
39 H.-J. Apell, M.M. Marcus, B.M. Anner, H. Oetliker and P. Läuger, J. Membrane Biol. 85 (1985) 49.
40 M.M. Marcus, H.-J. Apell, M. Roudna, R.A. Schwendener, H.-G. Weder and P. Läuger, Biochim. Biophys. Acta 854 (1986) 270.
41 C. Huang and J.T. Mason, Proc. Natl. Acad. Sci. U.S.A. 75 (1978) 308.
42 G.C. Newman and C. Huang, Biochemistry 14 (1975) 3363.
43 G. Obermeyer, Diplomarbeit, Universität Konstanz (1987).
44 G. Schwarz, Biophys. Chem. 26 (1987) 163.
45 G. Schwarz, H. Gerke, V. Rizzo and S. Stankowski, Biophys. J. 52 (1987) 685.
46 P. Woolley and H. Diebler, Biophys. Chem. 10 (1979) 305.
47 D.H. Haynes and P. Simkowitz, J. Membrane Biol. 33 (1977) 63.
48 J. Fumero, B.P. Bammel, H.P. Hopkins and J.C. Smith, Biochim. Biophys. Acta 944 (1988) 164.
49 D. Corda, C. Pasternak and M. Shinitzky, J. Membrane Biol. 65 (1982) 235.