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The kinetics of binding of *o*-methyl red to the outer surface of unilamellar spherical phospholipid vesicles

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Phospholipid vesicle; Adsorption; Interfacial reaction; Temperature-jump kinetics

The kinetics of adsorption of the proton carrier *o*-methyl red to the surface of unilamellar spherical phospholipid vesicles have been investigated by means of the temperature-jump relaxation technique with absorbance detection. Single-exponential relaxation curves were observed with time constants in the range 30–130 μs. *o*-Methyl red binds in both its anionic form A^- and protonated form AH. Adsorption-desorption of the two species is coupled by two fast protolytic reactions, occurring in the aqueous bulk phase and in the surface region of the membrane. The rate constants for adsorption and desorption of the two species were obtained from the dependences of the relaxation time on lipid concentration at different pH values. The analysis yielded apparent adsorption rate constants of $k_{AH}^{as} = 9.8 \times 10^6$ M^{-1} s^{-1} and $k_A^{as} = 1.3 \times 10^6$ M^{-1} s^{-1} (expressed in terms of monomeric lipid), and $k_{AH}^{as} = 1.2 \times 10^{11}$ M^{-1} s^{-1} and $k_A^{as} = 1.6 \times 10^{10}$ M^{-1} s^{-1} (expressed in terms of vesicle concentration). From the order of these rate constants it is concluded that adsorption of both species is actually diffusion-controlled. The peculiar pH dependence of the relaxation time is a consequence of the protolytic reaction in the surface region of the membrane. Its implication for the kinetics of adsorption-desorption processes are discussed.

1. Introduction

The basic framework of the plasma membrane which occurs in all present-day biological cells is a lipid bilayer. This is a structure in which two polar surface regions enclose a thin sheet of hydrocarbons forming a region of low dielectric constant. The lipid bilayer membrane is almost impermeable for most inorganic ions. One of the reasons for this is the high energy that is required to remove the ion's hydration shell partly or completely when transferring it from the polar aqueous bulk phase into the nonpolar interior of the membrane. Mirror image forces, which appear when ions are transferred from the aqueous bulk phase into a medium of lower polarity, also tend to exclude them from the membrane. Finally, it is the membrane's internal barrier structure which also determines the permeability for such compounds. In a certain sense, the membrane can be considered to be also impermeable for those compounds that have a strong tendency to dissolve in the membrane, such as lipid-like substances, small polypeptides composed mainly of hydrophobic amino acids, or integral membrane proteins. This is because of the large amount of energy which would be required in order to transfer these species into the aqueous bulk phase. Amphiphilic molecules, being considered among a series of small neutral organic molecules, and hydrophobic ions are intermediates between these two extremes. These have a certain tendency toward dissolving in both media. Therefore, the membrane permeability is expected to be rather high for these compounds. The mechanism by which these molecules

Dedicated to Professor Manfred Eigen on the occasion of his 60th birthday.

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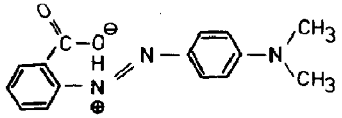

Fig. 1. Structure of *o*-methyl red in its uncharged (zwitterionic) form. From comparison of the spectral properties and structures of related compounds it has been concluded that the first proton is coordinated to the β-nitrogen of the azo group [3–8].

penetrate the membrane is of particular interest, because it may reflect the membrane's internal structure with respect to transport processes.

The three-layered structure of the membrane suggests the description of transfer across the membrane to a first approximation by a sequence of three steps: adsorption from the aqueous bulk phase to the membrane surface, translocation across the nonpolar interior to the opposite surface, and subsequent desorption to the adjacent aqueous bulk phase. The present paper deals with the kinetics of adsorption to and desorption from spherical unilamellar lipid vesicles of the azo dye *o*-methyl red (fig. 1), which acts as a proton carrier on planar black lipid membranes [1]. This compound, a well-known pH indicator, was chosen for its optical properties, which allow one to record binding to the membrane of lipid vesicles by means of absorption spectrophotometry in the visible range. Accordingly, the kinetics of these processes were studied by temperature-jump relaxation spectrometry [2] with absorption detection.

2. Materials and methods

o-Methyl red (*o*-dimethylaminophenylazobenzoic acid, sodium salt, Fluka) was used without further purification. KCl was from Merck ('Suprapur' grade). Buffers (Sørensen's citrate II) were obtained from mixtures of disodium citrate and NaOH (Merck, analytical grade). Water was used directly from a quartz double still. *o*-Methyl red stock solutions (1×10^{-3} M) were kept at high pH values (pH > 9) in order to avoid precipitation. Stock solutions of buffers, KCl and *o*-methyl red were degassed. It was then found to be unnecessary to degas dispersions in the measuring cell for temperature-jump experiments which usually requires some care.

In aqueous solutions *o*-methyl red binds two protons with pK values of 5 and 2.4 [8]. Since the kinetic experiments were restricted to the range pH > 5, only the anionic and zwitterionic forms need be considered. The pK was redetermined by spectrophotometric titrations to be 4.9 [1] for the ionic composition of the dispersions used for the kinetic experiments. The binding of protons to *o*-methyl red in this pH range is a diffusion-controlled process with rate constants of 3.5×10^{10} M^{-1} s^{-1} for recombination and 4.8×10^{5} s^{-1} for dissociation [9].

Lecithin was isolated from chicken egg yolk according to the method of Singleton et al. [10]. Vesicles were prepared by sonication following the method of Huang [11,12]. Stock dispersions were found to be stable for more than 2 weeks when stored at 5°C. However, for precautional reasons, sets of kinetic experiments were carried out in the shortest feasible time (< 5 days) using the same stock solution. The phosphorus content of dispersions was determined according to the method of Eibl and Lands [13]. The size of vesicles was determined from electron micrographs [1].

Kinetic studies were carried out by means of the temperature-jump relaxation method with absorption detection [2,14]. The cell had a heating volume of about 0.8 ml and an optical path length of 0.7 mm. A capacitor of 0.05 μF charged to 25 kV thus gave a temperature jump of about 4 degrees. All kinetic experiments were carried out in dispersions containing 60 mM KCl, and 30 mM disodium citrate-NaOH buffers. The concentration of *o*-methyl red was varied between 5×10^{-5} and 7×10^{-5} M. In order to avoid precipitation the stock solution of *o*-methyl red was added stepwise with thorough mixing of the dispersion.

3. Results and discussion

The scheme for the binding of *o*-methyl red to the membrane of spherical unilamellar phospholipid vesicles inferred from the results of spectrophotometric titrations and temperature-jump relaxation experiments [1] is shown in fig. 2.

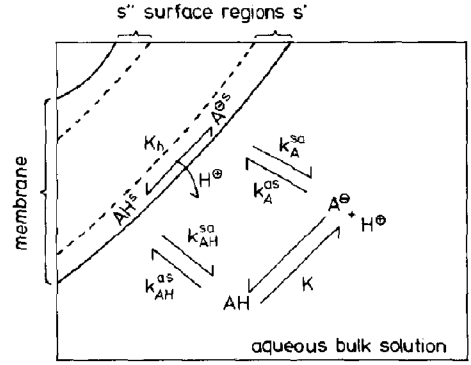

Fig. 2. Reaction scheme for binding of *o*-methyl red anions A^- and protonated molecules AH to the outer surface of spherical lipid vesicles. k_A^{as}, k_{AH}^{as}, k_A^{sa} and k_{AH}^{sa} denote the rate constants for adsorption and desorption. K and K_h are the equilibrium constants for protolytic reactions in the aqueous bulk phase and surface region, respectively.

Accordingly, *o*-methyl red is bound to the membrane surface in both its anionic form A^- and neutral form AH. In addition, the neutral form is dissolved to a small extent in the nonpolar interior of the membrane. Since this process will not be discussed here, it has been omitted from fig. 2. The binding constants for adsorption (based on the total lipid concentration, c_{Lip}°), obtained from spectrophotometric titrations, are $K_{AH}^{as} = 2 \times 10^3$ M^{-1} and $K_A^{as} = 16$ M^{-1}. This indicates that the affinity for the neutral form is about two orders of magnitude higher than that for the anionic species. In addition to the adsorption-desorption processes there exist two protolytic reactions, characterized by the equilibrium constants K and K_h, by which *o*-methyl red exchanges protons with buffer or water molecules. The equilibrium constant for the protolytic reaction of the surface-bound species, K_h, is equally based on the proton activity in the aqueous bulk phase, since it is difficult to measure proton activity in the surface region. The difference from the bulk activity is then included in the constant K_h and this reaction, therefore, is termed a 'heterogeneous' reaction. The system describing the binding of *o*-methyl red is thus a cyclic system consisting of four reactions, in which one of the four equilibrium constants is determined by the other three according to the relation $K_{AH}^{as}K = K_A^{as}K_h$. By using the binding constants it was found that the pK value of the protolytic reaction increases from pK 4.9 in the aqueous bulk phase to pK_h 7 in the surface region.

For quantitative description of the binding at equilibrium, a model was used that assumed that *o*-methyl red molecules are partitioned between the aqueous bulk phase and the phase of head groups of the lipid molecules which form a thin layer of thickness d^s (about 1/4 of the membrane's total thickness). The volume of the head group phase is proportional to the number of lipid molecules in a dispersion. Provided the volume occupied by the bound *o*-methyl red molecules is small compared to the total volume of this phase, partitioning between the two phases can be treated in the same manner as for ideal solutions. Thus, in the limit of small surface coverages, the ratio of bound to unbound *o*-methyl red molecules is simply proportional to the lipid concentration. The binding constants K_{AH}^{as} and K_A^{as} are in this sense partition coefficients which are based on the total lipid concentration. This binding model is almost equivalent to a model treating the polar head group region as a two-dimensional surface to which molecules are adsorbed in an ideal nonlocalized manner (e.g., ref. 15), i.e., where the co-area of the adsorbed molecule is neglected and where the molecules are assumed to have high lateral mobility. Binding of small molecules to the surface of lipid vesicles has also been treated by assuming binding sites, which are formed by a certain number of lipid molecules [16–19]. The term binding site, however, has two different meanings depending on whether or not the adsorbed molecule is assumed to be fixed to this site. In the first case, strong attractive interactions would be required. For the second, where the adsorbed molecules are assumed to have high lateral mobility, the number of lipid head groups forming a binding site merely corresponds to the co-area covered by the adsorbed molecule. Binding in this case will be quantitatively described by the adsorption isotherm for a nonideal, nonlocalized monolayer (e.g., ref. 15) However, in the limit of small surface coverages, the binding characteristics of the three models become identical, and no decision can be made in favor of one of them. For *o*-methyl red binding no deviation from linear behavior could be detected in the experimentally accessible concentration range and therefore the simplest binding model was used.

The kinetic experiments performed at pH values around 5 reveal a relaxation spectrum of three processes that are well separated in time [1]. The fastest process, which could not be resolved in time, is due to the rapidly equilibrating diffusion-controlled protolytic reactions. The shift of the protolytic reactions to the new equilibrium state indicated by a large amplitude change decays with the time characteristics of the heating process. The second process with relaxation times ranging from 30 to 130 μs for the chosen conditions is related to equilibration of the adsorption-desorption processes as concluded from the characteristics of the kinetics and amplitude changes [1]. A typical relaxation curve for this process, which is described well by a single exponential, is shown in fig. 3. In order to determine the rate constants of these processes the relaxation kinetics were investigated as a function of lipid concentration at various pH values. The results are shown in fig. 4, where the reciprocal relaxation time τ^{-1} is plotted vs. total lipid concentration.

In analogy to the treatment of the kinetics of fast ionic reactions in solution [20], the adsorption of a small molecule to the surface of a vesicle can be described by a two-step process. The molecule approaches a vesicle by diffusion and forms an encounter complex from which it either enters the surface region and becomes adsorbed, or diffuses back into solution. As long as the entry step is fast compared to diffusional separation, the diffusion-controlled encounter determines the adsorption rate. Conversely, when the entry step is rate-determining an activation barrier must exist in the surface region. For these two limiting cases, the attainment of equilibrium of the two-step system is characterized by one relaxation time only [21], and therefore adsorption or desorption can be characterized by one rate constant each ($k^{\mathrm{as}\prime}$ and $k^{\mathrm{sa}\prime}$). In our case, the small molecules 'react' with large vesicles, which are able to bind a large number of them. In the limit of small surface coverages, signifying that the free binding sites are in excess, the probability of a molecule impinging on an area that is already occupied is negligibly small, and thus each molecule will be adsorbed with the same probability. The vesicle concentration, therefore, can be considered to be constant during the relaxation process, and as a consequence the reciprocal relaxation time for this limit, given by eq. 1 [22],

$$\tau^{-1} = k^{\mathrm{as}\prime} c_{\mathrm{Ves}} + k^{\mathrm{sa}\prime}, \tag{1}$$

will depend on the vesicle concentration only (the prime indicates that the rate constants are based on the vesicle concentration). This situation corresponds to that of a bimolecular reaction where one of the two reactants is buffered [2]. For vesicles

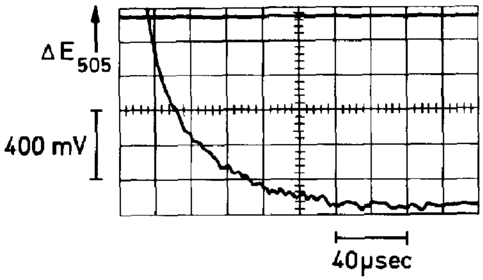

Fig. 3. Typical relaxation curve seen following a temperature-jump performed with a lipid dispersion (5×10^{-3} M lecithin, pH 5.08) in the presence of o-methyl red (5×10^{-5} M). The rapid amplitude change at the beginning of the relaxation curve, being of comparable size, was compensated by electronic means.

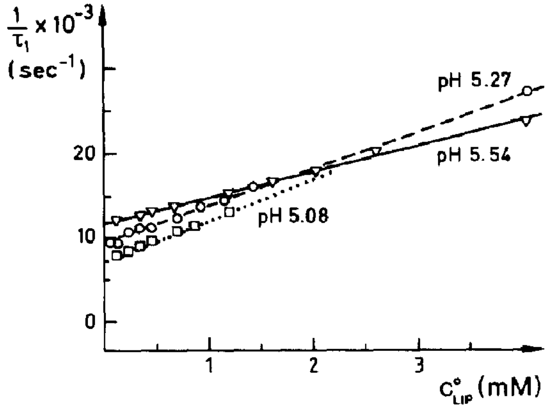

Fig. 4. Plot of reciprocal relaxation time τ^{-1} vs. total lipid concentration c^{0}_{Lip} for pH 5.08, 5.27 and 5.54. Lines represent the best linear fit to the experimental data.

of uniform size the vesicle concentration is proportional to the lipid concentration, i.e., $c_{\mathrm{Ves}} = c^{\mathrm{o}}_{\mathrm{Lip}}/n$, where $n = 4\pi(R'^2 + R''^2)/a_{\mathrm{L}}$ is the number of lipid molecules in each vesicle, R' and R'' the outer and inner radii of a vesicle, respectively and a_{L} the area per lipid molecule. Accordingly, eq. 1 can be expressed in terms of lipid concentration [22], where the corresponding rate constants are related by $k^{\mathrm{as}} = k^{\mathrm{as}'}/n$ and $k^{\mathrm{sa}} = k^{\mathrm{sa}'}$.

In the case of *o*-methyl red two adsorption-desorption processes for A^- and AH have to be considered, which are coupled by two protolytic reactions (see fig. 2). For the assumption of rapidly equilibrating protolytic reactions, it has been shown [1] that for vesicles of uniform size the attainment of equilibrium of these coupled adsorption-desorption processes may again be characterized by one relaxation time. The reciprocal relaxation time for this coupled reaction system is given by

$$\tau^{-1} = k^{\mathrm{sa}}_{\mathrm{A}} \frac{1}{1 + K_{\mathrm{h}}\bar{c}_{\mathrm{H}}} + k^{\mathrm{sa}}_{\mathrm{AH}} \frac{K_{\mathrm{h}}\bar{c}_{\mathrm{H}}}{1 + K_{\mathrm{h}}\bar{c}_{\mathrm{H}}} + \left\{ k^{\mathrm{as}}_{\mathrm{A}} \frac{1}{1 + K\bar{c}_{\mathrm{H}}} + k^{\mathrm{as}}_{\mathrm{AH}} \frac{K\bar{c}_{\mathrm{H}}}{1 + K\bar{c}_{\mathrm{H}}} \right\} \times (V/V') c^{\mathrm{o}}_{\mathrm{Lip}} \quad (2)$$

where $k^{\mathrm{as}}_{\mathrm{A}}$, $k^{\mathrm{as}}_{\mathrm{AH}}$, $k^{\mathrm{sa}}_{\mathrm{A}}$ and $k^{\mathrm{sa}}_{\mathrm{AH}}$ are the rate constants for adsorption and desorption of the species A^- and AH (based on the lipid concentration). $\bar{c}_{\mathrm{H}}$ is the proton activity in the aqueous bulk phase, the overbar indicating buffering. V denotes the total volume of the dispersion, V' being the volume of the aqueous bulk phase that is not enclosed by the vesicles. For the vesicle concentrations used here the ratio V/V' van be set to 1. The model is in good agreement with the experimental results shown in fig. 4. The relationship depends linearly on lipid concentration and also describes the peculiar pH dependence, which is discussed below in more detail. The rate constants determined from analysis of the experimental data are listed in table 1.

The question as to whether adsorption is diffusion- or activation-controlled cannot be resolved from the characteristics of the dependence of the reciprocal relaxation time τ^{-1} on lipid concentration. It has been shown that this is linear in both cases [22]. One of the criteria for a diffusion-controlled reaction, however, is the order of the rate constant. For the diffusion-controlled adsorption of small molecules to spherical vesicles, the rate constant can be calculated theoretically by solving the diffusion equation in polar coordinates [22,23] in analogy to the treatment of coagulation of colloid particles described by von Smoluchowski [24]. Neglecting electrostatic interactions, this is given by $k^{\mathrm{as}} = 4\pi DLR'/1000$, where D is the diffusion coefficient of *o*-methyl red molecules, R' the radius of collision which is to a good approximation the radius of the large vesicles, and L Avogadro's constant. By assuming for *o*-methyl red molecules a reasonable value of $D = 7 \times 10^{-6}$ cm^2 s^{-2}for the diffusion coefficient, the adsorption rate constant for vesicles of radius $R' = 20$ nm becomes $k^{\mathrm{as}'}_{\mathrm{AH}} = 1.06 \times 10^{11}$ M^{-1} s^{-1}. Comparison with the experimental results given in table 1 indicates that the adsorption of *o*-methyl red molecules is indeed a diffusion-controlled process. This finding agrees with results from similar investigations, which showed that the adsorption to lipid vesicles of other small molecules such as *N*-phenylnaphthylamine [22], phloretin [25], and anilinonaphthalenesulfonate [26] is also diffusion controlled. It should be noted that the above rate constant is of the same order of magnitude as the corresponding one for the fastest protolytic reaction in solution [21], namely, the recombination of H^+ with OH^- whose diffusion coefficients exceed that for *o*-methyl red molecules by more than one

Table 1

Rate constants for adsorption and desorption of the *o*-methyl red forms AH and A^-

Rate constants are based on lipid concentration (1) and vesicle concentration (2) or are calculated (3). Mean vesicle radius $R' = 20$ nm [1], diffusion coefficient $D = 7 \times 10^{-6}$ cm^2 s^{-1}, area per lipid molecule $a_{\mathrm{L}} = 0.66$ nm^2 [28] and membrane thickness $R' - R'' = 4.2$ nm [29] were used for calculations.

	(1)	(2)	(3)
$k^{\mathrm{as}}_{\mathrm{AH}}$ (M^{-1} s^{-1})	$(9.8 + 3) \times 10^6$	1.2×10^{11}	1.06×10^{11}
$k^{\mathrm{as}}_{\mathrm{A}}$ (M^{-1} s^{-1})	$(1.3 + 0.4) \times 10^6$	1.6×10^{10}	
$k^{\mathrm{sa}}_{\mathrm{AH}}$ (s^{-1})	$(4.9 + 0.3) \times 10^3$		
$k^{\mathrm{sa}}_{\mathrm{A}}$ (s^{-1})	$(2.1 + 0.5) \times 10^3$		

order of magnitude. The large value for the adsorption rate constant found here is due to the large radius of collision R', which compensates for the lower diffusional mobility of *o*-methyl red molecules. The rate constant for adsorption of *o*-methyl red anions is clearly smaller than that for neutral *o*-methyl red molecules. This may be caused by a smaller diffusion coefficient for the charged form or may indicate that the vesicles, which have been assumed to be neutral, bear a small negative charge.

The vesicles in a real dispersion are usually not uniform in size but have a more or less broad size distribution. Therefore, the attainment of equilibrium of the diffusion-controlled adsorption-desorption processes in such a vesicle dispersion is characterized by a spectrum of relaxation times reflecting the size distribution [22]. The findings that the relaxation curves of the present experiments could to a good approximation be fitted by one exponential only indicates that the vesicles used here were fairly uniform in size.

The pH dependence of the relaxation time (fig. 4) shows a peculiarity. At low vesicle concentrations the equilibration process becomes faster with increasing pH values, a feature that has also been found for the adsorption-desorption kinetics of phloretin [25], whereas at high vesicle concentrations the reverse is observed. This is a consequence of the protolytic reaction in the surface region of the membrane. In order to clarify this statement, we begin with an explanation of the course of the relaxation amplitude shown in fig. 3. This will be understood when taking into account that at the wavelength $\lambda = 505$ nm, the extinction coefficients of the four *o*-methyl red species follows the order $AH > AH^s \gg A^- > A^{-s}$, and that the concentration of surface-bound anions A^{-s} is negligibly small, as can be concluded from the binding constants. The rapid temperature change causes in the first instance a rapid shift of the protolytic equilibria in favor of the anionic forms. This results in a large decrease in absorbance and is expressed in the large amplitude change at the beginning of the relaxation curve (mostly omitted from fig. 3). During the slower equilibration of the adsorption-desorption processes a net number of surface-bound molecules AH^s are then transferred into the aqueous bulk phase, where at the pH value used here (pH > 5) these are transformed predominantly into anions. This results in the decrease in absorbance shown in fig. 3. Since the amount of A^{-s} molecules is about two orders of magnitude smaller than that of AH^s molecules, the amplitude change indicates essentially transfer from the surface-bound state AH^s to states A^- and AH in the aqueous bulk phase. Thus, for discussion of the magnitude of the amplitude the surface-bound state A^{-s} can be treated exactly as if it were not present. In contrast, the determined pH dependences of the relaxation process cannot be explained when this state is omitted. According to the model given here, surface-bound molecules AH^s can be transferred to the state A^- via two pathways: (1) by desorption from the surface to give AH which subsequently dissociates to form A^-, and (2) by dissociation of the proton in the surface region yielding A^{-s} which is then desorbed into the aqueous bulk phase. Due to the higher probability of anions leaving the surface region it follows that the second pathway is more rapid. For the inverse process, namely, transfer from A^- to AH^s, the two pathways are used in reverse order. From the values of the corresponding rate constants it is concluded that for adsorption the first pathway is faster. Now, the extent to

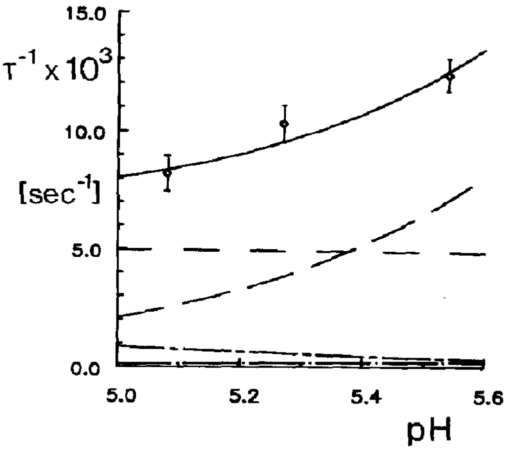

Fig. 5. Contributions of the four terms of eq. 2 to the reciprocal relaxation time τ^{-1} (———) at low lipid concentrations ($c^{o}_{\mathrm{Lip}} = 2 \times 10^{-4}$ M). k^{sa}_{A} (— — —), k^{sa}_{AH} (- - - - - -), k^{as}_{A}(–·–·–), k^{as}_{AH} (——-——-——); (◇) experimental data.

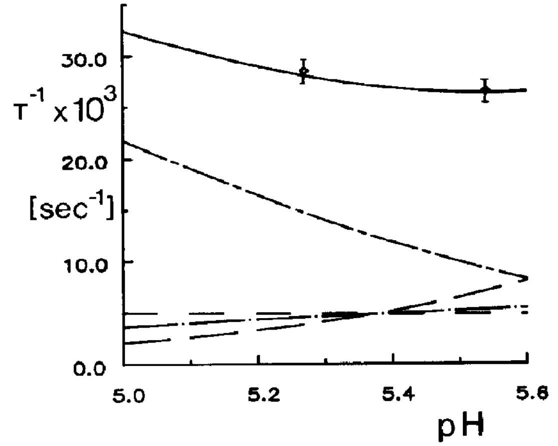

Fig. 6. Contributions of the four terms of eq. 2 of the reciprocal relaxation time τ^{-1} (———) at high concentrations ($c^{\circ}_{\mathrm{Lip}} = 5\times10^{-3}$ M). $k^{\mathrm{sa}}_{\mathrm{A}}$ (— — —), $k^{\mathrm{sa}}_{\mathrm{AH}}$ (- - - - - -), $k^{\mathrm{as}}_{\mathrm{A}}$ (–·–·–), $k^{\mathrm{as}}_{\mathrm{AH}}$ (— - — - —); (◇) experimental data.

which the four pathways are used to adjust the system to the new equilibrium situation is dependent on the pH value in the aqueous bulk phase. The amount being transferred on the fast 'desorption pathway', for example, is dependent on the fraction of surface-bound *o*-methyl red molecules that are in the form of anions. This fraction becomes larger the higher the pH value, and correspondingly this pathway will be used to a greater extent when the pH value increases.

The four pathways correspond to the four terms in the expression for the reciprocal relaxation time τ^{-1} (eq. 2), each of which has its own characteristic dependence on pH. The characteristics of the sum, i.e., the reciprocal relaxation time, are essentially those of the dominating terms. This is illustrated in figs. 5 and 6. Here, the contribution of each term on eq. 2 is shown in the pH range between 5 and 5.6 for two different vesicle concentrations. At low vesicle concentration (2×10^{-4} M lipid) the desorption terms dominate. Since the desorption term for the AH form is nearly constant in this range (decreasing slightly with increasing pH) the characteristics of the desorption term for A^- determine the pH characteristics of τ^{-1}. At high vesicle concentrations (5×10^{-3} M lipid), on the other hand, the adsorption term for AH dominates up to a pH value of about 5.5. Its characteristics of decreasing with increasing pH determine the behavior of τ^{-1} up to this pH value. At higher pH values the desorption term for A^- then dominates. The reciprocal relaxation time τ^{-1} thus passes through a minimum, whose position on the pH scale is determined by the actual vesicle concentration. The feature of the relaxation process becoming faster with increasing pH values can be considered as experimental proof of the existence of a protolytic reaction in the surface region. If this reaction were not to exist, the relaxation process would become slower with increasing pH, and furthermore the relaxation process would be characterized by two relaxation times, since then the adsorption-desorption reactions for A^- and AH would be coupled by the protolytic reaction in the aqueous bulk phase only.

4. Conclusions

The analysis of the kinetic data shows that the adsorption of *o*-methyl red molecules to the surface of a lipid membrane is a diffusion-controlled process, which implies that no substantial activation barrier exists in the surface region. Because this has also been found for other molecules [22,25,26], it is very likely that this will be true in general for small amphiphilic molecules. From these findings it can be concluded that the barriers determining the permeability of a membrane for these compounds are not located at the interface between the aqueous bulk phase and polar head group region, and therefore have to be localized in the membrane interior. As a consequence of the diffusion-controlled adsorption and desorption, the kinetics of binding to vesicles will depend strongly on the vesicle's size. Therefore, for experiments in which the kinetics of these processes can influence the results, it is necessary to work with vesicles of size as uniform as possible in order not to overcomplicate the situation, and to determine the size distribution. The latter has to be determined anyway for calculating vesicle concentrations, which cannot be inferred from the lipid concentration alone. The drastic differences which will be obtained for the relaxation times when temperature-jump experiments are performed at constant lipid concentration with vesicles of different sizes have been demonstrated elsewhere [27].

Finally, the kinetics of adsorption and desorption of *o*-methyl red to lipid membranes provide an illustrative example of the case where a weakly occupied state present in almost negligible quantities in the reaction system has a considerable effect on the kinetics of the processes. Such states, which often can barely be established by other means, may strongly determine the characteristics of complex transport systems. The results of the present kinetic studies show that analysis of the kinetics provides a tool by which the existence of such states can be demonstrated.

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