The Adsorption of Phloretin to Lipid Monolayers and Bilayers Cannot Be Explained by Langmuir Adsorption Isotherms Alone

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ABSTRACT Phloretin and its analogs adsorb to the surfaces of lipid monolayers and bilayers and decrease the dipole potential. This reduces the conductance for anions and increases that for cations on artificial and biological membranes. The relationship between the change in the dipole potential and the aqueous concentration of phloretin has been explained previously by a Langmuir adsorption isotherm and a weak and therefore negligible contribution of the dipole-dipole interactions in the lipid surface. We demonstrate here that the Langmuir adsorption isotherm alone is not able to properly describe the effects of dipole molecule binding to lipid surfaces—we found significant deviations between experimental data and the fit with the Langmuir adsorption isotherm. We present here an alternative theoretical treatment that takes into account the strong interaction between membrane (monolayer) dipole field and the dipole moment of the adsorbed molecule. This treatment provides a much better fit of the experimental results derived from the measurements of surface potentials of lipid monolayers in the presence of phloretin. Similarly, the theory provides a much better fit of the phloretin-induced changes in the dipole potential of lipid bilayers, as assessed by the transport kinetics of the lipophilic ion dipicrylamine.

INTRODUCTION

The change in Volta potential observed by spreading lipid monolayers at air-water interfaces is caused by the uniform orientation of the lipid molecules and/or their ability to alter the orientation of water dipoles. The magnitude of change in potential depends on lipid structure (Paltauf et al., 1971) and on the surface density of the lipid molecules, which means that it increases dramatically when the monolayer turns from the gas phase (no uniform lipid orientation) into the liquid phase (uniform lipid orientation perpendicular to the surface), e.g., while compressing it on a Langmuir trough (Mozaffary, 1991). This confirms the role of dipole moments attached to the lipid molecules as being responsible for the observed change in surface potential of monolayers from neutral lipids. Bilayers should exhibit similar potentials because they consist of two monolayers with essentially the same properties. In fact, permeability properties of lipid bilayer membranes for anions and cations suggest that the membrane interior is positive by several hundred millivolts with respect to the surface (Haydon and Myers, 1973; Hladky and Haydon, 1973; Szabo, 1976; Pickar and Benz, 1978; Flewelling and Hubbell, 1986; Brockman, 1994). This potential is caused by the orientation of dipoles within the lipid-water interface and is therefore correctly named the dipole (or dipolar) potential (Haydon and Hladky, 1972).

Several molecules have been found to change the dipole potential within monolayers and bilayers. One of them, phloretin, has been described as a molecule that reduces the existing positive dipole potential by its adsorption to the membrane (or monolayer) and induces a dipole potential of polarity opposite that of the preexisting one (Andersen et al., 1976; Melnik et al., 1977; Reyes et al., 1983). This means that the positively charged end of phloretin is directed toward the aqueous phase and the negatively charged end toward the hydrocarbon layer. The effect on the alteration of dipole potential is observed only with uncharged forms of phloretin and its analogs (Andersen et al., 1976; Reyes et al., 1983). In agreement with this, Andersen et al. (1976) found that phloretin does not change the conductance of lipid bilayers to either anions or cations when the aqueous pH is close to 10. Furthermore, only the uncharged form is able to adsorb to human red cell membranes (LeFevre and Marshall, 1959). This indicates the role of phloretin both as adsorbate with lipophilic character and as a molecule bearing a dipole moment.

Clearly, the change in dipole membrane potential depends on 1) the magnitude of the adsorbed molecule dipole moment, 2) the angle between the direction of the dipole moment vector and the water/lipid interface, 3) the dielectric constant of the environment, and 4) the surface density of the adsorbed molecule. Whereas 1) and 3) are constants for a given molecule and for the medium of the adsorption plane, respectively, 2) and 4) are unknown. When we assume that the adsorbed molecules are aligned parallel to each other, the change in dipole potential is a function of their surface density. The effect of phloretin on natural and artificial membranes has been studied in detail (Owen, 1974; Jennings and Solomon, 1976; Andersen et al., 1976; Melnik et al., 1977; Cousin and Motais, 1978; Verkman, 1980; Verkman and Solomon, 1980; Awiszus and Stark, 1988). In particular, a saturation effect of the potential change has been observed for high aqueous phloretin concentrations. This led to the assumption that the interaction

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between phloretin and the membranes could preferentially be controlled by hydrophobic interaction between phloretin and membrane. Although De Levie et al. (1979) have also discussed the possible influence of dipole-dipole interactions on phloretin adsorption, they have suggested that its adsorption is appropriately described by a Langmuir adsorption isotherm if the contribution of dipole-dipole interaction in the membrane interface is much weaker than Langmuir adsorption and therefore negligible.

In this study we investigated the adsorption of phloretin to lipid monolayers by surface potential measurements and its adsorption to bilayers by measuring the effect of increasing phloretin concentrations on the transport kinetics of lipophilic ions. We found significant deviations between the experimental results and a fit of them by using the Langmuir adsorption isotherm, especially for lipid monolayers. In particular, the use of the Langmuir adsorption isotherm leads to a lower maximum potential change ($\Delta\Psi_\infty$), as expected from the dependence of the experimental data on phloretin concentration. Furthermore, the concentration dependence of the dipole potential change was not well described by the Langmuir adsorption isotherm. For bilayers, the deviations between experiment and Langmuir adsorption isotherm were less pronounced but still present. A much better fit of the experimental data was achieved when we took into account a much stronger effect of the dipole-dipole interaction between membrane and phloretin than has previously been proposed (De Levie et al., 1979).

ELECTRICAL CONTRIBUTION OF DIPOLE MOMENT TO LANGMUIR ADSORPTION ISOTHERM

The Langmuir adsorption isotherm assumes a monomolecular adsorption layer with a maximum number of equivalent binding sites, each of which can be occupied by one molecule. The surface density Γ of adsorbed molecules is a function of their concentration, c, in the aqueous phase:

$$\Gamma = \frac{\Gamma_\infty c}{c + k} \tag{1}$$

where Γ_∞ is the maximum surface density and k is the dissociation constant. The change in the dipole potential, $\Delta\Psi$, by the adsorption of the molecules is a linear function of their surface density:

$$\Delta\Psi = \frac{4\pi\mu L\Gamma \sin\Theta}{\epsilon} \tag{2}$$

where μ is the dipole moment of one molecule, Θ is the angle between the direction of the dipole moment vector and the water/lipid interface, L is Avogadro's number, and ϵ is the effective dielectric constant in the adsorption plane. The surface density of the adsorbed molecules can be expressed by the potential change, and their maximum surface density by the maximum potential change:

$$\Delta\Psi = \frac{\Delta\Psi_\infty c}{c + k} \tag{3}$$

Equation 3 implicitly takes ϵ as constant (compare Equation 2). This might lead to some confusion, because phloretin, as a polar molecule, should alter the electric permittivity of the plane to which it adsorbs. This means ϵ in the adsorption plane should depend on the phloretin concentration. But we must keep in mind that we refer only to the changes in dipole potential (see Discussion), and therefore to the effects induced by single phloretin molecules. The permittivity of the region of one adsorbed dipole molecule has the same magnitude as the permittivity of many adsorbed dipole molecules (like a parallel circuit of capacitors, each with the same dielectric). In other words, as long as we consider the adsorption of dipole molecules as a uniform process with uniformly aligned dipoles, the electric permittivity of the region that contributes to the dipole potential change (i.e. the environment close to the adsorbed dipole molecules and the dipole molecules themselves) is not dependent on the concentration of adsorbed dipole molecules. Therefore it appears reasonable to take ϵ to be a constant.

The dissociation constant k is given by the free energy of adsorption:

$$k = \exp\left(\frac{-\Delta G_0}{RT}\right) \tag{4}$$

ΔG_0 is the standard free energy of adsorption; R and T have their usual meanings. In the following we separate the standard free energy of adsorption, ΔG_0, into two parts. One is the Langmuir interaction, ΔG_{00}, and the other is the standard free energy, ΔG_{el}, of the interaction of the dipole molecule with the existing dipole field in the membrane-water interface. The entire free adsorption energy is given by

$$\Delta G_0 = \Delta G_{00} + \Delta G_{el} \tag{5}$$

This means that the dissociation constant, k, can also be split into two parts. One of them is dependent on the dipole potential, and the other, k_0, is given by $\exp(-\Delta G_{00}/RT)$.

$$k = k_0 \exp\left(\frac{-\Delta G_{el}}{RT}\right) \tag{6}$$

ΔG_{el} has to be replaced by a potential term. For this we assume that the parallel aligned dipoles form a simple capacitor. The energy, W, in the electrical field can be expressed by the total dipole moment, μ_t, normal to the water/lipid interface and the field strength E:

$$W = \frac{\mu_t E}{2} \tag{7}$$

The field strength is the dipole potential divided by the thickness of the dipole layer or by the length of the dipole if the angle between the dipole moment vector and adsorp-

tion plane is 90°. When we use the ratio dipole moment divided by dipole length, we automatically normalize the angle of the dipole to the adsorption plane. Thus the energy per mole adsorbed dipoles is

$$\Delta G_{el} = \mu \frac{\Psi_0 - \Delta\Psi}{2l} L \tag{8}$$

Ψ_0 is the initial dipole potential of the membrane (monolayer), $\Delta\Psi$ is the change in dipole potential, l is the dipole length, and μ is the dipole moment of a single adsorbed molecule. When we combine Eqs. 6 and 8, it is evident that the dissociation constant, k, is a function of the dipole potential:

$$k = k_0 \exp\left(\frac{(-\Psi_0 + \Delta\Psi)\mu L}{2lRT}\right) \tag{9}$$

For simplification we define

$$\omega \equiv \frac{\mu L}{2lRT} \tag{10}$$

$$k_{00} \equiv k_0 \exp(-\psi_0\omega) \tag{11}$$

k_{00} contains both the contributions of Langmuir and the dipole field of the membrane. The combination of Eqs. 3, 9, 10, and 11 leads to

$$\Delta\Psi = \frac{\Delta\Psi_\infty c}{c + k_{00}\exp(\Delta\Psi\omega)} \tag{12}$$

According to Eq. 12, the change in dipole potential is dependent on both the aqueous concentration of the dipole molecule and the change in the dipole potential. The dissociation constant, k, should decrease when $\Delta\Psi$ increases, because of the adsorption of dipole molecules with a dipole moment opposite that of the membrane.

MATERIALS AND METHODS

Materials

Natural phospholipids, egg phosphatidylcholine (PC) and egg phosphatidylethanolamine (PE) from *Escherichia coli*, were obtained from Avanti Polar Lipids (Alabaster, AL). Phloretin was obtained from Sigma (St. Louis, MO). Benzene and *n*-decane were spectroscopically pure and ethanol was analytical grade (Merck, Darmstadt, Germany). Dipicrylamine was obtained from Fluka (Buchs, Switzerland; purissimum). Ultrapure water was obtained by passing deionized water through Milli-Q equipment (Millipore, Bedford, MA).

Buffers and solutions

In monolayer experiments, the lipids PC and PE were spread on the water-air surface in a mixture of benzene and ethanol (1:1 v/v) in a final concentration of 0.8 mM. In the lipid bilayer experiments, PC and PE were used as a 20 mg/ml solution in *n*-decane. The aqueous phase was in all experiments the same and contained 0.1 M NaCl and 20 mM NaH_2PO_4 dissolved in ultrapure water. Other salt concentrations tested showed that ionic strengths did not influence the dipole potentials. The pH was adjusted to 5.5, and the experiments were performed at 22°C throughout. Phloretin was dissolved in 1 M and 0.1 M NaOH; the stock solutions contained 1 mM, 10 mM, and 300 mM phloretin, respectively. Dipicrylamine was used as a 1 mM stock solution in ethanol. After membrane formation, dipicrylamine solution was added in final concentrations between 3×10^{-8} and 3×10^{-7} M. The ethanol content in the aqueous phase did not exceed 0.05% (v/v), which did not influence membrane properties.

Measurements of monolayer surface potentials

Lipid monolayers were formed on the water-air surface of a buffer-filled Teflon trough (surface area 120 cm^2, volume 300 ml). The lipids were spread on the surface with organic solvents, by the use of a Hamilton microsyringe (Hamilton, Bonaduz, Switzerland). The surface pressure of the monolayers was adjusted to 40 mN/m by adding the appropriate amount of lipid to the air/water interface. The surface pressure was measured by the Wilhelmy plate method (Allan, 1958; Gaines, 1966). Surface potential measurements of the monolayer were performed by the vibrating plate method originally introduced by Kelvin and improved by Yamins and Zisman (1933). This method has been described previously in detail (Brockman, 1994; Gaines, 1966). We used a 2-cm-diameter, gold-plated disk electrode adjusted to be less than 1 mm from the air-water interface. The plate vibrated at ~416 Hz, and the signal was measured with a laboratory-built lock-in amplifier (Bürner et al., 1994). The dipole potential was referenced to a Ag/AgCl electrode in the water phase. First the potential of the aqueous phase was measured. Then the plate was raised and the lipid was spread. After evaporation of the solvent, the plate was lowered to the same distance from the interface as before spreading, and the potential was recorded again. The Teflon trough contained a small hole in its side, through which small aliquots of stock phloretin solutions were introduced into the subphase. The same buffer volume was taken from the trough before the addition of phloretin solution to ensure a proper distance between plate and interface. Whenever phloretin stock solution was added to the subphase, the vibrating plate had to be raised and lowered to allow stirring with a Teflon stirrer bar. The change in dipole potential was taken after readjustment to zero as the difference between the new potential and the reference. Surface pressure and surface potential measurements showed standard deviations of less than ±1 mN/m and ±10 mV, respectively.

Estimation of phloretin-induced bilayer dipole potential change

The phloretin-induced change in the dipole potential of lipid bilayer membranes was measured from its influence on dipicrylamine transport parameters in charge pulse experiments (Benz and Cros, 1978; Pickar and Benz, 1978). These parameters are the translocation rate, k_i, and the partition coefficient, β, which were derived as follows. The decay of membrane voltage with time, $V(t)$, after a brief charge pulse is given by the sum of two exponential relaxations (Benz, 1988):

$$V(t) = V_0(a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2)) \tag{13}$$

a_1, a_2 (= 1 − a_1), τ_1 and τ_2 (> τ_1) are known functions of k_i, the total concentration of the lipophilic ions per unit surface, N_t, and the passive RC time constant, τ_m, of the lipid bilayer membrane. The inverse relations between the relaxation parameters and k_i, N_t, τ_m, and β are given by

$$k_i = (a_1/\tau_2 + a_2/\tau_1)/2 \tag{14}$$

$$N_t = 2RTC_m(1/\tau_1 + 1/\tau_2 - 2k_i - 1/(2\tau_1\tau_2 k_i))/F^2 k_i \tag{15}$$

$$\tau_m = 2k_i\tau_1\tau_2 \tag{16}$$

$$\beta = N_t/2c \tag{17}$$

C_m is the specific membrane capacitance and F is the Faraday constant.

Black lipid bilayer membranes were formed from a 2% (w/v) solution of PC and PE in *n*-decane. The membranes were formed across circular

holes with 1–2-mm diameter in the wall separating two aqueous compartments in a Teflon cell. Dipicrylamine was added to the aqueous phase in the form of concentrated (10^{-3} M) solutions in ethanol to give final concentrations in the aqueous solutions between 3×10^{-8} to 3×10^{-7} M. These concentrations were chosen to obtain a linear relationship between the concentrations of the lipophilic ions in the aqueous phase and in the membrane (Benz et al., 1976; Benz and Läuger, 1977) and to avoid boundary potentials (Andersen et al., 1978b).

The charge pulse experiments were carried out as described previously by using short current pulses of 5–10 ns duration (Benz et al., 1976). In brief: one Ag/AgCl electrode was connected to a fast commercial pulse generator (Philips PM 5712) through a fast diode (reverse resistance $> 10^{11}$ Ω), and the other electrode was grounded. A resistor of 10 MΩ was introduced between the two electrodes to define a passive RC time constant for the membrane (Benz, 1988). The voltage between these two electrodes was measured with a fast high-input-resistance voltage amplifier (bandwidth 200 MHz) based on a Burr Brown operational amplifier and a digital storage oscilloscope (Nicolet 4094). The voltage decay was analyzed using a personal computer, as has been described previously (Klotz and Benz, 1993). It should be noted that the specific capacitance of lipid bilayers is voltage dependent because of the pressure on the membrane caused by transmembrane potentials. However, the membrane capacitance is charged in the charge pulse to voltages of ~10 mV, which means that C_m can be taken as constant (Benz and Janko, 1976).

RESULTS

Phloretin-induced surface potential change of monolayers

Fig. 1 shows the phloretin-induced decrease in the surface potential, $\Delta\Psi$, of PC monolayers as a function of the phloretin concentration in the subphase (full points). The fit of the experimental data by a least-squares fit method using the Langmuir adsorption isotherm (Eq. 3) was not satisfactory, as Fig. 1 clearly shows. In particular, the deviation between experimental data and fit according to Langmuir was considerably high in the concentration range between 2×10^{-5} and 10^{-4} M phloretin and at a very high phloretin concentration, 3×10^{-4} M. A much better fit of the experimental results was achieved when we used Eq. 12 for the fit of the experimental data (see Fig. 2 *A*). The points of Fig. 2 *A* represent the experimental results taken from Fig. 1. Curve 1 shows the least-squares fit of the data points with the Langmuir adsorption isotherm using Eq. 3 ($\Delta\Psi_\infty = -246$ mV and $k = 5.4$ μM), and curve 2 the fit using Eq. 12 ($\Delta\Psi_\infty = -326$ mV and $k_{00} = 2.1$ μM). The fit was performed in a two-step procedure because the parameter ω includes the dipole moment ($\mu = 5.6$ D; Reyes et al., 1983) and its length, l, as constants. Because the dipole length was unknown, we fitted the data, including ω as a fit parameter in a first step, and then, in a second step, we used the mean value of ω as a constant. Using this procedure, we calculated a dipole length of 0.18 nm, which should be a realistic value.

It is noteworthy that curve 2 of Fig. 2 *A* provided a much better fit of the experimental data than curve 1. The fit of the

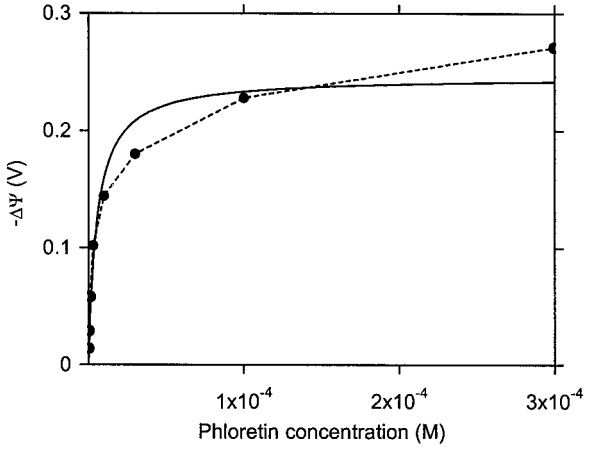

FIGURE 1 Change in dipole potential versus phloretin concentration of PC monolayers. The full line represents a Langmuir fit according to Eq. 3. The dashed line represents only the connection of the data points and has no physical significance. The fit shows typical deviations from the experimental data, which are observed at all monolayer and bilayer potential measurements without exception.

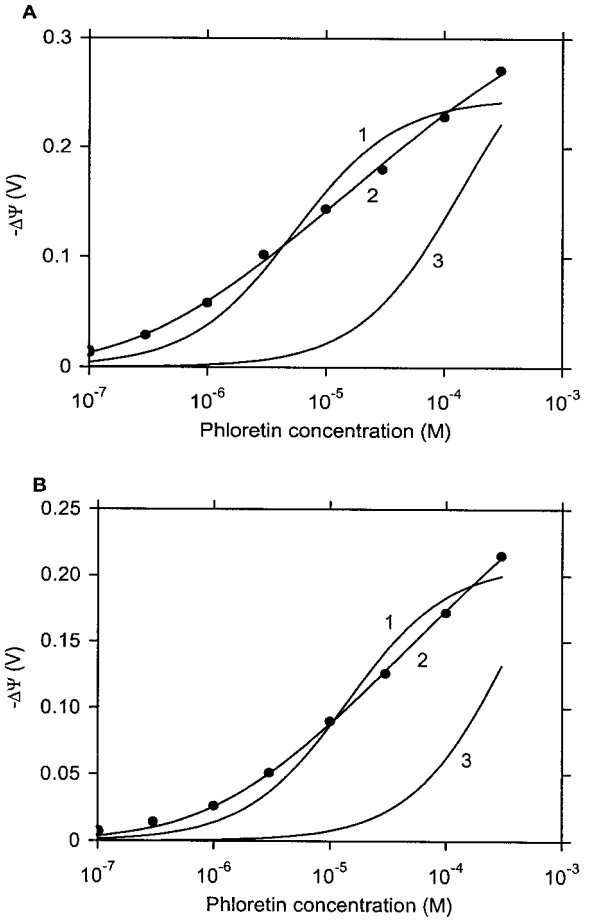

FIGURE 2 Change in dipole potential versus phloretin concentration of PC (*A*) and PE (*B*) monolayers. The surface pressure of the monolayers was 40 mN/m. The data represent the means of at least three individual experiments; the standard deviations were below ±10 mV. Curve 1 shows the Langmuir fit according to Eq. 3; curve 2 shows the improved fit according to Eq. 12. Curve 3 is obtained with Eq. 3, in which $\Delta\Psi_\infty$ is taken from the corresponding improved fit and k is from the calculated k_0 (see Table 1), which corresponds only to adsorbens-adsorbate interaction.

experimental data using Eq. 12 yielded two parameters, the maximum dipole potential change, $\Delta\Psi_\infty$, and the apparent dissociation constant, k_{00}. It is possible to calculate the maximum surface density, Γ_∞, of the adsorbed phloretin molecules from the maximum change in the dipole potential. For this it is necessary to know the angle between the dipole moment vector and the adsorption plane of the adsorbed phloretin molecule and the dielectric constant of its environment (see Eq. 2), which are both unknown. As an approach to the order of magnitude of Γ_∞, we assumed an angle of 90° between the dipole moment vector and the adsorption plane. The value of the effective dielectric constant can range from 2 (hydrocarbon region) to 20 (polar headgroups) (Coster and Smith, 1974). We assumed a medium value of 10 for the relative dielectric constant. It should be noted that the fit parameter k_{00} does not depend on the choice of ϵ and Θ, but only on the calculated maximum surface density Γ_∞. The apparent dissociation constant, k_{00}, contains two contributions (see Eq. 11). One is the dissociation constant of phloretin without the electrical contribution, k_0, and the other contains the dipole potential, Ψ_0, of the monolayer or of the bilayer times the fit parameter ω. This means that $\exp(-\Psi_0\omega)$ represents the contribution of the dipole potential to the adsorption of phloretin. Clearly, dipole potentials of monolayers and bilayers are positive and approximately on the order of $\Delta\Psi_\infty$, the exact values are not known. We assumed here that they are given by $\Delta\Psi_\infty$ (see Discussion).

The value of $\Delta\Psi_\infty = -326$ mV suggested that electrostatics made a considerable contribution to the free energy of phloretin adsorption. In fact, the contribution of Langmuir alone to the adsorption of phloretin to PC monolayers was presumably rather small, and k_0 had, under the conditions of Fig. 2 *A*, a value of 142 μM ($\Psi_0 = -\Delta\Psi_\infty$). This means that the contribution of electrostatics to phloretin adsorption was 70 times higher than that of Langmuir. This can also be derived from curve 3 of Fig. 2 *A*, which shows only the dependence of the Langmuir contribution on phloretin adsorption under the conditions described above (i.e., by assuming $\Psi_0 = 326$ mV).

Similar effects were observed for the adsorption of phloretin to PE monolayers (Fig. 2 *B*). Here $\Delta\Psi_\infty$ was -301 mV (fit with Eq. 12), whereas from Langmuir we derived a value of only -209 mV (using Eq. 3). Similarly, the combination of electrostatics and Langmuir also provided in this case a much better fit of the experimental data than Langmuir alone (compare curves 1 and 2 of Fig. 2 *B*). The Langmuir adsorption isotherm suggested a value of k equal to 14 μM, whereas k_{00} of the improved fit was 7.7 μM. Again, Langmuir alone contributed little to the treatment of the phloretin adsorption to PE monolayers (by assuming $\Psi_0 = -\Delta\Psi_\infty = 301$ mV). This means k_0 had a value of 385 μM that is almost two orders of magnitude larger than k_{00}. The data of the fit of phloretin adsorption to monolayers are summarized in Table 1, which also provides a comparison of the adsorption parameters of the Langmuir fits (Eq. 3) with those derived from fits using Eq. 12.

TABLE 1 Adsorption parameters of phloretin to PC, PE monolayers and bilayers derived from Langmuir adsorption isotherm and the improved adsorption isotherm

	$\Delta\Psi_\infty$ (V)*	k_{00} (μM)*,#	Γ_∞ (μmol/m^2)§	k_0 (μM)¶	k_0/k_{00}
Monolayer PC	—	—	0.202	142	69
Langmuir fit	−0.246	5.41	—	—	—
Improved fit	−0.326	2.05	—	—	—
Monolayer PE	—	—	0.186	385	50
Langmuir fit	−0.209	14.1	—	—	—
Improved fit	−0.301	7.7	—	—	—
Bilayer PC	—	—	0.12	50	12
Langmuir fit	−0.164	9.25	—	—	—
Improved fit	−0.188	4.31	—	—	—
Bilayer PE	—	—	0.1	187	8.8
Langmuir fit	−0.132	29.8	—	—	—
Improved fit	−0.167	21.4	—	—	—

The table also shows values for the dissociation constant, k_0, of only adsorbens-adsorbate interaction and the ratio k_0/k_{00} derived from a theoretical consideration. For details, refer to the text.

*The experimental data are fitted using the dipole moment for phloretin $\mu = 5.6$ D (Reyes et al., 1983). The value assumed for ω is 13 (see Eq. 10); the corresponding dipole length l is 0.18 nm.

#For Langmuir fits, k_{00} corresponds to k (see Eq. 3).

§The assumed angle, Θ, between the dipole moment vector and the adsorption plane is 90°; the assumed relative dielectric constant, ε, is 10.

¶The values assumed for Ψ_0 are the corresponding values for $\Delta\Psi_\infty$.

In addition to the experiments with phloretin, we also performed monolayer experiments with several phloretin analogs (Cseh and Benz, unpublished observations). Preliminary analysis of these data showed deviations similar to those of phloretin from the Langmuir isotherm and a better fit to the improved description according to Eq. 12. This indicates that the interaction of membrane surfaces with dipole molecules of different molecular structures can be treated in the same way as described here for phloretin (see Discussion).

Phloretin-induced dipole potential changes of lipid bilayer membranes

The dipole potential changes of lipid bilayer membranes were measured in an indirect way through the influence of phloretin on transport properties of the lipophilic ion dipicrylamine. For this purpose, charge pulse experiments were performed with PC- and PE-decane membranes at different concentrations of phloretin in the aqueous phase. These experiments yielded the parameters of two exponential voltage relaxations (Eq. 13), from which, in turn, the translocation rate constant, k_i, and the partition coefficient, β, could be calculated according to Eqs. 14–17. Both the translocation rate constant and partition coefficient were similar to those, which have been measured previously for the same systems (Benz and Gisin, 1978). When phloretin was added to the aqueous phase, both decreased. In previous

investigations (Szabo, 1974; Andersen et al., 1978a; Benz and Cros, 1978; Pickar and Benz, 1978) it has been demonstrated that the change in dipole potential can be detected by its influence on both the partition coefficient, β, and the translocation constant, k_i. The consideration of both is necessary because the location of the adsorption plane of lipophilic ions concerning the dipole potential is unknown. This means that the change in the phloretin-induced dipole potential is given by (Pickar and Benz, 1978)

$$\Delta\Psi = -\frac{RT}{F}\ln\frac{k_{i0}\beta_0}{k_i\beta} \tag{18}$$

$k_{i0}\beta_0$ is the product of both parameters for dipicrylamine before and $k_i\beta$ is the product after phloretin adsorption.

The dependence of k_i and β on the aqueous concentration of phloretin and the corresponding change in dipole potential are shown in Table 2. The results of phloretin-induced dipole potential change on PC bilayers are shown in Fig. 3 *A*. Again, the use of Eq. 12 provided a better fit of the data for dipole potential change than Langmuir alone, although the deviation from Langmuir was less pronounced than for monolayers (compare curves 1 and 2 of Fig. 3 *A*). This was presumably caused by the smaller phloretin-induced change in bilayer dipole potential as compared with the corresponding change in monolayer surface potential and the therefore smaller dipole potential (see Discussion). As in PC membranes, we observed a smaller effect of phloretin on the dipole potential of PE bilayers (Fig. 3 *B*). But again, the use of Eq. 12 provided a better fit of the experimental data, as compared with Eq. 3. The parameters for the fit of the phloretin-induced change in lipid bilayer membrane dipole potential are summarized in Table 1. The phloretin-induced

TABLE 2 Translocation rate constants, k_i, partition coefficients, β, and the changes in dipole potential, $\Delta\Psi$, dependent on the aqueous phloretin concentration, *c*, derived from charge pulse experiments at PC and PE membranes

c Phloretin (μM)	k_i (s^{-1})	β (10^{-3} cm)	$-\Delta\Psi$ (mV)
PC bilayer			
0	620	8.9	—
0.1	570	7.2	7.6
0.3	510	7.3	10
1	240	7.3	30
3	120	8.0	45
10	80	3.1	80
30	30	1.9	118
100	20	0.7	154
300	10	0.5	161
PE bilayer			
0	5988	5.2	—
0.1	5567	4.7	4.6
0.3	4128	5.8	6.5
1	3629	5.8	9.8
3	3247	5.2	15.6
10	3049	2.3	37.8
30	1051	2.6	61.5
100	359	1.8	98
300	106	2.3	123

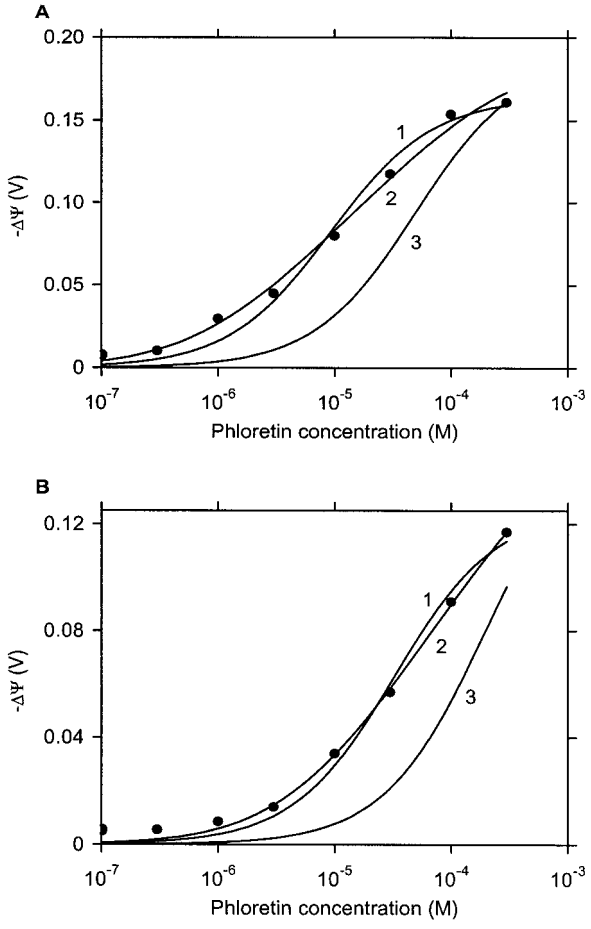

FIGURE 3 Change in dipole potential versus phloretin concentration of PC (*A*) and PE (*B*) bilayers. The data are the means of at least three individual experiments; the standard deviations were below ± 10 mV. Curve 1 shows the Langmuir fit according to Eq. 3; curve 2 shows the improved fit according to Eq. 12. Curve 3 is obtained with Eq. 3, in which $\Delta\Psi_\infty$ is taken from the corresponding improved fit and k is from the calculated k_0 (see Table 1), which corresponds only to adsorbens-adsorbate interaction.

dipole potential change in bilayers was approximately half of that derived from monolayers. This applied also to the constant k_{00} derived from the fit of the experimental results. Table 1 also shows an estimation of the maximum surface densities, Γ_∞, of bilayers, which were calculated using the same ϵ and Θ as assumed for monolayers (see above). ϵ and Θ are determined by material properties and the kind of lipid-phloretin interaction, but they are unknown. Note that the translocation rates, k_i, and the partition coefficients, β, presented in Table 2 do not depend on the choice of these values (see Eqs. 14–17).

DISCUSSION

Electrostatics has to be taken in account for phloretin adsorption to lipid monolayers and bilayers

The surface potential of lipid monolayers from PE and PC is positive by several hundred millivolts (Paltauf et al.,

1971; Haydon and Myers, 1973; Vogel and Möbius, 1988; Brockman, 1994). This is caused at least in part by the carbonyls of the lipid molecules, which become ordered when the area per lipid molecule is decreased by compression and the monolayer undergoes phase transition from the gas to the fluid state. The use of ether instead of ester lipid results in a strong decrease in the surface potential of monolayers from neutral lipids (Paltauf et al., 1971) and the dipole potential of neutral bilayers (Pickar and Benz, 1978), which indeed argues for an important role of ester carbonyls for the creation of a positive dipole potential. In fact, when we assume that only one of the two ester carbonyls of a lipid molecule (dipole moment μ = 1.8 D) (Flewelling and Hubbell, 1986) is oriented in a condensed monolayer ($\approx$ 0.6 nm^2 per lipid molecule) perpendicular to its surface and that the relative dielectric constant, ϵ, of the dipole layer is $\sim$10, the dipole potential of a monolayer is estimated to be $\sim$1.46 V. A dipalmitoyl phosphatidycholine (DPPC) molecule has a total dipole moment of 0.82 D (Vogel and Möbius, 1988), which means that the dipole potential of a DPPC monolayer is, under the conditions used above (ϵ = 10, 0.6 nm^2 per lipid molecule), $\sim$700 mV. Both dipole potentials are in qualitative agreement with permeability properties of positively charged lipophilic ions and their negatively charged structural analogs in neutral PC or PE bilayers. Several studies have demonstrated that the permeability of positively charged ions through lipid bilayer membranes is orders of magnitude smaller than that of their negatively charged analogs (Andersen et al., 1978b; Pickar and Benz, 1978; Flewelling and Hubbell, 1986).

When we accept the idea of positive dipole potentials in lipid monolayers and bilayers, we have also to accept that this potential represents a considerable driving force for the adsorption of negatively charged lipophilic ions and the rejection of the positively charged ones. Such a dipole potential also has a considerable influence on the adsorption of dipole molecules being dependent on the orientation of the dipole moment within the molecule. The driving force, aside from the Langmuir interaction, is for a small number of adsorbed dipole molecules, given by $\exp(-\Psi_0\omega)$ when the dipole moment has a direction opposite that of the dipole potential (see Eq. 11 of our theoretical treatment). This represents a considerable contribution to the adsorption of dipole molecules, such as phloretin and its analogs. Assuming a dipole potential Ψ_0 of 200 mV, a dipole length l of 0.2 nm, and a dipole moment μ of 5.6 D, which are all realistic values, the factor $\exp(-\Psi_0\omega)$ is $\sim$0.1, which means that the contribution of the interaction of the dipole with the dipole field cannot be neglected for such a case, as has been done in previous investigations of phloretin-mediated adsorption to monolayers (De Levie et al., 1979; Reyes et al., 1983). It is noteworthy that a Ψ_0 of 400 mV creates an electrical contribution of a factor of $\sim$0.01.

The combination of Langmuir and electric forces also provided another aspect of the explanation of the experimental data. Clearly, the use of Eq. 12 yielded much better fits of experimental data than those performed according to the Langmuir isotherm alone (see Figs. 2 and 3). The improved fits of monolayer results in particular definitely match the data better in the full concentration range and confirm our thesis that electrostatics has to be taken into account for the description of phloretin adsorption to lipid surfaces. Similar but less pronounced considerations also apply to the experiments with bilayers, where Eq. 12 also provides a better fit than Langmuir alone. However, we have to admit that a smaller difference exists between both fits (i.e., those by Eqs. 3 and 12) for bilayers. This may have led to the thesis of negligible electrical interactions for the adsorption of phloretin to bilayers (De Levie et al., 1979). However, we would be surprised if there were a difference between the forces of phloretin adsorption to monolayers and bilayers.

How high is the dipole potential of monolayers and bilayers?

Our simple estimation of the contribution of the carbonyls to the dipole potential demonstrated that the dipole potential of monolayers and bilayers is positive by at least several hundred millivolts toward the hydrocarbon chains. However, its exact value is not known in both cases. This represents a problem for the separation of hydrophobic interaction from the electrical contribution, because the fit parameter k_{00} depends on the value of Ψ_0 (see Eq. 11), which influences the adsorption of phloretin at small concentrations. To get an idea of its value, we consider the phloretin-induced maximum dipole potential change. It should depend on the preexisting potential, because the electrical part of adsorption energy depends on Ψ_0 (see Eq. 8). The surface potential of monolayers after the lipid was spread was $\sim$0.52 V for PC and 0.58 V for PE monolayers. When we assume that the contribution of water surface potential is ~ -0.2 V, we can use the maximum phloretin-mediated dipole potential change, $\Delta\Psi_\infty$, as a measure of the preexisting potential, Ψ_0, to estimate $\exp(-\Psi_0\omega)$ and k_0. (Until now there has been no agreement on the surface potential of the water-air interface. The published values range from 25 mV (Borazio et al., 1985) to 100 mV positive (Parfenyuk and Krestov, 1992) to several hundred millivolts negative (Davis and Rideal, 1961; Colacicco, 1988) toward air.) Although this represents an approximation, it is obvious that Ψ_0 should be at the right order of magnitude.

As shown in Table 1, the values of k_0 and k_{00} differ considerably. Following our approach, we conclude that the energy due to the adsorbens-adsorbate interaction is rather low compared to the electrical part of adsorption energy. It should be pointed out that the ratios between k_0 and k_{00} range from $\sim$10 at bilayers to 60 at monolayers, which indicates a smaller dipole potential of bilayers as compared to monolayers. However, also in this case, its exact value is not known. A rough estimate may be given if we assume that the Langmuir part of the interaction (i.e., k_0) between phloretin and membranes is the same as that between phlor-

etin and monolayers (e.g., 142 and 385 μM for PC and PE respectively; see Table 1) and calculate the corresponding preexisting potential by using Eq. 11. In this way we find values of 0.269 V and 0.222 V for PC and PE bilayers. These values are somewhat higher compared with the maximum dipole potential change, $\Delta\Psi_\infty$, of bilayers that we used in Table 1 as a measure for the preexisting potential. However, they are very similar to those that have been derived previously for positively and negatively charged lipophilic ions of the same structure (0.224 V and 0.215 V, respectively; Pickar and Benz, 1978). This means that the dipole potentials of monolayers are higher than those of bilayers, and we used for our calculation again the maximum phloretin-induced potential change as a measure for the dipole potential of PC and PE membranes.

The adsorption of dipole molecules changes the dipole potential of monolayers and bilayers

Our theoretical treatment takes into account the fact that the adsorbed dipole molecules change the existing dipole potential of monolayers and bilayers. Furthermore, it is also applicable when no dipole potential exists before the adsorption of the first dipole molecule or when the adsorption increases the dipole potential. The first case is demonstrated in Fig. 4. For this we assumed adsorption parameters similar to that of phloretin (dissociation constant $k_0 = 100$ μM, dipole moment $\mu = 5.6$ D, maximum dipole potential change $\Delta\Psi_\infty = -0.3$ V) and calculated the dipole potential change as a function of the aqueous concentration. Curve 1 of Fig. 4 shows the dipole potential change that should occur when the adsorption behavior follows the Langmuir adsorption isotherm alone without electrical contribution. Curve 2 shows the effect of the adsorption of dipole molecules on dipole potential according to Eq. 12 by using $\Psi_0 = 0$. The adsorption of dipole molecules hinders further adsorption of the dipole molecules to the surface, which results in a lower dipole potential change as compared with Langmuir. The dashed line is the Langmuir fit of the data of curve 2. It shows the typical deviations that were also observed in fits of our experimental data. It is noteworthy that also in the case of a preexisting dipole potential $\Psi_0 = 0$, Eq. 12 provides a much better fit than Langmuir alone. In particular, the maximum dipole potential changes differ from one another, in addition to the effect that apparent and real dissociation constants are different by a factor of ~5.

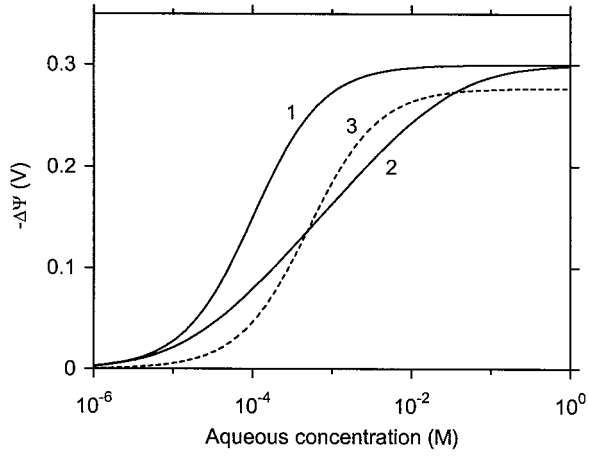

FIGURE 4 Change in dipole potential, plotted as a function of the aqueous concentration of a dipole molecule. For the plots it is assumed that the lipid layer does not possess a preexisting dipole potential, i.e., $\Psi_0 = 0$. The adsorption parameters of the dipole molecule were similar to that of phloretin (dissociation constant $k_0 = 100$ μM, dipole moment $\mu = 5.6$ D, maximum dipole potential change $\Delta\Psi_\infty = -0.3$ V). Curve 1 (plotted according Eq. 3) shows the dipole potential change created by adsorption of the dipole molecule following the Langmuir adsorption isotherm. Curve 2 shows the change of the dipole potential according to Eq. 12, using $\Psi_0 = 0$ (corresponding to $k_{00} = 100$ μM). The dashed line (*curve 3*) represents a fit of the data of curve 2 to Eq. 3 (Langmuir alone; $k = 501$ μM, $\Delta\Psi_\infty = -0.28$ V). The comparison between curves 2 and 3 demonstrates the typical deviations when the contribution of the dipole potential created by the adsorption of the dipole molecules is neglected.

Parameters of phloretin adsorption are different between monolayers and bilayers

We have already pointed out above that the adsorption of phloretin to monolayers and its adsorption to bilayers are different. Reyes et al. (1983) have reported the same result for phloretin and analogs in a Langmuir treatment. This is caused, to a certain extent, by the smaller dipole potential of bilayers, as we have already discussed above. The dipole potentials of monolayers and bilayers should be the same when normalized for lipid packing density. However, they are not. It has been discussed in several investigations that, e.g., a lipid packing-independent component of dipole potential measured in monolayers is responsible for this behavior (Brockman, 1994; Gawrisch et al., 1992). Vogel and Möbius (1988) assume a compensation of the dipole moments along the hydrophobic/hydrophobic contact line in bilayers, i.e., the contribution of the CH_3 groups of the hydrocarbon chain to dipole potential disappears. On the other hand, we cannot exclude the possibility that the adsorption of phloretin to monolayers occurs in a way that is different from its absorption to bilayers. The maximum surface densities of phloretin as shown in Table 1 are still calculated with a constant dipole angle. They can be corrected according to Eq. 2 (e.g., if we assume an angle of 90° in the case of monolayers, the corresponding angle at the PE bilayer is 32°, and that at the PC bilayer is 35° perpendicular to the membrane surface). However, we consider such different behaviors of adsorption to monolayers and bilayers to be extremely unlikely.

Limitations of our theoretical description of the phloretin adsorption to lipid surfaces

It is noteworthy that our theoretical treatment still represents a first-order approximation. First, the adsorption of phloretin is probably a discrete process, whereas we use a mac-

roscopic description of its effects on membrane properties and assume that the effects are smeared across a large surface. On the other hand, we measure the surface potential of monolayers with the Kelvin electrode, which averages the potential across a considerable area. Furthermore, we do not know whether the adsorption plane for phloretin molecules is the plane where the lipid dipoles reside. This means that the plane of the lipid and phloretin dipoles could be inhomogeneous, such that the phloretin dipoles are shifted along the axial direction of the lipid dipoles. But this would not affect our mathematical model, because we refer only to the changes in dipole potential (see Eqs. 2 and 12) and consider therefore only the potential effect caused by the phloretin dipoles. For this the exact alignment of phloretin dipoles in relation to the lipid dipoles is not critical. Bechinger and Seelig (1991) have observed that the phloretin adsorption does not affect the hydrocarbon region of the lipids, but only the polar headgroup region. It is therefore very likely that the adsorption plane of phloretin lies in this area. Another indication is given by surface pressure measurements on a Langmuir trough that we carried out (data not shown). Depending on the lipid and the surface pressure, we found an increase in the area per lipid molecule of 0.1–0.3 nm^2 at constant surface pressure when we added 0.1 mM phloretin to the buffered subphase. At constant area per lipid molecule, which corresponds to our experimental conditions, the surface pressure increased by ~10 mN/m. This clearly indicates an integration of phloretin into the lipid monolayer. At higher surface concentrations of phloretin, the assumption of uniform aligned dipoles could be critical, because the interaction between phloretin molecules could take precedence over the interaction between phloretin and lipid dipoles. The higher the phloretin surface concentration is, the lower is the free energy of adsorption. Thus the driving force that holds the dipole molecules uniformly aligned could vanish in favor of the repulsive force of dipoles close to each other. Consequently, neighboring adsorbed phloretin molecules could be ordered opposite one another. We cannot exclude such a behavior in principle, but it seems unlikely because of the following reason. The ratio of lipid molecules to adsorbed phlorein molecules at maximum surface coverage is still high, even if we assume an ϵ of 20 and a dipole angle of 45°. Using Eq. 2 and the lipid surface concentration (2.78 $\mu mol/m^2$), it is estimated to be ~5 at monolayers (using the maximum potential change, $\Delta\Psi_\infty$, as given in Table 1, the maximum surface concentration of phloretin is, under the conditions above, $\Gamma_\infty = 0.57\ \mu mol/m^2$); if we assume a low ϵ of 2 and a dipole angle of 90°, the ratio is almost 70 ($\Gamma_\infty = 0.04\ \mu mol/m^2$). This means that phloretin dipoles are probably completely surrounded by lipid dipoles. The direct dipole-dipole interaction between phloretin molecules, on the other hand, would require them to be in close proximity to each other. It is also crucial to our treatment that the exact dipole potentials of monolayers and bilayers are not known (see above). We must keep in mind that structural changes can also affect adsorption behavior. Reyes et al. (1983) concluded from their study that the change in dipole potential caused by the adsorption of phloretin and its analogs is not a simple function of the dipole moment, but the location, orientation, and/or maximum surface density of the dipole molecules also depend on their chemical structure. Bechinger and Seelig (1991) pointed out that phloretin rotates the N^+ end of the $^-P\text{-}N^+$ dipole of a phosphatidylcholine membrane closer to the hydrocarbon layer, which leads to a partial compensation of the electric field of the dipole agent. Besides this structural change, phloretin also modifies the hydration layer at the lipid-water interface. This means that the different effects of phloretin could influence our theoretical treatment.

CONCLUSION

The adsorption of phloretin to lipid monolayers and bilayers cannot be fully described by a Langmuir adsorption isotherm, because the electrical part of adsorption energy has to be taken in account. It depends on the dipole potential and the surface density of adsorbed dipoles, and therefore changes at various phloretin concentrations in the aqueous phase. When the electrical interactions between adsorbed molecule and dipole potential are taken into account, a modified theoretical description was proposed that matched the experimental data better than the Langmuir isotherm does. The fits of the experimental data suggest that the electrostatic contribution is higher than that of Langmuir alone. This means that the adsorption behavior of molecules with dipole moments, such as absorption of phloretin to membranes, is influenced more by electrical interactions, as has been discussed earlier.

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