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Phloretin and Phloretin Analogs: Mode of Action in Planar Lipid Bilayers and Monolayers

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Summary. Phloretin and other neutral phloretin-like molecules are able to decrease the electrostatic potential within neutral lipid bilayers and monolayers. The relationship between the change in the dipole potential and the aqueous concentration of the molecule is well described by a Langmuir isotherm. From the Langmuir isotherm, the apparent dissociation constants (K_D^A) and the maximum dipole potential change ($\Delta\Delta\psi_{max}$) are obtained for the different phloretin-like molecules tested. Considering the phloretin analogs as derivatives of acetophenone containing two kinds of substituents, one on the benzene ring and another on the carbon chain, it is found that (a) K_D^A is related to the hydrophobicity of the compound and is also a function of the position of the hydroxyl substituent in the ring; (b) from the dependence of K_D^A on the length of the acyl chain, it is estimated that the free-energy change is $\sim$650 cal/mole CH_2; (c) $\Delta\Delta\psi_{max}$ is not a simple function of the dipole moment of the molecule but depends on the substituent on the carbon chain and on the position and number of hydroxyl groups on the benzene ring; (d) phloretin adsorption parameters are a function of membrane lipid composition. The results are discussed in terms of the effect of these compounds on chloride transport in red blood cells.

Key words phloretin · lipid bilayers · lipid monolayers · membrane potentials · membrane ion transport

Introduction

Lipid bilayer membranes are several orders of magnitude more permeable to anions than cations. This had led to the conclusion that oriented dipoles at the membrane-solution interface impart to the membrane interior a high, positive electrostatic potential with respect to the aqueous phases (Liberman & Topali, 1969; LeBlanc, 1970).

Several dipolar molecules are able to modify the intrinsic dipole potential of lipid bilayer membranes (McLaughlin, 1973; Andersen et al., 1976; Smejtek & Paulis-Illangasekare, 1979; Tosteson & Wieth, 1979). One of these molecules, phloretin, is of special interest because it strongly inhibits facilitated transport of hexoses (LeFevre, 1961) and chloride transport in red blood cells (Wieth et al., 1973; Cousin & Motais, 1978). In addition, phloretin affects sugar and ion transport in several other systems (Bihler, Cavert & Fischer, 1965; Czech, Lynn & Lynn, 1973; Owen, 1974).

In planar lipid bilayers, phloretin increases cation conductance and decreases anion conductance (Andersen et al. 1976; Melnik et al., 1977). Andersen et al. (1976) concluded that these changes in bilayer conductance were due to a decrease in the positive dipole potential of the membrane due to phloretin orienting in the membrane and imparting a dipole potential of opposite polarity to the preexisting one. Furthermore, Melnik et al. (1977) showed that phloretin must give rise to a dipole potential lying entirely within the membrane.

Andersen et al. (1976) suggested that the phloretin-induced inhibition of chloride, urea and hexose transport is due to the effects of the high interfacial dipole fields on the translocator of those molecules. Cousin and Motais (1978) studied the effect of several phloretin analogs on chloride transport in red blood cells with the purpose of testing this hypothesis. They showed that the ability of phloretin analogs to inhibit chloride transport in red blood cells is a function of both the molecular dipole moment and the lipid solubility of the compound. However, the correlation found by Cousin and Motais (1978) could be fortuitous since they did not present independent measurements of the effect of phloretin analogs on the membrane dipole potential.

In this paper, we report studies on the effect of phloretin-like molecules with different dipole moments and hydrophobicities on planar lipid bilayers and monolayers. We show that most of the phloretin analogs are able to reduce the intrinsic dipole potential of lipid bilayers. The adsorption parameters calculated from the conductance measurements using ion-probes are in good agreement with those measured studying their interaction

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with lipid monolayers. We further show that the ability of a given compound to decrease the dipole potential is not a simple function of its dipole moment but depends to a great extent on the type of substituent on the benzene ring and on the carbon chain.

Materials and Methods

Surface Potential Measurements

The surface potential of bacterial phosphatidylethanolamine (PE) and *n*-dioleoyl-phosphatidylcholine (PC) monolayers and the changes induced by phloretin and phloretin analogs were measured in a two-compartment chamber (area monolayer compartment = 20 cm^2) as described by Reyes and Latorre (1979). The subphase composition was 1.0 M NaCl, 20 mM sodium phosphate, pH 5.5, unless otherwise indicated. The concentration of phloretin and phloretin analogs was varied by addition of the compounds in concentrated ethanolic solutions to the subphase of the monolayer-free compartment. The subphase was stirred with two magnetic bars. Control experiments showed that ethanol, at the concentrations used (<0.5% vol/vol) has no effect on the surface potential of the monolayers. The surface potential measurements were done at a surface pressure of about 45 dynes/cm by adding an excess of lipid to the air/water interface. The addition of phloretin to a final concentration of 2×10^{-5} M does not induce any appreciable change in surface pressure. Further, at the phloretin concentrations used in this work, surface pressure vs. area curves in the presence and in the absence of phloretin are not different within experimental error (~10%). This indicates that the monolayer fractional area occupied by phloretin molecules under the experimental conditions of the present work is small.

Membrane Formation

The membranes were formed at room temperature (22 ± 2 °C) according to the Montal and Mueller technique (1982). The aperture in the Teflon partition separating the two aqueous compartments was pretreated with a 2% solution of squalene in pentane. The membranes were made from bacterial phosphatidylethanolamine (PE). The lipids were spread on the surface of the electrolyte solution using 10 µl of a solution containing 12.5% mg/ml lipids in pentane. Tetraphenylborate ($TPhB^-$), tetraphenylarsonium ($TPhAs^+$), carbonylcyanide *m*-chlorophenylhydrazone ($CCCP^-$), and nonactin were added to both compartments in concentrated ethanolic solutions after the membrane was formed. $TPhB^-$, $TPhAs^+$, $CCCP^-$ and nonactin final concentrations were 6.6×10^{-8}, 10^{-3}, 10^{-6} and 10^{-7} M, respectively. The electrolyte solutions were symmetrical and consisted of 1 M NaCl or 1 M KCl (when nonactin was used) buffered at pH 5.5 with 20 mM phosphate. Unless otherwise indicated, phloretin and phloretin analogs were used after ethanol-hot water/cold water recrystallization and added in concentrated ethanolic solutions to the aqueous phases bathing the membrane. Control experiments showed that ethanol, at the concentrations used (<0.5% vol/vol), has no effect either on the bare membrane conductance and capacitance or the lipophilic ion-induced conductance.

Electrical Measurements

The system of measuring the electrical properties of the membranes has been described in detail by Alvarez and Latorre (1978). The capacitance of the membrane was measured by applying a 5 kHz, 10-mV peak-to-peak triangular voltage waveform. The area of the membrane was estimated from the capacitance value and the known value of the PE bilayer specific capacitance (0.68 µF/cm^2; Reyes & Latorre, 1979). After membrane formation and addition of the lipophilic ion, the aqueous phases were stirred for 45 min to ensure equilibrium before phloretin and phloretin analogs were added. The zero-voltage conductances induced by $TPhAs^+$, $CCCP^-$, and nonactin-K^+ were calculated from steady-state current-voltage curves recorded directly on an X-Y recorder. For $TPhB^-$, the current measurements were made using a voltage pulse technique (Ketterer, Neumcke & Lauger, 1971). The initial current induced by $TPhB^-$ was estimated from the current extrapolated at zero-time. To improve the signal-to-noise ratio of the $TPhB^-$-induced current transients, several current waveforms were converted to digital form and then added and stored with the help of a signal averager Model 1070 (Nicolet Instruments Corp., Madison, Wisc.). The digitized data were further analyzed with a Hewlett Packard 9825A calculator coupled to the signal averager.

Determination of the pK_a of the Different Phloretin Analogs

Phloretin and the phloretin analogs present a keto-enol tautomerism (Lambrechts, 1934; LeFevre & Marshall, 1959). Changes in the keto-enol equilibrium induced by pH were used to determine their acid dissociation constants (K_a). The compounds were added from concentrated ethanolic solutions to a low and constant ionic strength buffer (McKenzie & Elliott, 1969) to a final concentration of 3×10^{-5} M. Ultraviolet adsorption spectra were recorded in a Cary 15 recording spectrophotometer (Applied Physics Corp., Mourovia, Calif.) in the range 230 to 350 nm. The existence of a clear isobestic point was used as indication that a simple acid-base reaction was present. The pK_a values were calculated according to standard equations from the literature (Jaffe & Orchin, 1962) and they represent the average of the values obtained at three different pH values (7.68, 7.30 and 6.83). The adsorption spectra for the keto and enol forms were obtained at pH 4.30 and 10.38, respectively.

Materials

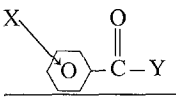
The PE used in these experiments was obtained from Supelco, Inc. (Bellefonte, Pa.). PC was obtained from Avanti Biochemicals (Birmingham, Ala.). Phloretin, phloretin analogs, $TPhB^-$ and $TPhAs^+$ were obtained from Aldrich Chemical Co. (Milwaukee, Wis.). $CCCP^-$ was obtained from Calbiochem (La Jolla, Calif.) and nonactin was obtained from E.R. Squibb (Princeton, N.J.). Pentane and squalene were obtained from Eastman Organic Chemicals (Rochester, N.Y.).

Results

Characteristics of Phloretin Analogs

The chemical structure of phloretin and the phloretin analogs is given in Table 1. In the Table, we have considered phloretin and its analogs as derivatives of acetophenone containing two kinds of substituents, one on the ring (X) and one on the carbon chain (Y) (Cousin & Motais, 1978). Thus, if the substituents in the ring are hydroxyls in posi-

Table 1. Chemical structure, dipole moment, and pK_a of phloretin analogs

Compound number[a]			Dipole moment (D)[b]	pK_a
	X	Y		
1	2,4,6-OH	$-CH_3$	5.5	7.28
2	2,4,6-OH	$-CH_2-CH_3$	5.5	7.45
3	2,4,6-OH		5.5	7.39
4	2,4,6-OH	$-CH_2-CH_2-$  $-OH$	5.6	7.35
5	2,6-OH	$-CH_3$	5.5	–
6	2,4-OH	$-CH_3$	3.7	7.48
7	2,4-OH	$-CH_2-CH_3$	3.7	7.47
8	2,4-OH		3.7	7.10
9	4-OH	$-CH_2-CH_2-CH_3$	3.8	7.76
10	4-OH	$-CH_2-(CH_2)_5-CH_3$	3.8	8.00
11	4-OH	 $-CH_3$	3.8	7.81
12	4-OH, $3CH_3$		–	7.99
13	2-OH	$-CH_2-(CH_2)-_3-CH_3$	2.9	–
14	2-OH	$-CH_2-CH_2-$ 	2.9	–
15	3-OCH_3	$-CH_3$	2.9	–
16	2-OCH_3	$-CH_3$	4.0	–

[a] Number of compound on the left side of the Table for identification purposes only. In the text, it appears as a number in parentheses after the name of the compound.
[b] Obtained from McClellan (1963) and Cousin and Motais (1978).

tions 2, 4 and 6 and the substituent in the carbon chain is a phenyl group, then the compound is designated 2,4,6-OH benzophenone (compound 3). Dipole moments and pK's for the different compounds are also shown in Table 1. Phloretin behaves as a weak acid with a pK = 7.3 (LeFevre & Marshall, 1959). This behavior is also followed by the phloretin analogs as shown in Table 1. The pK for the different compounds ranged from 7.1 to 8.0. For 2,6-OH acetophenone (5) and the 2-OH substituted compounds (13, 14), it was not possible to determine the pK as described in Materials and Methods, inasmuch as they did not undergo the characteristic shift in the ultraviolet spectrum induced by pH. At present, it is not clear why this is so, but it can be due to the absence of a resonant structure of the 4-OH substituted compounds.

Table 1 shows that the phloretin analogs can be divided into two main groups: those with a "high" dipole moment (5.5 to 5.6) and those with a "low" dipole moment (~2.9 to 3.8). In the former case, the substituents in the ring are hydroxyl groups in positions 2, 4 and 6 or in positions 2, 6. In the latter case, the substituents are hydroxyls in positions 2 and 4, a hydroxyl group in position 4, a hydroxyl group in position 2, and a methoxy group in positions 2 or 3.

Effect of Phloretin Analogs on Ion Transport through PE Bilayers

Figures 1 *a* and *b* show the effects of 2,4,6-OH propiophenone (2) and 2,4,6-OH benzophenone (3) on the conductance induced by the ions $TPhB^-$, $CCCP^-$, nonactin, and $TPhAs^+$ in PE membranes. For both compounds, we found that the conductance induced by positive ions (i.e., $TPhAs^+$ and nonactin-K^+) increased and that the conductance induced by negative ions (i.e., $TPhB^-$ and $CCCP^-$) decreased. On the average, 2,4,6-hydroxybenzophenone (3), at a concentration of 10^{-4} M in the aqueous phase, increased the cation conductance by about a factor of 35 and decreased the anion conductance by a factor of about 20. On the other hand, 2,4,6-OH propriophenone (2), at a concentration of 10^{-4} M, increased the cation conductance by a factor of about 80 and decreased the anion conductance by a factor of about 50. This behavior is found in most of the phloretin

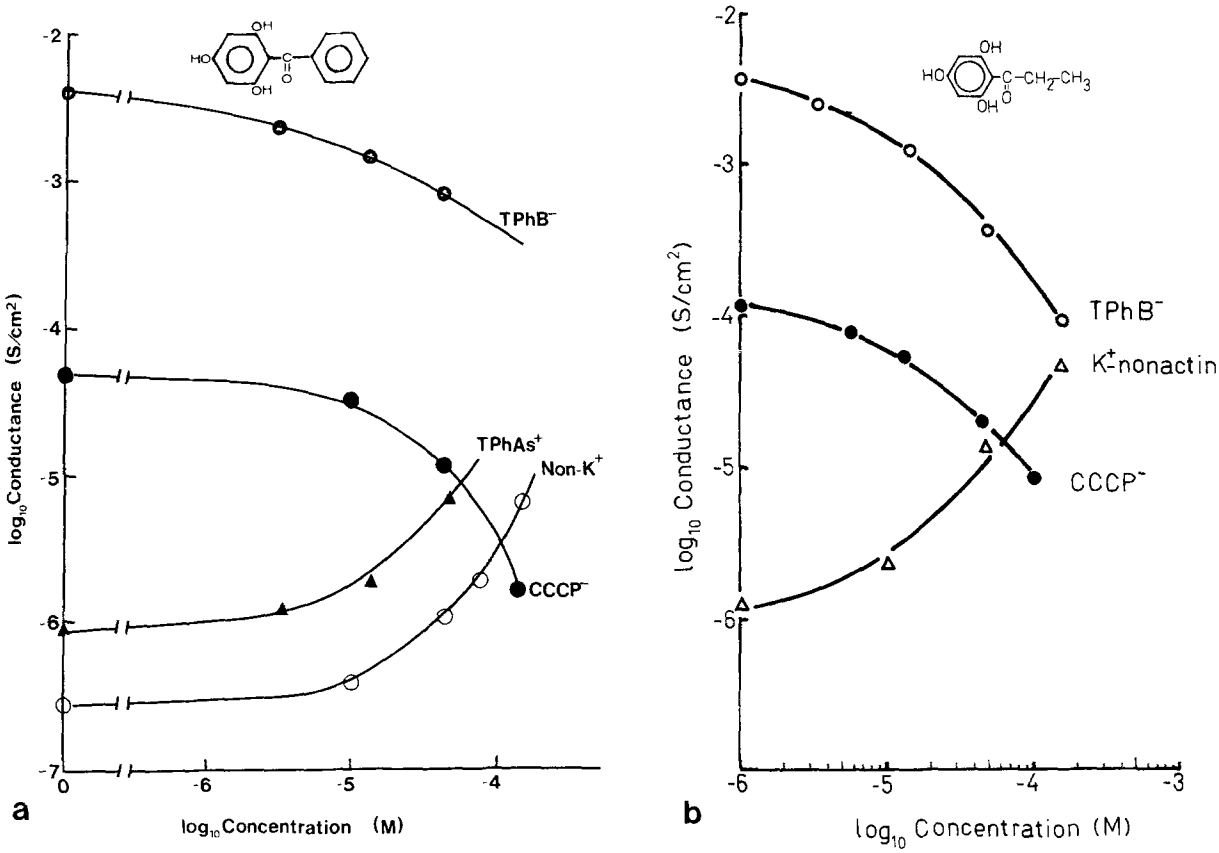


Fig. 1. *a.* Effect of 2,4,6-OH benzophenone (3) on the conductance induced by 6.6×10^{-8} M $TPhB^-$, 10^{-3} M $TPhAs^+$, 10^{-6} M $CCCP^-$, and 10^{-7} M Non-K^+ in PE membranes. The membranes were formed in 1 M NaCl, 20 mM phosphate, pH 5.5, or 1 M KCl, 20 mM phosphate, pH 5.5, when nonactin was used. The membranes were left for 45 to 60 min in the presence of the lipophilic ion or carrier with constant stirring before addition of the phloretin analog. Measurements were made when the changes in the membrane conductance reached a steady level (10 to 15 min) after addition of the compound. *b.* Effect of 2,4,6-OH propiophenone (2) on the conductance induced by $TPhB^-$, $CCCP^-$, and Non-K^+ in PE membranes. Other experimental conditions were similar to those of Fig. 1 *a*

analogs shown in Table 1[1]. However, the methoxy-substituted acetophenones (15, 16) produced practically no effect on the ion conductance up to a concentration of 10^{-4} M in the aqueous phases. As is the case with phloretin, the effect of phloretin analogs on the conductance induced by lipophilic ions and carriers can then be understood in terms of a decrease in the intrinsic positive internal membrane potential (Andersen et al., 1976; Melnik et al., 1977).

[1] If the effect of the different phloretin analogs tested is only to modify the intrinsic membrane dipole potential, then the decrease in anionic conductance must exactly match the increase in cationic conductance. However, we found slight asymmetries; e.g., the conductance of the cationic probe is affected more (Figs. 1 *a* and *b*) than that of the anionic probe, indicating that some other membrane parameter is affected by the phloretin analogs. Andersen et al. (1976) found that phloretin at a concentration of 2.5×10^{-4} M is able to increase threefold the permeability of acetamide. We found that phloretin and its analogs are able to increase electrical capacitance of PE membranes by as much as 30% when the concentration of these compounds in the aqueous phases is 10^{-4} M. An increase in fluidity and/or an increase in capacitance should increase the membrane permeability to both cations *and* anions. As discussed in the text, Szabo's (1974) analysis of the conductance data allows us in principle to calculate the contribution of the electrostatic potential term.

Magnitude of the Changes in Dipole Potential Promoted by Phloretin Analogs in Lipid Bilayers

Figures 1 *a* and *b* show that phloretin analogs produce opposite effects on anion and cation conductance regardless of the chemical structure of the ion probe. This makes unlikely specific interactions between these compounds and the current-carrying species, and also reveals a predominantly electrostatic effect. To further analyze the data of Figs. 1 *a* and *b*, we followed the approach of Szabo (1974; *see* footnote 1). Accordingly, the relative conductance $\bar{G}_1$, defined as the ratio between the conduc-

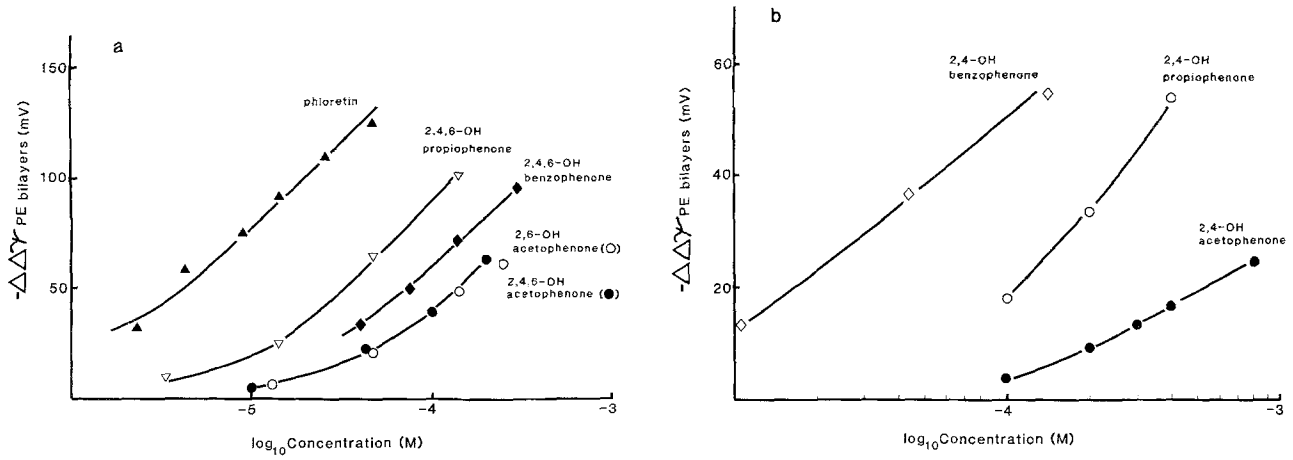


Fig. 2. *a* Calculated changes in the intrinsic dipole potential of PE bilayers induced by phloretin analogs with a dipole moment of 5.5 D. For details, *see* the text. *b*. Calculated changes in the intrinsic dipole potential of PE bilayers induced by the 2,4-OH substituted phloretin analogs ($\mu = 3.7$ D). For details, *see* the text

tance for a given current-carrying species i in the presence of the phloretin analog and the conductance for the same ion probe in the absence of the compound, is given by:

$$\bar{G}_i = \bar{\beta}_i \bar{\mu}_i \exp(-z_i F \Delta\psi_b / RT) \quad (1)$$

where $\bar{\beta}_i$ is the relative "chemical" partition coefficient, $\bar{\mu}_i$ is the relative mobility of the ion probe in the membrane, z_i is the valence of the ion, $\Delta\psi_b$ is the change in membrane dipole potential, and F, R and T have their usual meanings.

If we assume identical changes in mobility and in the partition coefficient for both cationic and anionic probes promoted by the addition of the phloretin analog, from Eq. (1), we have:

$$\left[\frac{\bar{G}_+}{\bar{G}_-}\right]^{-1/2} = \exp(F\Delta\psi_b/RT), \quad (2)$$

$$(\bar{G}_+ \times \bar{G}_-)^{1/2} = \bar{\beta}_i \bar{\mu}_i. \quad (3)$$

Equation (2) allows us to obtain the contribution to the overall conductance of the electrostatic potential term which affects, to the same magnitude but in opposite directions, the membrane conductance for negative and positive probes. Equation (3), on the other hand, allows us to obtain the contribution to those membrane structural parameters that affect the membrane conductance for positive and negative probes in the same magnitude and direction.

Figures 2*a* and *b* show the change in dipole potential of PE bilayers induced by various phloretin analogs calculated by using data such as that shown in Figs. 1*a* and *b* and Eq. (2). In Fig. 2*a*, we show the effect on the dipole potential of PE bilayers promoted by phloretin analogs with a dipole moment of 5.5 to 5.6 D and different hydrophobicities. Their ability to increase the intrinsic dipole potential follows the sequence 2,4,6-OH propiophenone (2) > 2,4,6-OH benzophenone (3) > 2,4,6-OH acetophenone (1) > 2,6-OH acetophenone (5). On the other hand, the 2,4-OH derivatives (~3,7 D) follow the sequence 2,4-OH benzophenone (8) > 2,4-OH propiophenone (7) > 2,4-OH acetophenone (6) (Fig. 2*b*). It is apparent from Fig. 2*b* that as long as the substituents in the ring are the same, increasing the hydrophobicity of the substituent in the carbon chain increases the effectiveness of the phloretin analog in decreasing the dipole potential of PE bilayers; *cf.* 2,4,6-OH propiophenone (2) and 2,4,6-OH acetophenone (1). However, we found that, if the substituent in the hydrocarbon chain is a phenyl, this rule is not followed. For example, 2,4,6-OH benzophenone (3) is *less* active than 2,4,6-OH propiophenone (2) despite the fact that a phenyl substituent is *more* hydrophobic than an ethyl substituent (Tanford, 1973). It is also of interest to note that if the *bulk* octanol-water partition coefficient is taken as an index of lipophilicity, then 2,6-OH acetophenone (5) is *more* hydrophobic than 2,4,6-OH acetophenone (1) (Cousin & Motais, 1978). Figure 2*a* shows, however, that 2,4,6-OH acetophenone (1) is equally "active" in lipid bilayers as 2,6-OH acetophenone (5).

Measurements of the Changes in Dipole Potential Induced by Phloretin Analogs in PE Monolayers

A direct method of estimating the dipole potential associated with lipids is to measure the changes

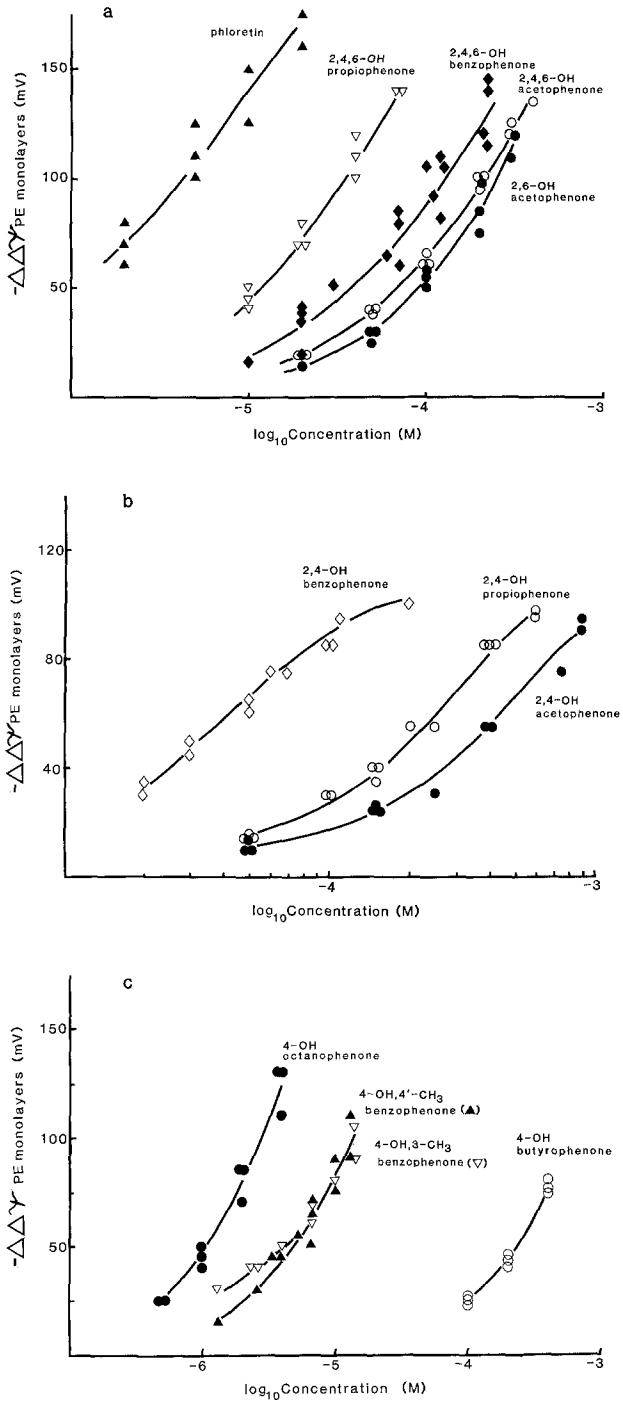


Fig. 3. *a.* Changes in the dipole potential of PE monolayers induced by phloretin analogs with a dipole moment of 5.5 D. The subphase was 1 M NaCl, 20 mM phosphate, pH 5.5. The phloretin analogs were added in concentrated ethanolic solutions to the monolayer-free compartment as described in Materials and Methods. *b.* Changes in the dipole potential of PE monolayers induced by phloretin analogs with a dipole moment of 3.7 D. Experimental conditions were the same as in Fig. 3*a*. *c.* Changes in dipole potential of PE monolayers induced by 4-OH substituted phloretin analogs ($\mu \sim 3.8$ D). Experimental conditions were the same as in Fig. 3*a*

in surface potential when a lipid monolayer is spread at the air/water interface. If PE is spread at the air/water interface, a change in surface potential of +460 mV is obtained (Reyes & Latorre, 1979) positive with respect to the aqueous subphase. Andersen et al. (1976) showed that, when phloretin (4) is added to the subphase of a PE monolayer to a final concentration of 10^{-4} M, surface potential decreases by 200 mV. We have confirmed this result.

Figures 3*a–c* show the effect of phloretin and phloretin analogs on the surface potential of monolayers made of PE. Inasmuch as PE monolayers are neutral at pH 5.5, we interpret this decrease in the surface potential of the lipid monolayer as a decrease in the dipole potential of the same. Figures 3*a–c* show that all of the phloretin analogs tested are able to decrease the dipole potential of PE monolayers to some extent. The data of Figs. 3*a* and *b* are in reasonable agreement with that obtained in bilayers. This correspondence will be addressed in more detail in the Discussion. It is worthwhile to mention here two points with respect to the 4-OH compounds. First, for these compounds, we found that the larger the hydrocarbon chain, the larger the dipole potential change the compound is able to induce when the phloretin analog concentration is far from saturation. Second, as mentioned above, compounds having a phenyl substituent behave differently from those having a linear hydrocarbon on the carbon chain (Fig. 3*c*). For example, the ability to reduce the dipole potential is the same for 4-OH,4′-CH_3 benzophenone (11) and 4-OH, 3-CH_3 benzophenone (12) in spite of the fact that the methyl group is in very different positions.

Effect of pH on the Mode of Action of Phloretin Analogs in PC Monolayers

It has been shown that only uncharged forms of phloretin are active in modifying the internal dipole potential of lipid bilayers (Andersen et al., 1976). From our lipid monolayer experiments (*see below*), it is apparent that this is also the case for the phloretin analogs tested here. Figure 4 shows that, when a PC monolayer is spread at the air/water interface, a change in surface potential of $+440 \pm 10$ mV is obtained.[2] This potential is not

[2] PC monolayers were used in these experiments because PE is an amphoteric lipid. At pH higher than 7, PE bilayers and monolayers have an appreciable negative surface charge (Bangham, 1968; McLaughlin et al., 1970). Phosphatidylcholine monolayers are even more sensitive to the action of phloretin than PE monolayers (*see* Discussion).

affected by a change in pH from 6 to 9.9. Moreover, at pH 9.9, 2,4-OH benzophenone (8), added to the aqueous subphase to a final concentration of 6.7×10^{-5} M, induces a change in surface potential of about 10 mV. Lowering the pH to 6.3 induces a further decrease in the surface potential of 55 mV. At pH 9.9, the concentration of undissociated 2,4-OH benzophenone is 1.1×10^{-7} M. 2,4-OH benzophenone, added to this final concentration to the subphase of a PC monolayer at pH 6.3, induces a change in surface potential of about 10 mV (*data not shown*). The same behavior found for 2,4-OH benzophenone was found for the other phloretin analogs tested. We conclude, therefore, that only the uncharged form of the phloretin analogs is able to change the dipole potential of monolayers. This is in agreement with the data that phloretin does not change the conductance of lipid bilayers to either cations or anions if the pH of the aqueous compartments is 10 (Andersen et al., 1976). These data are also in agreement with the evidence that only the uncharged form of phloretin is able to adsorb to the human red cell membrane (LeFevre & Marshall, 1959).

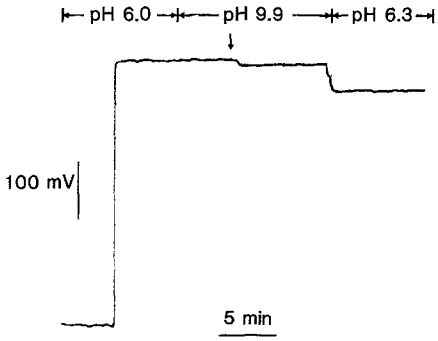


Fig. 4. Surface potential of a PC monolayer following changes in pH and additions of 2,4-OH benzophenone (8). The left portion of the record corresponds to the surface potential of unbuffered 10 mM NaCl, pH 6.0. Spreading a monolayer of PC causes a positive deflection of 455 mV. Phosphate buffer (final concentration 1.0mM, pH 9.9) has no effect on surface potential. Adding 2,4-OH benzophenone (arrow) to a final concentration of 6.7×10^{-5} M decreases the surface potential by 10 mV. Lowering the pH to 6.3 by adding 0.5 N HCl reduces the potential further by 45 mV. Since pH itself has no effect on surface potential in this range, this latter change must result from the re-equilibration (i.e., increased number) of neutral molecules of 2,4-OH benzophenone with the monolayer

Discussion

The experiments described in this paper were done with the purpose of finding the relationship between the dipole moment and hydrophobicity of phloretin analogs and their capacity to decrease the intrinsic dipole potential of lipid bilayers and monolayers. Furthermore, we decided to investigate this series of compounds in the hope of finding out whether the decrease in dipole potential promoted by them was related to their ability to inhibit the chloride transport in red blood cells (*cf.*, Cousin & Motais, 1978). Accordingly, we analyze below the adsorption parameters of phloretin and phloretin analogs obtained from the changes in the membrane dipole potential. Using the conclusions obtained from the analysis of our data, we discuss the current ideas for the mode of action of phloretin and its analogs on membrane transport systems.

Adsorption Parameters for Phloretin Analogs

The change in dipole potential of lipid bilayers and monolayers induced by phloretin and its analogs saturates with increasing concentration of the compound. This implies that the amount of adsorbed compound capable of altering the surface potential also reaches a maximum surface density. The simplest treatment of such an adsorption is the Langmuir adsorption isotherm (De Levie et al., 1979). In this treatment, the change in surface potential is given by an equation of the form:

$$\Delta\Delta\psi_D = \Delta\Delta\psi_{\max} \frac{[P]}{K_D^A + [P]} \tag{4}$$

where $\Delta\Delta\psi_{\max}$ is the maximum change in surface potential, $[P]$ is the phloretin or phloretin analog concentration in the aqueous phases, and K_D^A is the concentration at which half of $\Delta\Delta\psi_{\max}$ is obtained. Both $\Delta\Delta\psi_{\max}$ and K_D^A can be obtained by using Eadie's linear transformation of the data (Eadie, 1942). Figure 5 shows this type of plot for phloretin (4) and 2,4,6-OH propiophenone (2). Figure 5 also shows that the points lie on a straight line and, therefore, the data are consistent with the Langmuir adsorption isotherm given by Eq. (4).

Table 2 summarizes the adsorption parameters for phloretin and the analogs studied in this paper. Table 2 shows that the adsorption parameters obtained in monolayers and bilayers are in reasonable agreement. However, we found that monolayers tend to give higher values for $\Delta\Delta\psi_{\max}$.

Effects of Chemical Structure on $\Delta\Delta\psi_{\max}$. It can be shown from electrostatics that the potential

change, $\Delta\psi$ in crossing a sheet of aligned dipoles is given by:

$$\Delta\psi = \frac{4\pi n \mu \sin\theta}{\varepsilon} \quad (5)$$

where $\Delta\psi$ is the change in potential, n is the surface density of the dipoles, μ is the dipole moment of the molecules, ε is the effective dielectric constant, and θ is the angle between the direction of the dipole and the membrane/water interface.

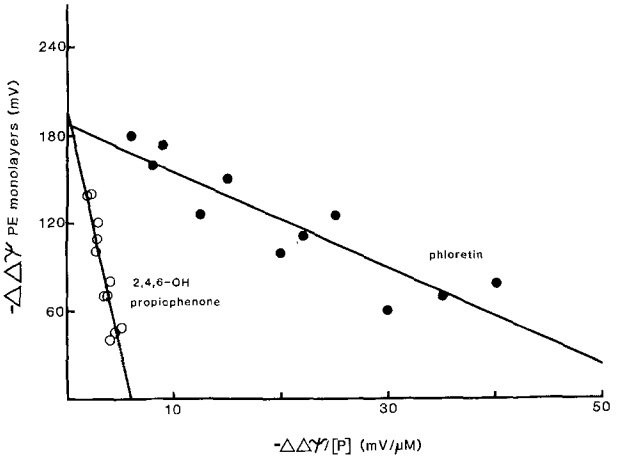


Fig. 5. Eadie's linear transformation of the data shown in Fig. 3*a* for phloretin (4) and 2,4,6-OH propiophenone (2). The continuous lines correspond to the regression parameters $-\Delta\Delta\psi_{max} = 188 \pm 12$ mV, $K_D^A = 3.3 \pm 0.5$ μM, $r = 0.90$ for phloretin, and $-\Delta\Delta\psi_{max} = 200 \pm 18$ mV, $K_D^A = 34 \pm 5$ μM, $4 = -0.91$ for 2,4,6-OH propriophenone. $\Delta\Delta\psi_{max}$ is the intercept of the regression line with the $\Delta\Delta\psi$ axis, K_D^A is the slope of the regression line

Equation (5) predicts that *if* the location, orientation, and maximum surface density of dipolar molecules are the same for two compounds of different dipole moment, the maximum $\Delta\Delta\psi_{max}$ the compound is able to induce would be larger for the molecule with the higher dipole moment.[3] However, Table 2 shows that some of the compounds with a dipole moment of 3.7 or 3.8 D show $\Delta\Delta\psi_{max}$'s equal to or larger than those compounds with a dipole moment of 5.5 or 5.6 D. This is more clearly seen when the compounds having an aliphatic substituent in the carbon chain are grouped and compared with respect to their $\Delta\Delta\psi_{max}$. For example, the 2,4,6-OH substituted compounds (5.5 D) induced about the same $\Delta\Delta\psi_{max}$ as the 4-OH substituted compounds (3.8 D). It is noteworthy that when compared to the 2,4,6-OH substituted compounds, 2-OH hexanophenone (13) induces a $\Delta\Delta\psi_{max}$ that is approximately the predicted value from its dipole moment (i.e., Eq. (5); *see* bottom of Table 2). However, the two 2-OH substituted compounds tested by us present a difference of about twofold in their $\Delta\Delta\psi_{max}$ in spite of the fact that they have the *same* dipole moment. We conclude, therefore, that $\Delta\Delta\psi_{max}$ is not a simple function of the dipole moment of the molecule. Hence, the location, orientation and/or maximum

[3] Both the orientation given by the angle and the location of the dipolar molecule in the bilayer are also determining the value of $\Delta\Delta\psi_{max}$. In principle, the value of the effective dielectric constant can range from 2 to 20, the former being the value for ε of the hydrocarbon region and the latter being the value for the polar group region (Coster & Smith, 1974).

Table 2. Adsorption parameters of phloretin analogs to PE bilayers and monolayers

Compound number	Compound name	Bilayers[a] $\Delta\Delta\psi_{max}$ (mV)	Monolayers $\Delta\Delta\psi_{max}$ (mV)	Bilayers K_D^A (μM)	Monolayers K_D^A (μM)
1	2,4,6-OH acetophenone	100±15	184± 9	174± 37	175 ± 14
2	2,4,6-OH propiophenone	124±18	200±18	45± 10	34 ± 5
3	2,4,6-OH benzophenone	131±18	144±18	117± 7	62 ± 14
4	phloretin	200±10	188±12	7± 2	3.3± 0.5
5	2,6-OH acetophenone	113±22	188±32	172± 51	243 ± 61
6	2,4-OH acetophenone	52±10	107±19	898±102	407 ±111
7	2,4-OH propriophenone	155±12	192±17	738±110	565 ± 65
8	2,4-OH benzophenone	72±11	134± 9	43± 10	57 ± 7
9	4-OH butyrophenone	–	217±32	–	770 ±146
10	4-OH octanophenone	–	213±32	–	3.4± 0.7
11	4-OH,4′CH_3 benzophenone	–	127±27	–	6.8± 2.7
12	4-OH,3-CH_3 benzophenone	–	110±13	–	4.0± 1.0
13	2-OH hexanophenone	–	84±19	–	5.9± 3.4
14	2-OH, β-phenylpropiophenone	–	47±10	–	2.5± 1.3

[a] For bilayers, the values of $\Delta\Delta\psi_{max}$ and K_A^D are the mean of pooled data of at least four different membranes. Values for monolayers are mean±SD of at least five different experiments

surface density of dipolar molecules in the membrane seems to be highly dependent on both the number and position of the hydroxyl groups in the ring. The type of substituent in the carbon chain (*cf.*, aliphatic and aromatic compounds) also appears to play an important role.

Effects of the Chemical Structure on K_D^A. Table 2 shows that the 2,4,6-OH compounds have larger apparent binding constants than the 2,4-OH compounds.[4] The exception to this "rule" is given by those phloretin analogs where the substituent in the carbon chain is a phenyl.[5] Thus, the differences in the ability of the "low" and "high" dipole groups of phloretin analogs to change the intrinsic dipole potential shown in Figs. 2*a* and *b* and Figs. 3*a–c* is not so much related to differences in dipole moment, but to differences in K_D^A's. It is apparent from Table 2 that the hydroxyls in positions 2 and 6 greatly influence the K_D^A's [*cf.*, 2,4,6-OH; 2,6-OH and 2,4-OH acetophenone(1,5,6)]. This is clearly demonstrated by the fact that 4-OH butyrophenone (9), a compound much more hydrophobic than 2,4,6-OH acetophenone (1), has a K_D^A fourfold larger than the latter compound.

Only when the substituent in the ring is kept constant do the K_D^A's follow a binding sequence expected from the lipid solubility of the aliphatic molecules. For example, from the relative K_D^A values of the 4-OH-substituted compounds, we calculated that each methylene group lowers the binding free energy by about 650 cal., in reasonable agreement with the hydrocarbon solubility (Tanford 1973). This is also followed by the 2,4,6-OH aliphatic-substituted compounds, but not by the 2,4-OH aliphatic-substituted compounds. The reason for this last finding is not clear.

Effects of Monolayer Composition on $\Delta\Delta\psi_{max}$ and K_D^A of Phloretin. The adsorption parameters of phloretin are also dependent on monolayer composition. For example, for dioleoylphosphatidylcholine monolayers, we found that $\Delta\Delta\psi_{max} = 162 \pm 6$ mV and $K_D^A = 0.47 \pm 0.04$ μM, whereas that for dioleoylphosphatidylcholine-cholesterol monolayers (molar ratio 1:1) was $\Delta\Delta\psi_{max} = 143 \pm 14$ mV and $K_D^A = 13.3 \pm 1.9$ μM. Table 2 shows that $\Delta\Delta\psi_{max}$ and K_D^A for PE are 188 ± 12 mV and 3.3 ± 0.5 μM, respectively.

Conclusions. (a) K_D^A depends primarily on the position and number of hydroxyl substituents on the ring. (b) If the substituent on the ring is the same and the substituent on the carbon chain is aliphatic, changes in K_D^A are in agreement with lipophilicity of the compound. (c) When the substituent on the carbon chain is a phenyl group, K_D^A appears to be less dependent on the number and position of the OH substituents on the ring. (d) K_D^A is dependent on the type of lipid used.

Phloretin Binding Parameters in Different Systems

Jennings and Solomon (1976) measured the binding of phloretin to both red blood cells and to sonicated extracts of lipid red cell ghosts. In the former, they found at least two saturable components with dissociation constants of 1.5 μM ("high affinity") and 54 μM ("low affinity"). In the sonicated lipids, they found a single set of binding sites with a $K_D^A = 44$ μM. More recently, Verkman and Solomon (1980) studied the binding of phloretin to unilamellar PC vesicles and found a $K_D^A = 8$ μM. We have found that phloretin binds 17-fold stronger to dioleoyl-PC monolayers than to PC vesicles and that the binding is influenced by lipid composition. Our results in monolayers show that phloretin binds 28-fold stronger to dioleoyl-PC monolayers than to dioleoyl-PC/cholesterol monolayers. Thus, if monolayers are taken as a good model of lipid bilayers, we can explain the differences between the binding of phloretin to PC and to red blood cell ghost lipids on the basis of the presence of cholesterol in the latter.

Mode of Action of Phloretin Analogs on Membranes and Model Systems

Our studies were prompted by those of Cousin and Motais (1978). They determined the concentration of phloretin and a variety of phloretin-like molecules able to halve the radioactive Cl^- efflux in red blood cells. Cousin and Motais (1978) concluded from their study that there is a significant correlation between inhibitory activity, dipole moment

[4] De Levie et al. (1979) found for phloretin in PE bilayers a $\Delta\Delta\psi_{max} = 220$ mV. From their data, a $K_D^A \sim 10$ μM can be calculated.

[5] In the case of 2,4,6-OH benzophenone (3) and 2,4-OH benzophenone (8) we found that K_D^A is essentially the same for both compounds. The anomalous behavior of these phloretin analogs may reside in their particular molecular structures with respect to the other analogs. In both compounds, the benzene ring containing the hydroxyls and the benzene ring having the role of the substituent in the carbon chain are at an angle of about 120° with respect to each other.

of the molecule, and lipid solubility. They further concluded that inhibition of anion transport is well correlated with the ability of a given compound to alter the intramembrane dipole potential (*see also* Andersen et al., 1976). We want to first comment on the relationship between the change in dipole potential and the potency of a given phloretin analog to inhibit Cl^- transport in red blood cells. The magnitude of the change in dipole potential in phosphatidylethanolamine bilayers or monolayers produced by the concentrations required to reduce facilitated anion transport to one-half its maximum value is, for all the compounds tested, ≤ 20 mV. In other words, virtually no phloretin, or phloretin analog, is bound to PE lipid bilayers (or monolayers) at concentrations that significantly inhibit Cl^- transport in beef red cells. Second, the lipid bilayer and monolayer studies show that phloretin analogs with different dipole moments are able to induce about the same $\Delta\Delta\psi_{max}$ in bilayers and monolayers, but that their binding constants can be widely different. It is clear, then, that although two analogs of phloretin – for example, 2,6-OH acetophenone and 2,4-OH acetophenone – may have the same substituent in the hydrocarbon chain, their concentration in the bilayer far from saturation can be very different. Cousin and Motais (1978) assumed that, since the two compounds have about the same *bulk* lipid solubility, the differences in their potency in inhibiting Cl^- transport were due to their differences in dipole moment. In conclusion, it is not clear at present whether the action of phloretin on the transport of Cl^- in red blood cells is due to a change in the boundary potentials or whether a specific binding site for phloretin (and the analogs) in the transport protein needs to be invoked (e.g., Jennings & Solomon, 1976). Considering the dependence of phloretin binding with lipid composition discussed above, one alternative explanation is that the lipids in the surroundings of the transport pathway are able to bind phloretin much stronger than the bulk bilayer lipids do.

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