Phloretin-Induced Changes in Ion Transport across Lipid Bilayer Membranes

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ABSTRACT Phloretin, the aglucone derivative of phlorizin, increases cation conductance and decreases anion conductance in lipid bilayer membranes. In this paper we present evidence that phloretin acts almost exclusively by altering the permeability of the membrane interior and not by modifying the partition of the permeant species between the membrane and the bulk aqueous phases. We base our conclusion on an analysis of the current responses to a square voltage pulse obtained for the lipophilic anion, tetraphenylborate, and the cation complex, peptide PV-K^+. These results are consistent with the hypothesis that phloretin decreases the intrinsic positive internal membrane potential but does not modify to a great extent the potential energy minima at the membrane interfaces. Phloretin increases the conductance for the nonactin-K^+ complex, but above 10^{-5} M the steady-state nonactin-K^+ voltage-current curve changes from superlinear to sublinear. These results imply that, above 10^{-5} M phloretin, the nonactin-K^+ transport across the membrane becomes interfacially limited.

INTRODUCTION

Phloretin is a well-known inhibitor of electrolyte and nonelectrolyte transport across biological membranes. In red blood cells, this molecule at micromolar concentrations strongly inhibits facilitated transport of hexoses (Lefevre, 1961; Czech et al., 1973) and chloride transport (Wieth et al., 1973). Phloretin also alters the nonelectrolyte permeability. It increases the permeabilities of lipophilic molecules and decreases those of hydrophilic solutes (Owen and Solomon, 1972).

Phloretin modifies the conductance of artificial lipid bilayer membranes. In particular it increases carrier-mediated cation conductances and decreases lipophilic anion conductances (Cass et al., 1973; Andersen et al., 1976a). According to Andersen et al., 1976a the action of phloretin can be accounted for by a reduction of the dipole potential of the membrane interior. This potential is apparently several hundred millivolts positive with respect to that of the aqueous phases. These observations and the mode of action of phloretin in biological membranes have led us to investigate further the mechanism by which this compound affects ion transport across membranes.

In bilayers of well-defined composition, the use of ion carriers and lipophilic ions as probes of membrane interfacial potentials and the barrier to transport inside the membrane has been very successful (McLaughlin et al., 1970; Hladky and Haydon, 1973; Szabo, 1974, 1976; Hall et al., 1973; Andersen and Fuchs, 1975). Interfacial potentials may arise from the surface charge or from oriented dipoles in the membrane or as a combination of both effects. The large changes in conductance for the nonactin-K^+ complex with varying surface charge density and ionic strength seem to agree well with those predicted by the Gouy-Chapman theory for an aqueous diffuse double layer (McLaughlin et al., 1970). On the other hand, the relative conductances of the nonactin-K^+ complex in glycerolmono-oleate and phosphatidylcholine membranes are accurately predicted by the difference in their surface potentials measured at the air-water interfaces (Haydon and Hladky, 1973). This surface potential may arise from oriented water dipoles and other oriented dipoles of the film-forming molecules (Davies and Rideal, 1963). Liberman and Topaly (1969) and subsequently Le Blanc (1970) and Andersen and Fuchs (1975) have shown that lipid bilayer membranes are much more permeable to lipophilic anions than to cations ($\sim 10^6$-fold). These results are consistent with high positive internal membrane potentials that arise as a consequence of oriented dipoles.

In regulating ion transport, membrane composition can alter the surface concentration of permeant species or the rate at which these species are being translocated (see Szabo, 1976). Essentially, the height of the membrane energy barrier will control the rate of translocation, and the depth of potential energy minima at the membrane interfaces is related to the partition coefficient of the permeant species between membrane and the aqueous phases. From conductance relaxation data, obtained by voltage pulsing, both translocation rates and partition coefficients for ion carriers can be calculated separately. It is then possible to assess the relative contribution of the two processes to the overall permeability of the permeant ions (Stark et al., 1971; Szabo, 1976).

Using the concepts and techniques described above we have studied the effect of phloretin on cation-carrier complexes (peptide PV and nonactin) and on a lipophilic anion (tetraphenylborate) in artificial lipid bilayer membranes. We have confirmed the results of Andersen et al. (1976a) that phloretin markedly decreases anion and increases cation conductance. The main objective of this paper is to assess how phloretin affects the translocation rates and the partition coefficient for the different permeant species tested.

MATERIALS AND METHODS

Membranes were formed in a Teflon chamber with an 18-μm thick Teflon partition according to the technique described by Montal and Mueller (1972). The surface area of each compartment was 4 cm^2 and the volume 3 ml. The membrane area was 6×10^{-4} cm^2. All membranes were formed from bacterial phosphatidylethanolamine (Supelco, Inc., Bellefonte, Pa.). The phospholipid was spread on the surface of the electrolyte solution using 10 μl of a solution containing 12.5 mg lipid per 1 ml pentane. Nonactin (E.R. Squibb & Son, New York), PV (Gisin and Merrifield, 1972), and tetraphenylborate sodium salt (TPhB$^-$) (kindly supplied to us by O. S. Andersen) were used as concentrated solutions in ethanol. Samples of these solutions were added directly to both compartments. Phloretin (ICN — K. & K. Labs., Inc., Cleveland, Ohio), unless otherwise stated,

was added to both compartments in ethanolic solutions of various concentrations. Control experiments showed that ethanol at the concentrations used (<1% vol/vol) had no effect on membrane conductance. PV, TPhB⁻, nonactin and phloretin were always added after the membrane was formed. The aqueous electrolyte solutions were symmetrical and consisted of unbuffered (pH ~6) 1 M KCl for the nonactin and PV experiments and 1 M NaCl for the TPhB⁻ experiments.

The system for measuring the electrical properties of the membrane is essentially that described by Alvarez et al. (1975). A pair of Ag/AgCl electrodes was used to measure membrane potential and another pair was used to provide and measure current. For the nonactin experiments steady-state current-voltage curves were recorded on an X-Y recorder at a voltage sweep rate low enough to introduce negligible hysteresis. Since both PV and TPhB⁻ transport are unstirred layer limited (Ting-Beall et al., 1974; Le Blanc, 1969), current measurements were made with a voltage pulse technique. In these cases current was recorded 0.05–10 ms after the voltage pulse was applied. For both compounds the current decayed with time after its initial response to a voltage pulse (Benz et al., 1976; Andersen and Fuchs, 1975). Initial values of current (or conductance) were determined from membrane current extrapolated to zero time on a logarithmic plot. The membrane capacitance for the phosphatidylethanolamine membranes was about 3.7×10^{-10} F (membrane area $\simeq 6 \times 10^{-4}$ cm^2). In our experimental conditions the total resistance in series with the membrane (electrode resistance, R_e, plus electrolyte solution resistance R_s) was $\leq 1,000 \, \Omega$. The charging time of the membrane, τ_c, given by:

$$\tau_c = C_m(R_s + R_e),$$

was therefore $\leq 3 \times 10^{-7} \, \mu$s. The time constant of the current-measuring amplifier was 7 μs, and this limited the effective time resolution of the system to about 70 μs.

RESULTS

Effect of Phloretin on Induced Conductance by Negative and Positive Ions.

Phloretin alone has no effect on membrane conductance even when added in the aqueous phases at a concentration of 10^{-4} M. Fig. 1 shows the effect of increasing phloretin concentration on the conductances induced by TPhB⁻, PV-K⁺ complex, and nonactin-K⁺ complex. The most striking features of these results are that the conductance for the nonactin-K⁺ complex increases 220-fold and the conductance for the PV-K⁺ complex, measured by voltage pulse at short times, increases 197-fold when the phloretin concentration is varied from 0 to 3×10^{-5} M, whereas the conductance for the lipophilic anion TPhB⁻ decreases by 222-fold for the same change in phloretin concentration. These results are in agreement with those of Andersen et al. 1976a and have been extended also to peptide PV. Above a phloretin concentration of 10^{-5} M the curve for the nonactin-K⁺ complex blends towards the concentration axis. At 10^{-5} M phloretin the current-voltage relationship is only slightly superliner and above 2×10^{-5} M phloretin the current-voltage curve becomes clearly sublinear (See Fig. 5 and Discussion).

Effect of Phloretin on the Initial Current and Time Constants of the Current Relaxation for TPhB⁻ and PV-K⁺ Complex

TETRAPHENYLBORATE For TPhB⁻ and PV-K⁺ complex the membrane current decays with time. The time courses of the TPhB⁻-induced current in both the presence and the absence of phloretin are shown in Fig. 2. The current as a

function of time in the absence of phloretin is shown by the closed triangles and the current in the presence of 3×10^{-6} M phloretin by the closed squares. This concentration of phloretin promotes a decrease of fourfold in the initial current (the current extrapolated at $t = 0$). On the other hand, the time constant for the current decay in the absence of phloretin is 7×10^{-4} s, and in the presence of $3 \times$
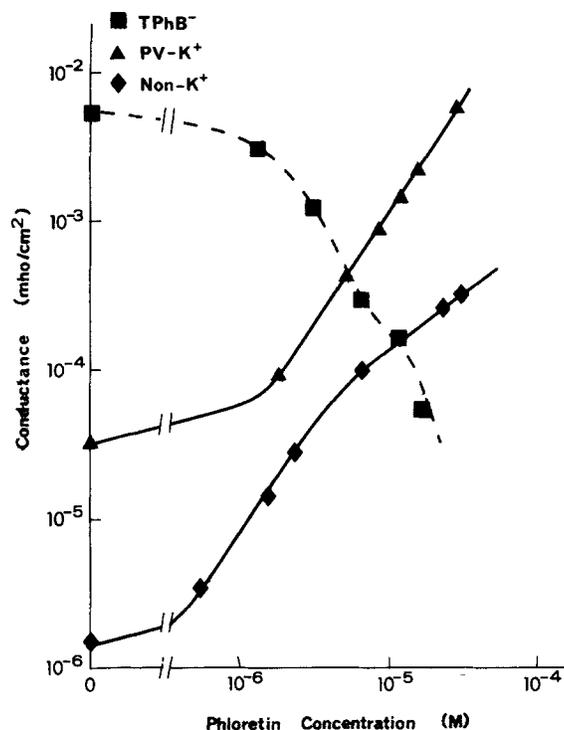

FIGURE 1. The effect of phloretin on the conductance induced by $TPhB^-$ (■), peptide PV (▲), and nonactin (◆) in PE membranes. In the $TPhB^-$ experiment the membrane was formed in unbuffered 1 M NaCl (pH ≈ 5.8), and $TPhB^-$ was added to both sides of the membrane to a concentration of 1.4×10^{-7} M. The initial membrane conductance before the addition of $TPhB^-$ was $<2 \times 10^{-8}$ mho/cm². Phloretin was added to both sides in the indicated increments. Nonactin and peptide-PV experiments were performed in a similar way, but in unbuffered 1 M KCl. Nonactin concentration: 3×10^{-8} M. Peptide-PV concentration: 8×10^{-6} M. The membrane area in all cases was 6×10^{-4} cm². Temperature, $22° \pm 1°C$.

10^{-6} M phloretin is 2.6×10^{-3} s. Thus the addition of 3×10^{-6} M phloretin in the aqueous phases promotes an increase of 3.7-fold in the time constant. For the two cases shown in Fig. 2, the straight lines connecting the experimental points have correlation coefficients greater than 0.99, indicating that the current decays exponentially with time. This observation is good evidence that the observed current relaxations are not due to diffusion polarization in the aqueous phases. If current magnitude were determined mainly by diffusion polarization, current would follow a time course roughly proportional to $1/\sqrt{t}$ (Haydon and Hladky, 1972; Läuger and Neumke, 1973). Furthermore, as discussed by Andersen and

Fuchs (1975), two stringent tests can be applied to determine if diffusion polarization is the limiting step in the current magnitudes. First, for short times, a plot of $I(0) - I(t)/I(0)$ vs. t should give a straight line with a slope of 0.5. Second, a plot of log $I(t)$ vs. log t should give a straight line with a slope of -0.5. If those
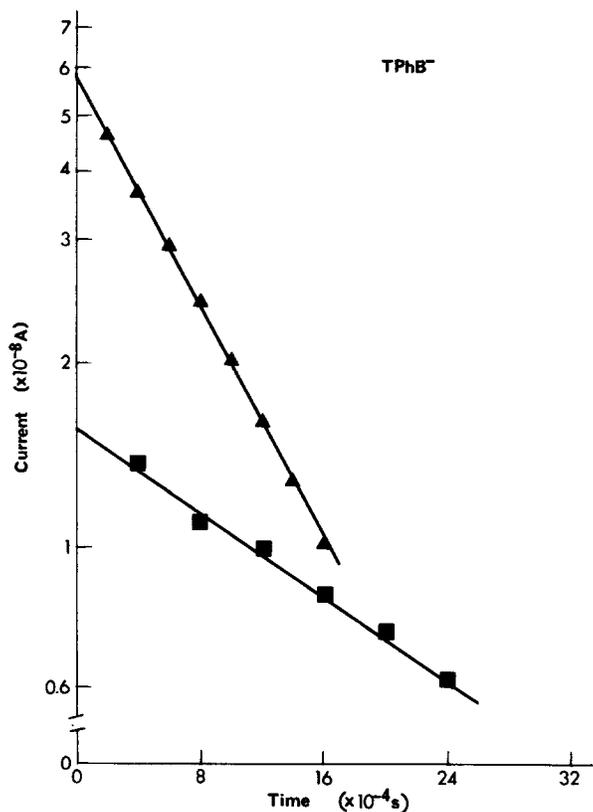

FIGURE 2. Time course of the TPhB$^-$ current in the absence ($\blacktriangle$) and in the presence of 3×10^{-6} M phloretin ($\blacksquare$). Potential 20 mV. The membrane was formed in unbuffered 1 M NaCl and TPhB$^-$ was added to both sides of the membrane to a concentration of 1.4×10^{-7} M. The time constants calculated from the regression lines (solid traces) were 7×10^{-4} s and 2.6×10^{-3} s in the absence and in the presence of phloretin, respectively. The regression lines computed from the data points have correlation coefficients >0.997. Membrane area 5×10^{-4} cm^2. Temperature, $22° \pm 1°$C.

tests are applied to our data for all the phloretin concentrations tested, they give results that are inconsistent with diffusion polarization as the limiting step determining the currents.

Fig. 3 A shows the effect of phloretin on TPhB$^-$-induced initial currents and time constants for a wide range of phloretin concentrations. In all cases both parameters were determined by applying a potential jump of 20 mV and observing the current relaxation with time. Fig. 3 A shows that increasing

phloretin concentration decreases the TPhB⁻-induced initial currents and increases the time at which the current has decreased by e-fold.

PV-K⁺ COMPLEX   As in the tetraphenylborate case, if a potential is suddenly applied to the membrane in the presence of PV, a large initial current is observed which declines to a much lower value with a time constant of 10 ms. The early decay is purely exponential and therefore the current is not limited by
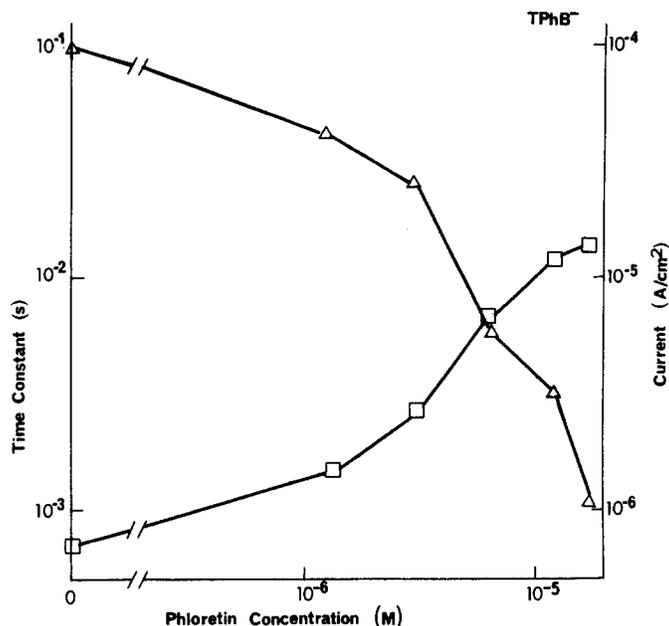

FIGURE 3 A.   The effect of phloretin on the TPhB⁻ initial currents (△, and right-hand scale) and on the time constants for the current decay (□, and left-hand scale). Initial currents and time constants were calculated from plots similar to those shown in Fig. 2. TPhB⁻ was added to both sides of the membrane to a concentration of 1.4×10^{-7} M. Unbuffered 1 M NaCl, temperature $21° \pm 1°C$.

diffusion polarization. Recently, evidence has been presented that PV acts almost exclusively by a solution-complexation mechanism which involves a complexation between ion and carrier in the aqueous phases and transport of the complex across the membrane (Benz et al., 1976). Fig. 3 B shows the effect of phloretin on the time constants for the current decay and on the initial currents induced by the PV-K⁺ complex. In contrast to TPhB⁻, the time constants decrease and the initial currents increase as phloretin concentration is increased.

In order to gain further insight about the mode of action of phloretin in lipid bilayer membranes, we will apply the theory derived from the generalized Nernst-Plank diffusion equation (Läuger and Neumcke, 1973; Hall et al., 1973; Hladky, 1974). We consider that the system is at equilibrium at any time before the voltage jump is applied, so that the concentration of current-carrying species (N) of either TPhB⁻ or PV-K⁺ is the same at both membrane interfaces.

Accordingly, the conductance at zero time [$G(0)$] is given by the equation (Hladky, 1974):

$$G(0) \equiv -\frac{J(0)}{\Delta V} = \frac{F^2 N P_m}{RT\delta} \alpha \, \sinh\left(\frac{u}{2}\right)\Big/ \sinh\left(\frac{\alpha u}{2}\right). \tag{1}$$
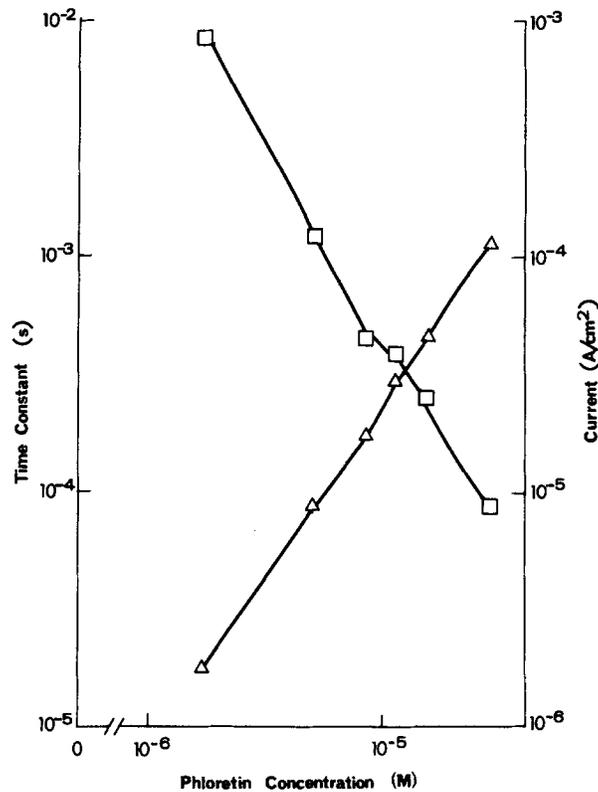

Phloretin Concentration (M)

FIGURE 3 B. The effect of phloretin on the PV-K$^+$ complex initial currents ($\triangle$, and right-hand scale) and on the time constants for the current decay ($\square$, and left-hand scale). Both parameters were calculated with the same procedures as that followed for TPhB$^-$. Peptide-PV was added to both sides of the membrane to a concentration of 8.3×10^{-6} M. Applied potential 20 mV. Unbuffered 1 M KCl. Temperature, 22° ± 1°C.

where $J(0)$ is the current at zero time; ΔV the applied potential; P_m is the permeability through the interior of the membrane for the current-carrying species; α is the width of the flat top of the barrier in relative units in the trapezoidal approximation (Hladky, 1974); u is the reduced potential ($u = F\Delta V/RT$); δ is the thickness of the absorption layers; and F, R, and T have their usual meanings.

If $|u| \ll 1$ Eq. (1) is reduced to:

$$G(0) = \frac{F^2 N P_m}{RT\delta}, \tag{2}$$

Eq. (2) can be expressed in terms of the concentration in the aqueous solutions of the permeant species through the partition coefficient (γ) between the membrane and the bulk aqueous phases (Läuger and Stark, 1970).

$$\gamma = \frac{2N}{\delta C_a},$$ (3)

where C_a is the concentration of current-carrying species in the aqueous solutions. Combining Eq. (2) and (3) we obtain:

$$G(0) = \frac{F^2 P_m \gamma}{2RT} (C_a).$$ (4)

Early in this paper we discussed the exponential current decay after a sudden change in potential. If the changes in concentrations of the current-carrying species in the unstirred layers are neglected, we can express the variation of current density $J(t)$ with time by the equation:

$$J(t) = J(\infty) + [J(0) - J(\infty)]e^{-t/\tau}.$$ (5)

If the ions are perfectly trapped in the membrane, Eq. (5) becomes:

$$J(t) = J(0)e^{-t/\tau}.$$ (6)

This is a good approximation since $J(0) \gg J(\infty)$ in all cases. It is noteworthy that, at least for short times, Eq. (5) applies for both TPhB$^-$ and the PV-K$^+$ complex. For the PV-K$^+$ complex this is due to the fact that the rate of dissociation of the complex is several orders of magnitude slower than is the case for valinomycin (Davis and Tosteson, 1975; Davis et al., 1976). Even for the K$^+$ complexes of nonactin and valinomycin, Eq. (6) applies over a very short time (Stark et al., 1971; Benz and Stark, 1975). If one assumes that the rate of desorption for the ions is much smaller than the translocation rate, τ can be expressed as (Ketterer et al., 1971):

$$\frac{1}{\tau} = \frac{2P_m}{\delta} \cosh(u/2).$$ (7)

For low potentials ($u \ll 1$) Eq. (7) becomes:

$$\frac{1}{\tau} = \frac{2P_m}{\delta}.$$ (8)

Combining Eq. (4) and (8) we obtain:

$$(\gamma\delta) = \frac{4RT\tau G(0)}{F^2(C_a)}.$$ (9)

Thus equations (4), (8), and (10) allow us to calculate in a separate way the rates of translocation (P_m/δ) across the membrane interior and the absorption coefficients $\gamma\delta$.

For TPhB$^-$ the amount of charge translocated reaches a limiting value at high potentials (Andersen and Fuchs, 1975). This can be interpreted as a complete depletion of TPhB$^-$ from one of the membrane interfaces and gives us an

independent method of calculation of $\gamma\delta$ from that shown in Eq. 9. In this case $\gamma\delta$ is simply given by the expression:

$$(\gamma\delta) = \frac{2Q_a}{FC_a},\tag{10}$$

where Q_a is the maximum charge translocated per unit area, and is obtained by the relation $Q_a = J(0)\tau$.

As shown in Fig. 4, the total charge adsorbed onto one membrane boundary is a linear function of the TPhB$^-$ concentration in the aqueous solution. If the concentration of TPhB$^-$ is increased further than 4×10^{-7} M, the decay of the
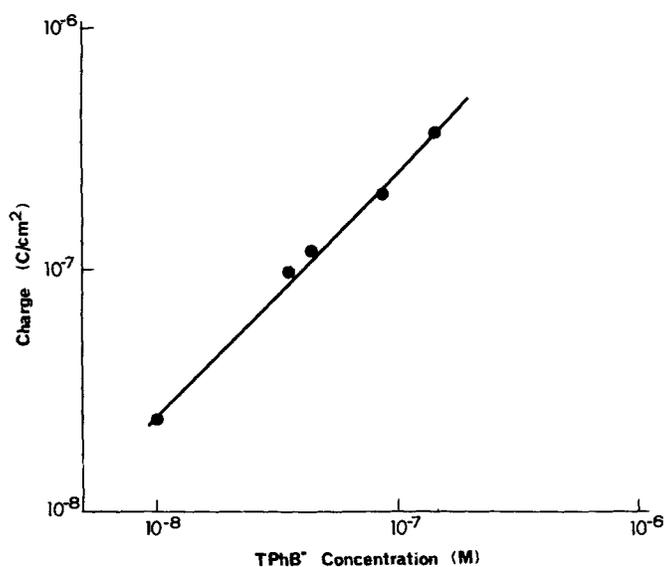

Figure 4. The effect of TPhB$^-$ concentration on the total charge moved through the membrane interior. The amount of absorbed charge was obtained from plots of charge transfer vs. applied potential at the TPhB$^-$ concentrations indicated in the figure. Unbuffered 1 M NaCl. Temperature, 22° ± 1°C.

current is no longer a single exponential, indicating that some process other than translocation of charge within the membrane, e.g., diffusion polarization, becomes important at concentrations of TPhB$^-$ higher than 4×10^{-7} M (see also Andersen and Fuchs, 1975). In order to calculate the total charge adsorbed, the concentration of TPhB$^-$ used was always less than 2×10^{-7} M.

Table I shows the effect of phloretin on the various steps of TPhB$^-$ permeation through the bilayer. Increasing phloretin concentration markedly decreases the initial conductances (see also Fig. 1), and the translocation rates (P_m/δ) derived by using Eq. (8). On the other hand, phloretin does not greatly alter partition between membrane and solution. We want to stress the point that $\gamma\delta$ was calculated by two independent methods, namely from the values for τ and $G(0)$ obtained for low potentials (Eq. 9) and from the measured amount of charge translocated (Eq. 10).

Table II shows the effect of phloretin on the various steps of permeation

through the bilayer for the PV-K complex. Unlike those of TPhB⁻, the initial conductance and the translocation rates increase with increasing phloretin concentration but, as for that of TPhB⁻, the partition coefficient remains nearly constant with varying phloretin concentration.

Effect of Phloretin on the Shape of the Current-Voltage Relationship in Membranes Treated with Nonactin

As we show in Fig. 1, the zero-potential membrane conductance for the nonactin-K⁺ complex tends to become constant above 10^{-5} M phloretin. To study

TABLE I

EFFECT OF PHLORETIN ON TPhB⁻ TRANSPORT PARAMETERS

[Phloretin] × 10^6	$G(0)$ × 10^3	$\tau(0)$ × 10^3	$(\gamma\delta)$ × 10^2 (from Eq. 9)	$(\gamma\delta)$ × 10^2 (from Eq. 10)	P_m/δ
M	mho/cm²	s	cm	cm	s⁻¹
0	5.3	0.7	2.80	2.7	720
1.3	2.6	1.5	2.90	2.4	340
3.0	1.3	2.6	2.51	1.7	190
6.4	0.3	6.9	1.54	1.3	80
12.0	0.16	12.0	1.43	1.0	40
17.0	0.05	15.0	0.54	—	25

Tetraphenylborate was added to both sides of the membrane to a concentration of 1.4×10^{-7} M; 1 M NaCl, pH 5.8. For an explanation of how different parameters were calculated see text. Temperature, $22° \pm 1°$C. $\tau(0)$ and $G(0)$ calculated from experiments like that shown in Fig. 2 for 20 mV applied potential.

TABLE II

EFFECT OF PHLORETIN ON PV-K⁺ COMPLEX TRANSPORT PARAMETERS

[Phloretin] × 10^6	$G(0)$ × 10^6	$\tau(0)$ × 10^3	$(\gamma\delta)$ × 10^4 (from Eq. 9)	P_m/δ
M	mho/cm²	s	cm	s⁻¹
0	2.7	18.0	6.30	30
1.7	8.9	8.50	9.85	59
5.0	44.0	1.50	8.60	420
8.3	86.0	0.44	4.91	1,100
11.0	150.0	0.38	7.41	1,280
15.0	235.0	0.24	7.33	1,970
28.0	577.0	0.095	7.12	5,810

PV was added to both sides of the membrane to a concentration of 8×10^{-6} M. 1 M KCl, pH 5.8. Temperature, 22°C. Parameters as described in the text.

further this phenomenon we examined the shape of the current-voltage relationship over the entire range of potential. Fig. 5 shows that increasing the concentration of phloretin in the aqueous solution changes the I-V relationship from superlinear (solid line) to sublinear (broken line). This change indicates that the movement of the nonactin-K⁺ complex through the membrane has become interfacially limited (see Discussion). The phloretin concentration at which the curve becomes sublinear is the same for nonactin concentrations between 10^{-9} M and 10^{-7} M. (See also Discussion).

DISCUSSION

Studies of un-ionized lipid monolayers spread at the air-water interface indicate that an electrical dipole potential exists at the interface with the air phase positive

relative to the aqueous subphase. This potential can be as high as 500 mV for bacterial phosphatidylethanolamine (Hladky, 1974). Since lipid bilayer membranes can be viewed as two monolayers with the nonpolar tails directed away from the aqueous phases, the existence of an electrical potential similar in nature to that found at the air-water interface in monolayers is to be expected. In neutral bilayer membranes this potential would define the electrostatic potential difference between the membrane interior and the bulk aqueous phases. In recent years, the influence of the dipole potential on ion transport across membranes
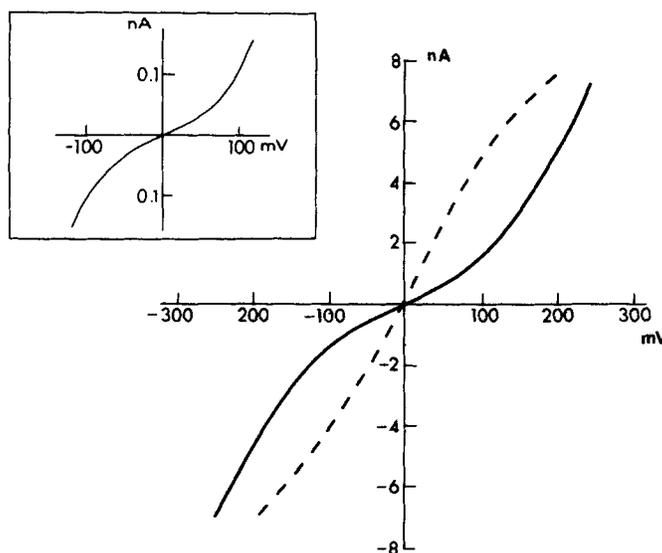

FIGURE 5. Current-voltage relationships for a PE membrane doped with nonactin at different phloretin concentrations. The membrane was formed in 1 M KCl and had an initial conductance of 10^{-8} mho/cm^2. Nonactin was added to both sides of the membrane to a concentration of 3×10^{-8} M and the zero voltage conductance changed to 2.6×10^{-6} mho/cm^2 (see *inset*). Phloretin was added to both sides of the membrane in increasing amounts. In this figure the solid line is the current-voltage relationship taken at a phloretin concentration equal to 6×10^{-6} M. The broken line is the current-voltage relationship taken at a phloretin concentration equal to 2.3×10^{-5} M.

has received much attention (Liberman and Topaly, 1969; Le Blanc, 1970; Haydon and Hladky, 1972; Hladky and Haydon, 1973; Szabo, 1974, 1976). In all cases this potential has been inferred through differential conductance measurements using either carrier-ion complexes or lipophilic ions.

As pointed out by Andersen and Fuchs (1975) the dipole potential is in principle not measurable but can be estimated by using extrathermodynamic assumptions (see also LeBlanc, 1970). In bacterial PE such estimation has been made by comparing the conductances of TPhB$^-$ and tetraphenylarsonium which is positively charged (Andersen and Fuchs, 1975). The reported value for the dipole potential, 310 mV, is much lower than the values of about 500 mV estimated from surface potential measurements made of monolayer of PE (Hladky, 1974). Although no quantitative agreement is reached with respect to

the magnitude of the intrinsic dipole potential, both kinds of measurements indicate that this must be positive and of more than 300 mV.

In this paper we have corroborated the results of Cass et al. (1973) and those of Andersen et al. (1976a) that phloretin modifies ion conductance in lipid bilayer membranes by decreasing anion conductance and increasing cation conductance. These results are in agreement with the hypothesis that phloretin decreases the positive dipole potential of the membrane.[1]

Several possible changes in intramembrane energy could explain the effects of phloretin shown in Fig. 1. The results presented in Tables I and II narrow the possibilities to only a few. Clearly the membrane energy profiles for nonactin, PV, and TPhB⁻ will all be different, but the present results, and those of Andersen et al. (1976a) have shown that phloretin acts on each of the current-carrying species mentioned above mainly electrostatically. One would expect therefore that the alteration of each barrier due to the presence of phloretin will be the same. By combining the data obtained for the three different probes, we can thus draw several conclusions about the additional term in the barrier induced by phloretin. First, phloretin alters only threefold the absorption coefficient for TPhB⁻ and does not affect the absorption coefficient for PV; for both compounds phloretin mainly affects the translocation rates. These observations indicate that phloretin must give rise to a dipole potential lying entirely within the membrane. This would indicate also that PV is adsorbed in the membrane totally outside of the dipole potential transition region, and that TPhB⁻ "sees" about 27 mV of the change in dipole potential induced by phloretin. Second, because the addition of phloretin does not cause the nonactin voltage curve to become steeper, the dipole cannot extend further into the membrane than the corner of the nonactin barrier. From experiments like that presented in Fig. 5, we have calculated that this corner lies at a distance of 0.32 times the membrane thickness down the electrical field (see also Hall and Latorre, 1976; Latorre and Hall, 1976). We can thus place unambiguous limits on the maximum extent of the phloretin dipole both toward the aqueous phase and toward the center of the membrane.

Obviously, the above analysis contains the assumption that the current-carrying species is located at the membrane boundaries. There are good reasons to believe that this is so. First, as discussed by Ketterer et al. (1971), the potential energy profile of a hydrophobic ion in the membrane may be represented as the sum position-dependent electrostatic term and a term which accounts for the hydrophobic interactions. When these two energy terms are added, and because they have opposite signs, the resulting potential profile shows two deep minima at the interfaces. Second, for TPhB⁻ Andersen and Fuchs (1975) have estimated

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[1] Cass et al. (1973) and Poznansky et al. (1975) have shown that phloretin also increases the membrane permeability to uncharged solutes. This effect has been interpreted as a change in "fluidity" of the membrane. We do not think that such a change in fluidity plays an important role in the present experiments. First, the effect of phloretin on the translocation rates is differential (P_m/δ increases for positive ions and decreases for negative ions). Furthermore, at a given phloretin concentration the increase in conductance for PV-K⁺ *equals* the decrease in P_m/δ for TPhB⁻. Second, the amount of phloretin needed to double the membrane permeability to acetamide is fourfold higher than the maximum phloretin concentration used in the experiments reported here.

that, for solvent-containing phophatidylethanolamine bilayers, the effective potential is 77% of the applied potential. Furthermore, by using the same treatment as Andersen and Fuchs, we have calculated that TPhB⁻ is sensitive to 90% of the applied potential in membranes formed in 1 M NaCl by the method of Montal and Mueller (1972).[2] As pointed out by Andersen and Fuchs (1975) the effective potential does not give an unequivocal position for the energy minima within the membrane. It is easy to explain, however, the fact that TPhB⁻ is sensitive to 90% of the applied potential by putting the energy minima at less than 0.1 times the membrane thickness towards the interior of the membrane.[3]

On the basis of our results we can speculate on the location in the membrane of the current-carrying species. The fact that $\gamma\delta$ for PV does not change with phloretin concentration and changes only threefold for TPhB⁻ indicates that the adsorption plane for these molecules must be different from that of the phloretin. A model in which the phloretin dipole lies closer to the membrane interior than do PV and TPhB⁻ would explain our experimental findings.

The relative positions of phloretin and TPhB⁻ are different in the present experiments from those reported by Andersen et al. (1976a). Andersen et al. (1976a) found that for TPhB⁻, phloretin affects both the interior permeability and the partition coefficient. These results may differ because of differences in lipid composition and/or differences in the membrane formation techniques. We used PE membranes and the Montal and Mueller (1972) technique, whereas Andersen et al. (1976a, b) used lecithin-cholesterol membranes and the Mueller and Rudin technique (Mueller et al., 1963).

We have shown in Results that above a concentration of 2.3×10^{-5} M phloretin, the current-voltage characteristics for the nonactin-K⁺ complex change from a superlinear to a sublinear shape. According to Läuger (1973) such a change would be expected if phloretin above a concentration of 2.3×10^{-5} M changed the membrane barrier profile from one in which the peak of the barrier in the center of the membrane is rate limiting to one in which the interfacial barrier becomes rate limiting.

Haydon and Myers (1973) have shown that if dipole potentials must be considered, the specific conductances $[G(0)]$ in the limit of zero applied potential of two membranes of different composition are related by the expression:

$$\Delta\phi = \frac{-RT}{F} \ln \frac{G_2(0)}{G_1(0)},$$

where $\Delta\phi$ is the surface potential difference.

The calculated dipole potential difference $(\Delta\phi)$ between the bilayer in the absence of phloretin and one treated with 2.3×10^{-5} M phloretin is approximately

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[2] It appears that the difference in magnitude between the effective potential calculated by us, and that of Andersen and Fuchs (1975) resides in the different ionic strength used in both cases. Thus, in PE membranes formed by the technique of Montal and Mueller (1972) but in 0.1 M NaCl the effective potential is about 78% of the applied potential (Donovan and Latorre, unpublished results).
[3] Recently Andersen et al. (1976b) have presented evidence that the apparent saturation in the absorption of TPhB⁻ into bilayers is due to electrostatic boundary potentials produced by the absorbed ions. If these potentials are considered in the effective potential analysis they would put the energy minima nearer the aqueous phases, thus strengthening our argument.

150 mV, and therefore the energy difference between the barrier height of the membrane relative to the aqueous phases is about 6 kT.

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