

# Effect of Phloretin on the Permeability of Thin Lipid Membranes

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**ABSTRACT** Phloretin dramatically *increases* cation conductances and *decreases* anion conductances of membranes treated with ion carriers (nonactin, valinomycin, carbonyl-cyanide-*m*-chlorophenylhydrazone [CCCP], and  $\text{Hg}(\text{C}_6\text{F}_5)_2$ ) or lipophilic ions (tetraphenylarsonium [ $\text{TPhAs}^+$ ] and tetraphenylborate [ $\text{TPhB}^-$ ]). For example, on phosphatidylethanolamine membranes,  $10^{-4}$  M phloretin increases  $\text{K}^+$ -nonactin and  $\text{TPhAs}^+$  conductances and decreases CCCP $^-$  and  $\text{TPhB}^-$  conductances  $10^3$ -fold; on lecithin:cholesterol membranes, it increases  $\text{K}^+$ -nonactin conductance  $10^5$ -fold and decreases CCCP $^-$  conductance  $10^3$ -fold. Similar effects are obtained with *p*- and *m*-nitrophenol at  $10^{-2}$  M. These effects are produced by the *un-ionized* form of phloretin and the nitrophenols. We believe that phloretin, which possesses a large dipole moment, adsorbs and orients at the membrane surface to introduce a dipole potential of opposite polarity to the preexisting positive one, thus *increasing* the partition coefficient of cations into the membrane interior and *decreasing* the partition coefficient of anions. (Phloretin may also increase the fluidity of cholesterol-containing membranes; this is manifested by its two- to three-fold increase in nonelectrolyte permeability and its asymmetrical effect on cation and anion conductances in cholesterol-containing membranes.) It is possible that phloretin's inhibition of chloride, urea, and glucose transport in biological membranes results from the effects of these intense interfacial dipole fields on the translocator(s) of these molecules.

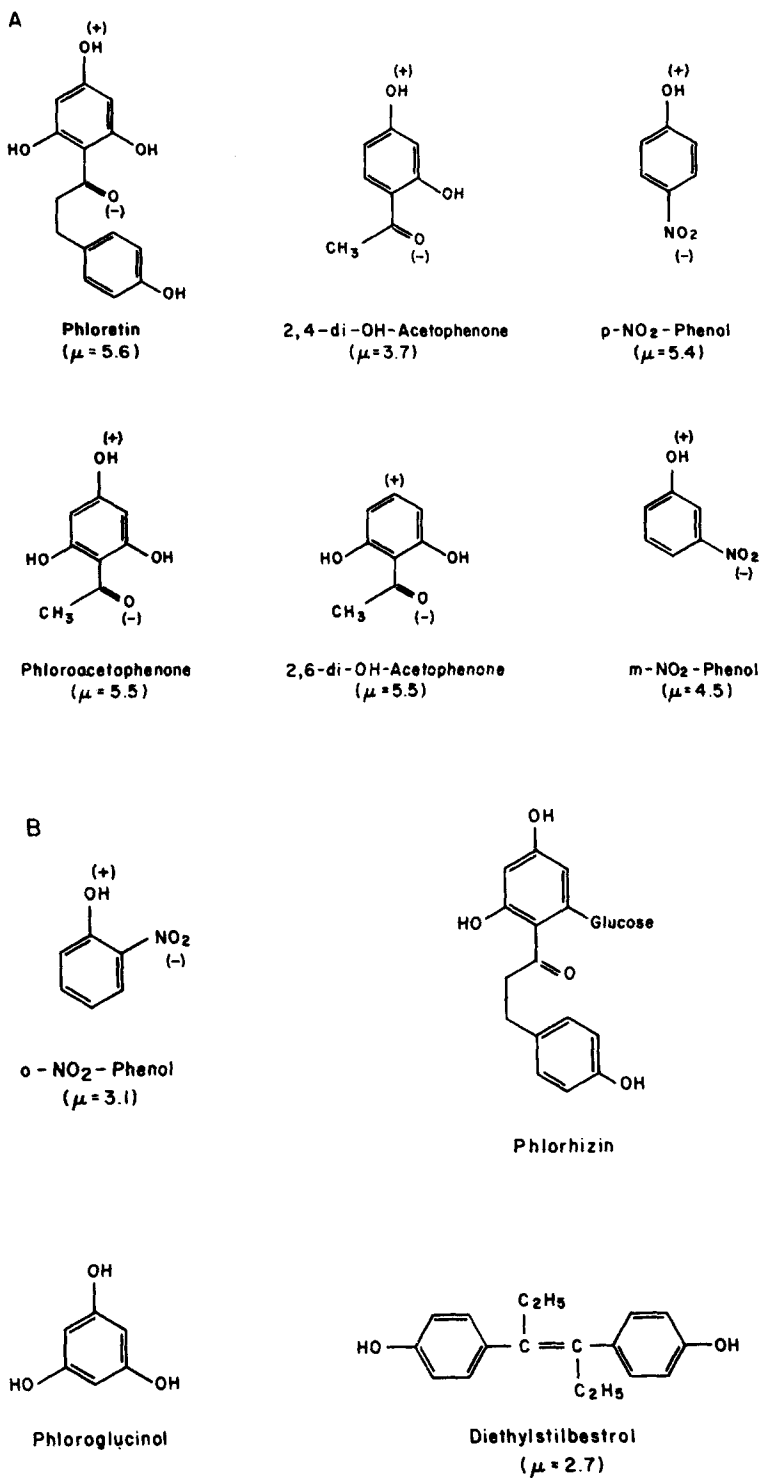
## INTRODUCTION

### *Interest in Phloretin*

Phloretin, the aglycone of phlorhizin, is the classical reversible inhibitor of the hexose transport system in the human red blood cell membrane (LeFevre, 1961); it also slows the movement of glycerol and urea (Macey and Farmer, 1970) and powerfully inhibits chloride movement (Wieth et al., 1973; Gunn et al., 1975). In addition, phloretin affects nonelectrolyte and ion transport across several other biological membranes (e.g. Kotyk et al., 1965; Batt and Schacter, 1971; Levine et al., 1973; Owen, 1974).

Since all biological membranes contain extensive regions of lipid bilayer, we decided to investigate the action of phloretin on bimolecular lipid membranes,

† Deceased



in the hope of finding a simple, physicochemical mechanism responsible for its effects on a diversity of membranes and transport systems. We report here that phloretin and certain other dipolar organic molecules (See Fig. 1) *increase* carrier-mediated and lipophilic cation conductances and *decrease* carrier-mediated and lipophilic anion conductances of lipid bilayers. (A preliminary note of these findings appeared earlier [Cass et al., 1973].) The mechanism of action of these dipolar molecules on lipid bilayers may have physiological significance.

#### *Factors Determining the Conductance of Lipid Bilayers*

The permeability of an ion is dependent on the product of its *concentration* and *mobility* in the membrane phase. Changes in either of these will change the membrane conductance. We focus here mainly on factors that change ion concentration in the membrane.

To a first approximation the conductance of an unmodified lipid bilayer is that of a thin layer of liquid hydrocarbon. It is essentially impermeable to small ions (e.g.  $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Cl}^-$ ), because of the enormous electrostatic energy required to transfer them from an aqueous solution into the low dielectric constant interior of the membrane. On the other hand, larger ions possessing many nonpolar groups (e.g. tetraphenylarsonium [ $\text{TPhAs}^+$ ], tetraphenylborate [ $\text{TPhB}^-$ ], carbonylcyanide-*m*-chlorophenylhydrazone [ $\text{CCCP}^-$ ],<sup>1</sup> and the potassium complexes of nonactin [ $\text{K}^+$ -nonactin] and valinomycin [ $\text{K}^+$ -valinomycin]) are much more lipid soluble and can therefore impart large conductances to the membrane (Lieberman and Topaly, 1969; Le Blanc, 1971; Haydon and Hladky, 1972; Lauger, 1972).

At least two features of the membrane, other than its hydrocarbon interior, determine the permeability of these lipid soluble ions. If the membrane has a surface charge, the concentration of ions at the membrane-solution interface is altered by the resulting surface potential (McLaughlin et al., 1970). For example, if the surface charge is negative, the concentration of cations at the surface will be greater than at remote regions of the aqueous phases, whereas the concentration of anions will be lower. Consequently, cation conductance is increased and anion conductance decreased as a function of the negative surface potential.

<sup>1</sup> CCCP, an uncoupler of oxidative phosphorylation, acts as a hydrogen ion carrier through lipid bilayers. The charged form ( $\text{CCCP}^-$ ), however, is the current-carrying species in the membrane (Le Blanc, 1971).

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FIGURE 1. (*Opposite*) Chemical structure of molecules investigated. The dipole moments,  $\mu$ , (measured in dioxane) of the nitrophenols are from McClellan (1963); all others are from Dr. K. Matsuo of the Department of Chemistry, Dartmouth College. (These are actually root-mean-square dipole moments; they are the weighted average of the *squares* of the dipole moments of all possible confirmations. This is why  $\mu$  for diethylstilbestrol, a symmetrical molecule, is not zero.) We have indicated where we believe the formal plus (+) and minus (-) ends of the dipoles are located. (A) Molecules that are active in increasing cation conductances and decreasing anion conductances of lipid bilayers. (B) Molecules that are inactive, or only slightly active, in increasing cation conductances and decreasing anion conductances of lipid bilayers.

A second feature, on which we focus in this paper, is that oriented dipoles at the membrane-solution interface impart to the membrane interior a high, positive, electrostatic potential with respect to the adjacent aqueous phases, and hence affect partitioning of ions into the hydrocarbon interior (Lieberman and Topaly, 1969; Le Blanc, 1970). Consequently, lipid bilayers are intrinsically more permeable to anions than to cations, a fact most striking in lecithin:cholesterol membranes, which are more than  $10^9$  times more permeable to the negative TPhB<sup>-</sup> than to its positive, isosteric counterpart TPhAs<sup>+</sup>.<sup>2</sup> This positive potential can be lowered by phloretin and certain other dipolar molecules added to the aqueous phases, with a consequent alteration of relative cation and anion conductances by factors of as much as  $10^8$ , through the simultaneous increase of cation conductances and decrease of anion conductances. Quite different substances that modify the dipole potential, such as  $\omega$ -substituted fatty acids, Cu<sup>+</sup> complexes of polyhalogen benzimidazole, and zwitterionic surfactants have been studied by Demin et al. (1972) and by Haydon and Myers (1973).

#### METHODS AND MATERIALS

Membranes were formed at room temperature by the brush technique of Mueller et al. (1963) across a hole (1–1.5 mm<sup>2</sup>) in a Teflon partition separating two Lucite or Teflon chambers, containing symmetrical NaCl or KCl solutions buffered at various pH's. In most experiments the membrane-forming solution was an *n*-decane solution of either bacterial phosphatidylethanolamine (PE), bacterial phosphatidylethanolamine:cholesterol (PE:C) (molar ratio 1:4), or egg lecithin:cholesterol (L:C) (molar ratio 1:4).<sup>3</sup> Other lipids used were phosphatidylglycerol (PG) and glycerylmonooleate (GMO).

After the membrane formed, one of the ionophores or lipophilic ions was added to both chambers, which were continuously stirred throughout the experiment. The membrane conductance reached a steady level after a few minutes. A small aliquot of an ethanolic solution of either phloretin, *p*-, *m*-, *o*-nitrophenol, 2,4, or 2,6 dihydroxyacetophenone, phloracetophenone, phloroglucinol, diethylstilbestrol, or phlorhizin was then added (generally) to both sides of the membrane. (Stock ethanolic solutions were stable indefinitely at -20°C except for CCCP, which was freshly prepared every 3 days.) After the membrane conductance reached its new steady level, a further addition of the same molecule was made, and so forth. Often a full current-voltage characteristic was obtained at each concentration, although we report here mainly on the ohmic (small signal) conductance. Ethanol alone at 3% vol/vol, the highest concentration used, had no significant effect on membrane conductance. The major conductance changes occurred within 1–2 min after addition of these agents; however, smaller changes, threefold or so, could occur over the next 10–15 min.

Except for TPhB<sup>-</sup> experiments, conductance was measured either by passing a known step of current,  $\Delta I$ , through a pair of electrodes and recording the resulting steady-state potential difference,  $\Delta V$ , across the membrane through the same or different pair of electrodes (current clamping), or by applying a known step of voltage,  $\Delta V$ , across the membrane through a pair of electrodes and recording the resulting steady-state current,

<sup>2</sup> Conductance is approximately  $3 \times 10^{-3}$  mho/cm<sup>2</sup> in  $10^{-7}$  M TPhB<sup>-</sup>, but only  $8 \times 10^{-8}$  mho/cm<sup>2</sup> in  $5 \times 10^{-3}$  M TPhAs<sup>+</sup>. See also Le Blanc (1970) and Andersen and Fuchs (1975).

<sup>3</sup> These solutions were 2% PE, 2% PE + 4% cholesterol, and 2% lecithin + 4% cholesterol. We were unable to carry out satisfactory experiments on cholesterol-free lecithin membranes, because they became heavily infiltrated with thick lenses after exposure to phloretin.

$\Delta I$ , through the same electrodes (voltage clamping). Conductance was then given by  $\Delta I / \Delta V$ . The electrodes were either Ag/AgCl wires in direct contact with the solutions or calomel electrodes coupled to the solutions through saturated KCl junctions.

In experiments with TPhB<sup>-</sup>, the conductance was obtained under voltage clamp conditions from the initial current ( $\Delta I$ )<sub>0</sub> flowing across the membrane in response to a small voltage step,  $\Delta V$  ( $\leq 60$  mV). ( $\Delta I$ )<sub>0</sub> was obtained by plotting the logarithm of the current versus time, and extrapolating back to  $t = 0$  (Andersen and Fuchs, 1975). The exponential decay of the current results from an internal redistribution of TPhB<sup>-</sup> ions in the membrane (Ketterer et al., 1971; Andersen and Fuchs, 1975). In the present experiments, current was exponential with time for about four time constants.

We are concerned primarily with the initial conductance ( $(\Delta I)_0 / \Delta V$ ). The extrapolated conductance at  $t = 0$  differs at most by a factor of 1.2 from the earliest recorded value at 10–250  $\mu$ s. Most experiments were done at TPhB<sup>-</sup> concentrations low enough ( $< 10^{-6}$  M) that membrane conductance is directly proportional to concentration (see also Andersen and Fuchs, 1975). At these low concentrations, measurements must be made at “zero time;” values obtained after a second or more reflect only diffusion polarization in the aqueous phases (Haydon and Hladky, 1972). The electrodes were either calomel electrodes, with NaCl agar bridges of the same concentration as the aqueous solutions bathing the membrane, or Ag/AgCl electrodes.

Surface potentials at the air-water interface were measured as follows.<sup>4</sup> A paraffin-coated Petri dish was divided into two compartments by a paraffin-coated glass barrier that separated the two surfaces but did not extend to the bottom of the dish; thus the subphases were in contact. The surface of the larger compartment (area  $\approx 60$  cm<sup>2</sup>) was cleaned until a potential more negative than  $-225$  mV was recorded (by a Kiethly electrometer) between the reference electrode (saturated calomel electrode with a saturated KCl junction) and the radium electrode positioned about 3 mm above the surface. From 5 to 50  $\mu$ l of the same solutions as used to form bilayers were spread on this surface, and within seconds a stable value of potential was reached. The difference between the potentials in the absence and the presence of the spread film is by definition the surface potential of the film. Ethanolic solutions of phloretin were then injected into the small compartment, which contained the reference electrode, and stirred into the subphase (either unbuffered 0.1 M KCl [pH 5.2] or 0.1 M KCl + 5 mM NaAc/HAc [pH 5.1]) of the large compartment by a magnetic stirring bar on the bottom of the latter; a new stable surface potential was achieved within 2 min. Ethanol alone to a final concentration of 1%, the highest ever used, produced no significant change in surface potential.

The membrane permeability coefficient,  $P_a$ , to [<sup>14</sup>C]acetamide was measured by methods described previously (Holz and Finkelstein, 1970).

Egg lecithin was obtained from Sylvana Chemical Company Millburn, N. J.; cholesterol was purchased either from Eastman Kodak Co. (Rochester, N. Y.) and recrystallized twice from ethanol, or from Fluka (purissimum grade) (supplied by Tridon Chemical Inc., Hauppauge, N. Y.) and used as supplied. Bacterial PE and PG were obtained from Supelco Inc. (Bellefonte, Pa.) and GMO from Sigma Chemical Co. (St. Louis, Mo.). *n*-Decane was gas chromatographic standard from either Merck & Co. (Rahway, N. J.) or Chemical Samples Company (99.9%) (Columbus Ohio). Valinomycin was obtained from Eli Lilly & Co., Indianapolis, Ind., nonactin from Miss Barbara Stearns of the Squibb Institute for Medical Research Princeton, N. J., TPhB<sup>-</sup> Na<sup>+</sup> from Merck, TPhAs<sup>+</sup> Cl<sup>-</sup> from Aldrich Chemical Co., Inc., Milwaukee, Wis., CCCP from Sigma, and Hg(C<sub>6</sub>F<sub>5</sub>)<sub>2</sub>

<sup>4</sup> We thank Dr. G. Colacicco and Dr. A. K. Ray of his laboratory for their assistance with these experiments and for the use of their equipment.

from Dr. E. A. Liberman.  $^{14}\text{C}$ -labeled acetamide was obtained from New England Nuclear Corp., Boston, Mass. Phloretin, phlorhizin, phloroacetophenone, 2,4 and 2,6 dihydroxyacetophenone, and phloroglucinol were obtained from K and K Laboratories (Plainview, N. Y.); phloretin was either used as supplied or recrystallized from ethanol. Diethylstilbestrol was from Calbiochem, San Diego, Calif.; *o*-, *m*-, and *p*-nitrophenol were from British Drug Houses (supplied by Gallard-Schlesinger Mfg. Co., Carle Place, N. Y.); *o*-nitrophenol was recrystallized from ethanol and *p*-nitrophenol from toluene. Water was doubly distilled; all other chemicals were reagent grade.

## RESULTS

### *Phloretin*

**UNMODIFIED MEMBRANES** At the highest attainable concentration in aqueous solutions ( $\approx 2.5 \times 10^{-4}$  M), phloretin produces trivial changes in the conductance of lipid bilayers separating NaCl or KCl solutions, and small increases in nonelectrolyte permeability. (In L:C bilayers, phloretin ( $1 \times 10^{-4}$  M) increases  $P_d$  [acetamide] threefold when added to both sides of the membrane and twofold when added to one side.)

### MODIFIED MEMBRANES

**General Results** Phloretin increases cation conductances and decreases anion conductances of membranes treated with ion carriers or lipophilic ions. This was found for  $\text{K}^+$ -nonactin,  $\text{K}^+$ -valinomycin,  $\text{TPhAs}^+$ ,  $\text{TPhB}^-$ ,  $\text{CCCP}^-$ , and  $[\text{Hg}(\text{C}_6\text{F}_5)_2]_2 \text{Cl}^-$ . (Because of possible direct interactions with  $\text{I}_2/\text{I}^-$ , it was not possible to interpret unambiguously or quantitatively the conductance decreases produced by phloretin on this system.) Moreover, these conductance changes are very large (Fig. 2 and Table I). For example,  $1.5 \times 10^{-4}$  M phloretin increases  $\text{K}^+$ -nonactin conductance  $10^5$ -fold and decreases  $\text{CCCP}^-$  conductance  $10^3$ -fold on L:C membranes. These conductance changes clearly reflect changes in permeability to the original conducting species, since ion selectivity is not altered. Furthermore, these effects cannot be attributed to specific interactions between phloretin and the current-carrying species, because (a) their magnitude is a function of bilayer composition (see Table I), (b) they occur with chemically quite different current carriers, (c) conductances continue to vary linearly with concentration of either ion, ionophore,<sup>5</sup> or lipophilic ion, and (d) the effects are obtainable at current-carrier concentrations greatly in excess of phloretin's concentration. (This last was established with both  $\text{CCCP}^-$  and  $\text{TPhAs}^+$ .)

**Un-Ionized Phloretin is the Active Species** Since phloretin is a weak acid ( $\text{pK} = 7.3$  [LeFevre and Marshall, 1959]), the above-described conductance changes, which were obtained on uncharged membranes (PE, PE:C, or L:C), might result from surface potentials generated by adsorbed phloretin anion. This possibility is excluded for several reasons. (a) The surface potential required to increase  $\text{K}^+$ -nonactin conductance and decrease  $\text{CCCP}^-$  conductance  $10^3$ -fold is  $-180$  mV; to obtain this with free charges requires a prohibitively large surface charge density (one electronic charge/25  $\text{\AA}^2$  in 0.1 M KCl). (b) The effect of phloretin on

<sup>5</sup> Conductance continues to vary as the square of  $\text{Hg}(\text{C}_6\text{F}_5)_2$  concentration.

K<sup>+</sup>-nonactin conductance is independent of pH between 4 and 6, even though the concentration of phloretin anion changes 100-fold. (c) At pH 10, where virtually all of the phloretin is ionized, phloretin does *not* increase K<sup>+</sup>-nonactin conductance nor decrease TPhB<sup>-</sup> conductance. Thus, un-ionized phloretin is active, whereas phloretin anion is inactive. (This is consistent with evidence that un-ionized phloretin is the species that adsorbs to the human red cell membrane [Le Fevre and Marshall, 1959].)

*One-Sided Versus Two-Sided Effects of Phloretin* Phloretin is more effective when added to both aqueous solutions than when added to only one, in agreement with the theory presented later. For example, on L:C bilayers,  $5.5 \times 10^{-5}$  M phloretin from one side increases K<sup>+</sup>-nonactin conductance 500-fold but from both sides increases conductance 10<sup>4</sup>-fold.

Also in agreement with the theory is the observation that addition of phloretin to only one aqueous solution causes the current-voltage characteristics to show rectification at high potentials (experiments with K<sup>+</sup>-nonactin, K<sup>+</sup>-valinomycin, and TPhB<sup>-</sup>). For voltages of equal magnitude, currents are larger when the phloretin-containing side is positive than when it is negative.

*Phloretin Action Is Reversible* The attainment of steady-state conductances within minutes after additions of phloretin to the aqueous phases strongly implies that phloretin's action is reversible. This is confirmed by our observation that the increase in K<sup>+</sup>-nonactin conductance produced by addition of phloretin to the solution on one side of a membrane is reversed by exchanging that solution with one that is phloretin free.

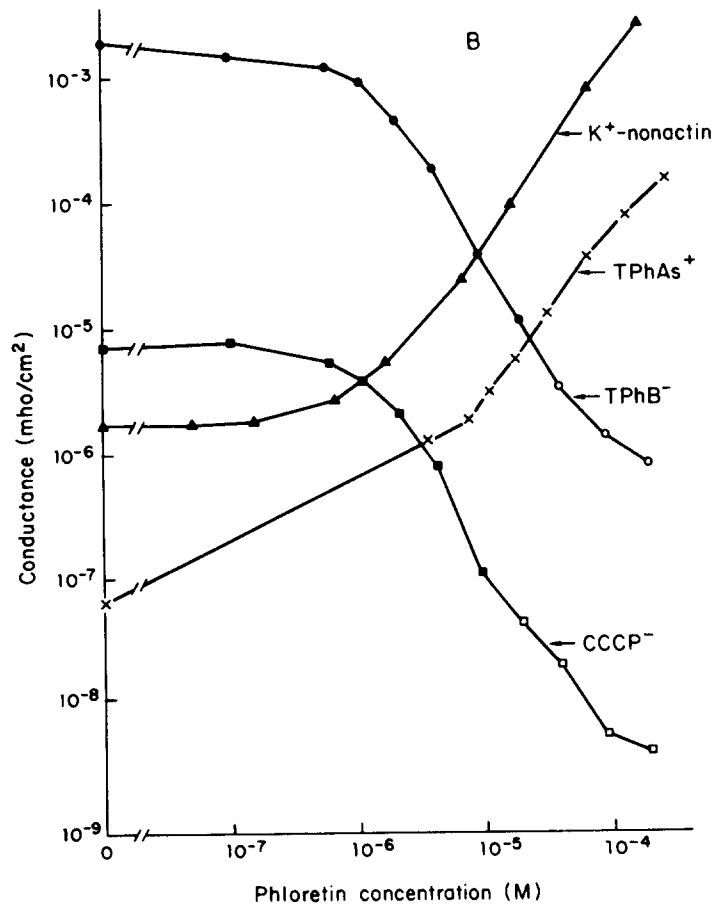
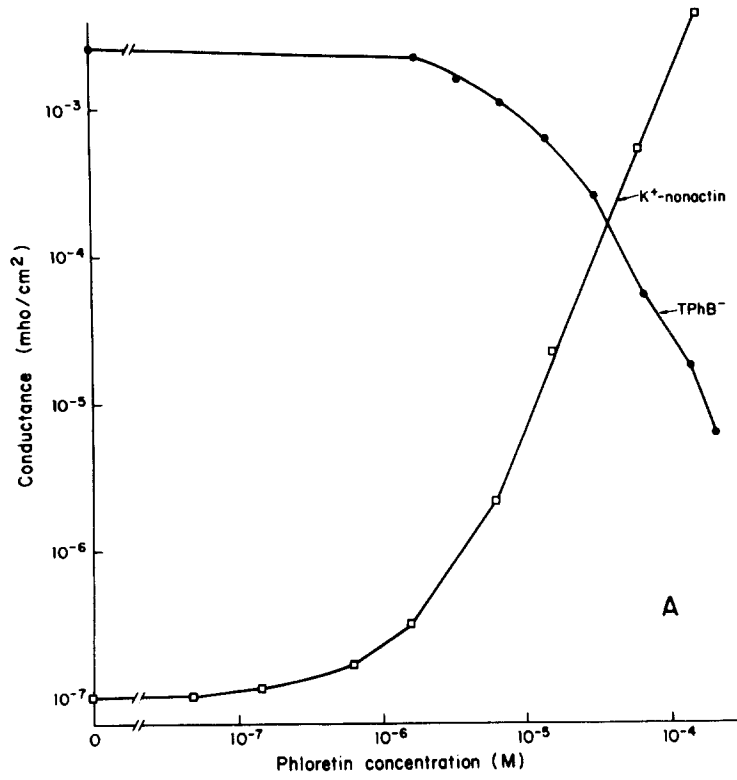
#### *Qualitative Interpretation of Phloretin Effect on Ion Conductance*

Before proceeding further, we think it helpful to offer our explanation for phloretin's action; a more quantitative treatment occurs in the Discussion section. We believe that there exists a positive potential difference of several hundred millivolts between the hydrocarbon interior of a membrane and the adjacent aqueous phases (Fig. 3 A). This potential difference arises from oriented dipoles (of either the lipids themselves or water) near the lipid-water interface. Phloretin adsorbs at this interface and, probably by the orientation of its own large dipole moment, introduces a dipole potential of opposite polarity to the preexisting one. Thus the positive potential in the membrane interior is reduced (Fig. 3 B). Consequently, the membrane becomes *more* permeable to cations, because of their *increased* partition coefficient into the membrane interior, and *less* permeable to anions, because of their *decreased* partition coefficient.

#### *Nitrophenols*

*p*- and *m*-Nitrophenol have qualitatively similar effects to those of phloretin; i.e. they increase cation conductances and decrease anion conductances. This was found for K<sup>+</sup>-nonactin, K<sup>+</sup>-valinomycin, TPhAs<sup>+</sup>, and TPhB<sup>-</sup>. But, whereas phloretin exerts its large effects at concentrations between 10<sup>-6</sup> and 10<sup>-4</sup> M, *p*- and *m*-nitrophenol act in the range 10<sup>-4</sup> to 10<sup>-2</sup> M.<sup>6</sup> (Their lesser potency may

<sup>6</sup> *o*-Nitrophenol is relatively ineffective in this range.





simply reflect a smaller adsorption coefficient at the membrane-solution interface.)

As with phloretin, the effects are large (see Fig. 4).  $2 \times 10^{-2}$  M *p*-nitrophenol increases  $K^+$ -nonactin and TPhAs<sup>+</sup> conductances  $10^5$ -fold and decreases TPhB<sup>-</sup> conductance 300-fold on L:C bilayers. The same criteria cited for phloretin establish the un-ionized form of the nitrophenols as the active species.<sup>7</sup>

<sup>7</sup> The nitrophenol anions are somewhat permeant and can themselves contribute to the membrane conductance. Most experiments, however, were done at pH 3.5, where virtually all of the nitrophenol is in the undissociated form, and the molecule alone produces no significant conductance.

FIGURE 2. (*Opposite*) (A) The effect of phloretin on cation and anion conductances of lecithin:cholesterol (molar ratio 1:4) membranes. In the  $K^+$ -nonactin experiment, the membrane was formed in 0.1 M KCl + 0.01 M KAc/HAc (pH 5.0); the membrane conductance was  $<10^{-8}$  mho/cm<sup>2</sup>. Nonactin was then added to both sides of the membrane to a concentration of  $1.67 \times 10^{-6}$  M, and the conductance immediately rose to  $1.05 \times 10^{-7}$  mho/cm<sup>2</sup>. In the TPhB<sup>-</sup> experiment, the membrane was formed in 1.0 M NaAc + 0.2 M HCl (pH 5.3); the membrane conductance was  $<10^{-8}$  mho/cm<sup>2</sup>. Sodium tetraphenylborate was then added to both sides of the membrane to a concentration of  $10^{-7}$  M, and the conductance immediately rose to  $2.7 \times 10^{-3}$  mho/cm<sup>2</sup>. In both experiments, phloretin was subsequently added to both sides of the membrane in successive increments; after each increment, a new stable conductance was achieved after several minutes. (B) The effect of phloretin on the cation and anion conductances of phosphatidylethanolamine (PE) membranes. In all experiments, the membrane conductance was  $<10^{-8}$  mho/cm<sup>2</sup> before addition of the ionophore or lipophilic ion. After their addition, the conductance immediately rose to the value shown for 0 M phloretin. Phloretin was then added to both sides in successive increments, and a new stable conductance was achieved within several minutes after each increment.  $K^+$ -nonactin experiment: Membrane was formed in 0.1 M KCl + 0.01 M KAc/HAc (pH 5.0); nonactin was then added to both sides to a concentration of  $1.67 \times 10^{-6}$  M. TPhAs<sup>+</sup> experiment: Membrane was formed in unbuffered 0.1 M NaCl (pH  $\approx$ 6). (Phloretin and TPhAs<sup>+</sup> formed a precipitate in other solutions studied.) TPhAsCl was then added to both sides to a concentration of  $1.3 \times 10^{-3}$  M. CCCP<sup>-</sup> experiment: Membrane was formed in 0.1 M NaCl + 0.12 M NaAc/HAc + 0.08 M sodium phosphate (pH 6.0); CCCP<sup>-</sup> was then added to both sides to a concentration of  $10^{-6}$  M. TPhB<sup>-</sup> experiment: Membrane was formed in unbuffered 0.1 M NaCl (pH  $\approx$ 6); NaTPhB was then added to both sides to a concentration of  $2 \times 10^{-7}$  M. In both the CCCP<sup>-</sup> and the TPhB<sup>-</sup> experiments, it was necessary to increase the current-carrier concentration in the course of the experiment when the conductance either approached that of the unmodified membrane (CCCP<sup>-</sup> experiment) or became too small to resolve at  $t = 0$  (TPhB<sup>-</sup> experiment). The open symbols represent normalized conductances at the higher current-carrier concentrations. (The normalization was achieved by dividing the conductance at the higher current-carrier concentration by the factor that the conductance jumped when the current-carrier concentration was raised. Thus, at  $9.1 \times 10^{-6}$  M phloretin, CCCP<sup>-</sup> conductance was  $1.25 \times 10^{-7}$  mho/cm<sup>2</sup>; when the CCCP<sup>-</sup> concentration was raised at this point from  $10^{-6}$  to  $4.1 \times 10^5$  M, the conductance jumped by a factor of 17 to  $2.1 \times 10^{-6}$  mho/cm<sup>2</sup>. At  $1.19 \times 10^{-5}$  M phloretin, TPhB<sup>-</sup> conductance was  $1.3 \times 10^{-5}$  mho/cm<sup>2</sup>; when the TPhB<sup>-</sup> concentration was raised at this point from  $2 \times 10^{-7}$  to  $1.2 \times 10^{-5}$  M, the conductance jumped by a factor of 16 to  $2.1 \times 10^{-4}$  mho/cm<sup>2</sup>.)

*Evidence that Phloretin and Nitrophenols Reduce the Positive Dipole Potential in Bilayers*

INDIRECT Our major finding is that un-ionized phloretin and *m*- and *p*-nitrophenol greatly *enhance* cation conductances and greatly *depress* anion con-

TABLE I  
EFFECT OF PHLORETIN ( $1.5 \times 10^{-4}$  M) ON CATION AND ANION CONDUCTANCES

| Membrane | Factor by which conductance is increased (cations) |                             |                    | Factor by which conductance is decreased (anions) |                       |
|----------|--|-----------------------------|--------------------|---|-----------------------|
|          | K <sup>+</sup> -nonactin                           | K <sup>+</sup> -valinomycin | TPhAs <sup>+</sup> | TPhB <sup>-</sup>                                 | CCCP <sup>-</sup>     |
| PE       | 10 <sup>3</sup>                                    | 10                          | 10 <sup>3</sup>    | 10 <sup>3</sup>                                   | 1.5 × 10 <sup>3</sup> |
| PE:C     | 10 <sup>4</sup>                                    | —                           | —                  | —   | 3 × 10 <sup>2</sup>   |
| L:C      | 10 <sup>5</sup>                                    | 10 <sup>3</sup>             | —                  | 5 × 10 <sup>2</sup>                               | 10 <sup>3</sup>       |

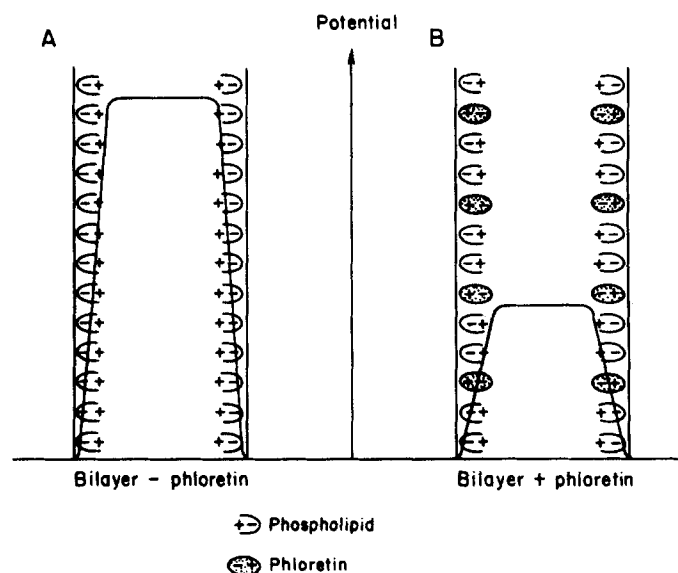


FIGURE 3. Schematic representation of the electrical dipole potential profile within a lipid bilayer membrane in the absence (A) and presence (B) of phloretin. (We believe that the polar, phloroglucinol end of phloretin sits at the interface, and the more lipophilic, phenolic end of the molecule extends into the membrane.)

ductances in lipid bilayers. This reciprocal action on positive and negative species implies that these molecules act mainly by reducing the electrostatic potential of the membrane interior with respect to the aqueous phases. As this is *not* achieved from changes in membrane surface charge (see Results, *Un-Ionized Phloretin Is the Active Species*), it must result from changes in the dipole potential at the membrane interface.<sup>8</sup> (Possible second-order effects on membrane fluidity

<sup>8</sup> Surface charges and surface dipoles can only affect the interior potential of *thin* membranes (thickness less than the Debye length within the membrane); in thick membranes they are screened by compensating ions in the diffuse double layers.

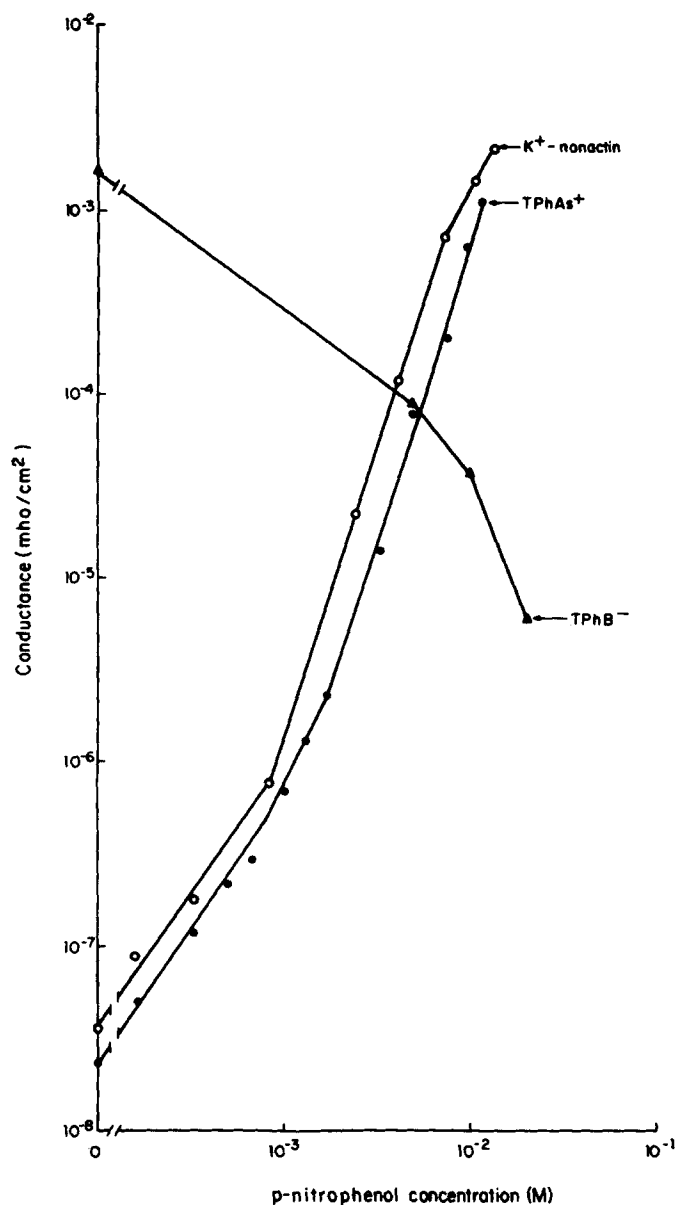


FIGURE 4. The effect of *p*-nitrophenol on the cation and anion conductances of lecithin:cholesterol (molar ratio 1:4) membranes. In all experiments the membrane conductance was  $<10^{-8}$  mho/cm<sup>2</sup> before the addition of the ionophore or lipophilic ion. After their addition, the conductance immediately rose to the value shown for 0 M *p*-nitrophenol. *p*-Nitrophenol was then added to both sides in successive increments, and a new stable conductance was achieved within several minutes after each increment. K<sup>+</sup>-nonactin experiment: Membrane was formed in 0.1 M KCl + 0.033 M NaAc/HAc (pH 3.3); nonactin was then added to both sides to a concentration of  $1.67 \times 10^{-6}$  M. TPhAs<sup>+</sup> experiment: Membrane was formed in 0.1 M KCl + 0.033 M NaAc/HAc (pH 3.5); TPhAsCl was then added to both sides to a concentration of  $5 \times 10^{-3}$  M. TPhB<sup>-</sup> experiment: Membrane was formed in 1.0 M NaCl + 0.2 M NaAc/HAc (pH 4.5); NaTPhB was then added to both sides to a concentration of  $5 \times 10^{-8}$  M.

and dielectric constant will increase or decrease *both* cation and anion conductances.)

**DIRECT** It is possible to demonstrate directly this change of dipole potential both on lipid monolayers and on bilayers.

*Monolayers* Films formed from 2% PE in decane had surface potentials of +425 mV. Phloretin at  $1.5 \times 10^{-4}$  M reduced this by 200 mV. (Phloretin had no significant effect on the surface potential of a clean air-water interface.) The sign and magnitude of the phloretin effect is almost exactly that predicted from the  $10^3$ -fold increase in  $K^+$ -nonactin and TPhAs<sup>+</sup> conductances, and the  $10^3$ -fold decrease in CCCP<sup>-</sup> and TPhB<sup>-</sup> conductances produced by  $1.5 \times 10^{-4}$  M phloretin on PE bilayers. (The reason for only a 10-fold increase in  $K^+$ -valinomycin conductance is given in the Discussion, *Phloretin Effect on  $K^+$ -Nonactin and  $K^+$ -Valinomycin Conductances*.)

(Films formed from PE:C and L:C had surface potentials of about +400 mV. Phloretin ( $1.5 \times 10^{-4}$  M), however, produced virtually no change in this value, even though it is very active on PE:C and L:C bilayers.)

*Bilayers* There can be no directly measurable potential difference *at equilibrium* across a membrane separating identical salt solution, even if there is an asymmetry in surface potentials. If, however, such an asymmetry is suddenly introduced, there will *initially* appear a potential difference, equal to the difference in surface potentials, which decays to zero at a rate determined by the membrane time constant. (See MacDonald and Bangham, 1972.) Such transient potential differences, of the proper sign and magnitude, occur on PE, PE:C, and L:C bilayers when phloretin is introduced on one side of the membrane (Fig. 5).

## DISCUSSION

### *Relative Magnitude of Phloretin Action on Cation and Anion Conductances*

If we assume that the conductance ( $g$ ) of a given ion is proportional to the product of its concentration ( $c_m$ ) and mobility ( $u_m$ ) within the hydrocarbon region of the bilayer, and that  $c_m$  is related to the membrane's surface dipole potential ( $V_d$ ) and the ion's concentration in the aqueous phases ( $c_{aq}$ ) by the Boltzmann distribution, then

$$g \propto u_m c_m \propto u_m c_{aq} e^{-zqV_d/kT}, \quad (1)$$

where  $q$  is the electronic charge,  $z$  is the valence of the ion,  $k$  is the Boltzmann constant, and  $T$  is the temperature in degrees Kelvin. A given concentration of phloretin should therefore increase all cation conductances and decrease all anion conductances by the same factor. To a large extent this expectation is realized.

**CHOLESTEROL-FREE MEMBRANES** The data in best agreement with our theory of phloretin action are those from cholesterol-free PE membranes. There, at  $1.5 \times 10^{-4}$  M phloretin,  $K^+$ -nonactin and TPhAs<sup>+</sup> conductances are

increased  $10^3$ -fold<sup>9</sup> and CCCP<sup>-</sup> and TPhB<sup>-</sup> conductances are decreased by the same factor. Furthermore, the surface potential change of  $-200$  mV produced by  $1.5 \times 10^{-4}$  M phloretin on PE monolayers and the peak transient transmembrane potential of  $-150$  mV produced by  $10^{-4}$  M phloretin on one side of PE

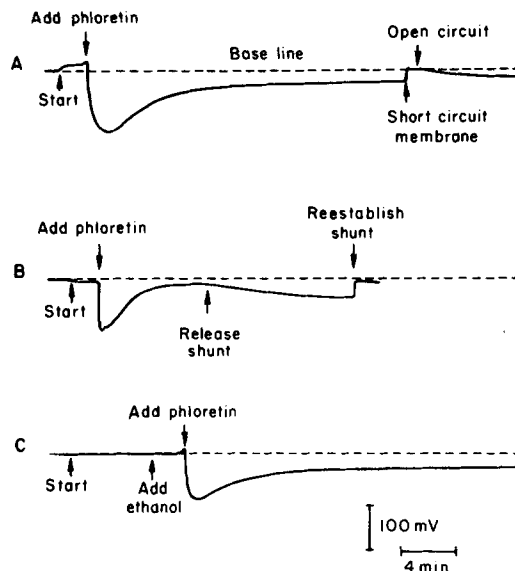


FIGURE 5. Transient potentials produced by addition of phloretin to one side of unmodified lipid bilayer membranes. The membranes ( $1 \text{ mm}^2$ ) were formed in  $0.1 \text{ M NaCl} + 0.2 \text{ M NaAc/HAc}$  ( $\text{pH } 4.6$ ). Phloretin was then added with rapid stirring to one aqueous phase and the resulting potential changes monitored. (A downward deflection means that the side *not* containing phloretin is negative.) (A) Experiment on PE membrane. The membrane conductance was  $2 \times 10^{-11} \text{ mho}$  and there was a slight asymmetry potential of about  $+10 \text{ mV}$ . Upon addition of  $10^{-4} \text{ M}$  phloretin to one side, the potential rapidly went to  $-140 \text{ mV}$  (total  $\Delta V = -150 \text{ mV}$ ) and then slowly decayed back to  $-25 \text{ mV}$ . (This latter is a *steady-state* potential that reappears after removal of a short circuit. Its origin is unknown; it is independent of  $\text{pH}$  at  $\text{pH} < 6.0$  and disappears at high  $\text{pH}$  [ $\approx 12$ ].) (B) To test that these transient potentials actually represent dipole potential changes and not some obscure diffusion potentials, the input of the amplifier (input impedance  $> 10^{12} \Omega$ ) was shunted with a  $10^{10} \Omega$  resistor. (Membrane resistance was  $3.3 \times 10^{10} \Omega$ .) The peak of the transient response in response to  $10^{-4} \text{ M}$  phloretin is only reduced by about 30% (from  $-150$  to  $-110 \text{ mV}$ ), but the steady-state potential is reduced by about a factor of 4 (from  $-41$  to  $-11$ ). Note that the steady-state potential increases back to  $-41$  when the  $10^{10} \Omega$  shunt is released. (C) Demonstration that similar potential changes occur on a cholesterol-containing membrane (PE:C) when  $10^{-4} \text{ M}$  phloretin is added to one side. The peak potential change was  $-110 \text{ mV}$ . (Note the ethanol control before addition of phloretin.)

bilayers are almost exactly what are required by relation 1 for the conductance changes. Whatever other actions phloretin may have on PE membranes (e.g. changing membrane fluidity), their effects on  $\text{K}^+$ -nonactin, TPhAs<sup>+</sup>, CCCP<sup>-</sup>,

<sup>9</sup> See p. 763 for an explanation of why  $\text{K}^+$ -valinomycin conductance is increased only 10-fold.

and TPhB<sup>-</sup> conductances are insignificant compared to the dipole potential effect.

**CHOLESTEROL-CONTAINING MEMBRANES** The data from cholesterol-containing membranes (PE:C and L:C) also agree with our theory, but there are quantitative discrepancies: the phloretin-induced increases in cation conductances are even larger than those on cholesterol-free PE membranes, but the decreases in anion conductances are the same or less. For example, on L:C membranes, K<sup>+</sup>-nonactin conductance is increased 10<sup>5</sup>-fold, yet CCCP<sup>-</sup> and TPhB<sup>-</sup> conductances are reduced only (!) 10<sup>3</sup>-fold; on PE:C membranes, K<sup>+</sup>-nonactin conductance is increased 10<sup>4</sup>-fold, but CCCP<sup>-</sup> conductance is reduced only 300-fold. Some or all of this difference may reflect phloretin-induced fluidity increases, produced either by a general disruption of structure or by direct interaction with cholesterol, in the "tighter" cholesterol-containing membranes. This is certainly suggested by phloretin's 10-fold *greater* effect on K<sup>+</sup>-nonactin conductance in PE:C membranes than in PE membranes, but its 5-fold *smaller* effect on CCCP<sup>-</sup> conductance in PE:C membranes than in PE membranes (Table I). A 10-fold increase in the mobility (or potential-independent partition coefficient) of these large ions in the membrane would account for the results on L:C membranes. (That is, the dipole effect alone increases cation conductances and decreases anion conductances by 10<sup>4</sup>; the fluidity increase *multiplies* by 10 the dipole effect on cations but *divides* by 10 the dipole effect on anions.)<sup>10</sup> That phloretin induces a threefold increase in the permeability coefficient of the small acetamide molecule makes this possibility not unreasonable.

The most disturbing aspect of phloretin's action on cholesterol-containing bilayers is its failure to induce the expected surface potential decreases in cholesterol-containing monolayers. Since these decreases *are* seen in bilayers, it appears, contrary to popular belief (MacDonald and Bangham, 1972; Haydon and Myers, 1973) that monolayers at the air-water interface are not (in some respects) good models for half of a bilayer.<sup>11</sup> (It is possibly relevant that there appeared to be a slick on the water surface when films were spread from *cholesterol-free*, phospholipid-decane solutions but none when spread from *cholesterol-containing*, phospholipid-decane solutions. Thus, the cholesterol-free monolayers may actually have existed at an oil-water interface, whereas the cholesterol-containing monolayers were at an air-water interface. A priori the former should be a better model for half of a lipid bilayer, although it has been claimed that the latter is equally good [Haydon and Myers, 1973].)

#### *Phloretin Effect on K<sup>+</sup>-Nonactin and K<sup>+</sup>-Valinomycin Conductances*

The simple prediction of relation 1 does not completely hold for phloretin's effect on these similar cation complexes. Although at low phloretin concentra-

<sup>10</sup> Fluidity increases might have a larger effect on nonactin and its complex than on CCCP<sup>-</sup> or TPhB<sup>-</sup>, both because of differences in their sizes and because of possibly different rate-limiting barriers to transport through the bilayer.

<sup>11</sup> Bilayers formed from two monolayers (Montal and Mueller, 1972) are fully sensitive to phloretin. K<sup>+</sup>-nonactin conductance in PE:cholesterol membranes (molar ratio 1:1) is increased 10<sup>3</sup>-fold by 8 × 10<sup>-5</sup> M phloretin.

tions the conductance increases for the two complexes are equal, at higher phloretin concentrations, K<sup>+</sup>-nonactin conductance continues to rise steeply, but K<sup>+</sup>-valinomycin conductance levels off and actually declines slightly (Fig. 6 A). Thus, in PE membranes, phloretin maximally increases K<sup>+</sup>-nonactin conductance 10<sup>3</sup>-fold, but K<sup>+</sup>-valinomycin conductance only 10-fold. (In lecithin:cholesterol membranes, phloretin maximally increases K<sup>+</sup>-nonactin conductance 10<sup>5</sup>-fold, but K<sup>+</sup>-valinomycin conductance only 10<sup>3</sup>-fold.)

The reason for the difference in the response of K<sup>+</sup>-nonactin and K<sup>+</sup>-valinomycin to phloretin is suggested by their *I-V* characteristics. In the absence of phloretin, both *I-V* characteristics bend toward the current axis (Fig. 6 Ba). The K<sup>+</sup>-nonactin characteristic retains this form at all phloretin concentrations, but the K<sup>+</sup>-valinomycin characteristic bends toward the voltage axis at phloretin concentrations where K<sup>+</sup>-valinomycin conductance levels off and falls (Fig. 6 Bb, c).<sup>12</sup> An *I-V* characteristic that bends toward the current axis indicates that ion translocation through the bilayer is rate limiting, whereas the converse characteristic implies that ion transfer across the interface is rate limiting or rate contributing (Läuger, 1972).

Thus phloretin is not as effective on (and may even depress) the interfacial transfer of K<sup>+</sup> by valinomycin as it is on the transport of the complex through the bilayer interior. A quantitative theory of phloretin (and *m*-, *p*-nitrophenol) action must therefore include not just the *magnitude* of the dipole potential, as in relation 1, but also its spatial profile near the interface and the kinetics of the transport system. These subtleties are neglected in relation 1, which implicitly assumes that the barrier to ion movement is translocation through the membrane interior. (See Appendix.)

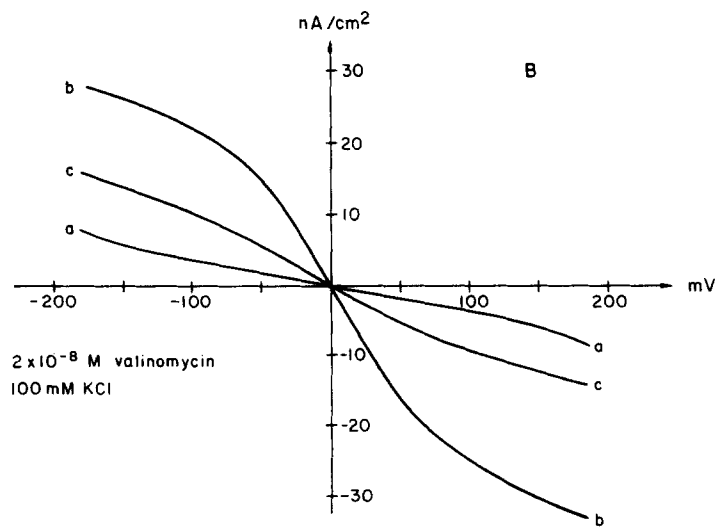
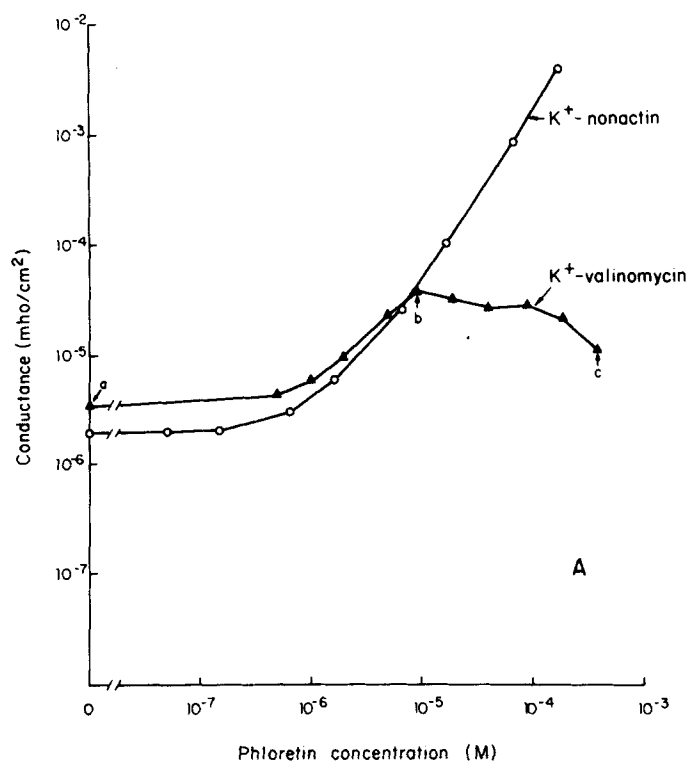
#### *Magnitude of the Dipole Potential*

If phloretin increases K<sup>+</sup>-nonactin conductance by decreasing the potential difference between membrane interior and aqueous phases, a potential decrease of 300 mV is necessary to explain a 10<sup>5</sup>-fold increase in conductance (seen in lecithin:cholesterol membranes).<sup>13</sup> Such a large potential change is attainable from alignment of phloretin dipoles (dipole moment = 5.6 D [K. Matsuo, personal communication]) at the interface, if phloretin can achieve interfacial concentrations of about one molecule per 600 Å<sup>2</sup>, a value perfectly reasonable in view of the red cell data (LeFevre and Marshall, 1959).<sup>14</sup> The preexisting dipole

<sup>12</sup> The *I-V* characteristics for the anions (CCCP<sup>-</sup> and TPhB<sup>-</sup>) bend toward the current axis at all phloretin concentrations.

<sup>13</sup> If part of the increase in K<sup>+</sup>-nonactin conductance results from fluidity changes (Discussion, CHOLESTEROL-CONTAINING MEMBRANES), the change in potential is less than 300 mV.

<sup>14</sup> Potential changes of several hundred millivolts are also attainable with *p*-nitrophenol (dipole moment = 5.4 D [McClellan, 1963]), if it can achieve comparable interfacial concentrations. The relative ineffectiveness of *o*-nitrophenol may be due to its smaller dipole moment (3.11 D [McClellan, 1963]). It may also result, however, from poor adsorption of the molecule or from misalignment of the dipole moment at the interface. Both effects could be due to the location of the dipole moment with respect to the phenyl group, which is energetically favored in the hydrocarbon region of the membrane. This is less of a problem for *m*-nitrophenol and none at all for *p*-nitrophenol, which can achieve complete alignment of its dipole moment perpendicular to the plane of the membrane by having the polar OH group at the interface and the remaining, relatively nonpolar nitrobenzene part of the molecule extending directly into the membrane.





potential in bilayers may be even larger than this. Monolayers of sterols and phospholipids at the air-water interface have dipole surface potentials of around 400 mV, air phase *positive* (Adam, 1941; Papahadjopoulos, 1968). If, as a first approximation, a bilayer is two monolayers back to back, one might expect a priori a large positive potential within the hydrocarbon interior.<sup>15</sup> The much greater permeability to TPhB<sup>-</sup> than to TPhAs<sup>+</sup> is certainly consistent with this.

It is noteworthy that phloretin does not increase K<sup>+</sup>-nonactin conductance in GMO membranes. These membranes have much smaller dipole potentials than do phospholipid membranes (Hladky and Haydon, 1973), which suggests that the intrinsic positive dipole potential of the bilayer may promote the adsorption of phloretin and/or the orientation of its dipole moment at the interface. In GMO:cholesterol membranes (molar ratio 1:2), which have a larger positive dipole potential (Szabo, 1974),  $1.5 \times 10^{-4}$  M phloretin increases K<sup>+</sup>-nonactin conductance 50-fold.

#### *Biological Implications*

EFFECT OF PHLORETIN AND NITROPHENOLS ON NONACTIN- AND VALINOMYCIN-INDUCED <sup>42</sup>K<sup>+</sup> EXCHANGE IN THE RED CELL MEMBRANE Since plasma membranes contain extensive regions of lipid bilayer, phloretin (and the nitrophenols) should produce effects on them similar to those seen with artificial bilayers. This has been confirmed on human red blood cells. Valinomycin-induced <sup>42</sup>K<sup>+</sup>

<sup>15</sup> There is a problem in relating monolayer potentials to potentials within bilayers. Monolayer surface potentials represent the *difference* between the potential at a clean air-water interface, and the potential after the monolayer is spread; we are concerned with the *absolute value* of the potential difference between the interior of the bilayer and the adjacent aqueous phases. This is the famous (or infamous) Galvani potential between two dissimilar phases; although not directly measurable, it must be reflected by the difference in TPhB<sup>-</sup> and TPhAs<sup>+</sup> permeability. (See Andersen and Fuchs [1975] for the use of TPhB<sup>-</sup> and TPhAs<sup>+</sup> in determining Galvani potentials.)

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FIGURE 6. (*Opposite*) (A) Comparison of the effects of phloretin on K<sup>+</sup>-nonactin and K<sup>+</sup>-valinomycin small signal conductance in phosphatidylethanolamine (PE) membranes. In both experiments the membrane conductance was  $<10^{-8}$  mho/cm<sup>2</sup> before addition of the ionophore. After their addition, the conductance immediately rose to the value shown for 0 M phloretin. Phloretin was then added to both sides in successive increments, and a new stable conductance was achieved within several minutes after each increment. K<sup>+</sup>-nonactin experiment: Membrane was formed in 0.1 M KCl + 0.01 M KAc/HAc (pH 5.0); nonactin was then added to both sides to a concentration of  $1.67 \times 10^{-6}$  M. K<sup>+</sup>-valinomycin experiment: Membrane was formed in unbuffered 0.1 M KCl (pH  $\approx$ 6); valinomycin was then added to both sides to a concentration of  $2 \times 10^{-8}$  M. (B) Current-voltage characteristics of a valinomycin-treated PE membrane at various phloretin concentrations. The membrane is the same one whose small signal conductance is plotted in Fig. 6 A; curves *a*, *b*, and *c* were taken at the phloretin concentrations (and corresponding small signal conductances) marked in Fig. 6 A. (The current voltage characteristic of the nonactin-treated membrane in Fig. 6 A was similar in shape to curve *a* at all phloretin concentrations.)

exchange is increased 7-fold (Wieth et al., 1973) and nonactin-induced  $^{42}\text{K}^+$  exchange 27-fold by  $2.5 \times 10^{-4}$  M phloretin (Cass and Wieth, unpublished observations). *p*- and *m*-Nitrophenol at  $10^{-2}$  M enhance nonactin-induced  $^{42}\text{K}^+$  exchange by 200–300-fold, whereas *o*-nitrophenol is virtually inactive, as it also is on artificial lipid bilayers (Cass and Wieth, unpublished observations). Thus, phloretin and the nitrophenols have similar actions on natural and artificial bilayers.

**EFFECT OF PHLORETIN ON NONELECTROLYTE PERMEABILITY OF CELL MEMBRANES** Phloretin has two different actions on nonelectrolyte transport in cell membranes (Owen et al., 1974). For molecules that cross the membrane simply by partitioning into and diffusing through the bilayer, phloretin *increases* permeability by about twofold (Owen et al., 1974), just as it does on artificial lipid bilayers. On the other hand, for molecules such as urea, acetamide, and glucose that cross the membrane by specific pathways, phloretin dramatically *decreases* permeability (Macey and Farmer, 1970; Owen and Solomon, 1972; Levine et al., 1973; LeFevre, 1954).

**MECHANISM OF PHLORETIN ACTION ON TRANSPORT ACROSS CELL MEMBRANES** Phloretin powerfully inhibits glucose and chloride transport (LeFevre, 1961; Gunn et al., 1975) and slows urea, acetamide, and glycerol transport (Macey and Farmer, 1970; Owen and Solomon, 1972) across the human red blood cell membrane; it also inhibits urea and acetamide transport across the toad urinary bladder (Levine et al., 1973). Its striking effects on ion transport across lipid bilayers occur over the same concentration range effective on these natural transport processes (Fig. 7).<sup>16</sup> Therefore, when we discovered the phloretin-like effect of *p*- and *m*-nitrophenol on lipid bilayers, we tested their action on  $^{36}\text{Cl}^-$  exchange across the human red cell membrane and found that there too they were powerful inhibitors at the same concentrations effective on bilayers (Cass and Wieth, unpublished observations). This prompted us to investigate, both on artificial bilayers and on the human red cell membrane, the action of several other molecules chemically related to phloretin. These studies, summarized in Table II, reveal a striking parallelism between the effectiveness of these molecules on  $\text{K}^+$ -nonactin conductance in lipid bilayers and their ability to inhibit  $^{36}\text{Cl}^-$  exchange in the red cell membrane.

Can phloretin's effect on membrane dipole potential be responsible for its inhibition of the chloride and other transport systems?<sup>17</sup> At present we can only speculate. The presumed positive dipole potential of the red cell membrane interior, generated by surface dipoles, is dropped over very short distances, a few angstroms at most. In this transition region, field strengths are very intense ( $\sim 10^7$ – $10^8$  V/cm). Such fields may profoundly affect molecules (charged, dipolar, or polarizable) involved in the translocation of substances such as  $\text{Cl}^-$  and

<sup>16</sup> The un-ionized form of phloretin is the species that inhibits glucose transport (LeFevre and Marshall, 1959), but it has not yet been definitely established that this is the form that inhibits chloride and urea transport.

<sup>17</sup> Conceivably, all molecules whose transport is inhibited by phloretin (glucose, chloride, urea, acetamide, glycerol) permeate via the same transport system.

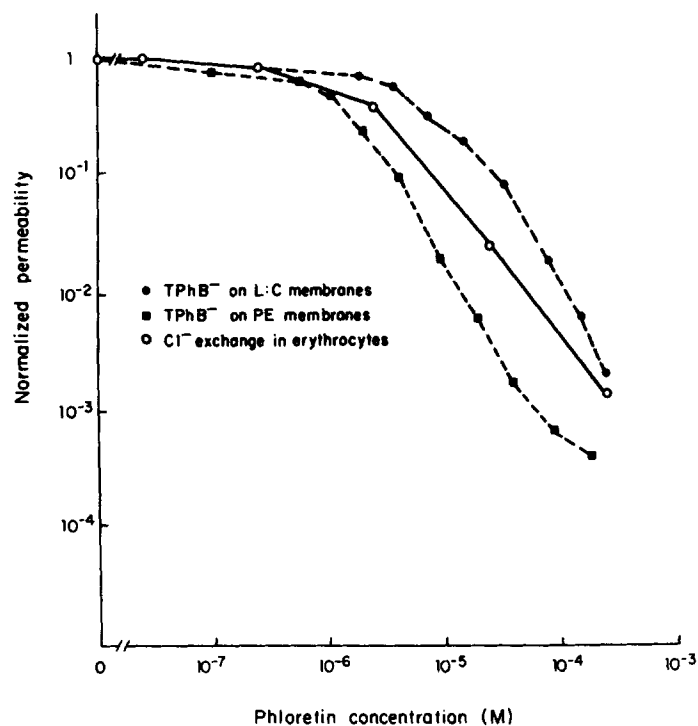


FIGURE 7. Comparison of the effect of phloretin on the chloride exchange across the human red blood cell membrane to the effect of phloretin on TPhB<sup>-</sup> conductance in PE and L:C membranes. The TPhB<sup>-</sup> data are from Fig. 2; the chloride exchange data are from Wieth et al. (1973). All data are normalized to 1 for zero concentration of phloretin.

TABLE II  
RELATIVE EFFECTIVENESS OF PHLORETIN AND RELATED  
MOLECULES ON TRANSPORT ACROSS LIPID BILAYERS AND ACROSS THE  
HUMAN RED BLOOD CELL MEMBRANE

| Concentration<br><i>M</i> | Molecule                | Factor by which K <sup>+</sup> -nonactin<br>conductance is increased in<br>L:C membranes | Factor by which Cl <sup>-</sup> trans-<br>port is decreased in the hu-<br>man red cell membrane |
|---------------------------|-------------------------|--|---|
| 2.5 × 10 <sup>-4</sup>    | Phloretin               | 10 <sup>5</sup>  | 780*  |
| 3 × 10 <sup>-3</sup>      | 2,6 Di(OH)acetophenone  | 10 <sup>4</sup>  | 500‡  |
| 3 × 10 <sup>-3</sup>      | Phloracetophenone       | 10 <sup>4</sup>  | 200‡  |
| 10 <sup>-2</sup>          | <i>m</i> -Nitrophenol   | 10 <sup>5</sup>  | 190§  |
| 10 <sup>-2</sup>          | <i>p</i> -Nitrophenol   | 10 <sup>5</sup>  | 144§  |
| 10 <sup>-2</sup>          | 2,4 Di(OH) acetophenone | 10 <sup>3</sup>  | 50‡   |
| 10 <sup>-2</sup>          | <i>o</i> -Nitrophenol   | 10 <sup>2</sup>  | 31§   |
| 10 <sup>-4</sup>          | Diethylstilbestrol      | 10 <sup>2</sup>  | 4§  |
| 10 <sup>-4</sup>          | Phlorhizin              | 10   | Inactive <sup>d</sup>   |
| 10 <sup>-2</sup>          | Phloroglucinol          | Inactive   | —   |

\* Wieth et al., 1973.

‡ Cass and Gunn, unpublished observations.

§ Cass and Wieth, unpublished observations.

<sup>d</sup> There is a 15-fold decrease at 2 × 10<sup>-3</sup> M phlorhizin (Schnell et al., 1973).

glucose.<sup>18</sup> By changing the magnitude (or even the sign) of the field, phloretin could alter the orientation and conformation of these molecules,<sup>19</sup> thus affecting translocation mechanisms. Since, however, phloretin is taken up so avidly at the membrane surface (concentrations of 1 phloretin molecule per 10 phospholipid molecules can be achieved [LeFevre and Marshall, 1959]), it could act in other ways, e.g., by changing the molecular packing of phospholipids and translocators at the interface. (The effects of phloretin on membrane fluidity in cholesterol-containing membranes may reflect this aspect of its action.) In any case, the implications that intense interfacial electrical fields have for biological transport processes deserve further consideration.

#### APPENDIX

Any attempt to refine the model that we have developed for phloretin's action depends upon specific information obtained with different current carriers. (It should of course be realized that it now becomes a matter of philosophy [or prejudice] whether one chooses the current carrier to study the spatial profile of the phloretin dipole potential, or chooses phloretin to study the kinetics of the current carrier.) For example, the results we have obtained for phloretin action on  $K^+$ -nonactin and CCCP<sup>-</sup> conductance can simply be interpreted in terms of the change in the concentration of the current-carrying species in the center of the membrane ( $c_m$  in Eq. 1). The data obtained with TPhB<sup>-</sup>, on the other hand, can be dissected further; indeed, this has already been done in the absence of phloretin (Andersen and Fuchs, 1975). There it was shown that conductance is proportional to the product of the number of ions adsorbed at the membrane-solution interface and the reciprocal of the time constant for their transfer from one interface to another. Although the latter quantity can be formally treated as a mobility term, it actually depends upon both the fluidity of the interior of the membrane *and* the height of the energy barrier that the ion must traverse in the center of the membrane to move from one interface to the other.

The 500-fold reduction of TPhB<sup>-</sup> conductance by phloretin ( $1.5 \times 10^{-4}$  M) in L:C bilayers results from a 17-fold decrease in the number of ions adsorbed at the interface and a 29-fold increase in the relaxation time constant for transport through the bilayer. Both effects are due to the dipole potential; their relative magnitude is determined by how far into the dipole-potential transition region the TPhB<sup>-</sup> adsorbs. If adsorbed TPhB<sup>-</sup> sat in the membrane totally within the dipole-potential transition region of Fig. 3, the entire phloretin-induced conductance change would result from a reduction in interfacial concentration, and the relaxation time constant would be independent of phloretin concentration. Conversely, if adsorbed TPhB<sup>-</sup> sat in the aqueous phase totally outside this transition region, the entire conductance change would result from an increase in the relaxation time constant, and the interfacial concentration of TPhB<sup>-</sup> would be independent of phloretin. The actual effect of phloretin on both interfacial concentration and relaxation time constant implies that TPhB<sup>-</sup> is adsorbed part way

<sup>18</sup> The above calculation assumes that there is a *uniform* layer of oriented dipoles at the membrane-solution interface. However, a large protein that penetrates through the interface, and hence displaces the surface dipoles, will feel a quite different dipole field. A simple solid angle calculation shows that the field in such a region may extend through the entire thickness of the membrane (and not be localized to the interfacial region) and will be 10-100-fold smaller than the above estimate.

<sup>19</sup> Phloretin's large effect (20-fold in PE membranes and 10<sup>3</sup>-fold in L:C membranes) on gramicidin A-induced conductance may be a prototype for this phenomenon, as the effect results primarily from an increase in the number and lifetime of the gramicidin A channels.

within the dipole-potential transition region. It is difficult, however, to assess its exact location in this region, because of, among other things, the possible effect of phloretin on membrane fluidity.

Another illustration of the importance of locating the position of the phloretin-induced dipole potential is the following observation. The steady-state  $g$ - $V$  characteristic of a monazomycin-treated membrane is shifted only marginally by the addition of phloretin to the side opposite to that containing monazomycin. In contrast, the  $g$ - $V$  characteristic is profoundly shifted (in negatively charged membranes) by surface potential changes generated by the addition of divalent cations to that side (Muller and Finkelstein, 1972). This can be understood if the mechanism for the monazomycin-induced voltage-dependent conductance involves the movement of the positive charge on monazomycin completely through the membrane into the aqueous phase on the other side, outside of the dipole transition region shown in Fig. 3 but within the space charge region of a negatively charged membrane. This latter region, not shown in Fig. 3, extends into the aqueous phase. Thus, monazomycin does not "see" changes in the dipole potential term contributing to the overall surface potential, but it does not see changes in the diffuse double-layer contribution to the overall surface potential.

We thank Drs. J. O. Wieth of the Department of Biophysics, University of Copenhagen and R. B. Gunn of the Department of Pharmacological and Physiological Sciences, University of Chicago for allowing us to quote from their unpublished experiments with Albert Cass on the action of phloretin and related molecules on the red cell membrane, and Dr. Keizo Matsuo of the Department of Chemistry, Dartmouth College for his determinations of the dipole moment of phloretin and several related molecules.

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