A Stepwise Mechanism for the Permeation of Phloretin through a Lipid Bilayer

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ABSTRACT The thermodynamics of interactions between phloretin and a phosphatidylcholine (PC) vesicle membrane are characterized using equilibrium spectrophotometric titration, stopped-flow, and temperature-jump techniques. Binding of phloretin to a PC vesicle membrane is diffusion limited, with an association rate constant $>10^8 \text{M}^{-1}\text{s}^{-1}$, and an interfacial activation free energy of $<2 \text{kcal/mol}$. Equilibrium binding of phloretin to a vesicle membrane is characterized by a single class of high-affinity (8 $\mu$M), noninteracting sites. Binding is enthalpy driven ($\Delta H = -4.9 \text{kcal/mol}$) at 23°C. Analysis of amplitudes of kinetic processes shows that 66 ± 3% of total phloretin binding sites are exposed at the external vesicle surface. The rate of phloretin movement between binding sites located near the external and internal interfaces is proportional to the concentration of un-ionized phloretin, with a rate constant of $5.7 \times 10^4 \text{M}^{-1}\text{s}^{-1}$ at 23°C. The rate of this process is limited by a large enthalpic (9 kcal/mol) and entropic (−31 entropy units) barrier. An analysis of the concentration dependence of the rate of transmembrane movement suggests the presence of multiple intramembrane potential barriers. Permeation of phloretin through a lipid bilayer is modeled quantitatively in terms of discrete steps: binding to a membrane surface, translocation across a series of intramembrane barriers, and dissociation from the opposite membrane surface. The permeability coefficient for phloretin is calculated as $1.9 \times 10^{-3} \text{cm/s}$ on the basis of the model presented. Structure-function relationships are examined for a number of phloretin analogues.

INTRODUCTION

There has been considerable biological interest in phloretin, a small dipolar nonelectrolyte that exerts many diverse effects on membrane transport. In a wide variety of biological systems, phloretin strongly inhibits monosaccharide and anion transport (LeFevre, 1961; Czech et al., 1973; Wieth et al., 1974), accelerates lipophilic nonelectrolyte transport and inhibits hydrophilic nonelectrolyte transport (Macey and Farmer, 1970; Owen and Solomon, 1972), and alters ion transport (Levine et al., 1973; Owen, 1974) in a complex way.
Although the mechanism of action of phloretin on these systems remains obscure, specific effects of phloretin on membrane fluidity, membrane potential (Andersen et al., 1976; Cousin and Motais, 1977), and transport proteins (Jennings and Solomon, 1976; Forman et al., 1981) have been observed.

In artificial lipid bilayers of uniform composition, phloretin binds to a set of well-defined, independent sites (DeLevie et al., 1979; Verkman and Solomon, 1980). Phloretin also increases carrier-mediated cation conductivity while decreasing lipophilic anion conductivity (Andersen et al., 1976; Melnik et al., 1977), which suggests that phloretin reduces the positive electrical potential of the membrane interior with respect to the aqueous phase. These results imply that phloretin orients itself at each membrane interface in a direction opposite to the dipole moment of membrane phospholipids.

We have previously reported temperature-jump studies of phloretin binding to phosphatidylcholine (PC) vesicles (Verkman and Solomon, 1980) and showed that the rate of binding of phloretin to the vesicle membrane surface was diffusion limited, with an association rate constant of $2.7 \times 10^{8} \text{ M}^{-1} \text{s}^{-1}$ and a dissociation rate constant of $2,900 \text{ s}^{-1}$ at pH 7.3 and 23°C.

A stepwise model for the permeation of phloretin and phloretin-like dipolar nonelectrolytes across a lipid bilayer is presented in this paper. The model for permeation consists of the diffusion-limited approach of phloretin to a membrane surface, followed by passage over a small interfacial barrier and binding to a set of potential minima near the membrane-solution interface. Phloretin then passes into and diffuses through a large, rate-limiting intramembrane barrier, and then binds to a set of potential minima near the opposite interface. This step is followed by dissociation and passage across the internal interfacial potential barrier. The thermodynamic properties for each step are characterized using temperature-jump, stopped-flow, and equilibrium binding techniques.

**MATERIALS AND METHODS**

Phloretin was purchased from K & K Laboratories (Plainview, NY) and its purity was checked by thin-layer chromatography in chloroform-propanol (3:1). Naringenin was purchased from Sigma Chemical Co. (St. Louis, MO) and was used without further purification. The other phloretin analogues, including (2,4,4'-OH, 2,6-OH, 2,4,6-OH) benzophenone, (2,4-OH, 2,4,6-OH) acetophenone, 2,4,6-OH propiophenone, and benzaldehyde were kindly provided by Dr. Ramon Latorre, Harvard Medical School. These compounds were added to buffers of the following composition: 0.1 M Tris (Fisher Scientific Co., Fair Lawn, NJ) or 0.1 M sodium phosphate (Sigma Chemical Co.) and 0.1 M KCl (Sigma Chemical Co.) from a 0.1-M stock solution in ethanol.

The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH; Gold Label; Aldrich Chemical Co., Milwaukee, WI) was stored as a 1-mM ethanol solution at 0°C and was added to vesicles while vortexing. Buffers having different viscosities were prepared by adding sucrose or glycerol (both Fisher Scientific Co.) to the buffer before adding vesicles or phloretin. Egg PC was obtained from Makor Chemicals Ltd. (Jerusalem, Israel) and gave a single spot after thin-layer chromatography on silica gel G using chloroform-methanol-water (65:25:4).
Lipid Preparation

Unilamellar vesicles were prepared by sonication using the method of Huang and Thompson (1974). Lipid stored at −20°C in ethanol was spun in a rotary evaporator to remove ethanol and suspended in buffer at a lipid concentration of ~25 mM. The suspension was sonicated at 4°C under N₂ for 1 h using a model W 185 sonicator (Branson Ultrasonics Corp., Shelton, CT). The vesicles were then centrifuged at 40,000 g for 1 h to remove titanium particles and lipid debris. Vesicles were stored under N₂ at 5°C and used within 1 wk of preparation. For some experiments, a completely homogeneous solution of single-walled vesicles was prepared by molecular-sieve chromatography on Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) as described by Huang and Thompson (1974). The results of equilibrium and kinetic experiments did not depend upon whether the additional chromatography step was performed.

Equilibrium Binding Studies

Spectrophotometric binding studies were performed on a spectrophotometer (model DU; Beckman Instruments, Inc., Fullerton, CA) using a semiautomatic titrator described previously (Verkman and Solomon, 1980). Temperature control was maintained using a Gilford model 2220 adapter (Oberlin, OH). For temperature-dependent studies, a buffer consisting of 0.1 M sodium phosphate plus 0.1 M KCl at pH 7.3 was used to minimize pH changes. The pKₐ of phloretin was found to vary <0.1 U over a 20°C temperature interval. For the phloretin analogues, equilibrium titrations were performed at 23°C in 0.1 M Tris, 0.1 M KCl, at a pH equal to the pKₐ for the analogue. The wavelength used for equilibrium titrations and kinetic measurements was chosen to maximize the difference in optical density between free and bound analogue.

Temperature-Jump Studies

Temperature-jump studies were performed on an apparatus that has been described in detail elsewhere (Verkman et al., 1980). A 4°C temperature increment was obtained within 4 μs by discharging a 0.1-μF capacitor charged to 15,000 V across a 0.8-ml solution volume. The instrument resolution time is ~5 μs. The initial sample temperature was controlled to within 0.1°C by circulating 20% glycerol in water through the stainless steel electrodes, which were hollowed out to within 60 μm of the tip. The binding of phloretin and naringenin was measured by the absorption of light at 328 nm. Data were recorded on a waveform recorder (Biomation 805; Cupertino, CA) that has an adjustable sampling rate of 0.2 μs to 100 ms per point. Data were then transferred to a PDP 11/34 computer (Digital Equipment Corp., Maynard, MA) for storage on disk and numerical analysis.

Stopped-Flow Studies

Stopped-flow experiments were performed on an apparatus consisting of two hand-driven syringes that propelled solutions into a dual jetstream mixing chamber and observation cell. The mixing chamber and observation cell were designed to replace the temperature-jump electrodes so that the optics and fast electronics developed for the temperature-jump apparatus could be used. The stopped-flow instrument was calibrated by mixing 0.01 M Fe(NO₃)₃ in 0.1 M H₂SO₄ with 0.01 M KSCN, as suggested by the Durrum Instrument Co. (Palo Alto, CA). The dead time of this instrument was <40 ms.
The amount of phloretin bound to vesicle membranes was determined by measuring either light absorption (328 nm) of phloretin or fluorescence (excitation 352 nm, emission 420 nm) of the intramembrane probe DPH. DPH fluorescence is quenched by phloretin by a collisional mechanism and the degree of quenching is proportional to the amount of membrane-bound phloretin (Verkman, 1980).

**RESULTS AND DISCUSSION**

**Binding at the Vesicle/Solution Interface**

**EQUILIBRIUM MEASUREMENTS** Phloretin is a weak acid (HPhl $\leftrightarrow$ H$^+$ + Phl$^-$; p$K_a = 7.3$) in which the uncharged species, HPhl, binds tightly to sites, $S$, on a lipid membrane (LeFevre and Marshall, 1959; Jennings and Solomon, 1976; Verkman and Solomon, 1980) according to the equation
The binding of phloretin to a PC vesicle membrane has been described in terms of a single class of noninteracting sites with an apparent dissociation constant, $K_D$, of 8 μM and a lipid per site ratio, $L:S$, of 4 at 23°C (Verkman and Solomon, 1980). An Arrhenius plot of $K_D$ as a function of temperature, in the range 12 to 50°C, is shown in Fig 1. The data are described well ($r = 0.99$) by a single binding enthalpy of $-4.9 \pm 0.9 \text{ kcal/mol}$ and entropy of $4 \pm 1 \text{ eu}$.

Thus, binding of phloretin to PC vesicles is primarily enthalpy driven at physiological temperatures.

Because the binding of phloretin to a vesicle membrane is characterized by a single apparent affinity and enthalpy, it follows that the binding sites for phloretin are noninteracting. The presence of almost one phloretin molecule per four phospholipid molecules does not affect the affinity for the binding of additional phloretin molecules. These results are in agreement with studies of phloretin binding to phosphatidylethanolamine bilayers (DeLevie et al., 1979), phlorizin binding to PC vesicles (Ehrenspeck, 1975), and phloretin binding to vesicles prepared from red blood cell lipids (Jennings and Solomon, 1976).

Equilibrium determination of a single class of sites gives no information about the distribution of sites within the membrane or of the presence of multiple potential barriers distributed throughout the membrane. A kinetic
approach was therefore used to obtain additional information that is not accessible from equilibrium measurements alone.

**MICROSECOND BINDING PROCESS**
The kinetics of the interactions of phloretin with PC vesicle membranes were investigated using the temperature-jump and stopped-flow techniques. The upper trace in Fig. 2 shows the time course of phloretin binding to PC vesicles after a 4°C temperature jump. The decrease in transmittance of light at 328 nm corresponds to the unbinding of uncharged phloretin from the vesicle. The initial decrease in light transmittance is a single, exponential, concentration-dependent process in the 30-200-μs time range, which has already been described (Verkman and Solomon, 1980). This process is the diffusion-limited binding of phloretin to exposed sites at the outer surface of a vesicle membrane. This binding process has been
characterized at 25°C in terms of a bimolecular association rate constant \( k_1 = 2.8 \times 10^8 \text{ M}^{-1}\text{s}^{-1} \) and a unimolecular dissociation rate constant \( k_{-1} = 2,900 \text{ s}^{-1} \). The association rate constant is inversely proportional to buffer viscosity and is strongly dependent upon buffer pH \( (k_1 \propto 1 + 10^{\beta-H-pK_a}) \), as would be expected for the diffusion-limited binding of the uncharged form of a weak acid.

The temperature dependence of \( k_1 \) is given in Fig. 1. From the slope of this plot, the activation enthalpy is calculated from the Arrhenius equation; the activation entropy is calculated from the intercept of the Arrhenius plot as \( 1/T \) approaches zero (Glasstone et al., 1941). The data fall along a straight line \( (r = 0.98) \) with an activation enthalpy of 9 ± 1 kcal/mol and activation entropy of ~12 eu.

The Transmembrane Process

The slower process (~300 ms) in the upper trace of Fig. 2 is quite different from the fast, diffusion-limited binding process. A detailed consideration of the slow process in Fig. 2 has led us to conclude that it is an intramembrane translocation corresponding to movement of phloretin from a set of potential minima near the external vesicle surface to a set of potential minima near the internal vesicle surface. The arguments that support this conclusion are presented in the following sections.

The movements of phloretin that occur during temperature-jump and stopped-flow experiments are shown schematically in the top section of Fig. 3. Before the temperature jump, the phloretin concentration has been equilibrated with the vesicles. When the solution temperature is suddenly raised, the \( K_D \) for binding is increased and phloretin molecules dissociate from the vesicle. The molecules bound at the external vesicle surface equilibrate with the solution rapidly (<1 ms), whereas the molecules bound at internal sites remain initially fixed. The intravesicular volume is so small that it would contain <1 phloretin molecule if the solution phloretin concentration were 0.5 mM, so that dissociation from internal vesicle sites into the intravesicular volume is not significant. The re-equilibration of internally and externally bound phloretin then occurs over tens and hundreds of milliseconds until the phloretin present in solution is again at equilibrium with the external and internal vesicle sites.

The transmembrane motion of phloretin is also observable using the stopped-flow technique as shown in the bottom of Figs. 2 and 3. A solution containing phloretin is mixed with a vesicle solution and subsequent binding processes are observed optically. There is a fast equilibration (<10 ms) between the solution and external binding sites, as deduced by the difference in light transmittance between a mixture of phloretin and buffer (flat trace at the bottom of Fig. 2) and the beginning of the phloretin-vesicle trace. This initial equilibration is followed by a movement of phloretin from external to internal vesicle sites. An analysis of the amplitudes of the fast and slow processes will be used to establish the distribution of binding between the internal and external vesicle sites. The concentration dependence of the transmembrane
time constants will be used to determine the mechanism and rate of phloretin motion between these two interfacial potential minima.

**DISTRIBUTION OF PHLORETIN BINDING SITES**  For a small perturbation, as in a temperature-jump experiment, the amplitude of the relaxation process is proportional to the number of binding sites that are involved in the re-equilibration of the system at a higher temperature (Hammes, 1974). Hence the amplitude of the fast component is proportional to the number of external binding sites and the total amplitude is proportional to the total number of vesicle binding sites. Therefore, the ratio of the amplitude of the fast surface

![Diagram](attachment:figure_3.png)

**FIGURE 3.** Schematic of phloretin-PC kinetics. In the temperature-jump experiment, phloretin in solution is initially at equilibrium with phloretin bound at external and internal sites on a vesicle. When the temperature is raised, phloretin in solution rapidly equilibrates with the external vesicle sites; this is followed by a slower process in which all three binding compartments equilibrate. In the stopped-flow experiment, the external vesicle sites are filled first, followed by an equilibration among the solution and external and internal vesicle binding sites. At equilibrium, the numbers of solution and external sites are depicted to be equal and twice the number of internal sites.

binding component to the total amplitude is equal to $f_0$, the fraction of total phloretin binding sites that are exposed at the external vesicle surface. 12 temperature-jump experiments performed at 4 different phloretin and vesicle concentrations gave $f_0 = 0.7 \pm 0.07$ (SE).

1 This assumes that the optical densities of phloretin bound at external and internal sites are equal. We reported previously (Verkman and Solomon, 1980) that the optical density of bound phloretin, consisting of contributions from phloretin bound at both external and internal sites, 0.0016 OD/µM, was equal to the optical density of non-ionized phloretin, [HPhl], present in solution. Therefore, a stopped-flow experiment performed at pH 4, where [HPhl] predominates, should give a nonzero amplitude only if external and internal optical densities were different. Because no process is observed experimentally at pH 4, the optical density of bound phloretin is independent of location within the membrane.
The value of $f_0$ can be determined more accurately using the increased signal-to-noise capabilities of a stopped-flow experiment. $f_0$, as defined above, is equal to the ratio of the fast to total amplitude in a stopped-flow measurement as in the bottom of Fig. 2, multiplied by a correction factor that takes the finite concentration changes and the detailed characteristics of the absorption measurement process into account. The correction factor alters the value

\[ f_0 = \frac{A_{\text{fast}}}{A_{\text{total}}} \times \text{Correction Factor} \]

**Figure 4.** Concentration dependence of the intramembrane translocation. The rate of the translocation process, $r^{-1}$, is plotted as a function of the concentrations of uncharged phloretin and naringenin in the external solution. Data for phloretin were obtained using 10-$\mu$M PC vesicles at low pH (Δ; pH 4.0–7.5), high pH (▼; pH 7.5–9.3), in buffer made three times more viscous than water by addition of sucrose (□), and at higher concentrations of PC vesicles (200–800 $\mu$M) at pH 7.3 (●). Naringenin data (○) were obtained at a 20 $\mu$M PC vesicle concentration at pH 7.3. Each data point represents the average of four stopped-flow experiments in 0.1 M Tris + 0.1 M KCl at 23°C and the error bars indicate 1 SD from the mean. The insert shows additional data with a tenfold expansion of the $y$ axis and threefold expansion of the $x$ axis. The plotted lines are weighted least-squares fits to the data.

The correction factor was determined from a calculated calibration curve of fast:total amplitude ratio vs. $f_0$ generated for each total phloretin and vesicle concentration. The calibration curve is determined as follows; for given values of $f_0$ and total phloretin and vesicle concentrations, the concentrations of free and bound phloretin (both ionized and non-ionized) present after the fast and total equilibration are calculated as in Eq. 3. The observed light
of $f_0$ by <10% at the concentrations used and approaches unity for $[\text{HPhl}] >> S_t$. Six stopped-flow experiments performed at two different phloretin concentrations gave $f_0 = 0.66 \pm 0.03$ (SE). This value is consistent with the geometric requirement that the outer surface area of a 200-Å-diam vesicle is 64% of the total surface area (Huang and Mason, 1978) and with the experimental observation that ~70% of total vesicle lipid is present in the outer bilayer (Rothman and Dawidowicz, 1975).

CHARACTERIZATION OF THE TRANSMEMBRANE PROCESS  The time course of the movement of phloretin from one membrane interface to the opposite interface was measured for several phloretin and vesicle concentrations. The results of stopped-flow measurements for phloretin and naringenin (see Fig. 6) are summarized in Fig. 4. The ordinate in Fig. 4 is the observed rate of translocation, given as the reciprocal time constant of a fitted single exponential obtained using the nonlinear Newton’s method. As the legend to Fig. 4 shows, some of the experiments are performed at very low PC concentrations (10 μM), using DPH as an intramembrane fluorescent indicator of phloretin binding to vesicles. DPH at concentrations <1 probe per 100 phospholipid molecules (~30 probe molecules/vesicle) does not alter the equilibrium affinity or kinetics of phloretin binding to vesicles (Verkman, 1980). The abscissa values for the data obtained with 10 μM PC are calculated from the total phloretin added, $P_t$, and $pK_a$ for phloretin because depletion of solution phloretin due to binding is negligible at a site concentration of 2 μM.

$$[\text{HPhl}] = P_t/(1 + 10^{pH-pK_a}).$$

Similar data were obtained using buffers to which sucrose or glycerol were added to give a threefold increase in solution viscosity.

For data obtained at high PC concentrations (200–800 μM), where depletion of solution phloretin is non-negligible, the abscissa values are calculated from the concentration of uncharged phloretin present in solution just after the external sites, $f_0S_t$, have equilibrated with the solution, using the equation:

$$Ee \cdot \text{absorption ratio} = \text{OD} = \varepsilon_b[\text{bound}] + \varepsilon_f[\text{free}],$$

where $Ee$ and $\varepsilon_f$ are the optical densities of the bound and free forms of phloretin determined from equilibrium titrations (Verkman and Solomon, 1980). The OD ratio then determines the measured fast:total amplitude ratio.

The determination of $f_0$ does not in itself prove that the slow process in Fig. 2 is a transmembrane process; for example, the data are consistent with a slow conformational change in the lipid structure induced by phloretin, which makes additional phloretin binding sites available. The most direct evidence for a transmembrane process comes from multilamellar liposome permeability measurements in which phloretin is mixed rapidly with liposomes and the movement of phloretin across multiple bilayers is followed (Verkman, 1980). At 10–100 μM phloretin, the fastest time constant, reflecting the rate of transport across the first layer, is approximately equal to the rate obtained from vesicle experiments. Therefore the slow process in the vesicle experiments represents a transmembrane phloretin equilibration.

$K_D = [\text{HPhl}] [S]/[\text{bound}]$ with $[S] = f_0S_t = [\text{bound}]$ and $P_t = (1 + 10^{pH-pK_a}) [\text{HPhl}] + [\text{bound}]$. 

$3$ The determination of $f_0$ does not prove that the slow process in Fig. 2 is a transmembrane process. 

$4$ Eq. 3 is the solution to the equilibrium equation, $K_D = [\text{HPhl}] [S]/[\text{bound}]$ with $[S] = f_0S_t = [\text{bound}]$ and $P_t = (1 + 10^{pH-pK_a}) [\text{HPhl}] + [\text{bound}]$. 

$5$ Similar data were obtained using buffers to which sucrose or glycerol were added to give a threefold increase in solution viscosity.
\[ [\text{HPhI}] = (P_t + f_0S_t + K_D^{(\text{pH})}) - \left(\frac{(P_t + f_0S_t + K_D^{(\text{pH})})^2 - 4f_0P_tS_t)^{1/2}}{2}\right) \\
K_D^{(\text{pH})} = K_D(1 + 10^{\text{pH}-pK_a}), \tag{3} \]

where \( S_t \) is the total concentration of vesicle sites.

All of the stopped-flow data for phloretin, obtained under very different experimental conditions, as described above, fall along the straight line in Fig. 4 \( (r = 0.98) \) with slope, \( k_{\text{eff}} = (5.7 \pm 0.5) \times 10^4 \text{M}^{-1}\text{s}^{-1} \), and intercept not significantly different from zero \( (0.001 \pm 0.004 \text{s}^{-1}) \). \( k_{\text{eff}} \) is the effective rate constant for translocation over the intramembrane barrier, which will be defined in Eq. 4, below.

Because the translocation rate is a function only of \([\text{HPhI}]\) over a pH range 4.0-9.3, the uncharged form of phloretin is transported through a bilayer many orders of magnitude more rapidly than charged phloretin. The lack of effect of buffer viscosity on translocation rate suggests that the observed process occurs primarily within the membrane and does not involve a bimolecular collision between phloretin in free solution and vesicles. The data taken at high PC concentrations show that the translocation rate depends upon the concentration of uncharged phloretin in solution exclusively and is therefore independent of the phloretin/PC ratio. Experiments performed with 10 \( \mu \text{M} \) PC and 50 \( \mu \text{M} \) \([\text{HPhI}]\) in the presence of 100 \( \text{mM} \) butanol or 20 \( \text{mM} \) halothane, agents known to alter membrane permeability properties (data not shown), gave \( 2 \pm 0.2 \)-fold decreases in the phloretin translocation time constant. These accelerating effects of butanol and halothane on translocation rate add further support to the view that phloretin is translocated through the membrane lipids.

The linear dependence of translocation rate on phloretin concentration is quite surprising. If the movement of phloretin between interfacial potential minima is describable by a unimolecular rate process over a simple thermodynamic barrier, the translocation rate would be independent of phloretin concentration. 1-anilino-8-naphthalenesulfonate (ANS), a negatively charged fluorescent probe, is similar to phloretin in that it binds to two sets of interfacial binding sites with a lipid per site ratio of 4:1 (Haynes and Simkowitz, 1977). In contrast to phloretin, the rate of movement of ANS across the intramembrane barrier is constant for high ANS concentrations, which implies a concentration-dependent permeability coefficient.

Equilibrium binding experiments, in the range \([\text{HPhI}] = 0-100 \mu \text{M} \), have shown that phloretin binds to a single class of sites with an affinity of 8 \( \mu \text{M} \). Because phloretin translocation across the membrane is linear to at least \([\text{HPhI}] = 150 \mu \text{M} \), translocation cannot occur from the 8-\( \mu \text{M} \) binding site, since, for \([\text{HPhI}] \gg 8 \mu \text{M} \), the site should saturate and the observed translocation rate would become independent of phloretin concentration.

It is possible to devise a kinetic scheme to account for the observed linearity between the translocation rate and phloretin concentration by modifying the multibarrier model, in which phloretin encounters a series of potential minima and potential barriers as it crosses the bilayer (Zwolinski et al., 1949; Scheu-
In quantitative terms, consider a model having two phloretin binding sites, \( S_0 \) and \( S_i \), at each membrane interface,

\[
\begin{align*}
HPhl & \overset{k_1}{\rightarrow} P_{S_0} \overset{k_0}{\leftarrow} P_{S_i} \overset{k_{a}}{\rightarrow} P'_{S_0} \overset{k_{a}^{-1}}{\leftarrow} P'_{S_i} \\
S_0 & \quad S_i \quad S_0 \quad S_0' \quad S_i' 
\end{align*}
\]

Primed quantities denote substances present at the internal membrane interface. The observed translocation rates, \( k_{\text{eff}} \) and \( k_{-\text{eff}} \), are the result of a combination of processes: a rapid exchange of phloretin from a high-affinity membrane site, \( Ps_0 \) (equilibrium constant, \( K_1 \)), to a low-affinity site at the foot of the barrier, \( Ps_i \); a unimolecular translocation step with rate constants, \( k_a \) and \( k_{a}^{-1} \); and an exchange between high- and low-affinity sites at the opposite interface. \( K_o \) (also \( K_s' \)) is the equilibrium constant for the distribution of phloretin between the high- and low-affinity sites.

An expression for the translocation rate, \( 1/\tau \), as a function of phloretin concentration is derived in the Appendix for the mechanism in Eq. 4, leading to

\[
\frac{1}{\tau} = \frac{[\text{HPhl}]}{K_1} + \frac{K_1}{K_0}.
\]

For \([\text{HPhl}] \ll K_1/2K_o\), this becomes,

\[
\frac{1}{\tau} = \frac{2k_0K_o}{K_1} [\text{HPhl}].
\]

Therefore, the condition for linearity between \( 1/\tau \) and \([\text{HPhl}]\) is \( K_o \ll K_1/2[\text{HPhl}] \). Because \( K_1 = 8 \mu M \) and because linearity is observed to \([\text{HPhl}] = 150 \mu M \), \( K_o \ll 0.03 \). In energetic terms, the potential minima for the \( S_i \) site must be at least 4 kcal/mol higher than the potential minima for the \( S_0 \) site at room temperature.

It is possible to express the observed bimolecular rate constant, \( k_{\text{eff}} \), in terms of the intrinsic constants, \( K_o, K_1, \) and \( k_a \). Because \( 1/\tau = k_{\text{eff}}[\text{HPhl}] \), \( k_{\text{eff}} = 2k_aK_0/K_1 \). Because the translocation rate does not deviate from linearity over measurable phloretin concentrations, it is not possible to extract exact values for \( k_a \) and \( K_o \); consequently, \( k_{\text{eff}} \) will be taken as an apparent bimolecular rate constant providing a quantitative description of the translocation process.
In qualitative terms, the critical feature of the model given in Eq. 4 is that it is necessary that phloretin binds to a weak site at the "foot" of the large potential barrier to translocation. Phloretin arrives at the weak site only after first binding to the deep potential minima near the membrane interface. This model is consistent with our data, but not unique, because other more complex barrier profiles are also possible.

Permeability Model

The well-defined equilibrium and time-dependent interactions between phloretin and a PC vesicle have made it possible to characterize the individual steps of the overall permeation process in a quantitative manner. Fig. 5 shows a schematic of the barrier to phloretin permeation as derived from the equilibrium and kinetic data presented. The first process is the diffusion-limited association of HPhl onto the external interface of the lipid membrane. $S_0$ represents the high-affinity phloretin binding site, depicted as a deep, interfacial potential minimum embedded among multiple, shallow minima. $S_i$ represents the low-affinity phloretin binding site. The translocation step is the rate-limiting process to permeation; it consists of a high, wide barrier through the hydrophobic membrane interior. $S'_i$ and $S''_i$ are the binding sites for phloretin at the inner interface. The final permeation process is the dissociation of phloretin from the membrane, which occurs within 1 ms, followed by diffusion away from the membrane.

**ASSOCIATION PROCESS**

The association rate constant ($K_1$; process A) has been found to be the same for phloretin and naringenin, $\sim 2.8 \times 10^8$ M$^{-1}$s$^{-1}$, consistent with diffusion-limited binding of a molecule with a diffusion coefficient of $\sim 10^{-5}$ cm$^2$/s (Verkman and Solomon, 1980). The activation enthalpy for phloretin association, 9 kcal/mol, consists mainly of a $\sim 5.5$ kcal/mol contribution from the activation energy for viscosity and diffusion. The positive activation entropy of 12 eu for binding may imply that phloretin is in an ordered state in solution due to alignment of adjacent water molecules, or that, as will be discussed below, the activated binding state may be

For example, a Nernst-Planck model would provide an unstructured "black box" description of the inner portion of the bilayer membrane that would encompass the bracketed quantities in Eq. 4. The physical basis of a Nernst-Planck model is simple diffusive flow within the black box region resulting in a steady state linear HPhl concentration profile across the inner portion of the membrane. Because the concentrations at the edges of the black box are proportional to solution HPhl in the steady state, the translocation rate becomes a linear function of HPhl. In the Nernst-Planck model for bilayer permeability, the partition coefficient, $K_0$, is equal to $dP/D_m$, where $d$ is membrane thickness, $P$ is the permeability coefficient, and $D_m$ is the diffusion coefficient of phloretin within the membrane. Assuming $d$ is $4 \times 10^{-5}$ cm, $D_m$ is $10^{-7}$ cm$^2$/s, and $P$ is $10^{-9}$ cm s$^{-1}$, the partition coefficient becomes $4 \times 10^{-3}$, equal to 3.3 kcal/mol at 23°C, given $\Delta G = -RT \ln K_0$. Because the intramembrane free energy of phloretin in the square barrier Nernst-Planck model, 3.3 kcal/mol, differs significantly from the calculated free energy, 12 kcal/mol at 23°C, for phloretin translocation (from the height of the intramembrane barrier in Fig. 8), there must be additional fine structure within the membrane that is not accounted for by the simple Nernst-Planck description. In particular, the presence of multiple barriers could account for a difference between measured free energy and free energy calculated from an assumed square potential.
degenerate due to multiple, rapid collisions between phloretin and the vesicle surface.

Although the rate of phloretin binding to a PC vesicle membrane is very fast and the measured activation energies are small, there may still be an unmeasured, very large, interfacial potential barrier. The reason that a binding experiment may fail to detect a large, intrinsic membrane barrier is that when a phloretin molecule approaches a vesicle, a successful binding event is likely because phloretin will make many hundreds of attempts to bind before leaving the vicinity. This is similar to the solvent entrapment effect described by chemists to explain the observation that, for reactions in solution, the steric factor used in kinetic calculations is frequently unity. The effects of an interfacial barrier on observed binding can be quantified using classical diffusion theory.

Consider a vesicle of radius $r_0$, having an interfacial potential barrier, $V(r)$. For steady state diffusion, the flux of particles, $J$, proportional to the associa-
The permeation rate, \( J \), is (Amdur and Hammes, 1966)

\[
J = -4\pi Dc_0 \int_{r_0}^{\infty} e^{-v_0/r^2} dr
\]

(7)

where \( c_0 \) is the concentration of particles in solution and \( D \) is the diffusion coefficient. In the absence of a potential when \( V(r) = 0 \), this expression simplifies to the well-known diffusion-limited flux, \( J = -4\pi Dc_0r_0 \).

To illustrate the dependence of the observed binding rate on the radius of the sphere explicitly, the square barrier can be considered,

\[
V(r) = \begin{cases} 
A & r_0 < r < r_0 + L; \\
0 & r > r_0 + L.
\end{cases}
\]

(8)

For this square potential, Eq. 9 gives the ratio of flux in the presence of the potential to the flux in the absence of any potential,

\[
\frac{J_{\text{potential}}}{J_{\text{no potential}}} = \frac{1 + L/r_0}{1 + L\sqrt{A}/r_0}.
\]

(9)

The dimensions of the barrier, \( A \) and \( L \), are intrinsic membrane properties and do not vary with the vesicle radius. Eq. 9 therefore demonstrates an important result: a given interfacial barrier becomes less effective in decreasing binding flux as the vesicle radius increases, at constant total vesicle lipid content. This shows that measurements of binding rates are poor indicators of the intrinsic interfacial barrier. Binding rates may be diffusion limited even though a considerable intrinsic interfacial barrier may exist. The same conclusion may be reached by a stochastic description of the binding process (see Appendix).

Kinetic studies cannot localize binding sites spatially, although kinetic measurements have indicated that two symmetric sets of minima exist and that each set is actually composed of many, rapidly equilibrating local minima. The physical basis for the equilibrium binding enthalpy of \(-4.9\) kcal/mol is some combination of electrical dipole interactions, hydrophobic interactions and hydrogen bonding effects. The interaction energy of the 5.6-debye phloretin dipole with a maximum \( 10^7 \) \( \text{V/cm} \) intramembrane electric field is \(<1\) kcal/mol. Therefore, the dipole interaction alone cannot account for the observed binding affinity between phloretin and a PC vesicle membrane.

COMPARISON WITH PHLORETIN ANALOGUES Some insight into the molecular determinants of binding can be obtained by comparing the binding affinities of various phloretin analogues. Table I shows equilibrium binding and kinetic results for a series of phloretin-like molecules using the equilibrium titration, temperature-jump binding, and stopped-flow translocation techniques developed for phloretin. Fig. 6 shows the molecular structure of the compounds studied. With the exception of benzaldehyde, each compound consists of a hydroxy-substituted phenone moiety coupled with an aceto,
proprio, or benzo group. Phloretin is a 2,4,6-OH phenone coupled by a two-carbon chain with a 4'-OH benzo group, allowing rotational freedom of the 4'-OH benzo group with respect to the phenone. Naringenin and benzophenones do not have this rotational freedom. Benzaldehyde, which was studied as a control, is a nonphenone compound having acid-base and optical properties similar to the phenones; it did not bind to PC vesicles and did not give rise to any observable kinetic processes.

The eight phloretin analogues studied were classified into two types on the basis of binding affinities and translocation rates. Type I analogues bound weakly to PC vesicles ($K_D \geq 50 \mu M$) and had a slow translocation rate relative to the type II analogues. The second column of Table I shows the relative lipid affinity for each of the analogues as approximated by octanol:water partition coefficients.

Each of the type I and type II analogues decreases the dipole potential of a lipid bilayer (R. Latorre, private communication; see also Andersen et al., 1976) and is expected, by analogy with phloretin, to align in the membrane in a direction opposite to the intrinsic membrane potential. The phenone moiety faces the membrane surface, whereas the substituent group is partially immersed in a relatively hydrophobic membrane interior region. Inspection of Fig. 6 and Table I shows that the type II (high-affinity) analogues have a relatively lipophilic substituent group compared with the type I analogues, whereas there is little correlation between overall relative lipid affinity with binding affinity. Therefore, the qualitative picture suggested is an aligned

| Table I
| BOUNDING AND KINETIC PARAMETERS FOR A SERIES OF PHLORETIN-LIKE MOLECULES |
|-----------------|-----------------|-----------------|
| **Compound**    | **Binding**     | **Relative**    | **Translocation** |
|                 | dissociation    | octanol:water   |                   |
|                 | constant*       | partition        |                   |
|                 | µM              | coefficient*‡  |                   |
|                 | $M^{-1}$        | Lipids          | Assoc.          | Relative       | $\Delta H^\ddagger$ | $\Delta S^\ddagger$ | $\Delta G^\ddagger$ |
|                 | $K_D$           | rate*           | rate*           | rate*          | kcal/mol         | m               | kcal/mol         |
| Phloretin       | 8               | 4               | $2.7 \times 10^8$ | 1              | 9±1             | -31±3           | 18±1             |
| Type I analogues|                 |                 |                 |                |                 |                 |                 |
| 2,4,6-OH acetophenone | 165          | 0.24           | 3.2             | $2.9 \times 10^8$ | 13             | 16±1            | 1±4              | 16±2            |
| 2,4,6-OH propionophenone | 79           | 0.31           | 2.1             | 17             |                 |                 |                  |
| 2,4-OH acetophenone | 50            | 0.72           | 60              | 12             |                 |                 |                  |
| Type II analogues|                 |                 |                 |                |                 |                 |                 |
| Naringenin      | 24              | 0.31           | 4               | $3 \times 10^8$ | 33             | 19±3            | 11±3             | 16±3            |
| 2,4,4'-OH benzophenone | 13            | 0.41           | 15              | $4 \times 10^8$ | 42             | 18±3            | 7±3              | 16±3            |
| 2,4,6-OH benzophenone | 12            | 0.41           | 17              | 58             |                 |                 |                  |
| 2,6-OH benzophenone | 6**           | 0.91           | ~10             | 73             |                 |                 |                  |
| Control         |                  | 7,000          | No processes    | observed       |                 |                 |                  |

* Given at 23°C
‡ Given by Cousin and Motais (1977).
§ The value for the activation entropy, $\Delta S^\ddagger$, has been calculated on the assumption that transmission coefficient in the Eyring equation is unity ($A^\ddagger = 1$; Glasstone et al., Eq. 112, Chap. IX [1941]).
¶ Estimated using general partitioning rules (Hansch, 1973).
** Binding curve is nonlinear; estimate of highest affinity site is given.


phloretin analogue having a binding affinity determined in part by ability of the substituent group to "anchor" within a relatively hydrophobic area.

The movement of phloretin from its binding site at one membrane interface to the opposite interface occurs many orders of magnitude slower (∼1 s) than
simple diffusion would predict ($\sim 10^{-7}$ s). The membrane interior presents a large, hydrophobic barrier to phloretin because of the dipolar and hydrophilic properties of phloretin. The amount of energy required to move a 5.6-debye dipole from a dielectric of 80 (water) to a dielectric of 2 (lipid) is $\sim 10$ kcal/mol, assuming a 5-Å dipole radius. In addition, the water:hexane partition coefficient for phloretin is $>5,000:1$ (Jennings, 1976).

The concentration dependence of the observed translocation rate was measured at four temperatures and the results are shown in Fig. 7 for phloretin and naringenin, as well as for two phloretin analogues. The activation enthalpies in Table I are calculated from the slope of the Arrhenius plot and activation entropies are calculated as described previously. Fig. 8 shows the enthalpic and entropic contributions to the free energy for the equilibrium and kinetic processes that have been characterized for phloretin.
Figure 8. Schematic energy profile for phloretin permeation through PC vesicle membranes. The enthalpic and entropic contributions to the free energy profile for phloretin permeation are shown at 23°C using the values already given. The dashed potential barrier labeled “intramembrane” barrier has been arbitrarily shown as a simple square barrier; however, as Fig. 5 points out, there must be additional fine structure not resolved from these experiments (see also footnote 5).

It is instructive to compare the thermodynamic characteristics of phloretin with those of the other analogues. The equilibrium binding of phloretin (Fig. 1) has an association enthalpy of -4.9 kcal/mol, which should be generally representative to the entire class of analogues, because the differences in binding affinity in Table I are equivalent to <2 kcal/mol in $\Delta G$.

The striking difference among the analogues is in translocation rate, which is one to two orders of magnitude slower for phloretin than for any of the others. Table I shows large differences in both the enthalpy and entropy of
activation. $\Delta H^\ddagger$ for the phloretin translocation is 7–10 kcal/mol smaller than for any of the analogues. Because the interaction energy of the dipole is small, this difference has been attributed to hydrogen bonding and indicates that phloretin has to break approximately two fewer hydrogen bonds than any of the others. Phloretin is unique in that its two benzene rings can rotate about the C-C single bond connecting them so that the two benzene rings can come into apposition with one another, forming a U-shaped molecule. This process would bring the 4-OH and 4'-OH groups very close together so that they could hydrogen bond with a single H$_2$O molecule, as can be shown with a Corey-Pauling-Koltun model. The resultant bimolecular complex would then cross the membrane as a whole. Formation of the complex would require that two fewer hydrogen bonds be broken. Furthermore, such a bimolecular complex would be a very ordered structure whose formation could account for the observed $\Delta S^\ddagger$ of $-31$ eu for phloretin, an increase in order that sets phloretin apart from all the other analogues, for which $\Delta S^\ddagger$ is either $\sim 0$ or positive.

As Fig. 6 shows, the structure of naringenin is particularly close to that of phloretin, but the binding dissociation constant of phloretin to PC vesicles is 8 $\mu$M compared with 24 $\mu$M for naringenin. Furthermore, the relative solubility of phloretin in octanol is twice that of naringenin. These differences may be explained by the ability of phloretin to partition into nonpolar environments in the form of a bimolecular complex containing one water molecule.

**TRANSLOCATION PROCESS** Of the three kinetic processes characterized, binding, translocation, and unbinding, the translocation step is clearly rate limiting. At 100 $\mu$M phloretin, for example, the binding time is $3 \times 10^{-5}$ s and the unbinding time is $3 \times 10^{-4}$ s, whereas the translocation time is 0.18 s. Under these conditions, when the translocation time is rate limiting, the phloretin flux, $J$, is equal to $(dA/dt)_{t=0}$, as given in Eq. A2 in the Appendix:

$$J = k_a S_{tot} \frac{[HPhl]}{[HPhl] + K_1/K_0}$$

(10)

in which $S_{tot}$ is the membrane density of total phloretin binding sites at the external membrane surface. Because $k_a = k_{eff}K_1/2K_0$, as shown below Eq. 6, and $[HPhl] << K_1/K_0$,

$$J = \frac{k_{eff} S_{tot}}{2} [HPhl].$$

(11)

The permeability coefficient, $P$, is defined by the relation $J = P\Delta C = PA[HPhl]$. Therefore,

$$P = \frac{k_{eff} S_{tot}}{2}.$$

(12)

Given $S_{tot} \sim 4 \times 10^{13}$ cm$^{-2}$ or $6.7 \times 10^{-11}$ mol cm$^{-2}$ and $k_{eff} = 5.7 \times 10^4$ M$^{-1}$s$^{-1}$, $P$ becomes $1.9 \times 10^{-3}$ cm s$^{-1}$. This value compares reasonably well...
with the calculated permeability coefficient of $1.1 \times 10^{-3}$ cm s$^{-1}$, obtained from measurements of the red cell permeability coefficient given by Jennings and Solomon (1976).

The rate of access of phloretin to surface binding sites on a lipid bilayer is extremely fast and is limited primarily by diffusion of phloretin in solution. Although phloretin partitions strongly into binding sites near the membrane surface, a large energetic barrier, throughout the inner, hydrophobic portion of the bilayer, limits the rate at which phloretin travels between binding sites located at opposite membrane surfaces. Because the rate of unbinding of phloretin from the surface binding site is extremely fast, the permeability of phloretin across a lipid bilayer is limited by the intramembrane potential and not by surface binding and unbinding rates. The empirical relation, $J = P\Delta C$, describes the phloretin permeation through a lipid bilayer well for the concentration range studied. The linearity between transmembrane equilibration rate and external phloretin concentration results from the presence of multiple barriers. Thus, even though binding of phloretin to a lipid membrane is a saturable process, the permeation kinetics appear to be unsaturable. The permeability of phloretin and similar dipolar, nonelectrolyte molecules are describable by the classical permeability equation, $J = P\Delta C$, because the binding of these molecules does not alter membrane properties and the presence of multiple barriers raises the concentration at which deviations from linearity between $J$ and $\Delta C$ occur.

**APPENDIX**

**Derivation of $1/\tau$ for the Mechanism Shown in Eq. 4**

We wish to derive an expression for $1/\tau$ as a function of [HPhl] when HPhl is mixed with $S_o$ in a stopped-flow experiment for the mechanism given in Eq 4. Because under experimental conditions [HPhl] $\gg$ [$S_o$], [$PS_l$] is effectively buffered, which gives a single exponential time course. $A = A_o (1 - e^{-t/\tau})$ for the reaction. A represents the total phloretin present at internal vesicle binding sites, $[P'S'_1] + [P'S'_0]$. A method to calculate an expression for $1/\tau$ is to observe

$$\frac{1}{\tau} = \left(\frac{dA}{dt}\right)_{t=m}/(A)_{t=m}.$$  \hfill (A1)

Given the equilibrium conditions [PS$_1$] = $K_o[S_o]/[PSo]$ and [PS$_0$] = [HPhl][S$_o$]/$K_1$ and the conservation of binding sites condition $S_{tot} = [S_l] + [PS_l]$

6 Jennings and Solomon (1976) found that the permeability coefficient for phloretin in red blood cell membranes is $2 \times 10^{-4}$ cm s$^{-1}$ for [HPhl] $\sim 100$ $\mu$M, pH $= 6.9$. At pH 6.9, [HPhl] = 0.72 of the total phloretin so that the apparent red cell permeability coefficient would be $2.8 \times 10^{-4}$ cm s$^{-1}$ considering [HPhl] as the driving force. Because binding to red cell lipids is characterized by lipids/site ratio $\sim 8$ and because $\sim 50\%$ of the membrane is lipid, this value for the apparent red cell permeability coefficient, $2.8 \times 10^{-4}$ cm s$^{-1}$, should be multiplied by four to compare with the value for PC vesicles given in the text. The correction factor of four gives $(2.8 \times 10^{-4})(4) = 1.1 \times 10^{-3}$ cm s$^{-1}$ for the calculated phloretin permeability coefficient through red cell lipid membranes.


\[
\frac{dA}{dt} = k_o [PS_i] = k_o S_{\text{tot}} \frac{[\text{HPhl}]}{[\text{HPhl}] + K_1/K_0}. \tag{A2}
\]

Assuming that the membrane is symmetric, \(k_a = k_{-a}\) and \(K_0 = K'_0\), and that \(S_{\text{tot}}^o = S_{\text{tot}}^o/2\) as discussed in the text,

\[
(A)_{t=\infty} = \frac{1}{2} S_{\text{tot}} \left( \frac{[\text{HPhl}]}{[\text{HPhl}] + K_1} + \frac{[\text{HPhl}]}{[\text{HPhl}] + K_1/K_0} \right). \tag{A3}
\]

**Figure 9.** A stochastic model for binding to a sphere. A particle positioned at \(r\) binds to a sphere of radius \(r_0\) by a random walk path. The sphere has an interfacial potential barrier; if a particle collides with the sphere, it will either bind or continue diffusion from a radius \(r_*\).

Combining Eqs. A1, A2, and A3,

\[
\frac{1}{\tau} = k_a \frac{[\text{HPhl}]}{[\text{HPhl}] + K_1(K_1 + K_0)/2}. \tag{A4}
\]

Because \(K_1 = 8 \mu M\) and \(K_0 << 1\), and \([\text{HPhl}] >> 8 \mu M\) in most experiments,

\[
\frac{1}{\tau} = k_a \frac{[\text{HPhl}]}{[\text{HPhl}] + K_1/2K_0}. \tag{A5}
\]

**Effect of an Interfacial Barrier on Binding Rates: A Qualitative Description**

Fig. 9 shows a qualitative, molecular explanation of the dependence of binding rates on radius for a constant interfacial potential. The potential, \(V\), acts by allowing only
a fraction, $e^{-v}$, of collisions between the diffusing particles and vesicle to be successful in binding. A successful binding event only occurs in this simplified model by a collision at $r_0$; when the collision is unsuccessful, the particle begins diffusion from radius $r_*$. $r_* - r_0$ is a characteristic of the membrane and does not scale with $r_0$. For a particle at radius $r$, the probability that the particle collides with the vesicle and binds on the first hit is $r_0 e^{-v} / r$ (Berg and Purcell, 1977); the probability that it binds on the second hit is $r_0^2 e^{-v} (1 - e^{-v}) / r^2 r_*$; on the third hit, $r_0^3 e^{-v} (1 - e^{-v})^2 / r^3 r_*$, etc. This infinite geometric series may be summed to give the probability, $P$, that a particle at $r_*$ binds to a vesicle membrane eventually,

$$P = e^{-v} \left( 1 + \sum_{n=1}^{\infty} \left[ \frac{(r_0/r_*) (1 - e^{-v})}{r_0} \right]^n \right) = e^{-v} / [1 - r_0 (1 - e^{-v}) / r_*]. \quad (A6)$$

For a particle that binds successfully, the average number of hits, $H$, that it makes before binding is the total binding probability divided by the probability of a successful collision, $e^{-v}$,

$$H = \left[ 1 - r_0 (1 - e^{-v}) / r_* \right]^{-1}. \quad (A7)$$

Eqs. A6 and A7 demonstrate that an interfacial potential may be overcome partially by making many hits near the membrane surface. If we choose $r_* - r_0 = 1.5$ Å (approximately one-half of a hydrogen bond length) and a 100-Å radius vesicle, $r_0 / r_* = 0.985$. If only 1 out of 20 hits is successful in binding ($e^{-v} = 0.05$), the number of hits, $H$, given by Eq. A7, becomes 15. Therefore the observed binding rate will be 54%, $[1 - (0.95)^{15}]$, of the maximum diffusion-limited binding rate.

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