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The Effect of Phloretin on Red Cell Nonelectrolyte Permeability

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Summary. The effect of phloretin on permeability of small nonelectrolytes into human red cells was shown to be bimodal for hydrophilic molecules and nonbimodal for lipophilic molecules. At low phloretin concentrations (<0.1 mM) hydrophilic as well as lipophilic nonelectrolyte permeation was increased. At high phloretin concentrations (>0.1 mM) the permeability of hydrophilic molecules was decreased, whereas lipophilic molecule permeability continued to be increased. These results suggest that the mechanism for phloretin acting on red cell nonelectrolyte pathways is different for hydrophilic molecules, as compared to lipophilic permeant molecules. The pH of the red cell buffer suspension also influenced the effect of phloretin on nonelectrolyte permeability and the keto or un-ionized form of phloretin (present at low pH), which is known to have a greater affinity for the membrane, had a larger effect on hydrophilic nonelectrolyte permeability than the ionized form of phloretin.

Macey and Farmer (1970) reported that 0.5 mM phloretin inhibits urea, methylurea and glycerol permeability into human red blood cells. More recently, Owen and Solomon (1972) showed that 0.25 mM phloretin also inhibits urea permeability as well as other hydrophilic molecules such as formamide and acetamide, and that 0.25 mM phloretin enhances the permeability of lipophilic molecules such as propionamide and 2,3-butanediol. Macey and Farmer (1970) suggested the possibility that phloretin was inhibiting a specific facilitated transport system for urea. On the other hand, Owen and Solomon (1972) proposed that the effect of phloretin was a general one, influencing the red cell permeability to most nonelectrolytes and showed that 0.25 mM phloretin inhibits permeation of hydrophilic molecules indirectly proportionately to the ether partition coefficient, k_{ether}, of the permeant molecule, whereas phloretin enhances permeation of lipophilic molecules in direct proportion to k_{ether}. Similar observations were

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also detected by Owen and Solomon at 0.1 mM phloretin, but to a lesser extent; i.e., the permeability of hydrophilic molecules was inhibited less and the permeability of lipophilic molecules was enhanced less in 0.1 mM phloretin than in 0.25 mM phloretin. From this brief symmetrical dose-response observation they suggested that the mechanism for the action of phloretin on red cell hydrophilic and lipophilic pathways might be similar. We have investigated in greater detail the dose-response effect of phloretin on red cell nonelectrolyte permeability, and we have found that the effect of lower concentrations (< 0.1 mM) of phloretin on hydrophilic molecule permeation reverses from an inhibition to an enchancement.

Materials and Methods

Red cells were obtained from healthy male or female human donors drawn by venipuncture. The heparinized blood was centrifuged to separate red cells from plasma, and the cells were washed twice with isosmolar buffer. The stock solution contained red cells which were resuspended in buffer to make a red cell volume fraction of about 1%. The isosmolar buffer had a pH of 7.4 and contained 4.7 mM KH_2PO_4, 12 mM Na_2HPO_4, and enough NaCl ($\sim$135 mM) to give a 300-milliosmoles/kg (moSM) solution, unless otherwise specified. The pH of the buffer was varied in some experiments by adding either NaH_2PO_4 to lower the pH, or Na_2HPO_4 to raise the pH, to 4.7 mM KH_2PO_4 and enough NaCl to give a 300-moSM solution. All of the nonelectrolytes were reagent grade. The phloretin was from K and K Laboratories, Plainview, New York, and was used without further purification. The phloretin was dissolved in ethanol and all experiments (including controls) were done at an ethanol concentration of 0.085 M (0.5% by volume). The nonelectrolyte permeability results obtained with red cells incubated in 0.085 M ethanol did not differ significantly from permeability results with cells without ethanol. The red cells were incubated in the phloretin solutions for approximately 30 min at room temperature, unless otherwise specified.

A Durrum stopped-flow apparatus, adapted to monitor the relative intensity of 550 nm wavelength light scattered at 90°, was used to measure the relative effects of phloretin on the permeability of nonelectrolytes. A monochromator slit width of 5 mm was used in the light scattering experiments. The red cell suspension was rapidly mixed (in $\sim$2 msec) with an equal volume ($\sim$0.3 ml) of a hyperosmolar solution containing 750 moSM nonelectrolyte and 100 moSM NaCl. To measure water osmotic permeability, a hypoosmolar solution containing 200 moSM NaCl was rapidly mixed with the isosmolar red cell suspension. In some experiments 0.3 M nonelectrolyte was used instead of 0.75 M in order to minimize the efflux of water prior to the influx of nonelectrolyte. If we assumed no efflux of water occurred with 0.3 M urea it would be necessary for urea to have a reflection coefficient of unity, which is a poor assumption according to the data of Goldstein and Solomon (1960). This approximation is adequate for our purposes since we were only interested in partially reducing the initial osmotic water permeability.

When an isosmolar suspension of red cells is mixed with a hyperosmolar solution of a nonelectrolyte, the red cells shrink as water flows out of the cells down its concentration gradient. At the minimum volume nonelectrolyte followed by water, flow down their concentration gradients into the cells and the cells swell. Typical stopped-flow oscilloscope traces of urea permeating red cells are shown in Fig. 1. As the red cells

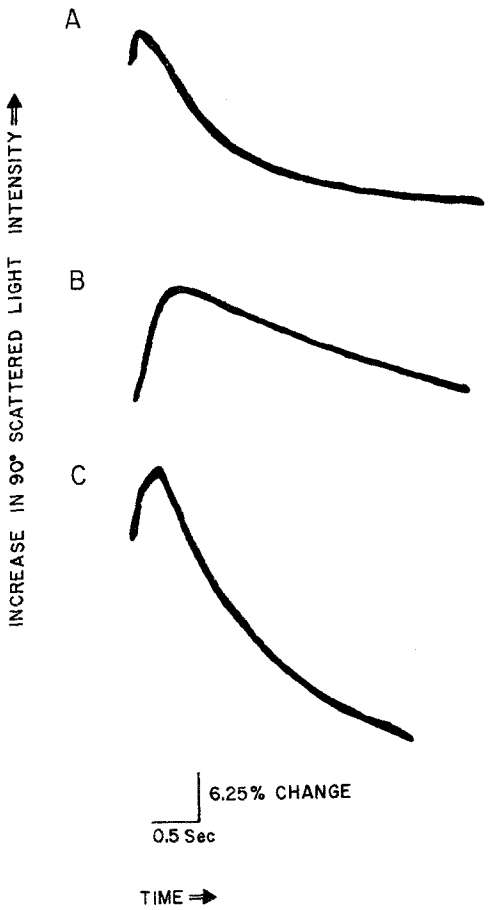

Fig. 1. (*A*) Durrum stopped-flow oscilloscope trace representing an equal volume (0.3 ml) of a 1% by volume suspension of human red cells in isosmolar saline (300 moSM) and a hyperosmolar (750 moSM urea plus 100 moSM NaCl) permeant molecule solution which were rapidly (2 msec) mixed. The initial rapid upward portion of the trace depicts the red cells shrinking due to the osmotic response to the hypertonic urea solution. The later slower downward portion represents the swelling of the red cells as the urea begins to enter the cells. (*B*) Same as (*A*) except the red cells were incubated in 2.5×10^{-4} M phloretin for 1 hr. (*C*) Same as (*A*) except the red cells were incubated in 2.0×10^{-5} M phloretin for 1 hr

shrink, the intensity of scattered light rapidly increases to a maximum value and then slowly decreases as the red cells swell when the nonelectrolyte enters the cells. This is in contradistinction to the previously reported (Sha'afi, Rich, Sidel, Bossert & Solomon, 1967) stopped-flow red cell shrinking-swelling traces which showed a decrease in 90° scattered light as the red cells decreased in volume. This was probably due to an unreported preamplifier on the CAT (Computer of average transients) used to record the stopped-flow oscilloscope traces (Owen, 1972, *personal observation*). This inversion problem in no way detracts from the accuracy of the data of Sha'afi *et al.* (1967), since relative changes in the scattered light were utilized in the permeability coefficient calculations.

Table 1. Comparison between the maximum slope procedure and the minimum volume method at two different phloretin concentrations

Nonelectrolyte	$(X_{phloretin}/X)$ at 0.1 mM/$(X_{phloretin}/X)$ at 0.25 mM		k_{ether} [c]
	Present data $(X \equiv S)$ [a]	Previously reported data $(\Phi \equiv \omega)$ [b]	
urea	2	1.35	0.00047
acetamide	1.4	1.99	0.0025
water	1.3	0.99	0.003 [d]
propionamide	1	0.84	0.013
2,3-butanediol	1	0.76	0.029

[a] Data extrapolated from Figs. 1 through 4.
[b] Data from Owen and Solomon (1972).
[c] Data from Collander (1949).
[d] Data from Sha'afi, Gary-Bobo and Solomon (1971).

Our experimentally obtained light-scattering curves of red cells shrinking and swelling (Fig. 1), as the cells are exposed to a hypertonic permeant nonelectrolyte solution, qualitatively agree with the theoretically obtained data of Latimer and Pyle (1972). Using the Mie equations and assuming red cells behave optically like simple homogeneous spheres of proteins and carbohydrates dissolved in water, Latimer and Pyle (1972) calculated that as cells shrink the amount of 90° scattered light increases.

The rate of volume change at the minimum volume point has been utilized by Sha'afi, Rich, McKulecky and Solomon (1970) to measure red cell permeability, while the maximum swelling rate just after the minimum volume has been utilized by Cohen and Bangham (1972) to measure liposome nonelectrolyte permeability. Hill and Cohen (1972) have developed equations for using the maximum slope of their absorbance time curve after the minimum volume as a measure of nonelectrolyte permeability. Rewriting their Eq. (6b)

$$\left(\frac{dV_a}{dt}\right)^{-1} = \left(\frac{C_i}{C_s}\frac{1}{A\omega\sigma RT} + \frac{1}{A\omega RT}\right)\left(1 - \frac{n_s}{V_a C_s}\right)^{-1} \tag{1}$$

where V_a is the cell volume, C_i and C_s are the outside concentrations of the impermeable and the permeable species, respectively, A is the cell area, ω is the permeability coefficient, σ is the reflection coefficient, R is the gas law constant, T is the temperature, and n_s is the number of permeable solute molecules inside the cell. If we assume that $n_s/V_a \ll C_s$ in our experiments, Eq. (1) becomes

$$\left(\frac{dV_a}{dt}\right)^{-1} = \left(\frac{C_i}{C_s}\frac{1}{A\omega\sigma RT} + \frac{1}{A\omega RT}\right) \tag{2}$$

or rearranging yields

$$\frac{dV_a}{dt} = \omega ART \frac{1}{1 + \dfrac{C_i}{\sigma C_s}}. \tag{3}$$

Although the change in cell volume (as we measured by the change in 90° scattered light in units of volts/sec) is a composite of both the permeability and the reflection coefficient in Eq. (3), the maximum slope of the cell volume change after the minimum volume point agrees closely with the previously reported coefficient data (*see* Table 1).

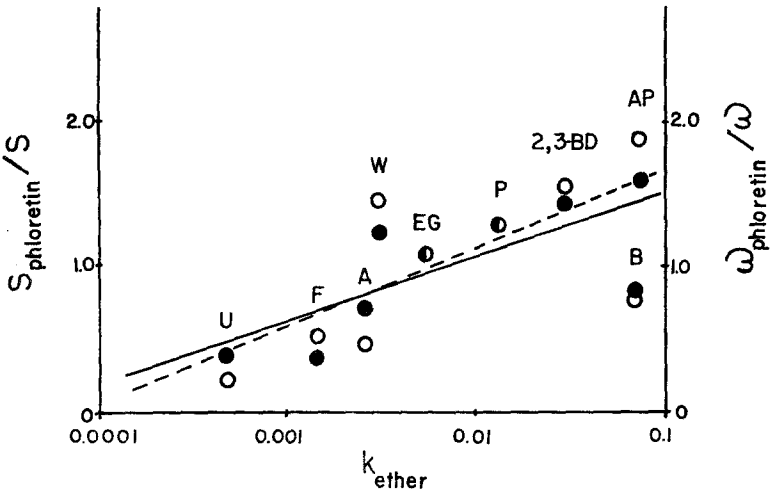

Fig. 2. The comparison between the permeability coefficient ratio data ($\omega_{phloretin}/\omega$) [represented by the open circles and dashed line], and the maximum slope ratio data ($S_{phloretin}/S$) [represented by the solid circles and solid line], using the stopped-flow equipment previously described (Owen & Solomon, 1972). The data is from a typical experiment (Exp. No. 97, 8-23-72) and the lines drawn through the points are least-squares lines. For ($S_{phloretin}/S$) the equation for the solid line is $y=0.44x+0.19$ and for ($\omega_{phloretin}/\omega$) the equation for the dashed line is $y=0.55x+0.027$. The concentration of the phloretin was 0.25 mM. The symbols are: *U*, urea; *F*, formamide; *A*, acetamide; *W*, water; *EG*, ethylene glycol; *P*, propionamide; 2,3-*BD*, 2,3-butanediol; *B*, butyramide; *AP*, antipyrine

Fig. 2 shows the comparison of the minimum method which yields absolute values for the nonelectrolyte permeability coefficient ω with the maximum slope S method which give relative permeability values. For a series of nonelectrolytes treated with phloretin, and using the previously described apparatus (Owen & Solomon, 1972), the minimum method permeability coefficient ratio, ($\omega_{phloretin}/\omega$) [represented by open circles], is compared with the maximum slope method ratio, ($S_{phloretin}/S$) [represented by solid circles], as a function of the nonelectrolyte k_{ether}. From Fig. 2 there appears to be fair agreement between the minimum (dashed line) and the maximum (solid line) method obtained with the previously described equipment (Owen & Solomon, 1972).

A further comparison between these two procedures (minimum volume and maximum slope methods) is made in Fig. 3, which shows our data obtained with the Durrum stopped-flow apparatus using the maximum method. The solid line is the least-squares line drawn through the maximum slope ($S_{phloretin}/S$) data points, while the dashed line is the least-squares line drawn through the numerous ($\omega_{phloretin}/\omega$) data points, previously reported (Owen & Solomon, 1972). Since the agreement between these two procedures and two types of stopped-flow equipment is good, the maximum slope method was used to measure the relative effects of phloretin on the nonelectrolyte permeability in red cells.

Our current phloretin data (Fig. 3) agree with that of Owen and Solomon (1972) except for the two nonelectrolytes, ethylene glycol and 1,3-propanediol. For ethylene glycol our ($S_{phloretin}/S$) of 1.3 (Fig. 3) is lower than the previously reported ($\omega_{phloretin}/\omega$) of 1.8 ± 0.4 (Owen & Solomon, 1972); and for 1,3-propanediol our ($S_{phloretin}/S$) of 1.9 (Fig. 3) is higher than the ($\omega_{phloretin}/\omega$) of 1.3 ± 0.2 (Owen & Solomon, 1972). A closer

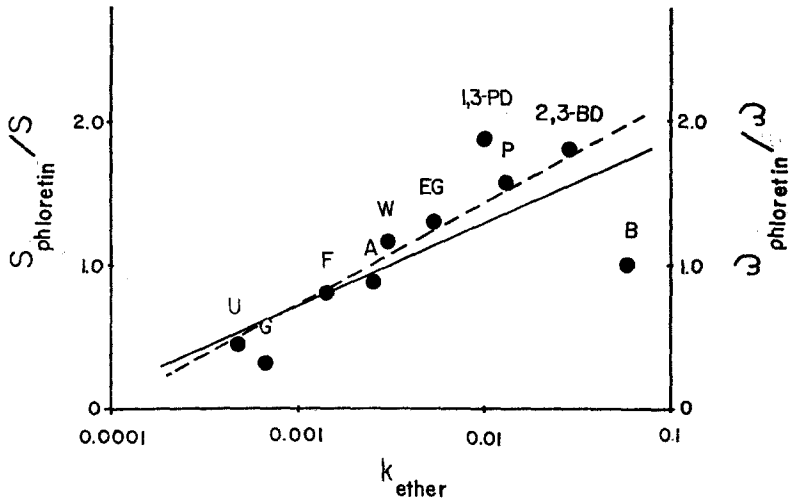

Fig. 3. Same as Fig. 2 except the ($S_{\text{phloretin}}/S$) data points were obtained from a Durrum stopped-flow apparatus. The dashed line is a least-squares line obtained from the several ($\omega_{\text{phloretin}}/\omega$) data previously reported (Owen & Solomon, 1972). The equation for the dashed line is $y=0.73x+0.024$. The solid line is a least-squares line for the ($S_{\text{phloretin}}/S$) data points. The equation for the solid line is $y=0.58x+0.14$. The concentration of the phloretin was 0.5 mM

comparison can be made with our other nonelectrolyte-phloretin data (Fig. 3), although we only wanted to show that we could obtain data with our equipment that compared qualitatively with previously published work.

Results

The phloretin dose-response curve for urea is shown in Fig. 4. The ordinate is the slope ratio ($S_{\text{phloretin}}/S$), which represents the effect of phloretin on the maximum slope, S, as the red cells swell when urea enters the cells. The slope ratio value at 0.25 mM phloretin (extrapolated from Figs. 1 and 4) was qualitatively similar to the previously reported (Owen & Solomon, 1972) permeability coefficient ratio ($\omega_{\text{phloretin}}/\omega$) and at 0.5 mM phloretin, to the ($P_{\text{phloretin}}/P$) value of Macey and Farmer (1970), where P represents the nonelectrolyte permeability as measured with a perturbation technique. At these concentrations, all evaluations showed a significant decrease in urea permeability. Our present investigations indicate that at low phloretin concentrations C_0 ($0.02 \leqq C_0 < 0.06$ mM), urea permeability into red cells was increased (Figs. 1 and 4). A similar bimodal observation was seen with two other hydrophilic molecules, acetamide and glycerol (Fig. 5).

The bimodal effect of phloretin on hydrophilic nonelectrolyte permeability was contrasted with its nonbimodal effect on the lipophilic molecules

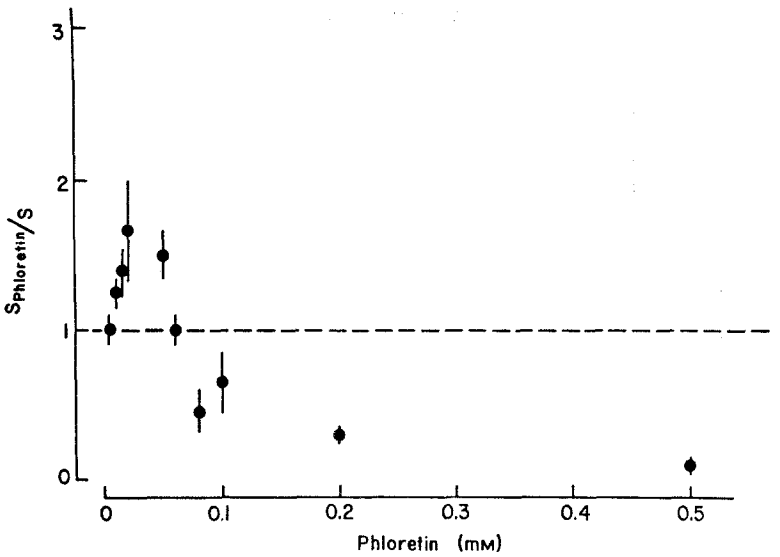

Fig. 4. The dose-response effect of phloretin on urea permeability across human red cells suspended in isosmotic saline buffer at pH = 7.4. The data points represents the mean ± SEM of at least three separate experiments. The ordinate, ($S_{phloretin}/S$), represents the effect of phloretin on the maximum slope which was utilized as a measure of the relative permeability of red cells to nonelectrolytes The dashed line at the ordinate value equal to one denotes the theoretical level at which the phloretin-treated red cell permeabilities are indistinguishable from the untreated or control red cells. Urea is a hydrophilic molecule with a k_{ether} value of 0.00047 (Collander, 1949). A hydrophilic molecule is defined as having a k_{ether} < the $k_{ether} = 0.003$ for water (Sha'afi, Gary-Bobo & Solomon, 1971). A lipophilic molecule has a k_{ether} > the k_{ether} for water

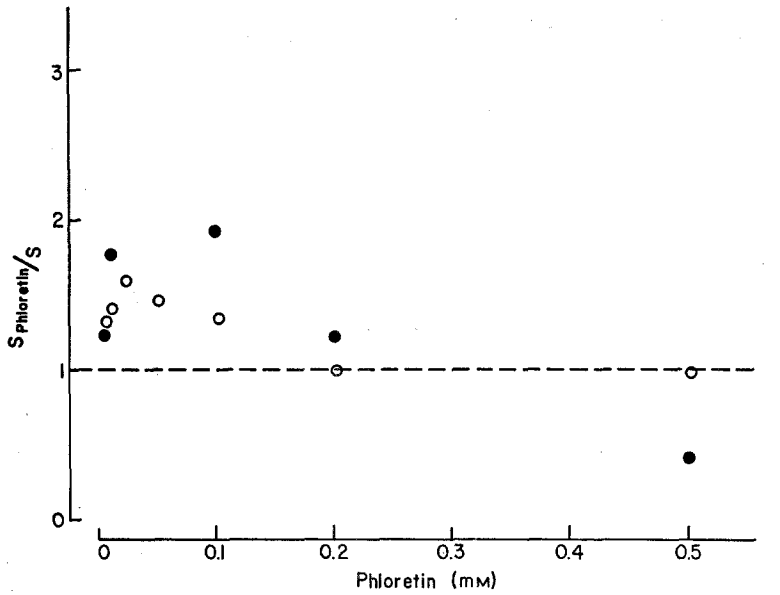

Fig. 5. A single representative experiment depicting the dose-response effect for phloretin on acetamide (○) and glycerol (●) permeabilities into red cells at pH = 7.4. The k_{ether} for acetamide is 0.0025 and the k_{ether} for glycerol is 0.00066 (Collander, 1949)

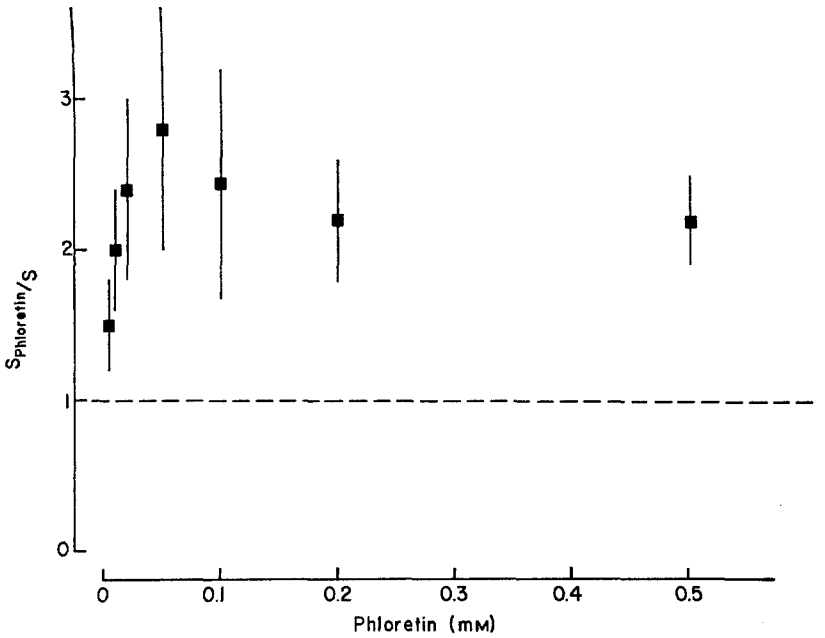

Fig. 6. The phloretin dose-response curve for 2,3-butanediol obtained from at least three experiments. The k_{ether} for the lipophilic molecule 2,3-butanediol is 0.029 (Collander, 1949)

2,3-butanediol (Fig. 5) and propionamide (Fig. 7). The permeability of both of these molecules was enhanced at all phloretin concentrations tested ($0.02 < C_0 < 0.5$ mM). In Fig. 7 the phloretin dose-response effect on osmotic water permeability is also depicted. Phloretin is shown to exhibit essentially no effect on water permeability at low concentrations ($0.02 < C_0 < 0.1$ mM), although it slightly enhanced water permeability at concentrations above 0.2 mM. Although water permeability did not appear to be affected at low phloretin concentrations ($0.02 < C_0 < 0.1$ mM), some experiments were done with 0.3 M urea instead of 0.75 M urea plus 0.05 M NaCl in order to reduce water efflux prior to urea influx. The results with 0.3 M urea at different phloretin concentrations were qualitatively similar to the 0.75 M urea results, and therefore indicated that the apparent increased urea permeability observation at low phloretin concentrations was not due to an increased water permeability.

Since only single representative experiments were reported in Figs. 5 and 7, very little significance can be placed on these nonelectrolyte permeability values at low phloretin concentrations (< 0.1 mM). The experimental data represented in Figs. 5 and 7 show only the qualitative similarity be-

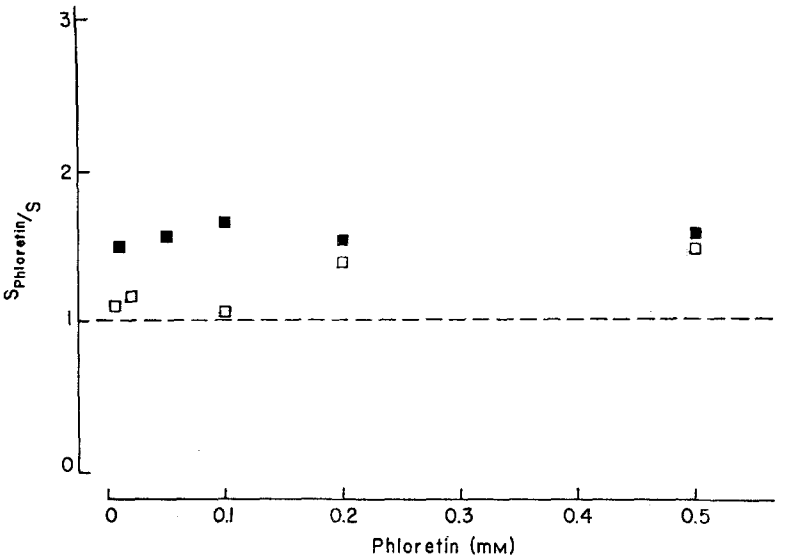

Fig. 7. A single typical experiment which illustrates the phloretin dose-response curves for propionamide (■) and water (□). The k_{ether} for propionamide is 0.013 (Collander, 1949) and for water is 0.003 (Sha'afi *et al.*, 1971)

tween the different hydrophilic nonelectrolyte permeability data obtained with phloretin-treated red cells (Figs. 4 and 5), and the qualitative similarity between the different lipophilic permeant molecule data (Figs. 6 and 7).

Table 1 summarizes the qualitative agreement between our present data and the previously reported (Owen & Solomon, 1972) data for hydrophilic and lipophilic nonelectrolytes at two different phloretin concentrations.

Fig. 8 shows how pH influences the effect of 0.25 mM phloretin on urea permeability. At pH ≅ 6.0 to 7.6, the permeability of urea was greatly inhibited; at pH ≅ 7.6 to 8.0 urea permeability was slightly inhibited; and at pH ≅ 8.3 urea permeability was increased.

In Fig. 9 the effect of pH on the permeability of 2,3-butanediol in the presence of 0.25 mM phloretin is depicted. As the pH was increased from 6.0 to 7.8 the permeability of 2,3-butanediol also increased. At pH > 8 the permeability of 2,3-butanediol continued to increase in phloretin-treated cells as compared with the control, but to a lesser extent than at pH 7.8.

The rate at which phloretin affects red cell nonelectrolyte permeability has not been previously reported. Phloretin is known to react rapidly, and it has been shown elsewhere (LeFevre & Marshall, 1959; Beneš, Kolínská & Kotyk, 1972) that phloretin binds to the red cells immediately after it is mixed with a red cell suspension. This rapid binding has been shown to be exclusively on the membrane surface, and phloretin molecules do not

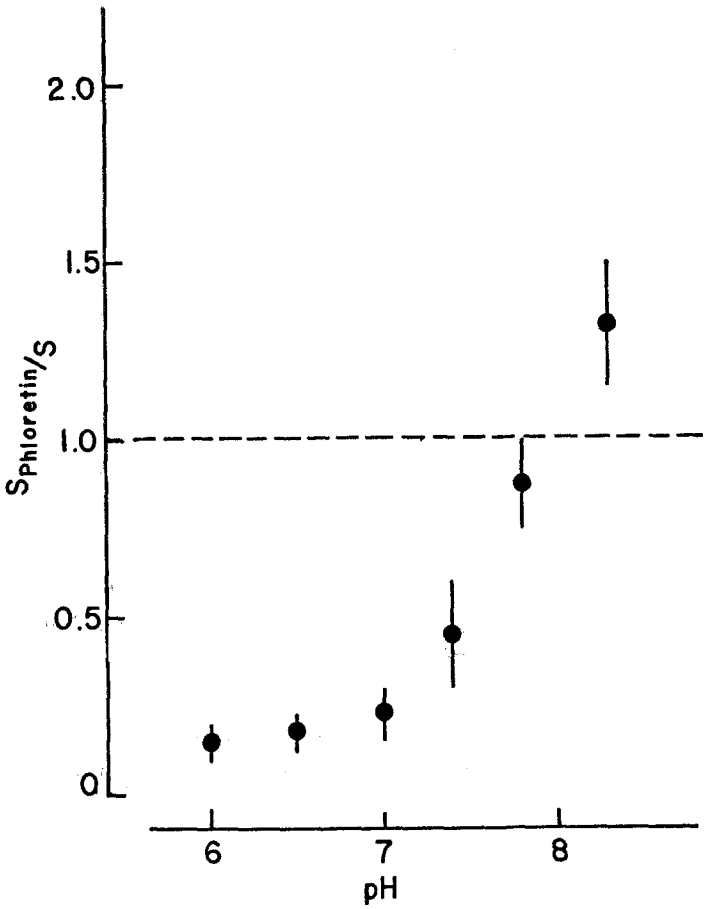

Fig. 8. The effect of buffer pH on the phloretin effect on 0.75 M urea permeability. The concentration of phloretin was 0.25 mM. At least three experiments are summarized by the data points (mean ± SEM)

penetrate the red cell after long incubation times (Beneš *et al.*, 1972). Our current experiments show that at "zero" incubation time, which denotes having the phloretin and nonelectrolyte together in one stopped-flow syringe and the red cell suspension in another syringe, 0.25 mM phloretin had essentially no effect on urea permeability; i.e., $(S_{phloretin}/S) \cong 1.0$. At incubation times of approximately 30 sec the effect of phloretin on urea permeability was similar to the effect observed after 1-hr incubation times. The "zero" incubation time maximum slope value for glycerol with 0.25 mM phloretin was greater than the control; i.e., $(S_{phloretin}/S) \cong 1.8$. After incubating the red cells for either 30 sec or 1 hr in 0.25 mM phloretin an approximate 50% inhibition of glycerol permeability was manifested. Since

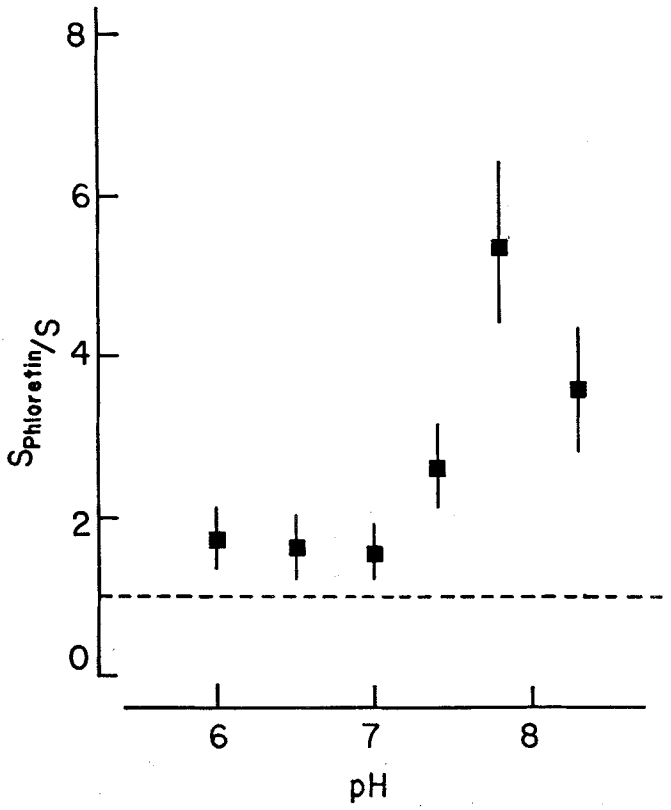

Fig. 9. The dependence of the phloretin effect on 0.75 M 2,3-butanediol upon buffer pH. The concentration of the phloretin was 0.25 mM. The data points represent the mean ±SEM for at least three experiments

the "zero" incubation time effect of phloretin is an increase in glycerol permeability, it appears as if a small portion of the 0.25 mM phloretin contacts the red cells during the rapid mixing process. This is also indicated in Fig. 2 where phloretin concentrations < 0.2 mM yielded an increase in glycerol permeability.

Table 2 depicts the effect of "washing off" phloretin from red cells, where washing denotes resuspending red cells in buffer not containing phloretin. Phloretin has been reported elsewhere (Beneś *et al.*, 1972) to reversibly bind to the red cell surface. Although we were able to partially reverse the effect of phloretin on urea permeability, the permeability never returned completely to the control state. This suggests that either some phloretin molecules remained bound to the cell surface or the effect of reversibly binding phloretin onto the cells left the membrane permanently affected.

Table 2. Effectiveness of repeated washings in buffer for reducing phloretin effect on urea permeability into red cells

Conditions	$(S_{\text{phloretin}}/S)_{\text{urea}}$
1. 1% RBC in buffer incubated 30–60 min in 0.25 mM phloretin	0.16
2. 1st (extra)[a] wash of 1	0.34
3. 2nd (extra)[a] wash of 1	0.53
4. 3rd (extra)[a] wash of 1	0.61

[a] Samples were normally washed twice to separate red cells from plasma. The extra washes were done on samples after the initial washes and incubation in 0.25 mM phloretin.

Discussion

Phloretin molecules exhibit keto-enol tautomerism over the pH range 6 to 9 (LeFevre & Marshall, 1959), where the keto or the un-ionized form of phloretin is predominant at $pH < pK$ ($pK \cong 7.5$) (Owen, 1974), and the enol or the ionized form of phloretin is mainly present at $pH > pK$. The structures of the keto and enol forms of phloretin are shown in Fig. 10. The value of the phloretin cell/medium binding ratio has been previously reported to be proportional to the phloretin molecule keto/enol concentration ratio (LeFevre & Marshall, 1959). The phloretin keto form also appeared to be responsible for the effect of phloretin on urea permeability. At pH 8.3 and a total phloretin concentration of 0.25 mM (Fig. 8) the keto-form concentration was approximately the same as when the pH was 7.4 and the total phloretin concentration was 0.04 mM (Fig. 4).[1] At each of these two different total phloretin concentrations, but similar keto-form phloretin concentra-

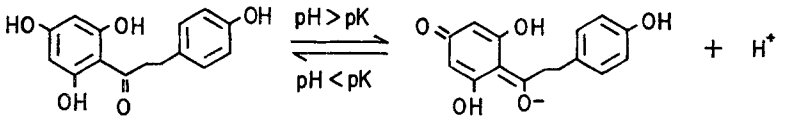

Fig. 10. The effect of pH on the structure of phloretin

1 The phloretin structures in Fig. 10 can be abbreviated by

$$HP \rightleftarrows P^- + H^+ \tag{a}$$

where HP and P^- represent the un-ionized forms of phloretin, respectively. The phloretin dissociation constant K can be written from Eq. (a)

$$K = \frac{a_{H^+}\, a_{P^-}}{a_{HP}} = \frac{[H^+]\, f_{H^+}\, [P^-]\, f_{P^-}}{[HP]\, f_{HP}}. \tag{b}$$

By taking the negative logarithm of Eq. (b) and setting the $\log f_{HP}$ equal to zero according to the extended Debye-Huckel theory, Eq. (b) becomes

$$pK = pH - \log\left(\frac{[P^-]}{[HP]}\right) - \log f_{P^-}. \tag{c}$$

tions, urea permeability was increased. A similar comparison cannot be made with the lipophilic nonelectrolyte, 2,3-butanediol, permeability since its permeability was increased at all phloretin concentrations and pH values investigated and the precision of the data (Figs. 6 and 9) was poorer than the urea data (Figs. 2 and 8).

At low concentrations phloretin may affect red cell nonelectrolyte permeability by specifically interacting with a lipoprotein complex which causes a conformational change in the permeant molecule's pathways (Owen & Solomon, 1972), but at higher phloretin concentrations (> 0.1 mM) there are enough phloretin molecules present to essentially cover the entire red cell surface.[2] It is also at this higher concentration range (> 0.1 mM) that the effect of phloretin shifted from an enhancement of urea permeability at lower phloretin concentrations to an inhibition.

Since the effect of phloretin was bimodal for hydrophilic nonelectrolyte permeability (Figs. 4, 5 and 8) and nonbimodal for lipophilic molecule permeability (Figs. 6, 7 and 9), the mechanism for its effect is probably different for each of these two types of nonelectrolytes.

It is interesting to note that phloretin, at similar concentrations, also has a bimodal effect on the K^+-dependent phosphatase activity in rat brain

The activity coefficient for the ionized form of phloretin f_{P^-}, can be calculated from the Davies equation (Robinson & Stokes, 1959) as shown below

$$\log f_{\pm} = \frac{-A|Z_1|Z_2|\sqrt{I}}{1+\sqrt{I}} + bI \tag{d}$$

where A is a constant $=0.5$, Z_1 and Z_2 are the ionic charges of P^- and H^+, b is a constant $= 0.2$ and I is the ionic strength which can be calculated from

$$I = \tfrac{1}{2}\Sigma C_i Z_i^2 \tag{e}$$

where C and Z are the concentration and charges, respectively, of the various ions in the red cell buffer solution. By calculating values of $I=0.22$ for Eq. (e) and $\log f_{P^-} = 0.12$ for Eq. (d) and substituting these, along with pK $=7.5$ (Owen, 1974), into Eq. (c) and rewriting

$$\log\left(\frac{[HP]}{[P^-]}\right) \cong 7.4 - \text{pH}. \tag{c'}$$

The total concentration of phloretin $[P_T]$ can be written as

$$[P_T] = [HP] + [P^-]. \tag{f}$$

At pH $=8.3$ and $[P_T]=0.25$ mM (Fig. 8), values of $[HP]=0.027$ mM and $[P^-]=0.223$ mM can be calculated from Eq. (c') and Eq. (f). Similarly, at pH $=7.4$ and $[P_T]=0.04$ mM (Fig. 4) values of $[HP]=0.0197$ mM and $[P^-]=0.0203$ mM can be calculated.

2 This follows if: 1) There are 2 million phloretin molecules per red cell when the cells are suspended in 10^{-6} M phloretin solution (LeFevre & Marshall, 1959); 2) if a red cell is assumed to have a 1.67×10^{-6} m^2 surface area (Sha'afi *et al.*, 1967); and 3) if a phloretin molecule is assumed to bind to a red cell in such a manner to cover approximately 85 A^2 (depending upon binding orientation this appears reasonable from CPK models).

(Robinson, 1969). K^+-dependent phosphatase is the terminal hydrophilic step of ATPase, after a Na^+-dependent phosphorylation of the enzyme (Robinson, 1969). The effect of phloretin on urea permeability across toad bladders has been shown to be nonbimodal, and only an inhibition of permeability was detected (Levine, Franki & Hays, 1973). A nonbimodal effect of phloretin on potassium conductance in *Aplysia* neurons has also been reported (Owen, 1974), where an increase in conductance was elicited with phloretin.

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