

Interaction between Phloretin and the Red Blood Cell Membrane

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ABSTRACT Phloretin binding to red blood cell components has been characterized at pH 6, where binding and inhibitory potency are maximal. Binding to intact red cells and to purified hemoglobin are nonsaturable processes approximately equal in magnitude, which strongly suggests that most of the red cell binding may be ascribed to hemoglobin. This conclusion is supported by the fact that hemoglobin-free red cell ghosts can bind only about 10% as much phloretin as an equivalent number of red cells. The permeability of the red cell membrane to phloretin has been determined by a direct measurement at the time-course of the phloretin uptake. At a 2% hematocrit, the half time for phloretin uptake is 8.7 s, corresponding to a permeability coefficient of 2×10^{-4} cm/s. The concentration dependence of the binding to ghosts reveals at least two saturable components. Phloretin binds with high affinity ($K_{\text{diss}} = 1.5 \mu\text{M}$) to about 2.5×10^6 sites per cell; it also binds with lower affinity ($K_{\text{diss}} = 54 \mu\text{M}$) to a second (5.5×10^7 per cell) set of sites. In sonicated total lipid extracts of red cell ghosts, phloretin binding consists of a single, saturable component. Its affinity and total number of sites are not significantly different from those of the low affinity binding process in ghosts. No high affinity binding of phloretin is exhibited by the red cell lipid extracts. Therefore, the high affinity phloretin binding sites are related to membrane proteins, and the low affinity sites result from phloretin binding to lipid. The identification of these two types of binding sites allows phloretin effects on protein-mediated transport processes to be distinguished from effects on the lipid region of the membrane.

Phloretin and phlorizin are inhibitors of membrane transport processes in a variety of systems. Phlorizin is the more potent inhibitor of the active transport of hexoses which occurs in the kidney (1) and small intestine (2). Phloretin is more potent in the inhibition of energy-independent transport processes: sugar transport in the erythrocyte (3), fat cells (4), and rabbit heart muscle (5), anion exchange in the erythrocyte (6), and urea transport in red cells (7) and toad bladder (8). Specific phlorizin receptors, observed in the membranes of fat cells (9) and in kidney brush border membranes (10, 11), are believed to be associated with phlorizin inhibition of sugar transport across these membranes. LeFevre and Marshall (12) have shown that phloretin is bound strongly to red cells and that the extent of binding, in phloretin analogs, parallels potency as glucose transport inhibitors. However, LeFevre and Marshall were not able to detect specific, saturable binding sites for phloretin on the red cell membrane. We have studied the details of phloretin binding to the red cell membrane in an attempt

to identify the interactions responsible for the various effects of phloretin on red cell transport processes.

MATERIALS AND METHODS

Phloretin binding to red cells was measured by the method described by LeFevre and Marshall (12). Fresh blood from healthy donors was drawn by venipuncture into heparin and used within 2 days. Red cells were washed four times in isotonic phosphate buffer before use. Phloretin was obtained from K and K Laboratories (Plainview, N. Y.) and its purity was checked by thin-layer chromatography in 3:1 chloroform:propanol (13). Phloretin was dissolved first in a small volume of 0.1 N NaOH, added to buffer, and titrated to pH 6. No ethanol was used to dissolve the phloretin. All binding experiments were performed at $22 \pm 2^\circ\text{C}$.

Hemoglobin-free red cell ghosts were prepared by the method of Dodge et al. (14). Cells were lysed in 20 vol of 20 mosM phosphate buffer, pH 8, 4°C , washed three times, and resealed by restoring normal osmolality with NaCl and incubating at 37°C for 1 h. 60–70% of the ghosts were resealed by this procedure, as determined by the accessibility of the internal marker enzyme glyceraldehyde-3-phosphate dehydrogenase, as used by Steck (15). The ghost concentration in each experiment was assayed by the acetylcholinesterase content (acetylcholinesterase activity is not lost during ghost preparation) by the method of Ellman et al. (16). Since the acetylcholinesterase activity per cell varied somewhat among donors, a known quantity of red cells was assayed for this enzyme just before lysis, and the enzyme activity per cell from a given donor was used to determine the ghost concentration in suspensions from the same donor.

Phloretin binding to ghosts was measured optically by absorbance loss from the supernatant after a 10-min centrifugation at 35,000 *g*. At concentrations above about 10 μM phloretin, the optical density was measured directly at 286 nm, the absorption maximum for phloretin at pH 6. At lower concentrations, the supernatant phloretin concentration was measured by extraction into ether, drying, and redissolving in a smaller volume of pH 10 carbonate buffer. The phloretin concentration was then measured at 323 nm, which is the absorbance peak at pH 10. The high pH was used in the final step of the assay because phloretin is quite soluble at this pH and also because the extinction coefficient at the high pH peak is higher than that at the low pH absorbance peak. This ether extraction method recovered at least 97.5% of the phloretin present, as judged from extraction of phloretin from solutions of known concentration. Such controls were performed for each experiment, and the losses due to the extraction method did not depend on phloretin concentration.

Phloretin binding to total lipid extracts of red blood cells was measured by the column chromatography method of Hummel and Dreyer (17) using a $30 \times 1\text{-cm}$ column containing Sephadex G50 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). Lipids were extracted from ghosts by a modification of the method of Burger et al. (18). Five volumes (95 ml) of methanol were added with stirring to 19 ml of packed ghosts in a 250-ml centrifuge bottle. The suspension was allowed to stand 5 min at room temperature. Then 95 ml of chloroform were added, and the protein precipitate was spun out after 10 min. The supernatant was decanted into two centrifuge bottles, and chloroform and water were added to a final ratio, chloroform:methanol:water = 10:5:1. The resultant two-phase system was mixed by vortexing and centrifuged for 10 min at 1,000 *g*. The top (aqueous) phase was removed and discarded, and the organic phase, which contained the lipid, was saved. Thin-layer chromatography in chloroform:methanol:water (65:25:4) revealed that all of the major lipid components of the red cell membrane were present. The lipid extract was dried in a flash evaporator, dispersed in a small volume of distilled

water, and sonicated for 25 min at 50 W in a Branson sonicator (Branson Sonic Power Co., Danbury, Conn.) under N_2 , with the temperature controlled at 4°C. The Hummel-Dreyer binding experiments were performed as follows: between 1 and 200 μM phloretin in phosphate buffer at pH 6 was dripped through the column at a constant flow rate. The absorbance at 286 nm of the material emerging from the column was recorded continuously. At the beginning of an experiment, flow was interrupted, a known quantity of lipid, measured as organic phosphate by the method of Gomori (19), was added to the top of the column, and flow was resumed. As the lipid emerged (in the void volume) from the column, the absorbance increased rapidly to a value too large to measure. After the lipid had all passed out of the column, the absorbance returned to its initial value for a minute or two, then decreased to a minimum value, and subsequently returned to the original value. The area of this "depletion trough" was found to be directly proportional to the quantity of lipid added to the column. These control experiments show that, though phloretin is retarded by the column (that is, elutes at a volume larger than the total column volume), the depletion trough area is a quantitative measure of the amount of phloretin bound by the lipid, after a small correction for the volume occupied by the added lipid suspension. The bound phloretin was calculated from the trough area as follows: Bound phloretin (nanomoles) = $acbf/s$, where a = trough area (square centimeters), c = phloretin concentration (nanomoles/milliliter)/absorbance unit, b = number of absorbance units per centimeter, f = flow rate (milliliters per minute), s = chart speed (centimeters per minute).

Phloretin binding to human hemoglobin (crystalline from Sigma Chemical Co., St. Louis, Mo.) was measured by equilibrium dialysis. Dialysis tubing ($1/4$ inch, A. H. Thomas, Philadelphia, Pa.) was washed three times by soaking for 1 h in distilled water at 80°C, and then filled with 2 ml of hemoglobin solution, with a known initial phloretin concentration in isosmolar phosphate, at pH 6. The contents of the dialysis bag were then dialyzed overnight against 23 ml of the same phloretin solution in phosphate buffer at pH 6.0, $22 \pm 2^\circ C$. Equilibrium was completely established by the next morning. Phloretin binding by the hemoglobin was measured as the disappearance of phloretin from the medium external to the bag. Corrections were necessary because of phloretin binding to the rubber stopper in the test tube containing the dialysis tubing and to the dialysis tubing itself. The validity of the dialysis method was checked by performing a binding experiment by the dialysis method, using whole red cells instead of hemoglobin. At 35 μM free phloretin, pH 6, the cell:medium distribution ratio determined by dialysis is 120 ± 15 (two experiments), which agrees well with the value of 124, which we obtained by the direct method of LeFevre and Marshall (12).

The rate of phloretin uptake by red cells was measured by three methods. Two of the methods employed rapid sampling of the extracellular medium after red cells had been suspended in a phloretin solution. Red cells, washed in pH 6 isotonic phosphate, were suspended in 100 μM phloretin, in pH 6 phosphate buffer. In the first method, the cells were rapidly sedimented in a Brinkmann centrifuge 3200 (Brinkmann Instruments, Inc., Westbury, N. Y.) at various time intervals after the resuspension. The supernatants were then assayed (by absorbance at 286 nm) for phloretin. A satisfactory separation of cells from medium could be obtained in 3 s, but the exact time points had an error of about 2 s.

The second method of sampling the supernatant was the Millipore filtration technique described by Dalmark and Wieth (20). Filtration assemblies were constructed exactly as specified by Dalmark and Wieth (20) with the omission of the ring of ice in the filter holders. Because phloretin adsorbs to the filters, only a very rough time-course for the uptake could be determined by this method.

The third method for measuring the rate of phloretin uptake relied on the fact that the

acid (uncharged) form, but not the dissociated (anionic) form of phloretin is taken up by the cells. Uptake of phloretin from an unbuffered phloretin solution therefore results in a pH rise. The rate of pH rise is a measure of the rate of phloretin uptake. Red cells were washed four times in 154 mM NaCl, titrated with HCl to an external pH of 6.90, and resuspended in 200 μ M phloretin, pH 6.90, $26 \pm 1^\circ\text{C}$. The extracellular pH was measured continuously with a pH meter and strip chart recorder. The response time of the system to a pulse of NaOH was 1.5–2.2 s. This finite response time introduced negligible error in the measured time-course of phloretin entry into the red cells. We also attempted to measure the time-course of phloretin binding to lipid model membranes by this method. The lecithin used was prepared from egg yolks by the procedure of Singleton et al. (21), lyophilized, dispersed in 154 mM NaCl, and sonicated for 40 min at 4°C under N_2 in a Branson sonicator.

RESULTS AND DISCUSSION

Phloretin Binding to Red Cells

Phloretin, a weak acid ($\text{pK} \cong 7.3$), is present in two forms in neutral pH: uncharged and negatively charged. It is the uncharged form which inhibits red cell glucose transport (12) and chloride exchange (22). The pH dependence of phloretin binding to red cells indicates that it is the uncharged form which binds to the cells (12). For these reasons, phloretin binding experiments were performed at pH 6, where over 90% of the phloretin is uncharged. The results are plotted in Fig. 1, showing bound phloretin in molecules per cell as a function of free concentration. These results are in quantitative agreement with those obtained by LeFevre and Marshall; the average cell:medium distribution ratio for phloretin in our experiments is 124 ± 4 , compared with 110 reported by LeFevre and Marshall at pH 6. The number of phloretin molecules bound per red cell is a linear function of free concentration, with no saturable binding process detectable within the accuracy of our measurements.

Phloretin Binding to Hemoglobin and to Red Cell Ghosts

The binding of phloretin to hemoglobin-free red cell ghosts and to purified human hemoglobin was determined in order to assess the relative contributions of the membrane and the major intracellular constituent to the total phloretin binding capacity of the intact red cell. The conditions of the binding experiments were identical with those in Fig. 1: pH 6 and $22 \pm 2^\circ\text{C}$. The results are plotted in Fig. 2, in the same coordinates as Fig. 1, using 328-mg Hb/ml packed cells (1.1×10^{10} cells) (23) to normalize the data for hemoglobin.

The data in Fig. 2 show that phloretin binding to hemoglobin is an apparently nonsaturable process of very similar magnitude to phloretin binding by intact cells. The error bars are a reflection of the uncertainty of the phloretin binding by the dialysis apparatus used in the experiment. The similarity between phloretin binding by red cells and hemoglobin suggests that the majority of the phloretin binding to red cells results from phloretin binding to hemoglobin. If most of the binding is intracellular, then isolated membranes should bind only a small fraction of the phloretin bound by intact cells. This is the case, as shown for ghosts in the lower portion of Fig. 2. These data show that about 90% of the

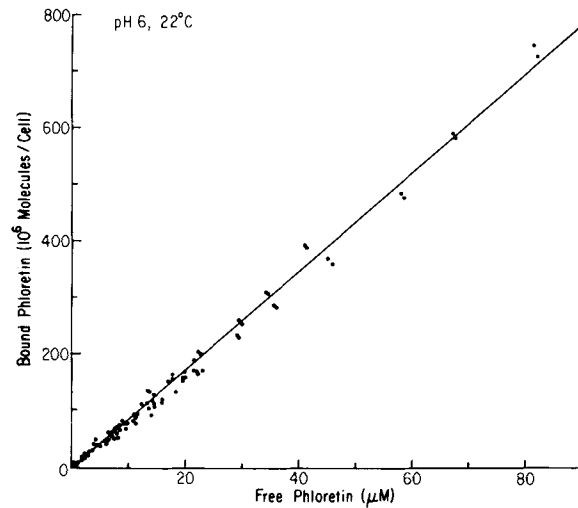


FIGURE 1. Phloretin binding to intact human red cells. Bound phloretin (10^6 molecules per cell) is plotted as a function of free phloretin concentration (micromolar). The data represent the results of 12 separate experiments. The slope of the line is 8.7 ± 0.3 (10^6 molecules/cell, micromolar).

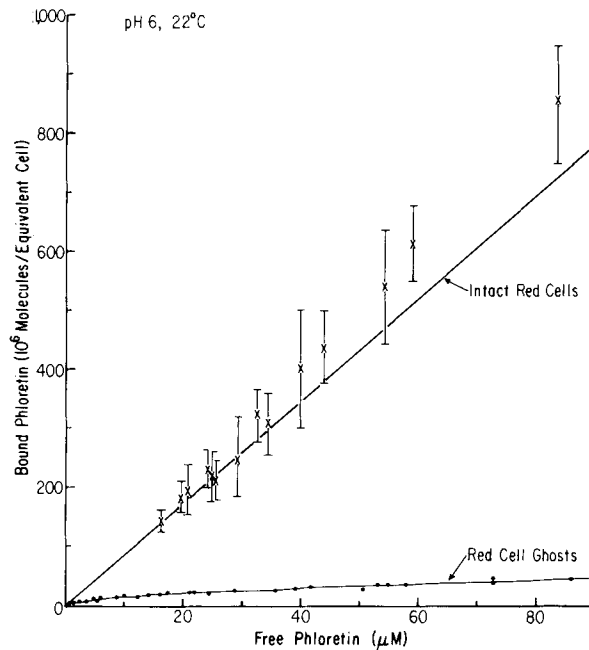


FIGURE 2. Phloretin binding to human hemoglobin (\times) and to hemoglobin-free red cell ghosts (\bullet). Bound phloretin (10^6 molecules per equivalent cell) is plotted as a function of free phloretin concentration (micromolar). The straight line represents the phloretin binding to intact red cells from Fig. 1. The data points for hemoglobin are results of two sets of dialysis experiments. Ghost data points are from 11 separate experiments on three different ghost preparations.

phloretin binding capacity of red cells is lost when hemoglobin and the other intracellular constituents are removed, and that this lost binding can be accounted for by phloretin binding to hemoglobin. This implies that phloretin is able to penetrate the red cell membrane.

The fact that the total phloretin binding to ghosts is a small fraction of the binding to red cells means that studies of the binding of phloretin to ghosts may reveal specific, saturable binding sites which were undetectable in red cells because of the large hemoglobin contribution. The Scatchard plot of phloretin binding to red cell ghosts shown in Fig. 3 has been made from the data for ghosts in Fig. 2. It reveals at least two classes of saturable binding sites for phloretin on the membrane. The steeply sloping left-hand region of the plot indicates the presence of high affinity binding sites. The moderately sloping midregion of the plot indicates saturable binding sites of lower affinity. At the uppermost bound levels, the bound/free ratio tends toward a constant, suggesting that there is also a nonsaturable binding process. As shown in the horizontal line, the contribution of this apparently nonsaturable process is small except at the highest phloretin concentrations. The binding parameters for the two saturable proc-

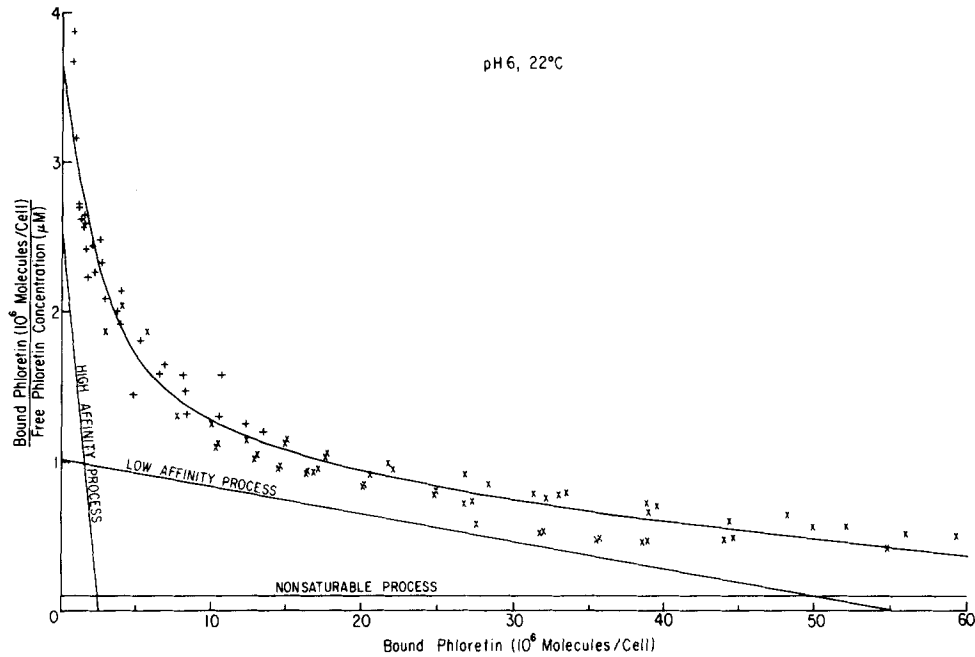


FIGURE 3. Scatchard plot of phloretin binding to hemoglobin-free red cell ghosts. The abscissa is bound phloretin (10^6 molecules per equivalent cell), and the ordinate is bound phloretin (10^6 molecules per equivalent cell) divided by a free phloretin concentration (micromolar). The separation of the Scatchard plot into high affinity, low affinity, and nonsaturable binding processes is discussed in the text, and the curve through the data represents the sum of the three separate binding processes shown. Data were obtained using both direct absorbance assay (\times) and ether extraction assay ($+$).

esses were determined by a nonlinear regression analysis of the data (after the nonsaturable component was estimated and subtracted from the data): the high affinity sites number $2.5 \pm 1.5 \times 10^6$ per cell and have an apparent dissociation constant for phloretin of $1.5 \pm 1 \times 10^{-6}$ M. The low affinity sites are much more numerous: $5.5 \pm 0.5 \times 10^7$ per cell, with an apparent dissociation constant of $5.4 \pm 0.5 \times 10^{-5}$ M. The large uncertainty in the total number of high affinity sites is a consequence of the very much larger number of low affinity sites, which means that a 10% error in the number of low affinity sites causes a much higher uncertainty in the number of high affinity sites.

Phloretin Binding to Red Cell Lipids

We measured the binding of phloretin to sonicated total lipid extracts of red cell ghosts to determine the contribution of membrane lipids to the total phloretin binding by ghosts. As in the other binding experiments, the phloretin binding to lipid was determined at $22 \pm 2^\circ\text{C}$, pH 6, in isotonic phosphate buffer. A Scatchard plot of the results is shown in Fig. 4, using 4.01×10^{-10} μmol lipid phosphorus per ghost (24) to normalize the data to the same coordinates used in Fig. 3. The experimental points fall on a single straight line, indicating that the binding is a simple, saturable process, with an apparent dissociation constant of $4.4 \pm 0.3 \times 10^{-5}$ M and a total number of $5.2 \pm 0.2 \times 10^7$ sites per equivalent cell. At maximum binding, there is one phloretin molecule for every 8.5 lipid molecules, including cholesterol. The binding parameters do not differ significantly from those of the low affinity binding process found in ghosts. Thus the low affinity sites can be identified with red cell lipids. The high affinity binding is absent in red cell lipids, indicating that the high affinity binding sites for phloretin are associated with membrane proteins.

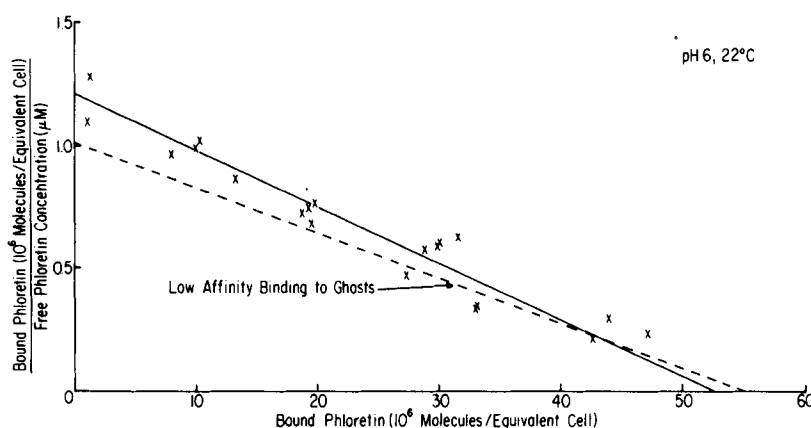


FIGURE 4. Scatchard plot of phloretin binding to sonicated red cell lipids, in the same coordinates as in Fig. 3. Each point is the result of a single Hummel-Dreyer experiment. Two different lipid preparations were used. The solid line is the least-squares fit for the experimental points. The dashed line is the Scatchard plot of the low affinity binding of phloretin to red cell ghosts, obtained from Fig. 3.

Rate of Phloretin Permeation

Our results show that only a small fraction of the phloretin binding by red cells is associated with the membrane. The rest of the binding can be accounted for by phloretin binding to hemoglobin, indicating that phloretin is able to penetrate the red cell membrane. This result contradicts the conclusion of Beněs et al. (25) that the red cell membrane is impermeable to phloretin. They based their conclusion in part on monosaccharide transport inhibition experiments which indicated that external phloretin has no effect on monosaccharide influx into ghosts. Taverna and Langdon (26), however, have shown that external phloretin strongly retards glucose entry into red cell ghosts. Beněs et al. present further evidence derived from phloretin binding and trapping experiments which show phloretin binding to intact red cells to be similar in magnitude to that in ghosts. This result is at variance with our results and also with earlier observations by LeFevre and Marshall (12), who reported that ghosts partially depleted of hemoglobin bind much less phloretin than do intact cells.

The belief that phloretin does not penetrate the red cell membrane was also based on the observations that phloretin uptake by red cells is rapid (12, 25) and that phloretin effects are exerted almost immediately after it is added to red cell suspensions (22, 27). Our argument that phloretin penetrates the membrane would be further strengthened if (a) a time-course of phloretin uptake were measured, and (b) this time-course is shown to be much slower than the rate of phloretin binding to the membrane surface.

Initial experiments showed, as reported earlier by LeFevre and Marshall (12), that the phloretin uptake by red cells is too rapid to be studied by conventional centrifugation techniques. We found that the phloretin uptake is complete 2 min after the cells are suspended in the phloretin-containing solution. It was necessary, therefore, to employ techniques which are capable of more rapid sampling of the extracellular medium. The first method we used was rapid centrifugation in a Brinkmann centrifuge 3200, which is capable of sedimenting red cells in less than 3 s. By this method we obtained a half time of 5–10 s for the uptake of phloretin by red cells (4% hematocrit), although there was a 2-s uncertainty in the exact times the cells were pelleted.

The second method for sampling the supernatant was the Millipore filtration technique employed by Dalmark and Wieth (20) to measure anion efflux from red cells. By this method, the time points could be obtained with an error of less than 1 s. However, phloretin adsorbs to the Millipore filters, creating an uncertainty in the phloretin concentration in the medium, and, therefore, in the half time for the uptake. The half time for phloretin penetration obtained by this method was between 5 and 15 s, in agreement with the centrifugation method.

A more precise method for following the phloretin uptake was based on the fact that phloretin is a weak acid ($pK \cong 7.3$). It is the undissociated form which is taken up by the cells. Therefore, if red cells are suspended in an unbuffered solution at a pH near the pK of phloretin, the extracellular pH will rise. This pH rise results from the fact that the acid (undissociated) form of phloretin is leaving the unbuffered extracellular medium, which is the equivalent of adding base to the medium. Specifically, suppose that initially 200 μM phloretin is present,

unbuffered, at, for example, $\text{pH}_o = 6.9$. At this pH, the ratio of uncharged phloretin (HA) to charged phloretin (A^-) is

$$HA/A^- = 10^{\text{pK}-\text{pH}_o} = 10^{7.3-6.9} = 10^{0.4} \quad (1)$$

So, initially, there is $200/[1 + 10^{-0.4}] = 143 \mu\text{M}$ uncharged phloretin, and $57 \mu\text{M}$ charged phloretin. If red cells (whose equilibrium external pH is 6.90, unbuffered) are suspended in this medium, they will take up an amount, X , of the uncharged phloretin, so that the final free concentration of phloretin is $200 - X$, and the final concentration of uncharged phloretin is $143 - X$. The new pH is given by

$$10^{\text{pK}-\text{pH}} = HA/A^- = [143 - X]/57. \quad (2)$$

If we define the change in extracellular pH as $Y = \text{pH} - 6.90$, Eq. 2 becomes

$$10^{0.4-Y} = [143 - X]/57. \quad (3)$$

If Y is small, 10^{-Y} can be expanded to

$$10^{-Y} = e^{-2.3Y} \cong 1 - 2.3Y, \quad (4)$$

and when Eq. 4 is substituted into Eq. 3 the result is

$$Y = X/330. \quad (5)$$

So Y is a linear function of X , which means that the time-course of the uptake X is the same as the time-course of the pH change Y .

Note that this method assumes that changes in the extracellular pH are independent of the strong buffering capacity of the cell contents. This is not generally the case in a red cell experiment, because rapid chloride-bicarbonate exchange produces pH equilibration between inside and outside media. However, phloretin itself is a powerful inhibitor of anion exchange (6), and therefore should inhibit pH equilibration. In fact, we have found that in the presence of $200 \mu\text{M}$ phloretin, the half time for pH equilibration is 10 min or longer (28), which is quite slow on the time scale of the phloretin uptake experiments. Therefore, during the phloretin influx, the external pH is independent of the intracellular pH.

The phloretin influx experiments using the pH method were carried out as follows. Red cells were washed four times in 154 mM NaCl, and titrated with HCl to an equilibrium external pH of 6.90. These cells were suspended in an unbuffered, $200 \mu\text{M}$ phloretin solution in 154 mM NaCl, pH 6.90, $26 \pm 1^\circ\text{C}$. The extracellular pH was measured by a glass electrode, pH meter, and strip chart recorder. The external pH, subsequent to the addition of the cells, rises with a single exponential time-course ($t = 10\text{--}20$ s) to a value between 7.05 and 7.25, depending on the number of cells added, and then falls with a much slower time-course toward the equilibrium pH of 6.90 (Fig. 5). The pH drop toward 6.90 results from the fact that the buffering power of the cell contents is stronger than that of the phloretin, so that the equilibrium pH is specified mainly by the cell contents.

In order to confirm further that the time-course of the pH rise represents

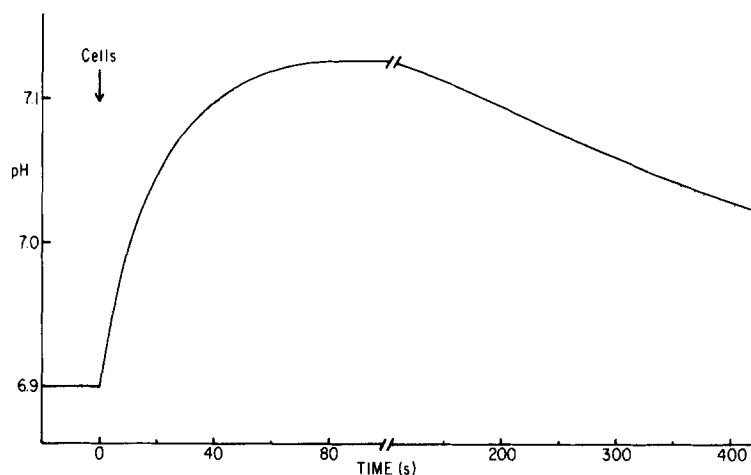


FIGURE 5. Time-course of the extracellular pH of an unbuffered $200 \mu\text{M}$ phloretin solution to which red cells are added (final hematocrit 0.0092). The rapid pH rise represents the uptake of undissociated phloretin. The slower pH drop toward 6.9 results from pH equilibration (by Cl-HCO_3 exchange) between the extracellular medium and the heavily buffered cell contents. The cells had been previously titrated to an equilibrium extracellular pH of 6.9.

penetration of phloretin through the membrane rather than the time-course of surface binding, we tried, using the same method, to measure the rate of phloretin binding to sonicated phosphatidyl choline vesicles. Addition of vesicles to the phloretin solution resulted in a pH rise the time-course of which was not distinguishable from a rapid pulse of NaOH. That is, the time-course of the phloretin binding to the surface of the lipid membranes is too rapid to be measured by this method. The much slower pH rise resulting from red cell additions therefore does not represent surface binding.

The rate constant for the pH rise (and hence for the phloretin uptake) is shown in Fig. 6 as a function of hematocrit. The rate is a linear function of hematocrit with a positive slope and positive intercept. If the line is extrapolated to a 4% hematocrit (the hematocrit used in the rapid centrifugation experiments), the rate constant is $0.125 \pm 0.013 \text{ s}^{-1}$. This corresponds to a half time of $5.5 \pm 0.5 \text{ s}$, in agreement with the centrifugation method.

The reason that the rate constant depends on hematocrit even at low cell concentrations is that the majority of the intracellular phloretin is bound to hemoglobin. From the measured cell:medium concentration ratio for uncharged phloretin (120) and the measured binding of phloretin to the membrane (10% of the total cell binding), it is inferred that the total intracellular phloretin concentration is about 108 times the free (uncharged) phloretin concentration. This means that the intracellular pool of phloretin is about 108 times larger (per unit volume) than the extracellular pool of phloretin. Since cell water constitutes about 70% of the cell volume, the internal compartment per unit solvent volume is 155 times as large as the external pool.

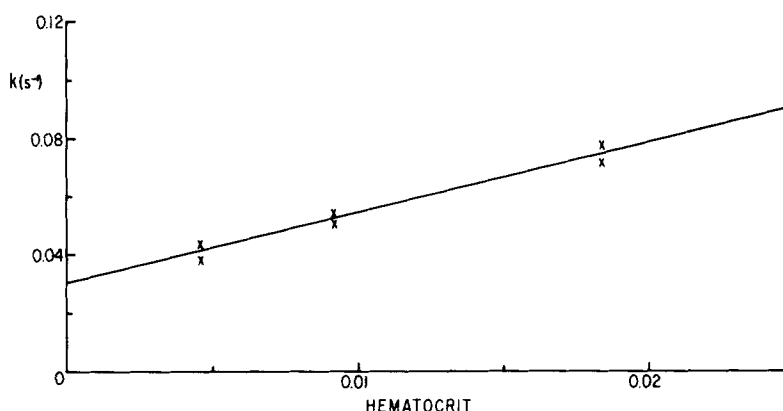


FIGURE 6. Rate constant (s^{-1}) for phloretin uptake by red cells, plotted as a function of suspension hematocrit. The solid line is a least-squares fit of the data.

If there were no binding of phloretin to the hemoglobin, the rate constant in s^{-1} for the uptake would be given by

$$k = [PA][1/V_m + 1/V_{cw}], \quad (6)$$

where P is the permeability coefficient (centimeters per second), A is the membrane area (square centimeters), V_{cw} is the volume of cellular water (cubic centimeters), and V_m is the volume of extracellular water (cubic centimeters). However, the bound phloretin effectively increases V_{cw} by a factor of 155, so the actual rate constant for uptake is

$$k = [PA][1/V_m + 1/155 V_{cw}], \quad (7)$$

which can be written

$$k = [PA/155 V_{cw}][1 + 155 V_{cw}/V_m]. \quad (8)$$

Since P and A/V_{cw} are constants, Eq. 8 shows that the rate of phloretin uptake should be a linear function of the ratio V_{cw}/V_m . The slope divided by the intercept is 155. If the data in Fig. 5 are plotted as K vs. V_{cw}/V_m , the slope:intercept ratio is 114 ± 30 , which is in reasonable agreement with the predicted value of 155. The intercept is $k = 0.030 s^{-1}$. Taking $A/V_{cw} = 2.33 \times 10^4 cm^{-1}$ (22), $P = 2.0 \times 10^{-4} cm/s$. This permeability coefficient is comparable to the permeability coefficient of the red cell membrane for urea ($3 \times 10^{-4} cm/s$; 22) and is perhaps surprisingly high for a solute whose molecular weight is 274. However, phloretin is very lipid soluble, as shown by its large binding by red cell lipids. Consistent with this high lipid solubility is the fact that phloretin has ether-water and octanol-water partition coefficients in excess of 100 (28). It is this high lipid solubility which is responsible for the comparatively large permeability.

Relationship Between Phloretin Binding and Transport Inhibition

Our results show two classes of saturable binding sites for phloretin on the erythrocyte membrane: high affinity protein sites and low affinity lipid sites.

Hence, the various phloretin effects may be classified on the basis of the concentrations at which they occur. The low affinity lipid binding sites are half saturated with phloretin at 50 μM free phloretin at pH 6 (about 100 μM at pH 7.4), so that phloretin effects observed at concentrations of 100 μM or above at pH 7.4 may be attributed to phloretin binding to the lipid region of the membrane.

At high concentrations (100 μM and above), phloretin has an accelerating effect on the transport of several lipophilic nonelectrolytes (29), valinomycin-mediated K exchange, and acetate self-exchange (6) across the red cell membrane. At these concentrations the low affinity binding dominates, indicating that these accelerating effects result from phloretin action on the lipid region of the membrane. There are two mechanisms by which phloretin may interact with the membrane lipids. It has been suggested by Cass et al. (30) that phloretin may change the electrostatic configuration of the lipid polar head groups. This mechanism was proposed to account for the fact that phloretin increases carrier-mediated cation conductance but decreases the conductance of lipophilic anions across planar lipid bilayer membranes.

From the increase in membrane permeability to noncharged solutes a second mechanism may be inferred: an increase in the fluidity of the hydrocarbon chains of the lipid molecules. This increase is consistent with the observation of Cass et al. of an increased carrier-mediated cation conductance. A fluidity increase is also consistent with the fact that phloretin protects red cells against hypotonic lysis (28), as do anesthetics (31). In the case of anesthetics this protection against lysis is believed to result from a hydrophobic expansion of the membrane interior, resulting in an increased membrane fluidity (32). Further evidence of the effect of phloretin in fluidizing the membrane is afforded by the results of Cass et al. (30) who have observed that phloretin at 100 μM doubles the permeability of lecithin-cholesterol bilayers to acetamide. Similar effects have been observed by Poznansky et al. (33) on the permeability of egg lecithin spherical bilayers to urea, formamide, acetamide, and valeramide.

Phloretin effects which occur at concentrations lower than 10–20 μM at pH 7.4 are probably mediated by protein rather than lipid, since only 20% or less of the lipid binding sites are occupied at these concentrations. The protein binding sites for phloretin number between 1 and 4 million per cell and exhibit an apparent dissociation constant of 0.5–2.5 μM at pH 6 (about 1–5 μM at pH 7.4). It is of interest to enumerate those transport processes which are associated with these high affinity binding sites.

Phloretin inhibits red cell glucose transport competitively with an apparent K_i of 0.5–2.5 μM (34, 12). Under the conditions of our binding experiments (pH 6, $22 \pm 2^\circ\text{C}$), we have found that phloretin inhibits glucose efflux from red cells half maximally at 0.6 μM (28). This is in reasonable agreement with the dissociation constant for the high affinity sites. Some subset of these sites is therefore responsible for the action of phloretin on glucose transport. Since phloretin is a competitive inhibitor of glucose transport, large concentrations of glucose should be able to displace phloretin from the glucose transport system. In two experiments, however, we have found that 100 mM glucose is unable to displace

detectable amounts of phloretin from the membrane at free phloretin concentrations of 0.42 and 0.6 μM . Current estimates of the number of glucose transport sites per cell range from 2×10^5 to 3.3×10^5 (35–37). The presence of a total of 300,000 glucose-inhibitable phloretin binding sites would have caused only a 10% reduction in phloretin binding at these concentrations. Our experimental variation in such experiments is about 10%, so 300,000 glucose-inhibitable phloretin binding sites could be present but not detected.

Another phloretin effect which occurs at low concentrations is inhibition of anion exchange across the red cell membrane. At pH 7.7, 0°C, half-maximal inhibition of chloride exchange occurs at about 2 μM (6). At pH 6.2, 23°C, phloretin inhibits chloride-bicarbonate exchange half maximally at 3–5 μM (28). The fact that phloretin inhibits anion transport at such low concentrations indicates that some of the high affinity phloretin sites are related to anion transport. Cabantchik and Rothstein (38) have shown that there are 300,000 anion transport systems on the human red cell membrane, and that anion transport is associated with a single membrane polypeptide. Therefore, 300,000 of the high affinity sites for phloretin may be attributed to the anion transport protein.

The effect of phloretin on urea transport is less well defined than that on glucose transport and anion exchange. Most authors report an inhibition (7, 29), though Owen et al. (27) report an acceleration at concentrations up to about 40 μM and an inhibition at higher concentrations. Wieth et al. (22) and Kaplan et al. (39) have presented evidence that there are two transport pathways for urea across the human red cell membrane. One is not saturable, is not inhibited by phloretin, and is thought to be a result of passive diffusion through the lipid portion of the membrane. The other pathway is saturable, at least for thiourea, exhibits mutual competition between urea and thiourea, and is inhibited by phloretin. Wieth et al. (22) have shown that the saturable pathway (in the case of thiourea transport) is over 95% blocked by 250 μM phloretin at pH 7.2, suggesting that half-maximal inhibition occurs at concentrations well below 50 μM . In separate experiments in our own laboratory we have determined a K_i of about 10 μM at pH 7 for phloretin inhibition of thiourea efflux (28). This K_i is much lower than the apparent dissociation constant for phloretin binding to red cell lipids, indicating that inhibition by phloretin of thiourea transport is not the result of its action on the lipid, but rather results from phloretin binding to a protein component of the membrane. The $2.5 \pm 1.5 \times 10^6$ high affinity sites for phloretin, therefore, represent a heterogeneous group of transport systems, which include about 300,000 anion transport proteins, about 300,000 glucose transport proteins, and an unknown number of thiourea transport pathways.

In addition, the high affinity sites may include the transport pathways for the small hydrophilic nonelectrolytes glycerol, formamide, and acetamide, whose penetration across the human red cell membrane is also inhibited by phloretin (7, 29). A complete dose-response relationship for phloretin inhibition of the permeation of these solutes is necessary in order to establish whether the high affinity phloretin sites are responsible.

The detailed mechanism of the action of phloretin on these transport systems

is as yet unclear. The phloretin binding is noncovalent, in contrast to the binding of amino or sulfhydryl reagents to membrane proteins. The amphipathic character of phloretin as well as the ability of phenolic compounds to form hydrogen bonds are likely the important determinants of phloretin inhibition of protein-mediated transport processes.

Because phloretin inhibits processes which are believed to be protein mediated (e.g., glucose transport and anion exchange) and accelerates passive diffusion of several solutes across the lipid region of the membrane, it is difficult to interpret phloretin effects on the transport of solutes which may penetrate the membrane by two pathways. In the case of thiourea, phloretin inhibits the saturable fraction of the transport but not the nonsaturable fraction. At 500 mM thiourea, i.e., when the nonsaturable transport dominates, 250 μM phloretin has no effect on the transport rate, even though it has completely inhibited the saturable fraction of the flux (22). Phloretin, then, may appear to have very little effect on the transport of a given substance because it inhibits one fraction of the flux and accelerates the other.

This may be the case for water, whose permeation through the red cell membrane is not affected by 250 or 500 μM phloretin (29, 7). The recent results of Brown et al. (40) implicate a membrane protein in the water permeation process. The permeability coefficient of water through lipid bilayers of various compositions has been found to be of the order of $1.3 \times 10^{-3} \text{ cm s}^{-1}$ (41, 42) which is about 40% of the diffusion permeability coefficient of the human red cell to water. This indicates that there may be significant contributions to water transport across the membrane from both lipid and protein components. Since Poznansky et al. (33) have shown that 250 μM phloretin doubles the permeability of egg lecithin bilayers to urea and short chain aliphatic amides, phloretin may also increase the importance of the lipid pathway for water diffusion through the human red cell membrane. The lack of an effect on water transport by 250 μM phloretin, therefore, could result from an increase in the lipid fraction of the flux and an inhibition of the protein-mediated fraction. In order to establish whether this is the case, it will be necessary to compare the effect of various phloretin concentrations on water transport through both lipid bilayers and the red cell membrane.

A further complication in interpreting phloretin effects on red cell membrane transport results from the presence of the nonsaturable phloretin binding sites shown in Fig. 3. Since the nonsaturable binding is not present in isolated lipids, it probably results from nonspecific attachment of phloretin to membrane proteins, similar to the nonsaturable binding of phloretin to hemoglobin. At a phloretin concentration of 200 μM , pH 6, this protein binding represents roughly 30% of the total binding to the membrane. This indicates that, although the majority of the phloretin binding is to the lipid, the nonspecific protein binding could contribute to phloretin effects which occur at concentrations of 200 μM and above. Thus, interpretation of the action of phloretin on cell membranes requires a detailed study of the dose-response curve in order to identify the membrane components responsible for the phloretin effects.

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