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Control of nonelectrolyte permeability in red cells

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SUMMARY

Phloretin is shown to alter the permeability coefficients, ω, of both hydrophilic and lipophilic solutes in human red cell membranes. At a phloretin concentration of 0.25 mM, the hydrophilic solute, urea, has an $\omega = 0.34$ control whereas the lipophilic solute, 2,3-butanediol, has an $\omega = 2.1$ control. Thus, phloretin, to which the membrane is impermeable, affects both hydrophilic and lipophilic permeation pathways. Our results are consistent with the view that phloretin interacts with a membrane protein to cause allosteric actions in which the conformational change effective in the aqueous path also controls lipid permeation.

Macey and Farmer[1] have shown that phloretin inhibits the permeation of human red cells by small hydrophilic nonelectrolytes such as urea and glycerol, whereas it exercises little or no effect on the permeation of other small molecules such as 1,3-dimethylurea and ethylene glycol. They also found that phloretin did not affect water flow as measured by the hydraulic conductivity of the membrane. These observations led Macey and Farmer to suggest that the transport of urea and glycerol into the red cell takes place by a phloretin inhibited facilitated diffusion system. In order to seek a more general explanation of this phenomenon, we have studied the effect of phloretin on the permeability coefficients of lipophilic as well as hydrophilic solutes.

Our observations show that phloretin exercises a general and far-reaching effect on the permeability coefficients, ω, of both lipophilic and hydrophilic solutes. The ratio $\omega_{\text{phloretin}}/\omega$ is dependent upon the logarithm of the ether:water partition coefficient, k_{ether}, in a symmetrical way so that the permeability coefficient of lipophilic molecules is increased by phloretin, whereas permeation by hydrophilic solutes is inhibited.

The permeability coefficients were measured on freshly drawn human red cells

(heparin 10 000 units; 0.2 ml/100 ml blood) by the minimum method of Sha'afi *et al.*[2]. The blood was suspended in a buffer (108 mM NaCl, 4.00 mM KCl, 1.54 mM $NaH_2PO_4 \cdot H_2O$, 24.9 mM $NaHCO_3$, 1.27 mM $CaCl_2$ and 1.19 mM $MgSO_4 \cdot 7H_2O$) to give a 3% suspension (by volume of whole blood). pH was adjusted to approximately 7.4 by aerating with 5% CO_2–96% air. The experiments lasted for many hours and pH rose by about 0.2 pH unit during this time. The osmolality was about 270 mosM, as measured with a Fiske G 62 osmometer (Fiske Associates, Inc., Uxbridge, Mass.). Phloretin was prepared by hydrolysis[3] of phlorizin obtained from Fluka (Buchs, Switzerland) and added to produce a final concentration of 0.25 mM in the buffer in which the cells were suspended, unless otherwise noted. 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 cells were incubated with phloretin for periods of 75 min or more. All solutes were reagent grade obtained either from Aldrich Chemical Co. (Milwaukee, Wisc.) or Eastman Kodak Co. (Richester, N.Y.).

Fig. 1 shows the least squares line relating the ratio, $\omega_{phloretin}/\omega$, taken from our data in Table I, to the logarithm of k_{ether} as given by Collander[4]. The point for butyramide is an exception to the general linear trend for reasons that we do not understand. Nonetheless, the correlation is significant with a P of 0.01. We have computed relative permeability coefficients from the histogram of Macey and Farmer[1] at 0.5 mM

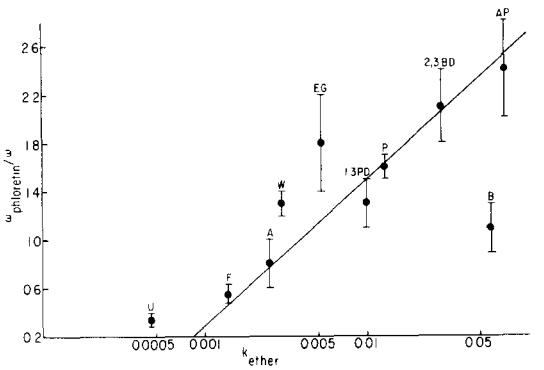

Fig. 1. Effect of phloretin on nonelectrolyte permeability coefficients as related to the ether:water distribution coefficient. The symbols are: U, urea; F, formamide; A, acetamide, W, water; EG, ethylene glycol; 1,3 PD, 1,3-propanediol; P, propionamide; 2,3 BD, 2,3-butanediol; B, butyramide; AP, antipyrine. The line has been drawn according to the method of least squares.

phloretin (*plus* 0.5% ethanol) and the least squares line relating these coefficients to log k_{ether} is shown in Fig. 2. The slopes of the least squares lines in Figs 1 and 2 are the same (0.8 ± 0.2 for Fig. 1; 1.0 ± 0.2 for Fig. 2), as are the intersection with the abscissa [$(0.6 \pm 0.2) \cdot 10^{-3}$ for both]. In this concentration range, Macey and Farmer found phloretin to have little or no effect on the hydraulic conductivity, L_p. We have found that phloretin at 0.25 mM increases L_p by a factor of 1.3 as shown in Table I. The hydraulic conductivity is a different parameter than the permeability coefficient, ω_{H_2O}, but the

TABLE I

EFFECT OF PHLORETIN ON NONELECTROLYTE PERMEABILITY COEFFICIENTS

The ratios are averages ± S.E. The number of experiments is in parentheses.

Solute	$\omega_{phloretin}/\omega$	k_{ether}	$\omega_{phloretin(0\ 1\ mM)}$ / $\omega_{phloretin(0\ 25\ mM)}$
Urea	0.34 ± 0.05 (10)	0.00047	1.35
Formamide	0.55 ± 0.08 (5)	0.0014	1.18
Acetamide	0.8 ± 0.2 (6)	0.0025	1.99
Ethylene glycol	1.8 ± 0.4 (5)	0.0053	0.88
1,3-Propanediol	1.3 ± 0.2 (2)	0.010	
Propionamide	1.6 ± 0.1 (5)	0.013	0.84
2,3-Butanediol	2.1 ± 0.3 (7)	0.029	0.76
Butyramide	1.1 ± 0.2 (9)	0.058	0.59
Antipyrine	2.4 ± 0.4 (5)	0.073	
	$L_{p(phloretin)}/L_p$		$L_{p(phloretin,\ 0.1\ mM)}$ / $L_{p(phloretin,\ 0.25\ mM)}$
Water	1.3 ± 0.1 (7)	0.003*	0.99

*C.M. Gary-Bobo, private communication.

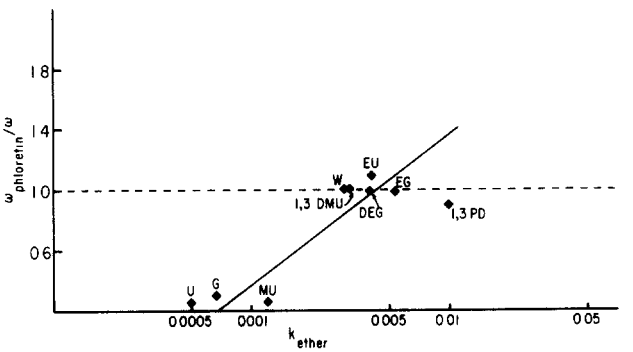

Fig. 2. Data of Macey and Farmer[1] plotted as in Fig. 1. The additional symbols are: G, glycerol; MU, methylurea; 1,3 DMU, 1,3-dimethylurea; DEG, diethylene glycol; EU, ethylurea. The line has been drawn from their histogram by the method of least squares.

two coefficients may well relate to properties of the same channel. We have therefore, treated the ratio, $L_{p(phloretin)}/L_p$ as equivalent to $\omega_{phloretin}/\omega$ in Figs 1 and 2. The value of 0.003 for k_{ether} for water has been determined by C.M. Gary-Bobo (private communication).

Lefevre and Marshall[5] have pointed out that phloretin is concentrated at the red cell surface. The pH-sensitive distribution ratio has a value of about 30 at pH 7.4 and is not affected by 0.085 M ethanol. This resultant high concentration of phloretin molecules

on the red cell surface would make it possible for different mechanisms to control the permeation of lipophilic and hydrophilic solutes. Therefore, in one experiment, the results of which are given in the last column in Table I, we compared the effect of phloretin at 0.1 mM with that at 0.25 mM. The dependence of k_{ether} is also apparent at 0.1 mM phloretin though the slope is smaller as shown from the ratio $\omega_{phloretin(0.1\ mM)}/\omega_{phloretin(0.25\ mM)}$ which is greater than unity for all the hydrophilic solutes and less than unity for the lipophilic ones. This symmetry in dose response is important because it supports the view that the same process is responsible both for promoting lipophilic permeation and inhibiting hydrophilic permeation.

An examination of the interactions of phloretin and its analogues in other systems has led us to the view that the effect of phloretin on nonelectrolyte permeability of red cells is actuated through an allosteric interaction with a surface extension of a membrane protein. Beneš *et al.*[6] have shown that phloretin can not penetrate across human red cell membranes so that the interaction controlling permeability must take place on the outside of the membrane. The evidence that phloretin can interact with a red cell protein arises from a comparison of specific effects on sugar transporting systems in human red cells with those in *Escherichia coli*. Evidence for an allosteric mode of action is provided by a study of the interaction between (Na^+–K^+)-activated ATPase from rat brain and phlorizin, which is phloretin-2′-β-glucoside.

Facilitated hexose transport across human red cell membranes and its inhibition by phloretin at 10^{-5} to 10^{-6} M has been studied in detail by Lefevre[7]. Batt and Schachter[8] have pointed out that facilitated β-galactoside diffusion into *Escherichia coli* is a useful model for, and shares many characteristics with, red cell hexose transport including: complete reversibility on washing, the effects of pH, the sensitivity to molecular analogues of phloretin such as diethylstilbestrol and the presence of counter transport. Studies of Fox and Kennedy[9] in *Escherichia coli* have indicated that a sulfhydryl containing protein is intimately associated with the transport of galactoside across the membrane. These several results have led us to infer that the specific action of phloretin in inhibiting hexose transport across human red cell membranes involves an interaction between phloretin and a membrane protein.

At higher concentrations, similar to those used in the present study, phloretin and its analogues have been shown to inhibit other transport related systems. Poznansky and Solomon[10] have shown that 0.5 mM phloretin inhibits red cell Na^+ and K^+ transport. In a similar concentration range, phlorizin has been shown by Britten and Blank[11] to inhibit (Na^+–K^+)-activated ATPase obtained from rabbit kidney and this observation has been confirmed by Robinson[12] for (Na^+–K^+)-activated ATPase from rat brain. Robinson has shown that the ATPase inhibition involves a resetting of the Na^+ and K^+ optima for enzyme activity, an explanation which is consistent with the findings of Poznansky and Solomon. Robinson advances persuasive evidence to support his conclusion that the effect is the result of an allosteric interaction between inhibitor and enzyme.

Protein control of an aqueous pathway would require a protein or protein complex in contact with the aqueous medium on both the inside and the outside of the

cells and Bretscher[13,14] has presented evidence that a protein with these properties traverses the human red cell membrane.

Fig. 1 shows that phloretin affects both hydrophilic and lipophilic molecules. These classes of solutes have been shown to cross cell membranes by separate pathways, as for example: in Chara by Collander and Bärlund[15], across the gall bladder epithelium by Smulders and Wright[16] and across the red cell membrane by Solomon[17]. Phloretin could affect these separate pathways by separate actions on the protein and lipid portions of the cell membrane but the preliminary dose response data has not given clear evidence for more than one site of action. The alternative explanation is that phloretin acts at a single site which controls the permeation of both hydrophilic and lipophilic solutes. This would require a high degree of organization within the lipid portion of the membrane, a possibility which is supported by the very high activation energies of 19 kcal·mole^{-1} and more which Galey *et al.*[18] have shown to characterize red cell membrane permeability to lipophilic solutes. According to this view, the control of both lipophilic and hydrophilic permeation would be mediated by a protein or a protein complex through allosteric actions in which the configurational change that was effective in the aqueous path would also affect lipophilic permeation probably by interactions with membrane lipids. A conformational change of this kind could well be relevant to other processes which are critically dependent upon the physical and chemical state of the membrane.

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