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Effect of Phloretin on Monosaccharide Transport in Erythrocyte Ghosts

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Summary. ^{3}H-labelled phloretin was shown to be bound reversibly by human erythrocyte and ghost membranes but not to penetrate across them in either direction. Kinetic parameters of D-xylose and D-galactose transport in intact cells and in ghosts, as well as the inhibition by phloretin of these transports were found to be in fair agreement. By enclosing phloretin in ghosts, its inhibition of monosaccharide transport was found to be symmetrical and thus an equivalence of the outer and the innner membrane sides of the human erythrocyte was demonstrated.

An interesting phenomenon observed in monosaccharide transport by erythrocytes is an asymmetric inhibition thereof by phloretin. This aglycone of phlorizin inhibits the efflux of monosaccharides from erythrocytes and has practically no effect on their uptake. Some of its derivatives show an identical type of inhibition but differ in their potency (LeFevre & Marshall, 1959; Kotyk, Kolínská, Vereš & Szammer, 1965). A detailed analysis of this inhibition (Rosenberg & Wilbrandt, 1962) shows that it might be due to two factors: (1) impermeability of erythrocyte membranes to phloretin; (2) an inhibitory trans-effect of the compound. The above authors advanced the hypothesis of an enzyme-plus-carrier transport system where the association and the dissociation of the carrier substrate complex is catalyzed by one or two different enzymes. In this model, phloretin reacts both with the carrier and with the enzyme. But, either alone or in a complex with the carrier, it cannot pass across the membrane. With respect to the monosaccharide substrate, phloretin behaves as a competitive inhibitor, similarly to phlorizin (LeFevre, 1954) and polyphloretin phosphate (Bowyer & Widdas, 1958), although more modern theories (Krupka, 1971) suggest that

it may play a role in stabilizing the less reactive of two postulated carrier states.

An attractive model for demonstrating the equivalence of both membrane faces with respect to the action of phloretin are erythrocyte ghosts. It was observed by Teorell (1952) that these membranes absorb glucose from the medium while sucrose is not taken up. Subsequently, erythrocyte ghosts were analyzed in great detail (for a review *see* Dodge, Mitchell & Hanahan, 1963) and the membrane was found to be basically unchanged in its qualitative content of lipids and proteins while the content of ions and water undergoes substantial changes. Still, transport of glucose surviving after preparation of ghosts exhibits all the features of mediated diffusion, as observed in intact erythrocytes (LeFevre, 1961).

A bilateral effect of phloretin on monosaccharide transport was reported in a preliminary way by Kotyk and Bosáčková (1964) who filled ghosts with a solution of 4-deoxyphloretin and showed it to depress the uptake of D-xylose by the preparation.

It was set out to establish the degree of similarity of monosaccharide transport between intact erythrocytes and their ghosts and to assess the degree of symmetry of erythrocyte membrane with the aid of inhibition by phloretin.

Materials and Methods

Blood was obtained from healthy donors by venous punction. For kinetic experiments the blood was always from the same donor. The blood was defibrinated with a wooden spatula, the erythrocytes were washed 3 to 4 times with isotonic saline containing sodium phosphate buffer of pH 7.4 and centrifuged at $1000 \times g$. The washed erythrocytes were stored at 2 to 4 °C for at most 3 days.

Ghosts were prepared according to Dodge *et al.* (1963). To enclose a given solution within the ghosts the appropriate solution was prepared in the 20 mOsm medium used for washing and sealing the ghosts.

Erythrocytes or ghosts suspended in approximately 20% (v/v) concentration in the NaCl-NaH_2PO_4 buffer were incubated aerobically at 30 °C in a Dubnoff incubator. Phloretin, dissolved in a small amount of ethanol and diluted with the medium was added to a final concentration of 10^{-5} M. This was followed after 10 to 15 sec by a solution of monosaccharide. Erythrocyte samples withdrawn at suitable time intervals were pipetted into small centrifuge tubes containing 0.5 ml silicone oil H (Merck 550, $\rho = 1.06$). After centrifuging for 30 sec at $10{,}000 \times g$ the incubation medium and the oil were removed and the erythrocyte sediment was washed with 0.2 ml ice-cold medium. The erythrocytes were then hemolyzed with 0.5 to 0.7 ml distilled water for 10 to 15 hr. An aliquot part was then transferred to a scintillation flask and 0.5 ml of 5% citric acid and 0.5 ml of 30% H_2O_2 were added. The mixture was evaporated at 90 °C almost to dryness and, after cooling, 1 ml of 99% ethanol and 10 ml toluene scintillation solution were added.

When incubating the suspension of ghosts the centrifuge tubes were not filled with the silicone oil but the oil was spread over their inner surface and they were heated for

2 to 3 hr at 95 °C. The ghost sediment after centrifugation adhered well to the bottom of such tubes. After hemolysis, 0.2 ml 0.3 N $ZnSO_4$ and 0.2 ml 0.3 N $Ba(OH)_2$ were added to adsorb the last remnants of hemoglobin and to facilitate sedimentation of the ghost membranes.

To study the efflux of sugars, erythrocytes or ghosts were incubated for 30 to 50 min with the sugar, centrifuged, washed with ice-cold medium and transferred to an equal volume of sugar-free medium at the appropriate temperature. Samples were then withdrawn and centrifuged and the supernatant separated.

Radioactivity was determined in a Nuclear Chicago Mark 1 scintillation spectrometer. The values obtained were referred to the dry weight of erythrocyte membranes as established by drying at 95 °C and after taking corrections for salts contained in the medium.

Nonlabelled phloretin was prepared from phlorizin isolated at the Isotope laboratory, Czechoslovak Academy of Sciences, by hydrolysis with HCl at 60 °C. The purity of the product was determined chromatographically. ^{3}H-labelled phloretin was prepared at the Isotope laboratory by a partial synthesis from phloroglucinol, applying tritiation of the intermediate chalcone. ^{14}C-D-galactose (3.7 mC/mmole) and ^{14}C-D-xylose (3.4 mC/mmole) were made at the Radiochemical Centre, Amersham, England. All other chemicals were of reagent-grade purity.

Results

Fig. 1 shows the binding and subsequent release of phloretin labelled with ^{3}H by intact erythrocytes and by ghosts. It follows that the binding

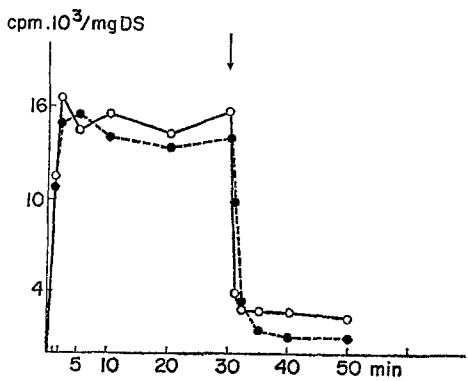

Fig. 1

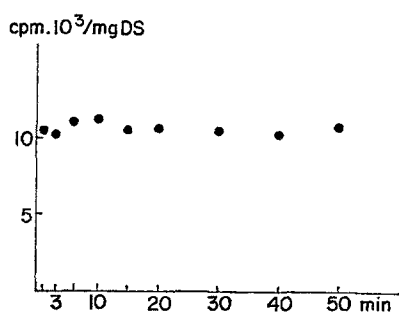

Fig. 2

Fig. 1. Binding of ^{3}H-phloretin by intact erythrocytes (o) and their ghosts (•) at 30 °C, aerobically. 10^{-5} M phloretin was added at time zero and the cells or ghosts were transferred to phloretin-free buffer at 30 min. DS membrane dry weight

Fig. 2. Level of phloretin enclosed in human erythrocyte ghosts. Ghosts were sealed in the presence of 10^{-5} M ^{3}H-phloretin and transferred to a phloretin-free medium (time zero). Activity due to phloretin was estimated after hemolysis of centrifuged ghosts. DS membrane dry weight

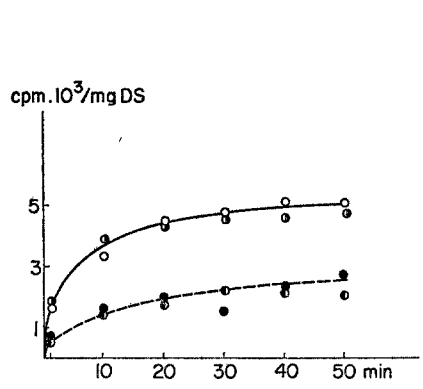

Fig. 3

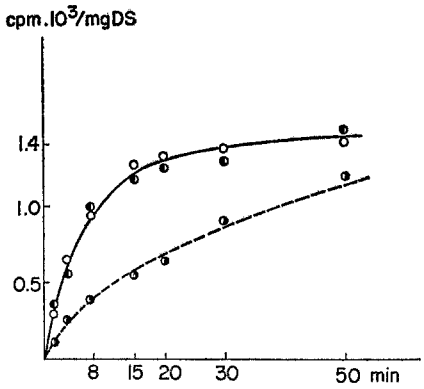

Fig. 4

Fig. 3. Effect of 10^{-5} M phloretin on 5 mM xylose uptake by erythrocyte ghosts. (○), no phloretin added; (◑), phloretin added to the outside medium; (◐), phloretin sealed inside the ghosts; (●), phloretin both outside and inside. DS membrane dry weight

Fig. 4. Effect of 10^{-5} M phloretin on efflux of 5 mM ^{14}C-D-xylose from erythrocyte ghosts. (○), control without phloretin inside the ghosts; (◑), phloretin added to the outside medium; (◐), phloretin sealed inside the ghosts. DS membrane dry weight

of phloretin is readily reversible, probably of the same type as that assumed for the binding of substrate with a carrier.

In the experiment depicted in Fig. 2, phloretin was sealed inside the ghosts which were then washed with ice-cold medium and resuspended at 30 °C. It will be seen that intracellular activity remained constant for 50 min. No activity was detected in the medium. It can thus be concluded that the ghost membrane is impermeable to phloretin.

Figs. 3 and 4 show the time curves of D-xylose transport. They provide evidence that phloretin is active only at the trans side of the membrane, i.e., at the side toward which the transport is proceeding and where, in terms of the carrier theory, the substrate-carrier complex dissociates. It is noteworthy that addition of phloretin to both membrane sides has no additional effect on the inhibition. Practically identical results were obtained with D-galactose.

It was of interest to compare the results obtained with ghosts with those in intact erythrocytes. Hence, the kinetic parameters of influx and efflux of the two sugars were estimated in intact erythrocytes, the K_m, K_i and V_{max} values obtained being shown in Table1. The effect of phloretin on V_{max} was negligible. It will be seen that the apparent K_i values of phloretin are rather similar for all the cases tested.

Table 1. Kinetic parameters of monosaccharide transport inhibition by phloretin in human red blood cells and ghosts[a]

Cell type	Sugar added	Direction of flux	K_m (mM)	V_{max}[b]	K_i (phloretin) (mM)
Erythrocytes	D-galactose	influx	13.0[c]	—	—
		efflux	12.5	7.6	0.0019
	D-xylose	influx	21.0[c]	—	—
		efflux	19.1	12.6	0.0015
Ghosts	D-galactose	influx	7.1	1.8	0.0008
		efflux	10.0	1.3	0.0015
	D-xylose	influx	13.8	7.1	0.0018
		efflux	23.5	5.1	0.0024

[a] The values were obtained from Lineweaver-Burk plots of the initial velocity of uptake or release measured at 1-min intervals after sugar addition (or transfer to sugar-free medium) and extrapolated to time zero. The influx and efflux curves were found to be practically linear up to 5 min of incubation. The flux of sugar always started toward a sugar-free compartment (cell or medium).

[b] Expressed in μmole sugar taken up or released in 1 min by 1 mg membrane dry weight.

[c] Values from Wilbrandt (1961).

Discussion

The principal objective of the work described here was to provide kinetic evidence, whether the "one-sided" effect of phloretin observed in monosaccharide transport by whole erythrocytes is due to intrinsic differences of the two sides of the plasma membrane or due to its impermeability toward phloretin. On the basis of the present results it appears that: (a) the erythrocyte membrane is impermeable toward phloretin, and (b) the two membrane sides are equivalent with respect to sugar transport and its sensitivity to phloretin. This is what one would expect from an almost classical system of mediated diffusion such as the transport of monosaccharides in human erythrocytes. To be sure, there is no definite knowledge of the actual mechanism of transport in this cell type. Attempts at isolating a membrane protein that would show affinity for glucose (Bobinski & Stein, 1966) have failed, perhaps because of the very low affinity of the membrane binding sites for glucose but perhaps also because there are no isolatable binding components in the erythrocyte membrane (*see also* LeFevre & Masiak, 1970). This has led to the suggestion of non-carrier, lattice-type models (Lieb & Stein, 1970; Naftalin, 1970) which can do without the classical carrier concept, and still account for phenomena like counter-transport and competitive acceleration.

The present results do not contribute to our knowledge of the molecular mechanism but are rather convincing for the assumption of true symmetry of the erythrocyte plasma membrane with respect to monosaccharide transport. The half-saturation constants are very similar in both ways across the membrane and so are the inhibition values of phloretin. The present values of K_m are in fair agreement with values obtained by other authors using different methods (Wilbrandt, 1961; LeFevre, 1962; Miller, 1965). The similarity of values of K_m obtained in erythrocytes and in ghosts suggests that the transport systems of monosaccharides are not altered on using a preparation of ghosts but it is rather likely that a fraction of the carrier molecules may be lost during the procedure, which might account for lower values of V_{max} found in the ghosts. The values of V_{max} are not expressed in terms of cell water, this being a rather inaccurate quantity particularly with the ghosts used. Moreover, their true values are probably much higher since the 1-min intervals used here for sampling are too long for a rigorous estimation (Graepel, 1966). However, the aim of the present investigation was a comparison of flux values inward and outward rather than an attempt at estimating the actual maximum rates.

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