The Attachment of Phloretin and Analogues to Human Erythrocytes in Connection with Inhibition of Sugar Transport

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In passing through the surface of mammalian erythrocytes, the common monosaccharides evidently make use of a special "carrier" system (1-6) which is strongly inhibited by phloretin, the aglucone of phlorhizin (2, 7). In most species, the activity of this mechanism subsides to a vestigial level shortly after birth (8), but in man and some other primates it is peculiarly persistent in the adult. This system is often cited as the clearest example of that type of membrane transport which has been designated as "facilitated diffusion" (9) or as the "reaction-diffusion type" (10). A process with comparable properties has been described in the free cells of the mouse Ehrlich ascites tumor (11, 12) and the Gardner lymphosarcoma (13).

Recently it has been found that some other diphenolic compounds, notably diethylstilbestrol, are comparable to phloretin as reversible inhibitors of sugar transport through the human red cell membrane (14). In showing far greater sensitivity to this type of molecule than to related glucosides, this sugar-carrier system differs diagnostically from the truly active sugar transport process in the renal tubule: in the latter system, not only is phloretin relatively inert (15-17), but a comparative study of many homologues points to the heteroside linkage as one of the molecular structural necessities for phlorhizin-like glucosuric activity (18). On the other hand, the type of comparative sensitivity seen in the red cell system has been reported also for at least two other biological processes: phosphorylation in the course of glycogenolysis in rabbit muscle (19), and multiplication of many varieties of bacteria (20). For both of these, phlorhizin is quite inactive, but the aglucone is strongly inhibitory; stilbestrol also is reported to exert strong general bacteriostatic action (21). Moreover, kidney phosphatase is reported to be 3 to 4 times as sensitive to phloretin as to phlorhizin (22).

The degree of inhibition by phloretin of sugar permeation in human red cells was previously shown (2) to depend systematically on the sugar concentration, in the manner expected if the inhibitor competes directly with the sugar for a limited number of carrier sites on the cell membrane. This kinetic analysis implied a rather tight binding of the drug, as expressed in a phloretin-carrier-complex apparent dissociation constant in the micromolar range.\(^1\) Examination of the characteristics of this apparent binding was therefore undertaken as a potential guide toward understanding of the sugar-carrier combination, and the nature of the membrane sites involved. The present report concerns the direct demonstration of a reversible attachment of the most active inhibitors to the red cells, and some implications arising from examination of factors influencing this attachment.

METHODS

Human blood obtained by venipuncture was either defibrinated with glass rods, or treated with ethylenediaminetetraacetate, citrate, or heparin. When not used on the same day, the blood was stored at 2-4°. Just before use, cells separated by centrifugation were taken through at least three successive washes in relatively large volumes of the artificial medium before final suspension. In the course of such preparation, a major fraction of the white cells was discarded.

The medium routinely used was a mixture of the chlorides of Na, K, Ca, and Mg in the molar ratio of about 150:6:3:2, buffered at pH 7.4 with 32 mM tris(hydroxymethyl)aminomethane, and with a total tonicity of 300 to 305 milliosmoles per liter as determined with the Fiske osmometer. In the studies involving variation in pH, other buffers were used in this same basic electrolyte mixture, as noted in the legend of Fig. 2. The Cambridge Instrument pH meter was used for all pH determinations.

The inhibitory compounds were obtained from K & K Laboratories with the following exceptions: diethylstilbestrol U.S.P. powder from Suffolk Drug Supplies and Surgical Company, naringenin generously donated by Sunkist Growers Products Department, and phlorpropophenone which was prepared by condensation of phloroglucinol and propionitrile (23). Many of these compounds were nearly insoluble in water, and were first taken up either in a small volume of concentrated NaOH which was neutralized with HCl after dilution in the medium, or in a minimal volume of ethanol subsequently diluted in the medium to at most 2 volume % (usually less than 0.5 volume %). The presence of such quantities of ethanol did not measurably alter either the fixation of the drugs by the cells, or the potency of the compounds as inhibitors of sugar transport. The latter potency was estimated in terms of the delay of glucose exit from the cells in the standard test previously adopted (14).

\(^1\) The originally reported figure of about 4.5 \(\mu\)M (2) was based on use of a commercial preparation of phloretin later found to be grossly contaminated with inert material. Purification by precipitation with water from ethanolic solution gave a product which was spectrophotometrically and cutoxically indistinguishable from better commercial preparations, and showed the same carrier-complex apparent dissociation constant of about 2.5 \(\mu\)M, at pH 7.4.
centrifuged human red cell suspensions proved to be a simple and adequate means for following the removal by the cells of such compounds added to the medium. The spectrum of phloretin in aqueous media has been thoroughly studied (24), and those of stilbestrol and hexestrol proved not to differ markedly at ordinary pH ranges from those reported for alcoholic solutions (25); the other related compounds showed comparable peaks in the range of 280 to 290 mp. With cuvettes of 1-cm optical path, analysis was feasible down to levels approximating the apparent half-saturation points kinetically indicated for the most powerful inhibitors.

No isolation of the drugs from the supernatant solutions before analysis was undertaken. Either washes, such as used to extract such agents from urine (26), are cumbersome and subject to uncertain losses; and standard precipitation methods for removal of products leaking from the cells into the suspension medium proved also to take out appreciable fractions of the agents under study. Therefore, absorption by the contaminants at the wave lengths used for the drug analyses was taken into account by parallel readings at a higher wave length (usually 412 mp) at which the drugs did not appreciably register. A Beckman DK-2 ratio-recording spectrophotometer was used for preliminary scanning in this connection to ascertain the most useful wave lengths and to establish the simple additivity of the component absorptions throughout the range employed. The actual calibrations and analyses were then carried out with the more precise Beckman DU spectrophotometer. In nearly all instances, the correction necessary for the cell products at the drug's absorption peak was only a minor contribution to the total optical density reading.

The ultraviolet absorption spectra of certain of the agents were distinctly sensitive to small pH changes in the physiological range, so that the samples were routinely treated with a fixed quantity of NaH₂PO₄, bringing the mixture to about pH 4.8, in order to stabilize the absorption behavior of the drug under study.

Conventional cell counts were carried out in a Levy hemacytometer chamber, and the relative volume of packed cells (in the initial concentrated suspensions) determined in capillary tubes in an air-turbine microcentrifuge (27). Concentrations of materials in or on the cells are expressed in terms of one or the other of these measures.

RESULTS

The basic expectation of a strong reversible attachment between the red cells and the most active inhibitors was readily verified by the direct analytic approach. However, there was no evidence of the saturation behavior anticipated by the presumption of a fixed number of sites of high affinity. In fact, no meaningful limit at all could be demonstrated in the number of inhibitor molecules with which a cell could be loaded. This is illustrated for phloretin in Fig. 1, which shows a reasonably constant, high distribution ratio (cells:medium) at all phloretin concentrations from the lowest adequately measurable (about 2 μm) up to near the solubility limit (about 0.25 mm). This

\* At the lowest drug concentrations, the correction for absorption by the contaminants of course became proportionately larger, as reflected in the high scatter of the points in Fig. 1 at the lower range. Consequently, other techniques are being adopted for further study at these levels where the specific inhibitory attachment may be expected to be demonstrable.

![Fig. 1. Distribution of phloretin between human erythrocytes and medium. Four different experiments indicated by four types of symbols. Cells were suspended at 1.5 to 3 volume % in standard medium, at pH 7.3 to 7.4.](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Approximate distribution ratio (cells:medium)</th>
<th>Inhibitory potency*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phloretin</td>
<td>35-50</td>
<td>100</td>
</tr>
<tr>
<td>Phlorhizin</td>
<td>0</td>
<td>1.2</td>
</tr>
<tr>
<td>Phlorpropiophenone</td>
<td>0-8</td>
<td>7</td>
</tr>
<tr>
<td>Phloroglucinaldehyde</td>
<td>2-3</td>
<td>0.2</td>
</tr>
<tr>
<td>Naringenin</td>
<td>7-9</td>
<td>7</td>
</tr>
<tr>
<td>Hexestrol</td>
<td>35-40</td>
<td>70</td>
</tr>
<tr>
<td>Stilbestrol</td>
<td>70-90</td>
<td>160</td>
</tr>
</tbody>
</table>

* Reciprocals of approximate millimolarity at level producing 50% inhibition in standard test system (14). distribution was attained so rapidly that even at 2-4°C the time course could not be properly studied by ordinary centrifugal separation of the cells; the fall in supernatant phloretin level was already about 80% completed when centrifugation was begun immediately upon mixing the drug with the suspension. The final distribution at 2-4°C was identical with that in the same medium at 27°C.

Similar behavior was observed with the other inhibitors structurally related to phloretin; Table I lists the approximate partition ratios for each of this group of compounds previously reported as significantly active (14).

In the hope of developing a clue as to the nature of the physico-chemical binding which may be presumed to underlie the cellular fixation of these transport inhibitors, alteration of the equilibrium distributions was attempted by modification of the medium.

The only analogue of significant activity for which no distribution data can be given here is dienestrol; "solutions" of this agent at concentrations feasible for these experiments proved to be finely dispersed suspensions even when appreciable concentrations of ethanol were present. Such preparations changed progressively in the course of an experiment, so that analyses were essentially uninterpretable.
Ca++ has been found to retard the slow progressive hemolysis of human red cells by stilbestrol (28); but the attachment of these drugs did not appear to involve in any way the electrolytes in the medium. Omission or excess of Ca++, Mg++, or K++, or addition of ethylenediaminetetraacetaete, or replacement of all electrolytes (except the buffer) by sucrose, led to no detectable change in the distribution. Moreover, addition of glucose even up to twice the isosmotic level, which on the basis of kinetically determined dissociation constants would be expected to displace nearly all of the inhibitor attached at the glucose-transfer sites, did not measurably alter phloretin fixation. Because of the marked inhibitory action on sugar transfer shown by some sulphydryl reagents, and the inactivity of others (29), a number of these were also examined, but none found to be consequential.

Only one parameter tested proved to influence the phloretin distribution significantly; this was the hydrogen ion concentration. The marked dependence on pH in the physiological range is shown in Fig. 2a; a shift of one pH unit toward the acid side doubled or tripled the cell:medium concentration ratio, but on the alkaline side the ratio fell precipitously. At pH 9 there was virtually no uptake by the cells at all. The inhibitory potency of phloretin in the sugar-transfer system showed this same pattern of pH-dependence. The special suggestiveness of this particular pattern is appreciated by reference to Fig. 2b; this shows the changing ultraviolet spectrum of phloretin at various H+ concentrations, which has been attributed to the keto-enol tautomerism of this compound (24). The peak at 294 mμ, taken to represent the ketonic form, varies inversely with the enolic peak at 320 mμ. The height of the ketonic peak closely parallels in its pH-dependence the cell:medium distribution ratio and the inhibitory potency of the phloretin. This clearly suggests that it is the ketonic form which is in direct equilibrium with the cell-fixed material, the enolic form being unable as such to attach appreciably to the cell sites involved. This interpretation of the correlation is supported by the facts that hexestrol and stilbestrol, which do not display any such tautomerism in the physiologically significant pH range, are correspondingly insensitive to pH in respect to their fixation to the cell and their potency as inhibitors of sugar movements; whereas the weaker inhibitors which share phloretin's pattern of tautomerism (naringenin, phlorpropophenone, phloroglucinaldehyde) also show a parallel relation between pH and their cellular fixation.

However, it is plain that the atomic grouping involved in this tautomerism does not in itself provide the essentials for the attachment process, since nearly identical ultraviolet spectral changes are shown by the glucoside phlorhizin (30), which does not appear to attach to the cells perceptibly at any tolerable pH. The finding that the proportional distribution between cells and medium does not depend systematically on the drug concentration over the range studied implies that the sites of inhibition of the sugar transport must represent only a tiny fraction of the potential sites for phloretin attachment, since the inhibition kinetics show that the transport sites are saturated even near the lower end of this concentration range. A corollary implication is that the fixation at the "carrier" loci is much tighter than at the nonspecific sites. A major difference between the two sets of sites is probably also shown in the failure of overwhelming doses of glucose to displace grossly the drug from the cells; however, as Bowyer and Widdas (31) have pointed out, it is possible that the inhibitor may act by combining not directly at the glucose site, but at an adjacent point where it tends
sterically to interfere with glucose adsorption, and thus could conceivably show competition kinetics while not being actually displaced by excess glucose. It is clear from the data of Table I that, in comparing the various compounds, the relative degree of fixation to the cells runs fairly parallel to the inhibitory potency. Similarly, the alterations in gross drug attachment with pH were always accompanied by corresponding changes in the activity of the sugar-transfer system. Thus, although the inhibitory sites do show a distinctively high tenacity, their comparative reactivity seems to be indistinguishable from that of the nonspecific sites.

Since both experimental estimates given in Table I for each compound are subject to a fairly large uncertainty, it appears that a comparison of the inhibitory activities at a given cellular load would show no noteworthy differences between the several analogues. The 50% inhibition level in the standard test system is seen to correspond to a cellular fixation of the general order of 5 to 10 $\times 10^{-4}$ mole of inhibitor per liter of cells, or about 50 million molecules per cell. This strongly suggests that the wide range of apparent “inhibitory potencies” is in a sense a secondary reflection of differences in the degree to which the several drugs become fixed to the cells, rather than to intrinsic differences in effectiveness at the site.

The experiments do not show whether the cells are actually penetrated by the drugs. The high speed of the attachment, together with the fact that the material is readily released back into the medium upon resuspension, suggests that the bulk of the attachment is at the cell surfaces. On the other hand, the sheer quantity of material which can be involved weighs against this: the uptake shown in Fig. 1 amounts to approximately 2 million molecules of phloretin per cell per micromolar unit of concentration in the suspending medium. Thus, at the uppermost levels shown, each red cell carried on the order of half a billion molecules of the drug, an amount which, if entirely superficial, could approximately cover the entire cell with a monolayer.

4 The glucoside phlorhizin is the one analogue for which the degree of fixation at a standard inhibitory level appears to be markedly out of line: even when much higher concentrations of cells were used than in any experiments with the other compounds, no measurable attachment whatever of phlorhizin could be demonstrated. Lambrecht (32, 33) has previously shown that in dog blood phlorhizin is completely restricted to the plasma, both in vitro and in vivo; in this case, the plasma proteins were found to have associated with the drug. The apparent small activity of phlorhizin, however, might be entirely attributable to its contamination with, or hydrolysis into, a small proportion of phloretin; Kalckar (22) has suggested such hydrolysis as the basis for phlorhizin’s action on kidney phosphenase. In the present experiments, the apparent effectiveness of phlorhizin could be thus accounted for if only 1 to 2% of the nominal content had been hydrolysed. Fixation of a fraction of such a small component (with the same ultraviolet characteristics as the parent substance) would not have been noticeable in the analyses.

5 A largely intracellular fixation is also suggested by the fact that erythrocyte “ghosts,” prepared by hypotonic hemolysis, washed, and reconstituted in isotonic media, bind much less phloretin than do the intact cells from which they are prepared. However, if the comparison is made in terms of residual hemoglobin content or the volume of packed material in the microhematocrit, the phloretin-fixing capacity of such ghosts exceeds that of the intact cells. Extrapolation from observations on a series of partially depleted ghosts suggests that a completely hemoglobin-free stroma would show about 15% of the phloretin-fixing capacity of the whole cell.

The high inhibitory activity in the sugar-transfer system of some of the diphenolic synthetic estrogens raises the question of the possible involvement of such cellular permeation by carbohydrates in the estrogenic action of these compounds; but there is no consistent parallel in potency ratings of the various homologues in these two respects. Although definite alterations in blood sugar levels and urinary sugar losses in animals on various stilbestrol or hexestrol regimes have been noted frequently (34–41), even the direction of these changes depends on the experimental details. The reported relations are clearly too complex to be accounted for by any simple competitive inhibition of membrane transfer of sugars in the renal tubule or elsewhere.

With such high cell:medium concentration ratios as found here for the strongest inhibitors, it becomes important, in analyzing experiments in vitro with such agents, to consider whether the amount of fixed drug constitutes a significant fraction of the total quantity present, since the effective dose then becomes dependent on the relative amounts of tissue used in different runs or samples. Thus, Reilly’s finding (42) that the depression of dehydrogenase activity in cat heart homogenates by a given nominal concentration of stilbestrol was greater, the less tissue used per flask, is almost certainly attributable to this consideration. No appreciable reassessment of apparent dissociation constants of these inhibitors in the red cell’s sugar-transfer system is indicated, however, because of the extreme dilution of the cell suspensions required in the Šrňkov densitometric procedure used in this work.

SUMMARY

1. Human red cells rapidly and reversibly bind phloretin, stilbestrol, and similar compounds which display strong competitive inhibition of the monosaccharide carrier system in the red cell membrane.

2. This binding proceeds to approximately the same distribution ratio throughout a very wide concentration range, extending far above the level at which the inhibitory kinetics show virtual saturation of the transfer sites; these specific sites thus constitute only a very small fraction of the total population of inhibitor-fixing sites, but are distinguished by a much higher tenacity.

3. Differences in cell:medium distribution ratio among the several related sugar transport inhibitors parallel the differences in their inhibitory potency, so that both types of sites show the same comparative specificity.

4. Those agents in which keto-enol tautomerism is shifted by pH changes in the physiological range show corresponding pH dependency in their fixation to the cells and in their inhibitory potency, such as to suggest that only the ketonic form is directly reactive with the cells.

5. The cellular binding of these inhibitors is unaffected by the ionic constitution of the suspension medium, the presence of unrelated sugar transport inhibitors of the sulfhydryl inactivating type, or addition of massive concentrations of a competing transported molecule (glucose).

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