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HYPOTHESIS FOR THE INTERACTION OF PHLORIZIN AND PHLORETIN WITH MEMBRANE CARRIERS FOR SUGARS*

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SUMMARY

Using 6-deoxy-D-glucose and D-xylose as substrates, it is shown that phlorizin, but not its aglycon, phloretin, is a fully competitive inhibitor of sugar active transport in hamster small intestine. The results indicate that: (1) phlorizin competes with glucose and its analogs for a common membrane binding site; and (2) phloretin inhibits sugar transport allosterically through binding to a different, albeit closely associated binding site. To explain these findings, a kinetic model has been constructed in which the carrier is assumed to possess two different sites, namely a sugar- and a phenol-binding site. Phlorizin binds simultaneously to both of these sites. The synergistic effect of this double binding causes the overall affinity of phlorizin for the sugar carrier to be three to four orders of magnitude higher than that of the physiological substrate, glucose. Phloretin binds only to the phenol site. The differences among tissues in their relative responses to phlorizin and phloretin are attributed to differing spatial relationships between the sugar site and the phenol site.

INTRODUCTION

Some time ago we reported that phlorizin acts as a competitive inhibitor of sugar active transport in hamster small intestine with the non-metabolizable compounds, 6-deoxy-D-glucose and 1,5-anhydro-D-glucitol (ref. 1). This observation was later extended to other substrates and to other animal species[2-5] and it has been confirmed in a variety of ways, with both intestine and kidney[6-9]. CARLISKY AND HUANG[10] independently obtained evidence to indicate that the situation may be similar in the intestine of some fishes. In light of these observations the conclusion seemed reasonable that phlorizin, which possesses a β-D-glucopyranoside ring, competes with actively transported sugars and sugar analogs for a common membrane

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binding site[2]. This conclusion was further strengthened by the observation that phlorizin also elicits countertransport of sugar transport substrates[3,11], as would be consistent with its being a substrate for the same carrier. However, newer observations have convinced us that somewhat more information would be required in order to demonstrate conclusively whether a membrane binding site is shared in common.

We have recently found that sugars, neutral amino acids and basic amino acids act as reciprocal partially competitive inhibitors and elicit countertransport of one another[12]. As is permitted by theory, these results have been interpreted as evidence for a common, polyfunctional carrier with separate class-specific binding sites, which are closely linked and capable of affecting each other in an allosteric way[12,13]. Under these circumstances, neither the usual kinetic evidence for 'competitive' inhibition nor the counter-transport phenomenon constitute by themselves unequivocal proof that a carrier binding site is shared. The inhibition must also be shown to be 'fully' rather than 'partially' competitive. This has now been done. More significantly, however, the enriched data and some new findings permit elaboration of a model for the interaction of phlorizin with the membrane carrier which indicates the presence of two specific sites on the membrane surface, one of which is coincident with the sugar-specific site.

METHODS AND MATERIALS

Transport was measured as described previously[2]. Hamsters were fasted overnight. Randomized rings from everted small intestine were incubated for 10 min in KREBS AND HENSELEIT[15] bicarbonate buffer saturated with O_2 *plus* 5% CO_2 and containing a concentration ratio Na/K of 22. The amount of substrate accumulated per ml tissue water in 10 min was calculated as usual, assuming an 80% tissue water content and correcting the results for the 2-deoxy-D-galactose space[2]. These values were used directly as equivalent to initial velocities[2,16].

6-Deoxy-D-glucose was prepared in this laboratory and was determined as methylpentose[17]. 6-Deoxy[^{3}H]glucose prepared in this laboratory and D-[^{14}C]xylose (Calbiochem) were determined by liquid-scintillation spectrometry.

Phlorizin and phloretin were from commercial sources; they were recrystallized before use and their purity tested by paper chromatography[2].

THEORETICAL CONSIDERATIONS

Basic to the present treatment of the data is the concept that the active transport of sugars in the small intestine may be explained in terms of a one-step, single mobile carrier[11,18]. According to this concept, the phenomenon of transport occurs in three stages; namely, (1) formation of a complex, *S–C*, between the substrate, *S*, and a binding site of the membrane carrier, *C*, at the outer side of the membrane; (2) translocation of *S–C* across the lipidic barrier of the membrane; and (3) dissociation of *S–C* at the inner side to regenerate free carrier and release *S* into the intracellular fluid. The process is assumed to be freely reversible and *S* may move back to the outer side of the membrane by reversal of these stages. The formation of *S–C*, in analogy to the formation of an enzyme–substrate complex, is described

by a constant, K_m, which is the reciprocal of the affinity between substrate and carrier. As shown elsewhere, accumulation of substrate against a concentration difference by means of this mobile carrier results from the fact that there is a gradient of substrate–carrier affinity across the membrane[18].

The activity of the carrier should conform to Michaelis–Menten kinetics when, (1) S, C and S–C are in equilibrium at the outer side of the membrane; (2) the translocation of S–C is the rate-limiting process in transport; and (3) the reverse reaction is quantitatively unimportant, as during the initial time periods before substantial accumulation has occurred. Evidence that all three conditions are met in our experiments is at hand and will be presented in detail elsewhere.

RESULTS

The effect of phlorizin on 6-deoxyglucose transport

When analyzed by the method of LINEWEAVER AND BURK[19], 6-deoxyglucose transport exhibits apparently typical saturation kinetics[1,2] with the following constants based on recalculation of the results of 8 different experiments: K_m, 1.91 mM (range: 1.51–2.42) and $v_{max.}$, 2.44 μmoles/ml tissue water per min (range: 2.19–2.72). Phlorizin inhibits this transport competitively with a K_i of about $8 \cdot 10^{-7}$ M (ref. 1).

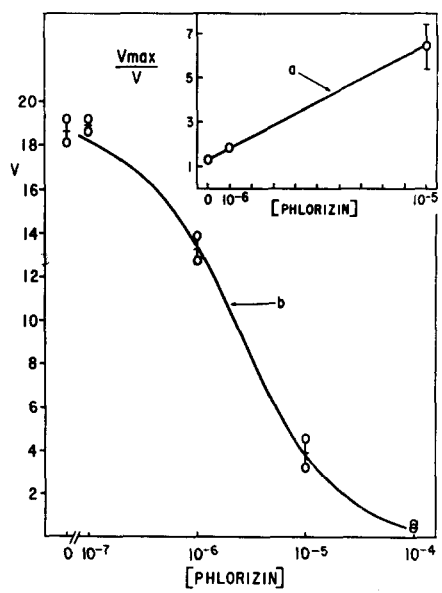

Fig. 1. Effect of phlorizin on 6-deoxyglucose transport. Intestinal rings were incubated[14] for 10 min in O_2 atmosphere, in 4 ml bicarbonate buffer[15] containing 5 mM 6-deoxyglucose and the indicated concentrations of phlorizin. Results were calculated and processed as described[2]. In main body of figure, velocities are plotted against the inhibitor concentration. In the inset, the same results were plotted according to THORN[21], assuming a $v_{max.}$ of 24.4 (see text). The slope of the straight line a was calculated to be $5.15 \cdot 10^5$ by the method of averages, the results at the highest phlorizin concentration being not used for this calculation. Assuming the average 6-deoxyglucose concentration was 4.35 mM, the K_m for 6-deoxyglucose was calculated to be 1.35 mM. From these data the K_i for phlorizin was calculated to be $6 \cdot 10^{-7}$ M (see text). Using this value of K_i and the equation for type Ia inhibition (Eqn. VIII. 7 in ref. 20), Curve b was calculated.

There is a high probability that this inhibition is fully competitive, *i.e.*, type Ia of DIXON AND WEBB[20], as suggested by the fact that essentially the same K_i value was found in separate experiments at different phlorizin concentrations[1]. Also, as the phlorizin concentration is increased, 6-deoxyglucose transport decreases proportionally toward, and eventually becomes, zero (Fig. 1). Concentrations of phlorizin as low as 10^{-4} M are known to completely block all transport of such sugars as glucose, methyl-α-glucoside, arbutin, xylose and others. This fact is especially significant since the equation for fully competitive inhibition demands that the velocity of an enzymatic reaction tends toward zero when the inhibitor concentration is increased at constant substrate. Furthermore, if our results are plotted according to THORN[21], that is, the ratio $v_{max.}/v$ is plotted against $[I]$, as in the inset of Fig. 1, a straight line is obtained from which a K_m/K_i ratio of about $2.24 \cdot 10^3$ can be calculated. If the K_m of 6-deoxyglucose is taken to be 1.91 mM (see above), the K_i for phlorizin based on this ratio would be about $8.6 \cdot 10^{-7}$ M. If the K_m for 6-deoxyglucose is taken to be 1.35 mM, as was assumed in order to fit the results in Fig. 1, then the K_i for phlorizin would be $6 \cdot 10^{-7}$ M, which is substantially the same value. The agreement between the values for the K_i of phlorizin calculated by the ratio method ($6–8.5 \cdot 10^{-7}$ M) and those calculated by the method of LINEWEAVER AND BURK ($8 \cdot 10^{-7}$ M) is excellent and may be taken as proof that not only does 6-deoxyglucose transport follow Michaelis–Menten kinetics but that phlorizin inhibition is fully competitive. It may then be concluded that phlorizin and 6-deoxyglucose share a common binding site. Since similar results have been obtained with 1,5-anhydroglucitol, methyl-α-glucoside, arbutin and xylose among others, the same conclusion would apply to all actively transported sugars.

The effect of phlorizin on D-*xylose transport*

Among the actively transported sugars studied, special mention needs to be made of xylose. Using the method of LINEWEAVER AND BURK[19], it was shown by ALVARADO[3] that phlorizin competitively inhibits xylose transport, as should be expected if this sugar shares a common transport mechanism with glucose and its analogs. In these experiments, however, the K_i for phlorizin appeared to be about two orders of magnitude higher than that calculated for other, perhaps more typical, substrates (see Table VII in ref. 3). No explanation could be offered at the time for this anomalous result.

The case of xylose has now been reinvestigated with calculations being made by the method of THORN[21] because of the necessity to distinguish between fully and partially competitive inhibition. From the typical result shown in Fig. 2 it seems evident that phlorizin is a fully competitive inhibitor of xylose transport. Furthermore, in several experiments using xylose concentrations ranging from 2 to 10 mM, the calculated K_i values for phlorizin ranged from 1.85 to $2.9 \cdot 10^{-7}$ M. This new and, we believe, more reliable value is of the same order of magnitude as that found with all other substrates tested and appears to confirm the assumption that xylose binds to the same site as phlorizin and 6-deoxyglucose.

The effect of phloretin on 6-deoxyglucose transport

Quantitatively, as compared to phlorizin, the effect of phloretin on sugar transport in the intestine would seem to be unimportant[1,22]. Qualitatively, however,

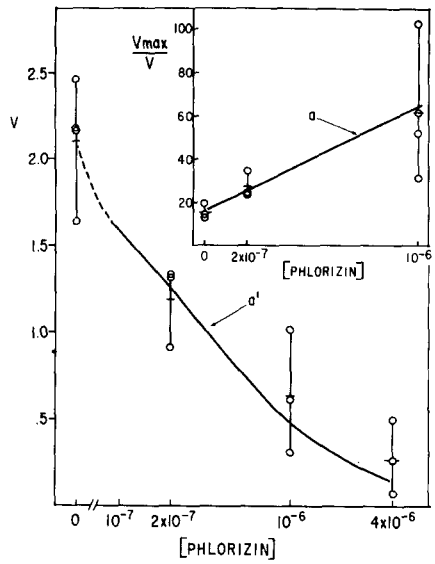

Fig. 2. Effect of phlorizin on D-xylose transport. Incubations were for 10 min in 5 ml bicarbonate buffer containing 7.2 mM [^{14}C]xylose *plus* phlorizin as indicated. In the inset, the results are plotted according to THORN[21]. Assuming that K_m for xylose is 100 mM (ref. 3), $v_{max.}$ was calculated to be 31.9 μmoles/ml tissue water per 10 min. The slope of the straight line a was calculated to be $4.9 \cdot 10^7$ by the method of averages. With these data and an average xylose concentration of 7.08 mM, K_i for phlorizin was calculated to be $2.88 \cdot 10^{-7}$ M. Using again these constants, and assuming fully competitive inhibition (Eqn. VIII. 7 in ref. 20), Curve a' was calculated.

and especially with regard to our conception of binding sites, the effect of phloretin is highly significant. As shown in Fig. 3, phloretin is unable to block sugar transport completely. As the phloretin concentration is increased, inhibition does not increase proportionally, but approaches an apparent limiting value of about 50%. This result, which has been confirmed using other substrates, together with the fact that THORN's plot gives a curvilinear relationship (inset, Fig. 3) stands in marked contrast to the results found with phlorizin and clearly indicates that phloretin inhibition occurs through a different mechanism. DIEDRICH[8] also reported substantially identical results with phloretin, using hamster intestine and glucose as substrate, but did not interpret them beyond the above conclusion, namely, that phlorizin and phloretin inhibitions are different.

What the present kinetic analysis seems to indicate is that phloretin does not compete with sugars for a common binding site but rather binds to a site which is separate from, albeit closely associated with, the sugar-binding site; that is, phloretin may be an allosteric inhibitor of sugar transport (see later). A similar phenomenon has been seen with amino acids as inhibitors of sugar transport and *vice versa*[12].

It also has recently been suggested that phloretin may inhibit sugar transport as measured in rat small intestine through an indirect effect on metabolism[23]. This would appear not to be the case, at least in the hamster. In our experiments, 6-deoxyglucose was transported against a concentration gradient in the presence of the highest phloretin concentrations used. For example, in the experiments in Fig. 3 and Table I, the per cent fillings attained were of about 340.

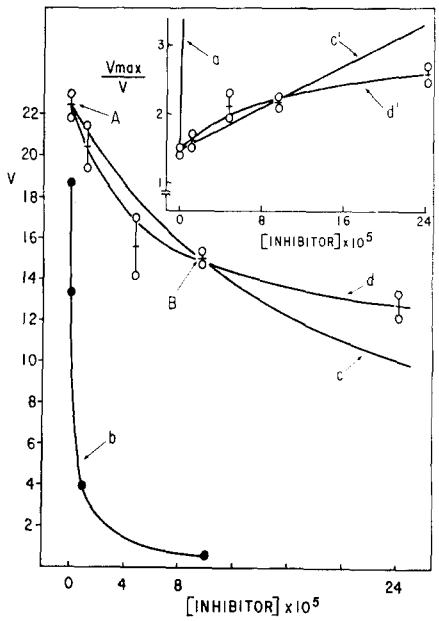

Fig. 3. Effect of phloretin on 6-deoxyglucose transport. The experimental procedure and presentation of results are the same as in Fig. 1, the difference being that here the initial 6-deoxyglucose concentration was 4.8 mM and the inhibitor was phloretin rather than phlorizin. For comparison purposes, however, the results found with phlorizin in Fig. 1 are shown here again; Curves a and b. Using Point A and assuming a K_m for 6-deoxyglucose of 1.91 mM (see text) and an average 6-deoxyglucose concn. of 4.1 mM, $v_{max.}$ was calculated to be 32.8: using this value of $v_{max.}$, the results shown in the inset were calculated. Using Points A and B, and assuming type Ia inhibition (Eqn. VIII. 7 in ref. 20), a K_i value of $6.2 \cdot 10^{-5}$ M was calculated for phloretin; using now this K_i value and the same equation, Curves c and c' were calculated; it seems clear that these theoretical curves do not fit the experimental results. Tentative curves d and d' were drawn by eye; for further explanation see text.

A model of mechanism of phlorizin inhibition of sugar active transport in the small intestine

Based on these findings: (1) that phlorizin is a fully competitive inhibitor of sugar transport with an affinity three to four orders of magnitude greater than the sugars and (2) that phloretin is an allosteric inhibitor of the same process, a model for phlorizin and phloretin interaction with the membrane carrier may be construed.

TABLE I

THE EFFECT OF PHLORETIN ON THE INHIBITION OF THE ACTIVE TRANSPORT OF 6-DEOXYGLUCOSE BY PHLORIZIN

Substrate, 3 mM 6-deoxy[^{3}H]glucose in 5 ml bicarbonate buffer; 10-min incubations. Phloretin was $5.7 \cdot 10^{-4}$ M; phlorizin was $8 \cdot 10^{-6}$ M. Results are means of quadruplicate determinations.

Condition	% *filling*	% *inhibition*
Control	600	
plus phloretin	338	38.5 ± 25
plus phlorizin	138	74.0 ± 3
plus phloretin *plus* phlorizin	131	75.2 ± 12

(a) Interactions of glucose and phloretin with the carrier.

It is postulated that the carrier, $-C-$, is bifunctional, possessing two different but closely associated binding sites; namely, (1) a sugar site for glucose and its analogs, represented by $-C$; and (2) a phenol site for phloretin and its analogs, represented by $C-$. The following equations would then define the interactions between C and each of its two classes of substrates; glucose, G; and phloretin, P.

$$G + C \rightleftharpoons GC; \quad K_G = \frac{[G]\,[C]}{[GC]} \tag{1}$$

$$P + C \rightleftharpoons CP; \quad K_P = \frac{[P]\,[C]}{[CP]} \tag{2}$$

$$G + P \quad -//\rightarrow \tag{3}$$

$$GC + P \rightleftharpoons GCP; \; K'_P = \frac{[GC]\,[P]}{[GCP]} \tag{4}$$

$$CP + G \rightleftharpoons GCP; \; K'_G = \frac{[CP]\,[G]}{[GCP]} \tag{5}$$

Substrate translocation is described by the following:

$$GC \xrightarrow{k_1} C + G_{(\text{intracellular})} \tag{6}$$

$$CP \xrightarrow{k_2} C + P_{(\text{intracellular})} \tag{7}$$

$$GCP \xrightarrow{k_3} C + G_{(\text{intracellular})} + P_{(\text{intracellular})} \tag{8}$$

where k_1, k_2 and k_3 are rate constants. For the purpose of the present discussion it does not matter whether k_2 is or is not equal to zero: that is, whether phloretin is actually translocated. Our concern is with the velocity of transport of G, v_G, which is given by:

$$v_G = k_1[GC] + k_3[GCP] \tag{9}$$

Substituting in (9) one obtains:

$$v_G = \frac{v_{\max.}\left(1 + \dfrac{k_3[P]K_G}{k_1K_PK'_G}\right)}{1 + \dfrac{K_G}{[G]} + \dfrac{[P]}{K_P}\cdot\left\{\dfrac{K_G}{[G]} + \dfrac{K_G}{K'_G}\right\}} \tag{I}$$

This equation would describe a general case of 'allosteric inhibition' if this term is used in its original sense, namely: "an indirect interaction between distinct specific binding sites" (ref. 24). It is equivalent to Eqn. 1 of FRIEDEN[25] to whom reference may be made for a full discussion of its significance.

What is presently relevant is the fact that Eqn. I will fit the case of phloretin inhibition of 6-deoxyglucose transport (Fig. 3) but not that of phlorizin inhibition. By making $k_3 = 0$ and $K_G = K'_G$, Eqn. I could be transformed into another (Eqn. VIII. 42 in ref. 20) that would fit the phlorizin data in Fig. 1. However, such an equation is one of non-competitive inhibition and cannot be applied to competitive inhibition of sugar transport by phlorizin[1].

Of special interest with regard to the meaning of Eqn. I are some of its special solutions which would equally well fit the phloretin data in Fig. 3. On the one hand, by making $k_1 = k_3$ and $K_G \neq K'_G$ one obtains the equation for partially competitive inhibition or type Ib of DIXON AND WEBB (Eqn. VIII. 28 in ref. 20). On the other, by making $k_1 \neq k_3$ and $K_G = K'_G$, one obtains an equation for non-competitive inhibition; type IIb of DIXON AND WEBB (Eqn. VIII. 47 in ref. 20). Both of these solutions have the following in common with Eqn. I: (1) all are based on the assumption of the formation of ternary complexes of the type G–C–P; (2) all give straight lines in plots of the LINEWEAVER–BURK type, compatible with either 'competitive'; 'non-competitive' or 'mixed type' inhibition; (3) in no case does the velocity fall to zero as the inhibitor concentration increases; rather, a limiting value greater than zero is reached; and (4) all give parabolic curves in plots of the DIXON[26] or THORN[21] type.

What the foregoing appears to mean is that unless inhibition is fully competitive; *i.e.*, type Ia of DIXON AND WEBB, little significance can be attached to the behavior of an inhibitor as described by a plot of the LINEWEAVER–BURK type. Lack of recognition of this fact appears to have contributed some confusion, possibly as a consequence of the application of the term, 'partially competitive' to inhibitions of type Ib; these inhibitions would be more aptly named 'pseudocompetitive'.

(b) Interactions of phlorizin and glucose with the carrier

In the same terms as above, phlorizin would be G–P and its interactions with the carrier would be the following:

$$GP + C \rightleftharpoons PG\text{–}C \; ; \; K_{GP} = \frac{[GP]\,[C]}{(PGC)} \tag{10}$$

$$GP + C \rightleftharpoons C\text{–}PG \; ; \; K'_{GP} = \frac{[GP]\,[C]}{[CPG]} \tag{11}$$

$$GP + C \rightleftharpoons \underset{\diagdown\,C\,\diagup}{G\text{——}P}; \; K''_{GP} = \frac{[GP]\,[C]}{[\overline{GCP}]} \tag{12}$$

The complex $\overline{GCP}$ ($\underset{\diagdown\,C\,\diagup}{G\text{——}P}$) is probably not formed in a single step as in (12) but results from rearrangement of either of the other two, as in the following:

$$PGC \rightleftharpoons \overline{GCP} \; ; \; K_H = \frac{[PGC]}{[\overline{GCP}]} \tag{13}$$

$$CPG \rightleftharpoons \overline{GCP} \; ; \; K'_H = \frac{[CPG]}{[\overline{GCP}]} \tag{14}$$

If phlorizin and glucose are present simultaneously, the following reactions among others may take place:

$$\overline{GCP} + G \text{ -//-}\rightarrow \tag{15}$$

$$PG\text{–}C + G \text{ -//-}\rightarrow \tag{16}$$

$$C\text{–}PG + G \rightleftharpoons GCPG \; ; \; K''_G = \frac{[CPG]\,[G]}{[GCPG]} \tag{17}$$

$$GC + PG \rightleftharpoons GCPG \; ; \; K''_{GP} = \frac{[GC]\,[PG]}{[GCPG]} \tag{18}$$

Lack of reaction at (15) and (16) is synonymous to saying that phlorizin behaves as a fully competitive inhibitor of sugar transport through formation of complexes PG–C and $\overline{GCP}$. Reaction (17) indicates phlorizin may also act as an allosteric inhibitor of sugar transport through formation of complex C–PG which is analogous to the phloretin–carrier complex, C–P (see above).

If the two pairs of reactions, 10 *plus* 13 and 11 *plus* 14 are considered as the same overall reaction through two separate pathways, it seems clear that the two sequences should have the same overall equilibrium constant, K''_{GP}:

$$GP + C \rightleftharpoons PG\text{–}C \rightleftharpoons \overline{GCP} \; ; \; K''_{GP} = K_{GP} \cdot K_H \tag{19}$$

$$GP + C \rightleftharpoons C\text{–}PG \rightleftharpoons \overline{GCP} \; ; \; K''_{GP} = K'_{GP} \cdot K'_H \tag{20}$$

It may then be argued that it is the dissociation constant of the complex $\overline{GCP}$ (K''_{GP}) which defines the overall affinity of phlorizin for the carrier; *i.e.*, K''_{GP} is equivalent and equal to K_i for phlorizin. Also, it may be assumed that the dissociation constant of the complex PG–C (K_{GP}) will have a numerical value of the same order of magnitude as that of the phenylglucoside, arbutin, which in turn has a K_m essentially identical to that of 6-deoxyglucose (ref. 2).

Therefore, (19) may be rearranged as follows:

$$\frac{K_{m(\text{arbutin})}}{K_{i(\text{phlorizin})}} \cong \frac{K_{GP}}{K''_{GP}} = \frac{1}{K_H} \tag{21}$$

By substitution of known values the constant K_H may be assessed to be about $3.3 \cdot 10^{-4}$. This value does not seem unreasonable and provides a quantitative basis for the present hypothesis of phlorizin interaction with the sugar carrier.

As an aid to understanding the above presentation it may be noted that inhibition of sugar transport by phlorizin as described by Eqn. (21) seems to have a counterpart in the interactions between acetylcholine and the enzyme, acetylcholinesterase. Acetylcholinesterase possesses two different but closely associated sites to which the different functional groups of its substrate, acetylcholine, may bind simultaneously. WILSON[27] has shown the existence of compounds that, according to their structure, can bind to only one or the other of these specific binding sites. For example, ethyl acetate can bind only to the 'esteratic' site since it lacks the quaternary ammonium group necessary for binding to the 'anionic' site. Consequently, the overall affinity of ethyl acetate for the esterase is smaller than that of acetylcholine; the ratio $K_{m(\text{ethyl acetate})}/K_{m(\text{acetylcholine})}$ is about 10^3 (ref. 27). There is an analogy between the pair arbutin–phlorizin interacting with the sugar carrier and that of the pair ethyl acetate-acetylcholine interacting with acetylcholinesterase.

The effect of phloretin on the inhibition of 6-deoxyglucose transport by phlorizin

As a first test of the present hypothesis, it may be noted that it predicts that phloretin and phlorizin should compete for binding to the phenol site. Thus, if the

model is correct, phloretin and phlorizin inhibitions of sugar transport should not be additive; indeed phloretin should antagonize phlorizin inhibition. On the other hand, if phlorizin and phloretin do not bind to the same site, their respective inhibitory effects should be additive.

Table I provides data from a representative experiment. Phlorizin and phloretin were tested as inhibitors of 6-deoxyglucose transport, both together and separately. The results seem clear in indicating that the effects of these phenols are not additive. Phloretin did not antagonize the inhibitory effect of phlorizin. However, its low solubility in water precludes use of phloretin/phlorizin concentration ratios high enough to compensate for the probably tremendous difference in affinity; which, similar to the sugars, should be of the order of three to four orders of magnitude in favor of phlorizin. Further work along these lines is in progress.

DISCUSSION

The allosteric effect of phloretin on sugar transport in hamster intestine seems very similar to the recently described interactions between sugars, amino acids and Na^+ in the same tissue[12,13]. More work will be required to establish unequivocally which solution of equation I could better fit the case of phloretin, but, independently

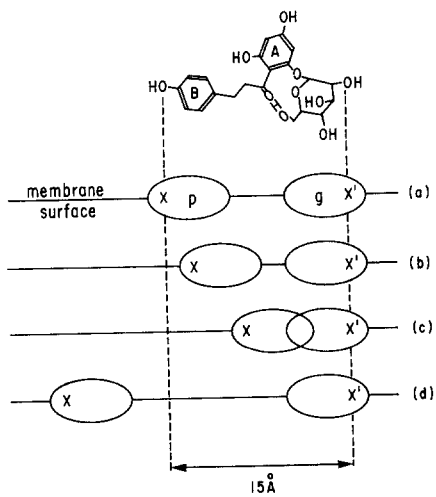

Fig. 4. Interactions of phlorizin with cell membranes. Phlorizin is depicted in its restricted, hydrogen-bonded conformation, as suggested by DIEDRICH[7]. The membrane surface is schematically shown to possess two separate regions (p, g) for specific binding to the phloretin and to the glucose moieties of phlorizin, respectively. Main points of interaction (x, x') are suggested to involve the oxygen at the B ring of phloretin and the –OH at C-2 of glucose. However, additional interactions probably also occur. For instance, glucose may bind through C-6 and through the axial –OH groups at C-3 and C-4. Phloretin may also bind through the B ring; and it is also conceivable that the A ring and/or some of its –OH groups could also contribute to binding. As suggested in the figure, which was inspired by actual atomic models, the phlorizin molecule may lie almost flat on top of the membrane surface, thus exposing all of its –OH groups for interaction. The particular situation suggested to occur in hamster small intestine is shown in (a), where the acceptors x and x' are shown to be separated by a distance (about 15 Å; ref. 7) that permits optimal simultaneous binding of phlorizin at each of the two sites, p and g. Other possible situations are also schematized (b through d); for further explanations, see text.

of this, the conclusion seems warranted that the 'phenol site' postulated above must be an integral part of the 'mosaic' of specific binding sites that have recently been postulated to occur in the membrane matrix of hamster small intestine[12]. The physiological significance of the phenol site, however, remains to be elucidated.

The present hypothesis for the action of phlorizin on sugar active transport in hamster small intestine can be schematized as in Fig. 4 a, where the glucose and phloretin moieties in the phlorizin molecule are shown to be separated by a fixed distance which is about the same as that separating their respective binding sites in the membrane, g and p. Several lines of evidence from this and other laboratories justify this model, as will be discussed below. Also, there are indications that this situation is not unique to hamster intestine. What may be unusual in hamster intestine is the spatial relationship between the two types of sites. As is also illustrated in Fig. 4 b, c and d, it seems conceivable that, in other cells or animal species, differences in the spacing between these binding sites may occur which would lead to operationally very different situations.

Both phlorizin and phloretin inhibit sugar transport in a variety of cells. This effect is known to occur at the membrane, that is, inhibition of transport may be shown with phenol concentrations much lower than those needed to affect metabolism. Phlorizin, but not phloretin, is a strong inhibitor of sugar transport in intestine and kidney whereas phloretin is the strongest inhibitor toward sugar transport in the red blood cell[28–31]. This difference suggests that the glucose moiety of phlorizin plays an important role in its interactions with membrane receptors in the intestine and kidney; but not in red blood cells. On the other hand, the fact that the apparent affinity of phlorizin for the sugar transport process in hamster intestine was three to four orders of magnitude greater than that of various free sugars[1] clearly suggested that the polyphenolic group attached at C-1 of the pyranose ring improves affinity. It was for this reason that a series of phenylglucosides were tested as substrates for intestinal sugar transport. It was found that the structure of the aglycon was important in determining the overall behavior of these compounds with respect to the system. For instance, phenyl-β-D-xylopyranoside exhibited an affinity for the sugar carrier at least 5 times greater than that of its parent sugar, D-xylose (ref. 2).

Independently, DIEDRICH studied a series of phlorizin analogs as inhibitors of renal glucose reabsorption in the dog *in vivo*[7] and later extended this work to hamster intestine *in vitro*[8]. We are in agreement in concluding that glucose and phlorizin compete for some membrane receptor or receptors. DIEDRICH also presented clear evidence that the structure of the aglycon is important in determining the extent of the inhibition. He suggested that intramolecular hydrogen bonding fixes phlorizin in a "specific three-dimensional pattern which favors an association with the membrane receptor" (ref. 7). This association would involve at least two 'loci', namely, a 'primary bond' through the glucose moiety and a 'secondary bond' involving the aglycon; but he did not relate the secondary bond to a specific phenol-binding site. For the secondary bond he singled out the oxygen atom (not necessarily –OH) at C-4 of the B ring and predicted that 4-deoxyphlorizin should not be a strong inhibitor[7]. This prediction was later borne out by KOTYK *et al.*[32].

KOTYK and his co-workers compared the effects of phlorizin, phloretin, and some analogs as inhibitors of sugar transport into human red cells, hamster small intestine, and rabbit kidney cortex slices. They concluded from their own obser-

vations and from data from the literature that a single type of carrier for sugar transport is involved in all of these cells. However, they were not able to decide whether the "membrane receptor, localized at or near the sugar carrier, is identical both for phlorizin and for phloretin" (ref. 32). They showed that the hydroxyl group at C-4 of the B ring is not necessary for phloretin inhibition. And since this same group is necessary for phlorizin to inhibit, they suggested that "the binding loci of the phlorizin molecule with the receptor are different from those of the phloretin molecule" (ref. 32). According to our model (Fig. 4), phlorizin binds to the phenol site through the aforsaid oxygen atom which is fixed in the appropriate position by the anchoring of phloretin to the glucose moiety. However, free phloretin is obviously not so restricted and could be expected to bind to the same site (x in Fig. 4) through any one of its four phenolic oxygens. It is possible that the phenol site here postulated is relatively little specific. In fact, it is possible that any phenol may bind to this site, as work in progress in this laboratory appears now to suggest.

Recently, SAHAGIAN[33] has published experiments in support of the view that phloretin is a competitive inhibitor of sugar transport in guinea pig intestine, with an affinity only about 5 times smaller than that of phlorizin. This is a most surprising result, both qualitatively and quantitatively, and more work will be required to confirm SAHAGIAN's results. They are unfortunately open to the special criticisms that the source of the phloretin used was not made known and, more importantly, that the kinetic analysis was carried with 'velocity' values obtained under steady-state rather than initial rate conditions. Nevertheless, it seems possible that SAHAGIAN's results may turn out to be essentially correct. It is also possible to explain them in terms entirely in accord with the present hypothesis. As discussed above, it is conceivable, that, in the guinea pig, sites g and p of the membrane may be closer together than they are in the hamster. In that case (Fig. 4 b and c) it can be visualized that phloretin, by binding to its specific site, could interfere with glucose binding to its own. Kinetically, phloretin could now appear as a fully competitive inhibitor of glucose transport.

The situation as described above would seem very similar to the case of phloretin inhibition of sugar transport in red blood cells. Some years ago, LEFEVRE[34] found that phloretin acts as a truly competitive inhibitor of glucose transport in human red blood cells; this work was later extended to include other polyphenols such as certain steroids[35]. The lack of structural relationship between these compounds and the sugars led LEFEVRE to suggest that the red cell membrane possesses groups for reversibly associating or binding to phenolic groups; and some of these groups may be the same involved in sugar binding[35]. From our point of view, the question now becomes not whether sugars and phenols share some common points of attachment to the red cell membrane, but whether simultaneous binding of sugars and phenols to the membrane is or is not possible. Simultaneous binding is assumed possible in case (a) of Fig. 4; which appears to represent hamster intestine. If this is, indeed, the relationship of the two membrane sites in hamster intestine and perhaps dog kidney[7], simultaneous binding of sugars and phenols should be not only possible but indeed synergistic. On the other hand, simultaneous binding would be difficult or impossible in cases (b) and (c) of Fig. 4. It is one of these relationships we would suggest for the red blood cell and, perhaps, guinea pig intestine. A similar suggestion was made by BOWYER AND WIDDAS[36] for the red cell, based on LEFEVRE's findings.

For completeness, it is to be noted that if the distance between sites g and p in the membrane were to increase above a critical limit, case (d) of Fig. 4, simultaneous binding would be possible but no effect of phloretin on sugar transport should be expected.

As we see it, the possible merit of the present model would appear to consist in providing a unifying hypothesis for the effect of phloretin and phlorizin on different types of animal cells. The difference in transport mechanisms in cells from different organs and animal species may be much smaller than has been ordinarily assumed.

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