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INHIBITION OF [^{3}H]PHLORIZIN BINDING TO ISOLATED KIDNEY BRUSH BORDER MEMBRANES BY PHLORIZIN-LIKE COMPOUNDS*

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SUMMARY

1. Earlier work has demonstrated that (a) isolated brush border membranes from rat kidney cortex possess a high-affinity receptor for phlorizin; binding is dependent upon the presence of sodium and is competitively blocked by D-glucose, (b) phlorizin competitively inhibits active transport of glucose across rat kidney proximal tubules and (c) a series of phlorizin-like compounds possess a characteristic, wide range of inhibitory potency as glucose transport poisons in kidney and intestinal systems.

2. ^{3}H-labeled phlorizin binding to the brush border membrane site has now been shown to be inhibited by this series of phlorizin analogs, and the critical finding is that the previously established relative potencies of these compounds as transport inhibitors parallel their ability to block phlorizin binding to the specific membrane receptor.

3. These results increase the weight of evidence for the view that the glucose-transport system is retained as a structurally unaltered component of these isolated brush border fragments.

4. A view of the conformation, which the phlorizin receptor could assume in the matrix of the outer membrane, is presented in the discussion.

INTRODUCTION

The immediate goal of an increasing number of investigators is to isolate and characterize the membrane component responsible for the transport of glucose into mammalian cells. This represents a redirection of effort in this research area and underscores the view that a deeper insight into the molecular mechanism of carrier transport cannot be gained solely from studies of a purely kinetic and descriptive nature. The exciting progress which has been made and the approaches which have been employed in the identification of various so-called membrane transport proteins, primarily from microorganisms, have recently been appraised by Kotyk[1]. The bio-

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chemical literature related to this field, including that dealing with mammalian transport systems, has also recently been reviewed[2]. Some efforts to identify specifically the glucose carrier in fragments of disrupted mammalian cells have been reported[3-6], but other work casts serious doubt on the significance of these findings and their interpretation[7-10]. Fruitful investigation of these mammalian sugar transport systems has been hindered because of the low affinity which the sugar substrate possesses for the membrane carrier. For this and other reasons glucose is of limited usefulness as a marker substance in binding studies designed to detect transport protein(s). On the other hand, the use of phlorizin affords particular advantages especially in work with intestinal and kidney membranes, since the glycoside has a much greater affinity than glucose for the carrier system in these tissues[11-15]. Indeed, a binding site having high affinity for phlorizin has already been characterized in isolated brush border membrane preparations from rat kidney epithelia[16]. This phlorizin binding was dependent upon the presence of sodium and was competitively inhibited by D-glucose but not by the weakly transported L-glucose[17]. A confirmation and expansion of this early work has recently been reported by other workers who used a different technique to study the adsorption characteristics of the phlorizin receptors in renal brush borders[18]. It therefore appears that the procedures which have been employed to isolate these membrane fragments do not cause the loss of the glucose carrier construction—a view which is further supported by our present work. A series of structurally related phlorizin-like compounds, which exhibits a characteristic range of potency as competitive inhibitors of glucose transport in dog kidney and hamster intestine[14,15], has now been found to have the same rank order of effectiveness as inhibitors of the specific phlorizin binding to these brush border membranes.

METHODS

Brush border membrane preparation

Male, Wistar rats of 160–200 g body weight were maintained on a standard diet and tap water. For each membrane preparation 20 animals were either anesthetized with thiobutabarbital (Inactin) or sacrificed by stunning, then decapitated and exsanguinated. Subsequent steps of the preparation were carried out in a 2–4 °C cold room. Kidneys placed in cold buffer containing 0.25 M sucrose, 10 mM triethanolamine–HCl, 5 mM EDTA, 2 mM dithiothreitol, adjusted to pH 7.6 at 20 °C, were immediately decapsulated, and thin slices of cortex were cut with a razor blade. The resulting tissue was further processed according to a modified procedure[19] of the original Kinne and Kinne-Saffran[20] method. In brief, the tissue was first hand-homogenized with the above-mentioned buffer using a Potter–Elvehjem system with Teflon pestle. This was followed by a brief and gentle motor-driven step yielding the whole homogenate. The suspension was differentially centrifuged to obtain a membrane fraction containing about 15 % of the brush borders originally present in the whole homogenate; this fraction was characterized by the specific activity of the brush border marker enzyme, alkaline phosphatase (EC 3.1.3.1). The membrane preparation used throughout this study had an alkaline phosphatase specific activity seven times greater than that in the whole homogenate. However, glucose-6-phosphatase (EC 3.1.3.9), which is specifically localized in endoplasmic reticulum, was

enriched only about 20 % whereas the specific activity of mitochondrial glutamic dehydrogenase (EC 1 4.1.2) was reduced 4-fold.

Materials and analyses

D-[^{14}C]Mannose was used as reference substance to correct for dead space between the sedimented membrane fragments since it was not significantly bound[17]. It had a specific activity of 48.8 mCi/mmole and was stored frozen as a 2.5 μM aqueous solution. The [^{3}H]phlorizin was uniformly labeled and had a specific activity of 176 mCi/mmole. Its radiochemical purity was established to be greater than 97 % by using the previously described thin-layer silica gel chromatographic system[21]. Both compounds were obtained from New England Nuclear, Boston, Mass.

Six concentrations of [^{3}H]phlorizin were stored frozen as stock solutions in parcels large enough to perform 2–3 experiments. When diluted 1 to 5 by the other additions to the incubation mixture, these solutions gave the following respective end phlorizin concentrations: 0.1, 0.5, 1, 5, 10 and 100 μM. The highest concentration only was prepared by mixing doubly recrystallized, unlabeled phlorizin with the tritiated material in a 10:1 ratio to keep the amount of radioactive compound at a reasonable level.

All of the phlorizin-like analogs tested as binding inhibitors were synthetized as reported earlier[22] with the exception of deoxycorticosterone-β-D-glucoside. This was a gift generously supplied by Dr R. Gaunt of CIBA Pharmaceutical Company, Summit, N. J.

Alkaline phosphatase was measured using *p*-nitrophenylphosphate as substrate[16]. The assay for glucose-6-phosphatase has already been described[19] and the method of Bücher *et al.*[23] was followed to determine glutamic dehydrogenase activity. Bovine serum albumin was used as standard to measure protein by the procedure of Lowry *et al.*[24].

Binding studies

The methods used were those previously adopted[17] with some modifications. Aliquots of 100 μl of the following substances were pipetted into polypropylene microtubes (Eppendorf) to form the standard incubation mixture: (1) Buffer; (0.5 M NaCl, 0.04 triethanolamine–HCl, 0.02 M Na_2EDTA, made to pH 7.6 at 20 °C) with and without D-glucose (2.0 or 3.0 M). (2) [^{14}C]Mannose, 2.5 μM. (3) [^{3}H]Phlorizin, at one of the concentrations listed above. (4) Water or an aqueous solution of the phlorizin analog to be tested.

The tube contents were mixed for 2–3 min in a rotary shaker and then 100 μl of the brush border preparation, suspended in a 1:4 dilution of the incubation buffer without glucose, was added. Tube contents were again agitated for 2 min, allowed to stand for 5 min, and then centrifuged for 10 min (15000 rev./min, Eppendorf Microsystem). All steps were performed at room temperature.

A 100-μl aliquot of each supernatant was added to a liquid scintillation vial which contained 200 μl of a 1:1 (v/v) mixture of isopropanol–Soluene (Packard Instrument Co., Downers Grove, Ill.) and 10 ml of a commercial scintillation fluid Insta-Gel (Packard) to which had been added 50 ml of 0.5 M HCl per l. A Pasteur pipette was used to carefully remove and dispose of the remaining supernatant without disturbing the membrane pellet. 200 μl of the isopropanol–Soluene mixture was then

added to this sediment, and the suspension was agitated until solubilization of the membrane occurred. The mixture was quantitatively transferred to a scintillation vial which contained only the adulterated Insta-Gel. All samples were analyzed simultaneously for ^{3}H and ^{14}C using the Packard Scintillation Spectrophotometric System, model 3380 equipped with external standard counting to correct for quenching.

Our present incubation medium and procedure were somewhat simplified and altered from that previously described[17], but these differences did not have a major influence on the calculated kinetic parameters of phlorizin binding. For example, Haemacel was omitted in the present work, while temperature and time of incubation were slightly altered. D-Glucose was used to block the high affinity receptors (earlier identified as the R_1 sites[16,17]) at a final concentration of either 0.4 or 0.6 M. No significant difference in the regression lines through both sets of these data (12 and 16 experiments respectively) was found, indicating that indeed, full blockade of the R_1 receptor was achieved with 400 mM glucose. Since the brush borders were isolated in a medium containing sucrose, some of which could have been carried over to the incubation despite our usual final wash in the sucrose-free buffer, we tested whether the disaccharide would effect phlorizin binding. Sucrose, added to the normal incubation mixture so that its final concentration was 250 mM, had no influence whatsoever (2 experiments) in our system.

Calculations

Some of the ^{3}H found in the sediment represents unbound phlorizin present in the dead space between the membrane fragments. We corrected for this by estimating the volume of entrapped supernatant from the ^{14}C content of the pellet and assuming that a constant ratio of [^{14}C]mannose to [^{3}H]phlorizin exists in both supernatant and dead space. Phlorizin adsorption was assessed according to a linear form of the Langmuir mass-law equation[25,26]:

$$\frac{b}{f} = -\frac{1}{K} \cdot b + \frac{n}{K}$$

where b is the amount bound per mg membrane protein, f is the concentration of phlorizin remaining free in the supernatant, K is the dissociation constant and n represents the number of binding sites per mg protein. A straight line with a slope of $1/K$ and an ordinal intercept equal to n/K will be obtained in the Scatchard plot only if a single receptor type is present. Our brush border membrane preparation possesses more than one phlorizin binding site[16,17], and a marked curvature in the plotted data is obtained. A linear component of this curve (hereafter called the composite) having a shallow slope is readily identified however (see Fig. 1), and it describes a low affinity receptor. The values for this nearly horizontal limb were subtracted from the composite curve yielding difference data which give a second straight line with steep slope. This line, which will always be shown in the insets of the figures, represents a high-affinity binding constituent in the membrane preparation. It is this specific binding site, R_1, which is sodium-dependent, competitively inhibited by D-glucose and, as will be shown, is depressed by some of the phlorizin-like analogs which have been currently tested.

Potency of the analogs, as phlorizin-binding inhibitors, was determined by estimating their inhibition constant, K_i, as described by Dixon[27]. In this method the

inhibitor concentration is plotted against the reciprocal of the amount of phlorizin bound at the various concentrations of free phlorizin tested. Amount bound refers only to the difference between the total and non-specific adsorption.

It should be mentioned that all of these data were also calculated as amount phlorizin bound per unit alkaline phosphatase activity instead of per mg protein. The values of the kinetic binding parameters determined in this manner did not differ from those cited here. This is probably due to the relative constancy of the alkaline phosphatase activity in our day-to-day preparation (mean 76, range 55 and 112 μmoles substrate split per h per mg protein) and the narrow range of brush border protein added per incubation for the experimental series (0.5–1.2 mg; mean, 0.77 mg).

RESULTS

The present series of experiments were completed with 32 membrane preparations. The results of our phlorizin-binding tests are shown in Fig. 1 as the solid curve connecting six points, each representing the mean of 32–41 separate observations*

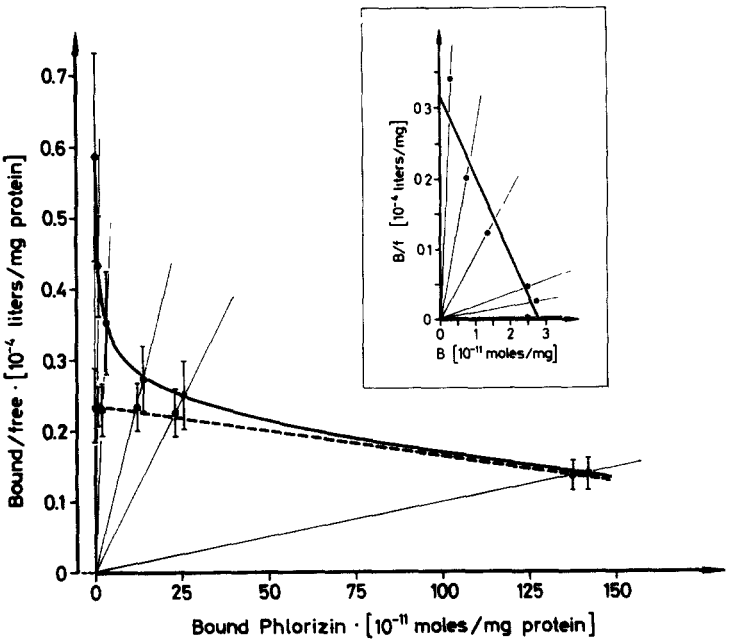

Fig 1 [^{3}H]Phlorizin binding to brush borders isolated from rat kidney. Each point represents the mean and standard deviation value of from 32–41 separate observations at each concentration of phlorizin tested. ●—●, phlorizin binding with no additions; ●---●, phlorizin binding in the presence of 0.4 or 0.6 M D-glucose The inset panel illustrates the data representing the high-affinity phlorizin-binding component; each point represents the difference between the dotted-line values and the composite phlorizin binding curve in the main figure along each of the diagonals intersecting at the origin (see the text and refs 26 and 28) The equation of the regression line in the inset is $y = -0.123x + 0.34$ The calculated K_D for this high affinity receptor is 0.81 μM.

* The thin lines radiating from the origin connect the values of bound phlorizin determined at the six concentrations which we used For example, the line most parallel to the ordinate describes data obtained with phlorizin at an initial concentration of 0.1 μM, while the one most parallel to the abscissa represents the 100 μM results

The dotted line in this illustration is the best least-squares fit to the data obtained when D-glucose was present in the incubation mixture at a final concentration of 0.4 or 0.6 M. The fact that the binding data are now representable by a straight line suggests that only one receptor was titrated; glucose preferentially inhibits phlorizin binding to the high-affinity receptor sites and has no influence on the adsorption to the low-affinity receptors. Thus, the amount of phlorizin bound in the presence of this high glucose concentration can be taken as the baseline adsorption to non-specific sites. When this amount is subtracted from the composite curve, the difference data representing the so-called R_1 binding site is obtained (see the inset panel of Fig. 1). The number of R_1 receptor sites, n, which is estimated from the intercept value on the abscissa, is 27.5 pmoles/mg membrane protein. The calculated dissociation constant of phlorizin for the high-affinity site is 0.81 μM.

Before describing the effects of the various phlorizin-like test compounds, an important point pertaining to these control studies should be made in view of earlier unexpected results of similar experiments with intestinal brush borders[21]. The brush border membranes, as we have isolated them from rat kidney, do not posses β-glucosidase (phlorizin hydrolase[21,29]) activity. This was unequivocally demonstrated in a separate series of experiments in which a very sensitive and specific assay for the enzyme was developed (D. F. Diedrich, unpublished results).

Inhibitory potency of the phlorizin-like glycosides

All of the potent glycosidic analogs at the concentrations which we tested failed to have a significant influence on phlorizin's binding to the low-affinity, non-specific receptor sites. Some of them were found to be potent inhibitors of the high affinity adsorption locus, and this was our most important finding. One of the potent glycosides was 4'-deoxyphlorizin[22]. Data for this analog are presented in Fig. 2 and the results are typical of those found for all the active glycosides in our test series. The binding of 0.1 μM phlorizin is inhibited by 4'-deoxyphlorizin at 1 μM about 50 % and at a concentration of 20 μM almost completely. In order to estimate the inhibitor constant, K_i, these data were plotted as shown in Fig. 3. The regression lines intersect at an approximate point above the horizontal axis. A perpendicular dropped from this intersection to the abscissa gives the K_i value of about 0.9–1.0 μM. Each of the analogs was tested in this manner and all of the potent ones gave straight lines in the Dixon plot.

Phloretin action

The data presented in Fig. 4 illustrate that the effect of phloretin on the phlorizin binding phenomenon was quite unlike that found for the potent glycosides. At 2 μM, phloretin failed to inhibit phlorizin's specific R_1 site whereas the low affinity receptors were partially blocked.

These effects were accentuated when phloretin was tested at 10 μM, but at this higher concentration, the aglycone also poisoned some of the R_1 sites. This latter action was quantitated in another series of experiments. Fig. 5 represents these results in which both 0.6 M glucose and 10 μM phloretin were simultaneously present in the incubation mixtures. As we expected, glucose inhibits the remaining specific R_1 sites which were not blocked by phloretin at these concentrations, but the sugar again failed to affect the low-affinity receptor. If the phloretin *plus* glucose data line,

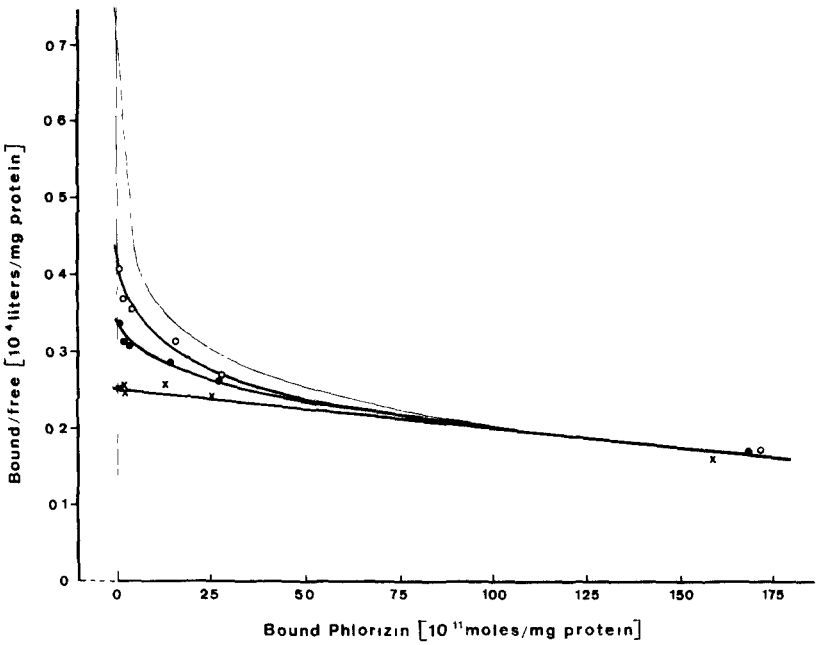

Fig 2 [^{3}H]Phlorizin binding in the presence of varying concentrations of 4'-deoxyphlorizin ○—○, 1 μM; ●—●, 2.7 μM; ×—×, 20 μM. The light solid line represents control phlorizin bindings for this series of experiments Notice that the high-affinity receptor is fully inhibited by 20 μM of the analog while no significant depression of the non-specific phlorizin binding was found at this concentration

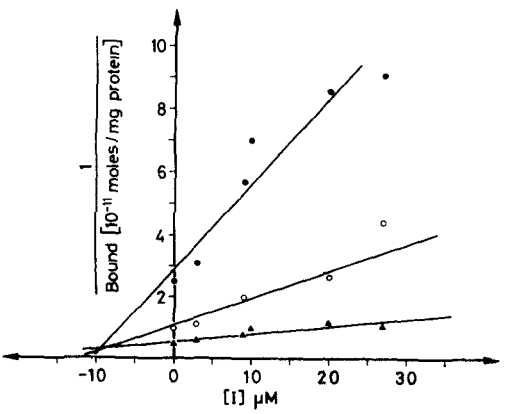

Fig 3 Estimation of inhibitor constant, K_i, for 4'-deoxyphlorizin The amount of bound phlorizin in the presence of various inhibitor concentrations, (I), is plotted according to the method of Dixon[27] Data were taken from Fig 2 The value for K_i is represented by the horizontal distance on the abscissa corresponding to the point of intersection of the regression lines Initial phlorizin concentrations ●—●, 0.1 μM; ○—○, 0.5 μM; △—△, 1.0 μM

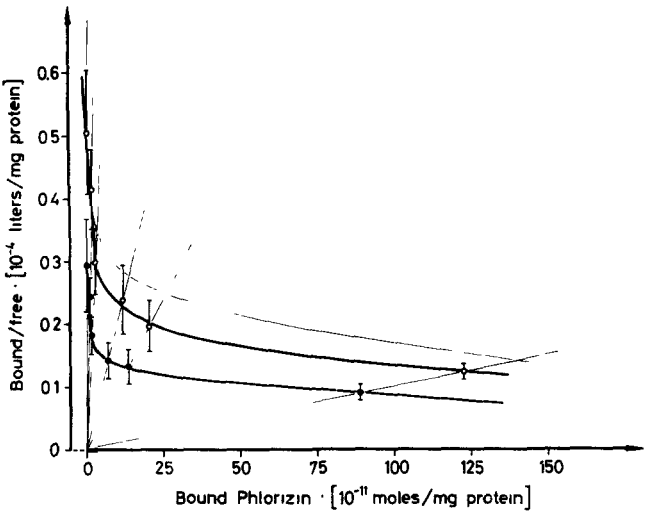

Fig 4 [^{3}H]Phlorizin binding in the presence of phloretin The thin, solid curve represents the same control data as that shown in Fig 1 ○—○, 2 μM phloretin, each point is the mean standard deviation of 4 observations ●—●, 10 μM phloretin, each point is the mean of from 14 to 20 observations Notice that the major effect of phloretin is to block phlorizin's low-affinity receptor site

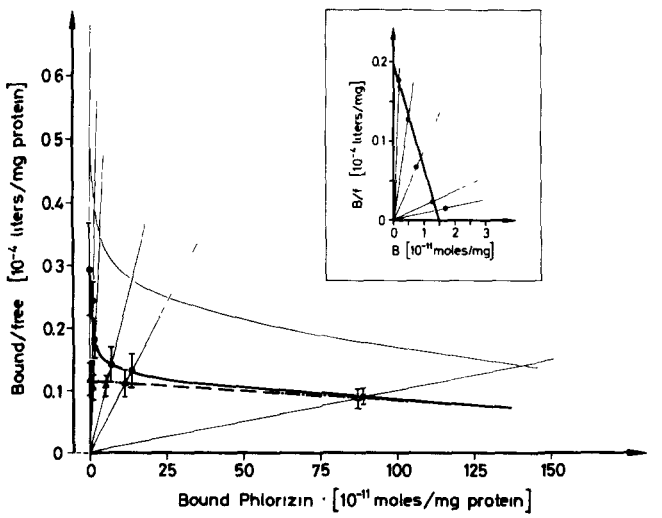

Fig 5 The effect of phloretin *plus* glucose on [^{3}H]phlorizin binding The thin, solid curve represents the same control data as that shown in Fig 1 Each point of the dot-dashed regression line represents the mean and standard deviation of 13 observations of phlorizin binding in the presence of both 10 μM phloretin and 0 6 M D-glucose Note that the slope of this line is identical with the nearly horizontal limb of the 10 μM phloretin only curve, ●—● The inset panel shows the difference data obtained when this dot-dashed baseline is subtracted from the composite 10 μM phloretin curve and represents the specific phlorizin receptor which is susceptible to full blockade by glucose but is uninfluenced by phloretin The regression line in the inset was calculated from only the 5 lowest phlorizin concentrations tested since no difference was measured with or without phloretin at the highest phlorizin concentration. The equation of the line is $y = -0\ 132\ x + 0\ 195$ and K_D, as determined from the intercepts, is 0.77 μM.

which is comparable to the linear component of the 10 μM phloretin curve, is substracted as usual from the phloretin-only composite, the difference data shown in the inset of Fig. 5 are obtained. Notice that about 45 % of these R_1 sites are depressed by the 10 μM phloretin (maximal phlorizin binding is reduced to 15 pmoles/mg protein) but no influence on the dissociation constant of this high affinity site was found (K_D with phloretin present is 0.77 μM). This type of activity is characteristic of a non-competitive inhibitor.

Effect of phlorizin chalcone

One of the first phlorizin analogs which we tested was the chalcone. This compound differs structurally from phlorizin only in having a double bond between the α and β carbon atoms which connect the aromatic rings of the molecule (A–CO–CH=CH–B; compare with phlorizin's structure in Fig. 8). This was a crucial experiment, since the analog has (surprisingly) no detectable inhibitory effect on glucose transport in either the intestine or kidney[14,15]. Consequently, despite its structural similarity to phlorizin, the chalcone should fail to poison the high affinity receptor if indeed this binding site in our brush border membrane preparation represents the sugar carrier transport system.

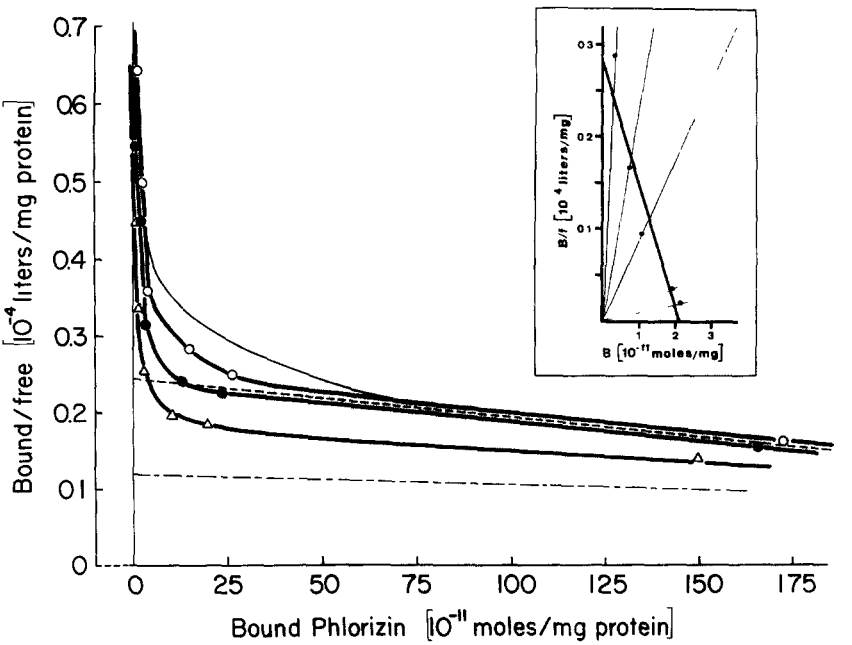

Fig 6. The effect of phlorizin chalcone on [^{3}H]phlorizin binding ○—○, 20 μM chalcone, mean of two experiments; ●—●, 100 μM chalcone, one experiment; △—△, 210 μM chalcone, mean of 5 experiments. The light solid curve represents phlorizin binding with no additions and is the mean control of the 8 experiments represented here Similarly, the binding plots of the glucose only and glucose *plus* phloretin data for this set of 8 experiments are presented as they were in Fig. 1. The inset panel shows the difference data which were obtained when a line tangential to the nearly horizontal limb of the 210 μM chalcone curve was subtracted from the composite Therefore, only 5 values were obtained to gain the data of the inset panel which represents the high-affinity receptor that remains uninhibited by 210 μM chalcone The equation of the regression line is $y = -0.130\,x + 0.278$ and calculated K_D is 0.76 μM

The chalcone's effect on phlorizin binding was complex. At sufficiently high levels, the analog mimics the action of phloretin and inhibits the non-specific adsorption sites (see Fig. 6). The receptor population which remains unaffected by 210 μM chalcone (but which is susceptible to full blockade by glucose) was determined as previously described and is represented by the regression line in the inset panel. The calculated constants for the high affinity receptor are K_D = 0.76 μM and n = 21 pmoles/mg protein. Thus, the chalcone has essentially no effect on phlorizin's affinity for the R_1 binding sites, even at a concentration of over 200 times the K_D, but appears to block 23 % of them, which suggests that the inhibition, like that exerted by phloretin, is non-competitive. It is important to point out, however, that this decrease in phlorizin binding is selectively expressed; the experimental chalcone plots possess a more acute curvature than the mean control binding curves. We had this same but less definite impression from the phloretin binding curves displayed in Fig. 4 where the high affinity limb of the experimental plots is steeper than in the controls. The results with the chalcone at lower concentrations make this point clear. For example, at 20 μM (Fig. 6, open circles) the analog has no effect on either the very high or the very low affinity receptors; yet phlorizin binding to some component of our system, which is titrated at the 1.5 and 10-μM phlorizin concentrations, is inhibited. These latter data were treated as usual to obtain the characteristics of the high-affinity receptor population. Fewer binding sites were available (n = 26 pmoles/mg), but phlorizin appeared to have essentially the same attraction for them (K_D = 0.62 μM). These results provoke a suspicion which will be discussed in some detail later; more than the two previously described[16,17] receptor types are contained

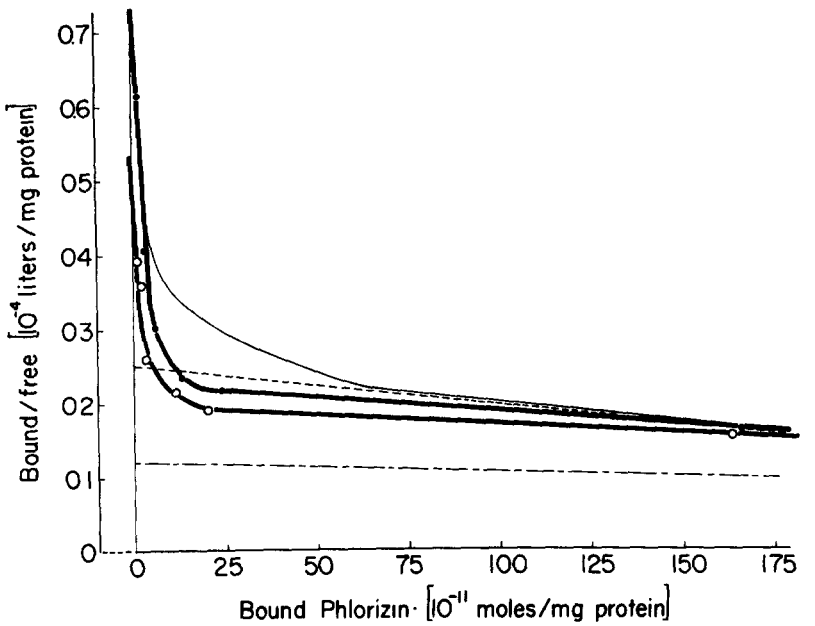

Fig. 7 Effect of D-mannose and 2-deoxygalactose on [^{3}H]phlorizin binding Each solid point represents the mean of 4 observations of binding experiments conducted in the presence of 0 4 M D-mannose The open circles are the mean data with 0 4 M 2-deoxygalactose present (3 experiments) The lightly drawn solid, dotted and dot-dashed curves are respectively the phlorizin control, phlorizin *plus* glucose and phlorizin *plus* both glucose and 10 μM phloretin data

in our brush border preparation, a third (hereafter referred to as R_3) may be present. It has an intermediate affinity for phlorizin, is selectively blocked by low concentrations of chalcone and is especially sensitive to phloretin inhibition. Although R_3 is not affected by even high concentrations of D-glucose (0.6 M), the results of a limited number of tests described below suggest that free sugars other than D-glucose have a preferential affinity for this site.

Effect of mannose and 2-deoxygalactose

The influence of D-mannose on phlorizin binding was tested early in our study, inasmuch as we contemplated using trace amounts of it as a ^{14}C marker to identify dead space in the sedimented membranes. We established the fact that mannose, at a final concentration as high as 0.4 mM, had no effect whatsoever on phlorizin binding (we therefore could use marker [^{14}C]mannose at our 0.5-μM level). Unexpectedly however, this sugar, at considerably higher concentrations, had actions which resembled those of the chalcone (see Fig. 7); at 0.4 M it blocked phlorizin binding to sites which glucose was unable to influence. Similar results were obtained for 2-deoxy-D-galactose at 0.4 M, although this sugar also seemed to inhibit the very high affinity site. However, the number of observations were too few to quantitate this latter effect.

DISCUSSION

The major objective of the present work was to determine the relative potency of a series of phlorizin-like compounds as inhibitors of a high-affinity phlorizin receptor in isolated membranes. We have accomplished this and have summarized our findings in Table I in which the K_i values of the analogs are compared. The results of some earlier studies, which illustrate the effectiveness of these compounds as transport inhibitors in hamster intestine and dog kidney are also shown for easy comparison. The satisfactory relationship between their relative effectiveness as inhibitors of glucose transport and phlorizin binding can be seen and indicates to us that the high-affinity phlorizin receptor present in our brush border preparation is either the sugar carrier itself or is one of a series of constituent moieties of the intact sugar transport system.

The single discrepancy in the inhibitory potency classification is exhibited by 4'-deoxyphlorizin. That is, in the hamster intestine test system, this analog was 55 and 70% more potent than phlorizin in its capacity to inhibit glucose transport. On the other hand, the derivative was only as potent as phlorizin when these compounds were tested as glucose transport poisons in dog kidney. Furthermore, we are currently testing the deoxy analog as an inhibitor of the active transport component for D-glucose in rat kidney proximal tubules. Our preliminary results indicate that phlorizin has a considerably greater potency than this derivative. Although we wish to reserve the privilege of discussing this matter in detail when our *in vivo* rat kidney studies are presented (H. Vick, D. F. Diedrich and K. Baumann, unpublished results), we would like to acknowledge the possibility that this apparent difference in potency of the 4'-deoxyphlorizin as a competitor for the D-glucose–phlorizin receptor in the brush border membrane from kidney and intestine may relate to a difference in receptor construction in the two organs.

TABLE I

THE COMPARATIVE INHIBITORY POTENCY OF PHLORIZIN-LIKE COMPOUNDS ON GLUCOSE TRANSPORT AND PHLORIZIN BINDING TO BRUSH BORDER

The potency of each inhibitor is listed relative to phlorizin which was arbitrarily given a value of 100 Transport System A Renal reabsorption of glucose by the dog[14]. Potency was measured as percentage decrease in maximal tubular capacity to reabsorb glucose caused by equal dosage of each compound infused intravenously Transport System B Glucose uptake by hamster intestine[15] Potency was determined from apparent K_i values found from experiments in which the *in vitro* accumulation of glucose by pieces of hamster small intestine was competitively inhibited Transport System C Similar to System B K_i data were obtained from a separate series of experiments in which glucose transport by the intestine was measured as the rate of loss of sugar from the incubation medium[15] K_D is the dissociation constant of the phlorizin-high affinity receptor complex as determined by methods described in the text K_i is the apparent competitive inhibition constant determined according to the method of Dixon[27]. The values for K_i are given as a range inasmuch as the lines formed in the Dixon plot (see Fig 3) only approximate a common intersection point and thus the maximum and minimum values are cited The mean phlorizin dissociation constant, K_D, was calculated from the intercepts on the x and y axes as described in the text. A range of each intercept was estimated with 95 % confidence limits by applying assumptions commonly employed for a simple least squares fit model of the mean difference data shown in the set panel of Fig 1 The range of K_D cited represents the values obtained when the maximum and minimum of each intercept were used to calculate the constant

Compound tested	*Inhibitory potency*				
	Transport system			*Phlorizin binding*	
	A	*B*	*C*	*Relative*	K_D *or* K_i (μM)
4'-Deoxyphlorizin	89–100	155	170	80–90	0 9–1 0
Phlorizin	90–100	100	100	100	0 81 (0 45–1 9)
4-Methoxyphlorizin	70–86	54	59	41–54	1 5–2 0
Deoxycorticosterone glucoside	24–50	16	12	14–20	4–6
Phloretin 2'-galactoside	13–27	16	6	4–5	16–22
Phloretin 2'-(3-methoxyglucoside)	10	13	8	1 5–2	40–50
Phloracetophenone 2'-glucoside	0	2	1	< 1	> 200; 200 μM inhibits R_1 receptor 12 %
Phlorizin chalcone	0	0	—	< 1	Non-competitive, 210 μM inhibits R_1 receptor 23 %
Phloretin	< 10	4	4	< 10 (?)	Non-competitive; 10 μM inhibits R_1 receptor 45 %

During the course of the present investigations, we obtained some results which were difficult to explain on the basis of the restriction that the brush border preparation possessed only two types of phlorizin binding sites, previously identified as R_1 and R_2[16]. Our data re-confirm the existence of the aforementioned high affinity R_1 receptor, which, in the present series of experiments, has a calculated dissociation constant for phlorizin of 0.8 μM. We have now also shown that it is this population which is selectively blocked by the phlorizin-like glycosides in a manner illustrated by a potent representative of the group, 4'-deoxyphlorizin (Fig. 2). Secondly, we have again demonstrated a relatively low affinity (K_D is approx. 100 μM), high capacity (n is approx. 3 nmoles/mg) adsorption site*. We have been referring to this

* These binding constants of the R_2 receptor were obtained from the intercept values of the extrapolated, dotted regression line in Fig 1

receptor population, R_2, as the non-specific pool. This term may be misleading in view of the current observation that low concentrations of phloretin and chalcone block the positions, whereas even a larger concentration of phloracetophenone glucoside (200 μM) has no effect on this receptor population. Yet, to come to the point of this discussion, we suspect the presence of a previously unidentified, third receptor type (R_3) and propose that it would (1) have an intermediate affinity for phlorizin (*i.e.* a K_D between 1 and 100 μM,) (2) possess a high affinity for phloretin and phlorizin chalcone and (3) adsorb free sugars such as D-mannose and 2-deoxy-D-galactose in preference to D-glucose.

Unfortunately, no simple and rapid technique is presently available to prepare large quantities of brush border membranes completely uncontaminated with any other cellular fragment. Plasma membranes from the lateral and basal borders of the cell, as well as lysosomes and other endoplasmic reticulum, cannot be completely removed by differential centrifugation[19,20,30]. These foreign membranes could obviously constitute contaminating binding sites for phlorizin with characteristics like those we have newly identified. It seemed futile, therefore, to extend our present study and establish the credibility of this third receptor. Our reasons for postulating it in the first place (the finding of a more acute hyperbolic binding curve caused by phoretin and phlorizin chalcone in the Scatchard plot) do not provide an attractive basis for an assay method. Furthermore, we do not have the answer to the critical question: Is the proposed receptor a component of the major membrane fragment in our preparation (the brush border), or is it associated with the above mentioned contaminating fragments? Further work on this problem should depend on the development of methods designed to isolate uncontaminated membranes such as that recently described by Kinne *et al.*[31,32].

Although we do not wish to over-emphasize the importance of the so-called R_3 receptor by an unjustifiably long discussion, the effects of phloretin and phlorizin chalcone on the binding system may perhaps be better understood if these proposed sites could indeed be characterized.

This is an especially provocative point since Glossmann and Neville (ref. 18, and unpublished results) have also reached the conclusion from their phlorizin binding studies that a receptor, like our proposed R_3 with a greater affinity for mannose than glucose, is also present in their brush borders isolated from rat kidney. Moreover, our present notation that, there seems to be in our brush border preparation, a D-glucose-preferring site to which phlorizin has a high affinity, as well as a D-mannose (and 2-deoxygalactose) receptor having a lesser compatibility for phlorizin binding is in complete accord with the interesting *in vivo* study of Silverman *et al.*[33] on the specificity of monosaccharide transport in dog kidney. These workers also propose the presence of two transport sites in kidney, *i e.* a glucose-preferring and a mannose-preferring carrier.

We have demonstrated that phloretin at 10 μM blocks 45 % of the available higher-affinity receptor population. However, the aglucone differs from the other potent phlorizin-like glycosides in its mode of inhibition*. Instead of causing an apparent decrease in phlorizin's affinity, phloretin, if anything, decreased the cal-

* This was also indicated in tests of phloretin's potency as a glucose transport inhibitor in intestine[34] Of all the phlorizin-like compounds tested, only the aglycone failed to inhibit sugar uptake in an apparently competitive manner (see also ref 35)

culated K_D value. We did not test its effects at higher concentrations because of its solubility limitations in the way we set up the assay system. We, therefore, failed to obtain the additional results which may have confirmed our suspicion that the major portion of the receptors blocked by the aglycone were of the newly proposed R_3 type. Only one other argument, which may bear on this point, can be offered. In every series of experiments the slope of the regression line for the phloretin *plus* glucose data (the dot-dashed line in Fig. 5) was significantly less than that for the glucose only data (the dotted line in Fig. 1). Clearly, under conditions when presumably all of the susceptible high affinity sites were fully blocked by 0.6 M D-glucose, the addition of phloretin to the system should have decreased only the ordinal intercept of the binding curve; the slope should not change if indeed the only receptor that phloretin additionally inhibited was the non-specific R_2 adsorption site described by the dotted line. A decrease in slope, as we have found, would be expected only if a population of high affinity, phloretin inhibitable receptors were present, which had not been blocked by 0.6 M glucose.

Our results may have some bearing on the recent reports of Kleinzeller[36]. He has described a sugar-carrier system present in kidney cortex preparations different from the Na^+-dependent, phlorizin-sensitive glucose transport process in the brush border. Two of Kleinzeller's findings were of particular interest to us: (1) his carrier system had a high affinity for 2-deoxygalactose which could not be inhibited by D-glucose even at a molar ratio of 1:50, and (2) phlorizin is a considerably less effective inhibitor of the deoxy sugar-transport system than the normal glucose carrier[37]. Furthermore, of even greater interest in this context is Kleinzeller's finding (personal communication) that, in his system, phloretin is a more effective inhibitor than phlorizin. Further developments are necessary for us to propose our newly described R_3 receptor as a candidate for the role of Kleinzeller's carrier.

Perhaps the most interesting observation of our present study is that phlorizin chalcone is unable to effectively block the specific, high affinity receptor for phlorizin. Because of the structural similarity of the two compounds, this must be considered as an unexpected finding. However, it is in complete accord with earlier results where the chalcone failed to inhibit glucose transport in the dog kidney[14] and hamster intestine[34]. The chemical identity of the chalcone is unquestionable, it is routinely prepared and serves as the immediate precursor in the synthesis of phlorizin Although both molecules have identical functional groupings, an outstanding difference is easily recognized if molecular models of the compounds are constructed and compared. The chalcone is an α,β-unsaturated ketone (compare this with phlorizin in Fig. 8) and it experiences resonance phenomena not shared by phlorizin. The conjugation of the chalcone imposes a restriction on the conformation which the B ring can assume with respect to the remainder of the molecule, *i.e.* the B ring cannot swing out of the plane of the spatially fixed glucoside moiety. The aromatic rings are consequently coplanar and the molecule is relatively flat[38].

Some of the structural features of phlorizin necessary for it to inhibit active glucose transport have been established[14,15,36,39]. These structure–activity relationship studies require the membrane receptor to have a critically-spaced functional group which will form a hydrogen bond with the phenolic hydroxyl on the B ring of the inhibitor molecule. Our current and unpublished results suggest that the phlorizin receptor portion of the sugar transport system is on the external surface

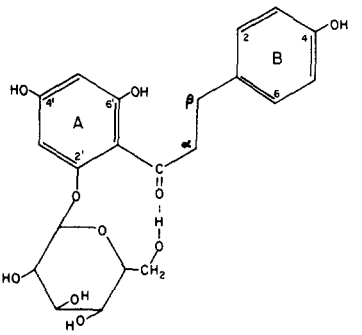

Fig 8. The structure of phlorizin.

of the membrane and the sugar moiety of the molecule is bound in a plane nearly perpendicular to that which binds the phenolic B ring. The chalcone fails to inhibit both phlorizin binding and sugar transport since it cannot interact simultaneously at both planes. The argument that the chalcone should, nevertheless, be able to attach to the glucose-binding locus, even though its tail system would remain unattached, is not supported by our findings. If this were possible, then surely a compound having no tail ring at all, but with identical remaining structure, should also bind to the plane composing the glucose receptor. This is not the case; phloracetophenone 2'-glucoside, which seems to meet the structural criteria necesssary for it to react at the glucose site, is ineffective as either a transport poison or a binding blocker. The critical nature of the B ring tail bonding is therefore unequivocal.

It is superfluous to even suggest that more information is needed before any further insight in this problem is gained. Further purification and isolation of the sugar-carrier complex is essential. We feel that sufficiently strong evidence has now been accumulated indicating that the isolated brush border membranes from kidney tubular cells contain at least the first part of the transport system. We offer this proposal with some confidence in view of our as yet unpublished current work in which we are testing this series of phlorizin analogs as renal glucose transport inhibitors in the same strain of rat from which we prepared our brush border membranes (H. Vick, D. F. Diedrich and K. Baumann, unpublished results). Our preliminary results indicate that the derivatives have essentially identical K_i values as inhibitors in both the *in vivo* renal transport and *in vitro* phlorizin binding system.

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