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THE BINDING OF PHLORIDZIN TO THE ISOLATED LUMINAL MEMBRANE OF THE RENAL PROXIMAL TUBULE

RUSSELL CHESNEY, BERTRAM SACKTOR* and ARNOST KLEINZELLER

Laboratory of Molecular Aging, Gerontology Research Center, National Institute of Child Health and Human Development, National Institutes of Health, Baltimore City Hospitals, Baltimore, Md, 21224 (U.S.A.); and Department of Physiology, University of Pennsylvania, School of Medicine Philadelphia, Pa. 19104 (U.S.A.)

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SUMMARY

The binding of [^{3}H]phloridzin by isolated luminal membranes of the rabbit proximal tubule and by slices of rabbit kidney cortex was studied.

Kinetic analyses of the relationship between the concentration of phloridzin in the incubation medium and the binding of phloridzin to the membrane indicated two distinct classes of receptor sites. One class, comprising high affinity sites, reached saturation at 20–25 μM phloridzin, had a K(phloridzin) of 8 μM, and $8 \cdot 10^{-2}$ nmoles interacted with 1 mg of brush border protein. The other class, comprising low affinity sites, had a K(phloridzin) of 2.5 mM, and the number of binding sites was 1.25 nmoles/mg. Na^+ was required for the binding of phloridzin at the high affinity sites. Na^+ decreased the apparent K_m for phloridzin; the apparent V of binding was not altered. Binding at the low affinity sites was independent of Na^+. Ca^{2+} was necessary for maximal binding at the high affinity sites. Binding of phloridzin at high affinity sites was more sensitive to *N*-ethylmaleimide and mersalyl than was binding at low affinity sites. Binding at high affinity sites, but not at low affinity sites, was temperature dependent.

D-Glucose was a competitive inhibitor of the high affinity binding of phloridzin. The apparent K_i was 1 mM. D-Glucose inhibited non-competitively at the low affinity sites. L-Glucose had no influence on phloridzin binding. Phloretin was a competitive inhibitor of high affinity phloridzin binding with an apparent K_i of 16 μM. Phloretin inhibited low affinity bindings of phloridzin non-competitively. Binding of phloridzin at high affinity sites was completely reversible. Binding at low affinity sites was only partially reversed. Phloridzin bound at high affinity sites on the brush border was displaced by phloridzin and phloretin but not by D-glucose.

The mechanism of the high affinity binding of phloridzin was distinguished from that of the initial interaction of D-glucose with the membrane. Binding of phloridzin required Na^+, whereas the interaction of D-glucose with the membranes had a prominent Na^+-independent component.

Intact renal cells in cortical slices accumulated phloridzin. The uptake did not saturate, was Na^+ independent, and was not competitively inhibited by sugars. These

* To whom correspondence should be addressed.

characteristics resemble those for the low affinity binding of phloridzin by isolated membranes. It is suggested that low affinity binding may represent an initial binding followed by uptake of the glycoside into membrane vesicles.

INTRODUCTION

Phloridzin is a potent inhibitor of the tranport of glucose. This inhibitory effect was described for the up-hill, energy-requiring translocation of sugar, as found in kidney [1–4] and intestine [5–7], and for the facilitated diffusion or passive transport of sugar, as found in ascites tumor cells [8], muscle [9] and red blood cells [10]. Phloridzin inhibited the active transport of sugar competitively [5,11]. In addition it was reported that inhibition occurred in the absence of detectable levels of the glycoside in cells, suggesting that the site of phloridzin action was at the membrane level [12]. Since inhibition was competitive and phloridzin did not penetrate the membrane, it was postulated that phloridzin interacts with and binds to the proposed glucose carrier [13,14]. Thus, a study of the binding of phloridzin to the renal proximal tubule luminal membrane may provide insight into the mechanism whereby D-glucose is translocated across this membrane.

In a previous paper [15], we described the stereospecific reaction of D-glucose with isolated brush border membranes. Dual affinity systems for the interaction of D-glucose with the membrane were proposed, in accord with the suggestion of Busse et al. [16]. Phloridzin was a potent competitive inhibitor of the uptake of D-glucose by the membranes [15]. Somewhat analogously, Kinne and associates [13,17] demonstrated two classes of affinity binding sites for phloridzin in rat kidney brush border preparations. Binding of phloridzin at the high affinity sites was inhibited competitively by D-glucose. The low affinity class of phloridzin binding was reported to be uninfluenced by the sugar.

This paper describes studies on the interactions of phloridzin with two preparations of rabbit kidney cortex, i.e. brush border membranes and slices. Kinetics of binding of phloridzin to isolated membranes were measured. Investigations with slices provided evidence for the uptake of the glycoside in the tissue. Additionally, binding of phloridzin to brush borders was compared with the interaction of D-glucose with these membranes, and the effects of D-glucose and phloritin on binding of the glycoside were determined. These observations permit some tentative conclusions to be drawn on the validity of phloridzin as a model for D-glucose and on the nature of the proposed sugar carrier in the renal tubule.

METHODS

The preparation of slices of kidney cortex and their use for studying binding of ^{3}H-labelled reagents were described [18,19]. Other essential experimental details are given in the text.

Renal brush border membranes were isolated by a described procedure [20], as modified [15]. Purity of preparations was monitored routinely by phase microscopy and occasionally by electron microscopy. Essentially pure preparations were observed. In addition, membranes were randomly evaluated by specific enzyme

markers, as reported previously [20]. For example, values for trehalase greater than 50 μmoles of glucose formed/min per mg protein $\times 10^{-2}$ were found, indicating brush border preparations of high purity.

Brush border pellets were washed and resuspended in Krebs–Ringer bicarbonate buffer, pH 7.4, as described [15]. In sodium-free buffers, NaCl was replaced with either choline chloride or LiCl, as indicated, and $KHCO_3$ substituted for $NaHCO_3$. When noted, equivalent Tris–Cl, pH 7.4, was used as a substitute for both NaCl and $NaHCO_3$.

Methods for measuring the binding of [^{3}H]phloridzin and/or other isotopically labelled compounds to membranes were described previously [15]. In experiments to test the reversibility of the binding of phloridzin, the reaction was incubated for 1 min (at which time the steady state was reached). Then, unlabelled phloridzin, sugar or buffer was added and the flasks reincubated as detailed [15]. Non-specific binding of phloridzin to membranes was estimated similarly to that of D-glucose [15]. Binding of phloridzin to membranes, previously treated with 10% trichloroacetic acid or 8% $HClO_4$, was approx. 15% that bound by untreated control preparations. This binding of phloridzin by membranes deproteinized by acid plus that by the Millipore filter was considered to be non-specific binding. Therefore, in most of the experiments reported in this paper, 15% of the measured phloridzin bound was subtracted from the total phloridzin bound to give that bound as a specific process. Except where noted, all results presented represent the specific binding, or 85% of the total amount bound.

[^{3}H]Phloridzin, labelled in the aglycone moiety, was kindly prepared by Dr R. L. Sullivan and was provided by Smith, Kline and French labs. Infrared analyses indicated that the [^{3}H]phloridzin was essentially pure. D-[^{14}C]Glucose was purchased from Schwartz BioResearch. Both labelled glucose and phloridzin migrated to spots with the same R_F values as authentic compounds. Unlabelled phloridzin and phloretin were obtained from Mann Research Labs.

EXPERIMENTAL RESULTS

Absence of phloridzin hydrolase activity in renal brush border

The rapid hydrolysis of phloridzin by hamster intestinal brush border preparations with the release of phloridzin and glucose was reported [21]. Although no evidence for the presence of a β-glucosidase in rabbit renal brush border membranes was found [20–22], 5 mM phloridzin was incubated for 5 min with these membranes in the standard incubation mixture used for binding studies. No free glucose was detected. This experiment provides no evidence for the presence of a phloridzin hydrolase in rabbit renal brush borders, and, thus, is in accord with the recently reported absence of the enzyme in rat renal membranes [14] and in cortical tubules of the rat [23].

Time-course of binding of phloridzin

Binding of phloridzin to membranes was determined after incubations for different periods of time. Approx. 75% of maximal binding occurred in the first 20 s of incubation (Fig. 1). Steady-state levels were attained in 45 s and no further increase in binding was seen at 5 min. The time-course of binding was seen for different concentrations of phloridzin, ranging from 20 to 200 μM. The extremely rapid binding

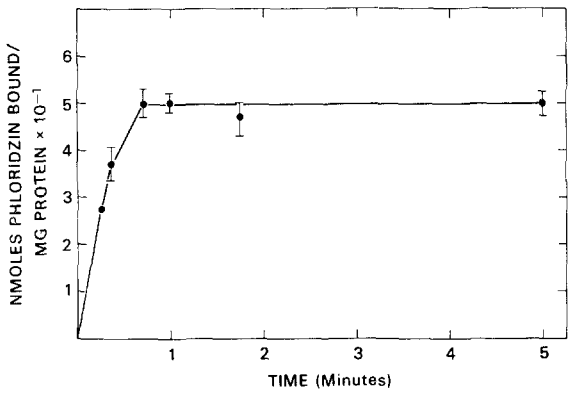

Fig. 1. The time-course of the binding of phloridzin to isolated renal brush border membranes. Binding was carried out as described in the text. The concentration of phloridzin was 200 μM.

of phloridzin contrast with the relatively slow interaction of D-glucose with this membrane preparation, the steady state not being attained with the sugar for several minutes [15].

Effect of phloridzin concentration on binding

Binding of phloridzin, expressed as nmoles bound per mg of membrane protein in 60 s, was greatly dependent on the concentration of phloridzin in the medium. When concentration was varied from 0.5 μM to 5 mM, two distinct binding systems were found. One system reached saturation at 20–25 μM phloridzin, with a concentration at half saturation of 8 μM. There was no further increase in binding until the concentration of phloridzin reached 150 μM. Then, increased binding was again observed with increased concentrations. Complete saturation of this second system was not obtained experimently because of a limitation in the solubility of phloridzin.

The two binding systems for phloridzin were characterized more definitively by plotting the ratio of bound phloridzin to free phloridzin against the amount per mg protein in 1 min, according to Scatchard et al. [24]. As shown in Fig. 2, the curve describing this relationship consisted of two components each with a different slope, clearly indicating the presence of at least two receptor sites for phloridzin in the brush border. For the binding system that was saturated at low levels of phloridzin, the K(phloridzin) was 8 μM, the stability constant was 0.125 l/μM, and $8 \cdot 10^{-2}$ nmoles (i.e. $4.8 \cdot 10^{14}$ molecules) could interact with 1 mg of membrane protein. At the other receptor site, the K(phloridzin) was 2.5 mM, the stability constant was $0.4 \cdot 10^{-3}$ l/μM, and 1.25 nmoles (i.e. $7.5 \cdot 10^{17}$ molecules) interacted with 1 mg of membrane. The binding system, which was saturated at low concentrations of phloridzin, had a low K(phloridzin), but had a smaller capacity for binding, is considered to have high affinity classes of binding sites. The binding system which had the high K(phloridzin) but had the greater number of binding sites per unit of membrane is designated as having low affinity classes of binding sites. The presence in this membrane of high and low affinity receptor sites for phloridzin is in general agreement with the two binding systems for the glycoside, as reported in rat kidney preparations [13,14,24].

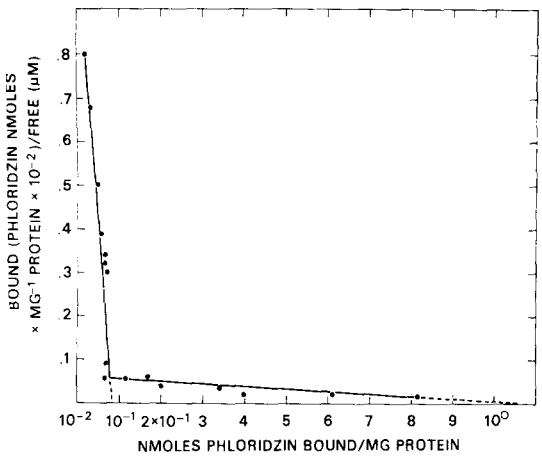

Fig. 2. A Scatchard plot characterizing the relationship between the concentration of phloridzin and its binding. Binding was carried out for 60 s as described in the text.

Ionic requirements for phloridzin binding

Na^+ was required for binding of phloridzin at its high affinity sites. Binding of phloridzin at high affinity sites (2 μM phloridzin) was reduced by approx. 90% when Na^+ was deleted from the medium and replaced with equivalent Li^+ or choline (Table I). On the other hand, Na^+ was not required for binding of phloridzin at its low (1 mM) affinity sites. In other experiments, not illustrated, the efficacy of Tris ions as a replacement for Na^+ was tested at concentrations of phloridzin characteristic of high and low affinity binding. At the high affinity sites, binding of phloridzin was markedly reduced when Tris ions replaced Na^+, as was the case when Li^+ or choline was the replacement. At low affinity sites, the substitution of Tris ions for Na^+ resulted in a moderate 30% inhibition of phloridzin binding. This contrasts with the finding

TABLE I

EFFECTS OF Na^+ ON THE HIGH AND LOW AFFINITY BINDING OF PHLORIDZIN

The binding of phloridzin was measured after 60 s in normal Krebs–Ringer bicarbonate buffer containing NaCl or the equivalent amount of choline or Li^+, as chloride salts, as indicated. $KHCO_3$ replaced $NaHCO_3$ in the sodium-free media. Each datum represents the mean ± S.E of the mean.

Phloridzin concn	Medium	Phloridzin bound (nmoles/mg protein)
2 μM	Sodium	$2.85 \pm 0.20 \times 10^{-2}$
2 μM	Lithium	$0.32 \pm 0.09 \times 10^{-2}$
2 μM	Choline	$0.26 \pm 0.08 \times 10^{-2}$
1 mM	Sodium	$3.03 \pm 0.46 \times 10^{-1}$
1 mM	Choline	$3.12 \pm 0.67 \times 10^{-1}$
2 μM	Sodium	$3.05 \pm 0.04 \times 10^{-2}$
2 μM	Sodium + 1 mM ouabain	$3.20 \pm 0.05 \times 10^{-2}$

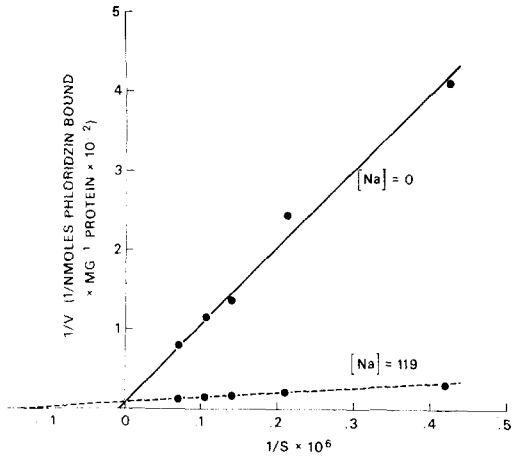

Fig. 3. A Lineweaver–Burk plot describing the action of Na^+ on the binding of phloridzin. The concentrations (mM) of Na^+ are given in the plot. Binding was measured after 60 s of incubation, as described in the text.

(Table I) that at the low affinity binding sites substitution of Li^+ or choline ions for Na^+ was without effect. Thus, the action of Tris ions in inhibiting low affinity binding of phloridzin resembles the action of this ion in inhibiting the interaction of D-glucose with brush borders [15].

The mode of action of Na^+ on binding of phloridzin was to increase the affinity of phloridzin to the receptor in the membrane (Fig. 3). The apparent V of binding was not altered by Na^+.

Table I also shows that ouabain, tested at a concentration in excess of that needed to block the Na^+-dependent accumulation of sugars in renal cortical cells [19], had no effect on the Na^+-dependent binding of phloridzin at the luminal membrane of the proximal tubule. As reported previously [15], ouabain did not inhibit the uptake of D-glucose by these membranes.

Since binding of phloridzin at its high affinity receptor sites in the membrane was dependent on Na^+, whereas uptake of D-glucose by these membranes did not require Na^+, per se* [15], at least with these membrane preparations, the present finding may suggest a distinction between the interactions of phloridzin and D-glucose with the presumed glucose carrier**.

Examination of the effects of other ions on binding of phloridzin to brush borders indicated that deletion of Ca^{2+} from the buffer caused a marked lowering of binding. For example, at a phloridzin concentration of 8.5 μM the bindings were $7.41 \cdot 10^{-2}$ and $2.39 \cdot 10^{-2}$ nmoles/mg protein in the presence and absence of Ca^{2+}, respectively. This 67% inhibition of high affinity binding of phloridzin is similar to

* Recent findings indicate that, although Na^+ per se was not required for D-glucose uptake by renal brush border membrane vesicles, uptake was enhanced by a Na^+ gradient from the outside to the inside of the vesicle [25]. Somewhat analogous observations were reported by Hopfer et al. [26] with isolated brush border membrane vesicles from rat intestine.

** This view assumes that the uptake of D-glucose by the renal tubule membrane vesicles was mediated via the carrier.

the 65% inhibition of the interaction of 1 μM D-glucose with the membrane caused by removal of Ca^{2+} [15]. In contrast, deletion of Mg^{2+} from the medium had no effect on high affinity binding of phloridzin (4 μM), $4.27 \cdot 10^{-2}$ and $4.38 \cdot 10^{-2}$ nmoles/mg protein in the presence and absence of Mg^{2+}, respectively. Substitution of Na_2SO_4 for $MgSO_4$ caused a modest decrease in uptake of D-glucose [15].

Reversibility of phloridzin binding

Reversibility of the binding of phloridzin is shown in Table II. After 1 min of incubation, when binding of labelled phloridzin had reached a steady state, unlabelled phloridzin or other test substances were added. The flasks were then re-incubated

TABLE II

THE REVERSIBILITY OF THE BINDING OF PHLORIDZIN

The reversibility of the binding of phloridzin was tested as described in the text. The concentrations of the substance bound and the reversing compound are given in parentheses. After the initial incubation, either 0.1 ml of Krebs–Ringer bicarbonate (control) or buffer containing unlabelled reversing substance was added to the 0.125-ml reaction mixture. The flasks were vigorously agitated, re-incubated for an additional minute, and the reaction terminated as described in the text. Each datum represents the mean ± S.E. of the mean. In those experiments in which the standard error is not given, the values reported represent the mean of three measurements.

Substance bound	Reversing compound	Substance bound (nmoles/mg protein)	
		Control	Reversed
Phloridzin (4 μM)	Phloridzin (1 mM)	$2.18 \pm 0.14 \times 10^{-2}$	$0.35 \pm 0.04 \times 10^{-2}$
Phloridzin (1 mM)	Phloridzin (2.8 mM)	7.36×10^{-1}	2.48×10^{-1}
Phloridzin (10 μM)	D-Glucose (50 mM)	$4.90 \pm 0.05 \times 10^{-2}$	$4.97 \pm 0.14 \times 10^{-2}$
Phloridzin (1 mM)	D-Glucose (10 mM)	$5.27 \pm 0.83 \times 10^{-1}$	$3.13 \pm 0.22 \times 10^{-1}$
Phloridzin (9.5 μM)	Phloretin (50 μM)	$8.26 \pm 0.17 \times 10^{-2}$	$4.53 \pm 0.15 \times 10^{-2}$

for an additional minute. Bound [^{3}H]phloridzin was readily displaced by unlabelled phloridzin. Compared to its paired control, 85% of the phloridzin bound at high affinity sites (4 μM phloridzin) was removed in 1 min. Binding of phloridzin at low affinity sites (1 mM phloridzin) was reversed, in part, the addition of non-labelled phloridzin effecting a reduction in bound [^{3}H]phloridzin from $7.36 \cdot 10^{-1}$ to $2.48 \cdot 10^{-1}$ nmoles/mg protein.

The time-course of reversal of binding of phloridzin to the membranes by phloridzin is illustrated in Fig. 4. There was initially a rapid reversal of binding. Approx. 50% of the phloridzin bound at high or low affinity sites was displaced in the first 15 s. After 1–2 min little additional phloridzin was removed. Thus, the rate of phloridzin release approximated the rate of binding (Fig. 1). A small fraction of phloridzin, about 10–20%, remained firmly bound. In the experiments described in Fig. 4, values for binding were not corrected for non-specific binding. If the non-specific bound glycoside (approx. 15% of the total phloridzin bound) had been deducted, practically all the phloridzin bound in a specific manner at high affinity sites would be reversibly bound and displaceable by phloridzin.

Phloridzin bound at high affinity sites (10 μM phloridzin) was not displaced

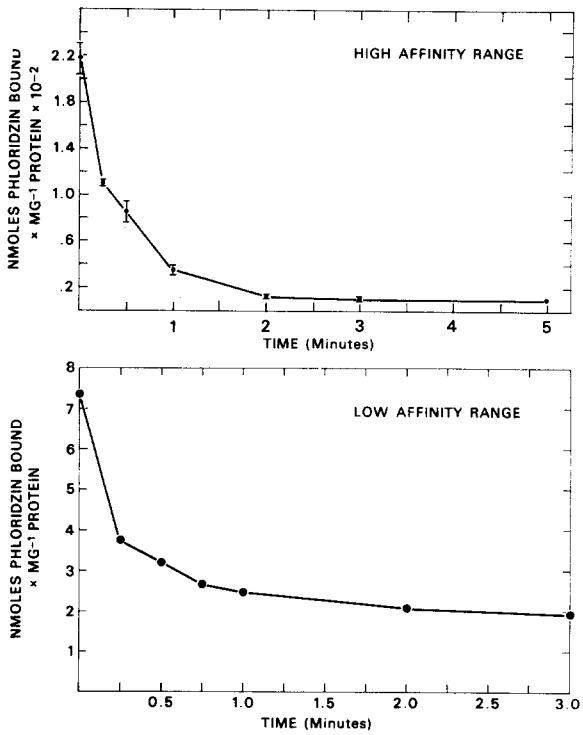

Fig. 4. The time-courses of the reversal of the binding of [^{3}H]phloridzin by unlabelled phloridzin The top curve describes the reversal of the binding of 4 μM phloridzin (high affinity range) by 1 mM phloridzin. The bottom curve describes the reversal of the binding of 1 mM phloridzin (low affinity range) by 2.8 mM phloridzin. The 0-time values represent the [^{3}H]phloridzin bound during incubations with brush border membranes for 1 min. At zero time, the unlabelled phloridzin was added. Each value indicates the amount of [^{3}H]phloridzin still bound at the different times after addition of the non-radioactive phloridzin. Additional details of the experiment are given in the text. Values for the binding were not corrected for non-specific binding, as described in the text.

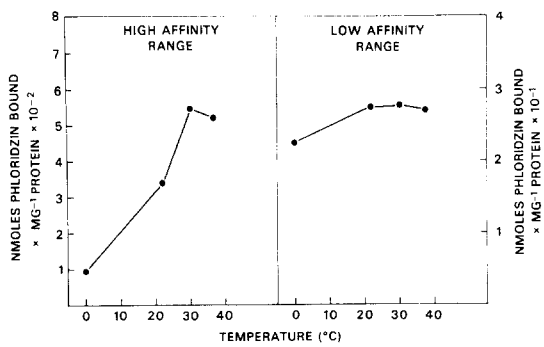

Fig. 5. The effect of temperature on the binding of phloridzin to renal brush border membranes at the high (8.5 μM) and low (1 mM) affinity binding sites. Binding was carried out for 60 s.

by D-glucose, however, even when tested at a sugar concentration 5000-fold that of phloridzin (Table II). In contrast, D-glucoside was able to reverse partially binding of phloridzin at low affinity sites (1 mM phloridzin). Phloretin, the aglycone moiety of phloridzin, reversed the high affinity binding of phloridzin.

Effect of temperature on phloridzin binding

The high affinity binding of phloridzin (8.5 μM) to the brush borders was highly sensitive to temperature (Fig. 6). Increases in temperature were correlated with increases in binding, although there was no additional increase binding when temperature was elevated from 30 to 37 °C. Low affinity binding of phloridzin (1 mM) was not temperature dependent, however.

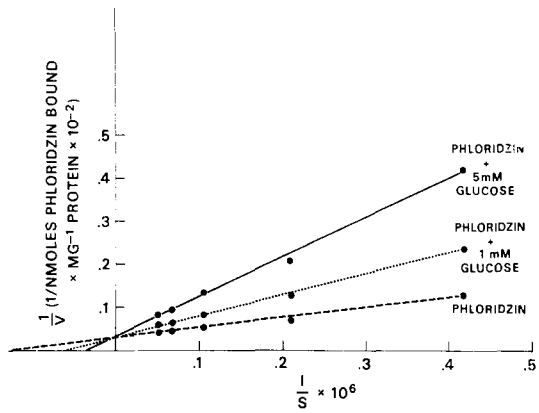

Fig. 6. Lineweaver–Burk plots describing the kinetics of the inhibition of the high affinity phloridzin binding by D-glucose. Binding was carried out for 60 s.

Effect of D-glucose on phloridzin binding

Phloridzin inhibited the interaction of D-glucose with renal membranes [15]. In the present studies the converse was found; namely that D-glucose inhibited binding of phloridzin to brush borders. For high affinity binding, kinetics typical of competitive inhibition were observed (Fig. 6). The apparent K_i was approx. 1 mM. Thus, these data tend to support the view that phloridzin and D-glucose have common or closely associated receptor sites on the membrane. In the present experiments phloridzin and sugar were added simultaneously. This was required because, as shown in Table II, D-glucose, if added to the reaction after the high affinity binding of phloridzin had already occurred, would not measurably reverse the binding of phloridzin.

Inhibition by D-glucose of the low affinity interaction of phloridzin with membranes was less pronounced and not consistent with competitive inhibition. For example, 10 mM D-glucose effected a 40% inhibition of phloridzin binding at 200, 500 and 1000 μM phloridzin (Table III). The non-competitive inhibition by D-glucose of the low affinity binding contrasted with the competitive inhibition of binding at the high affinity sites. On the other hand, the finding was in accord with the non-competitive inhibition by phloridzin of uptake of D-glucose, when millimolar concentrations of sugar were used [15].

In other experiments, 1 mM L-glucose had no influence on the high affinity

binding of phloridzin. The paired control value for binding of 2 μM phloridzin was $3.05 \pm 0.04 \times 10^{-2}$ nmoles/mg protein and it was 3.20 ± 0.08 in the presence of 1 mM L-glucose. As reported previously [15], phloridzin had no effect on the interaction of L-glucose with brush borders. D-Galactose, like D-glucose, inhibited binding of phloridzin at high as well as low affinity sites. When compared at a concentration of 10 mM, D-galactose was not as effective an inhibitor of phloridzin binding as was D-glucose (Table III).

TABLE III

INHIBITION OF THE LOW AFFINITY BINDING OF PHLORIDZIN BY D-GLUCOSE AND D-GALACTOSE

The binding of phloridzin was carried out for 60 s as described in the text. Each datum represents the mean ± S.E. of the mean.

Phloridzin concn (μM)	Phloridzin bound (nmoles/mg protein $\times 10^{-1}$)				
	Control	Glucose			Galactose 10 mM
		1 mM	5 mM	10 mM	
200	1.29 ± 0.08	1.13 ± 0.03	0.97 ± 0.05	0.81 ± 0.07	
500	2.61 ± 0.20		1.78 ± 0.04	1.46 ± 0.16	1.83 ± 0.27
1000	4.30 ± 0.24	3.58 ± 0.24	2.70 ± 0.05	2.48 ± 0.21	3.49 ± 1.03

Effect of sulfhydryl reagents on phloridzin binding

N-Ethylmaleimide and the organomercurial diuretic, Mersalyl, inhibited binding of phloridzin (Table IV). Bindings at both high and low affinity sites were blocked. High affinity classes of receptors, however, were significantly more sensitive to inhibition than were low affinity sites. With 5 mM *N*-ethylmaleimide, high affinity binding (8.5 μM phloridzin) was inhibited by about 80% whereas low affinity binding (1 mM phloridzin) was reduced by only about 30%. With lower concentrations of *N*-ethylmaleimide (0.02 mM), high affinity binding was inhibited by approx. 30%

TABLE IV

INHIBITION OF PHLORIDZIN BINDING BY *N*-ETHYLMALEIMIDE AND MERSALYL

The binding of phloridzin was carried out for 60 s as described in the text. Each datum represents the mean ± S.E. of the mean. In those experiments in which the standard error is not given, the values reported represent the mean of three measurements.

Phloridzin concn	Inhibitor	Phloridzin bound (nmoles/mg protein)	
		Control	Control + inhibitor
8.5 μM	*N*-Ethylmaleimide, 5 mM	$2.04 \pm 0.63 \times 10^{-2}$	$0.39 \pm 0.06 \times 10^{-2}$
8.5 μM	*N*-Ethylmaleimide, 0.02 mM	$4.23 \pm 0.46 \times 10^{-2}$	$3.05 \pm 0.21 \times 10^{-2}$
8.5 μM	Mersalyl, 1 mM	4.72×10^{-2}	0.50×10^{-2}
1.0 mM	*N*-Ethylmaleimide, 5 mM	$5.48 \pm 1.10 \times 10^{-1}$	$3.66 \pm 0.41 \times 10^{-1}$
1.0 mM	*N*-Ethylmaleimide, 0.02 mM	$3.78 \pm 0.12 \times 10^{-1}$	$3.62 \pm 0.10 \times 10^{-1}$

whereas binding at low affinity sites was only slightly, if at all, decreased. This difference between high and low affinity bindings of phloridzin in their sensitivity to sulfhdryl reagents is in agreement with the findings of Bode et al. [17] on the effect of *p*-chloromercuribenzoate on the bindings of phloridzin by rat renal membranes. The present results are also consistent with findings that *N*-ethylmaleimide inhibited uptake of D-glucose [15].

Effect of phloretin on phloridzin binding

Phloretin inhibited the binding of phloridzin. Both the bindings at high and low affinity sites were affected (Fig. 7). However, kinetics describing the inhibitions at the two sites differed. In the high affinity range kinetics resembled that for competitive inhibition. A secondary plot of slopes from Fig. 7 against the concentrations of phloretin yielded a straight line, from which an apparent K_i of 16 μM for phloretin was estimated. Inhibition by phloretin in the low affinity range of phloridzin binding was apparently non-competitive (Fig. 7). The interaction of D-glucose with renal membranes [15] as well as the transport of sugars in preparations of intestinal rings [27] were also found to be non-competitively inhibited by phloretin.

Interaction of [^3H]phloridzin with kidney cortex slices

Preliminary experiments with slices established that phloridzin was taken up by the tissue at a linear rate in the first 30 min of aerobic incubation at 25 °C. Subse-

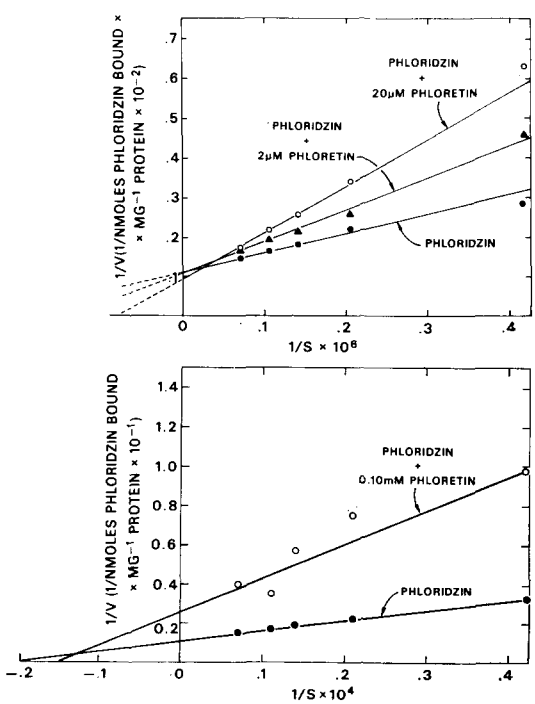

Fig. 7. Lineweaver–Burk plots describing the kinetics of the inhibition of the high (top) and low (bottom) affinity binding of phloridzin by phloretin. Binding was carried out for 60 s.

quently, uptake slowed and approached a steady-state level after 90 min incubation. As shown in Fig. 8, the level of tissue phloridzin increased linearly with glucoside concentration in the medium and slices accumulated the glucoside seemingly against 10-fold concentration gradient. Thus, no indication was obtained for saturation of tissue binding sites. Efflux experiments not reported here in detail showed that

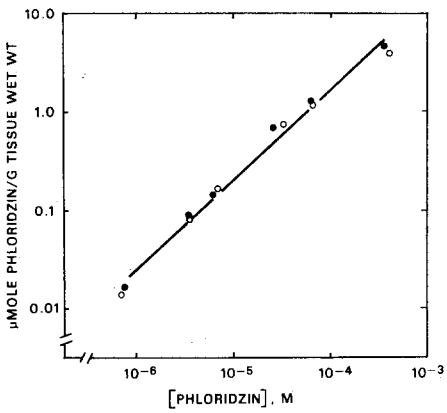

Fig. 8. The uptake of phloridzin by slices of rabbit kidney cortex. Groups of slices were pre-incubated aerobically (O_2) for 45 min in Krebs–Ringer saline at pH 7.2 (for composition see ref. 18) in order to obtain a steady state of tissue electrolytes. Subsequently, slices were incubated for 90 min under the above conditions in salines containing [^{3}H]phloridzin at concentrations varying from 1 to 500 μM (0.1 μCi/ml). The blotted and weighed slices were digested with 1 ml 1 M NaOH; the activity was measured in a portion of the acidified digest. Portions of the media at the end of incubation were treated similarly. Values are the means of four analyses. ○, control; ●, 5 mM α-methyl-D-glucoside present.

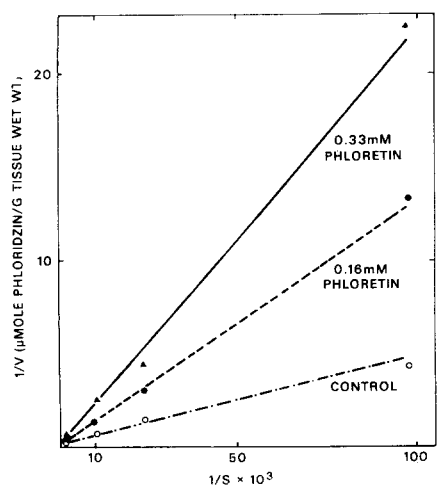

Fig. 9. Competitive effect of phloretin on the uptake of [^{3}H]phloridzin by kidney cortex slices. General experimental conditions are given in Fig. 8. Phloridzin concentrations were varied from 10 to 300 μM. ○, control (no phloretin added); ●, 0.16 mM phloretin; ▲, 0.33 mM phloretin. All values are the mean of three analyses.

phloridzin taken up by the tissue was rather firmly bound since less than 30% of the label could be washed out from the slices in 60 min and the percentage was only slightly increased by a relatively high concentration (0.1 mM) of phloridzin in the wash-out medium. Because of this interaction of phloridzin with tissue components no attempt was made to relate uptake of the glycoside by slices to cellular volume; hence, the accumulation of phloridzin against a concentration gradient may be only apparent.

Uptake of phloridzin (100 μM) by slices was not affected by the presence of 5 mM of some sugars (α-methyl-D-glucoside, D-galactose, 2-deoxy-D-glucose, 2-deoxy-D-galactose), the active transport of which is known to be inhibited by the glycoside [18]. Moreover, as compared with controls, the absence of external Na^+ did not affect uptake of phloridzin. Uptake of phloridzin in slices was competitively inhibited by phloretin (Fig. 9). These findings with renal slices, demonstrating a non-saturable, slow, uptake of phloridzin, which was not inhibited by the absence of Na^+ nor the presence of sugars, suggests that experiments with slices are correlated more closely with the low affinity than the high affinity bindings of phloridzin to isolated renal membranes.

DISCUSSION

The experiments in this paper describe the binding of phloridzin to renal proximal tubule luminal membranes isolated from the rabbit. Two binding systems for phloridzin, comprising high or low affinity sites, were found. This is in accord with the reports of Frasch et al. [13], and recently those of Glossmann and Neville [14] and Heath and Aurbach [23], which suggested the presence of two binding systems for phloridzin in rat kidney. The two binding systems have now been characterized further. The high affinity sites had a K(phloridzin) of 8 μM but had only a limited capacity, 80 pmoles/mg of membrane protein. The low affinity sites had a K(phloridzin) in the millimolar range but possessed over a 100-fold greater number of binding sites per unit of membrane. Other distinctive properties of the high and low affinity mechanisms of binding were found. Binding of phloridzin at high affinity sites was dependent on Na^+. In contrast, Na^+ was not required for binding at low affinity sites. High affinity binding was significantly more sensitive to inhibition by *N*-ethylmaleimide than was low affinity binding. At high affinity sites, increases in temperature were correlated with increases in binding, whereas low affinity binding was not temperature dependent. Phloridzin bound at high affinity sites was not displaced by the subsequent addition of D-glucose. In contrast, D-glucose was able to reverse, in part, binding of phloridzin at low affinity sites. Lastly, binding of phloridzin at high affinity sites was inhibited competitively by D-glucose, whereas inhibition by the sugar of low affinity binding was not.

The observations reported in this paper provide some evidence consistent with the view that phloridzin and D-glucose share a common receptor on the brush border membrane. D-Glucose was found to be a competitive inhibitor of high affinity binding of phloridzin, and phloridzin inhibited D-glucose uptake by the membrane vesicles at low substrate concentrations. Moreover, L-glucose had no effect on binding of phloridzin, and phloridzin had no effect on uptake of L-glucose by these membranes [15]. Additionally, binding of phloridzin and interaction of D-glucose with

the membranes were equally sensitive to *N*-ethylmaleimide and to the deletion of Ca^{2+} from the media.

On the other hand, other experiments tend to argue that the mechanism of high affinity binding of phloridzin was distinct from the initial interaction of D-glucose with the membrane. The apparent K_m of phloridzin for binding at high affinity sites was approx. 8 μM but the apparent K_i of phloridzin for the D-glucose interaction was 0.2 mM [15]. Similar discrepancies were reported between the K_m and K_i for the competitive inhibition between sugars in kidney cortex slices, however [28,29]. The higher apparent K_i for phloridzin than that predicted for simple binding equilibria may be the consequence of sequestering of phloridzin at low affinity sites or accumulation of the glycoside inside membrane vesicles. Perhaps the most irreconcilable finding for a common mechanism for the initial interactions of phloridzin and D-glucose with the membranes is that the high affinity binding of phloridzin required Na^+. The uptake of D-glucose by identical membrane vesicles had a prominent Na^+-independent component.

It is of interest to consider the interactions of phloridzin, phloretin and D-glucose with renal membranes in light of the proposal of Alvarado [27] on dual binding sites on the phloridzin molecule, i.e. the glycosidic and phenolic sites. As shown in Fig. 6, D-glucose, when incubated together with phloridzin, inhibited competitively the high affinity binding of phloridzin. The sugar, however, did not displace previously bound phloridzin (Table II). Phloridzin inhibited the interaction of D-glucose with membranes [15]. Phloretin was a competitive inhibitor of high affinity binding of phloridzin (Fig. 7) and was able to displace bound phloridzin (Table II). However, phloretin inhibited D-glucose uptake by membrane vesicles in a non-competitive fashion [15]. It is tempting to speculate on a D-glucose interaction with phloridzin at the glycosidic end of the phloridzin molecule and a phloretin interaction with phloridzin at the phenolic end, thus accounting for the competitive nature of the D-glucose–phloridzin and phloretin–phloridzin relationships. The interaction between D-glucose and phloretin might be expected to be non-competitive because of the 15 Å distance between the phenolic and glycosidic ends, as suggested from the Alvarado model of phloridzin [27]. A two-pronged attachment of phloridzin to the brush borders could also provide the basis for an interpretation of the finding that D-glucose did not displace bound phloridzin. One can also envision that the Na^+ requirement for phloridzin binding may be related to the influence of this ion at the phenolic binding site, and, thus offer an explanation for the distinction between the mechanism of the reactions of phloridzin and D-glucose with the membranes, with respect to their Na^+ dependence.

In light of the information presented on the interaction of phloridzin with the brush border membrane, the data obtained on uptake of the glycoside with intact cells in slices may indicate that phloridzin interacts with a quantitatively large number of binding sites of lesser specificity, localized elsewhere in the cells. Such assumption would be borne out by the lack of saturation of slices by the glycoside and the absence of other characteristics for binding observed in the brush border, i.e. Na dependence and competitive behaviour with sugars. These results are generally consistent with findings describing the low affinity binding of phloridzin to membranes. Thus, it is entirely feasible that the so-called "low affinity binding" to isolated brush borders may represent an initial binding followed by uptake of the glycoside into vesicles.

That this perhaps may be the case is suggested by the intracellular accumulation of phloridzin by cortical slices, as presented here, and by the apparent uptake of the glycoside in isolated renal tubules, as recently reported by Heath and Aurbach [23].

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