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# THE COMPARATIVE EFFECTS OF SOME PHLORIZIN ANALOGS ON THE RENAL REABSORPTION OF GLUCOSE

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## SUMMARY

A number of glycosides structurally related to phlorizin have been tested for biological activity. Their influence on the renal tubular reabsorption of glucose in the dog has been compared with the inhibitory capacity exhibited by phlorizin. Some conclusions concerning the nature of the critical structural and geometrical configuration of the inhibitor molecule are presented. Some information has been obtained on the mechanism of phlorizin inhibition at a molecular level and on the nature and topography of the chemical groups constituting the receptor site on the cell membrane to which the inhibitor is bound.

## INTRODUCTION

The penetration of various monosaccharides through a number of cell membranes is blocked by the hydroxydihydrochalcone glucoside, phlorizin (Fig. 1), and for this reason the compound has been used extensively to implement research on the mechanism of sugar transport. A number of investigations have been directed specifically towards the elucidation of the mechanism of transport inhibition by phlorizin. The results of some recent studies have indicated that its influence on sugar entry into cells is not related to any action it may have on the energy yielding processes of the cell.

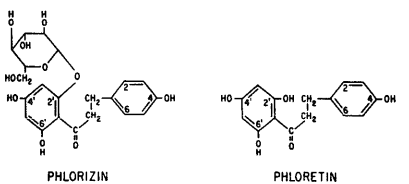


Fig. 1. A structural representation of phloretin (2',4,4',6'-tetrahydroxydihydrochalcone) and phlorizin (2'-β-D-glucopyranosyloxy-4,4',6'-trihydroxydihydrochalcone; phloretin 2'-glucoside).

Abbreviations: TmG, maximal renal tubular reabsorption capacity for glucose.

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For example, the inhibitor has been observed to impede the penetration of hexoses through the cell membranes of a variety of tissues despite the fact that the transport is not dependent on an energy source[1-3]. In addition, experiments with intestine[4] and kidney-cortex slices[5] have shown that phlorizin interferes with the entry of glucose even after the accumulation step has been blocked by metabolic inhibitors. These results indicate that the influence of phlorizin at low concentrations on sugar transport is not related to any action it may have on the energy yielding processes of the cell. Furthermore, on the basis of a number of investigations, it is doubtful that the effect of phlorizin is primarily on any intracellular process. Thus, PONZ AND LLUCH[6] found that phlorizin inhibition in intestine could be reversed by simply washing the mucosa with a glucose solution. Also, NEWEY *et al.*[7] have reported that phlorizin inhibits the intestinal transport of glucose at concentrations which do not affect the intracellular metabolism of this hexose. Furthermore, CRANE, FIELD AND CORI[1] have observed that the penetration of a number of sugars into Ehrlich ascites cells is blocked in spite of the fact that phlorizin did not enter the cells. Finally, ALVARADO AND CRANE[8] have recently reported that the influx of the actively transported compounds 1,5-anhydro-D-glucitol and 6-deoxy-D-glucose into intestinal epithelial cells is competitively inhibited by phlorizin. Thus, although its inhibitory action has not previously been analyzed on a molecular level, it seems most likely that phlorizin exerts its effects by competing with glucose for the membrane receptor site which normally binds or orients this hexose; it blocks the first step in the chain of reactions which lead to the transport of the sugar.

This report describes the results and interpretation of experiments which were designed to compare the effects of a number of phlorizin analogs on renal glucose reabsorption in order to determine the critical structure of the inhibitor molecule. The work is in accord with the well-known concept that the topography of a receptor site and/or the type of its constituent groups can be determined by elucidating the essential structural features of agents which react with it. A preliminary report on the activity of the galactose analog of phlorizin has already been published[9]. Additional references and discussion of the mechanism of phlorizin action can be found in the review articles by CRANE[10] and WILBRANDT AND ROSENBERG[11] and the recent Harvey Lecture by LOTSPEICH[12].

## METHODS

### *Experimental procedure*

All of the experiments reported here were performed on three female dogs (15–22 kg) over a two-year period. The influence of the phlorizin-like glycosides on the transport of glucose was determined by measuring their effect on the maximal renal tubular reabsorption of this hexose. The method used by LOTSPEICH AND WORONKOW[13] was employed. The phlorizin analogs were administered by constant intravenous infusion at low levels to glucose-loaded dogs. All experiments were performed under conditions used to cause 90–100 % TmG depression with phlorizin.

Following an 18-h fast, the animal was anesthetized with intravenous sodium pentobarbital (30 mg/kg). An initial infusion of 300–350 ml isotonic saline was administered to expand the extracellular fluid volume. This was done to avert dehydration which results from the profound glucosuria occurring during the subsequent

hypertonic glucose infusions. After the saline administration*, a priming dose of creatinine and glucose was injected intravenously. At the same time, the control infusion was begun. This solution contained glucose and creatinine for measuring glomerular filtration rate. It was infused at a constant rate of 5 ml/min. Mannitol, which was used by LOTSPEICH AND WORONKOW as an osmotic diuretic, was not included in this and subsequent infusion mixtures. The composition of the infusion was calculated to maintain the plasma concentration of creatinine at 25–35 mg/100 ml and glucose at 400–650 mg/100 ml; under these conditions saturation of the glucose reabsorptive mechanism was insured since the ratio of filtered to reabsorbed glucose (load/TmG) was approximately 2 in all experiments. After a sufficient equilibration time (1.5–2.0 h), three 10-min control collections were made to determine TmG. Thereafter, an identical infusion incorporating the glycoside under investigation** was begun without an equilibration period, and blood and urine samples were collected during three subsequent 10-min periods. The original glucose–creatinine infusion, which did not contain the glycoside, was then substituted and given for 45 min in order to determine the rate of recovery from any inhibition.

*Analytical methods and materials*

Glucose was determined in diluted urine and barium hydroxide–zinc sulfate filtrates of plasma[14] by means of the glucose oxidase reaction using the glucostat reagent supplied by Worthington Biochemical Corporation. Determination of creatinine in diluted urine and tungstic acid filtrates of plasma was done by a modification of the method of BONSNES AND TAUSSKY[15].

In order to obviate the use of the lengthy chemical nomenclature and also to emphasize their structural relationship to phlorizin, the compounds listed below have been assigned trivial names. (a) Phloretin 2'-galactoside for 2'-β-D-galactopyranosyloxy-4,4',6'-trihydroxydihydrochalcone. (b) Phloretin 2'-(3-methoxyglucoside); 2'-(3-methoxy-β-D-glucopyranosyloxy)-4,4',6'-trihydroxydihydrochalcone. (c) 4'-Deoxyphlorizin; 2'-β-D-glucopyranosyloxy-4,6'-dihydroxydihydrochalcone. (d) 4-Methoxyphlorizin; 2'-β-D-glucopyranosyloxy-4',6'-dihydroxy-4-methoxydihydrochalcone. (e) 4-Methoxyphlorizin chalcone; 2'-β-D-glucopyranosyloxy-4',6'-dihydroxy-4-methoxychalcone. (f) Phloracetophenone 2'-glucoside; 2'-β-D-glucopyranosyloxyphloracetophenone. (g) Phlorizin chalcone; 2'-β-D-glucopyranosyloxy-4,4',6'-trihydroxychalcone.

A description of the syntheses and the physical characteristics of the above glycosides has been reported elsewhere[16]. Phlorizin and its chalcone derivative (the α,β-unsaturated ketone) were prepared according to ZEMPLÉN AND BOGNÁR[17]. Deoxycorticosterone-β-D-glucoside was generously supplied by Dr. R. GAUNT of CIBA Pharmaceutical Company, Summit, New Jersey. The phloretin 4'-glucoside used in these studies was prepared by hydrogenating the chalcone form of partially hydrolyzed naringin (Eastman Organic Chemicals, Rochester, New York) according to JORIO[18]. The identity of the product was verified by comparison with a sample of phloretin

* This step differed from the procedure of LOTSPEICH AND WORONKOW in that no water was given by gavage prior to the experiments.

** The anhydrous substances were solubilized by triturating with 1 ml 95% ethanol and a small volume of warm glucose–creatinine infusion mixture. The 4-methoxyphlorizin and its chalcone analog were especially difficult to dissolve and in this case, a few drops of sodium hydroxide solution were added.

4'-glucoside isolated from the leaves of *Malus trilobata* by WILLIAMS[19]. Receipt of a gift sample from Dr. WILLIAMS is gratefully acknowledged.

## RESULTS

The data, summarized in Table I, are presented as the maximal inhibitory effect obtained after 30 min of infusion of each compound. Fig. 2 graphically illustrates the results of one typical experiment which shows the rate and extent of TmG inhibition and recovery for each compound tested.

TABLE I

COMPARISON OF THE EFFECTS OF PHLORIZIN ANALOGS ON THE MAXIMAL RENAL TUBULAR REABSORPTION CAPACITY FOR GLUCOSE

The per cent inhibition is the maximal degree attained after 30 min of glycoside infusion. The administered dosage and inhibition values represent 2–4 assay experiments for each compound.

| *Compound* | *Per cent reduction in TmG* | *Dosage × $10^2$ μmoles/kg body weight/min* |
|---|---|---|
| A. Phlorizin | 90–100 | 5.6–6.4 |
| B. 4'-Deoxyphlorizin | 89–100 | 6.2–7.1 |
| C. 4-Methoxyphlorizin | 70–86 | 6.2–7.3 |
| D. Deoxycorticosterone glucoside | 24–50 | 7.1–7.8 |
| E. Phloretin 2'-galactoside | 13–27 | 5.3–6.4 |
| F. Phloretin 2'-(3-methoxyglucoside) | 10 | 5.7–7.0 |
| G. Phloretin 4'-glucoside | 10 | 7.0–7.2 |
| H. Phloracetophenone 2'-glucoside | 0 | 9.0–9.3 |
| I. Phlorizin chalcone | 0 | 6.2–7.2 |
| J. 4-Methoxyphlorizin chalcone | 0 | 6.7–7.0 |

It will be noted that three of the analogs tested, *viz.* phloracetophenone 2'-glucoside, phlorizin chalcone and 4-methoxyphlorizin chalcone showed no inhibitory effect at the levels administered. The influence of phloretin 2'-galactoside on the capacity of the kidney to reabsorb glucose is less than that for phlorizin but still significant; in four separate experiments it depressed TmG by 13–27 % when administered at $5.3–6.4 \cdot 10^{-2}$ μmoles/kg body weight/min. The activity of phloretin 2'-(3-methoxyglucoside) and phloretin 4'-glucoside is quite low and the observed 10 % depressions are of questionable significance since the inherent error of the assay procedure was found to be approximately at this level. On the other hand, the inhibitory capacity of 4'-deoxyphlorizin is equal to that of phlorizin while 4-methoxyphlorizin is only slightly less active (75 % TmG depression). Desoxycorticosterone β-D-glucoside was intermediate in activity. In two experiments at low doses, the steroid depressed TmG from 24–50 %.

## DISCUSSION

### *General*

The interpretation of these experimental findings is based upon the thesis that phlorizin and the effective phlorizin analogs compete with glucose for a specific binding site or "carrier" on the renal tubular epithelia. Competitive inhibition of

intestinal glucose transport has been well documented[8] and the available evidence supports the assumption that the phlorizin effect on the kidney is similar. The ability of a compound to depress TmG is here interpreted to mean that it has a much greater affinity for the transport receptor site than glucose and is preferentially bound. Since phlorizin was found to be the most potent inhibitor of all the compounds tested, it may be assumed that its molecular architecture best complements that of the receptor and leads to the most stable intermolecular binding with the membrane. The rapid recovery of TmG after the experimental challenge (see Fig. 2) is consistent with competitive inhibition. Glucose, in high concentration in the glomerular filtrate,

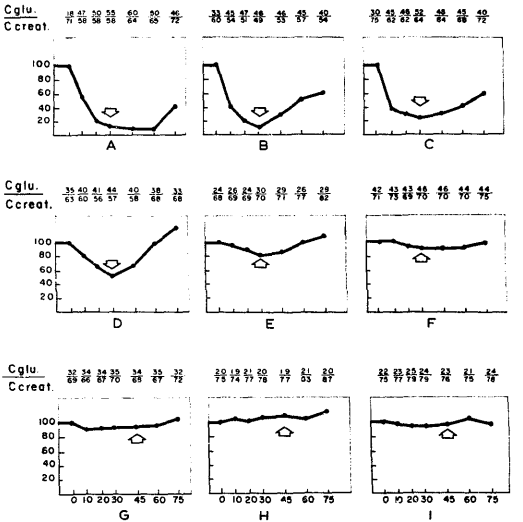


Fig. 2. The figure graphically illustrates the results of one typical clearance experiment for each compound tested. The rate and degree of TmG inhibition and recovery after 30 min of glycoside infusion is shown. The results are expressed as the per cent depressionof renal reabsorptive capacity observed during the control period (ordinate); the abscissa shows the time in minutes. At the arrow, the infusion of the respective glycoside was stopped. Above each curve appear the ratios of glucose clearance (Cglu.) and creatinine clearance (Ccreat.) found during each experimental period. The rate of infusion for each compound is uniformly expressed in $\mu$moles ($\times 10^{-2}$)/kg/min. A; phlorizin, 6.4. B; 4'-deoxyphlorizin, 7.1. C; 4-methoxyphlorizin, 7.3. D; deoxycorticosterone glucoside, 7.8. E; phloretin 2'-galactoside, 6.0. F; phloretin 2'-(3-methoxyglucoside), 7.0. G; phloretin 4'-glucoside, 7.2. H; phloracetophenone 2'-glucoside, 9.3. I; phlorizin chalcone, 7.2.

apparently displaces the inhibitor from the receptor site in the kidney. The phenomenon of reversibility appears identical to that observed in the intestine where phlorizin inhibition is reversed by washing the mucosa with a fresh glucose solution[8].

The compounds having little or no effect on TmG may indeed be active at slightly higher concentrations. However, the purpose of these experiments was not to deter-

mine the absolute activity of these analogs but only their potency relative to phlorizin. Although much can be learned about the kinetics of inhibition from the clearance technique, a detailed study employing a more easily controlled, *in vitro* assay system is required to quantify the comparative activity of all the phlorizin-like glycosides.

It may be argued that differences in the distribution of the analogs could account for their diverse inhibitory capacities. Thus, the relative inactivity of a compound might be the result of its failure to reach an inhibitory concentration in the glomerular filtrate. This could be the effect of detoxification and/or excretion by the liver, adsorption by plasma protein or absorption by blood cells to a greater degree than those compounds which exhibited inhibitory potency. For the sake of discussion, however, it was assumed that these distribution factors do not vary significantly from compound to compound in the series tested. It is anticipated that these questions will be answered by performing the activity determinations *in vitro*.

*Specificity of the glucose moiety*

It has been repeatedly observed that the intestinal and renal transport of glucose is unaffected by low concentrations of the aglucone phloretin, while phlorizin exerts a significant degree of inhibition at a comparable level[20]. The inference has been drawn that the glucose moiety of phlorizin is an essential substituent of the inhibitor molecule. It remained to be determined whether the specific stereochemical configuration of glucose as the glycosidic group was an indispensable feature. In view of the hypothesis of competition between phlorizin and glucose for the receptor site, one would expect that substitution of the hydroxyl groups of the sugar moiety or any change in their steric configuration should result in a decreased activity of the analog. The experimental results bear out this postulate and clearly demonstrate the prerequisite atomic groupings of at least a part of this moiety. An axial configuration of the hydroxyl group on C-4 of the pyranose ring (as in the galactoside) results in a decreased affinity of the analog for the membrane site. The critical nature of the free —OH function at C-3 of the glycosidic moiety (presumably in the equatorial configuration) is also indicated, since the 3-methyl ether of glucose as the glycoside is inactive at the concentrations used.

JORGENSEN, LANDAU AND WILSON[21] have determined the relative affinities of a few sugars for the transport site in hamster intestine. The affinity constant (analogous to the Michaelis constant) estimated for glucose was $2.5 \pm 0.5$ mM; galactose, $12 \pm 3$ mM; and 3-methoxyglucose, $10 \pm 5$ mM, indicating that the interaction between glucose and the receptor is most stable, while galactose and 3-methoxyglucose are bound less firmly. It is presumed that the affinity of these hexoses for the renal transport receptor are similar to those of the intestine. Under this assumption and in view of the large standard error of the above values for the affinity constants, the following postulate is consistent with the competitive inhibition thesis. Viewing the phlorizin-like glycosides as C-1 substituted hexoses, it follows that the relative activities of the various phloretin glycosides parallel the affinity of the parent free sugars for the transport site.

*Specificity of the aglycone moiety*

The affinity of phlorizin for the transport site in both the intestine[8] and kidney[22] has been estimated to be at least 1000 times greater than that shown by glucose.

Clearly, some feature of the aglucone moiety of the inhibitor facilitates the interaction of the glucosidic portion of the molecule with the receptor and as a result the stability of the complex is much greater than that formed with the free sugar. It is most likely therefore that a potent inhibitor possesses structural and conformational features which make it capable of aligning on the cell membrane so that it is tightly bound at two (or more?) points, one of which is at the glucose transporting site*. This multiple-point attachment proposal is in accord with the idea of competitive inhibition in that a sufficiently high concentration of glucose at the hexose specific site should readily effect the displacement of the pyranosidic moiety of the inhibitor. It seems apparent that secondary interactions of the aglucone moiety with complementary membrane loci facilitate the formation of this primary bond. The activities determined for the analogs which differ from phlorizin only with respect to the aglucone portion of the molecule corroborate this postulate. The discussion which follows will elaborate on the nature of the secondary bond formation and point out the observations which led to the above thesis.

In order to visualize the three-dimensional configuration of phlorizin, a model was constructed with the Godfrey Molecular Model kit (Bronwill Scientific Division, Will Corporation, Rochester, New York). The structural representation of this compound shown in Fig. 1 is based on preliminary studies. If the model is manipulated in a way such that the pyranose group is tilted out of the plane of the aromatic ring, the primary hydroxyl group on C-6 of the glucoside moiety lies in close proximity to the 4'-hydroxyl function. Hydrogen bond formation at this position seemed at the time to be likely since the three atoms involved (O–H···O) can be made to assume an alignment of 180°. This configuration was thought to gain stability from a second intramolecular hydrogen bonding between the carbonyl oxygen and the 6'-phenolic hydroxyl**. Thus, the formation of these intramolecular bonds would restrict the rotation about the $\beta$-glucosidic linkage. As a consequence, the pyranoside group would tend to be held approximately coplanar with the aromatic ring and the location of the 4-hydroxyphenyl group would be restricted. As a result of the primary bond formation, the aglucone moiety would be directed to its complementary locus on the cell membrane and the formation of secondary attachments would be imminent. On the other hand, if the pyranosidic group were not intramolecularly fixed in position, the remainder of the molecule could rotate freely about the glucosidic linkage and fail to interact with the membrane because of a lack of the directive force.

In order to evaluate these structural features, 4'-deoxyphlorizin was synthesized and tested for pharmacological activity. It was reasoned that if the spatial orientation of the pyranoside was dependent upon the presence of the 4'-hydroxylic function, the deoxy analog should be relatively inert. On the contrary, however, the analog was found to be as active as phlorizin in blocking glucose reabsorption. This observation made it necessary to visualize a conformation which was common to both phlorizin and 4'-deoxyphlorizin.

Based on a projection of the molecular model, the illustration in Fig. 3 represents

* The interaction of the glycosidic moiety of the inhibitor with the glucose transporting site will henceforth be referred to as the primary bond.

** This latter linkage would normally be considered to be fairly unstable since the angle between the direction of the atoms involved deviates considerably from linearity. However, the results of studies on the alkylation of polyhydroxyphenols of the phlorizin type indicate that the 6'-hydroxyl group is chelated with the carbonyl to a significant degree[23].

the configuration of phlorizin which can be assumed by the 4′-deoxy analog as well. It should be appreciated that the pyranoside ring assumes a configuration which is completely strain-free, coplanar with the aromatic A ring, and precisely oriented with respect to the remainder of the molecule. This conformation is presumed to derive a great deal of stability from the strong hydrogen bond formed between the carbonyl oxygen and the primary hydroxyl at C-6 of the pyranose group. The O-H···O grouping in this case is in an ideal 180° alignment. It is significant that the model assumes this configuration when floated in water; this operation obviates the exertion of any artificial external stress on the structure which often occurs when the model is handled or placed on a solid surface. From the evidence at hand, it appears that the illustrated configuration in Fig. 3 is the most stable and represents the form which best complements the receptor site on the cell membrane.

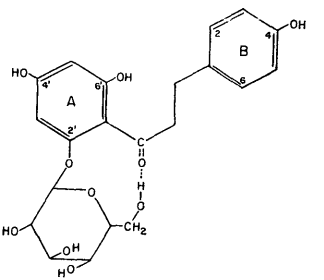


Fig. 3. The structural representation of phlorizin based on a projection from a molecular model. This geometrical configuration is presumed to be the preferred form which complements the receptor surface.

The relative inactivity of phloretin 4′-glucoside is a finding which lends support to the concept just described. When the model of the isomer is allowed to assume its natural conformation (by floatation in water) its shape is nearly linear. The glucose moiety is relatively "free-swinging" since hydrogen bond formation with the carbonyl oxygen is not possible and the group is not spatially fixed with respect to the aglucone portion of the molecule as it is in phlorizin. The likelihood of the aglycone moiety interacting with the membrane is therefore decreased and accounts for the relative inactivity of the 4′-glucoside.

If the model is properly manipulated however, the C-6 hydroxyl group of the sugar moiety can be made to assume a position which would suggest the formation of a hydrogen bond with the 6′-(or 2′) hydroxyl group (Fig. 4). As previously mentioned for the preliminary studies on the phlorizin model construction, one is required to tilt the pyranoside ring out of the plane of the aromatic ring A. Despite the slight degree of bond bending involved, the molecule can attain a configuration comparable with that of phlorizin (Fig. 3) which probably accounts for its slight inhibitory activity.

Another reason could be offered to explain the relative inactivity of this analog. The presence of an unsubstituted phenolic –OH at C-4′ would be an essential feature if

this group contributed to the secondary combination of the inhibitor to the cell membrane. However, the fact that the 4'-deoxyphlorizin is fully active proves that this reasoning is incorrect and clearly demonstrates the dispensability of this group.

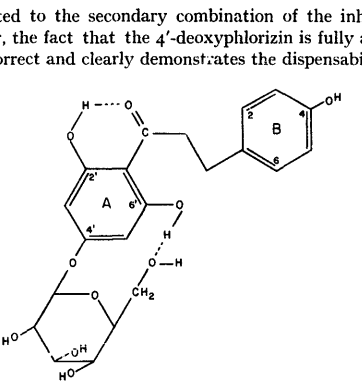


Fig. 4. The structure of phloretin 4'-glucoside. The representation is a projection from a molecular model and illustrates that the compound can assume a geometrical configuration similar to the preferred form of phlorizin shown in Fig. 3. However, this configuration is not the preferred form; the molecule naturally assumes a linear structure with free rotation about the $\beta$-glucosidic linkage.

I would like to re-emphasize the interpretation of the data discussed thus far. Phlorizin inhibition is not simply due to a process whereby the glucosidic portion of the molecule enters into the transport machinery in the same manner as the free sugar and merely impedes the transport process by virtue of the presence of a non-specific, bulky aglucone moiety. If this were the case, no obvious reason could be offered to explain the inert character of phloretin 4'-glucoside which differs from phlorizin only in the position of the pyranoside ring. It follows that not only is the stereochemical configuration of the glucoside moiety an essential feature for activity but, in addition, its orientation with respect to the remainder of the molecule is critical.

*Nature of the membrane locus*

At the concentrations tested, phloracetophenone 2'-glucoside failed to restrict renal tubular glucose reabsorption*. This was an extremely significant result inasmuch as it unequivocally established the 4-hydroxyphenyl moiety to be an indispensable feature of the inhibitor. The fact that this analog is inert indicates that all or a portion of the 4-hydroxyphenyl group (hereafter referred to as the B ring system) interacts with a membrane constituent adjacent to the glucose receptor. Although the nature of the secondary attachment has not been ascertained, as a working hypothesis it is proposed that a pivotal hydrogen bond is formed between the oxygen at position 4 of

* It would not be an unexpected result to find that this analog is an inhibitor at slightly higher concentrations since the compound does possess one essential feature for inhibition, *viz.* the glucosidic moiety. Indeed, it would be of interest to determine whether the compound could enter the glucose transporting machinery and be absorbed. WILSON[24] has cited the results of some recent work which showed that a number of glucosides, including $\beta$-phenyl-, $\beta$-*p*-chlorophenyl- and $\beta$-hydroquinone-D-glucoside, were actively transported by hamster intestine.

the inhibitor and a complementary group on the biological surface. An oxygen, nitrogen, or less likely, sulfur atom located at a precise distance from the actual glucose transporting site could serve as the membrane constituent. This distance would be equivalent to the intramolecular spacing between the oxygen at position 4 and the pyranosidic moiety, *i.e.* approx. 12–15 Å (determined from the center of the glucoside ring).

The results of experiments with 4-methoxyphlorizin afford some information about the chemical nature of the membrane locus binding the B ring system. At comparable dosages, the inhibitory activity of the methoxy analog was found to be almost as great as that observed for phlorizin (approx. 80 %)*. Presumably, the cell membrane locus to which the 4-methoxyphenyl moiety is attached is the same as that which binds the B ring system of phlorizin. The results of the assay therefore place a limitation on the character of the postulated secondary hydrogen bonding. Since 4-methoxyphlorizin lacks a potential proton at position 4, the ethereal oxygen must be the electron pair donor; this requires the subjoining membrane substituent to be one which can donate hydrogen to form the bond. This prerequisite makes it highly improbable that a carboxyl or a phosphate group serves as the membrane reactant because at physiological pH, both would be virtually completely ionized. Since the inhibitor is probably bound to the outermost layer of the cell membrane (*i.e.*, protein) the side chain of serine, histidine or other protonated amines could be included in the list of membrane groups which might function as the hydrogen donor.

There exists the possibility that the B ring system is attached to the biological surface by means other than hydrogen bonding. For example, a strong fixation could occur as a result of the incorporation of the lipophilic moiety into a lipid area or layer of the membrane. However, this type of association seems rather unlikely on consideration of the relative activity of 4-methoxyphlorizin. Since the methoxyphenyl moiety is much more hydrophobic than the hydroxyphenyl group, 4-methoxyphlorizin should be expected to exhibit the greater lipid affinity and thus greater inhibitory potency than phlorizin. However, since phlorizin was found to be the most potent inhibitor of all the compounds tested, it follows that the greatest membrane affinity and bonding stability is associated with the 4-hydroxyphenyl group.

A second alternative to hydrogen bonding which could account for the combination of the B ring system with the membrane is a $\pi$–$\pi$ bond formation. It is easy to visualize the flat hydroxyphenyl group with its extensive assemblage of $\pi$-electrons lying parallel and in close proximity to another aromatic ring system of the receptor surface. The overlapping of the $\pi$-electron orbitals resulting from this type of association would favor the formation of a $\pi$–$\pi$ bond. An interaction of this kind might contribute a considerable amount of binding force to the secondary attachment. However, in view of the relatively good inhibitory capacity of deoxycorticosterone glucoside which lacks the extensive cluster of $\pi$-electrons to form such a union, it is doubtful that the $\pi$–$\pi$ bond is the only interaction involved**.

* The bulky methoxy group could be an impediment to the proper alignment of the moiety on the biological surface and account for the slightly lower activity. The Van der Waals radius of a methyl group is 2.0 Å while that for hydrogen is 1.2 Å (see ref. 25).

** The nature of the secondary bond would be readily ascertained by estimating the activity of 4-deoxyphlorizin. Obviously, no hydrogen bonding between the receptor surface and the B ring can occur in this analog. Numerous attempts to synthesize and isolate this compound have thus far been unsuccessful.

The depression of TmG in dogs by deoxycorticosterone-β-D-glucoside was reported by DESPOPOULOS AND KAUFMAN[26]. It was important to extend the studies of these workers since the steroid glucoside had been administered in relatively large doses. The effect of this inhibitor was therefore determined at concentrations comparable to those of phlorizin used in the experiments described here. The results of two such determinations (Table I) clearly demonstrate the inhibitory activity of the agent at these low levels, but the effect is not as great as that seen with phlorizin.

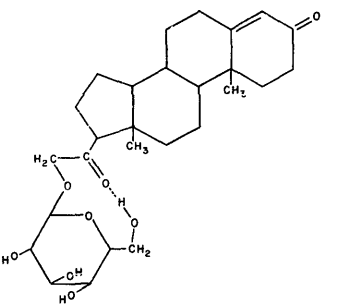


Fig. 5. The structural representation of deoxycorticosterone-β-D-glucoside.

The similarity to phlorizin in the spatial orientation of the critical groups is apparent (compare Figs. 3 and 5). Each pyranoside moiety has a restricted orientation by virtue of hydrogen bonding with the adjacent carbonyl group and the tailpieces of both compounds locate at approximately the same point. The critical intramolecular distance of the steroid does vary slightly, however; the spacing of the carbonyl oxygen to the pyranoside moiety is about 14–16 Å (in phlorizin the analogous spacing ranges from 12–15 Å). However, this difference becomes negligible if the tailpieces of both compounds are maneuvered out of the plane of the fixed pyranoside ring. Under these conditions, the carbonyl oxygen at position 3 of the steroid and the phenolic oxygen at position 4 of phlorizin occupy the same point in space relative to the glucoside moiety. The effectiveness of deoxycorticosterone glucoside at low levels indicates that the structural prerequisites for secondary bond formation are met by this compound. Since a flat aromatic ring is absent as a critical grouping, it is most likely that hydrogen bonding occurs with a membrane group capable of donating hydrogen.

The observation that phlorizin chalcone is pharmacologically inert is quite significant since all of the structural entities possessed by phlorizin itself are present in this compound. To account for the inactivity, it could be argued that the chalcone is unable to interact with the receptor because of an ionic repulsion between the cell membrane and the anionic form of the chalcone. As illustrated in Fig. 6, the compound can exist in at least two stable resonance forms. As a result of this resonance, the $pK_a$ of the hydroxyl group at position 4 is lowered and the compound could be anionic at physiological pH. The likelihood that this phenomenon accounts for the chalcone's inactivity is reduced in view of the results obtained with 4-methoxyphlorizin chalcone.

This analog has no ionizable hydrogen at position 4 and thus cannot carry a negative charge. Yet, this derivative is also without effect on glucose reabsorption.

The chalcones have in common a structural feature which limits their geometrical conformation, *viz.* the double bond (R'–CO–CH=CH–R). Consequently, the position of the aromatic ring systems A and B with respect to each other is restricted to the coplanar configuration and both molecules are relatively flat (*cf.* ref. 27). Thus, the B ring system cannot swing out of the plane of the spatially fixed glucosidic moiety.

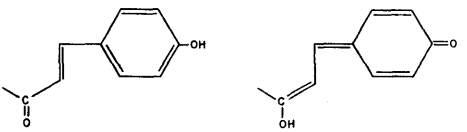


Fig. 6. Two stable resonance forms of the hydroxychalcone.

These considerations, together with the observations on the deoxycorticosterone glucoside, strongly suggest that the receptor locus involved in the secondary binding of the inhibitor molecule is out of the plane of the glucose transporting site.

The combination of many compounds with a receptor surface is usually explained in terms of the drug having three sites which are geometrically oriented so as to form bonds with the receptor (*cf.* ref. 28). The data presented here can be interpreted within the context of this three-point attachment theory. Thus, one of the interaction points described here and termed the primary bond actually represents two points of attachment (the –OH groups on C-3 and C-4 of the pyranoside moiety). However, it is not unlikely that supplementary points of attachment contribute to the binding of phlorizin to the receptor. This is suggested by the observation that the glucosidic moiety of the inhibitor must be spatially fixed with respect to the remainder of the molecule. This intramolecular fixation results in the molecule being non-linear and thus unsymmetrical in shape (see Fig. 3). It has already been noted that the inactive phloretin 4'-glucoside normally does assume a linear form. It could be argued that its lessened inhibitory capacity is due to its inability to present supplementary interaction sites to the receptor. On the basis of these limited data, it is interesting to speculate that an additional interaction of phlorizin with the receptor occurs at the hydroxyl group at C-6'. If this were the case, 6'-deoxyphlorizin should exhibit decreased activity and also, the relative inactivity of phloretin 4'-glucoside would easily be explained (compare Figs. 3 and 4).

As a result of these experiments, several features which describe the critical structure of a potent inhibitor of the renal tubular reabsorption of glucose may be stated. The inhibitory agent is one which possesses atomic groupings in a specific three-dimensional pattern which favors an association with the membrane receptor. The molecule is visualized to be bound at two (or more) loci; (a) a primary bond is formed through the interaction of the glycosidic moiety with appropriate groups of the membrane constituent. The linkage presumably is formed through hydrogen bonds which involve at least the hydroxyl groups on C-3 and C-4 of the sugar group. The most stable primary interaction occurs when these hydroxyl groups are situated in the more chemically reactive equatorial position; (b) a secondary bonding of the aglucone

portion of the inhibitor molecule to a locus on the biological surface which is adjacent to, but removed from the plane of the glucose transporting site. The linkage is probably also through hydrogen bonding with a membrane constituent capable of serving as a hydrogen donor at physiological pH. This locus is approx. 13–16 Å removed from the transport site to which glucose is normally bound. The data do not rule out the possibility that the secondary binding is accomplished through a $\pi$–$\pi$ bonding with a complementary group of the membrane and perhaps both types of interaction are involved.

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