Biochimica et Biophysica Acta, 693 (1982) 379–388
Elsevier Biomedical Press

BBA 71456

SYNTHESIS OF PHLORIZIN DERIVATIVES AND THEIR INHIBITORY EFFECT ON THE RENAL SODIUM/D-GLUCOSE COTRANSPORT SYSTEM

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(Received March 24th, 1982)
(Revised manuscript received August 13th, 1982)

Key words: Phlorizin derivative; Na^+/D-glucose cotransport; Brush border membrane; (Kidney)

To characterize further the Na^+/D-glucose cotransport system in renal brush border membranes, phlorizin – a potent inhibitor of D-glucose transport – has been chemically modified without affecting the D-glucose moiety or changing the side groups that are essential for the binding of phlorizin to the Na^+/D-glucose cotransport system. One series of chemical modifications involved the preparation of 3-nitrophlorizin and the subsequent catalytic reduction of the nitro compound to 3-aminophlorizin. From 3-aminophlorizin, 3-bromoacetamido-, 3-dansyl- and 3-azidophlorizin have been synthesized. In another approach, 3′-mercuryphlorizin was obtained by reaction of phlorizin with Hg(II) acetate. The phlorizin derivatives inhibit sodium-dependent but not sodium-independent D-glucose uptake by hog renal brush border membrane vesicles in the following order of potency: 3′-mercuryphlorizin = phlorizin > 3-aminophlorizin > 3-bromoacetamidophlorizin > 3-azidophlorizin > 3-nitrophlorizin > 3-dansylphlorizin. 3-Bromoacetamidophlorizin – a potential affinity label – also inhibits sodium-dependent but not sodium-independent phlorizin binding to brush border membranes. In addition, sodium-dependent phosphate and sodium-dependent alanine uptake are not affected by 3-bromoacetamidophlorizin. The results described above indicate that specific modifications of the phlorizin molecule at the A-ring or B-ring are possible that yield phlorizin derivatives with a high affinity and high specificity for the renal Na^+/D-glucose cotransport system. Such compounds should be useful in future studies using affinity labeling (3-bromoacetamido- and 3-azidophlorizin) or fluorescent probes (3-dansylphlorizin).

Introduction

Studies of D-glucose transport in brush border membrane vesicles isolated from intestine and kidney have shown that the transport process is sodium dependent and phlorizin sensitive [1–4]. The affinity of phlorizin for the transport system has been found to be about 1000-fold higher than the affinity of D-glucose. In hog renal brush border membranes, for example, the apparent K_m for D-glucose is 1.3 mM; the K_i for phlorizin is 1.2 μM [5]. Phlorizin or its derivatives can thus be used as marker molecules for the identification, characterization and isolation of the D-glucose-transport system.

In studies on the interaction of the Na^+/D-glucose cotransport system with phlorizin analogues, it was revealed by Diedrich and co-workers [6,7] that the glucose configuration of the sugar moiety, the phenoxy groups and the carbonyl group in the aglucone part of the phlorizin molecule as well as the conformational arrangement within the aglucone plane are essential for the binding to the glucose-tranport system. Therefore, in chemical

manipulations used to obtain phlorizin derivatives, these positions should not be blocked or removed.

For the synthesis of phlorizin derivatives, essentially two routes can be followed: (1) the total synthesis similar to the method of Zemplén and Bognar [8] can be attempted, or (2) the phlorizin molecule can be modified directly. In this paper, the selective introduction of a nitro group at position 3 in the ring B of phlorizin and of mercury at position 3 of ring A are described. The nitro group was then converted into an amino group. Using mild reaction conditions several activated residues could be coupled to this amino group. Thereby, oxidation and polymerization of phlorizin as well as hydrolysis of the glycosidic bond are kept to a minimum and the modification occurs at a defined position of the molecule. It is demonstrated that such derivatives still interact specifically and with a high affinity with the Na^+/D-glucose cotransport system. They thus comprise interesting tools to characterize further this transport system.

Materials and Methods

Phlorizin obtained from Fluka, F.R.G., was dissolved in small amounts of ethanol and decolored with charcoal. Appropriate amounts of hot water were then added to the ethanol solution for recrystallization at 4°C. The other chemicals used were of analytical grade.

The melting points of chemical compounds were measured with the Model SMP 20 (Buechi Technik AG, F.R.G.) and were not corrected. Infrared spectra were recorded in a Perkin-Elmer 457 spectrophotometer; ultraviolet spectra of compounds were measured in a Beckman Model 25 spectrophotometer; NMR spectra were registered in a WH-270 (Bruker-Physik AG, F.R.G.). The chemical shifts in NMR spectra were expressed as ppm (δ) using tetramethylsilane as internal standard. Thin-layer chromatograms (TLC) were developed either in (a) chloroform/ethanol/butan-2-one (60 : 26 : 14, saturated with water vapor), (b) isobutyl alcohol/glacial acetic acid/water (6 : 2 : 2) or (c) toluene/chloroform/acetone (6 : 1 : 3).

Protein assay. The protein content of the samples was determined by the method of Lowry et al. [9].

Membrane preparation. Brush border membranes from the hog kidney were isolated according to the method of Vannier et al. [10] as modified by Riedel [11].

Transport assay. Two methods were employed for determining the D-glucose-transport activity of membrane vesicles in the presence or absence of inhibitors.

(a) Gradient conditions. 20 μl of membrane vesicles (100 μg membrane protein) suspended in vesicle buffer (200 mM mannitol, 20 mM Hepes-Tris, pH 7.4) were added to 100 μl transport medium containing 100 mM NaSCN or KSCN, 100 mM mannitol, 20 mM Hepes-Tris (pH 7.4) and radioactively labeled substrates (D-glucose, L-alanine, phosphate). The final concentration of substrate in the transport medium was 0.1 mM. After 15 s, 1, 2 and 90 min, substrate uptake was terminated by rapid filtration technique (Millipore filter GSWP, 0.45 μm pore size) [1]. The filters were transferred into liquid scintillation fluid and radioactivity was counted by liquid scintillation spectrometry. The values obtained were corrected for the amount of radioactivity remaining on the filter in the absence of membrane vesicles in the incubation medium. Using the amount of counts present in a defined volume of the incubation medium, the specific activity of the substrate was calculated. With the aid of the specific activity the counts obtained were transformed into pmol of substrate taken up per mg of membrane protein.

(b) Tracer exchange. The membrane suspension (5 mg protein/ml) was preloaded with the same medium as described above for the transport experiments, but without radioactive substrate. To start the experiment, a trace of radioactive substrate was added and after different incubation times, the tracer exchange was determined by using the same filtration technique described in Section (a).

Binding assay

For determination of phlorizin binding to brush border membranes, [^{3}H]phlorizin with a specific radioactivity of 45 Ci/mmol, which had been synthesized in our laboratory (Lin, J.T., unpublished data) was used. Aliquots of membrane vesicles were incubated in media (200 mM NaCl or KCl, 100 mM mannitol, 20 mM Hepes-Tris (pH

7.4) containing various amounts of phlorizin (0.1, 0.5, 1.0, 5.0 μM, etc.) and various concentrations of phlorizin derivatives at 25°C for 20 min. The final protein concentration was 1.25 mg/ml. 20 μl of each pre-incubated membrane fraction were pipetted directly on a filter (Millipore, GSWP 0.45 μm pore size) under 0.5 kP/cm^2 vacuum followed by rapid washing with 5 ml cold incubation medium used above (K^+ or Na^+ without phlorizin). Phlorizin bound to the membranes was determined in terms of counts of [^{3}H]phlorizin remaining on the filter. The values were corrected for binding of phlorizin to the filters in the absence of membranes; this filter blank constituted about 2–10% of the total amount bound.

Chemical modification of phlorizin (see also Fig. 1)

Synthesis of 3-aminophlorizin. (a) 472 mg phlorizin were dissolved in 10 ml glacial acetic acid. The temperature of the solution was brought to 15°C. 75 μl of nitric acid (specific gravity 1.4) were added dropwise to the solution under vigorous stirring. At the end of the reaction, the color

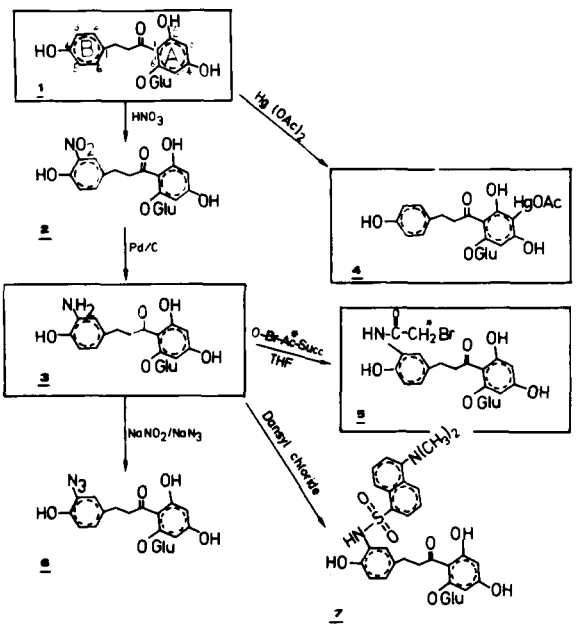

Fig. 1. Reaction sheme for the synthesis of phlorizin derivatives: compound 1, phlorizin; compound 2, 3-nitrophlorizin; compound 3, 3-aminophlorizin; compund 4, 3'-mercuryphlorizin; compound 5, 3-bromoacetamidophlorizin; compound 6, 3-azidophlorizin; compound 7, 3-dansylphlorizin.

of the solution turned to red-brown. The solution was then poured onto 20 g ice. The green precipitates were collected and dried under vacuum. For further purification, the dry precipitates were dissolved in tetrahydrofuran. The solution was passed through an aluminum oxide column (2 × 2 cm). The yellow solution was then concentrated and a small amount of petroleum ether (30–60°C) was added until the solution became slightly turbid. After some time crystals developed. Melting point: 217–218°C; elemental analysis: calcd.: C 52.4%, H 4.8%, N 2.9%; found: C 52.4%, H 4.8%, N 3.0%.

(b) Reduction of 3-nitrophlorizin to 3-aminophlorizin. 482 mg 3-nitrophlorizin were dissolved in 10 ml ethanol and mixed with 150 mg palladium/carbon (10% Pd). Hydrogen gas was gently introduced into the solution for a period of 1 h at room temperature. The catalyst was then removed by filtration. The ethanolic solution was diluted with 20 ml deoxygenated water and frozen immediately. Solid aminophlorizin was obtained by freeze-drying. The yield of the reduction product was almost 100%. Melting point: 91–93°C. During the whole procedure the reaction mixture was protected from light by aluminum foil.

Preparation of 3-bromoacetamidophlorizin. (a) *O*-Bromoacetyl-*N*-hydroxysuccinimide: 1.39 g bromoacetic acid and 1.38 g *N*-hydroxysuccinimide were dissolved in 10 ml dry dioxane. To the solution 2.1 g dicyclohexylcarbodiimide were added. The reaction was allowed to proceed for 1 h. The urea derivative formed is not soluble in dioxane and can be separated by filtration. To the filtrate (dioxane solution), petroleum ether (60–80°C) was added until the solution became slightly turbid. White crystals developed overnight at 4°C. Melting point: 118°C.

(b) Reaction of *O*-bromoacetyl-*N*-hydroxysuccinimide with 3-aminophlorizin. 91 mg 3-aminophlorizin and 48 mg *O*-bromoacetyl-*N*-hydroxysuccinimide were dissolved in 5 ml dry tetrahydrofuran. The solution was saturated with nitrogen and stirred under nitrogen atmosphere for 16 h. Afterwards, 20 ml of water were added and tetrahydrofuran was evaporated with slight vacuum. The precipitate was collected and recrystallized in an isopropanol/water mixture. Melting point: 148–150°C. Elemental analysis ($C_{23}H_{26}NO_{11}Br$): Calcd.: C 48.3%, H 4.6%, N

2.5%, Br 14.0%; found: C 48.4%, H 4.6%, N 2.5%, Br 13.8%.

Preparation of 3-azidophlorizin. 45 mg of 3-aminophlorizin were dissolved in 3 ml of 0.5 M ice-cold HCl. The solution was cooled down to −5°C. To this solution 0.5 ml of 0.2 mol/l cold $NaNO_2$ were added. The reaction was allowed to proceed at −5°C for 10 min. A deep green color developed. Subsequently, 0.5 ml of 0.2 mol/l sodium acetate were added. The solution changed its color rapidly to red-brown while bubbling occurred because of nitrogen release. A gel-like precipitate was obtained by centrifugation (low speed). Water was removed from the precipitate by freeze-drying. The dry precipitate was then dissolved in an excess of tetrahydrofuran. The deep brown solution was decolored by passing through a short silica gel column (5 × 2 cm). After part of tetrahydrofuran had been evaporated, a few drops of water were added to obtain the 3-azidophlorizin precipitate. Melting point: 85°C (decomposed).

Preparation of 3-dansylphlorizin. Under protection against light, 50 mg of dansylchloride (5-dimethylaminonaphthalenesulfonic acid) and 91 mg of 3-aminophlorizin were dissolved in 15 ml tetrahydrofuran and 5 ml of 1 M sodium acetate (pH 5). The reaction was allowed to proceed under an N_2 atmosphere for 30 h at room temperature. Subsequently, 100 ml of water were added and the organic solvent was evaporated. A green solid residue was obtained when the aqueous solution was lyophilized. The organic substances were extracted from the pellet with dry acetone. The acetone solution was then concentrated and chromatographed on an acetylated polyamide column (SC 6, 10 × 2 cm). The fluorescent product eluted with acetone was precipitated by adding water to the acetone solution. Melting point: 207–208°C.

Preparation of 3'-mercuryphlorizin. 710 mg phlorizin were dissolved in 20 ml boiling water. During boiling and stirring, 5 ml of Hg(II) acetate solution (320 mg) were added slowly to the solution. The white precipitate was sedimented after a few minutes. The reaction proceeded further for 45 min under reflux. With the aid of a funnel the precipitates were separated by vacuum filtration. The precipitates were washed with hot water, the dried precipitates were suspended in acetone, collected again on filter paper and dried. This solid substance is not soluble in usual solvents except in alkaline solutions. Melting point: 178°C.

Identification of mercuryphlorizin by substitution of mercury by iodine. 70 mg mercuryphlorizin were mixed with 1.1 ml of 0.1 M KI solution. A red precipitate formed immediately and redissolved by adding more KI. When the solution was cooled down, fine aggregates formed which could be separated by centrifugation. The sediments were redissolved in ethanol. By adding a small amount of water, a white precipitate was obtained which gave a positive reaction in the iodine test.

TABLE I

PROPERTIES OF PHLORIZIN DERIVATIVES

TLC: Reagents which positively react with the substance spots on the chromatogram are indicated as A, B, C, D, etc. (A) Paul's reagent (sulfanilic acid/sodium nitrite/sodium bicarbonate), for detection of phenolic groups; (B) naphthoresorcine/sulfuric acid, for detection of glucose residue; (C) Ninhydrin/ethanol, for detection of amino groups; (D) silver nitrate/hydroperoxide, for detection of halogen compounds; (E) Diphenylcarbazide/NH_4OH/ethanol, for detection of Hg groups. Infrared spectra were measured on KBr tablets. Appropriate amounts of solid sample were mixed well with dry KBr and pressed to a thin tablet. BAA-phlorizin, bromoacetamidophlorizin.

Compound	Melting Point (°C)	TLC	Infrared
Phlorizin	108–109	A,B	CO, -OH, -aryl,
3-Nitrophlorizin	217–218	A,B	CO, -OH, -aryl, $-NO_2$
3-Aminophlorizin	91– 93	A,B,C	CO, -OH, -aryl, $-NH_2$
3-BAA-phlorizin	148–150	A,B,D	CO, -OH, -aryl, CONH, C-Br
3-Dansylphlorizin	207–208	A,B	CO, -OH, -aryl, SO_2
3-Azidophlorizin	85 (decomp.)	A,B	CO, -OH, -aryl, $-N_3$
3'-Mercury phlorizin	178	A,B,E	–

Results and Discussion

Synthesis and identification of phlorizin derivatives

The existence of multiple functional groups in the phlorizin molecule raises problems with respect to the identification of phlorizin derivatives. Spectroscopic measurements, such as nuclear magnetic resonance (NMR), ultraviolet and infrared spectrophotometry were mainly used to identify the substituted or residual functional groups on the molecule. The chemical features of the phlorizin derivatives are summarized in Table I. With NMR spectroscopy (see Table II and Fig. 2), the position of substitution in the phlorizin molecule can be estimated on the basis of chemical shifts and proton coupling. In the infrared spectra, the functional groups are characterized by absorption bands.

(a) 3-Nitrophlorizin. The nitration of phenolic organic compounds [12] is an electrophilic substitution and can take place at the *o*-phenoxy positions on ring A (positions 3′ and 5′), the *o*-phenoxy positions on ring B (3 and 5) and the *m*-phenoxy positions on ring B (position 2 and 6). The infrared absorption at 1530 cm^{-1} indicates the vibration energy for C-NO_2. Theoretically, the *ortho* positions on ring A (positions 3′ and 5′) are expected to be substituted predominantly by electrophilic reagents because the former are activated by two adjacent phenoxy groups. However, and in contrast to mercury phlorizin (see below), the nitration of phlorizin with 65% HNO_3 gives only one product. In Fig. 2A and B, the NMR spectra of phlorizin and 3-nitrophlorizin are compared.

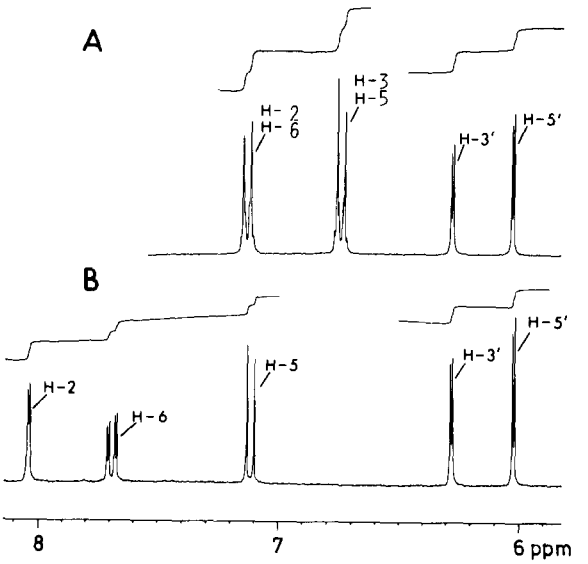

Fig. 2. Evidence of selective nitration of phlorizin by ^{1}H-NMR spectra (270 MHz; δ in ppm). (A) Phlorizin (H-3′ 6.27; H-5′ 6.02; H-2 and H-6 7.12; H-3 and H-5 6.74). (B) 3-Nitrophlorizin (H-3′ 6.27; H-5′ 6.02; H-2 8.04; H-6 7.69; H-5 7.11). As solvent [$^2H_6$]acetone was used. Tetramethylsilane was used as standard.

TABLE II

ESTIMATION OF THE EFFECT OF NO_2 SUBSTITUTION ON PROTONS IN THE PHLORIZIN MOLECULE ACCORDING TO THE METHOD OF JACKMAN AND STERNHELL (INCREMENT CALCULATION)

The chemical shifts in ppm are given for the protons on aromatic ring A (C-3′, C-5′) and ring B (C-2, C-6, C-3, C-5). As reference the signal of tetramethylsilane was used. As solvent [$^2H_6$] acetone was used. The letters in parentheses show the coupling type of the corresponding proton.

	Phlorizin		Nitrophlorizin		
	Calcd.	Obsd.	Calcd.		Obsd.
			C-3(NO_2)	C-2(NO_2)	
C-3′	6.03	6.27	6.03 (m)	6.03 (m)	6.27
C-5′	5.99	6.02	5.99 (m)	5.99 (m)	6.02
C-2	6.98	7.12	7.93 (m)	no signal	8.04
C-6	6.98	7.12	7.31 (o/m)	7.15 (o)	7.69
C-3	6.71	6.74	no signal	7.66 (m)	no signal
C-5	6.71	6.74	6.88 (o)	7.04 (o/m)	7.11

The chemical shifts of the aromatic protons show (1) that only one aromatic proton has been substituted, (2) that the protons 3′ (δ 5.93 ppm) and 5′ (δ 6.16 ppm) on ring A are unaltered compared to phlorizin, and (3) that the symmetry of the protons in ring B has disappeared. The chemical shift of the protons has moved toward a lower field strength and the spin coupling has become more complicated. On ring B, two different positions, namely *ortho* and *meta* positions to hydroxy groups, can be nitrated. From a chemical point of view, the orientation effect of the phenoxy group favors an electrophilic substitution in an *ortho* position. In Table II, the chemical shifts of aromatic protons are calculated according to Ref. 13. According to this calculation, if the NO_2 group is in an *ortho* position to the hydroxy group in ring B, three signals, 6.88, 7.31 and 7.93 ppm with coupling of *ortho*, *ortho-meta* and *meta*, respectively, were to be expected. If the NO_2 group is, however, in the *meta* position to the hydroxy group in ring B, three signals, 7.04, 7.15 and 7.66 ppm with coupling of *ortho-meta*, *ortho* and *meta*, respectively, were to be expected. The data obtained agree well with the values calculated for case 1. This confirms that the NO_2 group is located in the *ortho* position to the hydroxy group in ring B.

(b) Reduction of 3-nitrophlorizin to 3-aminophlorizin. Two main difficulties were encountered during reduction of 3-nitrophlorizin to 3-aminophlorizin. One was that reduction of the carbonyl group of phlorizin in addition to the reduction of the nitro group had to be prevented. This was achieved by the use of palladium carbon as catalyst and H_2 gas. The simplest approach, i.e., the use of dithionite [14] in the presence of sodium bicarbonate, failed because a by-product, 3-amino-6-sulfophlorizin, was obtained. The second difficulty arose from the high susceptibility of aminophlorizin to photooxidation and autooxidation, both processes, similar to the reactions observed with *o*-aminoquinodimethane [15,16], lead to polymerization. Therefore, freeze-drying under protection from light and air had to be employed.

(c) Coupling of specific reagents to 3-aminophlorizin to study the D-glucose-transport protein in brush border membranes. Three different active groups were coupled to 3-aminophlorizin: bromoacetic acid to obtain an affinity label, an azido group for future photoaffinity labeling and dansyl chloride for fluorescence studies. With respect to 3-bromoacetamidophlorizin, similar to the methods used in peptide chemistry [17], bromoacetic acid had to be activated to react with the amino group. In view of future studies with radioactive compounds the activated hydroxysuccinimide ester was preferred.

It has to be pointed out that bromoacetamidophenol derivatives in neutral or alkaline solution form cyclic products. Thus, it has been observed [18] that iodoacetamidonitrophenol at a neutral pH forms a stable ring product, 3,4-dihydro-6-nitro-1′, 4′-benzoxazol -3′-one. We also found that bromoacetamidophlorizin rearranges into other products when stored in aqueous solutions at neutral or alkaline pH.

The transformation of 3-aminophlorizin into 3-azidophlorizin by reacting it in the cold with dilute HCl and $NaNO_2$ followed by addition of NaN_3 to the diazonated phlorizin represents a classical reaction in organic chemistry. The infrared absorption at 2170 cm^{-1} of the final product is characteristic for the azido-group (see Table I).

The coupling reaction of aminophlorizin with dansyl chloride to obtain 3-dansylphlorizin was carried out at pH 5. Because of the low solubility of the reactants in water, tetrahydrofuran had to be added to the reaction mixture. Two main products were obtained. Dansylphlorizin has a yellow fluorescence, whereas the major by-product, a sulfonic acid derivative, shows a blue fluorescence.

(d) 3′-Mercuryphlorizin. 3′-Mercuryphlorizin represents a white solid substance that is not soluble in water or organic solvents. Hence, a direct identification of the product was difficult. Therefore, mercury was substituted by iodine [19]; the resulting iodophlorizin can be studied by NMR and from the location of the iodine molecules conclusions on the position of mercury in the mercuryphlorizin can be drawn. According to the spectroscopic data the mercury atom is located in the *ortho* position of the phenoxy group in ring A which is the most probable position for electrophilic substitution.

TABLE III

RELATIVE INHIBITORY EFFECT OF PHLORIZIN DERIVATIVES ON THE D-GLUCOSE UPTAKE BY HOG RENAL BRUSH BORDER MEMBRANES

D-Glucose uptake was determined in the presence of 100 mM NaSCN and after incubation for 15 s. The concentration of inhibitors in the incubation media was 10^{-5} mol/l. One representative experiment with measurements in triplicate is shown. Data in brackets indicate the relative inhibitory strength of phlorizin derivatives compared to phlorizin. Apparent K_i values were obtained from two to four experiments using uptake after 15 s and examining the data in a Dixon plot. n.d., not determined.

Compounds	D-Glucose uptake (pmol/mg protein) after 15 s	K_i (μM)
Control	312 (–)	
Phlorizin	61.8 (100%)	1.5 ± 0.3
3'-Mercuryphlorizin	60.5 (100.1%)	1.1 ± 1.0
3-Aminophlorizin	97.9 (85.2%)	3.8 ± 0.7
3-Bromoacetamidophlorizin	153.4 (63.1%)	7.7 ± 1.5
3-Azidophlorizin	111.5 (79.8%)	8.6 ± 3
3-Nitrophlorizin	173.6 (55.1%)	21.6 ± 1.6
3-Dansylphlorizin	178.5 (53.1%)	27.5 ± 3
3-Amino-6-sulfophlorizin	294.3 (7%)	n.d.

Inhibition of Na^+/D-glucose cotransport in renal brush border membrane vesicles by phlorizin derivatives

Most of the phlorizin derivatives inhibit Na^+/D-glucose cotransport in renal brush border membrane vesicles. The relative inhibitory effect of the various phlorizin derivatives at the same concentration (10^{-5} M) is shown in Table III. From the results shown in Table III and the apparent K_i values as determined from Dixon plots of the results of a series of transport studies using different concentrations of phlorizin derivatives and 0.1 and 0.2 mM D-glucose, the following order of potency is found: phlorizin = 3'-mercuryphlorizin > 3-aminophlorizin > 3-bromoacetamidophlorizin > 3-azidophlorizin > 3-nitrophlorizin = dansylphlorizin. 3-Amino-6-sulfophlorizin, a by-product of the reduction of nitrophlorizin to aminophlorizin with dithionite, shows the lowest affinity to the Na^+/D-glucose system.

Although the above-mentioned experiments were performed under gradient conditions involving a 15 s incubation time and thus provide only limited information about time affinities of the various phlorizin analogues, the following conclusions on the interaction of the Na^+/D-glucose transport with the phlorizin derivatives seem to be justified.

(1) Almost all phlorizin derivatives, independent of the kind of substituent, show a lower affinity for the Na^+/D-glucose cotransport system in brush border membrane than phlorizin, i.e., the substituents change to a certain extent the conformation or the electrical charge of the phlorizin molecule, which are responsible for the interaction with the transport system [6,20]. (2) Phlorizin derivatives which have lower pK values than phlorizin, such as 3-nitrophlorizin, 3-azidophlorizin and 3-bromoacetamidophlorizin, exhibit a lower inhibition of the D-glucose uptake. The derivatives which have higher pK values or have been tested in an electroneutral form, such as 3-aminophlorizin and 3'-mercuryphlorizin, show an inhibitory potency similar to phlorizin. (3) The derivatives which have bulky substituents such as 3-dansylphlorizin or which have strong anionic charges on the aromatic ring such as 3-amino-6-sulfophlorizin inhibit D-glucose uptake poorly. Thus, the interaction of the aglucone moiety of phlorizin with the glucose-transport system seems to involve not only hydrophobic interactions but also hydrophilic interactions with defined spatial requirements.

The specificity of phlorizin derivatives

The specificity of D-glucose-uptake inhibition in the brush border membranes was further investigated for two representative derivatives, 3-bromoacetamidophlorizin and 3'-mercuryphlorizin. Thereby, two different aspects were tested: (1) Is the inhibition of the D-glucose transport due to a direct interaction of the inhibitor with the Na^+/D-glucose cotransport system or due to a change in membrane properties affecting the driving forces of the Na^+ gradient? (2) Is the inhibition observed specific for the phlorizin derivatives or can it also be elicited by the reactive groups introduced into the molecule?

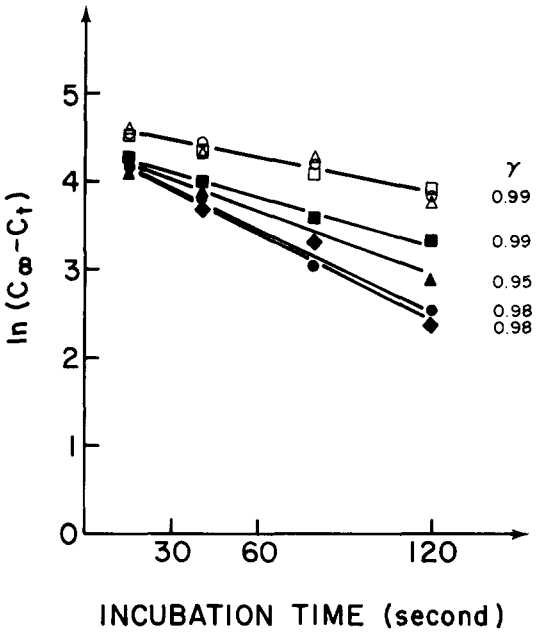

Fig. 3. Tracer-exchange experiments of brush border membranes in the presence of mercury compounds. Membrane vesicles were preincubated with 0.1 mM D-glucose in Hepes-Tris-mannitol buffer (20 mM Hepes-Tris (pH 7.4), 100 mM mannitol) containing in addition 100 mM NaSCN or KSCN, at 25°C for 1 h. The final protein concentration was 4 mg/ml. To start the experiments, 20 μl vesicle suspension were added to 100 μl of the same buffer as before containing in addition a trace of [^{3}H]glucose and 2 μM of the mercury compounds indicated: (-●-, -○-) control (-▲-, -△-) phlorizin, (-■-, -□-) mercuryphlorizin, and (-◆-, ◇-) PCMB. The open symbols are for potassium-containing media, the closed symbols are for sodium-containing media. Results from four experiments are compiled. Straight lines are calculated regression lines. All of the γ values are higher than 0.95. The values of the scatter among the individual experiments with different inhibitors are all less than 5%.

TABLE IV

THE INHIBITORY EFFECT OF MERCURY COMPOUNDS ON THE APPARENT EXCHANGE RATE (min^{-1}) OF D-GLUCOSE ACROSS BRUSH BORDER MEMBRANE VESICLES

The data are evaluated from Fig. 6.

Reagents (2 μM)	Na^+	K^+
Control	1.0 ±0.002	0.35±0.01
Phlorizin	0.71±0.018	0.38±0.02
3'-Mercuryphlorizin	0.57±0.041	0.35±0.005
PCMB	0.97±0.004	0.37±0.047
Hg(II) acetate	0.99±0.046	0.37±0.007

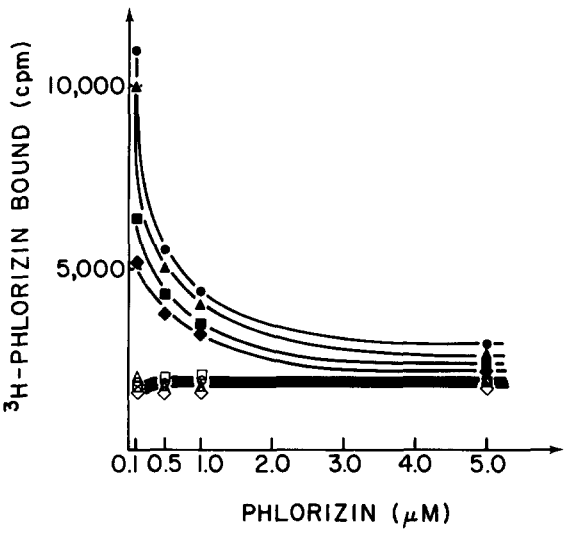

Fig. 4. Inhibitory effect of 3-bromoacetamidophlorizin on phlorizin binding to the brush border membranes. (-●-, -○-) Control values without 3-bromoacetamidophlorizin, (-▲-, -△-) in 5 μM, (-■-, -□-) in 15 μM and (-◆-, -◇-) in 30 μM of 3-bromoacetamidophlorizin. The closed symbols indicate the incubation medium with sodium and the open symbols the incubation medium with potassium. Points represent average values of four experiments.

3'-Mercuryphlorizin. Fig. 3 shows tracer-exchange experiments in which the rate of exchange of radioactively labeled D-glucose was determined in the presence of sodium and potassium but in the absence of any ion or D-glucose gradient. It is evident from the figure that sodium stimulates the

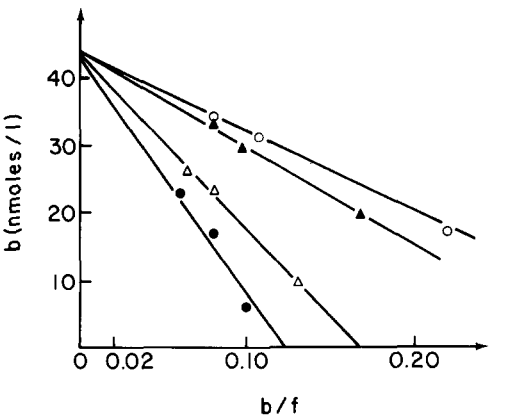

Fig. 5. Scatchard plot of sodium-dependent phlorizin binding in the presence of 3-bromoacetamidophlorizin from the data obtained from Fig. 4. (-○-) Control without 3-bromoacetamidophlorizin; (-▲-) in 5 μM; (-△-) in 15 μM; (-●-) in 30 μM 3-bromoacetamidophlorizin.

TABLE V

COMPARISON OF EFFECT OF 3-BROMOACETAMIDOPHLORIZIN ON Na^+/D-GLUCOSE, Na^+/PHOSPHATE AND Na^+/L-ALANINE COTRANSPORT SYSTEMS IN RENAL BRUSH BORDER MEMBRANES

Aliquots of membrane vesicles were preincubated with 10^{-5} mol/l of 3-bromoacetamidophlorizin at 37°C for 30 min. As control, membranes were incubated under the same experimental conditions without 3-bromoacetamidophlorizin (10 mg/ml membrane protein). For transport experiments, the conditions in Fig. 3 were adopted except 0.1 mM ^{3}H-labeled KH_2PO_4, 0.4 mM [^{14}C]alanine were used to replace 0.1 mM [^{3}H]glucose. Data given in the table indicate the uptake in pmol/mg protein after 20 s. Mean values ±S.D. compiled of four experiments are given. Data in brackets indicate the uptake after 90 min (equilibrium).

	Control	3-Bromoacetamidophlorizin
D-Glucose		
Na^+	512.8± 3.8 (134)	240.8± 7.4 (128)
K^+	26.0± 0.3 (135)	26.8±12.8 (133)
Phosphate		
Na^+	270.6± 6.4 (178)	289.2±14.7 (183)
K^+	83.5± 9.4 (189)	100.6±22.4 (181)
L-Alanine		
Na^+	1150 ±16 (1200)	1175 ±21 (1400)
K^+	–	–

uptake of D-glucose into the vesicles significantly and that this stimulation is strongly inhibited by 3′-mercuryphlorizin. It is noteworthy that the D-glucose transport in the presence of potassium is not affected. The corresponding apparent rate constants are given in Table IV. This table also indicates that *p*-chloromercuribenzoate (PCMB), a mercuric atom linked to a phenolic residue as well as free mercuric compound at 2 μM do not inhibit D-glucose transport whereas 3′-mercuryphlorizin inhibits D-glucose transport significantly. These results demonstrate that the inhibition of D-glucose transport by mercuryphlorizin in due to a direct interaction of this compound with the transport system. The question as to whether the inhibitory effect of mercuryphlorizin is due to (1) the phlorizin moiety, (2) mercury or (3) both is difficult to answer. The slightly higher affinity of mercuryphlorizin compared to the parent compound might suggest that indeed the mercury function is important to compensate for a decrease in affinity that would have been expected to occur due to steric hindrance.

The specificity of the interaction of 3-bromoacetamidophlorizin with the D-glucose-transport system was investigated by determining the effect this compound on the sodium-dependent phlorizin binding to the brush border membrane. This phlorizin-binding site is postulated to be closely related to or part of the Na^+/D-glucose transporter. As shown in Figs. 4 and 5, 3-bromoacetamidophlorizin inhibits the sodium-dependent binding of phlorizin in a competitive manner during short time incubations. The number of binding sites of 31 pmol/mg protein remains constant whereas the apparent affinity decreases from 0.07 μM in the control to 0.34 in the presence of 30 μM 3-bromoacetamidophlorizin. The sodium-independent binding of phlorizin that has been related to hydrophobic interactions of phlorizin with the membranes remains unchanged. Data shown in Table V demonstrate that the sodium-dependent

TABLE VI

EFFECT OF BROMOACETIC ACID COMPOUND TRANSPORT BY BRUSH BORDER VESICLES

Data show D-glucose uptake after 15 s expressed as % of equilibrium (incubation time after 90 min). D-Glucose uptake at equilibrium was 130.15±0.96 pmol/mg protein per 15 s. Average values of four determinations are compiled.

	Concentration (M)	Na^+ gradient	K^+ gradient
Control	–	390±2.5	20 ±2
Phlorizin	10^{-5}	58±2.5	20 ±0.5
3-Bromoacetamidophlorizin	10^{-5}	182±3.2	20.9±1.5
Bromoacetamidonitrophenol	10^{-4}	350±7	24.6±1.2
Bromoacetic acid	$2\cdot10^{-5}$	365±4	–

uptake of alanine and of phosphate is not affected by 3-bromoacetamidophlorizin. These experiments provide strong evidence that 3-bromoacetamidophlorizin inhibits Na^+/D-glucose cotransport by interacting directly with the transport system. The lack of inhibition of other sodium-gradient-driven transport systems supports this assumption. Any interference with the sodium permeability of the membranes or with the conductivity should also be reflected in the uptake of alanine and phosphate. In Table VI, the effect of phlorizin, bromoacetamidonitrophenol and 3-bromoacetamidophlorizin on D-glucose transport are compared. At a concentration of 10^{-5} M, 3-bromoacetamidophlorizin inhibits glucose transport by about 50% whereas bromoacetamidonitrophenol, which represents a bromoacetamido group and a benzene ring, at a 10-fold higher concentration inhibits only by 10%. This finding indicates that the phlorizin moiety is necessary for the high-affinity interaction of 3-bromoacetamidophlorizin with the sodium/D-glucose cotransport system, the kind of interaction remaining to be determined. Preliminary experiments with iodoacetamidophlorizin indicate that the compound interacts in an irreversible manner with SH groups, that are essential for D-glucose transport [21–23]. However, this reaction requires prolonged incubation. Therefore, in our experiments where short incubation times are employed, the interaction of the transport molecule with the phlorizin moiety of 3-bromoacetamidophlorizin is probably the main determinant for the inhibition.

Acknowledgements

This work was supported by Deutsche Forschungsgemeinschaft grant Fa 48/21, F.R.G., by the start-up fund of Albert Einstein College of Medicine of Yeshiva University (BRSG 6134564) and by NIH grant No. GM 77859. The authors wish to thank Dr. Fasold for his continuous support and interest and Ms. A. Stroh for her skillful technical assistance. Part of the results were obtained by K.-D. H in fulfillment of his Diplom-Thesis at the Max-Planck Institute of Biophysics, 7 Heinrich-Hoffman Str., 6000 Frankfurt/M-71, F.R.G.

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