Biochimica et Biophysica Acta, 688 (1982) 547–556
Elsevier Biomedical Press

BBA 71195

4-AZIDOPHLORIZIN, A HIGH AFFINITY PROBE AND PHOTOAFFINITY LABEL FOR THE GLUCOSE TRANSPORTER IN BRUSH BORDER MEMBRANES

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(Received December 10th, 1981)

Key words: Glucose transport; Azidophlorizin; Photoaffinity label; (Brush border membrane)

A new phlorizin derivative (2′-*O*-(β-D-glucopyranosyl)-4-azidophloretin, 4-azidophlorizin) has been synthesized and its affinity for the D-glucose, Na^+ co-transport system in brush border vesicles from intestinal and renal membranes has been compared with that of phlorizin. The extent of the reversible interaction of the ligand with the transporter in dim light has been evaluated from three separate measurements: (1) K_i', the constant for fully-competitive inhibition of (Na^+, $\Delta\psi$)-dependent D-glucose uptake, (2) K_d', the dissociation constant of 4-azido[^{3}H]phlorizin binding in the presence of an NaSCN inward gradient, and (3) K_i'', the constant for fully-competitive inhibition of the specific ((Na^+, $\Delta\psi$)-dependent, D-glucose protectable) high-affinity [^{3}H]phlorizin binding. In experiments with vesicles derived from rat kidney, all three constants (K_i', K_d' and K_i'') were essentially equal and ranged between 3.2 and 5.2 μM, that is, the azide derivative has almost the same affinity for this transporter as phlorizin itself. On the other hand, compared to phlorizin, the 4-azidophlorizin has a lower affinity for the transporter in vesicles prepared from rabbit; its K_i' values are some 15–20-times larger than those determined with rat membranes. However, the affinity of the azide for the sugar transporter in membranes from either the intestine or kidney of the same animal species (rabbit or rat) was essentially the same. In spite of the lower affinity for the transporter in either membrane system from the rabbit, results described elsewhere (Hosang, M., Gibbs, E.M., Diedrich, D.F. and Semenza, G. (1981) FEBS Lett., 130, 244–248) indicate that 4-azidophlorizin is an effective photoaffinity label in this species also. Photolysis of the azide yields a reactive intermediate which reacts with a 72 kDa protein in rabbit intestine brush borders. Covalent labeling of this protein occurred under conditions which suggests that it is (a component of) the glucose transporter.

Introduction

The stable, osmotically active vesicles prepared from both intestinal and renal brush border membranes [1–4] retain the protein(s) responsible for Na^+ and D-glucose co-transport (see, for example Refs. 5–7) as well as the high affinity, Na^+-dependent phlorizin-binding site. Strong evidence

Abbreviations: buffer A, 300 mM (or when indicated, 100 mM) mannitol, 10 mM Hepes/Tris, pH 7.5, and 0.02% KN_3; buffer B, 300 mM mannitol, 10 mM Tris-HCl, pH 7.0, 0.75 mM dithioerythritol and 0.02% KN_3.
K_i' and K_i'', are the respective inhibition constants of (Na^+, $\Delta\psi$)-dependent D-glucose uptake and D-glucose protectable [^{3}H]phlorizin or 4-azido[^{3}H]phlorizin binding. K_d', is the apparent dissociation constant at the indicated pH. All refer to the total (ionized + unionized) ligand concentration.

has been provided by many investigators that the phlorizin receptor and glucose transporter in this membrane are identical [8–11]. Although the kinetics of the transport system, its dependency on electrochemical membrane potential, and the susceptibility to competitive ligand interaction have been extensively studied, the identity of the carrier system and its relationship to other components in the membrane remain uncertain.

Several strategies have been developed in attempts to distinguish the sugar transporter from the many other protein constituents of the brush border: (1) Membranes have been solubilized in detergents to fractionate their components; the partially purified proteins have then been reconstituted into artificial liposomes and tested for functional transporter activity [12–14]. (2) Alkylating or other agents have been used to radioactively label the transporter in intact membranes; in attempts to make the reaction specific, the ligands selected were either sugar substrate derivatives [15] or general protein reagents used under conditions which could discriminate D-glucose and phlorizin protectable sites [16–21] and (3) the increase in specific, Na^+-dependent, reversible phlorizin receptor density has been monitored while negatively purifying membrane vesicles [22]. Although these approaches are useful and have provided important information, each has its limitations and inherent difficulties. Furthermore, the reports are sometimes contradictory as to which band in the SDS-polyacrylamide gel electrophoresis patterns is (a part of) the transporter protein.

Photoaffinity labeling should be a powerful method to identify the sugar carrier, if the photolabile probe has (1) high specificity, (2) a small binding constant and (3) suitable photo-reactivity. We have adopted this approach to ultimately identify the transporter and have synthesized an azide derivative of phlorizin which, on the basis of the experimental results described in this report, appears to be potentially useful to photoaffinity label the sugar carrier in brush border membranes.

Experimental procedures

Materials

Hydrocinnamonitrile and phloroglucinol were purchased from Eastman Organic Chemicals or from Matheson, Coleman and Bell. Silica Gel 60 was obtained from E. Merck, Darmstadt. Phlorizin dihydrate was obtained from Carl Roth, Karlsruhe or Fluka AG. and was recrystallized before use. [^{3}H]Phlorizin (6.3 Ci/mmol) and D-[1-^{3}H]glucose (30.0 Ci/mmol) were purchased from New England Nuclear Corp.

Methods

General. Melting points were taken on a Thomas Hoover capillary melting point apparatus and are uncorrected. TLC was performed on Silica Gel GF precoated plates using the following solvent systems: A, 3% ethanol in benzene plus 1% acetic acid; B, C and D, 1%, 3% and 30% *n*-propanol in chloroform, respectively. Infra-red spectra were taken in KBr pellets on a Beckman IR-8 spectrometer. ^{1}H-NMR spectra (100 MHz) were recorded on a Varian HA-100 using $(CH_3)_4Si$ ($\delta = 0.00$) as internal standard and acetone-d_6, $C^2H_3O^2H$ or C^2HCl_3 as solvents. Chemical shifts are given in ppm. Elemental and functional group analyses were performed by Galbraith Laboratories, Knoxville, TN and by the Laboratorium für Organische Chemie, Eidgenössische Technische Hochschule-Zentrum, Zürich, Switzerland.

Preparation of membrane vesicles. Brush border membrane vesicles from frozen rabbit small intestines were prepared daily by the calcium precipitation method of Schmitz et al. [1] as modified by Kessler et al. [4]. For some experiments, scrapings of intestinal mucosa were used to yield vesicles with enhanced stability of D-glucose transport activity (Takesue, Y., unpublished data). The yield of one preparation, 20–30 mg of membrane protein (Lowry et al. [23]), was usually suspended in 1–2 ml of buffer A for each experiment.

Only fresh mucosal scrapings (about 6 g of wet tissue from four animals) were used to prepare rat intestinal vesicles since brush border membrane vesicles derived from frozen rat intestines were of differing quality; the intravesicular volume was often below 10% of the normal value (1 μl/mg protein) and the D-glucose accumulation rate was strongly reduced [24] perhaps due to diminished vesicularization.

Brush border membrane vesicles were prepared from renal cortical slices of 2–4 male Sprague-Dawley rats or one rabbit by slightly modifying an

adaptation [3] of the protocol of Booth and Kenny [2]. The initial homogenate (300 ml) was centrifuged 15 min at 3000 × *g* to give the supernatant. The pellet obtained by centrifuging the supernatant for 30 min at 27000 × *g*, was homogenized in 75 ml buffer A (10 strokes with a motor-driven Teflon pestle), and again made 10 mM in $MgCl_2$. The steps used to obtain this pellet were repeated to give the final pellet which was suspended in 1–2 ml of appropriate buffer and passed 10 times through a 25 gauge needle before being used in the experiments.

Transport and binding experiment. Concentrative uptake of D-[^{3}H]glucose (in the presence of an inwardly directed NaSCN gradient) was determined by the rapid filtration technique [5] and performed as previously described [4]. To initiate transport, 10 μl of a vesicle suspension were rapidly mixed with buffer A (early tests were with the 100 mM mannitol buffer) containing 200 mM NaSCN, 0.2–1 μCi D-[^{3}H]glucose and the additions indicated in the legends to Fig. 2 and Table II. The incubation, performed at room temperature (and under subdued light if 4-azidophlorizin was used), was stopped by adding 2–2.5 ml ice-cold 0.9% saline buffered with 1 mM Tris-HCl, pH 7.5. For long incubations, the process was carried out manually while for the 2-s uptake measurements, the apparatus described by Kessler et al. [25] was employed. In both cases the diluted sample was immediately transferred onto a Sartorius filter (0.65 μm pore size) and rapidly washed with 5–8 ml of the stop solution. Blanks accounting for tritium trapped on the filter were determined by adding the vesicle suspension and the substrate medium separately to the stop solution.

(Na^+, $\Delta\psi$)-dependent [^{3}H]phlorizin and 4-azido[^{3}H]phlorizin binding experiments were conducted according to a modification [22] of the procedure described by Toggenburger et al. [11]. The tests were performed under two conditions; vesicles were either in the presence of an NaSCN gradient as described in the transport study or at equilibrium, in which case, they were preincubated for at least 30 min at room temperature with 100 mM of either NaCl or KCl in buffer B. The binding reaction was initiated by mixing 10-μl aliquots of these membrane suspensions with 10 μl

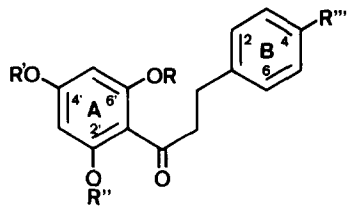

Fig. 1. Structural identification of the phlorizin derivatives.

I R = R′ = R″ = H; R‴ = NO_2
IIa R = R″ = H; R′ = β-D-glucopyranosyl $(OAc)_4$; R‴ = NO_2
II R = R″ = H; R′ = β-D-glucopyranosyl; R‴ = NO_2
IIIa R = β-D-glucopyranosyl $(OAc)_4$; R′ = R″ = H; R‴ = NO_2
III R = β-D-glucopyranosyl; R′ = R″ = H; R‴ = NO_2
IV R = R′ = β-D-glucopyranosyl $(OAc)_4$; R″ = H; R‴ = NO_2
Va R = β-D-glucopyranosyl; R′ = R″ = H; R‴ = NH_2
V R = β-D-glucopyranosyl; R′ = R″ = H; R‴ = N_3
VIa R = R″ = H; R′ = β-D-glucopyranosyl; R‴ = NH_2
VI R = R″ = H; R′ = β-D-glucopyranosyl; R‴ = N_3
VII R = R″ = H; R′ = β-D-glucopyranosyl; R‴ = OH (*p*-phlorizin)
VIII R = R′ = H; R″ = β-D-glucopyranosyl; R‴ = OH (phlorizin)

of the appropriate medium containing twice the desired final tritiated ligand concentration. After vigorous mixing and further incubation for the times indicated, the mixture was diluted with 2.5 ml of an ice-cold solution containing 250 mM KCl, 0.1 mM nonradioactive phlorizin and 1 mM Tris-HCl, pH 7.5, and then filtered and washed once with 5 ml of the stop solution. Specific binding was defined as the difference in the amount of ligand bound when K^+ replaced Na^+ in the medium and/or the binding in the presence of 25 mM glucose compared to 25 mM fructose. Filter blanks (which were relatively high for the tritiated azide) were determined by adding the membrane suspension and the incubation medium separately to the stop solution.

Synthesis of 2′,4′,6′-trihydroxy-4-nitrodihydrochalcone (4-nitrophloretin), I (Fig. 1). Anhydrous phloroglucinol (21 g, 170 mmol) and 30 g (170 mmol) 4-nitrohydrocinnamonitrile (26) in 2 liter diethyl ether were condensed as in the Hoesch preparation of phloroacetophenone [27]. The ketimine hydrochloride, collected after one week at 0°C, was boiled in 4 liter water to form the insoluble crystalline ketone I (22 g; 43%) of high purity (m.p. 268–272°C). The analytical sample was re-crystallized (without charcoal) from aqueous methanol to yield yellow needles, m.p.

274–276°C; $R_F = 0.20$ in solvent A.

Anal. Calcd. for $C_{15}H_{13}O_6N$: C, 59.4; H, 4.32; N, 4.62. Found: C, 59.3; H, 4.40; N, 4.69. The structure for I was confirmed by comparing the ^{1}H-NMR spectrum with that of phloretin: The B ring AA'BB'-system was shifted from δ 6.85 (ν_A, $\nu_{A'}$ = 7.01, ν_B, $\nu_{B'}$ = 6.68, J_{AB} = 9 Hz) to 7.80 (ν_A, $\nu_{A'}$ = 8.12, ν_B, $\nu_{B'}$ = 7.48, J_{AB} = 9 Hz). The AB-system, H-C (3', 5'), at δ 5.80 remained unchanged.

The original strategy to synthesize specifically the 2'-glucoside of I was to first acetylate or benzoylate the 4'-hydroxyl before glycosylation. Mixtures of acetate (or benzoate) esters were prepared which could be fractionated by selective alkaline extraction [28] or by Silica gel column chromatography using chloroform-methanol solvent mixtures. However, when glycosylation of either the 4'-acetate or benzoate ester was carried out as previously described [29], inadequate yields of the respective glycosides were obtained and the approach was abandoned. The melting points and R_F values in two solvent systems of some acetate and benzoate derivatives Ia–Ig are given in Table I.

Nitrophlorizin, III. I (4.5 g, 15 mmol) was glycosylated directly with 9.3 g (22.6 mmol) acetobromglucose in 120 ml acetone and 90 ml of cold 0.25 M KOH. After 24 h in the dark at 20°C, the mixture was poured into 2 liter ice containing 2 ml acetic acid. The product was collected after 4 h, air dried, and extracted twice with 50 ml of chloroform to recover 3.7 g of insoluble I. The extract, containing a mixture of IIa, IIIa, IV and various glucose acetates (3.1 g), was concentrated to 10 ml and subjected to flash chromatography [30] on a column of Silica Gel 60 (4.5 × 26 cm), pressure packed with chloroform. Elution was conducted with 1 liter 1.5% *n*-propanol in chloroform followed by 500 ml of 1.8% and then 1 liter of 3% propranol in $CHCl_3$. TLC was used to monitor the eluate; volumes between 280 and 360 ml, 380 and 510 ml, and 680 and 1600 ml were pooled, evaporated to dryness and the residues crystallized from methanol to yield 0.20 g (8%) of IV (m.p. 197–198°C), 0.39 g (23%) of IIa (m.p. 185–186°C) and 0.31 g (18%) of IIIa (m.p. 88–92°C), respectively. R_F values of the three products in solvent system C were 0.40, 0.29 and 0.14. Each derivative was ultrapurified on small Silica gel columns and crystallized from methanol.

Anal. Calcd. for $C_{29}H_{31}O_{15}N$: C, 54.98; H, 4.93; N, 2.21. Found, for IIa: C, 55.06; H, 5.02; N, 2.20. Found, for IIIa: C, 54.80; H, 5.06; N, 2.04. Calcd. for $C_{43}H_{49}O_{24}N$: C, 53.58; H, 5.12; N, 1.45. Found, for IV: C, 53.61; H, 5.17; N, 1.39.

Structures for IIIa, IV (and after saponification, for II) were confirmed from ^{1}H-NMR spectra. IIIa gave signals characteristic of a mono-(tetraacetyl)glucopyranoside; 2'-substitution was demonstrated by the AB-system at δ 6.03 (ν_A = 6.03, ν_B = 5.98, J = 2 Hz, H-C(3',5')), the signal of

TABLE I

MELTING POINTS AND R_F VALUES FOR SOME ACETATE AND BENZOATE DERIVATIVES OF 4-NITROPHLORETIN (I)

Elemental analyses (C, H and N) of each derivative were within 0.25% of the theoretical values. Acetyl group analysis of Ia, Ib and Ic were within 0.38% of theoretical; Id was 4.1% low. NMR analysis was used to decriminate between the 2'- and 4'-isomers. Solvent systems: A, 3% ethanol in benzene plus 1% acetic acid; B, 1% *n*-propanol in chloroform.

	R	R'	R''	m.p. (°C)	R_F values in	
					Solvent A	Solvent B
Ia	CH_3CO	H	H	184–186	0.30	0.12
Ib	H	CH_3CO	H	175–176	0.32	0.20
Ic	CH_3CO	CH_3CO	H	118–120	0.87	0.48
Id	CH_3CO	CH_3CO	CH_3CO	166–168	0.73	0.41
Ie	C_6H_5CO	H	H	202	0.42	0.21
If	H	C_6H_5CO	H	240–242	0.44	0.25
Ig	C_6H_5CO	C_6H_5CO	H	205–207	0.82	0.58
Ih	C_6H_5CO	C_6H_5CO	C_6H_5CO	130–131	0.85	0.58

the corresponding H-ligands of I being a singlet at δ 5.80. IV displayed the signals of di(tetraacetyl)glucopyranoside; the AB-system at δ 6.13 ($\nu_A = 6.23$, $\nu_B = 6.03$, H-C(3',5')) indicated substitution in positions 2' and 4'. II showed the signals of a monoglucopyranoside; substitution in 4' was again evidenced from the signal of H-C(3',5'), this time being a singlet at δ 6.08.

To saponify either IIa or IIIa, 1 mmol was treated in vacuo, in subdued light, with 2 ml cold 1 M sodium methoxide in methanol. After 15 min at room temperature, the mixture was neutralized under nitrogen with 0.3 M HCl. Each free glucoside crystallized in about 90% yield; both were recrystallized as trihydrates from 20% aqueous methanol. The analytical sample of II was obtained from ethylacetate as white plates, m.p. 200–202°C and III crystallized from methanol as hard, pale yellow prisms, m.p. 225°C. Their respective R_F-values in solvent system D were 0.29 and 0.39. *Anal.* Calcd. for $C_{21}H_{23}O_{11}N$: C, 54.19; H, 4.98; N, 3.01. Found for II: C, 54.32; H, 5.02; N, 2.91. Found for III: C, 54.01; H, 5.11; N, 2.92.

2'-O-(β-D-glucopyranosyl)-4-aminophloretin (4-aminophlorizin), Va. The trihydrate of III (200 mg; 0.38 mmol) was hydrogenated at atmospheric pressure in 40 ml methanol with 40 mg of 10% Pd/C as catalyst. The tetrahydrate of Va was obtained from dilute methanol as pale yellow prisms ($R_F =$ 0.30 in solvent D) which after drying in vacuo at 60°C melted at 139–142°C. The product was converted to the azide without further purification.

The Amersham Corporation, Arlingon Heights, IL, tritiated anhydrous Va by their TR-7 procedure in which benzylic hydrogens are catalytically exchanged. The labeled amine (65 mg) was chromatographed on a Silica gel 60 column (2 × 25 cm) in chloroform; consecutive 100 ml volumes of 10, 20 and 30% methanol in chloroform eluted the radiochemically pure 4-aminophlorizin which crystallized from aqueous methanol as colorless needles (48 mg).

The isomeric, 4-amino-*p*-phlorizin (VIa), prepared by hydrogenation of II as desribed above for III, was crystallized from aqueous methanol; after drying at 100°C in vacuo its m.p. was 159–162°C. $R_F = 0.17$ in system D.
Anal. Calcd. for $C_{21}H_{25}O_9N$: C, 57.93; H, 5.79; N, 3.22. Found: C, 57.73; H, 5.90; N, 3.08.

4-Azidophlorizin (V). All steps were performed in subdued light. Va (43.5 mg) in 5 ml acetone and 2 ml 0.5 M HCl (0°C) was treated with 0.5 ml of 0.54 M sodium nitrite added over a 15 s period. After 6 min at 0°C or less, 0.5 ml of cold 2.0 M sodium azide was added dropwise. After stirring for 10 min, 60 mg solid urea was added to bring the pH to about 6.5. Acetone was then removed in vacuo and the azide was extracted into ethyl acetate, dehydrated and then the solvent was evaporated to gain crude V (39 mg; 85%). Soft colorless needles were obtained from dilute methanol as the monohydrate; m.p. 150–155°C, after softening at 108–110°C. Anhydrous V could not be obtained without decomposition. $R_F = 0.42$, in system D. The structure of V was confirmed by NMR analysis. Its IR spectrum included the sharp 2130 cm^{-1} and 1280 cm^{-1} absorption peaks characteristic of the azide group and was otherwise very similar to that of phlorizin. V has two ultraviolet absorption maxima in ethanol or water; one at 251 nm, attributable to the azide group and the second at 285 nm which shifts to 328 nm in alkaline buffer ($\epsilon = 2.75 \cdot 10^{-3}$ $cm^{-1} \cdot M^{-1}$, in 0.05 M sodium borate, pH 9.3). This bathochromic shift is characteristic of those analogs possessing an ionizable 4'-hydroxy [31]; the 285 nm peak of the 4'-glucoside (VIa, see below) did not shift in borate buffer.

Radiochemically pure V, prepared from tritiated Va by the above procedure, was obtained by chromatography on a Silica gel 60 column equilibrated in chloroform. A 10% methanol in chloroform eluant delivered radiochemically pure azide (TLC radioautography) preceding the residual amine and other products. The pure azide (405 mCi/mmol) was stored in the dark at −70°C as a 10% ethanol in benzene solution.

4-Azido-p-phlorizin (VI). Essentially the same procedure to prepare V was used to convert VIa to the azide; the reaction was performed in aqueous methanol from which crude VI precipitated. It was purified by Silica gel 60 chromatography (chloroform/methanol) in 40% yield and crystallized from 50% methanol. After drying to constant weight at 20°C, m.p. 168–170°C. $R_F = 0.38$ in system D.
Anal. Calcd. for $C_{21}H_{23}O_9N_3$: C, 54.66; H, 5.02; N, 9.11. Found: C, 54.50; H, 5.24; N, 8.99.

Results

Inhibition of D-glucose uptake

The time-course of D-glucose uptake into vesicles prepared from the rat in the presence of an initial NaSCN gradient was similar to that reported earlier [4,11]; the peak of the labeled glucose overshoot occurred between 10 and 30 s and intravesicular levels exceeded that in the medium by 10–15-fold. When sufficiently high levels of 4-azidophlorizin were added to the incubation medium (50 μM), the overshoot was completely blocked as has been shown for phlorizin by many investigators. To quantitate the inhibition, initial D-glucose uptake rates were measured after either 2- or 7-s incubations in subdued light with varying sugar and 4-azidophlorizin concentrations; data from experiments using rat kidney vesicles are shown in Fig. 2, A in the form of a Dixon plot. The secondary Dixon plot (inset) and a Cornish-Bowden plot (S/V vs. I; not shown) indicated that the derivative acts strictly as a competitive inhibitor. In this particular experiment, the apparent K_i of 6.6 μM was the largest of all the values found for the azide in rat kidney but even so, it is only 3–4-times greater than that determined for phlorizin (Fig. 2, B) under identical conditions. All of our results are summarized in Table II, A. The mean of our two determinations for phlorizin with rat intestine (4.2 μM) compares with Hopfer's [32] value of 2.7 μM which he obtained under essentially the same conditions as ours. The mean K_i' value for 4-azidophlorizin as uptake inhibitor is only 2–3-times greater than that for phlorizin in either intestine or kidney vesicles from the rat.

Results with rabbit membranes were different. Phlorizin, with a K_i' of about 8 μM, is a less potent competitive inhibitor of glucose uptake in rabbit brush border membrane vesicles than in the rat

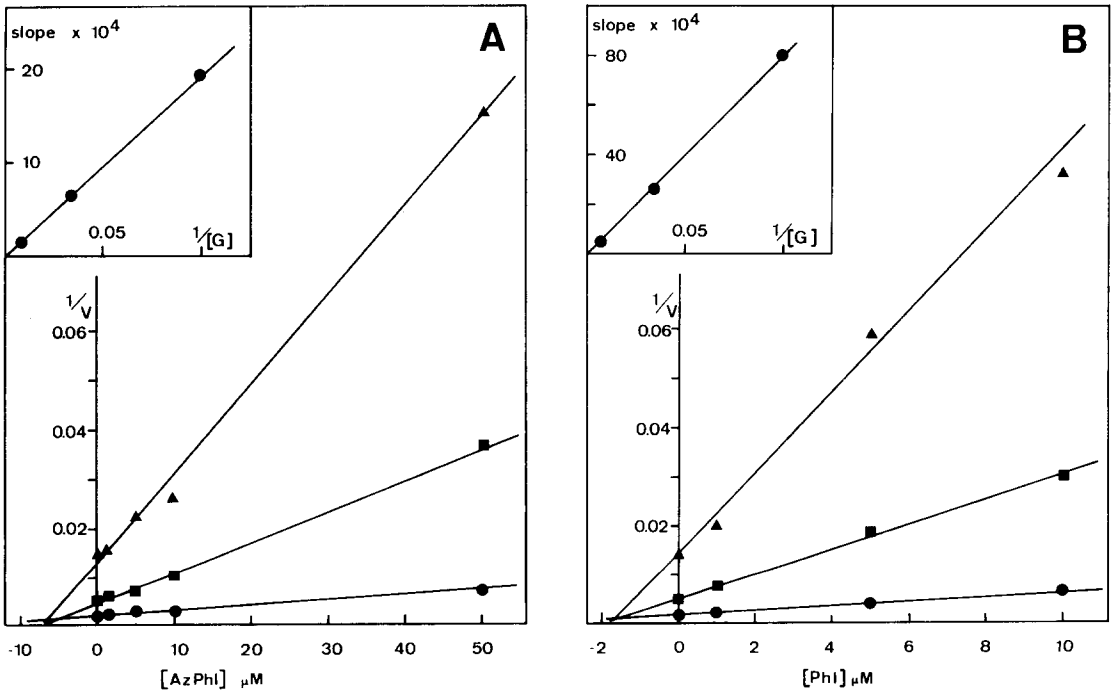

Fig. 2. Dixon plots for the inhibition of D-glucose uptake by 4-azidophlorizin (AzPhl) and phlorizin (Phl) into rat kidney brush border membrane vesicles (pH 7.5, 100 mM inward NaSCN gradient, 22°C; incubation time, 7 s). D-Glucose (G) concentrations; 10 μM (▲), 30 μM (■) and 150 μM (●). V is expressed as pmol D-glucose uptake/mg vesicle protein. K_i' for 4-azidophlorizin (Panel A) and phlorizin (Panel B) are 6.6 μM and 1.7 μM, respectively, and were derived from the intersection of the least-squares regression lines. The inset of each panel shows the slopes of these lines plotted against the inverse of the D-glucose concentrations; straight lines through the origin indicate that the inhibition is strictly competitive.

TABLE II

HALF-SATURATION CONSTANTS OF PHLORIZIN AND 4-AZIDOPHLORIZIN FOR THE GLUCOSE TRANSPORTER IN BRUSH BORDER MEMBRANE

Brush border vesicles were prepared and incubated in the presence of varying levels of phlorizin and 4-azidophlorizin as in Methods to determine: (A) effect of each ligand on the 2 s (rabbit) and 7 s (rat) rate of D-glucose uptake; mean K_i' values ± S.D. were determined from (n) individual Dixon plots. (B) Specific, (Na^+, $\Delta\psi$)-dependent binding of each ^{3}H-labeled ligand. Mean ± S.D. (n) of the K_d' values were determined from Scatchard plots at pH 6.5 and 7 s for rat kidney and at pH 7.5 and 2 s for rabbit intestine; other values as indicated were taken from the literature under varying conditions. Estimation of the dissociation constant for 4-azido[^{3}H]phlorizin with brush border membrane vesicles from rat intestine and rabbit kidney was not attempted. The value for rabbit intestine could not be measured because of very high non-specific binding which occurred at the ligand concentration required. (C) Effect of each ligand on (Na^+, $\Delta\psi$)-dependent [^{3}H]phlorizin binding. K_i'' values ± S.D. obtained from rat kidney brush border membrane vesicles were determined as described in Methods at pH 6.5 and 7 s, and for rabbit intestine at pH 7.5 and 2 s.

	Rat		Rabbit	
	Intestine	Kidney	Intestine	Kidney
A. Inhibition of D-glucose uptake by	K_i' values (μM)			
Phlorizin	4.2 ± 2.5 (2) 2.7 ± 0.2 [a]	2.1 ± 1.2 (4)	8 ± 1.2 (3)	7.5
4-Azidophlorizin	10	5.2 ± 1.9 (3)	139 ± 9 (3)	140
4-Nitrophlorizin (III)	–	17	445 ± 50 (2)	–
4-Aminophlorizin (Va)	–	2.8	44 ± 7 (2)	–
B. Specific, Na^+-dependent binding of	K_d' values (μM)			
Phlorizin	1.43 [b]	1.1 ± 0.3 (4) 0.25 [c], 0.8 [d]	4.6 ± 0.85 (5) [e]	8 [f]
4-Azidophlorizin	–	3.2 ± 0.7 (3)	–	–
C. Inhibition of specific [^{3}H]phlorizin binding by	K_i'' values (μM)			
Phlorizin	–	0.9 ± 0.08 (2)	12 ± 2 (2)	–
4-Azidophlorizin	–	3.8 ± 1.7 (3)	156 ± 40 (2)	–

[a] Hopfer [32]; brush border membrane vesicles with Na^+ gradient, pH 7.5, 12 s.
[b] Franklin et al. [33]; brush border membrane vesicles, 150 mM NaCl.
[c] Frasch et al. [8]; with intact brush borders, pH 7.5, 5 min.
[d] Bode et al. [34]; with intact brush borders, pH 7.5, 5 min.
[e] Toggenburger et al. [11]; brush border membrane vesicles with NaSCN gradient, pH 7.5, 2 s.
[f] Chesney et al. [35]; with intact brush borders, pH 7.5, 1 min.

membrane system. This species difference is more conspicuous when the K_i' values of 4-azidophlorizin are compared; the azide is almost 20-times less potent in the rabbit membrane system (Table II, A, B and C).

The time-course of phlorizin and 4-azidophlorizin binding to brush border membrane vesicles

There is very rapid interaction between phlorizin and the high affinity binding site of brush border membrane vesicles derived from rabbit intestine [11]. If measurements are made much after 2 s, this (Na^+, $\Delta\psi$)-dependent binding becomes masked by a delayed, non-specific, low-affinity absorption process. However, with kidney vesicles, the specific high-affinity phlorizin binding is much slower; in our present work with rat brush border membrane vesicles, the half-time of binding was

about 5 s in the presence of a 100 mM NaSCN gradient and this was increased to about 15 s in 100 mM NaCl equilibrated vesicles. Our results agree with the time course of phlorizin binding reported by Chesney et al. [35] using rabbit kidney membranes, with the report of Turner and Silverman [36] who worked with vesicles from dog kidney and with the results of Kessler et al. (1980, unpublished data) who compared the time course of phlorizin binding to intestine and kidney brush border membrane vesicles from the same species.

Binding of 4-azidophlorizin to brush border membrane vesicles from rat kidney cortex

In the case of phlorizin, a difference (initial NaSCN vs. initial KSCN gradient) in the amount bound to kidney brush border membrane vesicles could be measured for at least 90 min. However, in the case of 4-azidophlorizin, the (Na^+, $\Delta\psi$)-dependent binding could be detected only when incubation times were brief ($\leq$ 15 s) and when ligand concentrations were kept low. These limitations were necessary because of the much greater non-specific binding of the derivative to the Sartorius filters used in the assay and especially because of its extensive association with membrane lipids. The partition coefficient of 4-azidophlorizin in two systems (olive oil/water and phosphatidylcholine liposomes/water) was 7–8-times that found for phlorizin (data not shown).

Scatchard analysis of [^{3}H]phlorizin and 4-azido[^{3}H]phlorizin binding to rat kidney brush border membrane vesicles gave curvilinear plots from which high-affinity, (Na^+, $\Delta\psi$)-dependent sites could be identified. The K'_d values for each ligand are listed in Table IIB, together with previously reported values for phlorizin's dissociation constant derived under a variety of conditions. The values are essentially identical to the respective inhibition constants found in the glucose uptake study with these vesicles; the affinity of the azide is again seen to be about one-third that of phlorizin. The number of high affinity 4-azidophlorizin binding sites was not statistically different from that found for phlorizin (88 ± 7 and 91 ± 4 pmol/mg protein (± S.E.), respectively).

We were unable to determine the specific binding of 4-azidophlorizin to vesices prepared from either intestine or kidney of the rabbit. Because of the lower affinity of the azide for the transporter in this membrane, relatively high levels of ligand had to be tested and under these conditions, the high affinity binding was swamped by the extensive non-specific binding (i.e., under KSCN gradient) which occurred even at short time incubations.

Inhibition of [^{3}H] phlorizin binding

Our final evidence that 4-azidophlorizin is avidly bound to the phlorizin inhibitable Na^+, D-glucose transporter is based on the derivative's ability to competitively inhibit the high affinity phlorizin binding to brush border membrane vesicles. Its K'_i value of 3.8 μM in the rat kidney system (Table IIC) is the same as the K'_i and K'_d values found with these membranes. It has considerably less but still significant affinity for the high affinity phlorizin binding site in rabbit intestine (156 μM versus 3.8 μM for phlorizin).

Other phlorizin analogues

With the exception of the 4-amino (Va) and the 4-nitro (III) phlorizin derivates (Table II, A), none of the free glucosides identified in Fig. 1 had any significant interaction with the transporter. In particular, the *p*-phlorizin analogues (R' = β-D-glucopyranosyl, Fig. 1) interacted poorly or not at all (data not shown). *p*-Phlorizin (VII) itself has a very low affinity [31,37–39]; it has a K'_i value of about 420 μM [40].

Discussion

The results reported above show that 4-azidophlorizin (Fig. 1, V) interacts with the Na^+, D-glucose transporter of the brush border membranes from the small intestine and the kidney cortex of both the rat and the rabbit. This was demonstrated by our finding that for a given type of brush border membrane vesicles (for example, rat kidney), the K_i values for fully competitive inhibition of Na^+,D-glucose transport and Na^+-dependent phlorizin binding, as well as the K'_d value of its own Na^+-dependent binding when it could be estimated, were all essentially identical (Table II).

One of us [37,41] suggested that a site on the protein assembly which constitutes the Na^+,D-

glucose transporter donates a hydrogen that interacts with the -OH group on the B ring of phlorizin (VIII, Fig. 1) to form a strong H-bond (2.4–2.75 kcal/mol). Since azido groups can also act as the H-acceptor in hydrogen bond formation [42], we reasoned that the replacement of the phenolic group at position 4 with a photoreactive moiety ($-N_3$) might have only a minimal effect in reducing the binding energy between ligand and the transporter molecule. In fact, the affinity of 4-azidophlorizin for the transporter in membranes from the rat was reduced very little, but more so in the rabbit (Table II). However, even in the latter species, the observed decrease in binding energy (by approx. 1.7 kcal/mol) is compatible with a H-bond still being formed, albeit weakly. This type of interaction between a group in position 4 of the ligand and the binding site is also consistent with our findings (Table IIA) that 4-aminophlorizin (Va), which is probably deprotonated at pH 7.5, has significant affinity while that of the nitro derivative (III) is low.

The precise structure of the subsite with which the B ring of phlorizin and congeners interact (and which may be located in a relatively hydrophobic region of the transporter assembly) appears to be somewhat different in the two animals we have studied. Within the same species, however, the affinities of phlorizin and 4-azidophlorizin for the transporter in brush border membrane vesicles derived from either small intestine or kidney are essentially identical. This occurs in spite of the different time-course of binding in this tissues and the well known differences in the substrate specificity for monosaccharide transport.

Whatever the nature of the bonds involved in the interaction of phlorizin and 4-azidophlorizin with the Na^+,D-glucose transporter, it is clear from the data in Table II that 4-azidophlorizin, as well as the phlorizin derivatives substituted in position 6 of the glucopyranosyl moiety [40], meets 'in the dark' requirements for a potential photoaffinity label of the transporter: Its K_i' values, even with rabbit brush border membrane vesicles, are reasonably small. Thus, the occupation time of 4-azidophlorizin (as well as the photogenerated, reactive intermediates, mainly the 4-nitrene) should be long enough to lead to covalent labeling of the Na^+,D-glucose transporter. Indeed, we have already performed photolysis experiments using 4-azido[^{3}H]phlorizin and brush border membrane vesicles from the rabbit small intestine and found [43] that a number of polypeptides, identified as bands in SDS-polyacrylamide gel electrophoresis, become labeled. One of these membrane proteins, with an apparent M_r of 72000, fulfills the criteria for it to be (a part of) the Na^+,D-glucose transporter in brush border vesicles: (1) its labeling is strongly reduced by the presence of phlorizin, yet not by the isomeric *p*-phlorizin (VII) which has similar chemical and optical properties but very poor affinity for the transporter; (2) its labeling is also strongly reduced if choline$^+$ is substituted for Na^+ as the major cation; (3) its degree of labeling increases with the degree of negative purification in passing from brush border membrane vesicles to deoxycholate-extracted and then to deoxycholate plus KI-extracted membranes. In addition, the amount of label bound is compatible with the transporter density present in this membrane [43].

Photolysis experiments are also in progress with 4-azido[^{3}H]phlorizin using rat kidney brush border membrane vesicles. Preliminary results indicate that a number of proteins are also labeled in these membranes. Most of the label is incorporated into three bands, with M_r at about 70000, a broad 55000 and a 20000 region. These results will be published in detail elsewhere.

Acknowledgement

James O. Evans made important contributions in the synthesis and purification of the 4-azidophlorizin. The authors also wish to acknowledge the excellent technical assistance of Susanne L. Diedrich. Supported in part by Grant AM 06878, National Institutes of Health and by the Swiss National Science Foundation.

References

1 Schmitz, J., Preiser, H., Maestracci, D., Ghosh, B.K., Cerda, J.J. and Crane, R.K. (1973) Biochim. Biophys. Acta 323, 98–112
2 Booth, A.G. and Kenny, A.J. (1974) Biochem. J. 142, 575–581
3 Evers, C., Haase, W., Murer, H. and Kinne, R. (1978) Membrane Biochem. 1, 203–219
4 Kessler, M., Acuto, O., Storelli, C., Murer, H., Müller, M.

and Semenza, G. (1978) Biochim. Biophys. Acta 506, 136–154
5 Hopfer, U., Nelson, K., Perotto, J. and Isselbacher, K.J. (1973) J. Biol. Chem. 248, 25–32
6 Murer, H. and Hopfer, U. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 484–488
7 Tannenbaum, C., Toggenburger, G., Kessler, M., Rothstein, A. and Semenza, G. (1977) J. Supramol. Struct. 6, 519–533
8 Frasch, W., Frohnert, P.P., Bode, F., Baumann, K. and Kinne, R. (1970) Pflügers Arch. 320, 265–284
9 Vick, H., Diedrich, D.F. and Baumann, K. (1973) Am. J. Physiol. 224, 552–557
10 Aronson, P.S. (1978) J. Membrane Biol. 42, 81–98
11 Toggenburger, G., Kessler, M., Rothstein, A., Semenza, G. and Tannenbaum, C. (1978) J. Membrane Biol. 40, 269–290
12 Malathi, P., Preiser, H. and Crane, R.K. (1980) Ann. N.Y. Acad. Sci. 358, 253–266
13 Lin, J.T., DaCruz, M.E.M., Riedel, S. and Kinne, R. (1981) Biochim. Biophys. Acta 649, 43–54
14 Koepsell, H., Menuhr, H., Wissmüller, T.F., Ducis, I. and Haase, W. (1980) Ann. N.Y. Acad. Sci. 358, 267–281
15 Arita, H. and Kawanami, J. (1980) J. Biochem. 88, 1399–1406
16 Thomas, L. (1973) Biochim. Biophys. Acta 291, 454–464
17 Smith, M.W., Ferguson, D.R. and Burton, K.A. (1975) Biochem. J. 147, 617–619
18 Lemaire, J. and Maestracci, D. (1978) Can. J. Physiol. Pharmacol. 56, 760–770
19 Poiree, J.C., Mengual, R. and Sudaka, P. (1979) Biochem. Biophys. Res. Commun. 90, 1387–1392
20 Biber, J. and Hauser, H. (1979) FEBS Lett. 108, 451–456
21 Klip, A., Grinstein, S., Biber, J. and Semenza, G. (1980) Biochim. Biophys. Acta 598, 100–114
22 Klip, A., Grinstein, S. and Semenza, G. (1979) J. Membrane Biol. 51, 47–73
23 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
24 Kessler, M. (1978) Dissertation 6209, ETH Zürich
25 Kessler, M., Tannenbaum, V. and Tannenbaum, C. (1978) Biochim. Biophys. Acta 509, 348–359
26 Zemplén, G., Csürös, Z., Gerecs, A. and Aczél, S. (1928) Ber Deut. Chem. Ges. 61, 2486–2497
27 Gulati, K.C., Seth, S.R. and Venkataraman, K. (1935) Org. Synth. 15, 70–71
28 Canter, F.W., Curd, F.H. and Robertson, A. (1931) J. Chem. Soc., 1245–1255
29 Diedrich, D.F. (1962) J. Med. Pharm. Chem. 5, 1054–1062
30 Still, W.C., Kahn, M. and Mitra, A. (1978) J. Org. Chem. 43, 2923–2925
31 Evans, J.O. and Diedrich, D.F. (1980) Arch. Biochem. Biophys. 199, 342–348
32 Hopfer, U. (1977) J. Supramol. Struct. 7, 1–13
33 Franklin, J.E., Luk, G. and Isselbacher, K.J. (1976) Clin. Res. 24, A284
34 Bode, F., Baumann, K. and Diedrich, D.F. (1972) Biochim. Biophys. Acta 290, 134–149
35 Chesney, R., Sacktor, B. and Kleinzeller, A. (1974) Biochim. Biophys. Acta 332, 263–277
36 Turner, R.J. and Silverman, M. (1981) J. Membrane Biol. 58, 43–55
37 Diedrich, D.F. (1963) Biochim. Biophys. Acta 71, 688–700
38 Diedrich, D.F. (1965) Am. J. Physiol. 209, 621–626
39 Newey, H., Sanford, P.A., Smyth, D.H. and Williams, A.H. (1963) J. Physiol. London 169, 229–236
40 Hosang, M., Vasella, A. and Semenza, G. (1981) Biochemistry 20, 5844–5854
41 Diedrich, D.F. (1966) Arch. Biochim. Biophys. 117, 248–256
42 Boyer, J.H., Canter, F.C., Hamer, J. and Putney, R.K. (1956) J. Am. Chem. Soc. 78, 325–327
43 Hosang, M., Gibbs, E.M., Diedrich, D.F. and Semanza, G. (1981) FEBS Lett. 130, 244–248