Specific Photoaffinity Inactivation of the D-Glucose Transporter in Small Intestinal Brush Border Membrane Using New Phlorizin Analogues†

Markus Hosang, Andrea Vasella,‡ and Giorgio Semenza*

ABSTRACT: We have synthesized a series of photolabile derivatives of phlorizin, 6-azido-6-deoxyphlorizin (azido-Phlz), 6-*O*-[*N*-(2-nitro-4-azidophenyl)-β-alanyl]phlorizin (NAP-β-Ala-Phlz), *N*-(2-nitro-4-azidophenyl)-6-amino-6-deoxyphlorizin (NAP-N-Phlz), and *N*-[*N*-(2-nitro-4-azidophenyl)-β-alanyl]-6-amino-6-deoxyphlorizinamide (NAP-β-Ala-N-Phlz), as photoaffinity labels of (the phlorizin binding site of) the Na^+,D-glucose cotransporter in small intestinal brush border membrane. Binding of the phlorizin derivatives in the dark was monitored by (i) their ability to competitively inhibit D-glucose uptake in rabbit intestinal brush border membrane vesicles in the presence of a NaSCN gradient ($K_i' \approx 10$, 48, 40, and 148 μM, respectively, K_i' of phlorizin being 8 μM) and (ii) their inhibition of specific [^{3}H]phlorizin binding to vesicles ($K_i'' \approx 12$, 60, 80, and 122 μM, respectively) and to deoxycholate (DOC) extracted membranes ($K_i''' \approx 9$, 31, and 79 μM, respectively). Repeated short time photolysis of NAP-β-Ala-Phlz with visible light under anaerobic conditions in the presence of membrane vesicles resulted in a specific (phlorizin protectable) reduction of $\Delta\tilde{\mu}_{Na^+}$-driven accumulative D-glucose uptake. When photolysis time was increased, a considerable nonspecific (phlorizin nonprotectable) decrease in D-glucose uptake was found in addition, concomitant to a nonspecific depression of Na^+,Δψ-dependent L-methionine uptake. D-Glucose tracer exchange was inactivated to a similar extent after repeated short time photolysis of vesicles in the presence of NAP-β-Ala-Phlz. In addition, Na^+-dependent binding of [^{3}H]phlorizin to DOC-extracted membranes was also specifically and irreversibly blocked under the same photolysis conditions. Since only phlorizin, but not its 4′ isomer (*p*-phlorizin; a very weak inhibitor of D-glucose uptake), protects from inactivation of D-glucose tracer exchange and phlorizin binding, we conclude that NAP-β-Ala-Phlz specifically inactivates the small intestinal Na^+,D-glucose transporter by binding covalently at the substrate binding site. Finally, the "in dark" reversible inhibition potencies of the various phlorizin derivatives provide some indirect information on the interaction of C-6 of the sugar moiety with the corresponding subsite at the surface of the transporter.

D-Glucose transport across the brush border of intestinal epithelial cells is Na^+ and Δψ dependent (Riklis & Quastel, 1958; Bihler & Crane, 1962; Crane, 1962, 1965; Schultz & Curran, 1970; Rose & Schultz, 1971; White & Armstrong, 1971) and is inhibited fully competitively by phlorizin, the β-D-glucopyranoside of phloretin (Alvarado & Crane, 1962). In vesicles prepared from brush border membranes, these characteristics are preserved (e.g., Hopfer et al., 1973; Murer & Hopfer, 1974; Tannenbaum et al., 1977).

Various attempts have been made to identify the phlorizin-sensitive, Na^+,Δψ-dependent D-glucose transporter by labeling with thiol reagents (Thomas, 1972; Smith et al., 1975; Lemaire & Maestracci, 1978), which, however, led to conflicting results. Other approaches perhaps show more promise, e.g., solubilization and reconstitution (Crane et al., 1976) or negative purification and selective thiol labeling (Klip et al., 1979a–c). In spite of these efforts, final identification of the Na^+,Δψ-dependent D-glucose transporter has not been achieved yet. It seemed pertinent, therefore, to pursue an additional, different approach, i.e., photoaffinity labeling. The criteria and advantages of this technique over conventional labeling have been discussed (Bayley & Knowles, 1977). Photoaffinity reagents have been used to probe many diverse biological systems (Bayley & Knowles, 1977; Chowdry & Westheimer, 1979); however, very few successful photoaffinity probing studies of (membrane embedded) transport systems have been reported, most of them using photolabile derivatives of the substrate. Among these systems are the glucose transporters in the plasma membrane of erythrocyte (Farley et al., 1976) and of adipocytes (Trosper & Levy, 1977), the β-galactoside transport system in *Escherichia coli* membrane vesicles (Rudnick et al., 1975a,b), and the dipeptide transporter in *E. coli* (Staros & Knowles, 1978). In addition, various photolabile D-glucose derivatives were described as potential photoaffinity reagents for sugar transport systems (Perry & Heung, 1971; Ramjeesingh & Kahlenberg, 1977; Hagedorn et al., 1978). A valuable alternative to the use of a transportable substrate analogue as photoaffinity probe of a transport protein is the use of a nontransportable, competitive inhibitor, e.g., arylazido atractylosides as labels for the mitochondrial adenine nucleotide carrier (Lauquin et al., 1976). Therefore, as a first step toward photoaffinity labeling of the Na^+,Δψ-dependent D-glucose transporter of small intestine, we have synthesized a number of potential photolabels starting from phlorizin (Scheme I). This glucoside is a fully competitive inhibitor and a ligand with a rather high affinity for the transporter, the K_i' and K_d values being in the range 4–10 μM in the presence of both optimal Na^+ and Δψ gradients across the membrane (Tannenbaum et al., 1977; Toggenburger et al., 1978).

For reasons which will be discussed later, we focused on the synthesis of derivatives at position 6 of the β-D-glucopyranosyl moiety, which is also more reactive toward acylation and alkylation than position 2, 3, or 4 (Cramer, 1963). The photosensitive group chosen was the 2-nitro-4-azidophenyl (NAP)[1] group. This photolabile group exhibits excellent

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‡ Present address: Department of Organic Chemistry of the University of Zürich, Zürich-Irchel, Switzerland.

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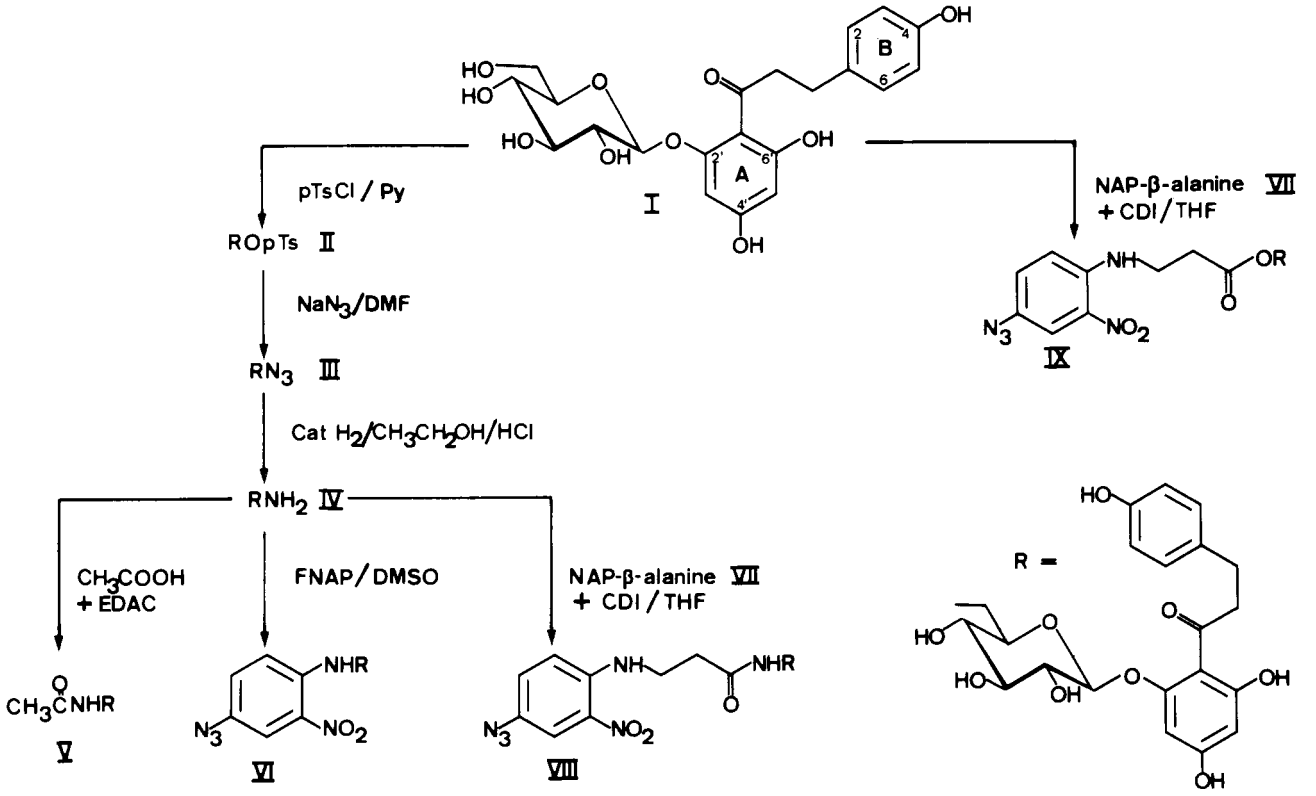
Scheme I



stability in the dark and can be photolyzed with light of $\lambda > 390$ nm, which is not likely to cause protein damage (Bayley & Knowles, 1977). Comparison of the inhibitory potencies of the phlorizin derivatives synthesized gave as some information as to their potential use as photolabels. In particular, 2-nitro-4-azidophenyl-β-alanyl-6-*O*-phlorizin ester specifically inactivates the Na^+,$\Delta\psi$-dependent D-glucose cotransporter in small intestinal brush border membrane vesicles. Some of our results have been presented in preliminary form (Hosang et al., 1980).

Experimental Procedures

Materials

D-[1-^{3}H]Glucose (5.2 Ci/mmol) and L-[*methyl*-^{3}H]-methionine (12 Ci/mmol) were purchased from Amersham Radiochemical Centre, Ltd., Bucks. [G-^{3}H]Phlorizin (lot no. 1109-267) was obtained from New England Nuclear (Boston, MA). Phlorizin dihydrate was from Fluka AG [Buchs (SG), Berne, Switzerland]. After treatment with charcoal, it was recrystallized from hot water. For synthetic purposes, it was recrystallized from hot dry ethyl acetate and dried at 10^{-3} torr in the presence of P_2O_5 for 72 h. Its authenticity was established by ^{1}H NMR. The spectrum (in acetone-d_6) showed a monohydrate and served as the standard for the interpretation of the spectra of the phlorizin derivatives. 1,1′-Carbonyldiimidazole (CDI) and β-alanine were obtained from Fluka AG, and *N*-ethyl-*N*′-[3-(dimethylamino)propyl]carbodiimide hydrochloride was obtained from Merck-Schuchardt (Munich, Germany). 4-Fluoro-3-nitrophenyl azide (FNAP) was prepared from 4-fluoro-3-nitroaniline by the method of Fleet et al. (1972). *p*-Phlorizin (phloretin 4′-glucoside) was a gift from Dr. D. Diedrich (Department of Pharmacology, University of Kentucky Medical School, Lexington, KY). All other chemicals were of highest quality available.

Thin-layer chromatography (TLC) was performed on silica gel plates or strips (Merck, F_{254}, 0.25-mm thickness) by using the following solvent systems: solvent A, methylene chloride–methanol (4:1 v/v); solvent B, 1-propanol–water–ethyl acetate (7:2:1 v/v). Visualization of the various compounds on TLC was carried out by using the following reagents: (1) 16 N sulfuric acid for sugars, (2) vanillin (1% in sulfuric acid, w/v) for phenols, and (3) ninhydrin spray for compounds containing free amino groups. Preparative layer chromatography was carried out on silica gel plates (Merck, F_{254}, 2-mm thickness); for column chromatography, silica gel (Merck, 60, 0.040–0.063 mm) was used routinely.

^{1}H NMR spectra (100 MHz) were recorded on a Varian HA-100 by using $(CH_3)_4Si$ ($\delta = 0.00$) as internal standard. Chemical shifts are given in parts per million (ppm). IR spectra were measured on a Perkin-Elmer 283 spectrometer and ATR–IR (Harrick, 1967) spectra on a Perkin-Elmer spectrograph 225, equipped with a Wilks 50 ATR mirror assembly. UV spectra were recorded on a Pye Unicam UV spectrometer (SP 1700) and optical rotations on a Perkin-Elmer polarimeter. Melting points are uncorrected.

Chemical Syntheses. The synthesis of the key compound 6-amino-6-deoxyphlorizin (IV) and of the NAP derivatives of phlorizin was accomplished as shown in Scheme I.

[1] Abbreviations used: NAP, 2-nitro-4-azidophenyl; β-Ala, β-alanine; Phlz, phlorizin; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; CDI, 1,1′-carbonyldiimidazole; DMF, dimethylformamide; Me_2SO (DMSO in figures), dimethyl sulfoxide; THF, tetrahydrofuran; pABA, 4-aminobenzoic acid; DOC, deoxycholate; DTE, 2,3-dithioerythritol; pCMBS, sodium *p*-(chloromercuri)benzenesulfonate; pTs, *p*-toluenesulfonyl; ATR, attenuated total reflection; IR, infrared; UV, ultraviolet; ^{1}H NMR, proton nuclear magnetic resonance; TLC, thin-layer chromatography; K_i', K_i'', and K_i''', inhibition constants of analogue as determined from D-glucose uptake, Na^+,$\Delta\psi$-dependent, D-glucose protectable phlorizin binding to vesicles, and Na^+-dependent phlorizin binding to DOC extracted membranes, respectively, and referred to the total (ionized + un-ionized) concentration of the analogue (Toggenburger et al., 1978); *p*-phlorizin, the 4′ isomer of phlorizin, i.e., the β-glucopyranoside of phloretin at position 4′ (rather than 2′) of the phloroglucitol ring (A).

Synthesis of 6-Amino-6-deoxyphlorizin. 6-O-(p-Toluenesulfonyl)phlorizin (pTs-Phlz, II). To a solution of phlorizin monohydate (I) (4.67 g, 10.7 mmol) in 195 mL of dry pyridine at 2 °C was added under stirring 4.09 g (20.3 mmol) of solid *p*-toluenesulfonyl chloride in small portions. After being stirred at room temperature in the dark for 20 h, the yellow solution was evaporated in vacuo to 40 mL and poured into 200 mL of ice-cold water containing 20 mL of 6 N HCl. The oil which separated was collected, washed with cold dilute HCl, and taken to dryness. Then it was applied to a silica gel column and chromatographed with methylene chloride–methanol, 6:1, as eluant. The fractions containing 6-*O*-tosylphlorizin (II) (R_f 0.39 on TLC, solvent A) were combined and evaporated to dryness. Unreacted phlorizin (I) was recovered from later fractions. The reaction was not brought to completion since the amount of ditosylated compounds increased strongly toward the end of the reaction. No attempts were made to further characterize the ditosylates and monosylated isomers, which all showed higher R_f values on TLC. The colorless product was redissolved in dry acetone, filtered, and dried in vacuo to a resin, which was pure on TLC but did not crystallize (3.43 g, 63% (based on starting material reacted)): IR (KBr) 3420 (br, OH), 1628 (C=O *o*-hydroxyaryl ketone), 1605, 1515 (aromatic), 1350, 1175 cm^{-1} (—SO_2O—); ^1H NMR (acetone-d_6) δ 2.31 [s, 3, CH_3(tosylate)] and 4.1–4.5 (AB system, 2, ν_A = 4.44, ν_B = 4.24, J_{AB} = 11 Hz, J_{AM} = 3 Hz, J_{BM} = 5.5 Hz, $CH_2(6)OSO_2$—); $[\alpha]^{25}_D$ −39.3° (EtOH, 1.74).

6-Azido-6-deoxyphlorizin (III). 6-*O*-(*p*-Toluenesulfonyl)-phlorizin (II) (1.95 g, 3.31 mmol) in 75 mL of freshly distilled DMF was stirred with sodium azide (0.645 g, 9.93 mmol) for 26 h at 80 °C in the dark. The brown reaction mixture was evaporated to dryness and redissolved in 30 mL of dry acetone with gentle heating. Insoluble salts were removed by filtration and washed with an additional 20 mL of acetone. The combined acetone fractions were taken to dryness and chromatographed on a column of silica gel, with methylene chloride–methanol, 5:1, as eluant. The fractions containing the product (TLC, R_f 0.36, solvent A) were pooled and taken to dryness. The resulting azide III, a pale yellow resin (1.16 g, 80.2%), was directly used in the next step; IR (KBr) 2105 cm^{-1} (s, azido group). The tosyl group signals at 1350 and 1175 cm^{-1} were missing. ^1H NMR (acetone-d_6) spectrum showed a shift of the signals at δ 4.1–4.5 ($CH_2(6)OSO_2$—) to 3.3–3.8 (m, 8, H—C(2,3,4,5), $CH_2(6)N_3$, CH_2CO); $[\alpha]^{25}_D$ −72.0° (2.08, ethanol).

6-Amino-6-deoxyphlorizin Hydochloride (IV). A solution of 6-azido-6-deoxyphlorizin (III) (732 mg, 1.59 mmol) in 8 mL of ethanol and 0.69 mL of 2 N HCl (1.75 mmol) was hydrogenated under atmospheric pressure at room temperature in the presence of 80 mg of 10% Pd/C catalyst for 3 h. After filtration through Celite and treatment with charcoal, the pale yellow solution was slowly cooled to 4 °C. 6-Amino-6-deoxyphlorizin hydrochloride (IV) crystallized as hygroscopic hairlike white needles. The mother liquor was purified by preparative LC using solvent A as eluant. A 654-mg sample of product IV was isolated (overall yield 91%). It showed only one spot on TLC (R_f 0.51, solvent B) and was ninhydrin positive: mp 135 °C (soften); mp 165–167 °C dec; IR (KBr) 3420, 3320 (br, OH, NH_3), 2920, 2700 (br, OH in intramolecular H bond), 1628 (C=O of *o*-hydroxyaryl ketone), no band at 2105 cm^{-1}; ^1H NMR (MeOH-D_6) δ 2.87 (t, 2, benzylic CH_2), 3.0–3.60 (m, 6, H—C(2, 3, 4, 5), CH_2CO), 3.60–3.90 (AB system, 2, $CH_2(6)NH_3^+$); $[\alpha]^{25}_D$ −32.7 ± 0.5° (1.71, ethanol).

6-Acetamido-6-deoxyphlorizin (V). For further characterization of amine IV, the acetamide V was prepared by applying the procedure for acylation of amino acids (Sheehan et al., 1965), using *N*-ethyl-*N'*-[3-(dimethylamino)propyl]-carbodiimide hydrochloride (EDAC) as coupling reagent and methanol–water (5:1 v/v) as solvent. The product (70% yield) was crystallized from ethanol–water as white fine fibers. It showed one spot on TLC (R_f 0.21, solvent A) and was ninhydrin negative; mp 228–230 °C dec: IR (KBr) 3440 (br, OH, NH), 3220, 3120 (sh, NH association), 1655 (sh, secondary amide association), 1570 cm^{-1} (sh, secondary amide association); ^1H NMR (acetone-d_6) δ 1.95 (s, 3, CH_3CON), 7.54 (br, 1, exchangeable with D_2O, NHCO); $[\alpha]^{25}_D$ −57.9° (1.85, ethanol). Anal. Calcd for $C_{23}H_{27}NO_{10}\cdot H_2O$: C, 55.76; H, 5.90; N, 2.82. Found: C, 55.54; H, 5.72; N, 2.65.

Synthesis of the NAP Derivatives. N-(4-Azido-2-nitrophenyl)-6-amino-6-deoxyphlorizin (VI). To a solution of the hydrochloride IV (94 mg, 0.2 mmol) and triethylamine (31 μL, 0.22 mmol) in Me_2SO (500 μL) was added 4-fluoro-3-nitrophenyl azide (FNAP, 110 mg, 0.6 mmol) as a solid. The mixture was heated under stirring at 52 °C for 24 h in the dark, affording a deep red solution which was lyophilized. The resulting red solid was dissolved in 2 mL of dry acetone. Precipitating salts were removed by filtration and washed with additional acetone. The combined filtrate and wash were evaporated in vacuo to a red resin. Chromatography on a preparative silica gel plate, using methylene chloride–methanol, 5:1, as eluant, and crystallization of the main fraction from ethanol–water gave 96 mg (80.3%) of VI as silky red crystals. The compound showed one spot on TLC (R_f 0.39, solvent A) and was ninhydrin negative; mp 130 °C dec; IR (KBr) 3380 (br, OH, NH), 2120 (s, aryl azide), 1570 (NH deformation), 1520, 1350 cm^{-1} (NO_2); UV_{max} (MeOH) 228 nm (ε 23 300), 268 (sh) (ε 29 000), 458 (ε 4750); ^1H NMR (acetone-d_6) δ 3.7–4.05 (AB system, 2, $H_2C(6)N$—), 7.00–7.25 [AB system, 2, H(5), H(6) (NAP)], 7.67 [d, 1, H(3) (NAP)], 8.35 (t, br, 1, exchangeable with D_2O, —NH—). Anal. Calcd for $C_{27}H_{27}N_5O_{11}\cdot H_2O$: C, 52.68; H, 4.75; N, 11.37. Found: C, 52.68; H, 4.60; N, 10.81.

N-(4-Azido-2-nitrophenyl)-β-alanine (VII) was synthesized by a modification of a previously published procedure (Jeng & Guillory, 1975), using Me_2SO/triethylamine instead of water–ethanol/sodium carbonate and stirring at 60 °C for 24 h in the dark. Crystallization in ethanol–water yielded VII as clusters of dark red crystals (77%) (TLC: R_f 0.43, solvent A): mp 138–140 °C dec (lit. mp 142.5–145 °C) (Jeng & Guillory, 1975); IR (KBr) 2120 (aryl azide), 1715 (COOH saturated), 1520, 1350 cm^{-1} (NO_2); ^1H NMR (acetone-d_6) δ 2.78 [t, 2, J = 7 Hz, H_2—C(2)], 3.74 (q, 2, J = 7 Hz, H_2—C(3)], 7.25 [m, 2, H(5), H(6) (NAP)], 7.75 [d, 1, H(3) (NAP)], and 8.20 (br, t, HN); UV_{max} (MeOH) 260 nm (ε 21 900), 280 (sh), 460 (ε 5300). Anal. Calcd for $C_9H_9N_5O_4$: C, 43.03; H, 3.61; N, 27.88. Found: C, 43.13; H, 3.74; N, 27.79.

N-[N-(4-Azido-2-nitrophenyl)-β-alanyl]-6-amino-6-deoxyphlorizinamide (VIII). To a solution of CDI (35 mg, 0.22 mmol) in dry THF (0.5 mL) was added NAP-β-alanine (VII) (55 mg, 0.22 mmol) as a solid. The reaction mixture was stirred for 15 min at 22 °C. Then, after the mixture was treated with triethylamine (21 μL, 0.22 mmol), 6-amino-6-deoxyphlorizin (hydrochloride) (95 mg, 0.20 mmol) in THF (0.8 mL) and DMF (0.2 mL) was added and allowed to react for 26 h at 50 °C in the dark. After evaporation in vacuo, the red residue was dissolved in dry acetone (3 mL) and filtered from precipitated salts. Then the solution was taken to dryness,

washed 3 times with chloroform (15 mL) to eliminate unreacted β-alanyl derivatives, and dried. Repetitive chromatography on a preparative silica gel plate (methylene chloride–methanol, 7:1; three runs) and crystallization from methanol–water gave pure VIII as soft red needles (82 mg, 60%) (TLC, R_f 0.37, solvent A): mp 125–130 °C dec; IR (KBr) 3380 (NH, OH), 2120 (arylazide), 1650 (sh, secondary amide), 1570 (amine), 1560 (sh, secondary amide), 1520, 1355 cm^{-1} (NO_2); UV_{max} (MeOH) 228 nm (ε 23 300), 268 (sh), 282 (ε 29 500), 458 (ε 4760). ^1H NMR assessed a mono-NAP-β-alanylamide, in position 6: δ 7.54 (br, 1, NHCO), 3.40–3.60 (m, 10, among other signals, $H_2C(6)N$]. Anal. Calcd for $C_{30}H_{32}N_6O_{12}·H_2O$: C, 52.48; H, 4.99; N, 12.24. Found: C, 52.92; H, 4.88; N, 12.01.

6-O-[N-(4-Azido-2-nitrophenyl)-β-alanyl]phlorizin (IX). IX was synthesized as described for the preparation of the amide (VIII). As decomposition occurred, the reaction was stopped before all phlorizin had reacted, as evidenced from TLC. The reaction mixture was purified by silica gel column chromatography, using a gradient of methylene chloride–methanol 12:1 to 4:1 as eluant. The fractions containing pure IX (R_f 0.44, solvent A; vanillin positive) were combined and taken to dryness. Mixed fractions were purified by repetitive TLC (three runs), using methylene chloride–methanol 7:1 as solvent. No attempts were made to further characterize the diesters and monoester isomers, which all showed higher R_f values on TLC. Crystallization of the red residue from ethanol–water gave 592 mg (80.5% relative to reacted starting material; 56.7% relative to total starting material) of cottonlike, red crystals: mp 88–90 °C (soften); 116–118 °C dec; IR (KBr) 3380 (br, OH), 2120 (aryl azide), 1730 (ester), 1570 (NH), 1520, 1350 cm^{-1} (NO_2); UV_{max} (MeOH) 228 nm (ε 23 300), 268 (sh), 282 (ε 29 500), 459 (ε 4900). ^1H NMR (acetone-d_6) showed a mono-NAP-β-alanyl ester of phlorizin in position 6 of the glucose moiety: δ 4.2–4.6 [AB system, 2, ν_A = 4.47, ν_B = 4.28, J_{AB} = 12 Hz, J_{AM} = 2.5 Hz, J_{BM} = 6.5 Hz, $COOCH_2(6)$]. Anal. Calcd for $C_{30}H_{31}N_5O_{13}·H_2O$: C, 52.40; H, 4.84; N, 10.19. Found: C, 52.60; H, 4.61; N, 9.93.

Methods

Preparation of Membranes. Brush border membrane vesicles from frozen small intestines were prepared daily by the calcium precipitation method of Schmitz et al. (1973), as modified by Kessler et al. (1978a). For some experiments, fresh or frozen scrapings of rabbit intestinal mucosa were used for the preparation, yielding vesicles which exhibited an enhanced stability of D-glucose transport activity (Y. Takesue, unpublished experiments). Final pellets were usually resuspended in 300 mM mannitol, 10 mM Hepes/Tris (pH 7.5), and 0.02% KN_3 and immediately used for transport, binding, or photolysis experiments. DOC extraction of membranes was carried out as described by Klip et al. (1979b), using 0.5 mg of DOC/mg of protein. DOC pellets were finally resuspended in 300 mM mannitol, 10 mM Tris-HCl (pH 7.0), 1 mM DTE, and 0.02% KN_3. DTE was omitted from membranes used for photolysis studies (see below).

Transport and Binding Measurements. Uptake of radioactive D-glucose and L-methionine in and binding of phlorizin to brush border membrane vesicles (untreated or photolyzed) was measured by the membrane filtration technique (Hopfer et al., 1973; Toggenburger et al., 1978), as automated by Kessler et al. (1978b). All measurements were carried out at room temperature (20–22 °C). Uptake (at 2 s) in vesicles in the presence of an initial, inwardly directed NaSCN gradient was initiated by the addition of vesicle buffer containing in addition (final concentrations) NaSCN (100 mM), D-[^3H]glucose or L-[^3H]methionine (100 μM), and inhibitors (NAP derivatives up to 150 μM and other analogues up to 1 mM). D-Glucose tracer exchange was performed as described (Klip et al., 1979c). Vesicles were preequilibrated with (final concentrations) D-glucose (100 μM) and either NaCl or KCl (100 mM for 30 min at room temperature. D-[^3H]-Glucose tracer exchange was initiated by mixing equal aliquots of preequilibrated vesicles and of the same buffer containing 1 μCi of the isotope. For solubility reasons, inhibitors were routinely added as aliquots from ethanolic stock solutions (stored at −20 °C) which were periodically checked for purity by TLC. Final concentration of ethanol was always 2%.

[G-^3H]Phlorizin binding to vesicles under NaSCN gradient conditions (at 2 s) in the presence of the inhibitors to be tested was assayed according to Toggenburger et al. (1978) whereas binding of [^3H]phlorizin to DOC-extracted membranes was determined essentially as described by Klip et al. (1979b). Specific binding in the latter case was defined as the difference between total (in the presence of 100 mM KCl) and unspecific binding (in the presence of 100 mM KCl) of [^3H]phlorizin (5 μM) measured after a 15-s incubation in 300 mM mannitol, 10 mM Tris-HCl (pH 7.0), and 0.02% KN_3. Specific binding activity represents the amount of Na^+-dependent bound [^3H]phlorizin divided by the protein content of the sample. Protein was determined according to Lowry et al. (1951).

Photolysis Experiments. Photolysis and photoinactivation studies were carried out in a water-jacketed circular quartz cell (5-mm path length, 2-mL volume) cooled at 20 °C by circulating water. A 350-W mercury short arc lamp (Illumination Industries Inc., Sunnyvale, CA) in a lamp house equipped with a collimating lense and a filter system [liquid filter cell (1 M $NaNO_2$, 15-mm thickness); UV cut-off filter, Balzers] to cut off light below 390 nm was used as the light source. The beam was focused on the photolysis cell (22-mm diameter) which was placed 20 cm from the light source. During irradiation, the sample was mixed and kept anaerobic with a stream of argon directed onto its surface.

A 100 μM solution of the corresponding NAP-glucoside in buffer was photolyzed in the absence and presence of membrane vesicles to monitor the photolysis rate of the NAP-group. At recorded time intervals, aliquots were removed, and their absorbances at 482 nm were recorded. The aliquots were then dried onto a germanium plate under vacuum, and ATR–IR (Harrick, 1967; Fringeli & Günthard, 1981) spectra were recorded.

Photoinactivation assays were usually carried out as follows: All membrane suspensions and media were deoxygenated prior to photolysis by bubbling argon through [~0.2 mL of argon mL^{-1} s^{-1}, according to Bayley & Knowles (1977)] for 30 min (see Results). Membrane vesicles were then preincubated with the appropriate ligands for 5 s in the dark, under the conditions used for reversible uptake inhibition studies (see above). In addition 10 mM pABA was included as a scavenger (Ruoho et al., 1973). This preincubation in the presence of an inwardly directed gradient of NaSCN (100 mM out, 0 in) allows maximum binding of all ligands to take place prior to photolysis, assuming that the NAP derivatives display the same biding kinetics as Na^+,Δψ-dependent phlorizin binding (Toggenburger et al., 1978; Toggenburger, 1979). DOC-extracted membranes were photolyzed at pH 7.0 either after mixing them with NaSCN (as above) or in the presence of 100 mM NaCl [after a 30-min preequilibration of the membranes with NaCl (Klip et al., 1979a)] after being preincubated for 15 s in the dark. DTE (0.75 mM) was omitted from the DOC membrane suspension because aryl azides are rapidly

reduced by thiols (Staros et al., 1978). After photolysis, samples were immediately diluted with 20 volumes of cold washing buffer [300 mM mannitol, 10 mM Hepes/Tris (pH 7.5) or Tris-HCl (pH 7.0), and 0.02% KN_3] to reduce post-photolytic reactions, with long-lived species originating from aryl nitrenes as observed (Mass et al., 1980). Washing by centrifugation and resuspension was repeated twice. In some experiments, free glucosides were efficiently separated by a Sephadex G-10 chromatography step (Evans & Diedrich, 1980) substituting for one washing cycle. The final pellets were resuspended in the appropriate buffers and immediately assayed for uptake or binding (as described above). Generally vesicles kept on ice for several hours showed a time-dependent loss in transport and binding activity and a concomitant reduction of their osmotic space (Y. and S. Takesue, unpublished experiments; see also Trüeb, 1978).

Results

(A) Synthesis. Direct condensation of NAP-β-alanine with phlorizin using CDI (Gottikh et al., 1970) as the coupling agent gave NAP-β-alanyl-6-*O*-phlorizin (IX) (80.5% yield). Its potential susceptibility to esterolytic activities present in the intestinal mucosa (Fernandez-Lopez et al., 1976; Nêgrel et al., 1976) led us to prepare the corresponding amide VIII via 6-amino-6-deoxyphlorizin (IV). The latter compound was synthesized in a three-step sequence. Direct sulfonylation (Cramer, 1963) of phlorizin gave the 6-*O*-tosylate II which was converted to the 6-azido sugar III. Subsequent catalytic hydrogenation led to the amino hydrochloride IV in an overall yield of 46%. By coupling IV and NAP-β-alanine with CDI as the coupling agent, the amide VIII was obtained. Acetylation of IV gave the corresponding acetamide V (Scheme I). Condensation of the amine IV with 4-fluoro-3-nitrophenyl azide (FNAP) yielded the short spaced NAP-aminophlorizin (VI).

(B) Interaction of Phlorizin Analogues with the Na^+-Dependent, D-Glucose Transporter in the Dark. Phlorizin is a fully competitive inhibitor of Na^+-dependent, D-glucose transport across the brush border membrane (Alvarado & Crane, 1962; Toggenburger et al., 1978). The interaction of phlorizin derivatives with the Na^+, D-glucose transporter was measured from (i) their inhibition of D-glucose uptake into brush border membrane vesicles and (ii) their inhibition of Na^+-dependent, D-glucose protectable phlorizin binding to membrane vesicles or to DOC-extracted membrane fragments.

(i) As we pointed out elsewhere, the vesicle preparation which we used (Kessler et al., 1978a) lends itself very well to the determination of K_i and K_d values also under conditions of a partially dissipating NaSCN gradient, if proper precautions are taken [for a detailed discussion, see Toggenburger et al. (1978)]. One of the conditions is that the initial velocities must be measured while the uptake is still linear with time, i.e., within the first 4 s of the incubation. Routinely, we determined overall kinetic constants from incubations lasting 2 s [see also Kessler et al. (1978b)].

The K_i' values of the various phlorizin derivatives, obtained from Dixon plots, are summarized in Table I. Typical Dixon plots for NAP-N-Phlz (VI) and for NAP-β-Ala-Phlz (IX) as inhibitors are shown in Figure 1. The K_i' of phlorizin (8 μM) agrees with the value of 7.8 ± 1.4 μM obtained by Tannenbaum et al. (1977) and Toggenburger et al. (1978) (and served as a reference).

(ii) The inhibition of Na^+-dependent phlorizin binding was measured by using the same brush border membrane vesicles (Kessler et al., 1978a) and also DOC-extracted membrane fragments, a preparation in which the specific phlorizin binding site(s) is (are) enriched 2-fold (Klip et al., 1979b). In each case, the inhibition by the phlorizin derivatives was fully competitive in nature. The inhibition constants (K_i'' and K_i''', respectively) are reported in Table I. They compare favorably with one another and with the corresponding K_i' values from

Table I: Reversible Inhibition of D-Glucose Uptake in and of Specific Phlorizin Binding to Brush Border Membrane Vesicles and DOC-Extracted Membranes by Phlorizin and Some 6-Substituted Analogues

	inhibition of		
		specific phlorizin binding[b] to	
compd	D-glucose uptake[a] (K_i') (μM)	vesicles (K_i'') (μM)	DOC-extracted membranes (K_i''') (μM)[c]
phlorizin (I)	8 ± 1.2 (4)	12 ± 2 (2)	9 ± 2 (2)
azido-Phlz (III)	10 ± 1.8 (3)	12 ± 2 (2)	ND
pTs-Phlz (II)	14 ± 2.5 (2)	ND	
NAP-β-Ala-Phlz (IX)	48 ± 5 (3)	60 ± 6 (3)	31 ± 4 (3)
NAP-N-Phlz (VI)	40 ± 7 (3)	80 ± 5 (3)	79 ± 10 (2)
NAP-β-Ala-N-Phlz (VIII)	148 ± 17 (2)	122 ± 10 (2)	ND
acetamido-Phlz (V)	315 ± 41 (3)	ND	ND
amino-Phlz (IV)	554 ± 20 (2)	ND	ND

[a] Uptake of D-glucose (at 2 s, 22 °C, pH 7.5) was performed as under Methods under an inwardly directed gradient of NaSCN (100 mM out, 0 in). K_i' values were determined from Dixon plots (Figure 1). Primed K_i values always refer to the total concentration (charged + uncharged) of the inhibitor. [b] Binding of [^{3}H]-phlorizin (5 μM) to membrane vesicles at 2 s was performed under the same conditions as D-glucose uptake. Specific binding was determined by subtracting unspecifically bound (25 mM D-glucose present) from total [^{3}H]phlorizin bound (25 mM D-mannitol present). Specific [^{3}H]phlorizin (5 μM) binding to DOC-extracted membranes (at 15 s, 22 °C, pH 7.0) was obtained by subtracting unspecifically bound (100 mM KCl) from total [^{3}H]phlorizin bound (100 mM NaCl). [c] Apparent inhibition constants of binding (K_i'' and K_i''') were calculated for fully competitive reversible inhibition of specific [^{3}H]phlorizin binding ($K_d \approx 5$ μM; Toggenburger et al., 1978) in the presence of 4–6 analogue concentrations. Data are represented as mean ± SD of (n) single plots or experiments; ND, not determined. For preparations and details, see Methods.

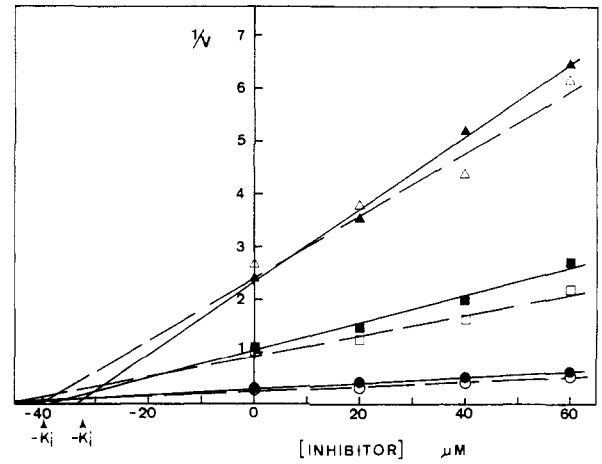


FIGURE 1: Dixon plots of NAP-6-amino-6-deoxyphlorizin (VI, filled symbols and solid lines) and NAP-β-Ala-Phlz (IX, open symbols and broken lines) inhibitions of D-glucose uptake into brush border membrane vesicles (pH 7.5, 100 mM to 0 inwardly directed initial NaSCN gradient, 25 °C). Incubation time, 2 s. D-Glucose concentrations were 10 (▲, △), 30 (■, □), and 150 μM (●, ○). The behavior is characteristic of fully competitive inhibition; the K_i' values were 34 ± 3 μM for compound VI and 39 ± 4 μM for compound IX (means ± SD). Single determinations were carried out in quadruplicate. The reciprocal uptake velocity is expressed in arbitrary units.

inhibition of D-glucose uptake into brush border membrane vesicles.

We conclude, therefore, that phlorizin and these derivatives bind to the same site(s), i.e., to (a part of) the Na^+, D-glucose transporter. The photolabile derivatives can thus be used as potential photoaffinity labels of the transporter.

(*C*) *Photoreactivity of the Compounds.* The visible spectrum of the NAP derivatives in aqueous solution exhibited a λ_{max} at 482 nm, corresponding to the absorption of the nitrophenyl group. The decrease in absorbance of this band with time of irradiation with visible light was taken as a measure of the photolysis rate of the NAP compounds. A decomposition half-time of 90 s was obtained for NAP-β-Ala-Phlz in buffer. A similar result was obtained when decomposition was monitored by recording the disappearance of the 2120 cm^{-1} band in ATR-IR (Harrick, 1967; Fringeli & Günthard, 1981). Interestingly, the decomposition rates was significantly increased when photolysis was carried out in the presence of membrane vesicles under otherwise identical conditions. Depending on the vesicles' concentration, the sensitivity of NAP-β-Ala-Phlz to irradiation was increased up to 40-fold (at 2 mg of protein/mL), as evidenced from the decrease of half-times. A similar effect was seen when photolysis was carried out in the presence of liposomes of either lecithin or asolectin (2 mg of lipid/mL), the half-time being then about 3 s. The reason for this finding was not investigated. Probably binding of the NAP label to the lipid core of the vesicle membrane led to their enhanced sensitivity to photolysis and similarly to the increase in sensitivity found with other aryl azido ligands when bound to their corresponding membrane receptors (Witzemann & Raftery, 1977). Since the increase in photolysis rate was observed by protein-free lipsomes, it is unlikely that sensitization of the labels was due to the chromophores known to be present in the brush border membranes (Bruder et al., 1980).

(*D*) *Photoinactivation of the Na^+-Dependent D-Glucose Transporter by NAP Derivatives of Phlorizin.* Three types of assays were used: (a) D-glucose uptake into brush border vesicles in the presence of a NaSCN gradient (out → in), (b) D-glucose tracer exchange at $\Delta\tilde{\mu}_{Na^+} \simeq 0$, and (c) phlorizin binding to DOC-extracted membranes. Each of the three assays has pros and cons and requires appropriate, "tailor-made" controls.

(*a*) *Photoinactivation of D-Glucose Uptake in the Presence of an Initial NaSCN Gradient.* $\Delta\tilde{\mu}_{Na^+}$-driven D-glucose uptake in vesicles (e.g., its initial velocity or the height of the "overshoot" peak) could be affected by at least three mechanisms or a combination thereof: (i) inactivation of the Na^+, D-glucose cotransporter, (ii) collapse of $\Delta\tilde{\mu}_{Na^+}$, and (iii) leakiness of the vesicles. Some considerations allow one to distinguish among these possible mechanisms: (i) Inactivation of the transporter should be delayed by the presence of competitive inhibitor(s) or substrate(s) ("specific protection"); (ii) a collpase of $\Delta\tilde{\mu}_{Na^+}$ should lead to a parallel decrease in other $\Delta\tilde{\mu}_{Na^+}$-dependent systems; (iii) leakiness of the vesicles should show up both in an apparent decrease of other transport systems and in a reduction of the osmotic space (equilibrium uptake). With appropriate controls ruling out other mechanisms, D-glucose uptake in the presence of the combined $\Delta Na^+, \Delta\psi$ gradients can thus be made into a specific test for the photoinactivation of the Na^+, D-glucose transporter. Its major advantage is that of being the most sensitive of the tests available.

It was soon found that irradiation alone (i.e., in the absence of phlorizin derivatives) could lead to severe reduction of

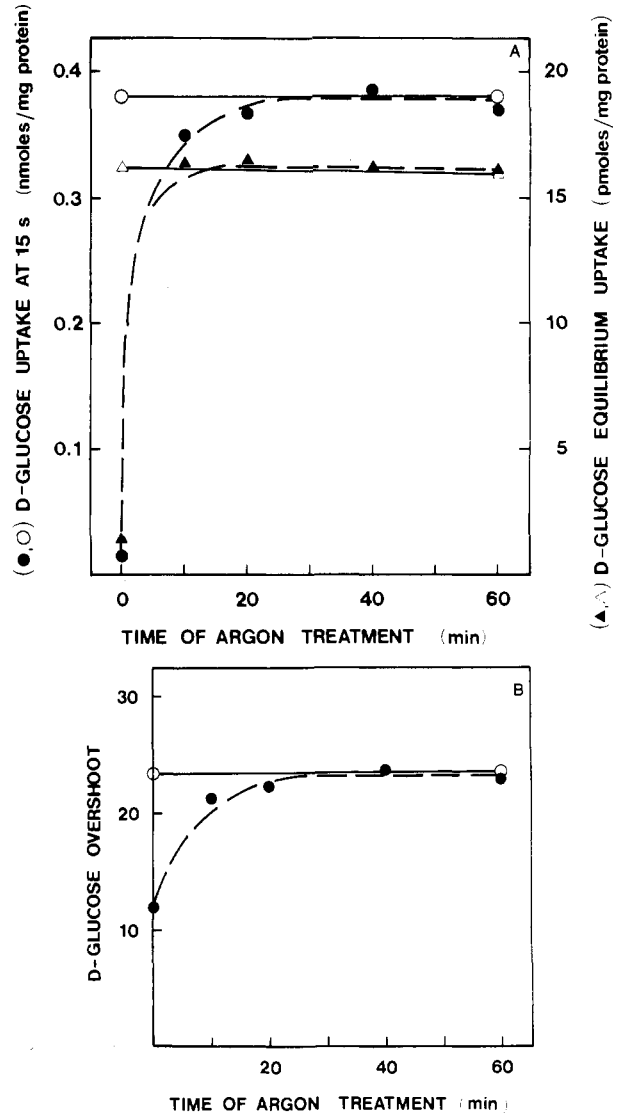


FIGURE 2: Effect of argon pretreatment on D-glucose uptake in brush border membrane vesicles illuminated by visible light. (A) Uptake of D-glucose at 15 s (●) or 90 min (▲) in brush border membrane vesicles which were (solid lines) or were not (dashed lines) photolyzed (for 15 min, 22 °C) after treatment with argon for the times indicated. (B) Overshoot of D-glucose uptake of vesicles in (A), expressed as ratio of uptake at 15 s (overshoot peak)/uptake at 90 min (equilibrium). For details, see Methods.

D-glucose uptake. As shown in Figure 2, D-glucose uptake at 15 s (overshoot peak) and equilibrium uptake (after 90 min) were both strongly depressed when vesicles were illuminated under aerobic conditions for 5 min (a time sufficient for complete photolysis). This general depression of uptake could be completely prevented by gassing membrane suspensions and media prior to photolysis with argon [as described by Bayley & Knowles (1977)] for at least 30 min (Figure 2). Therefore, a 30-min pretreatment was routinely carried out in all subsequent experiments. The use of antioxidants (e.g., (+)-catechin, 50 μM, pyrogallol 50 μM, or 2-hydroxyestradiol, 20 μM), instead or argon, was only partially successful. It is noteworthy that phlorizin (1 mM) also brought about some "protection", probably due to its pyrogallol-like nature or absorbancy of light (data not shown).

The inactivation of D-glucose overshoot produced by irradiation alone in the presence of oxygen is probably due to photooxidative damage to (a component of) the membrane leading to a collapse of $\Delta\tilde{\mu}_{Na^+}$. In fact, (1) Na^+-dependent, L-methionine uptake in these vesicles was similarly affected,

Table II: Effect of Short-Time Photolysis with NAP-β-Ala-Phlz on the Uptake Overshoot of D-Glucose and L-Methionine[a]

concn during photolysis (μM)		remaining overshoot[b] (%) of	
NAP-β-Ala-Phlz	Phlz	D-glucose ($n = 3$)	L-methionine ($n = 3$)
none, photolyzed	none	100 ± 4	100 ± 6
75, photolyzed	none	77 ± 7	ND
150, photolyzed	none	74 ± 8	100 ± 2
150, photolyzed	200	100 ± 3	94 ± 3

[a] Argon pretreated brush border membrane vesicles (1–2 mg of protein/mL) were preincubated with the ligands indicated for 5 s in the dark (22 °C, pH 7.5). After 15 s of photoirradiation, membranes were washed twice in Na^+-free buffer. After a second cycle of preincubation, photolysis, and washing, aliquots from the same membrane suspension were used for D-glucose and L-methionine uptake, respectively, under an NaSCN gradient as described under Methods. [b] Refers to the overshoot [expressed as the ratio of the substrate taken up at 15 s (D-glucose) or at 5 s (L-methionine) to the uptake at equilibrium (see also Figures 2 and 3)]. It is expressed as the percent of the illuminated membrane controls. The overshoot values of illuminated and non illuminated controls were not statistically different. Similar results were obtained when the overshoot values were expressed as picomoles of substrate taken up per milligram of protein. The figures are the means ± SD of (n) experiments. ND, not determined.

and (2) significantly increased Na^+ fluxes (at 15 s) were measured after photolysis under aerobic conditions, as compared to argon pretreated photolysates and to nonilluminated controls (data not shown). The decrease in equilibrium uptake might reflect increased loss by efflux during washing (on the filter) or reduction of internal vesicular space, due to general photoinduced leakiness of membranes to larger molecules, e.g., D-glucose, complete rupture of vesicles, or a combination of various mechanisms. In addition, photooxidation of (components of) the transporter may also occur, as reported for photoinactivation of amino acid and sugar transport in *E. coli* by near-UV and visible light (Koch et al., 1976; Sprott et al., 1976). Routinely, photooxidative damage (of the membrane and of the transporter) was thus kept within acceptable limits by pretreatment with argon.

Photoinactivation of the Na^+, D-glucose transporter with NAP derivatives of phlorizin was carried out in two cycles; i.e., after a first irradiation, the vesicles were washed (partially) free of photoproducts by centrifugation, regassed with argon, and irradiated again. This improved the efficiency. As shown in Table II, a concentration-dependent irreversible inactivation of overshoot of D-glucose was observed after photolyzing twice for 15 s (~6 times the $t_{1/2}$; see Results, section C) in the presence of NAP-β-Ala-Phlz, 23% (75 μM) and 26% (150 μM), with 150 μM being the solubility limit of this label.

This reduction in D-glucose overshoot was clearly due to a partial specific inactivation of the Na^+, D-glucose transporter since (Table II) 200 μM phlorizin fully protected from the inactivation by NAP-β-Ala-Phlz and since L-methionine uptake was not affected (L-methionine uptake is also Na^+ dependent but, contrary to D-glucose uptake, is unaffected, in the dark also, by either phlorizin or NAP-β-Ala-Phlz). Finally, NAP-β-Ala, i.e., an analogue lacking the site-directing phlorizin moiety, failed to produce any photoinactivation of D-glucose uptake (Figure 3A).

Changing the pH of photoinactivation from 7.5 to 6.5 did not result in any increase in specific inactivation. [It is the undissociated form of phlorizin, a weak acid with $pK_a' \approx 7.4$, which binds to the transporter (Toggenburger et al., 1978).] Also, attempts to photoinactivate the Na^+, D-glucose transporter by the use of NAP-*N*-phlorizin (an analogue with a K_i' value similar to that of NAP-β-Ala-phlorizin; see Table I) met

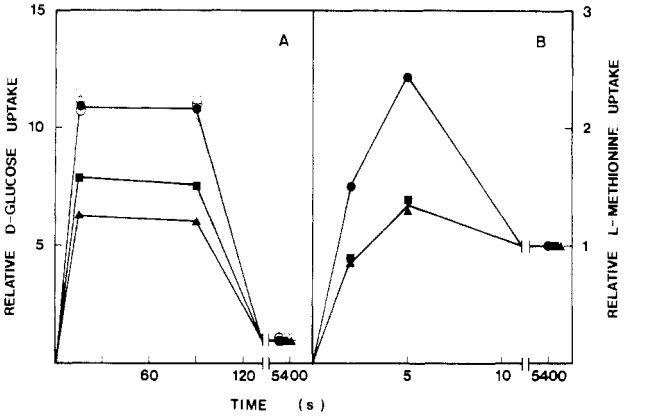


FIGURE 3: Effect of long-time photolysis with NAP-β-Ala-Phlz on the uptake overshoot of D-glucose (A) and L-methionine (B). (A) Photoinactivation of D-glucose uptake by long-time photolysis in the presence of NAP-β-Ala-Phlz (150 μM) was performed in two cycles of preincubation, photolysis, and washing, as described in the legend to Table II and under Methods; one photolysis lasted 5 min. D-Glucose overshoot (see Figure 2B) of vesicles illuminated in the absence (●) or in the presence of 150 μM NAP-β-Ala-Phlz (▲) or NAP-β-Ala-Phlz plus 250 μM phlorizin (■) was assayed; vesicles irradiated in the presence of 150 μM NAP-β-Alanine (○) and vesicles irradiated in the presence of 250 μM phlorizin (□). NAP-β-Ala-Phlz was photolyzed prior to incubation with the (photolyzed) vesicles (△). (B) Vesicles were illuminated in the presence of NAP-β-Ala-Phlz as described in (A) and assayed for L-methionine uptake at the times indicated; L-methionine uptake in vesicles illuminated in the absence (●) or illuminated in the presence of 150 μM NAP-β-Ala-Phlz (▲) or NAP-β-Ala-Phlz plus 250 μM phlorizin (■). For details, see Methods.

with little success, the specific inactivation being somewhat less than that with NAP-β-Ala-Phlz (data not shown).

Increasing the illumination time from 15 s to 5 min per cycle in the presence of NAP-β-Ala-Phlz led to a strong reduction of the uptake overshoots of *both* L-methionine *and* D-glucose (Figure 3). Phlorizin (200 μM) failed to protect the former and only slightly protected the latter. Thus, under these conditions, much of the reduction of D-glucose transport into the vesicles was due to mechanism(s) other than the inactivation of the Na^+, D-glucose transporter per se.

When vesicles and photolabel were illuminated separately and mixed immediately thereafter, no inactivation of D-glucose uptake was observed. This "prephotolysis control" ruled out long-lived intermediates generated in the bulk phase as being responsible for either the transporter-specific or the unspecific inactivation of D-glucose uptake. Therefore, the conditions described in Table II were used in all subsequent experiments, unless indicated otherwise.

(*b*) *Inactivation of* D-*Glucose Tracer Exchange.* The tracer exchange assay is a less sensitive but more reliable alternative for monitoring the inactivation of the transporter by site-specific photoinactivation. In fact, since functioning of the transporter in this case is assayed in the absence of $\Delta\tilde{\mu}_{Na^+}$, apparent inactivation as a consequence of a collapse if $\Delta\tilde{\mu}_{Na^+}$ is eliminated. In addition, for differentiation with even greater security between true site-specific protection and nonspecific protection by phlorizin (as light absorbent or scavenger), *p*-phlorizin, the 4′ isomer of phlorizin, was used as a control: due to its low inhibitory potency on D-glucose uptake (K_i' = 420 μM), this glucoside can be assumed to bring about only marginal site-specific protection at 200 μM but to otherwise display the general properties of phlorizin responsible for possible unspecific protection. The signficant difference ($P < 0.001$) in D-glucose tracer exchange activity found after D-glucose was photolyzed with NAP-β-Ala-Phlz in the presence of phlorizin (site-directed protection) as compared to that

Table III: Photoinactivation by NAP-β-Ala-Phlz of D-Glucose Tracer Exchange into Brush Border Membrane Vesicles and of Phlorizin Binding to DOC-Extracted Membranes[a]

photolysis conditions		remaining activity[b] (%) of		
			specific phlorizin binding after m cycles	
NAP-β-Ala-Phlz (μM)	protector (μM)	D-glucose tracer exchange	(m = 1, n = 6)	(m = 2, n = 2)
none, photolyzed	250 Phlz	100	100	100
100, photolyzed	250 p-Phlz	87 ± 4	88 ± 4	85
100, photolyzed	250 Phlz	107 ± 3	102 ± 3	106

[a] Native and DOC-extracted brush border membrane vesicles were illuminated once or twice as described in Table II and under Methods, with minor modifications. DOC-extracted vesicles were preincubated for 15 s in the dark; a single photolysis lasted 25 s. [b] Refers to the activity of membranes photolyzed in the presence of 250 μM Phlz (light control) as 100%. Activities of nonilluminated controls were not statistically different. Data are represented as the mean ± SD of (n) independent experiments; m indicates the number of photolysis cycles (for details, see Methods).

in the presence of p-phlorizin (unspecific protection), as shown in Table III, corresponded again to (at least) 20% true site-specific inactivation. The 7% higher exchange activity measured after irradiation in the presence of both label and protector as compared to irradiation in the presence of protector alone clearly demonstrated the necessity of correcting for unspecific protection by light absorption and scavenging by the label as a chromophore and phenol.

(*c*) *Inactivation of Phlorizin Binding to DOC-Extracted Membrane.* This preparation displays a 2–3-fold enrichment in Na^+-dependent phlorizin binding activity, as compared to intact vesicles (Klip et al., 1979b), but does not retain measurable D-glucose transport activity due to the irreversible disruption of membranes. As evidenced from Table III, Na^+-dependent phlorizin binding to DOC pellets was inactivated by photolysis with NAP-β-Ala-Phlz as the label. Again, specificity of inactivation was established by comparing site-directed protection by phlorizin (250 μM) with unspecific protection by its 4′ isomer p-phlorizin (250 μM); 14% specific inactivation of specific phlorizin binding was observed after one labeling cycle and 21% after two cycles ($P < 0.001$ in both cases). This is in good agreement with the specific inactivation of D-glucose tracer exchange (20%) obtained after two labeling cycles (previous paragraph).

Discussion

Choice and Synthesis of the Ligands. Phlorizin is the ligand with the highest (or second highest, see below) affinity for the Na^+, D-glucose transporter of intestinal brush border membranes. Its K_i' and K_d values (as measured from its inhibition of Na^+, $\Delta\psi$-dependent, D-glucose transport or from Na^+,$\Delta\psi$-dependent, D-glucose protectable binding, respectively) are in the range 4–10 μM (in the presence of an initial NaSCN gradient; Tannenbaum et al., 1977; Toggenburger et al., 1978). For comparison, the K_m for D-glucose uptake under identical conditions is approximately 100 μM. Thus, in designing potential (photo)affinity labels for this D-glucose transporter, it seemed appropriate to use phlorizin as the starting material (even if, of course, K_m and K_i or K_d do not have the same kinetic meaning).

In principle, the (photo)reactive group could be introduced into the aglycon or into the glucopyranosyl moiety of phlorizin or into both. Diedrich (1966) has investigated a number of phlorizin analogues of which the aglycon moiety was modified. With the single exception of 4′-deoxyphlorizin, which was some 50–70% more effective than the parent compound, no derivative was better inhibitor than phlorizin itself. We decided, therefore, to modify the glucopyranosyl moiety, the more so since other β-D-glucopyranosides (Alvarado & Crane, 1964) are transported by the same agency as D-glucose. Thus, it seemed logical to assume that the glucopyranosyl moiety of phlorizin interacts with the D-glucose binding site of the small-intestinal Na^+,$\Delta\psi$-dependent transporter and that it does so in a manner analogous to that of D-glucose itself.

Of the possible positions on the β-D-glucopyranosyl moiety of phlorizin, C-6 was chosen for attachment of the reactive groups, because this position is not critical or strictly necessary for free monosaccharides to interact with the transporter [e.g., 6-deoxy-D-glucose is an excellent substrate (Bihler & Crane, 1962; Honegger & Semenza, 1973), and D-xylose *is* transported, albeit with a much larger K_m value than D-glucose (Csáky & Lassen, 1964; Csáky & Ho, 1966; Alvarado, 1966)] and because the sugar binding site of the transporter may tolerate substituents in this position of the monosaccharide. The 6-deoxyfluoro derivatives of D-glucose and D-galactose are transported[2] (Wilson & Landau, 1960; Barnett et al., 1968). The minimum structural requirements for intestinal Na^+-dependent monosaccharide transport have been discussed by Crane (1960).

We have therefore synthesized photoactivable analogues of phlorizin by direct attachment of either the photoreactive NAP group to the C-6 position of the glucopyranosyl moiety of 6-aminodeoxyphlorizin or β-alanine as spacer to the same position of (i) phlorizin and (ii) 6-amino-6-deoxyphlorizin. Variation in distance of the NAP group from C-6 can be anticipated to add further possibilities of covalent reaction within or near to the substrate binding site region. We have chosen the NAP group as the photoreactive group, because aryl azides have proved very useful as the photoreactive moiety in a variety of photoaffinity probes (Bayley & Knowles, 1977). Aryl azides are unreactive in the dark; however, upon photolysis they generate aryl nitrenes, which may form covalent adducts with their targets by insertion reactions (Smith, 1970) or may react with nucleophilic residues [Chapman & Le Roux, 1978; reviewed by Staros (1980)], probably via long-lived intermediates.

At least one reaction, however, is worth mentioning. The imidazolide method [Gottikh et al., 1970; as modified by Jeng & Guillory (1975)] used for direct coupling has great potential use in that it may be applied for the synthesis of a variety of phlorizin derivatives; e.g., in addition to our photolabels, spin labels and fluorescent adjuncts may also be prepared in the same way.

To the best of our knowledge, only three other phlorizin derivatives have been described in addition to compounds II–IX (Scheme I) in which the glucopyranosyl moiety of phlorizin had been modified: the β-galactopyranosyl analogue, the 3-methoxide (Diedrich, 1963, 1966), and a 4-azido-2-nitrobenzoate derivative of phlorizin (Kaplan & Fasold, 1976), in which, however, the location of the photoreactive group was not precisely known.

Reversible Inhibition of D-Glucose Transport and Phlorizin Binding. In order for a reagent to be a photoaffinity inhibitor of a transporter, it should at least (i) reversibly inhibit its function in the unphotolyzed state and (ii) lead to irreversible

[2] The corresponding deoxyiodo and *O*-methyl derivatives are not transported. However, these derivatives most probably do bind to the carrier and act as competitive inhibitors, in analogy to what we have observed with the derivatives of phlorizin at position 6 (see Table I).

inhibition of the transporter upon photolysis. Further, the natural ligand for the receptor should protect against photoinactivation by the photolabile ligand.

The data in Table I indicate that the potential (photo)affinity ligands which we have prepared from phlorizin indeed satsify the "in dark requirements". They are reversible, fully competitive inhibitors of the Na^+,$\Delta\psi$-dependent D-glucose uptake, and they compete with phlorizin for Na^+,$\Delta\psi$-dependent phlorizin binding to brush border vesicles and to DOC-extracted membranes. Their K_i' values for transport and their K_i'' and K_i''' values for phlorizin binding increase in the following order: azido-Phlz (III) < NAP-β-Ala-Phlz (IX) ≤ NAP-N-Phlz (VI) < NAP-β-Ala-N-Phlz (VIII). Unfortunately, azido-Phlz (III), in spite of its excellent inhibitory potency ($K_i' \sim 10\ \mu M$), could not be further considered, because the light necessary to activate aliphatic azides ($\lambda \sim 253$ nm; Chakrabarti & Khorana, 1975) is likely to cause photochemical damage of proteins.

Independently of their potential use as photoaffinity labels, the phlorizin derivatives substituted at position C-6 of the glucopyranosyl moiety allow some inference to be made on the possible interaction of this position with the corresponding subsite of the Na^+, D-glucose transporter.

Barnett et al. (1968) have suggested, from the uptakes of 6-deoxy-D-galactose and various 6-deoxy-6-halo-D-galactose derivatives, that a hydrogen bond may be formed between the atom(s) bound to C-6 of the sugar (acting as the acceptor) and a corresponding group at the surface of the transporter (acting as the donor). If we now consider the K_i values of the phlorizin derivatives in Table I, we find indeed some indication for such a hydrogen bond.

In fact, the ability to accept an H bond should decrease in the series arylamide > amide > ammonium group, and the affinities of compounds VI (VIII and V) and IV decrease in this very order (Table I; compound IV is likely to be protonated at the pH at which phlorizin binding was measured). Likewise, the ability to accept a hydrogen bond should be larger for the ester oxygen than for the amide nitrogen, and compound IX has a larger affinity for the transporter than the corresponding amide (compound VIII). Of all compounds in Table I, the range of affinities corresponds to a difference in binding energy of about 2.4 kcal/mol (between compounds I and IV), which would be in keeping with a hydrogen bond being involved. The formation of an H bond between C-6 of the sugar and the Na^+, D-glucose transporter is thus fairly likely.[3]

Photolysis Conditions. The conditions for photolysis have been carefully optimized. Particularly the following conditions had to be considered:

(i) High-affinity, Na^+,$\Delta\psi$-dependent, D-glucose protectable phlorizin binding goes through a maximum at about 2–5-s contact time (Toggenburger et al., 1978; Toggenbnurger, 1979). Since the K_i' and K_i'' values of the best NAP-phlorizin derivatives are in the same order of magnitude as the K_i' and K_d values of phlorizin itself, the kinetics of binding of these derivatives to the brush border membranes can be expected to follow a similar time course. In addition, the specific binding of these lipophilic phlorizin derivatives to the D-glucose transporter is likely to be masked even more than in the case of phlorizin by a slow, nonspecific uptake into the lipid phase of the membrane. Therefore, short contact and short irradiation times are mandatory for photolabeling with these NAP-phlorizin derivatives.

(ii) The half-times of photodecomposition of the aryl azido derivatives were reduced severalfold, depending on the vesicle concentration, by the presence of natural or artificial membranes.

(iii) Also illumination per se, i.e., in the absence of photolabels, produced nonspecific photooxidative damage of the vesicles, leading to a severe reduction of D-glucose uptake. Only pregassing the vesicle suspension with argon was found to be really effective in preventing this. The mechanism and nature of the photooxidative damage were not investigated in detail. One factor was certainly the faster dissipation of $\Delta\tilde{\mu}_{Na^+}$, presumably due to increased Na^+ permeability, a phenomenon also observed after treatment of the vesicles with low concentrations of pCMBS or Cu (*o*-phenanthroline)$_2$ (Klip et al., 1979c; Biber & Hauser, 1979; Klip et al., 1980; Will & Hopfer, 1979). Chromophores known to be presnt in the brush border membrane (Bruder et al., 1980) may possibly act as sensitizers.

Photoinactivation of the Na^+, D-Glucose Transporter. Of all compounds investigated (Table I), NAP-β-Ala-Phlz (IX), due to its small K_i values in the dark, showed the most promise. Indeed, under carefully chosen photolysis conditions, it produced specific inactivation of the Na^+, D-glucose transporter. The three major criteria met for establishing the photoinactivation of the transporter, as opposed to nonspecific (apparent) inactivation of D-glucose transport or of phlorizin binding, were (i) complete protection from photoinactivation by phlorizin, (ii) little or no inactivation of another Na^+-dependent transport system present in the same membranes (that of L-methionine), and (iii) lack of protection by *p*-phlorizin as opposed to the protection by phlorizin. The last criterion is particularly important since the two compounds are very similar in having essentialy identical UV spectra at the pH of photolysis and nearly identical chemical properties as phenols. The important difference, for the purpose of the present work, was the very poor affinity of *p*-phlorizin for the Na^+, D-glucose transport system ($K_i' \approx 420\ \mu M$) (Tables II and III). Thus, the protection afforded by phlorizin, but not by *p*-phlorizin, is not due to phlorizin absorbing the light or acting as a scavenger; it must truly reflect the competition of phlorizin and NAP-β-Ala-Phlz for the same binding site, i.e., for the Na^+, D-glucose transporter. Note that, as expected, essentially the same conclusions were reacted no matter whether D-glucose uptake under NaSCN gradient, D-glucose equilibrium exchange (in vesicles), or phlorizin binding (to DOC-extracted membrane fragments) was the parameter eventually measured.

It seems safe to conclude, therefore, that NAP-β-Ala-Phlz photoinactivates small-intestinal Na^+, D-glucose transporter specifically and is thus most likely to be a suitable photoaffinity label.

Added in Proof

Attempts at Labeling the Na^+,D-Glucose Cotransporter Using (^3H)-NAP-β-Ala-Phlz. The results presented and discussed above showed that photolysis in the presence of NAP-β-Ala-Phlz specifically inactivated the small-intestinal Na^+,D-glucose cotransporter. We thus investigated also whether under conditions yielding optimum inactivation of the transporter one or more bands in the $NaDodSO_4$ pattern was (were) labeled specifically. No obvious specific labeling was found, however (Figure 4). In fact: (i) the labeling pattern of DOC-extracted membranes in the absence of phlorizin (lane 4) was qualitatively similar to that obtained in the presence of phlorizin (lane 6) or of *p*-phlorizin (lane 7). Thus, phlorizin

[3] Formation of hydrogen bonds in which an azido (Boyer et al., 1956) or a *p*-toluenesulfonyloxy (Fischli et al., 1976) group acts as the acceptor has been reported. It is not known, however, how good acceptors they are, as compared with the other atoms or groups at the C-6 position of the derivatives in Table I.

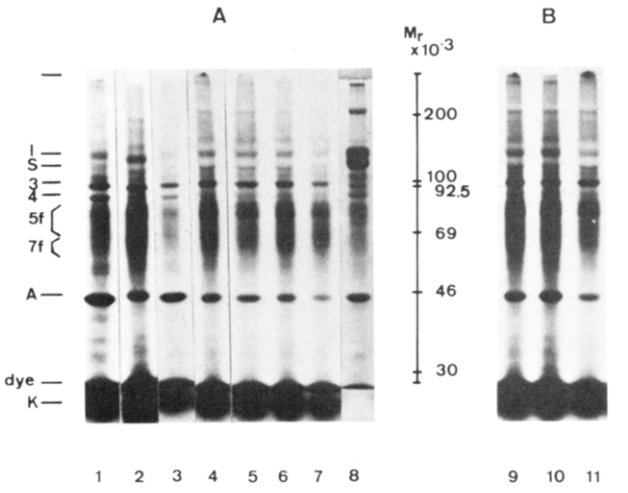


FIGURE 4: $NaDodSO_4$–polyacrylamide gel electrophoresis patterns of DOC-extracted membranes photolyzed in the presence of (^{3}H)-NAP-β-Ala-Phlz. (Lane 8 is a Coomassie blue stain of DOC-extracted membranes; all other lanes are fluorograms.) (A) Either intact brush border membrane vesicles or DOC-extracted membranes (1.4 mg of protein mL^{-1}) were photolyzed in the presence of 100 μM (lanes 1 and 2) or 50 μM (lanes 3 through 7) (^{3}H)-NAP-β-Ala-Phlz, for 25 s as described under Methods; 40 μg of sample protein was subjected to electrophoresis (8.4 × 2.7). Lanes 1 and 3 were obtained from vesicles; all other lanes were from DOC-extracted membranes. Lane 5: as in lane 4, but with 100 mM choline SNC, instead of NaSNC. Lane 6: as in lane 4, but in the presence of added 0.5 mM phlorizin. Lane 7: as in lane 4, but in the presence of added 0.5 mM *p*-phlorizin. Approximately 500 000 cpm was applied onto lanes 1 and 2; 220 000–250 000 cpm onto lanes 4 through 7. Fluorography [according to Bonner & Laskey (1974)] lasted 72 h. The numbering of the bands (at left) follows Klip et al. (1979b). (B) Effect of water-soluble scavangers on the labeling pattern. DOC-extracted membranes were photolyzed as in (A) in the presence of 50 μM (^{3}H)-NAP-β-Ala-Phlz and the following scavangers: 10 mM *p*-aminobenzoate (lane 9), none (lane 10), or 10 mM GSH (lane 11). The reduced glutathione (buffered) was added 30 s before photolysis; 40 μg of sample protein (160 000–220 000 cpm) was run, and the gels were exposed as in (A).

did not afford any obvious specific (as compared with *p*-phlorizin) or nonspecific protection against labeling with NAP-β-Ala-Phlz. As mentioned above, phlorizin has a fairly high affinity for the transporter, *p*-phlorizin a very low one. (ii) If choline was substituted for Na^+, again no obvious qualitative difference in labeling patterns was observed (compare lanes 4 and 5).

The reason for this failure to label unequivocally the Na^+,D-glucose cotransporter lies probably in the complicated electrophoresis pattern of this membrane, in particular in the extensive labeling of the 5f region [approximately (60–80) × 10^3 daltons]. In this area indirect evidence was presented earlier for the cotransporter (or one of its subunits) to occur (Kilp et al., 1979b). Indeed, using a less lipophilic phlorizin derivative, i.e., 4-azidophlorizin, Hosang et al. (1981) did label a sharp thin band of 72 000 daltons. The criteria for identifying it as (a part of) the Na^+,D-glucose cotransporter were fulfilled, i.e., protection by phlorizin but not by *p*-phlorizin, decrease of labeling in the absence of Na^+, relative increase in labeling along with partial negative purification of the transporter, etc. It seems clear, therefore, that a clear-cut labeling of the small-intestinal Na^+,D-glucose cotransporter using NAP-β-Ala-Phlz must await a further, if partial, purification of the system, in particular, a "clearing up" of the 5f region. Work along this line is presently in progress.

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