Phlorizin derivatives as photoaffinity labels of the Na+, D-glucose transporter in brush border membranes

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PHLORIZIN DERIVATIVES AS PHOTOAFFINITY LABELS OF THE 
Na\(^+\), D-GLUCOSE TRANSPORTER IN BRUSH BORDER MEMBRANES

A dissertation submitted to the 
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH 
for the degree of Doctor of Natural Sciences

presented by
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Prof. Dr. H. Murer, co-referee 
1981

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und meiner Frau
in Dankbarkeit gewidmet
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# CONTENTS (All titles)

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>II. Abbreviations and symbols</td>
<td>7</td>
</tr>
<tr>
<td>III. Materials, methods and syntheses</td>
<td>9</td>
</tr>
<tr>
<td>III.1. Materials</td>
<td>9</td>
</tr>
<tr>
<td>III.2. Methods</td>
<td>10</td>
</tr>
<tr>
<td>III.2.1. Preparation of brush border vesicles</td>
<td>10</td>
</tr>
<tr>
<td>III.2.2. Preparation of PC vesicles</td>
<td>13</td>
</tr>
<tr>
<td>III.2.3. DOC extraction</td>
<td>13</td>
</tr>
<tr>
<td>III.2.4. KI extraction</td>
<td>14</td>
</tr>
<tr>
<td>III.2.5. Transport experiments</td>
<td>14</td>
</tr>
<tr>
<td>III.2.6. Binding assays</td>
<td>15</td>
</tr>
<tr>
<td>III.2.7. Photolysis experiments</td>
<td>17</td>
</tr>
<tr>
<td>III.2.8. Photoinactivation and photolabeling experiments</td>
<td>18</td>
</tr>
<tr>
<td>III.2.9. SDS-Polyacrylamide gel electrophoresis</td>
<td>20</td>
</tr>
<tr>
<td>III.2.10. Determination of protein</td>
<td>23</td>
</tr>
<tr>
<td>III.3. Syntheses</td>
<td>24</td>
</tr>
<tr>
<td>III.3.1. General</td>
<td>24</td>
</tr>
<tr>
<td>III.3.2. Purification and analytical data of phlorizin</td>
<td>25</td>
</tr>
<tr>
<td>III.3.3. Synthesis of 6-amino-6-deoxyphlorizin</td>
<td>26</td>
</tr>
<tr>
<td>III.3.4. Synthesis of the NAP-derivatives of phlorizin</td>
<td>29</td>
</tr>
<tr>
<td>III.3.5. Synthesis of 4-azidophlorizin</td>
<td>31</td>
</tr>
<tr>
<td>III.3.6. Preparation and stability of ( ^3 \text{H} )-labeled phlorizin derivatives</td>
<td>35</td>
</tr>
<tr>
<td>III.4. Comments on the syntheses</td>
<td>40</td>
</tr>
<tr>
<td>IV. Results and discussion/part 1. The use of phlorizin derivatives modified in position 6 of the glucopyranosyl moiety</td>
<td>46</td>
</tr>
<tr>
<td>IV.1. Choice and characterization of the membrane vesicle preparation</td>
<td>47</td>
</tr>
<tr>
<td>IV.1.1. Polypeptide composition of intact and DOC extracted membranes</td>
<td>48</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>------</td>
</tr>
<tr>
<td>IV.1.2. Purity of the membrane preparation</td>
<td>51</td>
</tr>
<tr>
<td>IV.1.3. Stability of membranes with respect to proteolytic degradation</td>
<td>51</td>
</tr>
<tr>
<td>IV.1.4. Functional stability of membrane vesicles</td>
<td>54</td>
</tr>
<tr>
<td>IV.2. Interaction of (photoreactive) phlorizin analogues with brush border membranes in the dark</td>
<td>61</td>
</tr>
<tr>
<td>IV.2.1. Inhibition of D-glucose uptake in brush border membrane vesicles</td>
<td>62</td>
</tr>
<tr>
<td>IV.2.2. Inhibition of specific phlorizin binding to brush border membranes</td>
<td>65</td>
</tr>
<tr>
<td>IV.2.3. Binding of (3H)-NAP-β-Ala-Phlz to brush border vesicles and to PC vesicles</td>
<td>68</td>
</tr>
<tr>
<td>IV.3. Photoinactivation experiments</td>
<td>70</td>
</tr>
<tr>
<td>IV.3.1. Control experiments</td>
<td>70</td>
</tr>
<tr>
<td>IV.3.1.1. Photoreactivity of the NAP-derivatives alone</td>
<td>70</td>
</tr>
<tr>
<td>IV.3.1.2. Photoreactivity of the NAP-derivatives in the presence of membranes</td>
<td>72</td>
</tr>
<tr>
<td>IV.3.1.3. Irradiation of vesicles in the absence of photolabels</td>
<td>75</td>
</tr>
<tr>
<td>IV.3.2. Photoinactivation assays utilizing NAP-derivatives of phlorizin</td>
<td>78</td>
</tr>
<tr>
<td>IV.3.2.1. Irreversible inhibition of the overshoot of ΔΨNa+-driven D-glucose uptake</td>
<td>78</td>
</tr>
<tr>
<td>IV.3.2.2. Inactivation of D-glucose tracer exchange</td>
<td>82</td>
</tr>
<tr>
<td>IV.3.2.3. Inactivation of specific phlorizin binding to DOC extracted membranes</td>
<td>83</td>
</tr>
<tr>
<td>IV.4. Photolabeling with (3H)-NAP-β-Ala-Phlz</td>
<td>87</td>
</tr>
<tr>
<td>IV.4.1. Labeling of intact brush border vesicles with (3H)-NAP-β-Ala-Phlz</td>
<td>87</td>
</tr>
<tr>
<td>IV.4.2. Labeling of DOC extracted membranes with (3H)-NAP-β-Ala-Phlz</td>
<td>91</td>
</tr>
<tr>
<td>IV.4.2.1. Comparison of the labeling pattern of DOC extracted membranes with that of intact vesicles</td>
<td>91</td>
</tr>
</tbody>
</table>
IV.4.2. Specificity of (3H)-NAP-β-Ala-Phlz as a label for the phlorizin binding component of the transporter in DOC extracted membranes

IV.4.2.3. Effect of scavengers

IV.4.3. The location of labeled actin

IV.5. Discussion

IV.5.1. Choice and synthesis of the ligands

IV.5.2. Reversible inhibition of D-glucose transport and phlorizin binding

IV.5.3. Photolysis and photoinactivation studies

IV.5.4. Photolabeling studies

IV.5.5. Possible involvement of an H-bond between a group in C-6 of the glucopyranosyl moiety and the transporter

V. Results and discussion/part 2. The use of phlorizin derivatives modified in the aglycone moiety

V.1. Interaction of phlorizin derivatives modified in the aglycone moiety with brush border vesicles in the dark

V.1.1. Inhibition of D-glucose uptake in, and phlorizin binding to, rabbit small-intestinal brush border vesicles

V.1.2. Comparison of the inhibitory potencies of 4-azidophlorizin and phlorizin on D-glucose uptake in, and phlorizin binding to, brush border vesicles derived from rabbit and rat small intestines and kidneys

V.1.3. Binding of (3H)-4-azidophlorizin to brush border membrane vesicles

V.2. Photoinactivation experiments

V.2.1. Irreversible inhibition of D-glucose tracer exchange across brush border membrane vesicles

V.2.2. Irreversible inhibition of Na⁺-dependent phlorizin binding to DOC extracted membranes
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>V.3. Photolabeling with $^3H$-4-azidophlorizin</td>
<td>117</td>
</tr>
<tr>
<td>V.3.1. Labeling of intact brush border membrane vesicles with $^3H$-4-azidophlorizin; time and concentration dependence of labeling</td>
<td>117</td>
</tr>
<tr>
<td>V.3.2. Specific labeling of (a part of) the Na$^+$, D-glucose transporter in DOC extracted membranes</td>
<td>120</td>
</tr>
<tr>
<td>V.3.3. The effect of scavengers on the labeling of DOC extracted membranes</td>
<td>124</td>
</tr>
<tr>
<td>V.3.4. $^3H$-4-Azidophlorizin as label of other proteins present in the brush border membrane</td>
<td>124</td>
</tr>
<tr>
<td>V.3.5. Location of labeled actin</td>
<td>127</td>
</tr>
<tr>
<td>V.4. Discussion</td>
<td>128</td>
</tr>
<tr>
<td>V.4.1. Interaction of phlorizin derivatives modified in the aglycone moiety with brush border membranes in the dark</td>
<td>128</td>
</tr>
<tr>
<td>V.4.2. Photoinactivation of the transporter</td>
<td>131</td>
</tr>
<tr>
<td>V.4.3. Labeling of (a part of) the Na$^+$, D-glucose transporter in DOC extracted small-intestinal brush border membranes</td>
<td>131</td>
</tr>
<tr>
<td>V.4.4. Some tentative conclusions on the nature and the mode of functioning of the Na$^+$, D-glucose transporter and on its interaction with the aglycone moiety of phlorizin and its derivatives</td>
<td>135</td>
</tr>
<tr>
<td>VI. Final discussion</td>
<td>137</td>
</tr>
<tr>
<td>VI.1. Reactions of arylazide photolabels</td>
<td>137</td>
</tr>
<tr>
<td>VI.2. Modes of reversible and irreversible association of phlorizin and photolabile analogues with small-intestinal brush border membranes</td>
<td>141</td>
</tr>
<tr>
<td>VI.3. Final remarks and perspectives</td>
<td>146</td>
</tr>
<tr>
<td>VII. Appendix A</td>
<td>149</td>
</tr>
<tr>
<td>VIII. References</td>
<td>154</td>
</tr>
<tr>
<td>Summary</td>
<td>163</td>
</tr>
<tr>
<td>Zusammenfassung</td>
<td>165</td>
</tr>
<tr>
<td>Curriculum vitae</td>
<td>167</td>
</tr>
</tbody>
</table>
I. INTRODUCTION

Transport of D-glucose across the brush border of intestinal (and renal proximal tubular) epithelial cells is a Na\textsuperscript{+}-dependent (Riklis & Quastel, 1958; Bihler & Crane, 1962; Crane 1962 and 1965; Schultz & Curran, 1970) and \(\Delta\Psi\)-dependent (Rose & Schultz, 1971; White & Armstrong, 1971) process, which is inhibited in a fully competitive manner by phlorizin (Fig. 1), the \(\beta\)-D-glucopyranoside of phloretin (Alvarado & Crane, 1962).

Crane (1962 and 1965) formulated the theory of Na\textsuperscript{+}, D-glucose-"co-transport", in which D-glucose (and similar sugars) and Na\textsuperscript{+} are translocated across the membrane together and by the same system. The coupling of the D-glucose flux to that of Na\textsuperscript{+} explained the well known accumulation of D-glucose against its own concentration gradient; since the concentration of Na\textsuperscript{+} inside an epithelial cell is lower than in the lumen accumulation of D-glucose can be powered by the Na\textsuperscript{+} gradient.

In isolated vesicles, prepared from both small intestinal and renal brush border membranes, the characteristics of this transport process were found to be preserved (e.g., Hopfer et al., 1973; Murer & Hopfer, 1974; Aronson & Sacktor, 1974; Tannenbaum et al., 1977; Kessler et al., 1978a). This, firstly, confirmed the theory of Crane and, secondly proved that the energy required for this secondary-active transport originates from the electrochemical potential gradient of Na\textsuperscript{+} across the plasma membrane. It could also be demonstrated by the use of such vesicles that Na\textsuperscript{+}, D-glucose cotransport is a rheogenic (electrogenic) process (Murer & Hopfer, 1974), i.e., the compensation for the transfer of the positive charge of Na\textsuperscript{+} across the membrane does not occur through the same transporter. A more detailed analysis of the kinetic properties of the transport system, which allowed more detailed kinetic models to be developed, was subsequently made possible by refining the vesicle preparations and assay procedures (e.g., Kessler et al., 1978a,b).
However, despite their great importance for the characterization of the transporter, the kinetic models derived bear certain limitations: they can only lead to a partial understanding of the molecular mechanisms underlying the transport process.

An important step in this direction would be the identification of the, so far unknown, membrane protein(s) mediating this process. This would facilitate the isolation and subsequent reconstitution of the transport system; it would then be easier to elucidate the molecular mechanisms.

Several strategies have been used in our, and in other, laboratories in attempting to distinguish the proteins of the Na+, D-glucose co-transporter from the many other protein constituents of the small intestinal (and renal proximal tubular) brush border. Most of them are based on classical chemical methods which have successfully been applied in the identification of a variety of other membrane proteins.

(i) Membranes (small intestine and kidney) have been solubilized in detergents and their components have been fractionated; the partially purified proteins have then been reconstituted into artificial liposomes and tested for functional activity (e.g. Crane et al., 1976; Fairclough et al., 1979; Malathi et al., 1980; Köpsell et al., 1980; Lin et al., 1981). These studies provided evidence that membrane transport proteins may retain their activity after being solubilized from the membrane by detergents and after being reconstituted; however, the results reported by the various groups are conflicting.
(ii) Radioactive alkylating agents have been used to label the transporter in intact membranes; in order to make the reaction specific, the ligands selected were either sugar substrate derivatives (Arita & Kawanami, 1980), or general protein reagents, used in the presence of the native ligands to discriminate D-glucose and phlorizin protectable sites (binding proteins) (Thomas, 1973; Smith et al., 1975; Lemaire & Maestracci, 1978; Poiree et al., 1979; Biber & Hauser, 1979). Here again, the results reported are conflicting.

(iii) A relative increase in specific, $\text{Na}^+$-dependent, reversible phlorizin binding activity was achieved by removing unrelated proteins from the membrane ("negative purification"; Klip et al., 1979b).

In spite of these efforts, the final identification of the protein components of the $\text{Na}^+$, $\Delta\Psi$-dependent D-glucose transporter in small-intestinal and renal brush border membranes has not yet been achieved. The reasons for this are probably manyfold, but at least two are clear: carrier density is very low (approximately 10-100 pmoles $\cdot$ mg$^{-1}$protein; Toggenburger et al., 1978; Bode et al., 1970; cf also Kinne, 1976) and the transporter has a poor stability when solubilized in detergents (S. and Y. Takesue, unpublished results, 1979),

Therefore the technique of photoaffinity labeling is used in the present work as an additional and different approach in attempting to tag the transporter. The principle of photoaffinity labeling is schematically given in Fig. 2.

Fig. 2. Schematic representation of photoaffinity labeling:
(a) formation of a reversible label-receptor complex in the dark, (b) activation of the label (L) by light, (c) formation of a covalent bond between label (L) and receptor (R).
In this approach use is made of ligands carrying a chemically "inert" function which can be activated by light to give a species with a high chemical reactivity (radicals or excited states).

This technique therefore allows the experimenter to activate the label (reagent) at a particular time and place, and thereby in principle to circumvent the major limitations of classical affinity labeling (e.g., Baker et al., 1961; Wofsy et al., 1962; see also Wold (1977) and references cited therein). These are (i) competition between water and the sites to be labeled for the (mostly) electrophilic labels and (ii) activation of the labels cannot be controlled. In addition photogenerated species (such as nitrenes and carbenes, see Bayley & Knowles, 1977) are expected to be of a relatively indiscriminate reactivity (Knowles, 1972). Ideally the reactive label is only generated at the (ligand) site of interest and reacts with whatever group it finds there. The masked reactivity of photolabile reagents has an additional advantage over classical chemical affinity labels: binding or activity assays can be performed with modified ligands prior to photoactivation, and this can give some information as to the site specificity of the ligand analogue.

Among the criteria which must be met to establish the specificity of such a labeling reaction are (see also Bayley & Knowles, 1977): (i) in the dark the label should act as a reversible ligand of the binding site, and, upon photolysis an irreversible ligand-receptor complex should be formed (see also Fig. 2); (ii) the labeling reaction should be saturable with respect to label concentration; (iii) the labeling should be protected by the (photoinert) natural ligand (substrate, inhibitor) and by non-photolabile ligand analogues; (i) ideally there should be a stoichiometric relationship between incorporation of marker and inactivation of the binding site.

In practice, however, labeling also occurs outside the receptor site. This can be on the same macromolecule or in more complex systems on different "irrelevant" macromolecules (as is shown in the present work, both difficulties are inherent in the labeling of membrane embedded receptors). The use of proper scavenging agents (which are intended to destroy (all) photogenerated intermediates at places other than the ligand binding site) may sometimes help to increase the proportion of specific receptor site labeling.
Many diverse biological systems have been probed by photolabels (for a survey, see the recent reviews of Bayley & Knowles (1977) and of Chowdry & Westheimer (1979)); however, only few successful attempts in photoaffinity labeling of membrane transport systems have been reported; most of them used photolabile derivatives of the transported substrate. Among these systems are the glucose transporters in the plasma membrane of erythrocytes (Farley et al., 1976) and of adipocytes (Trosper & Levy, 1977), the β-galactoside transport system in E. 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., 1979; Arita & Kawanami, 1980).

A valuable alternative to the use of a transported substrate analogue as a photoaffinity probe of a transport protein is the use of a non transported, competitive inhibitor; e.g., arylazido atractlyosides as labels for the mitochondrial adenine nucleotide carrier (Lauquin et al., 1976). For the photoaffinity labeling of the Na⁺,D-glucose cotransporter, phlorizin (see Fig. 1) was the most obvious ligand on which to base the synthesis of potential photolabels: strong evidence has been provided by many investigators that the phlorizin receptor and the glucose transporter in the brush border membrane are identical (Frasch et al., 1970; Vick et al., 1973; Aronson, 1978; Toggenburger et al., 1978). Furthermore, phlorizin has the highest or second highest known affinity for this transporter (Diedrich, 1966; Kinne, 1976): the Ki- and Kd-values in small-intestinal brush border membrane vesicles being in the range of 4-10 μM in the presence of both optimal Na⁺ and ΔΨgradients across the membrane (Tannenbaum et al., 1977; Toggenburger et al., 1978).

The aim of the present work, therefore, was to evaluate the use of photolabile derivatives of phlorizin as potential photolabels of the (phlorizin binding component of the) small-intestinal Na⁺, D-glucose cotransporter.
As a first step towards this, a number of photolabile derivatives of phlorizin were synthesized. For reasons which will be discussed later (see IV.5.1, page 98), we started with the synthesis of derivatives modified in position 6 of the β-D-glucopyranosyl moiety. Later on we also prepared a photolabile derivative modified in the aglycone (phloretinyl) moiety (4-azidophlorizin; see V.4.1).

After establishing the inhibitory potencies of the derivatives in the dark, promising derivatives (on the basis of their affinities for the transporter) were used for photolabeling. Careful refinement of the photolysis conditions was required not only to minimize photochemical damage of brush border vesicles, but also to fulfill the experimental conditions of phlorizin binding (Toggenburger et al., 1978). Taking the above specificity requirements for photolabeling into account, we decided to initially demonstrate the formation by photolysis of an irreversible label-transporter complex. This was done by monitoring the irreversible inhibition of sugar transport by, and phlorizin binding to the Na⁺, D-glucose transporter. Only derivatives which proved to be potential photolabels with respect to photoinactivation were then used in radiolabeled form in attempting to label specifically the Na⁺, D-glucose transporter in small-intestinal brush border membranes.

Further, irrespective of the suitability of the synthesized photolabile derivatives as potential labels of the transporter, a comparison of the inhibitory potencies of these (and of other derivatives synthesized) concurred to a knowledge of their chemical structure could be expected to give some information on their mode of interaction with the transporter.
## II. ABBREVIATIONS AND SYMBOLS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABG</td>
<td>$\alpha$-acetobromoglucose (2,3,4,6-tetra-O-acetyl-$\alpha$-D-glucopyranosyl bromide)</td>
</tr>
<tr>
<td>Ac-</td>
<td>acetyl-</td>
</tr>
<tr>
<td>$\beta$-Ala</td>
<td>$\beta$-alanine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CDI</td>
<td>N,N'-carbonyl diimidazole</td>
</tr>
<tr>
<td>D</td>
<td>dalton</td>
</tr>
<tr>
<td>DFP</td>
<td>diisopropyl fluorophosphate</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DOC</td>
<td>deoxycholate</td>
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<tr>
<td>DTE</td>
<td>dithioerythritol</td>
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<td>EDTA</td>
<td>ethylenediamine-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol bis($\beta$-aminoethyl ether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione (reduced)</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>IR</td>
<td>infrared</td>
</tr>
<tr>
<td>MES</td>
<td>2-($N$-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>mol wt</td>
<td>molecular weight</td>
</tr>
<tr>
<td>Ms-</td>
<td>methane sulfonyl- (mesyl-)</td>
</tr>
<tr>
<td>NAP-</td>
<td>2-nitro-4-azidophenyl-</td>
</tr>
<tr>
<td>$^1$H NMR</td>
<td>proton nuclear magnetic resonance</td>
</tr>
<tr>
<td>pABA</td>
<td>4-aminobenzoic acid</td>
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<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>para-Phlz</td>
<td>para-phlorizin (4' isomer of phlorizin, i.e., 4'-$\beta$-D-glucopyranosyl-phloretin)</td>
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<tr>
<td>pCMBS</td>
<td>p-chloromercuri benzenesulfonic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>PC</td>
<td>phosphatidyl choline</td>
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<td>Phlz</td>
<td>phlorizin</td>
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<tr>
<td>PLC</td>
<td>preparative layer chromatography</td>
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<tr>
<td>PPO</td>
<td>2,5-diphenyloxazole</td>
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<tr>
<td>POP</td>
<td>1,4-di-2(5-phenyloxazolyl)benzene</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
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<td>THF</td>
<td>tetrahydrofurane</td>
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<tr>
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</tr>
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<tr>
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</tr>
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<td>UV</td>
<td>ultraviolet</td>
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<tr>
<td>vic</td>
<td>vicinal</td>
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<td>Vis</td>
<td>visible</td>
</tr>
</tbody>
</table>

\[ M_r \]

(apparent) relative molecular weight

\[ K_i', K''_i, K_i'' \]

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 + unionized) concentration of the analogue (Toggenburger et al., 1978).

\[ \Delta \tilde{\psi}_{Na^+} \]

gradient in electrochemical potential of Na\(^+\)

\[ \Delta \psi \]

electrical transmembrane (diffusion) potential
III. MATERIALS, METHODS AND SYNTHESSES

III.1. MATERIALS

The commercial sources and the purification procedure of unlabelled phlorizin are given on page 25. l,l'-Carbonyldiimidazole (CDI) and β-alanine were from Fluka, AG (Buchs, SG). N-Ethyl-N'(3-dimethylamino-propyl) carbodiimide hydrochloride was from Merck-Schuchardt (Munich, Ger.). 4-Fluoro-3-nitrophenyl azide (FNAP) was either prepared from 4-fluoro-3-nitroaniline by the method of Fleet et al., (1972) or purchased from BDH Chemicals Ltd. (Poole, England). Hydrocinnamonic acid was obtained from Eastman Organic Chemicals and phloroglucinol from Matheson, Coleman & Bell. SDS, acrylamide and bis-acrylamide were from Serva (Heidelberg, Ger.) and Sephadex G-10 was from Pharmacia. All other chemicals used were of the highest purity available.

Radiolabeled compounds

D-(l-³H)-Glucose (5.2 Ci/mmol), L-(methyl-³H)-methionine (12 Ci/mmol), β-(3-³H)-alanine (32 Ci/mmol) and a mixture of (¹⁴C)-methylated proteins as molecular weight markers were purchased from Amersham Radiochemical Centre, Ltd. (Buckinghamshire, England). (³H(G)-phlorizin (Lots no. 1070-031 (2.06 Ci/mmol), 1109-267 (5.2 Ci/mmol) and 1302-199 (6.3 Ci/mmol)) was obtained from New England Nuclear (Boston, Mass.). The synthesis of the radioactive photolabels is given on page 35.

Purity of ³H-phlorizin

The radioactive phlorizin gave a single spot (chemical purity 98%). with unlabelled phlorizin by both radioactive and UV-detection, when the following chromatographic systems were used:

1) chromatography on Whatman 3 MM paper, with water as the solvent,
2) TLC on silica gel 60, using
   a) methylene chloride-methanol = 4 : 1 (solvent A)
   b) chloroform-n-propanol = 3 : 1 as the eluents.

The radiochemical purity initially was greater than 97 %, except for lot no. 1302-199 which was approximately 90 %, as determined by TLC, scraping out segments from the plate and liquid scintillation counting. This lot was rechromatographed by TLC using solvent A.
III.2. METHODS

III.2.1. Preparation of brush border membrane vesicles

Brush border membrane vesicles derived from small intestines of rabbits were prepared daily by the calcium precipitation method of Schmitz et al. (1973) as modified by Kessler et al. (1978a), unless otherwise stated.

Either frozen or fresh small intestines were used for preparation. The former were obtained fresh from a rabbit slaughterhouse, where animals were killed by a gunshot in the head. The small intestines were quickly rinsed with ice-cold isotonic saline, everted and washed again with ice-cold saline. After removal of excess fluid, the tissue was frozen on dry-ice (package size approximately 500 g) and kept at -80°C (at -20°C for not longer than two months).

All the treatments described below were performed at 0 to 4°C unless otherwise stated.

The mucosa of frozen intestines was obtained as follows: after thawing 20 - 25 g of frozen intestine (in pieces of 1 g) in 60 ml 300 mM mannitol, 12 mM Tris-HCl, pH 7.1, the resulting "goulash" was subjected to vibration (100 Hz) using a vibromixer (Model El, Chemap AG, Männdorf ZH) fitted with a 2 cm diameter vibrating perforated plate for 90 s. This treatment released the mucosal cells and other soft components of the tissue almost completely. To separate from the gross pieces of connective tissue, the suspension was passed through a Büchner funnel with 1-mm holes. The filtrate was diluted 1 : 6 with ice cold 300 mM mannitol and homogenized in a mixer (Waring blender) at maximum speed for 3 min.

If fresh intestine was used as the starting material, the animal was killed by a blow in the neck, the small intestine removed as quickly as possible, rinsed with cold saline and cut open lengthwise. Then it was cut into pieces of 10 cm which were blotted repeatedly with hard household paper for the complete removal of mucus. The mucosa was scraped off gently with a stainless steel spatula, attention being paid of not taking along fat and muscle tissue also. Routinely
8 g scrapings (yield from 1 animal, approximately 20 g) were suspended in 360 ml ice-cold 300 mM mannitol, 2 mM Tris/HCl, pH 7.1, and homogenized in the mixer as described above. Mucosal scrapings could be stored at -20°C for up to 2 months.

To the homogenates was added solid CaCl₂, final concentration 10 mM, in order to precipitate non brush border material (nuclei, mitochondria, most of the basolateral membranes and other cell debris). After standing in the cold for 20 min the suspension was spun down at 3000 x g for 15 min. The supernatant was then spun down at 27,000 x g for 30 min. The pellet which contained purified brush border vesicles was resuspended in 40 ml of the appropriate buffer (100 mM or 300 mM mannitol, 0.02% KN₃, and either 10 mM HEPES/Tris, pH 7.5, 10 mM Tris-HCl, pH 7.0 or 10 mM MES/Tris, pH 6.5), homogenized in a Teflon Potter-Elvehjem homogenizer (with ten strokes) and spun down once more at 27,000 x g for 30 min. The pellet was homogenized in a small volume of the buffer required for further experiments (20 - 30 mg of protein/ml) by sucking the suspension 20 times through a hypodermic needle (25 G, 5/8) into a plastic syringe. This suspension contained almost pure vesicles from brush border membranes which could be used as such for transport and binding studies, or diluted, for photoinactivation experiments.

Occasionally further purification steps such as repetition of the Ca²⁺-precipitation-fractionated centrifugation-step (instead of the final centrifugation) were used. However, the additional purification achieved thereby was small (Kessler, 1978) and therefore these steps were not usually carried out.

When Ca²⁺ as the precipitating agent was to be replaced by other divalent cations, the precipitation method was modified according to Trüb (1978):

a) "Mg²⁺/EGTA-preparation":
Mg²⁺ (50 mM) instead of Ca²⁺ (10 mM) was used for precipitation. To all buffers was added EGTA (at 5 mM in all preparation buffers and at 0.1 mM in the buffers used for labeling and uptake experiments).
b) "Ba^{2+}/EGTA-Preparation":

Ba^{2+} (50 mM) was used, and all buffers contained EGTA at concentrations as in (a).

For the preparation of brush border vesicles from rat small intestine only fresh mucosal scrapings (from 4 to 8 rats) were used: with frozen intestine as the starting material vesicles of varying quality were obtained (Kessler, 1978). The vesicular space often was below 10% of the value normally measured (approximately 1 µl/mg protein). and the accumulation rate of D-glucose was strongly reduced (Kessler, 1978). This might be indicative of a diminished degree of vesiculardization.

Brush border membrane vesicles derived from kidneys of rabbits and rats were also prepared by the precipitation method using divalent cations. The procedure essentially followed the protocol by Booth & Kenny (1974) as adapted by Evers et al. (1978). Briefly, the kidneys of freshly killed animals (one rabbit or 4 to 6 rats) were placed as rapidly as possible into ice-cold mannitol buffer (10 mM mannitol, 2 mM Tris-HCl, pH 7.1). They were decapsulated, and the cortex was carefully dissected away from the medulla with a razor blade (thickness of the slices approximately 1 - 2 mm) and homogenized in 200 ml of the same mannitol buffer in a Waring blender run at full speed for 2 min. Solid MgCl_2 \cdot 6 H_2O was added to the homogenate to give a final concentration of 10 mM and then let to stand for 15 min at 4°C. A first vesicles pellet was obtained by fractionated centrifugation as described above. After resuspending in the same mannitol buffer (40 ml) and homogenizing with a Teflon Potter-Elvehjem homogenizer, MgCl_2 was added as before to 10 mM concentration. Fractionated centrifugation (a spin at 2200 x g, 15 min, to remove pelleted precipitates, followed by a centrifugation at 15,000 x g for 12 min) yielded a second vesicle pellet which was resuspended in 40 ml of the buffer needed for further experiments (100 mM or 300 mM mannitol, 10 mM HEPES/Tris pH 7.5 or 10 mM Tris-HCl pH 7.0 and 0.02% KN_3) with the glass Teflon homogenizer. The suspension was centrifuged at 48,000 x g for 20 min, and the final pellet was resuspended in a small volume of the appropriate buffer.
III.2.2. Preparation of phosphatidyl choline (PC) vesicles

Vacuum-dried (10⁻³ Torr, 12h) phosphatidyl choline (40 mg) was vortexed with 10 ml buffer (300 mM mannitol, 100 mM NaCl, 10 mM Tris-HCl, pH 7.0, 1 % ethanol, 0.02 % KN₃) until a homogeneous opaque suspension was obtained. This suspension was ultrasonically irradiated (Branson 100-W microtip sonifier, power setting: 100) for 20 min in a glass tube kept in an ice bath. The resulting clear suspension was centrifuged at low speed to sediment all metal débris.

III.2.3. DOC extraction

The extraction procedure was essentially as described by Klip et al. (1979b). Prior to DOC extraction, the membranes were washed once with a solution containing 250 mM KCl and 10 mM Tris-HCl, and then resuspended to about 2 - 3 mg protein/ml in a similar solution buffered to pH 8.5. The solution was then made 0.75 mM in DTE, and finally DOC (0.5 mg DOC/mg protein; in some experiments 0.65 mg/mg protein) was added. The suspension was shaken, incubated on ice for 15 min, and finally centrifuged at 60,000 x g for 30 min. The supernatant was discarded and the membranes were washed once in 300 mM mannitol, 10 mM Tris-HCl pH 7.0 and 0.75 mM DTE. The final pellet was resuspended in the same buffer and immediately used for binding experiments.

Since aryl azides are reduced by thiols (Staros et al., 1978), DTE was omitted when DOC extracted membranes were used for photolabeling experiments.

1) Control experiments showed that the presence of DTE was not critical for the functional stability of DOC extracted membranes: membranes kept on ice without DTE present displayed a phlorizin binding activity which was not significantly different from that of DTE controls, when binding was measured after certain time intervals. In both cases, a parallel time dependent decrease in binding activity was observed (possible explanations for this will be discussed later).
111.2.4. KI extraction

KI extraction was performed essentially as described by Bretscher & Weber (1980a): brush border vesicles or DOC extracted membranes (0.5-1 mg protein/ml) were extracted for 30 min at 0°C in 0.6 M KI, 1 mM CaCl₂, 0.2 mM ATP, 20 mM Tris-HCl (pH 7.8). Following extraction, the membranes were pelleted by centrifugation at 60,000 x g for 30 min, resuspended in the appropriate buffer and centrifuged again. The final pellets were resuspended in the same buffer, diluted to the concentration required, and immediately used for binding or labeling experiments.

111.2.5. Transport experiments

The uptake of substrates by brush border membrane vesicles was determined by the Millipore filtration technique as described by Hopfer et al. (1973) and refined by Kessler et al. (1978b).

All preincubation and incubation media contained 100 mM or 300 mM mannitol, 0.02 % KN₃, and 10 mM buffer. Further additions are described in the legends for the figures and tables. All measurements were performed at room temperature (approximately 20°C).

D-(1⁻³H) Glucose uptake in the presence of a NaSCN gradient was determined essentially as described by Kessler et al. (1978a,b). In the following the standard procedure is briefly described: a certain volume of vesicles (normally 10 µl, 10 - 20 mg protein/ml) was carefully placed at the bottom of a clear polystyrene test tube fitted into a vibration device controlled by an electric timer. A separate drop (incubation medium, normally 10 µl) containing the radioactively labeled ligand(s) and further additions was placed close (2 mm) to the vesicle drop. At the start of the timer, the shaking of the vibrator (frequency about 60 Hz) rapidly mixed the two drops (less than 80 ms for complete mixing (Kessler et al. 1978b)). At the chosen time 2.5 ml of ice cold 0.9 % saline solution buffered with 1 mM Tris-HCl, pH 7.5 (= stop solution) was automatically injected into the incubation tube slowing down the reaction. Then the sample was quickly filtered through a prewetted Sartorius microfilter (0.6 µm pore size, type SM 11305) and washed with further 5ml of the same cold solution. The time required for the entire washing procedure did not exceed 10 s.
Then the filter was dissolved in 10 ml scintillation fluid (toluene-Triton X-100-acetic acid, 6:3:0.45 (v/v), plus 5.08 g Butyl-PBD/l of mixture) and the radioactivity was counted. Filter blanks were determined by adding the vesicle suspension and the incubation cocktail separately to the stop solution.

Uptake of D-glucose under equilibrium conditions (tracer exchange) was determined after equilibrating the vesicles (20 mg protein/ml), in 300 mM mannitol, 10 mM Hepes/Tris pH 7.5 with 0.1 mM D-glucose and NaCl (100 mM) for 30 min at room temperature. D-(1-3H) glucose uptake was initiated by mixing 10 µl aliquots of the suspension with 10 µl of the same buffer containing 1 µCi of the isotope. Transport was stopped at the times indicated (usually 8 s) by diluting with 3 ml of ice-cold 250 mM KCl, 1 mM HEPES/Tris pH 7.5 followed by filtration as described. In some experiments carrier unrelated D-glucose influx was corrected for by subtracting the uptake in vesicles preequilibrated with 100 mM KCl (Klip et al., 1980). However, the fraction of carrier unrelated tracer exchange never amounted to the values obtained by those authors (i.e., 25 % after 8 s of incubation).

III.2.6. Binding assays

Three types of phlorizin binding measurement were performed: (i) Na\(^+\), Δψ-dependent, D-glucose protectable phlorizin binding to intact brush border vesicles, measured in the presence of an inwardly directed gradient of NaSCN, (ii) Na\(^+\)-dependent phlorizin binding to intact vesicles and to DOC extracted membranes preequilibrated in 100 mM NaCl, and (iii) equilibrium binding of phlorizin (and its photolabile analogues) to PC vesicles, determined by equilibrium dialysis.

(i) Phlorizin binding in the presence of a NaSCN gradient was determined as described by Toggenburger et al. (1978): NaSCN (100 mM), phlorizin, and either D-glucose or D-fructose (25 mM, each) were all in the incubation medium. The vesicles were thus exposed to all ligands simultaneously at the start of the incubation.
(ii) Phlorizin binding measurements under equilibrium conditions were performed according to Toggenburger et al. (1978) as modified by Klip et al. (1979b): Intact brush border vesicles (final concentration 10 - 20 mg protein/ml) or membranes extracted with DOC (5 - 15 mg protein/ml) were preincubated for 30 min at room temperature with 100 mM of either NaCl or KCl in 300 mM mannitol, 10 mM Tris-HCl, pH 7.0 and 0.02 % KN₃ with or without DTE present. The binding reaction was initiated by mixing 10-µl aliquots of these suspensions with 10 µl of the same medium containing twice the final (³H)-phlorizin and ligand concentration desired. After 5 s of vigorous mixing and a further 10 s of incubation, binding was stopped by diluting 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) followed by filtering as described and washing once with 5 ml of the same solution.

Specific binding was defined as the difference between total (incubation in the presence of 100 mM NaCl) and unspecific binding (in Na⁺-free solutions containing 100 mM KCl) of (³H)-phlorizin (5 µM). Specific binding activity represents the amount of Na⁺-dependent bound (³H)-phlorizin divided by the protein content of the sample.

(iii) equilibrium binding of (³H)-phlorizin and its (³H)-labeled photo-labile analogues to single-bilayer PC-vesicles was measured by equilibrium dialysis, using a Dianorm GD-3 dialysis apparatus equipped with 1 ml Teflon chambers. A cellulose standard dialysis tubing (Spectrapor, mol wt cut off: 12,000 - 14,000) was used as the membrane. Experiments were performed at 25°C in 300 mM mannitol, 100 mM NaCl, 10 mM Tris-HCl, pH 7.0, 1 % ethanol and 0.02 % KN₃; the PC concentration was 4 mg/ml and the (³H)-ligand concentration was 10 µM or 100 µM. The distribution of the ligand was determined by withdrawing duplicate samples (50 µl) from each compartment after 10 h and 24 h and counting them in the liquid scintillation cocktail.
III.2.7. Photolysis experiments

All photolysis and photolabeling experiments were carried out in the photolysis apparatus schematically drawn in Fig. 3. A 350-W mercury short arc lamp A (Illumination Industries Inc., Sunnyvale, CA) in a lamp house equipped with a mirror B and a collimating lense C was used as the light source. The focussed light was filtered through a 3 cm thick water-filled quartz cell D and a 1.5 cm thick liquid filter quartz cell E fitted into a cylindrical holder F. The filter solutions used were: (i) 1 M NaNO₂, for the photolysis of NAP-compounds (λ>390 nm); (ii) sat. Cu(II)SO₄, for the photolysis of the 4-azide (λ>315 nm (Katzenellenbogen et al., 1974)). For the photolysis of NAP-compounds a Balzers 390 nm cut-off filter G was used in addition. The light was focussed onto a water jacketed circular quartz photolysis cuvette H (4 mm path length, 2 ml volume) which was cooled at 20°C by circulating water I. The distance between the cuvette and the lamp was 25 cm. During irradiation the sample was mixed with and kept anaerobic by a gentle stream of argon or N₂.

To the monitor the photolysis rate of the NAP-group, a 100 μM solution of the corresponding NAP-derivative in 10 mM pABA/NaOH (pH 6.5), 4 % ethanol as the buffer was photolyzed in the presence or absence of membrane vesicles. At measured time intervals, aliquots were recorded on a Pye Unicam (SP 1700) spectrometer. Then the aliquots were dried onto a germanium plate by a stream of N₂ and ATR (= attenuated total reflection: Harrick 1967; Fringeli 1981) IR-spectra were recorded in the laboratory of PD Dr. H. Fringeli (Physikalische Chemie, ETH-Z) on a Perkin-Elmer spectrograph 225, equipped with a Wilks 50 ATR mirror assembly.

In preliminary experiments a 300-W xenon short arc lamp was also tested. However, the photolysis rate of NAP-compounds achieved was about 6 times lower than with the 350-W mercury lamp. Therefore, only the latter lamp was used further.
III.2.8. Photoinactivation and photolabeling experiments

All membrane suspensions and media were routinely deoxygenated by bubbling argon through (approximately 0.2 ml·ml⁻¹·s⁻¹; according to Bayley & Knowles (1977)) for at least 30 min (see also results). Deoxygenation by blowing a stream of argon onto the surface of the sample (R. Cherry, personal communication) was similarly effective, whereas repeated evacuation followed by flooding with N₂ (S. Takesue, personal communication) was not suited, in particular not for degassing larger sample volumes.

Photolysis of membranes in the presence of an inwardly directed gradient of NaSCN was carried out as follows: an aliquot of a degassed vesicle suspension was transferred into the photolysis cell (Fig. 3, H) and rapidly mixed with an equal volume of the same buffer containing in addition NaSCN, scavenger (pABA or GSH) and the ligands at twice the final concentration desired. After a 5 s incubation in the
dark\(^1\), the sample (0.2 – 1.5 ml) was irradiated for the time indicated in the text (the vesicle photolysate finally contained 300 mM mannitol, 100 mM NaSCN (out, zero in at t=0), 10 mM pABA or GSH/Tris (pH 6.5, 7.0 or 7.5), 1 % ethanol, 0.02 % KN\(_3\) and the ligands at concentrations indicated in the text or figure legends).

When vesicles preequilibrated in 100 mM NaCl (or KCl) for 30 min at room temperature were used for photolysis, dark incubation lasted 15 s and NaSCN in the incubation medium was replaced by 100 mM NaCl (or KCl). Photolysis of DOC extracted membranes was performed at pH 7.0 either in the presence of 100 mM NaSCN or, after preincubation with 100 mM NaCl for 30 min at room temperature, in the presence of 100 mM of this salt. DTE (0.75 mM) normally included in the buffer for DOC extracted membranes (see III.2.3.) was omitted because aryl azides are reduced by thiols (Staros et al., 1978). To reach maximal specific binding of phlorizin (see Fig. 10) under these conditions, dark incubations of DOC extracted membranes prior to photolysis lasted 15 s.

After photolysis, samples were immediately diluted with 30 ml of ice-cold washing buffer (300 mM mannitol, 10 mM of the appropriate buffer, 0.02 % KN\(_3\)) and spun at 27,000 x g for 30 min. In some experiments BSA (1 %, w/v) was added to the washing buffer to reduce postphotolytic (dark) reactions by long-lived species originating from arylnitrenes (see Staros, 1980 and page124). Washing by centrifugation and resuspension was repeated twice. The final pellets were resuspended in a small volume (100 – 200 µl) of the medium required and immediately assayed for uptake or binding (see III.2.5 and III.2.6).

When photolysis was repeated, membranes were washed only once after the first labeling. Then they were resuspended, deoxygenated and photolyzed again, as described.

An alternate procedure to remove free glucosides was gelfiltration on Sephadex G-10 as described by Evans & Diedrich (1980), with the following modifications: photolyzed samples were diluted to 2 ml with washing buffer (with or without BSA present) and applied onto a disposable Bio Rad column (0.8 x 4 cm) filled with Sephadex G-10 equilibrated in 300 mM mannitol, 100 mM KCl, 10 mM HEPES/Tris (pH 7.5) and 0.02 % KN\(_3\).

---

1) This assures maximum binding of all ligands when photolysis is started, assuming that the phlorizin derivatives display the same binding kinetics as Na\(^+\), Δψ-dependent phlorizin binding (Toggenburger et al., 1978).
The membranes (migrating with the void volume) were eluted with additional 3 ml of column buffer, collected in totally 10 ml of ice-cold washing buffer and centrifuged at 27,000 x g (intact vesicles) or at 60,000 x g (DOC extracts) for 30 min. Washing by resuspension and centrifugation was repeated, and the final pellets were proceeded as above.

Photolabeling of membranes with (3H)-labeled phlorizin derivatives followed the above procedure for photoinactivation, except that smaller volumes (100 - 300 μl) were photolyzed. To monitor the "wash-out" of (3H)-label, aliquots were withdrawn after each purification step (washing cycle) and counted. The final pellets were resuspended in a small volume (50 - 100 μl) of washing buffer, and, after withdrawing aliquots for protein determination and liquid scintillation counting, the membrane suspension was immediately solubilized in SDS.

III.2.9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was performed on 8.4 % or 15 % (w/v) slab gels according to Lämmli (1970), using a discontinuous Tris-glycine buffer system (Thoma, 1976).

a) Preparation of slab gels

The 2 mm thick slab gels consisted of

(i) a sampling (stacking) gel (track length, 1 cm);
   composition: (3.6 x 2.7), 58 mM Tris-HCl(pH 6.8), 0.1 % SDS

(ii) a running gel (length, 10 cm);
   composition: (8.4 x 2.7) or (15 x 2.7), 380 mM Tris-HCl(pH 8.8)
   0.1 % SDS

and were routinely prepared according to the mixing recipe given below:
### mixing recipe

<table>
<thead>
<tr>
<th></th>
<th>stacking gel</th>
<th>separating gels</th>
</tr>
</thead>
<tbody>
<tr>
<td>(3.6 x 2.7)</td>
<td>(8.4 x 2.7)</td>
<td>(15 x 2.7)</td>
</tr>
<tr>
<td>acrylamide</td>
<td>1.65 (^2)</td>
<td>8.25</td>
</tr>
<tr>
<td>Tris pH 8.8</td>
<td>--</td>
<td>7.5</td>
</tr>
<tr>
<td>pH 6.8</td>
<td>1.88</td>
<td>--</td>
</tr>
<tr>
<td>SDS</td>
<td>0.075</td>
<td>0.15</td>
</tr>
<tr>
<td>H(_2)O</td>
<td>9</td>
<td>12.75</td>
</tr>
<tr>
<td>glycerol</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>persulfate</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>TMED</td>
<td>0.007</td>
<td>0.018</td>
</tr>
</tbody>
</table>

\(^1\) The designation (A x V) is according to Hjertén (1962) and indicates:
- A: total monomer concentration (% w/v)
- V: degree of crosslinking (ratio of N, N'-methylenebis-acrylamide to acrylamide (in mole-%))

\(^2\) The numbers designate ml of stock solution and were calculated for one 2 mm thick gel.

The **stock solutions** used were:

- acrylamide: 30 g acrylamide, 0.81 g N,N'-methylene bis-acrylamide in 100 ml H\(_2\)O
- Tris-buffers: 1.545 M Tris-HCl (pH 8.8) or 0.50 M Tris-HCl (pH 6.8)
- glycerol: 80 % (v/v) in H\(_2\)O
- SDS: 20 % (w/v) in H\(_2\)O
- ammoniumpersulfate: 1.6 % (w/v) in H\(_2\)O (freshly prepared)

**b) Solubilization and denaturation**

The protein samples were solubilized for 60 s \(^1\) at 95°C in a solution of 10 mM Tris-HCl (pH 7.0), 10 % (v/v) mercaptoethanol, 10 % (v/v) glycerol, 4 % (w/v) SDS and 0.002 % bromphenol blue (SDS/protein ratio was approximately 2.5 g SDS/g protein). The solubilized samples were either directly applied onto the gel or frozen at -20°C \(^2\).

\(^1\) Prolonged boiling (>3 min) led to increased formation of aggregates (see Fig. 16B).

\(^2\) When frozen denatured samples were kept for longer than 4 weeks, degradation (proteolysis) and aggregation of proteins was observed sometimes.
c) Electrophoresis

Routinely 20 - 60 µg of membrane proteins (Lowry) were applied per slot. The electrophoresis buffer was 0.25 M Tris, 0.188 M glycine (pH 8.8) and 0.1 % SDS. Gels were run overnight at constant current (2 - 3 mA/cm²). A mixture of the following (¹⁴C)-methylated proteins (Amersham Radiochemical Centre, Ltd, Buckinghamshire, England) was used to calibrate the gel (fluorograms): myosin (M_r = 200,000 D), phosphorylase b (M_r = 100,000 and 92,500 D), bovine serum albumin (M_r = 69,000 D), ovalbumin (M_r = 46,000 D), carbonic anhydrase (M_r = 30,000), lysozyme (M_r = 14,300 D).

d) Staining and destaining of the gels

Gels were stained overnight in 25 % methanol, 7 % acetic acid and 0.1 % Coomassie Brilliant Blue R-250. Destaining was done in the same solution without Coomassie blue present, under several changes of the destaining solution.

e) Documentation

Gels were photographed with a Polaroid-Land-camera (MP 4) equipped with a yellow filter, using Polaroid film 665.

f) Fluorography

The DMSO/PPO procedure by Bonner & Laskey (1974) was followed for fluorography of stained and unstained gels, with minor modifications: Treated gels were sandwiched between two sheets of transparent household foil (Celloclair) and dried in a Pharmacia slab gel drier overnight (drying time could be reduced to 4 - 6 h by the use of a heat lamp, at 50 cm distance). Dried gels were exposed to a Kodak X-Omat R film at -70°C for up to 60 days.

g) Determination of radioactivity in (PPO treated) SDS-gels

The procedure of Schweizer et al. (1981) was followed for the determination of radioactivity in dried PPO-treated gels: Pieces of gels (max. 7 x 1 cm) were cut into 1 - 2 mm thick strips. The strips were rehydrated with 250 µl H_2O for 30 min at 40°C in a glass vial. 750 µl of NCS tissue solubilizer (Amersham)/H_2O (9 : 1, v/v) was added. After incubating for 5 h at 40°C, 15 ml scintillation cocktail (5.6 g PPO, 80 mg POPOP per 1 liter of toluene-ethanol (8 : 2, v/v) was added. After shaking for 5 h, the vials were counted.
III.2.10. Determination of protein

Protein was determined by the method of Lowry et al. (1951) using BSA as the standard (\(\Delta E_{280} = 6.6\) (1 cm, 1 %)). For a rapid estimation of protein (e.g. before DOC treatment) the method of Bradford (1976), the "Bio Rad assay", was used. To ensure complete solubilization of proteins, the membranes were dissolved in 50 µl of formic acid prior to the addition of the Bio Rad reagent.
III.3. SYNTHESES

III.3.1. General

Thin layer chromatography (TLC) and preparative layer chromatography (PLC) were performed on silica gel plates or strips (Merck, F_{254}', 0.25 mm and 2 mm thickness, respectively), using the following solvent systems:

solvent A = methylene chloride-methanol (4 : 1, v/v)

solvent B = n-propanol-water-ethylacetate (7 : 2 : 1, v/v).

In addition to fluorescence quenching at 254 nm, the following reagents were used to visualize the various compounds on TLC:

1) 16 N sulfuric acid, Δt (for sugars)
2) vanillin (1 % in sulfuric acid, w/v), Δt (for phenols)
3) ninhydrin spray, Δt (for compounds containing free amino groups).

For column chromatography silica gel 60 (Merck; 0.040 - 0.063 (230 - 400 mesh ASTM) or 0.063 - 0.200 mm (70 - 230 mesh ASTM) was used.

¹H NMR 100 MHz-spectra were recorded on a Varian HA-100, 90 MHz-spectra on a Bruker Spectrospin WH-90 spectrometer, using (CH₃)₄Si as internal standard. Chemical shifts are given in ppm and the coupling constants J in Hz. The multiplicity of the signals is given as follows: s = singlet, d = doublet, t = triplet, m = multiplet. The signals of acyl-, hydroxyl- and aminoprotons were eliminated by shaking in D₂O.

IR-spectra were measured on a Perkin-Elmer 283 spectrometer and UV-spectra on a Pye Unicam UV-spectrometer (SP 1700).

Optimal rotations were determined on a Perkin-Elmer polarimeter (M141) at 25°. The rotations were recorded at 365, 436, 546 and 589 nm. The reciprocal values were linearly extrapolated to the Na-D-line. The concentration (% w/v) of the solution and the solvent are given in brackets.

Massspectra were recorded on a Hitachi-Elmer RMU-6A or RMU-6D spectrometer in the laboratory of Prof. J. Seibl. The relative intensities of the masspeaks (m/e) are given in % of the basepeak (100 %) and are shown in brackets.
Melting points were taken on a Tottoli capillary melting point apparatus and are uncorrected.

All reactions involving arylazido compounds were carried out in subdued light.

III.3.2. Purification and analytical data of phlorizin

Unlabelled phlorizin (dihydrate; obtained from Fluka AG, Buchs (SG), ICN/K&K (Plainview, N.Y.) and Roth-Chemie GmbH Karlsruhe, Ger.) was separated from traces of phloretin and of coloured low-\( R_f \)-material (presumably polymers of the glucoside) by silica gel column chromatography, using solvent A as the eluent. Repetitive crystallization from hot water gave the dihydrate as fine white needles. For synthetic purposes it was recrystallized out of dry ethylacetate and dried for 72 h at room temperature at 10\(^{-3}\) Torr over P\(_2\)O\(_5\) to yield the monohydrate, as evidenced from \( ^1H \) NMR;

\[
\begin{align*}
\text{mp} & 120 - 124^\circ \text{C (Phlz • 2 H}_2\text{O)}, \text{lit. 108 - 112^\circ \text{C (Beilstein, 1920);}} \\
& 168 - 170^\circ \text{C (anhydrous Phlz), lit. 160 - 170^\circ \text{C (Kiss & Spiegelberg, 1964), different values;}} \\
& [\alpha]_D^{25} = -49.3^\circ \text{ (1.62, acetone), lit.: } [\alpha]_D^{25} = -51.2^\circ \text{ (acetone) (Beilstein, 1920);} \\
& \text{pk*}^{\text{MCS}} = 9.22 \text{ (in 1 ml MCS/H}_2\text{O, 80 : 20);} \\
& \text{IR (KBr) 3650-3100, 2910, 2700, 1628, 1515, 1455, 1440, 1400, 1372, 1250, 1202, 1175, 1078, 1038, 1024, 1000, 986, 900, 830, 815 cm}^{-1}; \\
& \text{MS 18(40), 27(12), 29(16), 31(15), 39(19), 41(10), 42(10), 43(32), 44(32), 55(14), 57(20), 60(30), 65(10), 69(10), 73(13), 77(19), 94(16), 107(100), 120(12), 166(20), 230(13)(M}+{^+}-206); \\
& \text{\( ^1H \) NMR (acetone-d}_6) \delta 2.88 \text{ (t, J=7.5, H}_2\text{C (benzylic)); 3.16 (s, 1 or 2 H}_2\text{O); 3.51 (t, J=7.5, H}_2\text{C (vic C}=0\); 3.4 - 4.0 (m, 7 H: H-C(2,3,4,5), H}_2\text{C(6), HO-C(6)); 4.4 - 4.7 (br, 3, HO-C(2,3,4)); 5.10 (d, br, J=8, H-C(1)); 6.13 (AB-system, }\nu_{\text{B}}=6.0, \nu_{\text{A}}=6.26, J=3, H-C(3',5')}; 6.91 (AA'BB'-system, }\nu_{\text{A''}}=7.10, \nu_{\text{B''}}=6.72, J_{\text{ortho}}=8, J_{\text{meta}}=2, H-C(2,6), H-C(3,5)(phenolic)); 8.03 (s, HO-C(4)(phenolic); 9.52 (s, HO-C(4')); 13.63 (s, sharp, HO-C(6'), chelated).} 
\end{align*}
\]
The IR and NMR-spectra served as standards for the interpretation of the corresponding spectra of the phlorizin derivatives synthesized. For brevity however, only the relevant signals of the spectra of the derivatives will be reported. On the advice of Prof. Seibl (Laboratorium für Organische Chemie ETH-Z) only the MS spectra of acetylated or protected derivatives were recorded.

III.3.3. Synthesis of 6-amino-6-deoxyphlorizin

The synthesis of 6-amino-6-deoxyphlorizin is shown in Fig. 4 on page 38.

6-O-Mesylphlorizin-(hydrate) (6):

The full details of the synthesis of 6 by introducing protecting groups first, i.e., via the reaction sequence 2 - 5, have been reported elsewhere (Hosang, 1976) and will not be repeated. However, this reaction sequence will be discussed briefly in III.4.1a (page 40).

The preparation of 6 by mesylation of non protected phlorizin was carried out as follows: To a stirred solution of phlorizin monohydrate (90 mg, 0.198 mmol) in 6 ml dry pyridine cooled to -20°C were added with a Hamilton syringe 28 μl (0.356 mmol) methanesulfonyl chloride. The reaction mixture was allowed to reach room temperature within 30 min, at which temperature it was kept for further 3 h. Then the solvent was removed by azeotropic distillation with toluene, and the resin-like residue obtained was chromatographed on a silica gel column using solvent A as the eluent. After drying for 24 h at room temperature over P₂O₅, the mesylate 6 was obtained as a glass-like resin (51% yield, based on starting material reacted). It was chromatographically pure as judged from TLC (Rₐ in solvent A, 0.36). No attempts were made to further characterize the dimesylates and monomesylated isomers, which all showed higher Rₐ-values on TLC. Crystallization of 6 out of the common solvents failed. The analytical data reported below are identical to the corresponding ones of the 6-mesylate as obtained via 2 - 5. This clearly establishes the authenticity of 6: mp, 80 - 85°C (hydrate?); mp, 140 - 142°C; IR (KBr) 1350, 1175 cm⁻¹ (SO₂). ¹H NMR (acetone-d₆) J 2.90 (t, 2, H₂C(benzylic)); 3.04 (s, 3, H₃C (mesyl-)); 3.49 (t, 2, H₂C(vic C=O)); 3.3 - 3.7 (m, 3, H-C(2,3,4,)); 3.75 (m, 1, H-C(5)); 4.52 (AB-system, νₐ=4.60, ν₃=4.44, J_gem=11, J_vic,A=2, J_vic,B=5, H₂C(6)); ν=5.22 (d, 1, J=7, H-C(1)); 6.17 (AB-system, ν=A=6.30, ν=B=6.04, J_meta=2, H-C(3',5')); 6.93 (AA'BB'-system, νₐ,νₐ₁=7.10, νₐ,νₐ₂=6.76, H-C(2,6), H-C (3,5), respectively); 4 - 7 (smeared, 5, HO-C(2,3,4), HO-C(4) phenolic, HO-C(4')); 13.69 (s, 1, HO-C(6'), chelated).
4'-O-Mesylphlorizin (7):

When the mesylation of non protected phlorizin monohydrate 1 was carried out in 1,2-dimethoxyethane/triethylamine instead of pyridine, the phenolic 4'-mesylate 7, a yellow resin, was isolated as the main product; TLC, Rf in 15 % (v/v) methanol-methylene chloride, 0.20 (indistinguishable from the Rf of the 6-mesylate 6); mp 108 - 110°C (hydrate?), mp 216°C (dec); pkMCS = 9.96 (in 1 ml MCS/H2O, 80/20; cf pkMCS of phlorizin, 9.22); IR (KBr) 1365, 1185 cm⁻¹ (-SO₂O-). ¹H NMR (acetone-d₆) δ 3.34 (s, 3, H-C(mesyl-)); 4.30, 4.52, 4.62 (s, br, HO-C (2,3,4), gluc); AB-system (H-C(3',5')) is shifted from 6.13 (υA=6.25, υB=5.99, phlorizin) to 6.67 (υA=6.77, υB=6.57); the signal at 9.52 (H0-C(4')) is missing.

6-O-p-Toluenesulfonylphlorizin (pTs-Phlz, 8):

Tosylation of non protected phlorizin 1 was essentially carried out as described for the direct preparation of the corresponding mesylate 6, with the following modifications: To a solution of phlorizin monohydrate 1 (4.67 g, 10.7 mmol) in 195 ml dry pyridine at 2°C were added under stirring 4.09 g (20.3 mmol) of solid p-toluenesulfonyl chloride in small portions. After stirring at room temperature in the dark for 20 h, the yellow solution was evaporated in vacuo to 40 ml and poured into 200 ml ice-cold water containing 20 ml 6N 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 the eluent. The fractions containing 6-O-tosylphlorizin 8 (Rf 0.39 on TLC, solvent A) were combined and evaporated to dryness. Unreacted phlorizin was recovered from latter fractions. The reaction was not brought to completion, since the amount of ditosylated compounds increased strongly towards the end of the reaction. No attempts were made to further characterize the ditosylates and monotosylated isomers, which all showed higher Rf values on TLC. The colourless 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 of o-hydroxyarylketone), 1605, 1515 (aromat), 1350, 1175 cm⁻¹ (-SO₂O-). ¹H NMR (acetone-d₆) δ 2.31 (s, 3, CH₃(tosylate)); 4.34 (AB-system, υA=4.44, υB=4.24, J₆,6=11, Jvic,A=3, Jvic,B=5.5, H₂C(6)OSO₂-); 4.4 - 4.8 (br, 3, HO-C(2,3,4,)); 6.10 (AB-system,
\[ \nu_A = 6.15, \nu_B = 6.05, \text{H-C}(3', 5'); 7.47 (\text{AA'BB'-system, } \nu_A \nu_A' = 7.70, \nu_B \nu_B' = 7.24, J = 8, 4 \text{ aromatic H (tosyl-)}); \text{the signals of the phenolic OH at 8.04 (HO-C(4)), 9.48 (HO-C(4')) and 13.64 (HO-C(6'), chelated) remain.} \]
\[ [\alpha]_{D}^{25} = -39.3^\circ (1.74, \text{ethanol}). \]

6-Azido-6-deoxyphlorizin (Azido-Phlz, 9):

6-O-p-Toluenesulfonylphlorizin (8) (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 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 the eluent. The fractions containing the product (TLC, \( R_f \) in solvent A, 0.36) were pooled and taken to dryness. The resulting azide 9, 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. \(^1\)H NMR (acetone-\( \text{d}_6 \)) spectrum showed a shift of the signals at \( \delta 4.1 - 4.5 \) (\( \text{H}_2\text{C}(6)\) OSO\(_2\)-) to 3.3 - 3.8 (m, 8, \text{H-C}(2,3,4,5), \text{H}_2\text{C}(6)\text{N}_3, \text{H}_2\text{C(vic C=O)}; [\alpha]_{D}^{25} = -72.0^\circ (2.08, \text{ethanol}). \]

6-Amino-6-deoxyphlorizin (hydrochloride) (Amino-Phlz, 10):

A solution of 6-azido-6-deoxyphlorizin 9 (732 mg, 1.59 mmol) in 8 ml ethanol and 0.69 ml 2 N HCl (1.75 mmol) was hydrogenated under atmospheric pressure at room temperature in the presence of 80 mg 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 10 crystallized as hygroscopic hairlike white needles. The motherliquor was purified by PLC using solvent B as the eluent. 654 mg of the product 10 were isolated (overall yield, 91%). It showed only one spot on TLC (\( R_f \) in solvent B, 0.51) and was ninhydrin positive; mp 135°C (soften), 165 - 167°C (dec); IR (KBr) 3420, 3320 (br, OH, NH); 2920, 2700 (br, OH in intramolecular H-bond), 1628 (C=O of o-hydroxyarylketone), no band at 2105 cm\(^{-1}\). \(^1\)H NMR (CD\(_3\)OD) \( \delta 2.87 \) (t, 2, \text{H}_2\text{C(benzylic)}); 3.0 - 3.60 (m, 6, \text{H-G}(2,3,4,5), \text{H}_2\text{C(vic C=O)}); 3.76 (AB-system, 2, \( \nu_A = 3.82, \nu_B = 3.69, J_{\text{gem}} = 9, J_{\text{vic,A}} = 2, J_{\text{vic,B}} = 3, \text{H}_2\text{C}(6)) \text{NH}_3^+\); 5.15 (dd, 1, \text{H-C}(1)); 6.11 (AB-sy-
stem, $\nu_A = 6.23$, $\nu_B = 5.99$, $J = 2$, H-C(3',5'); 6.84 (AA'BB'-system, $\nu_A, \nu_B = 7.01$, $\nu_{AB} = 6.67$, $J_{ortho} = 9$, $J_{meta} = 2$, H-C(2,6), H-C(3,5), phenolic); the signals of the phenolic OH-groups cannot be seen. $[\alpha]_{D}^{25} = -32.7 \pm 0.5^\circ$ (1.7, ethanol).

6-Acetamido-6-deoxyphlorizin monohydrate (Acetamido-Phlz, 11):

For further characterization of the amine 10, the acetamide 11 was prepared by applying the procedure for acylation of amino acids (Sheehan et al., 1965), using N-ethyl-N'-(3-dimethylaminopropyl) carbodi-imide 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 ($R_f$ in solvent A, 0.21) and was ninhydrin negative; mp 236°C (dec); IR (KBr) 3440 (br, OH, NH), 3220, 3120 (sh, NH ass.), 1655 (sh, sec. amide ass. 1620, 1605, 1570 cm⁻¹ (sh, sec. amide ass.). $^1$H NMR (acetone-d₆) $\delta$ 1.95 (s, 3, CH₃CON), 7.54 (br, 1, exchangeable with D₂O, NHCO). $[\alpha]_{D}^{25} = -57.9^\circ$ (1.85, ethanol).

Anal. Calcd for C₂₃H₂₇NO₁₀·H₂O: C, 55.76; H, 5.90; N, 2.82.

Found : C, 55.54; H, 5.72; N, 2.65.


N-(4-Azido-2-nitrophenyl)-β-alanine (NAP-β-Ala, 12):

NAP-β-alanine 12 was synthesized by a modification of a previously published procedure (Jeng & Guillory, 1975), using DMSO/triethylamine instead of water-ethanol/sodium carbonate and stirring at 60°C for 24 h in the dark. Crystallization in ethanol-water yielded 12 as clusters of dark red crystals (77 % yield). TLC: $R_f$ in solvent A, 0.43; mp 138 - 140°C (dec), lit. 142.5 - 145°C (Jeng & Guillory, 1975); IR (KBr) 3380, 2920, 2105, 2070 (arylazide), 1715 (COOH sat.), 1630, 1568 (sec. amine) 1520, 1350 cm⁻¹ (NO₂). $^1$H NMR (acetone-d₆) $\delta$ 2.78 (t, 2, J=7 Hz, H₂C(2)), 3.74 (q, 2, J=7 Hz, H₂C(3)), 7.25 (m, 2, H-C (5,6) (NAP)), 7.75 (d, 1, H-C(3), (NAP)), 8.20 (br, HN). UV max (MeOH): 260 (ε 21900), 280 (sh), 460 (ε 5300) nm.

Anal. Calcd for C₉H₉N₅O₄: C, 43.03; H, 3.61; N, 27.88.

Found : C, 43.13; H, 3.74; N, 27.79.
N-(4-Azido-2-nitrophenyl)-6-amino-6-deoxyphlorizin (NAP-N-Phlz, 13):

To a solution of the hydrochloride (94 mg, 0.2 mmol) and triethylamine (31 μl, 0.22 mmol) in DMSO (500 μl) was added 4-fluoro-3-nitrophenylazide (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 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 the eluent, and crystallization of the main fraction from ethanol-water gave 96 mg (80.3%) of 13 as silky red crystals. The compound showed one spot on TLC (Rf in solvent A, 0.39) and was ninhydrin negative; mp 130°C (dec); IR (KBr) 3380 (br, OH), 2120 (s, amylazide), 1570 (NH def), 1520, 1415, 1350, 1300, 1250 cm⁻¹ (NO₂).

UV max (MeOH): 228 (ε 23300), 268 (sh), 282 (ε 29000), 458 (ε 4750) nm.

1H NMR (acetone-d₆): 3.3 - 3.6 (m, 6, H-C(2,3,4,5), H₂C(vic C=O)); 3.6 - 4.05 (m, 2, H₂C(6)-N-); 7.12 (AB-system, H-C(5,6), NAP); 7.67 (d, 1, H-C(3), NAP): 8.35 (t, br, 1-NH-).

Anal. Calcd for C₂₇H₂₇N₅O₁₁·H₂O: 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-alanyl-6-amino-6-deoxyphlorizin (NAP-β-Ala-N-Phlz, 14):

To a solution of CDI (35 mg, 0.22 mmol) in dry THF (0.5 ml) was added NAP-β-alanine (55 mg, 0.22 mmol) as a solid. The reaction mixture was stirred for 15 min at 22°C. Then, after treating 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 three 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; 3 runs) and crystallization from methanol-water gave pure 14 as soft red needleless (82 mg, 60%); TLC: Rf in solvent A, 0.37; mp 125 - 130°C (dec); IR (KBr) 3380 (NH, OH),
2120 (arylazide), 1650 (sh, sec. amide), 1570 (amine), 1560 (sh, sec. amide), 1520, 1475(s), 1415, 1355(N0o), 1300, 1260 cm⁻¹. UV (MeOH): 228 (ε 23300), 268, (sh), 282 (ε 29500), 458 (ε 4760) nm. ¹H NMR δ 2.68 (t, 2, CH₂CO₂-(β-alanyl)); 3.0 – 3.8 (m, 10, H₂C(6)N, H₂CNH (β-alanyl), H₂C(vic C=O phlz), H-C(2,3,4,5)); 7.16 (AB-system, νₐ=7.23, νₗ=7.10, Jₐb=9, JₐA, long range=2.5, A=H-C(5), B=H-C(6), NAP); 7.54 (br, 1, NHCO); 7.70 (d, 1, H-C(3), NAP); 8.24 (NH(β-alanyl)).

Found : C, 52.92; H, 4.88; N, 12.01.

N-(4-Azido-2-nitrophenyl)-β-alanyl-6-O-phlorizin (NAP-β-Ala-Phlz, 15):

To a solution of CDI (370 mg, 2.28 mmol) in dry THF (5 ml) was added NAP-β-alanine (12) (570 mg, 2.28 mmol). After stirring for 15 min at 25°C, phlorizin monohydrate (693 mg, 1.52 mmol) was added in THF (4 ml) and allowed to react for 26 h at 50°C in the dark (as decomposition occurred, the reaction was stopped before all phlorizin had reacted, as evident from TLC). Evaporation in vacuo gave a red residue which was washed three times with chloroform (10 ml) to eliminate unreacted NAP-β-alanyl derivatives and then chromatographed over a silica gel column, using a gradient of methylene chloride-methanol, 12 : 1 to 4 : 1, as the eluent. The fractions containing pure 15 (R₇ in solvent A, 0.44; vanillin positive) were combined and taken to dryness. Mixed fractions were purified by repetitive PLC (3 runs), using methylene chloride-methanol, 7 : 1, or ethylacetate as the eluents (no attempts were made to further characterize the corresponding diesters and monoester isomers, which all showed higher R₇-values on TLC). Crystallization from ethanol-water gave 15 as cottonlike, red crystals (592 mg; 80.5 % relative to the reacted starting material, 56.7 % relative to the total starting material); mp 88 – 90°C (soften), 116 – 118°C (dec); IR (KBr) 3380 (br, OH), 2120 (arylazide), 1730 (ester), 1570 (amine), 1520, 1350 cm⁻¹ (NO₂). UV max (MeOH): 228 (ε 23300), 268 (sh), 282 (ε 29500), 459 (ε 4900) nm. ¹H NMR (acetone-d₆) δ 2.76 (t, 2, CH₂CO₂(β-alanyl)); 3.3-3.9 (m, 6, H-C(2,3,4,5), H₂C(vic C=O, phlz)); 3.69 (q, 2, CH₂NH(β-alanyl)); 4.39 (AB-system, 2, νₐ=4.48, νₗ=4.29, Jₗ_gem=12, Jₗ_vic,ₐ=2.5, J_vic,ₗ=6.5, H₂C(6)COO); 7.16 (AB-system, νₐ=7.23, νₗ=7.09, JₐB=9, JₐA, long range=2.5, A=H-C(5), B=H-C(6), NAP); 7.70 (d, 1, H-C(3), NAP); 8.18 (NH(β-alanyl)).

Anal. Calcd for C₃₀H₃₁N₅O₁₃·H₂O: C, 52.40; H, 4.84; N, 10.19.
Found : C, 52.60; H, 4.61; N, 9.93.
N-(4-Azido-2-nitrophenyl)-β-alanyl-6-O-para-phlorizin (NAP-β-Ala-p-Phlz, 16):

The synthesis of 16 followed exactly the procedure described for 15, except that it was performed on a microscale. 16 (Rf in solvent A, 0.33) was obtained in a yield of 36% (relative to the total starting material) and could not be crystallized. 1H NMR (acetone-d6) showed a monoester of NAP-β-alanine in position C-6 of the glucopyranosyl moiety of para-phlorizin: δ 4.32 (AB-system, 2, 2.40, 2.26, H2C(6)COO).

III.3.5. Synthesis of 4-Azidophlorizin (23)

The synthesis and use of phlorizin derivatives modified in the ring B of the aglycone moiety was part of a collaborative project with Dr. D.F. Diedrich (University of Kentucky, College of Medicine, Dept. of Pharmacology, Lexington, KY). The synthetic work was partially carried out in Lexington (Fig. 5, compounds 17, 22 and 23) and partially in Zurich (Fig. 5, compounds 18-21). For the sake of completeness the whole synthesis is reported (see also Gibbs et al., 1981).

2',4',6'-Trihydroxy-4-nitro-dihydrochalcone (4-nitrophloretin, 17):

Anhydrous phloroglucinol (21 g, 170 mmol) and 30 g (170 mmol) 4-nitrohydrocinnamonic nitrile (Zemplén et al., 1928) were condensed in a Hoesch reaction essentially as described for the preparation of phloroacetophenone (Gulati et al., 1935) except that 2 l of ether was used as solvent and the ketimine hydrochloride was allowed to precipitate for one week at 0°C. This solid was boiled in 4 l water to form the water insoluble crystalline ketone (22 g; 43%) which was of high purity (mp 268-272°C) and used directly in subsequent reactions. The analytical sample was recrystallized (without charcoal) from aqueous methanol to yield yellow needles, mp 274-276°C; Rf = 0.20 in benzene-ethanol-acetic acid, 96:3:1(v/v); 1H NMR (CD3OD)δ 3.24 (AA'BB'-system, 4, 3.40, 3.07, JAB=7.5Hz, A,A'=H2C(vic C=O), B,B'=H2C(benzylic)); 5.80 (s, 2, H-C(3',5')); 7.80 (AA'BB'-system, 4, 8.12, 8.48, JAB=9Hz, A,A'=H-C(3,5), B,B'=H-C(2,6)).

Anal. Calcd for $C_{15}H_{13}O_6$ N: C, 59.4; H, 4.32; N, 4.62

Found: C, 59.3; H, 4.40; N, 4.69.
2'-O-(β-D-glucopyranosyl)-4-nitrophloretin (4-nitrophlorizin, 21):

17 (4.5 g, 15 mmoles) was glycosylated directly with 9.3 g (22.6 mmoles) acetobromglucose in 120 ml acetone and 90 ml of cold 0.25 N KOH. After 24 hours in the dark at 20°, the mixture was poured into 2 l ice containing 2 ml acetic acid. The product was collected after 4 hours, air dried, and extracted twice with 50 ml of chloroform to recover 3.7 g of insoluble 17. The extract, containing a mixture of 18, 19, 20 (see Fig. 5) and various glucose acetates (3.1 g), was concentrated to 10 ml and subjected to flash chromatography (Still et al., 1978) on a column of Silica Gel 60 (4.5 x 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 % propanol in chloroform. TLC was used to monitor the eluate; volumes between 280 - 360 ml, 380 - 510 ml and 680 - 1600 ml were pooled, concentrated to dryness and the residues crystallized from methanol to yield 0.20 g (8 %) of 20 (mp 197 - 198°), 0.39 g (23 %) of 19 (mp 185 - 186°) and 0.31 g (18 %) of 18 (mp 88 - 92°) respectively. Rf values of the three products in 3 % n-propanol in chloroform were 0.40, 0.29 and 0.14. Each derivative was ultrapurified on small Silica Gel columns and crystallized from methanol.

Structures for 18 and 20 were confirmed from 1H NMR spectra. 18 gave signals characteristic of a mono-(tetraacetyl)-glucopyranoside; 2'-substitution was demonstrated by the AB-system at δ 6.03 (νA =6.06, νB =5.98, J=2 Hz, H-C(3',5')), the signal of the corresponding H-ligands of 17 being a singlet at δ 5.80. 20 displayed the signals of a di-(tetraacetyl)-glucopyranoside; the AB-system at δ 6.13 (νA =6.23, νB =6.03, H-C(3',5')) indicated substitution in positions 2' and 4'.

Found for 18 : C, 54.80; H, 5.06; N, 2.04.
19 : C, 55.06; H, 5.02; N, 2.20.

Calcd for C43H49O24N: C, 53.58; H, 5.12; N, 1.45.
Found for 20 : C, 53.61; H, 5.17; N, 1.39.

To saponify 18, 1 mmole was treated in vacuo, in subdued light, with 2 ml cold 1 N sodium methoxide in methanol. After 15 min at room temperature, the mixture was neutralized under nitrogen with 0.3 N HCl. The free glucoside 21 crystallized in about 90 % yield and was recrystal-
lized as trihydrate from 20% aqueous methanol; crystallized from methanol as hard, pale yellow prisms, mp 225°. \( R_f \) in 30% n-propanol in chloroform, 0.39.

Anal. Calcd for \( C_{21}H_{23}O_{11}N \): C, 54.19; H, 4.98; N, 3.01.

Found : C, 54.01; H, 5.11; N, 2.92.

2'-O-(\( \beta \)-D-glucopyranosyl)-4-aminophloretin (4-aminophlorizin, 22):

The trihydrate of 21 (200 mg; 0.38 mmole) was hydrogenated at atmospheric pressure in 40 ml methanol with 40 mg of 10% Pd/C as catalyst. The tetrahydrate of 22 was obtained from dilute methanol as pale yellow prisms (\( R_f \) in 30% n-propanol in chloroform, 0.30) which after drying in vacuo at 60°C melted at 139 - 142°. The product was converted to the azide without further purification.

2'-O-(\( \beta \)-D-glucopyranosyl)-4-azidophloretin (4-azidophlorizin, 23):

All steps were performed in subdued light. 22 (43.5 mg) in 5 ml acetone and 2 ml 0.5 N HCl (0°C) was treated with 0.5 ml of 0.54 N sodium nitrite added over a period of 15 s. After 6 min at 0°C or less, 0.5 ml of cold 2.0 N 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 23 (39 mg; 85%). Soft colorless needles were obtained from dilute methanol as the monohydrate; mp 150 - 155°C, after softening at 108 - 110°C. Anhydrous 23 could not be obtained without decomposition. \( R_f \) in 30% n-propanol in chloroform, 0.42; IR (KBr) 2130 and 1280 cm\(^{-1}\). \(^1\)H NMR spectrum was the same as that of phlorizin, except for the shifts of (i) the AA'BB'-system (H-C(3,5), H-C(2,6)) from \( \delta \) 6.91 (\( \nu_{A'A}, =7.10, \nu_{B'B'} ,=6.72 \)) to 7.13 (\( \nu_{A'A}, =7.31, \nu_{B'B'} ,=6.95 \)) and (ii) the triplet (H\(_2\)C(benzylic)) from 2.88 to 2.95. The signal of HO-C(4) was missing. UV\(_{\text{max}}\) (EtOH or H\(_2\)O) 251 (azido group), 285 (shifts to 328 nm in alkaline buffer (\( \varepsilon =2750, \text{in 0.05 M sodium borate, pH 9.3} \)).
III.3.6. Preparation and stability of (\(^3\)H)-labeled phlorizin derivatives

In the synthesis of radioactively labeled photoreactive ligands one is advised to introduce the radioactive marker in close proximity to the photoreactive group. This is to minimize loss of radiolabel from a covalently labeled protein during the procedures following photolysis.

In the case of NAP-\(\beta\)-Ala-Phlz the \(\beta\)-alanyl-spacer was used to introduce the radioactivity (\(^3\)H). Radiolabel on the glucopyranosyl moiety of phlorizin was likely to be cleaved by the esterolytic activities shown to be present in the intestinal mucosa (Fernandez-Lopez et al., 1976; Négrel et al., 1976); radiolabel located on the aglucone moiety was likely to be susceptible also to cleavage by the phlorizin hydrolase present in intestinal brush border membranes (Malathi & Crane, 1969).

In the case of 4-azido-phlorizin the radiolabel was for the same reason introduced in the aglucone moiety.

Tritium was chosen as the radiolabel in both syntheses because of the high specific activity available and the ease of incorporation.

a) Preparation of labeled compounds

All of the following steps were performed in subdued light.

NAP-\(\beta\)-(3-\(^3\)H)-alanine ((\(^3\)H)-12):

Commercially available \(\beta\)-(3-\(^3\)H)-alanine (32-36 Ci/mmol) was diluted with cold \(\beta\)-alanine to the specific activity desired, usually 2-4 Ci/mmol, concentrated in vacuo and redissolved in a small amount of DMSO (10-30 \(\mu\)l) containing 1.2 equivalents of triethylamine. After the addition of excess (5-10 fold) 4-fluoro-3-nitrophenyl azide, the suspension was heated in a sealed micro reagent tube for 20 h as described for 12. The resulting clear red solution was taken to dryness, extracted with acetone and then methanol and the combined solvents were dried down, yielding a solid residue. This residue was taken up in methanol and chromatographed on a TLC plate using A as solvent. Labeled NAP-\(\beta\)-alanine (\(R_f\) in solvent A, 0.43) was recovered from the plate by elution with methanol (55 - 60 \% yield). The pure compound was either stored in the dark at \(-20^\circ\)C as an ethanolic solution or, after carefully drying, immediately used in the next step.
NAP-β-(3-^3H)-alanyl-6-O-phlorizin((^3H)-15) and NAP-β-(3-^3H)-alanyl-6-O-para-phlorizin((^3H)-16):  

NAP-β-(3-^3H)-alanine (2-4 Ci/mmol was carefully dried at reduced pressure over P_2O_5 and redissolved in 15-30 µl of dry THF. After adding further 5-10 µl of dry THF containing 1.2 equivalents of freshly dissolved CDI and allowing the reaction mixture to stand at room temperature for 15 min, 2 equivalents of carefully dried (anhydrous) phlorizin or para-phlorizin in 5 µl of dry THF were added. The solution was allowed to react for 12 h at 50°C either under magnetic stirring on an oil bath, or with periodical shaking in a thermostated oven. If necessary small amounts of THF were added to the reaction mixtures to prevent them from coming to dryness. Then the reaction mixtures were directly applied onto TLC plates and chromatographed in two runs, using methylene chloride-methanol, 7 : 1 (v/v) as the eluent. (^3H)-15 and (^3H)-16 (R_f in solvent A, 0.44 and 0.33, respectively) were recovered from the plate by elution with ethyl acetate. After blowing the solution down with a stream of N_2, the red residue was redissolved in dry acetone and filtered through a Pasteur pipette with a plug of cotton-wool. This procedure was repeated several times to separate from traces of silica gel. The yields of these micro scale preparations never exceeded 15 % (as compared to 57 % obtained on a large scale, see 16). The radiochemical purity (initially > 98.3 %) was assessed by TLC (using ethyl acetate as the eluent) followed by monitoring the distribution of the radioactivity on the plate by scraping out appropriate segments and by liquid scintillation counting of powdered and methanol (150 µl) treated samples.  

The pure NAP-β-alanyl derivatives were stored in the dark at -20°C as diluted 10 % benzene in ethanol solutions. Radiochemical purity was periodically (every 3 - 6 months) rechecked and the compounds were rechromatographed if necessary.  

^1) There are several possible explanations for the low yields obtained: (i) decomposition of reactants and products in micro scale reactions seemed to be increased; (ii) for technical reasons micro scale reaction mixtures were 10 - 20 fold diluted with respect to reactant concentrations as compared to large scale preparations.
(3H)-4-Azidophlorizin (3H)-23:

The Amersham Corporation, Arlington Heights, IL., incorporated tritium into the anhydrous 4-amine 22 by their TR-7 procedure, which is thought to catalytically exchange benzylic hydrogens. The labeled amine (65 mg) was chromatographed on a silica gel 60 column (2 x 25 cm) in chloroform; 100 ml volumes of 10, 20 and 30 % methanol in chloroform were used to elute radiochemically pure (3H)-4-aminophlorizin. The compound crystallized from aqueous methanol as colorless well formed needles (48 mg).

Radiochemically pure 4-azide, prepared from the tritiated 4-amine as described for the synthesis of unlabeled 4-azide, was obtained by chromatography on a silica gel 60 column equilibrated in chloroform. 10 % Methanol in chloroform as eluent gave the tritiated azide 23 preceding, and sharply separated from, the residual amine and other products. The pure azide was stored in the dark at -20°C as a 10 % ethanol in benzene solution. Radiochemical purity was verified by TLC radiography. (3H)-23 had a specific activity of 0.405 mCi/mmol.

b) Stability of (3H)-phlorizin and its photolabile (3H)-derivatives in the presence of brush border membranes in the dark

Control experiments showed that, under the conditions chosen (100 µM label concentration, 2 mg protein/ml, pH 7.0), the (combined) esterase and phlorizin hydrolase activities were low: after a 5 min incubation at room temperature in the dark, 100 ± 2, 96 ± 1, and 96 ± 2 % of the initial label present was still identified with the original compound, i.e., (3H)-phlorizin, (3H)-4-azido-phlorizin and (3H)-NAP-β-Ala-Phlz, respectively, as determined by TLC and scintillation counting (see Methods). When incubation at room temperature in the presence of membranes lasted 120 min, 89 ± 2, 74 ± 3, and 69 ± 3 %, respectively, of the label molecules initially present were still intact.
Fig. 4. Synthetic route to the photolabile derivatives of phlorizin modified in position 6 of the glucopyranosyl moiety.
Fig. 5. Structural identification of the phlorizin derivatives modified in the aglycone moiety.
III.4. COMMENTS ON THE SYNTHESES


In principle, 6-0-p-toluenesulfonylphlorizin 8, a compound suited for further functionalization of phlorizin in position 6, could be prepared (i) by direct selective tosylation of unprotected phlorizin, or (ii) by introducing suitable protecting groups first (for (i), see Fig. 4).

Because of its straightforward route (i) was preferred. However several products (in addition to the 6-0-tosylate 8, probably several monotosyl isomers and ditosylated isomers) were isolated in preliminary attempts to prepare the 6-0-tosylate 6 by selective tosylation of 1. The procedure followed was the one described for the selective tosylation of glucose in position 6, using tosylchloride in pyridine at low temperature (Ball & Parrish, 1968). Therefore introduction of protecting groups seemed indispensable in order to guarantee selective functionalization in position 6.

a) Introduction of protecting groups

In order to protect selectively the hydroxyl groups in C-4 and C-6, phlorizin 1 was converted into 4,6-0-benzylidene phlorizin 2, using ZnCl₂ in benzaldehyde, according to the procedure (Zervas, 1931; Fletcher, 1962) used for the preparation of 4,6-0-benzylidene-D-glucopyranose. The yield of the acetal 2 never exceeded 59 %, due to the formation of a side product, probably 2,4'-0-benzylidene phlorizin, in yields of 30 to 60 %.

Acetylation of 2 using excess acetic anhydride in pyridine (Wolfrom & Thompson, 1963a) gave 4,6-0-benzylidene-2,3,4',4,6'-pentaacetylphlorizin 3 in a yield of 92 %.

2,3,4',4,6'-Pentaacetylphlorizin 4 was obtained by removal of the benzylidene group by catalytic hydrogenation over Pd/C in ethylacetate (Spichtig & Vasella, 1971). This compound was believed to lend itself well for selective functionalization in position 6, because a primary hydroxyl group should be more reactive than a secondary hydroxyl group. Since isolation of a homogeneous product failed,
probably due to acetyl group migration, the raw product 4 was used for the next step. In addition the hydrogenolysis reaction could not be reliably reproduced. In search of a better catalyst, only Pd-black in polar solvents was found to give adequate results, the other catalysts tested were Pt, PtO₂.

The pentaacetylderivative 4 was smoothly converted into the mesylate 5, by the use of mesylchloride/triethylamine in methylene chloride at low temperature (Crossland & Servis, 1970), whereas, for unknown reasons, the preparation of the corresponding tosylate, using tosylchloride in pyridine (Ball & Parrish, 1968), only gave unsatisfactory results. Surprisingly a considerable amount of dimesylate was already formed after 50 % turnover, the yield of 6-mesylate being 30 – 55 %, depending on the relative amount of mesylchloride used.

By deacylation of the pentaacetyl mesylate 5 in methanolic ammonia solution at low temperature (Wolfrom & Thompson, 1963b) 6-0-mesylphlorizin 6 was obtained in quantitative yield.

The overall yield of mesylate 6 as obtained from the above reaction sequence was in the range of 25 %. Nevertheless this pathway has the advantage of leading to a product which contains the mesyloxy group in position 6 and which, therefore, can serve as a reference.

b) Direct functionalization of unprotected phlorizin
As was reported above, the reaction of pentaacetyl-phlorizin 4 with tosylchloride in pyridine, surprisingly, gave unsatisfactory results, in contrast to the reaction with mesylchloride/triethylamine in methylene chloride. Therefore it seemed to be tempting to reevaluate direct mesylation of unprotected phlorizin using mesylchloride/triethylamine, especially because of the low overall yield of reactions 1 – 6.

When phlorizin 1 was reacted with mesylchloride/triethylamine in 1,2-dimethoxyethane as solvent at low temperature (Crossland & Servis, 1970), the main product obtained (29 % yield) was 4'-0-mesylphlorizin 7 as evidenced from ¹H NMR. The authenticity of 7 was further confirmed by comparing its pKₐ,MC₅ (9.96) with the value obtained for phlorizin (9.22), since one can assume that only the most acidic pK-value was recorded by this titration study. As had to be expected, there was no new product formed when the phenolic mesylate
2 was heated in DMF in the presence of NaN₃.

After optimizing the reaction conditions, treatment of phlorizin 1 with mesylchloride or tosylchloride in pyridine at low temperature gave the desired 6-0-mesylate 6 or 6-0-tosylate 8, respectively, as the main products in one step. The authenticity of 6 and 8 was established by comparing their Rf-values, ¹H NMR- and IR-spectra to the corresponding values and spectra of the reference sulfonyl-esters obtained via 1 → 6.

The AB-system at 4.5 ppm in ¹H NMR, reflecting 2 geminal (J=12 Hz) protons (H₂C(6)) in close proximity to an electron withdrawing group (= COO⁻), each of the protons coupling with a neighbouring (vicinal) proton (= H-C(5), J=3 Hz and 5 Hz, respectively), turned out to be the most helpful and reliable diagnostic of esterification in C-6 of the glucosyl moiety in general. The preparation of the azide 9 offered no problems. The tosylate 8 was preferred as the starting material, because the mesylate 6 and the azide 9 showed identical Rf-values on TLC using solvent A.

Catalytic hydrogenation using Pd/C as the catalyst and acidified ethanol as the solvent was found to be suitable for reduction of the 6-azide 9 to the amine 10. The reaction was carried out under slightly acidic conditions because the resulting amine 10 was rather unstable in polar solvents and turned out to be very labile under alkaline conditions (100 % decomposition of solid amine after an over-night exposure to an atmosphere of ammonia). The resulting aminohydrochloride of 10 was obtained in an overall yield of 46 %. For further characterization 10 was also converted into the corresponding acetamide 11 using EDAC (Sheehan et al., 1965) as the coupling agent.

Condensation of the amine 10 with 4-fluoro-3-nitrophenylazide (FNAP) (Fleet et al., 1972) in DMSO yielded the short spaced NAP-amino-phlorizin 13 in 80 % yield. A modification (Jeng & Guillory, 1975) of the imidazolide method of Gottikh and coworkers (1970) was applied to couple the amine 10 and NAP-β-alanine 12 to give the NAP-β-alanylamide 14: the utilization of N, N'-carbonyl diimidazole in THF facilitated the formation of an activated intermediate of the carboxylic acid 12 and the use of THF as solvent restricted the reac-
tion to the 6-amino group.

The same method was also used to esterify phlorizin 1 and its 4'-isomer paraphlorizin at the position 6 with NAP-β-alanine 12. This time, in the absence of a free amino group, the solvent THF was found to preferentially direct the acylation to the primary hydroxyl group, the NAP-β-alanylesters in position 6 of phlorizin and para-phlorizin, 15 and 16, respectively, being formed as the main products. The 6-esters could be separated by silica gel chromatography from the corresponding 2,3 or 4 monoacyl isomers and diacyl derivatives also formed by this esterification reaction. Under the reaction conditions chosen the phenolic hydroxyl functions appeared to be less reactive, as evidenced by the absence of any appreciable amount of products acylated in the aglycone moiety.

All the 6-esters were relatively stable when stored as solids at -20°C under N₂. Nevertheless, a slight darkening of the red colour of the compounds was observed after prolonged storage (several months to years). Therefore they periodically were either, prepared fresh, or purified by silica gel chromatography using ethylacetate as the eluent. In solution, in particular under slightly acidic conditions at room temperature, these compounds were less stable, most probably due to the conversion to isomeric esters by acyl migration, as reported for a number of other monoacyl derivatives of sugars (Zamecnik, 1962; Mc Laughlin & Ingram, 1965). However, this process became negligible when ethylacetate (or acetone) were used as solvents and when the solutions were stored at -20°C. In addition the solutions were periodically repurified as described above.

III.4.2 Synthesis of 4-azidophlorizin

In order to replace the hydroxyl function in C-4 of the phenolic ring B by another substituent, such as an azido group, the desired phlorizin analogue or appropriate precursors had to be resynthesized from D-glucose and a suitable aglucone (see Fig. 5).

The aglycone chosen was 2',4',6'-trihydroxy-4-nitro-dihydrochalcone
(4-nitrophloretin; 17), because the nitro group could easily be converted into an azido group at a later stage of the reaction sequence by conventional chemical methods. 17 was synthesized in the laboratory of Prof. D. F. Diedrich (University of Kentucky Medical School, Department of Pharmacology) by condensation of anhydrous phloroglucinol and 4-nitro-hydrocinnamonicitrile (Zemplén et al., 1928) in a Hoesch reaction essentially as described for the preparation of phloracetophenone (Gulati et al., 1935).

The original strategy for the (specific) synthesis of the 2'-glucoside of 17 was to initially block the 4'-hydroxyl group in order to direct the glycosylation reaction towards the 2'-position. Conventional acetylation and benzoylation reactions were therefore carried out with 17 to form mixtures of acetates and benzoates which were isolated by preferential alkaline extraction (Canter et al., 1931) and fractional crystallization from mixtures of chloroform or benzene and methanol; silica gel column chromatography using chloroform and methanol mixtures as eluents also was employed to isolate some of the derivatives.

Glycosylation of both the 4'-acetate and 4'-benzoate ester was attempted, as described for the 4'-benzoylphloracetophenone analogue (Diedrich, 1962); the results were poor. The alkaline conditions resulted in rapid saponification of the acetate and the benzoate was too insoluble to give adequate yields of the 2'-tetraacetylglucoside. Therefore this approach was abandoned and 17 was reacted with acetobromoglucose directly. 2'-O-(tetraacetyl-D-glucopyranosyl)-4-nitrophloretin 18 was obtained in 18 % yield, and in addition the 4'-isomer 19 (23 % yield) and the 2',4'-diglycoside 20 (8 %); the yields being based on unrecoverable 17.

Saponification of 18 with ice-cold 1 N sodium methoxide in methanol gave the free 2'-glucoside 21 in almost quantitative yield.

1) The characteristics of these compounds are presented elsewhere (Gibbs et al., 1981).

2) The free 4'-glucoside was also obtained by the same procedure and could have been used for the preparation of the corresponding azide. However the synthesis of this compound seemed to be beyond the scope of this work; the potential use of the derivatives of paraphlorizin only became evident later and is discussed on page 94.
Reduction of 21 by catalytic hydrogenation with 10 % Pd/C as catalyst at atmospheric pressure gave the 4-amine 22 which was converted to the 4-azide 23 by diazotization in acidified cold acetone, followed by treatment with sodium azide in the cold (85 % yield, according to the method popularized by Smith (1963).

III.4.3. General remarks on the handling of the aryl azides

Unlike diazocarbonyl compounds, most aromatic azides are reasonably stable and can be taken through multistep syntheses that do not involve excessive heating, or strong oxidizing or reducing conditions (Bayley & Knowles, 1977).

The nitroarylazides are, in general, less stable to heat and light and must be recrystallized with care to avoid thermal decomposition. About 50° C is considered to be the temperature limit for reactions involving the NAP-group.

Therefore, all arylazides were routinely handled in dim incandescent light, although it was found that short exposures (in particular of solids) to daylight did not cause considerable decomposition.

It has to be pointed out that low-molecular weight aryl azides are explosive when heated and should be handled carefully, especially in the solid state (Bayley & Knowles, 1977 and references quoted therein).
IV. RESULTS AND DISCUSSION / PART 1

THE USE OF PHLORIZIN DERIVATIVES MODIFIED IN POSITION 6 OF THE GLUCOPYRANOSYL MOIETY.

Phlorizin is known to inhibit Na\textsuperscript{+}-dependent D-glucose uptake by binding to the substrate site of the transporting protein (Alvarado & Crane, 1962; Toggenburger et al., 1978). If one intends to prepare (photo)-reactive analogues of phlorizin to label this binding site, one has to consider if it is better to introduce the (photo)-reactive group into the aglycone- or into the glucopyranosyl moiety of phlorizin, or into both. Examination of the structural requirements for phlorizin binding can be expected to give some information in this regard.

Diedrich (1966) has investigated a number of phlorizin analogues of which the aglucone moiety was modified. With the single exception of 4'-deoxy-phlorizin, which was found to be some 50 - 70 \% more effective, no derivative was a better inhibitor than phlorizin itself. On the other hand it was found (Alvarado & Crane, 1964) that other β-D-glucopyranosides 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\textsuperscript{+},ΔΨ-dependent transporter, and that it does so in a manner analogous to that of D-glucose itself. As a first approach, therefore, it was decided to modify the glucopyranosyl moiety, especially as the nature of the aglucone binding site is not clear (Diedrich, 1963).

Of the possible positions on the β-D-glucopyranosyl moiety of phlorizin, C-6 was chosen for attachment of the reactive groups. The reason for this choice will be discussed later.

The first section will report the work carried out with the 6-derivatives of phlorizin in detail. It will be preceded by an evaluation of the criteria which are of general importance in the choice of a membrane vesicle preparation suited for photolabeling experi-
ments. In addition, I discuss here some of the properties of the membrane preparation chosen, with respect to these requirements. This allows one to present the work with the other potential photolabels, e.g., 4-azidophlorizin and phlorizin itself, in the subsequent sections in a more concise way.

IV.1. CHOICE AND CHARACTERIZATION OF THE MEMBRANE VESICLE PREPARATION

Various criteria played an important role in the choice of the procedure for the preparation of membrane vesicles to be used for photoinactivation and photolabeling experiments. There were rapidity of preparation, yield and scaling up, and in particular tightness and sidedness of the vesicles. To a large extent these criteria are met by the Ca$^{++}$-precipitation procedure according to Schmitz et al. (1973) as modified by Kessler et al. (1978a). The procedure yields vesicles which are "macroscopically" sealed (Kessler, 1978) and exhibit a uniform orientation of the membrane (right side out) (Kessler, 1978; Haase et al., 1978). Further, using an impermeable NEM-derivative (MIMAX) Biber (1979) showed that approximately 98% of the membrane vesicles are sealed (on the assumption that non-muscular actin, the major soluble protein of intestinal brush borders (Tilney & Mooseker, 1971), and always present in these vesicles, presumably as a major component of the "dense core" seen on electron micrographs of all vesicle preparations (Louvard et al., 1973; Kessler, 1978), may be used as intravesicular marker protein; see also the following sections). The "EDTA-method" of Hopfer and coworkers (1973), on the other hand, yields vesicles whose tightness and sidedness is not clarified.

1) "Macroscopically" sealed means that these membrane vesicles are not permeable to high molecular weight substances, e.g., methoxy-inulin (M.W. approximately 5000 D).
IV.1.1. Polypeptide composition of intact and DOC extracted membranes.

An analysis of the brush border membrane proteins of rabbit small intestinal enterocytes by SDS-polyacrylamide gel electrophoresis under reducing conditions is presented in Fig. 6; the gel system is that of Lämmli (1970), as modified by Thoma (1976). Fig. 6A shows the Coomassie blue stained (8.4 x 2.7) electrophoretic pattern of the proteins of intact brush border membrane vesicles from rabbit small intestine (lanes V) and from DOC extracted membranes (lanes DOC). The vesicles were prepared from mucosal scrapings (fresh or frozen) by the Ca++-precipitation method. Vesicles prepared from frozen small intestines did not give strictly reproducible protein patterns (see also page 54 for a discussion of the functional instability of this preparation).

The molecular weight scale depicted to the right of the gels in Fig. 6A shows that only proteins of apparent mol wt of 200,000 D to approximately 25,000 D were resolved by this gel system. In order to analyze the polypeptides smaller than the lower limit, i.e. "the bromophenol-blue front band" on (8.4 x 2.7) gels, a (15 x 2.7) gel was used (Fig. 6B).

A nomenclature of the bands, to be used throughout this study, is given on the left of the gels. It essentially follows the nomenclature introduced by Klip et al. (1979b). Those bands which have previously been ascribed to identifiable proteins are labeled with capital letters; all other major bands are numbered; ill defined or fuzzy areas are followed by the letter f, and sharp contiguous bands (doublets) are labeled d. For the designation of low molecular weight components on (15 x 2.7) gels, the numbering by Klip et al. (1979b) was continued (Fig. 6B). A slightly different nomenclature of the banding pattern of small intestinal brush border membrane proteins was used by Biber (1979).

The protein pattern of intact (normal) brush border membranes shown in lanes V is very heterogeneous, as has to be expected from the large number of functions carried out by this membrane and the generally heterogeneous composition of plasma membranes (Guidotti, 1972). There are a few major bands (e.g., I,S,3,4,A,14) and a complex pattern of weakly staining, but distinct bands.
Fig. 6. Analysis of the protein pattern of intact and DOC extracted rabbit small intestinal brush border membranes by SDS-PAGE. The membrane samples were boiled for 60 s in a dissociating solution, containing mercaptoethanol as a reducing agent and bromophenol blue as a tracking dye. They were analyzed on (8.4 x 2.7) gels (A) and (15 x 2.7) gels (B), as described in Methods; the gels were stained with Coomassie blue. Lanes V: normal (intact) brush border membrane vesicles prepared from (fresh or frozen) scrapings of small intestinal mucosa by the Ca²⁺-precipitation method, 50 μg protein. The nomenclature used throughout this study is shown on the left of each set of gels: I = isomaltase-rich band, s = sucrase-rich band, A = actin-like protein. Designation of the bands is according to Klip et al. (1979b) and is presented in more details in the text. The molecular weight scale is shown on the right of each set of gels. Lanes DOC: Brush border membrane vesicles were treated with DOC (0.5 mg/mg protein) and centrifuged. After washing a sample (50 μg) was used for analysis.
The two main bands labeled I and S (mol wt 125,000 D and 112,000 D) have been previously reported to contain mainly isomaltase and sucrase, respectively (Semenza, 1976). In addition other proteins probably co-migrate with these bands, e.g., one of the aminopeptidases with S and the monomers of lactase-phlorizin hydrolase with I (Skovbjerg et al., 1981; additional evidence for this will be presented in Fig. 22B).

The monomers of alkaline phosphatase from pig small intestine were found to have mol wts of 61,000 D and 65,000 D (Colbeau & Maroux, 1978). Therefore these monomers can be attributed to bands 6d or 7d.

The most prominent band resolved by the gels V (about 20% of the total protein) appears to consist mainly of nonmuscular actin and is labeled A (mol wt 45,000). This actin was demonstrated to be the main constituent of the microfilaments (5 nm filaments), which as a bundle, run the whole length inside the microvillus (Tilney & Mooseker, 1971; Mooseker, 1976). The presence of actin in the vesicle suspension can be explained by its being trapped inside vesicles, assuming that they are formed during the first homogenization step.

For the same reason other proteins known to be components of the microfilaments may be present: Villin (95,000 D; Bretscher & Weber, 1979, 1980a), the 110,000 D (Crosslinking) protein of Matsudaira & Burgess (1979) and fimbrin (68,000 D; Bretscher & Weber, 1980b). In addition calmodulin (16,500 D) may be present as this has been shown to bind to microfilaments (Glenney & Weber, 1980). Further proteins from the terminal web, such as myosin (200,000 D; demonstrated to be present in brush border membrane fragments (Mooseker et al., 1978)), α-actinin (95,000 D and 105,000 D; Mooseker & Stephens, 1980) and non-muscular tropomyosin (30,000 D). Probably the bands 16 and 17 (mol wt 18,000 D and 16,000 D) are also structural proteins (associated with myosin; Mooseker et al., 1978).

The most conspicuous difference between gels V and DOC (Fig. 6A+B) is seen in the distribution of band A. This decrease in the relative content of A in DOC extracted membranes reflects the removal of this (and other cytoskeletal proteins) by DOC treatment, concomitant to a relative enrichment in bands S and I.

1) C-NEM Labeling studies of intact vesicles also revealed the presence of other (faint) bands in this region, which cannot be identical with sucrase-isomaltase, because this enzyme complex does not contain free SH-groups (Biber, 1979; Mosimann et al., 1974).

2) Trapping inside the vesicles of more than 90% of the actin present in the preparation has been inferred from proteolytic treatment (papain) of intact vesicles (Klip et al., 1979b). Evidence for exclusive location of actin inside the vesicles will be presented in Figs. 18B & 22C.
Using reference lipids and staining for glyco-compounds (PAS; Fairbanks et al., 1971) it was shown that the region K contains phospholipids and glycolipids (Biber, 1979). This is in agreement with some of the results from the labeling studies below (see Figs. 16 & 22).

IV.1.2. Purity of the membrane preparation.

According to Kessler (1978) approximately 90% only of the protein mass of the vesicles prepared by the Ca\(^{++}\)-method is derived from brush border membranes. The residual 10% can be attributed mainly to basal-lateral plasma membranes.

However, this 90% contains a variety of proteins which are not membrane bound; these are termed "endogenous impurities" and include in addition to some of the above cytoskeletal elements (actin, etc.) other, soluble cytoplasmic proteins. Assuming that all the protein components are stained equally and in a manner proportional to their protein content by Coomassie blue, the "endogenous impurities" were estimated to account for approximately 30% of the total protein mass of a brush border vesicle preparation (Biber, 1979). In DOC extracted membranes this impurity is reduced to approximately 5%.

Therefore, considering both the presence of basal-lateral membranes and the "endogenous impurities", only about 60% of the total Coomassie blue staining intensity on SDS gels of intact brush border vesicles can be ascribed to brush border membrane proteins. In the case of DOC extracted membranes, this fraction increases to approximately 85%.

IV.1.3. Stability of membranes with respect to proteolytic degradation.

Proteolytic degradation can also contribute to the observed complexity of SDS polyacrylamide gel electropherograms of biological membranes (for proteolytic degradation of membrane proteins as being responsible for functional instability of vesicles, see below). However, this could be largely ruled out in the electrophoretic analysis of the rabbit small intestinal brush border membrane when the vesicles were prepared from mucosal scrapings (and not from frozen intestines; see below).
In a suspension of intact vesicles there was no, or only very little, endogenous proteolytic activity when vesicles were kept at room temperature or on ice (in 300 mM mannitol, 10 mM HEPES/Tris, pH 7.5, 0.02 % KN\textsubscript{3}). The protein pattern on (8.4 x 2.7) and (15 x 2.7) gels did not show any detectable changes during a 24 h incubation (gel not shown). The same was true for vesicles which were subjected to long photolabeling experiments (see Fig. 16). This is in agreement with the findings of Biber (1979) who monitored the stability of the protein pattern of vesicles during a 100 h incubation.

It must be emphasized, however, that endogenous proteolytic activity in brush border vesicles was found to strongly increase in the presence of detergents (Biber, 1979). Exposure of vesicles to DOC (1 mg/mg protein) for 60 min at room temperature was shown to result in the disappearance of a series of protein bands, among them actin. There was less proteolytic breakdown after incubating at 4°C or when Triton X-100 was used instead. In addition this endogenous proteolytic activity was found to be only slightly inhibited by phenylmethyl sulfonfonylfluoride (PMSF) or N-p-tosyl-L-lysine chloromethyl-ketone (TLCK) (Biber, 1979)\textsuperscript{1}).

Nevertheless, proteolytic degradation by intrinsic proteases during the extraction of membrane vesicles as routinely performed in this study (0.5 mg/mg protein; for 15 min on ice) was found to be negligible (Klip et al., 1979 b). Proteolysis was also negligible during the subsequent experimental work, provided that DOC was carefully removed after extraction, as judged from SDS-PAGE: the protein patterns of these membranes on (8.4 x 2.7) and (15 x 2.7) gels did not differ detectably from those of controls which were solubilized and frozen immediately after DOC treatment and washing (gels not shown).

\textsuperscript{1)} Using the degradation of the actin band (A) to monitor detergent induced proteolysis in brush border membrane vesicles, Gains & Hauser (1981) recently demonstrated that only EDTA, iodoacetamide and diisopropyl fluorophosphate together, as inhibitors of the three main types of proteases, efficiently depressed proteolysis. These authors used frozen pieces of whole rabbit small intestine as the starting material for the membrane preparation. This tissue, however, might be particularly rich in proteases (Takesue et al., 1981; see also below).
It has to be admitted, however, that degradation of minor protein components, such as the D-glucose transporter (estimated amount in intact brush border membranes: 0.2 - 0.4 % of the total protein) probably would not be detectable by this approach and therefore cannot be ruled out (see also below: functional stability of membrane vesicles). In addition it has to be considered that removal of only a few amino acid residues from a protein may not result in a discernible change in the protein pattern on SDS gels, yet it may lead to its inactivation. On the other hand a protein might be split by proteases without any loss of its activity.

The origin and nature of the above proteases are not clear at the present. An influence of pancreatic proteases present during the preparation on the polypeptide pattern of brush border membranes has been ruled out (Biber, 1979; Wacker, 1979). The influence of proteases originating from subepithelial and muscle cells (Woodbury et al.,1978) is proposed to be responsible for the functional instability of vesicles prepared from frozen pieces of whole small intestine (Takesue et al 1981; see also below). This may be assumed to be minimized in the above preparation.

General remarks on SDS-PAGE:

"Molecular weights" of polypeptides as determined by either the above gel system must be considered as approximate since: (i) denaturation with SDS of hydrophobic proteins need not proceed in the same way as with well defined soluble proteins; (ii) membrane (glyco)proteins are known to bind different amounts of SDS and thus to exhibit different electrophoretic mobilities; (iii) binding of SDS to polypeptides (and the critical micelle concentration of SDS) depend on parameters, such as ionic strength and pH of the buffer, which change in a discontinuous system (Nielsen & Reynolds, 1978).
IV.1.4. Functional stability of membrane vesicles

The functional stability of the membrane vesicles is another important criterion to be met by the preparation procedure chosen, especially with regard to the long photolabeling experiments. For these, vesicles cannot be assayed for their transport ability until 12 - 24 h after the end of the preparation. Also in this respect the Ca\(^{++}\)-procedure is reported to be more suitable, yielding vesicles which are considerably more stable than EDTA vesicles; as evidenced by only a minor decrease of their transport ability after 90 min at 37°C (Kessler et al., 1975) or after 2 - 3 days at 4°C (Kessler et al., 1978a).

In the presence of an inwardly directed NaSCN gradient, D-glucose is known to be transiently accumulated inside brush border vesicles, attaining a concentration which is several fold higher than that in the medium (Kessler et al., 1978a). This phenomenon is referred to as "overshoot" (Kessler et al., 1978a).

Because of the duration of certain complicated labeling experiments, it seemed desirable to perform (a part of) the uptake experiments on the next day, i.e., 20 - 24 h after completion of the vesicle preparation. When preliminary control experiments showed a considerable decrease in the overshoot of D-glucose uptake after being kept on ice over night, it was decided to examine this decay in greater detail.

As shown in Fig. 7, vesicles tested immediately after preparation (t=1 h) exhibited a high overshoot peak of D-glucose, i.e. 20 - 30 times as high as the equilibrium value (at 90 min) using 20 \(\mu\)M D-glucose and 100 mM NaSCN. This overshoot reduced gradually with time if the vesicles were kept on ice (Fig. 7A). When the vesicles were stored at room temperature, the overshoot decayed rapidly (Fig. 7B). The half-times of overshoot were some 10 h in the former, and some 5 h in the latter case.

A more detailed analysis of the time and temperature dependent process is given in Fig. 8. It shows that Ca\(^{++}\)-vesicles (●) suffer a time dependent decrease of both their overshoot (A & B) and equilibrium of D-glucose uptake (C & D). The decrease of the latter was
Fig. 7. Stability of D-glucose transport activity of brush border membrane vesicles. Brush border membrane vesicles prepared by the Ca++-precipitation method (see Methods) were either kept on ice (A) or at room temperature (B). After the times indicated on the lines (numbers represent hours after end of preparation), D-glucose uptake under NaSCN-gradient conditions was measured at the times given on the abscissa. The results, determined as D-glucose taken up per mg protein, are plotted as % of maximal uptake in vesicles (at 15 s) measured 1 h after the end of the preparation. Composition of the incubation medium: 300 mM mannitol, 100 mM NaSCN (out, zero in), 10 mM HEPES/Tris, pH 7.5, 20 μM ($^3$H)-D-glucose, 0.02 % KN$_3$. Each point is the mean of 3 determinations.

Less dramatic and almost linear in time when vesicles were kept on ice (C), whereas, incubation at room temperature resulted in an accelerated reduction in equilibrium uptake (D). Overshoot on the other hand in both cases decreased rapidly with time (A & B), the half-life being reduced by a factor of 2 when vesicles were kept at room temperature.

In this context it must be mentioned that the transport "quality" of vesicles itself was found to strongly depend on the "quality" of the frozen tissue, an observation also made by others (Kessler, 1978). Frozen intestines stored in air-tight plastic bags at -20°C yielded vesicles of satisfactory transport activity after up to 6 - 9 months
Fig. 8. Stability of D-glucose transport activity of brush border membrane vesicles prepared by different procedures. Brush border membrane vesicles were prepared by the precipitation method using the following divalent cations/chelator: Ca\(^{++}\) (●, see also Fig. 6), Mg\(^{++}\)/EGTA (▲), or Ba\(^{++}\)/EGTA (■) (for details see Methods). Vesicles were either kept on ice (A, C) or at room temperature (B, D) for the times indicated and D-glucose uptake at 15 s (A, B) or D-glucose equilibrium uptake (C, D) were measured. The results, determined as D-glucose taken up per mg protein, are given as % of the corresponding uptake in Ca\(^{++}\)-vesicles (●) measured 1 h after the end of the preparation and are plotted against the hour of measurement. Composition of the incubation medium was as in Fig. 6. Determinations were carried out in triplicate.

of storage, if the bag was kept sealed. If a bag was opened frequently, however, the transport qualities of the individual preparations gradually decreased; the overshoot measured immediately after preparation fell from 40 fold to 8 fold when the bag was opened a dozen times (over a 2 month period), even if the bag was carefully resealed each time. Intestines stored at -20\(^\circ\) C, but improperly or not well wrapped, yielded membrane preparations which exhibited no, or only incomplete, vesicularization. The reason for this is not clear. Perhaps the tissue is oxidized (see below) by atmospheric
oxygen, or it just dries out.

The decrease in equilibrium uptake (Fig. 8C & D) is indicative of a "physical" defect of the vesicle membrane dependent on storage; be it complete rupture of vesicles resulting in a true loss of intravesicular (osmotically active) space, or be it increased leakiness of the membrane leading to an enhanced efflux of D-glucose during washing of the filter.

"General" leakiness of the membrane vesicles and/or an increase in the permeability of the membrane to ions (resulting in an accelerated collapse of \( \Delta \phi_{Na}^+ \)) may similarly be responsible for the decrease in D-glucose overshoot observed (Fig. 8A & B).

The idea that changes in the permeabilities of the membranes play an important role in this phenomenon is supported by the recent work of Takesue et al. (1981). These authors observed that vesicles prepared from frozen small intestine exhibited a large increase in L-glucose permeability (which is assumed to reflect "non-specific" permeability) as compared to vesicles prepared from fresh tissue (see also next sections).

In the following, several modifications of the vesicle preparation procedure were tested (i) to optimize the functional stability of the vesicles, and (ii) to get some information as to the mechanism underlying this phenomenon.

a) The influence of the divalent cation; the role of phospholipase(s)

Booth and Kenny (1974) have developed a procedure to prepare microvilli from rabbit kidney by Mg++-precipitation. If Ca++ was replaced by Mg++ in the preparation of rabbit small intestinal vesicles, there was no significant improvement in the purity of the product, the final Mg++ material being even somewhat less pure (Kessler et al., 1978a).

Lipids extracted from membranes prepared by the Ca++-precipitation method, however, exhibited exceptionally high levels of lysophospholipids 1) and free fatty acids (Hauser et al., 1980), which are most

1) The content in lysophosphatidylcholine and lysophosphatidylethanolamine was determined by those authors and was found to be 10 % as compared to the estimated 1 % in natural membranes (phosphatidylcholine and phosphatidylethanolamine account for approximately 65 % of the phospholipids present in small intestinal brush border membranes).
likely produced by the action of the Ca\textsuperscript{++}-activated brush border phospholipase A\textsubscript{2} (Subbaiah & Ganguly, 1970). Therefore it seemed worthwhile to test whether the activity of this phospholipase was responsible for the time and temperature dependent functional instability of vesicles observed.

Using EGTA to keep the free Ca\textsuperscript{++} concentration low so that phospholipases are inactivated, and employing Mg\textsuperscript{++} instead of Ca\textsuperscript{++} to aggregate contaminating membranes, Hauser and coworkers (1980) obtained membranes suitable for lipid analysis. Fig. 8 shows the effect of modifying the preparation by using Mg\textsuperscript{++}/EGTA (\(\text{A}\)) or Ba\textsuperscript{++}/EGTA (\(\text{■}\)) on the stability of D-glucose transport activity, compared with the stability of the standard Ca\textsuperscript{++}-vesicles (\(\text{•}\)). Direct comparison is possible because these preparations yield vesicles with similar transport properties and in similar yields. They have comparable initial maximum transport activities (\(\text{A}, t=1\ h\)) and comparable initial equilibrium volumes (\(\text{C}, t=1\ h\))\(^1\).

Neither of these modifications (\(\text{A, ■}\)) resulted in an increased stability of the vesicles, the rates of decrease in both their maximum transport activity (\(\text{A}\)) and their equilibrium uptake (\(\text{C}\)) were identical. When the vesicles were kept at room temperature, the rates of their loss in overshoot (\(\text{C}\)) and intravesicular volume (\(\text{D}\)) were comparable, the half-times being in the range of 5 - 10 h and 10 - 20 h, respectively. If the values are at all different, the stabilities of the single preparations decreased in the order Mg\textsuperscript{++}/EGTA > Ca\textsuperscript{++} > Ba\textsuperscript{++}/EGTA. From this, it is evident that the activity of the phospholipase A\textsubscript{2} cannot be the main reason for the time and temperature dependent loss in the transport ability of vesicles kept on ice; assuming a strict Ca\textsuperscript{++}-dependence of this phospholipase and complete removal of Ca\textsuperscript{++}. The parallel decrease in the transport abilities of the different types of vesicles when kept on ice (Fig. 8A & C) also supports this conclusion; it is known that phospholipase activity in a mucosal homogenate appears to continue both at low and high temperature (Eichholz, 1967).

1) In addition these vesicles also exhibited similar specific sucrase activities (1.3 - 1.6 U/mg) and they were obtained in comparable yields (1.2 - 1.3 mg of vesicle protein/g of frozen tissue) (Trüb, 1978).
Since the intestinal mucosa is one of the richest sources of phospholipases (Gallai-Hatchard & Thompson, 1965), the involvement of other minor (Ca\(^{++}\)-independent) phospholipases, i.e., phospholipase B (Marples & Thompson, 1960) cannot be completely ruled out at the present.

b) The role of proteases
Takesue et al. (1981) recently demonstrated that microvilli prepared from frozen and from fresh small intestines differ from one another with respect to functional deterioration. When measured immediately after isolation, the microvilli (prepared by the Mg\(^{++}\)/EGTA procedure in the presence of 0.75 mM DTT) from either fresh or frozen intestine showed a similarly high overshoot of Na\(^{+}\)-dependent D-glucose uptake. However, the preparations differed in their stability of the D-glucose transport activity, the activity of microvilli from fresh intestine (scrapings) being considerably more stable.

As a possible mechanism of the functional deterioration observed with material prepared from frozen pieces of (whole) intestine, proteolytic digestion from the cytosolic side of the membrane is suggested by these authors. Freezing and thawing of the tissue might liberate proteases from muscular and subepithelial cells, such as the group specific proteases (Katanuma et al., 1975; Woodbury et al., 1978) resulting in their subsequent trapping into the microvilli during the first steps of their isolation. Proteolytic digestion from the cytosolic side of the membrane is known to inactivate irreversibly the Na\(^{+}\), D-glucose cotransporter (Klip et al., 1979a; Klip et al., 1979b); it may also be responsible for the morphological changes of microvilli as observed by Takesue et al., (1981) and for the deterioration of other functional parameters.

This is consistent with the above results of Biber (1979) showing that there was no endogenous proteolysis detectable on the SDS-PAGE protein patterns of Ca\(^{++}\)-vesicles kept at room temperature as long as 100 h, when these vesicles were prepared from fresh scrapings of intestinal mucosa. Vesicles prepared from frozen whole tissue, on the other hand, exhibited protein patterns which were not strictly reproducible and the actin band of which was found to gradually decrease in intensity during the 100 h storage (Biber, unpublished experiments).
Gains & Hauser (1981), however, did not detect any, or only very little, endogenous proteolytic breakdown with membrane vesicles derived from frozen small intestine when kept at room temperature for not longer than 20 h. Therefore, any proteolytic activity responsible for the functional deterioration observed within this time period would have to be rather specific for the transporter.

c) Oxidative damage

It is established that SH groups are essential for the Na⁺-dependent D-glucose uptake by intestinal microvilli (Biber & Hauser, 1979; Klip et al., 1979c; Klip et al., 1980; Will & Hopfer, 1979). Addition of antioxidants, such as dithionite (1 mM) or ascorbate (10 mM), to microvilli isolated from frozen intestine failed to protect the transport activity during storage. The only factor which was found to be effective, and partially at that, in slowing down the decay of transport activity, was replacement of O₂ by N₂ in the gas phase (anaerobiosis) (Takesue et al., 1981). This might be indicative of an oxidative process as being (at least partially) responsible for the functional deterioration of vesicles shown in Figs. 7 & 8.

Conclusions drawn for further experimentation with brush border membrane vesicles:

Presumably the observed functional instability of membrane vesicles results from a combination of some or all of the above factors. Therefore, in order to optimize the quality of vesicles during experimentation, all subsequent experiments were carried out essentially as follows:
- Intestines were frozen in small batches; once opened, a batch was used for preparation for not longer than 2 - 3 months, care being taken to properly reseal the plastic bag after each use.
- the Ca²⁺-precipitation method was used unless otherwise stated.
- vesicles were always prepared fresh on the day of the experiment.

1) Due to their high functional instability frozen vesicles (prepared from frozen intestines and immediately frozen in dry-ice/isopropanol & stored at -80°C for only a short time) proved to be unsuited for long experiments. The addition of glycerol before freezing (Hilden & Sacktor, 1978) did not result in a detectable improvement of the transport stability.
- at a later stage of this work, fresh or frozen mucosal scrapings of intestine were used as the starting material for selected long photoinactivation and photolabeling experiments (Takesue et al., 1981). Frozen mucosal scrapings were kept at -20°C in air tight vessels and were used for preparation for not longer than 2-3 months.

- Long experiments were carried out without interruption, uptake or binding experiments being performed within 10 to 18 hours after the preparation.

IV.2. INTERACTION OF (PHOTOREACTIVE) ANALOGUES OF PHLORIZIN WITH BRUSH BORDER MEMBRANES IN THE DARK.

The identification of the binding site of the natural ligand by the modified (photolabile) analogues is a prerequisite for successful affinity labeling of an enzyme or a receptor. To obtain a measure of the affinity of the various phlorizin derivatives for the substrate site of the transporting protein, and thereby, to evaluate their use as potential photolabels, the binding capacity of these compounds in the dark was investigated. This was done by determining (i) their inhibitory potency on D-glucose uptake in brush border membrane vesicles, (ii) their reversible inhibition of Na⁺-dependent, D-glucose protectable phlorizin binding (specific phlorizin binding) to such vesicles and (iii) to DOC extracted membranes. Extraction with DOC yields membranes with a two fold increase in Na⁺-dependent phlorizin binding activity (Klip et al., 1979b). This enrichment of glycoside binding sites apparently reflects a partial, negative purification of the transporter and thus makes this preparation particularly suited for labeling studies.

Measurement of the reversible binding of (radiolabeled) phlorizin analogues to the membrane vesicles can be an alternative way to obtain a measure of the affinity of these derivatives for the substrate site of the transporter.
IV.2.1 Inhibition of D-glucose uptake in brush border membrane vesicles

As has been pointed out elsewhere, the vesicle preparation which was used in these experiments (Kessler et al., 1978a) lends itself very well to the determination of $K_i^1$ and $K_i^d$-values under conditions of a partially dissipated NaSCN gradient, provided that proper precautions are taken (for a detailed discussion, see Toggenburger et al., 1978). One of the conditions is that initial velocities must be measured while the uptake is still linear with time, i.e., within the first 4 s of the incubation. Routinely, overall kinetic constants were determined from incubations lasting 2 s (see also Kessler et al., 1978b).

The $K_i^1$-values of the various phlorizin derivatives, obtained from Dixon plots, are summarized in Table I. Typical Dixon plots for NAP-N-Phlz $13$ and NAP-$\beta$-Ala-Phlz $15$ as inhibitors are shown in Figure 9. The $K_i^1$ of phlorizin (8 $\mu$M) agrees with the values of 7.8 $\pm$ 1.4 $\mu$M obtained by Tannenbaum et al. (1977) and Toggenburger et al. (1978) and served as a reference. Replacement of the primary hydroxyl function by an azido group did not strongly affect the affinity of the azide $9$ ($K_i^1 = 10 \mu$M), whereas the primary amine $10$ showed a markedly decreased affinity ($K_i^1 \approx 550 \mu$M). This might be attributed to electrostatic repulsion, since the amino group must be fully protonated (charged) at pH 7.5. This explanation is supported by the observed larger inhibitory potency ($K_i^1 = 40 \mu$M) of the secondary aryl amine $13$ (which should be uncharged at pH 7.5). The sulfonyl ester $8$ and the NAP-$\beta$-alanylester $15$ showed considerable affinity, their $K_i^1$-values being 14 $\mu$M and 48 $\mu$M, respectively. A rationalization of the higher affinity of the former will be discussed later. The amides $11$ and $14$ showed a marked decrease in affinity ($K_i^1 \approx 315$ and 148 $\mu$M, respectively), as compared with the corresponding ester $15$ (Table I is on page 64; the structural formulas are given on page 38).
Figure 9: Dixon plots of NAP-6-amino-6-deoxyphlorizin (13: filled symbols and solid lines) and NAP-β-Ala-Phlz (15: 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 μM (▲, △); 30 μM (■, □); 150 μM; (●, ○). The behavior is characteristic of fully-competitive inhibition; the K_i' values were 34 ± 3 μM for 13 and 39 ± 4 μM for 15 (means ± SD). Single determinations were carried out in quadruplicate. The reciprocal uptake velocity is expressed in arbitrary units.
Table I

Reversible fully competitive inhibition (in the dark) of D-glucose uptake in, and of specific phlorizin binding to, brush border membrane vesicles and DOC extracted membranes by phlorizin, para-phlorizin and some 6-substituted analogues.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition of D-glucose uptake*</th>
<th>Specific phlorizin binding</th>
<th>DOC extracted membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_i$ (μM)</td>
<td>$K'_i$ (μM)$^c$</td>
<td>$K''_i$ (μM)$^c$</td>
</tr>
<tr>
<td>Phlorizin (1)</td>
<td>8 ± 1.2 (4)</td>
<td>12 ± 2 (2)</td>
<td>9 ± 2 (2)</td>
</tr>
<tr>
<td>Azido-Phlz (9)</td>
<td>10 ± 1.8 (3)</td>
<td>12 ± 2 (2)</td>
<td>n.d.</td>
</tr>
<tr>
<td>pTs-Phlz (8)</td>
<td>14 ± 2.5 (2)</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>NAP-β-Ala-Phlz (15)</td>
<td>48 ± 5 (3)</td>
<td>60 ± 6 (3)</td>
<td>31 ± 4 (3)</td>
</tr>
<tr>
<td>NAP-N-Phlz (13)</td>
<td>40 ± 7 (3)</td>
<td>80 ± 5 (3)</td>
<td>79 ± 10 (3)</td>
</tr>
<tr>
<td>NAP-β-Ala-N-Phlz (14)</td>
<td>148 ± 17$^d$ (2)</td>
<td>122 ± 10 (2)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Acetamido-Phlz (11)</td>
<td>315 ± 41 (3)</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>para-Phlz</td>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAP-β-Ala-para-Phlz (16)</td>
<td>n.d.</td>
<td>&gt;300 (1)$^d$</td>
<td>&gt;300 (1)$^d$</td>
</tr>
<tr>
<td>Amino-Phlz (10)</td>
<td>554 ± 20 (2)</td>
<td>n.d.</td>
<td></td>
</tr>
</tbody>
</table>

*Uptake of D-glucose (at 2 s, 22°C, pH 7.5) was performed, as in Methods, under an inwardly directed gradient of NaSCN (100 mM out, zero 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.

*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 ($K''_i$, $K'''_i$) were calculated for fully competitive reversible inhibition of specific ($^3$H)-phlorizin binding ($K_d$=5 μM; Toggenburger et al., 1978) at 4 – 6 different analogue concentrations. Data are represented as mean ± SD of (n) single plots or experiments; n.d. not determined. For preparations and details see Methods.

$^d$Due to limited solubility of labels (<150 μM), determination of $K_i$-values may be inaccurate.

$^d$Determined at pH 7.0.
VI.2.2. Inhibition of specific phlorizin binding to brush border membranes

The assumption that the various phlorizin analogues bind to the same site as phlorizin implies that their inhibitory effect on specific (Na\(^+\), Δψ-dependent, D-glucose protectable) phlorizin binding to vesicles, as measured by their K\(_{i}^{"}\) -values, corresponds to their inhibitory potency on D-glucose uptake in the dark (K\(_{i}\)\(^{'}\)). This should also be true for their reversible inhibition of Na\(^+\) -dependent phlorizin binding to DOC extracted membranes (K\(_{i}^{"'}\)).

IV.2.2.1 Inhibition of specific phlorizin binding to brush border membrane vesicles

As evidenced from Table I, the K\(_{i}^{"}\) -values corresponded closely to the K\(_{i}\)\(^{'}\) -values obtained from the kinetics of sugar uptake inhibition.

IV.2.2.2 Inhibition of specific phlorizin binding to DOC extracted membranes

a) Time course of phlorizin binding to DOC extracted membranes:
For the determination of specific phlorizin binding (under equilibrium conditions of NaCl or KCl) to DOC extracted membranes, Klip et al. (1979a,b) used 15 s as the incubation time, assuming that maximal specific binding was reached after this time.

The time course of phlorizin binding in Fig.10 shows that this assumption is valid. Specific (\(^3\)H)-phlorizin binding was found to be maximal after 15 s, as evidenced from its not being significantly different from the specific binding after a 180 s incubation. Therefore, 15 s were used as the incubation time for all subsequent measurements of phlorizin binding to DOC extracted membranes.

Unspecific binding, and thus also total binding of phlorizin, con-

1) The (maximal) specific binding usually was in the range of 12 - 20 pmoles of phlorizin bound/mg protein, depending on the quality of the vesicle preparation and on the DOC extraction.
Fig. 10. Time course of phlorizin binding under equilibrium conditions (100 mM NaCl or KCl) to DOC extracted membranes. DOC extracted membranes were equilibrated with 100 mM NaCl or KCl (300 mM mannitol, 10 mM Tris-HCl, pH 7.0, 0.02% KN3) for 30 min at room temperature. (3H)-Phlorizin binding (5 uM) was determined after the times indicated on the abscissa, as described in Methods. Specific phlorizin binding (●) was obtained by subtracting unspecific binding (in the presence of KCl; △) from total phlorizin bound (in the presence of NaCl; ▲). Determinations were carried out in pentuplicate; the values are given as mean ± SD (error bars were omitted when SD were smaller than the symbol size).
continuously increased with time, showing no steady state level. This rise in binding can, as will be discussed later, mainly be attributed to the slow uptake of $^3$H (as phlorizin and as phloretin$^1$) liberated by the phlorizin hydrolase$^2$ (Malathi & Crane, 1969)$^3$.

b) Inhibition of specific phlorizin binding to DOC extracted membranes: As evidenced from Table I, also the $K''_t$-values corresponded closely to the $K'_t$-values obtained from the kinetics of sugar uptake inhibition.

The minor differences found in some cases might, if at all significant, be due to the slightly different assay conditions used or might reflect the pH-dependence of ligand binding; the protonated form of phlorizin (and analogues) being the species bound (Toggenburger et al., 1978)$^4$.

As a whole, however, the data in Table I support the contention that phlorizin and its analogues modified in C-6 on the glucopyranosyl moiety bind to the same site. Therefore, the analogues may be used as potential photolabels of the D-glucose binding site of the sugar carrier.

1) Complete hydrolysis (0.7 N HCl, $80^\circ$ C, 80 min) of ($^3$H)-phlorizin showed that approximately 98 % of $^3$H was in the phloretin moiety.

2) As evidenced from control experiments (see also page 37), less than 0.5 % of phlorizin is hydrolyzed after a 3 min incubation at room temperature under identical conditions.

3) In addition, there is also evidence that phlorizin, after slowly penetrating the bilayer of (sealed) vesicles, is bound to cytoskeletal elements, such as actin (see page 149). Binding of phlorizin to other ATP-binding proteins, such as Na$^+$/K$^+$-ATPase in basal-lateral plasma membranes, has been reported (Rendi & Uhr, 1964).

4) Spectrophotometric (Trüb, 1978; Evans & Diedrich, 1980) and titrimetric determinations (Toggenburger, 1979) of the $pK'_a$ of phlorizin under conditions similar to those of the experiments on D-glucose uptake and phlorizin binding gave $pK'_a$-values of 7.15 and 7.36, respectively, a value similar to that reported for the aglycone phloretin, 7.3 (Jennings & Solomon, 1976). Evidence for the assignment of this $pK'_a$-value to the phenolic group in position 4 will be presented on page 110.
IV. 2.3. Binding of \(^3\text{H}\)-NAP-\(\beta\)-Ala-Phlz to brush border membrane vesicles and to phospholipid vesicles.

a) Binding to brush border membrane vesicles.

The measurement of the reversible binding of \(^3\text{H}\)-NAP-\(\beta\)-Ala-Phlz to membrane vesicles under the conditions used in the inhibition experiments was intended for the following reasons: (i) its dissociation constant \(K_d\) was assumed to match the \(K_d\)-values from the above inhibition experiments, and (ii) monitoring of the (total) binding of this compound was expected to give some information as to its specificity as photolabel.

The technique chosen to measure the binding of \(^3\text{H}\)-NAP-\(\beta\)-Ala-Phlz was the Millipore filtration technique, as used for the above inhibition experiments. In addition, this rapid ultrafiltration procedure was found to be the only way to measure high affinity phlorizin binding to intestinal brush border membrane vesicles (Toggenburger et al., 1978).

However, all attempts to measure specific binding of \(^3\text{H}\)-NAP-\(\beta\)-Ala-Phlz (at 10-100 \(\mu\)M) to membrane vesicles failed: in the presence of membranes >40% of the \(^3\text{H}\)-label was retained on the filter (in the absence of membrane vesicles 17% of the label adsorbed to the filter, regardless of the types of membrane filters used). Thereby the specific binding to the membrane vesicles was largely masked.

b) Binding to phospholipid vesicles

Since the aim of this work is to label a protein binding site exposed to the aqueous phase, a photoreactive label of this site ideally should have a strong preference for the aqueous phase, the more so as increased uptake of the photolabel into the membrane is likely to result in increased unspecific labeling of membrane proteins from within the lipid bilayer.

1) The measurement of the binding of \(^3\text{H}\)-4-azidophlorizin to membrane vesicles by this technique will be reported on page 113.

2) The membrane filters used were: Sartorius SM 11305 (0.65 \(\mu\)m), Millipore MF-HA (0.45 \(\mu\)m) and MF-DA (0.6 \(\mu\)m), and Cellpore CP-060.

3) The adsorption of \(^3\text{H}\)-phlorizin and \(^3\text{H}\)-4-azidophlorizin to these filters under similar conditions was about 0.3 % and 5 %, respectively (see page 113).
Phlorizin, however, is known to bind to brush border membrane vesicles (Toggenburger et al., 1978) and to phospholipid vesicles (Ehrenspeck, 1975). Attachment of the NAP-β-alanyl group had to be expected to increase the lipophilic character of the molecule, as suggested by the results in a).

Comparison of the partitioning into single bilayer phosphatidyl choline vesicles of NAP-β-Ala-Phlz with that of phlorizin showed that this indeed was so. The partition coefficients \( P_{DOPC} \) of these ligands, as obtained by equilibrium dialysis, were approximately 20,000 and 1,200, respectively. For comparison, the \( P_{DOPC} \) of 4-azidophlorizin (see Part II, page 129) was approximately 9,000. Artificial vesicles were used instead of brush border vesicles since during equilibration hydrolysis of the ligands by the phlorizin hydrolase (Malathi & Crane, 1969) and the esterases (Fernandez-Lopez et al., 1976) present in these vesicles can occur.

When the lipophilic character of the ligands was monitored by their partitioning between olive oil/water (see Seeman, 1972), similar relative partition coefficients were obtained (data not shown).

---

1) The partition coefficient for dioleoylphosphatidylyl choline vesicles was measured at 25°C, as described in Methods, and is defined as \((\text{ligand bound/mg of lipid})/(\text{free ligand/μl of external solution})\). The values are only approximate because there was a time dependent disappearance of the ligands from the dialysis incubations, probably due to their adsorption to the dialysis membrane or to the Teflon chamber.

2) For the stability of phlorizin and its derivatives in buffer in the presence of membrane vesicles, see page 37.
IV.3. PHOTOINACTIVATION EXPERIMENTS

IV.3.1. Control Experiments

IV.3.1.1. Photoreactivity of the NAP-derivatives alone

Prolonged photolysis can result in undesirable effects, such as slow nonspecific labeling by photolysis products and destruction or alteration of the binding site, as a result of which further photolysis can lead only to nonspecific labeling. In addition, the time dependence of phlorizin binding (see Discussion) requires short photolysis times.

Some idea of a reasonable photolysis time can be obtained by irradiating the photoreactive compound, under appropriate conditions, and by following the photolysis (a) by UV/Vis absorption spectroscopy, or (b) monitoring of the appropriate infrared band.

a) Absorption spectroscopy:

The UV/Vis-spectrum of the NAP-group in aqueous solution at pH 6.5 exhibits a strong absorption band with $\lambda_{\text{max}}$ 263 nm (ε20000) and a weaker band in the visible with $\lambda_{\text{max}}$ 480 nm (ε4500). The band with $\lambda_{\text{max}}$ 263 nm overlaps the band of (protonated)$^1$ phlorizin at $\lambda_{\text{max}}$ 284 nm (ε15800) to give a strong band with $\lambda_{\text{max}}$ 275 nm (ε21000).

The decrease in absorbance of the 480 nm band with time of irradiation with visible light was therefore used to monitor the photolysis of all the NAP-derivatives of phlorizin (Fig. 11A). As shown in Fig. 11A, the band at 480 nm did not completely disappear. A new broad band, most likely due to photoproduct(s), seemed to appear, gradually masking the disappearance of the monitored band. Therefore, to determine the photolysis rate of the NAP-group, it was assumed that photolysis was complete when the isosbestic point at 528 nm began to be unsharp and the absorbance started to increase again, i.e., at 8 min.

$^1$For pH-dependence of the UV absorption spectrum of phlorizin see page 110 and Evans & Diedrich, 1980.
Fig. 11A. Change of absorption spectrum of NAP-β-Ala-Phlz after irradiation. NAP-β-Ala-Phlz (75 μM) in 100 mM mannitol, 100 mM NaSCN, 10 mM pABA, 5 mM MES/Tris, pH 6.5, 4 % ethanol and 0.01 % KN₃ was irradiated at 24°C with visible light in the photolysis apparatus shown in Fig. 3 for the times indicated. The reaction was followed by measuring the visible absorbance of aliquots with a Pye Unicam spectrophotometer.

Fig. 11B. Photolysis of NAP-β-Ala-Phlz. The decrease in absorbance at 480 nm, characteristic of the nitrophenyl group, was measured at the times indicated until the isosbestic point at 528 nm was lost (at 8 min; see Fig. 11A). The half-time of decomposition was 90 s at room temperature.

In Fig. 11B the decrease in absorbance at 480 nm (from Fig. 11A) was plotted against the photolysis time. It showed that the time-course followed first-order kinetics, the decomposition half-time
being approximately 90 s\(^1\).

b) IR spectroscopy:
The azido group in NAP-derivatives exhibits a characteristic absorption band at 2120 cm\(^{-1}\).

ATR (= attenuated total reflexion)-IR spectroscopy (Harrick, 1967; Fringeli & Günthard, 1981) was chosen for monitoring this band because conventional equipment is not suited for measuring aqueous solutions and because there was not enough material to prepare KBr-pellets. Because of the strong intrinsic absorption of most of the buffer components used above, the solutions of the NAP-compounds (<270 µM, saturated) consisted only of 10 mM pABA/NaOH, pH 6.5 and 4% ethanol.

Photolysis was followed by subjecting photolyzed samples to both UV/Vis- and IR-measurements. The t\(_{1/2}\)-values obtained by the two methods were in good agreement, i.e., 73 s and 85 s, by the UV/Vis- and IR-method \(^2\), respectively.

In the following only the UV/Vis-method was used for the determination of photolysis rates.

IV.3.1.2 Photoreactivity of NAP-derivatives in the presence of membranes

The half-life of the ligand alone is only a very approximate guide to the behaviour of the ligand in a more complex environment. The sensitivity of the ligand to irradiation may change on binding, or, conversely, because of screening effects in biological preparations,

1) When NAP-β-alanine was photolyzed under identical conditions, a decomposition half-time of 78 s was obtained when the disappearance of both the band at 480 nm and at 263 nm (the absorption band of phlorizin is absent) was recorded. This suggests that following the disappearance of the 480 nm band as described is an adequate way to monitor the photolysis rate of NAP-derivatives of phlorizin.

2) To quantify the disappearance of the band at 2120 cm\(^{-1}\), the change in the transmission at this wavelength with photolysis time was monitored; two absorption bands which did not change upon irradiation were used as internal references.
Fig. 12. Dependence of photolysis rate of NAP-β-Ala-Phlz on the presence of membrane vesicles. Half-times of decomposition \( t_{1/2} \) of NAP-β-Ala-Phlz upon irradiation with visible light in the presence of membrane vesicles were determined for the vesicle concentrations (expressed by their protein content) indicated, according to Fig. 11B.

due to light absorption, the half-time of the label may be increased (Bayley & Knowles, 1977).

When photolysis of NAP-β-Ala-Phlz was carried out in the presence of brush border membrane vesicles, a significant increase in the decomposition rate as determined by the UV/Vis-method was observed. As shown in Fig. 12 there was a curvilinear relationship between the \( t_{1/2} \)-values determined and the concentration of membrane vesicles as expressed by their protein content. The half-time of approximately 2 s determined in the presence of 2.5 mg protein/ml corresponded to a 40 - 50 fold increase in sensitivity. This value is in good agreement with the half-time for photodecomposition of 4 s which was obtained when photolysis of \(^3\text{H}\)NAP-β-Ala-Phlz under similar conditions (2mg
protein/ml) was followed by TLC\textsuperscript{1).}

A similar effect was seen when photolysis was carried out in the presence of liposomes of either lecithin or asolectin (2 mg lipid/ml), the half time being about 3 s. The reason for this interesting finding is not clear. Probably binding of the NAP-labels to the lipid core of the vesicle membrane led to their enhanced sensitivity to photolysis. A similar increase in sensitivity has been found with other arylazido ligands when bound to their corresponding membrane receptors (Witzemann & Raftery, 1977). As the increase in photolysis rate was also observed by protein-free liposomes, it is unlikely that sensitization of the labels was due to chromophores present in the brush border membranes (Bruder et al., 1980).

In principle the photolysis efficieny could be further increased (i) by illumination with UV light (254 nm wavelength) to activate the 263 nm band ($E = 21000$) of the NAP-group \textsuperscript{2), or (ii) by increasing the radiation dose of visible light using light sources of higher intensity. (i) When NAP-$\beta$-Ala-Pblz was photolyzed as described, but without any filter present, the half-time of disappearance of the NAP-group was reduced to 1.2 s; corresponding to a 60 - 70 fold increase in sensitivity as compared to photolysis with visible light. However, destruction of the transport system under these conditions was considerable, there being a strong inactivation of D-glucose transport in control experiments (see below). This is in contrast to a number of other systems where it has been found (e.g., Cooperman & Brunswick, 1973) that the rate of ligand photolysis at 254 nm compares favorably with the rate of destruction of the biological system. (ii) The use of a high-pressure xenon lamp (300 W) resulted in a 5

\textsuperscript{1) After measured time intervals aliquots were spotted on TLC plates. After chromatography in the dark the appropriate region on the plate was scraped out and the silica gel was subjected to liquid scintillation counting.

\textsuperscript{2) Faster photolysis and higher labeling efficiencies of aryl azido compounds on irradiation at 254 nm rather than by illumination with light of longer wavelengths, have been reported (Bayley, 1975; Brunswick & Cooperman, 1971; Katzenellenbogen et al., 1974).}
fold increase in $t_{1/2}$ when photolysis of NAP-$\beta$-Ala-Phlz was carried out in the presence of vesicles (1 mg protein/ml), as compared to the photolysis rate observed with the mercury high-pressure lamp (350 W) under otherwise identical conditions. Preliminary experiments using a commercial photo-flash lamp as the light source showed a considerable increase in the photolysis rate, when the short duration of a single flash (in the range of ms) was taken into account (Trüb, 1978). However, the equipment was not suitable for achieving complete photolysis within a reasonably short time. The use of a single powerful xenon flash (< ms) to activate arylazides has been reported (Matheson et al., 1977; Cerletti & Schatz, 1979).

In view of these results, NAP-derivatives of phlorizin were, in the photolysis experiments, routinely irradiated with visible light from the 350 W Hg-lamp (see Fig. 3) for 15 - 25 s when membrane vesicles were present. This time is sufficient to activate > 99 % of the label molecules.

IV.3.1.3 Irradiation of membrane vesicles in the absence of photolabel.

Before performing photoinactivation and photolabeling experiments, the sensitivity of vesicles to irradiation with the high intensity (visible) light required for rapid photolysis (see above) had to be determined.

Because NaSCN-gradient ($\Delta\bar{\mu}_{Na}^+$) dependent D-glucose uptake had been chosen as a first screening test for the NAP-derivatives as irreversible photoinactivators of the D-glucose transporter, this assay was also employed to monitor photodestruction of the transporter. This assay is very sensitive, and, with the proper controls, allows various types of photochemical effects to be monitored and recorded. In fact $\Delta\bar{\mu}_{Na}^+$-driven D-glucose uptake in vesicles (e.g., its initial velocity or the height of the "overshoot" peak) can potentially be affected by at least three mechanisms or a combination of them: (i) inactivation of the $Na^+$, D-glucose cotransporter; (ii) collapse of $\Delta\bar{\mu}_{Na}^+$; (iii) leakiness of the vesicles. Some criteria can be applied to distinguish among the possible mechanisms, i.e., (i) inactivation of the transporter should be delayed by the presence of competitive inhibitor(s) or substrate(s) ("specific protection"); (ii) a collapse
Fig. 13A. Effect of argon pretreatment on D-glucose uptake in brush border membrane vesicles illuminated with visible light. 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 5 min, 22°C) after treatment with argon for the times indicated.

Fig. 13B. Overshoot of D-glucose uptake of vesicles, expressed as ratio of uptake at 15 s (overshoot peak)/uptake at 90 min (equilibrium). For details see Methods.

As shown in Figure 13A, D-glucose uptake at 15 s (overshoot peak) and equilibrium uptake (after 90 min) were strongly depressed when vesic-
les were illuminated under aerobic conditions for 5 min, a time sufficient for complete photolysis. The magnitude of the overshoot peak (measured as the ratio of D-glucose taken up after 15 s/equilibrium uptake) was also decreased (Figure 13B). This general depression of uptake could be completely prevented by bubbling argon through membrane suspensions and media prior to photolysis (as described by Bayley & Knowles, 1977) for at least 30 min (Figures 13A+B). Therefore a 30 min pretreatment with argon was routinely done in all subsequent photoinactivation studies.

In the presence of known antioxidants such as (+)-catechin (50 μM), pyrogallol (50 μM) and 2-hydroxy-estradiol (20 μM) photoinduced inactivation could be partially reduced. It is also noteworthy that phlorizin (1 mM) gave some "protection", probably due to its pyrogallol-like nature or due to its absorption of the light (Data not shown).

The observed photoinduced inactivation of D-glucose overshoot is probably due to photooxidative damage of (a component of) the membrane leading to a collapse of $\Delta_G^{\text{H+}}$ (see above, (ii)), as Na$^+$-dependent L-methionine uptake in these vesicles was similarly affected, and as significantly increased Na$^+$-fluxes (at 15 s) were measured after photolysis under aerobic conditions (as compared to argon pretreated photolysates and to nonilluminated control, 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 photo-induced leakiness of membranes to larger molecules e. g., D-glucose, or complete rupture of vesicles, or a combination of these mechanisms. Direct 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).
IV.3.2. Photoinactivation assays utilizing NAP-derivatives of phlorizin.

IV.3.2.1. Irreversible inhibition of the overshoot of $\Delta\mu_{\text{Na}^+}$-driven D-glucose uptake.

Having established the potency of the phlorizin analogues as reversible inhibitors of the Na$^+$, D-glucose carrier in the dark, the inactivation of NaSCN-gradient ($\Delta\mu_{\text{Na}^+}$) dependent D-glucose uptake was used as a first screening test for the NAP-derivatives as irreversible photoinactivators of the D-glucose transporter.

a) Short-time photolysis:
After suppressing the photooxidative effects described above by argon treatment, irreversible inhibition of D-glucose overshoot was monitored after a single period of short-time irradiation in the presence of NAP-derivatives of phlorizin. However, a series of such one-cycle-photolysis experiments using NAP-$\beta$-Ala-Phlz and NAP-N-Phlz as labels gave only unsatisfactory results. Irradiation for 15s, a time shown to be sufficient for activation of $>99\%$ of the label molecules present under the photolysis conditions chosen ($1 - 2$ mg of membrane protein/ml) gave only little inhibition of overshoot (in the range of 10 %), and it was not reliably reproducible. Varying the pH (6.5 - 7.5), the nature of the scavenger (none, pABA, BSA; see below) and the concentration of photolabel (up to its solubility limit, 200 $\mu$M) and of vesicles ($0.5 - 2.5$ mg of membrane protein/ml) did not improve the degree of inactivation.

Therefore, in order to increase the degree of inactivation (labeling efficacy), the labeling procedure was repeated after removal of free photoproducts by centrifugation-washing and by Sephadex G-10 chromatography (Evans & Diedrich, 1980).

As shown in Table II, a concentration dependent irreversible inhibition of overshoot of D-glucose uptake in brush border vesicles was observed after photolyzing twice for 15s (approximately 6 times $t_{1/2}$, see Fig. 12) in the presence of NAP-$\beta$-Ala-Phlz: 23 % (at 75 $\mu$M), 26 % (at 150 $\mu$M).

Two labeling cycles were found to be optimal, because after three cycles, the vesicles, due to their functional instability (see also Fig. 13) exhibited a markedly reduced transport activity (data not shown).

1 Only in later experiments.
Table II

Effect of short time photolysis with NAP-β-Ala-Phlz on the uptake overshoot of D-glucose and L-methionine.

<table>
<thead>
<tr>
<th>Photolysis conditions</th>
<th></th>
<th>% remaining overshoot of</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>concentration (µM)</td>
<td>D-glucose (n = 3)</td>
<td>L-methionine (n = 3)</td>
</tr>
<tr>
<td>None, photolyzed</td>
<td>None</td>
<td>100 ± 4</td>
<td>100 ± 6</td>
</tr>
<tr>
<td>75, photolyzed</td>
<td>None</td>
<td>77 ± 7</td>
<td>n.d.</td>
</tr>
<tr>
<td>150, photolyzed</td>
<td>None</td>
<td>74 ± 8</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>150, photolyzed</td>
<td>200</td>
<td>100 ± 3</td>
<td>94 ± 3</td>
</tr>
</tbody>
</table>

a) Argon pretreated brush border membrane vesicles (1 - 2 mg protein/ml) were preincubated with the ligands indicated for 5s in the dark (22°C, pH 7.5, 100 mM NaSCN-gradient). After 15 s of photoirradiation, membranes were washed twice in Na⁺-free buffer. After a second cycle of preincubation-photolysis-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) The overshoot (expressed as a ratio of the substrate taken up at 15 s (D-glucose), or at 5 s (L-methionine), to the equilibrium uptake; see also Figure 13B) is expressed as a % of the illuminated-membrane-control value. Illuminated and unilluminated controls were not statistically different. When the overshoot of D-glucose and L-methionine was expressed as ratio of pmoles substrate (taken up, at 15 s or at 5 s, respectively) per mg protein, a similar result was obtained. Data are represented as the mean + SD of (n) single experiments.
The binding site specificity of the inactivating reagent can be ascertained through the protective effect of the (photoinert) native ligand. This is shown in Table II, where inactivation of D-glucose overshoot by NAP-β-Ala-Phlz is protected by the presence of 200 μM unlabeled phlorizin during photolysis. Additional evidence for specificity of this process comes from the observation that the overshoot of Na⁺-dependent L-methionine uptake was not affected by this treatment (Table II). This was expected since in the dark neither phlorizin nor NAP-β-Ala-Phlz at the concentrations used for labeling measurably inhibited L-methionine uptake (data not shown).

Labeling at pH 6.5 instead of 7.5 did not result in a measurable increase in specific inactivation (data not shown). The use of NAP-N-Phlz (the \( K_i ' \) value of which is similar to that of NAP-β-Ala-Phlz, see Table I) gave less specific inactivation under the same conditions (data not shown, but see Fig. 15). Preliminary experiments with NAP-β-Ala-N-Phlz as label gave inconclusive results. Considering its considerably lower affinity (\( K_i ' \approx 150 \mu M \)) and its limited solubility, this label was not used for further photoinactivation studies.

All the photolabeling experiments described above were performed in the presence of 10 mM p-aminobenzoic acid (pABA; Ruoho et al., 1973; Rudnick et al., 1975a,b) as a scavenger, with the intention of destroying all photogenerated intermediates at places other than the ligand binding site. In previous control experiments pABA had been shown not to interfere with D-glucose uptake. Photolabeling with no pABA present resulted in a comparable degree of inactivation, ruling out pseudo photoaffinity labeling (Ruoho et al., 1973) as a major mechanism of the inactivation reaction observed. Therefore, unless otherwise stated, pABA was used as a scavenger in all subsequent experiments to reduce random labeling. BSA could not be considered as a scavenger (Ruoho et al., 1973), because it was found to bind (photolyzed) NAP-β-Ala-Phlz (see page 96).

1) The photoinertness of phlorizin is not absolute, as evidenced from its light absorbing properties (see page 77). For proper controls see below.

2) For pH-dependence of the binding potency of phlorizin and congeners, see Fig. 19.

3) The various affinity labeling mechanisms and the role of scavengers will be discussed in greater detail on page 137.
Fig. 14. 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 2 cycles of preincubation-photolysis-washing as described in the legend to Table II and Methods; a single period of photolysis lasted 5 min; D-glucose overshoot 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 (O) and vesicles irradiated in the presence of 250 μM phlorizin (□). NAP-β-Ala-Phlz was photolyzed prior to incubation with the (photolyzed) vesicles (△).

b) Long-time photolysis:
To rule out the possibility that the low degree of inactivation observed after (repeated) short time photolysis was due to incomplete activation of the photolabels, the photolysis time was prolonged. As shown in Fig. 14, both D-glucose (A) and L-methionine overshoot (B) were strongly reduced when photolysis in the presence of NAP-β-Ala-Phlz was increased to 5 min per cycle. However, inactivation of D-glucose overshoot under these conditions was only partially protected by the presence of 250 μM phlorizin during photolysis. Nevertheless, this partial protection by phlorizin must, to a major extent, result from competition for the same site, since inactivation of L-methionine overshoot was not protected at all (Fig. 14 B). A part of this protection might be attributed to unspecific protection due to
the light absorbing properties of the phenols, as evidenced from Fig. 14A: vesicles irradiated in the absence of NAP-label, but with phlorizin present, consistently showed a slightly higher overshoot than control vesicles (i.e., with no phlorizin present).

A carrier-unrelated effect of photolyzed label must account for the considerable unprotectable inactivation observed for both transport systems: covalent insertion into the membrane of lipophilic label photoproducts might cause a collapse of $\Delta\mu_{\text{Na}^+}$ or a general disturbance of the membrane similarly to the photoxidative effects occurring in the absence of label (see Figures 13A & B).

There was no inactivation when vesicles which had been illuminated in the absence of label were subsequently incubated with prephotolyzed label (immediately after its photoactivation). This "prephotolysis control" ruled out long-lived reactive intermediates of photolyzed arylazides (Chapman & Le Roux, 1977) as being responsible for the inactivation observed, as found for postphotolytic (dark) labeling of rhodopsin using NAP-taurine (Mas et al., 1980).

The lack of inactivation seen when free NAP-$\beta$-alanine was used as a label at the same concentration, 150 $\mu$M, suggests that the phlorizin moiety (and its site directing effect) is required for both specific and (some of) unspecific inactivation.

Neither the use of higher protector concentrations nor preincubation of vesicles and phlorizin prior to the addition of the NAP-label did measurably increase the protectable fraction of the decrease in D-glucose overshoot shown in Figure 14A.

IV.3.2.2. Inactivation of D-glucose tracer exchange

The tracer exchange assay is an alternative and probably more reliable method of monitoring the inactivation of the transporter by site-directed photo-attachment of nonlabeled photoligand. Since functioning of the transporter in this case is assayed in the absence of $\Delta\mu_{\text{Na}^+}$, apparent inactivation as a consequence of a collapse of $\Delta\mu_{\text{Na}^+}$ is eliminated (see above).

In addition, to differentiate between true site-specific protection and non-specific protection by phlorizin (as a light absorbent or scavenger), para-phlorizin, the 4'-isomer of phlorizin, was used as a control. This glucoside has a low inhibitory potency on specific
phlorizin binding to the carrier ($K_i \approx 420 \ \mu M$) and can therefore, at 250 $\mu M$, be assumed to bring about little site-specific protection, but to otherwise display the general properties of phlorizin responsible for possible unspecific protection.

The significant difference ($P < 0.001$) in D-glucose tracer exchange activity found after photolyzing with NAP-$\beta$-Ala-Phiz in the presence of phlorizin (site-directed protection) as compared to the presence of para-phlorizin (unspecific protection), as shown in Table III, corresponds to (at least) 20% true site-specific inactivation. The 7% higher exchange activity measured after irradiation in the presence of label and protector, as compared with irradiation in the presence of protector alone, clearly demonstrates the need of correcting for unspecific screening by light absorption and scavenging by both the phenol and chromophore.

IV.3.2.3. Inactivation of specific phlorizin binding to DOC extracted membrane vesicles.

If site-directed attachment of photolabel is the mechanism of the specific inactivation of D-glucose transport activity (as reported above), the Na$^+$-dependent, D-glucose protectable phlorizin binding (specific binding) to the carrier must be decreased similarly. To test this prediction, phlorizin binding to photolyzed DOC-extracted brush border membranes was monitored. These membranes display a 2 to 3 fold enrichment in Na$^+$-dependent phlorizin binding activity (as a preliminary step in purifying the Na$^+$, D-glucose cotransporter; Klip et al., 1979b), but do not retain measurable D-glucose transport activity, due to irreversible disruption of the membranes.

a) NAP-$\beta$-Ala-Phlz as photoinactivator of specific phlorizin binding:

As evidenced from Table III, Na$^+$-dependent phlorizin binding (at 15 s) to DOC extracted membranes was irreversibly reduced after photolysis in the presence of NAP-$\beta$-Ala-Phlz (100 $\mu M$) as label. Again specificity of irreversible inactivation was unambiguously established by comparing site-directed protection by phlorizin (250 $\mu M$) with unspecific protection by its 4'-isomer para-phlorizin (250 $\mu M$): 14% specific inactivation of specific phlorizin binding was observed after one short-time photolysis cycle, 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.
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

<table>
<thead>
<tr>
<th>Photolysis conditions</th>
<th>% remaining activity of D-glucose tracer exchange</th>
<th>% remaining activity of specific phlorizin binding after m cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAP-β-Ala-Phlz protector concentration (μM)</td>
<td>(m=1, n=6)</td>
<td>(m=2, n=2)</td>
</tr>
<tr>
<td>None, photolyzed 250 Phlz</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>100, photolyzed 250 p-Phlz</td>
<td>87 ± 4</td>
<td>88 ± 4</td>
</tr>
<tr>
<td>100, photolyzed 250 Phlz</td>
<td>107 ± 3</td>
<td>102 ± 3</td>
</tr>
</tbody>
</table>

aN Native and DOC extracted brush border membrane vesicles were illuminated once or twice as described in Table II and Methods, with minor modifications; DOC extracted vesicles were preincubated for 15 s in the dark, and a single photolysis lasted 25 s.

Tracer exchange of D-glucose was assayed after equilibrating the vesicles (30 min, room temperature) in 100 μM D-glucose and 100 mM NaCl or KCl, pH 7.5. Exchange was initiated by adding the same medium plus (3H)-D-glucose (approximately 10 μM (1 μCi/10 μl of medium) and incubating for 8 s. Carrier unrelated D-glucose influx (in the presence of KCl) was corrected for by subtracting the uptake in the presence of 100 mM KCl from the total uptake in the presence of 100 mM NaCl.

Phlorizin (5 μM) binding measurements (at 15 s) under equilibrium conditions (100 mM NaCl or KCl) were performed at pH 7.0; specific (Na⁺-dependent) phlorizin binding activity was determined as described in Methods.

bRefers 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 number of photolysis cycles (for details see Methods).
b) Long photolysis times in the irreversible photoinactivation of specific phlorizin binding by NAP-derivatives of phlorizin:

Prolonged photolysis (up to 120 s) of DOC membranes in the presence of NAP-β-Ala-Phlz (●) or NAP-N-Phlz (▲) did not result in a measurable increase in the inactivation of (initial) specific phlorizin binding, as compared to the inactivation observed after a 10 s photolysis (Fig. 15).

This is consistent with the above observations (see Table II) that specific photoinactivation of D-glucose overshoot with NAP-β-Ala-Phlz as the label was maximal after a 15 s under similar photolysis conditions.

c) Comparison of the labeling efficencies of NAP-β-Ala-Phlz and NAP-N-Phlz:

Because of the high degree of accuracy of the measurements achieved, phlorizin binding to DOC extracted membranes also proved to be well suited for monitoring minor differences between the inactivation efficiencies of the various labels.

A comparison of the potencies of NAP-β-Ala-Phlz (●) and NAP-N-Phlz (▲) as photoinactivators of the (initial) phlorizin binding (at 2 s) to DOC extracted membranes, after photolysis under identical conditions (at 120 µM), is presented in Fig. 15. It is evident that NAP-N-Phlz was less efficient under the conditions chosen. This is in agreement with earlier, preliminary experiments, where NAP-β-Ala-Phlz was also found to be more potent than NAP-N-Phlz, with respect to photoinactivation of D-glucose uptake in vesicles (see above).

Among other findings this is consistent with the preferential use of NAP-β-Ala-Phlz as label in photoinactivation and photolabeling experiments.

The reason for the high inactivation efficencies achieved with both labels after 10 s irradiation (i.e., 48 % and 29 % inactivation of the (initial) phlorizin binding, at 2 s, see Fig. 15) as compared to the inactivation efficiency of NAP-β-Ala-Phlz (as determined from inactivation of maximal specific phlorizin binding at 15 s, see above) is not clear.
Fig. 15. Comparison of the effectiveness of NAP-β-Ala-Phlz and NAP-N-Phlz as photoinactivators of specific phlorizin binding to DOC extracted membranes with respect to the influence of the photolysis time. DOC extracted membranes (1.75 mg protein/ml) were incubated in the absence of label (■), or in the presence of 120 μM NAP-β-Ala-Phlz (●) or 120 μM NAP-N-Phlz (▲) for 15 s in the dark (300 mM mannitol, 100 mM NaSCN, 10 mM Tris-HCl, pH 7.0, 1 % ethanol, 0.02 % KN₃). After a single photolysis, for the time indicated, membranes were separated from free photoproducts by Sephadex G-10 chromatography and repeated washing by centrifugation, as described in Methods. Membranes were then assayed for phlorizin binding under equilibrium conditions in NaCl or KCl (100 mM, pH 7.0) at 2 s, and specific phlorizin binding was determined as described in Methods. Determinations were carried out in pentuplicate; the values are given as the mean ± SD.
IV.4. PHOTOLABELING WITH $^{3}$H-NAP-β-ALA-PHLZ

IV.4.1. Labeling of intact brush border membrane vesicles with $^{3}$H-NAP-β-Ala-Phlz.

Slab gel electrophoresis followed by fluorography according to Bonner & Laskey (1974, 1975) was chosen to monitor covalent incorporation of the ligand.

Labeling of intact rabbit small-intestinal brush border membrane vesicles with $^{3}$H-NAP-β-Ala-Phlz is shown in Fig. 16A. Photolysis with 100 μM label, under conditions previously shown to cause 14 % site-directed inactivation of D-glucose uptake and phlorizin binding (see Fig.14 and Table III), resulted in the labeling of a great number of proteins (lane 2). Among the major protein bands labeled were I, 3, 4 and A (for comparison the Coomassie blue staining pattern of the same gel is given in lane 1). Notably there was also considerable labeling of a diffuse band(s) in the region of 60,000 D to 80,000 D, where a possible candidate for the phlorizin binding component of the brush border membranes has been located (Klip et al., 1979b). This diffuse staining pattern probably reflects labeling of intrinsic proteins, because these are known to display ill-defined contours in this electrophoresis system, whereas peripheral polypeptides yield sharp bands.

However, it appears that the labeling observed under these conditions is mainly unspecific, since there was no band of which the labeling intensity was reduced in the presence of phlorizin (lane 3) as compared to para-phlorizin (lane 2). In addition there was no detectable difference in the labeling pattern when choline SCN or KSCN were used instead of NaSCN, all at 100 mM (gels not shown).

Lowering the concentration of the $^{3}$H-label to 50 μM resulted in a general decrease of the labeling intensity (lane 4). However, there was no qualitative difference between this labeling pattern and the one observed after photolysis at the higher label concentration.

1) The occurrence of fuzzy bands in SDS-PAGE patterns of some proteins has been attributed to heterogeneity in the carbohydrate content of glycoproteins (Steck, 1974) and to the continuous formation of reversible disulfide bonds during the run (Lane, 1978).
Fig. 16 A. Fluorograms showing the labeling of intact small-intestinal brush border membrane vesicles with $^{3}$H-NAP-$\beta$-Ala-Phlz. Vesicles (1.5 mg protein/ml) were photolyzed for 25 s in the presence of 100 $\mu$M ($^{3}$H)-label plus 250 $\mu$M para-phlorizin (lane 2) or plus 250 $\mu$M phlorizin (lane 3), as described in Methods. In lane 4 photolysis was performed in the presence of 50 $\mu$M ($^{3}$H)-label plus 250 $\mu$M para-phlorizin. After Sephadex G-10 chromatography and extensive washing photolysates were boiled with solubilizing solution under reducing conditions for 60 s. 50 $\mu$g samples of protein were subjected to SDS-PAGE (8.4 x 2.7). Approximately 300,000 cpm were applied onto lanes 2 & 3, and 150,000 onto lane 4. After Coomassie blue staining (the labeling pattern is shown in lane 1) gels were proceeded for fluorography according to Bonner & Laskey (1974). For the nomenclature of some of the bands given or the left, see Fig 6. The molecular weight scale is shown to the right.

Fig. 16B. Heat-dependent aggregation of labeled vesicle proteins. The fluorograms of samples (50 $\mu$g protein) of the photolysate boiled for 1 min, and of a similar photolysate (from a different experiment, but carried out under the same conditions) boiled for 3 min are compared (Gels 1' and 3'). Both gels contain 300,000 cpm; a Kodak X-Omat R film was exposed to both gels for 90 h at -70°C. Note the disappearance of ($^{3}$H)-label in the 80,000 D to 60,000 D region and the appearance of stained aggregates on top of the gel 3'. Gel 1' is the same as in lane 2 of A.
Most of the radioactivity on the gel was associated with the region K, reflecting extensive labeling of lipids, and with the tracking dye front (the two regions are not well separated on these fluorograms; a cleaner separation is shown in Fig. 17). The latter included labeled polypeptides which were not resolved by the (8.4 x 2.7) gel, i.e., with an apparent molecular weight below 30,000 D. A (15 x 2.7) gel showed at least two labeled bands in the region of 20,000 D to 28,000 D and a weakly labeled band at 14,000 D. The labeling intensity of neither of these bands was reduced by the presence of protector or KSCN instead of NaSCN (gels not shown).

Most of the label comigrating with the dye front, however, was noncovalently bound and/or photoproducts (including adducts with low molecular weight compounds, such as the scavengers pABA and Tris), which were not removed by Sephadex G-10 gel filtration and centrifugation-washing because they were dissolved in the lipid bilayer. This was supported by control experiments where membranes were incubated (i) with prephtotolyzed label (= prephotolysis control, see below) or (ii) with intact label in the dark; subsequently both were washed as above. In both cases there was (~H)-label comigrating with the dye front; in (ii) the presence of (~H)-label (0.2 % or less of the label initially present, depending on the number of washing cycles) was restricted to the dye front, whereas in addition in (i) weak labeling of lipids (K) and protein bands such as A and I could be observed (in total approximately 1% of the initial label present). The reason for postphotolytic labeling of polypeptides in (i) will be discussed on page 140.

When photolysis in the presence of 100 μM (~H)-NAP-label was performed at a vesicle concentration of 2 mg protein/ml (corresponding to a concentration of total lipid of approximately 1 mg/ml (Hauser et al., 1980)), 10 - 15 % of the (~H)-label initially present remained associated with the vesicles after Sephadex G-10 gel filtration and washing by centrifugation. This corresponds to some 6 - 9 nmoles of label associated with the vesicles per mg of protein. If one corrects for free (~H)-photoproducts (30 %) comigrating with the dye front and for (~H)-label bound to lipids (region K on the gels in Fig. 16; 35 % of the total label present), there are still some 2 - 3 nmoles of label bound per mg of protein. This number exceeds by far the specific phlorizin binding capacity of this brush border preparation, which would
give the maximum number of label molecules that can be incorporated site-specifically as 10 - 40 pmoles/mg protein (Toggenburger et al., 1978; see also introduction and discussion below).

At a given vesicle concentration (as expressed by the protein content) and photolysis time there was a linear relationship between the label concentration (range tested: 50 - 150 \(\mu\)M) and the total amount of label associated with the vesicles after photolysis and washing (data not shown). This reflects incorporation of label in a nonsaturating process and is consistent with the finding that 65% of the \(^3\)H-label finally associated with vesicles is covalently bound to lipids or dissolved in the lipid bilayer.

A peculiar feature of the labeling pattern of these vesicles became apparent when the length of heating after addition of the electrophoresis solubilizer was varied (Fig. 16B). Boiling the samples for not longer than 1 min produced labeling patterns in which band 1 was the component of the highest molecular weight labeled; no significant amount of label was detectable on top of either the separating or the stacking gel. However, as the boiling period was prolonged, considerable amounts of \(^3\)H-label appeared on top of both the separating and the stacking gel (Fig. 16B, 3'). Interestingly, not all the labeled polypeptides contributed to the same extent to the aggregated material, since all bands did not fade to a similar degree. The labeled bands migrating in the 5 to 7 region (mol wt 80,000 D to 60,000 D) seemed to be the main components of the aggregate, whereas the rest of the stain was only minimally changed. These changes were barely detectable on the corresponding Coomassie blue stained gels, and thus show that this effect is restricted to a minority of (ill-staining) polypeptides (Coomassie blue stains of gels are not shown). This susceptibility to heat of a selected population of labeled protein bands was even more pronounced when DOC extracted membranes were used; among other reasons, probably because these bands were relatively enriched in this preparation. In contrast to this, with DOC extracted membranes these changes could also be detected on the Coomassie blue stained gels. This finding agrees with the observations of Klip et al. (1979b) who described similar changes in the Coomassie blue staining pattern of DOC extracted membranes after prolonged boiling. Since aggregation occurred even in the presence of re-
ducing agents, formation of disulfide bonds is an unlikely explanation. Heat-dependent changes in the aggregation state of other membrane proteins in SDS have been reported (Furthmayr & Marchesi, 1976; Saari, 1974). To prevent the formation of these aggregates, all the samples in the experiments described below were boiled for only 1 min. Shorter heating periods were avoided because incomplete dissociation of the sucrase-isomaltase complex was observed sometimes.

IV.4.2. Labeling of DOC extracted brush border membranes with (\(^3\)H)-NAP-\(\beta\)-Ala-Phlz.

DOC extracted brush border membranes were shown to exhibit a two to three fold increase in specific phlorizin binding activity, concomitant with a decrease in unspecific binding of the glucoside, as compared to intact membrane vesicles (Klip et al., 1979b). Considering the enormous amount of unspecific labeling observed with intact membrane vesicles, both these features seemed to make DOC extracted membranes a promising system for further photolabeling studies.

IV.4.2.1. Comparison of the labeling pattern of DOC extracted membranes with that of intact brush border vesicles.

A comparison of the labeling pattern of DOC extracted membranes after photolysis in the presence of 100 \(\mu\)M (\(^3\)H)-NAP-label (Fig. 17 A, lane 2) with the one of intact vesicles photolyzed under identical conditions (lane 1) shows that these were qualitatively similar. However, the former exhibited a marked decrease in the staining intensity of the bands 4 and \(\lambda\), concomitant to an increase in the labeling intensity of band I and the 60,000 D to 80,000 D region. These changes paralleled the changes in the Coomassie blue staining pattern (see also Fig. 6) and thus may merely reflect that extraction with DOC removes some, and enriches other, proteins susceptible to labeling with the NAP-label. There was no significant decrease in the labeling of lipids with the DOC extracted membranes (lane 2, K).

The labeling pattern observed after photolysis in the presence of 50 \(\mu\)M NAP-label is shown in Fig.17 A, lane 4; for comparison the corresponding pattern of labeled intact vesicles is shown in lane 3. It is obvious that the labeling pattern of DOC extracted membranes did not change qualitatively upon lowering the (\(^3\)H)-label concentration. Nevertheless photolysis at lower label concentrations could be
Fig. 17. Fluorograms showing the labeling of DOC extracted membranes with \((^3H)\text{NAP-}\beta\text{-Ala-Phlz}\). (A): Comparison of the labeling patterns of DOC extracted membranes with that of intact vesicles; specificity of labeling of DOC extracted membranes. DOC extracted membranes (1.4 mg protein/ml) were photolyzed for 25 s in the presence of 100 \(\mu\text{M}\) (lane 2) or 50 \(\mu\text{M}\) \((^3H)\text{NAP-}\beta\text{-Ala-Phlz}\) (lanes 4 - 7), as described in Methods; 40 \(\mu\text{g}\) samples of protein were subjected to SDS-PAGE (8.4 x 2.7). For comparison the labeling pattern of intact membrane vesicles in the presence of 100 \(\mu\text{M}\) (lane 1) or 50 \(\mu\text{M}\) \((^3H)\)-label (lane 3) is also shown (see Fig. 16A). Photolysis of the sample in lane 5 was performed in the presence of 100 mM choline SCN. The sample in lane 7 had 500 \(\mu\text{M}\) para-phlorizin and that in lane 6 had 500 \(\mu\text{M}\) phlorizin present in the incubation medium during photolysis. Lane 8 shows the Coomassie blue stained pattern of DOC extracted membranes. Approximately 500,000 cpm were applied onto lanes 1 and 2, and 220,000 to 250,000 cpm onto lanes 4 - 7. Fluorography lasted 72 h. (B): The effect of scavengers present during photolysis on the labeling pattern. DOC extracted membranes were photolyzed as in (A) in the presence of 50 \(\mu\text{M}\) \((^3H)\text{-NAP-}\)label and the following scavengers: 10 mM pABA (lane 9), none (lane 10), 10 mM GSH (lane 11). The reduced glutathione (buffered) was added 30 s before photolysis. 40 \(\mu\text{g}\) samples of protein (160,000 to 220,000 cpm) were electrophoresed and the gels were fluorographed (72 h exposure) as in (A).
expected to result in a reduction of unspecific labeling to an extent that compared favourably with the reduction of the possible specific labeling. This would allow the detection of changes in the labeling intensity (e.g. in the presence of protectors) of a minor, faint band or region which could be masked by the particularly strong unspecific labeling at high label concentration.

IV.4.2.2. The specificity of $^3$H)-NAP-β-Ala-Phlz as a label for the phlorizin binding site of the transporter in DOC extracted membranes.

The specificity of the photolabeling process with $(^3$H)-NAP-β-Ala-Phlz at 50 μM (a concentration corresponding to its $K_i$, see Table I) was carefully examined by monitoring firstly the protectability of the labeling reaction, secondly the Na⁺-dependence of the labeling reaction, and thirdly the labeling by other, less or not at all site directed, NAP-derivatives.

a) **Protectability:**

The labeling pattern observed after photolysis in the presence of 500 μM phlorizin as protecting agent is shown in Fig. 17A, lane 6. Para-Phlorizin at the same concentration was used as a control for possible unspecific protection by the phenol (lane 7). There was no detectable, selective decrease in the labeling intensity of a particular band in the presence of phlorizin. Note, however, a slight reduction in the staining intensity of bands A and I after photolysis in the presence of para-phlorizin. Neither did the presence of D-glucose (100 mM) result in the protection of any of these bands.

b) **Na⁺-Dependence:**

There was no selective change in the labeling pattern when photolysis of DOC extracted membranes was performed in the presence of 100 mM choline SCN instead of NaSCN (Fig.17A, lane 5).

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1) For the use of para-phlorizin as control for unspecific protection, see also page 83 and Table III.
c) Labeling with other NAP-derivatives:

NAP-\(\beta\)-Ala-para-Phlz has a much lower affinity for the binding site of the transporter \(K_i > 300 \ \mu \text{M},\) see Table I) than the corresponding NAP-derivative of phlorizin. Therefore, photolysis of DOC extracted membranes in the presence of this \((^3\text{H})\)-labeled glucoside is expected to result exclusively in unspecific labeling\(^1\). However, because this ligand is isomeric with NAP-\(\beta\)-Ala-Phlz, the labeling pattern observed with this ligand may be assumed to correspond to those parts of the labeling pattern of NAP-\(\beta\)-Ala-Phlz which result from unspecific labeling. This again could help to detect bands specifically labeled by NAP-\(\beta\)-Ala-Phlz. The same control can be made using NAP-\(\beta\)-alanine as an unspecific label.

The labeling patterns observed after photolysis in the presence of 100 \(\mu\text{M}\) NAP-\(\beta\)-Ala-para-Phlz (Fig. 18A, lane 1) and of 100 \(\mu\text{M}\) NAP-\(\beta\)-Ala-Phlz (lane 2) are in qualitative terms strikingly similar; there is a generally lower staining intensity with the former, concomitant to an increased formation of aggregates which do not enter the gel.

The labeling patterns of membranes photolyzed in the presence of \((^3\text{H})\)-NAP-\(\beta\)-alanine and \((^3\text{H})\)-phlorizin, both at 100 \(\mu\text{M}\), are shown in lanes 3 and 5, and 4 and 6, respectively. There was hardly any labeling detectable in lanes 3 and 4 which were fluorographed for 3 days which is the same time period as for lanes 1 and 2. When the time of fluorography was prolonged to 40 days, the labeling patterns shown in lanes 5 and 6 were obtained. Note the qualitative similarity of the pattern observed with NAP-\(\beta\)-alanine (lane 5) to the one observed with NAP-\(\beta\)-Ala-Phlz (lane 2).

Surprisingly, photolysis with visible light of membranes in the presence of \((^3\text{H})\)-phlorizin led to labeling of a few bands, among them 1, 3, A, and the 60,000 D to 80,000 D region (lane 6). This important finding will be examined in more detail in Appendix A.

IV.4.2.3. The effect of scavengers on labeling by \((^3\text{H})\)-NAP-\(\beta\)-Ala-Phlz.

All the above photoinactivation and photolabeling experiments were performed in the presence of pABA as a scavenger during photolysis. In order to monitor the influence of this agent on the labeling pattern, it was either omitted or replaced by reduced glutathione which is also

\(^1\) The possibility of unspecified labeling of the phlorizin binding component of the transporter by this ligand (and also by NAP-\(\beta\)-Ala-Phlz) cannot be ruled out (see also Discussion).
Fig. 18. (A): Fluorograms showing the labeling of DOC extracted membranes by various \(^3\text{H}\)-NAP-derivatives. DOC extracted membranes (1.4 mg protein/ml) were photolyzed in the presence of the following at 100 \(\mu\text{M}\) \(^3\text{H}\)-labeled compounds (specific activity: 4 Ci/mmol): NAP-\(\beta\)-Ala-para-Phlz (lane 1), NAP-\(\beta\)-Ala-Phlz (lane 2) and NAP-\(\beta\)-alanine (lanes 3 and 5); labeling in the presence of 100 \(\mu\text{M}\) \(^3\text{H}\)-phlorizin (6.3 Ci/mmol) (lanes 4 and 6). 40 \(\mu\text{g}\) samples of protein (cpm applied were: 425,000 (lane 1), 505,000 (lane 2), 27,500 (lanes 3 and 5) and 11,000 (lanes 4 and 6)) were subjected to SDS-PAGE (8.4 x 2.7), and gels were fluorographed, as described. Exposure of gel lanes 1 - 4 lasted 3 days; lanes 5 and 6 show the 40 days fluorograms of lanes 3 and 4, respectively. Lane 7 shows the Coomassie blue stained pattern.

(B): The effect of extraction with KI prior to photolysis on the labeling pattern of intact vesicles and DOC extracted membranes. DOC extracted membranes (lanes 8 and 9) and intact membrane vesicles (lanes 10 and 11) were (lanes 9 and 11) or were not (lanes 8 and 10) extracted with 0.6M KI, as described in Methods. Membranes (1.3 to 1.5 mg protein/ml) were then photolyzed in the presence of 50 \(\mu\text{M}\) \(^3\text{H}\)-NAP-\(\beta\)-Ala-Phlz for 25 s, electrophoresed on a (8.4 x 2.7) gel (40 \(\mu\text{g}\) of protein) and fluorographed as described (72 h exposure).
frequently used as a scavenger (Bayley & Knowles, 1977, 1978a,b).

The labeling pattern observed after photolysis in the presence of 10 mM pABA (Fig. 17B, lane 9) showed a slightly decreased staining intensity, as compared with the one observed in the absence of pABA (lane 10). The presence of 10 mM reduced glutathione resulted in a stronger general reduction of the labeling of DOC extracted membranes (lane 11). However, aryl azides are known to be rapidly reduced to the corresponding amines by (di)thiols at physiological pH and temperature; the rate of reduction of electron-deficient aryl azides, such as the NAP-derivatives, being relatively faster (Staros et al., 1978). Therefore, the decrease in labeling by the NAP-derivative might, in addition to the efficacy of the monothiol as a scavenger, also be due to a decrease in the concentration of unphotolyzed ligand by reduction prior to and during photolysis.

Based on this finding 10 mM pABA was used as the scavenger in subsequent experiments.

An interesting finding was that, BSA (10 mg/ml), added immediately after photolysis in order to trap non-covalently bound (3H)-ligand and photoproducts, became covalently labeled. This is in agreement with the recent observation of Mas et al. (1980) that incorporation of radioactivity from 35S-NAP-taurine into rhodopsin in rod outer segment disk membranes did not stop following cessation of illumination. This finding will be analyzed in more detail in the following section (see also Discussion, page 140).

IV.4.3. The location of labeled actin.

Labeling of the actin band A was observed when both intact and DOC extracted membrane vesicles were photolyzed in the presence of (3H)-NAP-β-Ala-Phlz (see Fig. 17A).

The labeling of actin, in particular when using intact membrane vesicles, raised the important question as to whether the labeled actin is located inside sealed vesicles, or is accessible to the bulk phase? Labeling of actin inside sealed vesicles would imply that NAP-β-Ala-Phlz was able to penetrate the membrane in sufficient amounts during

1) Buffered reduced glutathione was added to the photolysis incubation immediately (approximately 30 s) before photolysis.
the photolysis (25 s).

One way to answer this question was by extracting both, intact vesicles and DOC extracted membranes, with KI prior to the photolysis. This treatment is known to solubilize the majority of cytoskeletal proteins (Bretscher & Weber, 1980a).

The labeling of normal DOC extracted membranes by \((^{3}H)\)-NAP-\(\beta\)-Ala-Phlz is shown in Fig. 18B, lane 8. Brief extraction of these membranes with 0.6 M KI prior to photolysis resulted in a decrease in both the Coomassie blue staining and the labeling intensity of the actin band A (lane 9). Since the majority of DOC extracted membranes are known to be leaky and to expose both sides of the bilayer to the external medium (Klip et al., 1979c), this demonstrates, that, if accessible to the bulk phase, actin associated with brush border membranes can be extracted by KI.

Extraction of intact vesicles with KI prior to photolysis, however, did not result in a detectable decrease in the Coomassie blue staining and labeling intensity of the actin band (lane 11), as compared with the labeling intensity of untreated vesicles (lane 10). This demonstrates that actin labeled by NAP-\(\beta\)-Ala-Phlz is located inside sealed vesicles and, therefore, leads to the conclusion that this NAP-derivative is indeed able to penetrate the membrane bilayer in considerable amounts during photolysis.

2) Using an impermeable derivative of NEM (MIMAX) Biber (1979) showed that 98% of the actin associated with intact membrane vesicles was inaccessible to the bulk phase. However, the remaining 2%, either located inside "open" vesicles or adhering to the outer surface of closed vesicles, might be the material which becomes labeled by the NAP-derivative.
IV.5. DISCUSSION

IV.5.1. Choice and synthesis of the ligands.

Of the possible positions on the β-D-glucopyranosyl moiety of phlorizin, C-6 was chosen for attachment of the reactive group. 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 the sugar binding site of the transporter may tolerate substitutions in this position of the monosaccharide: the deoxy-fluoro derivatives of D-glucose and D-galactose are transported (Wilson & Landau, 1960; Barnett et al., 1968). The minimum structural requirements for intestinal Na^+-dependent monosaccharide transport have been discussed by Crane (1960).

Therefore photoactivatable analogues of phlorizin were synthesized either by direct attachment of the photoreactive NAP-group to the C-6 position of the glucopyranosyl moiety of 6-amino-deoxyphlorizin or via β-alanine as spacer to the same position of (i) phlorizin and (ii) of 6-amino-6-deoxyphlorizin (see Fig. 4). Variation in the distance of the NAP-group from C-6 might increase the possibility of covalent reaction within, or near to, the substrate binding site region. The potential susceptibility of NAP-β-alanyl-6-O-phlorizin to esterase activities present in the intestinal mucosa (Fernandez-Lopez et al., 1976; Négrel et al., 1976) necessitated the preparation of the corresponding amide.

The NAP-group was chosen as the photoreactive group, because aryl azides have proved very useful as the photoreactive moiety of a variety of photoaffinity probes (Bayley & Knowles, 1977). Aryl azides are unreactive in the dark, however, upon photolysis they generate aryl nitrenes; these may either directly form covalent adducts with their targets by insertion reactions (Smith, 1970) or rearrange to relatively long-lived reactive intermediates, e.g., cyclic ketenimines (Chapman & Le Roux, 1978; reviewed by Staros, 1980) which primarily attack nucleophilic residues.

1) The corresponding deoxy-ido 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 was observed with the derivatives of phlorizin at position 6 (see Table I).
Meta nitrophenyl azides are particularly suited for labeling studies because the substitution shifts the $\lambda_{\text{max}}$ into the visible, away from the UV absorption of the proteins, and decreases the half-life (i.e., increases the reactivity) of the nitrene (Bayley & Knowles, 1977). However, the nature of their intermediates which react with nucleophiles is not established at the present (Staros, 1980).

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 the above photolabels, spin labels and fluorescent adjuncts may also be prepared in the same way.

It has to be mentioned in this context that a 4-azido-2-nitrobenzoate derivative of phlorizin was previously used for photoinactivation of sulfate exchange in erythrocyte ghosts (Kaplan & Fasold, 1976), but the precise location of the photoreactive group on the phlorizin molecule was not established.

IV.5.2. Reversible inhibition of D-glucose transport and phlorizin binding.

In order for a reagent to be a photoaffinity inhibitor of a transporter, the reagent should (i) reversibly inhibit transport in the unphotolyzed state and (ii) result in irreversible inhibition on 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 were prepared from phlorizin do satisfy the "in dark requirements", albeit to different extents: they are reversible, fully-competitive inhibitors of the $\text{Na}^+$, $\Delta\psi$-dependent D-glucose uptake and they compete with phlorizin for $\text{Na}^+,(\Delta\psi)$-dependent phlorizin binding to brush border vesicles and to DOC extracted membranes. Their $K^*$ values for transport and their $K^*_t$ and $K^*_n$ values for phlorizin binding increase in the order: azido-Phlz (9) < NAP-$\beta$-Ala-Phlz (15) < NAP-N-Phlz (13) < NAP-$\beta$-Ala-N-Phlz (14). Unfortunately, azido-Phlz (9), in spite of its excellent inhibitory potency($K^*_t$ approx. 10 $\mu$M) could not be further considered, because the light necessary to activate aliphatic azides ($\lambda$ approx. 253 nm, Chakrabarti & Khorana, 1975) is likely to cause photochemical damage to proteins.
IV.5.3. Photolysis and photoinactivation studies.

The conditions for photolysis had to be carefully optimized, particularly with respect to (i) phlorizin binding and (ii) the rate of photodecomposition of the aryl azides. (i) High-affinity, Na\(^+\), Δψ-dependent, D-glucose protectable phlorizin binding goes through a maximum at about 2 - 5 s contact time (Toggenburger et al., 1978; Toggenburger, 1979). As the \( K_1 \) and \( K_0 \)-values of the best NAP-derivatives of phlorizin were in the same order of magnitude as the \( K_1 \) and \( K_0 \) values of phlorizin itself, the kinetics of binding of these derivatives to the brush border membranes could be expected to follow a similar time course. In addition, the specific binding to the D-glucose transporter of these lipophilic phlorizin derivatives (see page 68) had to be expected to be masked even more than in the case of phlorizin by slow, non-specific uptake into the lipid phase of the membrane. Therefore, short contact and short irradiation times seemed to be mandatory for photolabeling with these NAP phlorizin derivatives. (ii) The half-times of photodecomposition of the arylazido derivatives were reduced several fold, depending on the vesicle concentration, by the presence of natural or artificial membranes.

Brush border vesicles which had been irradiated with high intensity visible light in the absence of photolabel under aerobic conditions displayed a drastic reduction in accumulative D-glucose uptake (overshoot) in the presence of a NaSCN-gradient, concomitantly to an increase in Na\(^+\)-conductance. Pretreatment with argon completely prevented this. This points to photooxidative effects on the membrane as being responsible for, among other things, a collapse of Δψ\(^{Na\,+}\) leading to the (apparent) inhibition observed. The inhibitory effect of agents such as the organomercurial pCMBS (at low concentrations) and Cu-(o-phenanthroline)\(_2\) on active non-electrolyte transport has similarly been shown to be based on this mechanism (Klip et al., 1979c; Biber & Hauser, 1979; Klip et al., 1980; Will & Hopfer, 1980). Yet direct chromophore-sensitized photooxidation of (a component of) the transporter cannot be ruled out (Jori, 1975). Cytochromes (b\(_5\), P-420), NADH-cytochrome c reductase or other chromophores reported to be present in the plasma membranes of intestinal microvilli (Bruder et al., 1980) might act as membrane bound sensitizers. Vesicles irradiated in the presence of NAP-β-Ala-Phlz (15), the most promising of the labels studied here, exhibited an irreversible inactivation of Δψ\(^{Na\,+}\)-dependent D-glucose overshoot, the extent of inactivation depending
on the photolysis time. The inactivation observed after short photolysis times (2 x 15 s) seems to reflect mainly the reaction of the photolabel specifically with the D-glucose transporter(s), since (i) phlorizin yielded complete protection and (ii) L-methionine uptake was not significantly affected (Table II). On the other hand long irradiation times (2 x 5 min) led to considerable phlorizin non-protectable (=unspecific) inactivation of D-glucose and also of L-methionine uptake (Fig. 14A & B). The extent of phlorizin protectable (=specific) inactivation of D-glucose overshoot seems to be comparable in both instances. The nature of the unspecific inactivation observed after prolonged photolysis is not clear. Time-dependent uptake of the lipophilic NAP-label or photoproducts by the membrane might result in increased covalent insertion of the label into the membrane and thereby lead to disturbances in the bilayer (affecting $\Delta \psi_{\text{Na}^+}$).

When the D-glucose transport function in photolyzed vesicles is monitored by tracer exchange, the irreversible inactivation observed is fully protected by phlorizin (Table III). True site-directed protection by phlorizin (and thus site-specific inactivation (20 % in two cycles) by attachment of the label) is unambiguously controlled by the use of the 4'-isomer para-phlorizin. This glucoside is a weak inhibitor of D-glucose transport ($K_i \approx 420 \mu$M) but exhibits similar properties to phlorizin with respect to light absorption and scavenging, and therefore can serve as the control for possible unspecific screening by phenols (Table III).

The site-specific inactivation of tracer exchange of D-glucose corresponds to the specific inactivation of Na$^+$-dependent phlorizin binding to DOC extracted membranes, which, under the same conditions, was 14 % (1 cycle) and 21 % (2 cycles). The inactivation efficiencies of 20 % (2 labeling cycles) reported here for D-glucose transport activity and phlorizin binding are in the range often found for aryl azido derivatives (10 % to 30 %, cf. Forbush et al., 1978).

Theoretically the photolabeling cycles could have been repeated until 100 % inactivation was achieved. However, the time dependent decrease in functional stability of (treated) vesicles made the reliable determination of their transport and binding activity impossible after a certain time period of experimentation. In addition a larger
total radiation dose may itself finally have some deleterious effects on both carrier activity and ligand-binding properties.

Finally it has to be emphasized that site-directed (=specific) photo-inactivation of the transporter, as opposed to site-unrelated inactivation, does not infer that there is no labeling outside the binding site of the transporter. Incorporation of label into the transporter, but outside the binding site, and into other proteins would not necessarily be recognized by the above assays.

IV.5.4. Photolabeling studies. 

As shown in Figs. 16 & 17 photolysis of both, intact brush border membrane vesicles and DOC extracted membranes, in the presence of \( ^3H \)-NAP-\( \beta \)-Ala-Phlz resulted in the labeling of a variety of protein bands with different labeling intensities.

To differentiate between site-related (=specific) and site-unrelated (=unspecific) labeling of the transporter by this label, that is, to identify a particular labeled protein band(s) on a gel as the phlorizin binding component(s) of the transporter, the same criteria as for the specificity of photoinactivation of the carrier can be applied. These are: (i) protectability of the labeling reaction by another ligand; (ii) Na\(^+\)-dependence and (iii) membrane potential-dependence of the labeling reaction. In addition the extent of specific labeling by NAP-derivatives of phlorizin and congeners can be assumed to roughly parallel their affinity for the binding site. Finally, any specifically labeled protein band has to be relatively enriched in DOC extracted membranes, as compared to native brush border vesicles.

Failure to detect any specific labeling according to the above criteria (i) - (iii), even at subsaturating concentrations of \( ^3H \)-NAP-\( \beta \)-Ala-Phlz (see Figs. 16 & 17) must lead to the conclusion that the labeling patterns observed with this label mainly reflect unspecific labeling.

This is further substantiated by the finding that photolysis in the presence of the isomer \( ^3H \)-NAP-\( \beta \)-Ala-para-Phlz results in a labeling pattern which is, at least qualitatively, strikingly similar to the one with \( ^3H \)-NAP-\( \beta \)-Ala-Phlz. Since the NAP-derivative of para-phlorizin exhibits a low affinity for the substrate binding site of the transporter, it is expected to give rise only to site-unrelated la-
beling upon photolysis. This labeling pattern, however, may be assumed to comprise most of the bands arising from unspecific labeling by NAP-β-Ala-Phlz and, therefore, to serve as a stringent control for the labeling specificity of this ligand.

In addition, the similar labeling pattern obtained with \(^{3}H\)-NAP-β-alanine as label, but with a lower labeling intensity, suggests, that, to a certain extent, all these NAP-derivatives possess a common (similar) mechanism of (unspecific) labeling. A possible model for this common labeling mechanism will be given in the final discussion.

To obtain an estimate of the amount of \(^{3}H\)-label specifically incorporated into the transporter under the experimental conditions chosen, the following calculation can be made: if the 10 % specific photoinactivation of the transporter observed per photolysis cycle is equated with the efficiency of labeling, and assuming a glucoside binding capacity of DOC extracted brush border membranes of 20 pmoles/mg, then only some 2 pmoles \(^{3}H\)-label bound/mg protein can be attributed to specific labeling. This value compares unfavourably with the total \(^{3}H\)-label covalently bound to membrane proteins (2-3 nmoles/mg proteins), as calculated by slicing and counting the gels. This explained our inability to detect specific labeling in any band on the fluorograms; a minor band which is labeled (but is barely visible on a Coomassie blue staining pattern) is likely to be submerged by the massive amount of unspecific labeling. In addition site-directed labeling of the phlorizin binding component of the transporter might be masked by unspecific (non-protectable) labeling of this component (see final discussion).

Therefore, considering all the above findings, it can be concluded that NAP-β-Ala-Phlz will only be a useful label, if a higher degree of purification of the transporter is achieved.

IV.5.5. Possible involvement of an H-bond between a group in C-6 of the glucopyranosyl moiety and the transporter.

Independent of the use of the NAP-derivatives of phlorizin as photo-labels, it is possible from the results with these and other 6-deoxy-derivatives of phlorizin to draw some conclusions on their interaction
with the Na\textsuperscript{+}, D-glucose carrier.

Of the compounds listed in Table I, 6-amino-6-deoxy-phlorizin \textsuperscript{10} has the smallest affinity for the Na\textsuperscript{+}, D-glucose transporter. It is the only compound in the series that could possibly carry a positive charge at the pH used (7.5) and also the only one that could have a positive charge when bound to the transporter. In the absence of there being any obvious steric hindrance it is therefore tempting to correlate the poor affinity of this compound with it being positively charged; it may undergo unfavorable electrostatic interaction with some group(s) of the transporter and/or may be unable to undergo some critical interaction with the binding site (see below).

Barnett et al. (1968) have compared the intestinal uptake values of 6-deoxy-D-galactose with those of various 6-deoxy-6-halo-D-galactose derivatives and have suggested that an important role in their interaction with the sugartranslocator may be played by a hydrogen bond between the atom(s) bound to C-6 of the sugar (acting as the acceptor) and a corresponding group at the translocator's surface (acting as the donor). If the phlorizin derivatives listed in Table I are considered, there are some indications that a hydrogen bond may similarly be formed between the atom bound to position C-6 of the \(\beta\)-D-glucopyranosyl moiety of phlorizin (acting as the acceptor) and a corresponding group at the surface of the translocator.

The ability to accept a hydrogen bond should decrease in the series arylamine \textgreater amide \textgreater ammonium group; and the affinities of compounds \textsuperscript{13}, \textsuperscript{11} and \textsuperscript{14} decrease in this very order (Table I). Likewise the ability to accept a hydrogen bond should be larger for the ester oxygen than for the amide nitrogen; and compound \textsuperscript{15} has larger affinity for the transporter than the corresponding amide \textsuperscript{14}. The range of affinities of the 6-deoxy-derivatives of phlorizin in Table I lies between those of compounds \textsuperscript{1} and \textsuperscript{10}. The difference in the affinities of these two compounds corresponds to a difference in binding energy of about 2.6 kcal/mol, which would be in keeping with a hydrogen bond being involved.

The formation of hydrogen bonds in which an azido (Boyer et al., 1956) or a p-toluene-sulfonyloxy (Fischli et al., 1976) group acts as the acceptor has been reported. At present, however, it is difficult to make a comparison with the other atoms or groups in position C-6 of the compounds in Table I. The potential importance of other factors cannot be assessed: formation of a hydrogen bond at a neighbouring
group (e.g., the carbonyl) is also possible as well as some unspecified interaction of the bulky aromatic groups in compounds 8, 13, 14 and 15. However steric hindrance in the immediate neighbourhood of C-6 does not seem to play a significant role.

Finally, it has been proposed that the active site conformation of phlorizin involves an intramolecular H-bond between the primary hydroxyl group at C-6 (as the donor) and the carbonyl oxygen in the aglycone moiety (as the acceptor) (Diedrich, 1966). The small $K_l$-values of the tosylate 8 and the azide 9 render this rather unlikely.
V. RESULTS AND DISCUSSION / PART 2

THE USE OF PHLORIZIN DERIVATIVES MODIFIED IN THE AGLYCONE MOIETY.

V.1. INTERACTION OF PHLORIZIN DERIVATIVES MODIFIED IN THE AGLYCONE MOIETY WITH BRUSH BORDER VESICLES IN THE DARK.

Using a variety of phlorizin derivatives modified in the aglycone moiety, Diedrich (1966) showed that, with the exception of 4'-deoxyphlorizin, none of the analogues synthesized was a better inhibitor of the Na⁺, D-glucose cotransporter than phlorizin itself. This, together with the finding that the 4-hydroxyphenyl moiety (ring B) is an indispensable feature of the inhibitor, the hydroxyl group being involved in a H-bond (as the donor) with the binding site (Diedrich, 1963), has important consequences. It necessitates a careful analysis of the "in the dark" binding properties of 4-azidophlorizin in a variety of membrane systems with respect to its use as potential photolabel. Determination of the affinity of other derivatives modified in positions 4 and 4' of the aglycone moiety was expected to give additional information as to the mode of interaction of the aglycone moiety with its membrane binding site.

V.1.1. Inhibition of D-glucose uptake in, and phlorizin binding to, rabbit small intestinal brush border vesicles.

a) Influence of the nature of the substituent in position 4 (ring B) and 4' (ring A):

To obtain a measure of the affinity of the various phlorizin derivatives, the binding capacity of these compounds in the dark was investigated by determining: (i) their inhibition of D-glucose uptake in brush border membrane vesicles and (ii) their reversible inhibition of Na⁺-dependent, D-glucose protectable phlorizin binding (specific binding) to vesicles; these assays are the same as those used for determining the binding of the derivatives of phlorizin modified in position 6 of the glucopyranosyl moiety. $K_I^*$-and $K_r^*$-values described here can therefore be directly compared with the corresponding values listed in Table I.
Table IV
Reversible, fully-competitive inhibition (in the dark) of D-glucose uptake in, and of specific phlorizin binding to, small-intestinal brush border membrane vesicles by analogues of phlorizin modified in the aglycone moiety.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition of D-glucose uptake&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Specific phlorizin binding&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_i (\mu M)$&lt;sup&gt;c&lt;/sup&gt;</td>
<td>$K_i^&quot; (\mu M)$&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phlorizin (1)</td>
<td>R=OH, R'=OH</td>
<td>8 (7-9)</td>
</tr>
<tr>
<td>Modifications in ring B:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Aminophlorizin (22)</td>
<td>R=NH₂, R'=OH</td>
<td>44 (42-46)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>4-Azidophlorizin (23)</td>
<td>R=N₃, R'=OH</td>
<td>139 (130-150)</td>
</tr>
<tr>
<td>4-Nitrophlorizin (21)</td>
<td>R=NO₂, R'=OH</td>
<td>445 (395-495)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Modifications in ring A:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4'-Deoxyphlorizin</td>
<td>R=OH, R'=H</td>
<td>13 (11-15)</td>
</tr>
<tr>
<td>4'-mesylphlorizin (7)</td>
<td>R=OH, R'=OSO₂CH₃</td>
<td>110 (105-115)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Uptake of D-glucose in brush border vesicles (at 2 s, pH 7.5, 100 mM mannitol) was performed as in Methods under an inwardly directed gradient of NaSCN (100 mM out, zero in); D-glucose concentrations used were 10, 30, 60 and 150 μM out, zero in. Range of inhibitor concentrations tested was 0-500 μM (phlorizin and 4'-deoxyphlorizin: 0-40 μM). $K_i$-values were determined from Dixon plots (see also Fig. 9). Each number represents the mean of at least 2 independent plots. The range of the $K_i$-values obtained is given in brackets.

<sup>b</sup> 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). Apparent inhibition constant of binding ($K_i^"$) was calculated for fully-competitive, reversible inhibition of specific ($^3$H)-phlorizin binding ($K_d=5$ μM; Toggenburger et al., 1978) in the presence of 4 to 6 different analogue concentrations; n.d., not determined. For preparations and details see Methods.

<sup>c</sup> Primed $K_i$-values always refer to the total concentration (charged + uncharged) of the inhibitor.

<sup>d</sup> Measured under NaCl (100 mM)-equilibrium conditions (with kind permission from Reber, 1981).
As shown in Table IV, replacement of the hydroxyl group in position 4 (ring B) by an azido group resulted in a 15 - 20 fold decrease in the inhibitory potency of the resulting azide, as evidenced from its $K_1'$- and $K_2'$-values. The excellent agreement of $K_1'$ with $K_2'$ again supports the contention that phlorizin and its 4-azido-analogue bind to the same site. Therefore 4-azido-phlorizin may be used as a potential photolabel of the phlorizin binding site of the sugar carrier.

The presence of an amino group in position 4 resulted in a considerably smaller loss in inhibitory capacity of the amine as compared to the azide, whereas the corresponding nitro-analogue exhibited a markedly reduced inhibitory potency (Table IV).

The inhibitory capacity of 4'-deoxyphlorizin ($K_1'$-10 μM) as determined with rabbit small intestinal brush border vesicles, agrees with its reported good inhibitory potency on D-glucose uptake in surviving intestines from golden hamster (Diedrich, 1966). However, in contrast to the latter system, where its inhibition was shown to be more potent than phlorizin, 4'-deoxyphlorizin in brush border vesicles is at the best equipotent with phlorizin.

The introduction of a mesyl-group in the 4'-position, on the other hand, resulted in a marked decrease in the inhibitory activity of the 4'-mesylate, as evidenced from its $K_1'$ which was an order of magnitude higher than the $K_1'$ of the 4'-deoxyanalogue (Table IV).

b) pH-Dependence of the inhibitory potencies of 4-azidophlorizin, phlorizin and 4'-deoxyphlorizin:

Fig. 19 shows the pH-dependence of the inhibitory potency of 4-azidophlorizin (●). This is compared with the variation in $K_1'$ with pH of phlorizin (▲) and 4'-deoxyphlorizin (■). The $K_1'$-values of phlorizin at the pH-values indicated are in good agreement with the values reported by Toggenburger (1979) and served as a reference in each experiment.

1) The $K_1'$-values of the 4-amine and the 4-nitro-compound were determined after a 7 s incubation and are therefore somewhat too high. For comparison, the $K_1'$-values of phlorizin and 4-azidophlorizin determined under identical conditions were 10 μM and 225 μM, respectively (for the time-dependence of the $K_1'$-values of phlorizin, see Kessler, 1978).
Fig. 19. pH-Dependence of the inhibitory potency of 4-azidophlorizin (●), phlorizin (▲) and 4'-deoxyphlorizin (■) on Na⁺, D-glucose cotransport in small-intestinal brush border vesicles. $K_i$-values were determined from Dixon plots showing fully competitive inhibition (see also Fig. 9 and Tables I and IV) and were plotted against the (bulk) pH of the medium. Rabbit small-intestinal brush border vesicles were incubated at room temperature (under subdued light) for 2 s; medium composition: 300 mM mannitol, 10 mM buffer (MES/Tris (pH 6.5), HEPES/Tris (pH 7.5 and 8.0), Tris/HEPES (pH 8.5), KHCO₃/K₂CO₃ (pH 9.5), 2 % ethanol, 0.02 % KN₃ and inwardly directed gradients of NaSCN (100 mM out, zero in) and of D-glucose (10, 30, 60 and 150 μM out, zero in); ranges of inhibitor concentrations (4 – 5 different concentrations) tested were 0-50 μM (phlorizin), 0-150 μM (4'-deoxyphlorizin) and 0-300 μM (4-azidophlorizin).
As was expected, the 4-azide exhibited a pH-dependence of its inhibitory capacity which was essentially similar to the one observed for phlorizin (Toggenburger 1979). There was, however, a slight difference in their inhibitory activity in the pH-range 6.5 - 7.5. The 4-azide showed maximal inhibition of D-glucose transport at pH 6.5, the pH-dependence being linear over the whole range tested, whereas phlorizin was nearly equipotent at pH 7.5 and 6.5. This result clearly suggests that photolabeling studies using 4-azidophlorizin should be performed at neutral or slightly acidic pH (6.5), in order to assure maximal occupancy of the binding site by the label.

The finding that the inhibition constant $K_i^t$ of 4'-deoxyphlorizin remains unaltered over the pH-range tested (pH 7.5 - 9.5) is strong evidence that it is the 4'-phenolic group that makes phlorizin a weak phenolic acid ($pK_a$ of about 7.2 (Trüb, 1978; Evans & Diedrich, 1980) and which therefore is deprotonated first. Further it supports the conclusions by Toggenburger (1979) that it is the protonated (uncharged) form of phlorizin that interacts with the carrier, and that the phlorizin binding site of the carrier is not itself subjected to acid-base dissociation or altered otherwise in the pH-range monitored.

A possible use of the pH-independence of the inhibitory potency (and therefore also of the binding capacity) of 4'-deoxyphlorizin will be discussed on page 130.

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1) This need not imply, however, that this pH-range is also optimal for the photolabeling reaction (for discussion of the mechanism(s) of labeling reactions, see page 137).

2) Other evidence is the finding that derivatives of phlorizin which are missing this group, i.e., in addition to 4'-deoxyphlorizin the 4'-glucoside of phloretin (=para-phlorizin), do not exhibit a pH-dependent shift in UV-absorption which is characteristic of phlorizin (Evans & Diedrich, 1980).
V.1.2 Comparison of the inhibitory potencies of 4-azidophlorizin and phlorizin on D-glucose uptake in, and phlorizin binding to, brush border vesicles derived from rabbit and rat small intestines and kidneys

In the course of a collaborative project (together with the group of Prof. D.F. Diedrich, University of Kentucky Medical School, Lexington, KY) with the goal of using 4-azidophlorizin as a potential photolabel of the D-glucose carrier in both rabbit intestinal and rat renal brush border membranes, a surprisingly large discrepancy in the inhibitory potency of this compound in the two systems was found; the $K_I^*$-values being 139 $\mu$M and 5.2 $\mu$M, respectively (Table V).

This 25-fold difference in $K_I^*$ was considerably larger than the difference in $K_I^*$ of phlorizin observed between these two types of membranes, 8 $\mu$M and 2–3 $\mu$M, respectively$^{1)}$. To test whether this observation reflected a tissue or a species specific property, the inhibitory potency of the 4-azide on D-glucose uptake in rabbit kidney and rat intestine was also measured.

Table V clearly shows that the difference in inhibitory capacity of 4-azidophlorizin is related to the species. The glucoside was a similarly weak inhibitor of D-glucose transport in, and phlorizin binding to, vesicles derived from both rabbit tissues; in all instances it was about 15 to 20 times less potent than phlorizin itself. However with both rat renal and small intestinal vesicles its inhibitory potency on D-glucose uptake and phlorizin binding was consistently higher, the $K_I^*$ and $K''_I$-values being only about a factor of 2 smaller than those of phlorizin.

The reason for the higher affinity of the 4-azide for the transport systems in the rat, as compared to those of the rabbit, is not clear at the present. One may speculate on the possibility that the D-glucose transport systems of the two species tested are not quite identical, but possess minor (minute) differences in the aglucone binding subsite which are only detected by the interaction with the 4-azido-analogue, and not with the parent glucoside.

$^{1)}$ This difference in affinity of phlorizin was also observed by others (see Kinne, 1976).
Table V
Reversible fully competitive inhibition (in the dark) of Na', D-glucose cotransport across, and phlorizin binding to, brush border membrane vesicles derived from rabbit and rat small intestines and kidneys a.

<table>
<thead>
<tr>
<th>source of brush border vesicles</th>
<th>inhibitory potency of D-glucose phlorizin uptake</th>
<th>4-azidophlorizin uptake</th>
<th>binding</th>
<th>uptake</th>
<th>binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>animal tissue</td>
<td>D-glucose phlorizin uptake</td>
<td>D-glucose phlorizin uptake</td>
<td>phlorizin binding</td>
<td>phlorizin binding</td>
<td></td>
</tr>
<tr>
<td>rabbit intestine</td>
<td>8 ± 1.2 (3) 12 ± 2 (2) 139 ± 9 (3) 156 ± 40 (2)</td>
<td>n.d.</td>
<td>140</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>kidney d,e</td>
<td>7.5</td>
<td>n.d.</td>
<td>140</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>rat intestine</td>
<td>4.2 ± 2.5 (2) n.d.</td>
<td>10</td>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>kidney d,f</td>
<td>2.1 ± 1.2 (4) 0.9 ± 0.1 (2) 5.2 ± 1.9 (3) 3.8 ± 1.7 (3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Uptake of D-glucose in, and phlorizin binding to, the brush border vesicles (at 2 s, pH 7.5) were performed as described in Table IV and Methods. For preparation of vesicles see also Methods.

b \(K_i\)-values were determined from Dixon plots and are given as the mean ± SD of (n) independent plots.

c \(K''_i\)-values were determined as described in Table IV and Methods and are given as the mean ± SD of (n) independent experiments.

d Incubations lasted 7 s.

e Experiments were only performed once.

f Data, with kind permission, from Gibbs (1980).
V.1.3. Binding of (3H)-4-azidophlorizin to brush border membrane vesicles.

The Millipore filtration technique was used to determine reversible binding of (3H)-4-azidophlorizin to brush border membrane vesicles. Binding of the (3H)-4-azide over a concentration range of 10 μM to 400 μM under NaSCN gradient conditions (100 mM out, zero in; pH 6.5) at 2 s was 0.025 to 0.9 nmoles/mg protein; this corresponds to the binding of 4 to 5% of the label present. Determination of specific (= Na^+-dependent) binding of the (3H)-4-azide, however, failed: replacement of NaSCN by 100 mM KSCN did not result in a statistically significant reduction in (3H)-4-azide binding at the above concentrations.

When (3H)-label and vesicles were added separately to the same stop solution, 5 to 6% of the (3H)-label applied was retained by the filter (=filter blank). This value is of the same magnitude as the total binding of the label to the vesicles. For comparison, unspecific binding of (3H)-phlorizin to vesicles at 10 μM was approximately 10 pmoles/mg protein, and the filter blank was 0.2 to 0.3% of the (3H)-label applied.

When binding of the (3H)-4-azide to brush border vesicles equilibrated with NaCl (or KCl) was assayed at 15 s (the time period needed to reach maximum binding of phlorizin under these conditions, see Fig. 10), the total binding was increased, 60 pmoles/mg protein (at 10 μM) and 0.5 nmoles/mg (at 100 μM). Determination of specific (Na^+-dependent) binding of (3H)-4-azido-phlorizin again failed, since there was no statistically significant difference between the binding in the presence of NaCl or of KCl; the increase in binding under these conditions can be attributed to the longer contact time (15 s) allowing more (3H)-4-azide to be taken up by the bilayer (see final discussion).

The combination of both increased (total) binding to vesicles and decreased affinity of the 4-azide for the carrier, which is at least one order of magnitude lower than that of phlorizin (see Table V), is

1) Retention of label on the filter seems to be due to adsorption (binding) of the label to the filter, as similar values were measured when membrane vesicles were omitted from the stop solution.

2) The filter blanks of both labels were found to depend on the pH of the stop solution and on the presence of excess unlabeled phlorizin (Reber, personal communication).
certainly the reason for our inability to monitor the Na\(^+\)-dependent (specific) fraction of total binding of this ligand.

Analysis of the binding data of the 4-azide according to Scatchard (1948/51) gave a horizontal line (data not shown), as expected if indeed the binding observed is largely unspecific and unsaturable and completely masks any specific (Na\(^+\)-dependent) binding.

**V.2. PHOTOINACTIVATION EXPERIMENTS**

Control experiments in the absence of phlorizin derivatives showed that photolysis of brush border membranes vesicles with light of wavelengths > 315 nm, (as required for rapid activation of arylazides; Bayley & Knowles, 1977)), resulted in a large time dependent loss of their D-glucose tracer exchange activity and equilibrium volume\(^1\). In contrast to the (photooxidative) inactivation by visible light (see Fig.13A) this decrease could only be partially prevented by pretreatment with argon (see below).

**V.2.1. Irreversible inhibition of D-glucose tracer exchange across brush border membrane vesicles.**

As shown in Table VI, photolysis of vesicles for 1 min in the presence of 200 \(\mu\)M 4-azide resulted in a 16 % irreversible inhibition of D-glucose tracer exchange, as compared to illuminated controls. This inhibition was completely protected by the presence of 200 \(\mu\)M phlorizin during photolysis.

Under these photolysis conditions the inactivation of the transporter in the absence of label was itself approximately 25 %, as compared to dark-controls. Therefore, the above value for irreversible inhibition of the transporter by site-directed incorporation of label can only be considered as an estimation.

\(^1\) The half-time for photodecomposition of the 4-azide in the presence of membrane vesicles (2 mg protein/ml) with light of wavelengths > 315 nm was 15 - 20 s (using a 350 W Hg-lamp with a 3 cm liquid filter of sat. Cu(II)SO\(_4\) to cut off below 315 nm). It was determined by TLC and liquid scintillation counting.
Table VI

Effect of photolysis with 4-azidophlorizin on D-glucose tracer exchange across brush border vesicles.

<table>
<thead>
<tr>
<th>photolysis conditions</th>
<th>% D-glucose tracer exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td>illuminated controls</td>
<td>100</td>
</tr>
<tr>
<td>200 µM 4-azide</td>
<td>84 ± 5</td>
</tr>
<tr>
<td>200 µM 4-azide plus</td>
<td>103 ± 5</td>
</tr>
<tr>
<td>200 µM phlorizin</td>
<td></td>
</tr>
</tbody>
</table>

aVesicles were photolyzed for 1 min with light of wavelengths >315 nm, as described in Methods.

bD-glucose tracer exchange (at 8 s) was assayed as described in Table III and in Methods, and is expressed as a % of the illuminated-membrane-control value. Data are represented as the mean ± SD of 3 independent experiments, each carried out in duplicate. Measurements were carried out in pentuplicate.

V.2.2. Irreversible inhibition of Na⁺-dependent phlorizin binding to DOC extracted membranes.

As shown in Table VII, photolysis of DOC extracted membranes in the presence of 200 µM para-phlorizin plus 100 or 200 µM 4-azide for 1 min resulted in 27 ± 6 and 34 ± 15 % irreversible inhibition of Na⁺-dependent phlorizin binding, as compared to illuminated controls with 300 µM para-phlorizin present. The presence of 200 µM phlorizin instead of para-phlorizin during photolysis, however, gave only partial protection.

Similar to the results with D-glucose tracer exchange, there was a 30 - 40 % irreversible photoinactivation of phlorizin binding in the absence of label, as compared to nonilluminated controls. Therefore also the values for site-directed photoinactivation of the specific phlorizin binding activity have to be considered as approximate.
Table VII
Effect of photolysis with 4-azidophlorizin on Na\textsuperscript{+}-dependent phlorizin binding to DOC extracted membranes.

<table>
<thead>
<tr>
<th>photolysis conditions</th>
<th>% phlorizin binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>illuminated controls (300 μM para-phlorizin)</td>
<td>100</td>
</tr>
<tr>
<td>100 μM 4-azide plus 200 μM para-phlorizin</td>
<td>73 ± 6</td>
</tr>
<tr>
<td>100 μM 4-azide plus 200 μM phlorizin</td>
<td>93 ± 15</td>
</tr>
<tr>
<td>200 μM 4-azide plus 200 μM para-phlorizin</td>
<td>66 ± 15</td>
</tr>
<tr>
<td>200 μM 4-azide plus 200 μM phlorizin</td>
<td>75 ± 6</td>
</tr>
</tbody>
</table>

\textsuperscript{a}DOC extracted membranes were photolyzed for 1 min as described in Table VI and in Methods.

\textsuperscript{b}Na\textsuperscript{+}-dependent phlorizin binding (at 15 s) was assayed as described in Table III and in Methods, and is expressed as a % of the illuminated-membrane-control value (with 300 μM para-phlorizin present). Data are represented as the mean ± SD of 3 photoincubations. Measurements were carried out in pentuplicate.

Milder photolysis conditions causing less photoinactivation of the transporter, however, required considerably longer photolysis times\textsuperscript{1}) which were found to give rise to increased unspecific labeling of brush border membranes (see below). Therefore, the subsequent labeling experiments were performed under the photolysis conditions used for rapid activation of the 4-azide, despite the extensive inactivation of the transporter by this light.

\textsuperscript{1}) The photolysis conditions tested to determine the half-times (given in brackets) in the presence of membranes were: (i) 350 W Hg-lamp/365 nm interference filter (Balzers) (290 s); (ii) 350 W Hg-lamp/365 nm interference filter & sat. Cu(II)SO\textsubscript{4} - liquid filter (300 s); (iii) 350 W Hg-lamp/313 nm interference filter (Balzers) (1000 s); (iv) Sylvania (GTE, Manchester, N.H., USA) 275 W sun lamp, at 20 cm distance (900 s). (i)-(iii) were performed in the photolysis apparatus shown in Fig. 3. For comparison \textsubscript{1/2} for photolysis with light of wavelengths >315 nm (sat. Cu(II)SO\textsubscript{4} - liquid filter) in the absence of membranes was 53 s. In the presence of membranes (2 mg protein/ml) these half-times for photodecomposition were decreased by a factor of about 2.5 (see Footnote on page 114; for accelerated photodecomposition of NAP-derivatives of phlorizin in the presence of membranes see page 73).
V.3. PHOTOLABELING WITH (3H)-4-AZIDO-PHLORIZIN

V.3.1. Labeling of intact brush border membrane vesicles with (3H)-4-azidophlorizin; time and concentration dependence of labeling.

The labeling of intact small-intestinal brush border membrane vesicles with (3H)-4-azidophlorizin at various concentration at pH 7.0 is shown in Fig. 20A. Photolysis for 1 min with label at 50 μM (lane 2), 100 μM (lane 3) and 200 μM (lane 4) resulted in labeling patterns which did not differ qualitatively. The 5 major bands labeled were: I, 3, 4, upper band of 5d and A. In addition there was a series of minor bands labeled (for comparison the Coomassie blue staining pattern of the same gel is given in lane 1).

The amounts of (3H)-label present in the electrophoresis samples after photolysis at 50 μM, 100 μM and 200 μM (3H)-label and at a protein concentration of 2 mg/ml were 2.4, 3.5 and 5.8 nmoles (3H)-label per mg protein, respectively. These values correspond to 5 to 7% of the label initially present. For comparison the amounts of (3H)-NAP-β-Ala-Phlz present in the electrophoresis samples after photolysis under similar conditions and at 50 μM and 100 μM label were 4 and 6 to 9 nmoles/mg, respectively.

Similar to the result with (3H)-NAP-β-Ala-Phlz (see Figs. 16 & 17) a large fraction of the total radioactivity present was in a band, comigrating with the dye front of the (8.4 x 2.7) gel. As evidenced from fluorograms of (15 x 2.7) gels, this band included a few (minor) labeled polypeptides not resolved by the (8.4 x 2.7) gel (e.g. of 27,000 D, 17,000 D and 14,000 D, gels not shown), however, the majority of the (3H)-label was due to the presence of non-covalently bound (3H)-ligand and/or photoproducts.

The time-dependence of the labeling is shown in Fig. 20B. There was a general increase in the labeling when the photolysis time was increased from 1 min (1') to 5 min (5'); corresponding to 4.7 and 6.7 nmoles of
Fig. 20. (A): Fluorograms of brush border membrane vesicles labeled with various concentrations of $^{3}$H-4-azidophlorizin. Vesicles (1.5 to 2 mg protein/ml) were photolyzed for 1 min (at room temperature, pH 7.0) in the presence of 50 $\mu$M (lane 2), 100 $\mu$M (lane 3) and 200 $\mu$M $^{3}$H-label (lane 4) with light of $\lambda$ > 315 nm, as described in Methods. After Sephadex G-10 gel filtration and repeated washing by centrifugation, photolysates were boiled with solubilizing solution under reducing conditions for 60 s. 50 $\mu$g samples of protein were subjected to SDS-PAGE (8.4 x 2.7); the cpm applied were 17,000 (lane 2), 35,000 (lane 3) and 72,000 (lane 4). After Coomassie blue staining (the protein staining pattern is shown in lane 1) gels were fluorographed as described. The fluorographs are from different experiments, but performed under identical conditions. Exposure of the films lasted 50 days at -70°C. (B): Time dependence of the labeling. Vesicles were photolyzed as above in the presence of 200 $\mu$M $^{3}$H-4-azide for 1 or 5 min (lanes 1' and 5') and treated as in (A). In the prephotolysis experiment (lane P) the $^{3}$H-label was photolyzed for 5 min in the absence of vesicles. Immediately after photolysis vesicles were added and the mixture was incubated for 5 min at room temperature (at 200 $\mu$M $^{3}$H-label). The subsequent treatment of the membranes was as in (A). The samples applied (50 $\mu$g protein) contained 11,000 cpm (P), 55,000 cpm (1') and 72,000 cpm (5'). Exposure of the film lasted 14 days.
label present per mg of protein respectively. Since photolysis for 1 min was found to be sufficient for activation of at least 90% of the label molecules initially present, then it is likely that this 40% increase in the total amount of ($^3$H)-label associated with the photolysate and in particular with the labeling of single protein bands (I and A) is mainly due to incorporation of ($^3$H)-label via postphotolytic reactions.

This is also supported by the prephotolysis experiment shown in Fig. 20B. Where vesicles were incubated for 5 min with prephotolyzed label immediately after photolysis, and subsequently treated like a normal photolysate, 0.9 nmoles ($^3$H)-label/mg protein were associated with the membranes. This label was either incorporated into lipids (region K) or was present as free ($^3$H)-label comigrating with the dye front. Prolonged fluorography (50 days) was needed to detect faint labeling of polypeptide bands A and I (fluorogram not shown).

The large amounts of ($^3$H)-label associated with membranes after photolysis (2 to 5 nmoles/mg protein) exceed by far the (maximum) amount of ($^3$H)-label that can be specifically incorporated (10 to 40 pmoles/mg protein). This leads to the conclusion that, similar to the labeling with ($^3$H)-NAP-β-Ala-Phlz, the large majority of the labeling was not associated with the binding site of the transporter.

In order to optimize specific labeling, the photolysis time in the further experiments was restricted to 1 min and the label was used at 100 μM.

1) The half-time of photodecomposition was approximately 20 s.

2) "Postphotolytic" labeling is also likely to occur during photolysis (for a detailed description of the various photochemical reactions, see final discussion).
V.3.2. Specific labeling of (a part of) the Na⁺, D-glucose transporter in DOC extracted membranes.

Clearly, unspecific labeling has to be expected also when DOC extracted membranes are photolabeled with (³H)-4-azidophlorizin. As shown in Fig. 21 the labeling pattern of DOC extracted membranes (e.g. lane 2) was very similar to that of intact vesicles (Fig. 21, lane 8). Similar to the labeling with (³H)-NAP-β-Ala-Phlz, the decrease in the labeling of the bands A and 4 and the increase in the labeling of the band I in DOC extracted membranes reflected the selective removal and enrichment, respectively, of these proteins by this extraction.

Some important requirements must be met, therefore, before a labeled band in the SDS-PAGE pattern can be identified as the Na⁺, D-glucose transporter or a part thereof. Accordingly, in planning the representative experiment shown in Fig. 21, the following (independent) criteria were applied for differentiating: (i) protection by the native ligand; (ii) Na⁺-dependence; (iii) relative enrichment in DOC extracted membranes; (iv) further enrichment in membranes which had been extracted with DOC plus KI.

(i) The labeling of the transporter should be prevented or reduced by other ligands known to compete with 4-azidophlorizin (e.g., by phlorizin, see Fig. 21, lane 3) but not by other compound(s) which, although chemically related, have little or no affinity for the transporter (e.g., para-phlorizin, the 4'-isomer of phlorizin, see Fig. 21, lane 4). This control is particularly important, because prevention or reduction of labeling by phlorizin may have been due to optical shielding (absorption) and/or to a scavenging effect of phlorizin, rather than to competition for the same binding site on the transporter. Para-phlorizin has a very similar UV/Vis spectrum (at the pH used) and nearly identical properties as phlorizin, but, unlike phlorizin, has about 100 times lower affinity for the Na⁺, D-glucose transporter (Kᵢ approximately 420 µM, see Table I; for the use of para-phlorizin as control for "true" protection by phlorizin from photoinactivation of the transporter, see also Table VII). Thus the reduction in the labeling of a minor band with an apparent molecular weight of approximately
Fig. 21. Fluorograms of DOC extracted membranes labeled with (\(^3\)H)-4-azido-phlorizin in the presence of various protectors and cations. Membranes (1.7 mg protein/ml) were photolyzed in the presence of 100 \(\mu\)M (\(^3\)H)-4-azide and 50 \(\mu\)g samples of protein were analyzed on a (8.4 x 2.7) gel, as described in Methods. The protectors and/or cations present were (cpm are given in brackets): (2) no addition (31,000 cpm); (3) 200 \(\mu\)M phlorizin (29,400 cpm); (4) 200 \(\mu\)M para-phlorizin (24,700 cpm); (5) 1 mM phlorizin (18,200 cpm); (6) 1 mM para-phlorizin (13,000 cpm); (7) 100 mM choline SCN instead of NaSCN (29,800 cpm); (8) intact vesicles were labeled in the presence of 100 \(\mu\)M (\(^3\)H)-label and no further addition. For comparison the labeling pattern of DOC extracted membranes photolyzed in the presence of 100 \(\mu\)M (\(^3\)H)-NAP-\(\beta\)-Ala-Phlz is shown in (9). The Coomassie blue staining pattern of DOC extracted membranes is given in (1). The figure is a composite of two separate fluorographs from two different experiments, lanes 1, 2, 5-9 from one and lanes 3 and 4 from the other. Fluorography at -70° C lasted 50 days, except for lane 9 where it lasted only 3 days. The arrow indicates the radioactive band specifically labeled.
72,000 D \(^1\) (Fig. 21, arrow) in the presence of 200 \(\mu\)M phlorizin (Fig. 21, lane 3) but not in the presence of 200 \(\mu\)M para-phlorizin (Fig. 21, lane 4) strongly suggested that this band is labeled specifically. Note that this differential effect of phlorizin and para-phlorizin on labeling by (\(^3\)H)-4-azidophlorizin is not apparent in any other band than the 72,000 D band (neither is there any difference in the labeling of bands not resolved by this 8.4 % gel, as evidenced from 15 % gels (not shown)). At a high enough concentration to nearly saturate the carrier (i.e., at 1 mM, for a \(K'_i\) of \(\approx 420\ \mu\)M) para-phlorizin, as expected, protected the 72,000 D band from being labeled (Fig. 21, lane 6) to a similar extent as phlorizin, at the same concentration did (Fig. 21, lane 5). There was a concomitant decrease in the labeling of other bands, such as I and A, in the presence of high concentrations of both glucosides. Interestingly, however, para-phlorizin under these conditions in all experiments protected the labeling of bands I and A better than phlorizin (compare lanes 5 and 6 in Fig. 21); thereby adding more significance to the stronger labeling of the 72,000 D band in the presence of this glucoside. There was also a strong decrease in the labeling of lipids after photolysis in the presence of high concentrations of both glucosides (region K in lanes 5 and 6); again para-phlorizin was a better "protecting agent".

(ii) The presence of Na\(^+\) is necessary for the interaction of phlorizin with the carrier, as measured via inhibition of D-glucose transport (Bihler, 1969) or via phlorizin binding (Toggenburger et al., 1978; Kinne, 1976). Likewise, Na\(^+\) is assumed to be needed for the interaction of 4-azidophlorizin with the carrier in small-intestinal brush border membranes in the dark\(^2\). Accordingly, in the absence of Na\(^+\) (replaced by choline\(^+\)) less labeling of the 72,000 D band by (\(^3\)H)-4-azidophlorizin was observed (Fig. 21, lane 7). The sensitivity of the

\(^1\)One has to bear in mind the anomalous migration of membrane (glyco)proteins in SDS-PAGE. In addition it has to be considered that the labeled polypeptide may exhibit an electrophoretic mobility which is different from the one of the unmodified polypeptide (not only due to the molecular weight of the covalently bound ligand, approximately 700 D, but also due to the nature of the modified residue).

\(^2\)For our inability to measure Na\(^+\)-dependent (\(^3\)H)-4-azidophlorizin binding to small-intestinal brush border membranes, see page 113; Na\(^+\)-dependent (\(^3\)H)-4-azidophlorizin binding to kidney brush border membranes could be demonstrated (Gibbs E.M., unpublished results, 1980).
labeling to ΔΨ could not be monitored because DOC extracted membranes are not sealed (Klip et al., 1979b). Nevertheless SCN− was used as the anion, in order to use labeling conditions which were comparable to the ones with intact vesicles. Labeling of DOC extracted membranes in the presence of 100 mM NaCl resulted in a labeling pattern which was not different from the one with NaSCN (gel not shown).

Procedure(s) known to lead to a negative purification of the Na+, D-glucose transporter should also lead to a similar purification of the 72,000 D band. This is indeed the case:

(iii) DOC extraction: The labeling of the 72,000 D band is considerably stronger with DOC extracted membranes (Fig. 21, lane 2) than with intact membrane vesicles (Fig. 21, lane 8). In the latter the labeling is barely detectable.

(iv) KI extraction: DOC extracted membranes can be further treated with KI, which removes the majority of the cytoskeletal proteins (Bretscher & Weber, 1980a), without inactivating or releasing the Na+, D-glucose transporter (Klip et al., 1979b). Accordingly, the labeling of the 72,000 D band remains constant or even increases in KI treated, DOC extracted membranes (Fig. 22C, compare lanes 8 and 7).

Last but not least, labeling of this band was totally dependent on photolysis, since a control not exposed to light showed no such labeling (= dark control); nor did incubation of membranes with prephotolyzed photolabel (gels not shown, but see also Fig. 20B).

The labeling patterns obtained with the (3H)-4-azide (Fig. 21, lane 2) and with (3H)-NAP-β-Ala-Phlz (Fig. 21, lane 9) are very similar, there being only one major difference: the diffuse and, most likely, unspecific labeling of the bands in the 5 to 7 region (mol wt 80,000 D to 60,000 D) is much stronger with the latter label. Since the 72,000 D band comigrates with this region (with band 5f), it is very likely that, if labeled at all by the NAP-derivative of phlorizin, the (specific) labeling of this band is masked by this strong (unspecific) labeling and therefore is not borne out clearly when (3H)-NAP-β-Ala-Phlz is used as the label.

As observed before, there was an increase in the formation of labeled protein aggregates migrating only a short distance into separating gel when DOC extracted membranes were used. It is not clear, however, whether they originate from labeled (membrane) proteins migrating in the 60,000 to 80,000 D region, similar to the heat dependent formation of protein aggregates labeled with (3H)-NAP-β-Ala-Phlz (see Fig. 16B).
V.3.3. The effect of scavengers on the labeling of DOC extracted membranes.

The above photolysis experiments were performed in the presence of 10 mM pABA as a scavenger. Therefore, the possible influence of scavengers on the labeling with \( ^3\text{H} \)-4-azidophlorizin was examined, in particular with respect to the specific labeling of the 72,000 D band (for the influence of scavengers on the labeling with \( ^3\text{H} \)-NAP-\( \beta \)-Ala-Phlz, see Fig. 17B).

Omission of pABA did not result in a detectable change in the labeling pattern of DOC extracted membranes (Fig. 22A, lane 1), whereas replacement of pABA by reduced glutathione led to a general decrease in the labeling (lane 3), similar to when glutathione was used as the scavenger for \( ^3\text{H} \)-NAP-\( \beta \)-Ala-Phlz (see Fig. 17B). Reduction of the 4-azide by the thiol is likely to be (at least partially) responsible for this decrease in labeling.

Addition of an excess of an ice cold solution of bovine serum albumin (BSA) (12 mg/ml) to the photolysate immediately after photolysis did not result in a detectable reduction in the labeling of the membranes when photolysis was performed either in the presence or in the absence of pABA (gels not shown). However, covalent incorporation of \( ^3\text{H} \) label into BSA clearly showed that postphotolytic labeling did occur and that BSA in principle was able to act as a postphotolytic scavenger of free long-lived reactive intermediates of the arylazide\(^1\). Photolysis of BSA in the presence of 100 \( \mu \text{M} \) \( ^3\text{H} \)-4-azide resulted in the incorporation of large amounts of \( ^3\text{H} \)-label, showing that BSA is a potent ligator of phlorizin and congeners.

V.3.4. \( ^3\text{H} \)-4-Azidophlorizin as label of other proteins present in the brush border membrane.

In addition to its inhibition of \( \text{Na}^+ \), D-glucose cotransport, phlorizin is known to inhibit other enzymes present in the intestinal brush border membrane. These include the intestinal phosphatase, the mutaro-

\(^1\) \( ^3\text{H} \)-Labeled BSA was recovered by TCA precipitation from the supernatant obtained after the first washing by centrifugation. In some experiments it could also be detected as a faint band at 66,000 D on Coomassie blue stained gels and on fluorograms of these gels when it was not completely removed by the washing by centrifugation prior to SDS-PAGE.
Fig. 22. (A): The effect of scavengers on the labeling pattern of DOC extracted membranes. Membranes were photolyzed as in Fig. 21 in the presence of 100 μM (3H)-label and the following scavengers: (1) none (32,000 cpm); (2) 10 mM pABA (31,000 cpm); (3) 10 mM reduced glutathione (16,100 cpm). (B): The effect of various carrier-unrelated inhibitors on the labeling pattern of DOC extracted membranes. Membranes were photolyzed in the presence of 100 μM (3H)-4-azide and the following compounds: (4) 100 μM deoxynojirimycin (31,000 cpm); (5) 2.5 mM theophylline (28,600 cpm); (6) 2 mM of each AMP and ADP (29,500 cpm). (C): The effect of extraction with KI prior to photolysis on the labeling pattern of intact vesicles and DOC extracted membranes. DOC extracted membranes (lanes 7 and 8) and intact brush border vesicles (lanes 9 and 10) were (lanes 8 and 10) or were not (lanes 7 and 9) extracted with 0.6 M KI, as described in Methods. Membranes (1.7 to 2.0 mg protein/ml) were then photolyzed in the presence of 100 μM (3H)-4-azide. For A, B and C 50 μg samples of protein were electrophoresed on a (8.4 x 2.7) gel and the gels were fluorographed (50 days of exposure). The arrow indicates the radioactive band specifically labeled.
tase, the phlorizin hydrolase and those enzyme systems which require or are stimulated by adenine nucleotides (these effects of phlorizin have been reviewed by Crane (1960) and will be discussed later).

To find out if labeling of some of the bands in the above photolysis experiments reflected protectable labeling of one of these enzymes, a series of ligands were tested as potential protectors. These were: para-phlorizin (Evans & Diedrich, 1980) and deoxynojirimycin (Niwa et al., 1970) as known or potential inhibitors, respectively, of phlorizin hydrolase, theophylline as inhibitor of alkaline phosphatase (Ghijsen et al., 1980) and AMP/ADP as ligands of the nucleotide binding site(s) of nonmuscular actin (Wallace & Frazier, 1979).

The labeling patterns obtained are shown in Fig. 22 B and should be compared with the labeling pattern in Fig. 22A, lane 2. Photolysis in the presence of 100 μM deoxynojirimycin did not result in a selective change in the labeling pattern (Fig. 22 B, lane 4). In particular there was no protection of any band in the region of 140,000 to 160,000 D, where the phlorizin hydrolase monomer of the lactase-phlorizin hydrolase complex in human small intestine has been located (Skovbjerg et al., 1981).

The same was true for theophylline (2.5 mM, lane 5) and AMP plus ADP (each at 2 mM, lane 6) as potential protectors for alkaline phosphatase and the nucleotide binding site of actin, respectively, since the presence of these compounds during photolysis did not result in a detectable, specific change in the labeling pattern.

As shown above, photolysis in the presence of both 1 mM phlorizin and para-phlorizin resulted in a marked decrease in the labeling of band I (Fig. 21 lanes 5 and 6). This could lead to the conclusion that labeling of the band designated I reflected labeling of phlorizin hydrolase, since both para-phlorizin and phlorizin are potent inhibitors of this enzyme (Evans & Diedrich, 1980). However, the decrease in the labeling of band I was consistently stronger in the presence of para-phlorizin (lane 6) than in the presence of phlorizin (lane 5). This renders the above conclusion questionable, since phlorizin has a higher affinity for the phlorizin hydrolase than para-phlorizin (Evans & Diedrich, 1980). An alternative explanation for these findings will be given in the final discussion.
V.3.5. The location of labeled actin.

The actin band A was labeled when either intact or DOC extracted membrane vesicles were photolyzed in the presence of \(^{3}\)H-4-azido-phlorizin (see Fig. 21, lanes 2 and 8), similar to what was observed with \(^{3}\)H-NAP-\(\beta\)-Ala-Phlz as label (see Fig. 17A).

Treatment of DOC extracted membranes with KI prior to the photolysis in the presence of \(^{3}\)H-4-azide resulted in a marked decrease in the labeling of the actin band (Fig. 22C, lane 8), as compared to the labeling of untreated DOC extracts (lane 7). This is assumed to reflect the removal of this protein by the KI treatment. However, when intact membrane vesicles were used there was no difference between the labeling pattern of KI treated (lane 10) and of untreated membranes (lane 9).

This supports the conclusion drawn from the labeling experiments with \(^{3}\)H-NAP-\(\beta\)-Ala-Phlz, namely, that with intact membrane vesicles most of the labeling of actin occurs inside sealed vesicles. Therefore, not only the lipophilic NAP-derivative of phlorizin, but also the 4-azido analogue, or reactive intermediates of it, must be able to diffuse across the lipid bilayer in sufficient amounts during the photoincubation.

The nature of the interaction of phlorizin (analogues) with actin, which leads to the labeling of this protein, is not clear. As mentioned before, nonmuscular actin is known to possess an ATP/ADP binding site(s) (Straub & Feuer, 1950), and probably in addition a distinct ADP/AMP binding site(s) (Wallace & Frazier, 1979). Since phlorizin can bind to enzyme systems which require or are stimulated by adenine nucleotides (see the review by Crane (1960)), it is possible that it is to these sites of actin to which phlorizin and its photolabile derivatives bind. On the other hand actin, due to its abundance, might simply act as a high-capacity, low affinity sink for the amphiphilic/lipophilic derivatives of phlorizin.

The lack of protection of actin by ADP and AMP (see Fig. 22B, lane 6) supports the latter rationalization, the reduction in its labeling in the presence of high concentrations of para-phlorizin and phlorizin is consistent with both (see Fig. 21, lanes 5 and 6).
Finally note that the 72,000 D band was still present in KI treated DOC extracts and that its labeling was at least as strong as in DOC extracted membranes (Fig. 22C, lanes 7 and 8).

V.4. DISCUSSION

V.4.1. Interaction of phlorizin derivatives modified in the aglycone moiety with brush border membranes in the dark.

It is generally accepted that it is the additional interaction of the aglycone moiety with one more subsite on the membrane which makes phlorizin a potent, non-transported inhibitor of the Na⁺, D-glucose cotransporter in brush border membranes. However, neither the nature of the aglycone binding site nor its location with respect to the sugar binding site of the transporter are clear. In particular it is not known whether these two binding subsites are located on the same component (subunit) of the transporter.

The tight binding of phlorizin to the carrier could also occur as a result of an interaction of (a part of) the lipophilic aglycone moiety with the lipid bilayer of the membrane. However, this type of association appears unlikely because of the strict requirements necessary for phlorizin derivatives to be inhibitors (Diedrich, 1963; Diedrich, 1966) and because of the lower affinity of certain more lipophilic derivatives of phlorizin modified in the aglycone moiety (Diedrich, 1963).

Substitutions in ring B:

A hydrogen bond between the phenolic hydroxyl group in position 4 of ring B and a component of the receptor has been proposed to be an important factor in the interaction of the aglycone moiety of phlorizin with its binding site of the receptor (Diedrich, 1963 & 1966). This view was supported by the work of Kotyk et al. (1965) who showed that 4-deoxyphlorizin exhibited a marked decrease in inhibitory activity on D-galactose uptake in rabbit kidney cortex and hamster jejunum. Therefore, substitution of this function is likely to affect the affinity of the phlorizin derivative for the transporter.

The differing affinities of the various derivatives in position 4, as determined by their $K_i$-values (see Table IV), are consistent with this idea: replacement of the hydroxyl group by an azido- or an amino group results in a decrease in $\Delta\Delta G^*$ of approximately 1.7 and 1.0 kcal/
mole, respectively. The decrease in binding energy of these compounds is consistent with both the role of the hydroxyl group as a donor and an acceptor of the hydrogen for bond formation. From the relatively high potency of 4-methoxyphlorizin as an inhibitor of D-glucose uptake in surviving intestines of golden hamsters, Diedrich (1966) concluded that the reactive group on the receptor is the one which donates the hydrogen for bond formation. The considerably lower affinity of the 4-nitro-derivative (ΔΔG° = 2.5 kcal/mole) is probably due to electrostatic repulsion.

The data in Table IV show that 4-azidophlorizin satisfies the "in dark requirements" of a potential photolabel of the rabbit small intestinal transporter: it is a reversible, fully-competitive inhibitor of Na^+/ΔΨ-dependent D-glucose uptake and it competes with phlorizin for Na^+-dependent phlorizin binding.

The pH-dependence of inhibition of D-glucose uptake by the 4-azide is similar to that of phlorizin. The comparatively higher inhibitory potency at pH 6.5 to 7.0 suggests that photolabeling experiments should be performed in this pH-range (Fig. 19).

However, as had to be expected from theory (Hansch & Fujita, 1964) substitution of the hydroxyl group in C-4 by an azido group resulted in an increased lipophilicity of the 4-azido derivative, as evidenced from its approximately 8 fold higher partitioning into monolamellar liposomes (K ≈ 9,000, see page 69). This enhanced lipophilicity of the 4-azide is likely to be responsible for its approximately four fold higher unspecific (=total) binding than that of phlorizin at the same concentration.

Furthermore, the affinity of the 4-azide for the transporter, which is at least one order of magnitude lower than that of phlorizin (see Table IV), makes it necessary to use correspondingly higher 4-azide concentrations in order to achieve comparable occupation of the binding site of the transporter. This must result in a further increase in unspecific binding of the 4-azide, as compared to phlorizin.

The free energy of binding is calculated from the K_i'-values by the equation:

\[ ΔG° = 1.422 \log K_i' \]
Considering the above large unspecific component of the binding of the 4-azide at concentrations required for the photolabeling of the rabbit small-intestinal transporter, the data in Table V deserve particular attention. They clearly show that the affinities of the 4-azide for both, the small-intestinal and the renal D-glucose transporter of the rabbit, are considerably smaller than those for the corresponding systems of the rat. This obviously species and not organ related difference in the inhibitory capacity of the 4-azide clearly makes this compound a more promising photolabel of the transport systems of the rat.

In addition the 15 to 20 fold difference in affinity of the 4-azide for the transporters of the two animals significantly exceeds the corresponding 3 fold difference found for phlorizin. The reason for this is not clear at the present. One may speculate on the possibility that the D-glucose transport systems of the two animals tested are not quite identical, but possess slight differences in the aglycone binding subsite which are more prominent in the interaction with the 4-azido-analogue than in that of the parent glucoside.

Substitutions in ring A:

4'-Deoxyphlorizin:

The high inhibitory potency of 4'-deoxyphlorizin on D-glucose uptake in rabbit small-intestinal brush border membrane vesicles (see Fig. 19) is consistent with the idea that the 4'-phenolic group is not required for optimal binding of phlorizin to the transporter (Diedrich, 1963; Diedrich, 1966). However, the 4'-deoxy-derivative at pH 7.5 was at the best equipotent with phlorizin (see Table IV). This is in contrast to its inhibitory potency in whole (surviving) small intestines of golden hamsters which at pH 7.4 has been reported to be 155 to 170 % relative to that of phlorizin (Diedrich, 1966).

The pH-independence of the binding of 4'-deoxyphlorizin has a great advantage: using (photo)reactive derivatives of this ligand certain labeling reactions can be performed at alkaline pH, where the affinity of the corresponding phlorizin derivatives would decrease.

To these labeling reactions belong all those which involve a nucleophilic attack on the label by an appropriate group on the protein. Not only a variety of "classical" chemical (affinity) labeling reactions are known to proceed by this mechanism (e.g., Powers, 1977), but it was
recently proposed that also an important reaction pathway(s) leading to covalent incorporation of photoactivated arylazides involves nucleophilic attack (Staros, 1980; see also Fig. 23 and final discussion). The considerably lower affinity of the 4'-mesylate is indicative of steric repulsion by the mesyl group and shows that the possibility of introducing reactive groups in this position of the phlorizin molecule is limited.

V.4.2. Photoinactivation of the transporter.

Because of the extensive photoinactivation of the transporter by the light required for rapid photolysis, the 10 to 20 % labeling efficiency of the 4-azide, as determined from site-directed inactivation of the transporter (see page 114), can only be considered as approximate. Nevertheless it may be concluded that it lies in the same range as has been observed with NAP-β-Ala-Phlz as photolabel (see Table III). The inactivation of the transporter by UV-light is independent of whether the photolysis mixture is deoxygenated prior to photolysis. This leads to the conclusion that this process is different from the photooxidative damage of vesicles caused by visible light (see IV.3.1.3.). Nevertheless these photolysis conditions were preferred to "softer" ones: the longer photolysis (and contact) times required for activation of the label under the latter conditions (see footnote on page 116) were found to result in increased unspecific labeling.

V.4.3. Labeling of (a part of) the Na⁺, D-glucose cotransporter in DOC extracted small-intestinal brush border membranes.

When intact small-intestinal brush border membrane vesicles and DOC extracted membranes were photolyzed in the presence of (³H)-4-azido-phlorizin, a variety of protein bands were labeled (see Fig. 21). However, only one minor band with an apparent mol wt of approximately 72,000 D was found to be labeled specifically: using DOC extracted membranes labeling of this band was largely suppressed when (i) phlorizin, but not para-phlorizin, at 200 µM was present in the photolysis
mixture, and when (ii) Na\(^+\) was replaced by choline in the photolysis medium. In addition, relative enrichment of the transporter by (iii) DOC-extraction or (iv) DOC-extraction plus KI extraction led to a more prominent labeling of band 72,000 D. Let us discuss the extraction procedures first.

As shown before, extraction of membrane vesicles with DOC results in a negative purification of the phlorizin binding component of the transporter. This occurs, mainly, by an opening of the brush border membrane vesicles which allows the selective release of proteins which are not related to the phlorizin binding component of the transporter; these are in particular loosely bound proteins such as some of the cytoskeletal proteins (actin and probably villin, see below).

Therefore enhanced labeling of a specifically labeled band in DOC extracted membranes is a further criterion for specific labeling. This is indeed met by the labeling of the 72,000 D band, since it is strongly enhanced in DOC extracted membranes with respect to native brush border membrane vesicles as the controls (compare lanes 2 and 8 in Fig. 21).

The phlorizin binding component of the transporter was also shown to be enriched when DOC extracted membranes were subjected to various procedures known to remove selectively externally and internally adsorbed proteins (Klip et al., 1979b). Among these procedures was extraction with membrane perturbing agents such as NaI (Hatefi & Hanstein, 1969; Kahlenberg, 1976). KI, a similar perturbing agent, has proved particularly selective in solubilizing cytoskeletal proteins in chicken small-intestinal brush borders, leaving (integral) membrane proteins in the pelletable fraction (Bretscher & Weber, 1980a). The resistance of the 72,000 D protein in DOC extracted membranes to treatment with this agent (see lanes 8 & 9 in Fig. 22C) is consistent with this protein being an integral membrane protein, as is expected for (some of the components of) a transport protein.

According to the above "enrichment" criterion, it may be concluded that those bands which, after extraction of membranes with DOC (and KI), are labeled less than in untreated membranes are not essential components of the binding site. Among the bands which by this means can be eliminated as candidates for the phlorizin binding component are some of the major bands labeled, A, 3 and 4.
Finally the amount of \(^3\text{H}\)-label specifically incorporated into the 72,000 D band of DOC extracted membranes was estimated to be 2-5 pmoles/mg protein. This figure is in good agreement with the amount of label expected to be specifically incorporated into the (phlorizin binding component of the) transporter, 1 to 2 pmoles/mg protein, assuming a carrier density of 20 pmoles/mg protein in these membranes and a labeling efficiency of 10 to 20% under the conditions chosen.

It is very likely, therefore, that the 72,000 D band is (a component of) the \(\text{Na}^+\), D-glucose transporter of this brush border membrane. This agrees with previous indirect conclusions of this laboratory: from partial negative purification (Klip et al., 1979b) or semi-selective labeling with \(\text{HgCl}_2\) (Klip et al., 1980) the polypeptide bands 5f and 7f (the 60,000 to 70,000 D region; Klip et al., 1980), along with bands 1 and A, were proposed as likely candidates. The 72,000 D band would migrate within band 5f. Our conclusions are compatible also with the figures reported by other laboratories from solubilization-reconstitution of kidney cortex membranes (60,000 to 70,000 D under denaturing conditions (Kinne et al., 1981) or approximately 150,000 D under non-denaturing conditions (Malathi & Crane, 1981)).

The notion that the (labeled) phlorizin binding component is a part of the \(\text{Na}^+\), D-glucose cotransporter in both, intestinal and renal brush borders has been well substantiated (Diedrich, 1966; Bode et al., 1972; Kinne, 1976; Silverman, 1976; Tannenbaum et al., 1977; Toggenburger et al., 1978). Whether additional components exist and whether they copurify with the glycoside binding element during the extraction procedures described, is not known. Be that as it may, due to the use of SDS for solubilization of the membranes, a characterization of a possible transporter macromolecule would be possible neither with intact membrane vesicles nor with DOC extracted membranes. Boiling with SDS abolishes non-covalent interactions among proteins and precludes the detection of proteins whose functional state depends on a complex subunit structure.
Furthermore, since SDS-PAGE was routinely performed under reducing conditions (with mercaptoethanol as the reducing agent), it cannot be ruled out that the labeled protein is only an element of (the phlorizin binding component of) the transporter which may be linked to other elements by S-S bonds.

Considering the property of KI as a selective solubilizer of cytoskeletal proteins, it is possible from the results in Fig. 22C to speculate on the nature and identity of two of the major bands labeled, bands 3 and 4. These bands, along with residual actin, are released quite selectively from DOC extracted membranes when these are treated with KI. This suggests that, in addition to the well known actin band A, also these two bands can be attributed to cytoskeletal proteins. Since the protein in band 4, but not in band 3, is easily extracted by DOC, and always to a similar extent to actin, it is very likely that this protein is physically linked to actin. Villin is an important cytoskeletal protein which has been shown to be linked to actin (Bretschcher & Weber, 1979). Therefore, and because of the similar mol wts (approximately 90,000 D for band 4 and 95,000 D for villin (Bretschcher & Weber, 1979)) of these proteins in the (different) gel systems used, it is tempting to identify band 4 as villin. The resistance of band 3 to DOC-, but not to KI-extraction (see lanes 7 and 8 in Fig. 22C), suggests that this (cytoskeletal) protein is more tightly associated with the membrane than band 4. This property of band 3, together with its apparent mol wt of approximately 105,000 D in the gel system used, leads to the assumption that it is the 105,000 to 110,000 D protein of Matsudaira & Burgess (1979). This protein is proposed to be (a component of) the lateral arm linking the core of the actin filaments to the membrane, probably by direct interaction with the microvillar membrane.

Possible explanations for the labeling of these and other carrier-unrelated proteins will be discussed below.
Some tentative conclusions on the nature and the mode of functioning of the Na⁺, D-glucose transporter and on its interaction with the aglycone moiety of phlorizin and of its derivatives.

It is possible from the identification of the 72,000 D band with (a part of) the Na⁺, D-glucose transporter to draw some general conclusions on its nature and its mode of functioning. A first one is neither novel nor surprising: as the band has an apparent mol wt of approximately 72,000 D and stains with Coomassie blue (Fig. 21, lane 1), it is in all likelihood proteinaceous. Secondly, a polypeptide chain of 72,000 D can (although admittedly need not) span the membrane, a feature for which some evidence has been presented (Klip et al., 1979 a,c); it is large enough to build (a part of) a "gated pore" (see below).

The mode of functioning of this membrane transporter, indeed, that of all "mobile carriers", is still matter of speculation. Since it shows a stable structural (Klip et al., 1979 a,c) and functional (Kessler & Semenza, 1981) asymmetry, a "rotational" or "diffusive" mechanism can be ruled out. Whether monomeric or oligomeric, the most likely mode of operations seems to be that of a "gated channel" (Semenza, 1981), which is a limiting case of the membrane transporter (Lauger, 1980). Such a model is in keeping of course, with the current ideas on the mode of functioning of a "mobile carrier" (Singer, 1974 & 1977; Lauger, 1980).

Further it is possible from the fact that a reactive group at position 4 of ring B does indeed react with (a part of) the Na⁺, D-glucose carrier to make some inference on the mode of the interaction of phlorizin and its derivatives with it. The $K_i$-values of phlorizin for D-glucose transport and the $K_d$-values of phlorizin binding to brush border membrane vesicles are approximately 4 - 7 μM (Toggenburger et al., 1979). These values are considerably smaller than the $K_m$-values of monosaccharides or most β-D-glucopyranosides for transport which lie in the mM-range (the smallest value being perhaps that of D-glucose "initial" uptake into brush border vesicles, 0.1 mM (Kessler et al., 1978b)). Also, the $K_i$-value of the only monosaccharide known to us to act as a fully competitive inhibitor (L-fucose, in intestinal rings, 20 mM; Caspary et al., 1969) is much larger than that
of phlorizin. Therefore, in order to explain the higher binding energy of phlorizin Diedrich (1963 & 1966) suggested that this glucoside binds to the carrier both at its sugar binding site (via the glucopyranosyl moiety) and, in addition, at a (presumably hydrophobic) site (via the aglycone moiety). As mentioned before, the phenolic group in ring B modified by us is proposed to contribute importantly to the interaction of phlorizin with the latter subsite: this group is assumed to be involved in a hydrogen bond. Indeed the additional binding energy of this proposed H-bond at position 4 may account by itself for the observed difference between the $K_i'$ of phlorizin and the $K_m$ of glucose (if $K_m$ can be equated to $K_s$). The subsite(s) interacting with the aglycone moiety of phlorizin could in part be lipids. The observations of Fig. 21 do not rule out the occurrence of lipids at the aglycone binding site(s), but they demonstrate that the reactive group in position 4 of ring B reacts covalently with a polypeptide.
VI. FINAL DISCUSSION

VI.1. REACTIONS OF ARYL AZIDE PHOTOLABELS

Aryl azides were introduced as photoactivatable precursors of arylnitrilenes in biochemistry little more than a decade ago (Fleet et al., 1969). The breadth of their use is indicated by the extensive list of references to their use in two recent comprehensive reviews (Bayley & Knowles, 1977; Chowdry & Westheimer, 1979). As mentioned before, the popularity of the arylazido group is largely due to its relative ease of synthesis, stability in storage, and lack of reactivity under physiological conditions in the absence of light.

When this work was started in 1976/77, it was generally believed, based on some precedent from the organic chemical literature (Smith, 1970), that the major route to covalent adducts of aryl azide photolabels with their target molecules was through the insertion of the photolytically generated nitrene into a nearby C-H bond to give a secondary aryl amine (Fig. 23, route a). Furthermore, aryl nitrenes photogenerated in the hydrophobic regions of biological membranes would lead to aziridines by their addition to double bonds of unsaturated fatty acids (Fig. 23, route b). In addition some of the photolabel could ring expand and react with amines to form azepines (Fig. 23, route c). Until quite recently, however, this route was not seriously considered in the design of photolabeling experiments. All of the above reactions can be considered productive, i.e., they all result in a covalent adduct between the photolabel and target. A number of nonproductive reactions have also to be considered. The most prominent of these is a pair of hydrogen abstraction reactions leading to an unreactive primary aryl amine (Fig. 23, route d). Dimerization can also occur, to produce azobenzenes (Fig. 23, route e). The recently recognized nonphotochemical reduction of aryl azides, e.g., by thiols (Staros et al., 1978) has been mentioned before (Fig. 23, route f).

Of course, in any experiment, there may be nonproductive products of the reactions outlined in a-c, i.e., some photolabel molecules will react covalently with water, buffer molecules, scavengers (see below), or with other components of the system rather than the target structure. This can be particularly important for a photolabile analogue that retains good functional and binding properties, because the modification
Fig. 23. Some important reactions of aryl azide photolabels. Possible covalent adducts with target structures include secondary aryl amines (a), aziridines (b), and azepines (c). Nonproductive reactions include formation of primary aryl amines (d and f) and azobenzenes (e). For some pathways, e.g., (c) the intermediacy of the nitrene is open to question (Chapman & Le Roux, 1978). For further details, see the review by Staros (1980).

of the natural ligand that gives the photolabile analogue involves part of the ligand that is unimportant in recognition and binding. This means that the modified part of the molecule may be exposed to the bulk solvent, to the lipid phase of a membrane, or to a neighbouring macromolecule, and that little labeling of the target molecule will occur upon photolysis. This might be responsible for the low labeling efficiencies observed with the NAP-derivatives of phlorizin modified in C-6 of the glucopyranosyl moiety (see TableII).
Another property of aryl azides that many investigators have relied upon is the short lifetimes of the aryl nitrenes in solution. These have been estimated to be in the 0.1 - 1 ms range, based on model systems (Reiser et al., 1968). Short lifetimes are required if one wants the covalent reaction of a photolabel to be fast relative to the rate of dissociation of the label-binding site complex (Ruoho et al., 1973), and in crosslinking studies if one wants to distinguish between relatively stable structures and collision complexes (Kiehm & Ji, 1977).

Recent work from several laboratories has led to a reevaluation of the assumptions concerning (i) the relative importance of the various routes leading to covalent products, and (ii) the lifetimes of the reactive intermediates.

(i) Products of photolabeling.
Bayley & Knowles (1978a) using phenyl azide as a label for the hydrophobic domain of synthetic phospholipid vesicles and reduced glutathione as a nucleophilic scavenger found that reactive intermediates from photolyzed phenyl azide did not insert into C-H bonds, and when generated in the presence of both, C-H bonds and nucleophiles, showed a greater preference for the latter. When gramicidin A in synthetic phospholipid bilayers was labeled with a phenyl azide covalently attached to the \( \omega \)-position of a fatty acyl chain of a phospholipid, then only the single nucleophilic amino acid residue, Trp, present in the polypeptide, was modified (Brunner & Richards, 1980).

These studies suggest that aryl azide photolabels probably do not insert to any great extent into C-H bonds in heterologous systems. This lack of C-H insertion reaction, however, does not preclude the use of aryl azido derivatives as photoprobes. In highly constrained systems, e.g., a photoaffinity label bound very tightly to its target binding site, C-H insertion may play a role. However, the general preference of unconstrained aryl azide photoproducts (such as phlorizin derivatives used in this work) for nucleophiles, once realized, can be useful in the interpretation and the design of labeling experiments.
(ii) Lifetimes of reactive intermediates.

When the lifetimes of reactive intermediates from photolysis of aryl azides are of consequence, the assumption is usually made that the reactive intermediate of importance is the aryl nitrene. Consequently lifetimes for triplet aryl nitrenes in ethanol (0.1 - 1 ms; Reiser et al., 1968) are often cited to suggest the rate expected for the coupling reactions in photolabeling experiments. However, these data were obtained from model systems using organic solvents and may not be relevant for biological labeling experiments.

From the lipid labeling experiments in (i) it was evident that the lifetimes of the reactive intermediates were sufficiently long to allow relatively rare collisions to occur between these intermediates and nucleophiles in the system. Even more surprising in this respect was the finding by us (see page 96) that BSA added postphotolytically to photolyzed membranes was covalently labeled, as well as membranes which were added to photolyzed label immediately after photolysis. These findings are corroborated by the recent report by Mas et al., (1980). They used NAP-taurine [N(4-azido-2-nitrophenyl)-2-aminoethylsulfonate] a watersoluble aryl azide probe designed for topological labeling of membrane surfaces (Staros & Richards, 1974), to label rhodopsin in outer segment disk membranes. Covalent incorporation of radiolabel into rhodopsin continued for several minutes after photolysis.

It seems unlikely that the nitrene produced by photolysis could be responsible for such long-lived reactivity. While such long lifetimes of reactive intermediates may not be of particular concern in the study of the topology of membrane proteins, they are of great importance in the labeling of proteins (receptors, transporters etc) using labels with a moderate affinity for the binding site, such as the above phlorizin derivatives. It is clear that optimized photolysis conditions, in combination with suitable scavengers are required, in order to minimize co- and postphotolytic incorporation of long lived reactive intermediates into sites not related to the target (= unspecific labeling, see below).
As to the nature of these long lived intermediates, the cyclic ketenimine 1-aza-1,2,4,6-cycloheptatetraene has been proposed as the primary product on the route to the formation of azepines (Chapman & Le Roux, 1978). These would be formed by the attack by a nitrogen nucleophile at C₂ followed by proton migration.

While the cyclic ketenimine route to azepines may explain some of the photoproducts of aryl azides in biochemical systems and some of the preference for nucleophiles shown by reactive intermediates of aryl azide photolysis, it is not clear whether this route has anything to do with the long-lived intermediates observed by the group of Hargraves (Mas et al., 1980) and by us. In addition it has been recently suggested (Nakayama et al., 1979) that photolysis of meta or para nitrophenyl azides (NAP-) does not lead to the formation of azepines, implying that the cyclic ketenimine pathway may not be an important intermediate in the photolysis of such compounds. The effects of substituents on the aromatic ring on the reaction pathways and on the lifetimes are still largely unknown (Staros, 1980).

VI.2. MODES OF REVERSIBLE AND IRREVERSIBLE ASSOCIATION OF PHLORIZIN AND PHOTOLABILE ANALOGUES WITH SMALL-INTESTINAL BRUSH BORDER MEMBRANES.

Despite the specific labeling of a minor band (at 72,000 D) with 4-azidophlorizin, photolabeling with this ligand, and even more so with the NAP-derivative(s) of phlorizin, predominantly results in transport-site unrelated, nonprotectable labeling of brush border membranes.

A deeper understanding of the biochemical and chemical properties of the system leading to the observed labeling patterns is required not only to evaluate the (further) use of the above photolabile analogues, but also to learn more about possible limitations of phlorizin and its analogues as affinity labels of the D-glucose transporter in general. This is indispensable for the design of new (photo)reactive analogues of this glucoside.

Since the light induced incorporation of photolabile derivatives of phlorizin into components of the brush border membrane is preceded by
reversible binding (to the membrane), this binding must be examined in greater detail.

There are at least three ways by which phlorizin and its derivatives can reversibly associate with brush border membranes, and therefore there are probably at least as many ways in which labeling can occur.

(i) Phlorizin and its derivatives bind stoichiometrically to the substrate binding site of the transporter in a Na\(^+\)-dependent and D-glucose protectable way. This type of binding, and of labeling, is called **specific**. (ii) Phlorizin (and derivatives) probably bind to substrate or cofactor binding sites of other brush border proteins. This type of binding is called **unspecific** and is said to lead to **unspecific labeling**. (iii) Phlorizin and its derivatives bind to the lipid bilayer and, possibly, to various membrane proteins via their hydrophobic (nitro azido) aryl residues. This type of binding is termed **general unspecific binding** and it leads to **general unspecific labeling**.

In the following the two types of unspecific labeling (ii and iii) of brush border membranes or vesicles by phlorizin and its analogues will be discussed in more detail.

(ii) Phlorizin, beside its relatively specific inhibitory action on intestinal (and renal) D-glucose absorption, is known to exhibit at least four additional sites of action as an inhibitor or as a substrate in these membranes. These have been reviewed by Crane (1960) and are: (1) intestinal phosphatase(s); (2) the mutarotase; (3) the phlorizin hydrolase (only present in small intestinal brush borders; Malathi & Crane, 1969)); and (4) enzymes and enzyme systems which require or are stimulated by adenine nucleotides, including ATPases. Phlorizin also induces an ATP-reversible swelling of mitochondria (Keller & Lotspeich, 1959).

The concentration of phlorizin required to inhibit these systems or to bind to them is considerably higher (>0.1 mM) than that required to inhibit intestinal D-glucose absorption (in the range of 10 \(\mu\)M). However, the relatively high concentrations at which the photolabile derivatives have to be used because of their lower affinities might lead to increased binding to and labeling of these systems.

The failure of known ligands, such as theophylline (for alkaline phosphatase; Ghjisen et al., 1980) and AMP/ADP (for nucleotide binding
enzymes), to protect any band from being labeled suggests that labeling of these systems does not contribute to the labeling patterns observed, unless the phlorizin analogues and these ligands bind to different sites on these systems. Reduction of labeling of the band I with both, NAP-β-Ala-Phlz and the 4-azide as labels, in the presence of high concentrations of para-phlorizin and phlorizin, could lead to the conclusion that this protein is actually the phlorizin hydrolase which had been labeled at the catalytic site: this enzyme from human small intestine is known to migrate in the same region of an SDS gel as isomaltase (apparent mol wt of the former is 160,000 D; Skovbjerg et al., 1981) and to exhibit comparable affinities for both glucosides (Evans & Diedrich, 1980). However, the finding that there is always less labeling of the band "I" (and also of band A and of lipids) in the presence of para-phlorizin, as compared to phlorizin, suggests, that this is probably not so, since the affinity of para-phlorizin for the phlorizin hydrolase is lower than that of phlorizin (Evans & Diedrich, 1980). An additional, not alternative explanation for these findings will be given below.

(iii) Due to the relatively high concentrations which have to be used and due to the higher lipophilicity, the photolabile phlorizin analogues exhibit considerably more unspecific binding to brush border membranes than phlorizin. This, among other things is likely to be responsible for the excessive unspecific labeling observed with both photolabels.

Since photolysis of brush border membranes in the presence of 4-azide, NAP-β-Ala-Phlz and NAP-β-Ala-para-Phlz results in strikingly similar (unspecific) labeling patterns, it may be concluded that these labels, although they are structurally and chemically quite different, behave similarly with respect to unspecific labeling.

Two modes of general unspecific labeling of membrane proteins can be differentiated, according to the compartment from which it occurs: (a) from the aqueous (bulk) phase, and (b) from within the lipid core of the membrane.

The failure of the two hydrophilic scavengers pABA and BSA to reduce significantly co- and postphotolytic (unspecific) labeling of membrane
proteins by either label, although these agents become labeled themselves (for the postphotolytic labeling of BSA, see page 96), suggests that with both labels (b) is responsible for the majority of the (general) unspecific labeling observed.

This can be rationalized if phlorizin, due to its amphiphilic character, binds to a membrane predominantly by inserting its aglucone moiety into the lipid bilayer. As to the 4-azido derivative, this would mean that the photoreactive groups of molecules bound to the membrane are buried inside the lipid bilayer, and hence inaccessible to the hydrophilic scavengers. The same must be true for the NAP-β-alanyl group of NAP-β-Ala-Phlz. This group can be assumed to be able to "bend back" from the glucopyranosyl moiety exposed to the aqueous phase into the lipid phase. Or else, NAP-β-Ala-Phlz has completely lost the amphiphilic character of the parent glucoside. The above idea that most of the unspecific labeling of membrane proteins by the NAP-derivative of phlorizin occurs via membrane-embedded NAP-groups is further supported by the finding that NAP-β-alanine, an amphiphilic label expected to bind to the membrane via immersion of the NAP-group into the bilayer, produces a labeling pattern which is qualitatively very similar to those obtained with the photolabile phlorizin analogues.

It is very likely that long-lived photoproducts of the (nitro) arylazido labels (see above) contribute to this predominant labeling from within the lipid bilayer. It can be imagined that these intermediates, due to their long lifetimes, are able to diffuse laterally in (and also perpendicular to) the plane of the bilayer until they encounter a nucleophilic group (e.g., -SH, -NH₂) on the hydrophobic segment of a membrane protein or a lipid molecule.

Further, similar to photolyzed phenyl azides (Bayley & Knowles, 1978a), the phlorizin analogues and/or some of their long-lived reactive intermediates must have enough time to leave the bilayer on both sides of the membrane and to label membrane proteins and cytosolic proteins from the bulk phase. This is demonstrated by the massive labeling of actin located in the intravesicular space of sealed vesicles by both phlo-
rizin (see Appendix A) and its photolabile analogues\(^1\).

The reduction in the labeling of the proteins "I" and A in intact vesicles in the presence of high concentrations of phlorizin or its para-isomer can also be explained in the frame of the "labeling from within the lipid bilayer"-model for unspecific labeling: these glucosides, according to their partition coefficients, would be dissolved in the lipid bilayer in large amounts, and, thereby might reduce the uptake of label molecules into the membrane bilayer (saturate the binding sites of the label molecules in the membrane bilayer). This would then result in a decrease in the labeling of the hydrophobic segments of membrane proteins (such as the anchor of isomaltase).

This view is supported by the concomitant reduction in the labeling of lipids. Further, an impaired uptake of label into the bilayer would result in a decrease of the equilibration rate of the label molecules across the bilayer, and hence lead to a reduction in the (unspecific) labeling of proteins trapped inside, such as actin. As mentioned above, however, some site-directed protection of the proteins "I" and A by these glucosides at high concentrations cannot be ruled out completely, since both, "I" (or, rather comigrating phlorizin hydrolase) and actin, possess or may possess, respectively, a phlorizin binding site(s). Finally, light screening by these phenols may also be responsible for some of the protection observed.

It has to be emphasized that general unspecific labeling of the very transporter can also occur via mode (b), since it is an integral membrane protein. Such a labeling might, to a certain extent, mask specific labeling of this component, since it is likely to be Na\(^+\)-independent and only moderately "protectable" by phlorizin and paraphlorizin at the concentration normally used, 200 \(\mu\)M (for the decrease in general unspecific labeling of membrane proteins in the presence of high concentrations of these glucosides, see above).

\(^1\)Transport of phlorizin into the intravesicular space by the D-glucose carrier has been shown to be unlikely (Toggenburger, 1979; Kessler et al., 1981, in preparation).
In future experiments with aryl azido derivatives of phlorizin, or with phlorizin itself (see Appendix A) as photolabels, lipophilic nucleophiles as the scavengers might be used to reduce this type of general unspecific labeling. Alternatively phlorizin derivatives could be rendered more hydrophilic by attachment of a polar (charged) group in close proximity to the photolabile function. Of course the use of different photoreactive groups, such as carbene generating functions has to be considered (see below).

Finally the similarity in the development and use of photolabile analogues of phlorizin, in particular NAP-β-Ala-Phlz, and of NAP-Taurine [N(4-azido-2-nitrophenyl)-2-aminoethylsulfonate] should be noted. The latter photolabile reagent has been developed as a nonspecific label to monitor surface features of cell membranes (Staros & Richards, 1974). However, it was realized later (Dockter, 1979) that this reagent, being amphipathic, can orient at the membrane surface with the reactive nitrene group inside the bilayer and, therefore, can also label membrane proteins from inside the bilayer.

VI.3. FINAL REMARKS AND PERSPECTIVES

From the above results it is evident that photolabile phlorizin analogues, such as the 4-azide, can be used as photolabels of the phlorizin binding component of the D-glucose transporter. However, the use of this label, and even more so of the NAP-derivative of phlorizin, suffers from the considerable unspecific labeling of brush border membrane proteins which results.

The following means can be envisaged to improve the use of phlorizin and derivatives as labels of the phlorizin binding component of the D-glucose transporter.

(i) The use of the above azido analogues (or other azido derivatives, which, by the attachment of a polar group(s) have been rendered more hydrophilic) in combination with lipophilic nucleophiles as the scavengers. In the presence of such scavengers (but most likely not in the presence of the hydrophilic ones used in the present work) flash photolysis (Matheson et al., 1977; Kiehm & Ji, 1977) might further aid
in optimizing specific labeling.

(ii) The use of phlorizin derivatives which carry a carbene generating group. Carbenes (see Jones & Moss, 1973 & 1975) react very rapidly with a variety of chemical functions, such as nucleophilic centers, double bonds (including those of aromatic systems), single bonds (including insertion into C-H bonds), and by hydrogen abstraction. However, some species can possess limitations: (1) if a H-ligand is on the carbon atom in α-position to the carbene group, hydrogen migration occurs, leading to an unreactive olefin; (2) the α-ketocarbene species may undergo the intramolecular Wolff rearrangement, resulting in a ketene; (3) α-diazocarbonyl compounds are susceptible to heat and acid and (4) can require long photolysis times.

Nevertheless the very high indiscriminate reactivity of the initially formed carbene makes its precursors very potent labels (a number of convenient precursor species for the photochemical generation of carbenes are listed in the reviews by Bayley & Knowles (1977) and by Chowdry & Westheimer (1979)). The superiority of phenylcarbene over phenyl-nitrene as a reagent in photolabeling hydrophobic regions of biological systems has been demonstrated by Bayley and Knowles (1978a,b) (see above). It may be mentioned in this respect that the very first example of photolabeling involved the use of a diazoacetyl compound by Westheimer and his group (Singh et al., 1962).

(iii) Preliminary photolysis experiments with phlorizin (see Appendix A) have shown that this ligand itself, under certain conditions, can be a photolabel of brush border membrane proteins. Since the advantages of an unmodified, "natural" ligand as the label for its receptor are obvious, this promising approach has to be pursued. It can be improved with respect to the use of suitable (lipophilic) scavengers and to the photolysis conditions.

(iv) Conventional affinity labeling (e.g., Singer (1967)) with suitable reactive derivatives of phlorizin could be another approach to label the phlorizin binding site of the transporter. Preliminary experiments with 6-O-pTs-phlorizin as an affinity label have resulted in irreversible inhibition of D-glucose uptake in and phlorizin binding to brush border vesicles. However, this process(es) was not protectable by phlorizin or D-glucose (these experiments are not repeated in this
Dissertation). This raises some questions as to the nature of this inactivation process. Among the frequently used reactive groups that are likely to give a clearer answer are the isothiocyanato- and the haloacetamido groups. Maltosyl isothiocyanate has been reported recently to be a successful affinity label of the sugar transporter in red blood cells (Mullins & Langdon, 1980). The amino groups of the amino analogues 10 and 23 of phlorizin would lend themselves for the introduction of these reactive groups.

Clearly the characterization of a specifically labeled binding protein on the basis of its migration distance in polyacrylamide gels (or in gel filtration of sedimentation experiments) is not sufficient to answer the question whether this polypeptide by itself alone is responsible for the (transport) function in the membrane. It can at the best be operationally defined as the species capable of specifically binding the ligand. Therefore, the identification of the phlorizin binding component as (a component of) the functional transporter will ultimately depend on the purification of the binding activity and the demonstration of the transport activity in a reconstituted system. The method of photoaffinity labeling with 4-azidophlorizin (and probably also other photolabile analogues, including phlorizin itself) in conjunction with SDS-PAGE (under denaturing or under nondenaturing conditions) or two-dimensional gel electrophoresis (e.g., O'Farrell (1975)) should be a very useful tool to analyze the (phlorizin binding component of the) Na+, D-glucose cotransporter at various stages during purification. It is evident that other photolabile derivatives, such as the NAP-derivatives, will only be useful tools (e.g., to map the binding site) when the transporter has been purified to some extent.
APPENDIX A

Phlorizin as it is an \(\alpha,\beta\)-unsaturated ketone can itself be considered a potential photolabel. Such ketones are the best understood members of a class of photoactivatable reagents which can couple to a receptor by a radical mechanism (for a review, see Bayley & Knowles, 1977). Excitation in the \(n-\pi^*\) band produces a diradical triplet state which is an efficient hydrogen abstractor (Turro, 1965). The abstraction is selective, and C-H bonds are normally attacked in preference to the stronger O-H bonds of the solvent water. This newly generated radical may then couple with the other half of the initially generated pair, resulting in the covalent attachment of the ligand.

Compounds that so far have been successfully used as photolabels include both natural ligands that already contain an \(\alpha,\beta\)-unsaturated carbonyl function (e.g., ketosteroids (Martyr & Benisek, 1974; Katzenellenbogen et al., 1974) and quercetin (Farley et al., 1976)), and ligands to which such a function has been attached (e.g. acetylbenzoyl- and benzoylbenzoyl derivatives of gastrin (Galardy et al., 1974)).

The use of native phlorizin as the photolabel seemed particularly promising for the following reasons: (i) it is the ligand with the highest known affinity for the transporter; (ii) it is considerably less lipophilic than the photolabile derivatives synthesized, as judged from their relative partition coefficients (see page 69), and (iii) the preference of \(\alpha,\beta\)-unsaturated ketones for C-H bonds could be an advantage, since nitrenes (and also carbenes) may fruitlessly react with the solvent and result in low labeling efficiencies.

Preliminary photolysis experiments, however, performed under the conditions required for the activation of acetophenones (irradiation with light of wavelengths \(> 280\) nm for 1 h at room temperature; Galardy et al., 1974) resulted in a strong irreversible inhibition of the transporter (data not shown). Therefore, and because of the long photolysis times required for complete activation of these compounds, this approach was not pursued at first.

Later, when photolysis of vesicles in the presence of 100 µM \(^3\text{H}\)-phlorizin under relatively mild photolysis conditions (irradiation with light of wavelengths \(> 315\) nm, for 1 min) was found to result in the
incorporation of \(^{3}\text{H}\)-label (see Fig. 18A), the use of phlorizin as a photolabel was reexamined.

The labeling patterns of membranes obtained after photolysis in the presence of low concentrations of \(^{3}\text{H}\)-phlorizin (specific activity: 6.3 Ci/mmol) are shown in Fig. 24.

Photolysis of intact vesicles in the presence of 6.8 \(\mu\text{M}\) \(^{3}\text{H}\)-phlorizin (corresponding to its \(K_{d}\)), using pABA (10 mM) as the scavenger, resulted in the faint labeling of several bands (lane 2). When the concentration of phlorizin was increased to 13.6 \(\mu\text{M}\), the labeling pattern in lane 3 was obtained. The major bands labeled were I, S, 3, 4, 5d and A.

Replacement of NaSCN by choline SCN (100 mM) did not result in a detectable specific change in the labeling pattern (lane 4).

When both (cold) phlorizin (lane 5) and para-phlorizin (lane 6) at 250 \(\mu\text{M}\) were used as "protectors", labeling of proteins was completely abolished.

Omission of pABA as the scavenger in the photolysis incubation resulted in the labeling pattern shown in lane 7. Note the selective decrease in the labeling of the bands I, S, 4 and 5d. When pABA was replaced by 10 mM reduced glutathione, labeling of proteins was largely suppressed (lane 8).

Photolysis of DOC extracted membranes in the presence of 13.6 \(\mu\text{M}\) \(^{3}\text{H}\)-phlorizin resulted mainly in the labeling of bands I and 3 (lane 9).

Analysis of the above photolysates on (15 x 2.7) gels did not show labeling of further (low molecular weight) bands (gels not shown). There was no labeling of lipids (region K on the fluorograms in Fig. 24).

The total amounts of \(^{3}\text{H}\)-label associated with the vesicles after photolysis and extensive washing were 20 pmoles/mg protein (at 6.8 \(\mu\text{M}\) \(^{3}\text{H}\)-phlorizin) and 40 to 45 pmoles/mg protein (at 13.6 \(\mu\text{M}\) \(^{3}\text{H}\)-phlorizin) when pABA was present as the scavenger. Omission of pABA and replacement by 10 mM reduced glutathione resulted in the incorporation of 35 and 23 pmoles of label per mg of protein, respectively. These numbers are very much smaller than those obtained for \(^{3}\text{H}\)-NAP-\(\beta\)-Ala-Phlz and \(^{3}\text{H}\)-4-azide, which, after photolysis at 50 \(\mu\text{M}\) label, were 3 nmoles and 2.5 nmoles/mg of protein, respectively.
Fig. 24. Fluorograms of intact brush border membrane vesicles and DOC extracted membranes photolyzed in the presence of $^3$H-phenoliz in under various conditions.

Intact vesicles (1.65 mg protein/ml) were photolyzed in the presence of 6.8 $\mu$M (lane 2) or 13.6 $\mu$M $^3$H-phenoliz (lanes 3 - 9) for 1 min with light of wavelength $\lambda > 315$ nm (saturated Cu(II)SO$_4$ solution as the filter), as described in Methods. Unless indicated the composition of the photolysis incubation was: 300 mM mannitol, 100 mM NaSCN (out, zero in), 10 mM pABA, 10 mM Tris-HCl, pH 7.0, 0.02% KN$_3$. After washing by centrifugation 60 $\mu$g samples of protein were analyzed on a (8.4 x 2.7) cm gel. The cpm applied are given in brackets. (2) & (3) no additions (2800 cpm & 6250 cpm, respectively); (4) 100 mM choline SCN instead of NaSCN (6050 cpm); (5) 250 $\mu$M phenolizin (2800 cpm); (6) 250 $\mu$M para-phenolizin (2400 cpm); (7) no pABA present (4850 cpm); (8) 10 mM GSH red. instead of pABA (3150 cpm); (9) DOC extracted membranes, no addition (3800 cpm). The Coomassie blue protein stained pattern of intact membrane vesicles is shown in lane 1. Fluorography was performed as described; exposure at $-70^\circ$ C lasted 55 days. The numbering shown to the left of the labeled bands follows the nomenclature given in Fig. 6; the molecular weight scale is shown to the right.
The final amount of (\(^3\)H)-label present corresponds to 0.3 to 0.8 % of the label initially present in the photolysis incubation. Therefore, the presence of (reactive) contaminants as being responsible for the labeling observed was ruled out by TLC\(^1\).

Among the major bands labeled in the presence of pABA as the scavenger are I, S, 3 and A. These proteins are the most abundant and most likely are not related to the phlorizin binding site of the carrier. This and the inability to detect any specific labeling of one of the remaining bands leads to the conclusion that the labeling observed under the conditions chosen is mainly unspecific. It has to be emphasized, however, that the labeled band 5d lies in close proximity to the 72,000 D band which was labeled specifically with 4-azidophlorizin (see above).

Photolysis of vesicles in the absence of pABA seems to result in a selective reduction in the labeling of the bands I and S (lane 7). Therefore, it is tempting to conclude that pABA, by an unknown mechanism, is responsible for the (unspecific) labeling of these two proteins which are the most abundant proteins accessible from the luminal space. The complete suppression of labeling in the presence of reduced glutathione probably reflects preferential abstraction of the hydrogen from the SH group. This suggests the use of lipid soluble thiols to reduce possible unspecific labeling from within the lipid bilayer.

The strong labeling of the actin band A, even at a concentration of phlorizin as low as 6.8 \(\mu\)M, clearly shows that phlorizin itself is capable of penetrating the membrane bilayer during the 1 min photolysis. This is consistent with the ideas that (i) binding to actin accounts for a considerable amount of reversible unspecific (Na\(^+\)-independent, D-glucose non-protectable) binding of (\(^3\)H)-phlorizin to intact vesicles even at short incubation times, and that (ii) the removal of this protein contributes to the reduction in unspecific binding of phlorizin to DOC extracted membranes, as observed (Klip et al., 1979b) (see also page 49). Further these results suggest that actin is indeed a high affinity ligator of phlorizin, in agreement with the \(^1\)\(^3\)H-Phlorizin was purified by repeated TLC (using methanol-methylene chloride, 1:4 (v/v) as the eluent). The radiochemical purity achieved was \(>99.5\%\).
proposal by Crane (1960) that many enzyme systems which require or are stimulated by adenine nucleotides can bind phlorizin (see also page 142).

Further photolysis experiments under refined conditions will be required to decide whether phlorizin itself can be used to label specifically its binding component of the transporter. In particular reduction of unspecific labeling (by omitting pABA and by using lipid soluble antioxidants and thiol derivatives as the hydrogen donors), prolonged photolysis times and the use of D-glucose and other photo-inert ligands as the protectors can be expected to help at answering this question.
VII. REFERENCES


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SUMMARY

A series of photolabile derivatives of phlorizin have been synthesized as photoaffinity labels of the (phlorizin binding component of the) Na\(^+\) D-glucose transporter in brush border membranes. These derivatives carry the photoreactive (nitroaryl)azido group either in position 6 of the glucopyranosyl moiety (6-azido-6-deoxyphlorizin, N-(2-nitro-4-azidophenyl)-\(\beta\)-alanyl-6-O-phlorizin (NAP-\(\beta\)-Ala-Phlz), N-(2-nitro-4-azidophenyl)-6-amino-6-deoxyphlorizin and N-(2-nitro-4-azidophenyl)-\(\beta\)-alanyl-6-amino-6-deoxyphlorizinamide) or in the ring B of the aglycone moiety (4-azidophlorizin).

Like phlorizin (\(K'_i\approx8 \, \mu\text{M}\)), these derivatives competitively inhibited D-glucose uptake in rabbit small-intestinal brush border membrane vesicles in the presence of a NaSCN-gradient (\(K'_i\approx10, 48, 40, 148\) and \(139 \, \mu\text{M}\), respectively). In addition they inhibited specific phlorizin binding to vesicles and to DOC extracted membranes to a similar extent.

Repeated short-time photolysis of NAP-\(\beta\)-Ala-Phlz with visible light under anaerobic conditions in the presence of membrane vesicles resulted in a specific (phlorizin-protectable) reduction of \(\Delta\bar{V}_{\text{Na}^+}\)-driven accumulative D-glucose uptake and of D-glucose tracer exchange in these vesicles. Also Na\(^+\)-dependent binding of \(^3\text{H}\)-phlorizin to DOC extracted membranes was specifically and irreversibly blocked under the same photolysis conditions. Since only phlorizin, and not its 4'-isomer (para-phlorizin; a very weak inhibitor of D-glucose uptake) protected the inactivation of D-glucose tracer exchange and phlorizin binding, it is concluded that NAP-\(\beta\)-Ala-Phlz specifically inactivates the small-intestinal Na\(^+\) D-glucose transporter by binding covalently to the substrate binding site.

Photolysis of \(^3\text{H}\)-NAP-\(\beta\)-Ala-Phlz in the presence of both intact vesicles and DOC extracted membranes resulted in the labeling of a variety of protein bands as shown when the membranes were analyzed by SDS-polyacrylamide gel electrophoresis. However, specific labeling (phlorizin but not para-phlorizin protectable, Na\(^+\)-dependent) of a band could not be detected.
Photolysis of 4-azidophlorizin with light of wavelength $>$ 315 nm in the presence of brush border vesicles or DOC extracted membranes led to an irreversible inhibition of D-glucose tracer exchange and Na$^+$-dependent phlorizin binding, respectively; there being a stronger unspecific photoinactivation (in the absence of photolabel) of the transporter by this light than by visible light. Photolysis of ($^3$H)-4-azidophlorizin in the presence of DOC extracted membranes resulted, besides the (unspecific) labeling of a variety of bands, in the specific labeling of a minor protein band with an apparent molecular weight of approximately 72,000 D: (i) the presence of 200 $\mu$M phlorizin during photolysis, but not, or only to a lesser extent, the presence of 200 $\mu$M para-phlorizin, prevented the band from being labeled; (ii) labeling of this band was reduced when Na$^+$ in the photolysis incubation was replaced by choline$^+$ or K$^+$; (iii) labeling of this band was increased when membranes were used in which the "carrier density" was enriched by "negative purification", i.e., by DOC extraction or by DOC extraction plus KI extraction. It is proposed therefore, that the 72,000 D band is the Na$^+$, D-glucose transporter or a part of it.

Finally the "in dark" reversible inhibitory potencies of the various phlorizin derivatives provide some indirect information on the mode of interaction of phlorizin and its derivatives with the binding site of the transporter.
ZUSAMMENFASSUNG

Eine Reihe von photoreaktiven Derivaten von Phlorizin wurden als mögliche Photoaffinitätsmarker des Na⁺, D-Glukose Transporters (oder seiner Phlorizin-bindenden Untereinheit(en)) in Bürstensaummembranen synthetisiert. Diese Derivate tragen die photoreaktive (Nitroaryl)azido Gruppe entweder in Position 6 des Glukopyranosylrests (6-azido-6-deoxyphlorizin, N-(2-nitro-4-azido phenyl)-β-alanyl-6-0-phlorizin (NAP-β-Ala-Phlz), N-(2-nitro-4-azidophenyl)-6-amino-6-deoxyphlorizin und N-(2-nitro-4-azidophenyl)-β-alanyl-6-amino-6-deoxyphlorizinamide) oder im Ring B des Aglykon-Rests (4-Azidophlorizin).

Im Dunkeln hemmten diese Derivate die D-Glukoseaufnahme in Bürstensaummembranvesikel von Kaninchendünndärmen unter einem 100 mM NaSCN Gradienten in vollständig kompetitiver Weise (K₁' = 10, 48, 40, 148 und 139 µM, respektive). Der K₁'-Wert von Phlorizin war 8 µM. Im weiteren hemmten diese Derivate die spezifische Phlorizinbindung an Vesikel und an DOC-extrahierte Membranen in ähnlichem Ausmass.

Photolyse von \(^3\text{H}\)-NAP-\(\beta\)-Ala-Phlz in Anwesenheit von Membranen führte zur Markierung einer Vielzahl von Membranproteinen, wie aus SDS-Polyacrylamid Gel Elektrophorese ersichtlich war, unabhängig davon, ob intakte oder DOC-extrahierte Membranen markiert wurden. Spezifische Markierung einer Bande (Phlorizin-, aber nicht Para-Phlorizin schützbar, \(\text{Na}^+\)-abhängig) konnte jedoch nicht beobachtet werden.

Bestrahlung von 4-Azidophlorizin in Gegenwart von Vesikeln oder DOC-extrahierten Membranen mit Licht mit \(\lambda\geq315\ \text{nm}\) führte ebenfalls zu einer irreversiblen Hemmung des D-Glukose "Tracer Exchange" und der Phlorizinbindung. Die unspezifische Beschädigung des Transporters durch dieses Licht (in Abwesenheit von Label) war jedoch stärker als diejenige, welche durch sichtbares Licht verursacht wurde. Photolyse von DOC-extrahierten Membranen in Anwesenheit von \(^3\text{H}\)-4-Azidophlorizin führte neben beträchtlichem (unspezifischem) Labeling, zur spezifischen Markierung einer feinen Proteinbande mit dem scheinbaren Molekulargewicht von 72,000 D: (i) Die Markierung dieser Bande wurde in der Gegenwart von 200 \(\mu\text{M}\) Phlorizin stark unterdrückt; in der Gegenwart von 200 \(\mu\text{M}\) para-Phlorizin ging die Reduktion nicht vorhanden oder sehr viel schwächer. (ii) Die Markierung dieser Bande wurde vermindert, wenn \(\text{Na}^+\) (100 mM) im Photolysepuffer isoosmolar durch \(\text{K}^+\) oder Cholin\(^+\) ersetzt wurde. (iii) Die Markierung dieser Bande war verstärkt, wenn Membranen verwendet wurden in denen der Transporter durch "negative Reinigung" "angereichert" war, d.h. nach DOC oder DOC und KI Extraktion.

Aus den relativen Hemmvermögen der einzelnen Phlorizininderivate konnten indirekte Hinweise auf die Art der Wechselwirkung von Phlorizin und seinen Derivaten mit der Bindungsstelle des Transporters erhalten werden.
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