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N-ETHYLMALEIMIDE LABELING OF A PHLORIZIN-SENSITIVE D-GLUCOSE BINDING SITE OF BRUSH BORDER MEMBRANE FROM THE RAT KIDNEY*

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SUMMARY

A glucose-sensitive high-affinity receptor for phlorizin is present in isolated brush border membrane of rat kidney and is thought to be the first station in a chain of processes comprising "transmembrane transport of glucose".

This receptor was labeled with *N*-[^{14}C]ethylmaleimide for identification and future isolation according to the substrate protection effect. Three molecules of *N*-[^{14}C]ethylmaleimide are inhibited from the reaction with the brush border membrane if one high-affinity binding site is protected by phlorizin. D-Glucose also protects the high-affinity phlorizin receptor from the reaction with *N*-[^{14}C]ethylmaleimide, suggesting that the site labeled is not only part of a phlorizin receptor but also of a glucose binding protein in the brush border membrane.

INTRODUCTION

Glucose reabsorption of the renal proximal tubule can be inhibited effectively by a relatively small amount of a glycoside, phlorizin[1]. Proximal tubular brush border has been isolated in this laboratory from a homogenate of rat renal cortex[2] and was shown to bind phlorizin with high affinity. This association could be inhibited competitively and reversibly by D-glucose[3,4]. Binding and competitive inhibition were dependent on the presence of Na^+ in the incubation medium. These properties of the binding site conformed well with those attributed to a hypothetical receptor of D-glucose at the renal brush border membrane which is thought to be the first station in a chain of processes comprising "transmembrane transport of glucose" (refs 5 and 6).

Attachment of phlorizin to the receptor appeared to involve free sulfhydryl groups at the membrane since sulfhydryl group inhibitors *p*-chloromercuribenzoate (PCMB) and *N*-ethylmaleimide blocked phlorizin binding[7]. It was conceivable that *N*-ethylmaleimide could be used to label the phlorizin binding site for future isolation

Abbreviation. PCMB, *p*-chloromercuribenzoate

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and identification of the receptor using a method described by Kennedy and coworkers[8,9] as "substrate protection" experiments.

By this method, substrate initially protects its specific binding site while other *N*-ethylmaleimide-sensitive groups are saturated with the unlabeled inhibitor which is present in a low concentration. Subsequently, substrate is washed off its reversible bond and excess inhibitor is removed by the same washing procedure. Radioactively labeled inhibitor is then added to the membrane and associates with those binding sites previously occupied by substrate.

This paper describes irreversible labeling of the specific phlorizin binding site of renal tubular brush border with *N*-[^{14}C]maleimide. Three sulfhydryl groups seem to be protected by one molecule of phlorizin. The effect is caused by the glucose moiety of the glycoside rather than by its aglucon phloretin.

MATERIALS AND METHODS

(1) *Brush border membrane preparation*

Homogenization of rat renal cortex, fractionation and purification of brush border fraction have been described in detail previously[2]. Alkaline phosphatase is known to be located predominantly in the brush border[10,11] and was therefore used as marker enzyme for this structure. Constant relation of alkaline phosphatase activity to protein content of the fraction indicated reproducibility of our isolation procedure and permitted expression of all data in terms of membrane protein content.

(2) *Protection experiments*

Brush border fraction corresponding to approximately 30 mg of protein was incubated in 20 ml of sucrose–triethanolamine–HCl–Haemaccel–NaCl buffer (0.25 M sucrose, 0.01 M triethanolamine–HCl, 0.7 % Haemaccel, 0.15 M NaCl, of pH 7.4 at 20 °C) containing 0.005 mM phlorizin and 0.5 mM unlabeled *N*-ethylmaleimide, at 28 °C for 70 min. Incubation was terminated by high speed centrifugation (35000 × *g*, for 20 min) yielding membrane sediment and supernatant which was discarded. The sediment was resuspended and washed in sucrose–triethanolamine–HCl buffer (0.25 M sucrose, 0.01 M triethanolamine–HCl, of pH 7.6 at 20 °C), centrifuged and taken up in sucrose–triethanolamine–HCl–Haemaccel–NaCl buffer. Labeling was achieved by incubation in 0.5 mM *N*-[^{14}C]ethylmaleimide alone or with added 0.08 mM phlorizin, 0.45 M D-glucose, phlorizin and D-glucose, or with 0.01 mM phloretin at 37 °C for 30 min. *N*-[^{14}C]Ethylmaleimide solutions contained 2.7 μM [^{3}H]mannose of adequate activity to permit calculation of the dead-space between brush border particles after centrifugation and of its *N*-[^{14}C]ethylmaleimide content. After centrifugation at 13000 rev./min for 10 min, the supernatant was discarded and the sediment washed twice in sucrose–triethanolamine–HCl–EDTA–dithiothreitol buffer (0.25 M sucrose, 0.01 M, triethanolamine–HCl, 5 mM EDTA, 2 mM dithiothreitol, of pH 7.6 at 20 °C) alone or with added phlorizin, D-glucose, or phloretin depending on the preceding incubation. Final sediment and aliquots of the last supernatant were transferred to counting vials for liquid scintillation counting[3].

(3) *Phlorizin binding studies*

[^{3}H]Phlorizin binding studies were done in a previously described fashion[3] in order to determine the number of specific phlorizin binding sites (n_{phl}) and affinity constants (K_{phl}) of the pre-incubated and washed membrane fraction. The incubation media had the following phlorizin concentrations: 0.1, 0.5, 1.0 or 10 μM. 0.4 M D-glucose was used for complete inhibition of the high-affinity phlorizin-specific binding site.

(4) *Materials*

N-[^{14}C]Ethylmaleimide (spec. act. 5.95 Ci/mole), D-[^{3}H]mannose (spec. act. 562.5 Ci/mole) and D-[^{14}C]mannose (spec. act. 48.8 Ci/mole) were obtained from NEN, Boston, Mass. [^{3}H]Phlorizin was prepared by Farbwerke Hoechst, Frankfurt, Germany, with a spec. act. of 59 Ci/mole. All chemicals were of analytical grade and purchased commercially.

(5) *Calculations*

Radioactively labeled D-mannose served in all experiments as marker substance of deadspace between membrane particles of the sediment and its content of *N*-[^{14}C]ethylmaleimide or [^{3}H]phlorizin. Previous studies had shown no appreciable binding of mannose to brush border membrane[3]. It is assumed that supernatant and deadspace have a constant concentration of mannose and *N*-ethylmaleimide or phlorizin, respectively. Thereby, total sediment activity of *N*-[^{14}C]ethylmaleimide or [^{3}H]phlorizin can be corrected for that present in the deadspace and the amount is obtained which is truly membrane bound.

RESULTS

(1) *N-Ethylmaleimide binding to phlorizin-sensitive receptors*

Pre-treatment of brush border with *N*-ethylmaleimide in low concentration reduces subsequent phlorizin binding by 60 %. If, however, brush border is incubated with both *N*-ethylmaleimide and phlorizin simultaneously (Column C, Table I), no reduction of phlorizin binding is observed. This preliminary experiment established that *N*-ethylmaleimide in low concentration (0.5 mM) will not appreciably interfere with simultaneous phlorizin binding of the brush border membrane.

An indirect measure of *N*-ethylmaleimide-sensitive groups of the phlorizin receptor should therefore be derived from the difference in *N*-[^{14}C]ethylmaleimide activity of a membrane previously incubated with 0.5 mM *N*-[^{14}C]ethylmaleimide alone and of a membrane sample incubated with 0.5 mM *N*-[^{14}C]ethylmaleimide and 0.08 mM phlorizin (corresponding to Columns B and C, Table I). Two experiments of this type showed, however, that *N*-[^{14}C]ethylmaleimide bound to the phlorizin receptor accounted for only 2 % of the total ^{14}C activity of the sediment. Thus, statistically significant results would have been difficult to obtain.

In order to reduce the prohibitively high "*N*-ethylmaleimide background", brush border was treated first with unlabeled *N*-ethylmaleimide in the presence of phlorizin for 70 min at 28 °C, then washed and finally incubated with *N*-[^{14}C]-ethylmaleimide alone or in combination with phlorizin. This manoeuver increased the *N*-[^{14}C]ethylmaleimide activity attributed to the phlorizin receptor to 9 %

TABLE I

EFFECT OF *N*-ETHYLMALEIMIDE (NEM) ON [^{3}H]PHLORIZIN BINDING TO ISOLATED BRUSH BORDERS

The number of binding sites (n_{phl}) and the dissociation constant of the phlorizin receptor complex (K_{phl}) were determined in a binding study as described under Materials and Methods 3 n is expressed in 10^{-10} moles/mg brush border protein, K_{phl} in 10^{-6} M

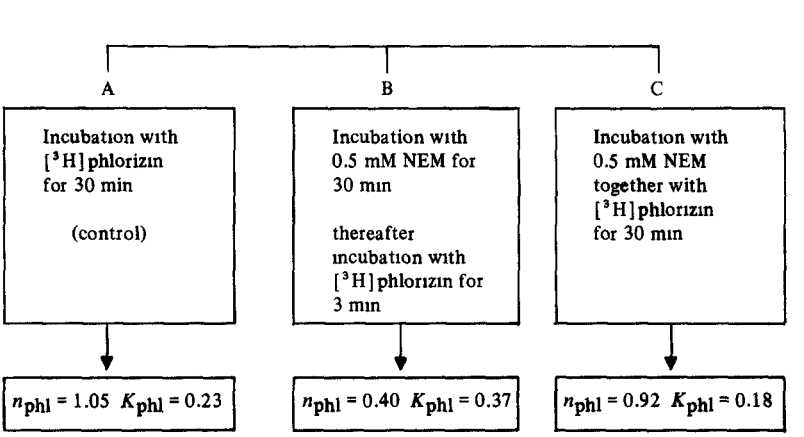
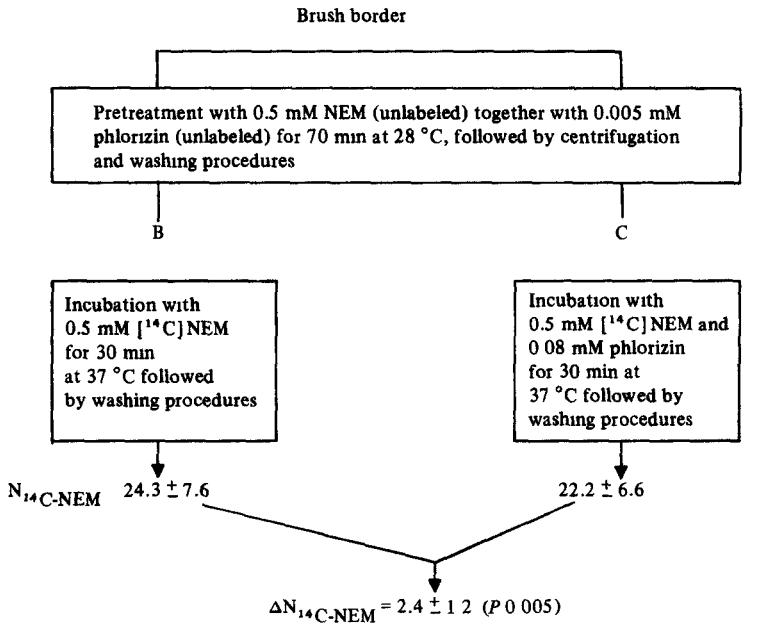


TABLE II

DETERMINATION OF *N*-[^{14}C]ETHYLMALEIMIDE BINDING ($N_{14C-NEM}$) TO PHLORIZIN-SENSITIVE SITES OF ISOLATED BRUSH BORDER

$N_{14C-NEM}$ is given in 10^{-10} moles/mg brush border protein The mean values of 24 experiments are given with the standard deviations $\Delta N_{14C-NEM}$ and P are derived from paired data NEM, *N*-ethylmaleimide.



of the entire ^{14}C activity or $2.4 \cdot 10^{-10}$ moles/mg of brush border protein (Table II).

In five experiments, membrane was prepared according to the scheme in Table II, but using only unlabeled *N*-ethylmaleimide. Instead of counting ^{14}C activity at the end of the experiment, the sediments were used for [^{3}H]phlorizin binding studies to obtain the actual number of phlorizin binding sites (n_{phl}) and their dissociation constant (K_{phl}) under these experimental conditions (Table III). Incubation with *N*-ethylmaleimide alone did not eliminate all phlorizin binding sites (residual of $0.62 \pm 0.19 \cdot 10^{-10}$ moles/mg protein) while simultaneous incubation with *N*-ethylmaleimide and phlorizin and subsequent washing yielded $1.44 \pm 0.14 \cdot 10^{-10}$ moles/mg protein. The difference of $0.82 \cdot 10^{-10} \pm$ S.D. moles/mg protein ($P < 0.05$ paired data) represents the number of binding sites protected by the substrate (*i.e.* phlorizin) against *N*-ethylmaleimide during the incubation procedure. There was no recognizable difference of the dissociation constants of the receptor–substrate complex.

TABLE III

[^{3}H]PHLORIZIN BINDING TO ISOLATED BRUSH BORDERS TREATED AS SHOWN IN TABLE II BUT WITH UNLABELED *N*-ETHYLMALEIMIDE AT INCUBATION

The number of binding sites (n_{phl}) and the dissociation constant of the phlorizin receptor complex (K_{phl}) were determined in binding studies as described under Materials and Methods 3. n_{phl} is expressed as 10^{-10} moles/mg protein, K_{phl} in 10^{-6} M. Means and standard deviations are given for 5 experiments.

Treatment according to:	K_{phl}	n_{phl}
Table II, Column B	0.52 ± 0.12	0.62 ± 0.19
Table II, Column C	0.59 ± 0.26	1.44 ± 0.14

(2) *Protection of the phlorizin binding site by various substrates*

N-Ethylmaleimide reactive groups of the phlorizin receptor of brush border membrane could have been protected by the glucose moiety of the glycoside, its aglucon moiety (*i.e.* phloretin) or both. Simultaneous incubation of pre-treated membrane with *N*-[^{14}C]ethylmaleimide and phloretin resulted in enhancement of *N*-ethylmaleimide binding by the membrane ($25 \cdot 10^{-10}$ moles/mg protein). The data showed a wide scatter and were equivocal at statistical evaluation. In contrast, D-glucose protected more *N*-ethylmaleimide sites of the membrane than phlorizin ($12.6 \cdot 10^{-10}$ moles/mg protein) as shown in Table IV. Phlorizin had no additive effect on the D-glucose protection of *N*-ethylmaleimide binding sites.

TABLE IV

AMOUNT OF *N*-[^{14}C]ETHYLMALEIMIDE ($\Delta N_{^{14}C\text{-NEM}}$) BOUND TO PHLORIZIN- AND TO D-GLUCOSE-SENSITIVE *N*-[^{14}C]ETHYLMALEIMIDE BINDING SITES OF ISOLATED BRUSH BORDER

Phlorizin was used in a concentration of 0.08 mM, D-glucose in a concentration of 0.45 M. $\Delta N_{^{14}C\text{-NEM}}$ was determined as described in Table II. The mean values of n experiments are given with standard deviations.

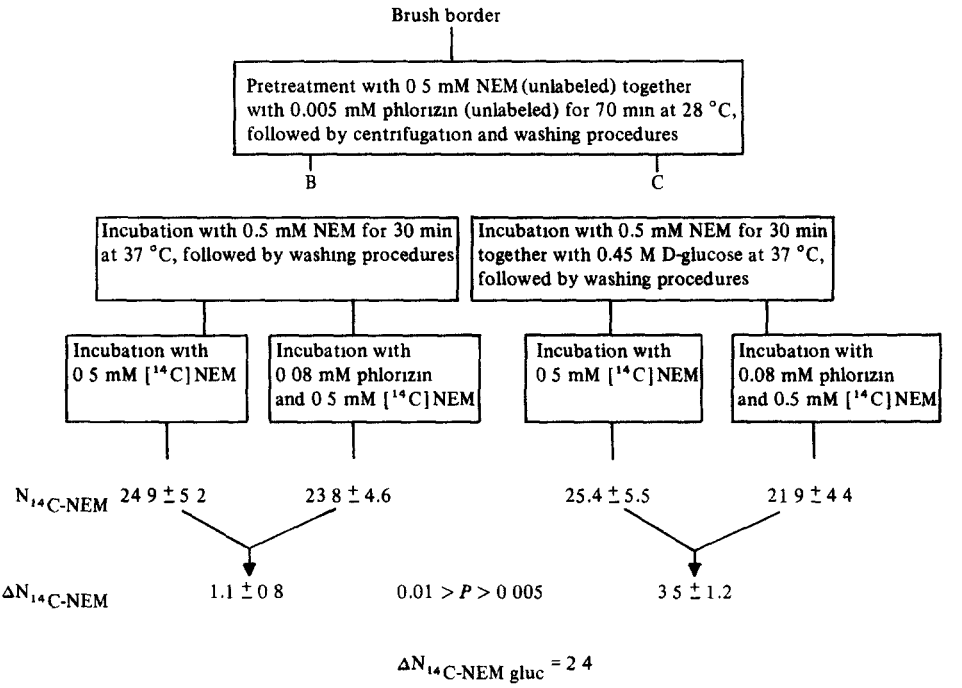
Substance present in the incubation mixture	$\Delta N_{^{14}C\text{-NEM}}$ (10^{-10} *moles/mg protein*)	n
Phlorizin	2.4 ± 1.2	24
D-Glucose	12.6 ± 5.0	6
D-Glucose and phlorizin	12.2 ± 3.5	6

Another experimental approach was taken to ascertain that *N*-ethylmaleimide binding sites protected by glucose included those which are sensitive to phlorizin. Pretreated membrane was incubated first with *N*-ethylmaleimide alone or with *N*-ethylmaleimide and glucose (0.45 M). After washing, *N*-[^{14}C]ethylmaleimide binding was examined in presence or absence of phlorizin (Table V). Incubation with *N*-ethylmaleimide alone left a residual of $1.1 \cdot 10^{-10}$ moles/mg of phlorizin-sensitive *N*-ethylmaleimide binding sites while prior glucose protection yielded a significantly higher number of phlorizin-sensitive sites, *i.e.* $3.5 \cdot 10^{-10}$ moles/mg. The difference of $2.4 \cdot 10^{-10}$ moles *N*-ethylmaleimide/mg represents those phlorizin-sensitive *N*-ethylmaleimide site which had been protected by D-glucose. It correponds well with the number of sites obtained by a different method (Table II).

TABLE V

PROTECTION OF PHLORIZIN-SENSITIVE *N*-[^{14}C]ETHYLMALEIMIDE BINDING SITES BY D-GLUCOSE ($\Delta N_{^{14}C\text{-NEM gluc}}$)

$N_{^{14}C\text{-NEM}}$ is expressed as 10^{-10} moles *N*-[^{14}C]ethylmaleimide per mg protein. The mean values of 5 experiments are given with the standard deviations. $\Delta N_{^{14}C\text{-NEM}}$ and *P* are derived from paired data. NEM, N-ethylmaleimide



DISCUSSION

The results show that *N*-ethylmaleimide in low concentration (0.5 mM) inhibits two thirds of the glucose-sensitive phlorizin receptors of renal tubular brush border (Table III), or in absolute numbers $0.82 \cdot 10^{-10}$ moles/mg brush border protein. Under our experimental conditions, *N*-ethylmaleimide is known to react mainly

with sulfhydryl groups of amino acids located at the surface of polypeptide chains[12–14]. Since PCMB inhibits the glucose-sensitive phlorizin binding of brush border membrane as effectively as *N*-ethylmaleimide[7], one may conclude that inhibition of phlorizin binding is due to blockade of certain sulfhydryl groups at the membrane.

Reaction of *N*-ethylmaleimide with sulfhydryl groups of the brush border membrane decreases the number of binding sites without influencing the affinity between receptor and phlorizin molecule (Table III). This blockade can be prevented by the presence of phlorizin or D-glucose during incubation. Results of experiments with *N*-[^{14}C]ethylmaleimide binding (Table II) and of [^{3}H]phlorizin binding studies (Table III) showed, that $2.4 \cdot 10^{-10}$ moles of *N*-ethylmaleimide prevented $0.82 \cdot 10^{-10}$ moles of phlorizin to attach to brush border equivalent to 1 mg of protein. The question whether each phlorizin molecule protects three sulfhydryl groups or whether the 3:1 ratio is coincidental representing the mean of a number of different combinations, will remain unresolved though the absence of a sizeable effect of *N*-ethylmaleimide on the phlorizin-receptor affinity (Table I, Column A and C) seems to favor the former alternative.

Now the question arises to which part of the binding protein the *N*-ethylmaleimide molecules are attached. The substrate protection effect could indicate that the functional sulfhydryl groups belong to the active center of the binding protein. In favor of this assumption will be the finding of Silverman *et al.*[15] who could show that for active hexose transport through the brush border membrane of the kidney four hydroxyl groups of the pyranose are important, two being essential. One could speculate that these two hydroxyl groups may interact with the sulfhydryl groups by hydrogen bonding in the active center.

On the other hand, it has to be considered that the labeling studies presented here were performed on a molecule incorporated into a membrane system which is thought to undergo transformational changes during its function as a carrier. In microperfusion studies the application of D-glucose to the outside of the cell apparently changes the affinity of the transport molecules towards L-glucose inside the cell[16]. The same effect could be observed in binding studies where L-glucose enhanced the affinity of isolated brush border membranes to phlorizin which only binds to the outside of the membranes[3]. Probably the presence of L-glucose inside the microvilli has a profound effect on the molecular structure of the binding site facing the outside of the membranes. Also Caspary *et al.*[17] provided evidence in transport studies in the gut that binding of hexose to the brush border is followed by a transformational change of the carrier prior to the translocation of the sugar molecule. If these considerations are applied to the studies presented here the change of conformation could alter the reactivity of sulfhydryl groups of the binding protein. These groups then should be located within the membrane and should not be freely accessible from the surface, an assumption which could explain the failure to demonstrate sulfhydryl groups in the brush border membranes which can react with high molecular Hg compounds like Hg–phenylferritin[18]. The exact determination of the location and function of the sulfhydryl groups requires, however, an isolation of the protein in an active form. D-Glucose protects more *N*-ethylmaleimide binding sites than phlorizin, part of them are identical with the phlorizin binding site. The sulfhydryl groups protected in excess could be part of other glucose binding proteins, like the disaccharidase maltase, or D-glucose may also protect, due to

its penetration into the microvillus, the glucose transport protein at the inside of the membrane.

Two experimental approaches have been employed to specifically label certain molecules of the transmembranous transport system of sugars. One method uses the protective effect of substrate on its "carrier" while saturating non-specific receptors with the irreversible inhibitor in order to label the carrier later with radioactive inhibitor[6,9]. This method was used here. The other approach is taken mainly for bacterial systems where induced and non-induced populations show different rates of synthesis of a certain transport system[19]. Tritiated and ^{14}C-labeled amino acids can be incorporated into these systems and serve as marker substances. Both methods have shortcomings, the latter, for instance, yields different results when a tritiated amino acid is taken instead of a ^{14}C-labeled or *vice versa* under otherwise similar experimental conditions. The reason is thought to be the difference in location of the label resulting in "crossing over" of different portions of the molecule during metabolism of the transport protein[19].

The substrate protection method faces different problems. From a great number of sulfhydryl groups of the membrane only very few may be part of the prospective receptor site. Fox and Kennedy[8] could relate about 60% of all labeled groups to the transport sites in bacterial membrane. In this study, only 2% of all surface sulfhydryl groups were associated with the hypothetical D-glucose carrier and a special pre-loading step had to be used in order to reduce non-specific *N*-ethylmaleimide binding sites and increase the percentage of carrier related sulfhydryl groups to 9% of the total The difference between both membrane systems reflects the relative sparsity of glucose transport sites of the renal brush border as compared to the bacterial wall

Attempts to increase the efficiency of labeling by changes of incubation temperature, duration of incubation or variation of phlorizin and *N*-ethylmaleimide concentration failed. Phlorizin concentrations in excess of $8 \cdot 10^{-5}$ moles had to be avoided since previous binding studies had shown the presence of phlorizin binding sites of great capacity but low affinity (K_m $3 \cdot 10^{-4}$ M) at the brush border surface which could not be inhibited by D-glucose[7]. This binding site was not thought to be part of the glucose transport system. Increase of the *N*-ethylmaleimide concentration of the incubation medium by a factor of 10 resulted in loss of the protective effect of phlorizin on its binding sites. Reduction of non-specific sulfhydryl groups by further fractionation of the brush border prior to substrate protection is currently being investigated. In future, affinity labeling with pseudo-substrates will be considered[20].

This study has added new information on the inhibitory nature of phlorizin as a glycoside. Its aglucon phloretin is known to inhibit renal glucose transport in the cat only one tenth as effectively as phlorizin in similar concentration[21]. Thus, the interaction of phloretin and glucose carrier of renal brush border is rather weak. *N*-Ethylmaleimide binding of the isolated brush border was actually enhanced by phloretin (10^{-5} M) as compared to the unequivocal reduction caused by phlorizin. This may be explained by an unspecific unfolding of membrane proteins due to the polyphenolic nature of phloretin resulting in an increased number of accessible sulfhydryl groups. On account of the potentially unspecific effect of phloretin on *N*-ethylmaleimide binding, no unequivocal answer can be given

as to which portion of the phlorizin molecule provides the sulfhydryl group protection. The experimental evidence (Tables IV and V) suggests strongly that the protective group is the glucose moiety of the glycoside. Glucose and phlorizin had no additive effect on sulfhydryl group blockade (Table IV) and the number of sulfhydryl groups protected by phlorizin was not altered when the membrane was pre-incubated with *N*-ethylmaleimide and glucose (Table V). Therefore, we conclude that we successfully labeled those sulfhydryl groups which are part of a phlorizin-sensitive receptor of brush border membrane for D-glucose. Previous studies suggested that this receptor is part of the transmembrane transport system of glucose[3].

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