*Biochimica et Biophysica Acta*, 692 (1982) 210–217
Elsevier Biomedical Press

BBA 71363

# RENAL SODIUM-D-GLUCOSE COTRANSPORT SYSTEM

# INVOLVEMENT OF TYROSINE RESIDUES IN SODIUM-TRANSPORTER INTERACTION

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(Received June 10th, 1982)

*Key words: $Na^+$/glucose cotransport, $Na^+$-binding site; Brush border membrane; Tyrosine residue; (Calf kidney)*

**Using brush-border membrane vesicles isolated from calf kidney cortex the effect of tyrosine-reactive reagents on sodium-dependent D-glucose transport was investigated. Treatment of the membranes for 60 min with NBD-Cl (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole), *N*-acetylimidazole or tetranitromethane decreased D-glucose uptake 50, 70 and 40%, respectively. Tracer exchange experiments revealed that the inhibition of transport is due to a direct modification of the sodium-D-glucose cotransport system. The modification by NBD-Cl decreases the apparent $V_{max}$ of the transport system with respect to its interaction with sodium. In addition, the rate of inactivation of the transport system by NBD-Cl is reduced in the presence of high concentrations of sodium. The results indicate that tyrosine residues play an essential role in sodium-D-glucose cotransport and are probably involved in the binding and/or transport of sodium by the sodium-D-glucose cotransport system.**

## Introduction

Studies employing group specific reagents that interact reversibly or irreversibly with SH-groups of the brush-border membrane have shown that sodium-dependent D-glucose transport as well as phlorizin binding to the sodium-D-glucose cotransport system are markedly inhibited by a modification of SH-groups [1–3]. Substrate protection experiments indicated furthermore that the highly reactive SH-groups are located close to or are part of the sugar-binding site, since the inhibition of phlorizin binding by *N*-ethylmaleimide was partly prevented by the presence of high concentrations of D-glucose during the modification period [4,5].

In recent years evidence has been accumulated that sodium transport systems such as the sodium channel in the squid axon [6], the amiloride-sensitive sodium channel in toad bladder [7], and the $(Na^+ + K^+)$-ATPase [8,9] contain tyrosyl groups that appear to be essential for the interaction of the cation with its binding site. Since sodium-D-glucose cotransport involves an interaction of the transport system with both sugar and sodium it was of interest to determine whether tyrosine residues are essential for the function of this transport system, too. In the following it will be demonstrated that tyrosine-reactive reagents inhibit sodium-D-glucose cotransport and that tyrosyl groups play an important role in the binding and or transport of sodium by the transport system.

Part of the results has been published in abstract form at the 32nd Annual Fall Meeting of the American Physiological Society, No. 198, October, 1981, Cincinnati, U.S.A., and at the 66th Annual Meeting of the Federation of American Societies for Experimental Biology, No. 12054, April, 1982, New Orleans, U.S.A.

## Materials and Methods

Dithiothreitol, NBD-Cl, *N*-acetylimidazole and tetranitromethane were purchased from Sigma Chemical Company, U.S.A. D-[$^3$H]Glucose (spec. act. 15 Ci/mmol), $^{22}$Na (carrier free) and scintillation fluid Formula 963 for $\beta$-counting were obtained from New England Nuclear, U.S.A. Other chemicals were of the highest purity commercially available. Sorvall ARC-1 (Du Pont) was used for high-speed centrifugation and Sorvall RC-5B for low-speed centrifugation.

*Preparation of brush border membranes from calf kidney cortex*

Calf kidney was obtained from a private slaughter house. The kidneys were essentially free of blood, thus, a perfusion [10] was not necessary. Slices of kidney cortex were prepared and stored at −70°C before use. To prepare brush-border membrane vesicles, a modified $CaCl_2$-precipitation method described previously was used [11]. The recovery of the membrane protein was on the average 2.45% of the cortex homogenate protein. The enrichments of marker enzymes [12] were 11–12-fold for alkaline phosphatase with about 28% recovery and 14-fold for aminopeptidase M with about 30% recovery. These results are similar to those reported for hog kidney brush-border membranes [10]. The specific enzyme activities in the final membrane fraction were 430 mU/mg protein for alkaline phosphatase and 1000 mU/mg protein for aminopeptidase M. After isolation, the membrane vesicles were suspended in 20 mM Hepes-Tris (pH 7.4)/200 mM mannitol (vesicle buffer) and stored at −70°C in small portions (1–2 ml). No loss in transport activity was found after 3 months storage at −70°C.

*Transport studies*

The uptake of D-glucose or sodium by membrane vesicles and vesicles treated with tyrosine-reactive reagents was determined by using the rapid filtration technique described previously [13]. 0.45 $\mu$m pore size Millipore filter and 600 mmHg vacuum were used during the filtration experiments. The experiments were performed at 25°C in duplicate or triplicate. Mainly, two different transport conditions were employed:

*(a) Net transport.* 20 $\mu$l of membrane vesicles (100–200 $\mu$g membrane protein) suspended in vesicle buffer were added to 100 $\mu$l transport medium which contained 100 mM NaSCN or KSCN, 100 mM mannitol, 20 mM Hepes-Tris (pH 7.4) and radioactively labeled D-glucose with a final concentration of 0.1 mM. After 15 s, 1 min, 2 min, 90 min D-glucose uptake was terminated by a rapid filtration technique. The filters were transferred into liquid scintillation fluid and counted by liquid scintillation spectrometry. The values obtained were corrected for the amount of radioactivity remaining on the filter in the absence of membrane vesicles in the incubation medium. Using the amount of counts present in a defined volume of the incubation medium, the specific activity of the substrate was calculated. With the aid of the specific activity the counts obtained were transformed into pmol substrate taken up per mg membrane protein.

*(b) Tracer-exchange.* The membrane suspension (5 mg protein/ml) was preloaded with the same medium as described for the net transport experiments but without radioactive D-glucose. To start the experiment a trace of radioactive D-glucose was added and after different incubation times the tracer-exchange rate of D-glucose was determined by using the same filtration technique described in (a). These experimental conditions were used to evaluate the kinetic constants of the sodium-D-glucose cotransport system [18]. In the time periods investigated the rate of tracer influx was constant.

*Preincubation of membrane vesicles with tyrosine-reactive reagents*

Except for special conditions described in the text or legends to the figures, the following conditions were used. To the brush-border membrane appropriate amounts of NBD-Cl, *N*-acetylimidazole or tetranitromethane (dissolved in ethanol), as indicated in the legend to the figures, were added. The final protein concentration was adjusted to 4–5 mg/ml with vesicle buffer. The preincubation was allowed to proceed at 25°C for 60 min, with gentle shaking. In order to remove the free reagents after incubation, the treated membranes were diluted with an excess of cold (4°C) reagent-free buffer followed by centrifugation, or by Sephadex G-100 filtration.

*Protein determination*

The protein in the samples was precipitated with 10% trichloroacetic acid and determined according to the method described by Lowry et al. [14].

## Results

*Effect of tyrosine reactive reagents on sodium-D-glucose cotransport*

When renal brush border membranes are incubated for 60 min with the tyrosine-reactive reagents *N*-acetylimidazole, NBD-Cl or tetranitromethane the results shown in Fig. 1A and B are obtained. In the presence of a sodium thiocyanate gradient D-glucose uptake into control membranes (incubated for the same time period but without the reagents) shows a typical overshoot, the uptake after 1 min exceeding the equilibrium uptake about 8-fold. All three compounds decrease the overshoot, D-glucose uptake after 20 s is inhibited 50 ± 1.5% with 5 mM *N*-acetylimidazole, 60 ± 6% with 0.17 mM NBD-Cl (see Table I) and 40% with 0.8 mM tetranitromethane . In the latter case, the pretreatment was performed at pH 8.0 instead of pH 7.4; this explains why the sugar uptake of the control membranes shown in Fig. 1 is different. Incubation of the membranes with *N*-acetylimidazole, NBD-Cl or tetranitromethane does, however, not affect the amount of D-glucose taken up at equilibrium, indicating that the chemical modification does not alter vesicle size or the degree of vesiculation of the membranes. Similarly, sodium-dependent alanine uptake by the brush-border membranes was only slightly effected by treatment with 0.15 mM NBD-Cl.

The inhibition of sodium-D-glucose transport increases when the concentrations of *N*-acetylimidazole, NBD-Cl or tetranitromethane in the incubation medium are increased. For an incubation time of 60 min an apparent $K_i$ of 58 $\mu$M was obtained for NBD-Cl, of 20 $\mu$M for tetranitromethane and of 13.5 mM for *N*-acetylimidazole. A representative Dixon-plot is shown in Fig. 2

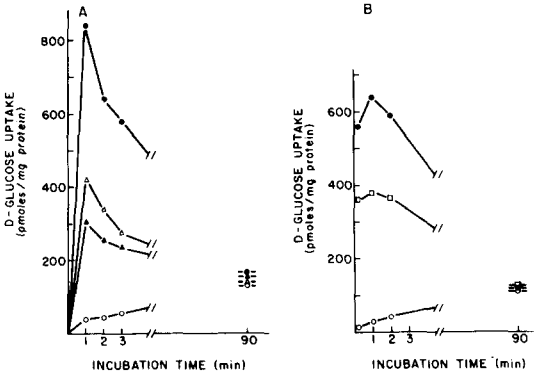


Fig. 1. A. Effect of NBD-Cl and *N*-acetylimidazole on D-glucose uptake by renal brush border membranes. Membranes were pre-incubated for 60 min at pH 7.4 and 25°C with 0.17 mM NBD-Cl or 5 mM *N*-acetylimidazole, respectively. D-Glucose uptake was determined under gradient conditions: ●——●, control membrane in 100 mM NaSCN; ▲——▲, NBD-Cl-treated membrane in 100 mM NaSCN; △——△, *N*-acetylimidazole-treated membrane in 100 mM NaSCN; ○——○, control membrane in 100 mM KSCN. B. Effect of tetranitromethane on D-glucose uptake by renal brush-border membranes. The membranes were preincubated for 60 min at pH 8 and 25°C with 0.8 mM tetranitromethane. D-Glucose uptake was determined under gradient conditions: ●——●, control in NaSCN; □——□, tetranitromethane-treated membrane in presence of NaSCN; ○——○, control membrane in 100 mM KSCN. For A and B, one representative experiment is shown.

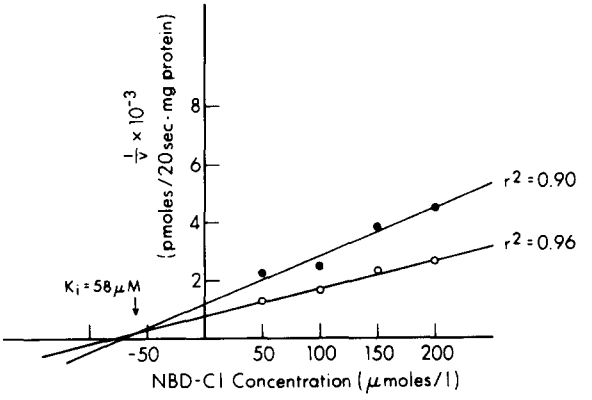


Fig. 2. Inhibition of sodium-dependent D-glucose uptake by various concentrations of NBD-Cl. Membrane vesicles were incubated with NBD-Cl concentrations from 50 $\mu$M to 200 $\mu$M for 60 min at pH 7.4. After incubation the vesicles were washed with NBD-Cl free buffer. D-Glucose uptake after 20 s of incubation was measured at 0.1 mM (●——●) and 0.2 mM (○——○) D-glucose in the presence of a NaSCN or KSCN gradient. The difference in uptake is defined as sodium-dependent D-glucose uptake. Mean values from four experiments are given, the standard deviation was about 10%. Regression lines were calculated using the least-squares method.

TABLE I

REVERSIBILITY OF D-GLUCOSE TRANSPORT INHIBITION BY NBD-Cl AND *N*-ACETYLIMIDAZOLE

Calf brush-border membrane vesicles were treated with 0.15 mM (in the case of dithiothreitol (DTT) treatment) or 0.2 mM (in the case of dilution-maneuver) NBD-Cl or 5 mM *N*-acetylimidazole as described in Fig. 1. After incubation, vesicles were treated with 1 mM DTT at 25°C for 10 min or diluted 10-fold with reagent-free vesicle buffer and incubated at 4°C for 15 min, respectively. The vesicles were spun down at 100000× *g* for 1 h and resuspended in vesicle buffer. D-Glucose uptake at 20 s in the presence of a sodium gradient was determined as described in Materials and Methods. The uptake by membrane vesicles incubated under the same conditions but in tyrosine-reactive reagent-free buffer is given as 100% (corresponding to 700 pmol/mg protein). Mean values of data derived from three experiments are given with the standard deviation.

| Membranes | Effect of 1 mM DTT (10 min at 25°C) (%) | | Effect of dilution (15 min at 4°C) (%) | |
|---|---|---|---|---|
| | −DTT | +DTT | before dilution | after dilution |
| Control | 100 | 95±3 | 100 | 100 |
| NBD-Cl-treated | 40±4.6 | 52±1.8 | 28±1.2 | 25±0.5 |
| *N*-Acetylimidazole-treated | - | - | 50±1.5 | 70±3 |

for the concentration dependence of the NBD-Cl inhibition. The plot of the data obtained for the *N*-acetylimidazole inhibition showed similarly an intercept of the two straight lines above the abscissa, the values for the tetranitromethane inhibition (data not shown) yielded an intercept on the abscissa. In Table I are shown results of experiments in which the reversibility of the inhibition of sodium-dependent D-glucose uptake was investigated. Dilution of the membranes after modification by NBD-Cl into buffer alone or buffer containing 1 mM dithiothreitol does not reverse the inhibition. In *N*-acetylimidazole-treated membranes, however, the inhibition of the transport is

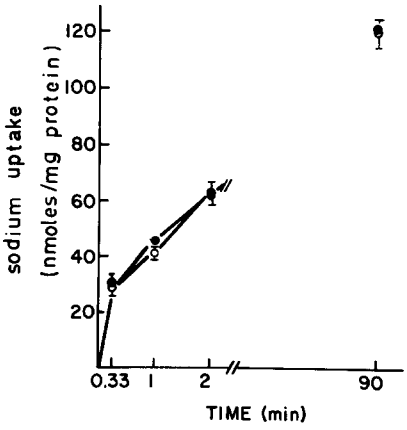


Fig. 3. Lack of effect of NBD-Cl on D-glucose-independent sodium uptake by brush-border vesicles. Membrane vesicles were modified with 0.15 mM NBD-Cl and washed. Sodium uptake was determined in the presence of mannitol buffer and a 100 mM NaSCN gradient. ○——○, NBD-Cl modified vesicles; ●——●, control vesicles without NBD-Cl treatment. Mean values± S.D. compiled from four experiments are given.

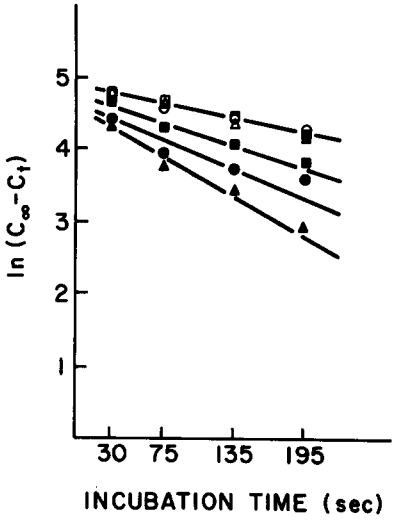


Fig. 4. Effect of NBD-Cl on D-glucose transport in tracer-exchange experiments. Vesicles were modified with 0.15 or 0.075 mM NBD-Cl, washed, and loaded with 0.1 mM D-glucose, 100 mM NaSCN or 100 mM KSCN. Uptake was started by addition of a small amount of D-[$^3$H]glucose to the incubation medium. Control membrane in NaSCN (▲——▲) or KSCN (△——△), membrane treated with 0.075 mM NBD-Cl and uptake in NaSCN (●——●) or KSCN (○——○), membrane treated with 0.15 mM NBD-Cl and uptake in NaSCN (■——■) or KSCN (□——□).

significantly lower after dilution of the membranes. These data suggest that the incubation of the membranes with NBD-Cl leads predominantly to a modification of tyrosine residues, SH residues would be reactivated by dithiothreitol. Incubation with *N*-acetylimidazole, however, modifies both tyrosyl and SH-groups, the latter are reactivated by dilution of the membranes.

The transport experiments presented above have been performed in the presence of a sodium thiocyanate and a glucose gradient across the brush-border membrane. Under these circumstances it cannot be distinguished whether the inhibition of sodium-dependent D-glucose transport observed after chemical modification of the membranes is due to a direct modification of the sodium-D-glucose cotransport system or due to a change in membrane permeability or conductivity. In the latter case, an increase in ion permeability could lead to a faster dissipation of the sodium thiocyanate gradient, this would result in an inhibition of D-glucose uptake owing to reduced driving forces. Therefore the effect of NBD-Cl on sodium uptake by the brush-border membrane vesicles was investigated. As shown in Fig. 3 treatment of brush border membranes with NBD-Cl did not alter the rate of sodium uptake, suggesting that the sodium-D-glucose cotransport system was modified directly. This assumption was supported in tracer exchange experiments where no gradients for sodium, thiocyanate and D-glucose exist across the membrane. Fig. 4 compiles the effect of various concentrations of NBD-Cl on the exchange rate of D-glucose in the presence of sodium and in the presence of potassium. It is evident that D-glucose exchange in the presence of sodium is decreased with increasing concentrations of NBD-Cl, whereas D-glucose exchange in the presence of potassium is not affected by NBD-Cl. These results indicate that NBD-Cl interacts directly with the sodium-D-glucose cotransport system.

*Evidence for modification of the sodium-binding site*

As mentioned above, tyrosine residues have been found to be involved in the interaction of sodium with a variety of transport systems. The inhibition of sodium-dependent D-glucose transport by NBD-Cl raised the possibility that also in this transport system the sodium binding site might

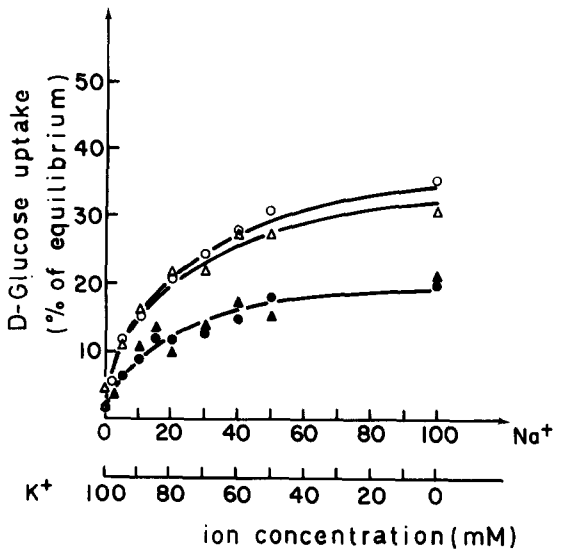


Fig. 5. Effect of NBD-Cl on the sodium dependence of D-glucose transport. Membranes were modified with 0.15 mM NBD-Cl, washed, and sodium-dependent D-glucose uptake was measured under tracer-exchange conditions. Tracer uptake by control membrane (○———○) and NBD-Cl-treated membrane (●———●) after 20 s of incubation; control membrane (△———△) and NBD-Cl treated membrane (▲———▲) after 1 min incubation. Mean values of three experiments are given. The standard deviation was about 10%.

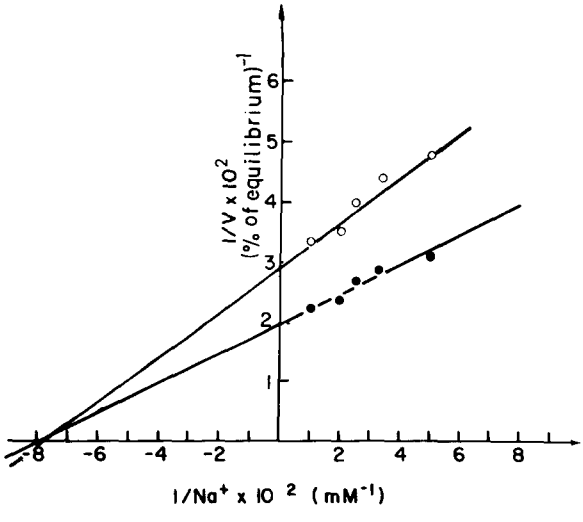


Fig. 6. Lineweaver-Burk plot of the data given in Fig. 5 on sodium dependence of D-glucose transport. (●———●), control membrane, $R^2$ of linear regression is 0.98; (○———○) NBD-Cl treated membrane, $R^2$ of linear regression is 0.90.

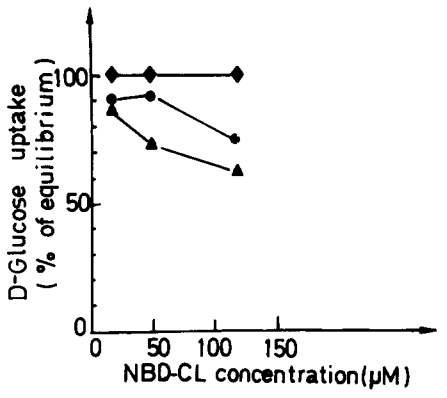


Fig. 7. Effect of sodium on the time course of inactivation of sodium-D-glucose cotransport system by NBD-Cl. Membrane vesicles were preincubated with 50 μM NBD-Cl in the presence of 500 mM NaSCN or 500 mM KSCN for 15 min. After intensive washing, D-glucose uptake was determined in the presence of 100 mM NaSCN gradient. As control, membrane vesicles were incubated under identical condition, i.e. 500 mM NaSCN, but without NBD-Cl (◆——◆). Membrane with NBD-Cl and 500 mM NaSCN (●——●); membrane with NBD-Cl and 500 mM KSCN (▲——▲). Points represent mean values of four experiments.

be modified by NBD-Cl. In order to test this hypothesis, the effect of NBD-Cl on the kinetic properties of the sodium-D-glucose cotransport system was investigated under tracer exchange conditions. As shown in Fig. 5, sodium-D-glucose cotransport is saturated with sodium at about 40 mM, the apparent $K_m$ is 14 mM. In NBD-Cl-treated membranes an identical $K_m$ is obtained; the maximal velocity is, however, decreased. These results were independent of the incubation time after which the amount of tracer exchanged was determined; this indicates that the rate of tracer exchange is affected in a similar manner. The Lineweaver-Burk plot (Fig. 6) reveals that NBD-Cl inhibits sodium-D-glucose cotransport in a non-competitive manner. One plausible explanation for such inhibition is that NBD-Cl reacts with tyrosine residues which form part of the sodium-binding site of the transport molecule. If this assumption were correct, one would expect that a high sodium concentration, at which the sodium site is saturated, should interfere with the interaction of

TABLE II

EFFECT OF D-GLUCOSE AND SODIUM ON MEMBRANE MODIFICATION BY NBD-Cl

Membrane vesicles were incubated with 50 μM or 120 μM NBD-Cl in the presence or absence of D-glucose (100 mM D-glucose or mannitol, 50 mM NaSCN, 20 mM Hepes-Tris, pH 7.4) or in the presence or absence of sodium (500 mM NaSCN or KSCN, 20 mM Hepes-Tris, pH 7.4), respectively, at 25°C for 15 min. After incubation, D-glucose or sodium was removed by gel-filtration with Sephadex G-100 followed by centrifugation of the filtrate at 100000× *g* for 1 h. The pellets were suspended in vesicle buffer. D-Glucose uptake was determined under sodium gradient conditions as described in Material and Methods. Mean values of three determinations are given and represent % of uptake by control membrane vesicles (no NBD-Cl) at 1 min. Values in brackets indicate the ratio of D-glucose uptake at 1 min to the uptake at equilibrium.

| | NBD-Cl reaction in D-glucose (100 mM) | | | NBD-Cl reaction in sodium or potassium (500 mM) | | |
|---|---|---|---|---|---|---|
| NBD-Cl (μM): | 0 | 50 | 120 | 0 | 50 | 120 |
| D-Glucose present | 100 (4.72) | 61.8±0.8 (2.92) | 44.7±1.5 (2.11) | – | – | – |
| D-Glucose absent | 100 (4.09) | 61.6±1.7 (2.52) | 44.3±4.5 (1.65) | – | – | – |
| NaSCN | – | – | – | 100 (3.08) | 93±0.6 (2.87) | 66.8±2.1 (2.06) |
| KSCN | – | – | – | 100 (3.06) | 72±2.6 (2.21) | 54.9±2.6 (1.68) |

NBD-Cl with the tyrosine residues. As shown in Fig. 7, the presence of 500 mM sodium indeed partially protects the sodium-D-glucose cotransport system from its modification by NBD-Cl. At relatively low NBD-Cl concentrations (50 $\mu$M), maximum substrate protection is observed after 15 min of incubation (Table II). On the other hand, incubation of the membranes with NBD-Cl in the presence of 100 mM D-glucose and 50 mM sodium does not reveal any substrate protection.

## Discussion

One of the major problems that arises in studies with so-called side-group-specific reagents is the limited specificity of the reagents employed. Tyrosine possesses a phenoxyhydroxyl group that can be modified by acetylation, esterification and ether formation. However similar chemical reactions are possible at side-chains carrying a free SH group. Possibilities for distinguishing the site at which the modification occurs include the proper choice of the pH at which the modification is performed and a test of the stability of the reaction products. Thus, the optimum pH for the reaction of NBD-Cl with tyrosine residues is 7.4 [9,15] and for the reaction with tetranitromethane the pH optimum is 8.0 [7,15]. These reaction conditions were used in the studies reported above. In addition, it is known that the reaction products between NBD-Cl and tyrosine residues are in general more stable than the thioether obtained when NBD-Cl reacts with SH groups (for references see Ref. 9). The experiments with dithiothreitol show that the inhibition of D-glucose transport by NBD-Cl is not reversed by short time incubation with dithiothreitol. On the contrary, the inhibition by *N*-acetylimidazole is to a large extent reversed by exposure of the membranes to reagent-free buffer. Under these conditions the covalent bonds between *N*-acetylimidazole and SH groups are hydrolyzed [15]. It should be noted, however, that a residual inhibition of sugar transport by *N*-acetylimidazole remains even after thorough washing of the membranes, indicating that also a reaction between tyrosine residues and *N*-acetylimidazole has taken place. In addition to nitration, tetranitromethane changes side-chain groups by oxidation [15], at pH 8.0 predominantly, but not exclusively, tyrosine residues are modified. The reactions (nitration, oxidation) are irreversible and thus the side-groups involved cannot be distinguished further under our experimental conditions. The fact that three reagents known to attack tyrosyl-phenoxy groups inhibit sodium-dependent D-glucose transport under conditions were contributions from other side-groups have been minimized strongly suggests that tyrosine residues are involved in sodium-D-glucose cotransport.

Further support for our assumption that a tyrosyl group is modified has been obtained recently in our laboratory in iodination experiments [16,17] where it was observed that sodium-D-glucose cotransport was inhibited 80% in membranes iodinated with 0.25 mM potassium iodide (KI) in the presence of peroxidase for 2 h at room temperature, compared to the control membranes treated in the absence of KI (Lin et al., unpublished observations).

When the interaction of the NBD-Cl modified transport system with sodium is investigated, a noncompetitive inhibition pattern is obtained. Such pattern could be explained by an irreversible attachment of NBD-Cl to the sodium-binding site [18]. The maximum number of sodium-binding sites in the membranes is reduced, whereas the affinity ($K_m$) of the free sodium sites remains unchanged. This assumption is further substantiated by the finding that high sodium concentrations – in the absence of D-glucose, but not D-glucose in the presence of low sodium concentrations – decrease the rate of inactivation of the transport system by NBD-Cl. Such a substrate protection provides strong evidence for a modification of the transport molecule at or close to the sodium-binding site. It should be mentioned in this context that attempts to protect the sodium-binding site with harmaline, shown to inhibit the sodium-D-glucose transport system in rabbit renal brush border by competing with sodium [19], failed because the interaction of harmaline and the transport system was in our hands irreversible.

In the course of our studies we observed that D-glucose-independent sodium uptake into brush-border membrane vesicles, which occurs mainly via sodium-proton exchange, was not inhibited by treatment of the membranes with NBD-Cl. This finding suggests that the sodium-binding site of

the sodium-proton exchanger differs from the sodium-binding site of the D-glucose cotransport system. Such a difference is already evident from the higher affinity of the former for sodium, and its lower specificity [12,20–22]. The sodium-proton exchanger also interacts with lithium, ammonium and amiloride, whereas the sodium-D-glucose cotransport system is highly specific for sodium and is not inhibited by amiloride.

In conclusion, our studies indicate that in addition to SH groups that have been found to be essential for the interaction of phlorizin and/or D-glucose with the sodium-D-glucose cotransport system [3–5] also tyrosine residues are involved in the interaction of sodium with the transport system. The nature of the interaction remains to be elucidated. It might be an electrostatic interaction between a negatively charged tyrosine residue and the sodium cation, although the p$K$ values of the tyrosine residues modified in the protein are not known.

**Acknowledgements**

This work was supported by NIH grant GM 27859 and start-up funds provided to Dr. Jiann-Trzuo Lin by the Albert Einstein College of Medicine of Yeshiva University (BRSG No. 613-4564).

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