Sodium-induced Conformational Changes in the Glucose Transporter of Intestinal Brush Borders*

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Using brush-border membranes prepared from rabbit small intestine by Ca\textsuperscript{2+} precipitation and KSCN treatment, we have studied the kinetics and conformational changes of the glucose carrier. Na\textsuperscript{+} behaves as a competitive activator of glucose transport under zero-trans conditions. Phenyl isothiocyanate and fluorescein isothiocyanate (FITC) inhibit Na\textsuperscript{+}-dependent transport in an irreversible but substrate-protectable manner. Vesicles pretreated with phenyl isothiocyanate in the presence of substrates were then selectively labeled at the glucose carrier with FITC. Competition experiments with Na\textsuperscript{+} and phlorizin or glucose indicated that FITC binds to the glucose site on the carrier. Carrier-bound FITC displays a saturable quenching of fluorescence in the presence of Na\textsuperscript{+}. The $K_a$ of the Na\textsuperscript{+}-specific quench is $25\text{ m}\mu$, which is similar to the apparent $K_a$ for Na\textsuperscript{+} activation of glucose transport. Two tyrosine group-specific reagents, N-acetylhomosildenafil and tetranitromethane, inhibit glucose uptake and fluorescent quenching in a Na\textsuperscript{+}-protectable fashion. FITC labeled a 75-kilodalton peptide on sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a substrate-sensitive manner. We conclude that Na\textsuperscript{+} binds to the glucose symporter of intestinal brush borders, a 75-kilodalton peptide, and this induces a rapid conformation change in the transporter which increases its affinity for D-glucose.

Entry of glucose into intestinal and renal epithelial cells occurs as the result of coupling to Na\textsuperscript{+} transport down its electrochemical potential gradient by a cotransporter in the brush-border membrane. Kinetic studies of the cotransporter indicate that the carrier has an Ordered Bi Bi reaction mechanism (1-3) with Na\textsuperscript{+} the obligate first substrate (2). This is consistent with an increase in the apparent carrier affinity ($K_a$) for glucose with Na\textsuperscript{+} (2). Information concerning the molecular events involved in Na\textsuperscript{+} alteration of carrier glucose affinity is incomplete, although we have recently demonstrated a Na\textsuperscript{+}-dependent fluorescent quench of fluorescein isothiocyanate bound to the glucose carrier (4).

In this paper, we extend our earlier findings with FITC and present results which confirm that FITC binds to the glucose site and that the Na\textsuperscript{+}-induced fluorescence quenching is the result of interaction with the Na\textsuperscript{+} site on the glucose carrier. We also demonstrate that the Na\textsuperscript{+}-dependent fluorescence quenching is dependent on a sulfhydryl group somewhat removed from the Na\textsuperscript{+} and glucose-active sites.

EXPERIMENTAL PROCEDURES

Preparation of Membranes—Ca\textsuperscript{2+}-precipitated brush-border membrane vesicles were isolated from rabbit small intestine by the method described by Stevens et al. (5). The Ca\textsuperscript{2+}-precipitated vesicles were further purified by a KSCN procedure (4) and were stored at $-180^\circ\text{C}$ until use. Briefly, the Ca\textsuperscript{2+}-precipitated vesicles were exposed to 0.6 m KSCN for 10 min on ice, then diluted 20-fold with 0.3 m mannitol + 10 mM HEPES/Tris, pH 7.5, and centrifuged for 10 min at 6,000 x g. These supernatants were centrifuged for 30 min at 38,000 x g, and the resulting pellets were harvested. The pellets were washed and resuspended in 300 mM mannitol + 10 mM HEPES/Tris, pH 7.5, and pelleted again. This was repeated twice. The membranes were routinely 70-fold enriched in alkaline phosphatase and 130-fold enriched in γ-glutamyltranspeptidase and sucrase compared to the initial homogenate (4).

Glucose Transport—Glucose uptake was measured by a rapid quench, rapid filtration method as described by Stevens et al. (5). Na\textsuperscript{+}-dependent glucose uptake (nanomoles mg$^{-1}$ s$^{-1}$) is defined as the uptake seen with 100 mM cis-NaCl minus uptake seen with 100 mM cis-KCl. All uptakes were measured at 3 s at room temperature ($22^\circ\text{C}$) using 100 μg of vesicle protein. Phlorizin Binding—[3H]Phlorizin binding was performed according to the method of Toggenburger et al. (6) at room temperature and pH 7.5. Na-dependent phlorizin binding is defined as the total phlorizin bound in the presence of 100 mM NaCl minus the phlorizin bound in the presence of 100 mM KCl. Preliminary experiments indicated that phlorizin binding attained a steady state within 3 s, and all results presented were obtained from 3-s incubations with a phlorizin concentration (20 μM) well above the $K_a$.

FITC and FITC Treatment—Vesicles to be studied by fluorescence were pretreated with 2 mM phenyl isothiocyanate in the presence of 100 mM NaCl and 10 mM glucose, and 50 mM Tris-Cl, pH 9.2, + 2 mM EDTA for 30 min at room temperature. Exposure of vesicles to FITC or PITC at basic pH favors binding to the ε-amino group of lysine. The reaction was stopped by the addition of a 10-fold excess of ice-cold 50 mM Tris-Cl, pH 9.2, + 2 mM EDTA for 30 min at room temperature. Exposure of vesicles to FITC or PITC at basic pH favors binding to the ε-amino group of lysine. The reaction was stopped by the addition of a 10-fold excess of ice-cold 50 mM Tris-Cl, pH 9.2, + 2 mM EDTA for 30 min at room temperature. Exposure of vesicles to FITC or PITC at basic pH favors binding to the ε-amino group of lysine. The reaction was stopped by the addition of a 10-fold excess of ice-cold 50 mM Tris-Cl, pH 9.2, + 2 mM EDTA for 30 min at room temperature. Exposure of vesicles to FITC or PITC at basic pH favors binding to the ε-amino group of lysine. The reaction was stopped by the addition of a 10-fold excess of ice-cold 50 mM Tris-Cl, pH 9.2, + 2 mM EDTA for 30 min at room temperature. Exposure of vesicles to FITC or PITC at basic pH favors binding to the ε-amino group of lysine. The reaction was stopped by the addition of a 10-fold excess of ice-cold 50 mM Tris-Cl, pH 9.2, + 2 mM EDTA for 30 min at room temperature. Exposure of vesicles to FITC or PITC at basic pH favors binding to the ε-amino group of lysine. The reaction was stopped by the addition of a 10-fold excess of ice-cold 50 mM Tris-Cl, pH 9.2, + 2 mM EDTA for 30 min at room temperature. Exposure of vesicles to FITC or PITC at basic pH favors binding to the ε-amino group of lysine. The reaction was stopped by the addition of a 10-fold excess of ice-cold 50 mM Tris-Cl, pH 9.2, + 2 mM EDTA for 30 min at room temperature. Exposure of vesicles to FITC or PITC at basic pH favors binding to the ε-amino group of lysine. The reaction was stopped by the addition of a 10-fold excess of ice-cold 50 mM Tris-Cl, pH 9.2, + 2 mM EDTA for 30 min at room temperature. Exposure of vesicles to FITC or PITC at basic pH favors binding to the ε-amino group of lysine. The reaction was stopped by the addition of a 10-fold excess of ice-cold 50 mM Tris-Cl, pH 9.2, + 2 mM EDTA for 30 min at room temperature. Exposure of vesicles to FITC or PITC at basic pH favors binding to the ε-amino group of lysine. The reaction was stopped by the addition of a 10-fold excess of ice-cold 50 mM Tris-Cl, pH 9.2, + 2 mM EDTA for 30 min at room temperature. Exposure of vesicles to FITC or PITC at basic pH favors binding to the ε-amino group of lysine. The reaction was stopped by the addition of a 10-fold excess of ice-cold 50 mM Tris-Cl, pH 9.2, + 2 mM EDTA for 30 min at room temperature. Exposure of vesicles to FITC or PITC at basic pH favors binding to the ε-amino group of lysine.

Vesicles were then exposed to 50 μM FITC (dissolved in dimethylformamide) in 50 mM Tris-Cl, pH 9.2, and 2 mM EDTA for 15 min at 22°C in the dark. The reaction was stopped by the addition of a 20-fold excess of ice-cold 50 mM Tris-Cl, pH 9.2, + 2 mM EDTA and centrifuged for 30 min at 38,000 X g. The pellets were resuspended in a minimum volume of 300 mM mannitol + 10 mM HEPES/Tris, pH 7.5.

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Studies examining the ability of substrates or inhibitors to protect against FITC inhibition of transport or FITC binding were performed with vesicles pretreated with FITC and substrates. The appropriate concentration of substrates was added during exposure to FITC. Phlorizin, pCMBS, and N-acetylimidazole were added to vesicles 30-60 min prior to FITC treatment in the presence or absence of NaCl. Tetranitromethane was added to vesicles 30 min prior to the addition of FITC in 300 mM mannitol and 10 mM phosphate buffer, pH 8.8. All treatments were performed at 22°C. For transport studies, reversal of phlorizin inhibition was accomplished by washing, pelleting.

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* The abbreviations used are: FITC, fluorescein isothiocyanate; PITC, phenyl isothiocyanate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; pCMBS, p-chloromercuribenzenesulfonic acid; SDS, sodium dodecyl sulfate.
and resuspending the pellets twice in 300 mM mannitol and 10 mM HEPES/Tris, pH 7.5. For reversal of pCMBS inhibition, 1 mM dithiothreitol was added to the wash.

**Fluorescence**—Fluorescence experiments were performed on an Aminco-SLM SPF 500 spectrofluorometer at room temperature set in the ratio mode. The excitation and emission wavelengths were 455 and 525 nm, with slit widths of 2 nm. All fluorescence quench results were corrected for dilution by addition of an equal volume of either buffer or KCl to the reference cuvette containing an equal amount of substrate-protected FITC-labeled vesicles and are reported as uncorrected fluorescence emission spectra.

**SDS-Polyacrylamide Gel Electrophoresis**—SDS-polyacrylamide gel electrophoresis was performed on 10–15% linear gradient slab gels or 10% slab gels according to the method of Laemmli (7). Samples were solubilized in 1% SDS in 10 mM Tris–Cl, pH 6.8 ± 0.4% β-mercaptoethanol. The suspension was then centrifuged for 120 min at 50,000 × g, and the supernatant was collected. Following electrophoresis, 0.3-cm slices of the FITC-labeled tracks were mashed and suspended after Comassie Blue-staining by absorbance measurements at 550 nm. Molecular weight standards obtained from Bio-Rad were run in parallel.

**Enzyme Assays**—Brush-border marker enzymes were routinely used to monitor vesicle purification. Alkaline phosphatase was assayed according to the method of Mircheff and Wright (8); sucrase was measured using the Bio-Rad protein assay with γ-globulin as the protein standard.

**Materials**—FITC was purchased from Molecular Probes, Inc., Junction City, OR. N-Acetylimidazole and PITC were purchased from American Scientific Products, Los Angeles, CA.

## RESULTS

Brush-border membrane vesicles prepared by a divalent metal precipitation procedure (5) are routinely 30-fold enriched in alkaline phosphatase and 40-fold enriched in sucrase above that found in the total homogenate. Our adaptation of the KSCN treatment procedure of Hopfer et al. (11) resulted in a 3–4-fold further enrichment of the brush-border enzyme markers alkaline phosphatase, sucrase, and glutamyltranspeptidase, as well as an increase in the Na+-dependent transport velocity (4) and an increase in the Na+-dependent phlorizin binding. Following KSCN treatment, Na+-dependent phlorizin binding increased 9-fold, and the maximum velocity of Na+-dependent glucose uptake increased 11-fold (Table I).

**Kinetics**—Thiocyanate-treated brush-border membrane vesicles contain a saturable Na+-dependent carrier and a Na+-independent glucose uptake system. The apparent affinity of the Na+-dependent uptake (K<sub>a</sub>) was 85 μM, similar to the K<sub>a</sub> of Ca<sup>2+</sup>-precipitated vesicles (2). The V<sub>max</sub> of Na+-dependent uptake was 10 times that previously reported (0.1 nmol/mg/s) for Ca<sup>2+</sup>-precipitated vesicles. Fig. 1 is a Woolf-Augustinsson-Hofstee plot of the effect of Na<sup>+</sup> on carrier glucose affinity at three sodium concentrations. As the Na<sup>+</sup> concentration increased from 25 to 100 mM, the glucose K<sub>a</sub> decreased from 200 to 79 μM. Increasing the glucose concentration from 40 to 500 μM did not affect the Na<sup>+</sup> affinity: the apparent K<sub>a</sub> for Na<sup>+</sup> activation of glucose uptake was 34 μM at 40 μM glucose and 36 μM at 500 μM glucose (data not shown). Hill plots gave a slope of 1, indicating a single class of noninteracting Na<sup>+</sup> sites.

**PTIC and FITC Inhibition of Transport**—Fig. 2a demonstrates that PITC inhibits Na+-dependent glucose uptake. Incubation for 30 min results in inhibition of Na+-dependent glucose uptake with no effect on Na+-independent uptake. Inhibition is dependent on the concentration of PTIC and reaches a maximum of 65% at 2 mM. The addition of Na<sup>+</sup> and D-glucose during exposure to PITC protects the Na+-dependent glucose uptake. Fig. 2b shows the Na+-dependent uptake following exposure for 30 min at room temperature to 2 mM PTIC and substrates. In the presence of 10 mM glucose and 100 mM NaCl, PITC inhibition is less than 2% of that seen in the absence of Na<sup>+</sup> and glucose. Substitution of K<sup>+</sup> for Na<sup>+</sup> or L-glucose for D-glucose results in no protection of transport. Similar results were obtained with FITC.

Fig. 3 demonstrates the reduction in FITC binding with PTIC pretreatment in the presence of substrates. Both PTIC and FITC in the absence of substrates inhibit Na+-dependent glucose uptake approximately 60%. Pretreatment of membranes with FITC in the presence of 100 mM NaCl and 10 mM glucose does not inhibit Na<sup>+</sup>/D-glucose uptake compared to control uptakes in the absence of PTIC (0.83 ± 0.06 versus 0.84 ± 0.04 nmol/mg/s). FITC treatment in the absence of

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**TABLE I**

| Glucose transport | Phlorizin binding |
|-------------------|-------------------|
| V<sub>max</sub> | pmol/mg |
| Controls | 0.15 ± 0.01 | 32 ± 3 |
| (n = 3) | (n = 3) |
| KSCN treated | 1.75 ± 0.17 | 294 ± 23 |
| (n = 6) | (n = 3) |

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**Fig. 1. Kinetics of Na<sup>+</sup>-dependent glucose uptake as a function of cis-Na.** Vesicles (100 μg) in 300 mM mannitol and 10 mM HEPES/Tris, pH 7.5, were added to uptake buffer which included 25, 50, or 100 mM NaCl, 10 mM HEPES/Tris, pH 7.5, 50 μM d-[6-<sup>3</sup>H]glucose. The d-glucose concentration was varied between 25 μM and 10 mM, and the osmolarity was maintained with mannitol. Na<sup>+</sup>-independent uptake was determined by subtraction of KCl for NaCl in the uptake buffer and has been subtracted out. Uptake was terminated following a 3-s incubation. The results (means of duplicates) are presented as a Woolf-Augustinsson-Hofstee plot where uptakes (J) are plotted against uptake/glucose concentrations. The plot is representative of three separate experiments.
PITC pretreatment inhibits Na\(^+\)-dependent D-glucose uptake by 60% and results in 24 ± 2 nmol of FITC bound per mg of protein. Pretreatment with PITC in the presence of substrates followed by exposure to FITC does not affect FITC inhibition of Na\(^+\)-dependent glucose uptake, but does reduce the amount of FITC bound per mg of membrane protein, 90 ± 5%. Inclusion of substrates during exposure to FITC returns transport to control levels (0.8 ± 0.06 nmol/g/s) and results in a further 60% reduction in FITC binding. The difference between FITC binding in the presence and absence of substrates following PITC pretreatment is 0.8 ± 0.2 nmol/mg protein.

**Identification of FITC-binding Site**—Phlorizin and pCMBS were examined for their ability to protect against FITC inhibition of Na\(^+\)-dependent glucose uptake and ability to decrease carrier-specific FITC binding. The isothiocyanate derivatives are irreversible inhibitors, as judged by the lack of reversal upon repetitive washing (not shown). In contrast, both pCMBS and phlorizin inhibition are reversible (12, 13). Fig. 4 shows that phlorizin, whose interaction with the carrier is thought to be at the glucose site (6), gives 95% protection in a Na\(^+\)-dependent manner against FITC inhibition of transport and reduces FITC binding by 1.2 nmol. In contrast, pCMBS affords no protection against FITC inhibition of transport and does not significantly affect FITC binding to the vesicles. This result is in agreement with Semenza (14), who reported that the essential sulfhydryl group cannot be protected by substrates. An estimate of the number of FITC sites related to the carrier glucose site may be determined by competition between phlorizin and FITC. Fig. 5 illustrates the results of these experiments in which vesicles were titrated with various concentrations of the irreversible inhibitor FITC and then Na\(^+\)-dependent phlorizin binding was determined. The results indicate that 242 pmol of phlorizin/mg of protein bind to the carrier in a FITC-sensitive manner. The FITC-sensitive phlorizin binding is 98% of the Na\(^+\)-dependent phlorizin binding. The plot of phlorizin bound versus FITC bound (Fig. 6) is a straight line, the slope of which gives the stoichiometry between phlorizin and FITC binding. Since only Na\(^+\)-dependent phlorizin binding is thought to reflect specific carrier binding, the Na\(^+\)-dependent phlorizin binding was used. The plot is a straight line with a slope of 2.7 ± 0.3 which suggests about three FITC sites/phlorizin-binding site. This is in contrast to the effect of substrates on FITC inhibition of transport which results in a slope of 1 (Fig. 6b).

**Fluorescence**—Bound FITC undergoes a fluorescence quench upon addition of Na\(^+\) but not Rb\(^+\), Cs\(^+\), K\(^+\), or glucose. A typical response is shown in Fig. 7. Addition of 50 mM NaCl to 100 μg of membranes labeled with FITC results in an immediate 19% quench of the FITC fluorescence. Addition of 50 mM K\(^+\) has no effect on the observed fluorescence. The K\(_{0.5}\) for Na\(^+\)-induced fluorescence quenching is 25

![Graph](image-url)
mM as compared to 34 mM (average of three determinations) for glucose uptake. FITC bound in the presence of substrates does not undergo a fluorescence quenching regardless of the ion added.

To examine the site of Na⁺ interaction with FITC-bound vesicles, two reagents, N-acetylimidazole and tetranitromethane, were examined for their effect on Na⁺-induced FITC fluorescence quenching and Na-dependent glucose uptake. Fig. 8 indicates that both of these reagents inhibit Na⁺-dependent glucose uptake. N-Acetylimidazole inhibits transport with an apparent \( K_{0.5} \) of approximately 50 \( \mu M \), and inhibition is sensitive to the presence of Na⁺ during exposure to the reagent. Maximum inhibition is 68% (average of four determinations). Sodium at 100 mM protects 98% against inhibition by N-acetylimidazole with an apparent \( K_{0.5} \) of 18 mM, similar to the response seen for Na⁺ quenching of FITC fluorescence (Fig. 9). The addition of glucose during exposure to N-acetylimidazole does not decrease inhibition in the absence of Na⁺ or increase protection in its presence. This is consistent with N-acetylimidazole acting at the carrier Na⁺ site. The other tyrosine group-specific reagent, tetranitromethane, inhibits transport with a \( K_{0.5} \) of 10 \( \mu M \). Inhibition is not dithiothreitol-sensitive, nor is it sensitive to dilution and washing, indicating that the reagent binds irreversibly to the carrier. Na⁺ also protects against tetranitromethane inhibition of transport (not shown).

The effect of N-acetylimidazole on the Na⁺-induced FITC

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**Fig. 4.** Effect of pCMBS and phlorizin on FITC-induced inhibition of Na⁺-dependent glucose uptake and FITC binding. Vesicles (500 \( \mu g \)) were pretreated with FITC in the presence of 100 mM NaCl and 10 mM glucose as described in Fig. 2. Following removal of substrates and unreacted FITC by centrifugation, the vesicles were exposed to varying concentrations of phlorizin and pCMBS for 30 min at 22 °C and then 50 \( \mu M \) FITC in 50 mM Tris-Cl, pH 9.2, and 2 mM EDTA for 15 min in the dark at 22 °C. Unreacted FITC was removed by centrifugation. Vesicles were washed twice with 300 mM mannitol and 10 mM Hepes/Tris, pH 7.5. Phlorizin binding was determined in the presence of 100 mM NaCl (solid line) and 100 mM KCl (broken line) at 22 °C. The results are means ± S.E. of a single experiment performed in triplicate, and each is representative of the results in three separate experiments.

**Fig. 5.** Effect of FITC on phlorizin binding. Vesicles (500 \( \mu g \)) were treated with FITC in the presence of substrates and processed as described in Fig. 5. Following removal of substrates and unreacted FITC by centrifugation, vesicles were treated with varied concentrations of FITC for 15 min in the dark at room temperature. Unreacted FITC was removed by centrifugation, and the vesicles were resuspended in 300 mM mannitol and 10 mM Hepes/Tris, pH 7.5. Phlorizin binding was determined in the presence of 100 mM NaCl (solid line) or 100 mM KCl (broken line) and 20 \( \mu M \) phlorizin. Binding was performed using 3-h incubations. Results are means of two samples, and the plot is representative of three separate experiments.

**Fig. 6.** Stoichiometry of FITC binding to phlorizin binding. Vesicles (500 \( \mu g \)) were treated with FITC in the presence of substrates and processed as described in Fig. 5. Following removal of substrates and unreacted FITC by centrifugation, vesicles were treated with varied concentrations of FITC for 15 min in the dark at room temperature. Unreacted FITC was removed by centrifugation, and the vesicles were resuspended in 300 mM mannitol and 10 mM Hepes/Tris, pH 7.5. Phlorizin binding was determined in the presence of 100 mM NaCl (solid line) or 100 mM KCl (broken line) and 20 \( \mu M \) phlorizin. Binding was performed using 3-h incubations. Results are means of two samples, and the plot is representative of three separate experiments.
fluorescence-quenching is shown in Fig. 10. N-Acetylimidazole inhibits FITC fluorescence quenching with a $K_{0.5}$ of 35 $\mu$M similar to its apparent $K_{0.5}$ for inhibition of transport (35 $\mu$M). Inclusion of 100 mM Na during exposure to N-acetylimidazole protects the Na$^+$-induced fluorescence quenching in agreement with Na$^+$’s effect on N-acetylimidazole inhibition of Na$^+$-dependent glucose uptake. Addition of glucose during Na$^+$ protection against N-acetylimidazole inhibition does not significantly affect inhibition of transport or Na$^+$-induced FITC fluorescence quenching. The effect of phlorizin and pCMBS on Na$^+$-induced FITC fluorescence quenching is shown in Fig. 11. Both reagents inhibit transport with $K_{0.5}$ values of 25 $\mu$M for pCMBS (see also Ref. 12) and 1 $\mu$M for phlorizin (15). In contrast, only pCMBS affects the Na$^+$-dependent FITC fluorescence quenching.

**Carrier Structural Studies**—FITC-labeled KSCN brush-border membrane vesicles were examined on 10–15% linear gradient SDS-polyacrylamide slab gels according to the method of Laemmli (7). Fig. 12 shows the Coomassie Blue-staining pattern at the top of the figure and the FITC-binding pattern at the bottom. The solid line is the FITC-binding pattern following FITC pretreatment in the presence of substrates followed by FITC treatment. The dashed line is substrate-protected vesicles. Only one polypeptide band is protected by the presence of substrates during exposure to FITC, the 75,000-dalton band. In five experiments on three preparations, the molecular mass of the FITC-binding polypeptide band was 75 ± 2 kDa.

**DISCUSSION**

Na$^+$-dependent organic solute transporters have been shown to increase their affinity for their second substrate on exposure to Na$^+$. Na$^+$-induced decreases in carrier $K_m$ for organic solute have been reported for glucose (9), succinate (16), and proline (17). We have examined the mechanism for this increase in carrier glucose affinity using a fluorescent group specific reagent, FITC.

FITC has been demonstrated in a number of enzyme systems (18–22) to be a highly sensitive reporter of changes in protein conformation. In these studies, relatively pure enzyme preparations were employed, while the glucose carrier is only...
FIG. 9. Effect of Na\(^+\) on N-acetylimidazole inhibition of glucose transport. Vesicles were treated with 2 mM N-acetylimidazole in the absence of Na\(^+\) or in the presence of varied NaCl concentrations in 10 mM potassium phosphate buffer, pH 7.5, for 60 min at 22 °C. Osmolality was maintained with mannitol. Unreacted reagent was removed by centrifugation and Na\(^+\)-dependent glucose uptake determined as in Fig. 8. Control Na\(^+\)-dependent glucose uptake was 1.24 nmol/mg/s. Results are means of duplicate estimates, and the plot is representative of four separate experiments.

FIG. 10. Effect of N-acetylimidazole on Na\(^+\)-induced FITC fluorescence quench. Vesicles (500 µg) labeled with FITC as described in Fig. 7 were subsequently treated with varied concentrations of N-acetylimidazole in the presence and absence of 100 mM NaCl in 10 mM potassium phosphate buffer, pH 7.5, for 60 min at room temperature. Membranes were recovered by centrifugation and were resuspended in 300 mM mannitol + 10 mM HEPES/Tris, pH 7.5. The FITC fluorescence quenching by Na\(^+\) was determined as described in Fig. 7. Results are means of two estimates, and the plot is representative of three separate experiments.

FIG. 11. Effect of N-acetylimidazole, phlorizin, and PCMBs on the Na\(^+\)-dependent FITC fluorescence quenching. Vesicles (500 µg) were treated with PITC in the presence of substrates, followed by FITC as described in Fig. 3. Following removal of unreacted FITC by centrifugation, vesicles were treated with variable concentrations of N-acetylimidazole in 10 mM potassium phosphate buffer, pH 7.5, for 60 min at 22 °C or with variable concentrations of pCMBS or phlorizin in 10 mM Hepes/Tris, pH 7.5, for 30 min at room temperature. Fluorescence quench by 50 mM Na\(^+\) was then measured as described in Fig. 7. Results are expressed as means ± S.E. of four estimates.

a minor component of brush-border membranes (0.1-0.3% (12)). To overcome this problem, we used brush-border membranes enriched in carrier and selectively labeled the carrier by pretreatment of the membranes with a nonfluorescent FITC analogue, PITC, in the presence of substrates. Carrier appeared to be selectively purified by an adaptation of the KSCN procedure of Hopfer (11), as previously described (4). As judged by the increase in \(V_{\text{max}}\) of Na\(^+\)-dependent glucose uptake and Na\(^+\)-dependent phlorizin binding (Table I), the carrier is 10-fold enriched in KSCN-treated vesicles as compared to Ca\(^++\)-precipitated brush-border membrane vesicles. In contrast, the brush-border membrane markers alkaline phosphatase, \(\gamma\)-glutamyltranspeptidase, and sucrase were only 3- to 4-fold enriched. Following KSCN treatment, we estimate from the number of phlorizin-binding sites (~300 pmol/mg protein) and the molecular mass of the carrier (~75,000 daltons) that approximately 2.5% of the membrane protein is glucose carrier. This is similar to the enrichment reported following papain digestion, deoxycholate extraction, and alkali extraction (23). The selective enrichment of the glucose transporter suggests that this polypeptide is more firmly anchored to the membrane lipid than the marker enzymes.

Selective FITC binding to the carrier was accomplished by pretreatment of the vesicles in the presence of substrate with a nonfluorescent irreversible, but substrate-protectable, FITC analogue, PITC. Fig. 3 shows a 90% reduction in FITC labeling of membranes following PITC pretreatment. Pretreatment with PITC in the presence of substrates does not affect Na\(^+\)/D-glucose uptake, nor does it affect subsequent FITC inhibition. Maximum inhibition by both isothiocyanate derivatives is approximately 65% which is somewhat larger than that reported by Weber and Semenza (24). This may
reflect the increased purification of carrier in KSCN-treated vesicles, or the higher pH of the reaction medium. PITC reacts with unprotonated amino groups and so even greater inhibition may be seen at higher pH values.

The site of FITC interaction with the carrier was identified to be at, or near, the glucose site. Glucose in the presence of Na+ completely protects against inhibition of Na+-dependent glucose uptake by both isothiocyanate derivatives with an apparent $K_{0.5}$ for glucose of 50 μM, similar to the apparent $K_{m}$ for transport of 85 μM in the presence of 100 mM Na+. Phlorizin, which has been identified as a competitive inhibitor of glucose uptake with an apparent $K_{0.5}$ of 8 μM in rabbit small intestine brush-border membrane vesicles (25), protects against FITC binding and inhibition of Na+-dependent glucose uptake with a $K_{0.5}$ of 15 μM. The reduction in FITC binding by Na+ and phlorizin is similar to the reduction in binding seen with Na+ and glucose. These results indicate that FITC binds near to or at the carrier glucose-binding site.

The slope of FITC binding versus phlorizin binding is approximately 3 (Fig. 6), indicating that FITC competes for the same sites which bind phlorizin in the presence of Na+. At this juncture, we have no definitive explanation for the stoichiometry between FITC and phlorizin binding. Weber and Semenza (24) have suggested that a lysine is at or near the glucose-binding site on the basis of inhibition of glucose uptake by lysine group-specific reagents. FITC is also thought to react with ε-NH$_2$ groups of lysine in a number of enzyme systems.

Fig. 12 indicates that FITC binds specifically to a 75-kilodalton polypeptide band on SDS-reducing gels. The presence of substrates during exposure to FITC specifically reduces the amount of FITC bound to this polypeptide 60% without significantly affecting FITC binding to any other polypeptide. At present, it is not known if this band contains more than one polypeptide. In light of the results with 4-azidophlorizin (26) and with a monoclonal antibody against the glucose carrier (27), we feel that this polypeptide band is a good candidate for the carrier. The results of Malathi and Preiser (28) suggest that the transport competent carrier is a dimer composed of two 85,000-dalton monomers, but we have no direct information bearing on this point. Other reports have suggested molecular masses of 50,000 to 360,000 daltons (29). The identity of the Na+-binding site is at present unknown.

The fluorescence of FITC bound to the carrier-enriched vesicles is quenched in a specific saturable fashion by Na+ (Ref. 4 and Fig. 4). Li+, Cs+, Rb+, K+ and choline+ have no effect on FITC fluorescence. The Na+ quench is saturable with a $K_{0.5}$ of 25 mM which is similar to the $K_{0.5}$ for Na+-dependent transport (34 mM, Fig. 1 and Table I). The quench of FITC fluorescence by Na+ indicates a rapid change in the carrier conformation at the glucose site.

The inhibition of transport and the Na+-induced FITC fluorescence quench by N-acetylimidazole confirms that FITC fluorescence is indeed monitoring glucose carrier conformation. The tyrosine group-specific reagent N-acetylimidazole (30) inhibits both transport and Na+ quench with similar $K_{0.5}$ values (50 and 35 μM, respectively), while Na+ in the absence of glucose protects N-acetylimidazole inhibition of transport and the Na+ quench with $K_{0.5}$ values of 18 and
TABLE II
Summary of Na/glucose transport parameters

| Sodium          | 1. Activations of glucose transport | 2. Protection against N-acetylimidazole | 3. Quench of FITC fluorescence |
|-----------------|------------------------------------|-----------------------------------------|-------------------------------|
|                 | 3.5 mM                             | 18 mM                                   | 9.5 mM                        |
| Glucose         | 1. Transport with 100 mM NaCl       | Protection against FITC                 | Na⁺-induced fluorescence      |
|                 | 80 µM                              | 50 µM                                   | 15 µM                         |
| Phlorizin       | 1. Protection against FITC inhibition | 50 µM                                   | Na⁺ quench of FITC            |
|                 | 2. Reduction of FITC binding        | 35 µM                                   | Na⁺ quench of FITC            |
| N-acetylimidazole| 1. Inhibition of glucose transport  | 50 µM                                   | 35 µM                         |
|                 | 2. Inhibition of Na⁺ quench of FITC | 25 µM                                   | 35 µM                         |
| pCMBS           | 1. Inhibition of glucose transport  | 50 µM                                   | 35 µM                         |
|                 | 2. Inhibition of Na⁺ quench of FITC | 25 µM                                   | 35 µM                         |

25 mM. Two other tyrosine group-specific reagents, tetrani- tromethane (31) and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole, (32) also inhibited glucose uptake in a Na⁺-sensitive manner. Glucose transport in renal brush-border vesicles was also inhibited by 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (33). These experiments indicate that a tyrosine residue is close to the Na⁺ site and that the Na⁺ quench of FITC fluorescence is directly related to Na⁺ binding to the glucose carrier.

A Na⁺-dependent conformational change of the glucose carrier was anticipated from the kinetics of glucose uptake. Na⁺ increased the apparent affinity of the carrier for glucose without a change in the maximum velocity under identical experimental conditions (i.e. zero-trans conditions, Fig. 1). It should now be possible to monitor the kinetics of this conformational change using stop-flow spectroscopy. Our experiments also suggest that a sulfhydryl group may be essential for the transition from the low-affinity to the high-affinity form of the carrier. pCMBS inhibited the Na⁺-induced fluorescence quench and glucose uptake. It has long been shown that a wide variety of sulfhydryl reagents (e.g. N-ethylmaleimide and Hg²⁺, see Ref. 34) inhibits transport and that these effects are reversed by dithioerythritol. No protection by substrates has been observed, which leads us to infer that the sulfhydryl groups are remote from the active site (see also Ref. 35).

There are few additional clues available about the mechanism of Na⁺/glucose cotransport. On the basis of kinetic experiments of glucose transport and phlorizin binding, Kessler and Semenza (1) proposed a gated channel model. This is an attractive model compared to the more classical carrier models, but the cotransporter may have more in common with pumps (Na⁺/K⁺, Ca²⁺, and H⁺/K⁺ ATPases) than ion channels. For example, the turnover of the glucose symporter at 22 °C is more comparable with pumps (5–30 s⁻¹) than channels (~10⁶ s⁻¹). From our binding (phlorizin and FITC) and transport data, the turnover of the glucose carrier is in the range of 3–6 s⁻¹ (Jmax/density = 2 nmol/mg⁻¹/s⁻¹/0.3–0.8 nmol/mg⁻¹).

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