

Demonstration of Electrogenic Na⁺-dependent D-glucose Transport in Intestinal Brush Border Membranes

(membrane vesicles/active transport/electrogenic)

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Communicated by Albert L. Lehninger, September 26, 1973

ABSTRACT Na⁺-coupled D-glucose transport was studied in isolated membrane vesicles from intestinal brush borders. Concentration gradients of SCN⁻, K⁺, and H⁺ were established between the intravesicular solution and the incubation medium and their influence on D-glucose uptake from the medium was measured. A gradient (medium > vesicle) of NaSCN, but not of KSCN, produced a transient overshoot of D-glucose uptake above the equilibrium level. Similarly, an increase of the membrane conductance with valinomycin (K⁺-conductance) or with uncoupling agents of oxidative phosphorylation (H⁺-conductance) induced an overshooting D-glucose uptake, provided a (vesicle > medium) K⁺-gradient or a H⁺-gradient, respectively, was present in each case. The transient overshoot is evidence that D-glucose was taken up against its concentration gradient (up to 10-fold). The gradients of SCN⁻, K⁺ (in the presence of valinomycin), and H⁺ (in the presence of uncouplers) are thought to contribute to the "driving" force for this "active" D-glucose transport by changing the electrical potential across the vesicle membrane and thus making the inside more negative (with respect to the medium). These experiments, therefore, provide evidence that the Na⁺-coupled D-glucose translocation across the brush border membrane is an electrogenic process, i.e., the positive charge associated with Na⁺ is not compensated by the co-movement of an anion or the counter-movement of a cation *via* the glucose "carrier". The results imply that an electrical potential across the brush border membrane may play an important role in determining the transport of D-glucose by intact cells.

Of the various tissues of higher animals; only the small intestine, the kidney, and the choroid plexus have the ability to absorb D-glucose "actively", i.e., against a glucose concentration gradient. The importance of Na⁺ for "active" sugar absorption has been recognized for a long time (1-3). After mechanisms involving phosphorylation and mutarotation were shown to be incompatible with existing evidence, the Na⁺-dependence became the basis for the "ion-gradient" hypothesis of "active" glucose transport (4-6). This model suggests that Na⁺ is coupled to sugar translocation across the brush border membrane of epithelial cells. Specifically, the model proposes the formation of a ternary complex of sugar-Na⁺-carrier at either interface of the brush border membrane prior to simultaneous transfer of both substrates. The role of the "carrier" is the same as in "facilitated diffusion", ex-

cept that D-glucose as well as Na⁺ are substrates and that their transfer across the membrane is coupled. Under physiological conditions, the intracellular Na⁺-concentration is lower than the luminal one; thus, a movement of Na⁺ down a concentration gradient could provide the energy to "drive" sugar movement against a gradient. A low concentration of intracellular Na⁺, and with it "active" glucose absorption, is maintained by a Na⁺-pump (7, 8).

An important, albeit often neglected aspect of the "ion-gradient" model, is the question of whether Na⁺-coupled glucose transfer is an electroneutral or an electrogenic process. Is the positive charge, associated with Na⁺-transfer, compensated by the co-movement of an anion or the counter-movement of a cation *via* the same glucose-carrier (electroneutral transfer)?* Or is the charge compensation achieved by transfer of an ion at a different site in the plasma membrane (electrogenic transfer)? Microelectrode studies with recordings of the potential across the brush border membrane of rabbit or bullfrog small intestine have suggested an electrogenic mechanism (9-11); however, results from rabbit or bullfrog are at variance with those from rat and tortoise (12-14), so that the above question has not been solved satisfactorily.

An intact glucose transport system has recently been demonstrated in isolated brush border membranes from rat intestine (15). This was possible because the membrane fraction contains an osmotically active space, presumably the membrane vesicles seen in electron microscopy. Glucose transport in the isolated membrane has the same features as in preparations with intact mucosa in that the transport kinetics are faster for D-glucose than for L-glucose and the D-glucose uptake is stimulated by Na⁺ and inhibited by phlorizin (a specific inhibitor of intestinal and renal glucose absorption). In the isolated brush border membrane, glucose transport has the characteristics expected for "facilitated diffusion" of glucose coupled to Na⁺-translocation.

The availability of this rather simple *in vitro* system stimulated us to reinvestigate the problem of electrogenicity of intestinal glucose transport. Valinomycin and uncoupling agents of oxidative phosphorylation were used as specific tools to study the effects of changes in membrane conductance

Abbreviations: Tris, tris(hydroxymethyl)amino methane; HE-PES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid; MES, 2(*N*-morpholino)ethane-sulfonic acid; CF-CCP, carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone; S 13, 3-*t*-butyl, 5-chloro, 2'-chloro, 4'-nitrosalicylanilide

* For example, an electroneutral transfer could be provided by a carrier forming a complex with either K⁺ (K⁺-carrier) or with sugar and Na⁺ (sugar-Na⁺-carrier). The movement of sugar and Na⁺ in one direction would thus be coupled to the movement of K⁺ in the other direction resulting in zero charge transfer.

on glucose uptake. These compounds are known to increase the permeability of lipid bilayers and biological membranes to K⁺ and H⁺, respectively (16–18), and the effects appear to be exerted *via* the lipid phase of membranes. As lipid constitutes about 35% of the weight of intestinal brush border membranes (19), it could reasonably be expected that the properties of this plasma membrane would be altered in the same manner as all other biological membranes tested (16, 17, 20, 21).

METHODS AND MATERIAL

Brush border membranes were prepared as described by Hopfer *et al.* (15) with the modification that D-mannitol was used to a concentration of 500 mM instead of only 100 mM.

D-Glucose uptake was measured by a Millipore filtration technique as follows. Membranes were incubated at 25° in a buffer of the composition indicated in the figure legends. The buffer contained, as labeled substrate, 1 mM D-[U-¹⁴C]glucose (20 Ci/mol). The uptake of glucose was terminated by the removal of an aliquot (20–80 μg of protein) which was diluted 40-fold with ice-cold buffer containing 150 mM NaCl, 50 mM MgCl₂, 30 mM D-mannitol, 10 mM Tris-HEPES (10 mM HEPES adjusted with Tris-hydroxide to pH 7.5), 0.2 mM phlorizin, and 1 μCi/ml D-[1-T]mannitol. The membrane was immediately collected on a Millipore filter (HA 0.25, 0.45 μm) and washed once with 4 ml of ice-cold buffer from which the labeled D-mannitol had been omitted. The amount of labeled glucose and mannitol on the filter were determined by liquid scintillation techniques (15). The glucose uptake by the membranes was corrected for any trapped glucose as measured by the retention of mannitol. Usually this correction was negligible. Single experiments are presented in this paper; however, all experiments were repeated at least three times with similar results.

Alloxan diabetic rats (male Sprague-Dawley of 200 g) were employed for most experiments, since some effects were more pronounced in these animals. The difference appeared to be due to a decreased shunt pathway for Na⁺ in diabetic animals (Hopfer, in preparation). As qualitative results were always similar for diabetic and healthy animals, the conclusions about the mechanism of glucose transport were thought to be unaffected by the use of diabetic rats. Experimental diabetes was induced by the intravenous injection of 50 mg of alloxan per kg of body weight. The rats were used 5–6 days after injection, provided the blood sugar was above 300 mg%.

Other methods were reported previously (15).

Carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone (CF-CCP) was a gift of Dr. P. G. Heytler (DuPont de Nemours and Co., Wilmington, Del.), and 3-*t*-butyl,5-chloro, 2'-chloro,4'-nitrosalicylanilide (S 13) was kindly given to us by Monsanto Chemical Co. (St. Louis, Mo.). Valinomyacin was obtained from Sigma Chemical Corp. (St. Louis, Mo.).

RESULTS AND DISCUSSION

The membrane fraction employed in these studies consisted of highly purified brush border membranes that were essentially free of soluble enzymes and other types of membranes (22). Sucrase (sucrose α-glucohydrolase, E.C 3.2.1.48), a specific marker enzyme for intestinal brush border membrane, was enriched about 30-fold in the final membrane preparation as compared with the starting homogenate.

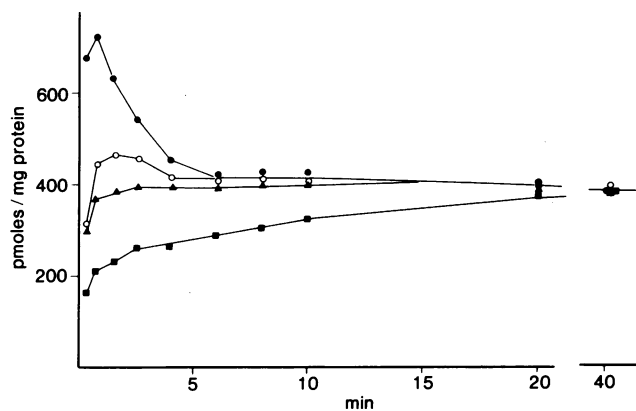


FIG. 1. Anion requirement of D-glucose uptake: D-Glucose uptake in the presence of a NaCl gradient (○—○), a NaSCN gradient (●—●), and a KSCN gradient (■—■), and after abolition of a NaSCN gradient (▲—▲). The membranes were prepared in a buffer containing 100 mM D-mannitol, 0.1 mM MgSO₄, and 1 mM Tris-HEPES (pH 7.5). D-Glucose uptake was initiated by the addition of the membranes to the incubation medium which had the following final composition: 1 mM D-[U-¹⁴C]glucose, 100 mM D-mannitol, 0.1 mM MgSO₄, and 1 mM Tris-HEPES (pH 7.5) with either 100 mM NaCl, or 100 mM NaSCN, or 100 mM KSCN. The NaSCN gradient was abolished by preincubation of the membranes with 100 mM NaSCN for 10 min at 25°; D-glucose uptake was initiated subsequently in 100 mM NaSCN as indicated above. (The numbers on the ordinate indicate pmoles of D-glucose per mg of membrane protein.)

Effect of Anions. The cation requirement for D-glucose transport in the isolated membrane has been investigated previously (15). D-Glucose uptake was found to be stimulated specifically by Na⁺. This result is confirmed in Fig. 1, which shows the difference between D-glucose uptake in medium with NaSCN and in medium with KSCN. Interestingly, experimental conditions were found in which the nature of the accompanying anion also strongly influenced D-glucose uptake. If a salt gradient between the intravesicular buffer and the medium existed at the beginning of the incubation, then initial D-glucose uptake was much higher with NaSCN than with NaCl (Fig. 1). A concentration of 100 mM NaSCN induced a rapid overshoot in the uptake of D-glucose, which was present in the medium at a concentration of 1 mM. Preincubation of the membrane with NaSCN abolished the salt gradient, as measured by the uptake of labeled ²²Na⁺ (data not shown), and also the overshooting part of the sugar uptake (Fig. 1). The same result was obtained after preincubation with NaCl, i.e., glucose transport by membranes preincubated with either NaSCN or NaCl was indistinguishable.

Fig. 1 also demonstrates that the final uptake for D-glucose was the same regardless of the presence of a salt gradient and the nature of the anion or of the cation. In Na⁺-containing media, the equilibrium was already reached after about 6 min of incubation, and uptake remained constant up to the measured 40 min. These results suggest that further D-glucose uptake or efflux stopped when the intravesicular D-glucose concentration was the same as that of the medium. This conclusion is also supported by experiments in which transport of amino acids, other sugars, and Na⁺ was measured in the same membrane preparation (ref. 15 and Murer and Hopfer, in preparation). The equilibrium values were nearly identical for D-glucose, L-glucose, D-alanine, L-alanine, D-galactose,

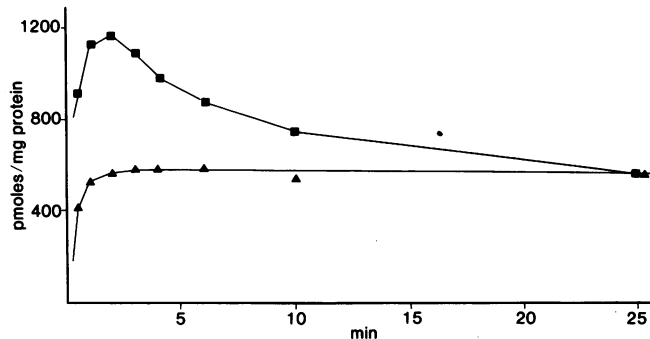


FIG. 2. Effect of valinomycin on the D-glucose uptake of K^+ -preloaded vesicles: D-Glucose uptake in the presence of valinomycin (■—■) or in the absence of valinomycin (▲—▲). The membranes were prepared in a medium containing 500 mM D-mannitol, 0.1 mM $MgSO_4$, and 1 mM Tris-HEPES (pH 7.5) and preloaded with K_2SO_4 by mixing 1 volume of membrane suspension with 6 volumes of a solution containing 50 mM K_2SO_4 , 1 mM Tris-HEPES (pH 7.5), and 0.1 mM $MgSO_4$. The membranes were subsequently collected by centrifugation and resuspended in a solution containing 100 mM D-mannitol, 50 mM K_2SO_4 , 0.1 mM $MgSO_4$, and 1 mM Tris-HEPES (pH 7.5). D-Glucose uptake was initiated by addition of 1 volume membrane suspension to 8 volumes incubation medium with the final composition: 100 mM D-mannitol, 50 mM Na_2SO_4 , 0.1 mM $MgSO_4$, 1 mM D-[$U-^{14}C$]glucose, 5.6 mM K_2SO_4 , and 1 mM Tris-HEPES (pH 7.5) with or without valinomycin (8 μg /mg of membrane protein). (The numbers on the ordinate indicate pmoles of D-glucose per mg of membrane protein.)

and Na^+ , provided all compounds were compared at the same concentration. These findings are to be expected if all the tested molecules equilibrate with the same intravesicular space. It follows from this interpretation of the equilibrium that the observed overshoot represents a transient movement of D-glucose against its concentration gradient ("active" transport). The conditions of the experiment in Fig. 1 are such (100 mM salt, 1 mM glucose) that enough free energy is available in the salt gradient to support an "active" glucose transport of the observed magnitude. The discharge of the salt gradient with time explains the efflux of D-glucose from the vesicles after about 45 sec and the disappearance of the "overshoot" by preincubation of the membrane with salt.

Anions are presumed not to interact with the intestinal glucose "carrier" (6), which is consistent with the indifference of the glucose uptake to the nature of the anion after abolition of the salt gradient, i.e., in the virtual absence of a net movement of the added ions. On the other hand, the experiments with salt gradients show that $NaSCN$ is more potent than $NaCl$ in supporting D-glucose uptake above equilibrium. Under the conditions of net ion fluxes into the vesicles, the anion could exert an indirect influence on D-glucose movement *via* an electrical membrane potential. SCN^- is known to permeate biological membranes predominantly in the charged form at pH 7.5 (23). Therefore, diffusion of SCN^- into the vesicles could make the inside electrically negative with respect to the medium and thus provide an extra "pull" on an electrogenic Na^+ -coupled D-glucose uptake by the vesicles. In other words, the efficacy of the SCN^- anion can be explained in terms of a lipophilic anion with an electrogenic mode of translocation coupled to a second electrogenic transport process across the same membrane. The prominent overshoot of D-glucose uptake, as induced by a $NaSCN$ gradient,

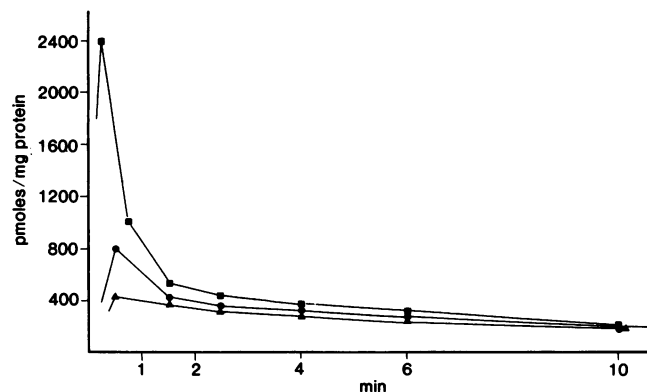


FIG. 3. Effect of uncouplers on the D-glucose uptake of H^+ -preloaded vesicles: D-Glucose uptake in the presence of a pH-gradient with CF-CCP (■—■) and without CF-CCP (▲—▲) and in the absence of a pH-gradient but with CF-CCP (●—●). The membranes were prepared, preloaded, and resuspended as in Fig. 2, but with 50 mM Tris-MES (50 mM MES adjusted with Tris-hydroxide to pH 5.25) instead of K_2SO_4 for the experiments with a pH-gradient. D-Glucose uptake was initiated by addition of 1 volume of membrane suspension to 8 volumes of incubation medium. The final composition of the medium was 100 mM D-mannitol, 1 mM D-[$U-^{14}C$]glucose, 0.1 mM $MgSO_4$, 50 mM Na_2SO_4 , 50 mM Tris-HEPES, and 5.6 mM Tris-MES (final pH 7.5) with CF-CCP (87 μM) or without CF-CCP. D-Glucose uptake in the absence of a pH-gradient was achieved by replacing Tris-MES by 50 mM Tris-HEPES (pH 7.8) during preloading and resuspending of the membrane. The composition of the final medium for uptake was as above, but with 50 mM Tris-HEPES (pH 7.8) as the buffer and CF-CCP (87 μM) as the uncoupler. (The numbers on the ordinate indicate pmoles of D-glucose per mg of membrane protein.)

provides the first indication that the charge associated with Na^+ coupled to D-glucose translocation is not compensated by the movement of another ion *via* the glucose carrier.

Effect of Valinomycin. In order to test the proposal that the charge associated with Na^+ -coupled D-glucose translocation is not compensated by movement of another ion *via* the glucose carrier, valinomycin was used to increase membrane conductance for K^+ . Fig. 2 presents the results of D-glucose uptake by membrane vesicles preloaded with 50 mM K_2SO_4 . Dilution of the membranes with K^+ -free medium resulted in a K^+ -gradient of 50 mM inside to 5.6 mM outside the membrane. The addition of valinomycin produced an overshoot in D-glucose uptake of 2–3 times that at equilibrium. The final uptake of D-glucose was independent of valinomycin, as expected if equilibration of the glucose between medium and intravesicular buffer takes place. The addition of valinomycin in the absence of K^+ had no effect on the D-glucose uptake.

These results indicate that valinomycin contributes to the overshooting D-glucose uptake by providing a pathway for the discharge of the K^+ -gradient (vesicle > medium). K^+ -flux *via* valinomycin is known to be an electrogenic process (16, 17, 24). The efflux of K^+ has to be coupled to the flux of another ion because of the requirement of overall electroneutrality. Therefore, the results in Fig. 2 strongly suggest that the D-glucose uptake coupled to the K^+ -efflux in the presence of valinomycin proceeds *via* an electrogenic mode of translocation.

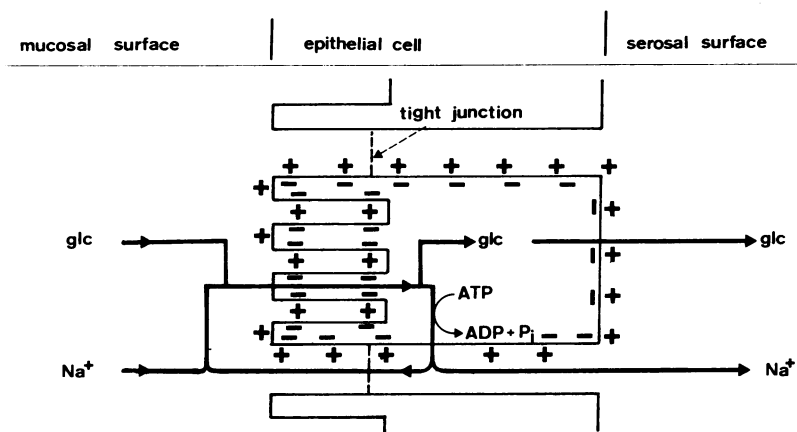


FIG. 4. Model for glucose transport across epithelial cells: This model for glucose absorption is based on the assumption that an electrogenic Na⁺,K⁺-ATPase creates the membrane potential (glc = glucose, P_i = inorganic phosphate).

Effect of Uncouplers of Oxidative Phosphorylation. Fig. 3 presents analogous experiments to those of Fig. 2, but with uncouplers and a H⁺-gradient. Brush border membranes were preloaded by osmotic shock with a pH 5.25 buffer, followed by an incubation at pH 7.50 with D-glucose and Na₂SO₄ in the medium. As seen in Fig. 3, the combined energy of the H⁺-gradient and Na⁺-gradient was able to support a transient D-glucose uptake with a maximum of 10 times the equilibrium level provided CF-CCP was present. That intrinsic H⁺-conductance is small compared to the uncoupler-catalyzed conductance is indicated by the smaller glucose uptake in the absence of uncouplers. Glucose uptake of about 4 times the equilibrium value was observed with a Na⁺-gradient alone, provided an uncoupler was added and charge compensation could take place by the efflux of protons. The two other uncouplers tested besides CF-CCP, namely S 13 and dinitrophenol, gave similar results with respect to a glucose overshoot. On a concentration basis, S 13 was the most effective uncoupler; CF-CCP and dinitrophenol were less effective, in that order.

The catalytic effect of uncoupling agents rather than a stoichiometric involvement in glucose transfer is indicated by the fact that S 13 at a concentration of 1 μM, i.e., 1000 times lower than the glucose concentration, is about as effective as CF-CCP (Fig. 3) at 87 μM.

In these experiments, valinomycin and CF-CCP allow coupling of the free energy existing in the K⁺-gradient and H⁺-gradient, respectively, to the transport of glucose against a concentration gradient. That this coupling of flows occurs *via* the electric field across the membrane is strongly suggested by their known ability to increase the K⁺-conductance or H⁺-conductance, respectively (16, 18).

The above experiments with such structurally unrelated compounds as SCN⁻, valinomycin, and uncouplers all point to the same conclusion: that a positive charge, presumably Na⁺, is translocated together with D-glucose by the glucose "carrier" across the brush border membrane, i.e., that the translocation mechanism is electrogenic.

Such a mechanism implies that an electrical potential across the brush border membrane will influence the direction and rate of glucose transport in the intact cell. Microelectrode studies of rabbit and bullfrog intestine have revealed potentials of 34–40 mV across the brush border membrane (negative inside the cell) *in situ* (9, 10). These potentials were

responsive to changes of the luminal glucose concentration, which suggests an electrogenic mode of glucose transfer, in agreement with the investigations on isolated membranes. The values for rabbit and bullfrog intestine appear to be better estimates for the conditions in intact cells than the low and unresponsive potentials of less than 10 mV reported for rat and tortoise intestine (13, 14). The results from studies of rat and tortoise intestine could be a consequence of insufficient re-sealing of the brush border membrane after penetration of the electrode. An electrical potential of 35 mV compares favorably in magnitude with a measured (25) 3-fold Na⁺-concentration gradient (lower inside the cell) as the driving force for active glucose transport. Indeed, a membrane potential may explain accumulation of sugars against a concentration gradient in isolated epithelial cells, even though the Na⁺-concentration is higher within the cell than in the medium (26). Kimmich, who carried out these latter experiments, indicates such an explanation in a recent review article (27). Several questions arise with respect to transcellular glucose transport *in vivo*. For example, the source of the electrical potential across the brush border membrane is not known. Fig. 4 depicts a model for glucose absorption derived under the assumption that an electrogenic Na⁺,K⁺-ATPase creates the membrane potential. An intestinal Na⁺,K⁺-ATPase (ATP phosphohydrolase, EC 3.6.1.3) has been shown to be located in the lateral-basal plasma membrane, but absent from the brush border membrane (28–30). Another important feature incorporated into the model is the high Na⁺-conductance of the "tight" intercellular junction suggested by electrical and isotope flux measurements of Na⁺ (31, 32). Both properties together would enable the enterocyte to pump Na⁺ into the intercellular space; Na⁺ could return via the "tight" junction into the lumen and then back into the cell *via* the brush border membrane. At the level of the brush border membrane, nutrients could "hook on" to Na⁺ to be carried into the cell. A circular Na⁺-current, as indicated in Fig. 4, would be expected to be important at low luminal Na⁺-concentration. The magnitude of the Na⁺-current with respect to trans-epithelial Na⁺-movement would determine to what extent Na⁺ would be involved catalytically rather than stoichiometrically in transcellular glucose movement.

Indeed, a circular Na⁺-current may account for the apparent Na⁺-independence of glucose absorption *in vivo*, which contrasts sharply with the findings, *in vitro* (33–37).

Glucose exit from the cell at the lateral-basal (serosal) side of the plasma membrane may proceed *via* a Na⁺-independent "carrier", similar to that in erythrocytes. This last supposition is supported by measurements of glucose transport into intestinal epithelial tissue under conditions where glucose entry across the brush border membrane was blocked (38).

This investigation was supported by a grant from the Swiss National Science Foundation, Grant no. 3.511.71. The authors are indebted to Dr. H. Leese for many fruitful discussions and to Prof. G. Semenza for his continued interest in the work.

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