THE ROLE OF SH-GROUPS IN THE CONCENTRATIVE TRANSPORT OF D-GLUCOSE INTO BRUSH BORDER MEMBRANE VESICLES

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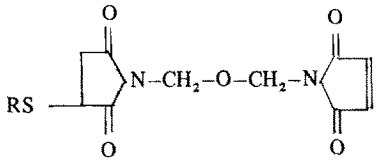
1. Introduction

D-Glucose transport across the brush border membrane of intestinal epithelial cells is tightly coupled to sodium [1,2]. The preparation of closed and right side out [3] brush border membrane vesicles made it possible to investigate the transport of D-glucose and other solutes under well-defined conditions [4].

Here we investigate the role of SH-groups in the transport of D-glucose using brush border membrane vesicles from rabbit small intestine. Evidence will be presented to show that certain SH-reagents such as Cu–(*o*-phenanthroline)$_2$ inhibit the concentrative D-glucose transport in the presence of a Na^+ gradient, but do not affect the transport in the absence of such a gradient. Results indicate that one or several free SH-groups located either on the cytoplasmic side of the membrane or within the membrane itself are essential for concentrative D-glucose transport. Oxidation, alkylation or chelation of these SH-groups lead to a specific increase in the Na^+ permeability of the membrane and in turn to a rapid dissipation of the Na^+ gradient driving concentrative D-glucose transport [2]. The increase in the Na^+ permeability is specific and is not due to the membrane becoming leaky. Apart from the structural changes responsible for the increase in Na^+ permeability the integrity of the membrane structure seems to be preserved.

Abbreviations: DTBN, 5,5'-dithio-bis(2-nitrobenzoic acid)
MIMAX, Glutathione–maleimide derivative (cf. [5])



R = $^-O_2C–CH_2–NH–CO–\underset{\displaystyle -CH_2}{\underset{|}{CH}}–NH–CO–CH_2–CH_2–\underset{\displaystyle NH_2}{\underset{|}{CH}}–CO_2^-$;

NEM, *N*-ethylmaleimide; 4-PDS, 4,4'-dithiodipyrimidine; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Diamide, diazinedicarboxylic acid bis-dimethylamide

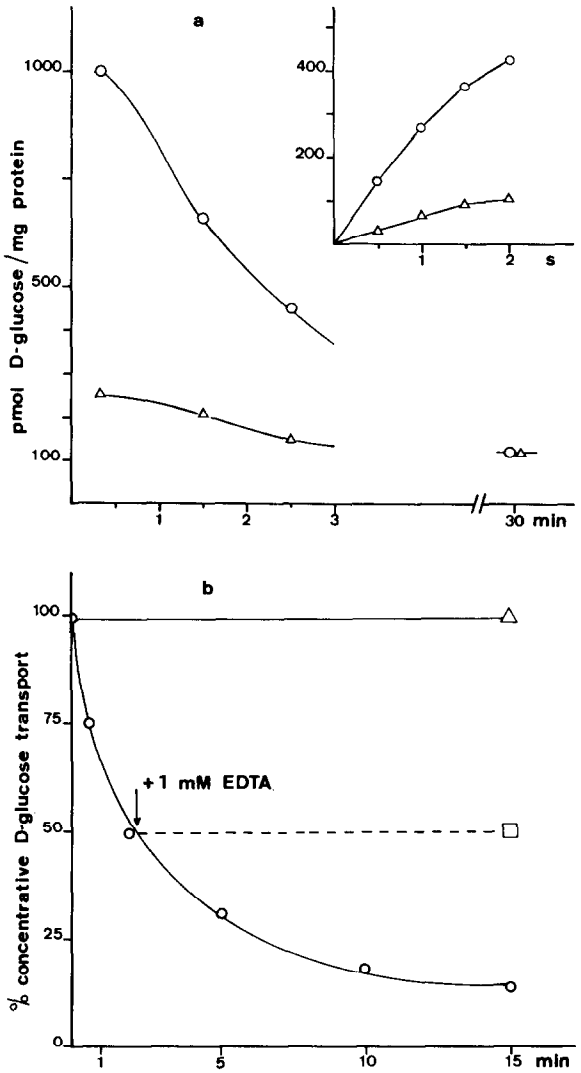
2. Materials and methods

D-[1-^{3}H]Glucose and ^{22}NaCl were obtained from New England Nuclear and Amersham, respectively. MIMAX was synthesized according to [5]. Brush border membrane vesicles were prepared from rabbit small intestine (frozen at –70°C) according to [6]. Unless stated otherwise the buffer used was 0.01 M Hepes/Tris (pH 7.5), 0.1 M mannitol. D-Glucose transport was measured as in [6,7].

3. Results and discussion

The effect of the Cu–(*o*-phenanthroline)$_2$ complex on the Na^+-dependent D-glucose transport into brush border membrane vesicles in the presence of a NaSCN gradient (0.1 M outside) is depicted in fig.1. Figure 1a shows that 10 μM Cu–(*o*-phenanthroline)$_2$ almost completely inhibits the concentrative uptake of D-glucose (referred to in [4] as the overshoot). The effect on D-glucose uptake at very short times is

shown as an inset. From fig.1b it is clear that the inhibition of the overshoot is essentially complete after 15 min incubation with 10 μM Cu–(*o*-phenanthroline)$_2$. The concentration dependence of Cu–(*o*-phenanthroline)$_2$ on the overshoot is shown in fig.1c. In table 1 the effect of Cu–(*o*-phenanthroline)$_2$ is compared to that of other SH-reagents. It can be seen that at concentrations at which Cu–(*o*-phenanthroline)$_2$ inhibited the overshoot neither Cu^{2+} nor *o*-phenanthroline were active. In contrast, *o*-phenanthroline was reported to have an inhibitive effect on D-glucose transport in kidney brush border membrane [13].



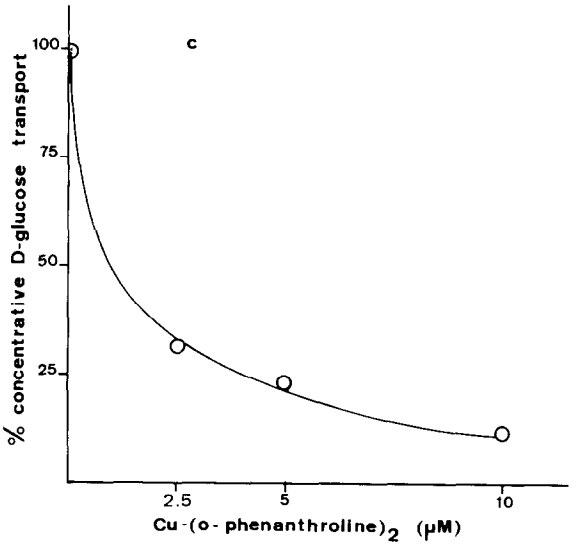


Fig.1. Concentrative transport of D-glucose (overshoot) into brush border membrane vesicles in the presence of a NaSCN gradient at 25°C. At zero time 0.1 M NaSCN and 0.25 mM D-[1-^{3}H]glucose were added to the external medium of the vesicles. (a) Time course of the glucose uptake in untreated vesicles (-○-) and in vesicles treated with 10 μM Cu–(*o*-phenanthroline)$_2$ (-△-). Inset: section of fig.1a giving the time course at very short intervals after adding NaSCN. (b) Effect of incubation time on the concentrative D-glucose transport. Brush border membrane vesicles were incubated with 10 μM Cu–(*o*-phenanthroline)$_2$ for various times. The reaction was stopped with 1 mM EDTA and the overshoot was determined at 15 s and plotted as % of the control experiment (-○-). Incubation in the absence of Cu–(*o*-phenanthroline)$_2$ had no effect on the overshoot (-△-). Once 1 mM EDTA was added no further decrease in the overshoot with time was observed (-□-). (c) Concentration dependence of the Cu–(*o*-phenanthroline)$_2$ effect. Membrane vesicles were incubated with increasing concentrations of Cu–(*o*-phenanthroline)$_2$ for 15 min. The reaction was stopped with 1 mM EDTA and the overshoot of D-glucose was measured at 15 s.

Figure 2 describes the effect of Cu–(*o*-phenanthroline)$_2$ on the D-glucose transport in the absence of a NaSCN-gradient, i.e., when NaCl or KCl are present in equal concentrations (0.1 M) on either side of the membrane. Under these conditions, when no overshoot was observed, Cu–(*o*-phenanthroline)$_2$ had little if any effect on D-glucose transport. This is also true for the other compounds in table 1 inhibiting the overshoot.

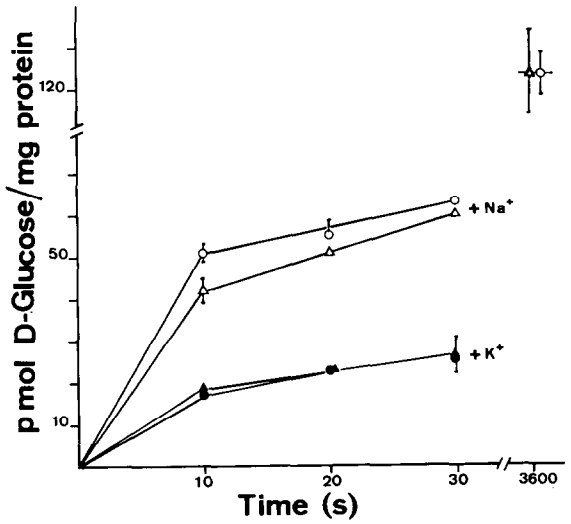
The Na^+ permeability of brush border membranes

Table 1
Effect of SH-reagents on the Na^+-dependent, concentrative transport of D-glucose into brush border membrane vesicles

Reagent[a]	% concentrative uptake of D-glucose	Relative rate constant k_1 of Na^+-efflux
Control	100 ± 1	1
$CuSO_4$ (10 μM)	100	1
o-Phenanthroline (100 μM)	100	1
Cu–(*o*-phenanthroline)$_2$ (10 μM)	18 ± 7	2.1
Diamide (2 mM)	100 ± 2	1
$NaAsO_2$ (0.2 mM)[b]	100 ± 1	1
$NaAsO_2$ (0.2 mM) + dimercapto-propanol (0.2 mM)[b]	47 ± 3	2
Dimercaptopropanol (0.2 mM)[b]	100 ± 1	n.d.
N-Ethylmaleimide (NEM; 3 mM)[c]	44 ± 3	1.4
MIMAX (10 mM)[c]	100 ± 3	1
5,5′-Dithio-bis-2-nitrobenzoic acid (DTNB; 1 mM)[d]	95	1.1
4,4′-Dithiodipyrimidine (4-PDS; 1 mM)[d]	15	1.7

[a] All reagents except MIMAX, $NaAsO_2$ and diamide were purchased from Fluka AG. $NaAsO_2$ and diamide (diazinedicarboxylic acid bis-dimethylamide) were bought from Merck and Cal-Biochem, respectively
[b] At 37°C
[c] 0.05 M K^+-phosphate (pH 6.9)
[d] 10 min incubation

Unless stated otherwise brush border membrane vesicles (1–2 mg protein/ml 0.01 M K^+-phosphate, pH 7.7) were incubated with various reagents at 25°C for 15 min. The reaction mixture was diluted with Hepes/Tris buffer containing 1 mM EDTA, and the membranes were spun down at 30 000 × *g* for 30 min. The pellet was resuspended in Hepes/Tris buffer and the D-glucose uptake was measured after 15 s. Data are presented as the mean ± SD of 3–6 expt. Alternatively, the values given are the mean of 2 measurements



was measured under conditions described in fig.3. The efflux of Na^+ from untreated membrane vesicles is compared to that from vesicles treated with Cu–(*o*-phenanthroline)$_2$. Results are plotted according to eq. (1).

Fig.2. Uptake of 0.1 mM D-[1-^{3}H]glucose into brush border membrane vesicles in the absence of a Na^+ gradient. Vesicles were equilibrated with either 0.1 M NaCl (open symbols) or KCl (full symbols) for 60 min at 25°C; untreated vesicles (-●-, -○-) and vesicles treated with 10 μM Cu–(*o*-phenanthroline)$_2$ (-△-, -▲-). The bar or the size of the symbols indicate the spread of four experiments. Equilibrium values for untreated (-○-) and treated (-△-) vesicles obtained after 60 min are included (top right).

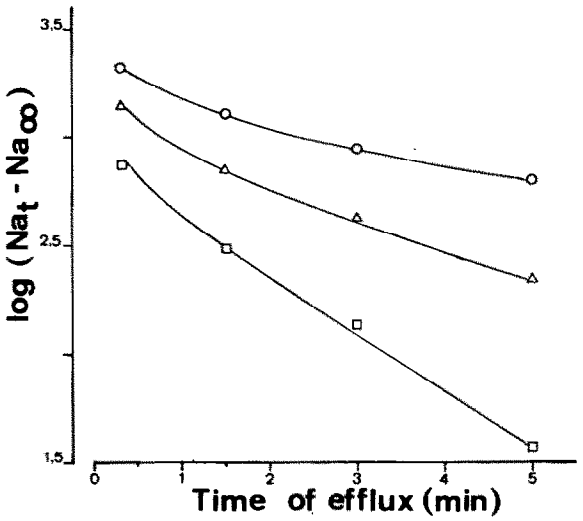


Fig.3. Na^+ permeability of brush border membrane vesicles. The efflux of $^{22}Na^+$ was determined at 25°C with membrane vesicles pre-equilibrated in Hepes/Tris buffer containing 0.1 M $^{22}NaCl$. At zero time an aliquot of the membrane suspension was diluted 50-fold with buffer containing no $^{22}NaCl$. At the time indicated the sample was rapidly filtered and the quantity of $^{22}Na^+$ associated with the membranes determined. Log ($Na_t - Na_\infty$) is plotted according to eq. (1) where Na_t is the amount of $^{22}Na^+$ associated with the membrane at time t and Na_∞ is that which remains attached to the membrane after equilibration; untreated membranes (-○-), membranes incubated with 10 μM Cu–(*o*-phenanthroline)$_2$ for 3 min (-△-) and for 15 min (-□-). For the purpose of comparing Na^+ permeabilities the initial slope of the curves was taken which is related to the first order rate constant k_1. The relative values thus obtained are 1, 1.4 and 2.1 from top to bottom, respectively.

$$\text{Log}\,(Na_t - Na_\infty) = C - \frac{k_1}{2{,}3}\,t \qquad (1)$$

where k_1 is the first order rate constant. Its value represented by the initial slope of the curves increased ~2-fold when brush border membrane vesicles were treated with 10 μM Cu–(*o*-phenanthroline)$_2$. Inspection of table 1 reveals a good correlation between the inhibition of the concentrative D-glucose uptake and the increase in the Na^+-permeability. All reagents reducing the overshoot significantly (> 50%) produced a 50–100% increase in the Na^+-permeability.

3.1. *Reaction mechanism*

Cu–(*o*-phenanthroline)$_2$ is known to catalyze the formation of S–S bonds [8]:

$$2\ \text{R-SH} + 2\ \text{Cu–(}o\text{-phenanthroline)}_2 \rightleftharpoons$$

$$\text{R–S–S–R} + 4\ (o\text{-phenanthroline}) + 2\ Cu^+ + 2\ H^+$$

$$4\ (o\text{-phenanthroline}) + 2\ Cu^+ + 2\ H^+ + 1/2\ O_2 \rightleftharpoons$$

$$2\ \text{Cu–(}o\text{-phenanthroline)}_2 + H_2O$$

The amount of O_2 taken up by brush border membranes in the presence of Cu–(*o*-phenanthroline)$_2$ was measured with a Clarke-electrode. It was found to be stoichiometrically related according to the above reaction scheme to the reduction in the number of free SH-groups measured. Determination of the free SH-groups before and after the reaction according to [9] revealed that ~1/3rd of the total number of free SH-groups (71 ± 1 nmol/mg protein) had interacted. In contrast to Cu–(*o*-phenanthroline)$_2$, no O_2 uptake was detected in the presence of the other reagents inhibiting concentrative D-glucose uptake (table 1). However, like Cu–(*o*-phenanthroline)$_2$ these reagents produced a 30–50% reduction in the total number of free SH-groups. From these results we conclude that one or several free SH-groups are essential for concentrative D-glucose transport and that blocking of the SH-group(s) inhibits this transport. The inhibitors listed in table 1 probably differ in their reaction mechanism in as much as they might oxidize, chelate or bind covalently with SH-groups.

3.2. *Location of SH-groups*

It can be shown by SDS–PAGE that an actin-like protein carrying many free SH-groups is a major protein of the brush border membrane. All SH-groups of this protein were alkylated when permeable NEM was added to brush border membrane vesicles, whereas only ~2% of the SH-groups were accessible and interacted with the membrane-impermeable maleimide derivative MIMAX [10]. After solubilization of the membrane with Triton X-100 all SH-groups interacted with MIMAX. From this we conclude that:

(1) Practically all brush border membrane vesicles are closed;
(2) This actin-like protein can be used as a marker to monitor the membrane permeability of SH-reagents.

Using this marker protein it can be shown that the effective inhibitors listed in table 1 are membrane

permeable, e.g., Cu–(*o*-phenanthroline)$_2$, NEM, 4-PDS. The reactivity of AsO_2^- lends further support to the correlation between the permeability and the inhibiting effect of SH-reagents. The inhibition of AsO_2^- becomes only appreciable in the presence of equimolar concentrations of dimercaptopropanol. AsO_2^- is known to form complexes with vicinal SH-groups [11] and this complex is more hydrophobic and probably more permeable than the uncomplexed anion. Diamide is an exception to this correlation. Despite its permeability it has no inhibitory effect. The reason for this is unknown. The above findings may be rationalized by postulating that the SH-groups responsible for the transport inhibition are not readily accessible on the external surface of the brush border membrane vesicles, but are located either on the cytoplasmic side or within the membrane itself.

3.3. *Mechanism of the inhibition of the concentrative D-glucose transport*

The concentrative D-glucose transport has been shown to be directly related to the electrochemical gradient of Na^+ across the membrane [1,2]. Table 1 reveals that the inhibition is accompanied by a marked increase of the Na^+-permeability of the membrane. Based on this it seems reasonable to postulate that the modification of one or several SH-groups located within the membrane or at the internal surface produces a specific increase in the Na^+-permeability which in turn leads to a rapid dissipation of the Na^+-gradient [12]. The observation that the Na^+-dependent concentrative uptake of L-alanine and L-methionine was also inhibited by Cu–(*o*-phenanthroline)$_2$ is consistent with the above mechanism. Furthermore, the nature of inhibitors such as Cu–(*o*-phenanthroline)$_2$ and AsO_2^- suggest that more than one SH-group is involved and that those SH-groups are located in close proximity. The following lines of evidence indicate that the increase in Na^+-permeability is a specific effect and not due to the membrane becoming leaky:

(i) The kinetics and the equilibrium of the D-glucose transport (in the absence of an electrochemical gradient, fig.2) and of the D-fructose transport in brush border membrane vesicles treated with an inhibitor (table 1) were the same as in untreated vesicles;

(ii) The osmotic properties of treated vesicles were unchanged as compared to untreated ones;

(iii) As judged from SDS–PAGE no solubilization or cross-linking of membrane proteins was detectable;

(iv) ζ-Potential measurements show that the surface potential and hence surface charge density are the same in treated and untreated membranes.

From this we conclude that the interaction with Cu–(*o*-phenanthroline)$_2$ and other inhibitors does not affect the integrity of the membrane. A non-specific increase in membrane permeability can be ruled out. Instead we conclude that the reaction generates specific channels for Na^+.

In conclusion, we have shown that the interaction of one or several free SH-groups located on the cytoplasmic side or in the interior of the membrane generates specific channels for Na^+ through which the electrochemical gradient of Na^+ will quickly dissipate. This rapid breakdown of the Na^+ gradient is believed to be the reason for the inhibition of the concentrative D-glucose transport observed with the SH-reagents of table 1.

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