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Critical carboxyl group(s) in Na^+-dependent cotransporters of the intestinal brush-border membrane

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We have previously provided functional evidence for a role of carboxyl group(s) in the mechanism of coupling of Na^+ and D-glucose fluxes by the small-intestinal cotransporter(s) (Kessler, M. and Semenza, G. (1983) J. Membrane Biol. 76, 27–56). We present here a study on the inactivation of the Na^+-dependent transport systems, but not of the Na^+-independent ones, in the small-intestinal brush-border membrane, by hydrophobic carbodiimides. Although marginal or insignificant protection by the substrates or by Na^+ was observed, the parallelism between Na^+-dependence and inactivation by these carbodiimides strongly indicates the role of carboxyl group(s) previously indicated. Contrary to the carboxyl group identified by Turner ((1986) J. Biol. Chem. 261, 1041–1047) in the sugar binding site of the renal Na^+/D-glucose cotransporter, the carboxyl group(s) studied here probably occur elsewhere in the cotransporter molecule.

Introduction

The study of structure-function relationships in the Na^+/D-glucose cotransporters of the small intestine and of renal tubuli is in its infancy. One of the approaches has been to investigate the in situ effect of selective or semi-selective reagents on their functions, i.e., on Na^+-dependent D-glucose transport and on Na^+-dependent phlorizin binding. Thus, in the small intestinal Na^+/D-glucose transporter, the occurrence of essential SH group(s) at the cytosolic and/or hydrophobic surface was deduced from its inactivation by organic mercurials [1] and from reversion by thiols of the inactivation by $HgCl_2$ [2,3]. Other essential thiol(s) and amino groups have also been noted [4]. In the course of these studies evidence was also produced that this transporter, as expected, is a *trans*-membrane protein [1,3].

The occurrence of one or more amino group(s) [5,6] and of one carboxyl group (in the kindered renal outer cortical transporter, Ref. 7) in the sugar binding site was shown by the inactivation by appropriate reagents and by the protection afforded by transportable monosaccharides. As to the Na^+ binding site, inactivation by tyrosine-specific reagents and the moderate protection by Na^+ render likely the participation of a tyrosine residue in the Na^+ binding site (in the Na^+/D-glu-

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; DCCD, *N*,*N'*-dicyclohexylcarbodiimide; EDAC, *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide; WRK, Woodwards reagent K: *N*-ethyl-5-phenylisoxazolium-3'-sulfonate.

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cose cotransporter of renal outer medulla [8]).

We [9] have produced functional evidence that the small-intestinal Na^+/D-glucose cotransporter is an asymmetric mobile channel (or pore) responsive to the membrane potential difference ($\Delta\psi$). The portion of the cotransporter the movement of which is related to the transfer of the substrates (or to the change in their accessibility from the two sides of the membrane) carries one or more negative charges, as shown by the type of $\Delta\psi$-response of the transinhibition by substrates [9], and by the lack of effect of $\Delta\psi$ on the rate of dissociation of phlorizin ($+Na^+$) from the cotransporter [10] (see Ref. 11 for the renal transporter). If (one of) the negative charge(s) is a COO^-, it could also be the reason for the sharp increase in K_m for D-glucose which is observed at acidic pH values [12]. (The K_m for the sugar is strongly affected by Na^+, see, for example, Ref. 9).

In the present paper we show that dicyclohexylcarbodiimide (DCCD), a well-known carboxyl group reagent, inactivates a number of small-intestinal Na^+-dependent transport systems, while not affecting Na^+-independent ones. The substrates of Na^+-dependent systems afford only marginal protection against DCCD inactivation. In spite of this, the parallelism observed between Na^+-dependency and DCCD inactivation supports the suggestion made earlier [9,13] that one or more carboxyl group(s) may play a central role in the coupling of fluxes in Na^+-dependent transport systems. Some of our observations have been reported in a preliminary form [14–16].

Materials and Methods

L-[3-^{3}H]Alanine (75 Ci/mmol), D-[6(n)-^{3}H]glucose (33.1 Ci/mmol), D-[U-^{14}C]fructose (325.8 mCi/mmol), γ-amino[2,3(n)-^{3}H]butyric acid (28 mCi/mmol) and β-[3(n)-^{3}H]alanine (34 mCi/mmol) were purchased from New England Nuclear, *N,N'*-dicyclohexyl[^{14}C]carbodiimide (57 mCi/mmol, L-[4,5-^{3}H]lysine (75 Ci/mmol) and L-[U-^{14}C]proline (290 mCi/mmol) were from Amersham. Glycine methylester monohydrochloride, *N,N'*-diisopropylcarbodiimide, *N,N'*-dicyclohexylcarbodiimide, *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide, Woodwards reagent K (*N*-ethyl-5-phenylisoxazolium-3'-sulfonate) and hydroxylamine were from Fluka. D-Mannitol, potassium thiocyanate, sodium thiocyanate, D-fructose and D-glucose were from Merck. L-Alanine, L-proline, L-lysine, and Tris (tris(hydroxymethyl)aminomethane) were from Sigma, Sepharose 4B from Pharmacia. All chemicals were reagent grade.

Vesicle preparation. Brush-border membrane vesicles were prepared from frozen rabbit small intestine, according to the Ca^{2+} precipitation procedure, as described elsewhere and used immediately after preparation [17,18]. Alternatively, the final pellet was suspended in 33 ml of 150 mM KCl, 50 mM Tris-HCl, pH 7.4 (4.5 mg protein · ml^{-1}) and passed through a Sepharose 4B column (15 cm, ∅ 7.5 cm) at a flow rate of 136 ml · h^{-1}, at 4° C, in the same buffer [19]. The gel filtration step was found to increase the stability of the vesicles and improve their purity by a factor of approx. 2, as shown by the sucrase activity which increased to a specific activity of approx. 2.2 units · (mg protein)$^{-1}$. The eluate containing the brush-border membrane vesicles was concentrated by centrifugation at 60000 × *g* for 30 min, and washed three times in MHT buffer (300 mM D-mannitol/10 mM Hepes-Tris (pH 7.0). The final brush-border membrane vesicle preparation was suspended in MHT buffer (approx. 30–40 mg protein · ml^{-1}) to which protease inhibitors were added from stock solutions (to the following final concentrations: pepstatin A 0.01 mg, chymostatin 0.01 mg, aprotinin 2 KIU/mg, bacitracin 0.1 mg per ml), frozen and stored in liquid N_2. The brush-border membrane vesicles were thawn shortly before use; they were found not to lose transport activity for at least one year.

Protein. Protein was determined with the Bio-Rad Protein Assay kit (Coomassie blue binding) according to Bradford [20].

Uptake into brush-border membrane vesicles. Uptake was measured essentially as described elsewhere [21], using Kessler's rapid incubation apparatus *, followed by quick filtration through Sartorius filters (∅ 0.65 μm). (two washings with cold stop solution).

Prior to the uptake proper, brush-border membrane vesicles were preincubated in MHT contain-

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ing either 200 mM NaCl or 200 mM KCl (approx. 10–20 mg protein · ml^{-1}) on ice for at least two hours. The uptake was measured at room temperature, by mixing 10 μl of the vesicle suspension with 10 μl of a mixture composed of 300 mM D-mannitol, 200 mM NaCl (or KCl), 10 mM Hepes-Tris (pH 7.0) and the radioactive substrate (see legends of the figures).

When the uptake was measured in the presence of inwardly directed cation and anion gradients, brush-border membrane vesicles were pre-incubated on ice in MHT buffer alone. Incubation was carried out in the presence of an initial gradient of NaSCN or KSCN (100 mM out, zero in), at the substrate concentrations indicated in the legends.

Reaction of brush-border membrane vesicles with unlabelled DCCD. 2 ml of either freshly prepared brush-border membrane vesicles or freshly thawn (at 37 °C) brush-border membrane vesicles were treated (final protein concentration: 2 mg · ml^{-1}) in 20 mM Mes-Tris, 100 mM D-mannitol either with DCCD alone (final concentration 0.4 mM or 2 mM for 0 to 30 min, added in 10 μl dioxane; the same amount of dioxane was added to the blanks) or with DCCD plus 100 mM NH_2OH or 100 mM glycine methyl ester (HCl). Unless stated otherwise, the final pH of the mixture was adjusted to 5.0 with Tris. The reaction was carried out at room temperature. The incubations were terminated at selected times by diluting the samples with 2 vols. of ice-cold 300 mM D-mannitol, 10 mM Hepes-Tris (pH 7.5) (which brought the pH to approx. 7.0). The vesicles were sedimented at 60000 × g for 30 min and washed twice with the same buffer at pH 7.0. They were finally suspended to about 10 to 20 mg · ml^{-1} in MHT buffer alone, or in MHT buffer containing either NaCl or KCl (200 mM each) (see previous section).

When the possible protection from inactivation by substrates or other ligands was tested, the sugar (100 mM), or the amino acid (100 mM), or phlorizin (10 mM), or NaCl (up to 250 mM), or ammonium acetate (100 mM) were added to the vesicles 10 min prior to adding DCCD, at room temperature. The rest of the reaction was carried out as described above.

Reaction of brush-border membrane vesicles with radioactive DCCD. Brush-border membrane vesicles (2 mg/ml) in 200 mM mannitol, 20 mM Mes-Tris (pH 5.0) were incubated with 25 μM ^{14}C-DCCD (5 mM stock solution in dioxane) for 0 to 30 min at room temperature. Incubations were terminated by diluting with 2 vols of ice-cold MHT buffer (pH 7.5). The vesicles were spun down (30 min at 60000 × g), washed in MHT buffer, pH 7.0 and suspended to approx. 2 mg/ml with the same buffer (protein content was measured after diluting). 50 μl were filtered on Sartorius filters and washed once with 3 ml 20% dioxane in water, which removed both unreacted DCCD and dicyclohexylurea. The radioactivity retained by the filters from ^{14}C-DCCD-treated brush-border membrane vesicles was corrected for the radioactivity retained from samples with ^{14}C-DCCD but without brush-border membrane vesicles (amounting to less than 10%).

Reaction with N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide (EDAC) and N-ethyl-5-phenylisoxazolium-3′-sulphonate (WRK, Woodwards reagent K). Vesicles were incubated with EDAC (10 or 50 mM), or with WRK (10 or 50 mM) for 15 min at room temperature in 20 mM Mes-Tris, 100 mM glycine methyl ester (pH 6.5) in a manner similar to that described above; simultaneously samples of brush-border membrane vesicles were reacted with DCCD, also at pH 6.5. Care was taken to keep the pH of the reaction mixture constant at 6.5. (The stock solution of EDAC was 0.5 M and adjusted to pH 6.5 with KOH; that of WRK was 0.5 M in 1 mM HCl).

Results

All experiments were repeated at least three or four times, with consistent results. Individual, representative experiments are reported in the figures.

The brush-border membrane vesicles (prepared from rabbit small-intestine by the Ca^{2+} precipitation method), are rich in carboxylate groups: a minimum of 600 nmol per mg protein was roughly estimated by titration [14]. Of these, approx. 22 nmol per mg protein (i.e., approx. 3.6%) react with ^{14}C-DCCD (Fig. 1), under conditions similar to those used in experiments on transport inactivation. Still, a minute fraction only of these DCCD-reactive carboxyl groups can be expected to be parts of the substrate binding sites or of the

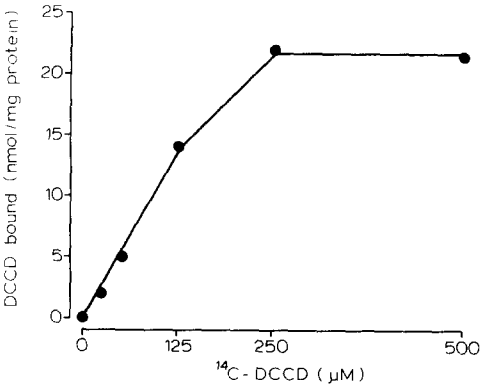

Fig. 1. Reaction of [^{14}C]DCCD with brush-border vesicles. The amount of irreversibly bound carbodiimide after 30 min at room temperature, as a function of the DCCD concentration is given. (Preliminary experiments had shown that, at each DCCD concentration, the reaction is complete within 30 min). For details, see Methods.

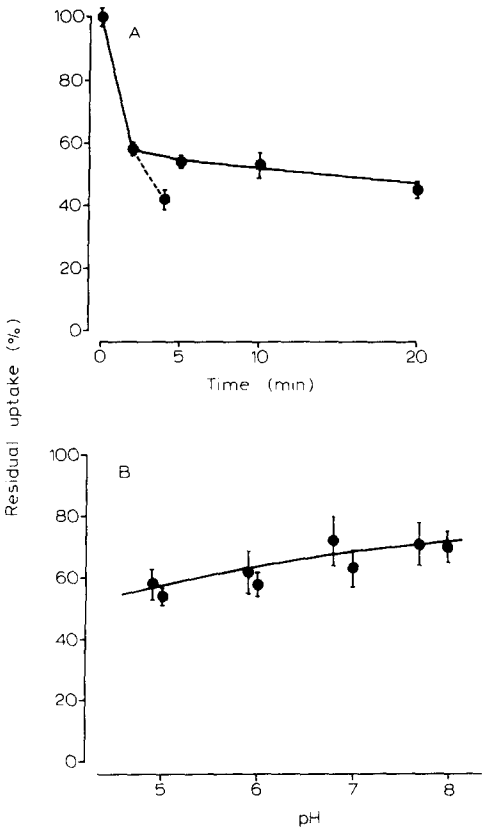

Fig. 2. Inactivation of initial Na^+-dependent D-glucose uptake by DCCD. The modification was performed in 20 mM Mes-Tris, 250 mM KCl, 100 mM glycine methyl ester, plus 100 mM D-glucose (identical results were obtained in the absence of glucose). For details, see Methods. (A) Residual D-glucose uptake (17 μM D-glucose influx after 2 s), after modification of brush-border vesicles by 400 μM DCCD for various times at pH 5.0. (●———●). Addition of an extra 400 μM DCCD after 2 min further decreases the uptake (●- - - - -●). (B) Residual Na^+-dependent D-glucose uptake after reaction of brush-border vesicles with 400 μM DCCD at different pH values. After reaction with DCCD the Na^+-dependent D-glucose influx was determined in each case at pH 7.0.

negatively charged (and presumably Na^+ binding) mobile portions of the cotransporters; these vesicles possess approx. 14 pmol of Na^+/glucose cotransporter per mg protein, as deduced from phlorizin binding [12].

Figs. 2 and 3A show the irreversible inactivation of Na^+-dependent D-glucose transport in vesicles following treatment with DCCD. The vesicles were first reacted with DCCD plus glycine methyl ester or NH_2OH in the absence of Na^+, washed free of excess reagents, preincubated in NaCl or KCl and finally tested for D-glucose uptake at low glucose concentration. Note that in this experimental setup a nonspecific change in Na^+ permeability or in electric conductance (which DCCD treatment may also produce) could not mimic an inactivation of the Na^+/D-glucose cotransporter(s) proper.

Furthermore, the nearly unchanged osmotic space of the vesicles (as deduced from the amount of D-glucose associated with them at the equilibrium, data not shown) and the unchanged low level of D-glucose uptake in the absence of Na^+ (Fig. 3A) shows that DCCD treatment did not grossly alter either the permeability or the geometry of the vesicles.

Although DCCD could in principle also react with groups other than COO^-, the inactivation of D-glucose transport by DCCD in Figs. 2 and 3A is, indeed, due to its reaction with carboxyl (or carboxylate) group(s). In fact: (i) The (limited) pH dependence of the reaction in the pH range 5–8, in which the vesicles are stable (Fig. 2A) is similar to that reported for the DCCD inactivation of proteins in which DCCD was found to react covalently with a carboxylate [22,23]. (ii) The conditions of the reactions were so as to favor specifically the reaction of DCCD with carboxyl, rather than with amino groups (low pH, presence of a

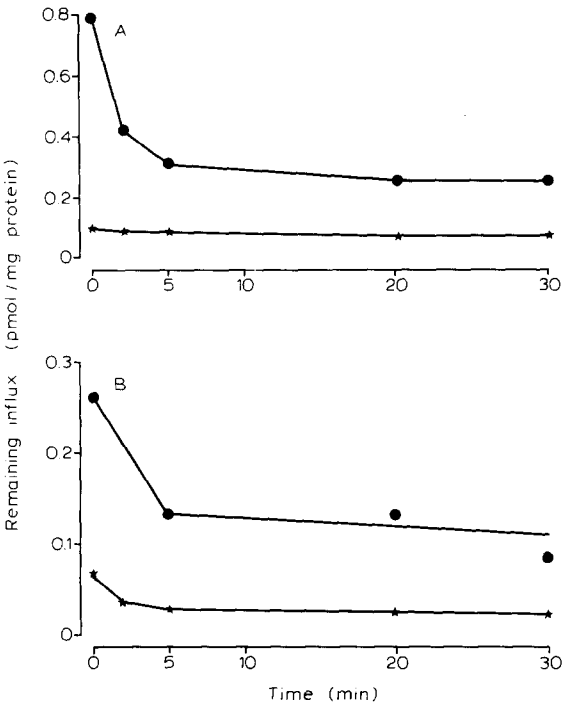

Fig. 3. Inactivation of two Na^+-dependent transport systems by DCCD. Brush-border vesicles were reacted with 400 μM DCCD for the times given in 20 mM Mes-Tris/100 mM mannitol/100 mM hydroxylamine (pH 5.0). After washing, the remaining influx (5 s) of D-glucose (1.5 μM, A) or of L-alanine (1.25 μM, B) was measured at pH 7.0, in the absence (★———★) or in the presence (●———●) of 200 mM Na^+.

TABLE I

INFLUENCE OF HYDROXYLAMINE ON DCCD-MODIFIED BRUSH-BORDER VESICLES

2 ml (4 mg protein) intestinal brush-border vesicles in 20 mM Mes/100 mM KCl/100 mM glycine methyl ester/100 mM mannitol (pH 5.0) were incubated for 30 min at room temperature in the presence or absence of 400 μM DCCD. After diluting with 5 ml ice-cold 300 mM mannitol and centrifugation (30 min at 60000 × g) the sedimented vesicles were resuspended either in 2 ml 10 mM Hepes-Tris/300 mM mannitol (pH 7.0) or in 2 ml 0.5 M hydroxylamine-hydrochloride (pH 7.0). The hydroxylamine containing fractions were incubated at room temperature for 20 or 60 min. All samples were subsequently diluted with 5 ml 300 mM mannitol/10 mM Hepes-Tris (pH 7.0), sedimented and washed twice in the same buffer. Initial D-glucose uptake was determined as described in Fig. 2. (Transport was measured in triplicate).

Treatment	Initial Na^+-dependent D-glucose influx ($pmol \cdot mg^{-1} \cdot (2\ s)^{-1}$)	Remaining transport activity (%)
Control	3.44 ± 0.01	
Control, then 20 min 0.5 M NH_2OH	3.04 ± 0.06	
Control, then 60 min 0.5 M NH_2OH	2.77 ± 0.14	
400 μM DCCD	1.49 ± 0.15	43 ± 4
DCCD, then 20 min 0.5 M NH_2OH	1.46 ± 0.06	48 ± 2
DCCD, then 60 min 0.5 M NH_2OH	1.25 ± 0.04	45 ± 3

large excess of appropriate nucleophiles). (iii) DCCD may have reacted with tyrosine or very reactive serine residues. However, reaction with these residues, if it had occurred, should have been reversed by hydroxylamine [24]. Table I shows, however, that NH_2OH failed to reactivate DCCD-inactivated vesicles (as compared to controls treated with NH_2OH alone). (iv) DCCD may have reacted with one or more of the essential thiols known to occur in this cotransporter [1–4]. As the reaction of these thiols with $HgCl_2$ is known to be – at least partially – reversible [2], the vesicles were reacted with $HgCl_2$ first, then with DCCD (or vice versa), then with the permeant dithioerythritol and tested for D-glucose uptake activity (Table II). Clearly, reaction of thiols with $HgCl_2$ prior to the reaction with DCCD did not lead to any protection of the cotransporter against inactivation by the latter reagent.

In a previous study [5] we observed that hydrophobic isothiocyanate derivatives reacted with the amino group(s) presumed to occur in the sugar binding site more efficiently than similar, but hydrophilic isothiocyanates. The difference was due neither to a difference in the intrinsic reactivity of the reagents nor in their different permeation across the membrane. It was thus taken to indicate the presence of hydrophobic structures in the neighbourhood of the reactive amino group(s). We thus tested, in addition to the hydrophobic DCCD, the equally hydrophobic, *N,N'*-diisopropylcarbodiimide which inactivated similarly (data not shown). In comparison, hydrophilic reagents, such as EDAC and Woodwards reagent K either did not inactivate the cotransporter, or were much less efficient than DCCD (Table III).

TABLE II

DCCD INACTIVATION IN THE ABSENCE OR PRESENCE OF MERCURIC IONS

Brush-border vesicles were reacted at pH 5.0 with 25 μM $HgCl_2$ (in analogy to Ref. 2), with 400 μM DCCD and with 20 mM DTE (5 min on ice) under the same conditions as described in Table I. Initial D-glucose uptake was determined as described in Fig. 2. (transport was measured in triplicate). DTE, dithioerythritol.

Treatment of the vesicles	Initial Na^+-dependent D-glucose influx ($pmol \cdot mg^{-1} \cdot (2\ s)^{-1}$)	Remaining transport activity (%)
Control	5.38 ± 0.03	
Control; DTE	5.53 ± 0.46	
$HgCl_2$ 30 min	2.05 ± 0.05	38 ± 1
$HgCl_2$ 30 min; DTE	3.47 ± 0.04	63 ± 5
DCCD 30 min	1.99 ± 0.05	37 ± 1
DCCD 30 min; DTE	2.17 ± 0.08	39 ± 4
Control; DTE	5.53 ± 0.46	
$HgCl_2$ 0 min; DTE	5.14 ± 0.11	
$HgCl_2$ 30 min; DTE	3.47 ± 0.04	
DCCD 30 min; DTE	2.17 ± 0.08	39 ± 4
DCCD 30 min; $HgCl_2$ afterwards; DTE	2.18 ± 0.10	42 ± 2
$HgCl_2$ first; then DCCD for 30 min; then DTE [a]	1.26 ± 0.23	36 ± 8

[a] In this sample, $HgCl_2$ which blocks SH-groups immediately [2] was added prior to DCCD in order to allow a complete reaction before DCCD begins to attack nucleophiles.

The small-intestinal brush-border membrane is endowed with a number of (co)transporters of different characteristics. Whereas the Na^+/D-glucose transport system(s) use Na^+ as a co-substrate, the transporter of D-fructose does not (see, for example, Refs. 25 and 26); likewise, whereas the transport of L-alanine is Na^+-dependent [27], those of L-lysine [28] of β-alanine [29] and of γ-aminobutyrate (Fig. 4) (see the points at zero reaction times in the DCCD experiment), are not. Now, if one or more carboxylate group(s) indeed play a central role in Na^+/substrate cotransport [9,10,13] one should expect that Na^+-dependent systems should generally be sensitive to DCCD,

TABLE III

EFFECT OF VARIOUS CARBOXYL GROUP REAGENTS ON THE Na^+-DEPENDENT INFLUX OF D-GLUCOSE IN BRUSH-BORDER MEMBRANE VESICLES

The modifications of brush-border vesicles by DCCD, EDAC and WRK for 15 min at pH 6.5 and the D-glucose influx determinations were done as described in the legend of Fig. 2 and under Methods. The pH of the WRK incubation mixture was held constant during the reaction by adding 1 mM HCl. After the 50 mM WRK-modification the brush-border vesicles looked grey-yellowish and the protein content was reduced by about 25%.

Reagent	Remaining transport activity (%)
Control	100
800 μM DCCD	41 ± 2
10 mM EDAC	91 ± 4
50 mM EDAC	75 ± 2
10 mM WRK	96 ± 4
50 mM WRK	46 ± 1

whereas Na^+-independent systems should not. This was found to be indeed the case: whereas the transport systems for D-glucose (Figs. 2 and 3A)

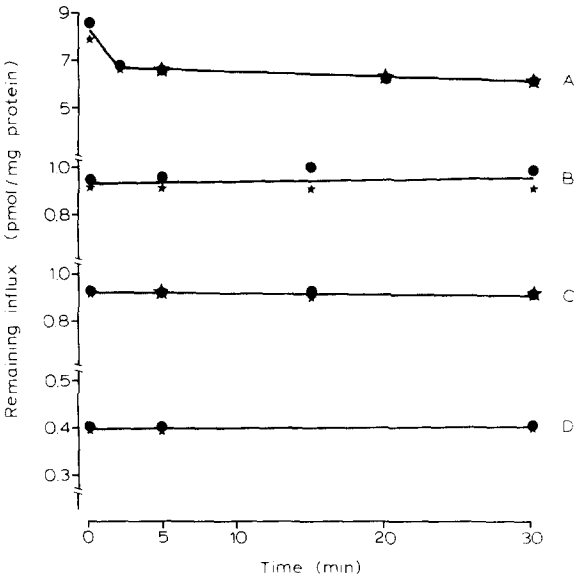

Fig. 4. Lack of effect of DCCD on some Na^+-independent transport systems of brush-border membranes. Brush-border vesicles were reacted for various times with DCCD, as in Fig. 3. After washing, the remaining influx (5 s) of D-fructose (87 μM, A), or β-alanine (1.48 μM, B), or γ-aminobutyrate (1.42 μM, C), or L-lysine (0.7 μM, D) was measured in the absence (★——★) or in the presence (●——●) of 200 mM Na^+.

or for L-alanine (Fig. 3B) or for proline (data not shown) are inactivated by DCCD, those for D-fructose, or for L-lysine, β-alanine or γ-aminobutyrate are not (Fig. 4).

Discussion

The results presented above clearly show that one or more carboxyl group(s) are essential for the functioning of Na^+-dependent D-glucose cotransporter, and also of at least another Na^+-dependent transport system of the same membrane (L-alanine). Contrary to this, none of the non Na^+-dependent transport systems investigated occurring in the same membrane and acting on similar substrates (D-fructose, L-lysine, β-alanine and γ-aminobutyrate) is inactivated by DCCD. These observations are circumstantial but fairly strong evidence for the postulated role of one or more carboxylate group(s) and for the suggested mode of operation of Na^+-dependent transport and flux coupling; they agree with and complement the functional criteria presented earlier [9,10,13].

Little information is presently available as to the location of this (these) carboxyl group(s) within (or in the immediate neighbourhood of) the cotransporter. In fact, only marginal protection against inactivation by DCCD or by EDAC was afforded by the simultaneous presence of up to 100 mM D-glucose or D-galactose and 250 mM NaCl (vs. KCl or choline chloride) at either pH 5.0 or 6.5; Na^+ being present at the same concentrations on both sides of the membrane with $\Delta\psi = 0$, or being present at the outer side alone with $\Delta\psi \ll 0$ (negative on the inside; these conditions should make the sites binding Na^+ and the glucose better accessible from the outside [9,12]). The small efficiency (if any) of Na^+ or the sugar in protecting the carboxylate(s) from reacting with DCCD may indicate that this (these) group(s) may not be involved in the binding of the cation or of the sugar. However, it may indicate instead that the diffusion of DCCD onto the Na^+ or sugar binding site is slow (involving perhaps a hydrophobic interaction) as compared with the reaction proper; if so, no detectable protection by the cation or the sugar should be expected for the slow rate-limiting step. Finally, one more possible mechanism whereby a substrate may not efficiently protect the substrate binding site from being irreversibly inactivated can be an (unsuspected) low affinity of the substrate for the site. In fact, a low affinity need not be reflected by the K_m: for example, lactose permease is not protected by lactose ($K_d/K_m = 56$) against inactivation by *N*-ethylmaleimide, whereas it is protected by other substrates, having a K_d/K_m equal to one, such as thiodigalactoside, *p*-nitrophenyl-β-galactoside etc. [30]. Thus, whatever the actual reason, lack of protection by Na^+ need not indicate that this carboxylic group is not a part of the Na^+ binding site(s) (along, of course, other ligands, such as Tyr-OH). It should be noted that the protection afforded by Na^+ against the inactivation of the kidney cotransporter by tyrosine reagents is also quite small [8].

An attractive possibility is that DCCD may react with a carboxylate of the 'Na^+ rail' (see Ref. 13) which, although partaking in a bucket-like fashion to the transport, may not to have a sufficiently high affinity for the cation. It can be ruled out fairly safely that the DCCD-reactive carboxyl group(s) reported in the present paper should correspond to the carboxyl group identified by Turner [7] in the renal Na^+/D-glucose cotransporter, since the latter reacts with water soluble carbodiimides, and is protected by D-glucose or phlorizin. (The carboxyl group(s) studied by us do neither). Turner's carboxyl group is probably involved in the interaction with the sugar.

Whatever the exact location of the DCCD-reactive carboxylate(s) with respect of the Na^+ binding site (or the 'Na^+ rail') may be, we favour the idea that it belongs to the cotransporter protein. However, we cannot rule out the possibility that it may belong to the polar head of a lipid (i.e., of a phosphatidylserine), perhaps lining a protein-lipid chain of ion pairs; i.e., the 'Na^+ rail' need not consist of protein alone. (Lev's group [31] has already indicated the possibility that phospholipids may occur in the walls of channels in membranes.) Whether or not lipids partake in the transport event, their proximity to the transport may be the reason why hydrophobic, but not hydrophilic carboxylate reagents inactivate the critical carboxylate(s) in Na^+/D-glucose cotransporter (Table III). Similar considerations can be made in connection with the amino group in-

volved in the binding of D-glucose which is also preferentially inactivated by hydrophobic, rather than hydrophilic amino group reagents [5].

Clearly, the mapping of cotransporters by way of inactivation by selective reagents is based on an inherently indirect approach. The individual details in the conclusions derived from the present and similar studies may well have to be revised in the future. As of to-day, however, all observations are compatible with the functional model of the Na^+/D-glucose cotransporter presented earlier [13]. It was suggested to be composed of two neighbouring 'rails' of functional groups bound to and supported by fairly rigid protein structures (e.g., helices), 'rails' and helices being perpendicular to the plane of the membrane; whenever one substrate (Na^+ or D-glucose) gets engulfed into its own rail, it would force the loosening up and opening of the other rail to the other substrate. Increase in affinity for co-substrate and flux coupling would thereby result. (See Ref. 13 for more details on the model.) In the absence of substrates, the functional groups in each rail would mutually saturate their valencies (by forming, e.g., ion pairs or H-bonds). Although limited, the information presently available on the functional groups in the Na^+/D-glucose cotransporter tends to support our model: in the binding (and/or transport) of D-glucose, amino [5] and carboxyl [7] groups are probably involved, whereas in the binding (and/or transport) of Na^+ tyrosine(s) [8] and carboxyl groups (present paper) are.

Also the recent identification of an essential Arg in the Na^+-dependent functioning of the renal D-glucose cotransporter(s) (and of the Na^+-dependent cotransporters for phosphate and for L-alanine [32]) agrees with and supports the model of Ref. 13: an Arg may be essential in transmitting the 'loosening up' of the one rail to the other and/or may occur in a critical position of one of the ion pairs exchanging Na^+ in a bucket-like fashion.

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