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Histidyl Residues at the Active Site of the Na/Succinate Cotransporter in Rabbit Renal Brush Borders

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Summary. Mono-, dicarboxylic acid-, and D-glucose transport were measured in brush border vesicles from renal cortex after treatment with reagents known to modify terminal amino, lysyl, ε-amino, guanidino, serine/threonine, histidyl, tyrosyl, tryptophanyl and carboxylic residues. All three sodium-coupled cotransport systems proved to possess sulfhydryl (and maybe tryptophanyl sulfhydryl, disulfide, thioether and tyrosyl) residues but not at the substrate site or at the allosteric cavity for the Na coion. Histidyl groups seem to be located in the active site of the dicarboxylic transporter in that the simultaneous presence of Na and succinate protects the transporter against the histidyl specific reagent diethylpyrocarbonate. Lithium, which specifically competes for sodium sites in the dicarboxylic acid transporter, substantially blocked the protective effect of Na and succinate. Hydroxylamine specifically reversed the covalent binding of diethylpyrocarbonate to the succinate binding site. The pH dependence of the Na/succinate cotransport is consistent with an involvement of histidyl and sulfhydryl residues. We conclude that a histidyl residue is at, or is close to, the active site of the dicarboxylate transporter in renal brush border membranes.

Key Words renal brush border membranes · carboxylic acid transport · glucose transport · Na-coupled cotransport · histidyl, sulfhydryl, tyrosyl and tryptophanyl residues

Introduction

The sodium-coupled transport systems for sugars, amino acids, and carboxylic acids found in brush border membranes of renal cortex are distinct entities with only minor overlapping substrate specificities (Kippen et al., 1979; Wright et al., 1980; Mircheff et al., 1982; Nord et al., 1982, 1983; Ullrich, Rumrich & Kloss, 1982; Sheridan, Rumrich & Ullrich, 1983). In the present study we have attempted to characterize the active sites for sodium and substrate binding to the transporters using group-specific reagents. Our approach was to determine the effects of reagents on Na-dependent transport of D-glucose, L-lactate and succinate, and to assess whether inhibitory effects were blocked by Na and/or substrate. Although a number of reagents blocked transport, in particular sulfhydryl, tyrosyl, and tryptophanyl group reagents, we have found that only the inhibition of diethylpyrocarbonate (ethoxyformic anhydride, DEP) of Na/succinate cotransport is prevented by a simultaneous presence of succinate and Na in the reaction medium. This suggests that histidyl residues are involved in the active site for succinate binding and/or transport.

Materials and Methods

Renal cortical brush border membrane vesicles (BBMV) were prepared from New Zealand white rabbits using a calcium-precipitation procedure previously described (Wright et al., 1980). Brush borders were resuspended at 15 to 20 mg protein/ml in a standard solution, BS, that contained 350 mM sorbitol and 20 mM KH_2PO_4/K_2HPO_4 buffered at pH 6.4. This pH stabilizes the N-carbethoxyhistidine residue. Tris buffers were avoided, since they were known to interact with DEP (Berger, 1975). The membranes were stored in liquid nitrogen for up to 3 weeks before carbethoxylation and transport studies without major loss of transport capacity. The purity of brush border membranes was determined as before (Wright et al., 1980) by assay of alkaline phosphatase—a marker for brush border membranes, ouabain-sensitive KpNPPase—a marker for basolateral Na,K-ATPase, and succinate dehydrogenase—a mitochondrial marker enzyme. Protein was assayed by BioRad method (BioRad Labs., Richmond, Calif.). Brush border membranes were enriched at least 10-fold and the other two membrane markers were reduced by factors of 2 or more compared with the original homogenate.

Carbethoxylation and Decarbethoxylation

The structure of DEP is shown in Fig. 6*a* and the reaction of diethylpyrocarbonate with imidazole is shown in Fig. 6*b*. The on and off reactions with amino acid residues in general are coined

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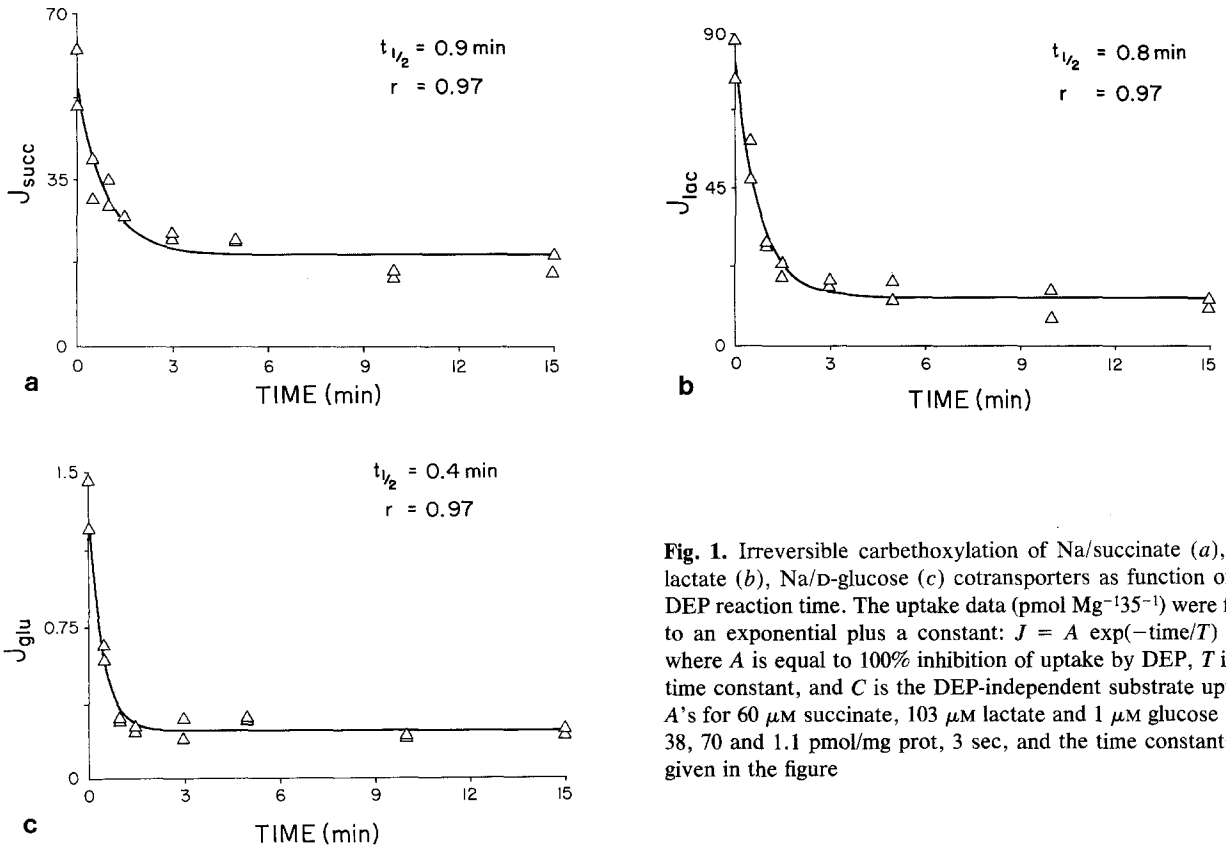

Fig. 1. Irreversible carbethoxylation of Na/succinate (*a*), Na/lactate (*b*), Na/D-glucose (*c*) cotransporters as function of the DEP reaction time. The uptake data (pmol $Mg^{-1}35^{-1}$) were fitted to an exponential plus a constant: $J = A \exp(-\text{time}/T) + C$ where A is equal to 100% inhibition of uptake by DEP, T is the time constant, and C is the DEP-independent substrate uptake. A's for 60 μM succinate, 103 μM lactate and 1 μM glucose were 38, 70 and 1.1 pmol/mg prot, 3 sec, and the time constants are given in the figure

carbethoxylation and decarbethoxylation (*see* e.g. Miles, 1977). Frozen membranes were thawed at 32°C and then kept at room temperature (20 to 22°C) for an hour. All following steps were performed at room temperature. 200 μl of the membrane protein suspension were pipetted into 50-ml centrifuge tubes. Fresh diethylpyrocarbonate was diluted 1 : 1 in ethyl alcohol. This solution was further diluted 1 : 250 in BS, mixed and 200 μl of this DEP buffer were added immediately to the vesicle solution and mixed. The reaction was usually allowed to proceed for 10 min and then stopped by addition of 40 ml BS at room temperature. In control experiments an equivalent amount of fresh DEP was added to the first wash solution. The membrane vesicles were pelleted, resuspended, washed and resuspended in BS. The wash procedure diluted the DEP by more than a factor of 10^5 and resulted in a loss of about 30% of the membrane protein. The concentration of the finally resuspended vesicles was about 15 mg/ml. In control experiments parallel to DEP treatment, 0.2% alcohol alone did not affect the transport of succinate, lactate, or glucose. After resuspension the membranes were held at room temperature for an hour before assay for transport activity.

Decarbethoxylation was produced by NH_2OH. 200 mM sorbitol in the first wash solution were replaced by 100 mM NH_2OH and both the control and the NH_2OH containing wash mixture were left for 30 min before continuing the wash procedure.

Transport Measurements

A rapid filtration technique described elsewhere (Wright et al., 1980) was employed. The final uptake buffer contained 67 mM NaCl or KCl, 225 mM sorbitol, and 20 mM KH_2PO_4/K_2HPO_4, pH 6.4, 103 μM L-lactate, or 60 μM succinate or 1 μM D-glucose. Uptake was terminated after 3 to 5 sec by addition of 1000 μl ice-cold BS-medium, filtering the cold suspension through a 0.45 μM SS filter (BA85, Schleicher & Schuell, N.H.) and washing the filter with an additional 4 ml of ice-cold BS. The radioactivity trapped on the filter was assayed by a conventional liquid scintillation counting procedure. The Na-dependent uptake of substrate was calculated as the difference between transport in presence of Na and K from duplicate determinations. All errors given as ± the standard error of the mean.

pH Dependence of Na-Coupled Substrate Transport

Membranes stored in 350 mM sorbitol, 50 mM KCl and 5 mM Tris/HEPES, pH 7.4, were used in a study of external pH on the Na-dependent uptake of succinate, lactate and glucose. The final composition of the uptake buffer was 225 mM sorbitol, 67 mM NaCl or KCl, 50 mM KCl, 20 mM buffer and substrates in tracer amounts. The pH was varied from 4.0 to 9.0 and from 8.0 to 11.5 using boric acid/MES/HEPES/Tris and CAPS/Tris/KOH buffers. The actual pH of the transport mixture was measured with a pH-meter by mixing membranes and transport solution in the right proportion without tracer substrates.

Materials

[2,3-^{14}C]-succinic acid (118 mCi/mmol) and D-[6-^{3}H(N)]-glucose (33.1 Ci/mmol) were purchased from New England Nuclear (Boston, Mass.). L-[U-^{14}C]-lactate (90 mCi/mmol) was obtained

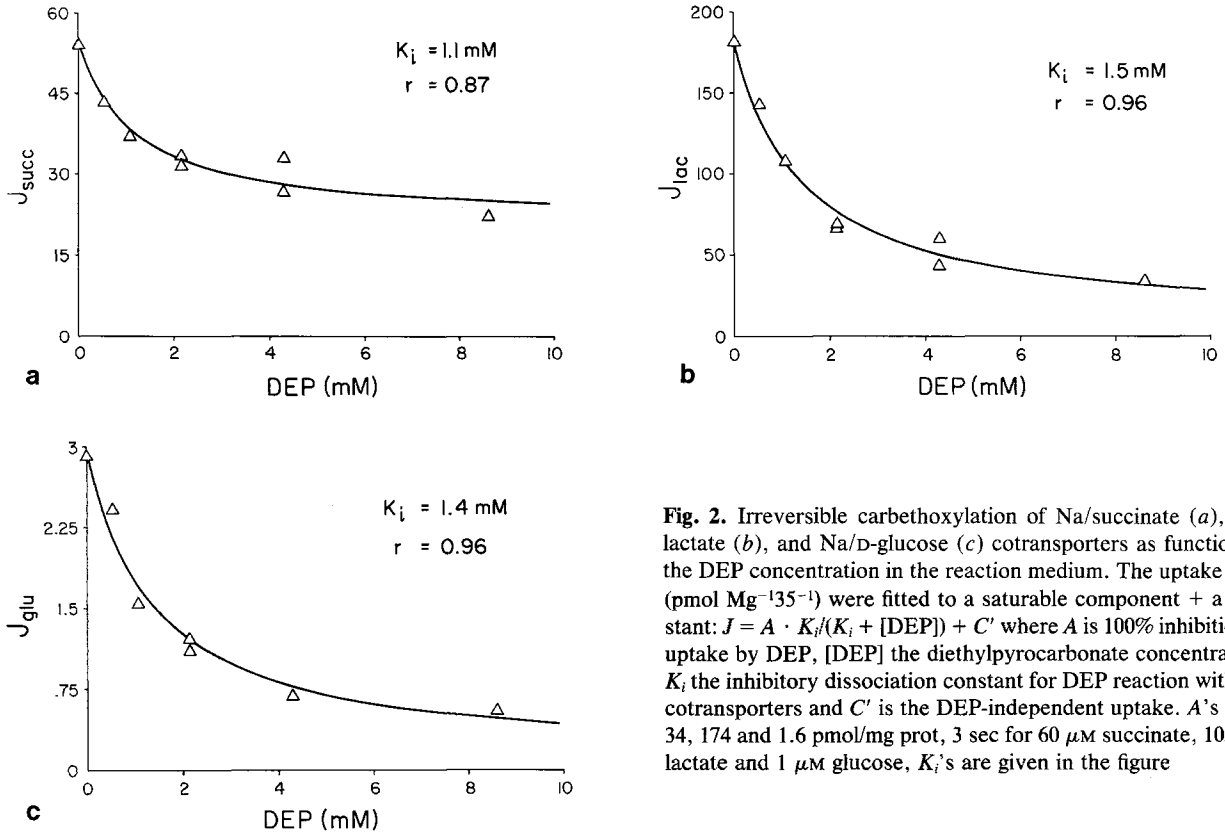

Fig. 2. Irreversible carbethoxylation of Na/succinate (*a*), Na/lactate (*b*), and Na/D-glucose (*c*) cotransporters as function of the DEP concentration in the reaction medium. The uptake data (pmol $Mg^{-1}35^{-1}$) were fitted to a saturable component + a constant: $J = A \cdot K_i/(K_i + [DEP]) + C'$ where A is 100% inhibition of uptake by DEP, [DEP] the diethylpyrocarbonate concentration, K_i the inhibitory dissociation constant for DEP reaction with the cotransporters and C' is the DEP-independent uptake. A's were 34, 174 and 1.6 pmol/mg prot, 3 sec for 60 μM succinate, 105 μM lactate and 1 μM glucose, K_i's are given in the figure

from ICN (Irvine, Calif.). Diethylpyrocarbonate (DEP), in flasks of 5 ml all with the same lot no., and DL-dithiotreitol (DTT) were bought from Sigma Chemical Co. (St. Louis, Mo.). DEP was checked spectrophotometrically according tp Roosemont (1978) and found 98% pure. Dichlorotriazinyl amino fluorescein was purchased from Molecular Probes (Junction City, Ore.). All other chemicals were of the highest commercial grade available.

Abbreviations

Tris-Tris (hydroxymethyl)aminomethane; MES-2-(N-morpholino)ethane sulfonic acid; CAPS-Cyclohexylaminopropane sulfonic acid; HEPES-N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid. Also *see* Appendix.

Results

In preliminary experiments we tested the reactivity of several group-specific reagents towards the Na/succinate and Na/lactate transporters. To find the residues most likely situated in the substrate- and/or Na sites we also looked for protection of the transporters by including substrate and Na in the reaction medium of parallel experiments. From these experiments it can be concluded that sulfhydryl groups (and maybe tryptophanyl and tyrosol groups) are important conformers but these are not located in the substrate or co-ion sites. A more detailed account of the experiments is given in the Appendix. The results in the Appendix are pertinent and support the conclusion we draw that histidyl residues are likely groups in the active site of the Na/succinate transporter. The following results focus on DEP, since the interaction between DEP and the Na/succinate transporter could be specifically protected by the presence of succinate and Na in the reaction medium.

Inhibition by Carbethoxylation

Figure 1 shows the time dependence of the irreversible inhibition of succinate, lactate and glucose transport by 8.6 mM DEP. The half-times for inhibition were 0.8 min for L-lactate, 0.4 min for glucose, and 0.9 min for succinate transport. The concentration dependence of DEP inhibition on transport was measured over a concentration range of 0.0 to 20 mM DEP. The K_i for DEP inhibition for all three systems was approximately 1.3 mM (*see* Fig. 2). 8.6 mM DEP and 10-min reaction time was used in all subsequent DEP experiments and the inhibitions obtained are given in Table 1. The incomplete inhibition of succinate transport by DEP may be due in part to reversibility of the reaction. Carbethoxyla-

Table 1. Inhibition of Na-dependent lactate, succinate and glucose uptake by DEP[a]

	DEP Inhibition		
	L-lactate	Succinate	D-glucose
%	80	54	75
SEM	±2	±3	±5
n	9	9	7

[a] BBMV were reacted with 8.6 mM DEP for 10 min. The concentrations of substrates were 103, 60, and 1 μM for lactate, succinate and glucose, respectively, and the transport rates were 120 ± 12, 130 ± 10, and 1.6 ± 0.2 pmol/mg prot, 3 sec. The controls are 0% inhibition. SEM is standard error of the mean; *n*, number of experiments.

tion of histidyl groups is a slowly reversible process (Miles, 1977) and decarbethoxylation may have taken place between the reaction with DEP and the actual measurement of transport (4 to 6 hr).

DTT (10 mM) was added to the first wash in three DEP inhibition experiments. The subsequent uptakes of succinate and lactate were equal to those in non-DTT-treated controls, which indicates that the inhibition by DEP is not due to a reaction with sulfhydryl groups.

Substrate Protection

Only succinate in the DEP-reaction medium gave protection of transport. Thus with 3.3 mM succinate and 50 mM Na in the reaction medium, the succinate transporter was protected 90 ± 8% (n = 9). Succinate at 3.3 mM did not protect either lactate or glucose transport and neither did lactate (50 mM) or glucose (10 mM) in the presence of 50 mM Na protect any of the three transporters. Specifically, 50 mM lactate plus 50 mM Na in the reaction medium only reversed the DEP inhibition of lactate transport by less than 2% (n = 2) and 10 mM glucose plus 50 mM Na reversed the DEP inhibition of glucose uptake less than 5% (n = 2). Figure 3 shows the concentration dependence of succinate protection from DEP attack at the succinate transporter based on data from 3 experiments where the succinate concentration in the reaction mixture ranged from 0.03 to 10 mM. The apparent dissociation constant for the protective action of succinate at the succinate transporter against irreversible inhibition by DEP was 0.45 mM. This value is comparable with the dissociation constant for succinate transport (Wright et al., 1983).

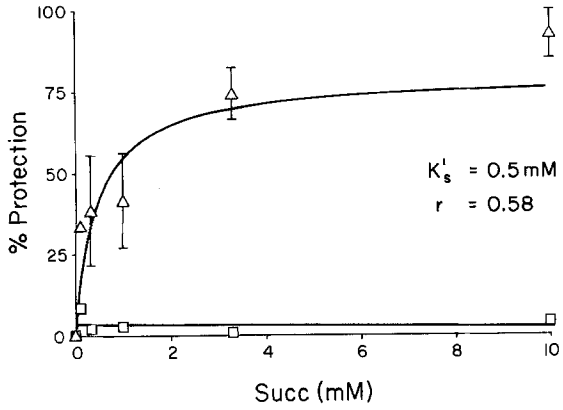

Fig. 3. Protection of the Na/substrate cotransporters for succinate, lactate and glucose by succinate in a concentration range from 0 to 10 mM with 50 mM Na in the diethylpyrocarbonate reaction medium. The DEP concentration was 8.6 mM and reaction time 10 min. The data for succinate uptake (△) were fitted to a hyperbola and the apparent dissociation constant for protection K_s was 0.45 mM succinate. Data for lactate and glucose uptake are lumped together (□) and show that succinate in the presence of sodium does not protect the lactate or glucose cotransporters. [The protection by succinate at 3.3 mM in the above 3 experiments (80%) was low compared to 6 other experiments in which succinate (3.3 mM) gave 100% protection.]

Na^+ Protection

Na^+ in the reaction medium is an obligatory requirement for the protection of the succinate transporter from DEP attack. Replacing all Na with K ions completely prevented the protective effect of succinate. Figure 4 depicts results from an experiment in which the final concentration of Na ranged from 0 to 88 mM in the DEP reaction medium and with a fixed succinate concentration of 3.3 mM. Data from two experiments were fitted to the Hill equation and mean values of the parameters were K_p^{Na} = 8.6 mM and n = 2.3; K_p^{Na} is the apparent dissociation constant for the protective action of Na and n is the Hill coefficient. The value of the dissociation constant compares with the K_m^{Na} of 32.5 mM for transport (Wright et al., 1983). A Hill coefficient of 2.3 is indicative of multiple sodium sites and this is in complete agreement with results obtained from kinetic studies that yielded a Hill coefficient of 2.3 (Wright, Kippen & Wright, 1982).

Malate Protection

A Na-coupled transport of malate presumably via the dicarboxylic cotransporter has been demonstrated by cyanine-dye technique for BBMV from kidney (Wright et al., 1981; Kragh-Hansen, Jørgen-

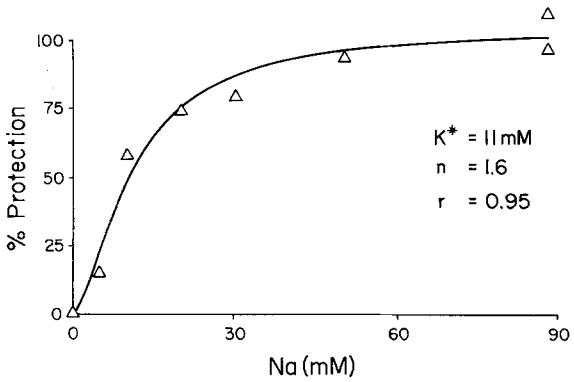

Fig. 4. Protection of the Na/succinate cotransporter by Na ions in the concentration range from 0 to 88 mM with 3.3 mM succinate in the DEP reaction medium. The reaction medium had 8.6 mM DEP and the reaction lasted 10 min. The data were fitted to Hill's equation. The protection was found to be 100% at 72 mM Na and going asymptotically towards 102% protection and with a dissociation constant for Na protection. K_m^* of 11.1 mM Na and a Hill coefficient of 1.6

Table 2. Reversal of DEP inhibition by NH_2OH[a]

	Decarbethoxylation with NH_2OH		
	L-lactate	Succinate	D-glucose
%	5.4	65	4.5
SEM	±1.4	±15	±1.9
n	4	4	3

[a] 100 mM NH_2OH replaced 200 mM sorbitol in the first wash. The mixture of wash medium (40 ml) and DEP reaction medium (400 μl) was left for 30 min at room temperature before carrying on with the rinse. The concentrations of lactate, succinate and glucose were 103, 60, 1 μM and the transport rates in the absence of DEP were 110 ± 20, 100 ± 20 and 1.8 ± 1.3 pmol/mg prot, 3 sec. The percent decarbethoxylation was obtained from the transport rates measured in membranes i) untreated with DEP, ii) treated with DEP, and iii) treated with DEP and then with NH_2OH.

sen & Sheikh, 1982). The specificity of succinate protection was tested by using DL-malic acid at 3.3 mM (pH 6.4) instead of succinate. Malic acid gave 83% protection. This is in accordance with an inhibitory dissociation constant for malate at the Na/succinate cotransporter; K_i, malic = 0.07 mM *vs.* K_i, succ = 0.05 at pH 7.5 (Wright et al., 1980). Surprisingly, malate (3.3 mM) in the presence of Na in the DEP reaction medium protected the lactate cotransport by 38% in contrast to lactate itself which, as mentioned earlier, did not protect at concentrations as high as 50 mM. Although malic acid is an analog of lactate the result is somewhat unexpected but has not yet been further investigated. Malic acid did not protect the Na/glucose cotransporter in the presence of Na.

Li Ions on Na Protection

The lithium ion is known to strongly inhibit the renal Na/succinate cotransporter with a K_i of 1 mM (Wright, Wright, Hirayama & Kippen, 1982). Li at 4 mM was included in the DEP reaction medium in two experiments with different batches of kidney membranes and the medium further contained the two mutually required protectants, Na and succinate at 50 and 3.3 mM. Li reduced by 35 and 72% the protective effect of Na and succinate. The effect of Li can simply be explained as preventing Na from inducing a conformational change which favors succinate binding at the site of DEP attack. As expected, 4 mM Li had no effect on glucose and lactate cotransporters in membranes reacted with DEP in the presence of 3.3 mM succinate and 50 mM Na.

Hydroxylamine Decarboxylation

The reversibility of DEP inhibition was tested using hydroxylamine for the three cotransporters. Hydroxylamine reversal of DEP effects in enzymes is considered indicative of reaction with histidyl groups (e.g. Miles, 1977). It can be seen from Table 2 that only DEP inhibition of succinate cotransport by DEP was significantly reversed (65%), thus supporting the notion of histidyl groups in the active site of the Na/succinate transporter.

pH Dependence of Na/Substrate Cotransport

The interpretation of effects of changing pH on enzyme kinetics and substrate transport is rather complex (Knowlers, 1976). The complexity increases even further when studying multi-sited cotransport systems such as the Na/succinate cotransport. In spite of these obstacles, slope changes in the initial rate of a cotransported substrate within a pH range may still be used in a cautious approach of identifying the amino acid residues that are likely to be involved in the active site of an enzyme (Tipton & Dixon, 1979) or a cotransporter.

Figure 5 shows the initial rates of succinate uptake by the Na/succinate cotransporter as a function of pH. The broken line is succinate cotransport after correcting for disappearance of the dibasic succinate species at low pH, which is taken to be

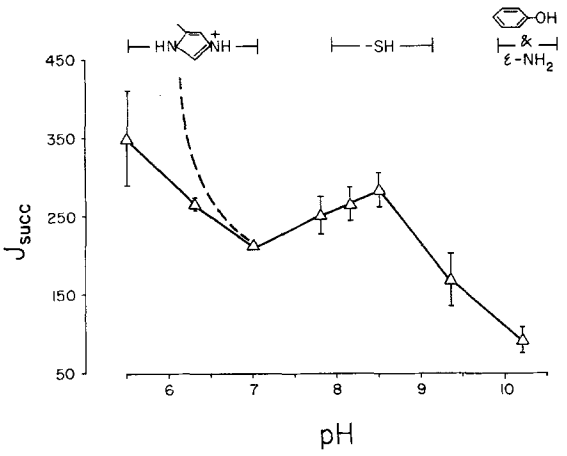

Fig. 5. Initial rates of Na-dependent succinate uptake (pmol/mg prot, 3 sec) in rabbit kidney BBMV as a function of pH. The concentration of succinate was 60 μM. The mean of duplicate determinations from two independent experiments are presented. The broken line gives uptake rates corrected for disappearance of dibasic succinate with falling pH. The fraction of succinate in dibasic form, Fract, at varying pH is given by: Fract $= (A \cdot B)/[1 + A \cdot B + B]$, where A is equal to $10^{(pH-4.2)}$, B to $10^{(pH-5.6)}$ and the pK_a's for succinate are 4.2 and 5.6. Negative slopes are likely to relate to succinate binding at the transporter and positive slopes to the Na cotransporter interaction. The pK_a interval for imidazole, sulfhydryl, tyrosine and lysyl ε-amino residue from model systems are indicated at the top. These intervals can be wider for residues in proteins. *See* text for further inferences

the only substrate for cotransport (Wright et al., 1980). The negative slope in Fig. 5 from 5.5 to 7.0 could be interpreted as titration of a histidyl residue involved in the binding of succinate, and the negative slope from 8.4 to 10.2 might be due to titration of a sulfhydryl residue participating in the binding of succinate or just involved in the overall conformation of the transporter as found in other studies (Appendix). The phenolic group in model systems usually titrate with a pK_a between 9.8 and 10.5, but the pK_a interval in proteins is broader. The lysyl ε-amino group is an unlikely candidate, since neither phenylglyoxalic acid nor fluorescein isothiocyanate exposure affected the succinate cotransport (Appendix). The decaying transport of succinate at high pH does not seem to be due to disruption of the vesicles, since both lactate and glucose cotransport in this pH region are stable (*not shown*).

Discussion

The reaction between proteins and so-called group-specific reagents is not necessarily very selective as noted by Cohen (1968): "the term group-specific represents a level of optimism not often achieved in practice." One example to support this statement is the interaction of *p*CMBS, which is generally thought of as a specific sulfhydryl reagent, with the sperm-whale met-hemoglobin. This protein does not contain a single sulfhydryl group. At a 2-Å level *p*CMBS was found bound to a histidyl and an asparaginyl residue (Watson, Kendrew & Stryer, 1964). Therefore, in the search for the amino acid residues that form the active sites of membrane-bound transporters, it is valuable to study the effects of a spectrum of group-specific reagents, compare the observations, and thereby establish an internally consistent picture of the involved residues. Accordingly we have studied group-specific reagents for all known residues (*see* Appendix). The effects of the group-specific reagents may involve protein residues that are important for the transport function but remote from substrate and allosteric sites. These are referred to as conformers.

Specificity of DEP for Histidyl Groups

In model systems only histidine reacts readily with DEP at pH 6 (Little, 1977). The reaction between DEP and imidazole groups is shown in Fig. 6*b*. The rate of reaction increases as pH rises from 6 to alkaline pH, but at alkaline pH DEP reactivity is much less specific and attacks amino-, sulfhydryl- and alcohol groups of tyrosine and serine (Mühlrad, Hegyi & Toth, 1967). It is also generally believed that DEP labels histidyl groups in macromolecules at acidic pH.

It was of particular importance in this study to compare three cotransporters for the action of DEP. Succinate buffers at 100 mM are known to interfere with the DEP reaction and cause sulfhydryl groups to become more susceptible to DEP attack (Garrison & Himes, 1975). The specific protection against DEP by Na and succinate (3.3 mM) only at the Na/succinate cotransporter (Table 1) is good evidence that the observed effects of succinate are not due to nonspecific buffer effects but rather a carbethoxylation of an imidazole residue in the active site of this transporter. A reaction between DEP and sulfhydryl groups seems unlikely since DTT did not reverse the DEP inhibition of Na/succinate and Na/lactate cotransport.

Further indication for involvement of an imidazole residue in the Na/succinate cotransporter came from studies on reversal of DEP binding, decarboxylation. This process is catalyzed by hydroxylamine, NH_2OH, close to neutral pH. The decarbethoxylation by hydroxylamine is rather specific for histidyl and tyrosyl residues (Melchior &

Fahrney, 1970; Burnstein, Walsh & Neurath, 1974). In the present study hydroxylamine 100 mM, pH 6.4, for 30 min reversed the carbethoxylation of the Na/succinate cotransporter by 65%, but had no effect on the inhibition of the lactate or glucose transporters by DEP (Table 2). From studies of the pH-dependence of the succinate transport systems (*see* Fig. 5), one would predict possible involvement of a histidyl and/or a sulfhydryl group. The results of titration and NH_2OH reversal does not eliminate tyrosine as a possible residue being present in the active site, but results presented below are clearly against such a possibility. So far our results are compatible with an imidazole group located in the active site of the Na/succinate transporter.

Additional Observation in Favor of Histidine

We have not found protection by Na and substrates against a host of other group "specific" reagents that inhibit the Na/succinate and Na/L-lactate transporters. We have tested for all the likely amino acid residues, including tyrosine by NBD · Cl, iodination and acetylation (75%, 90% and 15% inhibition), serine by NTCB (35 to 95% inhibition), indole by BNPS skatole and N-bromo succinimide (50–100% inhibition) and sulfhydryl groups by *p*CMBS, 3BrP and NEM (50 to 95% inhibition). Amino groups were reacted with FITC and guanidino groups with phenylglyoxal without any inhibition of succinate or lactate transport, while PITC produced a nonprotectable inhibition (*see* Appendix).

DEP does not always react with imidazole groups at low pH. Melchior and Fahrney (1970) have suggested labeling of a serine group in alpha-chymotrypsin at pH 4 with low concentrations of DEP and strangely enough, no labeling of the two histidyl residues in the active site of the enzyme. At concentrations of 10 to 20 mM DEP, amino groups also reacted with DEP at pH 4. But the authors found the decarboxylation by NH_2OH to be specific for histidyl residues (Melchior & Fahrney, 1970). We thus feel reasonably confident that the site of the succinate transport molecule contains a histidyl residue.

Only Partial Decarbethoxylation by NH_2OH

The lack of complete decarbethoxylation, i.e. 100% return of Na/succinate transport capacity with NH_2OH treatment, may be due to several factors. The simplest and most likely is that the concentration of NH_2OH was too low in the given time to remove all the DEP residues. The decarbethoxylation process although generally very fast, sensitive, and complete (Burstein et al., 1974; Cousineau & Meighen, 1976) can be very slow (Miles & Kumagai, 1974; Wiejnans & Muller, 1982). Another explanation for the lack of complete decarbethoxylation by NH_2OH is the possible irreversible carbethoxylation of the second nitrogen atom in the imidazole ring followed by an opening of the ring in the presence of NH_2OH (*see* Fig. 6*b*, step 3). This secondary reaction between DEP and histidyl residues has been shown to take place when high concentrations of DEP are used (Avaeve & Krasnova, 1975) as in this study.

It is unlikely that DEP reacts with functional sulfhydryl groups in the Na/succinate transporter. Several reagents known to affect such sulfhydryl groups irreversibly present no protection with Na and succinate in the reaction medium (Appendix) whereas Na and succinate together can protect completely against the DEP reaction. Also as men-

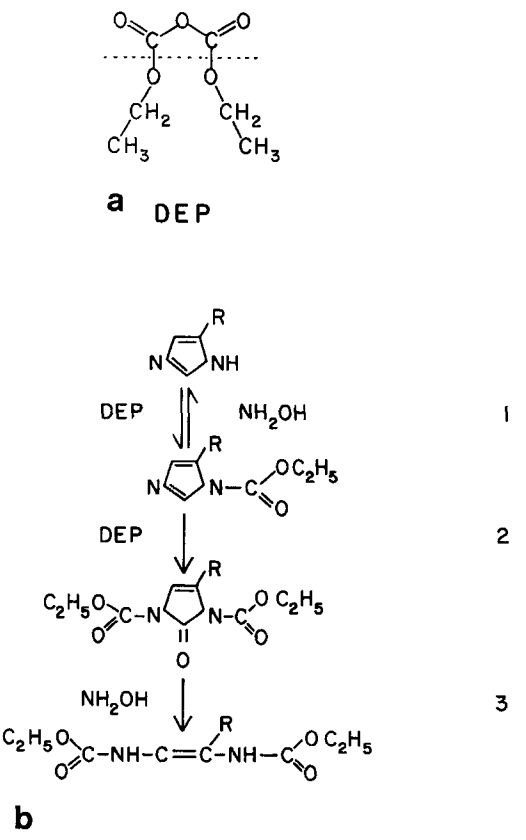

Fig. 6. (*a*) Configuration of diethylpyrocarbonate (DEP). (*b*) Reaction scheme for carbethoxylation of histidyl residues by diethylpyrocarbonate and the reversible decarbethoxylation catalyzed by hydroxylamine (NH_2OH). At higher concentrations of DEP, a second irreversible carbethoxylation may take place (step 2). Hydroxylamine will catalyze an opening of the dicarbethoxylated histidyl ring (step 3)

tioned DTT did not reverse DEP inhibition. Accordingly -SH groups reacting with DEP are not the reason for the lack of complete decarbethoxylation with NH_2OH.

DEP Interaction with Lactate and Glucose Transport

Although DEP is a potent inhibitor of lactate and glucose transport across renal brush borders (Figs. 1 and 2 and Table 1), there is good evidence that these DEP effects are not related to those on succinate transport: First, there was no protection of lactate and glucose transport by substrates (Fig. 3), i.e. the DEP reactions are unlikely to be at the active sites of these transporters; and second, NH_2OH did not reverse the DEP inhibition of glucose or lactate transport (Table 2). This indicates that DEP is not reacting with a histidyl residue or, if a histidyl group is involved, that irreversible carbethoxylation of the second nitrogen in the imidazole ring occurs. It is unlikely that a -SH group is involved as 10 mM DTT did not reverse DEP inhibition of lactate uptake.

Does DEP Bind to a Na or Succinate Site?

From kinetic analyses which showed that Na acts as a pure competitive activator of the Na/succinate cotransporter, it was concluded that the binding of Na ions and succinate is an ordered rapid equilibrium process in which 3 Na molecules bind first followed by succinate (Wright et al., 1983). As the prevention of DEP attacks requires both Na and succinate, it seems that the groups that react with DEP are also at the site for succinate rather than for sodium.

Other Cotransport Systems

Few studies have been conducted to identify the active sites of membrane-bound cotransport systems. The Na/glucose cotransporter in rabbit small intestine seems to have a lysine residue at the glucose site (Weber & Semenza, 1983; Peerce & Wright, 1984) and possibly a tyrosine residue at the Na site in both kidney and intestine (Lin, Stroh & Kinne, 1982; B.E. Peerce, *personal communication*). Evidence from Schmidt et al. (1983) and Peerce and Wright (1984) indicates that the Na/glucose transport protein has a molecular weight of 72,000 daltons. The H/monocarboxyl acid transporter in red blood cells, which is a possible cotransporter, may have a lysine/arginine group at the monocarboxyl site, since binding of DIDS can be specifically prevented by lactate. Using lactate-dependent ^{3}H-DIDS labeling this transporter has been identified as a 43,000-dalton protein (Jennings & Adams-Lackey, 1982). One of the residues in Na/amino acid cotransporters from rat liver seems to be a -SH group, since binding of NEM can be specifically prevented by L-alanine. A protein molecule with a weight of 20,000 daltons has been identified as a likely candidate for the amino acid transporter (Hayes & McGiven, 1983). Schaeffer, Preston and Curran (1973) found that phenylalanine could protect against *p*CMBS attack on the rabbit small intestine Na/phenylalanine cotransporter. This may be indicative of a -SH group at the phenylalanine site.

We have shown that all three entities have sulfhydryl groups that are essential for the transport function (Appendix). The sulfhydryl group(s), however, are remote from the active site, since neither Na nor substrate or in combination can prevent the sulfhydryl residue(s) from reacting with a host of sulfhydyl reagents. There are several examples of such sulfhydryl groups important for the overall conformation of enzyme molecules without being directly involved in the active site configuration (Gerhart & Schachmann, 1968; Yun & Suelter, 1979).

Possible Labeling of Na/Succinate Transporter with Tracer DEP

Unfortunately the N-carbethoxyimidazole is only stable in a narrow pH range from 5.0 to 7.5 (Melchior & Fahrney, 1970). This will cause problems in trying to isolate the transporter by conventional protein fractionation. Another problem which faces the cotransport researcher is the lack of a handle on the protein to be isolated as the purification procedure progresses. Our attempts to fluorescently label the succinate active site with dichlorotriazinylamino fluorescein, a reputed reagent for histidyl residues, have failed so far. At the moment only transport into vesicles can be used as a marker for the Na/succinate transport protein. Therefore unless other markers are developed, one is eventually forced to reconstitute isolated macromolecules to prove which ones are the Na/succinate transporter.

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Appendix

In preliminary studies we tested the sensitivity of the succinate and lactate Na-cotransporters towards a host of group-specific reagents, and along with these studies we probed for possible protection against the reagents by Na ions and substrates. In all experiments the scheme given in Materials and Methods was followed with minor modifications given below. Vesicles were suspended in a standard medium which consisted of 350 mM sorbitol, 50 mM KCl and 10 mM Tris/HEPES, pH 7.4, before storage in liquid nitrogen. All the steps following thawing of the membrane vesicles were carried out at room temperature, 20 to 23°C. All the reagents were prepared fresh just before use and protected from light. The preincubation solution had either 50 mM NaCl or KCl, 250 mM sorbitol, 50 mM KCl and 10 mM Tris/HEPES at pH 7.4, unless specified otherwise. In experiments where substrate protection was studied, succinate (33 mM) or lactate (50 mM) as sodium or potassium salts replaced NaCl or KCl in the preincubation medium. Preincubation was carried out in the dark. The group-specific reagent in question was always added to the first wash solution of all control tubes. The time and the concentration required to reduce the substrate transport to $1/e$ will be given as $T(1/e)$ and $C(1/e)$.

Reagents

3-Bromopyruvic acid (3BrP), 98% pure; *p*-chloromercuribenzene; sulfonic acid (*p*CMBS); benzoyl formic acid (phenylglyoxalic acid, FGO), 90 to 95% pure; N-acetylimidazole; N-ethylmaleimide (NEM), 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD · Cl); N-bromosuccinimide; 2-nitro-5-thiocyano benzoic acid (NTCB), fungal glucosoxidase (GO) specific activity 123 I.U./mg at 30°C; and lactoperoxidase (LPO) specific activity 67 units/mg were obtained from Sigma Chemical Co. (St. Louis, Mo.). N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinilone (EEDQ) and phenyl isothiocyanate (PITC) were from Aldrich (Milwaukee, Wis.) and fluorescein isothiocyanate (FITC) from Calbiochem (La Jolla, Calif.). ^{22}Na (carrier-free) was purchased from Amersham.

Sulfhydryl Groups

The possible presence of sulfhydryl groups in the Na/lactate and Na/succinate cotransporters was assayed with *p*CMBS, 3BrP, and NEM.

*p*CMBS: EDTA in equimolar amounts of *p*CMBS was included in all *p*CMBS studies to trap free mercury. EDTA in itself

did not affect the two cotransporters. $C(1/e)$ was found to be 155 μM for succinate and 215 μM for L-lactate uptake. $T(1/e)$ was 4 ± 1 min for succinate and 8 ± 3 min for lactate (n = 3). In subsequent experiments 20-min preincubations with 1 mM pCMBS were used. This reduced the uptake to 6 ± 1% and 5 ± 1% for succinate and lactate (n = 8). Neither of the substrates (33 mM succinate, 50 mM lactate) gave any protection in the presence of 50 mM Na (n = 2). 10 mM DTT/cystine in the first wash reversed the inhibition to 102% of control for lactate and 108% for succinate cotransport (n = 2). DTT/cystine did not affect the control uptake rates. Since pCMBS has been shown to make cell membranes leaky, specifically to small inorganic cations (Sutherland, Rothstein & Weed, 1967; Will & Hopfer, 1979) it was paramount to test for such an effect of pCMBS on the kidney membrane vesicles. Both the substrate-independent (amiloride-sensitive) and substrate-dependent ^{22}Na uptakes were reduced to zero after preincubating the vesicles with 1 mM pCMBS for 30 min while the nonspecific ^{22}Na flux did not change within experimental error. Thus pCMBS under given conditions attacked the Na/substrate cotransporters directly. By varying the substrate concentrations we found that preincubation with 1 mM pCMBS reduced the succinate and lactate V_{max}'s with no change in the K_t's. Varying the Na concentration at fixed substrate concentrations after pretreatment with 1 mM pCMBS also gave a reduction in the apparent V_{max} with no change in the Na dissociation constant at the allosteric sites on the transporters. This demonstrates that even within the short reaction time of a few minutes, the attack of pCMBS is mainly a "V_{max} effect."

3BrP, a haloketonic acid and a sulfhydryl reagent, is a nontransported inhibitor of both L-lactate and succinate cotransport in BBMV detected with the cyanine-dye technique (Schell, Wright & Bindslev, *unpublished*). 3BrP has been successfully used to label enzymes that have 3BrP analogs as substrates and for which the substrates protect the enzymes specifically against 3BrP attack (Meloche, 1970; Chang & Hsu, 1973; Yoshida & Wood, 1978; Yun & Suelter, 1979; Alliel, Mulet & Lederer, 1980). The specific interaction of 3BrP in all these studies was with sulfhydryl residues. Irreversible inhibition of succinate and lactate cotransport by 3BrP was tested over a pH range from 5.5 to 8.7 using Tris/HEPES/MES buffers. A concentration of 3 mM 3BrP was used throughout. 3BrP inhibited most effectively at high pH and least efficiently at pH 6.8. $T(1/e)$ at pH 8.7 was 43 ± 11 and at pH 7.0 it was 180 ± 56 min (n = 2) for lactate cotransport. The Na-dependent succinate and L-lactate uptakes were reduced to 40 ± 2% and 26 ± 7% (n = 4) of controls by preincubating for 75 min at pH 8.7. Adding lactate or succinate plus Na to the preincubation did not protect against the 3BrP inhibition (n = 4). DTT/cystine (10 mM) reversed the 3BrP inhibition completely, 98 ± 5% and 113 ± 4% (n = 2), for L-lactate and succinate. The pH optimum at high pH and the DTT/cystine reversal are good evidence that this haloketone attacks sulfhydryl groups in the cotransporters, although other mechanisms have been suggested for 3BrP's reaction with proteins (Hartman, 1977). Finally, NEM at 1 mM for 60 min at pH 7.4 reduced the succinate uptake to 75% and the L-lactate uptake to 30%, and 50 mM Na-nicotinate gave no protection. The two cotransporters thus seem to have sulfhydryl groups that are important for the overall conformation, but that these sulfhydryl groups are at some distance from the substrate and allosteric sites. Important -SH groups have also been found in the kidney (Bode et al., 1970; Turner & George, 1983) and intestinal (Klip, Grinstein & Semenza, 1979) glucose cotransporters, and may even be located near the substrate site of this transporter from horse kidney (Poiree, Mengual & Sudaka, 1979).

Disulfide and Thioether Groups

Disulfide bonds are unlikely to be of importance for the succinate and lactate transporters, since reducing the vesicles by treatment with DTT did not affect transport. Important disulfide bonds have been found in renal brush border Na/D-glucose cotransporters (Turner & George, 1983). Thioether groups are also unlikely residues because this group reacts readily with haloacids at acid pH, and contrary to this 3BrP was more reactive at alkaline pH.

Guanodino Groups

Phenylglyoxalic acid is a fairly specific reagent for guanodino- and terminal amino groups (Takahashi, 1968). Preincubating BBMV with 16 mM FGO for 45 sec in CAPS · Tris · KOH buffers at pH 10.4 or for 30 min at pH 8.7 did not inhibit either of the cotransporters.

Lysine and Terminal Amino Groups

The negative results with FGO were confirmed by attempts to inhibit the two cotransporters with FITC and PITC, amino reagents which at alkaline pH are thought to interact preferentially with the lysyl ε-amino groups in proteins. Using 100 μM FITC in a preincubation medium with 1 mM EDTA and 50 mM Tris buffer at pH 9.2 for 30 min left the Na-dependent uptake of succinate and L-lactate unchanged at 107 ± 8% and 98 ± 10% of control (n = 3). Including substrates and sodium also had no influence on the uptake. The negative results with FGO and FITC strongly suggest lack of amino groups including the guanidino residue in the active site of the two carboxyl acid transporters. However, 1 to 2 mM PITC inhibited the transport of succinate, lactate, and glucose (65 to 100%) under identical conditions to those with FITC, but there was little protection (<30%) by Na and the substrates.

Tyrosine Groups

Three different reagents for tyrosine residues were employed, NBD · Cl, iodination catalyzed by lactoperoxidase, and acetylimidazole. The ligands are, however, not exclusive for tyrosine residues. At alkaline pH, NBD · Cl reacts readily with -SH groups (Birkett et al., 1970) although this reaction is reversible with DTT (Cantley, Gelles & Josephson, 1978). Iodide halogenation catalyzed by LPO also involves histidine residues (Morrison & Bayse, 1970). NBD · Cl had a $T(1/e)$ of 20 min for both systems using 250 μM at pH 7.4. Preincubating for 30 min reduced the uptake of L-lactate and succinate to 20 ± 4% and 23 ± 6% (n = 5). There was no protection by substrate and Na (50 to 200 mM) in the preincubation medium (n = 3). DTT (5 mM) reversed the inhibition by 25 ± 8% and 31 ± 9% (n = 4) indicating a partial reaction of -SH groups with NBD · Cl. Iodination was performed using 2.5 mM NaI, 10 mM glucose, GO 67 I.U./ml and 25 μg/ml of LPO in the reaction medium. The preincubation lasted 5 min at pH 7.4. In three experiments lactate and succinate uptakes were reduced to 7 ± 3% and 8 ± 2% and in two experiments which included the substrates and Na for possible protection were reduced to 8 ± 1% and 10 ± 1%. DTT (5 mM) reversed by 15 and 10% the iodination effect on the two transporters. Thus tyrosine groups may be involved as conformers in the cotransport molecules but located remotely from the active sites for Na

and substrates. Consistent with this conclusion is the observation that N-acetyl imidazole (1 to 20 mM) also failed to inhibit succinate, lactate and glucose uptake even when the pH of the reaction medium was varied from 7.4 to 9.2 (*see* Cohen, 1968).

Hydroxyl Groups

Serine and threonine residues can react with NTCB (Liao & Wadano, 1979). The reaction medium had 10 mM NTCB and 1 mM $CaCl_2$ at pH 8.7 and we ran the reaction for 50 min. This reduced the L-lactate and succinate uptake to 29 ± 4% and 65 ± 3% (n = 3). DTT (5 mM) reversed the inhibition by 40 ± 9% and 74 ± 24% (n = 3). Substrates and sodium did not protect against the NTCB inhibition which can probably be ascribed partially to a reaction at -SH groups.

Tryptophan Groups

The possible involvement of tryptophan residues was explored using BNPS Skatole and N-bromosuccinamide. These are considered to react specifically with tryptophan if sulfhydryl groups are protected (*see* Fontana, 1972). Incubation of membranes with 0.2 mM BNPS Skatole for 15 to 30 min at pH 6 to 9.5 inhibited succinate and glucose transport 50 to 100% (n = 4). However, no specific protection was observed with 100 mM Na and 10 mM substrate (n = 4) and DTT failed to reverse the inhibition. Similar results were obtained with the harsher N-bromosuccinimide reagent. This suggests involvement of tryptophan residues, but, as with -SH groups, these are not at the active sites of the transporter.

Carboxyl and Phosphate Groups

EEDQ (1 mM), a carboxyl and phosphate group reagent (Belleau, DiTullio & Godin, 1969; Muren & Weissman, 1971) was reacted with BBMV for 60 min at pH 7.4 with or without 100 mM methylglycyl ester. This procedure resulted in a 9 and 24% drop in the uptake of L-lactate and succinate but no protection by substrates and sodium was observed.

In conclusion these preliminary experiments indicated a nonspecific involvement of -SH, tyrosyl, and tryptophanyl groups as conformers in the macromolecules for L-lactate and succinate cotransport. These groups did not seem to be near the active sites.