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Kidney brush-border membrane transporters: differential sensitivity to diethyl pyrocarbonate

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The effects of the histidine modifier, diethyl pyrocarbonate (DEPC), on brush-border membrane transport systems were studied in rat kidney. DEPC caused a strong inhibition of sodium-dependent phosphate and D-glucose uptake. Phosphate uptake remained linear up to 10 s in control and DEPC-treated membrane vesicles. The D-glucose carrier was more sensitive than the phosphate carrier with half-times of inhibition being 4 and 7 min, respectively. Sodium-independent phosphate and D-glucose uptake remained unaffected by DEPC. Intravesicular volume and two enzyme activities endogenous to the luminal membrane (alkaline phosphatase and aminopeptidase M) remained unaffected by DEPC. Increasing the preincubation pH from 5 to 9 increased phosphate transport inhibition caused by DEPC from 73 to 88% in the presence of DEPC. Hydroxylamine was able to completely reverse phosphate uptake inhibition by DEPC (100%), but only partially reversed the D-glucose uptake inhibition (16%). Sodium or substrate (D-glucose or phosphate) in the preincubation media were unable to protect their respective carriers from DEPC. Sodium-dependent transport of L-glutamine, L-phenylalanine, L-leucine, L-alanine, L-glycine, β-alanine and L-proline were inhibited at different levels ranging from 70 to 90%. Three transport processes were found insensitive to DEPC modification: L-glutamate, L-lysine and D-fructose. None of the amino acid transporters was protected against DEPC by sodium and/or their respective substrates. Sodium influx was inhibited by DEPC (47%) in the absence of any substrate. Our results show a differential sensitivity of sodium-dependent transporters to DEPC and suggest an important role for histidine residues in the molecular mechanisms of these transporters. More experiments are in progress to further characterize the residue(s) involved in these transport inhibitions by DEPC.

Introduction

Chemical modification of amino acid residues by specific reagents is a powerful tool in the study of structure–function relationships of enzymes or transport proteins. This approach is an interesting means of determining the substrate binding sites or sites responsible for conformational changes in transport proteins. This approach was useful in the kidney to characterize many transport systems [1–8]. The comparison of the differential sensitivity of these transport proteins to a single modifier is another way of approaching an understanding of these transport processes at the molecular level.

Histidine residues are good candidates for protonation sites in sodium-dependent transport proteins because their pK_a are in the physiological range. Diethyl pyrocarbonate (DEPC) is a well known specific modifier of histidine residues in proteins [9]. This reagent has been used in the determination of essential histidine residues in many transport processes such as β-lactam transporter [10], lactose/proton symporter [11], Na^+/H^+ exchanger [2,12], and renal L-proline [4], D-glucose [3] and *p*-aminohippurate carriers [6].

The purpose of this study is to compare the sensitivity of rat renal transporters to modification by DEPC. The results presented here show that amino acid

Abbreviations: BBMV, brush-border membrane vesicles; DEPC, diethyl pyrocarbonate.

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residues, sensitive to the modification by DEPC, are involved in the molecular mechanisms of the sodium-dependent transport of phosphate, D-glucose and amino acids (β-alanine, L-proline, L-leucine, L-alanine, L-glutamine, L-phenylalanine and L-glycine). In contrast, no inhibition was found for the facilitated transport systems of D-fructose, L-lysine and for the transport of L-glutamate, which is sodium-dependent, but stimulated by a potassium gradient. Sodium-dependent transport processes being more sensitive to DEPC, these results may suggest that common molecular characteristics for these transporters may exist.

Materials and Methods

Membrane preparation

Rat renal brush-border membrane vesicles (BBMV) were prepared as described by Booth and Kenny [13]. The final pellet was washed in 20 mM Mes-KOH buffer (pH 6.0) containing 300 mM mannitol to obtain a final protein concentration of 20 mg/ml. BBMV were frozen in liquid nitrogen until use. The enrichment for alkaline phosphatase [14], a specific marker enzyme for BBM, was 13-fold compared to the homogenate. Aminopeptidase M activity was determined by the method of George and Kenny [15].

Transport studies

The rapid filtration technique [16] was used for uptake studies. Incubation media were in a 30 μl final volume. After incubation, the reactions were stopped by adding 1 ml of an ice-cold stop solution and the suspension was applied on a 0.45 μm pore size Millipore filter under vacuum. The filters were washed with 8 ml of the stop solution and processed for liquid scintillation counting. Non-specific absorption of radioactive substrates by vesicles or filters, measured by adding the stop solution directly to the incubation media, was subtracted from transport measurements. Incubation media and stop solutions are described in the legends of the figures and tables. Phosphate transport stop solution was prepared as already described [17].

Modification with diethyl pyrocarbonate

Stock solutions (0.1 to 1.0 M) of DEPC were freshly prepared for each experiment in absolute ethanol. The control was made with 1% ethanol, final concentration. BBMV (1 mg protein/ml) were incubated 0 to 10 min with appropriate concentrations of DEPC. The reactions were stopped by adding 10 volumes of a medium containing 300 mM mannitol and 20 mM Hepes-Tris (pH 7.5). The BBMV were then centrifuged at 35 000 $\times g$ for 30 min at 4°C and this step was repeated twice.

Materials

Radioactive chemicals were obtained from DuPont-New England Nuclear. Diethyl pyrocarbonate and hydroxylamine were obtained from Sigma. All other chemicals were reagent grade.

Results

Fig. 1 illustrates the initial rate of sodium-dependent phosphate uptake following DEPC modification of BBMV. Uptake remained linear for the first 10 s of incubation. DEPC caused an 85% inhibition of the initial rate of uptake. The inhibition of phosphate and D-glucose uptake by DEPC follows a mono-exponential kinetic pattern, as shown in Fig. 2. D-glucose transport was more sensitive to DEPC modification than phosphate transport, with $t_{1/2}$ (time at which 50% inhibition is observed) being about 7 min for the phosphate carrier and 4 min for the D-glucose carrier. The sodium-dependent uptake of phosphate or glucose could be inactivated completely when 5 mM DEPC was used in the pre-incubation (results not shown).

The physical integrity of the vesicles after DEPC treatment was evaluated by measuring the intravesicular volume (D-glucose uptake at equilibrium). It remained unchanged after the DEPC modification procedure (Table I). Sodium-independent D-glucose and phosphate uptake were unaltered by DEPC. Aminopeptidase M activity was not affected by DEPC treatment, while a small significant inhibition of alkaline phosphatase activity was found. However, sodium-de-

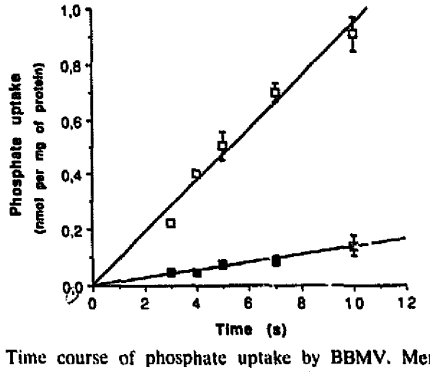


Fig. 1. Time course of phosphate uptake by BBMV. Membrane vesicles were preincubated in the presence (■) or absence (□) of 2.0 mM DEPC for 10 min at 25°C. Reactions were stopped by adding 10 volumes of a medium containing 300 mM mannitol, and 20 mM Hepes-Tris (pH 7.5) and BBMV suspensions were then centrifuged as described in Materials and Methods. The incubation media, for transport measurements, contained 100 mM mannitol, 100 mM $NaNO_3$, 20 mM Hepes-Tris (pH 7.5), and 200 μM [^{32}P]orthophosphate (5 μCi) (pH 7.5). The stop solution contained 150 mM KCl and 5 mM Hepes-Tris (pH 7.5). Values are the means ± S.E. of three experiments performed in triplicate.

pendent phosphate and glucose uptake were strongly inhibited by DEPC.

The inhibition of phosphate uptake was only slightly sensitive to the preincubation pH (Fig. 3); the inhibition varied from 73 to 88% for a pH from 5 to 9. The inhibitions observed at pH 6, 7, 8 and 9 were significantly higher ($P < 0.01$) than the one at pH 5. The inhibition of phosphate uptake at pH 6 also differs significantly from the one observed at pH 9. However, there was no significant difference between the inhibition at pH 6 and 9 for the uptake of D-glucose and L-amino acids (results not shown).

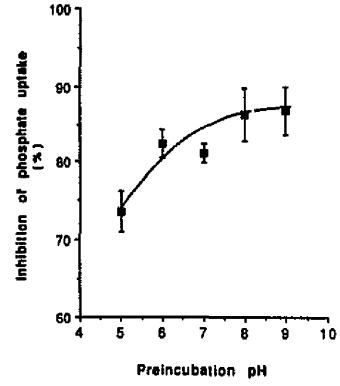
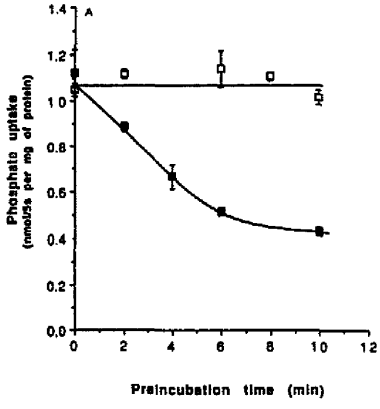
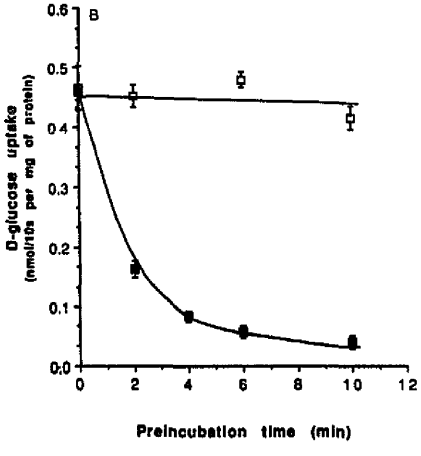
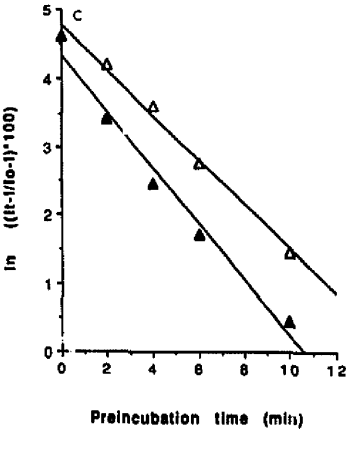


Fig. 3. Effect of preincubation pH on inhibition of phosphate transport by DEPC. Membrane vesicles (20 mg protein/ml) were diluted in 20 volumes of a medium containing 300 mM mannitol and 20 mM of the following buffers: Mes-Tris (pH 5.0), Mes-KOH (pH 6.0), Hepes-Tris (pH 7.0), Tris-HCl (pH 8.0) and CAPS-Tris (pH 9.0). BBMV were passed five times through a 27-gauge needle and equilibrated 20 min at 25°C before the DEPC-modification procedure. The incubation medium and stop solution are described in Fig. 1. Values represents the means ± S.E. of three experiments performed in triplicate.







Decrease of protein activity by DEPC can be correlated with the modification of histidine residues when hydroxylamine is able to restore the original activity of the protein modified by DEPC. Hydroxylamine was able to reverse completely (100%) the inhibition of phosphate transport by DEPC, but was much less effective (15–17%) in reversing the inhibition of glucose uptake (Table II). Moreover, hydroxylamine itself caused a strong inhibition of phosphate (79%) and glucose (58%) uptake.

Fig. 2. Inactivation of sodium-dependent phosphate (A) and D-glucose (B) transport by DEPC. BBMV were treated in the presence (■) or absence (□) of 1 mM DEPC from 0 to 10 min at 25°C as described in the legend of Fig. 1. The incubation medium, for glucose transport measurements, consisted of 100 mM mannitol, 100 mM $NaNO_3$, 20 mM Hepes-Tris (pH 7.5) and 100 μM D-[^{3}H]glucose (0.5 μCi) (pH 7.5). The stop solution contained 100 mM mannitol, 100 $NaNO_3$, 5 mM Hepes-Tris (pM 7.5), and 1 mM phloridzin. Fig. 2C shows the results of (A) and (B) as ln of the ratio between residual transport for phosphate (△) and D-glucose (▲) measured at the indicated preincubation time ($I_t - I$) and the residual transport at 0 min of preincubation ($I_0 - I$). 'I' represents transport values at 60 min of preincubation with DEPC-treated vesicles. Values represent the means ± S.E. of three experiments performed in triplicate.

TABLE I

Effects of DEPC modification on BBM enzymes and phosphate and glucose uptake

Membrane vesicles were preincubated for 10 min at 25°C in the presence or absence of 2 mM DEPC. Enzyme activities and transport measurements were performed as described in Material and Methods. Intravesicular volume was measured by the uptake of D-glucose at equilibrium. Values represent the mean ± S.E. of 3 to 11 experiments performed in triplicate.

Parameter	Control	2 mM DEPC	Effect (%)
Alkaline phosphatase (μmol of *p*-nitrophenol/min per mg of protein)	1.05 ±0.10	0.88 ±0.08	16 *
Aminopeptidase M (μmol of *p*-nitroaniline/min per mg of protein)	1.29 ±0.10	1.17 ±0.14	9
Intravesicular volume (μl/mg of protein)	1.99 ±0.15	1.92 ±0.18	4
Na^+-independent phosphate uptake [a]	0.076±0.016	0.072±0.011	15
Na^+-dependent phosphate uptake [a]	1.176±0.106	0.342±0.092	77 *
Na^+-independent D-glucose uptake [a]	0.026±0.005	0.029±0.008	0
Na^+-dependent D-glucose uptake [a]	0.263±0.037	0.038±0.013	86 *

[a] Transport in nmol/10 s per mg of protein. * $P < 0.005$ paired Student's *t*-test.

As shown in Table III, neither 150 mM sodium or 10 mM phosphate alone or combined were able to protect the phosphate carrier from DEPC modification. Increasing the phosphate concentration up to 100 mM had no significant effect on the inhibition by DEPC. The same results were obtained for the D-glucose transport process, with an 85% inhibition observed for both the control and the protection medium containing 150 mM sodium and 10 mM D-glucose.

A variety of cotransport processes were very sensitive to DEPC modification, as shown in Table IV. Transport of proline, glutamine, phenylalanine, leucine, alanine and β-alanine were strongly inhibited, while the transport of glutamate, fructose and lysine were not inhibited by DEPC. Protection experiments for amino acid transport were attempted in order to determine whether DEPC reacts at the binding site of amino acid or sodium ions. None of the amino acid carriers studied was protected by the addition of both the respective substrate and sodium to the preincubation medium (Table V). Sodium influx was studied after modification of the membranes by DEPC. The sodium flux across the membrane decreased in the absence of other substrates (Fig. 4) with, a significant inhibition of 47% compared to untreated control vesicles.

TABLE II

Reversal of DEPC inactivation of phosphate and D-glucose uptake by hydroxylamine

Membrane vesicles were treated with or without 2 mM DEPC as described in Fig. 1 and washed twice in a medium containing 300 mM mannitol and 20 mM Hepes-Tris (pH 7.5). The pellets were diluted in 5 ml of a medium containing different concentrations of hydroxylamine. The osmolarity was balanced with mannitol, and the solution was buffered with 20 mM Hepes-Tris (pH 7.5). After a 20 min incubation, the reactions were stopped by adding 10 volumes of a medium containing 300 mM mannitol and 20 mM Hepes-Tris (pH 7.5). BBMV were washed twice in the same medium before the transport measurements. Values are the mean ± S.E. for two experiments performed in triplicate.

	Inhibition (%)	
	phosphate uptake	D-glucose uptake
Control	85±3	57±16
Hydroxylamine (25 mM)	0±2	42± 4
Hydroxylamine (50 mM)	10±2	40± 4

Discussion

Treatment of brush-border membrane vesicles with the histidine-specific modifier DEPC resulted in a significant inhibition of sodium-dependent phosphate uptake and D-glucose uptake (Figs. 1 and 2). Phosphate uptake remained linear for 10 s with control or modified membrane vesicles, indicating no dissipation of the

TABLE III

Effect of protection by substrate and/or sodium on phosphate and D-glucose uptake inhibition by DEPC

BBMV were treated with 2 mM DEPC in a medium containing the indicated concentrations of substrate and/or cosubstrate. The modification procedure is described in the legend for Fig. 1. The wash was repeated three times with an incubation of 20 min between each wash. Data are expressed as the mean ± S.E. of two experiments performed in triplicate.

Substrate	Uptake (nmol/5 s per mg of protein)		
	control	2 mM DEPC	inhibition (%)
Phosphate			
control	1.144±0.071	0.180±0.034	84
NaCl 100 mM	1.076±0.049	0.258±0.035	76
Phosphate 10 mM	0.998±0.115	0.111±0.056	89
NaCl 100 mM + Phosphate 10 mM	1.280±0.079	0.114±0.059	91
D-Glucose			
control	0.219±0.019	0.032±0.002	85
NaCl 150 mM + D-glucose 10 mM	0.224±0.024	0.034±0.009	85

TABLE IV

Effect of DEPC modification on amino acid and D-fructose brush-border membrane transport systems

Membrane vesicles were treated with 2 mM DEPC or 1% ethanol as described in the legend to Table I. The incubation media for amino acid transport measurements contained 150 mM NaCl, 20 mM Hepes-Tris (pH 7.5) and 25 μM [^{3}H]amino acid, except for L-lysine transport measurements where KCl (150 mM) replaced NaCl. Incubation media for D-fructose transport contained 150 mM KCl, 20 mM Hepes-Tris (pH 7.5) and 50 μM D-[^{14}C]fructose. The stop solution contained 150 mM KCl and 5 mM Hepes-Tris (pH 7.5). Values represent the mean ± S.E. of three to five experiments performed in triplicate.

Substrate	Substrate uptake (pmol/10 s per mg of protein)		
	control	2 mM DEPC	inhibition (%)
L-Proline	105 ±7	5 ±1	90 *
β-Alanine	31 ±2	3 ±1	90 *
L-Glycine	18 ±2	2.5±0.7	86 *
L-Alanine	95 ±4	17 ±4	82 *
L-Leucine	117 ±6	27 ±4	77 *
L-Phenylalanine	57 ±4	14 ±1	75 *
L-Glutamine	34 ±2	10 ±1	70 *
L-Lysine	14 ±2	12.5±0.5	13
D-fructose	1.6±0.1	1.5±0.3	4
L-Glutamate	6.1±1.4	7.5±1	0

* $P < 0.005$ paired Student's t-test.

sodium gradient (Fig. 1) which could have caused secondary inhibition of transport by decreasing the driving force. The D-glucose carrier was more sensitive to DEPC than the phosphate carrier, with $t_{1/2}$ of 4 min for D-glucose compared with 7 min for phosphate.

Numerous controls were made to ensure that the transport inhibitions observed were due to chemical modification of one or more amino acid residues on the transport proteins and not to an indirect effect on membrane vesicles. First, DEPC treatment did not alter the physical integrity of membrane vesicles since the intravesicular volume remained unchanged (Table I). Sodium-independent transport of phosphate and D-glucose also remained unchanged (Table I), indicating no gross alteration in membrane permeabilities to substrates, induced by DEPC. Moreover, the presence of histidine in the preincubation media reversed the inhibition of phosphate and D-glucose uptake, indicating a strong competition for DEPC between exogenous free histidine and the carrier sites modified by DEPC. Furthermore, the electrophoretic pattern of membrane proteins obtained by SDS-PAGE remained unchanged by the DEPC modification procedure (data not shown). These data suggest that DEPC caused an inhibition of these transport systems by modifying one or more residues.

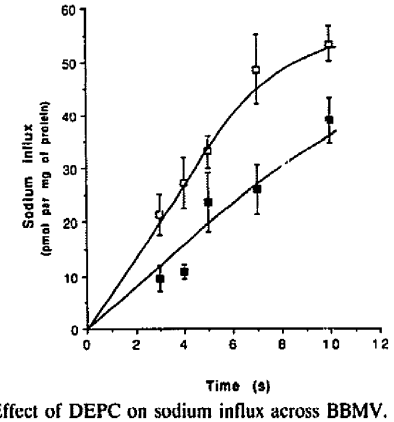


Fig. 4. Effect of DEPC on sodium influx across BBMV. Membrane vesicles were treated in the presence (■) or absence (□) of 2 mM DEPC as described in Fig. 1. Incubation medium contained 150 mM [^{22}Na]NaCl (1 μCi) and 20 mM Hepes-Tris (pH 7.5). The stop solution constained of 150 mM NaCl and 5 mM Hepes-Tris (pH 7.5). Values represent the means ± S.E. of two experiments performed in triplicate.

TABLE V

Effect of amino acid protection on respective amino acid transport by BBMV

Membrane vesicles (20 mg/ml) were diluted 20-fold in a medium containing 150 mM NaCl and 10 mM of the respective amino acids. After a 30 min equilibration period, membrane vesicles were treated with DEPC as described in Fig. 1. The wash was, however, done four times. The data are expressed as the mean ± S.E. of a representative experiment, done in quadruplicate. Incubation media and stop solution are described in Table V.

Substrate	Amino acid uptake (pmol/5 s per mg of protein)					
	control medium			protection medium		
	−DEPC	+DEPC	inhibition (%)	−DEPC	+DEPC	inhibition (%)
L-Alanine	108± 4	19 ±5	82	101± 8	18 ±2	82
L-Glutamine	64± 5	20 ±3	69	62± 5	26 ±2	58
L-Leucine	160±13	34 ±2	79	158±13	46 ±9	71
L-Glycine	18± 2.6	2.5±0.7	86	14± 3.3	4.2±0.5	71
L-Phenylalanine	92±26	4 ±2	95	97±10	4 ±3	96
L-Proline	71± 5	6 ±1	92	75± 6	9 ±1	88

Increasing the preincubation pH from 5 to 9 leads to a slightly higher inhibition for phosphate uptake (Fig. 3). Because the specificity of DEPC towards amino acid residues varies with pH, the results obtained as a function of modification of pH are somewhat difficult to interpret. No such difference was found for D-glucose transport (data not shown). It is difficult to conclude whether the residue(s) modified at pH 6 and at pH 9 are the same or not, considering the fact that DEPC can also react with other nucleophilic residues, such as lysine or arginine, at pH higher than 6.0 [9].

Transport inhibition by DEPC modification can be correlated to histidyl modification if hydroxylamine restores the activity, in part or completely [9]. Hydroxylamine was able to totally restore the phosphate transport, and partially the glucose transport (Table II). However, hydroxylamine itself caused an important inhibition of these transport processes. In contrast, Poirée et al. [3] have shown a partial reversal (20%) of D-glucose transport activity by hydroxylamine in rabbit kidney. More data are needed to evaluate the reactivity of hydroxylamine with these proteins. For the renal phosphate carrier, Pratt and Pedersen [1] have identified sulfhydryl groups essential for the transport activity. Disulfide bonds were also reported to be involved in the intestinal [18] and the renal [5] glucose transporter. However, the modification of sulfhydryl group(s) by DEPC is partially rejected because of the lack of protection offered by dithiothreitol (data not shown).

It seems unlikely that the site(s) modified by DEPC are part of the sodium, phosphate or glucose binding sites. Neither sodium or substrate (phosphate or glucose) were able to protect against DEPC. For the glucose carrier, our results differ from another study [3] where protection by sodium and glucose was obtained. Their study being performed on pig kidney, a variation in molecular characteristics of these proteins from one species to another is thus possible.

In addition to the phosphate and D-glucose cotransporters, a variety of amino acid transport systems are sensitive to DEPC (proline, β-alanine, glycine, alanine, leucine, phenylalanine and glutamine) (Table IV). Three transport activities were found to be unaffected by this histidine-specific reagent: lysine, glutamate and D-fructose. To avoid artifacts due to the modification procedure, which may alter the sodium gradient, sodium entry was measured in the absence of other substrates. Sodium flux was reduced in initial rate, probably indicating a reduction in membrane permeability (Fig. 4). Sodium channels present in this membrane can be inhibited by DEPC, resulting in a reduced entry of sodium into membrane vesicles [19–23]. A histidyl residue in the sodium channel was found in frog bladder [24]. Grillo and Aronson [2] found that the imidazole moiety of a histidine residue was the binding site of the proton for the Na^+-H^+ antiport system in rabbit kidney. Inhibition of the Na^+-H^+ antiport by DEPC may be responsible for the smaller rate of sodium entry, since this antiport is thought to be the major factor for sodium entry in epithelial cells of the proximal tubule [25–27].

Similar inhibitions by DEPC were observed for alanine (82%), leucine (77%), glutamine (70%) and phenylalanine (75%). These results can be related to the study of Lynch and McGivan [28] which proposed that these four neutral amino acids share the same transport protein. Glutamate reabsorption in the kidney is sodium-dependent and stimulated by potassium ions [29,30]. The absence of inhibition of the glutamate carrier by DEPC suggests that acidic amino acid reabsorption may occur by a transport system with molecular characteristics distinct from neutral amino acid transport systems. Furthermore, L-lysine uptake was also found insensitive to DEPC, in agreement with the fact that cationic amino acids are transported by a different transport system (Y^+) [31–34]. The renal reabsorption of β-alanine is driven by the sodium and the chloride gradients [35–37]. The strong inhibition observed for β-alanine transport by DEPC (90%), compared with neutral amino acids, supports the conclusion that the β-amino acid transport systems differs from L-amino acid transport systems. Moreover, a histidyl residue may be part of the active site of the renal sodium-coupled transport system of proline [4].

Protection studies were carried out to evaluate whether the modified residue(s) are part of, or near the substrate or sodium binding sites of the amino acid transporters. None of these substrates can afford protection of their transport proteins against DEPC (Table V). Increasing amino acid concentrations in the preincubation media seems to lead to a reaction between DEPC and the free amino acid (results not shown). Further experiments are in progress to characterize the residues involved in transport inactivation by DEPC.

In summary, the results presented in this paper suggest that a variety of sodium-dependent transport processes have a common sensitivity to DEPC modification. The differences in the sensitivity of these transport proteins to a single chemical modifier indicate that the reactivity of the sites modified in these carriers are distinct, since the inhibition range is from very low (fructose: 4%), to very high (proline and β-alanine: 90%). More data are needed to further characterize the residue(s) involved in these transport inhibitions by DEPC.

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