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THE EFFECT OF DIAMIDE ON AMINO ACID TRANSPORT BY RAT RENAL CORTEX SLICES

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Summary

Diamide directly added to renal cortical slices inhibits the uptake of amino acids. Steady-state kinetic analysis indicates an inhibition of α-amino acid influx without effect on efflux. The effect could be reversed by addition of pyruvate to the incubation medium. Although there was a good correlation of the transport effect of diamide with its ability to decrease cellular reduced glutathione concentration, there did not appear to be a necessary connection between them. This was shown by the fact that renal cortical slices stored at 4°C have no alteration in amino acid uptake despite the fact that GSH concentration is as low as that seen with diamide. Diamide was shown to have a direct effect on the uptake of glycine by isolated renal brush border membrane vesicles.

Introduction

The description of the thiol-oxidizing agent diazenedicarboxylic acid bis-(*N*,*N*-dimethylamide), called diamide, which readily oxidizes reduced glutathione (GSH) has stimulated great interest in the cellular metabolic consequences of the altered state. This stems from the high intracellular levels of GSH and the relation of GSH to such cellular processes as glucose metabolism [1], mitosis [2], regulation of protein synthesis [3], and response to irradiation [4]. Considerable attention has been paid recently to the effect of lowering the cellular concentration of GSH by diamide on the transport of amino acids [5,6] and α-methyl-D-glucoside [7] by rat kidney cortex slices as well as on the calcium transport by muscle [8] and isolated mitochondria [9].

Kosower et al. [10] demonstrated that diamide caused the rapid oxidation

of GSH to GSSG at 4°C and that the diamide effect was completely reversible. Hewitt et al. [5] and Pillion and Leibach [7] have examined the effect of diamide on the uptake of α-amino acids and of α-methyl-D-glucoside, respectively. Their protocol included incubation with diamide at 4°C, washout of the compound at 25°C, and subsequent measurement of substrate uptake at 37°C. They demonstrated that GSH level in the controls fell continuously and spontaneously during incubation at 37°C. During the same time, GSH levels in diamide-treated cells were rising in recovery. Thus transport was being assessed under two different patterns of changing GSH levels [5]. On the other hand, Carlen et al. [8] have reported the effects of diamide at 10^{-2} mM when frog muscle was incubated continuously with the chemicals. We have, therefore, studied the direct effect of diamide on the uptake of α-amino acids and α-methyl-D-glucoside by rat renal cortex slices at 37°C when the thiol oxidant was present during the entire incubation period. The kinetic aspects of the uptake process as well as the relationship with intracellular glutathione concentration were examined. The effect of diamide on the uptake of an amino acid by isolated rat kidney brush border membrane vesicles was also studied. Our results form the basis of this report.

Materials and Methods

Adult male Sprague-Dawley rats weighing 150—200 g were obtained from Charles River Breeding Laboratories and fed ad libitum on Purina rat chow until being killed by decapitation. Kidney cortex slices were prepared with a Stadie-Riggs microtome and handled as described in previous reports [11—13]. The technique for determining the in vitro uptake and intracellular content of ^{14}C-labeled amino acids in kidey cortex slices incubated in Krebs-Ringer bicarbonate buffer (pH 7.4) at 37°C has been described [11,14,15]. Vessels were gassed with O_2/CO_2 (95 : 5, v/v), sealed and incubated in a Dubnoff metabolic shaker for the times indicated. At the end of the incubation, slices were removed, weighed, extracted, and assayed for radioactivity as described previously [11,14]. The uptake was calculated by the technique of Rosenberg et al. [11,16] which has been employed in several reports from this laboratory [11—14]. The results are expressed as the distribution ratio, the ratio of cpm/ml intracellular fluid to cpm/ml medium. The fluid spaces used for the calculations were identical to those reported elsewhere [11,12,16]. Diamide does not alter the fluid spaces of the slice [5,6].

In studies for kinetic analysis, the slices were incubated with unlabeled glycine for 60 min with or without diamide. At that time the flasks were opened, tracer [^{14}C]glycine added, gassed, resealed, and incubated for the various times indicated. The fluxes were calculated using a two compartment analysis [17] based on the original formulation of Rosenberg et al. [18]. Efflux studies were performed according to Segal et al. [14]; slices were incubated with and without diamide to the steady state with radioactive substrate, transferred to new buffer with or without diamide, and the appearance of radioactive amino acid in the medium determined.

Reduced glutathione was determined in the slices by the method of Tietze [19] using 5,5′-dithiobis(2-nitrobenzoic acid). The GSH levels were also deter-

mined by using the glyoxylase method described by Paleka et al. [20]. The values reported here were those of the former method. Both methods gave results which were generally in agreement.

To determine the effects of storage at 4°C on glycine uptake and GSH levels, cortical slices were prepared as described previously and collected in bicarbonate buffer. Nine slices were placed in 10 ml bicarbonate buffer, the flasks gassed with O_2/CO_2, sealed and placed at 4°C [21—23] for the length of time indicated, after which uptake and GSH levels were measured as described above.

Isolated brush border membrane vesicles were prepared by the method of Booth and Kenny [24]. The uptake of glycine by these vesicles was performed by the methods reported earlier from this laboratory [25].

Unlabeled amino acids were purchased from Mann Research. These and all other compounds were of reagent grade. Diamide was obtained from Vega Fox Biochemicals, 5,5'-dithiobis(2-nitrobenzoic acid) from Sigma, and dithiothreitol from Calbiochem. Radioactive substrates: [^{14}C]glycine (109 Ci/mol), 3-*O*-[^{3}H]methyl-D-glucose (81 Ci/mmol), [^{14}C]valine (232 Ci/mol), [^{14}C]lysine (286 Ci/mol), α-amino[^{14}C]isobutyric acid (9 Ci/mol) were purchased from New England Nuclear Corporation; [^{14}C]methyl-D-glucoside (360 Ci/mol) was purchased from Calatomic.

Results

Effect of diamide on uptake

Table I shows the effect of 2 mM diamide on uptake as indicated by the distribution ratio of various amino acids and α-methyl-D-glucoside after 30 min of incubation at 37°C. The presence of the thiol oxidant during the entire incubation time resulted in decreases of 22—32% in the uptake of glycine, valine, α-aminoisobutyric acid, and the methylglucoside. Clear-cut relationship between diamide concentration and the distribution ratio of 0.065 mM glycine uptake is shown in Fig. 1. There was a variable response to diamide concentration of 1 mM or less but consistent inhibition at 2 mM diamide. The maximal

TABLE I

EFFECT OF DIAMIDE ON UPTAKE OF VARIOUS SUBSTRATES

Cortical slices were incubated for 30 min at 37°C in Krebs-Ringer bicarbonate buffer, pH 7.4, with 0.065 mM amino acids or 2.065 mM α-methyl-D-glucoside. Diamide concentration was 2 mM. Uptake is reported as the distribution ratio, which represents the mean ± S.E. for the number of determinations indicated in the parentheses. Student's *t*-tests were used to determine the significance of differences between control and diamide treated samples.

Substrate	Distribution ratio	
	Control	Diamide
Substrate	3.70 ± 0.26 (6)	2.88 ± 0.15 (6) *
Valine	2.53 ± 0.18 (4)	1.89 ± 0.08 (4) *
α-Aminoisobutyric acid	2.89 ± 0.23 (8)	1.96 ± 0.08 (6) *
α-Methyl-D-glucoside	2.37 ± 0.07 (8)	1.69 ± 0.07 (6) **

* $P < 0.02$.
** $P < 0.01$.

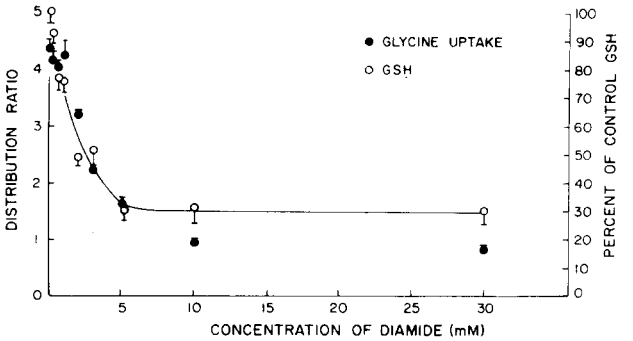

Fig. 1. The effect of diamide concentration on glycine uptake and GSH levels in cortical slices. Rat renal cortical slices were incubated for 30 min with 0.065 mM [^{14}C]glycine in Krebs-Ringer bicarbonate buffer, pH 7.4, with varying amounts of diamide present as described in the text. Glycine uptake (●) was measured as the distribution ratio of radioactivity in the cells. GSH levels (○) are expressed as the percent of control GSH where no diamide was present in the control incubations. The results given are the means of 4—10 determinations. Brackets indicate S.E.

effect of diamide on glycine uptake was reached between 5 and 10 mM diamide. At these concentrations of the drug, incubation of slices in the presence of diamide resulted in a distribution ratio for 0.065 mM glycine of about 1, indicative of the cessation of active transport.

The time course of uptake of 0.065 mM glycine is shown in Fig. 2A. 1 mM diamide (■) produced a significant decrease of the steady-state distribution

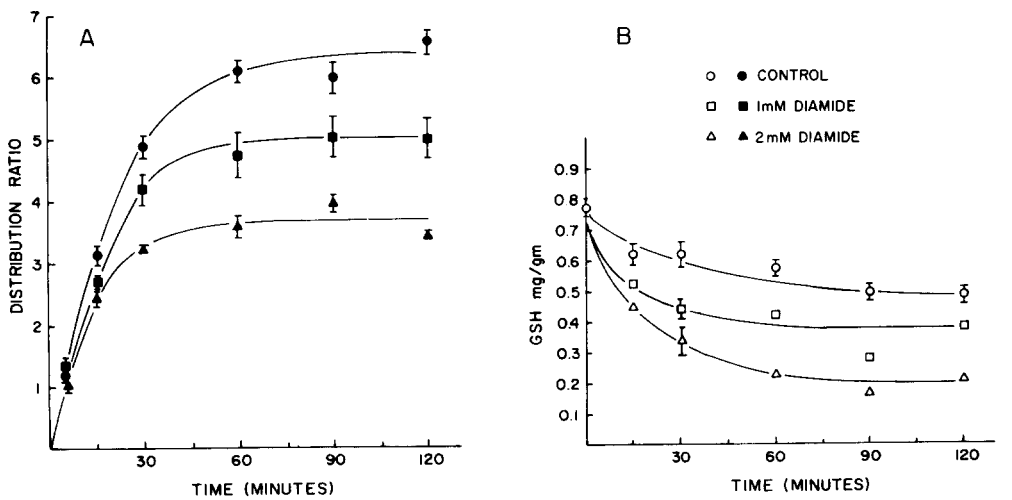

Fig. 2. The influence of diamide on the time course of glycine accumulation and GSH levels in rat renal cortical slices. (A) Cortical slices were incubated with 0.065 mM [^{14}C]glycine in Krebs-Ringer bicarbonate buffer at 37°C with (■,▲) and without (●) diamide as described in the text. Uptake was measured as the distribution ratio for ^{14}C which was determined for slices which were incubated for the length of time indicated with no diamide present (●) and with 1 mM (■) or 2 mM (▲) diamide. Results given are the mean of 3—6 determinations. (B) GSH levels were measured by the method of Tietze [19] in slices incubated at 37°C in Krebs-Ringer bicarbonate buffer alone (○) or in Krebs-Ringer bicarbonate buffer + 1 mM (□) or 2 mM (△) diamide for the times indicated. The means of 4—8 determinations are given. Brackets indicate S.E. Where no brackets appear, S.E. are included in the size of the point representing the means.

ratio. 2 mM diamide (▲) appeared to inhibit the initial rate of uptake as well as cause about a 40% decrease in the steady-state.

Kinetic analysis of the diamide effect

An analysis of the steady-state influx and efflux of glycine was performed for a two compartment system as described previously [17]. Cortical slices were incubated at 37°C for 30 min with 0.065 mM glycine in either buffer alone or buffer containing 2 mM diamide, after which the flasks were opened and a tracer quantity of [^{14}C]glycine added to each. The subsequent uptake of tracer glycine was determined at the intervals shown in Fig. 2A but is not presented in this figure. The distribution ratio of [^{14}C]glycine at 5 min of incubation thus determined was 1.58 ± 0.10 for the control and 0.84 ± 0.07 for the diamide-treated slices with values for later time points being similar to those in Fig. 2A. There appeared to be a greater diamide effect on the 5 min glycine uptake value after the 60 min preincubation to the steady state which suggests that time is required for the diamide to have an effect. Table II shows the calculated influx and efflux steady-state rate constants as well as the total flux of glycine for control slices and slices incubated with 2 mM diamide. The influx rate constant was reduced by about 50% while no dramatic effect was seen on the efflux rate constant. To substantiate the lack of effect of diamide on efflux, slices were incubated with [^{14}C]glycine in the absence and presence of diamide for 60 min to achieve a steady state, after which the slices were transferred to fresh buffer and the appearance of radioactive glycine in the medium determined as described previously [14]. The rate of loss of glycine from the cells was the same for the control and diamide-treated tissue, thus confirming the indication that the primary effect of diamide is on the process of substrate entry into cortical cells.

Reversal of the diamide effect

Since the diamide effect on thiol oxidation is known to be reversed by

TABLE II

INFLUENCE OF DIAMIDE ON KINETIC PARAMETERS OF STEADY-STATE GLYCINE TRANSPORT

Medium $\underset{\lambda_{MI}}{\overset{\lambda_{IM}}{\rightleftharpoons}}$ Intracellular space

All calculations are based on 100 mg of tissue and an intracellular space of 52.8% wet tissue weight.

Incubation conditions	Medium pool size (μmol)	Steady-state distribution ratio	Intracellular fluid pool (μmol)	Fractional turnover rate/min *		Net flux (μmol/min per 100 mg wet weight)
				λ_{IM}	λ_{MI}	
Control	0.13	6.25	0.0214	0.0083	0.0503	0.00108
Diamide (2 mM)	0.13	3.70	0.0127	0.0044	0.0454	0.00058

* The rate constants are related by equation $M \cdot \lambda_{IM} = ICFP \cdot \lambda_{MI}$, where M is medium pool size and $ICFP$ is the intracellular fluid pool.

TABLE III

EFFECT OF GLUCOSE AND PYRUVATE ON DIAMIDE INHIBITION OF GLYCINE UPTAKE AND GSH LEVELS

The distribution ratio for 0.065 mM glycine uptake represents the mean ± S.E. of the number of separate determinations indicated in the parenthesis for 30-min incubations of renal cortical slices in Krebs-Ringer bicarbonate buffer, pH 7.4, at 37°C. Diamide concentration was 2 mM and glucose or pyruvate concentration was 5 mM. The statistical differences from controls were determined by the Student's *t*-test for paired data.

Addition	Distribution ratio	mg GSH/g
None	3.69 ± 0.01 (4)	0.623 ± 0.059 (3)
Glucose	3.97 ± 0.22 (4)	0.549 ± 0.042 (3)
Pyruvate	3.92 ± 0.11 (5)	0.594 ± 0.043 (3)
Diamide	2.87 ± 0.11 (6) **	0.345 ± 0.057 (3) *
Diamide + glucose	2.86 ± 0.15 (5) **	0.444 ± 0.044 (3) *
Diamide + pyruvate	3.80 ± 0.18 (5)	0.566 ± 9,914 (3)

* $P < 0.05$.
** $P < 0.001$.

glucose [10], the effect of 5 mM glucose and pyruvate on preventing the diamide inhibition of glycine uptake was studied. The result of these experiments where the cortical slices were incubated together with diamide and glucose or pyruvate are shown in Table III. The addition of 5 mM glucose did not reverse the diamide effect, but the addition of 5 mM pyruvate completely returned the glycine uptake to the control level.

The addition of 5 mM dithiothreitol, which is a potent disulfide-reducing agent, also obviated the diamide effect (Table IV). The addition of GSH (not shown) to the incubation did not reverse the diamide effect. In fact, the addition of 2 mM GSH itself to buffer was found to inhibit amino acid uptake.

Relationship of diamide effect on transport to cellular GSH

GSH levels in renal cortical slices decreased on incubation in bicarbonate buffer as shown in Fig. 2B, an observation also reported by others [5,26]. The amount of decrease was variable, but by 90 min the levels were generally 25% below that observed in unincubated fresh tissue. GSH levels decreased further

TABLE IV

REVERSAL OF THE EFFECT OF DIAMIDE BY DITHIOTHREITOL (DTT)

Results are expressed as the mean ± S.E. for the number of determinations indicated in the parentheses.

Substrate	Distribution ratios after 30 min of incubation			
	Control buffer	Buffer + 5 mM DTT	Buffer + 2 mM diamide	Buffer + 2 mM diamide + 5 mM DTT
Glycine (0.065 mM)	4.11 ± 0.13 (5)	3.98 ± 0.31 (6)	3.26 ± 0.12 (5) *	4.44 ± 0.26 (6)
α-Methyl-D-glucoside (2.06 mM)	2.50 ± 0.05 (5)	2.44 ± 0.08 (3)	1.77 ± 0.09 (3) *	2.37 ± 0.08 (3)

* Levels differ from control levels at $P < 0.01$.

upon incubation in the presence of diamide. After 60 min, the GSH levels in tissues incubated with 1 and 2 mM diamide reached a plateau (Fig. 2B) at about 70 and 40%, respectively, of that observed in buffer alone. Comparison of glycine uptake with GSH concentration in Fig. 2 suggests that there is a relationship between uptake of the amino acid and the GSH concentration. This relationship was studied further by examining the changes in the 30 min distribution ratio for glycine uptake and corresponding GSH levels with increasing diamide concentration in the incubation, the results of which are shown in Fig. 1. The decrease in distribution ratio of uptake corresponds well with the decrease in cellular GSH levels. A significant decrease in GSH was observed between 0.5 and 1.0 mM diamide and the maximum effect was observed at 5 mM diamide. Diamide caused a significant decrease in glycine uptake at diamide concentrations between 1 and 2 mM with the maximal effect occurring between 5 and 10 mM diamide.

The ability of pyruvate to prevent the decreased uptake of glycine in cortical slices caused by diamide was paralleled by maintenance of GSH at levels found in control tissue (Table III). The addition of glucose, which failed to block the diamide effect on uptake, also failed to maintain the GSH at the level of untreated tissue.

In an attempt to find an experimental situation where the relationship of GSH to amino acid uptake could be studied without the use of a thiol oxidant, we examined stored renal cortical slices [21—23]. This model has been used in the past to show the dissociation of cellular ATP levels and transport [27]. Previous studies [21—23] have shown amino acid and sugar uptake by rat and human renal cortex slices stored for 24 h at 4°C is similar to that of freshly prepared tissue. In view of the decreased level of GSH as fresh tissue in incubated in buffer, it seemed likely that the GSH level of stored tissue would be low. This proved to be true as shown in Table V where the GSH level in stored tissue was low and remained at a level about 50% of fresh tissue during a 37°C incubation. The level of GSH in the stored slices was close to or lower than that of slices incubated with 2 mM diamide. Despite the low GSH levels in stored tissue, uptake of α-aminoisobutyric acid was not impaired. Thus, the stored slice demonstrates that partial depletion of cellular GSH need not necessarily be associated with impaired uptake of amino acids.

TABLE V

EFFECT OF TISSUE STORAGE AT 4°C ON α-AMINOISOBUTYRIC ACID (AIB) UPTAKE AND CELLULAR GSH LEVELS

n.s., not significant.

Time of incubation (min)	Distribution ratio for 0.065 mM AIB			mg GSH/g tissue	
	Fresh	Stored		Fresh	Stored
0	—	—		0.632 ± 0.037 (6)	—
30	2.34 ± 0.06 (9)	1.97 ± 0.27 (5)	n.s.	0.454 ± 0.021 (6)	0.221 ± 0.016(4) *
60	2.88 ± 0.41 (6)	2.04 ± 0.17 (9)	n.s.	0.310 ± 0.024 (3)	0.160 ± 0.026 (4) *
90	3.00 ± 0.21 (9)	2.49 ± 0.22 (6)	n.s.	0.322 ± 0.018 (5)	0.133 ± 0.011 (4) *

* Levels in stored tissue differ from levels in fresh tissue at $P < 0.001$.

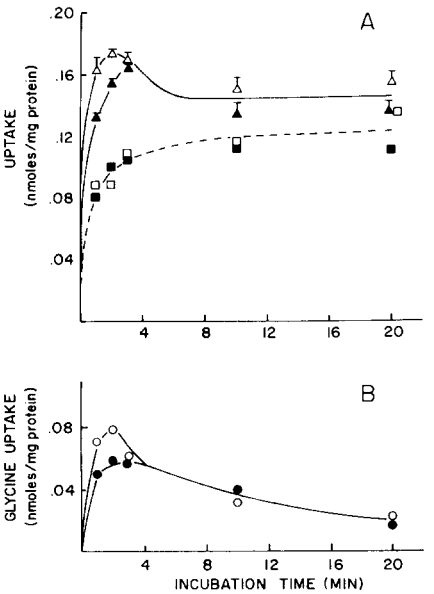

Fig. 3. Influence of 0.2 mM diamide on glycine uptake by isolated brush border vesicles. Uptake of 0.06 mM [^{14}C]glycine was measured by rapid filtration on Millipore filters (HAWP, 0.45 μm) as described previously [23]. Diffusion was assessed using 3-*O*-methyl-D-glucose (□,■) as previously discussed [23]. The time course of total [^{14}C]glycine uptake in the presence (▲) and absence (△) of 0.2 mM diamide is presented in (A), along with the diffusion component in the presence (■) and absence (□) of 0.2 mM diamide. Glycine uptake in excess of diffusion is presented in (B). The time course of uptake in the presence of 0.2 mM diamide (●) is compared to that when no diamide is present (○). The values given are means of 12 determinations. Brackets indicate S.E. Where no bracket appears, S.E. is included in the size of the symbol representing the mean.

Glycine uptake by isolated brush border membrane vesicles

Several reports have appeared that indicate diamide causes not only GSH oxidation but also oxidation and cross-linking of sulfhydryl groups in cellular proteins [28]. The possibility that diamide itself could cause alteration in the brush border membrane of renal tubule cells was therefore examined. Isolated brush border membrane vesicles were incubated with diamide and the uptake of glycine was compared with that of untreated membranes. Fig. 3 shows that diamide had a direct effect on the initial uptake of glycine by isolated membranes in the presence of a sodium gradient. This is consistent with the concept that diamide acts on the membrane surface, perhaps in a manner similar to the effect observed on sulfhydryl groups in cellular protein. Diamide had no effect on trapped and diffused space in the membrane vesicle, indicating that the membrane effect was primarily due to a reduced rate of Na^+ gradient-dependent uptake of glycine. Whether the focus for the effect was on Na^+ transport, glycine transport, or both was not determined.

Discussion

The description of diamide as a potent, membrane-penetrating GSH-oxidizing agent at 4°C has led to numerous investigations in which the experimental protocol has been the preincubation of tissues with large amounts of the

chemical followed by incubations in its absence [5,7,10,29]. With this type of protocol, Hewitt et al. [5] used 30 mM diamide to produce a 40% inhibition of glycine uptake by kidney cortex slices when incubated subsequently for 60 min in buffer at 37°C. The direct addition of diamide to muscle [8] and adipose tissue [30,31] has produced observable effects at $5 \cdot 10^{-5}$ and $1 \cdot 10^{-4}$ M, respectively. The presence of the drug during the entire incubation thus permits the use of smaller concentrations. In our experiments, the addition of 2 mM diamide during the uptake of glycine by renal cortical slices caused the same degree of inhibition, 40%, as 30 mM diamide pretreatment of Hewitt et al. [5]. Pillion et al. [32] have shown that 10 mM diamide did not reduce the steady-state levels of ATP in kidney cortex slices which had been incubated for as long as 90 min at 37°C in the presence of the compound. Thus the inhibition produced by 2 mM diamide in our experiments cannot be correlated with ATP levels. Since the effect of diamide is reversible, the pretreatment protocol produces an oxidized cellular environment from which the tissue recovers on subsequent incubation at 37°C. This results in assessing diamide effects in a non-steady state, especially with regard to GSH [5]. In the present studies, the addition of 1 or 2 mM diamide produces a new steady-state renal GSH level which is maintained for 2 h of incubation.

The protocol employed here permitted an assessment of the influence of diamide on kinetics of glycine flux. With the incubation of cortical tissue to a steady state with the drug and unlabeled glycine for 60 min, the measurement of tracer[^{14}C]glycine uptake could be analyzed by a two compartment model system [17,18]. The calculations showed that diamide caused a marked decrease in entry of amino acid without influencing the exit from the cells. This was substantiated by studies designed to measure efflux directly. Such a finding for α-amino acids is in contrast to that of Chesney and Jax [6] who reported that diamide cause an accelerated efflux of β-amino acids from renal tubule cells. It is not clear from their studies whether diamide affected entry also. Although they do show the low K_m but not the high K_m system for entry is impaired, the data are based on the uptake at 60 min, when the rapid efflux caused by diamide was profound. Diamide did not affect the entry or efflux of high taurine concentrations. In our own experiments, we focused on the uptake at near-physiological glycine levels [33].

The correlation of diamide medium concentration with transport and GSH levels was very good and pyruvate protection was associated with maintenance of GSH concentration. The fact, however, that α-aminoisobutyric acid, a non-metabolizable amino acid, is taken up by renal slices stored for 24 h in a similar fashion as fresh tissue, despite GSH being very low in the stored tissue, suggests that it may not be the glutathione decrease per se which is related to the diamide-induced impairment of transport. Diamide, which was previously thought to be a relatively specific oxidant for GSH, has been shown to have many other effects. In kidney slices, it inhibits protein kinases [29] and ($Na^+ + K^+$)-dependent ATPase [34]. In other systems, it has been shown to oxidize thiol groups of membranes [35] and cell proteins [28]. This may also be true in kidney membranes since the experiments reported here with isolated renal brush border membranes show a direct effect of diamide on the membrane uptake of glycine. However, since vesicle glycine uptake is electrogenic in

nature, the primary effect of diamide may be on sodium transport and the effect of diamide on vesicle uptake of glycine may be secondary. Indeed, Pillion et al. [34] have shown that high levels of diamide can increase the Na^+ content of cortical tissue thus reducing the electrochemical gradient for Na^+ in the cells.

Diamide can also oxidize NADH and NADPH [28] and alter calcium fluxes in muscle [8] and isolated mitochondria [9,36]. Indeed, diamide has other profound effects on mitochondria, uncoupling oxidative phosphorylation and stimulating mitochondrial ATPase activity [35]. These effects on mitochondria may be pertinent since other uncouplers of oxidative phosphorylation exhibit amino acid uptake [18,36]. Usually, however, uncouplers cause a marked acceleration of amino acid efflux [18,37] which was not observed here. Pyruvate protection of transport from the diamide effect may be related to oxidative metabolism and maintenance of the reduced state of pyridine nucleotide cofactors. Glucose itself was not beneficial which might be related to its impaired transport and/or breakdown to three-carbon fragments.

It is quite apparent that the effects of diamide on renal cells may be multiple [34] and that the primary events related to transport inhibition are not yet clearly discernable. It should be noted that diamide effects on transport and metabolism of various tissues may differ. Goldstein and Livingston [38] have shown a diamide-induced increase in passive permeability of adipocyte membranes to sugars. We have shown, in this report, that diamide produces no changes in passive permeability of renal brush border membranes with regard to 3-*O*-methyl-D-glucose, one of the sugars employed to measure diffusion in brush border vesicles [25]. Thiol oxidation may be an important feature. Czech et al. [30,31] indicate that fat cells respond to low concentrations of diamide by an increase in sugar transport and a disproportional increase in oxidation of D-[^{14}C]glucose over [6-^{14}C]glucose, an effect mimicked by H_2O_2. The data in fat cells indicate thiol oxidation is involved.

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