Structural requirements for protection by small amino acids against hypoxic injury in kidney proximal tubules[1]

JOEL M. WEINBERG,*·[2] MANJERI A. VENKATACHALAM,† RICARDO GARZO-QUINTERO,† NANCY F. ROESER,* AND JULIE A. DAVIS*

**Division of Nephrology, Department of Internal Medicine, University of Michigan and Veteran's Administration Medical Center, Ann Arbor, Michigan 48109-0676, USA; and †Departments of Pathology and Medicine, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284, USA*

Abstract Kidney proximal tubules are resistant to hypoxic injury if glycine or L-alanine is present in their incubation medium. Protection does not depend on the concentration or turnover of ATP in cells. We have investigated structure-function relationships that govern this protective activity. Among more than 45 amino acids and analogs examined, only glycine, L-alanine, D-alanine, β-alanine, and the neuronal glycine binding site agonist, 1-aminocyclopropane-1-carboxylic acid, were active. The protective effect could not be explained by amino acid metabolism. Ultrastructural features in protected cells were preserved to a degree which suggested that processes responsible for degradation during hypoxia were retarded. These results are consistent with stringent requirements of amino acid molecular structure for protection against hypoxia, and suggest the involvement of highly specific, acceptor-ligand effects on a process critical for maintaining cellular integrity. —WEINBERG, J. M.; VENKATACHALAM, M. A.; GARZA-QUINTERO, R.; ROESER, N. F.; DAVIS, J. A. Structural requirements for protection by small amino acids against hypoxic injury in kidney proximal tubules. *FASEB J.* 4: 3347-3354; 1990.

Key Words: hypoxic injury • kidney tubule cell • amino acids • glycine • alanine metabolism

MAMMALIAN CELLS THAT ARE STRICTLY aerobic, such as neurons and proximal tubule cells, are particularly susceptible to damage during hypoxia. High rates of oxidative metabolism are coupled to membrane-based transport functions in both cell types. These properties predispose them to rapid declines of adenosine triphosphate (ATP) during periods of respiratory arrest. Known strategies that protect cells from hypoxic injury involve energy turnover. For example, ectothermic animals survive hypoxia by down-regulating the rates of membrane transport and glycolysis in a coupled fashion, i.e., by metabolic arrest (1). Pharmacologic interventions that reduce work loads can protect certain mammalian cells from hypoxic injury (2-4). In contrast, we and others have reported that glycine and L-alanine, two small, neutral amino acids, confer upon kidney tubule cells striking tolerance to hypoxia and other forms of injury that is unrelated to improvement of cellular energetics (5-11). We now report stringent structural requirements for protection by the amino acid molecules not related to their metabolic interconversions. Also, we document the remarkable degree of ultrastructural preservation seen in protected cells. These observations suggest that hypoxia tolerance induced by amino acids is mediated by ligand-acceptor interactions with degradative processes responsible for lethal injury.

METHODS

Isolation and incubation of tubules

Proximal tubules were isolated from the kidney cortex of New Zealand white rabbits (≅ 2.5 kg; Shankin's Rabbitry, Warren, Mich.) by collagenase digestion and isopycnic centrifugation on Percoll gradients (5, 6). Final tubule pellets were resuspended in ice-cold medium gassed with 95% O_2/5% CO_2. The medium contained 105 mM NaCl, 2.6 mM KCl, 25 mM $NaHCO_3$, 2.4 mM KH_2PO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM $MgSO_4$, 0.6% dialyzed dextran (T40, Pharmacia, Piscataway, N. J.). The medium also contained 5 mM glucose, 4 mM sodium lactate, 1 mM L-

[1]A preliminary report of some of the data in this paper was included in the Minisymposium Molecular Targets of Cell Injury presented at the 74th Annual Meeting of the Federation of American Societies for Experimental Biology, April 2, 1990, Washington, D.C. Chaired by J. L. Farber and L. M. Buja.

[2]To whom correspondence and requests for reprints should be addressed, at: Nephrology Division, Rm. 1560, MSRB II, University of Michigan Medical Center, Ann Arbor, MI 48109-0676, USA.

0892-6638/90/0004-3347/$01.50.

alanine, and 10 mM sodium butyrate, a substrate combination providing excellent stability of the tubule preparation during in vitro study. Aliquots (5 ml) containing 5–7.5 mg tubule protein/ml were placed in siliconized 25-ml flasks that were gassed with 95% O_2/5% CO_2, sealed, and kept on ice until use. Flasks were gently shaken at 37°C in a water bath for warm incubation. Tubules were initially incubated at 37°C for 15 min. Then experimental additions were made and flasks were regassed with 95% N_2/5% CO_2 to produce hypoxia or 95% O_2/5% CO_2 to maintain oxygenated conditions. Flasks were then sealed and returned to the shaker. After 30 min of hypoxic incubation, tubules were sampled for analysis without a period of reoxygenation.

Analytical methods

Intracellular K^+ was measured by atomic absorption spectroscopy in tubules separated from their medium by centrifugation through bromododecane (Aldrich, Milwaukee, Wis.) (5). ATP was determined by HPLC[3] on trichloroacetic acid extracts (5). Lactate dehydrogenase (LDH) was measured in the medium before and after addition of 0.1% Triton X-100, as described (6). D-Alanine, L-alanine, and glutamate were assayed enzymatically (12, 13). Protein was measured by the Lowry method (14).

Morphology

Tubules were fixed in suspension or after pelleting at ≅12,000 g. Fixatives were 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 or 50 mM lysine-2% glutaraldehyde (15). Postfixation and tissue processing followed standard procedures.

Reagents

All reagents were obtained from Sigma (St. Louis, Mo.) unless otherwise indicated. They were added from 200-fold concentrated, neutral pH, aqueous stock solutions. Unless otherwise indicated, L-amino acids were used.

Statistics

Multiple groups were compared by analysis of variance for repeated measure designs and the Neuman-Keuls test for multiple comparisons (PC ANOVA, Human Systems Designs, Northridge, Calif.). Differences with $P < 0.05$ were considered statistically significant.

RESULTS

Protection by glycine, alanine, and 1-aminocyclopropane-1-carboxylic acid (ACPC)

Tubules supplemented with either 2 or 5 mM glycine, L-alanine, β-alanine, or D-alanine during 30 min hypoxia released less LDH than untreated tubules (**Fig. 1**). At 2 mM, glycine reduced LDH release to a greater extent than the other compounds. At 5 mM, L-alanine reduced LDH release to the same degree as glycine, and significantly more than β-alanine and D-alanine. β-Alanine was more effective than D-alanine. Among other compounds tested, another agent with protective effects was identified. ACPC, an agonist at the glycine modulatory site of the neuronal N-methyl-D-aspartate (NMDA) receptor (16, 17), had a dose-dependent effect similar to that seen with β-alanine (Fig. 1, insert).

Hypoxic tubules had sharp decreases of ATP and K^+ relative to controls (**Table 1**). Neither ATP nor K^+ levels were significantly affected by any of the treatments. We had shown previously that altered metabolic parameters (ATP, K^+) in hypoxic tubules treated with glycine or L-alanine revert to normal values after recovery in oxygenated medium, and that protection by the amino acids lasts for hypoxic periods of as long as 90 min (5, 7). Similar observations were made during the present study (data not shown).

Morphology

Unsupplemented hypoxic tubules with 70–80% LDH released to the medium were almost completely necrotic (**Fig. 2**). In contrast, hypoxic tubules with protective amino acids showed a degree of structural preservation that could not be distinguished readily from oxygenated controls by light microscopy (Fig. 2). Hypoxic tubules treated with D-alanine showed slightly greater numbers of damaged cells than those treated with glycine, L-alanine, β-alanine, or ACPC. By electron microscopy, most unsupplemented hypoxic tubules showed total disruption of cell structure (**Fig. 3*A***); a small minority showed severe damage not amounting to the necrosis shown in Fig. 3*A*. Figures 3*B–D* show the morphology of glycine-protected tubules. In Fig. 3*B*, structural preservation is apparent in every tubule profile as evaluated by light microscopy. The extent of protection may be judged from the overall appearance by electron microscopy and the preserved structure of microvilli, cell junctional complexes, clathrin-coated membrane pits, mitochondria, and peroxisomes (Fig. 3*C*, Fig. 3*D*). Focal areas of membrane fusion, swelling, and blebbing of microvilli, and dissociation of actin cores in microvilli attributable to ATP depletion were indeed occurring in this population of cells. However, the damage was minor, which indicates that progressive structural disruption had been retarded (Fig. 3*D*). A small minority of cells (≅10%) was necrotic, as might be expected from the LDH data, and a similarly small minority had intermediate forms of reversible damage (not shown).

[3]Abbreviations: LDH, lactate dehydrogenase; ACPC, 1-aminocyclopropane-1-carboxylic acid; HPLC, high-performance liquid chromatography; NMDA, N-methyl-D-aspartate.

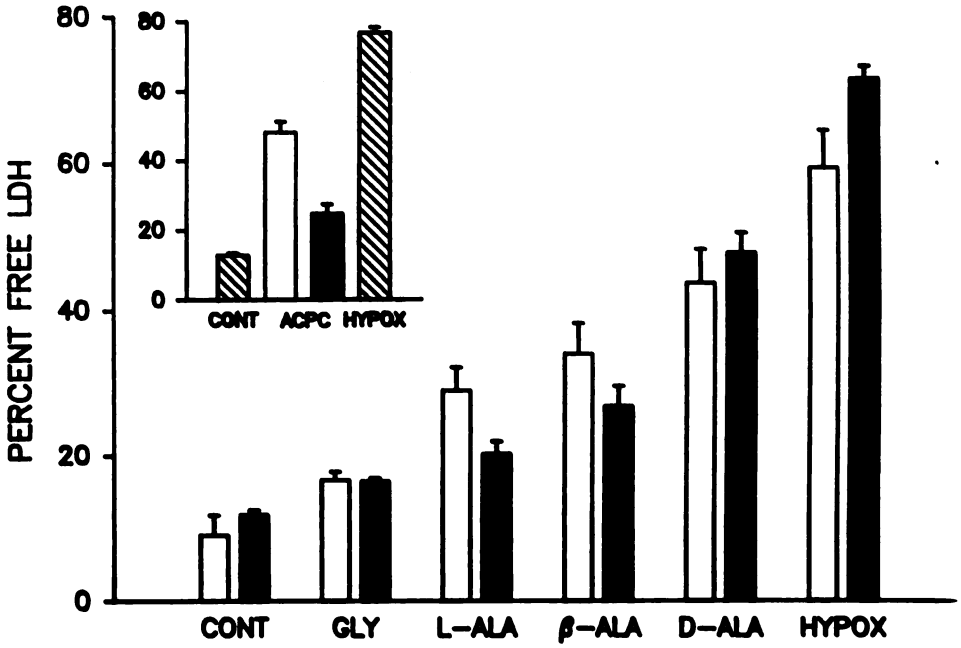

Figure 1. Release of LDH during hypoxia. Free LDH is shown as the percentage of total LDH (medium + cells). Tubules were incubated under oxygenated conditions for 15 min and then for 30 min with O_2/CO_2 (CONT), N_2/CO_2 without supplements (HYPOX), or N_2/CO_2 with glycine (GLY), L-alanine (L-ALA), β-alanine (β-ALA), or D-alanine (D-ALA). The agents were added at either 2 (open bars) or 5 mM (filled bars) at the start of hypoxia. There were separate oxygenated and hypoxic controls for the 2 and 5 mM groups. Values are means ± SE (n = 4). Differences between tubules with amino acids and those without were significant ($P < 0.01$; $P < 0.05$ for 2 mM D-ALA). At 2 mM, glycine was superior to all alanines ($P < 0.05$). At 5 mM, glycine and L-alanine were equally effective, and better than β-alanine and D-alanine ($P < 0.05$); β-alanine was superior to D-alanine ($P < 0.05$). Inset) Similar data for 1-aminocyclopropane-1-carboxylic acid (ACPC). 2 mM (open bars), 5 mM (filled bars). Oxygenated and hypoxic controls are shown as hatched bars. Values are means ± SE (n = 5-7). Differences among all groups, $P < 0.01$.

Relationship of protection to alanine metabolism

Since the standard incubation medium for all studies contained 1 mM alanine, we studied the possible contribution of alanine metabolism to the protective effects by measuring the amino acid in the medium. Oxygenated tubules consumed L-alanine rapidly; this metabolism was suppressed during hypoxia (**Fig. 4**). Glutamate appeared stoichiometrically as alanine disappeared (Fig. 4). Consumption of L-alanine ceased during hypoxia regardless of whether glycine, β-alanine, or D-alanine had been provided. This led to ≅500 μM L-alanine remaining in the medium during the 30-min hypoxic period (Fig. 4, Fig. 5). L-Alanine levels of tubules treated with additional L-alanine reflected the concentrations added (2 or 5 mM) plus the alanine remaining from that originally provided in the incubation medium (**Fig. 5**). D-Alanine concentrations at the end of hypoxia in tubules treated with 2 and 5 mM concentrations were 2.1 ± 0.3 and 5.2 ± 0.5 mM, respectively.

TABLE 1. *Cell ATP and K^+ during hypoxia with and without amino acids*[a]

	ATP	K^+
	nmol/mg cell protein	
Control	11.14 ± 1.18	346.5 ± 5.0
Hypoxia, untreated	0.44 ± 0.06	69.8 ± 2.5
Hypoxia, glycine	1.12 ± 0.22	67.1 ± 4.9
Hypoxia, L-alanine	1.00 ± 0.12	68.4 ± 3.9
Hypoxia, β-alanine	1.13 ± 0.18	72.3 ± 4.9
Hypoxia- D-alanine	0.76 ± 0.15	70.9 ± 5.6

[a] Values are means ± SE (n = 4). Control tubules were kept oxygenated throughout. All other groups were subjected to 30-min hypoxia in the presence of 2 mM concentrations of the indicated amino acids.

Concentration dependence of protection

The effects of glycine and alanine were also compared under conditions where alanine that was carried over from the preincubation period was removed by washing before hypoxia (**Fig. 6**). Glycine and alanine were similarly and virtually completely protective at 5 mM, but glycine was better at all lower concentrations ($P < 0.01$).

Effects of additional amino acids and related compounds

A large number of additional compounds structurally related to glycine and alanine were studied: other amino acids, agents with activity at neuronal glycine receptors, and metabolites and derivatives of glycine. **Table 2** shows the results and includes some related observations published earlier. None of these compounds provided protection.

DISCUSSION

Among the more than 45 amino acids and analogs examined, only glycine, L-alanine, D-alanine, β-alanine,

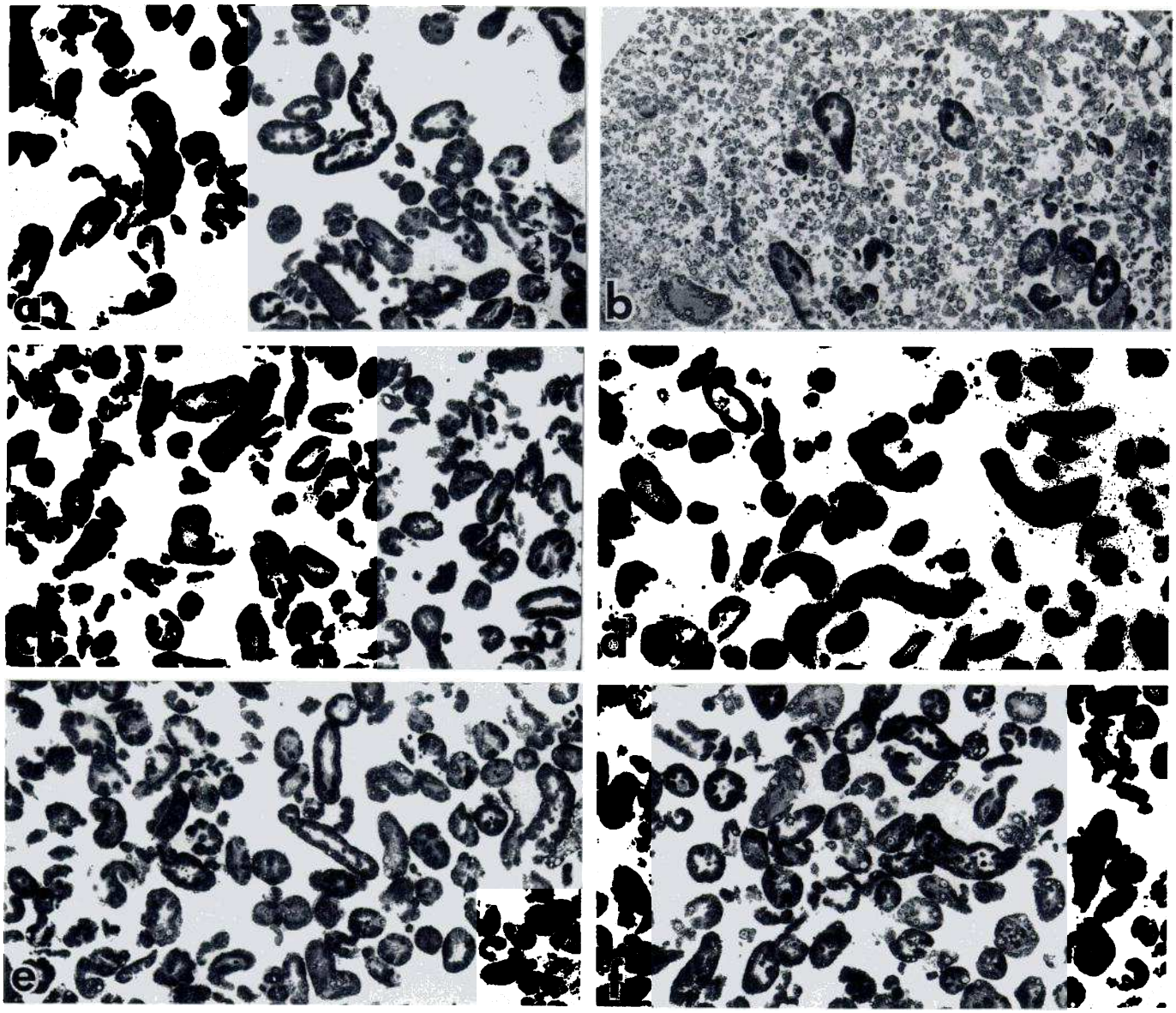

Figure 2. Low magnification photomicrographs of tubules fixed in suspension, pelleted, and processed for microscopy. Unsupplemented hypoxic tubules (*b*) show virtually complete necrosis with few survivors compared with oxygenated controls (*a*). Tubules supplemented with L-alanine (*c*), 1-aminocyclopropane-1-carboxylic acid (*d*), and β-alanine (*e*) show preservation of structure nearly as well as the controls. Tubules with D-alanine (*f*) show good preservation of structure, but not as well as that seen with the other agents (×130). All amino acids were 5 mM.

and ACPC were protective. **Figure 7** summarizes their structures and those of other closely related compounds that were inactive. Glycine and L-alanine were highly protective, as suggested by previous reports (5, 7, 8). Glycine was better than L-alanine at 2 mM, but not at 5 mM. Alanine differs from glycine only in the presence of a methyl group and in its chirality. L-Alanine was more effective than D-alanine. Inserting a methylene group in alanine to form α-aminobutyric acid eliminated protection. Addition of an α-methyl group to alanine to yield α-aminoisobutyric acid also made it inactive. However, the structurally similar cyclic compound, ACPC, had considerable activity. The presence of hydroxylmethyl (serine) or mercaptomethyl (cysteine) groups abolished activity. Transferring the amino group of alanine to carbon 2 to form β-alanine did not substantially impair protection, but adding a methyl group to β-alanine to form β-aminobutyric acid negated the effect.

Both amino and carboxyl groups in their free form are required to provide biological action. Thus taurine, cysteamine (not shown in the figure), sarcosine, propargylamine, betaine, aminoacetonitrile, ethanolamine and alaninol, all of which have variations in structure relative to glycine and alanine that are limited to either their amino or carboxyl groups, showed no activity. Propionic and acetic acid, which lack the amino groups, and methylamine, ethylamine, and isopropylamine (not shown in the figure), which lack the carboxyl groups, were also ineffective. Structurally unrelated amino acids such as glutamate, glutamine, methionine, phenylalanine, isoleucine, valine, aspartic acid, and arginine were without effect.

These data indicate that a highly specific, limited

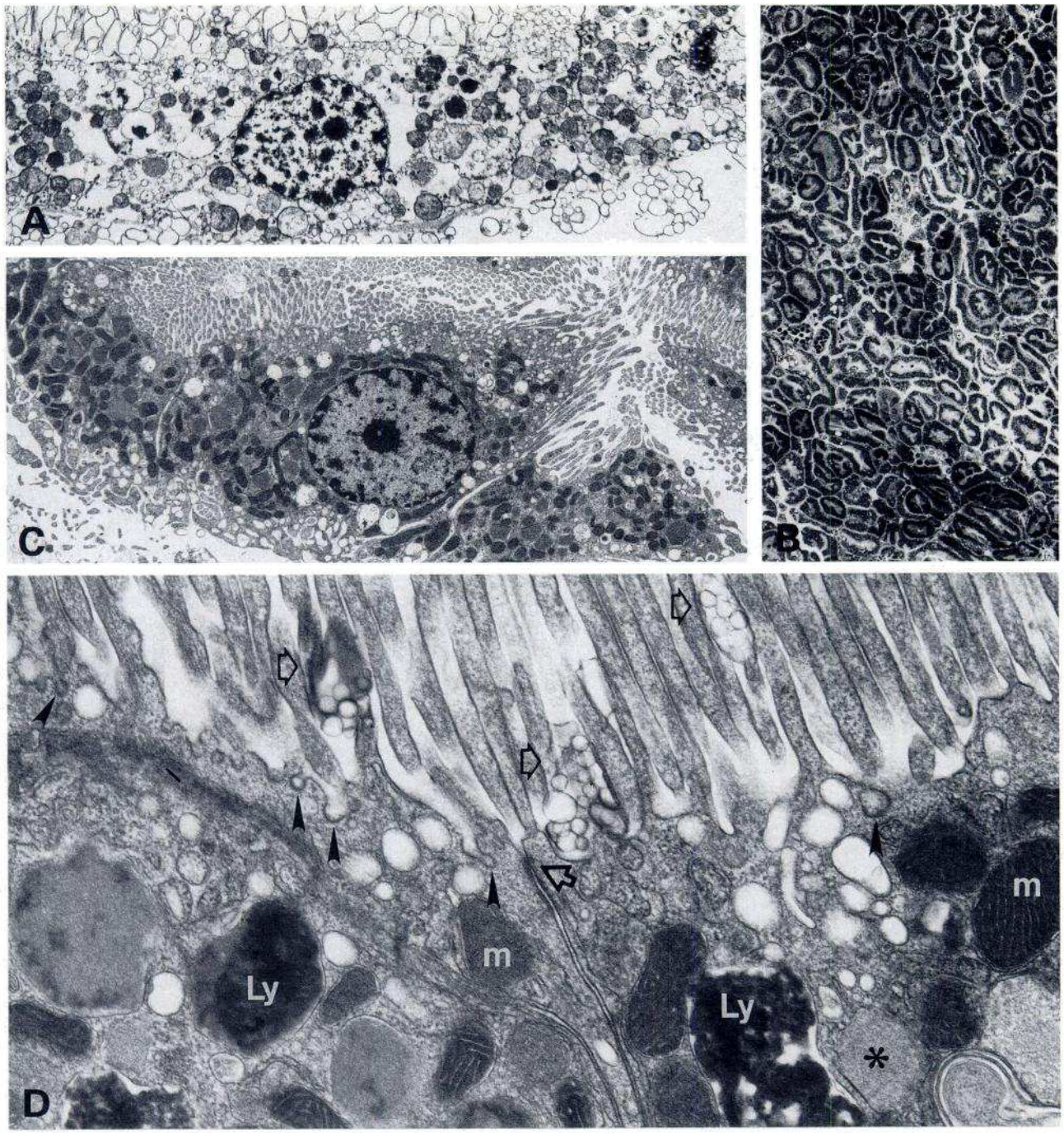

Figure 3. *A*) Electron micrograph representative of the majority of hypoxic tubules without supplements, corresponding to the light micrograph (Fig. 2*b*). There is total disruption of cell structure (×4000). *B*) Low magnification micrograph of hypoxic tubules given 2 mM glycine, fixed after centrifugation and pelleting. Many tubules are compacted together, and most have a recognizable structure (×130). *C*) Electron micrograph corresponding to the majority of tubules seen in Fig. 3*B*. There is excellent overall preservation of cell structure except for focal swelling of microvilli (×4000). *D*) Electron micrograph (×26,000) of cell from hypoxic tubules given 2 mM glycine. There is excellent preservation of cell ultrastructure including intact clathrin-coated membrane pit (small arrowheads), intercellular junctional complexes (short arrow), mitochondria (m), peroxisomes (*), and lysosomes (Ly). There are focal areas of membrane damage and blebbing in microvilli (wide arrows). Micrographs of higher resolution showed many areas of disruption of actin fibrils in the microvilli (not shown).

configuration is responsible for activity and that metabolic transformation of the protective agents is probably not required. This conclusion is based on the measurements of alanine in the present manuscript and previously reported glycine determinations (5). L-Alanine is metabolized by oxygenated tubules. The simultaneous and stoichiometric production of glutamate (Fig. 4) indicates that this metabolism involves transamination. However, L-alanine metabolism was completely inhibited during hypoxia. Moreover, both glycine and L-alanine were fully protective even if they were added only during hypoxia (refs 5, 7 and the present study). There was also no measurable consumption of L-alanine or D-alanine supplements. D-Alanine is metabolized by D-amino acid oxidase (12): this pathway is inoperative in the absence of oxygen.

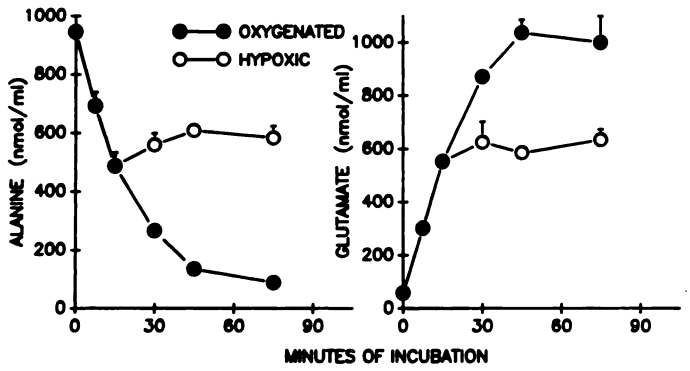

Figure 4. Tubule suspension L-alanine and glutamate levels. Tubules were incubated in the standard medium (1 mM alanine) either continuously under oxygenated conditions or under hypoxic conditions started after an initial 15 min of oxygenated incubation. Consumption of alanine is continuous during oxygenated conditions and ceases during hypoxia. Alanine is ≅500 μM after 15 min of oxygenated incubation. Glutamate increases stoichiometrically as alanine is consumed. Values are means ± SE (n = 3).

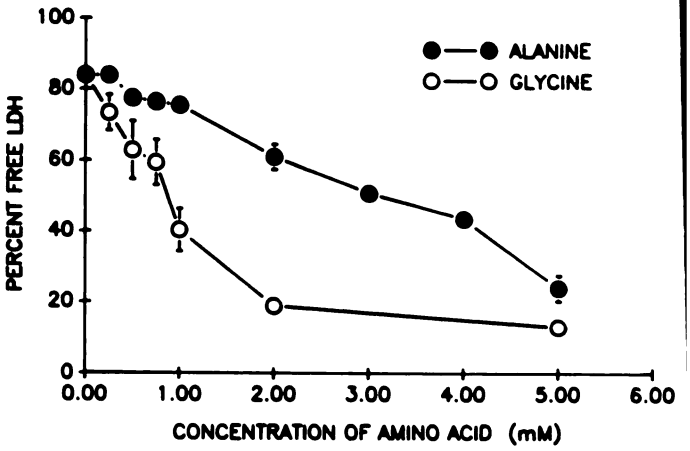

Figure 6. Concentration dependence of protection mediated by glycine and L-alanine. Tubules were incubated with O_2/CO_2 for 15 min. resuspended in fresh medium with either glycine or L-alanine in different concentrations from 0.25 to 5 mM, and immediately gassed with N_2/CO_2 for 30 min of hypoxia. Values are means ± SE (n = 4).

There are no known metabolic interconversions of ACPC.

Glycine in the kidney is mainly metabolized to serine by a two step process beginning with the glycine cleavage reaction (18, 19). Oxidative deamination by glycine oxidase to glyoxylate may also occur (19). Neither serine nor glyoxylate was protective. Thus, even if glycine had been partially metabolized to serine or glyoxylate, such a conversion cannot explain protection. Aminoacetonitrile, propargylamine, valproic acid, and cysteamine, which all inhibit the glycine cleavage system (20), neither protected against nor exacerbated injury. Incorporation of glycine into proteins, purines, and other complex molecules is an unlikely explanation, as these energy-dependent reactions would cease during hypoxia.

Glycine is actively transported and concentrated to high levels in oxygenated, metabolizing proximal tubule cells (21). However, our data show that the protective amino acids are effective even if they are present only during hypoxia when active transport is not a factor. Under these conditions, they rapidly reach intracellular concentrations similar to medium levels (22).

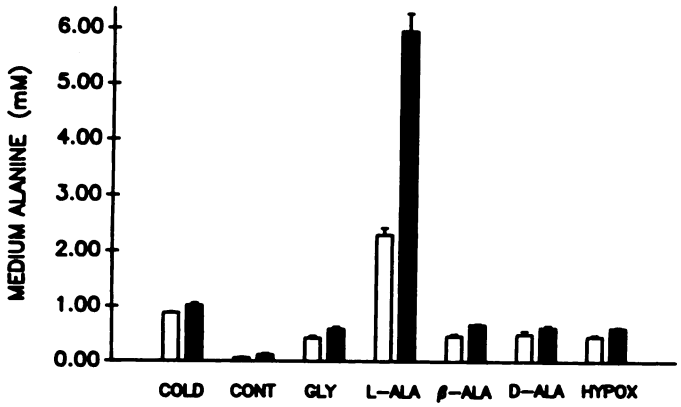

Figure 5. L-Alanine in the medium in the experiments described in Fig. 1. Measurement was made at the end of 15 min preincubation + 30 min hypoxia. Bars marked cold show alanine levels at the beginning of incubation when tubules were still in cold medium.

The protective effects were not mediated by cell ATP. Slightly higher ATP levels of glycine-protected cells in previous studies were likely an effect rather than the cause of structural preservation (6, 7, 9). Cell K^+, a sensitive indicator of energy availability (6, 9), fell to similar levels in protected and unprotected cells. We have also observed that the rates of decline of ATP mass and of ^{32}P (Pi)-labeled ATP are similar in protected and unprotected tubules (data not shown).

Based on the apparent lack of a requirement for metabolism for these protective effects, a comparison of structure activity relationships reported here with those defined for neuronal glycine receptors is relevant. Two classes of glycine receptors have been characterized in neuronal cells. The strychnine-sensitive, inhibitory glycine receptor is half-maximally activated at glycine levels of approximately 100 μM (23). L-Alanine, D-alanine, L-serine, taurine, and β-alanine are also agonists for this receptor (23, 24). L-Serine and taurine did not protect kidney tubules from hypoxia. Recently, a high-affinity neuronal glycine binding site, with effects to modulate activity of the N-methyl-D-aspartate subtype of glutamate receptors, has been described (17, 25–27). ACPC and D-alanine, which protected renal tubules from hypoxia, are agonists for the NMDA-modulatory site (16, 17, 28). However, D-serine is also a potent agonist at this site (28) and was ineffective in the tubule system. Two antagonists at the glycine-modulatory site, kynurenic acid (26, 29) and cycloleucine (30), lacked effects on the tubules, and in other studies (not shown) did not alter the effects of glycine.

Thus, the protective effects of glycine have overlapping degrees of specificity to those evident at neuronal glycine receptors. This comparison suggests that a reversible ligand binding site interaction with highly constrained steric, electronic, and conformational requirements accounts for protection as it does for neuronal binding. However, the interaction must be of

TABLE 2. *LDH release by hypoxic proximal tubules during treatment with additional amino acids and related compounds*[a]

	Percent free LDH
L-Glutamate	Not protective (ref 5)
L-Glutamine	Not protective (ref 7)
L-Cysteine	Not protective (ref 5)
Taurine	77.0 ± 6.8
L-Serine	Not protective (ref 5)
D-Serine	76.4 ± 6.6
L-Methionine	61.9 ± 5.8
L-Phenylalanine	67.3 ± 3.5
L-Isoleucine	63.9 ± 3.4
L-Valine	66.6 ± 4.4
L-Arginine	Not protective (ref 7)
L-Aspartate	Not protective (ref 7)
α-Aminoisobutyric acid	57.9 ± 4.0
L-α-Aminobutyric acid	71.1 ± 5.4
DL-β-Aminobutyric acid	63.8 ± 3.4
γ-Aminobutyric acid	60.1 ± 4.3
L-α-Aminovaleric acid	69.1 ± 5.6
δ-Aminovaleric acid	67.2 ± 3.3
Ethanolamine	59.7 ± 3.0
Alaninol	66.8 ± 4.1
Acetate	58.4 ± 8.2
Propionate	54.1 ± 4.2
Glyoxylate	66.7 ± 1.5
Sarcosine	72.2 ± 0.9
Propargylamine	67.1 ± 3.8
Betaine	69.4 ± 4.7
Acetonitrile	70.1 ± 4.5
Glycyl-sarcosine	64.2 ± 5.6
Glycine anhydride	72.8 ± 5.9
Iminodiacetic acid	75.4 ± 4.7
β-Chloro-L-alanine	76.9 ± 2.5
β-Chloro-D-alanine	75.8 ± 3.7
N-acetyl-L-cysteine	68.4 ± 2.8
Cysteamine	69.7 ± 4.6
Valproic acid	67.0 ± 3.4
Kynurenic acid	67.8 ± 6.2
Cycloleucine	89.5 ± 10.6

[a] Values are means ± SE of data from at least four experiments except for the acetate, propionate, glycine anhydride, and glycylsarcosine groups, which consisted of experiments on three tubule preparations. The experimental agents were studied in seven groups, each of which also included paired time control, untreated hypoxic, and glycine-treated hypoxic tubules. Average percent free LDH values from the seven experimental groups were: time control, 13.4; hypoxia, 66.1; hypoxia + 2 mM glycine, 19.7. Test agents in all groups were added at 2 mM coincident with the start of hypoxia. In at least two additional studies, the following compounds were also tested at 5 mM: glutamate, L-serine, L-cysteine, α-aminoisobutyric acid, L-α-aminobutyric acid, DL-β-aminobutyric acid, γ-aminobutyric acid, valine, and isoleucine. Several additional compounds not listed above were also tested in two studies: L-leucine (5 mM), L-threonine (5 mM), pyruvate (5 mM), methylamine (2 and 5 mM), ethylamine (2 and 5 mM), and isopropylamine (2 and 5 mM). None of these compounds provided protection relative to the paired, untreated hypoxic preparations.

lower affinity and does not correspond to either of the known receptor types.

Glycine and alanine are used as osmolytes in tissues of various organisms. They are nonperturbing for protein structure and function over a broad range of concentrations (31). Methylamines such as betaine and sarcosine, which serve as osmolytes in urea-rich tissues, including the renal medulla (32), counteract destabilizing influences of urea on protein structure (31). When they are present in concentrations of 0.5–1 M, glycine, alanine, β-alanine, betaine, L-serine, γ-aminobutyric acid, sarcosine, and taurine stabilize protein structure against thermal denaturation without changing hydration values. This indicates that they are excluded from the protein domain and are unlikely to be directly bound (33). These low-affinity protein interactions with diverse osmolytes are unlikely to explain the effects of glycine, alanine, and ACPC on proximal tubules. The latter are more likely mediated by specific ligand binding such as those of glycine with neuronal receptors.

Ischemia-reperfusion injury is now widely considered to result from both oxygen deprivation and reoxygenation components. Hypoxic injury to this tubule preparation results primarily from oxygen deprivation, as *1*) injury is very similar during severe hypoxia and complete anoxia (34), *2*) deliberate reoxygenation after hypoxia or anoxia does not produce further injury (5, 34, 35), and *3*) a variety of antioxidant treatments do not modify the development of injury (5). Thus, scavenging of reactive oxygen species by the amino acids is unlikely to be critical for protection. We have also considered whether formation of acyl-amino acid conjugates could contribute to protection by detoxifying free fatty acids released during hypoxic injury, but so far we have not been able to document this mechanism of action (35).

Important morphological features of protected tubules were the preservation of the internal structure of cell organelles, and cohesion of the organelles and cytoplasm to such a degree as to simulate superficially the normal state. Close inspection revealed that changes attributable to ATP depletion such as disassembly of actin fibrils and swelling of the microvilli were occurring in variable degrees. Nevertheless, we were struck by the overall structural integrity, and infer that degradative processes responsible for the disintegration of cells had been retarded. Whereas the degradative events must involve multiple targets, and are probably interactive in nature, the effects of glycine

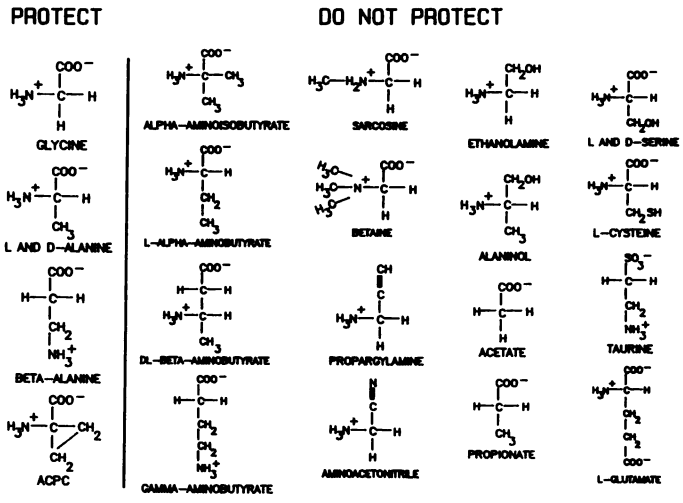

Figure 7. Structures of protective and nonprotective compounds.

analogs appear to be determined by strict requirements of molecular mimicry. Therefore, protection should involve acceptor-ligand interaction (or interactions) with binding sites that must meet correspondingly stringent requirements of structure. Also, such interactions must be close enough in the sequence of degradation to explain the apparent preemption of the entire cascade of injury. However, the nature of these putative molecular interactions and mechanisms that underlie protection remain to be completely defined. FJ

We appreciate the careful reading of this manuscript and many helpful comments by Dr. Richard Lawton of the Department of Chemistry, University of Michigan, Ann Arbor, Michigan and by Dr. Arun K. Roy, Division of Molecular Genetics, Department of Obstetrics and Gynecology, University of Texas Health Science Center, San Antonio, Texas. The assistance of Magaly Abarzua, Sandra Schultz, Uta Hussong, Smytha Rao, and Sujatha Sastry was invaluable. This work was supported by National Institutes of Health grants DK-34275 and DK-39255 (J. M. W.), and DK 37139 and DK 17387 (M. A. V.).

REFERENCES

1. Hochachka, P. W. (1986) Defense strategies against hypoxia and hypothermia. *Science* **231,** 234-241
2. Simon, R. P., Swan, J. H., Griffiths, T., and Meldrum, B. S. (1984) Blockade of N-methyl-D-aspartate receptors may protect against ischemic damage in the brain. *Science* **226,** 850-852
3. Brezis, M., Rosen, S., Silva, P., and Epstein, F. H. (1984) Renal ischemia: a new perspective. *Kidney Int.* **26,** 375-383
4. Cheung, J. Y., Leaf, A., and Bonventre, J. V. (1984) Mechanism of protection by verapamil and nifedipine from anoxic injury in isolated cardiac myocytes. *Am. J. Physiol.* **246,** C323-C329
5. Weinberg, J. M., Davis, J. A., Abarzua, M., and Rajan, T. (1987) Cytoprotective effects of glycine and glutathione against hypoxic injury to renal tubules. *J. Clin. Invest.* **80,** 1446-1454
6. Weinberg, J. M., Davis, J. A., Abarzua, M., and Kiani, T. (1989) Relationship between cell ATP and glutathione content and protection by glycine against hypoxic proximal tubule cell injury. *J. Lab. Clin. Med.* **113,** 612-623
7. Garza-Quintero, R., Ortega-Lopez, J., Stein, J. H., and Venkatachalam, M. A. (1990) Alanine protects rabbit proximal tubules against anoxic injury in vitro. *Am. J. Physiol.* **258,** F1075-F1083
8. Mandel, L. J., Schnellmann, R. G., and Jacobs, W. R. (1990) Intracellular glutathione in the protection from anoxic injury in renal proximal tubules. *J. Clin. Invest.* **85,** 316-324
9. Weinberg, J. M., Davis, J. A., Abarzua, M., and Kiani, T. (1990) Glycine-dependent protection of proximal tubules against lethal cell injury due to inhibitors of mitochondrial ATP production. *Am. J. Physiol.* **258,** C1127-C1140
10. Silva, P., Spokes, K. C., Rosen, S., and Epstein, F. H. (1989) Glycine protects the thick ascending limb from hypoxic damage. *Clin. Res.* **37,** 585 (abstr.)
11. Baines, A. D., Shaikh, N., and Ho, P. (1990) Mechanisms of perfused kidney cytoprotection by alanine and glycine. *Am. J. Physiol.* **259,** F80-F87
12. Grabl, M., and Supp, M. (1985) D-Alanine. In *Methods of Enzymatic Analysis. Volume VIII, Metabolites 3: Lipids, Amino Acids and Related Compounds* (Bergmeyer, H. U., Bergmeyer, J., and Grabl, M., eds) pp. 336-341, VCH Publishers, Deerfield Beach, Florida
13. Lowry, O. H., and Passonneau, J. V. (1972) *A Flexible System of Enzymatic Analysis* Academic, New York
14. Lowry, O. H., Rosebrough, A. L., Farr, A. L., and Randall, R. J. (1951) Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193,** 265-275
15. Boyles, J., Fox, J. E. B., Phillips, D. R., and Stenberg, E. (1985) Organization of the cytoskeleton in resting discoid platelets: preservation of actin filaments by a modified fixation that prevents osmium damage. *J. Cell Biol.* **101,** 1463-1472
16. Nadler, V., Kloog, Y., and Sokolovsky, M. (1988) 1-Aminocyclopropane-1-carboxylic acid (ACC) mimics the effects of glycine on the NMDA receptor ion channel. *Eur. J. Pharmacol.* **157,** 115-116
17. Marvizon, J. C., Lewin, A. H., and Skolnick, P. (1989) 1-Aminocyclopropane carboxylic acid: a potent and selective ligand for the glycine modulatory site of the N-methyl-D-aspartate receptor complex. *J. Neurochem.* **52,** 992-994
18. Lowry, M., Hall, D. E., and Brosnan, J. T. (1986) Serine synthesis in rat kidney: studies with perfused kidney and cortical tubules. *Am. J. Physiol.* **250:** F649-F658
19. Rowsell, E. V., Al-Naama, M. M., and Rowsell, K. V. (1982) Glycine metabolism in rat kidney cortex slices. *Biochem. J.* **204,** 313-321
20. Jordan, R. A., Colburn, W. A., and Chang, T. (1988) Development of a rapid and sensitive assay for characterizing inhibitors of the rat liver mitochondrial glycine cleavage system. *FASEB J.* **2,** A1764 (abstr.)
21. Schafer, J. A., and Barfuss, D. W. (1980) Membrane mechanisms for transepithelial amino acid absorption and secretion. *Am. J. Physiol.* **238,** F335-F346
22. Davis, J. A., Abarzua, M., Laughrey, E., and Weinberg, J. M. (1990) Cellular glycine fluxes during protection by glycine against renal proximal tubule cell injury. *FASEB J.* **4,** A1142 (abstr.)
23. Tokutomi, N., Kaneda, M., and Akaike, N. (1989) What confers specificity on glycine for its receptor site. *Br. J. Pharmacol.* **97,** 353-360
24. Marvizón, J. C. G., Vázquez, J., García Calvo, M., Mayor, F., Jr., Ruiz Gómez, A., Valdivieso. F., and Benavides, J. (1986) The glycine receptor: pharmacological studies and mathematical modeling of the allosteric interaction between glycine- and the strychnine-binding sites. *Mol. Pharmacol.* **30,** 590-597
25. Foster, A. C., and Kemp, J. A. (1989) Neurobiology: glycine maintains excitement. *Nature (London)* **338,** 377-378
26. Kessler, M., Terramani, T., Lynch, G., and Baudry, M. (1989) A glycine site associated with N-methyl-D-aspartic acid receptors: characterization and identification of a new class of antagonists. *J. Neurochem.* **52,** 1319-1328
27. Huettner, J. E. (1989) Indole-2-carboxylic acid: a competitive antagonist of potentiation by glycine at the NMDA receptor. *Science* **243,** 1611-1613
28. Kleckner, N. W., and Dingledine, R. (1988) Requirement for glycine in activation of NMDA-receptors expressed in xenopus oocytes. *Science* **241,** 835-837
29. Pullan, L. M., and Cler, J. A. (1989) Schild plot analysis of glycine and kynurenic acid at the *N*-methyl-D-aspartate excitatory amino acid receptor. *Brain Res.* **497,** 59-63
30. Snell, L. D., and Johnson, K. M. (1988) Cycloleucine competitively antagonizes the strychnine-insensitive glycine receptor. *Eur. J. Pharmacol.* **151,** 165-166
31. Somero, G. N. (1986) Protons, osmolytes, and fitness of internal milieu for protein function. *Am. J. Physiol.* **251,** R197-R213
32. Bagnasco, S., Balaban, R., Fales, H. M., Yang, Y. M., and Burg, M. (1986) Predominant osmotically active organic solutes in rat and rabbit renal medullas. *J. Biol. Chem.* **261,** 5872-5877
33. Arakawa, T., and Timasheff, S. N. (1985) The stabilization of proteins by osmolytes. *Biophys. J.* **47,** 411-414
34. Takano, T., Soltoff, S. P., Murdaugh, S., and Mandel, L. J. (1985) Intracellular respiratory dysfunction and cell injury in short-term anoxia of rabbit renal proximal tubules. *J. Clin. Invest.* **76,** 2377-2384
35. Weinberg, J. M. (1985) Oxygen deprivation-induced injury to isolated rabbit kidney tubules. *J. Clin. Invest.* **76,** 1193-1208

Received for publication July 10, 1990.
Accepted for publication August 15, 1990.