Mechanisms of perfused kidney cytoprotection by alanine and glycine

A. D. BAINES, N. SHAIKH, AND P. HO
Department of Clinical Biochemistry, University of Toronto, Toronto, Ontario M5G 1L5, Canada.

BAINES, A. D., N. SHAIKH, AND P. HO. *Mechanisms of perfused kidney cytoprotection by alanine and glycine.* Am. J. Physiol. 259 (Renal Fluid Electrolyte Physiol. 28): F80–F87, 1990.—We examined the cytoprotective action of individual amino acids in isolated perfused kidneys during perfusion with either 10 mM lactate or 5 mM glucose. In the absence of amino acids inulin clearance fell rapidly, whereas fractional excretion of phosphate, lactate, or glucose increased to more than 30%; lactate dehydrogenase was released into perfusate and alkaline phosphatase into the urine. Functional deterioration was less in kidneys from rats rendered chronically water diuretic by drinking 5% glucose. Adding 5 mM glycine, L-alanine, β-alanine, or D-alanine to the perfusate also prevented functional deterioration and release of enzymes. Glycine perfusion increased total phospholipid per microgram DNA by 6%. Aspartate, glutamate, glutamine, taurine, isoleucine, leucine, and valine were not protective. Serine, proline, and α-aminoisobutyric acid had small protective effects. Micropuncture measurements of proximal tubular free- and stop-flow pressures showed no effect of L-alanine on glomerular hemodynamics. L-Alanine increased oxygen consumption by both glucose- and lactate-perfused kidneys and increased gluconeogenesis by lactate-perfused kidneys but did not alter renal ATP content or energy charge. L-Alanine was not consumed during 70 min of perfusion and its protective action was not inhibited by blocking transamination with 0.5 mM amino-oxyacetate. The protective action of glycine was not inhibited by blocking glycine metabolism with 0.1 mM cysteamine. Thus the beneficial effects of L-alanine and glycine do not require their metabolism. These observations suggest that small neutral amino acids prevent tubular disruption through their physicochemical effects, which can stabilize membrane protein tertiary structure.

acute tubular necrosis; amino acids; alkaline phosphatase; lactate dehydrogenase

IT HAS BEEN SHOWN BY Brezis et al. (5–7) that proximal S3 and medullary thick ascending limbs (MTAL) deteriorate in rat kidneys perfused with no organic substrates other than glucose and albumin. The structural lesions in perfused kidneys can be prevented by increasing oxygen-carrying capacity of the perfusate (5) or reducing transport activity (6). Proximal tubular cells are more sensitive to hypoxia and ATP depletion than are MTAL cells but the latter are particularly susceptible to damage when there is an imbalance between oxygen delivery and energy consumption (7).

Damage to MTAL of isolated perfused kidneys can be reduced by adding a mixture of amino acids to the perfusate (5, 14). How amino acids protect the kidney is not known. They increase glomerular filtration rate (GFR) and reabsorptive work (1) without providing more oxygen. Thus their protective effect cannot be attributed simply to improved oxygen delivery relative to workload in the MTAL. Glutathione production cannot account for the bulk of the protective action (4).

The following experiments examine a number of questions related to the protective action of amino acids in perfused kidneys. Which amino acids are protective? What is their effect on proximal tubular function and renal metabolism? Are the protective amino acids metabolized? Do amino acids prevent phospholipase activation during isolated kidney perfusion?

METHODS

Male Wistar rats weighing 280–350 g were starved for 18–24 h but allowed free access to water. In some experiments animals were not starved but the results were similar therefore they have been pooled. Under pentobarbital sodium anesthesia (60 mg/kg ip) the right kidney was prepared for perfusion as previously described (1). The kidneys were perfused at 37.5°C and 100 mmHg pressure with a salt solution containing 10 mM Na L-lactate or 5 mM glucose and bovine serum albumin with an oncotic pressure of 23.5–24.5 mmHg (4100 Colloid Osmometer, Wescor) The albumin (Miles Laboratory or Sigma, fraction V reagent grade) was dialyzed for 48 h against a solution containing the following (in mM): 117 NaCl, 25 $NaHCO_3$, 2.3 KH_2PO_4, 3.5 KCl, 0.5 $MgSO_4$, and 1.2 $CaCl_2$. The perfusate was made by diluting dialyzed albumin with a similar salt solution except that the NaCl content was reduced to compensate for the addition of 10 mM Na lactate. The solution was gassed with 95% oxygen-5% carbon dioxide in a water-jacketed film oxygenator, and the kidney was rested in a water-jacketed glass receptacle. During perfusion the perfusate pH was 7.42 ± 0.007. To replenish losses by metabolism and urinary excretion, an infusion containing 20 mM KCl, 20 mM Na-lactate, and 20 mM NaCl was infused into the perfusate at 0.076 ml/min, no glucose or lactate was added to this solution when kidneys were perfused with glucose. Pressure in the renal artery was measured through a no. 30 needle inserted through the no. 18 needle used to cannulate the renal artery. The smaller needle was attached with polyethylene tubing to a pressure transducer. Flow was adjusted to maintain constant intra-arterial pressure. Flow through the arterial line was measured with an electromagnetic flowmeter (Carolina Medical Electronics). Dialyzed tritiated inulin (New

England Nuclear) was added to the perfusate for measurement of GFR. Samples of perfusate were collected at 10-min intervals beginning at 15 min, and urine was collected at 10-min intervals beginning at 20 min.

Na and K were measured by flame photometry, P_i by the method of ChandraRajan and Klein (11). Tritiated inulin was measured by scintillation counting in Aquasol (11.5 ml plus 2.5 ml water). Enzymatic methods of analysis were used to measure lactate, glucose, pyruvate, alanine, and aspartate (3). In addition, several samples of perfusate were analyzed for amino acid content by high-performance liquid chromatography (HPLC; courtesy of Drs. Verjee and Lehotay, Hospital for Sick Children, Toronto). Some kidneys were snap-frozen after 70 min of perfusion by clamping the cortex between two brass blocks chilled in liquid nitrogen. The frozen kidneys were pulverized in liquid nitrogen and the frozen powder added to chilled 2% perchloric acid. Wet weight was determined by weighing the acid solution before and after adding the kidney powder and protein was measured in the precipitate after redissolving in NaOH. ATP, ADP, and AMP in perchloric acid extracts of snap-frozen renal cortex were converted to fluorescent adducts and measured by HPLC (22). Lactic dehydrogenase was measured by NADH oxidation and alkaline phosphatase by *p*-nitrophenylphosphate hydrolysis with the use of a Hitachi 737 analyzer. *N*-acetylglucosaminidase was measured colorimetrically (N-A-G Kit, Thames Genelink, Surrey, UK). Renal phospholipid content was measured after homogenization of the entire kidney and extraction by a modified Folch method as described by Shaikh and Downar (26) except that the final extraction of the acidic lower phase was omitted. Results are expressed per milligram protein and per microgram DNA. Subfractions of phospholipid were quantitated by thin-layer chromatography.

Cumulative production and consumption of glucose and lactate were calculated from the perfusate concentration and volume at each sampling time. To obtain perfusate volume the volumes removed in urine and samplings were added to the volume present at the end of the perfusion. Consumption and production of lactate and glucose were corrected for urinary losses, which were considerable in kidneys perfused without protective amino acids. Results are expressed in terms of kidney wet weight. Because the kidney was frozen we used wet kidney wt/100 g body wt derived from other experiments to calculate the weight of the perfused kidney (1).

In some experiments the PO_2 of arterial and venous perfusate were measured continuously by Clarke-type electrodes incorporated into the perfusion circuit in the arterial and renal venous sides of the kidney. Each electrode was enclosed in a gas-tight Plexiglas chamber through which the perfusate passed; the polypropylene membranes of the electrodes formed one wall of the chamber. Before and after an experiment, the electrodes were calibrated in a calibration chamber with distilled water equilibrated with the following gases: 100% N_2, 20% O_2, 50% O_2, and 95% O_2. The electrodes were stored in glass chambers containing 5 ml distilled water with 0.3 mg penicillin.

Proximal tubular free-flow pressures were measured with a null-point pressure transducer. Early proximal tubular segments were selected for micropuncture by injecting lissamine green through a micropipette with a tip diameter of less than 6 μm. Stop-flow pressure was measured in the segments after retrograde injection of a viscous paraffin oil from a downstream loop of the tubule. Data are presented as means ± SD, and were analyzed by analysis of variance (ANOVA) with the Bonferroni correction and unpaired and paired *t* tests using SYSTAT.

RESULTS

We (1) have previously shown that inulin clearance falls rapidly while fractional sodium and phosphate excretion increase in kidneys perfused with either lactate or glucose alone. In the following experiments lactate was used so that we could measure gluconeogenesis, and glucose was used when we wished to measure free water clearance. In other respects there was no apparent functional difference between glucose- and lactate-perfused kidneys.

Figure 1 shows that neutral amino acids slow the rate of deterioration markedly. After 40 min of perfusion with lactate alone inulin clearance was 0.5 ± 0.1 $ml \cdot min^{-1} \cdot g^{-1}$ ($n = 25$). After 40 min of perfusion with lactate plus 5 mM glycine ($n = 11$), L-alanine ($n = 31$), β-alanine ($n = 6$), or D-alanine ($n = 6$) inulin clearance was close to 1 $ml \cdot min^{-1} \cdot g^{-1}$ (Fig. 1). Aspartate ($n = 14$), glutamate ($n = 2$), glutamine ($n = 7$), taurine ($n = 3$), serine ($n = 3$), proline ($n = 3$), or a mixture of isoleucine, leucine, and valine (1.3 mM of each, $n = 3$) did not increase inulin clearance (Fig. 1). There was no simple relationship between the effect of an amino acid on inulin clearance and its effect on fractional sodium excretion (Fig. 1) but in almost every instance an improvement in inulin clearance was associated with a major decrease in fractional phosphate excretion (Pearson correlation coefficient 0.58). Total phosphate excretion was also reduced; for example 5 mM L-alanine added to a lactate perfusate reduced inorganic phosphate excretion from 0.29 ± 0.08 μmol/min to 0.17 ± 0.06 μmol/min ($P < 0.001$). Serine and proline reduced fractional phosphate excretion even though they did not improve inulin clearance significantly.

Similar responses to protective amino acids were observed in glucose perfused kidneys when 2 mM L-alanine ($n = 8$) or 2 mM β-alanine ($n = 4$) were added to the perfusate (Fig. 2). Because amino acids prevent cell necrosis in the MTAL (5, 14) they might be expected to alter free water excretion; however, this was not observed. Fractional free water excretion correlated with fractional sodium excretion ($FE_{CH_2O} = 1.5 \pm 0.3 + 0.3 \pm 0.07 \times FE_{Na}$) but amino acids had no significant effect on the correlation ($P = 0.95$ by multiple regression analysis).

Fractional lactate excretion was 15% or greater in kidneys perfused with lactate alone or with aspartate, glutamate, or glutamine. Fractional lactate excretion was <3% in kidneys perfused with L-alanine, β-alanine, or glycine ($P < 0.001$). Fractional glucose excretion was 17

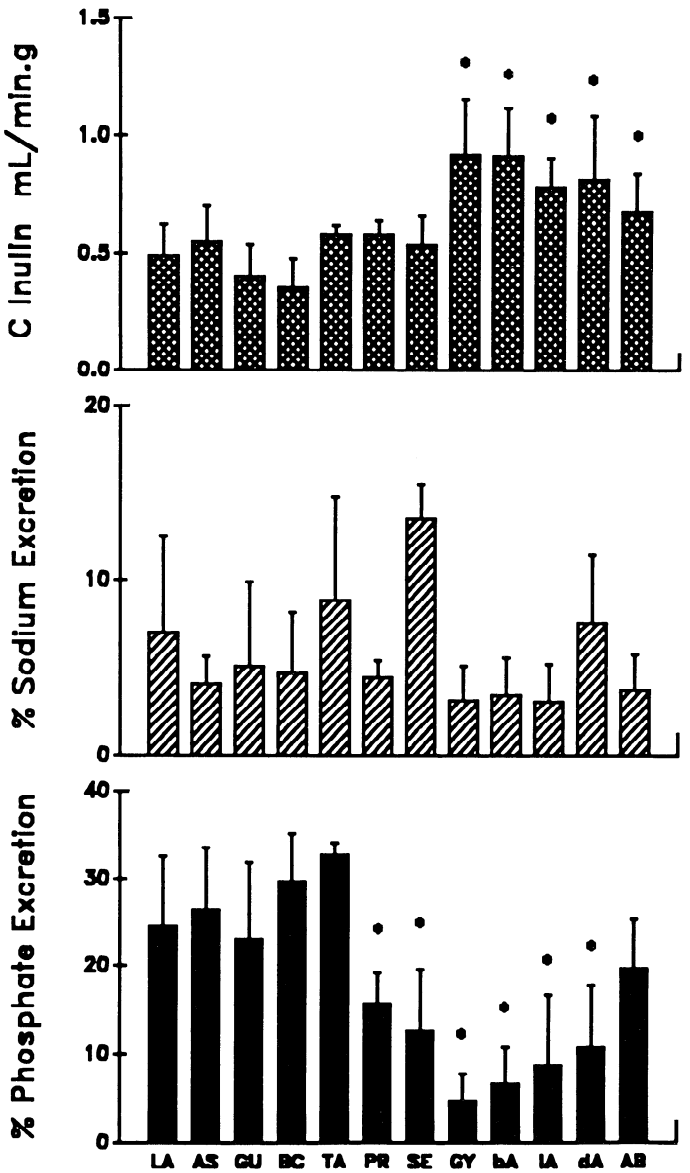

FIG. 1. Effect of amino acids on function of isolated rat kidneys perfused with 10 mmol/l lactate was measured by adding to perfusate 5 mM aspartate (AS), glutamine (GU), taurine (TA), serine (SE), glycine (GY), β-alanine (bA), L-alanine (lA), D-alanine (dA), α-aminoisobutyric acid (AB), or a mixture of 1.3 mmol/l leucine, isoleucine, and valine (BC). Results are compared with kidneys perfused with lactate alone (LA) by ANOVA with Bonferroni correction; $^*P < 0.05$. Measurements were made for 30- to 40-min period after beginning perfusion. Means ± SD.

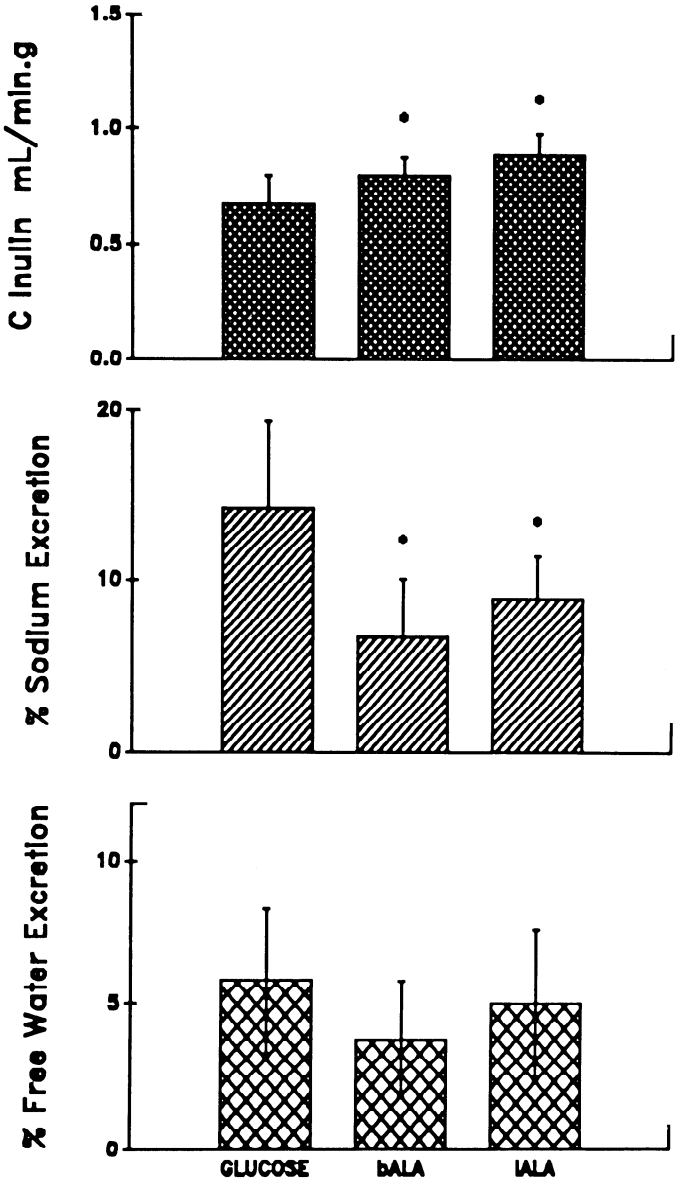

FIG. 2. β-Alanine (bALA, 2 mmol/l) or L-alanine (lALA, 2 mmol/l) was added to perfusate containing 5 mmol/l glucose. Measurements were made for the 30- to 40-min period after beginning perfusion. Means ± SD. $^*P < 0.05$ by ANOVA.

± 7% in kidneys perfused with glucose alone and 9 ± 1% in kidneys perfused with glucose plus 2 mM β-alanine.

Micropuncture revealed no effect of amino acids on glomerular hemodynamics that could account for the increased inulin clearance. Proximal tubular stop-flow and free-flow pressures were not significantly different in glucose-perfused kidneys compared with glucose plus L-alanine-perfused kidneys, although inulin clearance was significantly lower in the absence of L-alanine (Table 1). These kidneys were perfused at 85 mmHg instead of the usual 100 mmHg. Lissamine green was injected into proximal tubule to identify the micropuncture site. In at least two-thirds of the microinjected tubules of alanine-perfused kidneys, dye appeared in the distal convolution but in kidneys perfused without alanine less than one-half of the injections reappeared in distal convolutions. The lack of reappearance of dye suggests a leak or obstruction to flow through Henle's loop.

TABLE 1. *Proximal tubular pressures in glucose-perfused kidneys*

	No Alanine ($n = 7$)	L-Alanine, 5 mM ($n = 4$)
C_{inulin}, $\mu l \cdot min^{-1} \cdot g^{-1}$	430±30	560±20
Proximal pressure, mmHg	19±0.8	17±0.5
Stop-flow pressure, mmHg	29±0.8	29±1.2
Oncotic pressure, mmHg	22.2	21.9
Urine flow, $\mu l \cdot min^{-1} \cdot g^{-1}$	71±11	61±22

Values are means ± SD; *n*, no. of kidneys. Arterial perfusion pressure was 85 mmHg.

Deterioration of renal function was associated with evidence of cell and proximal tubular brush-border dis-

ruption (Fig. 3). Lactate dehydrogenase appeared in the perfusate of kidneys perfused with lactate alone or with lactate plus nonprotective amino acids. Protective amino acids reduced lactate dehydrogenase release into the perfusate by 70% and reduced urinary alkaline phosphatase by 90%. Urinary *N*-acetylglucosaminidase excretion was the same in kidneys perfused with lactate plus nonprotective glutamine (5.2 ± 0.4 mU/min) as in kidneys perfused with protective L- or β-alanine (8.3 ± 1.1 and 9.6 ± 3.6 mU/min, $n = 3$ in each group).

Recently published observations (27, 29–31) indicate that glycine may be as effective as alanine in renal cytoprotection. Preliminary experiments revealed a 7–13% higher phospholipid content per milligram protein in kidneys perfused with lactate and one of glycine, L-alanine, β-alanine, or D-alanine compared with kidneys perfused with lactate alone ($P = 0.02$ by ANOVA; $n = 3$ in each group). Therefore we examined the effect of glycine on renal phospholipid content in more detail. There was no net loss of phospholipids after 60 min from lactate-perfused kidneys compared with control kidneys that were washed with perfusate for 2 min. However, phospholipid content per milligram protein or microgram DNA was increased by 6–9% in kidneys perfused with glycine plus lactate (Table 2). Perfusion did not significantly alter the distribution of phospholipid among the various subclasses.

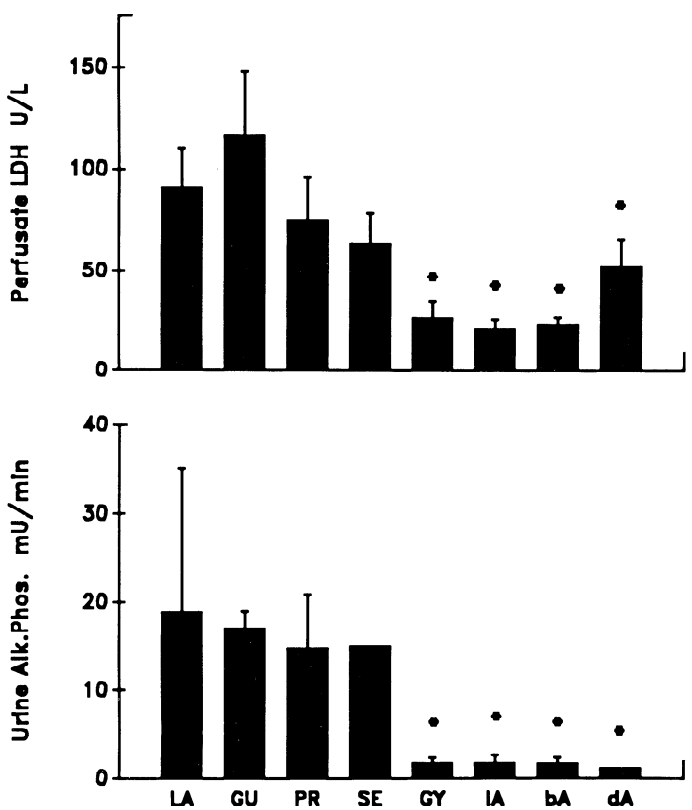

FIG. 3. Effect of amino acids on release of lactate dehydrogenase (LDH) into perfusate and alkaline phosphatase (Alk.Phos) into urine in the 50- to 60-min period after beginning perfusion with 10 mmol/l lactate. Perfusate contained lactate alone (LA) or lactate with 5 mmol/l of one of the following amino acids: glutamine (GU), proline (PR), serine (SE), glycine (GY), L-alanine (lA), β-alanine (bA), or D-alanine (dA). Results were compared by ANOVA. Each amino acid was compared with lactate control group using Bonferroni correction; $^*P < 0.05$).

TABLE 2. *Phospholipid content of rat kidneys after 60 min of perfusion*

	Control	Lactate, % Composition	Lactate + Glycine	
Phospholipid Fractions				
LPC	1.0–1.5	1.0±0.1	1.1±0.2	
SPH	13–13	13.1±0.2	13.2±0.5	
PC	30–34	34.6±0.9	33.7±0.8	
PI + PS	14–14	13.7±0.3	13.7±0.1	
PE	29–28	29.4±0.7	29.6±0.3	
TOP	8–8	8.0±0.2	8.2±0.1	
Total phospholipid, μg P				
per mg protein	10.9±0.4	10.6±0.5	11.8±0.9	$P = 0.01$
per 100 μg DNA	68±2	68±2	72±1	$P = 0.027$

Values are means ± SD. Total phospholipid/mg protein measured on individual samples from 10 mmol/l lactate ($n = 8$) and lactate + 5 mmol/l glycine ($n = 8$) and control perfusions ($n = 6$). Control kidneys were perfused for 1 min to remove red cells. Total phospholipid/100 μg DNA measured on 3 samples from each group. Fractionation by thin-layer chromatography was done in triplicate on 2 groups of pooled control experiments and on 3 groups of pooled experiments from lactate and lactate plus glycine experiments. LPC, lysophosphatidylcholine; SPH, sphingomyelin; PC, phosphatidylcholine; PI + PS, phosphatidylinositol plus phosphatidylserine; PE, phosphatidylethanolamine; and TOP, cardiolipin, phosphatidic acid, and phosphatidylglycerol.

Early work with perfused kidneys suggested that aspartate was essential for maintenance of function (25) but other experiments indicated that L-alanine alone was sufficient (20). These observations led us to compare the effects of alanine and aspartate on perfused kidney function and metabolism. AIB was also studied as an example of a nonmetabolizable amino acid. None of the amino acids had a significant effect on cortical ATP content or energy charge [ATP + ½ADP/(ATP + ADP + AMP)] (Table 3). Alanine increased gluconeogenesis in lactate-perfused kidneys from 0.38 ± 0.03 $\mu mol \cdot min^{-1} \cdot g^{-1}$ to 0.59 ± 0.06 ($P < 0.05$). AIB increased gluconeogenesis to the same extent (Table 3). Aspartate increased gluconeogenesis even more. Lactate consumption decreased by >50% during perfusion with lactate alone (from 3.0 ± 0.5 to 1.3 ± 0.2 $\mu mol \cdot min^{-1} \cdot g^{-1}$). There was no significant change in lactate consumption during perfusions with L-alanine, AIB, or aspartate. Thus there was no clear relationship between cytoprotection and the effect of an amino acid on gluconeogenesis or lactate consumption. Oxygen consumption was increased by L-alanine in both lactate- and glucose-perfused kidneys (Fig. 4); therefore cytoprotection was associated with increased oxygen consumption.

Protective amino acids may modify the redox state of some cells (28); however, alanine and AIB had little effect on the lactate-to-pyruvate ratio in perfusate. Aspartate reduced the lactate-to-pyruvate ratio ($P < 0.01$). In perfused kidneys cortical tissue lactate-to-pyruvate ratios correlate closely with tissue ratios (2).

There was no net consumption of 5 mM L-alanine during perfusion; after 70 min perfusion there was still 5 ± 0.2 mM alanine in the perfusate ($n = 4$). Traces (<0.05 mM) of some other amino acids were found in the perfusate after 70 min. Similar traces of amino acids were found after perfusion with lactate alone ($n = 3$). Urinary

TABLE 3. *Effect of amino acids on gluconeogenesis, lactate consumption, and ATP*

Perfusate	Glucose Production, $\mu mol \cdot min^{-1} \cdot g^{-1}$	Lactate Consumption, $\mu mol \cdot min^{-1} \cdot g^{-1}$	Perfusate Lactate/Pyruvate, mmol/l	Tissue	
				ATP, nmol/mg	EC, no units
Lactate	0.38±0.03 (13)	1.3±0.2	7.8±0.4/0.21±0.01	8.4±1.4	0.86±0.01 (6)
+1 Ala	0.59±0.06 (8)*	2.8±0.4†	7.5±0.4/0.19±0.01	9.4±1.7	0.86±0.01 (5)
+ AIB	0.59±0.03 (5)†	2.1±0.4†	8.4±0.3/0.20±0.01	10.4±0.8	0.87±0.01 (3)
+ Asp	0.86±0.01 (7)†	3.1±0.4†	7.0±0.2/0.23±0.01	8.5±1.6	0.87±0.01(4)

Values are means ± SD; no. of experiments is in parentheses. Gluconeogenesis and lactate consumption calculated for the 40- to 60-min period of perfusion. Perfusate lactate and pyruvate concentration measured after 70 min of perfusion. ATP, ADP, and AMP measured in snap-frozen renal cortex after 60-min perfusion. AIB, α-aminoisobutyric acid. Energy charge (EC) = ½ [(2ATP + ADP)/(ATP + ADP + AMP)]. * $P < 0.05$, † $P < 0.01$ by ANOVA.

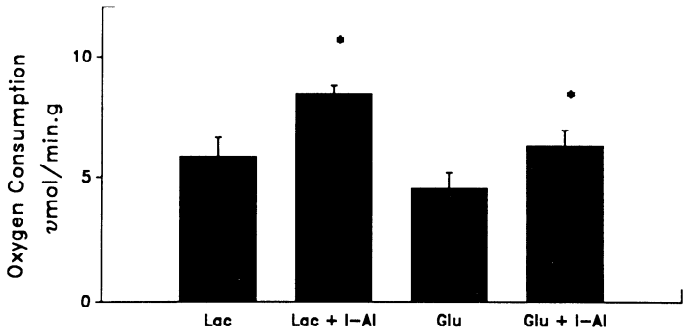

FIG. 4. Effect of 5 mmol/l L-alanine on oxygen consumption by kidneys perfused with 10 mmol/l lactate and of 2 mmol/l L-alanine on oxygen consumption by kidneys perfused with 5 mmol/l glucose. Measurements were made between 30 and 40 min after beginning perfusion. *$P < 0.05$ by unpaired t test.

fractional excretion of alanine was 4–9%. At the end of the aspartate perfusion aspartate concentration had decreased from 5 to 1.5 ± 0.1 mM, serine concentration was 0.3 ± 0.02 mM, glutamate concentration was 0.7 ± 0.1 mM, and glycine concentration was 0.1 ± 0.02 mM. There were traces of other amino acids as well. Urinary aspartate losses were large with fractional excretion ranging from 16 to 50% of the filtered load.

Kidneys perfused with pyruvate alone function almost as well as kidneys perfused with glucose or lactate plus amino acids (1, 2). Pyruvate could be produced by transamination of alanine; therefore we examined the effect of inhibiting transamination on perfused kidney function. Amino-oxyacetate (0.5 mM), which inhibits the malate-aspartate shuttle by blocking transamination (23), stopped gluconeogenesis (data not shown) and decreased lactate consumption by 53 ± 9%. Further evidence of the inhibition of the malate-aspartate shuttle was the dramatic drop in perfusate pyruvate concentration from 0.2 ± 0.02 to 0.05 ± 0.01 mM. Amino-oxyacetate decreased sodium reabsorption by kidneys perfused with lactate alone ($P < 0.05$) but did not alter the beneficial effects of alanine or pyruvate on inulin clearance and phosphate excretion (Fig. 5).

Cysteamine, which inhibits the major renal metabolic pathway for glycine [half-maximal inhibition at 60 μmol/l (19)], did not interfere with the beneficial actions of glycine on inulin clearance, phosphate excretion, alkaline phosphatase excretion, and lactate dehydrogenase release (Table 4). Cysteamine did, however, reduce inulin clearance ($P = 0.01$).

The effect of osmotic stress on perfused kidneys was

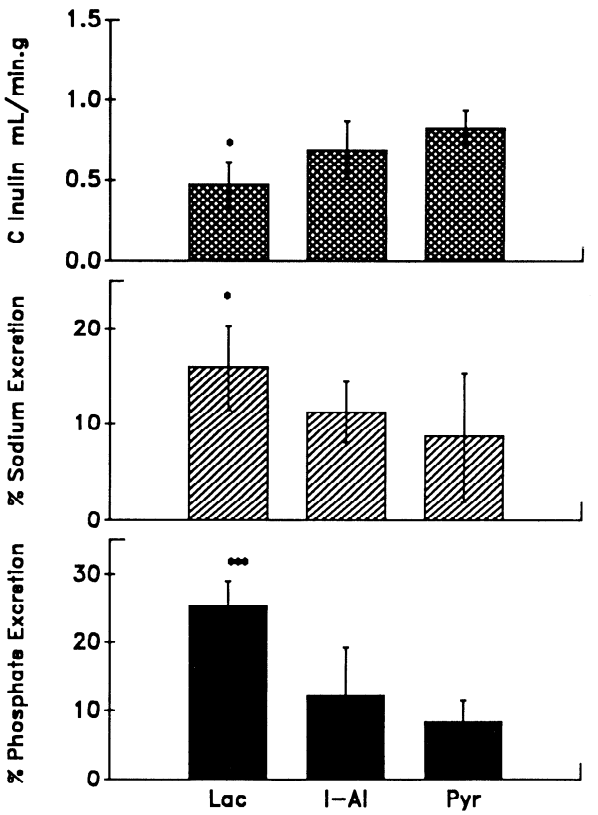

FIG. 5. Effect of inhibiting transamination in isolated perfused kidneys by adding amino-oxyacetate (0.5 mmol/l) to perfusate containing 10 mmol/l lactate alone or with 5 mmol/l L-alanine or to perfusate containing 10 mmol/l pyruvate. Means ± SD; *$P < 0.05$, ***$P < 0.001$ by ANOVA.

TABLE 4. *Effect of 0.1 mM cysteamine on the protective action of glycine in isolated lactate-perfused rat kidneys*

Perfusate	C_{inulin}, $ml \cdot min^{-1} \cdot g^{-1}$	FE_{Na}, %	FE_P, %	UV_{ALKP}, mU/min	$Perf_{LDH}$, U/l
Lac	0.49±0.13	7.0±5.4	24.5±7.8	19.5±19	91±19
Lac + Gly	0.92±0.22*	3.1±1.9	4.7±2.9*	1.8±0.5*	27±10*
Lac + Gly + Cys	0.63±0.12*†	4.5±1.6	5.4±1.2*	1.9±1.1*	26±16*

Values are means ± SD. Lac, 10 mM lactate; Gly, 5 mM glycine; Cys, 0.1 mM cysteamine. Functional values are for the 30- to 40-min period. Perfusate lactic dehydrogenase (LDH) measured at 60 min. Urine alkaline phosphatase excretion measured at 50–60 min. * $P < 0.001$ by ANOVA; † $P = 0.01$ comparing Lac + Gly with Lac + Gly + Cys.

TABLE 5. *Effect of chronic water diuresis on function of perfused rat kidneys*

Rats	C_{inulin}, ml·min^{-1}·g^{-1}	FE_{Na}, %	FE_p, %	UV_{ALKP}, mU/min	$Perf_{LDH}$, U/l	n
Water-diuretic	0.66±0.13	4.2±0.4	11.5±2.1	7±4	48±22	3
P	0.04	0.4	0.006	0.06	0.001	
Nondiuretic	0.49±0.13	7.0±5.5	24.5±7.8	21±14	99±19	25

Values are means ± SD; n, no. of kidneys. Water-diuretic rats were provided with 5% glucose to drink for 2 days before kidney perfusion with 10 mM lactate. No amino acids were added to the perfusate. Fractional sodium and phosphate excretion (FE_{Na} and FE_p) were measured after 30- to 40-min perfusion. Urinary alkaline phosphatase excretion (UV_{ALKP} measured from 50–60 min) and perfusate lactate dehydrogenase ($Perf_{LDH}$) measured at 60 min. Results compared by unpaired t test.

examined by using kidneys from water-diuretic rats. Rats were given 5% glucose in water to drink for 2 days before the experiment, which we have found increases urine flow from the normal rate of several milliliters per day to more than 40 ml/day. Table 5 shows that the water-diuretic kidneys perfused with lactate alone had significantly higher inulin clearance and lower fractional phosphate excretion. The higher inulin clearance implies that total sodium reabsorption and workload were higher in the diuretic kidneys than in the nondiuretic kidneys. Lactic dehydrogenase release was also significantly reduced. The reduction in alkaline phosphatase excretion was almost significant ($P = 0.06$).

DISCUSSION

Amino acids added to the perfusate after the initial few minutes do not prevent functional deterioration (unpublished observations and Ref. 8); therefore amino acids can only protect against progressive renal deterioration if they are present in the first few minutes of perfusion when the extracellular environment is changing most rapidly. Initiation of isolated perfusion exposes the kidney to sudden hypoxic, hydrostatic, pH, and osmotic stresses. Perfusate flow and intrarenal pressures are greatly increased. Osmotic, CO_2, and NH_3 gradients are rapidly washed out. All these stresses are greatest in the medullary region, which may explain the rapid deterioration of cells in the MTAL and S3 segment of the proximal tubule. Glycine or alanine in the initial perfusion solution could protect the kidney by counteracting one or more of these osmotic, hypoxic, pH, and hydrostatic stresses. A role for osmotic stress in the deterioration of perfused kidneys is suggested by the observation that kidneys from water-diuretic rats had higher inulin clearance and less evidence of cell breakdown than kidneys from nondiuretic rats (Table 5); however, reduced energy consumption by thick ascending limbs in the chronically water-diuretic rats might also protect against the stresses of isolated perfusion.

The cytotoxic onslaught of isolated perfusion on the MTAL can be curtailed by inhibiting energy-consuming transport processes or by increasing oxygen delivery (5, 6). These modifications succeed because the MTAL is damaged by an imbalance between energy-consuming transport activities and oxygen delivery. The proximal S3 is more sensitive than the MTAL to hypoxia but less likely to be damaged by an imbalance between workload and oxygen supply (7).

Amino acids increased inulin clearance and sodium reabsorption (Figs. 1 and 2) relative to perfusion flow; therefore they did not protect by reducing work-load relative to oxygen delivery. For example free water formation, which is a measure of sodium reabsorption in the TAL and collecting duct system, increased with inulin clearance (and fractional free water excretion was unchanged) when L-alanine was added to the perfusate (Fig. 2). L-alanine also increased oxygen (Fig. 4) and lactate consumption and stimulated gluconeogenesis (Table 3).

The nature of the functional defects indicates that the proximal tubule was a major site of tubular disruption during perfusion. Excretion of glucose, lactate, amino acids, and phosphate was high. All these substances are reabsorbed primarily in the proximal tubule. Breakdown of the proximal tubular brush border released alkaline phosphatase into the urine. Glycine and the alanines not only reduced alkaline phosphatase release by 90% but also enhanced glucose, lactate, and phosphate reabsorption, which provides further evidence that proximal tubular brush-border function was protected. Since the early work of Burg et al. (9), it has been known that alanine improves function of isolated perfused proximal tubules but amino acids are not necessary for maintenance of function in other nephron segments (24). Burg's observations on isolated tubule fragments suggest that alanine, and other protective amino acids, preserve function of the proximal tubule specifically. The micropuncture experiments with L-alanine indicate that protective amino acids do not alter glomerular hemodynamics (Table 1). Instead they probably slow the fall of inulin clearance by preventing the development of tubular leaks and obstruction.

Protective amino acids also prevented the cell destruction that released lactate dehydrogenase into the perfusate (Fig. 3). Release of lysosomal enzymes, represented by *N*-acetylglucosaminidase, was not altered by addition of amino acids.

Only small neutral amino acids were protective. They did not act as a source of metabolic energy and did not alter cortical ATP content or energy charge (Table 3). D-Alanine, which is oxidized by D-amino-oxidase in the peroxisomes of the S3 segment (10), was as protective as L-alanine or glycine, which are poorly metabolized by this enzyme (17). Glycine was protective even when its major metabolic pathway was inhibited by cysteamine (Table 4). There was no net consumption of L-alanine during the perfusion and its beneficial effects remained when amino-oxyacetate blocked its major route of metabolism (Fig. 5). Amino-oxyacetate inhibits the malate-aspartate shuttle (23) and the transfer of reducing equivalents into the mitochondria necessary for lactate oxidation. Amino-oxyacetate inhibits Na reabsorption slightly in perfused kidneys (Fig. 5) (23), which suggests that energy derived from lactate metabolism through the malate-aspartate shuttle is needed for a small portion of the total sodium reabsorption.

Shurek et al. (25) suggested that kidneys perfused with lactate or glucose alone lack malate-aspartate shuttle substrates. Adding aspartate to the perfusate rectified this deficiency and increased transfer of reducing equivalents from cytoplasm to mitochondria, so that lactate consumption, and gluconeogenesis increased while the lactate/pyruvate ratio decreased (Table 3); however, renal function was not improved (Fig. 1) and renal cells were not protected (Fig. 3).

Thick ascending limbs can be protected by reducing the transport demands placed upon them. Amino acids could reduce tubular fluid flow into the thick ascending limb by stimulating Na cotransport in the proximal tubule; however, aspartate decreased fractional sodium excretion without protecting the kidney, and D-alanine protected without reducing sodium excretion (Fig. 1). L-alanine in the proximal tubular lumen inhibits phosphate reabsorption by competing for sodium-cotransport pathways (13) thus reabsorption of amino acids could reduce reabsorption of glucose, lactate, and phosphate. The protective effect of L-alanine outweighed any competitive inhibition of reabsorption there may have been.

The stresses of isolated perfusion might be expected to activate phospholipases; however, we found no change in the total phospholipid content (expressed per mg protein or μg DNA) during perfusion with lactate alone (Table 2). We were surprised to find a small but significant increase in total phospholipid content when glycine or one of the alanines was added to the perfusate. There was no significant change in the distribution of phospholipid subtypes. These results were obtained for the entire kidney and we may therefore have missed significant changes in local regions of the nephron. A similar increase in phospholipid content has been reported by Humes et al. (18) in tubule fragments rendered hypoxic for 22.5 min and re-oxygenated for 37.5 min. Venkatechalam's group (21) found an insignificant increase in phospholipid content of kidneys after 15 min of anoxia and 2 h of reoxygenation. The overall increase in phospholipid content could be due to decreased phospholipid breakdown relative to synthesis or to loss of protein and DNA out of proportion to the phospholipid loss. Further studies will be required to delineate the mechanism.

Fatty acids that escaped into the perfusate were unlikely to have been the major destructive agent in perfused kidneys since the perfusate contained albumin, which is a very effective trap for harmful extracellular free fatty acids (18); however, albumin does not protect cells from fatty acids released internally by hypoxia. Fatty acids released into the tubular lumen would not be trapped by albumin and might have contributed to luminal membrane damage.

These results show that glycine, L-alanine, D-alanine, β-alanine (Fig. 1), and pyruvate (Fig. 5) protect proximal tubules in perfused kidneys. The protective effect requires neither transamination nor metabolism of the amino acid. Increased oxygen consumption in L-alanine-perfused kidneys was probably a result of preservation of cell structure rather than being the cause of it. The lack of metabolic component that can be linked to the protective action of these amino acids suggests that they catalyze essential enzymatic reactions. One example of alanine's catalytic effect was its stimulation of gluconeogenesis in the proximal tubule by inhibiting pyruvate kinase (15) (Table 3). We are unable to conceive of a mechanism through which this catalytic effect could contribute to preservation of proximal tubular function.

Amino acids might protect by virtue of their physical effects on protein tertiary structure. This protein structuring effect has been postulated to explain the beneficial actions of small amphipathic amino acids as intracellular osmolytes (28). These amino acids can be accumulated within the cell without disrupting pH or binding to reactive sites on intracellular proteins. They also are excluded from the structured water around protein surfaces which accounts for their capacity to stabilize protein tertiary structure by reducing the entropy of protein solutions. Taurine, serine, and proline, like glycine and alanine, are protein-stabilizing molecules but they were not effective in protecting perfused kidneys. We have no explanation for this discrepancy. Transport of amino acids does not appear to be a factor, since β-alanine, which is poorly reabsorbed (12), is as protective as L-alanine, which is avidly reabsorbed. Taurine and β-alanine are reabsorbed by the same transport system with similar kinetics; β-alanine is highly protective but taurine is not.

Glycine protects proximal tubules against hypoxia (29, 30), uranyl nitrate (16), and a variety of substances that reduce ATP production (4). Protection is not dependent on glutathione metabolism (4, 30, 31). Glycine also prevents histological deterioration in the MTAL of glucose-perfused kidneys (27). Weinburg (personal communication) has found that glycine, L-alanine, β-alanine, and to a lesser extent D-alanine prevent the release of lactate dehydrogenase from proximal tubule fragments during anoxia. The similarity of response to specific amino acids in Weinberg's experiments with proximal tubules and ours with isolated perfused kidneys indicates that we are examining the same phenomenon. We propose that these neutral zwitterionic amino acids protect the isolated perfused kidney by virtue of their unique ability to stabilize tertiary protein structure and membrane structure.

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Address for reprint requests: A. D. Baines, Dept. of Clinical Biochemistry, University of Toronto, 100 College St., Toronto, Ontario M5G 1L5, Canada.

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REFERENCES

1. BAINES, A. D., P. HO, AND H. JAMES. Metabolic control of renal vascular resistance and glomerulotubular balance. *Kidney Int.* 27: 848–854, 1985.
2. BAINES, A. D., AND B. D. ROSS. Gluconeogenesis and phosphate reabsorption in isolated lactate- or pyruvate-perfused rat kidneys. *Miner. Electrolyte Metab.* 10: 286–291, 1984.
3. BERGMEYER, H.-U. (Editor). *Methods of Enzymatic Analysis.* New York: Verlag Chemie, 1974.
4. BREZIS, M., S. ROSEN, P. SILVA, AND F. H. EPSTEIN. Selective glutathione depletion on function and structure of isolated perfused rat kidney. *Kidney Int.* 24: 178–184, 1983.
5. BREZIS, M., S. ROSEN, P. SILVA, AND F. H. EPSTEIN. Selective vulnerability of the medullary thick ascending limb to anoxia in

the isolated perfused rat kidney. *J. Clin. Invest.* 73: 182–190, 1984.
6. BREZIS, M., S. ROSEN, K. SPOKES, P. SILVA, AND F. H. EPSTEIN. Transport-dependent anoxic cell injury in the isolated perfused rat kidney. *Am. J. Pathol.* 116: 327–341, 1984.
7. BREZIS, M., P. SHANLEY, P. SILVA, K. SPOKES, S. LEAR, AND F. H. EPSTEIN. Disparate mechanisms for hypoxic cell injury in different nephron segments. Studies in the isolated perfused rat kidney. *J. Clin. Invest.* 76: 1796–1806, 1985.
8. BREZIS, M., P. SILVA, AND F. H. EPSTEIN. Amino acids induce renal vasodilation in isolated perfused kidney: coupled to oxidative metabolism. *Am. J. Physiol.* 247 (*Heart Circ. Physiol.* 16): H999–H1004, 1984.
9. BURG, M., C. PATLACK, N. GREEN, AND D. VILLEY. Organic solutes in fluid absorption by renal proximal convoluted tubules. *Am. J. Physiol.* 231: 627–637, 1976.
10. CHAN, A. W., S. G. PERRY, H. B. BURCH, AND S. FAGIOLI. Distribution of two aminotransferases and D-amino acid oxidase within the nephron of young and adult rats. *J. Histochem. Cytochem.* 27: 751–755, 1985.
11. CHANDRARAJAN, J., AND L. KLEIN. Determination of inorganic phosphorus in the presence of organic phosphorus and high concentrations of proteins. *Anal. Biochem.* 72: 407–412, 1976.
12. DANTZLER, W. H., AND S. SILBERNAGL. Renal tubular reabsorption of taurine, γ-aminobutyric acid (GABA), and β-alanine studies by continuous microperfusion. *Pfluegers Arch.* 367: 123–128, 1976.
13. DENNIS, V. W., AND P. C. BRAZY. Sodium, phosphate, glucose and alanine interactions in the isolated proximal convoluted tubule of the rabbit kidney. *J. Clin. Invest.* 62: 387–397, 1978.
14. EPSTEIN, F. H., J. T. BROSNAN, J. D. TANGE, AND B. D. ROSS. Improved function with amino acids in the isolated perfused kidney. *Am. J. Physiol.* 243 (*Renal Fluid Electrolyte Physiol.* 12): F284–F292, 1982.
15. FREIDRICHS, D., AND W. SCHONER. Regulation of gluconeogenesis by alanine. *Biochim. Biophys. Acta* 343: 341–355, 1973.
16. GABBAI, F. B., O. W. PETERSON, AND R. C. BLANTZ. Protective effect of glycine infusion in a single nephron model of acute renal failure (Abstract). *Kidney Int.* 35: 406, 1989.
17. HAMILTON, G. A. Peroxisomal oxidases and suggestions for the mechanism of action of insulin and other hormones. *Adv. Enzymol.* 57: 85–178, 1985.
18. HUMES, H. D., V. D. NGYUYEN, D. A. CIESLINSKI, AND J. M. MESSANA. The role of fatty acids in hypoxia-induced injury to renal proximal tubule cells. *Am. J. Physiol.* 256 (*Renal Fluid Electrolyte Physiol.* 25): F688–F696, 1989.
19. LOWRY, M., D. E. HALL, M. S. HALL, AND J. T. BROSNAN. Renal metabolism of amino acids in vivo: studies on serine and glycine fluxes. *Am. J. Physiol.* 252 (*Renal Fluid Electrolyte Physiol.* 21): F304–F309, 1987.
20. MAACK, T. Physiological evaluation of the isolated perfused rat kidney. *Am. J. Physiol.* 238 (*Renal Fluid Electrolyte Physiol.* 7): F71–F78, 1980.
21. MATTYS, E., J. PATEL, J. KREISBERG, J. H. STEWART, AND M. VENKATACHALAM. Lipid alterations induced by renal ischemia: pathogenic factor in membrane damage. *Kidney Int.* 26: 153–161, 1984.
22. RAMOS-SALAZAR, A., AND A. D. BAINES. Fluorometric determination of adenine nucleotides and adenosine by ion-paired, reverse-phase, high performance liquid chromatography. *Anal. Biochem.* 145: 9–13, 1985.
23. ROSS, B. D., P. SILVA, AND S. BULLOCK. Role of the malate-aspartate shuttle in renal sodium transport in the rat. *Clin. Sci.* 60: 419–426, 1981.
24. SCHAFER, J. A., AND T. E. ANDREOLI. Perfusion of isolated mammalian renal tubules. In: *Membrane Transport in Biology*, edited by G. G. Giebisch, D. C. Tosteson, and H. H. Ussing. New York: Springer Verlag, 1979, vol. 4B.
25. SCHUREK, H. J., J. P. BRECHT, H. LOHFERT, AND K. HIERHOLZER. The basic requirements for the function of the isolated cell free perfused rat kidney. *Pfluegers Arch.* 354: 349–365, 1975.
26. SHAIKH, N. A., AND E. DOWNER. Time course of changes in porcine myocardial phospholipid levels during ischemia. A reassessment of the lysophopholipid hypothesis. *Circ. Res.* 49: 316–325, 1981.
27. SILVA, P., K. C. SPOKES, S. ROSEN, AND F. H. EPSTEIN. Glycine protects the thick ascending limb from hypoxic damage (Abstract). *Clin. Res.* 37: 584A, 1989.
28. SOMERO, G. N. Protons, osmolytes, and fitness of internal milieu for protein function. *Am. J. Physiol.* 251 (*Regulatory Integrative Comp. Physiol.* 20): R197–R213, 1986.
29. WEINBERG, J. M., A. DAVIS, AND M. ABARZUA. Recovery of metabolic inhibitor-treated tubules protected by glycine or GSH (Abstract). *Kidney Int.* 35: 406, 1989.
30. WEINBERG, J. M., J. A. DAVIS, M. ABARZUA, AND T. KIANI. Relationship between cell adenosine triphosphate and glutathione content and protection by glycine against hypoxic proximal tubule cell injury. *J. Lab. Clin. Med.* 113: 612–622, 1989.
31. WEINBERG, J. M., J. A. DAVIS, M. ABARZUA, AND T. RAJAN. Cytoprotective effects of glycine and glutathione against hypoxic injury to renal tubules. *J. Clin. Invest.* 80: 1446–1454, 1987.