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ACETAZOLAMIDE INHIBITION OF RENAL γ-GLUTAMYL TRANSPEPTIDASE

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Abstract—The effect of acetazolamide (AZ) on renal γ-glutamyl transpeptidase (EC 2.3.2.2) activity (γ-GT) was studied with the purified enzyme, subcellular fractions, and in the isolated functioning kidney. Activity of γ-GT was assessed using either one of two γ-glutamyl donors, γ-glutamyl-*p*-nitroanilide (γGpNA) or glutamine, and either the γ-glutamyl acceptor glycylglycine (Gly-Gly) or methionine (Met). With the microsomal enzyme and β-GpNA, AZ was shown to inhibit *p*-nitroaniline (*p*-NA) formation; however, γ-GpNA K_m remained unchanged (1.8 mM), while the V_{max} was reduced significantly, 333 vs 200 μmoles · min^{-1} · mg^{-1}. Adding Gly-Gly removed AZ inhibition, while AZ elevated the apparent K_m for Gly-Gly from 16 to 48; AZ inhibition of γ-GT activity resulted in a decrease in γ-glutamyl-Gly-Gly formation consistent with interaction at the γ-glutamyl acceptor site. With glutamine as the β-glutamyl donor, AZ reduced NH_3 and apparent γ-glutamylmethionine formation in the purified enzyme in agreement with inhibition at the acceptor site. In the functioning kidney, perfused with 10^{-3} M L- or D-glutamine, AZ (10^{-3} M) markedly reduced NH_3 formation and increased glutamine excretion, results consistent with AZ inhibition of the *in situ* γ-GT.

Acetazolamide (AZ)† is a potent diuretic, reflecting carbonic anhydrase inhibition and reduced HCO_3^- reabsorption [1–3]. AZ also decreases renal NH_3 production, an effect not attributable to the shift of NH_3 from the relatively more alkaline urine to the renal venous blood [4–6]. Rather, AZ inhibits *in vivo* NH_3 formation from glutamine by either a direct action on a renal glutaminase [7] or by an indirect mechanism(s) [6, 8]. A direct effect of AZ on glutaminase activity of renal homogenates has been described previously [7]; in this report some 30% of the total homogenate activity could be inhibited, leading to the suggestion that more than a single enzyme could contribute to NH_3, one AZ sensitive and the other insensitive. The existence of an extramitochondrial glutaminase sensitive to AZ inhibition was demonstrated in kidney microsomes [5]; subsequent studies confirmed the identity as a reaction catalyzed by γ-glutamyl transpeptidase (γ-GT, EC 2.3.2.2) [9]. The purpose of this study was to elucidate the mode of AZ inhibition of γ-GT and possible physiological consequences in the isolated functioning kidney. To accomplish this, the glutamine analogue γ-GpNA was employed as the γ-glutamyl donor; in addition L-glutamine was utilized in purified enzyme preparation to confirm the mechanism of inhibition using a natural substrate. Finally, isolated functioning kidneys were perfused with D-glutamine to assess the effect of AZ on the *in situ* enzyme and glutamine excretion. The results to follow indicate that AZ interacts with the enzyme at the γ-glutamyl acceptor site, an action which correlates with increased glutamine excretion.

MATERIALS AND METHODS

Studies with γ-GT preparations. Kidneys were isolated from male Sprague–Dawley rats under sodium pentobarbitol anesthesia, 30 mg/kg, and homogenized in 0.44 M sucrose, 1 g cortex:9 volume. The post-mitochondrial supernatant and microsomal fractions were obtained by differential and ultracentrifugation, 105,000 *g* × 1 hr respectively. A more purified preparation (sp. act. 570 μmoles · min^{-1} · mg^{-1}) was obtained following papain treatment of the microsomes, gel filtration on Sephadex G-200, and affinity chromatography on concanavalin A-Sepharose 4B [9, 10]. Glutamyl transpeptidase activity was determined in the absence and presence of AZ (Sigma Chemical Co., St. Louis, MO) in the following assay system:

I. 5 mM γ-GpNA → γ-Glu-GpNA + *p*-NA
Ia. 5 mM γ-GpNA + Gly-Gly → γ-Glu-Gly-Gly + *p*-NA
II. 10 mM Gln + 60 mM maleate → NH_3 + Glu
IIa. 10 mM Gln + 60 mM maleate + 10 mM Met → NH_3 + Glu + γ-Glu-Met

Reactions I and Ia were followed by the formation of *p*-NA measured spectrophotometrically [11] and by thin-layer chromatography of the γ-glutamyl acceptor products [12]; formation of NH_3, II and IIa, was measured by the microdiffusion method [13], and glutamate was determined by enzymatic analysis [14]. Protein was measured by the method of Lowry *et al.* [15] using bovine serum albumin as the standard.

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† Symbols and abbreviations used: AZ, acetazolamide; γ-GT, γ-glutamyl transpeptidase; Gln, glutamine or glutaminyl; Glu, glutamic acid or glutamyl moiety; Gly, glycine or glycyl; Met, methionine or methionyl; and GpNA, γ-glutamyl-*p*-nitroanilide.

Studies with the isolated kidney. Kidneys were isolated and perfused with an artificial plasma solution [16] containing dialyzed bovine serum albumin, 6 g/100 ml, [^{14}C-methoxy]inulin (New England Nuclear Corp., Boston, MA) and 2 mM D- or L-glutamine plus 10 mM hippurate, pH 7.4. Urine and plasma collections were made at 15-min intervals with glomerular filtration rates estimated from [^{14}C]inulin clearance. Perfusate flow rate was maintained at 30 ml·min^{-1}·g^{-1} over the 60-min time course; results were obtained from the averages of four individual clearance periods per kidney. Ammonia procedure and glutamine uptake were calculated as described [17, 18], while urinary D-glutamine was measured as ammonia liberated after acid hydrolysis [19]. Differences between the means were analyzed by Student's *t*-test and judged different at the 0.05 percentile level.

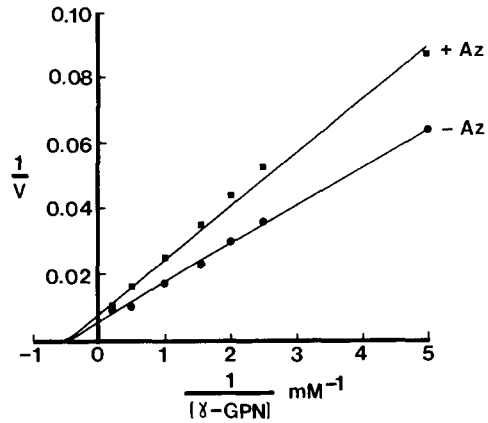

Fig. 2. Double-reciprocal plot, 1/*V* vs 1/[donor], of AZ, 5 × 10^{-3} M, inhibition of *p*-NA release from γ-GpNA.

RESULTS

Effect of AZ on γ-GT: γ-GpNA as the γ-glutamyl donor. The extent and mode of AZ inhibition were studied employing the microsomal fraction and γ-GpNA while monitoring the formation of *p*-NA, reaction I. The effect of AZ on this reaction is shown in Fig. 1. At 5 × 10^{-3} M AZ, *p*-NA production from 5 × 10^{-3} M γ-GpNA was reduced 35% and the maximum inhibition, 65%, occurred at 20 × 10^{-3} M AZ which is the limit of solubility for AZ in this system. AZ reduced *p*-NA formation by noncompetitive inhibition of γ-GpNA utilization as shown in a Lineweaver–Burk plot, Fig. 2; γ-GpNA K_m remained unchanged (1.8 mM), while the V_{max} decreased from 333 μmoles·min^{-1}·mg^{-1} to 200 μmoles·min^{-1}·mg^{-1}. The effect of increasing the concentration of Gly-Gly, the γ-glutamyl acceptor, on the AZ inhibition is shown in Fig. 3. In the absence of Gly-Gly, 10 × 10^{-3} M AZ reduced the reaction 56%, from 344 to 193 μmoles·min^{-1}·(mg protein)$^{-1}$, while increasing the acceptor concentration to 8 × 10^{-3} M returned the activity to the uninhibited level. The double-reciprocal plot, Fig. 4 (1/*V* vs 1/Gly-Gly), reveals a mixed type of inhibition with the K_m of Gly-Gly increasing from 16 to 48 × 10^{-3} M; the complicated interaction at the acceptor site apparently reflects autotransfer, track 2 of Fig. 5; in the presence of AZ (track 4), both γ-glutamyl acceptor products were reduced.

Effect of AZ on γ-GT: glutamine as the γ-glutamyl donor. AZ inhibition of γ-GT L-glutamine utilization and NH_3 production was studied using both the

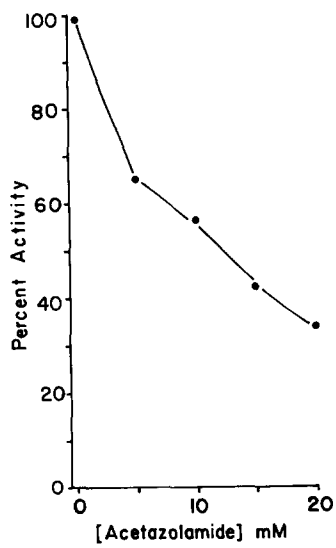

Fig. 1. Effect of acetazolamide (AZ) on γ-glutamyl-*p*-nitroanalide (γ-GpNA), conversion to *p*-nitroanaline (*p*-NA). The medium (1 ml) contained 5 mM γ-GpNA in 50 mM Tris buffer, pH 8.0, with the reaction initiated by the addition of the microsomal fraction (100 μg); the reaction was stopped after 2 min with 0.5 ml of 2 N acetic acid and the protein-cleared supernatant fractions were read at 412 nm against the appropriate blanks. The assay was carried out at 37°, with *p*-NA formation linear over the 2-min time course. The absolute value of activity (100%) was 333 μmoles·min^{-1}·mg^{-1}.

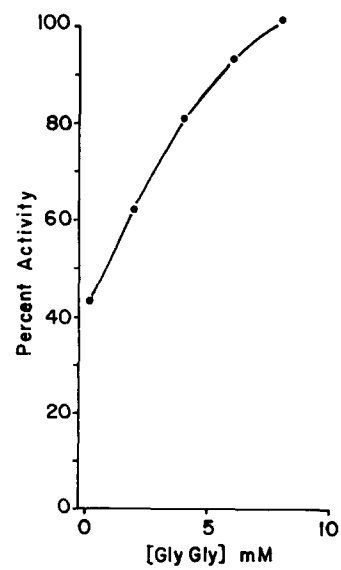

Fig. 3. Effect of increasing acceptor concentration, Gly-Gly, on inhibition by 5 × 10^{-3} M acetazolamide of *p*-NA from 5 mM γ-GpNA. The control (100%) level was 344 μmoles·min^{-1}·(mg protein)$^{-1}$.

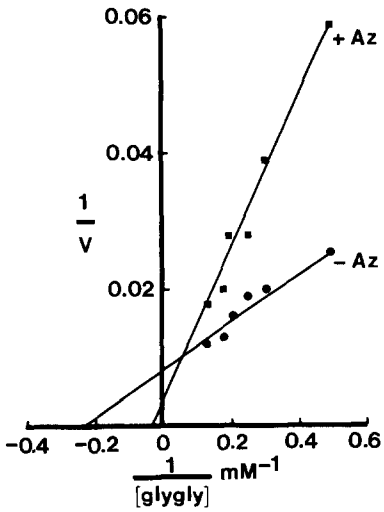

Fig. 4. Double-reciprocal plot of Gly-Gly effect on AZ inhibition of *p*-NA formation by 5×10^{-3} M AZ.

post-mitochondrial supernatant (PMS) fraction (limiting the presence of the mitochondrial phosphate-dependent glutaminase) and the purified enzyme preparation (see Materials and Methods). Table 1 shows the effect of AZ on NH_3 formation. With the PMS, NH_3 production was inhibited 25% in the present of equimolar AZ:Gln; glutamic acid formation was also reduced, confirming decreased glutamine hydrolysis. Maleate is known to stimulate glutamine utilization by γ-GT [20, 21]. A maleate concentration of 60×10^{-3} M more than doubled NH_3 formation to 5.1 from 2.2 μmoles/ml, while the addition of AZ decreased production 35% to 3.4 μmoles/ml. Interaction of AZ with the enzyme at the acceptor site was studied by converting the reaction to the transferase mode; this was accomplished by adding methionine, an excellent acceptor of the γ-glutamyl moiety of glutamine under these conditions [20], and measuring the formation of NH_3 and glutamic acid (the latter is inversely related to β-glutamylmethionine formation [20]). As in the PMS, maleate more than doubled NH_3 formation (0.85 to 2.0 μmoles/ml) while converting the γ-glutamyl moiety to glutamic acid; 10×10^{-3} M methionine further stimulated NH_3 production while decreasing glutamic acid formation consistent with the formation of γ-glutamylmethionine. Under these conditions, 10×10^{-3} M AZ resulted in a 38% inhibition of NH_3 formation, while glutamic acid production (1.22 vs 1.17 μmoles/ml) remained unchanged, consistent with inhibition of γ-glutamylmethionine formation.

Effect of AZ on the isolated kidney: NH_3 *production and glutamine excretion.* The intact kidney contains both the mitochondrial PDG and γ-glutamyl transpeptidase [9, 19]. To distinguish γ-GT-specific effects of AZ, kidneys were perfused with 2×10^{-3} M D-glutamine, a substrate not metabolized by the

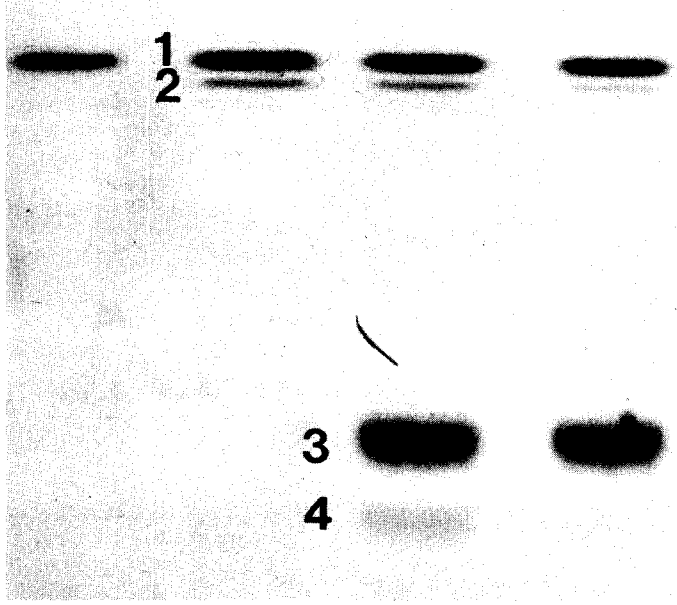

Fig. 5. Thin-layer chromatography of γ-glutamyl products from Figs. 1 and 3. From left to right: track 1: 5 mM γ-GpNA (1) minus the enzyme; track 2: γ-GpNA (1) plus enzyme, forming γ-Glu-GpNA (2); track 3: γ-GpNA (1), 4 mM Gly-Gly (3) plus enzyme, forming γ-Glu-GpNA (2) and γ-Glu-Gly-Gly (4); and track 4: effect of 5×10^{-3} M AZ on γ-Glu-GpNA (2) and γ-Glu-Gly-Gly (4) formation.

Table 1. Effect of acetazolamide on ammonia production from glutamine

Substrates	Products (μmoles/ml) NH_3	Glutamic acid
Post-mitochondrial supernatant fraction*		
Glutamine	2.22	0.43
Glutamine + AZ	1.67	0.24
Glutamine + maleate	5.06	
Glutamine + maleate + AZ	3.41	
Purified enzyme*		
Glutamine	0.85	0.44
Glutamine + maleate	2.00	2.00
Glutamine + maleate + Met	2.61	1.22
Glutamine + maleate + Met + AZ	1.62	1.17

* One milliliter of the various incubation media, with 50 mM Tris–HCl buffer, pH 7.45, and 5 mM $MgCl_2$ contained a 10 mM concentration of both substrate and AZ and 60 mM maleate where stated; reaction was started by adding 1.2 mg PMS fraction or 7 μg purified enzyme and stopped with 15% trichloroacetic acid (TCA), with ammonia and glutamate analyzed as described in Materials and Methods. Values are averages of duplicate determinations from a representative experiment.

stereospecific PDG, and with hippurate, an activator of γ-GT as is maleate but which unlike maleate is present *in vivo*. Hippurate has been shown to increase γ-GT catalyzed hydrolysis much like the mechanism of maleate [22]. The effect of 1×10^{-3} M AZ on renal function, urine acidification, NH_3 production, and D-glutamine excretion is shown in Table 2A. AZ exhibited effects on the functioning of the isolated kidney similar to those observed *in situ*, with significant elevations in urine pH (7.32 ± 0.08 vs 6.92 ± 0.10) and diuresis ($\dot{Q}$/GFR × 100 = 26 ± 8 rising to 60 ± 5%). AZ inhibited NH_3 formation from D-glutamine from 0.82 ± 0.05 to 0.41 ± 0.08 $\mu mole \cdot min^{-1} \cdot g^{-1}$ and increased urinary D-glutamine excretion from 26 ± 9 to 49 ± 14% of the filtered D-glutamine load, GFR × [D-Gln]p where *p* denotes perfusate. Identical experiments were then performed replacing D-glutamine with the natural L-isomer, Table 2B. Again AZ provoked a diuresis and an alkalinization of the urine. AZ reduced both NH_3 formation, 1.50 ± 0.11 to 0.59 ± 0.09 $\mu mole \cdot min^{-1} \cdot g^{-1}$, and glutamine utilization, 1.63±0.15 to 0.72±0.17 $\mu mole \cdot min^{-1} \cdot g^{-1}$. Excretion of L-glutamine almost doubled from 10 ± 4% of the filtered load to 18 ± 3%, similar to the doubling observed with D-glutamine.

DISCUSSION

These results demonstrate an inhibition of γ-glutamyl transpeptidase by acetazolamide using the purified enzyme (Table 1), subcellular fractions (Fig. 1 and Table 1), or the isolated functioning kidney (Table 2). The site of acetazolamide inhibition is apparently the γ-glutamyl acceptor site rather than the γ-glutamyl donor site since: (1) AZ had no effect on γ-GpNA K_m (Fig. 2), (2) Gly-Gly could restore the AZ inhibition to the uninhibited level (Fig. 3), (3) AZ elevated the apparent K_m of Gly-Gly (Fig. 4) and (4) AZ reduced the formation of both autotransfer and transfer products (Fig. 5). Similarly, with glutamine as the γ-glutamyl donor, AZ reduced both NH_3 production and apparent γ-glutamylmethionine formation (Table 1). The ability of AZ through its interaction with the γ-glutamyl acceptor site to reduce the rate of γ-glutamyl donor utilization

Table 2. Effect of AZ on NH_3 production and glutamine excretion*

		NH_3 production ($\mu moles \cdot min^{-1} \cdot g^{-1}$)	Glutamine filtered [$\mu moles \cdot (60\ min)^{-1} \cdot g^{-1}$]	Excreted	%
(A)	D-Glutamine				
	Control (4)	0.82 ± 0.05	63 ± 8	16 ± 4	26 ± 9
	AZ, 10^{-3} M	0.41 ± 0.08	51 ± 3†	25 ± 3†	49 ± 4†
(B)	L-Glutamine				
	Control (4)	1.50 ± 0.11	61 ± 6	6.3 ± 2	10 ± 4
	AZ, 10^{-3} M (4)	0.59 ± 0.09†	42 ± 3†	7.5 ± 2	18 ± 3†

* Four kidneys were in each group.
† Significantly different from control, $P < 0.05$.

is not too surprising in view of the remarkable effect of both maleate and hippurate, acting on the acceptor site, to stimulate glutamine utilization [21, 23]. Furthermore, the interaction of γ-glutamyl sulfonamide derivatives with γ-glutamyl transpeptidase has been reported previously [22], supporting the interaction of acetazolamide with the enzyme as we reported and possibly explaining some anomalous findings involving AZ effects on renal function other than those attributed to carbonic anhydrase inhibition [2].

These findings confirm the previous reports of reduction of NH_3 formation by acetazolamide [4–8] and, specifically, inhibition of γ-glutamyl transpeptidase [5]. They also explain why a previous study failed to observe γ-GT inhibition by 1 mM AZ in kidney homogenates incubated with 5 mM γ-GpNA and Gly-Gly [24]; in that study the failure to observe inhibition no doubt reflected the very high Gly-Gly to AZ ratio (approximately 20:1) that, thereby, eliminated AZ interaction with the γ-glutamyl acceptor site. In fact, a ratio of acceptor to AZ of 4:1 in our study or 3:1 in the previous work [7] is sufficient to remove the inhibition. The addition of 1×10^{-3} M AZ to isolated kidneys perfused with 2×10^{-3} M glutamine resulted in a significant reduction (22%) in NH_3 production, an effect attributed to a non-specific inhibition [8]. It was for this reason that we perfused kidneys with D-glutamine, since NH_3 formation from this isomer is limited to γ-GT [9, 19]; in addition, the potential usefulness of D-glutamine for studies of the physiological function of γ-glutamyl transpeptidase has been emphasized previously [25]. An important advantage of this approach is that effects of AZ upon intracellular variables, i.e. pH, would not be a factor, as γ-GT is an extrinsic membrane protein [26] that reacts with its substrate in the extracellular luminal fluid. Consequently, the 50% inhibition at a perfusate D-glutamine AZ ratio of 2:1 indicates a directional inhibition of the *in situ* γ-glutamyl transpeptidase. However, the actual AZ concentration at the luminal brush border enzyme site is considerably higher* due to secretion of the drug by the organic acid secretory system [23] localized in the proximal straight segment of tubule [28], the region of highest γ-GT concentration [29]. Ammonia production from L-glutamine is approximately double that from D-glutamine, reflecting the greater reactivity of the L-isomer with γ-glutamyl transpeptidase [9] and, as well, utilization by the mitochondrial glutaminase [19]. The 60% inhibition with AZ is consistent with the contribution from γ-GT although mitochondrial glutaminase inhibition cannot be ruled out; in contrast to inhibition, however, other works noted that AZ actually stimulates the mitochondrial glutaminase [30]. Another report of AZ inhibition of *in vivo* ammonia production in the dog [6] excluded γ-GT on the assumption that this enzyme is absent in this species; actually both dog kidney and human kidney [31] (in which AZ also inhibits NH_3 production) [4] contain considerable γ-GT activity. Nevertheless, measurement of the extent to which AZ inhibits γ-GT *in situ*, independently of the mitochondrial glutaminase, will ultimately depend upon the ability to differentiate the reaction products other than ammonia.

Besides inhibiting NH_3 formation by the isolated functioning kidney, AZ also increased both the excretion of filtered water (diuresis) and filtered glutamine. In the absence of AZ, 75% of the filtered D-glutamine was reabsorbed and/or converted to D-glutamic acid by the brush border γ-GT. In the presence of AZ, D-glutamine excretion increased to 48% of the filtered D-glutamine load while the ammonia formation decreased, suggesting inhibition of brush border γ-GT and excretion of the previously hydrolyzed D-glutamine. With L-glutamine, AZ also increased the excretion rate although the absolute amounts excreted were less than with the D-isomer. Conceivably L-glutamine could be converted to γ-glutamyl glutamine by the brush border γ-GT, crossing the brush border membrane in this form. In the presence of AZ and subsequent inhibition at the γ-glutamyl acceptor site of the enzyme, γ-glutamyl glutamine formation and hence glutamine reabsorption might very well have been reduced, giving rise to the increased excretion. In support of this, AZ was shown to reduce Gly-Gly-stimulated glutamine utilization in the perfused kidney [17] consistent with inhibition of γ-glutamyl acceptor formation. Although glutathione is much more reactive than equal molar amounts of glutamine at the γ-glutamyl donor site, evidence exists consistent with the *in vivo* utilization of glutamine as an auxillary source of γ-glutamyl moieties. In patients exhibiting 5-oxoprolinuria, large amounts of NH_3 and 5-oxoproline are found in the urine [32]; furthermore, the synthesis of glutathione is markedly depressed, reflecting a low synthetase activity [33] and suggesting that under these conditions glutamine is converted to NH_3 and 5-oxoproline. It is noteworthy that glutamine has been reported to reduce cystine excretion in cystinuria [34] while cystine is one of the best acceptors of γ-glutamyl moieties [35]. Finally, AZ increased amino acid excretion 38% in healthy individuals with the increase largely a result of cystine, lysine and arginine, although neither glutamine excretion nor ammonia formation was measured [36]. Interestingly, AZ administered to patients with cystinuria resulted in a reversal of this pattern with amino acid excretion rates, specifically cystine, arginine and lysine being reduced. These findings suggest an interaction of AZ with the amino acid transport system, although the nature of the interaction is clearly quite complicated. Further studies are required to determine the physiological effects of acetazolamide on the *in situ* enzyme.

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* Some estimate of the concentration of acetazolamide in the proximal tubule lumen relative to that in plasma can be gained from the study of Weiner *et al.* [27]; in dogs undergoing a massive diuresis, urine acetazolamide concentration was twelve times greater than the plasma concentration. The fact that urine acetazolamide concentration can be reduced to only three times the plasma level by probenecid is consistent with tubular secretion as the process by which high intraluminal concentrations are achieved in the functioning kidney.

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