The purine nucleotide cycle in the regulation of ammoniagenesis during induction and cessation of chronic acidosis in the rat kidney

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The effect of chronic acid feeding and its subsequent withdrawal was determined on the amounts of the metabolic intermediates and enzymic activities of the purine nucleotide cycle. Sprague–Dawley rats were given 1.5% (w/v) NH_4Cl in their drinking water for 5 days. The renal excretion of NH_3 rose 70-fold and the rats developed acidosis. The amount of renal IMP rose from a control value of 4.5 ± 2.2 to 20.4 ± 3.7 nmol/g of kidney after 48 h of acid feeding ($P < 0.001$) and fell to normal within 48 h of the recovery. Adenylosuccinate concentrations fell from a control value of 4.5 ± 0.9 nmol/g of kidney to 1.2 ± 0.3 nmol/g ($P < 0.005$) by day 5 of acidosis and continued to fall to undetectable values by 48 h after recovery. The amount of AMP remained constant through the acid-feeding and the recovery periods. The activity of adenylosuccinate synthetase, the rate-limiting enzyme of the purine nucleotide cycle, paralleled the rise and fall in NH_3 excretion. The activities of phosphate-dependent glutaminase and glutamate dehydrogenase were elevated during the acid-feeding and the recovery period. Thus changes in the purine nucleotide cycle correlate with changes in NH_3 excretion to a more parallel degree than does the activity of glutaminase or glutamate dehydrogenase.

Renal ammoniagenesis is a major regulator of acid–base balance in the body; NH_3 is excreted with excess H^+ ions as NH_4^+ ions in response to acid loads (Pitts, 1972). Much of the newly formed NH_3 is derived from the amide and amino nitrogen atoms of glutamine (Van Slyke *et al.*, 1943). The deamidation reaction, which converts glutamine into glutamate, is catalysed by phosphate-dependent glutaminase (EC 3.5.1.2). A major pathway of removal of the amino nitrogen is the deamination of glutamate to form NH_3 and 2-oxoglutarate via the glutamate dehydrogenase (EC 1.4.1.2) reaction.

A second pathway of renal NH_3 production from the amino nitrogen of glutamate involves transamination of glutamate to aspartate and entry into the purine nucleotide cycle (Bogusky *et al.*, 1976). This cycle produces ammonia from aspartate by using catalytic amounts of IMP, adenylosuccinate and AMP. The enzymes that catalyse the reactions are adenylosuccinate synthetase (EC 6.3.4.4), adenylosuccinase (adenylosuccinate lyase, EC 4.3.2.2) and adenylyate deaminase (EC 3.5.4.6). The net reaction for one turn of the cycle is:

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In the present paper we investigate the effect of the induction and the cessation of chronic acidosis on renal concentrations of the intermediates, as well as enzymic activities, of the purine nucleotide cycle.

Methods

Animal preparation

Male Sprague–Dawley rats (approx. 250 g), were obtained from Charles River Breeding Laboratories, Wilmington, MA, U.S.A. They were fed on Purina rat chow *ad libitum* and were given 1.0 M-sucrose to drink. After a control period of 2 days, 1.5% (w/v) NH_4Cl was added to their sucrose drinking solution; 5 days later the NH_4Cl /sucrose was replaced with the sucrose solution for another 2 days. Renal-venous blood was collected for NH_3 determination. On the morning of the study, the rats were sequestered in metabolic cages for 4 h of urine collection. The animals were then killed and their kidneys removed, freed from fat and connective tissue, and hemisected.

Determination of enzymic activities

Biochemicals were obtained from Sigma Chemical Corp., St. Louis, MO, U.S.A. The extraction and assay of enzyme activities of the purine nucleotide

cycle were performed as described previously by Bogusky *et al.* (1976). The activities were only determined under conditions of $V_{\max}$ when specified in the present paper. For measurement of optimal adenylosuccinate synthetase activity, renal cortices were extracted with 0.2 M-KCl containing 5.0 mM-EDTA, centrifuged as described by Bogusky *et al.* (1976), then chromatographed on Sephadex G-25 gel, with an elution buffer of 50 mM-imidazole/HCl, pH 7.2. The final assay mixture contained 2 mM-IMP and no KCl. For measurement of optimal adenylosuccinase activity, kidneys were homogenized in 60 mM-potassium phosphate buffer, pH 7.7, with 1.0 mM-dithiothreitol; this buffer was also used for the gel-filtration step. The final reaction mixture contained 30 mM-potassium phosphate buffer, pH 7.2. AMP deaminase was measured at optimal conditions as reported previously (Bogusky *et al.*, 1976).

Glutaminase and glutamate dehydrogenase activities were measured in kidneys homogenized in 10 vol. of 0.25 M-sucrose and then sonicated for 2 min at setting 3 with a Branson W350 sonicator. Phosphate-dependent glutaminase was measured by the method of Goldstein & Copenhaver (1970). Glutamate dehydrogenase was measured in the direction of oxidative deamination of glutamate (Seyama *et al.*, 1970) in an assay mixture that contained 0.1 mM-NAD⁺, 1 mM-ADP, 32 mM-potassium phosphate buffer, pH 8.2, and approx. 0.25 mg of protein extract in 3 ml. The reaction was initiated by the addition of 13 mM-glutamate at 25°C, and the increase in A_{340} was followed spectrophotometrically.

Metabolite determinations

Rats were stunned by a blow to the head, the abdomen was quickly opened, and the left kidney immediately frozen between aluminium blocks chilled in liquid N₂, and immersed in liquid N₂ until extraction with HClO₄ (Williamson & Corkey, 1969). Samples were then neutralized to pH 7.0 with 2.0 M-KOH. Adenylosuccinate was measured by using a Perkin-Elmer 356 double-wavelength spectrophotometer set at 0.01 absorbance unit full-scale expansion. The amount of adenylosuccinate in the sample was calculated from the increase in absorption ($\epsilon_{\text{mM}} = 10$) after the addition of purified adenylosuccinase, with the dual monochromators of the spectrophotometer set at 310 and 282 nm. Adenylosuccinase was prepared by the technique of Carter & Cohen (1955). IMP was measured on 0.1 deproteinized kidney extract by the method of Schultz & Lowenstein (1976).

Protein was determined by the method of Lowry *et al.* (1951). Hypoxanthine/xanthine, inosine and adenosine were measured by the method of Kalckar (1947), and ATP, ADP, AMP, 2-oxoglutarate,

aspartate, malate, glutamine and glutamate by standard spectrophotometric methods (Bergmeyer, 1974). Serum HCO₃⁻ concentration was determined by titration of blood, urinary NH₃ by the method of Seligson & Seligson (1951), and urinary creatinine by the method of Folin (1914). Student's *t* test was used for statistical analysis. At least six values were used for each mean, except where stated.

Results

Effect of acid feeding

After 48 h of acid feeding, urinary NH₃ excretion in the rats rose from a mean of 12.2 ± 4.5 (S.E.M.) to 862 ± 190 $\mu\text{mol/mg}$ of creatinine ($P < 0.001$); it remained high until acid feeding was stopped (Table 1). By 24 h later the NH₃ excretion fell to 44.5 ± 5.8 $\mu\text{mol/mg}$ of creatinine ($P < 0.01$), and by 48 h of recovery, it returned to control values. Acidosis occurred concomitantly with the rise in NH₃ excretion (Table 1).

Renal metabolites

IMP content in the kidney rose 5-fold after 48 h of acid feeding ($P < 0.005$) (Table 2) and rose still further on day 5 of acid feeding ($P < 0.01$). They fell to control values for 48 h of recovery. The mean adenylosuccinate content fell to 25% of control values by day 5 of acid feeding and continued to fall to undetectable values by 48 h of recovery. AMP contents remained constant throughout the acid-feeding and recovery periods. The hypoxanthine/xanthine content remained at control values during the acid-feeding period, but increased 8-fold during recovery. The tissue contents of adenosine and inosine remained unchanged throughout the study, at mean values of 13.9 ± 2.4 and 10.7 ± 1.3 nmol/g of kidney respectively. 2-Oxoglutarate content fell during the acid-feeding period and returned towards normal by 48 h of recovery (Table 2). Adenine nucleotide contents were comparable with previously reported data (Vinay *et al.*, 1980).

Enzymic activities in acid feeding

The activity of adenylosuccinate synthetase, the

Table 1. Urine NH₃ and serum HCO₃⁻ in acid-fed rats
Experimental details are given in the Methods section. Results are expressed as means $\pm$ S.E.M. for five experiments. * $P < 0.001$; † $P < 0.01$.

	Urine NH ₃ ($\mu\text{mol/mg}$ of creatinine)	Serum HCO ₃ ⁻ (mequiv./l)
Control	12.2 ± 4.5	24.0 ± 0.82
48 h of NH ₄ Cl	$862 \pm 190^*$	$6.6 \pm 1.5^*$
120 h of NH ₄ Cl	$771 \pm 31^*$	$18.6 \pm 1.7^\dagger$
24 h of recovery	$44.5 \pm 5.8^\dagger$	$37.3 \pm 1.2^*$
48 h of recovery	15.7 ± 3.1	27.5 ± 1.0

Table 2. *Effect of acid feeding on renal metabolites in rats*

Experimental details are given in the Methods section. Values represent means $\pm$ S.E.M. for six to ten experiments.

* $P < 0.050$; † $P < 0.020$; ‡ $P < 0.010$; § $P < 0.005$; || $P < 0.001$.

Treatment ...	Content (nmol/g of kidney)			
	Control	48 h of NH ₄ Cl	120 h of NH ₄ Cl	48 h of recovery
IMP	4.5 $\pm$ 2.2	20.4 $\pm$ 3.7§	37.5 $\pm$ 8.8‡	5.8 $\pm$ 1.9
Adenylosuccinate	4.5 $\pm$ 0.9	2.8 $\pm$ 0.8	1.2 $\pm$ 0.3§	<0.5
Hypoxanthine/xanthine	5.0 $\pm$ 1.8	6.3 $\pm$ 1.1	3.9 $\pm$ 0.9	42.6 $\pm$ 10.6§
Treatment ...	Content (μ mol/g of kidney)			
	Control	48 h of NH ₄ Cl	120 h of NH ₄ Cl	48 h of recovery
2-Oxoglutarate	0.52 $\pm$ 0.10	0.30 $\pm$ 0.04	0.059 $\pm$ 0.018	0.23 $\pm$ 0.04
ATP	1.45 $\pm$ 0.11	1.91 $\pm$ 0.11†	1.45 $\pm$ 0.10	1.78 $\pm$ 0.11*
ADP	0.74 $\pm$ 0.09	1.3 $\pm$ 0.08§	1.0 $\pm$ 0.09*	0.84 $\pm$ 0.20
AMP	0.47 $\pm$ 0.09	0.60 $\pm$ 0.07	0.37 $\pm$ 0.13	0.79 $\pm$ 0.12

Table 3. *Effect of acid feeding on the renal enzymic activities of ammoniogenesis in rats*

Experimental details are given in the Methods section. Each value is the mean $\pm$ S.E.M. for six to eight experiments:

* $P < 0.025$; † $P < 0.01$; ‡ $P < 0.005$; § $P < 0.001$.

Treatment ...	Activity (nmol/min per g of kidney)				
	Control	48 h of NH ₄ Cl	120 h of NH ₄ Cl	24 h of recovery	48 h of recovery
Adenylosuccinate synthetase	81.2 $\pm$ 14	260 $\pm$ 22§	164 $\pm$ 14§	132 $\pm$ 11‡	97 $\pm$ 11
Adenylosuccinase	134 $\pm$ 22	340 $\pm$ 23§	287 $\pm$ 29‡	273 $\pm$ 11§	230 $\pm$ 32*
Adenylate deaminase	966 $\pm$ 58	1150 $\pm$ 74	1091 $\pm$ 151	887 $\pm$ 109	1190 $\pm$ 88
Treatment ...	Activity (μ mol/min per g of kidney)				
	Control	48 h of NH ₄ Cl	120 h of NH ₄ Cl	24 h of recovery	48 h of recovery
Glutaminase	19.6 $\pm$ 0.6	29.7 $\pm$ 2.7‡	52.4 $\pm$ 2.5§	67.5 $\pm$ 2.5§	48.9 $\pm$ 6.4§
Glutamate dehydrogenase	8.2 $\pm$ 1.5	11.2 $\pm$ 0.6	15.9 $\pm$ 1.7†	15.2 $\pm$ 0.8‡	15.9 $\pm$ 1.1‡

rate-limiting enzyme of the purine nucleotide cycle, paralleled the rise and fall in NH₃ excretion. Changes in the activity were inversely proportional to the serum bicarbonate concentration ($r^2 = 0.99$), except after 24 h of recovery. The mean adenylosuccinate activity doubled after 2 days of acid feeding and remained elevated during the recovery period. Adenylate deaminase, which is not rate-limiting for the purine nucleotide cycle, showed no change during the acid-feeding or recovery periods.

Phosphate-dependent glutaminase activity was elevated at 48 h and 5 days of acid feeding. As observed by Parry & Brosnan (1978), the activity remained significantly elevated through the recovery period, at a time when NH₃ excretion had returned to normal (Table 3). Glutamate dehydrogenase activity significantly increased, to twice that in control rate, by day 5 of acid feeding. It remained elevated 48 h into the recovery period, when NH₃ excretion had returned to control values (Table 3).

Maximal renal enzymic activities

The maximal specific activities of enzymes of the purine nucleotide cycle from rats in normal acid-base balance were the measured activities of phos-

Table 4. *Activities, measured as V_{max} , of renal enzymes of ammoniogenesis*

Each value is the mean $\pm$ S.E.M. for six experiments.

See the Methods section for experimental details.

	Activity (μ mol/min per g of kidney)
Adenylosuccinate synthetase	0.3 $\pm$ 0.01
Adenylosuccinase	0.4 $\pm$ 0.01
Adenylate deaminase	3.5 $\pm$ 0.4
Glutaminase	20.0 $\pm$ 0.6
Glutamate dehydrogenase	8.2 $\pm$ 1.5
(direction of ammonia formation)	

phate-dependent glutaminase and glutamate dehydrogenase (Table 4).

Discussion

The results of this study suggest that the purine nucleotide cycle may play a regulatory role in renal ammoniogenesis. An increase in the activity of the rate-limiting enzyme of the purine nucleotide cycle, adenylosuccinate synthetase, was directly correlated

with an increase in NH_3 excretion; after withdrawal of the acid, both NH_3 excretion and the synthetase activity fell in parallel. On the other hand, glutamate dehydrogenase activity, measured in the direction of NH_3 formation from glutamate, and glutaminase activity increased in activity by day 5 of NH_4Cl feeding and remained elevated after the withdrawal of acid, although NH_3 production fell. Therefore adenylosuccinate synthetase activity correlated with renal ammoniogenesis more specifically than did phosphate-dependent glutaminase or glutamate dehydrogenase activity. In addition, the tissue contents of IMP changed in parallel with renal NH_3 production. The lack of stoichiometry between NH_3 formed and IMP content is indicative of the cyclic nature of the pathway, in that relatively small amounts of substrates are able to cycle larger, non-stoichiometric amounts of aspartate for ammoniogenesis.

2-Oxoglutarate concentrations in the freeze-clamped kidneys are higher than those reported by others in rats not drinking sucrose (Vinay *et al.*, 1980). The sucrose was added to make the NH_4Cl palatable, a common experimental practice in acid feeding. The animals are thus prevented from losing weight and water owing to the unpalatability of the NH_4Cl . However, the decrease in 2-oxoglutarate during acidosis is of the same magnitude as the values reported by others after acid feeding. Unfortunately, in studies by others, where NH_4Cl was given alone in the drinking water, the degree of dehydration or weight loss was often not recorded.

The significance of the purine nucleotide cycle pathway must be examined in the light of other currently proposed pathways for NH_3 production. Glutamine is the major substrate for renal ammoniogenesis. Its transport into mitochondria is a critical initial step, in which the HCO_3^- ion may play an important regulatory role (Simpson & Hager, 1979). After deamination of glutamine to glutamate, the subsequent metabolism of glutamate is a focal point in the regulation of ammonia production (Tannen, 1978) and regulation could occur via glutamate dehydrogenase and/or the purine nucleotide cycle. Although the glutamate dehydrogenase reaction is energetically poised for NH_3 utilization rather than formation (Lowenstein, 1972), glutamate dehydrogenase flux has been postulated as a key mechanism in the renal adaptation to chronic acidosis (Schoolwerth *et al.*, 1978).

The absolute contribution of glutamate transamination to overall ammoniogenesis by the purine nucleotide cycle cannot be determined from data

reported here. NH_3 production in rat kidneys *in vivo* ranges from $0.03 \mu\text{mol}/\text{min}$ per g of kidney in normal acid balance to 3.0 during chronic metabolic acidosis (Relman & Yablon, 1977). Of this, approximately one-third can be estimated to arise from the amino group of glutamate (Pitts, 1972). The cycle-enzyme rates are therefore in a range to produce a significant amount of NH_3 from the amino group of glutamate in the rat kidney *in vivo*.

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