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Orotate, Citrate, and Urea Excretion in Rats Fed Various Levels of Arginine (38432)

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Rats fed diets devoid of arginine have marked increases in urinary orotate, citrate and urea excretion within 24 hr (1, 2). Dietary arginine is not required for arginine balance by adult men (3), dogs (4) or rats (5) and it is believed that other mammalian species also meet their needs for this amino acid from tissue synthesis. Thus arginine occupies an intermediate position between the "essential" and "nonessential" amino acids for mammals (6). Kennan and Cohen (7) have suggested that adult rats have limited capacity for arginine synthesis beyond their needs and that the Krebs–Hensleit urea cycle is operating near its maximum capacity for ammonia (*i.e.*, the sum of $NH_3 + NH_4^+$) detoxification. Because of the metabolic relationship of the Krebs–Hensleit urea cycle and pyrimidine biosynthesis (8), arginine probably plays a key role in the balance between these two pathways.

The present studies were undertaken to determine the concentration of dietary arginine required to prevent increased orotate, citrate and urea excretion.

Materials and Methods. Thirty male, Sprague–Dawley[1] rats (45–50 g) were fed a control (Diet 1) Rogers and Harper L-amino acid diet (1, 9). After 3 days they were weighed and randomly assigned to five diets containing concentrations of arginine expressed as a percentage of the air dry diet: Diet 1, 1.12; Diet 2, 0.84; Diet 3, 0.56; Diet 4, 0.28 and Diet 5, 0.00. All experimental diets, prepared in dry form and suspended in 3% agar gel, were made isonitrogenous with the control (Diet 1) by substitution of glycine. The rats were housed individually in stainless steel metabolism cages in a controlled environment. Water was provided *ad libitum*.

Urine collected daily in bottles containing 6N H_2SO_4 was stored at $-20°$ until analyzed. All feces were collected from each animal from day 5–8 of the experiment.

Urine urea, ammonia and orotate were determined colorimetrically as previously described (1). Citrate was determined by the method of Camp and Farmer (10). Total nitrogen in urine and feces was determined by semi-micro and macro-Kjeldahl procedures (11). Statistical analysis of the data followed methods described by Steel and Torrie (12). Differences with ($P < 0.05$) were considered statistically significant.

After 13 days on the assigned diets the rats were fasted for 18 hr and injected once with 2 ml of either 0.9% NaCl or ammonium chloride (3 mmoles). Urine was collected for the next 4 hr.

Results. Removal of arginine from the diet had no significant effect on feed consumption unless the concentration of arginine was less than 0.28%. Weight gains after 8 days of feeding were: 48.0 ± 2.4, 43.4 ± 2.8, 44.1 ± 3.2, 28.8 ± 1.7 and 17.3 ± 0.7 for diets 1–5, respectively. Only rats consuming either diets 4 or 5 had significantly lower weight gains.

All animals were in a positive nitrogen balance. Only complete removal of arginine (Diet 5) caused a significantly lower *N* retention ($P < 0.05$) (Table I). As shown previously (13), total arginine deficiency caused a marked increase in urinary nitrogen as a percentage of nitrogen consumed (Table I). Diets containing 0.56% arginine or less increased urinary nitrogen loss which was principally due to increased urea excretion (Table I).

Increased urea excretion was evident after one day on diets 2, 3, 4, or 5 (Table II). However, animals consuming diet 2 (0.84% arginine) reduced their urea excretion within 48 hr indicating that compensatory mechanism(s) were operating probably to increase endogenous arginine synthesis. Animals fed diets containing

[1] ARS Sprague–Dawley, Madison, WI 53711.

TABLE I. Four Day Nitrogen Balance Data for Rats[1,2] Fed an L-Amino Acid Diet Containing Graded Levels of Arginine.

	Diet				
Arginine (%)	1 (1.12)	2 (0.84)	3 (0.56)	4 (0.28)	5 (0.0)
Intake *N* (mg)	590.4 ± 34.3[a]	635.4 ± 32.4[a]	608.5 ± 24.2[a]	563.5 ± 26.9[a]	418.3 ± 10.9[b]
Fecal *N* (mg)	42.2 ± 3.1	46.2 ± 6.6	48.0 ± 3.7	42.9 ± 2.5	37.8 ± 1.3
Fecal *N* as percentage of intake	7.1	7.3	7.9	7.6	9.1
Urinary *N*	124.2 ± 7.2	121.3 ± 8.6	131.0 ± 16.7	124.2 ± 6.1	156.4 ± 14.3
Urinary *N* as percentage of intake	19.0[a]	18.9[a]	21.5[b]	22.0[b]	37.4[c]
N retained during 4 days (mg)	424.1 ± 25.8[a]	468.0 ± 40.4[a]	429.5 ± 16.5[a]	389.6 ± 44.8[a]	223.8 ± 18.3[b]
Av body wt gain/4 days (g)	19.2 ± 1.0[a]	17.3 ± 0.6[a]	21.5 ± 2.0[a]	15.3 ± 0.9[b]	11.1 ± 0.5[b]

[1] Mean ± SEM for six animals per diet. Initial wt 45–50 g.
[2] Values with unlike superscripts (a, b, c, d) differ $P < 0.05$.

0.56% arginine were unable to reduce urea excretion indicating that they had reached their limit for arginine synthesis. Urea output tended to reach a maximum when the arginine content of the diet was reduced to 0.28%.

The pattern of orotate excretion was similar to that for urea. Orotate was significantly elevated after one day on the diet containing 0.84% arginine (Diet 2). By day 2 orotate excretion for rats consuming diet 2 was not significantly higher than for control animals (Fig. 1). The orotate excreted tended to increase as the percentage of dietary arginine in the diet decreased (Fig. 1).

Citrate excretion was dramatically increased on all diets containing less than the control intake of arginine (Table II). Citrate excretion, unlike urea and orotate, did not reverse in the rats consuming diet 2 (0.84% arginine) after 24 hr of feeding (Table II), and remained elevated for all animals consuming less than the control intake of arginine.

After 13 days of feeding, followed by 18 hr of fasting, rats force fed 3 mmoles of ammonium chloride excreted approximately twice as much ammonia in their urine as animals force fed saline regardless of the content of arginine in their assigned diet (Table III). Urea excretion was increased significantly after ammonia loading in all cases except in animals consuming diets 4 or 5 containing 0.28 and 0.0% arginine, respectively (Fig. 2). Orotate excretion for all

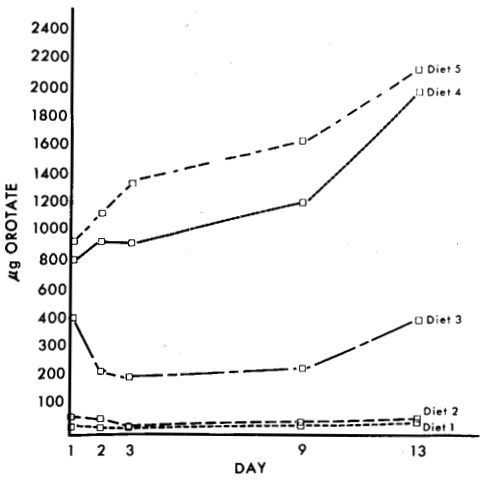

FIG. 1. Orotate excretion in rats consuming diets with graded concentrations of arginine. Diets 1–5 refer to 1.12, 0.84, 0.56, 0.28 and 0.0% arginine, respectively.

TABLE II. Urinary Urea and Citrate Excretion of Rats[1,2] Consuming L-Amino Acid Diets Containing Different Concentrations of Arginine.

	Diet				
Arginine (%)	1 (1.12)	2 (0.84)	3 (0.56)	4 (0.28)	5 (0.0)
24 hr urea excretion (mg)					
Day 1	70 ± 3[a]	84 ± 6[b]	86 ± 11[b]	139 ± 11[b]	156 ± 7[b]
Day 3	66 ± 7[a]	64 ± 4[a]	109 ± 29[ab]	130 ± 14[b]	201 ± 15[c]
24 hr citrate excretion (μg)					
Day 1	442 ± 40[a]	2050 ± 661[b]	4700 ± 1133[c]	5220 ± 545[c]	3750 ± 1189[c]
Day 13	446 ± 62[a]	1000 ± 217[b]	2175 ± 294[c]	3060 ± 411[d]	3660 ± 931[cd]

[1] Mean ± SEM for six animals per diet. Initial wt 45–50 g.
[2] Values with unlike superscripts (a, b, c, d) differ $P < 0.05$.

animals was significantly elevated by administration of ammonium chloride (Fig. 3). Urinary citrate was depressed after gastric intubation of 3 mmoles of NH_4Cl compared to saline (Fig. 4).

Discussion. The lack of change in feed consumption with partial removal of arginine from the diet indicates that marginal levels of dietary arginine satisfy satiation. Arginine at 0.28% of the diet or higher sustained normal food consumption and nitrogen balance. However, this content of arginine was not adequate to prevent metabolic changes as evidenced by the increased excretion of urea, citrate and orotate. Russek (14) has suggested the presence of hepatic glucoammonium receptors which monitor ammonia concentrations in the portal circulation. He attributed the strong satiating effect of high dietary protein to ammonia liberated in deamination of amino acids. Previous work in our laboratory (1) has shown a marked increase in portal blood ammonia in rats consuming an arginine deficient diet. Whether reducing dietary arginine influences the action of these postulated hepatic glucoammonium receptors or those of higher centers is unknown. However, our results would argue against a stimulation by either arginine or ammonia.

TABLE III. Ammonia Excretion (mg) by Rats[1,2] Consuming an L-Amino Acid Containing Different Concentrations of Arginine and Force-Fed Either NaCl or NH_4Cl (3 mmoles).

Diet	Arginine %	Treatment	Mean ± SEM
1	1.12	NaCl	4.9 ± 0.4[a]
		NH_4Cl	9.5 ± 1.1[c]
2	0.84	NaCl	3.5 ± 0.5[a]
		NH_4Cl	6.3 ± 3.9[c]
3	0.56	NaCl	2.3 ± 0.2[b]
		NH_4Cl	6.5 ± 0.5[c]
4	0.28	NaCl	3.5 ± 0.9[a]
		NH_4Cl	6.2 ± 0.4[c]
5	0.00	NaCl	2.3 ± 0.4[b]
		NH_4Cl	5.4 ± 0.3[c]

[1] Four hour urine collection from three animals per dietary treatment after 14 days of experimental feeding.
[2] Values with unlike superscripts (a, b, c) differ $P < 0.05$.

Kesner (15) showed that ammonia administration leads to marked excretion of orotate which agrees with the data obtained in these studies (Fig. 3). It is well known that ammonia intoxicated animals respond favorably to arginine (16–21). This response has normally been associated with an increased urea production. Therefore, it may be assumed that ammonia toxicity and arginine deficiency result in metabolic derangements associated with the urea cycle. Since the urea cycle and pyrimidine metabolism both require carbamyl phosphate, the data indicate that a derangement of urea formation predisposes a change in pyrimidine metabolism.

Force feeding of ammonium chloride markedly increased urinary NH_3–N regardless of the diet previously consumed (Table III). Urea excretion after ammonia loading, however, was highly dependent on the dietary regimen. Urea production was stimulated by ammonia loading in the rats consuming diets 1, 2 or 3 but was without effect in animals previously fed diets 4 or 5. On the basis of these results, the urea cycle must have been functioning maximally in animals consuming diets 4 or 5 before additional

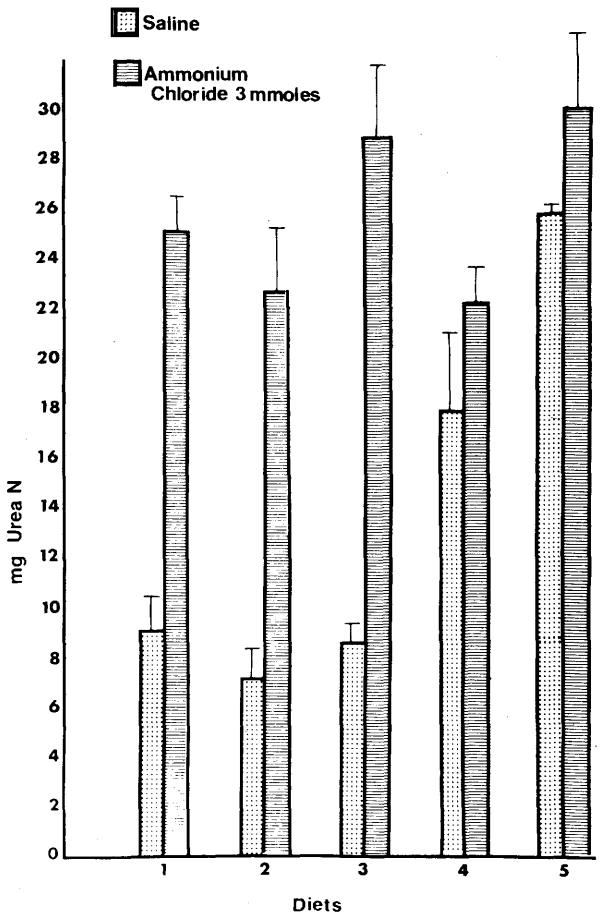

FIG. 2. Urea excretion in rats consuming diets with graded concentrations of arginine force fed either NaCl (0.154 m*M*) or NH_4Cl (3.0 mmoles) in 2 ml. Diets 1–5 refer to 1.12, 0.84, 0.56, 0.28 or 0.0% arginine, respectively.

ammonia was introduced (Fig. 2).

Citric acid may be a more sensitive indicator of arginine intake than orotic acid. However, conventional diets contain citrate. Thus orotate being present in the urine at minimal concentrations would appear to be a more reliable indicator of arginine deficiency when diets of conventional ingredients are fed. Experiments are presently underway to determine the validity of this hypothesis.

Milne *et al.* (22) and Grollman *et al.* (23) have developed the hypothesis that renal tubular reabsorption of citrate is controlled by the pH within renal tubule cells rather than by urine pH. We have not observed any differences in urinary pH between animals fed diets with or without arginine (unpublished data). An increased tubular intracellular pH may be responsible for increased excretion of citrate found in arginine deficient rats, however.

Kennen and Cohen (7) suggested that mature rats have limited reserves of arginine for urea synthesis. Our data agree with this hypothesis for rats ranging from 50–500 g (1). The present data provide further evidence supporting the therapeutic value of arginine for preventing hyperammonemia when the liver is capable of utilizing arginine. Arginine is believed to prevent hyperammonemia by supplying the Krebs–Henseleit urea cycle with essential substrate (18). Our data in agreement with others, show that dietary arginine may be a deciding factor in preventing death from hyperammonemia.

Orotate feeding has resulted in increased liver concentrations of uridine nucleotides and de-

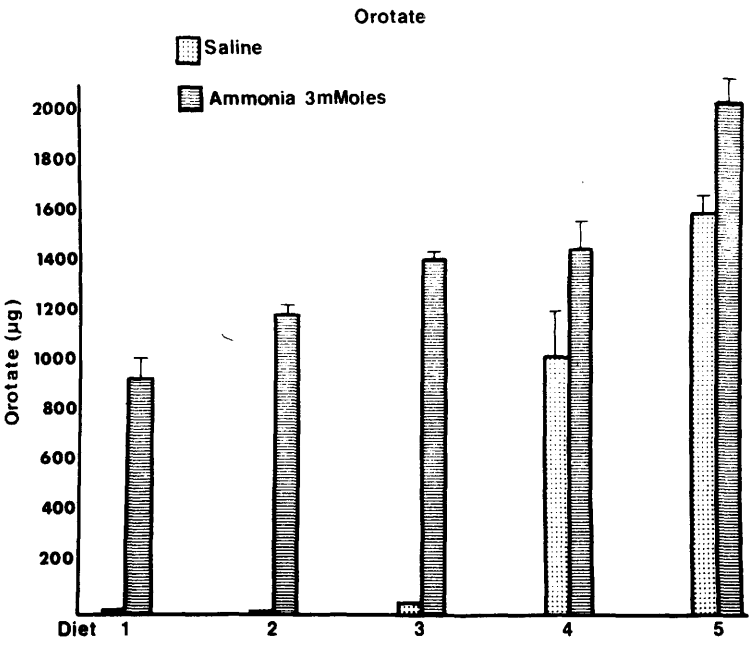

FIG. 3. Orotate excretion in rats consuming diets with graded concentrations of arginine force fed either NaCl (0.154 m*M*) or NH_4Cl (3 mmoles). Diets 1–5 refer to 1.12, 0.84, 0.56, 0.28 or 0.0% arginine, respectively.

pressed adenine nucleotides. Nucleic acid purine: pyrimidine base ratios were also altered with orotate feeding (24). In experiments comparing arginine deficiency to diets containing 1% orotate, similar quantities of orotate were excreted in the urine (our unpublished data). Investigations are presently underway to determine if arginine deficiency alters concentrations of purine and pyrimidine nucleotides as well as base ratios. Precedence for these studies is the recent finding that arginine or one of its metabolites initiate DNA synthesis (25).

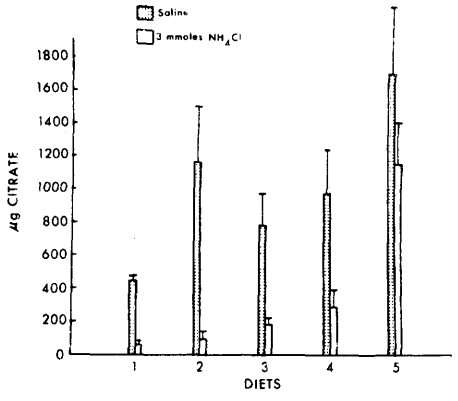

FIG. 4. Urinary citrate excretion in rats consuming diets with graded concentrations of arginine force fed either NaCl (0.154 m*M*) or NH_4Cl (3.0 mmoles) in a 2 ml vol. Diets 1–5 refer to 1.12, 0.84, 0.56, 0.28 or 0.0% arginine, respectively.

The data show that rats respond rapidly to arginine deficiency by changing the excretion rate or primary end products of nitrogen metabolism. The present studies with purified diets show that 0.84% dietary arginine is the minimal dietary concentration which will prevent metabolic changes. The present studies demonstrate that diets containing 0.28% arginine caused the urea cycle to operate maximally for ammonia detoxification in growing rats of the size used in these experiments.

Williams (25) using data on carcass analyses calculated the arginine requirement of the rat to be 0.77% of the diet. Ramasarma *et al.* (26) concluded that the dietary requirement of 0.2%, suggested by Borman *et al.* (27) may be insufficient for rapid growth. Clearly 0.84–1.12% of the arginine in the diet was required for excretion of normal concentrations of metabolites in the present experiments.

Summary. Thirty Sprague–Dawley male rats (50 g) were conditioned to an agar gel L-amino

acid diet containing 1.12% arginine (Arg) before random assignment to diets containing different concentrations of Arg (1.12, 0.84, 0.56, 0.28 or 0%). Feed intake or *N* balance were depressed only with 0% Arg. Day 13 urinary excretions of citrate were 446, 1002, 2175, 3056 and 3655 μg for 1.12, 0.84, 0.56, 0.28 and 0.0% Arg, respectively. With Arg concentrations of 0.56% or lower urinary orotate was dramatically increased throughout the 13 day experiment. Urea excretion was greater than control with 0.56, 0.28 or 0% Arg. Within 48 hr on the 0.84% Arg diet, urea, ammonia and orotate excretion returned to control values but citrate remained above control. NH_4Cl (3 mmoles) caused a doubling in ammonia excretion in all rats regardless of diet when compared to NaCl. Similarly, orotate excretion increased in all ammonia treated rats. Urea excretion was increased after NH_4Cl for rats fed 0.56, 0.84 or 1.12% Arg. We conclude that rapidly growing rats have limited reserves of Arg and that more than 0.84% dietary Arg was required to prevent abnormal loss of intermediary metabolites.

1. Milner, J. A., and Visek, W. J., Nature **245,** 211 (1973).

2. Milner, J. A., Wakeling, A. E., and Visek, W. J., J. An. Sci. **35,** 220 (1972a) (Abstract).

3. Rose, W. C., Haines, W. J., and Warner, D. T., J. Biol. Chem. **206,** 421 (1954).

4. Rose, W. C., and Rice, E. E., Science **90,** 186 (1939).

5. Wolf, P. A., and Corley, R. C., Amer. J. Physiol. **127,** 589 (1939).

6. Rose, W. C., Wixom, R. L., Lockhart, H. B., and Lambert, G. F., J. Biol. Chem. **217,** 987 (1955).

7. Kennan, A. L., and Cohen, P. P., Proc. Soc. Exp. Biol. Med. **106,** 170 (1961).

8. Jones, M. E., Adv. Enz. Reg. **9,** 19 (1971).

9. Rogers, Q. R., and Harper, A. E., J. Nutr. **87,** 167 (1965).

10. Camp, B. J., and Farmer, L., Clin. Chem. **13,** 501 (1967).

11. Skoog, D. A., and West, D. A., "Fundamentals of Analytical Chemistry", p. 351. Holt, Rinehart and Winston, Inc., New York (1966).

12. Steel, R. G. P., and Torrie, J. H., "Principles and Procedures of Statistics", pp. 43, 99. McGraw-Hill Book Co., New York (1960).

13. Milner, J. A., Wakeling, A. E., and Visek, W. J., Fed. Proc. **31,** 716 (1972b) (Abstract).

14. Russek, M., Neurosci. Res. **4,** 213 (1971).

15. Kesner, L., J. Biol. Chem. **240,** 1722 (1965).

16. Barak, A. J., and Beckenhauer, H. C., Biochem. Pharmacol. **15,** 1295 (1966).

17. Goldsworthy, P. D., Middleton, M. D., Kelley, K. A., Bombeck, C. T., Aoki, T., and Nyhus, L. M., Arch. Biochem. Biophys. **128,** 153 (1968).

18. Greenstein, J. P., Winitz, M., Gullino, P., Birnbaum, S. M., and Otey, M. C., Arch. Biochem. Biophys. **64,** 342 (1956).

19. Roberge, A., and Charbonneau, R., Life Sci. **8,** 369 (1969a).

20. Roberge, A., and Charbonneau, R., Rev. Can. Biol. **28,** 111 (1969b).

21. Roberge, A., and Charbonneau, R., Rev. Can. Biol. **28,** 1 (1969c).

22. Milne, M. D., Scribner, B. H., and Crawford, N. A., Amer. J. Med. **24,** 709 (1958).

23. Grollman, A. P., Harrison, H. C., and Harrison, H. E., J. Clin. Invest. **40,** 1290 (1961).

24. Marchetti, M., Puddu, P., and Caldarera, C. M., Biochem. J. **92,** 46 (1964).

25. Paul, D., and Walter, S., Proc. Soc. Exp. Biol. Med. **145,** 456 (1974).

26. Williams, H. H., Curtin, L. V., Abraham, J., Loosli, J. K., and Maynard, L. A., J. Biol. Chem. **208,** 277 (1954)

27. Ramasarma, G. B., Henderson, L. M., and Elvehjem, C. A., J. Nutr. **38,** 177 (1949).

28. Borman, A., Wood, T. R., Black, H. C., Anderson, E. G., Oesterling, M. J., Womack, M., and Rose, W. C., J. Biol. Chem. **166,** 585 (1946).

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