Regulation of urea and citrulline synthesis under physiological conditions

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Information on the regulation of urea synthesis *in vivo* was obtained by examining the relationship between ureagenesis *in vivo*, citrulline synthesis *in vitro*, and two factors currently hypothesized to exert short-term regulation of this pathway: the liver mitochondrial content of *N*-acetylglutamate (NAG) and substrate availability. Rats meal-fed for 4 h every day (4-20 schedule) or for 8 h every other day (8-40 schedule) were used. (1) The citrulline-synthesizing capacity of mitochondria from livers of rats on the 8-40 schedule exceeded the corresponding velocity of urea synthesis *in vivo* at all time points studied. (2) Mitochondrial NAG in these livers increased from 127 ± 32 pmol/mg of protein at 0 h to 486 ± 205 pmol/mg at 3 h after the start of a meal, and decreased thereafter, but the correlation between

NAG content and the velocity of citrulline synthesis was not simple, suggesting that NAG is not the only determinant of the state of activation of carbamoyl phosphate synthase I. (3) In rats on the 4-20 schedule killed 1 h after the start of the meal, the liver content of ornithine, citrulline, arginine, glutamate, alanine and urea increased 2.1–12-fold with respect to the values at 0 h; glutamine decreased by 39 %. (4) The combined findings indicate that *in vivo*, moment-to-moment control of the velocity of urea synthesis is exerted by substrate availability. (5) Digestion limits the supply of substrate to the liver, and prevents its ureagenic capacity from being overwhelmed following a protein-containing meal.

INTRODUCTION

Although the pathway of urea synthesis was discovered nearly 60 years ago [1], its mechanism of rapid regulation is not yet completely understood. Available data have been interpreted to indicate that the short-term regulation of urea synthesis is primarily brought about by (a) substrate availability [2–7], (b) changes in the activity of carbamoyl phosphate synthase I (CPS_I) caused by changes in the concentration of its activator, *N*-acetyl-L-glutamate (NAG) [8–10], and (c) acid–base status [11].

Studies using isolated liver, hepatocytes and mitochondria have yielded much information on the regulation of urea synthesis, but fail to account for structural and functional interactions within the liver and between organs, interactions that are important *in vivo*. The experimental conditions used *in vitro* are also often unphysiological. Many studies measured urea synthesis at saturating concentrations of ammonia, yet in normal animals the ammonia concentrations in arterial and portal blood are much less than saturating; furthermore, more than half of the nitrogen in urea is normally directed to it by way of glutamate, the source of both aspartate and some ammonia [2,3]. In general, the presence of substrates at saturating concentrations would obscure modulations in pathway flux which might occur at physiological concentrations (e.g., see [12]).

Studies *in vivo* have provided valuable information on this topic, but have also involved unphysiological conditions, including surgical trauma [13], infusions of ammonia [7,14], administration of mixtures of amino acids [15], and sudden large increases in the dietary protein of rats previously adapted to very low protein intakes, and vice versa [16].

The present paper describes our findings on the role of substrate availability, NAG and ornithine in the regulation of carbamoyl phosphate, citrulline and urea synthesis *in vivo* and *in vitro*. Using rats adapted to scheduled feedings, we performed experiments at

various times during and after the ingestion of a meal. The variables studied were urinary excretion of urea, ammonia and total nitrogen, rates of carbamoyl phosphate and citrulline synthesis in isolated liver mitochondria, NAG content of the latter, and liver content of amino acids, ammonia and urea.

EXPERIMENTAL

Materials

Male Sprague–Dawley-derived rats weighing 150–175 g were purchased from Charles River Breeding Co., Wilmington, MA, U.S.A., and from Simonsen Laboratories, Gilroy, CA, U.S.A. The animals were housed in plastic cages, four to a cage, in a room with a 12 h-light/12 h-dark cycle, and were meal-fed on a standard rat diet containing 23 % protein (Agway, Syracuse, NY, U.S.A.).

Lithium buffers and *o*-phthalaldehyde for h.p.l.c. of amino acids were from Pickering Laboratories, Mountain View, CA, U.S.A.; Brij 35 was from Fisher Scientific Co., Fair Lawn, NJ, U.S.A.; 2-mercaptoethanol from Eastman Kodak Co., Rochester, NY, U.S.A., and boric acid from J. T. Baker Chemical Co., Phillipsburg, NJ, U.S.A. Acylase type I was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; other reagents were of the highest quality commercially available.

Methods

Except where indicated, rats were adapted for 3 weeks to the meal-feeding schedule of Potter et al. [17], on which they have access to food for 8 h consecutively every other day (8-40 schedule). The rats ate during the dark cycle. At the end of this period they were transferred to individual stainless-steel metabolic cages. Urine was collected (in bottles containing 3 drops of 3 M H₂SO₄ as preservative) during consecutive 8 h intervals

Abbreviations used: CPS_I, carbamoyl phosphate synthase (ammonia), EC 6.3.4.16; NAG, *N*-acetyl-L-glutamate.

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throughout 48 h, and assayed for urea, ammonia and total nitrogen [18–20].

Isolation of mitochondria

Rats adapted to the meal-feeding regimen were killed by cervical dislocation at designated times, and the livers were excised, rinsed with cold distilled water, blotted dry, and weighed. Each liver was minced and rinsed twice with cold homogenizing medium [21], consisting of 0.21 M mannitol, 2 mM Na-Hepes, 0.5 g/l defatted BSA and 1 mM Na₂EGTA (final pH 7.4). The minced tissue was divided into thirds, and each was homogenized in 9 vol. (v/w) of cold homogenizing medium, with five strokes of a Teflon pestle rotating at 800 rev./min, in a Potter–Elvehjem homogenizer kept on ice. Mitochondria were isolated as described in [21]. The final suspension contained 30–60 mg of protein/ml; protein was measured by a biuret method [22]. The acceptor control ratio at 37 °C, with succinate as the respiratory substrate, was determined as described in [21] with a YSI model 53 oxygen monitor; only those preparations having a ratio greater than 4.5 were used.

Measurement of citrulline synthesis

Immediately after isolation, mitochondria (3–6 mg of protein) were incubated in a reaction mixture containing (final concns.): 50 mM Tris, pH 7.4, 15 mM KCl, 5 mM MgCl₂, 2 mM Na₂EDTA, 15 mM KHCO₃, 5 mM K₂HPO₄, 10 mM ornithine, and either 10 mM NH₄Cl and 10 mM succinate or 10 mM of an amino acid. Reaction vessels were equilibrated with O₂/CO₂ (19:1). The final volume was 2 ml. Incubations were performed in 25 ml Erlenmeyer flasks at 30 °C, in a shaking water bath. Reactions were started by addition of mitochondrial suspension, and the flasks were flushed with O₂/CO₂ (19:1) and stoppered. The reactions were terminated after 10 min by addition of 1 ml of 5 M HClO₄. Citrulline was measured in the deproteinized supernatants as described in [23].

Measurement of mitochondrial content of NAG

For this purpose, the liver was homogenized in a modified medium consisting of 0.21 M mannitol, 0.07 M sucrose, 2 mM Hepes and 1 mM-Na₂EGTA, final pH 7.4. Mitochondria were isolated as in [21] and the final pellet was deproteinized with 5 vol. (v/w) of 1 M HClO₄; the tube contents were mixed and centrifuged. Each supernatant was collected and neutralized to about pH 6 with a mixture of 3 M KOH and 1 M K₂CO₃, chilled and centrifuged to remove KClO₄, and dried under vacuum in a Speed-Vac centrifuge. The residue was dissolved in 3 ml of distilled water, and a sample representing 3 mg of mitochondrial protein was removed and dried as above.

NAG was measured as described in [24], with some modifications needed for good reproducibility and recovery. The dry samples were dissolved in 200 µl of deionized water and adjusted to about pH 3.5 with 1 M HCl; 100 µl of this solution was applied to a 0.2 ml column of Dowex AG50W-X8, washed with 0.4 ml of 10 mM HCl, and the combined effluents were collected. The column was shown to retain all of the glutamate present in the samples. Potassium phosphate, pH 6.5 (enough to yield a final concentration of 50 mM phosphate), 20 µl of 0.5 M KHCO₃, and 1200 units (µmol/h) of acylase (from a 10 mg/ml solution in 50 mM potassium phosphate, pH 6.5, prepared immediately before use) were added to the effluent, and the mixture was

incubated at 37 °C for 1 h; a control mixture without mitochondrial extract was also incubated. The reaction was stopped with HClO₄ (1.7 M final concn.). After centrifugation, the supernatant was collected, the precipitate was washed with 0.1 ml of 2.5 M HClO₄, and centrifuged, and the supernatants were combined. The supernatant was adjusted to about pH 3.5 with 10 M KOH and 2.5 M KHCO₃, centrifuged, and the supernatant was collected; the KClO₄ precipitate was rapidly washed with 0.2 ml of ice-cold water, centrifuged, and the supernatants were combined. Glutamate was isolated chromatographically from the pooled supernatants [24]; the fraction containing it was dried under vacuum and the residue dissolved in 0.1 ml of lithium diluent containing 1 nmol of norleucine as internal standard. Glutamate was measured by h.p.l.c. [6]; the recovery of 0.75–3 nmol of NAG added to mitochondrial pellets before deproteinization and processed as described above was 81.6 ± 4.2 %.

Measurement of amino acids, urea and ammonia in freeze-clamped liver

For these experiments, rats were meal-fed for 4 h every day (4–20 schedule) for 2 weeks. On the day of the experiment, animals were killed by cervical dislocation at the indicated times, and the livers were freeze-clamped and pulverized [25]. A 1 g sample of powder was rapidly mixed with 8 ml of ice-cold 60 g/l HClO₄ and homogenized with ten strokes of a Teflon pestle rotating at 6000 rev./min. The homogenates were kept on ice for 5 min, then centrifuged at 30000 g for 10 min at 4 °C. The supernatant was neutralized immediately to about pH 6 with a mixture of 5 M KOH and 2.5 M KHCO₃ (in the presence of 4 µl of universal indicator/ml) and kept on ice for 30 min; the suspension was then centrifuged and the supernatant collected. Glutamine and ammonia were measured immediately by the method of Lund [26]; recovery experiments showed that less than 10 % of glutamine added to frozen liver powder was hydrolysed under these conditions. Other amino acids were measured by ion-exchange h.p.l.c. as described in [6]. Values for proline were not obtained because proline does not react with *o*-phthalaldehyde (the derivative-forming reagent used), nor for serine and threonine, because they were incompletely resolved from one another during chromatography. Cysteine and cystine are reported as the sum of the two.

Liver mitochondria were isolated from a separate group of animals maintained on this feeding schedule, and used to measure rates of citrulline synthesis from NH₄⁺ and HCO₃⁻ as described in [5]. Protein was measured as in [27].

Statistical analyses

Dunnett's two-sided T test [28], the Student–Newman–Keuls test for pairwise comparison [29] and repeated measurements analysis of variance [30] were used where indicated.

RESULTS

Urinary excretion of urea, ammonia and total nitrogen

The amounts of urea (expressed in mg of N) excreted per 8 h throughout 48 h are shown in Table 1. When the velocity of urea excretion is plotted against time, it is clear that it decreased rapidly and linearly between 8 and 32 h (Figure 1). The net precursors for most of the urea synthesized during this period must have been dietary amino acids, since partially digested food was present in the stomach of the rats for at least 19 h after the beginning of the meal. After 32 h, the velocity decreased more slowly, and most likely reflected the degradation of endogenous

Table 1 Urinary excretion of urea, ammonia and total nitrogen during a 48 h feeding cycle

Rats were meal-fed for 8 h every other day (see under 'Methods'). Urine from each rat was collected during consecutive 8 h periods for 48 h, and urea N, ammonia and total nitrogen were measured as described under 'Methods'. The results shown are means $\pm$ S.D. of the amounts of each compound (in mg of N) excreted in 8 h. Statistical significance was established by the Student–Newman–Keuls test for pair-wise comparison [29]: values across a row not sharing a common superscript are significantly different at $P < 0.05$. The number of animals was 6 for urea and total N, and 5 for ammonia. A significant trend of urea, ammonia and total N excretion was established by repeated measurements analysis of variance [30] at $P < 0.0001$.

Compound	Excretion (mg)					
	0–7 h	8–15 h	16–23 h	24–31 h	32–39 h	40–47 h
Urea	158 $\pm$ 12 ^a	113 $\pm$ 7.8 ^b	81.7 $\pm$ 8.3 ^c	41.8 $\pm$ 16 ^d	37.9 $\pm$ 12 ^d	33.8 $\pm$ 7.4 ^d
Ammonia	2.1 $\pm$ 0.41 ^a	1.3 $\pm$ 0.57 ^b	0.59 $\pm$ 0.04 ^c	0.78 $\pm$ 0.04 ^{bc}	0.83 $\pm$ 0.07 ^{bc}	0.86 $\pm$ 0.08 ^{bc}
Total N	183 $\pm$ 29 ^a	141 $\pm$ 14 ^b	113 $\pm$ 21 ^c	54 $\pm$ 15 ^d	46.0 $\pm$ 8.1 ^d	39.0 $\pm$ 8.2 ^d

Figure 1 Urinary urea, mitochondrial NAG content, and maximal velocity of citrulline synthesis in mitochondria

Data on urinary urea and NAG contents are those given in Tables 1 and 4 respectively; maximal velocities of citrulline synthesis are those obtained with saturating NH_4^+ and HCO_3^- , given in Table 3. Statistical significance is reported in the respective Tables. Symbols: ●, urinary urea (mg of urea N excreted in 8 h); ▼, velocity of citrulline synthesis from NH_4^+ and HCO_3^- (nmol/min per mg of mitochondrial protein); ○, NAG content (pmol/mg of mitochondrial protein).

protein. Total urinary N excretion paralleled that of urea N; ammonia accounted for only 0.5–2.1 % of the total N excreted (Table 1).

Velocity of urea synthesis *in vivo*

A gross estimate of the velocity of urea synthesis in the liver can be made from the data in Tables 1 and 2, on the bases that under normal conditions: (a) urea synthesis *de novo* occurs only in the liver, and the velocity of synthesis does not oscillate measurably [7,13]; (b) urea equilibrates throughout body fluids [13] and is not sequestered other than in urine; and (c) large amounts of urea-cycle intermediates neither accumulate in the liver ([15] and Table 5) nor are released from it [31]. Hence over long periods of time such as were encompassed by these observations, the appearance of urea in urine closely reflects its synthesis in the liver. The calculation does not account for urea arising from the hydrolysis of dietary arginine or that excreted in faeces, which are small fractions of the total formed, or for the recycling of ammonia arising from the hydrolysis of urea in the intestine, which may be as much as 20 % of total urea synthesized [32,33].

The highest velocity of urea synthesis occurred during the 8 h feeding period: the rats synthesized about 6.4 mmol of urea, of

which 5.6 mmol (158 mg of N; Table 1) was excreted and an additional 0.8 mmol (22 mg of N) was distributed in body fluids [assuming that body weight was 250 g, water space was 160 ml, and the urea concentration in body fluids increased by an average of about 5 mM, as it did in the liver (Table 5)]. If the velocity had been constant throughout this period, and using a liver weight of 7.5 g (interpolated from the data in Table 2), the production of 6.4 mmol of urea per 8 h would be equivalent to 1.8 $\mu\text{mol}/\text{min}$ per g of liver. Since the velocity was not constant, the highest velocity *in vivo* was greater than the latter value; the data in Figure 1 suggest that the maximum had occurred by 3 h.

Between 8 and 16 h, the velocity of urea excretion was less than 0.71 mmol of urea/h (19.75 mg of N/h; Table 1) and more than 0.5 mmol of urea/h (14.13 mg of N/h; Table 1), equivalent to 1.18 and 0.84 μmol synthesized/min per g of liver respectively (calculated by using a liver weight of 10 g interpolated from values in Table 2). For the period between 32 and 48 h, similar calculations show that the velocity decreased to less than 0.39 $\mu\text{mol}/\text{min}$ per g.

Citrulline synthesis in isolated liver mitochondria

The velocity of citrulline synthesis from saturating NH_4^+ and HCO_3^- was about 9 nmol/min per mg at 0 h (Table 3); it increased to 48 nmol/min per mg within 3 h, remained high for at least 19 h, then decreased progressively to the fasting level. These differences are not the result of changes in the total activities of CPS_I and ornithine carbamoyltransferase, which do not vary detectably in 24 h (N. Cohen, C.-W. Cheung and L. Rajman, unpublished work; see [34]). At all times studied between 3 h and 48 h, mitochondria were capable of synthesizing citrulline at velocities much greater than occurred *in vivo*. For example, the velocity of 38 nmol/min per mg measured at 11 and 19 h is equivalent to 1.98 $\mu\text{mol}/\text{min}$ per g of liver {1 g of liver from fed rats contains 52.5 ± 3.1 ($n = 9$) mg of mitochondrial protein (C.-W. Cheung, N. Cohen and L. Rajman, unpublished work; see [35])}; this is 1.7–2.4-fold greater than the velocities of urea (and hence, of citrulline) synthesis *in vivo* calculated above for the interval between 8 and 16 h.

When amino acids were supplied as the source of energy as well as of ammonia, the velocities of citrulline synthesis were lower overall (Table 3), in agreement with earlier findings [36]. This is as expected for coupled mitochondria, for reasons of stoichiometry discussed in [36]. At 3 h, when the velocities with all substrates were highest, glutamine was 2-fold more effective than the mono-amino acids as a source of ammonia; the velocities obtained with glutamate, alanine and glycine at this time were similar. Qualitatively, the change with time in the rate of citrulline

Table 2 Body and liver weights during a 48 h feeding cycle

The body and liver weights are those of the animals used to measure mitochondrial NAG (see Table 4). Values at each time point are the mean weights in g ($\pm$ S.D.) for three rats. Statistical significance of changes in liver weight from 0 h was determined by Dunnett's two-sided T test [28]: * $P < 0.05$; ** $P < 0.01$.

	Time (h)						
	0	3	11	19	27	35	43
Body wt.	195 $\pm$ 12	233 $\pm$ 35	272 $\pm$ 8	268 $\pm$ 7	233 $\pm$ 15	230 $\pm$ 24	209 $\pm$ 12
Liver wt.	5.6 $\pm$ 0.5	7.0 $\pm$ 1.1	10.5 $\pm$ 0.4**	11.0 $\pm$ 0.2**	9.2 $\pm$ 0.8**	7.6 $\pm$ 1.3*	6.3 $\pm$ 0.3

Table 3 Velocities of citrulline synthesis in liver mitochondria isolated from rats during the 48 h feeding cycle

Liver mitochondria were isolated from rats killed immediately before the start of the meal (0 h) and at the indicated times during the 48 h feeding cycle. Incubation mixtures and conditions for the measurement of citrulline synthesis were as described under 'Methods'. The number of animals used for each time point is shown in parentheses under the respective column; the values shown are means $\pm$ S.D. of each group. Significant differences compared with 0 h were determined by Dunnett's two-sided T test [28]: * $P \leq 0.05$; ** $P \leq 0.01$.

Substrate	Time ...	Citrulline synthesis (nmol/min per mg of protein)						
		0 h	3 h	11 h	19 h	27 h	35 h	43 h
NH ₃ + HCO ₃ ⁻		9.4 $\pm$ 4.0	48.3 $\pm$ 18.0**	38.1 $\pm$ 6.2**	38.6 $\pm$ 9.3**	14.4 $\pm$ 7.0	12.1 $\pm$ 7.8	6.8 $\pm$ 4.5
Glutamine		0.6 $\pm$ 0.4	4.7 $\pm$ 1.6**	3.3 $\pm$ 1.0**	1.9 $\pm$ 1.0	1.0 $\pm$ 0.5	0.9 $\pm$ 0.4	0.7 $\pm$ 0.4
Glutamate		0.8 $\pm$ 0.5	2.2 $\pm$ 1.5**	1.6 $\pm$ 0.6	1.7 $\pm$ 0.7	1.6 $\pm$ 0.3	1.5 $\pm$ 0.3	1.1 $\pm$ 0.4
Alanine		0.4 $\pm$ 0.3	2.2 $\pm$ 0.3**	0.9 $\pm$ 0.4	1.1 $\pm$ 0.3*	0.9 $\pm$ 0.4	0.8 $\pm$ 0.3	0.3 $\pm$ 0.1
Glycine		0.3 $\pm$ 0.2	1.9 $\pm$ 1.2**	1.5 $\pm$ 0.7**	0.9 $\pm$ 0.4	0.8 $\pm$ 0.3	0.7 $\pm$ 0.4	0.4 $\pm$ 0.2
		(6)	(8)	(11)	(12)	(7)	(6)	(6)

Table 4 NAG content of liver mitochondria isolated from rats during the 48 h feeding cycle

Liver mitochondria were isolated from the rats described in Table 2, immediately before the start of the meal (0 h) and at the indicated times during the 48 h feeding cycle. NAG was measured as described under 'Methods'. Values (in pmol of NAG/mg of mitochondrial protein) are for each of three animals used per time point, and are means $\pm$ S.D. The values are not corrected for recovery of NAG which was 81.6 $\pm$ 4.2%. Significant differences of the mean values from those for 0 h was determined by Dunnett's two-sided T test [28]: * $P < 0.05$; ** $P < 0.01$.

Time ...	Mitochondrial NAG (pmol/mg of protein)						
	0 h	3 h	11 h	19 h	27 h	35 h	43 h
Expt. 1	153	501	263	247	330	84	155
Expt. 2	91	684	519	302	198	126	159
Expt. 3	136	274	393	230	220	143	164
Mean $\pm$ S.D.	127 $\pm$ 32	486 $\pm$ 205**	392 $\pm$ 128*	260 $\pm$ 38	249 $\pm$ 71	118 $\pm$ 30	159 $\pm$ 4

synthesis was the same whether amino acids or NH₄⁺ and HCO₃⁻ were substrates.

NAG content of liver mitochondria

The amount of NAG increased from 127 $\pm$ 32 pmol/mg of mitochondrial protein at 0 h to 486 $\pm$ 205 pmol/mg at 3 h, and decreased thereafter (Table 4). At its highest, NAG was approximately equimolar with CPS_I (about 500 pmol of enzyme/mg of protein in mitochondria from rats fed on a standard diet [37,38]). Between 3 and 19 h, the mean NAG content decreased more (47%; Table 4) than the velocity of citrulline synthesis (20%; Table 3).

Liver contents of some amino acids, urea and total ammonia

The values in Table 5 were obtained from rats meal-fed for 4 h every day; they are in good agreement with reported data

obtained under comparable conditions [14,15,39,40]. The values are not corrected for the contribution of blood and plasma in the vascular and extravascular spaces of the liver, which together amount to approx. 23% of its weight in fed rats [41].

The liver content of all amino acids measured except lysine changed during the time studied. The changes were most pronounced between 0 and 1 h, when liver glycine and glutamine concentrations decreased and those of the other amino acids increased; all except the changes in glycine were statistically significant. Between 1 h and 10–14 h, the concentrations returned to fasting values, with a small but statistically significant glutamine overshoot at 10 h. The concentrations at 5 and 8 h (not shown) were intermediate between the adjacent values. The slow return to fasting levels probably reflects the fact that the stomach of the animals contained food for at least 8 h after the beginning of the meal.

During the first 1 h, the most notable changes in amino acids closely related to urea synthesis were the increases in alanine (12-

Table 5 Urea, ammonia and amino acid concentrations in liver of rats meal-fed for 4 h every day

The livers were freeze-clamped at the indicated times, and extracts were prepared and analysed as described under 'Methods'. Methionine, tryptophan, valine and argininosuccinate were undetectable. Three animals per time point were used except where indicated. Concentrations are given in μmol per g wet wt. of liver. (In separate experiments it was determined that at saturating NH_4 and HCO_3^- the velocity of citrulline synthesis in isolated mitochondria was 15, 57, 57 and 42 nmol/min per mg at 0, 2, 7 and 12 h respectively.) Significant differences from 0 h values were determined by Dunnett's two-sided T test [28]: * $P \leq 0.05$; ** $P \leq 0.01$. The values for glutamine were also analysed by the Student-Newman-Keuls test [29] for pair-wise comparison: ‡ significantly different from one another ($P \leq 0.05$).

Compound	Time (h) ...	Concn. ($\mu\text{mol/g}$ wet wt.)					
		0	1†	4	6	10	14†
Urea		3.84 ± 0.38	$8.10 \pm 1.58^{**}$	$8.85 \pm 0.22^{**}$	$6.71 \pm 0.55^{**}$	3.70 ± 0.60	3.86 ± 0.46
Ammonia		0.69 ± 0.13	0.50 ± 0.03	0.47 ± 0.05	0.50 ± 0.02	0.61 ± 0.26	0.64 ± 0.18
Ornithine		0.19 ± 0.02	$0.44 \pm 0.09^{**}$	$0.33 \pm 0.07^*$	0.25 ± 0.04	0.17 ± 0.01	0.18 ± 0.02
Citrulline		0.070 ± 0.009	$0.313 \pm 0.004^{**}$	$0.25 \pm 0.03^{**}$	$0.21 \pm 0.04^{**}$	0.080 ± 0.021	0.12 ± 0.06
Arginine		0.044 ± 0.015	$0.13 \pm 0.01^{**}$	$0.079 \pm 0.003^*$	0.063 ± 0.010	0.049 ± 0.012	0.056 ± 0.023
Alanine		0.16 ± 0.02	$1.90 \pm 0.04^{**}$	$0.58 \pm 0.03^{**}$	$0.68 \pm 0.08^{**}$	$0.36 \pm 0.09^*$	0.27 ± 0.06
Aspartate		0.62 ± 0.07	0.92 ± 0.18	0.72 ± 0.11	0.41 ± 0.03	$1.01 \pm 0.20^*$	0.80 ± 0.18
Glutamate		1.60 ± 0.16	$3.13 \pm 0.56^{**}$	1.99 ± 0.24	1.86 ± 0.18	$2.76 \pm 0.66^*$	1.79 ± 0.38
Glutamine		4.10 ± 0.34	$2.49 \pm 0.36^{**}$	3.19 ± 0.30	$3.57 \pm 0.35^\ddagger$	$5.00 \pm 0.44^\ddagger$	4.56 ± 0.61
Glycine		2.04 ± 0.27	1.60 ± 0.13	2.09 ± 0.09	1.88 ± 0.08	1.64 ± 0.13	1.65 ± 0.34
Cysteine + cystine		0.20 ± 0.02	$0.36 \pm 0.03^{**}$	$0.34 \pm 0.02^{**}$	0.25 ± 0.06	0.21 ± 0.01	0.23 ± 0.04
Isoleucine		0.085 ± 0.014	$0.14 \pm 0.001^{**}$	$0.13 \pm 0.009^{**}$	0.094 ± 0.024	0.082 ± 0.001	0.083 ± 0.006
Leucine		0.13 ± 0.02	$0.27 \pm 0.01^{**}$	$0.23 \pm 0.02^{**}$	0.16 ± 0.04	0.13 ± 0.003	0.13 ± 0.01
Phenylalanine		0.075 ± 0.007	$0.11 \pm 0.007^*$	0.094 ± 0.001	0.077 ± 0.009	0.070 ± 0.004	0.061 ± 0.011
Tyrosine		0.071 ± 0.008	$0.18 \pm 0.01^{**}$	0.096 ± 0.006	0.074 ± 0.003	0.087 ± 0.008	0.079 ± 0.022
Lysine		0.41 ± 0.03	0.37 ± 0.004	0.40 ± 0.09	0.44 ± 0.14	0.49 ± 0.03	0.53 ± 0.04
Histidine		0.31 ± 0.01	$0.49 \pm 0.04^{**}$	$0.48 \pm 0.03^{**}$	$0.43 \pm 0.04^{**}$	$0.42 \pm 0.03^{**}$	0.37 ± 0.05

† Two animals used.

fold), glutamate (2-fold), ornithine (2.3-fold), citrulline (4.5-fold) and arginine (3-fold), and the 39% decrease in glutamine. These findings cannot be attributed to contamination of the liver with arterial and portal plasma [31,42,43]; glutamine, for example, changes in opposite directions in liver and blood after a meal.

The sum of ornithine, citrulline and arginine increased 2.9-fold within 1 h, from 0.30 to 0.88 $\mu\text{mol/g}$ of liver. This was not the result of a decrease in argininosuccinate, since its concentration in liver of fed rats is of the order of 0.03 $\mu\text{mol/g}$ [44]. The enlarged hepatic pool of ornithine-cycle-specific intermediates must have originated from dietary arginine and from the intestinal [45] synthesis of ornithine from glutamine.

Since glutamate is not readily taken up by the liver [46], the increase from 1.6 to 3.1 $\mu\text{mol/g}$ between 0 and 1 h is likely to reflect hepatic synthesis *de novo* from other amino acids and glucose. Glutamine decreased by 1.6 $\mu\text{mol/g}$ upon feeding, perhaps as a result of glutaminase activation; the decrease persisted for most of the absorptive period.

The liver content of alanine increased 12-fold, to 1.9 $\mu\text{mol/g}$, during the first 1 h of the meal, as observed previously [47]; contributors to the pool include dietary alanine, other amino acids (by transamination with pyruvate derived from dietary carbohydrate [47]), and perhaps alanine of intestinal origin [31].

The liver content of urea increased from 3.84 $\mu\text{mol/g}$ at 0 h to 8.1 $\mu\text{mol/g}$ at 1 h; it remained elevated for at least 8 h and returned to the fasting value by 10 h. Total ammonia, 0.69 ± 0.12 $\mu\text{mol/g}$ at 0 h, did not change significantly (see [2,3]).

Methionine, tryptophan, valine and argininosuccinate were undetectable in the small volume of extract that could be used for h.p.l.c. analysis.

Since the rats used for these measurements were on the 4-20 feeding schedule, the citrulline-synthesizing capacity of their liver mitochondria at various times was determined and compared with that of mitochondria from rats on the 8-40 schedule. The findings were similar to those shown in Table 3; with saturating

NH_4^+ and HCO_3^- , mitochondria from rats on the 4-20 schedule synthesized citrulline at the following velocities, in nmol/min per mg: 15 at 0 h, 57 at 2 and 7 h, and 42 at 12 h.

DISCUSSION

Factors that control the synthesis of carbamoyl phosphate and aspartate are likely to affect the synthesis of urea; these factors include the concentrations of NAG and ornithine, and the availability of substrate. Our findings using the meal-fed rat model indicate that the latter, substrate availability, is the primary regulator of the velocity of urea synthesis at any given instant.

Velocity of urea synthesis *in vivo*

Our experiments show that rats synthesize urea at 30% or less of the potential maximum velocity of about 6 $\mu\text{mol/min}$ per g of liver [48], which is the V_{max} of argininosuccinate synthase [49]. During the 8 h meal, the average velocity was about 1.8 $\mu\text{mol/min}$ per g of liver (consistent with velocities from endogenous substrates measured in fed rats, of about 1 $\mu\text{mol/min}$ per g, calculated from [7] on the assumption that the liver of the 360 g rats used weighed 15 g). This is sufficient to dispose of surplus nitrogen while maintaining normal concentrations of amino acids and ammonia in blood. It is conceivable, however, that for a very short time early during a meal rats may synthesize urea at or near the potential maximum velocity.

There was no steady-state of urea synthesis in the fed state or in short-term fasting (Figure 1). The velocity of synthesis decreased linearly with time even while the stomachs of the rats were replete with food, indicating that digestion limits the availability of ureagenic substrates to the liver. A near-steady-state was approached only 32 h after the start of the meal, in early starvation.

Effects of NAG and ornithine on CPS_I

The mitochondrial NAG content was positively correlated with the velocity of citrulline synthesis from NH_4^+ and HCO_3^- , and the changes in NAG throughout the feeding cycle were generally consistent with changes in urea synthesis. This supports the notion that NAG plays a role in the regulation of CPS_I *in vivo* [8–10].

The correlation between CPS_I activity and NAG content, however, is not simple. If matrix CPS_I and NAG are in solution, the approximate [total CPS_I], [total NAG] and [CPS_I · NAG] can be calculated as described in [50], on the bases of the mol of CPS_I and NAG per mg of mitochondrial protein, the matrix total water volume (approx. 1 μl /mg of mitochondrial protein [51] under the conditions used; Table 3), and the volume of free matrix water (approx. 50% of total matrix water [52,53]).

At 0 h, for 1 mM total CPS_I (500 pmol/mg), a mean of 0.254 mM total NAG (127 pmol/mg; Table 4) and a K_m of 0.1 mM [37,54], [CPS_I · NAG] is 0.226 mM. (This value would be slightly greater if the K_m in the matrix were 11 μM ; see [55].) Mitochondria isolated at 0 h and incubated with NH_4^+ and HCO_3^- should be capable of synthesizing carbamoyl phosphate at about 0.23 V_{max} of CPS_I, or 33 nmol/min per mg, rather than the observed 0.06 V_{max} , 9.4 nmol/min per mg (Table 3; V_{max} is approx. 145 nmol/min per mg under the conditions used; see [56]). Since carbamoyl phosphate was measured as citrulline, it is important to note that the former does not accumulate under the conditions used [4], and also that efflux of matrix NAG is undetectable under those conditions [4,57]. Similar calculations show that at 19 h, CPS_I · NAG should be 0.44 mM, 2-fold greater than at 0 h, yet the velocity of citrulline synthesis was 4-fold greater (Table 3).

These and other [57,58] pronounced discrepancies between the velocity of carbamoyl phosphate synthesis and the matrix NAG content suggest that the activity of CPS_I *in situ* is affected by variables which enhance, diminish, and sometimes nearly abolish, the effects of NAG. CPS_I may not be homogeneously distributed in the matrix [57]; association with other molecules may alter its kinetic properties, as may compounds other than NAG.

In situ, the V_{max} of CPS_I is low at low ornithine concentration even at high NAG, and in the virtual absence of carbamoyl phosphate [4,6,56]. Stated otherwise, CPS_I *in situ* has a nearly absolute requirement for ornithine. This determines that the potential upper limit of activity of CPS_I in various metabolic states is set by the concentration of ornithine as well as that of NAG. The activating function of ornithine is distinct from its function as a catalytic intermediate of the ornithine cycle.

Role of changes in the liver content of ornithine

Cohen et al. [4,6,59] have proposed that changes in the concentration of ornithine contribute to the regulation of urea synthesis *in vivo*. The following facts are pertinent: (a) ornithine has independent kinetic effects on CPS_I and the ornithine transporter/ornithine carbamoyltransferase system [6,59]; (b) the composite $S_{0.5}$ of ornithine for these parameters changes in direct proportion to the availability of substrate [6,58]; (c) the capacity of the ornithine transporter/ornithine carbamoyltransferase system is not greatly in excess of that of CPS_I, not even at saturating ornithine; under some conditions, the transporter or the carbamoyltransferase is limiting for citrulline synthesis [6,56,59]; (d) ornithine at physiological concentrations is non-saturating for both CPS_I and the transporter/carbamoyltransferase system [6,56,59].

It is known that ornithine at concentrations similar to that in liver at 0 h (Table 5) does not support citrulline synthesis at

velocities of 1.8 or more $\mu\text{mol}/\text{min}$ per g of liver [6,60]. The rats used in the present work synthesized citrulline at these high velocities for at least 4 h after the start of the meal, during which time the liver ornithine content was 100% higher than at 0 h (Table 5).

We propose that the observed changes in ornithine, by their effects on the V_{max} of CPS_I, and on the fractional saturation of the ornithine transporter and ornithine carbamoyltransferase, are required for the velocity of carbamoyl phosphate and citrulline synthesis to adjust to the different fluxes of ammonia which occur *in vivo* (see [4,6]; cf. [60–62]). Further support for this proposal is the fact that animals lacking an adequate endogenous source of ornithine, such as cats and ferrets, develop severe or lethal hyperammonaemia after ingestion of arginine-deficient diets [63].

Role of substrate availability

The velocity of urea synthesis, the citrulline-synthesizing capacity and NAG content of mitochondria, and the ornithine content of the liver increased several-fold within 1–3 h after the beginning of a meal. At all times studied, the citrulline-synthesizing capacity of mitochondria was substantially greater than the respective velocities *in vivo*; since amino acids, ammonia and the pathway-specific intermediates of urea synthesis do not accumulate in normal rats during a meal, the velocity of synthesis must have been limited by the supply of substrates to CPS_I (see [6,58]). Evidence points to ammonia as the substrate which kinetically controls CPS_I [5,7] under normal conditions.

Our findings are consistent with the mechanism proposed by Krebs [2,3,64] for the production of carbamoyl phosphate and aspartate in the 1:1 stoichiometry required for urea synthesis. The mechanism provides that when the pool of glutamate increases as a result of net carbon and nitrogen input, as during the meal (Table 5), net flux through the glutamate dehydrogenase/glutamate-oxaloacetate transaminase near-equilibrium system increases in the direction of NH_4^+ and aspartate formation, i.e. in the ureagenic direction; aspartate remains at a relatively constant concentration [64], as observed in this work (Table 5).

If increases in glutamate of the observed magnitude (Table 5) stimulate NAG synthesis *in vivo* as in isolated mitochondria [57,65] then substrate-driven increases in flux through CPS_I should be followed by an increase in the V_{max} of this enzyme [15], provided that ornithine is also present at sufficient concentration. The ureagenic capacity of the liver would thus tend to remain greater than the demand.

The ability of mammalian liver to cope with wide variations of surplus nitrogen [3] is a function of slow changes in the liver content of urea-cycle enzymes in response to the protein intake [66] and of somewhat faster changes in the concentrations of ornithine and NAG, and other variables. The present work indicates that substrate availability exerts immediate control over the velocity of urea synthesis *in vivo*. Since digestion limits the delivery of substrate, this may be quantitatively most important in preventing the ureagenic capacity of the liver from being overwhelmed upon the ingestion of protein.

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