The apparent K_m of ammonia for carbamoyl phosphate synthetase (ammonia) *in situ*

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Experiments with carbamoyl phosphate synthetase (ammonia) in solution and in isolated mitochondria are reported which show the following. (1) NH_3 rather than NH_4^+ is the substrate of the enzyme. The apparent K_m of NH_3 for the purified enzyme is about $38\text{ }\mu\text{M}$. (2) The apparent K_m for NH_3 measured in intact isolated mitochondria is about $13\text{ }\mu\text{M}$. This value was obtained for both coupled and uncoupled mitochondria and was unchanged when the rate of carbamoyl phosphate synthesis was increased 2-fold by incubating uncoupled mitochondria in the presence of 5 mM -*N*-acetylglutamate. (3) According to the literature, the concentration of NH_3 in liver is well below the measured apparent K_m . On the basis of this and previous work we conclude that, quantitatively, changes in liver $[\text{NH}_3]$ and [ornithine] are likely to be the most important factors in the fast regulation of synthesis of carbamoyl phosphate and urea. This conclusion is consistent with all available evidence obtained with isolated mitochondria, isolated hepatocytes, perfused liver and whole animals.

Carbamoyl phosphate synthetase (ammonia) (EC 6.3.4.16) catalyses the formation of carbamoyl phosphate (Jones *et al.*, 1955), the first intermediate in the pathway of urea synthesis. The activity of this enzyme *in vivo* is subject to both slow and fast regulation. The former, measured in days, occurs predominantly via changes in the amount of enzyme (Schimke, 1962), whereas the latter, measured in minutes, is thought to occur via alterations in the availability of some substrates (Raijman, 1976) or effectors (Shigesada & Tatabana, 1971). In addition, ornithine, a substrate in the second reaction of the pathway, has a great stimulatory effect on the activity of carbamoyl phosphate synthetase *in situ* (Cohen *et al.*, 1980, 1982).

The present paper reports our findings on the species of ammonia that is the true substrate of carbamoyl phosphate synthetase and on its affinity for the enzyme. The physiological response of the enzyme to increases in the availability of ammonia is consistent with its kinetic properties with respect to this substrate.

Abbreviation used: DMO, 5,5-dimethylloxazolidine-2,4-dione.

Materials and methods

Animals and reagents

Male rats of the Sprague–Dawley strain, weighing 150–250 g, were obtained from Simonsen Laboratories, Gilroy, CA, U.S.A.; they were fed *ad libitum* on standard laboratory chow.

Carbamoyl phosphate synthetase (specific activity $1.5\text{ }\mu\text{mol/min}$ per mg of protein) from rat liver was purified as described by Powers (1981), and ornithine carbamoyltransferase (specific activity $650\text{ }\mu\text{mol/min}$ per mg) from bovine liver by the method of Marshall & Cohen (1972). 5,5-Dimethyl[2- ^{14}C]oxazolidine-2,4-dione (^{14}C]DMO; sp. radioactivity 60 Ci/mol) was obtained from New England Nuclear, Boston, MA, U.S.A. ^{14}C]Acetate (sp. radioactivity 51 Ci/mol), ^{14}C]sucrose (sp. radioactivity, 350 Ci/mol), ^{14}C]ethanolamine (sp. radioactivity 95 Ci/mol) and $^3\text{H}_2\text{O}$ (sp. radioactivity 100 Ci/mol) were from ICN, Irvine, CA, U.S.A., and Hydrofluor was from National Diagnostics, Somerville, NJ, U.S.A. Dow Corning silicones 200 (1.5 cSt) and 550 were from William F. Nye, New Bedford, MA, U.S.A. All other reagents were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Preparation and incubation of mitochondria

The animals were killed by cervical dislocation. The liver was homogenized as described by Rajman & Bartulis (1979) in 9 vol. of 0.3M-mannitol/2mM-Hepes/KOH, pH 7.4. The homogenate was diluted 2-fold with the same medium, and centrifuged at 4°C for 5 min at 600g in a Sorvall RT 6000 centrifuge with a horizontal rotor; the post-nuclear supernatant, containing about 85% of the protein of the homogenate, was carefully decanted. [Similar results can be obtained by centrifuging for 5 min at <400g (about 1700 rev./min) in a Sorvall RC-5 centrifuge with an SS-34 rotor.] The mitochondria were pelleted by centrifugation at 4°C for 10 min at 3000g in a Sorvall RC-5 centrifuge with an SS-34 rotor, washed once with a volume of mannitol/Hepes equal to the original homogenization volume, and resuspended in the same medium at a protein concentration of about 25 mg/ml. This method yields a mitochondrial preparation minimally contaminated with lysosomes and endoplasmic reticulum, with a recovery of 70–75% of the total mitochondria (Katz *et al.*, 1983; Cohen & Cheung, 1984).

Mitochondrial incubations for measuring synthesis of carbamoyl phosphate and citrulline, matrix pH and mitochondrial volumes were performed in duplicate in 20 ml glass vials in a Dubnoff shaking bath at 30°C. The standard incubation medium contained (final concns.) 50 mM-Tris/HCl, pH 7.25–8.45, as required, 15 mM-KCl, 15 mM-KHCO₃, 10 mM-ornithine, 5 mM-MgCl₂, 5 mM-KH₂PO₄, 2 mM-EDTA, 0.1% defatted bovine serum albumin, 10 mM-succinate, 0 or 0.1–10 mM-NH₄Cl, and mannitol as required to maintain iso-osmolality. Parallel incubations for the measurement of mitochondrial volumes contained, in addition, 2.4 µCi of ³H₂O/ml and 1.25 µCi of [¹⁴C]sucrose/ml; for determination of matrix pH, 0.5 µCi of [¹⁴C]DMO/ml, 0.5 µCi of [¹⁴C]acetate/ml or 0.72 µCi of [¹⁴C]ethanolamine/ml were added to the standard medium. The final volume was 1–2 ml, and the final mitochondrial protein concentration was 2.0–2.5 mg/ml. The incubation mixtures were warmed for 3 min; the mitochondria, also prewarmed to 30°C, were added to start the reactions.

The incubations were terminated after 30 s by addition of HClO₄ (final concn. 1.7 M). Citrulline was measured in portions of the deproteinized samples; ammonia was measured in portions which were neutralized to about pH 6.5 with KOH and KHCO₃, and centrifuged to remove KClO₄. Incubations for the measurement of mitochondrial volumes and matrix pH were terminated after 30 s by separating the mitochondria from the medium

by centrifugation through silicone oil (Rajman & Bartulis, 1979) into 0.1 ml of 14% (w/v) HClO₄; samples of the supernatant were deproteinized with HClO₄ (final concn. 1.7 M). Duplicate 10 µl portions of each of the protein-free supernatant samples and of the mitochondrial extracts were counted for radioactivity in 4 ml of Hydrofluor in a Beckman LS 100 C liquid-scintillation counter. Mitochondrial volumes were measured exactly as described by Cohen & Cheung (1984), and matrix pH by the method of Addanki *et al.* (1968). The pH of the mixture after 30 s of incubation was measured in parallel incubations with a Radiometer PHM62 standard pH-meter.

Incubations with purified carbamoyl phosphate synthetase

Carbamoyl phosphate synthetase and ornithine carbamoyltransferase were dialysed against four changes each of 500 vol. of 20 mM-Hepes/1 mM-ornithine/0.5 mM-dithiothreitol, pH 7.6, immediately before use. The incubation mixtures contained 0.01 unit (assayed at pH 7.4; 1 unit = 1 µmol/min) of carbamoyl phosphate synthetase/ml, 18 units of bovine ornithine carbamoyltransferase/ml, 50 mM-Hepes/KOH, pH 7.23–8.08 as required, 15 mM-KHCO₃, 12 mM-MgSO₄, 2 mM-ATP, 5 mM-acetylglutamate, 5 mM-ornithine, 0.1% defatted bovine serum albumin, 5 mM-phosphocreatine (di-Tris salt), 5 units of creatine kinase/ml, and 0 or 0.1–10 mM-NH₄Cl, as required; KCl was added as required to maintain a constant ionic strength. The incubations were performed at 37°C, in duplicate, in closed 1.5 ml micro-centrifuge tubes in a total volume of 0.5 ml. The tubes, containing everything but ATP, were preincubated at 37°C for 5 min, and the reactions were started with ATP. After 5 min they were stopped with 0.25 ml of 5 M-HClO₄; portions of the deproteinized samples were used to measure citrulline and ammonia. The reactions were linear with respect to time. The pH of replicate incubation mixtures was measured at the end of incubation.

Measurement of metabolites and protein

Citrulline was measured by the method of Ceriotti & Spandrio (1963) as described previously (Cohen *et al.*, 1980), and ammonia was measured as described by Gutmann & Bergmeyer (1974). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Methods of calculation of [free NH₃] and [free NH₄⁺]

In experiments with purified carbamoyl phosphate synthetase, the measured pH of the incubation mixtures and the measured total ammonia were used to calculate the concentrations of NH₃

and NH_4^+ , by using a pK for ammonia at 37°C of 8.88 (Dawson *et al.*, 1969). In mitochondrial incubations, the concentrations of NH_3 and NH_4^+ in the medium were determined similarly, with a pK for ammonia at 30°C of 9.1 (Dawson *et al.*, 1969). Since NH_3 diffuses freely into the matrix (Chappell & Crofts, 1966; Chappell, 1968), the concentrations of free NH_3 should be the same in the matrix and medium, and a function of the pH of the medium. The matrix concentration of free NH_4^+ was calculated from $[\text{NH}_3]$ and the measured matrix pH .

Contamination of mitochondrial preparations with lysosomes (de Duve, 1965) can lead to inaccuracies in measurements of pH gradients, since the lysosomes contribute to the measured sucrose-impermeable space (Reijngoud & Tager, 1977). The lysosomal volume in the mitochondrial preparation used in the present studies, however, was no more than about 2% of the mitochondrial volume (Cohen & Cheung, 1984); the small error contributed by this contamination would not change the results obtained.

Results

The true substrate for carbamoyl phosphate synthetase is NH_3

Incubations with purified carbamoyl phosphate synthetase were performed at measured pH 7.23–8.08; this range was selected because at pH values much above 8.1 the activity of the purified enzyme decreases markedly (Lusty, 1978), and at pH values much below 7.2 only a very small percentage of total ammonia is present as NH_3 . For total ammonia the apparent K_m was calculated (by linear regression) as 1.85–0.391 mM, depending on the pH of the experiment (Table 1). When the apparent K_m was calculated on the basis of NH_4^+ , the values changed from 1.81 mM at pH 7.24 to 0.353 mM at pH 7.91; that is, the value decreased with increasing pH . In contrast, when the K_m was

calculated on the basis of NH_3 , the value was 29–41 μM , and was independent of pH . We interpret these findings to mean that NH_3 is the true substrate of carbamoyl phosphate synthetase (see below). In seven experiments at pH values in the range 7.23–8.08, the apparent K_m for NH_3 was $38.29 \pm 2.67 \mu\text{M}$ ($\pm$ S.E.M.). (The concentration of enzyme used in these experiments was 50 nM.)

The $[\text{ATPMg}]$ in these experiments was 2 mM, and was kept constant by the use of a regenerating system; with 5 mM-ATPMg and 15 mM- MgSO_4 , synthesis was inhibited by approx. 20% at all pH values, but the apparent K_m for NH_3 was not changed. It could be argued that changes in the ionization of ATPMg over the pH range used could have affected our kinetic observations. The pK value of $\text{ATPMgH}^- \rightleftharpoons \text{ATPMg}^{2-} + \text{H}^+$ is estimated to be 4.88 (O'Sullivan & Perrin, 1964), so that virtually all of the ATPMg would be fully ionized even at the lowest pH used. This suggests that fully ionized ATPMg is the species used by the synthetase, and shows that the availability of ATPMg did not affect our results. The latter conclusion is supported by experiments (results not shown) in which the apparent K_m of carbamoyl phosphate synthetase for ATPMg was measured and found to be constant over the pH range 7.31–7.83.

Elimination of the lag in citrulline synthesis observed in mitochondrial incubations

For the mitochondrial experiments described below, it was necessary to employ very short incubation times in order to avoid using a large percentage of the ammonia added. In incubations with intact mitochondria, however, a lag in citrulline synthesis of several minutes is generally observed (Charles *et al.*, 1967; Meijer & Van Woerkom, 1977; Cohen *et al.*, 1980). For the studies that we wished to do, it was obviously undesirable to work during a period when the rate of synthesis was changing. It was found that the lag could be eliminated by simply warming the mitochondrial suspension to 30°C before starting the reactions. This was done on a timed schedule so that the mitochondria in all experimentals were preincubated for the same length of time.

pH gradient established under the incubation conditions used

The pH gradient established by the mitochondria under the standard incubation conditions at an external pH of about 7.5 was generally 0.3–0.4 pH unit (alkaline inside in all cases), when either the weak acids, $[^{14}\text{C}]$ acetate or $[^{14}\text{C}]$ DMO, or the weak base, $[^{14}\text{C}]$ ethanolamine, were used as indicators. The absolute value obtained was slightly different (about 0.1 pH unit) with the

Table 1. Apparent K_m of ammonia for purified carbamoyl phosphate synthetase

Incubation conditions and methods for measuring citrulline synthesis, ammonia concentration, and pH were as described in the Materials and methods section.

pH	K_m calculated on the basis of:		
	NH_3 (μM)	NH_4^+ (mM)	Total ammonia (mM)
7.24	41	1.81	1.85
7.63	29	0.514	0.543
7.91	38	0.353	0.391

different indicators, as previously reported (Wilson & Forman, 1982). The ΔpH measured with DMO or acetate was unaffected by NH_4Cl (0–10 mM) and ornithine (0–5 mM). In contrast with most of the literature, Dodgson *et al.* (1983) reported an acidic-inside pH gradient in mitochondria, which was largest in the presence of ornithine; they suggested that DMO may not provide a valid measurement of mitochondrial pH, especially in the presence of ornithine, because of possible binding and trapping of it by ornithinium cation in the matrix. Our results do not support this suggestion. We do not know the reason for the findings of Dodgson *et al.* (1983); their method, however, depends on assumptions about the behaviour of carbonic anhydrase which have not been validated for the conditions obtaining in the mitochondrial matrix.

Apparent K_m of NH_3 for carbamoyl phosphate synthetase in mitochondria

We initially attempted to determine the K_m for NH_3 on the basis of measured mitochondrial ammonia. However, we consistently found 2–5 nmol (2.75 $\pm$ 0.43; $\pm$ S.E.M. for nine experiments) of total ammonia/mg of mitochondrial protein when none had been added to the incubation mixture; this ammonia was not utilized for carbamoyl phosphate synthesis. These findings are in agreement with the average of 5 nmol/mg reported by Wanders *et al.* (1980), who also found that this may represent a metabolically inert pool

of ammonia. Given these high background values, it was not possible to measure small increments in ammonia in mitochondrial pellets with sufficient reliability, especially at the lower ammonia concentrations employed. We therefore used $[\text{NH}_3]$ and $[\text{NH}_4^+]$ calculated as described in the Materials and methods section.

Experiments to determine the activity of carbamoyl phosphate synthetase at different concentrations of added NH_4Cl were performed at measured pH 7.25–8.45. The calculated matrix concentrations of NH_3 , NH_4^+ and $\text{NH}_3 + \text{NH}_4^+$ at which $\frac{1}{2}V_{\text{max}}$ was obtained are shown in Table 2. It should be noted that, in calculating these concentrations, the only assumption made was that NH_3 is the passively permeant species (Chappell & Crofts, 1966; Chappell, 1968). The apparent K_m for NH_3 for coupled mitochondria ranged from 10 to 25 μM (Table 2); there was no systematic change as a function of pH. In experiments with uncoupled mitochondria (using 2 μM -carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and 10 μg of oligomycin/ml), a similar apparent K_m for NH_3 was obtained (Table 2). This value was unchanged when the acetylglutamate concentration of uncoupled mitochondria was increased by incubation with 5 mM-*N*-acetylglutamate (Cohen *et al.*, 1982; Cohen & Cheung, 1984), indicating that acetylglutamate did not significantly change the affinity of the synthetase for NH_3 (Table 2), in agreement with the findings of Elliott & Tipton (1974). Values within this range were obtained for

Table 2. *Apparent K_m of ammonia for carbamoyl phosphate synthetase in mitochondria*

Mitochondrial incubations for the experiments in (a), and the measurement of citrulline, ammonia, and pH of medium and matrix were done as described in the Materials and methods section. For the experiments in (b), succinate was omitted and the incubation mixtures contained, in addition to the standard components, 2 μM -carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, 10 μg of oligomycin/ml, 2 mM-ATP, and an ATP-regenerating system consisting of 5 units of creatine kinase/ml and 5 mM-phosphocreatine. The mitochondria were preincubated for 5 min at 30°C with the uncoupler, the oligomycin, and all components of the incubation mixture except HCO_3^- , ornithine, and ATP; a mixture of these three was added to start the reactions. The V_{max} values given are measured. Abbreviation: N.D., not determined.

Mitochondria	Medium pH	Matrix pH	K_m calculated on the basis of:				V_{max} (nmol/min per mg)
			NH_3 (μM)	NH_4^+ (μM)	Total ammonia (μM)		
(a) Coupled	7.25	7.51	11	420	431		34
	7.50	7.99	14	178	192		33
	7.86	8.18	13	107	120		33
	8.05	8.34	14	80	94		19
	8.28	8.52	25	96	119		26
	8.45	8.65	20	57	77		15
(b) Uncoupled	7.50	N.D.	11				38
	7.50*	N.D.	13				70
	7.50*	N.D.	10				67

* These incubation mixtures contained 5 mM-acetylglutamate. In longer incubations, carbamoyl phosphate would have accumulated (in addition to citrulline) under these conditions (Cohen *et al.*, 1982); no carbamoyl phosphate was found in these experiments, in which incubation was for only 30s.

coupled and uncoupled mitochondria in many additional experiments not shown here; the apparent K_m in 14 experiments was $13.14 \pm 1.17 \mu\text{M}$ ($\pm$ S.E.M.). In contrast with the constancy of the apparent K_m for NH_3 at all pH values used, the value calculated for NH_4^+ decreased from 420 to $57 \mu\text{M}$ when matrix pH values increased from 7.51 to 8.65 (Table 2). These data suggest that NH_3 is the substrate of matrix carbamoyl phosphate synthetase.

Mechanistically, it is reasonable to assume that the same species of ammonia serves as the substrate for carbamoyl phosphate synthetase in solution and *in situ*; these experiments show that the affinity of the enzyme for NH_3 is similar under both conditions. Most K_m values for mitochondria were between 10 and $14 \mu\text{M}$. The range of K_m for NH_3 was greater in the experiments with mitochondria, however, than with the soluble enzyme (cf. Tables 1 and 2); this may reflect inherently larger experimental errors incurred in working with intact organelles at very low substrate concentrations. We do not know if the numerical differences between the K_m of the purified enzyme and the enzyme *in situ* are real.

The concentration of carbamoyl phosphate synthetase in the matrix is extremely high, about 0.5 mM (Raijman, 1976), whereas $\frac{1}{2}V_{\text{max}}$ was obtained at micromolar concentrations of NH_3 . In these experiments, $[\text{NH}_3]$ was not decreased significantly by binding to the enzyme, because (a) the total amount of synthetase was about 1–1.5 nmol/ml of incubation mixture, whereas the total amount of NH_3 was in large excess over the enzyme (since NH_3 diffuses freely into the matrix, the total pool of NH_3 in medium plus matrix was available to the synthetase); (b) at all pH values used, $[\text{NH}_4^+]$ in the medium was in large excess over $[\text{NH}_3]$, thereby buffering the latter.

Discussion

Jones (1976) pointed out that chemically, NH_3 , rather than NH_4^+ , can be expected to react with the enzyme–substrate intermediate containing some of the elements of HCO_3^- . Later, experiments using purified frog liver carbamoyl phosphate synthetase suggested that NH_3 , rather than NH_4^+ , reacts with previously formed enzyme-bound 'active CO_2 ' to yield carbamoyl phosphate (Rubio & Grisolia, 1977). We decided to determine the species of ammonia which is the true substrate for the overall reaction, in which case no assumptions need be made about the order of addition of substrates (Rubio & Grisolia, 1977; Raushel *et al.*, 1978). We conclude that NH_3 is the substrate for rat liver carbamoyl phosphate synthetase. [McGivan & Bradford (1983) determined that the species

responsible for the activating effect of ammonia on mitochondrial glutaminase is also NH_3 , not NH_4^+ .]

It is generally stated that ammonia is present in rat liver at about its K_m for carbamoyl phosphate synthetase. The concentration of total ammonia measured in freeze-clamped liver is about 0.7 mM (Brosnan *et al.*, 1970); this value is indeed similar to the K_m for total ammonia reported for the purified synthetase at pH 7.6 by Elliott & Tipton (1974), for the synthetase in permeabilized mitochondria at pH 7.4 by Lof *et al.* (1983), and for the purified synthetase at pH 7.63 in the present studies. The substrate for the synthetase, however, is NH_3 , the pK of ammonia at 37°C is 8.88 (Dawson *et al.*, 1969), and the pH of the cytoplasm measured in isolated hepatocytes has been reported to be 7.0 (Hoek *et al.*, 1980); at this pH, the $[\text{NH}_3]$ in the cell would be about 100-fold lower than the [total ammonia], or about $7 \mu\text{M}$, which is below the apparent K_m of $13 \mu\text{M}$ determined for the synthetase in intact mitochondria in the present work. In fact, [free NH_3] in liver must be less than $7 \mu\text{M}$; experiments by Wanders *et al.* (1980) indicate that a significant portion of the ammonia in extracts of isolated hepatocytes is metabolically inert (see also Harris & Bassett, 1971; Sainsbury, 1980), possibly bound. From an average value of metabolically inert total ammonia of 4 nmol/mg of mitochondrial protein (Wanders *et al.*, 1980; the present work), and assuming a total mitochondrial content of 60 mg/g of liver (Scholz & Bücher, 1965), the value of $0.7 \mu\text{mol/g}$ of liver given above for total ammonia (Brosnan *et al.*, 1970) would have to be decreased by $0.24 \mu\text{mol/g}$, leaving $0.46 \mu\text{mol/g}$ as metabolizable ammonia, of which only about 1% would be present as NH_3 . [Free NH_3] would therefore be no more than $5 \mu\text{M}$ in the cytoplasm and in the mitochondrial matrix of liver cells from normal fed animals, which is well below its apparent K_m for carbamoyl phosphate synthetase. Increases in [free NH_3], whether brought about by increases in total ammonia from any source, or in cellular pH, would exert a large stimulatory effect on the activity of the synthetase in normal liver. This is in agreement with experimental findings obtained with perfused liver (Hems *et al.*, 1966) and whole animals (Duda & Handler, 1958; Katunuma *et al.*, 1966; Saheki *et al.*, 1978), and also with the finding by Bean & Atkinson (1983) that the rate of urea synthesis in isolated hepatocytes increases with increasing pH.

The fast regulation of carbamoyl phosphate synthetase, involving changes in activity of 10–20-fold within 1 min (Hems *et al.*, 1966), can in theory be mediated by any of its substrates, cofactors and effectors. Of the cofactors, Mg^{2+} and K^+ are not likely to be significant regulators under most

physiological conditions, since the concentration of these cations in mitochondria is substantially greater than their kinetic constants for the synthetase (Meijer & Van Woerkom, 1977). Measurements of acetylglutamate in freeze-clamped liver of animals fed on a normal diet indicate that this cofactor is at about K_m or higher concentrations in mitochondria (Shigesada & Tatibana, 1971; Saheki *et al.*, 1978; Stewart & Walser, 1980; Van Dijk & Lund, 1984) (assuming that 65% of liver acetylglutamate is in the mitochondria; Hensgens *et al.*, 1980). It was proposed (Raijman, 1976) that increases in the acetylglutamate concentration alone could result in no more than a doubling (approximately) of carbamoyl phosphate synthetase activity in such animals. This inference has been confirmed in experiments using isolated mitochondria and hepatocytes (McGivan *et al.*, 1976; Meijer & Van Woerkom, 1978; Cheung & Raijman, 1980; Hensgens *et al.*, 1980). Furthermore, increases in the synthetase activity attributable to increases in matrix [acetylglutamate] are significant only after 10–15 min (Meijer & Van Woerkom, 1978; Hensgens *et al.*, 1980). Clearly, the large and essentially instantaneous increases in carbamoyl phosphate synthetase activity observed by Hems *et al.* (1966) and Krebs *et al.* (1973) cannot be explained by increases in matrix acetylglutamate (cf. Meijer & Verhoeven, 1984).

Changes in substrate concentrations could have rapid and large effects on the activity of carbamoyl phosphate synthetase. Bicarbonate is present in liver at saturating concentrations for the enzyme and is not limiting under most conditions (Rognstad, 1983). The activity of the synthetase in isolated mitochondria is affected by changes in matrix ATP/ADP ratio (Williamson *et al.*, 1981), but only when this ratio is well below the values found in mitochondria from isolated hepatocytes (Akerboom *et al.*, 1978; see also Raijman & Bartulis, 1979).

We conclude that NH_3 is the only substrate of carbamoyl phosphate synthetase whose affinity for the enzyme and concentration in normal liver are such that changes in its concentration can effect large and nearly instantaneous changes in the activity of the enzyme *in vivo*. It has been shown that ornithine exerts a large and rapid stimulatory effect on urea synthesis by perfused liver (Hems *et al.*, 1966; Krebs *et al.*, 1973) and isolated hepatocytes (Krebs *et al.*, 1974) and on carbamoyl phosphate synthesis by isolated mitochondria (Cohen *et al.*, 1980, 1982). Quantitatively, changes in liver $[\text{NH}_3]$ and [ornithine] (Raijman, 1976) are likely to be the most important factors in the fast regulation of carbamoyl phosphate synthesis, and to be equally important in determining the rate of urea synthesis. This conclusion is consistent with

all available evidence obtained with isolated mitochondria, isolated hepatocytes, perfused liver and whole animals.

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