Regulation of Activity of Carbamoyl Phosphate Synthetase from Mouse Spleen*

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ABSTRACT: Factors affecting the activity of glutamine-dependent carbamoyl phosphate synthetase from mouse spleen have been studied to determine the role of this enzyme in the control of *de novo* pyrimidine biosynthesis. Potassium ion markedly stimulated activity, but sodium did not. Both magnesium and ATP were required for enzyme activity and the optimal ratio was greater than 1.0 so that both free magnesium and the magnesium–ATP complex appear to bind to the synthetase. The curve of velocity *vs.* ATP concentration was sigmoidal and fit a 2:1 function. The pH optimum was 7.4. Kinetic determinations at this pH gave K_m values of 3.2 mM for ATP, 0.05 mM for glutamine, and 11 mM for bicarbonate. Synthetase activity was inhibited by UTP, and a K_i was calculated to be 0.11 mM. The inhibition was competitive with respect to ATP. The kinetics of inhibition suggested that UTP may be an allosteric inhibitor while ATP may be both a substrate and allosteric effector. The results suggest that the synthetase is subject to reciprocal control by ATP and UTP. Both the substrate ATP and inhibitor UTP protected against thermal inactivation of the enzyme, but ATP was more effective than UTP.

The glutamine-dependent carbamoyl phosphate synthetase (CPS)[1] derived from hematopoietic mouse spleen copurifies with aspartate transcarbamoylase (ATC, EC 2.1.3.2) (Hoogenraad *et al.*, 1971). The stoichiometry of the reaction catalyzed by mouse spleen CPS, established by Tatibana and Ito (1969) for partially purified enzyme, was shown to be similar to that obtained for the enzyme derived from *Escherichia coli* (Anderson and Meister, 1965):

$$2ATP + HCO_3^- + \text{L-glutamine} \longrightarrow \text{carbamoyl phosphate} + 2ADP + \text{L-glutamate} + P_i$$

Since CPS in mammalian cells catalyzes the first step unique to the *de novo* pyrimidine biosynthetic pathway, it is reasonable for control of the pathway to be exerted at this site (Umbarger, 1961; O'Donovan and Neuhard, 1970). In fact, CPS from mouse spleen and also from *Neurospora* have been shown to be inhibited by UTP (Tatibana and Ito, 1967; Williams and Davis, 1970). However, the mechanism by which UTP inhibits CPS from mouse spleen has not been established. In *E. coli* there is a dual control of the *de novo* pyrimidine pathway: feedback inhibition of CPS by UMP (Anderson and Meister, 1966b) and feedback inhibition of ATC by CTP (Yates and Pardee, 1956). The inhibition of ATC is competitive with respect to both substrates, carbamoyl phosphate and aspartate (Bethell *et al.*, 1968). The binding of CTP occurs at a site distinct from that of the two substrates (Gerhart and Schachman, 1965) and consequently is allosteric.

The present investigation shows that the inhibition of CPS from mouse spleen by UTP is competitive with respect to the substrate ATP, and that the relative concentrations of this purine nucleotide and the pyrimidine nucleotide UTP appear to be critical in control of the activity of CPS.

Experimental Section

The CPS source used in the present study was the dialyzed enzyme preparation prepared as described by Hoogenraad *et al.* (1971) and had a specific activity of 4.78 nmoles/min per mg of protein. Except as noted, materials and the CPS assay[2] were as previously described (Hoogenraad *et al.*, 1971). Hepes and Mes were obtained from Calbiochem. Sodium salts of solutions were used throughout.

Calculations and Data Processing. The kinetic analysis of CPS was carried out using initial velocity data obtained from time-progress studies. In those experiments which were too unwieldy to enable determinations at a number of different times, preliminary experiments were conducted to ensure that the reaction rates were linear during the time of incubation. In all experiments, less than 10% of the limiting substrate was consumed and a minimum of 500 cpm was recovered in the product. Data were analyzed as described by Porter *et al.* (1969), using an IBM 360/50 time-sharing system with on-line communication and programs written in the PL/ACME language. Least-squares fits were obtained by using the appropriate velocity equations which included

$$v = \frac{V(S)}{K + (S)} \quad (1)$$

$$v = \frac{V(S^2)}{a + b(S) + (S^2)} \quad (2)$$

$$v = \frac{V((S^2) + c(S))}{a + b(S) + (S^2)} \quad (3)$$

* From the Department of Pediatrics, Division of Developmental Biology, Stanford University Medical School, Stanford, California 94305. *Received April 28, 1971.* Aided by grants from the National Foundation-March of Dimes and National Institutes of Health Grants HD-00391 and HD-02147 from the U. S. Public Health Service. R. L. L. is a trainee of the Medical Scientist Training Program under National Institutes of Health Grant GM-1922. Presented in part at annual meeting of American Society of Biological Chemists, San Francisco, Calif., June 1971.

[1] Abbreviations used are: CPS, carbamoyl phosphate synthetase; ATC, aspartate transcarbamoylase; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

[2] At present the following substrate concentrations are being used in the standard assay: glutamine, 1.0 mM; bicarbonate, 10 mM; ATP, 7.5 mM.

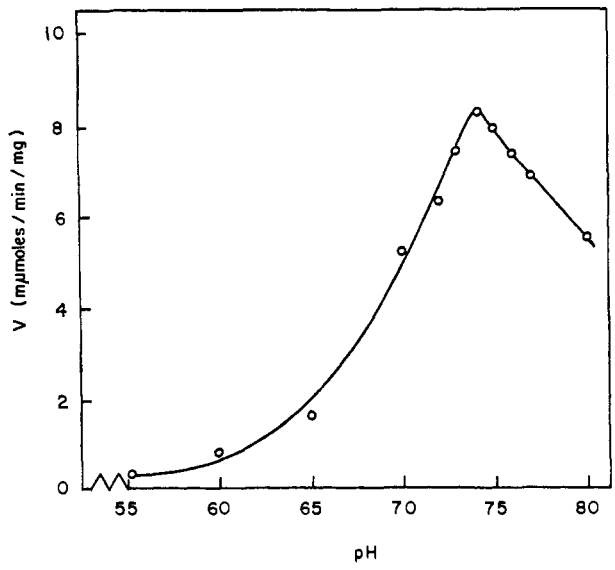

FIGURE 1: pH profile for CPS. The standard assay was employed with 50 mM buffer. The buffers were Mes (pH 5.5–6.5) and Hepes (pH 7.0–8.0). The pH values shown in the figure are those of the complete reaction mixture. Each assay contained 27 μg of protein.

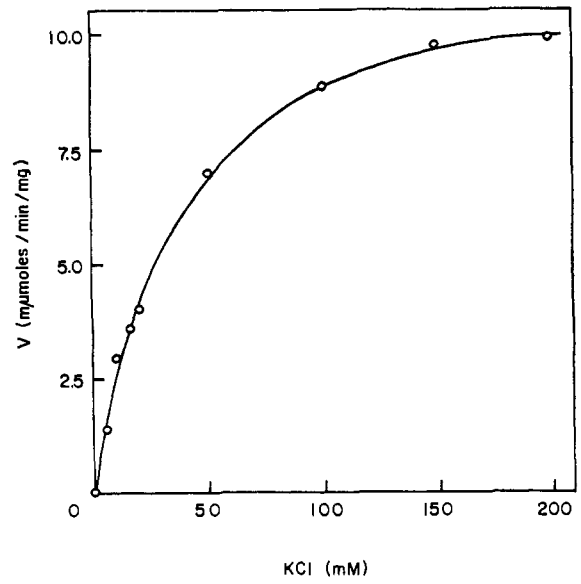

FIGURE 2: Effect of KCl on activity of CPS. Enzyme (27 μg of protein) was incubated with increasing concentrations of KCl and the standard assay was used.

$$v = \frac{V((S^2) + c(S))}{a + b(S) + (S^2)(1 + (S)/K_i)} \quad (4)$$

where v = velocity, V = maximal velocity, K = Michaelis constant, (S) = substrate concentration, a, b, and c are constants, and K_i is the inhibitor constant of substrate (S). Equation 1 describes the usual Michaelis–Menten kinetics; eq 2–4 represent sigmoidal curves; eq 3 and 4 are 2:1 functions (Cleland, 1963), and 4 includes a term for substrate inhibition. With ATP as the variable substrate, the initial velocity data fitted most accurately to eq 4. However, when K_i and (S) are large, eq 4 becomes:

$$(S^2)/v = a/V + (S^2)/V \quad (5)$$

so that a plot of $(S^2)/v$ *vs.* (S^2) gives a line of slope $1/V$ and intercept K_m^2/V.

Determination of Kinetic Parameters. When either glutamine or bicarbonate was the variable substrate, the data were fitted to eq 1. When the concentration of ATP was varied, eq 5 was used. The true K_m and V were estimated by successive extrapolation of each substrate to infinite concentration after the velocity data were fitted to either eq 1 or 5. No correction was made for small amounts of endogenous bicarbonate (Tatibana and Ito, 1969).

Feedback Inhibition Studies of CPS. In experiments where nucleotides were added, Mg^{2+} was added to the incubation medium with the nucleotides to maintain the ratio of magnesium to nucleotide at 1.5.

Results

pH Profile of CPS. Purified CPS has a single, sharp optimum at pH 7.4 (Figure 1). Consequently, all CPS assays were performed at pH 7.4.

Effect of Potassium Ion on CPS Activity. Potassium markedly stimulated CPS (Figure 2) while sodium was without effect. The curve was obtained by fitting the data to a hyperbola (eq 1).

Effect of Magnesium:ATP Ratio on CPS Activity. The requirement of magnesium and ATP for CPS activity is shown in Figure 3. Magnesium was varied at a fixed concentration of ATP (25 mM). Maximal activity was obtained when the ratio of Mg:ATP was between 1.2 and 1.5 with a peak at 1.4.

In view of the known binding of potassium to ATP (Rechnitz and Mohan, 1970) the possibility that potassium stimulates CPS activity by binding ATP and releasing magnesium

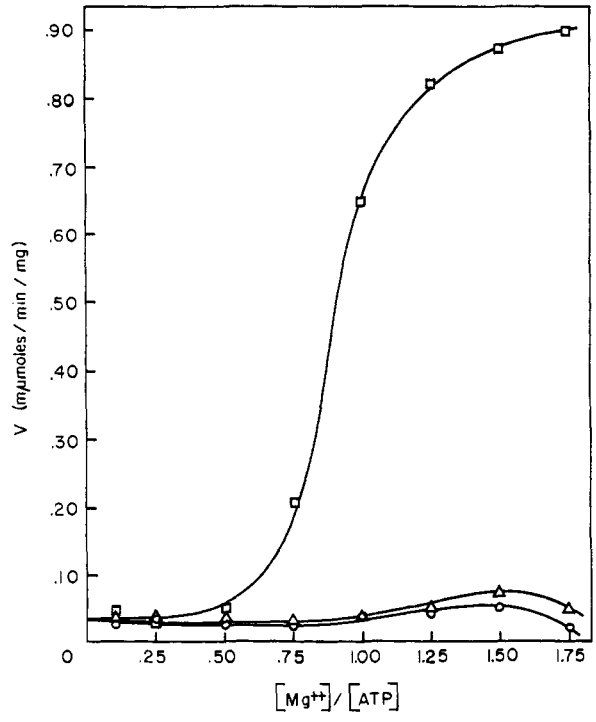

FIGURE 3: Effect of monovalent ion on CPS activity. Magnesium sulfate was varied while total ATP was held constant at 25 mM. Enzyme (27 μg of protein) was determined by the standard assay except that the bicarbonate concentration was 0.25 mM. No additions (△); +30 mM NaCl (○); +30 mM KCl (□).

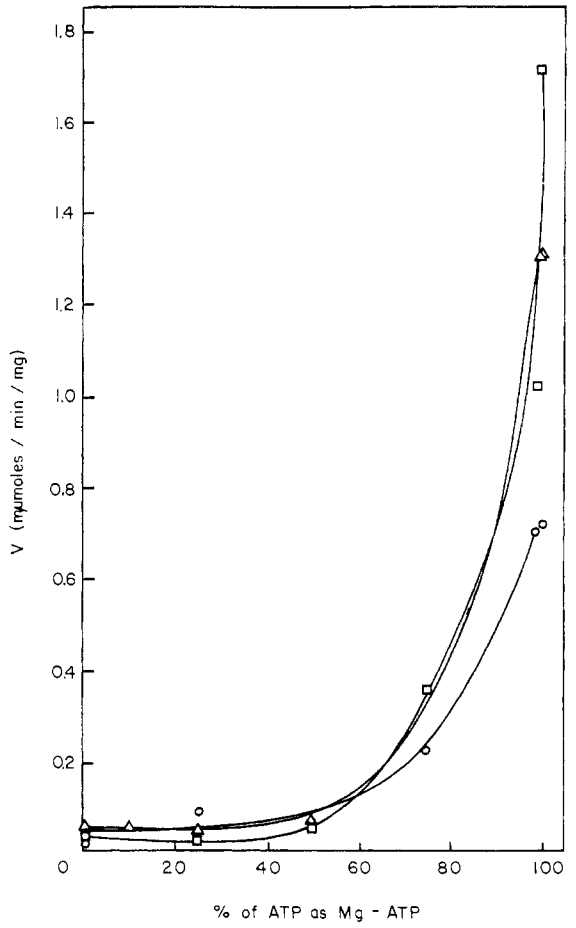

FIGURE 4: Effect of varying Mg–ATP on activity of CPS. Magnesium chloride was varied while total ATP was held constant at 2.5 mM (○), 12.5 mM (△), or 25 mM (□). The concentration of Mg–ATP was calculated by assuming a dissociation constant of 10^{-5} M for Mg–ATP (Dixon and Webb, 1964). Enzyme (14 μg of protein) was determined with the standard assay except that the bicarbonate concentration was 0.25 mM.

TABLE I: Effect of Nucleotides on CPS Activity.[a]

Nucleotide	% of Control Act.
None	100
UTP	8
dUTP	49
UMP	85
dUMP	110
CTP	47
dCTP	62
CMP	57
dCMP	56
TTP	106
TMP	124
GTP	81
GMP	102
AMP	90
3′5′-cAMP	87

[a] The CPS assay was carried out with 1 mM ATP, 0.25 mM HCO_3^-, and 1.25 mM glutamine. Nucleotides (1 mM) were added with magnesium (20 mM). One-hundred per cent CPS activity is equal to 0.34 nmole/min per mg of protein.

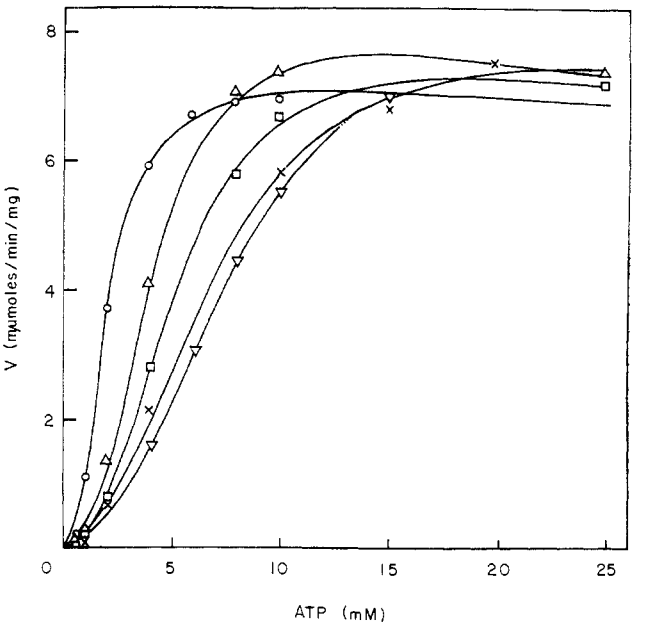

FIGURE 5: Effect of UTP on activity of CPS at varying concentrations of ATP. Enzyme (25 μg of protein) was determined with the standard assay except that the concentration of ATP was varied as shown. The concentration of glutamine was 1.0 mM and that of bicarbonate was 10.0 mM. UTP was added to a final concentration of none (○), 1 mM (△), 2 mM (□), 3 mM (×), or 4 mM (▽). The curves were constructed by fitting the data to eq 4.

was investigated. The presence of potassium ions had virtually no effect on the Mg:ATP ratio at which optimal CPS activity is obtained, whereas substitution of sodium for potassium ions resulted in practically no measurable CPS activity (Figure 3). Thus it is likely that stimulation of CPS by potassium involves mechansisms other than a simple interaction with ATP.

Magnesium concentration was varied at three different fixed concentrations of ATP (2.5, 12.5, and 25 mM) and the per cent of ATP present as the complex, Mg–ATP, was plotted against activity of CPS (Figure 4). There was a sharp increase in the activity of CPS when about 60% of the ATP was complexed with magnesium and this increase was independent of the total amount of ATP present in the incubation. Inhibition of the enzyme by magnesium (not shown in Figure 4) did not occur until Mg–ATP accounted for more than 99% of the total ATP.

Effect of Nucelotides on CPS Activity. Table I shows that that the most effective inhibitor of CPS is UTP. Cytidine nucleotides also inhibit the activity of the enzyme between 40 and 60%.

Kinetics of Inhibition of CPS by UTP. A plot of CPS activity *vs.* ATP concentration gave a sigmoid curve (Figure 5). This sigmoidality is consistent with an allosteric reaction, although it could result from the known stoichiometry of the CPS reaction, which requires the cleavage of two molecules of ATP for each molecule of carbamoyl phosphate produced (Anderson and Meister, 1965; Tatibana and Ito, 1969). Increasing the concentration of UTP increased this sigmoidality but did not affect the maximal velocity. The inhibition was therefore competitive with respect to ATP. Preliminary experiments indicated that the inhibition was noncompetitive with respect to bicarbonate and uncompetitive with respect to glutamine.

Choice of Equation for Fitting Initial Velocity Data. Since the initial velocity data with ATP as the variable substrate de-

TABLE II: Dependence of Kinetic Coefficients on Concentration of UTP.[a]

UTP (mM)	App K_m for ATP (mM)	App V (nmoles/min per mg)	a	b	c
0	2.1	7.9	3.30	−0.193	−0.350
1	3.8	7.9	23.3	−2.34	−0.0388
2	5.0	7.9	37.9	−2.57	−0.0472
3	6.4	7.9	89.3	−3.01	2.25
4	7.1	7.9	115.0	−5.42	1.90

[a] The data shown in Figure 5 were fitted to eq 4, giving the coefficients shown above. K_i for ATP as inhibitor = 219 mM. The apparent K_m were calculated using eq 6.

TABLE III: Estimates of True K_m and Maximal Velocity for CPS.[a]

Substrate	K_m (mM)	Max. Velocity (nmoles/min per mg)
ATP	3.2	15
Glutamine	0.05	15
Bicarbonate	11.0	15

[a] Initial velocity data were obtained using the standard assay medium which contains 7.5% (w/v) dimethyl sulfoxide, 2.5% (w/v) glycerol, Hepes buffer pH 7.4 (50 mM), K^+ (30 mM), and Mg^{2+} added to give a Mg–ATP ratio of 1.5, with the following combinations of substrate concentrations (millimolar): ATP, 1.67, 2.00, 2.50, 3.33, 5.00; glutamine: 0.0100, 0.0146, 0.0250, 0.100; bicarbonate: 5.00, 6.67, 10.0, 20.0. Data were processed as described in the Experimental Section.

scribed a sigmoidal curve, they were first fitted to eq 2. Fitting the data to a 2:1 function (eq 3) gave a better fit, and this was further improved by allowing for the inhibition by ATP (eq 4). The extent to which ATP inhibits the enzyme depends on the concentrations of the other substrates. At the concentrations of the standard assay (glutamine, 1.0 mM; bicarbonate, 10 mM), inhibition was observed at ATP concentrations as low as 7.5 mM. Thus all curves shown in Figure 5 were obtained by fitting the data to eq 4. The various coefficients which were calculated are shown in Table II. The K_m for a reaction described by eq 4 is given by

$$K_m = \frac{b - 2c + \sqrt{(b - 2c)^2 + 4a}}{2} \quad (6)$$

The coefficients b and c are relatively small and do not vary greatly with UTP concentration. However, the coefficient a does vary and becomes large as the concentration of UTP increases. Thus the value of the apparent K_m is mainly dependent on the value of coefficient a. When coefficient a was plotted against concentration of UTP, a straight line was obtained from which a K_i was calculated as 0.11 mM.

Estimation of Kinetic Parameters. The true K_m and V_{max} for each of the substrates of CPS were determined by varying one substrate at different fixed concentrations of the other substrates. When glutamine and bicarbonate were the variable substrates, initial velocity data were fitted to eq 1, and when ATP was the variable substrate the data were fitted to eq 5. The results are summarized in Table III.

Effect of Nucleotides on the Thermal Stability of CPS. The effect of UTP as a stabilizer of mouse spleen CPS was measured by incubating enzyme at 0 or 37° in the presence or absence of 10 mM UTP. Figure 6 shows that the UTP substantially stabilized the CPS and that the loss of activity of CPS in the presence or absence of UTP follows first-order kinetics.

ATP was more effective than UTP in preventing thermal inactivation of CPS (Table IV). When the concentration of each nucleotide was 0.5 mM or greater, UTP interfered with the protective action of ATP.

Discussion

The glutamine-dependent CPS from mouse spleen shares certain properties with enzyme obtained from other species. The pH optimum of CPS was 7.4, the same as that reported for CPS derived from Ehrlich ascites tumor and fetal rat liver (Hager and Jones, 1967a,b) and blowfly (Kameyama and Miura, 1970) and compares to a pH optimum of 7.5 reported for the enzyme from Walker carcinosarcoma 256 (Yip and Knox, 1970).

The Mg:ATP ratio required for optimal activity of mouse spleen CPS was found to be greater than 1. This result was similar to that obtained by O'Neal and Naylor (1969) using CPS from pea, who suggested the possibility that both free magnesium and the Mg–ATP complex were bound to the enzyme and required for activity, while free ATP was an inhibitor.

Potassium ions markedly stimulated CPS from mouse spleen, whereas sodium ions were without effect. This property was also observed with CPS from *E. coli* and pea (Anderson and Meister, 1966a; O'Neal and Naylor, 1969). In general, the mechanism by which potassium stimulates enzymes is not

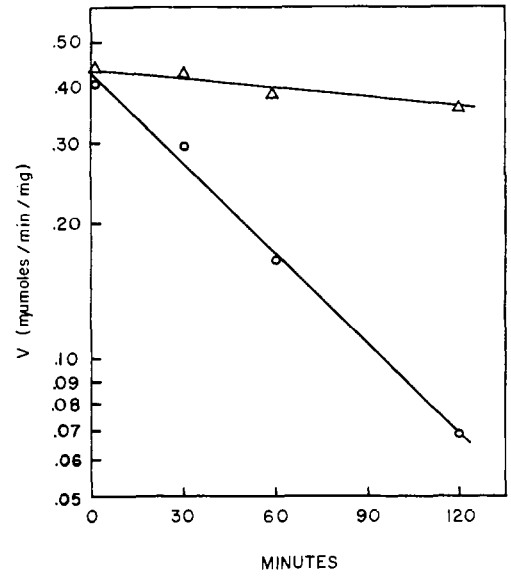

FIGURE 6: Stabilization of CPS by UTP. Enzyme was held at 37° in the presence (△) or absence (○) of 10 mM UTP as described in Table IV.

TABLE IV: Effect of ATP, UTP, and ATP + UTP on the Stability of CPS at 37°.[a]

Concn of Each Nucleotide (mM)	ATP		UTP		ATP + UTP	
	Half-Life (hr)	Stabilizing Factor[b]	Half-Life (hr)	Stabilizing Factor[b]	Half-Life (hr)	Stabilizing Factor[b]
0	0.52	1.0	0.52	1.0	0.52	1.0
0.1	4.45	8.6	2.09	4.0	10.24	19.7
0.5	15.98	30.7	2.67	5.1	9.29	17.9
1.0	17.10	32.9	3.73	7.2	8.83	17.0
10.0	22.54	43.4	3.27	6.3	5.20	10.0

[a] To 0.24 ml of thawed enzyme was added 0.06 ml of magnesium nucleotide (ratio of 1.5) sufficient to give a final concentration of nucleotide as shown. The protein concentration was 0.45 mg/ml. Tubes were incubated at 37° and 0.05 ml was withdrawn for assay at 0, 0.75, 1.5, 3, and 4 hr. The standard assay was employed except that UTP was added to make the final concentration 0.625 mM, ATP was added to make the final concentration 10 mM, and glutamine was 1.0 mM. Under these conditions UTP did not inhibit the enzyme. [b] Stabilizing factor = half-life with nucleotides/half-life without nucleotide.

known (Suelter, 1970), although it has been suggested that the potassium–ATP complex has a function in phosphoryl-transfer reactions (Lowenstein, 1960). Further, Melchior (1965) noted that the effect of magnesium on the velocity of the pyruvate kinase reaction is dependent on the concentration of potassium.

Because the velocity equation with respect to ATP is complex (eq 4), many data points are required to construct an accurately fit curve. With three substrates, more than 200 initial velocities at varying concentrations of the substrates would be required to accurately determine true K_m. By using eq 5 instead of 4, when ATP is the variable substrate, an accurate fit was obtained with only 80 determinations. To compare the two equations, apparent K_m were obtained by varying ATP at fixed concentrations of bicarbonate and glutamine. When the initial velocity data were processed using eq 5, the K_m was 2.29; when eq 4 was used, the K_m was 2.24. Subsequently, the true K_m for ATP was obtained at a range of concentrations of bicarbonate and glutamine using eq 5 (Table III).

UTP was the most effective inhibitor of mouse CPS (Table I) and protected the enzyme against thermal inactivation. However, the substrate ATP was more effective than UTP in stabilizing CPS. Hager and Jones (1967a,b) had previously found that high concentrations of Mg–ATP stabilized crude preparations of mammalian CPS, and Hoogenraad *et al.* (1971) subsequently used Mg–ATP to stabilize the enzyme during sucrose gradient centrifugation.

The curve representing velocity of CPS *vs.* ATP concentration is sigmoidal (Figure 5). Sigmoidal kinetics have also been reported for CPS from Ehrlich ascites cells (Hager and Jones, 1967a), fetal rat liver (Hager and Jones, 1967b), and Walker carcinosarcoma 256 (Yip and Knox, 1970). The sigmoidality observed in Figure 5 was increased by UTP. This curve is similar to that obtained for the feedback inhibition of *E. coli* ATC by CTP, where subunit interactions are responsible for the sigmoidal concentration curves for both aspartate (Gerhart and Pardee, 1962) and carbamoyl phosphate (Bethell *et al.*, 1968). The finding of a sigmoidal curve suggests that CPS is an allosteric enzyme. However, sigmoidal kinetics may simply result from the stoichiometric requirement of 2 moles of ATP/mole of carbamoyl phosphate synthetized (Anderson and Meister, 1966a; Tatibana and Ito, 1969). Many allosteric enzymes have been shown to have activators as well as inhibitors (Stadtman, 1966). The possibility exists that ATP may act both as a substrate and an activator, particularly since the ATP substrate curve fits a 2:1 function rather than a 2:0 function. Regardless of whether the interactions of ATP and UTP with CPS are allosteric, the relative concentrations of these two metabolites are important in the regulation of CPS activity. The K_m for glutamine was about 5×10^{-2} mM while that for bicarbonate was about 10 mM so that *in vivo* the enzyme would be essentially saturated with both substrates. In contrast, the true K_m for ATP was 3 mM, which was approximately equal to the concentration usually found in the cell, so that fluctuations in cellular ATP concentration could be important in the physiological control of CPS activity. Since UTP inhibition was competitive with respect to ATP, an increase in concentration of pyrimidines would inhibit *de novo* pyrimidine synthesis while an increase in the concentration of the purine nucleotide, ATP, would relieve the inhibition. A similar reciprocal control of pyrimidine biosynthesis by purine and pyrimidine nucleotides was proposed for CPS from *E. coli* by Anderson and Meister (1966b). It was suggested by Yip and Knox (1970) that regulation of CPS by nucleotides is likely to be less important than the actual amount of the enzyme in specific tissues. However, the K_i for UTP with respect to coefficient a obtained in the present study (0.11 mM) suggests that UTP may be a physiologic inhibitor of CPS. It is probable that both the amount of CPS and its control by nucleotides are significant in the regulation of CPS in mammals, as they are in *E. coli*.

In addition to the *de novo* pathway, biosynthesis of pyrimidine nucleotides may proceed by the utilization of preformed pyrimidine bases in a "salvage" pathway. It is difficult to assess the relative importance of the two pathways *in vivo* (Sköld, 1960; Galofré and Kretchmer, 1970). In Ehrlich ascites cells, the salvage pathway may be of major importance (Fridland and Scholefield, 1969) while in mouse spleen the *de novo* pathway may be quantitatively more important (Sköld, 1960).

In vivo evidence suggests that *de novo* biosynthesis of pyrimidine nucleotides is physiologically significant in humans. The hereditary disease, orotic aciduria, results from blocks in the *de novo* pyrimidine pathway at orotidylic pyrophosphorylase (EC 2.4.2.10) and orotidylic decarboxylase (EC 4.1.1.23) and is characterized by urinary excretion of orotic acid and also megaloblastic anemia. The anemia results from impaired DNA

synthesis in erythrocyte precursors (Wintrobe and Lee, 1970). The megaloblastic anemia and the orotic aciduria can be corrected by oral administration of uridine (Smith *et al.*, 1966). Presumably, the additional uridine is converted to UTP, which then inhibits CPS, thereby preventing the overproduction of orotic acid. Uridine probably relieves the megaloblastic anemia by serving as a source of pyrimidine nucleotides for nucleic acid biosynthesis. Consequently, in orotic aciduria the salvage pathway cannot completely compensate for the decreased *de novo* pyrimidine biosynthesis.

Another clinical observation provides additional evidence for the significance of the *de novo* pathway; in man, the turnover of purines estimated from the rate of uric acid synthesis is almost equal to the rate of *de novo* pyrimidine synthesis (Weissman *et al.*, 1962; Seegmiller *et al.*, 1961). Thus the *de novo* pathway could provide all of the pyrimidines required by man.

Since the *de novo* pathway appears to be quantitatively important in providing pyrimidines for nucleic acid synthesis, it is possible that the modulation of CPS activity by UTP and ATP is critical in the control of cellular proliferation.

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