Bicarbonate-Dependent Cleavage of Adenosine Triphosphate and Other Reactions Catalyzed by *Escherichia coli* Carbamyl Phosphate Synthetase*

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ABSTRACT: Purified carbamyl phosphate synthetase from *Escherichia coli*, which catalyzes the synthesis of carbamyl phosphate from bicarbonate, glutamine (or ammonia), and adenosine 5′-triphosphate (ATP), also catalyzes the following reactions: (1) bicarbonate-dependent cleavage of ATP to adenosine 5′-diphosphate (ADP) and inorganic phosphate (P_i); (2) stoichiometric formation of ATP and ammonia from carbamyl phosphate and ADP; and (3) ATP- and bicarbonate-dependent hydrolysis of γ-glutamyl hydroxamate not associated with stoichiometric formation of ADP. Potassium ions are required for all of the reactions catalyzed by the enzyme.

The present findings are consistent with the mechanism previously proposed according to which ATP reacts with bicarbonate to give enzyme-bound carbonate phosphate anhydride; this reacts with glutamine to yield enzyme-bound carbamate, which is phosphorylated by reaction with a second molecule of ATP to give carbamyl phosphate.

The authors reported previously that highly purified carbamyl phosphate synthetase from *Escherichia coli* catalyzes the reaction[1]

$$2ATP + \text{glutamine} + HCO_3^- + H_2O \longrightarrow 2\,ADP + \text{carbamyl phosphate} + P_i + \text{glutamate} \quad (1)$$

in which synthesis of one molecule of carbamyl phosphate is associated with cleavage of two molecules of ATP to ADP (Anderson and Meister, 1965a). The formation of carbamyl phosphate occurs more slowly when glutamine is replaced by ammonia, but the stoichiometry of the reaction is the same. Pulse-labeling experiments and other studies suggested that the synthesis of carbamyl phosphate takes place in at least three steps (Anderson and Meister, 1965b)

$$\text{enzyme} + ATP + HCO_3^- \rightleftharpoons \text{enzyme}\text{–}[CO_3PO_3^{3-}] + ADP \quad (2)$$

$$\text{enzyme}\text{–}[CO_3PO_3^{3-}] + \text{glutamine} + H_2O \longrightarrow \text{enzyme}\text{–}[NH_2CO_2^-] + \text{glutamate} + P_i \quad (3)$$

$$\text{enzyme}\text{–}[NH_2CO_2^-] + ATP \rightleftharpoons \text{enzyme} + NH_2\text{-}COOPO_3^{2-} + ADP \quad (4)$$

The present communication offers additional evidence for this sequence of reactions. Thus, the enzyme catalyzes a bicarbonate-dependent cleavage of ATP to ADP in the absence of added glutamine or ammonia. In addition, the enzyme can catalyze the synthesis of 1 mole of ATP from 1 mole each of carbamyl phosphate and ADP, a reaction that is the reversal of the third step (reaction 4) in the sequence of reactions given above. The enzyme also catalyzes ATP- and bicarbonate-dependent hydrolysis of γ-glutamyl hydroxamate not associated with stoichiometric formation of ADP. Several other characteristics of the enzyme are also described in this report; notable is the requirement for potassium ions. Significant similarities and differences between the present enzyme and vertebrate liver carbamyl phosphate synthetase (Cohen, 1962) are summarized here.

Experimental Section

Materials. ATP, ADP, lithium carbamyl phosphate, TPN, phosphoenolpyruvate, rabbit muscle pyruvate kinase [crystalline suspension in $(NH_4)_2SO_4$], rabbit muscle lactate dehydrogenase [crystalline suspension in $(NH_4)_2SO_4$], yeast hexokinase [crystalline suspension in $(NH_4)_2SO_4$], glucose 6-phosphate dehydrogenase (type V), and Tris were obtained from the Sigma Chemical Co.; γ-glutamyl hydroxamate and glutamine were obtained from General Biochemicals and Mann Research Laboratories, respectively; [^{14}C]Na_2CO_3 and [^{14}C]KCNO were obtained from New England Nuclear Corp.,; DPNH and bovine serum albumin were

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[1] Abbreviations used in this work: ATP, adenosine 5′-triphosphate; ADP, adenosine 5-′diphosphate; ATPase, adenosine 5′-triphosphatase; TPN, triphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide.

obtained from Calbiochem; [^{14}C]carbamyl phosphate (dilithium salt) was prepared from [^{14}C]KCNO by the method of Spector *et al.* (1957).

The purified enzyme preparation was obtained as previously described (Anderson and Meister, 1965b). The enzyme was stored at −20° in a solution containing 0.15 M potassium phosphate, pH 6.8, and 5×10^{-4} M EDTA. The frozen sample was rapidly thawed just prior to use and was either diluted for use with phosphate buffer or passed through a column of Sephadex G-25 equilibrated with the desired buffer.

Methods. DETERMINATION OF THE VARIOUS ENZYMATIC ACTIVITIES. The rate of the over-all reaction (synthesis of carbamyl phosphate) was determined by measuring the amount of [^{14}C]carbamyl phosphate formed from [^{14}C]$NaHCO_3$ after incubation for 10 min at 37° as previously described (Anderson and Meister, 1965b); the [^{14}C]urea formed from [^{14}C]carbamyl phosphate was eluted from a 6-ml column of Dowex 1-X8 with 11 ml of H_2O, and 1-ml aliquots of the eluate were counted in a Nuclear-Chicago liquid scintillation spectrometer. The rate of ADP formation was measured as follows. The reaction was stopped by adding 0.1 ml of 1 N HCl/0.3 ml of reaction mixture. After standing for 10 min at 0° the mixture was neutralized by addition of 0.1 ml of 1 M Tris. The ADP concentration was then determined by adding an appropriate volume (2–5 ml) of a solution containing 0.001 M phosphoenolpyruvate, 0.0002 M DPNH, 0.02 M $MgSO_4$, 0.05 M KCl, pyruvate kinase (1 unit/ml), lactate dehydrogenase (2 units/ml), and 0.15 M Tris–HCl buffer, pH 7.6, and measuring the net decrease in absorbance at 340 mμ in a Beckman DU spectrophotometer.

ATPase activity was determined by measuring the ADP formed after 10 min at 37° as described above. In these studies the samples were centrifuged at 15,000*g* for 15 min before measuring the absorbance at 340 mμ.

The enzymatic synthesis of ATP from ADP and carbamyl phosphate was carried out in reaction mixtures of final volume 0.3 ml at 37°. The reaction was stopped after 10 min by adding 0.1 ml of 1 N HCl, and the acidified solution was placed at 0° and neutralized after 10 min by addition of 0.1 ml of 1 M Tris. The ATP concentration was then determined by adding an appropriate volume (1.5–2.5 ml) of a solution containing 0.02 M glucose, 0.002 M $MgCl_2$, 0.0004 M TPN, bovine serum albumin (5 mg/ml), hexokinase (0.5 unit/ml), glucose 6-phosphate dehydrogenase (0.4 unit/ml), and 0.1 M glycylglycine buffer, pH 7.6, and measuring the net increase in absorbance at 340 mμ in a Beckman DU spectrophotometer after centrifugation at 10,000*g* for 15 min.

The rate of hydrolysis of γ-glutamyl hydroxamate was determined by measuring the decrease in absorbance at 535 mμ of the supernatant solution after addition of 0.8 ml of $FeCl_3$ reagent to 0.2 ml of reaction mixture followed by centrifugation (Pamiljans *et al.*, 1962).

Other Methods. Inorganic phosphate was determined by the method of Fiske and Subbarow (1925). The concentration of enzyme was obtained from its absorbance at 280 mμ (Layne, 1957).

The buffers and other solutions used in the studies on the effect of bicarbonate concentration on the rates of the reactions were prepared just prior to the experiments. When the data from these experiments were plotted by the method of Lineweaver and Burk (1934) a straight line was obtained for bicarbonate concentrations of 0.001 M and greater. The endogenous bicarbonate concentration was estimated by extrapolating this line to the reciprocal of the rate obtained in the absence of added bicarbonate.

Results

Bicarbonate-Dependent ATPase. When catalytic amounts of the enzyme were incubated with ATP there was a slow cleavage of ATP to ADP and inorganic phosphate; this reaction was accelerated considerably by addition of bicarbonate. Data on the cleavage of ATP in the presence and absence of added bicarbonate are given in Table I. The effect of bicarbonate concentration on the cleavage of ATP is described in Figure 1; the data lead to an apparent K_m value of 0.001 M. The maximal rate of the bicarbonate-dependent ATPase is about 10% of the maximal rate of carbamyl phosphate synthesis. Figure 2 describes the effect of

TABLE I: Cleavage of ATP in the Presence and Absence of Bicarbonate.[a]

Time (min)	ADP Formed (μmoles)	P_i Formed (μmoles)
	No $NaHCO_3$ Added	
5	0.08	0.10
10	0.25	0.25
20	0.50	0.45
	$NaHCO_3$ Added	
5	0.25	0.25
10	0.71	0.66
20	1.50	1.40

[a] The reaction mixtures contained ATP (6 μmoles), $MgCl_2$ (6 μmoles), KCl (60 μmoles), Tris–HCl buffer (30 μmoles, pH 8.2), enzyme (0.13 mg), and $NaHCO_3$ (12 μmoles) in a final volume of 0.6 ml. The reaction was initiated by adding enzyme to the appropriate mixture at 0°. The complete reaction mixture was then warmed to 37° and maintained at that temperature. At the indicated intervals an aliquot was removed for P_i and ADP determinations, which were carried out as described in the text. Analyses were also performed on aliquots removed from the cold reaction mixtures immediately after adding enzyme.

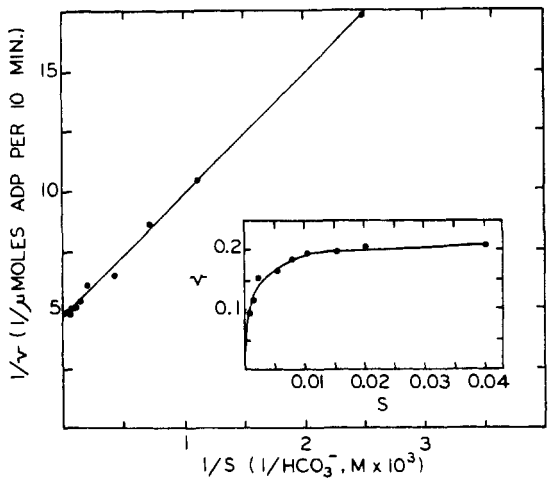

FIGURE 1: Effect of bicarbonate concentration on cleavage of ATP. The reaction mixtures contained ATP (3 μmoles), $MgCl_2$ (3 μmoles), potassium phosphate buffer (17 μmoles, pH 7.8), enzyme (0.12 mg), and $NaHCO_3$ as indicated in a final volume of 0.3 ml. The ADP formed after incubation for 10 min at 37° was determined as described in the text. The values for bicarbonate concentration were corrected for an estimated endogenous bicarbonate concentration of 0.0004 M.

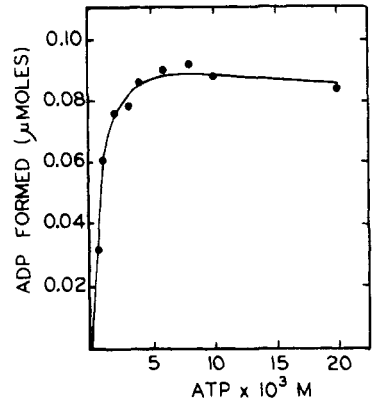

FIGURE 2: Effect of ATP concentration on bicarbonate-dependent ATPase. The reaction mixtures contained $MgCl_2$ (equimolar with ATP), $KHCO_3$ (30 μmoles), Tris–HCl (30 μmoles, pH 8.2), enzyme (0.015 mg), and ATP as indicated in a final volume of 0.3 ml. The ADP formed after incubation for 10 min at 37° was determined as described in the text.

ATP concentration on bicarbonate-dependent ATPase; the apparent K_m value is 0.0007 M. It is of interest that the concentration of ATP necessary for half-maximal velocity of the over-all carbamyl phosphate synthesis reaction is about ten times greater than this value (see below). The ratio of the values for the bicarbonate-dependent ATPase and the over-all synthesis reaction was the same in the various fractions of the effluent of the Sephadex G-200 column used in the last step of purification of the enzyme (Anderson and Meister, 1965b).

Synthesis of ATP from Carbamyl Phosphate and ADP. When the enzyme was incubated with ADP and carbamyl phosphate there was stoichiometric formation of ATP and ammonia (Table II). Presumably the reaction studied here leads initially to the formation of enzyme-bound carbamate, which reacts with water to yield ammonia and bicarbonate. Only 1 mole of ATP was formed per mole of ammonia produced and per mole of carbamyl phosphate utilized. The apparent decrease in ATP formation with time may be ascribed to the ATP-cleavage reaction described above. These findings indicate that a partial reversal of the over-all reaction occurs rather than complete reversal, which would be accompanied by synthesis of 2 moles of ATP/mole of carbamyl phosphate utilized. The initial rate of this reaction is approximately 14% of that of the over-all rate of carbamyl phosphate synthesis. Addition of glutamate (0.005 M) did not affect the rate of ATP

TABLE II: Stoichiometry of ATP Synthesis from Carbamyl Phosphate and ADP.[a]

Time (min)	ATP Formed (μmoles)	Ammonia Formed (μmoles)	[^{14}C]-Carbamyl Phosphate Utilized (μmoles)
2	2.3	2.1	2.4
4	4.2	3.2	4.1
6	5.4	5.3	5.4
8	6.2	6.4	6.8
12	6.4	7.4	7.6
16	5.7	8.1	8.4

[a] The reaction mixtures contained [^{14}C]carbamyl phosphate (10 μmoles, 330,000 cpm), ADP (20 μmoles), $MgCl_2$ (20 μmoles), potassium phosphate buffer (85 μmoles, pH 7.0), and enzyme (2.7 mg) in a final volume of 1.0 ml, 37°. The concentrations of [^{14}C]carbamyl phosphate and ammonia were determined at the indicated intervals by adding a 0.07-ml aliquot to a 20-ml glass vial containing 0.1 ml of 4 N KOH; the vial was immediately closed by application of a rubber stopper to which was attached a glass rod moistened with 0.02 ml of 1 N H_2SO_4 (Seligson and Seligson, 1951). After rotation on a wheel for 30 min at 26°, the ammonia was determined by immersing the rod in Nessler's reagent, and the [^{14}C]cyanate remaining in the vial was determined as described (Anderson and Meister, 1965b). The concentration of ATP was determined as described in the text after adding an 0.03-ml aliquot of the reaction mixture to 0.1 ml of 1 N HCl.

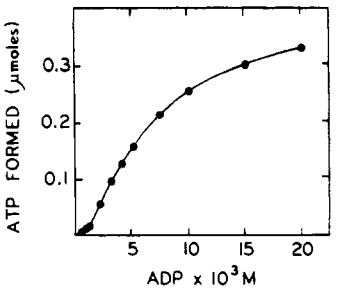

FIGURE 3: Effect of ADP concentration on the synthesis of ATP from ADP and carbamyl phosphate. The reaction mixtures contained $MgCl_2$ (equimolar with ADP), lithium carbamyl phosphate (3 μmoles), enzyme (0.036 mg), and potassium phosphate (20 μmoles, pH 7.8) in a final volume of 0.3 ml. The ATP formed after incubation for 10 min at 37° was determined as described in the text.

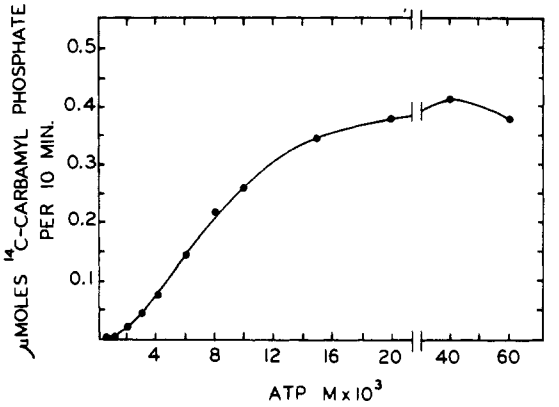

FIGURE 4: Effect of ATP concentration on the rate of the over-all synthesis reaction. The reaction mixtures contained $MgCl_2$ (equimolar with ATP), [^{14}C]$NaHCO_3$ (20 μmoles, 700,000 cpm), glutamine (10 μmoles), KCl (100 μmoles), enzyme (0.015 mg), and Tris–HCl (100 μmoles, pH 8.2) in a final volume of 1.0 ml. The [^{14}C]carbamyl phosphate synthesized after incubation for 10 min at 37° was determined as described in the text.

formation, and studies with [^{14}C]glutamate did not show detectable synthesis of [^{14}C]glutamine. The rate of the reaction was about the same in 0.05 M Tris–HCl–0.1 M KCl as in 0.1 M potassium phosphate buffer at pH 7.6.

The effect of ADP concentration on the rate of synthesis of ATP from ADP and carbamyl phosphate is described in Figure 3. The sigmoidal shape of this curve is similar to that observed in studies on the effect of ATP concentration on the over-all synthesis reaction (see below).

TABLE III: Dependence of the Hydrolysis of γ-Glutamyl Hydroxamate on ATP and Bicarbonate.

Addnl Components[a]	Concn (M × 10³)	γ-Glutamyl Hydroxamate Hydrolyzed (μmole)	ADP Formed (μmole)
ATP, $NaHCO_3$	10,10	0.39	0.06
ATP, $NaHCO_3$	1,10	0.04	—
ATP	10	0.08	—
$NaHCO_3$	10	<0.01	—
ADP, $NaHCO_3$	10,10	<0.01	—
ATP, $NaHCO_3$[b]	10,10	—	0.03
ATP, $NaHCO_3$, glutamine	10,10, 0.2	0.08	—
ATP, $NaHCO_3$, glutamine	10,10 0.5	0.01	—

[a] The reaction mixtures contained L-γ-glutamyl hydroxamate (0.8 μmole), potassium phosphate buffer (16 μmoles, pH 6.8), enzyme (6 μg), and additional components as indicated in a final volume of 0.2 ml. The disappearance of γ-glutamyl hydroxamate and the formation of ADP were determined as described in the text; incubated for 10 min at 37°. [b] γ-Glutamyl hydroxamate omitted.

Hydrolysis of γ-Glutamyl Hydroxamate. As indicated in Table III, the enzyme catalyzes the rapid hydrolysis of γ-glutamyl hydroxamate. Although this reaction is dependent upon the presence of ATP and is greatly stimulated by added bicarbonate, it is not accompanied by stoichiometric formation of ADP. The rate of this reaction is about 90% of that of carbamyl phosphate formation. The hydrolysis of γ-glutamyl hydroxamate is markedly inhibited by very low concentrations of L-glutamine. Glutamate was identified as a major product by high-voltage paper electrophoresis of the reaction mixture (Anderson and Meister, 1965b). After treatment of the paper strips with ninhydrin the glutamate was quantitated as described by Giri *et al.* (1952); these studies showed that the disappearance of γ-glutamyl hydroxamate is accompanied by stoichiometric formation of glutamate.

Studies of the Over-All Reaction (Synthesis of Carbamyl Phosphate). The effect of ATP concentration on the rate of the over-all reaction is described in Figure 4. The curve is sigmoidal and half-maximal velocity is achieved with a concentration of ATP of about 8×10^{-3} M. On the other hand, the effect of bicarbonate concentration yields a hyperbolic curve (Figure 5); when these data are plotted by the method of Lineweaver and Burk (1934), a straight line is obtained. The apparent K_m value is 0.0012 M, which is about the same as the apparent K_m value for bicarbonate in the ATPase reaction (see above). The effect of glutamine concentration is given in Figure 6; the apparent K_m value for glutamine is 3.8×10^{-4} M.

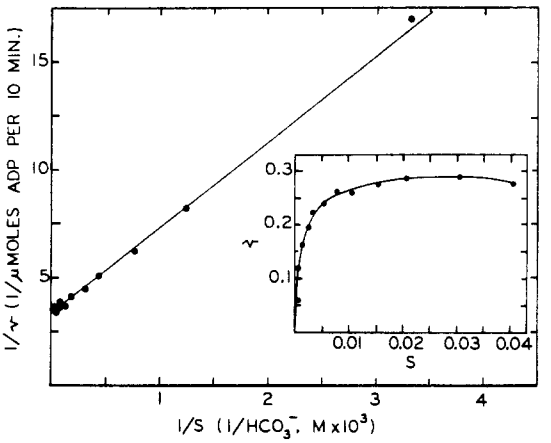

FIGURE 5: Effect of bicarbonate concentration on the rate of ADP formation in the over-all synthesis reaction. The reaction mixtures contained ATP (6 μmoles), $MgCl_2$ (6 μmoles), glutamine (3 μmoles), potassium phosphate buffer (17 μmoles, pH 7.8), enzyme (0.012 mg), and $NaHCO_3$ as indicated in a final volume of 0.3 ml. The ADP formed after incubation for 10 min at 37° was determined as described in the text. The values for bicarbonate concentration are corrected for an estimated endogenous bicarbonate concentration of 0.0003 M.

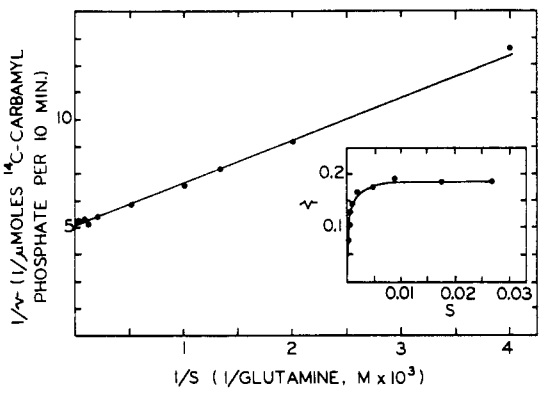

FIGURE 6: Effect of glutamine concentration on the rate of carbamyl phosphate synthesis. The reaction mixtures contained ATP (20 μmoles), $MgCl_2$ (20 μmoles), [^{14}C]-$NaHCO_3$ (20 μmoles, 700,000 cpm), Tris–HCl buffer (100 μmoles, pH 8.2), KCl (80 μmoles), enzyme (0.017 mg), and glutamine as indicated in a final volume of 1.0 ml. [^{14}C]Carbamyl phosphate formed after incubation for 10 min at 37° was determined as described in the text.

TABLE IV: Effect of Potassium Ions on the Reactions Catalyzed by Carbamyl Phosphate Synthetase.[a]

Expt	Reaction	Product Determined	Product (μmole) KCl	Product (μmole) NaCl
1	Over-all Reaction	ADP	0.23	<0.01
2	ATPase	ADP	0.08	<0.01
3	ATP synthesis	ATP	0.08	<0.01
4	Hydrolysis of γ-glutamyl hydroxamate	Glutamate	0.09	<0.01

[a] The reaction mixtures contained: expt 1, ATP (5 μmoles), $MgCl_2$ (5 μmoles), L-glutamine (3 μmoles), $NaHCO_3$ (6 μmoles), and enzyme (6 μg); expt 2, ATP (3 μmoles), $MgCl_2$ (3 μmoles), $NaHCO_3$ (6 μmoles), and enzyme (30 μg); expt 3, ADP (3 μmoles), $MgCl_2$ (3 μmoles), lithium carbamyl phosphate (3 μmoles), and enzyme (30 μg); expt 4, ATP (3 μmoles), $MgCl_2$ (3 μmoles), L-γ-glutamyl hydroxamate (0.9 μmole), $NaHCO_3$ (5 μmoles), and enzyme (6 μg). All of the reaction mixtures contained sodium phosphate buffer (30 μmoles, pH 7.8) and KCl or NaCl (30 μmoles) as indicated in a final volume of 0.3 ml. The products formed after incubation for 10 min at 37° were determined as described in the text.

The Effect of Potassium Ions. In the course of these studies it was found that potassium ions are required for maximum activity of the enzyme in all of the reactions studied. The effect on the over-all synthesis reaction is described in Figure 7; the optimal concentration was about 0.1 M. Although higher concentrations of potassium chloride were accompanied by decreased activity, the same inhibitory effect was observed with equivalent concentrations of sodium chloride in the presence of 0.1 M potassium chloride. This suggests that the inhibition observed with higher concentrations of potassium chloride probably reflects a nonspecific salt effect.

The effect of potassium ions on the synthesis of carbamyl phosphate from bicarbonate and ammonium chloride (in place of glutamine) is described in Figure 8. Relatively little carbamyl phosphate was formed with concentrations of ammonium chloride less than 0.01 M in the absence of added potassium ions. However, with increasing concentrations of ammonium chloride, the rate of synthesis of carbamyl phosphate in the absence of added potassium ions approached that in its presence suggesting that ammonium ions can replace potassium ions as an activator under these conditions. The effects of potassium ions on the other three reactions catalyzed by the enzyme are summarized in Table IV; potassium ions are required in each case.

Discussion

The present and earlier findings (Anderson and Meister, 1965a,b, 1966a) indicate that *E. coli* carbamyl phosphate synthetase differs in significant respects

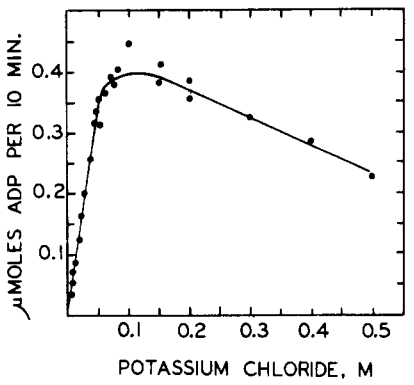

FIGURE 7: Effect of potassium ion concentration on the rate of ADP formation in the over-all synthesis reaction. The reaction mixtures contained ATP (6 μmoles), $MgCl_2$ (6 μmoles), $NaHCO_3$ (6 μmoles), L-glutamine (3 μmoles), enzyme (0.008 mg), Tris–HCl buffer (15 μmoles), and KCl as indicated in a final volume of 0.3 ml. The ADP formed after incubation for 10 min at 37° was determined as described in the text.

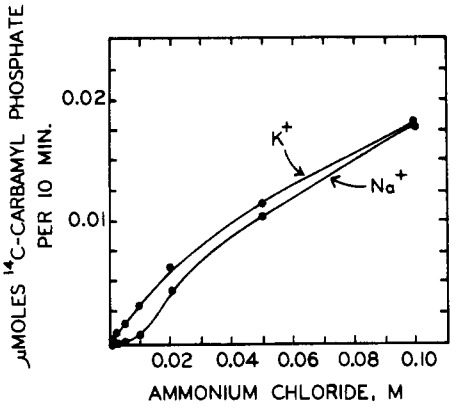

FIGURE 8: Effect of NH_4Cl concentration on the rate of carbamyl phosphate synthesis in the presence and absence of potassium ions. The reaction mixtures contained ATP (20 μmoles), $MgCl_2$ (20 μmoles), [^{14}C]$NaHCO_3$ (20 μmoles, 700,000 cpm), enzyme (0.017 mg), Tris–HCl (100 μmoles, pH 8.1), NaCl or KCl (80 μmoles), and NH_4Cl as indicated plus NaCl (total NH_4Cl + NaCl = 100 μmoles) in a final volume of 1.0 ml. The [^{14}C]carbamyl phosphate synthesized after incubation for 10 min at 37° was determined as described in the text.

from the vertebrate liver carbamyl phosphate synthetase (Cohen, 1962). For example, the enzyme from *E. coli* utilizes glutamine and, less effectively, ammonia, while the liver enzyme uses only ammonia. The liver enzyme requires *N*-acetylglutamate, which is not needed by the bacterial enzyme. In addition, the effect of ATP concentration on the over-all reaction catalyzed by the *E. coli* enzyme is described by a sigmoidal curve, an effect apparently not observed with the liver enzyme. On the other hand, the two enzymes are similar in respect to over-all stoichiometry (Anderson and Meister, 1965a,b; Kalman *et al.*, 1966), the requirement for potassium ions (which can be replaced by ammonium ions), the ability to catalyze bicarbonate-dependent cleavage of ATP, and the synthesis of ATP from ADP and carbamyl phosphate.

The present findings support the mechanism given above (eq 2–4). Thus, the requirement for bicarbonate for the ATP-cleavage activity can be explained in terms of formation of enzyme-bound carbonate phosphate anhydride, which (in the absence of glutamine or ammonia) reacts with water to yield inorganic phosphate and regenerate bicarbonate. It is notable that the effect of bicarbonate concentration on the ATPase reaction and on the over-all synthesis reaction is similar; the corresponding apparent K_m values are virtually identical. On the other hand, the effect of ATP concentration on the rates of these reactions is markedly different. The half-maximal velocity for the ATPase reaction occurs at a tenfold lower concentration of ATP than that required for half-maximal velocity of the over-all reaction. This indicates that the rate-determining step in the over-all reaction is probably not the formation of enzyme-bound carbonate phosphate anhydride. It is of interest in this connection that the various nucleotides which affect the rate of the over-all synthesis reaction do not significantly alter the rate of the ATPase reaction (Anderson and Meister, 1966b).

The ability of the enzyme to catalyze ATP formation from carbamyl phosphate and ADP (associated with the formation of ammonia) is in accord with reversal of reaction 4 and hence this observation appears to represent experimental evidence for one of the postulated partial reactions. Our findings indicate that 1 mole each of ATP and ammonia is formed per mole of carbamyl phosphate utilized, and therefore that complete reversal of the reaction has not occurred. Addition of glutamate did not accelerate the reaction nor was glutamine formation detected. Kalman *et al.* (1966) have also observed ATP synthesis from carbamyl phosphate and ADP catalyzed by *E. coli* carbamyl phosphate synthetase; although these workers concluded from their observations that the over-all reaction was reversible, they did not report studies on the stoichiometry of the reaction. Their finding that ATP synthesis was increased by addition of either glutamate or inorganic phosphate could not be repeated in our system. It seems possible that these effects are related to the fact that the studies of Kalman *et al.* were carried out with less than optimum concentrations of potassium ions. In addition, the acceleration of carbamyl phosphate synthesis by ammonia observed by Kalman *et al.* (in the presence of glutamine) may also be ascribed to the low concentration of potassium ions used; the present observations indicate that ammonium ions can replace potassium ions in activating carbamyl phosphate synthesis.

The rapid hydrolysis of γ-glutamyl hydroxamate catalyzed by the enzyme seems to reflect an aspect of the second step in the postulated sequence of reactions (reaction 3). Although the hydrolysis of this glutamine analog is dependent upon ATP and bicarbonate, the reaction is not accompanied by stoichiometric formation of ADP, indicating that the hydroxylamine moiety which is released does not react with enzyme-bound activated carbon dioxide. Other studies in our laboratory (P. M. Anderson and A. Meister, unpublished) indicate that the enzyme can react with glutamine in the absence of ATP to yield a glutamyl–enzyme–ammonia complex. The requirement for ATP and bicarbonate for the hydrolysis of γ-glutamyl hydroxamate suggests that formation of enzyme-bound carbonate phosphate anhydride (and perhaps the binding of a second molecule of ATP) may be necessary for release of the bound glutamyl moiety as free glutamate. Further studies on this aspect of the reaction will be presented in a later communication.

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