Evidence for an Activated Form of Carbon Dioxide in the Reaction Catalyzed by *Escherichia coli* Carbamyl Phosphate Synthetase*

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ABSTRACT: Carbamyl phosphate synthetase of *Escherichia coli* has been purified about 300-fold and the stoichiometry of the reaction catalyzed has been studied. The synthesis of one molecule of carbamyl phosphate from glutamine, carbon dioxide, and adenosine triphosphate (ATP) is associated with the cleavage of two molecules of ATP to adenosine diphosphate (ADP). Similar stoichiometry is obtained when glutamine is replaced by ammonia.

Evidence derived from pulse-labeling experiments and other studies is consistent with a mechanism involving at least three steps, the first of which is an ATP-dependent formation of enzyme-bound activated carbon dioxide.

The formation of carbamyl phosphate, a key intermediate in the biosynthesis of pyrimidines, arginine, and urea, is catalyzed by carbamyl phosphate synthetase of liver according to the following reaction.[1,2]

$$NH_4^+ + HCO_3^- + 2ATP \xrightarrow[N\text{-acetylglutamate}]{Mg^{2+}} NH_2COPO_3^{2-} + 2ADP + P_i \quad (1)$$

Metzenberg *et al.* (1958), who studied the enzyme purified from frog liver, have proposed that the reaction proceeds in two steps as follows.

$$ATP + CO_2 \xrightarrow{N\text{-acetylglutamate}} ADP + P_i + \text{"active } CO_2\text{"} \quad (2)$$

$$ATP + \text{"active } CO_2\text{"} + NH_4^+ \underset{}{\overset{N\text{-acetylglutamate}}{\rightleftharpoons}} NH_2COPO_3^{2-} + ADP \quad (3)$$

Carbamyl phosphate is also formed from carbon dioxide and ammonia in the reaction catalyzed by bacterial carbamate kinase. Jones and Lipmann (1960) have obtained evidence that carbamate, formed nonenzymatically according to reaction 4, is the substrate for phosphorylation (reaction 5).

$$NH_4^+ + HCO_3^- \rightleftharpoons NH_2COO^- + H_3O^+ \quad (4)$$

$$NH_2COO^- + ATP \overset{Mg^{2+}}{\rightleftharpoons} NH_2COPO_3^{2-} + ADP \quad (5)$$

The equilibrium of the reaction catalyzed by carbamate kinase favors adenosine triphosphate (ATP) synthesis; in contrast, the synthesis of carbamyl phosphate catalyzed by carbamyl phosphate synthetase (reaction 1) is essentially irreversible.

An additional reaction leading to carbamyl phosphate synthesis was discovered by Levenberg (1962), who found a glutamine-dependent carbamyl phosphate synthetase in mushrooms. This enzyme is much more active with glutamine than with ammonia. Piérard and Wiame (1964) later found a glutamine-dependent carbamyl phosphate synthetase in *Escherichia coli*. Kalman *et al.* (1965) recently described the partial purification of this enzyme and concluded that the enzyme is probably identical with carbamate kinase. It should be noted, however, that the published studies have not elucidated the stoichiometric relationships involved in the utilization of glutamine for carbamyl phosphate formation. The present studies were undertaken in an effort to clarify the stoichiometry of the glutamine-dependent reaction and also to seek information concerning the mechanism of this reaction. The studies reported here indicate that the glutamine-dependent synthesis of carbamyl phosphate catalyzed by a purified *E. coli* enzyme preparation is associated

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

[2] A valuable review of the literature of this area is available (Cohen, 1962).

with the cleavage of 2 molecules of ATP to adenosine diphosphate (ADP) and is therefore analogous to the reaction catalyzed by liver carbamyl phosphate synthetase. We also report evidence that the synthesis of carbamyl phosphate takes place in at least three steps, the first of which is the ATP-dependent formation of enzyme-bound activated carbon dioxide.

Experimental Section

Materials. L-Glutamine, phosphoenolpyruvate (trisodium salt), rabbit muscle lactate dehydrogenase, rabbit muscle pyruvate kinase, ATP (disodium salt), carbamyl phosphate (dilithium salt), and EDTA were obtained from Sigma Chemical Co. Protamine sulfate, Sephadex G-25 and G-200, and reduced diphosphopyridine nucleotide (DPNH) were obtained from Nutritional Biochemicals Corp., Pharmacia Fine Chemicals, Inc., and Calbiochem, respectively. Polyethylene glycol was purchased from City Chemical Corp. Uniformly labeled ^{14}C-L-glutamine and NaH-$^{14}CO_3$ were purchased from Schwarz Bioresearch and New England Nuclear Corp., respectively. AT^{32}P, uniformly labeled in the β and γ positions, was a gift from Dr. Abraham Novogrodsky.

Calcium phosphate gel was prepared by the method of Singer and Kearney (1950) and stored at 4° for at least 3 weeks before use; the suspension used contained about 28 mg of gel/ml. Reagent grade ammonium sulfate was recrystallized from 2×10^{-3} M EDTA. DEAE-cellulose was purchased from Brown Co. and was prepared for use according to the procedure of Peterson and Sober (1962).

Ornithine transcarbamylase was prepared from *E. coli* B (obtained as a frozen paste from Grain Processing Co.) according to the method of Rogers and Novelli (1962); the procedure was carried through the ammonium sulfate precipitation step.

Methods. Carbamyl phosphate and inorganic phosphate (P_i) were separated from ATP and ADP by paper electrophoresis in 0.05 M sodium acetate buffer, pH 5.4. Electrophoresis was carried out on Whatman 3 MM paper (25 × 96 cm) at 30 v/cm at 10° for 120 min in a Model D high-voltage electrophorator (Gilson Medical Electronics). The compounds were located on the paper by the spray reagent described by Leuthardt and Testa (1951). Under these conditions, carbamyl phosphate moved 37–39 cm, inorganic phosphate moved 29–31 cm, and ATP and ADP moved 14–24 cm toward the positively charged electrode. The radioactivity was determined after eluting the compounds from the paper with 0.005 M potassium phosphate buffer, pH 7.6.

^{14}C-Glutamate was separated from ^{14}C-glutamine by paper electrophoresis in 0.05 M sodium acetate buffer, pH 5.5. Electrophoresis was carried out on Whatman 3 MM paper (1 × 12 cm) at 30 v/cm for 50 min in a modified Durrum-type electrophoresis cell (Beckman, Model R) at room temperature. The areas containing the respective compounds were cut from the strip and counted on a planchet in a gas-flow counter.

ADP was determined from the decrease in absorbance at 340 mμ when a sample was added to a solution containing pyruvate kinase (2 units/ml), lactate dehydrogenase (4 units/ml), phosphoenolpyruvate (5×10^{-3} M), DPNH (1.5×10^{-4} M), and potassium phosphate buffer (0.1 M, pH 7.6) at 26°. Samples from reaction mixtures in which enzyme, glutamine, and NH_4Cl were separately omitted served as blanks.

Carbamyl phosphate was usually determined by conversion to urea (see below); in some cases, however, it was determined by adding the sample to a solution containing 0.01 M ornithine, 0.1 M potassium phosphate buffer, pH 7.8, and sufficient ornithine transcarbamylase to convert all of the carbamyl phosphate to citrulline in 2 min at 26°. The citrulline formed was then determined (after deproteinization with $HClO_4$) by the colorimetric procedure of Gerhart and Pardee (1962).

The protein obtained through step 5 in the purification outlined below was determined with the biuret reagent of Levin and Brauer (1951); after step 5, protein was determined from the absorbance at 280 mμ (Layne, 1957). Crystalline bovine serum albumin was used as the standard for both methods.

Determination of Enzyme Activity. The standard assay mixture contained ATP (20 μmoles), $MgCl_2$ (20 μmoles), NaH$^{14}CO_3$ (20 μmoles, 400,000 counts/min), glutamine (10 μmoles), potassium phosphate buffer (100 μmoles, pH 7.6), and sufficient enzyme to catalyze synthesis of 0.02–0.4 μmole of carbamyl phosphate in a final volume of 1.0 ml. The reaction was carried out at 37° for 10 min and stopped by adding 0.1 ml of a solution containing 0.7 N NH_4OH and 2.7 N KOH (prepared immediately prior to use). Under these conditions ^{14}C-carbamyl phosphate is quantitatively converted to ^{14}C-cyanate (Allen and Jones, 1964). After standing for an additional 10 min at 37°, the ^{14}C-cyanate was converted to ^{14}C-urea by adding 0.4 ml of 4 M NH_4Cl (pH 8.5), and heating at 100° for 10 min (Allen and Jones, 1964). The samples were applied to 6-m, columns of Dowex 1-X8, in the hydroxide form, and the ^{14}C-urea was eluted with 10 ml of H_2O. An aliquot (1 ml) was placed on a planchet containing 0.3 ml of 0.01 M $Ba(OH)_2$ and 1 drop of dilute detergent; after drying in a stream of air, the radioactivity was determined.

A unit of activity is defined as the amount of enzyme that catalyzes the synthesis of 1 μmole of carbamyl phosphate/hr. Specific activity is expressed in terms of units per milligram of protein.

Growth of Bacteria.[3] *Escherichia coli* B was grown aerobically on a minimal salt medium (Anderson, 1946) which contains NH_4Cl (2 g/l.), glucose (5 g/l.) as the major carbon source, and L-arginine (0.2 g/l.). The cells were harvested at the end of the log phase of growth by centrifugation and washed once with distilled water. The cell paste obtained after centrifugation with a

[3] The cells were grown in 580-l. batches at the New England Enzyme Center, Tufts University School of Medicine. Addition of arginine increased the amount of enzyme formed by a factor of 1.5.

TABLE 1: Purification of the Enzyme.[a]

Step	Volume (ml)	Total Protein (mg)	Total Units	Specific Activity (units/mg)[b]
1. Centrifuged extract	3300	79,000	87,000	1.1
2. Heat denaturation	2850	35,600	78,500	2.2
3. Protamine sulfate	3750	36,600	84,000	2.3
4. Ammonium sulfate precipitation	300	13,000	65,000	5.0
5. Calcium phosphate gel	3000	2,700	54,000	20
6. DEAE-cellulose	11	320	29,700	93
7. Sephadex G-200	70	69	20,000	300

[a] Experimental details are given in the text. [b] Activity values for steps 1–4 were obtained after dialysis against 0.15 M potassium phosphate buffer (pH 6.8) containing 5×10^{-4} M EDTA.

Sharples Super Centrifuge was frozen and stored at −20°.

Purification of the Enzyme. Except where indicated, all steps were carried out at 4°.

STEP 1. CENTRIFUGED EXTRACT. The cell paste (about 1 kg) was suspended in 3 l. of 0.1 M potassium phosphate buffer, pH 7.6. Aliquots (300 ml) of the suspension were sonicated with a 20 kc MSE ultrasonic disintegrator (Measuring and Scientific Equipment Co., Ltd., London) for 18 min at 8°. The sonicated mixture was centrifuged at 20,000*g* for 10 min; the precipitate was discarded.

STEP 2. HEAT DENATURATION. Sufficient glutamine was dissolved in the supernatant solution obtained in step 1 to yield a final concentration of 0.03 M. Portions of this mixture (1000 ml in 2-l. erlenmeyer flasks) were placed in a water bath at 67° and stirred vigorously until the temperature had increased to 54° (4–5 min). This temperature was maintained for 5 min; the mixture was then cooled rapidly to less than 15° by placing the flasks in an ice–water bath. The suspension was centrifuged at 10,000*g* for 25 min; the inactive precipitate was discarded.

STEP 3. PROTAMINE SULFATE. Protamine sulfate (1.5 ml of a 2% solution adjusted to pH 5.0 with 2 N KOH/4 ml of protein solution) was added with rapid stirring. The precipitate was removed by centrifugation at 10,000*g* for 25 min.

STEP 4. AMMONIUM SULFATE. EDTA (20 mg/100 ml) and ammonium sulfate (35 g/100 ml) were added to the supernatant solution with rapid stirring, which was continued for 10 min after all of the ammonium sulfate had dissolved. The suspension was centrifuged at 10,000*g* for 25 min. The supernatant solution was discarded and the precipitate was dissolved in the minimum volume (about 250 ml) of 0.15 M potassium phosphate, pH 6.8, containing 5×10^{-4} M EDTA. The purification was usually taken to this step in 1 day and then stored overnight at 4°.

STEP 5. CALCIUM PHOSPHATE GEL. The solution obtained in step 4 was applied to a column (4.8 × 56 cm) of Sephadex G-25 previously equilibrated with 0.02 M potassium phosphate buffer, pH 6.8, and then eluted with the same buffer. The protein eluate was diluted with buffer to give a solution containing 10 mg of protein/ml. Sufficient calcium phosphate gel (about 1.2 mg of gel/mg of protein) was added to adsorb 50% of the total protein. The suspension was stirred for 5 min and the precipitate was removed by centrifugation at 1500*g* for 10 min and discarded. An amount of gel equal to that added above was added to the supernatant solution and the stirring and centrifugation were repeated. The supernatant was discarded. The enzyme was eluted from the gel by four consecutive extractions with 0.05 M potassium phosphate buffer, pH 6.8. In each case the gel was suspended in a volume of the buffer equal to 0.55 the initial volume of protein solution and the suspension was stirred for 5 min before centrifuging.

STEP 6. CHROMATOGRAPHY ON DEAE-CELLULOSE. The combined extracts obtained in step 5 were applied under air pressure (10 psi) to a column (4.8 × 56 cm) of DEAE-cellulose equilibrated with 0.05 M potassium phosphate buffer, pH 6.8, containing 5×10^{-4} M EDTA. About half of the protein (including the enzyme) was adsorbed. The proteins were eluted from the column with a linear gradient of potassium phosphate buffer (pH 6.8) containing 5×10^{-4} M EDTA; the mixing vessel contained 6 l. of 0.05 M buffer and the reservoir contained 6 l. of 0.25 M buffer. The eluate was collected in 250-ml fractions at a rate of about 200 ml/hr. The peak of enzyme activity emerged with a phosphate concentration of about 0.15 M. The fractions containing most of the activity (about 1500 ml) were combined and added to 2.5 volumes of distilled water. The diluted solution was then passed through a small column (3.8 × 9 cm) of DEAE-cellulose, equilibrated as described above, under pressure (8 psi). The adsorbed protein was eluted with 0.25 M potassium phosphate buffer, pH 6.8, containing 5×10^{-4} M EDTA. The volume of the eluate (about 60 ml) was further reduced to 10–15 ml by dialysis against 0.15 M potassium phosphate buffer, pH 6.8, containing 5×10^{-4} M EDTA and polyethylene glycol (15% by weight).

STEP 7. SEPHADEX G-200. The concentrated protein

solution was applied to a column (5 × 80 cm) of Sephadex G-200 equilibrated with 0.15 M potassium phosphate buffer, pH 6.8, containing 5×10^{-4} M EDTA and eluted with the same buffer at a flow rate of about 20 ml/hr; 5-ml fractions were collected. The peak of enzyme activity emerged from the column when the volume of eluate was 35% of the column volume. The specific activity was usually constant in the fractions that contained most of the activity. Solutions containing enzyme were frozen in a Dry Ice–acetone bath and stored at −20°.

Table I gives a summary of the purification procedure. Preliminary experiments with disk electrophoresis on polyacrylamide gel at pH 8.3 have shown that the enzyme preparation contains one minor protein contaminant. The properties of the purified enzyme will be the subject of a later communication. All studies reported in this paper were carried out with enzyme processed through the final purification step.

Results

Stoichiometry. The data given in Table II (expt 1) show that within experimental error ADP, ^{14}C-glutamate, and carbamyl phosphate are formed in a ratio of 2:1:1, respectively, and that the reaction proceeds virtually to completion. Similarly, when the reaction was carried out with NH_4Cl in place of glutamine, ADP and carbamyl phosphate were formed initially in a ratio of 2:1, respectively (expt 2).

Evidence for Enzyme-Bound Activated Carbon Dioxide. When a relatively large amount of enzyme was preincubated with $H^{14}CO_3^-$ and ATP and then mixed with a solution containing glutamine and a large excess of unlabeled HCO_3^- the ^{14}C-carbamyl phosphate that was formed contained considerably more radioactivity than could be accounted for if it is assumed that all of the radioactive HCO_3^- equilibrated with the unlabeled HCO_3^-, and that all of the glutamine present was utilized (Table III, expt 1). The possibility that the excess radioactivity was a result of synthesis of a small amount of ^{14}C-carbamyl phosphate during preincubation was excluded by adding base before adding the solution containing glutamine and HCO_3^- (expt 4). Preincubation of the enzyme with $H^{14}CO_3^-$ in the absence of ATP, or with ATP followed by HCO_3^- + $H^{14}CO_3^-$, gave the amount of ^{14}C-carbamyl phosphate

TABLE II: Stoichiometry of the Reaction.[a]

Expt No.	Time (min)	ADP Formed (μmoles)	Glutamate Formed (μmoles)	Carbamyl Phosphate Formed (μmoles)
1	2	2.02	1.05	0.84
	4	2.75	—	1.20
	8	2.86	1.37	1.15
	16	3.14	1.37	1.15
2	4	1.35		0.65
	9	2.60		1.25
	15	3.50		1.50
	22	4.50		1.85

[a] Experiment 1: The reaction mixtures contained ATP (10 μmoles), $NaHCO_3$ (10 μmoles), $MgCl_2$ (20 μmoles), ^{14}C-L-glutamine (1.37 μmoles; 500,000 counts/min), potassium phosphate buffer (25 μmoles; pH 7.8), and enzyme (0.18 mg) in a final volume of 0.5 ml; 22°. Experiment 2: The reaction mixtures contained ATP (10 μmoles), $NaH^{14}CO_3$ (10 μmoles; 2×10^5 counts/min), $MgCl_2$ (10 μmoles), NH_4Cl (50 μmoles), potassium phosphate buffer (25 μmoles; pH 7.8), and enzyme (0.6 mg) in a final volume of 0.5 ml; 22°. At the indicated time intervals 0.1 ml of the reaction mixtures was added to 0.3 ml of cold 95% ethanol to stop the reaction. Analyses for ADP and ^{14}C-glutamate were carried out as described under Methods. In expt 1, carbamyl phosphate was determined by conversion to citrulline with ornithine transcarbamylase. In expt 2, ^{14}C-carbamyl phosphate was determined as ^{14}C-urea, as described under Methods.

TABLE III: Evidence for Enzymatic Activation of CO_2.

Expt No.	Reaction Mixtures[a]	^{14}C-Carbamyl Phosphate (counts/min)
1	(Enzyme + ATP + $H^{14}CO_3^-$), (HCO_3^- + glutamine)	16,800
2	(Enzyme + $H^{14}CO_3^-$), (HCO_3^- + glutamine + ATP)	600
3	(Enzyme + ATP), (HCO_3^- + $H^{14}CO_3^-$ + glutamine)	470
4	(Enzyme + ATP + $H^{14}CO_3^-$),[b] (HCO_3^- + glutamine)	690

[a] The reaction mixtures (final volume, 0.2 ml) contained enzyme (2.6 mg, 0.1 ml), ATP (2.13 μmoles), $MgCl_2$ (2.13 μmoles), $NaH^{14}CO_3$ (0.44 μmole; 8×10^5 counts/min), $KHCO_3$ (150 μmoles), L-glutamine (0.075 μmole), and potassium phosphate buffer (7.5 μmoles; pH 7.5). The components given in the first set of parentheses were mixed with phosphate buffer and $MgCl_2$ and incubated for 30 sec at 26°. Then the components given in the second set of parentheses were added together; after 10 sec the reaction was stopped by adding 0.2 ml of a solution containing 0.3 N NH_4OH and 1.3 N KOH followed by 0.2 ml of 0.001 M carbamyl phosphate. ^{14}C-Carbamyl phosphate was determined as ^{14}C-urea as described under Methods. [b] The solution containing NH_4OH and KOH was added before adding the components given in the second set of parentheses.

expected if the labeled HCO_3^- equilibrated with the unlabeled HCO_3^- before reaction with glutamine.

These results indicate that the radioactive HCO_3^- becomes bound to the enzyme during preincubation in a reaction that requires ATP, and that the binding is of such a nature as to permit the bound $H^{14}CO_3^-$ to be immediately available for reaction with glutamine. According to this interpretation the ^{14}C-carbamyl phosphate in expt 1 contains a large excess of radioactivity because the unlabeled HCO_3^- does not equilibrate with the radioactive enzyme-bound intermediate formed during preincubation.

Evidence for Cleavage of AT^{32}P in the Absence of Glutamine. As indicated in Table IV, when a relatively large amount of enzyme was preincubated with AT^{32}P and HCO_3^- followed by addition of a mixture of glutamine and a large excess of ATP, the supernatant solution obtained from the deproteinized reaction mixture after treatment with charcoal (to remove AT^{32}P and AD^{32}P) contained more radioactivity than would be expected if AT^{32}P and ATP had equilibrated before reaction with glutamine (expt 1). Thus, much less ^{32}P was found when AT^{32}P, ATP, and glutamine were added to enzyme at the same time (expt 2). Paper electrophoresis of the supernatant solution obtained after charcoal treatment in expt 1 carried out as described under Methods gave a clean separation of inorganic phosphate from carbamyl phosphate; in this experiment more than 85% of the radioactivity was found in the inorganic phosphate. Control studies have shown that carbamyl ^{32}P-phosphate can be recovered in satisfactory yield under these conditions.

The ^{32}P found in the supernatant solution in expt 1 could conceivably result from synthesis of carbamyl phosphate during preincubation if some ammonia were present or it could arise if the enzyme preparation contains ATPase. The first possibility is remote since electrophoresis of the supernatant solution obtained after charcoal treatment in expt 3 showed that less than 5% of the total radioactivity was present as carbamyl ^{32}P-phosphate; such carbamyl phosphate formation would give about equal amounts of carbamyl ^{32}P-phosphate and $^{32}P_i$. If the cleavage of AT^{32}P during preincubation is simply the result of contaminating ATPase activity, the ADP concentration in the preincubation mixture should increase linearly with time. However, as shown in Table V the ADP formed is produced in an initial "burst," and a significant increase in ADP does not occur after 30 sec. The finding of increased ^{32}P in expt 1 and 3 (Table IV) and the data on ADP formation are consistent with formation of an activated enzyme-bound intermediate associated with cleavage of ATP to ADP.

TABLE IV: Evidence for Cleavage of ATP in Absence of Glutamine.

Expt No.	Reaction Mixtures[a]	Formation of ^{32}P in Supernatant Solution (counts/min)
1	(Enzyme + HCO_3^- + AT^{32}P), (ATP + glutamine)	13,900
2	(Enzyme + HCO_3^-), (ATP + AT^{32}P + glutamine)	3,400
3	(Enzyme + HCO_3^- + AT^{32}P),[b] (ATP + glutamine)	13,400

[a] The reaction mixtures (final volume, 0.4 ml) contained enzyme (5 mg, 0.16 ml), potassium phosphate buffer (16 μmoles, pH 7.8), $NaHCO_3$ (4 μmoles), AT^{32}P (0.8 μmole, 244,000 counts/min), ATP (80 μmoles), L-glutamine (0.4 μmole), and $MgCl_2$ in concentration equimolar with ATP and/or AT^{32}P. The components given in the first set of parentheses were mixed with phosphate buffer and $MgCl_2$ and incubated for 30 sec at 4°. Then the components given in the second set of parentheses plus $MgCl_2$ were added together; after 10 sec, the reaction was stopped by adding 0.6 ml of 0.5 N $HClO_4$ followed by 0.1 ml of a solution containing 0.1 M carbamyl phosphate and 0.05 M potassium phosphate buffer, pH 7.8. The suspension was centrifuged immediately and a 0.85-ml portion of the supernatant solution was neutralized with 0.25 ml of 0.9 N KOH. After centrifugation to remove $KClO_4$, the nucleotides were removed by mixing with 5.15 ml of a suspension containing 540 mg of activated charcoal. After centrifugation, the radioactivity in the supernatant was determined. [b] Perchloric acid and carbamyl phosphate were added before adding the components given in the second set of parentheses.

TABLE V: Formation of ADP in Absence of Glutamine.[a]

Time (sec)	ADP (mμmoles)
30	38
60	44
120	44

[a] The reaction mixture contained enzyme (5 mg), potassium phosphate buffer (16 μmoles, pH 7.8), ATP (0.8 μmole), $MgCl_2$ (0.8 μmole), and $NaHCO_3$ (4 μmoles) in a total volume of 0.2 ml; 4°. Aliquots of the reaction mixture were added to equal volumes of 0.5 M $HClO_4$, and after centrifugation of the precipitated protein, ADP was determined as described under Methods.

Discussion

The studies on the stoichiometry of the reaction show that 2 molecules of ATP are utilized in the synthesis of

1 molecule of carbamyl phosphate. The data also indicate that the reaction proceeds virtually to completion. The same stoichiometry is obtained when NH_4Cl is substituted for glutamine. Thus, the reaction catalyzed by this enzyme is not analogous to that catalyzed by carbamate kinase, but rather to the reaction catalyzed by liver carbamyl phosphate synthetase. The *E. coli* enzyme differs from the liver enzyme in that glutamine and ammonia are both active substrates and that *N*-acetylglutamate does not affect the rate of the reaction (Anderson and Meister, 1965).

The data obtained in the pulse-labeling experiment with $H^{14}CO_3^-$ (Table III) provide clear evidence that the first step in the synthesis of carbamyl phosphate is formation of an enzyme-bound activated form of carbon dioxide and that this reaction requires ATP. Although the chemical nature of the activated carbon dioxide cannot be established from these experiments, the results are consistent with the following sequence of reactions.[4]

$$\text{enzyme} + \text{ATP} + CO_2 \rightleftharpoons \text{enzyme-}[^-OCOOPO_3^{2-}] + \text{ADP} \quad (6)$$

$$\text{enzyme-}[^-OCOOPO_3^{2-}] + \text{glutamine} \rightleftharpoons \text{enzyme-}[NH_2COO^-] + P_i + \text{glutamate} \quad (7)$$

$$\text{enzyme-}[NH_2COO^-] + \text{ATP} \rightleftharpoons \text{enzyme} + \text{ADP} + NH_2COOPO_3^{2-} \quad (8)$$

According to this mechanism, incubation of the enzyme with ATP and HCO_3^- in the absence of glutamine or ammonia results in cleavage of ATP to give ADP and enzyme-bound carbonate phosphate anhydride (reaction 6). The data show that a "burst" of ADP is produced under these conditions (Table V). Although the findings do not unequivocally exclude an intermediate involving formation of free inorganic phosphate, as proposed by Metzenberg *et al.* (1958), it might be expected that the formation of such an intermediate would be accompanied by rapid exchange between $AT^{32}P$ and the unlabeled phosphate buffer during preincubation with enzyme and HCO_3^- in the absence of glutamine; thus, there would be extensive formation of $^{32}P_i$, much greater than actually observed. Although it is not yet possible to obtain accurate values for binding of HCO_3^- and phosphate to the enzyme from the present experiments, estimates of such values based on the "extra" formation of ^{14}C and ^{32}P products (Tables III and IV) and the values observed for formation of ADP are of about the same order. Additional studies along these lines will be undertaken.

According to the proposed mechanism, if the molecule of ATP which is used for phosphorylation of carbamate is not bound to the enzyme until the intermediate has reacted with glutamine, *i.e.*, it is not bound during preincubation as described in expt 1, Table IV, then the radioactivity obtained in the supernatant solution should be found mainly in the P_i rather than in carbamyl phosphate. The experiments described here show that there is indeed much less radioactivity in the carbamyl phosphate than in the P_i formed. It is anticipated that additional studies and perhaps special techniques will be required to characterize the enzyme-bound intermediate as carbonate phosphate anhydride. However, the present findings are consistent with reactions 6–8, but do not unequivocally exclude other types of activated intermediates.

According to the proposal put forth here, the enzyme carries out two steps which are analogous to simple kinase reactions. Thus, carbonic acid is phosphorylated in the first step and carbamic acid is phosphorylated in the last step. In this connection the findings by Raijman and Grisolia (1964) that liver carbamyl phosphate synthetase phosphorylates formate and acetate in reactions involving cleavage of 1 molecule of ATP are of considerable interest. It would be of importance to do studies of the type described here on the liver enzyme; it seems probable that similar mechanisms are involved.[5] It would also be of interest to carry out such experiments with other enzymes capable of activating carbon dioxide, *e.g.*, the biotin-containing carboxylases.

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[4] Similar schemes have been proposed earlier (Meister, 1965; Jones, 1965).

[5] Although it has been reported that the liver enzyme does not utilize glutamine in place of ammonia, it is possible that the affinity or activity of the enzyme for glutamine is altered in the course of purification. It might be of interest to reexamine the question of the utilization of glutamine by liver carbamyl phosphate synthetase.

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A Kinetic Study of Nucleotide Interactions with Pyruvate Kinase*

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ABSTRACT: The apparent broad specificity of the enzyme pyruvate kinase for the nucleoside diphosphate substrate has been re-examined. Nucleoside diphosphokinase activity was found to be present but was active only when exogenous adenosine triphosphate was present.

The apparent K_m and V_{max} values were determined at several pH values between 6.0 and 9.0 for a number of nucleoside diphosphates. A doubly ionized phosphoryl group on the nucleotides and/or an imidazole group on the enzyme appear to be essential for nucleotide binding. Both the apparent K_m and V_{max} values decrease between pH 7.5 and 9.0, which suggests the involvement of an ionized α-amino group in the reaction. The results of the determinations of kinetic parameters in the pH range of 7–8 are discussed in relation to a proposed mechanism in which the nucleoside diphosphate is bound to the enzyme on two regions in an obligatory order, with the β-phosphoryl group binding first and the nucleoside portion binding second.

A broad specificity for the nucleoside diphosphate substrate in the pyruvate kinase catalyzed reaction has been reported previously (Strominger, 1955; Tietz and Ochoa, 1958; Adam, 1961). However, doubt has been cast on this specificity by Davidson (1959), who claimed that the apparent rate obtained, using pyrimidine nucleoside diphosphates, resulted from contamination of the reaction mixture with nucleoside diphosphokinase and adenosine phosphates. An upper limit for nucleoside diphosphokinase activity under varying conditions will be presented. Evidence will be given that two regions of the nucleotide are bound, that the phosphate must be doubly ionized for binding, and that an imidazole and an α-amino group on the enzyme are involved in binding.

Methods

Materials. The sodium salts of PEP,[1] AMP, ADP, ATP, GDP, IDP, UDP, CDP, dADP, dCDP, NADH, and $NADP^+$; crystalline lactic dehydrogenase, type II; and crystalline yeast hexokinase were purchased from the Sigma Chemical Co. The crystalline glucose 6-phosphate dehydrogenase was prepared by C. F. Boehringer und Soehn. The [^{32}P]ATP was prepared by mitochondrial phosphorylation of ADP by [^{32}P]inorganic phosphate. The [^{32}P]PEP was labeled by an exchange reaction using [^{32}P]ATP and pyruvate kinase by a method to be published elsewhere. Product separation was effected in both instances by chromatography on Dowex-1 chloride using the method of Khym and Cohn (1953).

Enzyme Preparation. Pyruvate kinase was prepared from rabbit muscle by the "fluorokinase" method of Tietz and Ochoa (1958). Two different preparations were used in this work. One batch had a specific activity of 60 μmoles of pyruvate formed/min/mg of protein at pH 7.0 and 25.0° and is referred to later

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[1] Abbreviations used in this work: PEP, phosphoenolypyruvate; AMP, ADP, ATP, monophosphate, diphosphate, and triphosphate of adenosine; GDP, IDP, UDP, CDP, dADP, dCDP, guanosine, inosine, uridine, cytidine, deoxyadenosine, and deoxycytidine diphosphates; NADH, reduced nicotinamide–adenine dinucleotide; $NADP^+$, oxidized nicotinamide–adenine dinucleotide phosphate.