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CARBAMYL PHOSPHATE SYNTHESIS IN *NEUROSPORA CRASSA*

I. PRELIMINARY CHARACTERIZATION OF ARGININE-SPECIFIC CARBAMYL PHOSPHOKINASE

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SUMMARY

An enzyme from *Neurospora crassa* which catalyzes carbamyl phosphate formation is reported. Carbamyl phosphate formation by the enzyme is shown to be dependent upon ATP, ammonia, bicarbonate, and magnesium ions. Glutamine and *N*-acetyl-L-glutamate, involved in carbamyl phosphate synthesis in other organisms, are not required for the reaction and do not influence it. While direct evidence for carbamate as the true substrate is lacking, the enzyme is considered similar to bacterial carbamyl phosphokinase (ATP:carbamate phosphotransferase, EC 2.7.2.2).

INTRODUCTION

Studies of Neurospora mutants deficient in early steps of pyrimidine and arginine synthesis have revealed a complexity in carbamyl phosphate metabolism[1–4]. The evidence indicates that there are two enzymes of carbamyl phosphate synthesis, one specific for each pathway dependent upon this compound. This paper describes some of the fundamental characteristics of the arginine-specific enzyme of carbamyl phosphate synthesis as an introduction to the following paper[5]. The latter gives evidence for the metabolic position of the enzyme specifically in the arginine pathway. The pyrimidine-specific system of carbamyl phosphate synthesis has not yet been demonstrated *in vitro*, even in mutant strains completely deficient in the arginine-specific enzyme.

Three enzymes of carbamyl phosphate synthesis have been described previously in other organisms. The first, carbamyl phosphokinase (ATP:carbamate phosphotransferase, EC 2.7.2.2), catalyzes carbamyl phosphate formation by the phosphorylation of carbamate by ATP in the presence of magnesium; carbamate is formed non-enzymatically from ammonium and carbonate ions[6,7]. This enzyme is found in bacteria. The second enzyme, carbamyl phosphate synthetase, is found in vertebrates[8] and at least one invertebrate, the earthworm[9]. It catalyzes a complex reaction of carbonate, ammonia, and ATP, in the presence of magnesium and acetylglutamate, to form carbamyl phosphate. In contrast to the bacterial enzyme, carbamyl phosphate synthesis here requires two moles of ATP per mole of carbamyl phosphate formed. The third enzyme, the carbamyl phosphate synthetase of *Agaricus bisporus*, catalyzes

carbamyl phosphate formation in the presence of magnesium ions, ATP, bicarbonate, and L-glutamine; the amide group of the last substrate serves as the nitrogen donor[10]. This enzyme has recently been shown to exist in bacteria[11] and yeast[12], although these reports are not yet definitive[13]. The data presented below indicate that the Neurospora enzyme requires bicarbonate, ammonia, ATP, and magnesium. It is clearly distinct from the carbamyl phosphate synthetases of vertebrates and *A. bisporus*. Because of the low activity of the Neurospora enzyme, however, and the difficulties of its purification, further work is required to define and describe the reaction more precisely. A problem of particular importance is whether the true substrate is carbamate, as it is in bacteria. For purposes of discussion, the enzyme will be referred to as carbamyl phosphokinase. A preliminary note on this work has appeared previously[14].

EXPERIMENTAL PROCEDURE

Materials

$NaH^{14}CO_3$ (80 mC/mmole) was obtained from Volk Radiochemical Co.; it was diluted to 0.05 mC/mmole with unlabelled $KHCO_3$ prior to use except where noted. ATP, GTP, CTP, and UTP were obtained from Sigma Chemical Co. Carbamyl phosphate (dilithium salt) was prepared chemically as described previously[15,16]. Sephadex G-25 (medium) was obtained from Pharmacia Fine Chemicals, Inc., and DEAE-cellulose was obtained from Eastman Chemical Co. and Bio-Rad Laboratories. Neurospora strains were those in the author's stock collection, and are available from the Fungal Genetics Stock Center, Dartmouth College, Hanover, N.H. (U.S.A.).

Partially purified ornithine transcarbamylase (carbamoylphosphate:L-ornithine carbamoyltransferase, EC 2.1.3.3) was prepared from arginine-limited cultures of Neurospora strain 30300 (*arg-3*) as described previously[16]; since this strain has a mutational deficiency for carbamyl phosphokinase[5], the ornithine transcarbamylase preparations used in assays were completely free of this enzyme. Conversely, carbamyl phosphokinase preparations were made from cultures of UM-3 (*arg-12*), a mutant strain devoid of ornithine transcarbamylase[17].

Methods

Carbamyl phosphokinase activity was usually measured by determining the amount of radioactivity, introduced as $H^{14}CO_3^-$, rendered acid-stable by its transformation to carbamyl phosphate and thence to citrulline. Citrulline, in contrast to the bicarbonate used as a substrate and the intermediates of citrulline synthesis derived from it, was acid-stable. The second reaction, the conversion of carbamyl phosphate to citrulline, was assured by including excess ornithine and ornithine transcarbamylase. Standard reaction mixtures contained the following ingredients in a volume of 0.5 ml: Tris–HCl buffer (pH 8.4), 50 μmoles; $MgCl_2$, 8 μmoles; NH_4Cl, 30 μmoles; ATP, 3 μmoles; $KH^{14}CO_3$, 10 μmoles (0.5 μC); ornithine·HCl, 2.5 μmoles; excess ornithine transcarbamylase; and a rate-limiting quantity of a carbamyl phosphokinase preparation. The final pH was 8.2. Incubations were carried out at 37° for 30 min, after which 0.4 ml of the reaction mixtures were plated into 0.15 ml of 2M HCl in 25 % ethanol on an etched glass planchet 1 in in diameter. The planchets were dried carefully under a spotlight, and the acid-stable radioactivity, identified as citrulline

chromatographically, was measured with a Nuclear Chicago D-47 gas flow detector. With 0.4 ml reaction mixture plated, self-absorption was 25 %; the correction was not made unless necessary. Under the conditions described, 1 μmole of citrulline is equivalent to approx. 25 000 counts/min (uncorrected). This value corresponds to the specific radioactivity of the bicarbonate used as substrate. Specific activities of carbamyl phosphokinase are given as counts/min of acid-stable radioactivity per mg protein per hour.

In several experiments involving no radioactive isotopes, a colorimetric assay for citrulline was used. Reaction mixtures were the same as those above except that the bicarbonate was unlabelled. They were stopped with the addition of 0.1 ml 2M $HClO_4$, and all or part of the reaction mixtures were used in half-volume colorimetric reactions for citrulline as described by KORITZ AND COHEN[18], and modified by DAVIS[16].

For the direct determination of carbamyl phosphate, non-radioactive reaction mixtures lacking ornithine and ornithine transcarbamylase were used. The reactions were stopped by adding 0.3 ml 1M NH_4Cl and placing them in a boiling-water bath. This treatment, devised by YASHPHE AND GORINI[13], transforms carbamyl phosphate largely to cyanate and the latter quantitatively to urea. The urea is measured by the same colorimetric test as that used for citrulline. A standard curve for carbamyl phosphate was determined by adding various amounts of this compound to time-zero reaction mixtures and measuring the urea formed. Color was proportional to carbamyl phosphate concentration over the range studied.

Protein was measured by the method of LOWRY *et al*[19]. Inorganic phosphate was measured by the method of LOWRY AND LOPEZ[20]. Carbonate and carbamate were measured by differential precipitation with barium, as described by YASHPHE AND GORINI[13].

Growth of mycelia and carbamyl phosphokinase purification

Mycelium of the *arg-12* strain, UM-3, was grown for enzyme purification in 700-ml shake cultures from heavy conidial inocula as described previously[21]. The cultures were grown to terminal weight in VOGEL's medium[22] containing 100 μg L-arginine·HCl/ml. This concentration of supplement is ultimately limiting to growth, and by the time of harvest, the specific activity of carbamyl phosphokinase rises two- to four-fold over the specific activity found in wild type mycelia[5]. Mycelia were harvested on a Buchner funnel, rinsed with water, and dried quickly with acetone. The dry pads were ground in cold (−10°) acetone with an Omni-Mixer blendor (Servall), and the suspended particulate matter was collected by filtration and dried in air. The acetone powder was used as the starting material in enzyme purification. This procedure, in which the material was exposed only momentarily to an acetone–water phase, did not lead to significant differences in carbamyl phosphokinase activity from preparations made without acetone treatment. All further steps were performed in the cold ($< 4°$).

9 g of dry acetone powder were suspended in 50 ml of 0.05 M potassium phosphate (pH 7.0) and centrifuged for 20 min at 3500 $\times$ *g*. The supernatant was collected; the residue was resuspended and again centrifuged. The combined extracts (about 80 ml) were dialyzed overnight against 2000 ml of 0.05 M phosphate buffer (pH 7.0). The dialyzed extract was made up to 150 ml with buffer, and fractionation with solid $(NH_4)_2SO_4$ was carried out. To the extract, 37.5 g $(NH_4)_2SO_4$ was added; after 20 min,

the preparation was centrifuged at 3500 × *g* for 30 min and the residue discarded. To the supernatant 6 g $(NH_4)_2SO_4$ was added, and the residue, after centrifugation, was again discarded. To the supernatant 16.5 g $(NH_4)_2SO_4$ was added and dissolved, the mixture centrifuged, and the residue was dissolved in 30 ml 0.02 M phosphate buffer (pH 7.0) and dialyzed overnight against 2000 ml of the same buffer with 0.02 % mercaptoethanol. $MgCl_2$ was added to a concentration of $5 \cdot 10^{-3}$ M, and the preparation was applied to a DEAE-cellulose column (2 × 7 cm) previously equilibrated with 0.02 M phosphate buffer (pH 7.0), $5 \cdot 10^{-3}$ M $MgCl_2$ and 0.02 % mercaptoethanol. The column was washed with 150 ml of this buffer, followed with 150 ml buffer containing 0.04 M NaCl. Elution of enzyme was carried out with buffer containing 0.075 M NaCl; one or two 70-ml fractions (including the void volume) were collected. These were each brought to 0.05 M phosphate buffer (pH 7.0) by the addition of 2.1 ml of 1 M buffer, and to each 70 ml, 28 g solid $(NH_4)_2SO_4$ was added and dissolved. After 1.5–2 h, the suspensions were centrifuged at 19000 × *g* for 30 min. The residues were dissolved in 4-ml volumes of 0.02 M phosphate (pH 7.0; without mercaptoethanol), and passed through a short Sephadex G-25 column equilibrated with the same buffer in order to remove $(NH_4)_2SO_4$. The final preparations were frozen in small volumes and stored at −15°, where they remained stable indefinitely. A typical purification is summarized in Table I.

TABLE I

SUMMARY OF CARBAMYL PHOSPHOKINASE PURIFICATION

Assays were done by the radioactive method described in the methods section. Specific activity = counts/min of citrulline per mg protein per hour; 25000 counts/min = approx. 1 μmole citrulline.

Step	*Volume*	*Total protein*	*Total units*	*Recovery*	*Specific activity*	*Purification*
Acetone powder extract	150	1704	4 905 000	100	2880	1.0
Ammonium sulfate fraction	30	380	4 100 000	83.5	10 800	3.75
DEAE-cellulose eluate	70	32.2	1 038 000	21.1	32 200	11.2
Final ammonium sulfate preparation (after Sephadex treatment)	5.2	15.7	600 000	12.2	38 200	13.25

The enzyme is quite unstable in many conditions; purifications cannot involve ethanol or acetone precipitations, nor precipitation with slightly acid buffers. Storage of the enzyme, even in the frozen state, in Tris–HCl buffer (pH 7.4) leads to the loss of over one-half the activity within two days. The low recovery from the DEAE-cellulose column is presumed not due to the loss of a second distinct enzyme, since no further activity was recovered with high NaCl concentrations, and a similar loss was seen when purified enzyme was applied to a DEAE-cellulose column and eluted a second time. Gradient elution from DEAE-cellulose yields the carbamyl phosphokinase activity in a single symmetrical peak between NaCl concentrations of 0.04 and 0.07 M.

Just prior to use in reactions, purified preparations were often combined with the appropriate amount of ornithine transcarbamylase and the mixture was passed through Sephadex G-25 equilibrated with 0.02 M Tris–HCl buffer (pH 7.4) to remove phosphate. This procedure led to no change in activity determinations (except by

dilution of enzyme), but eliminated precipitations by magnesium in certain experiments and allowed phosphate determination in others.

RESULTS AND DISCUSSION

Time, enzyme and pH dependencies

With crude extracts or the first $(NH_4)_2SO_4$ fraction, it was found that the formation of acid-stable radioactivity from $H^{14}CO_3^-$ is linear with respect to time and to the amount of enzyme added to reaction mixtures. Similarly, non-radioactive reaction mixtures gave a linear relationship between time and amount of citrulline, as measured colorimetrically. When ornithine transcarbamylase and ornithine are omitted from reaction mixtures containing purified enzyme, and the carbamyl phosphate formed is estimated after chemical transformation to urea, the time course is not linear after 10 min or so, because of thermal and enzymatic breakdown of carbamyl phosphate during the reaction. Parallel rate measurements of the same purified enzyme preparation by the urea method and by the transcarbamylase method show that the initial rates of citrulline formation are identical, within experimental error (Fig. 1).

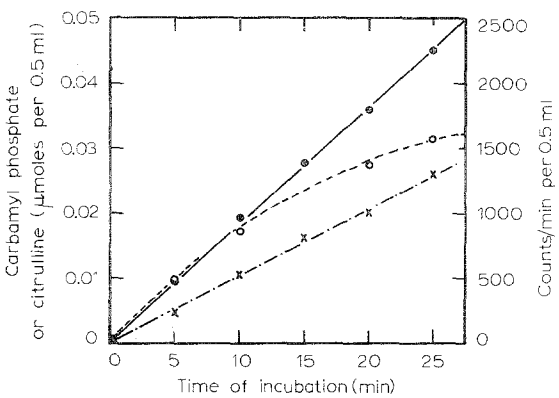


Fig. 1. Carbamyl phosphate production as a function of time of incubation in three types of reaction mixtures (see *methods*). ●—●, reaction mixture with unlabelled bicarbonate, ornithine and ornithine transcarbamylase; citrulline was measured colorimetrically; ○----○, reaction mixtures with unlabelled bicarbonate and without ornithine or ornithine transcarbamylase; urea, derived from carbamyl phosphate, was measured colorimetrically; ×---×, same as uppermost curve, but with radioactive bicarbonate; citrulline was measured as acid-stable radioactivity (right ordinate). All reaction mixtures were done simultaneously with the same amount of carbamyl phosphokinase added to each.

This indicates that the transcarbamylase does not influence the initial reaction rate, and in fact the transcarbamylase method allows more confidence in a linear time course under the usual conditions of assay. In all experiments reported, the amount of citrulline produced in the reaction mixtures, radioactive or non-radioactive, rarely exceeds 0.1 μmole.

The influence of the pH of the reaction mixtures is shown in Fig. 2, which indicates an optimal pH of 8.1 to 8.3.

Identification of the product

The overall reaction described required that ornithine and ornithine trans-

carbamylase be present for any radioactivity to be rendered acid-stable. The substitution of aspartate and Neurospora aspartate transcarbamylase, however, gave the same level of acid-stable counts. This is consistent with the hypothesis that the product of carbamyl phosphokinase is indeed carbamyl phosphate. No fixation of $H^{14}CO_3^-$ took place when dialyzed crude extracts of a strain lacking aspartate transcarbamylase (*pyr-3d*) or of a strain lacking ornithine transcarbamylase (*arg-12*) were used, if the amino acid substrate for the transcarbamylase reaction was that of the missing transcarbamylase.

A further test of the identity of the product of carbamyl phosphokinase was made by adding pure dilithium carbamyl phosphate to complete reaction mixtures.

TABLE II

EFFECT OF CARBAMYL PHOSPHATE ADDED TO REACTION MIXTURES

Reaction mixtures were standard except for the addition of dilithium carbamyl phosphate as noted.

Carbamyl phosphate added (*μmoles*)	*Counts/min per 0.4 ml per 30 min*
0	1520
1	1429
10	795
20	194

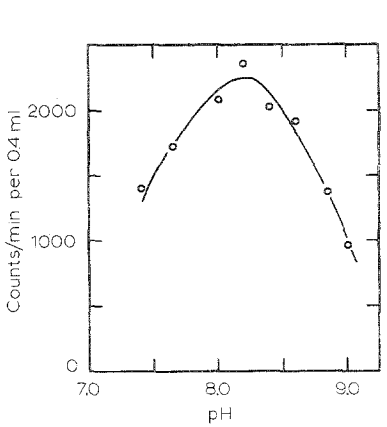
The data (Table II) show that as the carbamyl phosphate concentration is increased, the radioactivity in citrulline diminishes. Neither Li^+ nor cyanate (a decomposition product of carbamyl phosphate, particularly above pH 6.0) is inhibitory to the overall reaction. It is therefore probable that the unlabelled carbamyl phosphate added dilutes the radioactive product of carbamyl phosphokinase until the carbamyl phosphate added disappears by utilization and decomposition. Independent measurements of the activity of the ornithine transcarbamylase preparation used are consistent with this view. The addition of carbamyl phosphate to reaction mixtures in which aspartate transcarbamylase and aspartate were used in place of ornithine transcarbamylase and ornithine similarly reduced the acid-stable counts.

The product of the carbamyl phosphokinase reaction was acid-labile. In reaction mixtures lacking ornithine transcarbamylase and ornithine, the product capable of chemical transformation to urea disappeared upon 90 sec exposure to 100° in 0.1 M HCl. The product of reaction mixtures containing ornithine transcarbamylase and ornithine, however, remained unchanged after acid hydrolysis under conditions in which citrulline likewise is unaffected. These results are consistent with the identity of the product of the former reaction as carbamyl phosphate, and of the latter as citrulline.

Substrate dependencies

Omission of ATP or magnesium led to a complete inability of the enzyme to transform labelled bicarbonate to citrulline. When magnesium was held constant at 10 to $16 \cdot 10^{-3}$ M and ATP was varied, Lineweaver–Burk plots of ATP concentration and velocity yielded relatively high K_m values (above $1 \cdot 10^{-2}$) where the plot was

linear. Further, the reaction rate was optimal when ATP concentration was about one-half the magnesium concentration, and strong substrate inhibition was observed at higher ATP levels. When ATP was held constant at 6 to $10 \cdot 10^{-3}$ M, and magnesium was varied, no significant reaction took place until the magnesium concentration was brought nearly equimolar with ATP, at which point the reaction rate increased to a maximum. These results suggest that an ATP–magnesium complex is the effective molecule involved. When magnesium and ATP concentrations are varied simultaneously such that the molar ratio of magnesium to ATP is 2 to 1, a curve shown in Fig. 3 is obtained. A slight threshold effect is seen, and substrate inhibition still prevails.



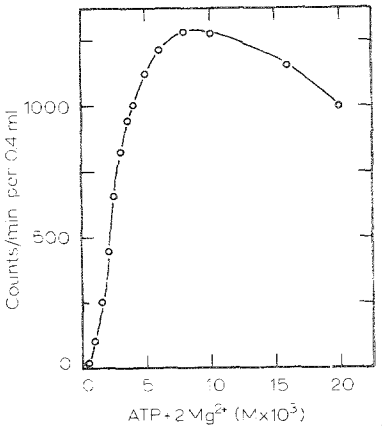


Fig. 2. Influence of pH upon the carbamyl phosphokinase reaction. Tris–HCl buffer was used in all reaction mixtures; assays were done by the radioactive method, as described in *methods*.

Fig. 3. Influence of ATP and Mg^{2+} concentration upon the reaction rate. The assays were performed with the normal amount of bicarbonate of twice the normal specific radioactivity. To minimize ATP decomposition, assays were done for 15 min. In all reaction mixtures, Mg^{2+} is twice the concentration of ATP.

The K_m values for ATP and magnesium derived from such data after extrapolation of the curve to the abscissa were not considered dependable; the K_m value is estimated at about $1–2 \cdot 10^{-3}$ M for ATP, without consideration of the form in which it is utilized. Further work with more highly purified enzyme preparations would be necessary in order to obtain more definitive kinetic data. The problems of ATP and magnesium interaction are discussed by KUBY AND NOLTMANN[23] for creatine phosphokinase.

In the course of reactions, the release of phosphate from ATP was studied. With the purified enzyme preparations used, the ammonium-dependent or bicarbonate-dependent phosphate liberation was one-third to one-fourth of the total. This made it impossible to determine stoichiometric ratios of phosphate to citrulline dependably in the course of the reaction. Nevertheless, the experiments showed that ATP concentration did not change markedly (less than 10 %) over the 30-min reaction time usually used.

No significant reaction was observed when ATP was replaced with UTP, CTP, or GTP. However, manganous ion could replace magnesium in the reaction, giving the

same maximal velocity measurements as magnesium at the same concentration.

Omission of ammonia or bicarbonate from standard reaction mixtures led to a complete inability of the enzyme to form citrulline, as measured isotopically or colorimetrically. Reciprocal plots of substrate–velocity curves for bicarbonate and ammonia are shown in Figs. 4 and 5. These curves were obtained by varying each while the other was held constant at the maximal value. The K_m values determined in this way were $1.6 \cdot 10^{-2}$ M for ammonium ions and $4 \cdot 10^{-3}$ M for bicarbonate, with slight substrate inhibition in the case of the latter compound. The K_m values for the two substrates were interdependent, the K_m for each substrate rising by a factor of 2 or 3 if the concentration of the other was reduced by a similar factor.

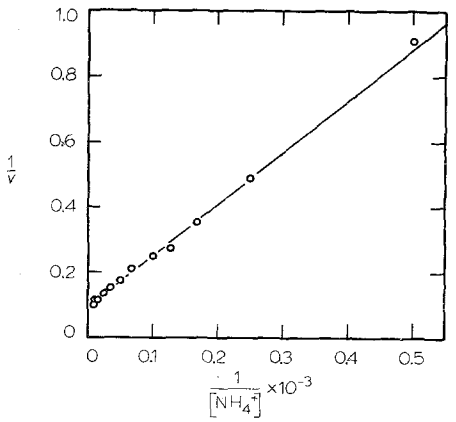


Fig. 4. Lineweaver–Burk plot of NH_4^+ concentration against reaction velocity. Assays were standard for the radioactive method with the exception of variations in NH_4Cl.

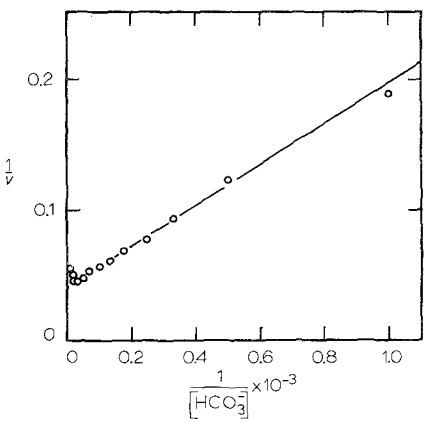


Fig. 5. Lineweaver–Burk plot of bicarbonate concentration against reaction velocity. Assays were standard for the radioactive method with the exception of variations in $KH^{14}CO_3$.

The high K_m for ammonium ions and the interaction of substrates implied by the interdependence of K_m values suggests that carbamate might be the true substrate of the enzyme, as it is for bacterial carbamyl phosphokinase. A mixture of NH_4Cl and $KHCO_3$ equilibrates rapidly and non-enzymatically to an equilibrium mixture of carbamate and bicarbonate. The estimated concentration of the former at equilibrium is approx. 3–5 % of the latter, as determined by direct measurement of the salts (in Tris buffer) in concentrations similar to those used in reaction mixtures. The optimal concentrations of ammonia and bicarbonate used, therefore, may merely be those leading to optimal conditions for carbamate formation. A number of experiments have been done, without success, to detect a period of carbamate formation by a slowly increasing rate of the reaction at 25° under conditions where NH_4Cl or $KHCO_3$ concentration is suboptimal. A representative experiment, comparing reaction mixtures in which the salts (with suboptimal NH_4Cl) were preincubated or not preincubated is shown in Fig. 6. No difference in the course of the first six minutes of the reaction is seen. Other experiments, comparing pure ammonium carbamate with NH_4Cl and suboptimal $KHCO_3$ (the latter two not preincubated) were carried out, using, of necessity, the less sensitive colorimetric method to measure citrulline formation. No variation of reaction rate in either condition was seen among successive 5-min intervals

of a 30-min reaction time. This suggests that rapid equilibration to the true substrate from one direction or the other had occurred. Whether the enzyme preparation is responsible for rapid equilibration of bicarbonate and carbamate is not known. If carbamate is the substrate, however, the apparent K_m of the enzyme for it is on the order of $2 \cdot 10^{-4}$ M or less. Further work is required, however, to define the true substrate of the enzyme. Until larger amounts of the purified enzyme are available,

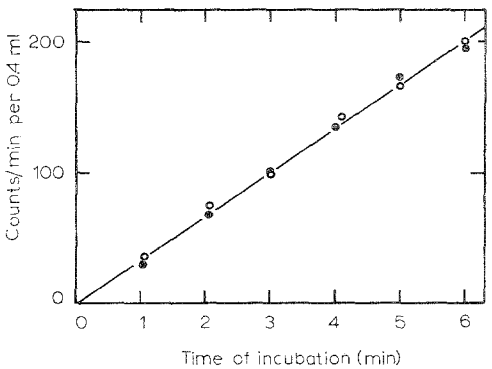


Fig. 6. Comparison of the early course of the carbamyl phosphokinase reaction where NH_4Cl and $KHCO_3$ are or are not incubated prior to the reaction. The reaction mixtures were identical in final ingredients. The specific radioactivity of the bicarbonate was double that normally used. NH_4Cl was added to $2 \cdot 10^{-2}$ M, rather than the standard $6 \cdot 10^{-2}$ M. The temperature of the reaction mixtures was 25°. O—O, salts not preincubated, ●—●, salts preincubated 40 min at 25°.

TABLE III

INABILITY OF L-GLUTAMINE TO REPLACE AMMONIA

Reaction mixtures (0.5 ml) were standard except for NH_4Cl and glutamine additions.

NH_4Cl added (μmoles per reaction mixture)	*Glutamine added (μmoles per reaction mixture)*	*Counts/min per 0.4 ml per 30 min*
0	0	7
30	0	620
0	10	6
30	10	651

critical experiments along these lines, similar to those reported by THORNE AND JONES[24] and by JONES AND LIPMANN[7] are not feasible. The designation of the enzyme as "carbamyl phosphokinase" is therefore provisional.

Twenty-nine possible nitrogen donors have been tested to see whether one or more would replace ammonia. These included all 20 of the protein amino acids, β-alanine, citrulline, ornithine, urea, hydroxylamine, α-amino-*n*-butyric acid, α-amino-isobutyric acid, γ-aminobutyric acid, and oxamic acid. None were effective at a level of 10 μmoles per reaction mixture in the isotopic assay. L-Glutamine deserves special note, since the *A. bisporus* enzyme of carbamyl phosphate synthesis uses the amide nitrogen of this compound as a nitrogen donor[10]. The Neurospora enzyme was unaffected by L-glutamine, when tested in the presence or absence of NH_4^+ (Table III).

The Neurospora enzyme reaction is not influenced significantly by *N*-acetyl-L-glutamate at levels of $0.2–20 \cdot 10^{-3}$ M. This distinguishes the Neurospora enzyme from the carbamyl phosphate synthetase of vertebrates.

The carbamyl phosphate produced by the enzyme described here is specifically destined for arginine synthesis in normal cells[5]. Arginine is not inhibitory to the carbamyl phosphokinase reaction, however, indicating that feedback inhibition does not prevail in this reaction. However, the following paper[5] gives evidence for changes of specific activity of the enzyme dictated by the arginine content of the medium.

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