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Partial Purification and Properties of Carbamoyl Phosphate Synthetase of Alaska Pea (*Pisum sativum* L. cultivar Alaska)

PURIFICATION AND GENERAL PROPERTIES

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1. Carbamoyl phosphate synthetase was purified up to 45-fold from Alaska pea seedling (*Pisum sativum* L. cultivar Alaska). 2. The enzyme was most active with and had the lowest K_m for L-glutamine as compared with NH_4^+. 3. The purest preparations utilized very poorly or not at all L-asparagine and urea as nitrogen donors. 4. At saturating concentrations of components of the reaction, the K_m for L-glutamine was $1{\cdot}2 \times 10^{-4}$M, and the K_m for ATP was approx. $3{\cdot}9 \times 10^{-4}$M. 5. Although the enzyme was very labile, stability was improved by glutamine, asparagine, ammonium sulphate, dithiothreitol and especially L-ornithine. 6. Free ATP was markedly inhibitory, and $MgATP^{2-}$ and Mg^{2+} appeared to be the actual substrates utilized. 7. Fe^{2+} and Mn^{2+} were also utilized, but not as readily as Mg^{2+} except at low concentrations. K^+ increased activity significantly. 8. Of the four nucleotides tested (ITP, ATP, GTP and UTP) only ATP served as an effective phosphate donor.

Carbamoyl phosphate is of particular importance because it is a precursor of both arginine and UMP. Many studies have been made in the past decade on the enzymology of carbamoyl phosphate synthesis by using mammalian sources, yeast and several species of bacteria. These studies revealed that at least three distinct enzymes have evolved that catalyse carbamoyl phosphate synthesis (see Meister, 1965).

In many species of bacteria the enzyme carbamate kinase (EC 2.7.2.2) catalyses carbamoyl phosphate synthesis as follows:

$$NH_2{\cdot}CO_2^- + ATP^{4-} \underset{}{\overset{Mg^{2+},\ K^+}{\rightleftharpoons}} NH_2{\cdot}CO{\cdot}O{\cdot}PO_3^{2-} + ADP^{3-}$$

This enzyme has been purified from *Streptococcus faecalis* (Kalman & Duffield, 1964) and *Saccharomyces cerevisiae* (Grisolia, Harmon & Raijman, 1962). The reaction is readily reversible, but the synthesis of ATP is favoured (Kalman & Duffield, 1964).

Genetic evidence (Pierard, Glansdorff, Mergeay & Wiame, 1965) indicates that the most important mechanism for carbamoyl phosphate synthesis in *Escherichia coli* and yeast utilizes L-glutamine as a nitrogen donor instead of NH_4^+. The enzyme involved, 'glutamino-carbamyl phosphate synthetase', was first detected in and partially purified from *Agaricus bisporus* (Levenberg, 1962) and has since been highly purified from *E. coli* (Anderson & Meister, 1965; Kalman, Duffield & Brzozowski, 1966). It catalyses the following reaction:

$$HCO_3^- + H_2O + \text{glutamine} + 2\ ATP^{4-} \underset{}{\overset{Mg^{2+},\ K^+}{\rightleftharpoons}} NH_2{\cdot}CO{\cdot}O{\cdot}PO_3^{2-} + 2\ ADP^{3-} + HO{\cdot}PO_3^{2-} + \text{L-glutamate}$$

'Glutamino-carbamyl phosphate synthetase' is a large molecule with a molecular weight of about 700000 (Kalman *et al.* 1966) as opposed to a molecular weight of 46000–66000 for carbamate kinase (Marshall & Cohen, 1966). Another major difference is that the forward reaction with 'glutamino-carbamyl phosphate synthetase' occurs ten times as fast as the reverse reaction (Kalman *et al.* 1966).

Carbamoyl phosphate synthetase (EC 2.7.2.*a*), an *N*-acetylglutamate-requiring enzyme, has been highly purified from the liver of several mammalian species (see Jones, 1965).

$$HCO_3^- + H_2O + NH_4^+ + 2\ ATP^{4-} \underset{N\text{-acetylglutamate}}{\overset{Mg^{2+},\ K^+}{\rightleftharpoons}} NH_2{\cdot}CO{\cdot}O{\cdot}PO_3^{2-} + 2ADP^{3-} + HO{\cdot}PO_3^{2-}$$

The frog liver enzyme has a molecular weight of 315000 and the equilibrium of the reaction lies strongly in favour of carbamoyl phosphate synthesis (Marshall, Metzenberg & Cohen, 1958, 1961).

Although knowledge of carbamoyl phosphate

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metabolism in mammals and bacteria is appreciable, the nature of the enzyme or enzymes responsible for carbamoyl phosphate synthesis in photosynthetic organisms is meagre. After confirming earlier work showing that addition of NH_4^+ to the culture medium of *Chlorella pyrenoidosa* stimulated dark fixation of $^{14}CO_2$ into citrulline, Hiller (1964) reported, without giving details, that cell-free preparations of *Chlorella* required ATP and glutamine in the carboxylation reaction. He considered the reaction to be similar to that described in *Agaricus bisporus* (Levenberg, 1962). Bone (1959) found that mung-bean-seedling mitochondria, when incubated with ATP, Mg^{2+}, ammonium chloride, L-ornithine and $^{14}CO_2$, yielded [^{14}C]citrulline. Glutamine was not tested as a substrate. In a survey of plant tissues for the enzyme Kleczkowski (1965) could not detect significant carbamoyl phosphate synthesis in seedling homogenates of lupin, barley, cucumber or bean, but acetone-dried powders derived from pea-seedling shoots (*Pisum sativum* L. cultivar Perla Szlachetna) formed significant amounts of citrulline in the presence of carbon dioxide, ATP, Mg^{2+}, ornithine and L-glutamine. Greatest activity was achieved when glutamine was the nitrogen donor, L-asparagine and NH_4^+ being only 30% and 9–12% as effective respectively.

Because of the lack of any intensive studies on the nature of the enzyme forming carbamoyl phosphate in higher plants, the present study was undertaken.

MATERIALS AND METHODS

Chemicals. Buffers and amino acids were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. and Calbiochem, Los Angeles, Calif., U.S.A., and purine and pyrimidine nucleotides (purity 95–99%) were obtained from Sigma Chemical Co. $BaH^{14}CO_3$ ($^{14}C/^{12}C$ ratio 83·6:16·4) was obtained from Oak Ridge National Laboratory, Oak Ridge, Tenn., U.S.A., and was converted into $NaH^{14}CO_3$ before use. Cellex P and D were purchased from Bio-Rad Laboratories, Richmond, Calif., U.S.A., and purified with NaOH, HCl and EDTA before use. DEAE-Sephadex A-50, CM-Sephadex C-50 and Sephadex G-25, G-150 and G-200 were purchased from Pharmacia, Piscataway, N.J., U.S.A. Calcium phosphate gel (Swingle & Tiselius, 1951) was obtained through the courtesy of Dr Ronald Greene, Duke University. To a suspension of 100ml. of 10% (w/v) cellulose powder (Whatman standard grade CF-11) 100ml. of calcium phosphate gel (30mg./ml.) was added and stirred. This was used for the preparation of a calcium phosphate gel–cellulose column. Calcium phosphate gel (aged 2 years) prepared by the method of Keilin & Hartree (1938) was also used. Aged alumina C_γ gel (A grade) was obtained from Calbiochem or from Dr R. Greene.

Assays. Protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin (Armour and Co., Chicago, Ill., U.S.A.) as standard. When thiols were used in purification studies, correction was necessary for their effect on extinction. Protein concentration was also determined on fractions from column eluates by measuring the extinction at 280nm.

L-Citrulline was assayed by the procedure of Archibald (1944) as modified by Ratner (1955), with a correction for ATP (Pierard *et al.* 1965).

P_i was assayed by the method of Lowry & Lopez (1946) at 700nm.

[^{14}C]Citrulline was identified by t.l.c. Silica gel G plates were employed for this purpose, and phenol–water (3:1, v/v) and methanol–chloroform–water (2:2:1, by vol.) were the solvents used for one-dimensional chromatograms. Identification was made by means of Ehrlich's reagent, ninhydrin and R_F values. Radioautographs were also made.

ADP was determined by the method of Adam (1963). The following compounds were present in Pyrex cuvettes of 1cm. light-path: 5mM-$MgSO_4$, 0·15mM-NADH, 2mM-phosphoenolpyruvate, 10mM-potassium phosphate buffer, pH7·6, 3 units of pyruvate kinase and excess of lactate dehydrogenase. The sample containing ADP was added last and the decrease in extinction measured.

Ornithine transcarbamoylase was purified 26-fold from arginine-adapted *Streptococcus faecalis* by the procedure of Jones (1962).

Enzyme assays. Urease and asparaginase activities were measured by the Conway procedure as performed by Sehgal & Naylor (1964), except that titration to the end point was done with 2mM-HCl. Ornithine transcarbamoylase activity was measured by the colorimetric determination of L-citrulline, where 1 unit of activity is defined as that amount catalysing the synthesis of 1·0μmole of citrulline/10min. at 37°. The buffer was 50mM-triglycine–KOH, pH8·1, and the substrates were 10mM-carbamoyl phosphate and 10mM-ornithine hydrochloride in a total volume of 1ml. Phosphatase activity was assayed by measuring the release of P_i from carbamoyl phosphate in triglycine–KOH buffer, pH8·1, with correction for non-enzymic degradation by using appropriate blanks.

Carbamoyl phosphate synthetase activity was measured in two ways.

(*a*) The carbamoyl phosphate produced was converted into L-citrulline in the presence of L-ornithine and ornithine transcarbamoylase. Citrulline was then assayed colorimetrically. This method as performed suffers from low sensitivity, lack of ease in manipulation and lack of precision.

(*b*) In this procedure $NaH^{14}CO_3$ served as the CO_2 source in the synthesis of [^{14}C]carbamoyl phosphate, which was immediately and continually converted into [^{14}C]-citrulline by the presence of excess of ornithine transcarbamoylase and ornithine. The reaction was stopped by pipetting a 0·2ml. portion from the assay tubes (total volume 0·25ml.) into a 2·5cm. stainless-steel planchet containing 0·25ml. of ethanol–2·5M-HCl (2:1, v/v) plus a drop of 0·1% Alconox detergent. The planchets were carefully dried under a 250w incandescent lamp, and the radioactivity was determined in a Baird–Atomic model 135 scaler and model 755 automatic sample-changer. For purification studies the reactions were run at 37 ± 0·2° for 8min. The components of the reaction mixture used were as follows: 5mM-ATP, 15mM-$MgSO_4$, 3mM-L-ornithine hydrochloride, 50mM-triglycine–KOH buffer, pH8·1, 0·7–1·4mM-2-mercaptoethanol or 0·09–0·12mM-dithiothreitol, 8–10 units of ornithine transcarbamoylase, 0·1mM-$NaH^{14}CO_3$ (specific radioactivity approx. $2·2 \times 10^6$c.p.m./

m-mole), 4mM-L-glutamine, less than 0·06mg. of enzyme and distilled water to a final volume of 0·25ml.

In the colorimetric assay of L-citrulline 10mM-$KHCO_3$ replaced $NaH^{14}CO_3$ as the CO_2 source.

Source of carbamoyl phosphate synthetase. Several species of plants were examined to find those with the greatest specific activity of carbamoyl phosphate synthetase. Extracts were made from acetone-dried powders of hydrated jack-bean seeds, jack-bean-seedling roots and shoots, hydrated Black Diamond watermelon seeds, watermelon-seedling shoots, Alaska pea seeds (hydrated) and seedlings (both roots and shoots) and Laxton Progress pea-seedling shoots. Extracts of these seedlings were tested with NH_4^+, glutamine and asparagine as nitrogen donors, with and without *N*-acetylglutamate (1mM). Only the acetone-dried powders of watermelon shoots, Alaska pea shoots and Laxton Progress pea shoots contained significant activity in the colorimetric assay. Alaska pea seedlings were selected for further investigation.

Enzyme preparation and purification. Alaska pea seeds, obtained from Burpee Seed Co., Philadelphia, Pa., U.S.A., were planted $\frac{1}{2}$in. deep in sandy loam soil and grown for 11–20 days in a greenhouse. At harvest the seedlings were cut about 3in. above the ground line and frozen at −20° for 1hr. or more. Except during preparation of the acetone-dried powder all subsequent operations were performed at 0·5–2°.

RESULTS AND DISCUSSION

Purification of the enzyme

Stage 1: preparation of acetone-dried powder. The seedlings (up to 300g.) were homogenized in redistilled acetone containing 14mM-2-mercaptoethanol at −20° in a large Waring Blendor (model B4); 6–10ml. of acetone–mercaptoethanol/g. fresh wt. was used for the initial extraction, which consisted of homogenization for three or four 20sec. periods at 19000rev./min. The slurry was then filtered under vacuum on a Buchner funnel containing two layers of Whatman no. 5 filter paper. The filter cake was resuspended (in 5–7ml./g. original fresh wt.) of acetone containing 14mM-mercaptoethanol at −20° and the conditions of the first extraction were repeated at 17000rev./min. The homogenate was then filtered on a Buchner funnel and the filter cake washed (with 2ml./g. original fresh wt.) of acetone–mercaptoethanol. Suction was continued until nearly all of the acetone had been removed. The filter cake was removed and spread to dry at room temperature for 2–5hr. The small lumps were then ground to a powder in a mortar and stored at −20°. Under these storage conditions the enzyme lost less than 2% of its initial activity/week.

Stage 2: ammonium sulphate fractionation. A 20g. sample of acetone-dried powder was suspended in 320ml. of 0·1M-triglycine–potassium hydroxide buffer, pH7·5, containing 14mM-mercaptoethanol and 2mM-glutamine. The thick suspension was stirred for 15–20min. and then centrifuged at 12000*g* at 0° for 12min. The supernatant was filtered through two layers of cheesecloth and saturated ammonium sulphate (pH unadjusted) was added slowly, with stirring, to give 31% saturation, the mercaptoethanol concentration being maintained at 14mM. After 20–25min. the solution was centrifuged at 12000*g* for 15min. To the supernatant ammonium sulphate was added to give 53% saturation, the mercaptoethanol concentration being maintained at 14mM. After standing for 25min. the preparation was centrifuged at 20000*g* for 15min. The supernatant was carefully decanted.

Stage 3: calcium phosphate gel adsorption and ammonium sulphate precipitation. (Removal of ammonium sulphate by gel filtration on Sephadex G-25 would probably make this step more reproducible, but units would be lost owing to enzyme lability while on the gel.) The protein pellets in the centrifuge buckets were rinsed twice with several millilitres of 20mM-potassium phosphate buffer, pH6·5, containing 7mM-mercaptoethanol, 2mM-ornithine hydrochloride and 1mM-glutamine. The protein pellets were then dissolved in 50ml. of the same buffer. Calcium phosphate gel (50mg./ml.) was slowly added in the optimum ratio, namely 4mg. of gel/mg. of protein. The suspension was stirred for 15min. and then centrifuged at 5000*g* for 6min. The supernatant was discarded. Approx. 30ml. of 65mM-potassium phosphate buffer (optimum concentration), pH7·6, containing 3mM-ornithine hydrochloride, 2mM-glutamine and 7mM-mercaptoethanol was added to the centrifuge tube containing the gel–protein pellet and stirred to a uniform suspension. After standing for 10–15min. the suspension was centrifuged at 4000*g* for 5min. and the supernatant saved. The enzyme was then precipitated by adding ammonium sulphate to give 55% saturation, the mercaptoethanol concentration being maintained at 7mM.

Stage 4: first gel filtration. The pellet from the preceding step was dissolved in 2·5ml. of 20mM-2,2,2-tris(hydroxymethyl)ethanesulphonic acid–sodium hydroxide buffer, pH6·8, containing 2mM-glutamine, 0·4mM-EDTA and 0·5–0·9mM-dithiothreitol. (The addition of ornithine, although not attempted, would in all likelihood increase stability during stages 4 and 5 and consequently increase purification.) To this was added 0·5vol. of 15% (w/v) sucrose. After mixing, the enzyme solution was applied to the top of a column (2·5cm. × 37cm.) of Sephadex G-150 (or G-200) equilibrated in the same buffer. Fractions (3ml.) were collected automatically at 5min. intervals. Maximum activity was in the fifth and sixth fractions following a large initial protein peak (Fig. 1). Maximal enzyme activity always corresponded to a second,

always small, protein peak. The initial large peak was separable into two strongly overlapping peaks by using a Sephadex G-200 column and collecting 2·5ml. fractions. Active fractions were pooled and brought to 56% saturation with ammonium sulphate (pH 7·0), the mercaptoethanol concentration being maintained at 14mM. After standing for 15–25min. the preparation was centrifuged at 20000*g* for 15min. and the pellets were either stored at −20° or used in the next step.

Stage 5: second gel filtration. The ammonium sulphate pellet was dissolved in 2ml. of the buffer used in the first gel filtration. A column (2·5cm. × 24cm.) of Sephadex G-200 was used for the second gel filtration. The enzyme supernatant (containing 5%, w/v, sucrose) was applied by syringe to the top of the column and 3ml. fractions were collected at 5min. intervals. Protein was determined by measuring the extinction at 280nm. and two overlapping peaks were seen. The second peak contained the greatest enzyme activity.

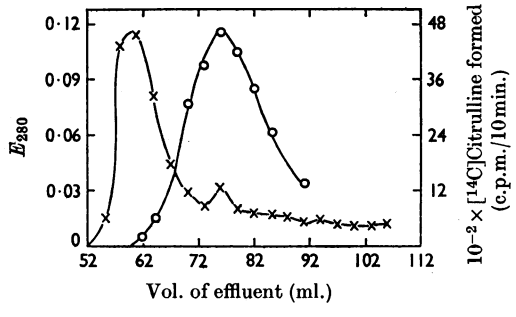

Fig. 1. Gel filtration of carbamoyl phosphate synthetase of Alaska pea seedlings. Enzyme (stage 3 preparation, purified 13-fold) was passed through a column (2·5cm. × 38cm.) of Sephadex G-150 at a flow rate of 35ml./hr.; approx. 3ml. fractions were collected. The void volume of the column was 53ml. ×, Protein concentration (E_{280}); ○, enzyme activity (c.p.m. of [^{14}C]citrulline formed/10min.).

Purification data are presented in Table 1. The enzyme remaining after stage 5 was purified about 49-fold, with a unit recovery of 6%. Instability made it difficult to carry the purification further. However, the addition of ornithine, the stabilizing effect of which was not discovered until purification studies were concluded, should help circumvent this problem.

To eliminate most of the ornithine transcarbamoylase activity, a 31–46%-saturated ammonium sulphate precipitation was used and the calcium phosphate gel was eluted with 45–50mM-potassium phosphate buffer, pH 7·6.

A large number of other purification techniques were employed without success. These involved chiefly stages 2 and 3 of the procedure (O'Neal, 1968).

Stability studies

A serious problem in the purification and assay of carbamoyl phosphate synthetase was its lack of stability. Numerous attempts were made to find stabilizing compounds and conditions. A concise summary of conditions contributory to stability follows.

Stability seemed to increase after the gel-filtration step. At protein concentrations of 0·2–0·5mg./ml., however, a loss of 3·2–7%/hr. occurred after 2–4hr. even though 7mM-mercaptoethanol or 0·5mM-dithiothreitol and 1mM-glutamine were present.

Stability was greatest at pH 6·3–6·8 in triglycine–potassium hydroxide buffer and at pH 6·8–7·3 in potassium phosphate buffer. Even at these pH values, however, stage 2 and 3 preparations often lost 10–20% of their initial activity/hr. over a 2–4hr. period, even in the presence of 3–50mM-mercaptoethanol plus glutamine.

Mercaptoethanol and dithiothreitol increased stability by 6–30%, dithiothreitol being the most effective protectant. Dithiothreitol was most effective in the 0·5–4mM range. GSH decreased

Table 1. *Summary of purification of Alaska-pea-seedling carbamoyl phosphate synthetase*

One unit of enzyme catalysed the formation of 1c.p.m. of [^{14}C]citrulline/min. at 37° under the conditions described.

Purification stage	Total protein (g.)	10^{-6}× Total activity (units)	Specific activity (units/mg. of protein)	Yield (%)	Purification	Range of purification
Green Plant	11·02	29·7	2700	100	1	1
1: Acetone-dried powder	3·99	15·08	3852	50·5	1·4	1·2–1·5
2: $(NH_4)_2SO_4$ precipitate	1·2	13·45	11286	45·3	4·2	3·9–4·4
3: $Ca_3(PO_4)_2$-gel eluate	0·29	9	31032	20·1	11·7	8·5–13
4: Sephadex G-150	0·0348	3·44	98690	11·4	37·2	29·5–45
5: Sephadex G-200	0·014	1·81	142560	6	48·7	38·2–57

Table 2. *Effect of* L-*ornithine on the stability of carbamoyl phosphate synthetase*

Enzyme was a stage 3 preparation, and was dissolved in 60mM-2-tris(hydroxymethyl)ethanesulphonic acid–NaOH buffer containing 1·5mM-glutamine. Expt. 1: pH7·2, 14mM-mercaptoethanol, protein concentration 14·7mg./ml. Expt. 2: pH6·4, 1·2mM-dithiothreitol, protein concentration 12·8mg./ml.

Expt. 1	Initial activity (c.p.m./10min.)	Activity after 2·3hr. (c.p.m./10min.)	Loss/hr. (%)	Activity after 47·8hr. (c.p.m./10min.)	Loss/hr. (%)
Minus ornithine	11350	9520	7·7	380	2
Plus 4mM-ornithine hydrochloride	11500	11450	0·1	4070	1·38
Plus 50mM-ornithine hydrochloride	11700	10430	5·4	—	—

Expt. 2	Initial activity (c.p.m./10min.)	Activity after 3·5hr. (c.p.m./10min.)	Loss/hr. (%)	Activity after 48·7hr. (c.p.m./10min.)	Loss/hr. (%)
Minus ornithine	8000	4800	12	185	2
Plus 4mM-ornithine hydrochloride	8300	6470	6·6	2850	1·4

stability. Stability was slightly improved when 0·5mM- rather than 5mM-EDTA was used.

Ammonium sulphate stabilized the enzyme at 1–3% saturation. The optimum concentration varied in two tests, being approx. 1% saturation in one case and 2·5% saturation in the other, with stability decreasing significantly on either side of these 'optima'. But ammonium sulphate decreased the initial activity of the enzyme. For example, when ammonium sulphate (66mM) was present in the assay medium, activity was decreased by 47% even though the glutamine concentration was 1mM. Perhaps there is competition of ammonium sulphate for the glutamine-binding site.

Magnesium sulphate at 2–7mM increased stability if glutamine and ornithine were not present, but no additive effect was noted.

ATP at 0·5mM increased stability slightly in the absence of amino acids.

A number of amino acids increased stability, but by far the most effective was L-ornithine hydrochloride, followed by glutamine and asparagine. Glutamine, in the absence of L-ornithine, increased stability by 15–40% over controls, and asparagine was nearly as effective. No increase in stability was noted when the concentration of glutamine was greater than 2mM. The effect of L-ornithine hydrochloride on stability is shown in Table 2.

The effect of protein concentration on activity was marked, but depended to some extent on the stage of purification. In general, and especially during stages 1–4, it was found that it was best to attempt to maintain the protein concentration at at least 5mg./ml.

Temperatures between 5° and 25·1° had a deleterious effect on stability. Freezing the enzyme solution at −20° and subsequent thawing resulted in losses up to 60% of the activity. Freezing in liquid nitrogen and thawing at room temperature resulted in significant but variable losses in units, but never as much as when a −20° freezer was used.

Significant losses in activity occurred when enzyme preparations in 55%-saturated ammonium sulphate were frozen at −20° and thawed. Yet, if the protein pellet derived from ammonium sulphate precipitation was frozen at −20°, loss in units was only 0·5–4% of the initial activity/day. On thawing, there was an initial loss of 8–33% of the units. In spite of the losses the frozen-pellet method was used as a routine for storage of the enzyme.

Table 3. *Requirements for enzymic synthesis of* [^{14}C]*citrulline in pea seedlings*

The complete system contained 3mM-L-ornithine, 5mM-ATP, 15mM-$MgSO_4$, 50mM-triglycine–KOH buffer, pH8·1, 10 units of ornithine transcarbamoylase, 4mM-L-glutamine, 0·7mM-2-mercaptoethanol and 0·1mM-$NaH^{14}CO_3$ (205000c.p.m.). The enzyme preparation (0·1mg. of protein/tube) was assayed after 11min. at 37°. The total volume was 0·25ml.

Incubation system	[^{14}C]Citrulline formed (c.p.m.)
Complete	9000
Complete minus L-ornithine	91
Complete minus L-glutamine	60
Complete minus Mg^{2+}	30
Complete minus ATP	36

Development of a citrulline-synthesizing system

General requirements for activity. The acetone-dried powder was found to contain sufficient enzymes to fix ^{14}C from $NaH^{14}CO_3$ into at least two

compounds in addition to citrulline. By using a 75%-saturated-ammonium sulphate precipitate, however, or the acetone-dried-powder eluate that had been passed through a column of Sephadex G-25, only citrulline and a trace of an unknown compound were seen. Ornithine and ATP were either present or generated in the system. However, a 30–57%-saturated-ammonium sulphate-precipitated fraction that had been passed through a column (2·5cm. × 19cm.) of Sephadex G-25 was (Table 3) essentially freed of L-ornithine, L-glutamine, Mg^{2+} and ATP but not ornithine transcarbamoylase. Additional purification steps involving ammonium sulphate precipitation and calcium phosphate gel adsorption were required to remove that enzyme.

Optimum pH *and buffer comparison.* At 37° in triglycine–potassium hydroxide buffer the optimum pH is 8·1–8·2 (Fig. 2). A comparison of relative activities in several buffers was made at pH 8·1 and 50mM. In those buffer systems ordinarily lacking K^+, 15mM-potassium sulphate was present to satisfy the K^+ requirement. The relative activities in 2-hydroxyethyl-2-phenylethanesulphonic acid–potassium hydroxide, glycylglycine, triglycine–potassium hydroxide and tris–sulphuric acid buffers were 100%, 94%, 93·5% and 71% respectively. Kleczkowski (1965) reported that phosphate buffer was 40% less effective than glycylglycine buffer. It is recognized that the optimum pH may vary according to the buffer and substrate concentration.

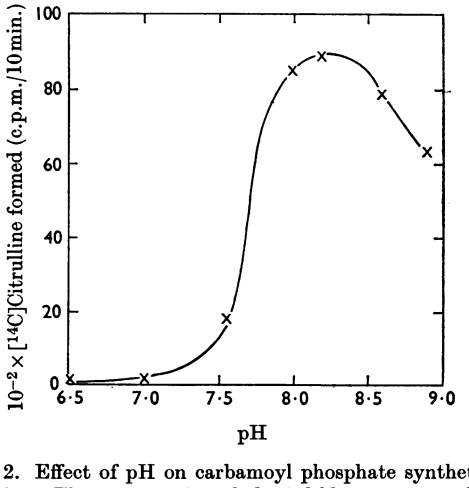

Fig. 2. Effect of pH on carbamoyl phosphate synthetase activity. The enzyme (purified 11-fold) was assayed in 50mM-triglycine–KOH buffer after 10min. at 37°. The assay mixture contained 1·5mM-ATP, 13mM-$MgSO_4$, 1mM-L-glutamine, 1mM-L-ornithine hydrochloride, 1·4mM-2-mercaptoethanol, 0·1mM-$NaH^{14}CO_3$ and 5 units of ornithine transcarbamoylase in a final volume of 0·25ml.

Effects of protein concentration and time. With a stage 3 preparation the reaction was linear only up to about 0·042mg. of protein/0·25ml. At protein concentrations below 0·025mg./0·25ml. enhancement of activity occurred in the presence of 0·2mg. of bovine serum albumin and was necessary to maintain linearity.

The reaction was linear only for about 7min., falling off rapidly after 11min.

It is our belief that the reaction is not linear at high protein concentrations and for longer periods for two reasons: (1) lability of the enzyme and (2) the production of ADP (or ADP and AMP in crude preparations). It is probable that ADP and AMP are the major factors, since both of these compounds are effective inhibitors of the enzyme (T. D. O'Neal & A. W. Naylor, unpublished work). Further, Kleczkowski (1965), using a different variety of pea and an ATP-generating system, found the reaction to be linear for much higher concentrations of protein and for a longer time.

Nucleotide specificity. GTP, ITP and UTP did not substitute for ATP with stage 4 preparations. Activity with these compounds at concentrations of 1–3mM was 0·2–2% that of ATP. That amount

Table 4. *Relative effectiveness of several nitrogen donors for carbamoyl phosphate synthesis with acetone-dried-powder extracts passed through Sephadex G-25*

The concentrations used were: L-glutamine, 5mM; L-asparagine, 5mM; $(NH_4)_2SO_4$, 20mM; urea, 15mM. Concentrations of other substrates were the same as in Table 3.

Nitrogen donor	Relative activity (%)
L-Glutamine	100
L-Asparagine	25–36
$(NH_4)_2SO_4$	32–55
Urea	35–45

Table 5. *Effectiveness of several nitrogen donors for carbamoyl phosphate synthesis with enzyme purified 52-fold*

The concentrations used were: L-glutamine, 1mM; L-asparagine, 3mM; $(NH_4)_2SO_4$, 10mM; urea, 1mM; L-arginine, 3mM. The enzyme was assayed at 37° after 11min. at pH 8·1 (triglycine–KOH buffer). Concentrations of other substrates were the same as in Table 3.

Nitrogen donor	Relative activity (%)
L-Glutamine	100
L-Asparagine	2·8
$(NH_4)SO_4$	30
Urea	1·1
L-Arginine	0·5

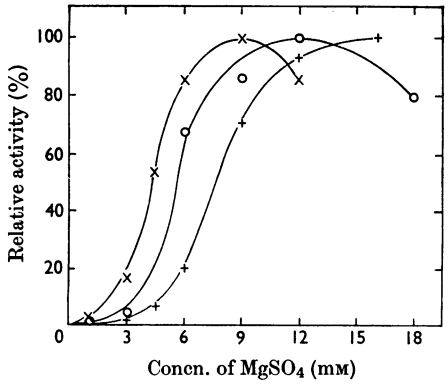

Fig. 3. Effect of $MgSO_4$ concentration on carbamoyl phosphate synthetase activity at three concentrations of ATP. The reaction was run for 12 min. at 37° in 50 mM-tris–H_2SO_4 buffer, pH 8·2, with enzyme that had been purified 10–12-fold. The assay mixture contained ATP (as indicated below), 1 mM-glutamine, 1 mM-ornithine hydrochloride, 1·4 mM-2-mercaptoethanol, 5 mM-K_2SO_4, 0·1 mM-$NaH^{14}CO_3$ and 10 units of ornithine transcarbamoylase in a final volume of 0·25 ml. ×, 1·2 mM-ATP; ○, 4·4 mM-ATP; +, 8 mM-ATP.

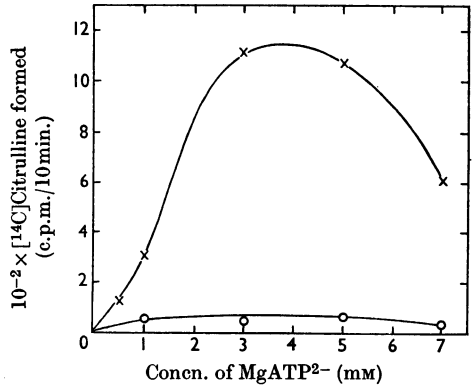

Fig. 4. Effect of 'free' (unbound) ATP on carbamoyl phosphate synthetase activity. The reaction was run for 10 min. at 37° in 50 mM-triglycine–KOH buffer, pH 8·1. The enzyme had been purified 11·2-fold. The assay mixture contained ATP and $MgSO_4$ (as indicated), 1 mM-glutamine, 1 mM-ornithine hydrochloride, 0·6 mM-2-mercaptoethanol, 0·1 mM-$NaH^{14}CO_3$ and 5 units of ornithine transcarbamoylase in a final volume of 0·25 ml. ×, ATP and $MgSO_4$ concentrations equal; ○, an additional 2·5 mM-ATP present.

of activity could have been caused by ATP contamination in the commercial preparations.

Nitrogen-donor specificity. Initial studies were made with acetone-dried powder extracts, some of which had been taken through the first ammonium sulphate precipitation step. In all cases the enzyme was passed through a column of Sephadex G-25 before being assayed to remove endogenous nitrogen compounds and ammonium sulphate. These studies with crude enzyme (Table 4) showed that a variety of nitrogen compounds other than glutamine were effective as nitrogen donors in the synthesis of carbamoyl phosphate. Activity in the presence of asparagine and urea may have resulted from NH_4^+ production by asparaginase and urease, likely contaminants of the acetone-dried powder. This conjecture is partially supported by experiments with the stage 4 preparation (Table 5). Apparently the enzyme can use nitrogen from asparagine to some degree. Kleczkowski (1965) reported that asparagine was used 30% as well as glutamine in carbamoyl phosphate synthesis in the variety of pea he had, whereas ammonium sulphate and ammonium chloride were only 12% as effective.

Metal ion requirement. Mg^{2+} resulted in the greatest carbamoyl phosphate synthetase activity, but Mn^{2+} and Fe^{2+} were effective to some degree. At optimum concentrations of bivalent ion, however, Mg^{2+} gave twice as much activity as Fe^{2+} or Mn^{2+}.

The optimum concentration of Fe^{2+} was 7–10 mM when the ATP concentration was 3·8 mM. As concentrations below 3 mM activity with Mn^{2+} and Fe^{2+} was more than double that with Mg^{2+}. When Mg^{2+} and Mn^{2+} but not Fe^{2+} concentrations were varied while that of ATP was held constant sigmoidal kinetics prevailed. Plots of the activity versus Mg^{2+} concentration at three concentrations of ATP are given in Fig. 3, which shows that the greater the ATP concentration the higher the optimum Mg^{2+} concentration. At 8 mM-ATP there was essentially no enzyme activity when the Mg^{2+} concentration was 1 mM, but if the ATP concentration was lowered to 1 mM there was very significant activity. Apparently 'free' ATP inhibits the enzyme.

The hypothesis of inhibition by ATP was checked and enzyme activity plotted against equimolar concentrations of Mg^{2+} and ATP such that the actual substrate should be $MgATP^{2-}$. Additional ATP (2·5 mM) did diminish activity of the enzyme at all concentrations of $MgATP^{2-}$, as shown in Fig. 4. These results indicate that the probable substrates are $MgATP^{2-}$ and Mg^{2+}. Results similar to these have been reported by Keech & Barritt (1967) for pyruvate kinase. At least two binding sites are present on pea carbamoyl phosphate synthetase, one binding Mg^{2+} and the other $MgATP^{2-}$. Free ATP apparently does not bind at the catalytic $MgATP^{2-}$ site, for there was no greater activity at 7 mM- than at 3 mM-$MgATP^{2-}$ when there was 2·5 mM-ATP (excess).

K^+ increases the activity of pea carbamoyl phosphate synthetase significantly. With tris-

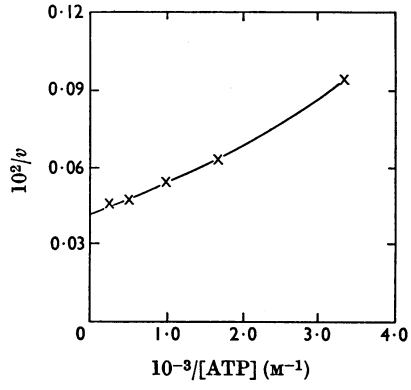

Fig. 5. Determination of a tentative K_m value of carbamoyl phosphate synthetase for ATP (at a given $MgSO_4$ concentration). The reaction was run at 37° for 10min. in 50mM-triglycine–KOH buffer, pH 8·1. The enzyme was a stage 5 preparation (purified 58-fold). The assay mixture contained ATP (as indicated), 14mM-$MgSO_4$, 4mM-glutamine, 1·2mM-ornithine hydrochloride, 0·3mM-dithiothreitol, 0·1mM-$NaH^{14}CO_3$ and 5 units of ornithine transcarbamoylase in a final volume of 0·25ml. Enzyme activity, v, is expressed as c.p.m. of [^{14}C]citrulline formed/10min.

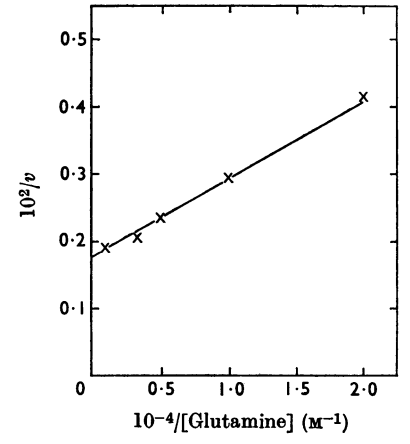

Fig. 6. Determination of a tentative K_m value of carbamoyl phosphate synthetase for L-glutamine. The reaction was run at 37° for 10min. in 50mM-triglycine–KOH buffer, pH 8·1. The enzyme was a stage 5 preparation (purified 51-fold). The assay mixture contained 3mM-ATP, 13mM-$MgSO_4$, 1mM-ornithine hydrochloride, 0·3mM-dithiothreitol, 0·1mM-$NaH^{14}CO_3$ and 5 units of ornithine transcarbamoylase in a total volume of 0·25ml. Enzyme activity, v, is expressed as c.p.m. of [^{14}C]citrulline formed/10min.

sulphuric acid buffer, pH 8·1, and a stage 2 preparation it was found that the addition of 30mM-K^+ increased the production of [^{14}C]citrulline threefold over the low-K^+ (0·3mM) controls. The nature of stimulation by K^+ is not known.

Effect of thiols. Most thiols increased both the stability and activity of the enzyme. Dithiothreitol (0·5–4mM), 2-mercaptoethanol (1mM) and GSH (0·5–1mM) all increased the activity by 20–35%.

Substrate saturation and Michaelis constants. With a stage 5 preparation the apparent K_m for ATP was approx. $3{\cdot}9 \times 10^{-4}$M (Fig. 5). The K_m for L-glutamine was approx. $1{\cdot}2 \times 10^{-2}$M (Fig. 6). Double-reciprocal plots of activity versus ATP concentration were often curvilinear. This could have been caused by the Mg^{2+} concentration not being optimum for each ATP concentration, or it could be due to an allosteric alteration of enzyme conformation, or both.

K_m determinations for Mg^{2+} could not be made owing to marked sigmoidal kinetics. However, the K_m for $MgATP^{2-}$ is probably in the range $1{\cdot}3 \times 10^{-3}$–$1{\cdot}7 \times 10^{-3}$M (Fig. 4), and the concentration of free Mg^{2+} required to cause a 50% stimulation of activity is calculated to be about 3·6–4·6mM.

Although the K_m for NH_4^+ was not determined, it was found that an increase in ammonium sulphate concentration from 2·1mM to 12·6mM increased enzyme activity 3·2-fold. Thus the K_m for NH_4^+ is probably greater than 3×10^{-3}M, i.e. over 15 times the K_m for glutamine.

The effect of carbon dioxide concentration on citrulline production was not determined. Even if no bicarbonate was added to the medium, however, the enzyme was 88–94% saturated with carbon dioxide. Therefore the components of the assay medium contained near-saturating amounts of carbon dioxide.

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