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# CARBAMYL PHOSPHATE BIOSYNTHESIS IN *BACILLUS SUBTILIS*

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SUMMARY

1. Two enzyme systems capable of synthesizing carbamyl phosphate in cell-free extracts of *Bacillus subtilis* were studied after separation from each other by fractionation with $(NH_4)_2SO_4$.

2. Carbamyl phosphate synthetase utilizes as substrates ATP ($K_m$ = 4.9 mM), glutamine ($K_m$ = 1.8 mM) and bicarbonate; and has a pH optimum of 7.5. It is inhibited by arginine and pyrimidines, and its formation is repressed by uracil, with a concerted effect of arginine at lower uracil concentrations. A biosynthetic role for this enzyme is thus indicated.

3. Carbamate kinase (ATP:carbamate phosphotransferase, EC 2.7.2.2) uses ATP ($K_m$ = 15 mM) and ammonium carbamate ($K_m$ = 8 mM), but not glutamine, as substrates. Its pH optimum is 8.5. It is more stable than carbamyl phosphate synthetase. It is partially inhibited by glutamine and arginine, and induced by arginine. This induction suggests a catabolic role.

4. Single mutational events lead to auxotrophy for arginine and pyrimidines, and marked reductions in the levels of carbamyl phosphate synthetase and carbamate kinase. The double requirement can be most simply understood if a single synthetase feeds into a carbamyl phosphate pool common to the arginine and the pyrimidine pathways. The pleiotropic effect on the two enzyme systems points to additional interrelationships. Perhaps the two enzymes share structural or regulatory elements.

## INTRODUCTION

The first enzyme systems shown to catalyze the synthesis of carbamyl phosphate *in vitro* were the acetyl glutamate-activated carbamyl phosphate synthetase of vertebrates[1] and the carbamate kinase of bacteria[2]. The early assumption that these systems were necessary steps in the normal pathway for arginine and pyrimidine biosynthesis in the respective organisms, soon became untenable in the light of contradictory evidence. For instance, bacterial mutants blocked in the synthesis of carbamyl phosphate have nevertheless normal levels of carbamate kinase[3], and vertebrate tissues synthesizing pyrimidines lack the acetyl glutamate-activated synthetase[4]. The contradictions were resolved with the discovery of a glutamine-

dependent carbamyl phosphate synthetase and the recognition of its anabolic role in pyrimidine and arginine metabolism. The glutamine-dependent enzyme has been identified in *Agaricus bisporus*[5], *Escherichia coli*[6], *Saccharomyces cerevisiae*[7], *Neurospora crassa*[8,9], *Coprinus radiatus*[10], Ehrlich ascites cells[11], rat liver[12], mouse spleen[13], and pea seedlings[14]. The anabolic role was established on the basis of the auxotrophy resulting from mutational loss, the regulatory control and the stoichiometry of the reaction[6–10,15–17]. By the same token, the carbamate kinase was relegated to a catabolic role; while the acetyl glutamate-activated system was assigned primarily to the ornithine cycle for ammonia detoxication in urotelic vertebrates[18].

Two different glutamine-dependent carbamyl phosphate synthetases have been identified in fungi: one enzyme has its regulatory controls tailored to meet the requirements of arginine biosynthesis, while the other responds to regulation by the pyrimidines[7–10]. In *E. coli*, on the other hand, a single carbamyl phosphate synthetase is regulated by both end products[15,19]. The present work shows that in *Bacillus subtilis* the biosynthesis of carbamyl phosphate and its regulation follow the same general pattern as in *E. coli*. A preliminary report has been published[9].

## MATERIALS AND METHODS

### *Reagents*

ATP, UTP, UMP, CTP and CMP, sodium salts, were obtained from Sigma Chemical Co. Streptomycin sulfate from E. R. Squibb & Sons, Argentina; L-glutamine, L-arginine, L-ornithine and uracil, from Nutritional Biochemicals. Bacto casamino acids and yeast extract, from Difco Laboratories. 1-Phenyl-1,2-propanedione-2-oxime, from Eastman Organic Chemicals. DEAE-cellulose, from Serva. Sephadex G-200, from Pharmacia. Resin AG 1-X8, from Bio-Rad Laboratories. The radioactive compounds from the Comisión Nacional de Energía Atómica (Argentina) and the Commissariat à l'Énergie Atomique (France). The specific activity of the [$^{14}$C]-bicarbonate was 0.1 and that of [$^{14}$C]urea 10.6 mC/mmole. Labeled carbamyl phosphate was prepared by the method of LOWENSTEIN AND COHEN[20], with a specific activity of 0.0028 mC/mmole.

### *Strains*

Two strains of *B. subtilis* (*try C2* and *try C2, ura*), were kindly provided by Dr. PIERRE SCHAEFFER. Wherever the strain is not specified, *try C2* was used. Two arginine-*plus*-pyrimidine auxotrophs (*cap*$_1$ and *cap*$_2$) and one arginine auxotroph (*arg*) were isolated following ultraviolet irradiation at the 1% survival level, and enrichment by the penicillin technique with recyclization[21], in the presence of indol. Selection was for arginine and/or pyrimidine auxotrophs.

Revertants from *cap* were isolated following ultraviolet irradiation at the 50% level of survival, and plating on minimal medium *plus* indol.

### *Media*

The minimal medium is that of GARY AND BARD[22], with 0.1% casamino acids instead of yeast extract, and 0.5% in glucose. It was supplemented as required. The complex medium (P. SCHAEFFER, personal communication, modified) contained: peptone broth, prepared in the laboratory, 1 l; yeast extract, 1 g; glucose, 5 g;

$MgSO_4 \cdot 7\ H_2O$, 0.25 g; $MnCl_2 \cdot 4\ H_2O$, 2 mg; $CaCl_2$, 55 mg; $FeSO_4 \cdot 7\ H_2O$, 0.28 mg. The glucose, the $CaCl_2$ and the $FeSO_4 \cdot 7\ H_2O$ were autoclaved separate from the rest of the ingredients, and at a lower temperature (105° for 30 min). The pH was adjusted to 7 before autoclaving.

*Cultures*

Inocula were prepared by culturing with agitation for 10 h in 100 ml of the same medium to be used subsequently. These inocula were added to 2-l batches of media, in 4-l erlenmeyers, and incubated with agitation for 7.5 h if the complex medium was used, and for 20 h with minimal medium. All incubations were at 37°. The cultures were in late log phase at the time of harvest, unless limited by a nutritional requirement.

*Cell-free extracts*

The cells were harvested by centrifugation, washed and suspended in 0.02 M Tris–HCl buffer (pH 7.5), and broken in the Virtis homogenizer or the Mickle disintegrator with the aid of glass beads. After centrifugation at 15 000 × *g* for 15 min, the supernatant was dialyzed for 3 h at 4° against the same buffer (containing 0.1 mM GSH unless otherwise indicated).

*Enzyme assays*

Carbamyl phosphate synthetase was assayed by following the formation of [$^{14}$C]carbamyl phosphate from $NaH^{14}CO_3$, glutamine and ATP. The [$^{14}$C]carbamyl phosphate was converted to urea before counting[16]. The incubation mixture contained, in μmoles: L-glutamine, 10; ATP, 20; $NaH^{14}CO_3$, 20; $MgCl_2$, 20; Tris–HCl (pH 7.5), 100; and enzyme preparation. Final volume, 1.6 ml. After 10 min at 37°, the reaction was stopped by the addition of 0.16 ml of a freshly prepared solution containing 0.7 M $NH_4OH$ and 2.7 M KOH. After 10 more min at 37°, 0.64 ml of 4 M $NH_4Cl$ (pH 8.5) were added, and the mixture was heated for 10 min at 100°. The excess bicarbonate was eliminated by means of an AG 1-X8 column[16]. 0.5 ml of the water eluate (12 ml) was placed on a planchet containing 0.15 ml of 0.01 M $Ba(OH)_2$ and a drop of dilute detergent solution. The planchet was dried in the cold under vacuum and counted in a gas-flow detector (Nuclear Chicago).

Carbamate kinase was determined by the same method, but with $NH_4Cl$ as substrate instead of L-glutamine. The incubation mixture contained, in μmoles: $NH_4Cl$, 50; ATP, 10; $NaH^{14}CO_3$, 20; $MgCl_2$, 10; Tris–HCl (pH 8.5), 100; potassium acetate, 160; and enzyme preparation. Final volume, 1.6 ml. The acetate prevents interference by acetate kinase[23,9]. The reaction mixture was then treated as in the carbamyl phosphate synthetase assay above.

An alternative method for kinase assay is based on the colorimetric estimation of urea[24]. The reaction mixture for the assay contained, in μmoles: ammonium carbonate, preincubated for 30 min at 37° in order to equilibrate the carbonate with ammonium carbamate[25], 187; ATP, 10; $MgCl_2$, 10; Tris–HCl (pH 8.5), 100; potassium acetate, 150; and enzyme preparation. Final volume, 1.5 ml. All the ingredients except the enzyme were preincubated for 10 min at 37°. After incubation for 15 min at 37° the reaction was stopped by the addition of 0.1 ml of 1 mM $HgCl_2$. 0.5 ml of 2 M $NH_4Cl$ (pH 8.5) was added and the mixture was heated for 10 min at 100°.

Aliquots of 1 ml were assayed colorimetrically for urea[24]. The results obtained by the isotopic and colorimetric methods are entirely comparable. Wherever the latter method is not specified, the former was used.

Ornithine transcarbamylase (carbamoyl phosphate:L-ornithine carbamoyltransferase, EC 2.1.3.3) was assayed by measuring the [$^{14}$C]citrulline formed from [$^{14}$C]carbamyl phosphate and L-ornithine[25] at pH 7.5. This pH is the optimum for the specified ornithine concentration (I. M. ISSALY, unpublished observations). The incubation mixture contained, in $\mu$moles: L-ornithine, 7.5; [$^{14}$C]carbamyl phosphate, 2; Tris–HCl (pH 7.5), 150; and enzyme preparation. Final volume, 0.425 ml. After incubation for 10 min at 37°, the reaction was stopped by addition of an equal volume of 1 M HCl and heated for 10 min at 100°. 0.2-ml aliquots were placed on planchets with detergent, dried by heating, and counted as indicated above.

Aspartate transcarbamylase (carbamoyl phosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) was determined by measuring the [$^{14}$C]carbamyl aspartate formed from [$^{14}$C]carbamyl phosphate and L-aspartic acid[26]. The incubation mixture contained, in $\mu$moles: potassium aspartate (pH 9.1), 7.5; [$^{14}$C]carbamyl phosphate, 2; Tris–glycine (pH 9.1), 100; and enzyme preparation. Final volume, 0.5 ml. After incubation for 10 min at 35° the reaction was stopped by addition of 0.25 ml of 1 M HCl. 0.2-ml aliquots were placed in planchets and treated as for ornithine transcarbamylase.

## RESULTS

### *Fractionation of carbamyl phosphate synthetase and carbamate kinase by $(NH_4)_2SO_4$*

All operations were performed at 0–4°. The buffer used was Tris–HCl, 0.02 M (pH 7.5), containing 0.1 mM GSH.

A cell-free extract from cells grown on minimal medium was dialyzed against buffer. After addition of streptomycin up to 5% and stirring for 15 min, the mixture was centrifuged for 15 min at 15 000 × *g* and the precipitate was discarded. Solid $(NH_4)_2SO_4$ was added slowly and with continuous agitation to the supernatant up to 1.5 M, and after stirring for 1 h in the cold, the mixture was centrifuged for 30 min at 25 000 × *g*. The precipitate was taken up in buffer and dialyzed against the same. This is Fraction I. The supernatant from the 25 000 × *g* centrifugation was made 2.5 M in $(NH_4)_2SO_4$ by the addition of solid salt, and the precipitate collected by centrifugation and dialyzed as for Fraction I. This is Fraction II. As shown in Table I, Fraction I contains all the carbamyl phosphate synthetase activity, and Fraction II most of the carbamate kinase activity. Assays of kinase activity at the pH optimum for the synthetase (7.5) and of synthetase activity at the pH optimum for the kinase (8.5) are also included in the table. The results rule out the possibility that enzyme systems indiscriminate as to substrate, but stringent as to pH, were being separated; and confirm that the carbamate-utilizing system (kinase) and the glutamine-utilizing system (synthetase) are different entities.

### *Purification of the carbamate kinase*

A typical run is described below. All operations were performed at 0–4°. The buffer used was Tris–HCl (pH 7.5), at the indicated concentrations, with 0.1 mM GSH. Solid $(NH_4)_2SO_4$ was added slowly and with continuous agitation, allowing

TABLE I

SEPARATION OF CARBAMYL PHOSPHATE SYNTHETASE AND CARBAMATE KINASE BY $(NH_4)_2SO_4$ FRACTIONATION

The reaction mixtures contained 1–2 mg of enzyme preparation.

| *Enzyme assayed* | *Expt. No.* | *Specific activities, assayed at the indicated pH values (units/mg protein)* | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Crude extract* | | *Fraction I** | | *Fraction II*** | |
| | | *pH 7.5* | *pH 8.5* | *pH 7.5* | *pH 8.5* | *pH 7.5* | *pH 8.5* |
| Carbamyl phosphate synthetase | 1 | 0.34 | | 0.75 | | 0.00 | |
| | 2 | 0.15 | | 0.11 | | 0.00 | |
| | 3 | 0.08 | | 0.17 | 0.00 | 0.00 | 0.00 |
| | 4 | 0.14 | | 0.43 | 0.00 | 0.02 | 0.00 |
| Carbamate kinase | 1 | | 0.46 | | 0.02 | | 2.50 |
| | 2 | | 0.21 | | 0.09 | | 0.31 |
| | 3 | | 0.15 | 0.02 | 0.03 | 0.09 | 0.80 |
| | 4 | | 0.23 | 0.00 | 0.01 | 0.02 | 1.30 |

* Precipitate at 1.5 M $(NH_4)_2SO_4$.
** Precipitate between 1.5 and 2.5 M $(NH_4)_2SO_4$.

30 min for precipitation. Centrifugations were carried out at 15 000 × *g* for 30 min.

3.4 ml of a 50% solution of streptomycin sulfate were added slowly and with agitation to 81 ml of cell-free extract, from 96 g of bacteria (wet wt.) grown on complex medium. The mixture was centrifuged and the supernatant made 1.8 M in $(NH_4)_2SO_4$. The supernatant obtained after centrifugation was made 2.4 M in $(NH_4)_2SO_4$. The mixture was centrifuged and the precipitate suspended in 0.02 M buffer, and dialyzed against the same buffer overnight. The dialyzate (56 mg protein) was chromatographed on a DEAE-cellulose column (1 cm × 20 cm) which had been previously equilibrated with 0.05 M buffer, and eluted with the same buffer in increasing concentrations: 0.05, 0.2, 0.4 and 0.8 M (80 ml of each). Fractions (5 ml each) were collected. The main peak of activity came out with the 0.2 M eluant. The fractions under this peak were pooled, made 3.4 M in $(NH_4)_2SO_4$ and centrifuged. The precipitate was dissolved in 0.05 M buffer and dialyzed overnight against the same buffer. The dialyzate was passed through a column of Sephadex G-200 (1 cm × 17 cm) which had been equilibrated with 0.05 M buffer, and eluted with the same buffer. The progress of the purification can be appreciated in Table II.

*Comparison of the properties of carbamyl phosphate synthetase and carbamate kinase*

In the studies reported below, the kinase was purified as in Table II and the synthetase as for Fraction I in Table I, unless otherwise specified. The greater lability of the latter enzyme precluded further purification.

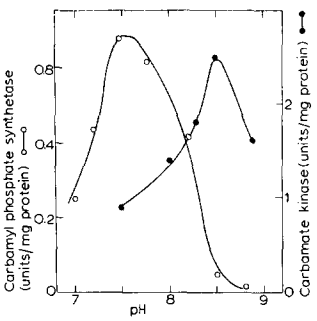
*pH optima.* They are 7.5 for the synthetase and 8.5 for the kinase (Fig. 1).

*Stability.* At 50° the half-life of the kinase is 12 min, while that of the synthetase is 5 min (Fig. 2). This difference in stability is more apparent in the cold. The synthetase inactivates overnight at either 2°, 0° or −20°, and no appreciable protection is afforded by the addition of $5 \cdot 10^{-4}$ M EDTA, 0.02% mercaptoethanol, 20% glycerol[17] or 0.03 M glutamine[16]; while 0.1 mM GSH has a small, but significant, stabilizing

TABLE II

PURIFICATION OF CARBAMATE KINASE

| *Fraction* | *Specific activity (units/mg protein)* | *Total activity in fraction (units)* | *Recovery (%)* | *Purification (×)* |
|---|---|---|---|---|
| I Cell-free extract | 0.46 | 310 | (100) | (1) |
| II 1.8–2.4 M $(NH_4)_2SO_4$ precipitate | 2.35 | 146 | 70 | 5 |
| III DEAE-cellulose eluate | 16.6 | 68 | 32 | 36 |
| IV Sephadex eluate | 22 | 38 | 18 | 48 |



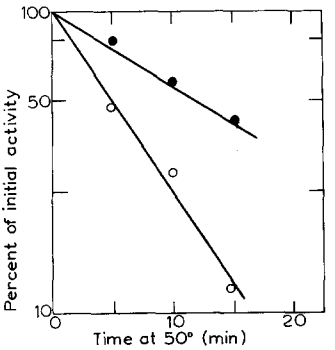
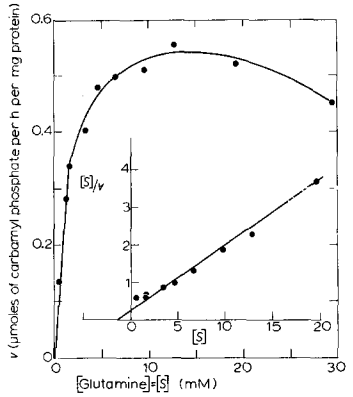




Fig. 1. Activities of carbamyl phosphate synthetase and carbamate kinase as a function of pH. Protein per incubation mixture: 2.5 mg (Fraction I) for the synthetase and 1.4 mg (Fraction II) for the kinase.

Fig. 2. Thermal inactivation of carbamyl phosphate synthetase (○—○) and carbamate kinase (●—●). Protein per incubation mixture: 2 mg.



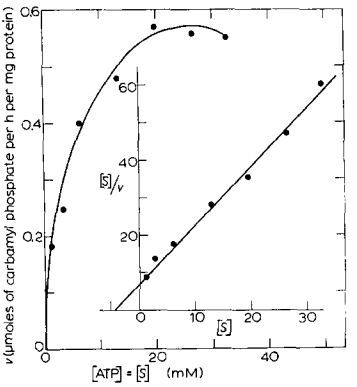
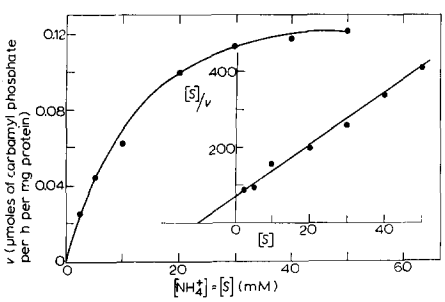




Fig. 3. Rate of carbamyl phosphate synthesis by carbamyl phosphate synthetase as a function of the concentration of glutamine. Protein per incubation mixture: 1.6 mg. $v$ = μmoles of carbamyl phosphate per h per mg protein; $[S]$ = glutamine concentration (mM).

Fig. 4. Rate of carbamyl phosphate synthesis by carbamyl phosphate synthetase as a function of the concentration of ATP. Protein per incubation mixture: 1.4 mg. The concentration of $MgCl_2$ was made equal to that of ATP.

effect. The carbamate kinase, on the other hand, was stable at 2° for at least 1 week in the presence of $5 \cdot 10^{-4}$ M EDTA *plus* 0.02% mercaptoethanol, and more than 3 weeks with 0.1 mM GSH.

*Substrates and Michaelis constants.* For the carbamyl phosphate synthetase the $K_m$ glutamine is 1.8 mM (Fig. 3), and the $K_m$ ATP is 4.9 mM (Fig. 4). $NH_4Cl$ can be substituted for glutamine. The resulting $K_m$ is high (10 mM), as shown in Fig. 5, and the maximal velocity ($v_{max}$) several fold lower than with glutamine (*cf.* Figs. 3 and 5). The possibility that this activity is due to contamination with the kinase cannot be excluded until methods for further purification of the synthetase become available. The effect of pH on this residual activity of ammonia with synthetase preparations (*cf.* carbamate kinase assay of Fraction I at the two pH values in Table I) would



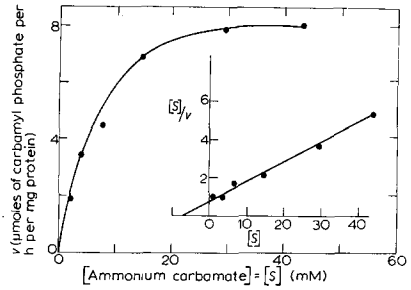




Fig. 5. Rate of carbamyl phosphate synthesis by carbamyl phosphate synthetase as a function of the concentration of $NH_4Cl$. The standard reaction mixture, with $NH_4Cl$ instead of glutamine and supplemented with 0.1 M potassium acetate, contained 1.9 mg protein.

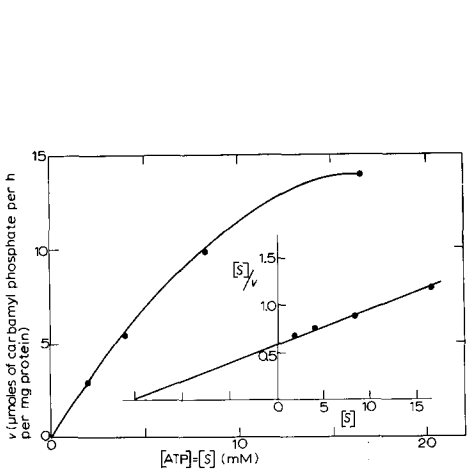
Fig. 6. Rate of carbamyl phosphate synthesis by carbamate kinase as a function of the concentration of ammonium carbamate. Protein per incubation mixture: 0.1 mg. Urea assayed colorimetrically. The carbamate concentration was assayed by the method of Faurholt as adapted by YASHPHE AND GORINI[27].

seem to encourage the contamination hypothesis. However, it may well be that pH optimum is also a function of the substrate, and hence an unreliable indication of which enzyme is involved. For the carbamate kinase the $K_m$ ammonium carbamate is 8 mM (Fig. 6) and the $K_m$ ATP is 15 mM (Fig. 7). Glutamine is not a substrate for this enzyme.

*Activation and inhibition.* Both enzymes require $Mg^{2+}$ for activity. A plateau in this activation is reached at 25 mM for the synthetase, and at 10 mM for the kinase. The $K_m$ $Mg^{2+}$ is 2.5 mM for the former enzyme, and 1.7 mM for the latter. $K^+$ (0.1 M) does not stimulate the synthetase.

The inhibition of carbamate kinase by glutamine can be appreciated in Fig. 8. The synthetase is not sensitive to $NH_4^+$ at the concentration tested (40 mM $NH_4Cl$).

As indicated by previous work with crude extracts[9], carbamyl phosphate synthetase is subject to feed-back inhibition by arginine and pyrimidines. These results were confirmed here using partially purified preparations. Table III shows the effect of arginine and various pyrimidines and derivatives, when added in mmolar concentrations to synthetase or kinase incubation mixtures. Notice that the synthetase is inhibited by UTP, arginine, uridine and CMP (in order of decreasing effect), while the kinase responds mostly to arginine, followed up by UMP and UTP. In



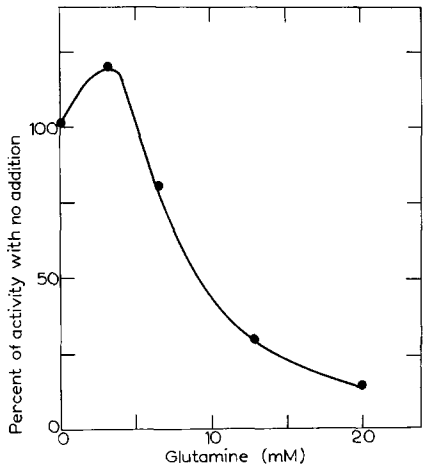




Fig. 7. Rate of carbamyl phosphate synthesis by carbamate kinase as a function of the concentration of ATP. The concentration of $MgCl_2$ was made equal to that of ATP. Protein per incubation mixture: 0.22 mg. Urea assayed colorimetrically.

Fig. 8. Inhibition of carbamate kinase by glutamine. Protein per incubation mixture: 2 mg.

considering the meaning of these differences, it should be born in mind that the two enzyme systems are assayed at different pH values.

The sum of arginine *plus* UTP gives a cummulative effect for the synthetase. The apparent cooperative effect observed in the assay made 24 h later, was not reproducible in further tests.

*Repression of carbamyl phosphate synthetase and induction of carbamate kinase.* The formation of carbamyl phosphate synthetase is repressed when arginine and uracil are added to the culture medium. If the arginine and uracil concentrations are

TABLE III

INHIBITION OF CARBAMYL PHOSPHATE SYNTHETASE AND CARBAMATE KINASE BY PYRIMIDINE DERIVATIVES AND ARGININE

The reaction mixtures contained approx. 2 mg of enzyme preparation. The two enzymes were fractionated as on Table I.

| *Additions to the incubation mixture (mM)* | *Relative activity** | | | | |
|---|---|---|---|---|---|
| | *Carbamyl phosphate synthetase* | | | *Carbamate kinase* | |
| | *Crude extract* | *Fraction I* | | *Crude extract* | *Fraction II* |
| Uridine | 0.72 | 0.76 | | 0.98 | 1.02 |
| UMP | 1.10 | 1.06 | | 0.67 | 0.70 |
| UTP | 0.18 | 0.20 | 0.25** | 0.70 | 0.80 |
| CMP | | 0.79 | | | 0.88 |
| CTP | | 0.92 | | | 0.87 |
| Citrulline | | 0.98 | | | 0.99 |
| Arginine | 0.70 | 0.69 | 0.77** | 0.65 | 0.60 |
| Arginine *plus* UTP | 0.08 | 0.09 | 0.03** | 0.60 | 0.58 |

* Relative to activity with no addition.
** Measured after storing for 24 h at 0–4°.

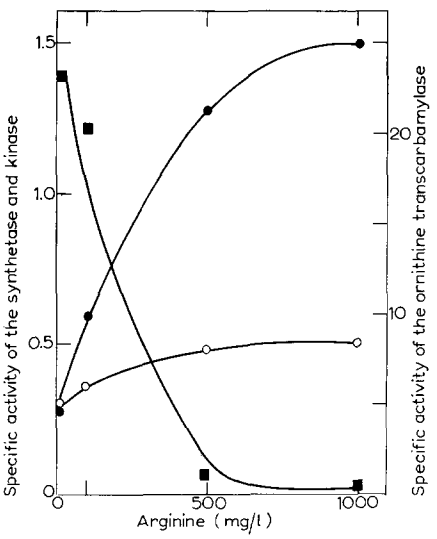
TABLE IV

REGULATION BY ARGININE AND URACIL OF THE LEVELS OF CARBAMYL PHOSPHATE SYNTHETASE AND CARBAMATE KINASE

The reaction mixtures contained 2.0–2.5 mg of enzyme preparation in carbamyl phosphate synthetase and carbamate kinase assays, and 0.2–0.3 mg in ornithine and aspartate transcarbamylase assays. The standard strain used is prototrophic for arginine and uracil.

| *Supplements added to the culture media (1 g/l of each)* | *Specific activities (units/mg protein)* | | | |
|---|---|---|---|---|
| | *Carbamyl phosphate synthetase* | *Carbamate kinase* | *Ornithine transcarbamylase* | *Aspartate transcarbamylase* |
| None | 0.30 | 0.33 | 1.60 | 4.35 |
| Arginine | 0.35 | 1.60 | 0.42 | 9.05 |
| Uracil | 0.03 | 0.36 | 1.30 | 0.34 |
| Arginine *plus* uracil | 0.02 | 0.16 | 0.44 | 0.27 |

low (100–200 mg/l), no repression is detected when either arginine or uracil are added alone, but a concerted effect becomes manifest in the presence of both[9]. At higher concentrations (1 g/l) the repressive effect of uracil alone overshadows that of arginine. This is shown in Table IV, together with the effect of the same nutrients on carbamate kinase. Notice that uracil by itself has no effect on the synthesis of the kinase. Arginine alone induces it, increasing 5-fold its specific activity, but this induction is abolished in the presence of uracil. Table IV also reports the levels of



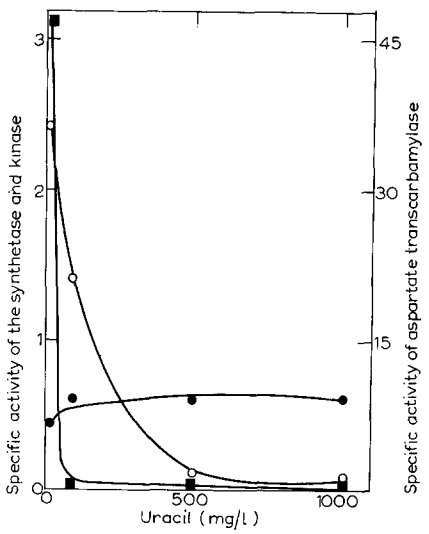




Fig. 9. Effect of the addition of arginine to the culture medium (minimal) on the specific activity of carbamate kinase (●—●), carbamyl phosphate synthetase (○—○) and ornithine transcarbamylase (■—■). Activity was measured on crude cell-free extracts of the *arg* strain. Protein per incubation mixture: 1.4–2 mg for carbamate kinase and carbamyl phosphate synthetase; 0.2–0.4 mg for ornithine transcarbamylase.

Fig. 10. Effect of the addition of uracil to the culture medium (minimal) on the specific activity of carbamyl phosphate synthetase (○—○), carbamate kinase (●—●) and aspartate transcarbamylase (■—■). The lowest uracil concentration is 2 mg/l. Activity was measured with crude cell-free extracts of the *try C2ura* strain. Protein concentrations similar to Fig. 9.

ornithine transcarbamylase and aspartate transcarbamylase in the same cultures. Since the synthesis of these enzymes is repressed by arginine and uracil, respectively, their levels provide independent indices of the sizes of the end-product pools.

The induction of carbamate kinase by arginine in an arginine auxotroph is described in Fig. 9. The effects of arginine on the synthetase and on ornithine transcarbamylase, are also included in this figure for the purpose of comparison. Notice that the level at which arginine induces the kinase is not significantly different from that repressing ornithine transcarbamylase. Fig. 10 shows the course of repression of carbamyl phosphate synthetase by uracil in a uracil auxotroph. Notice the absence of effect on carbamate kinase. This repression is not coordinate with that of aspartate transcarbamylase.

*Effects of mutations.* Mutants $cap_1$ and $cap_2$ were selected as described under MATERIALS AND METHODS. Since the selection was for arginine and/or pyrimidine requirement, the appearance of double auxotrophs among comparable numbers of single auxotrophs[9] is a strong indication that they originated by a single mutational step. The acquisition of a double requirement by single-step mutation is easily understood on the assumption of a single carbamyl phosphate synthetase serving both the arginine and the pyrimidine pathways. The reduced levels of synthetase found in these strains (approx. 10% the normal values) provide strong support for this hypothesis. Furthermore, four independently isolated revertants of $cap_1$, selected as prototrophs, displayed restoration of the synthetase to normal levels.

The situation is, however, more complicated. The *cap* mutants are also deficient in carbamate kinase when compared with their $cap^+$ counterparts, after growth in media with various concentrations of arginine and uracil (Table V). One mutant ($cap_1$) was tested for kinase and synthetase activities after growth on a far more extensive range of arginine and uracil concentrations than reported in the former table, yet the deficiency for both enzymes was confirmed in every culture. As expected, arginine starvation derepressed ornithine transcarbamylase in the arginine auxotrophs. It was surprising, however, to find that large excess of uracil reversed

TABLE V

EFFECTS OF THE *cap* MUTATION ON ENZYME LEVELS

Protein per incubation mixture, 1.5–2.5 mg in carbamyl phosphate synthetase and carbamate kinase assays, and 0.2–0.3 mg in ornithine transcarbamylase assay. Some values are averages of several experiments. $cap^+$ is the standard strain; $cap^{+r}$ is a revertant from $cap_1$. Supplements in the culture medium: For the prototrophic strains, low arg. (arginine) means no addition, high means addition of 1 g/l. For the *arg* and *cap* strains the medium was supplemented with 10 mg/l arginine at the low level, and 1 g/l at the high level. Uracil was added as indicated.

| *Genotype* | *Uracil (mg/l)* | *Specific activities (units/mg protein)* | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Carbamyl phosphate synthetase* | | *Carbamate kinase* | | *Ornithine transcarbamylase* | |
| | | *Low arg.* | *High arg.* | *Low arg.* | *High arg.* | *Low arg.* | *High arg.* |
| $cap^+$ | | 0.30 | 0.35 | 0.33 | 1.60 | 1.60 | 0.42 |
| $cap^{+r}$ | | 0.38 | 0.45 | 0.40 | 1.30 | 0.98 | 0.30 |
| *arg* | | 0.29 | 0.50 | 0.29 | 1.50 | 23.00 | 0.32 |
| $cap_1$ | 2 | 0.04 | 0.01 | 0.01 | 0.16 | 0.57 | 0.27 |
| | 100 | | | 0.04 | 0.14 | 5.60 | 0.25 |
| | 1000 | 0.00 | 0.02 | 0.03 | 0.04 | 1.50 | 0.45 |
| $cap_2$ | 2 | 0.06 | 0.01 | 0.01 | 0.12 | 0.41 | 0.17 |

this derepression in $cap_1$ (Table V). Less drastic instances of this effect have been observed in $cap^+$ strains.

As a test for the possibility that the kinase of *cap* was structurally altered, its pattern of inhibition by arginine and pyrimidine derivatives was compared with that of control strains. No difference was found.

The alternative that *cap* mutants are deficient in a thermostable cofactor was also tested by addition of boiled $cap^+$ preparations to fresh *cap* extracts, and assay of carbamyl phosphate synthetase and carbamate kinase activities. The boiled extracts failed to stimulate.

## DISCUSSION

The two enzyme systems from *B. subtilis* studied here, carbamyl phosphate synthetase and carbamate kinase, are capable of catalyzing the synthesis of carbamyl phosphate from $CO_2$ *in vitro*. There is also a third enzyme, acetate kinase, with the same activity in *B. subtilis*[23]. This latter enzyme does not interfere with the measurement of either of the former, so long as glutamine is used as source of nitrogen, or acetate is added to the medium[9,27].

Carbamate kinase and carbamyl phosphate synthetase were separated from each other by $(NH_4)_2SO_4$. Besides differing in pH optimum and nitrogenous substrate, the two systems differ in the pattern of inhibition. Could it be that the differences in the two enzyme systems are artifacts due to alterations in the structure of an originally homogeneous molecular population? This question is raised in view of the reported behavior of carbamyl phosphate synthetase from other organisms[8,28–30]. Such artifacts are excluded here by the observation that the ratio of the two activities is dependent, in a very reproducible fashion, upon the conditions under which the culture was grown. These same results give us the clue to the physiological role of each enzyme. The synthetase is repressed by growth on high concentrations of uracil. At subthreshold concentrations of uracil, addition of arginine elicits repression in a concerted fashion. *In vitro*, UTP is the main feed-back inhibitor, with arginine causing additional inhibition (cumulative, or nearly so). These patterns of repression and inhibition provide strong evidence in favor of a biosynthetic role for this enzyme.

Carbamate kinase, on the other hand, is induced by arginine. This suggests that this enzyme plays a role in arginine catabolism, possibly along the sequence indicated in the following scheme:

$$\text{arginine} \xrightarrow{1} \text{citrulline} \xrightarrow{2} \text{carbamyl phosphate} \xrightarrow{3} CO_2 + NH_3 + \text{ATP}$$

This sequence is supported by work with various microorganisms[31], and there is evidence for the occurrence of Reactions 2 (refs. 32, 33) and 3 (this paper) in the genus Bacillus. However, the precise nature of this pathway remains unclear because attempts to demonstrate the enzyme expected to catalyze Reaction 1 (L-arginine iminohydrolase, EC 3.5.3.6) have not been successful, and because of the existence of an alternative pathway of arginine degradation[32,34]. Furthermore, two of the properties of carbamate kinase reported in this paper are not the ones one would expect to find in a purely catabolic enzyme. One is its sensitivity to inhibition by arginine. The other is the ability of uracil to abolish the arginine induction of this enzyme. The latter effect may be rationalized as a mechanism to salvage carbamyl

phosphate from excessive depletion by the kinase when the synthetase is subject to concerted repression by uracil and arginine.

A single carbamyl phosphate synthetase, common for both the arginine and pyrimidine pathways, is indicated by the finding of concerted effects in repression. Furthermore, the pattern of feed-back inhibition in enzymes extracted from arginine- or uracil-supplemented cultures was the same regardless of the culture conditions[9]. If there is only one synthetase, we expect that single mutation at the synthetase locus would lead to simultaneous auxotrophy for arginine and uracil. The mutants $cap_1$ and $cap_2$ are precisely this kind of mutant: they are deficient in the synthetase and require both arginine and pyrimidines. Their frequence of forward mutation and of reversion leaves little doubt that they are single mutants (baring "concomitant mutations" of the type described by VOGEL AND BACON[35]). The value of these mutants as definitive argument in favor of the occurrence of a single carbamyl phosphate synthetase is blunted by the observation that they are also deficient in the kinase. The occurrence of such a pleiotropic effect, affecting enzymes with different regulatory regimes—hence not part of the same operon—raises questions but provides no answers. The possibility that the two enzymes may share structural or regulatory elements is worthy of consideration as a working hypothesis.

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