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Characterization of Pyrimidine-Repressible and Arginine-Repressible Carbamyl Phosphate Synthetases from *Bacillus subtilis*

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The number and properties of carbamyl phosphate synthetases in *Bacillus subtilis* have been uncertain because of conflicting genetic results and instability of the enzyme in extracts. The discovery of a previously unrecognized requirement of *B. subtilis* carbamyl phosphate synthetases for a high concentration of potassium ions for activity and stability permitted unequivocal demonstration that this bacterium elaborates two carbamyl phosphate synthetases. Carbamyl phosphate synthetase A was shown to be repressed by arginine, to have a molecular weight of about 200,000, and to be coded for by a gene that maps near *argC4*. This isozyme was insensitive to metabolites of the arginine and pyrimidine biosynthetic pathways. Carbamyl phosphate synthetase P was found to be repressed by uracil, to have a molecular weight of 90,000 to 100,000, and to be coded for by a gene that maps near the other *pyr* genes. This isozyme was activated by phosphoribosylpyrophosphate and guanine nucleotides and was strongly inhibited by uridine nucleotides. Other kinetic properties of the two isozymes were compared. *Bacillus* thus resembles eucaryotic microbes in producing two carbamyl phosphate synthetases, rather than the enteric bacteria, which produce a single carbamyl phosphate synthetase.

The aspartate transcarbamylase from *Bacillus subtilis* is unlike this enzyme from many other microbes in that it is not subject to feedback inhibition by the end products of the pyrimidine biosynthetic pathway (4, 15). Regulation of pyrimidine biosynthesis at the level of feedback inhibition in *B. subtilis* is therefore probably exerted on the synthesis of carbamyl phosphate, which is a precursor to both pyrimidine nucleotides and arginine. There are conflicting reports in the literature concerning the number and properties of carbamyl phosphate synthetase(s) in *B. subtilis* (10, 17). Issaly et al. (10) concluded that *B. subtilis* resembles the enteric bacteria (16) in containing a single carbamyl phosphate synthetase that is regulated jointly by the end products of the arginine and pyrimidine pathways. This proposal was largely based on the isolation of an apparent point mutation producing auxotrophy for both arginine and uracil. More recently, Potvin and Gooder (17) have argued that *B. subtilis* possesses two carbamyl phosphate synthetases, as has been shown in eucaryotic microbes (11, 24). Their evidence was also largely based on the properties of mutant strains, which displayed arginine-sensitive and uracil-sensitive phenotypes. Both mutants were capable of growth on minimal medium, but growth was inhibited by arginine or uracil, respectively. This growth inhibition was reversed in both strains by addition of uracil plus arginine to the medium, which would eliminate a requirement for carbamyl phosphate synthetase. These properties are readily explained by proposing that each mutant lacks one of two carbamyl phosphate synthetases and that addition of arginine or uracil represses synthesis of the remaining carbamyl phosphate synthetase, which leads to a requirement for the end product not added to the medium. A similar situation has been described in studies of *Saccharomyces cerevisiae* mutants (11).

These conflicting genetic studies have not been resolved. Potvin and Gooder were not able to demonstrate clearly that the two mutations described above map at different loci, as must be the case if they are mutations in structural genes for two carbamyl phosphate synthetases. Furthermore, both the arginine-sensitive and uracil-sensitive phenotypes have been described in *Salmonella typhimurium* (2), an organism that is known to contain a single carbamyl phosphate synthetase. In both of the previous studies an unequivocal resolution of the problem by biochemical studies was prevented by the apparent instability of the enzyme(s) and difficulty

in developing a reliable assay in cell extracts (10, 17).

Because of a continuing interest in the regulation of nucleotide biosynthesis in *B. subtilis* (20) we have reinvestigated the question of the number and nature of carbamyl phosphate synthetases. In this paper, we report the development of procedures for convenient and reliable assay of the enzyme in extracts of *B. subtilis* cells and demonstrate the existence of two functionally distinct carbamyl phosphate synthetases. Physical, kinetic, and regulatory properties of the two enzymes are reported. A preliminary report of these studies has appeared (T. J. Paulus, Fed. Proc. **37**:1798, 1978).

MATERIALS AND METHODS

Bacterial strains. The standard strain used in this investigation was *B. subtilis* 168 (Trp^-) received from J. Hageman, New Mexico State University, Las Cruces. The arginine-sensitive H37 and the uracil-sensitive H59 strains of *B. subtilis* were kindly provided by B. Potvin, Columbia University, New York (17). A second uracil-sensitive strain, JH861 (*trpC2 urs-1*), and its isogenic sibling were constructed and generously provided by J. A. Hoch, Research Institute of Scripps Clinic, La Jolla, Calif.

Media and culture methods. The bacteria were grown in buffered medium containing 0.1% glucose, 50 μg each of 19 amino acids (excluding arginine) per ml, and a salts mixture (3). When it was necessary to supplement with arginine or uracil, 50 or 100 μg/ml was added. Growth was monitored with a Klett-Summerson colorimeter using a no. 66 filter. Phenotypes of the mutant strains were checked on solid medium containing 0.1% glucose, 0.1% glutamate, and a salts mixture (5). Supplements were added at 50 μg/ml.

Preparation of extracts. Cells were harvested during log phase by centrifugation at 16,000 × *g* at 4°C. The pellets were washed with 50 mM potassium phosphate buffer (pH 7.6) containing 10% glycerol (by volume) and then recentrifuged as above. At this point the pellets could be frozen in liquid nitrogen and stored at −70°C for several weeks. The pelleted cells were suspended at a ratio of 40 ml of the original culture into 1 ml of 100 mM potassium phosphate buffer (pH 7.6) containing 10% glycerol. The cells were broken by sonic disruption at 4°C for a total of 2 min using 15-s bursts with a 1-min cooling period between bursts. These extracts were centrifuged at 17,000 × *g* for 15 min, and the supernatant solution, routinely about 8 mg of protein per ml, was saved for assay. In sonic extracts prepared by this procedure, carbamyl phosphate synthetase activity was stable at −20°C for several days.

Before assay, extracts were routinely dialyzed for 3 to 4 h against 100 volumes of 50 mM triethanolamine hydrochloride, 10% glycerol (by volume), 100 mM KCl, and 1 mM reduced glutathione. The final pH was 7.7 at 4°C. The buffer was changed once approximately halfway through dialysis. In later experiments, 25 mM potassium *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.7) was substituted for 50 mM triethanolamine.

Carbamyl phosphate synthetase assay. The standard reaction mixture for carbamyl phosphate synthetase activity contained 100 mM triethanolamine hydrochloride, 200 mM KCl, 36 mM $MgCl_2$, 24 mM ATP, 10 mM L-glutamine, 10 mM [^{14}C]$KHCO_3$ (100 to 400 cpm/nmol), 1 mM reduced glutathione, and extract. The final volume was 0.5 ml. The pH of the reaction mixture at 37°C was 7.6. During this investigation it was discovered that triethanolamine hydrochloride was inhibitory. Therefore, the characterization of the kinetic properties was performed using 50 mM potassium HEPES. The amount of [^{14}C]carbamyl phosphate formed was measured by two methods. Method I was used in all cases, unless otherwise noted.

(i) Method I. The carbamyl phosphate formed was converted enzymatically to carbamyl aspartate using a partially purified preparation of *B. subtilis* aspartate transcarbamylase (4). In this case 25 or 50 mM potassium L-aspartate and 1 IU of aspartate transcarbamylase activity (containing no carbamyl phosphate synthetase activity) was present in the standard reaction mixture during the assay. The reaction was terminated by the addition of 0.1 ml of 20% trichloroacetic acid. The mixtures were centrifuged, and a 0.5-ml portion was transferred to a 20-ml scintillation vial. The excess [^{14}C]$KHCO_3$ was removed from the acidified sample by evaporating the samples to dryness in a ventilated oven at 45 to 60°C. The samples were resolubilized by the addition of 1 ml of water, and 10 ml of a scintillation mixture containing 25% Triton X-114, 75% xylene, and 0.4% diphenyloxazole was added for scintillation counting.

(ii) Method II. In this method, carbamyl phosphate was converted chemically to hydroxyurea by the procedure of Levine and Kretchmer (13). This assay was used when testing carbamyl phosphate synthetase activity as a function of pH and when it was desirable to avoid addition of potassium L-aspartate. The reaction was terminated by the addition of 50 μl of 2 M hydroxylamine hydrochloride and placed in a boiling water bath for 10 min. After the samples were cooled on ice, they were acidified by the addition of 50 μl of 50% trichloroacetic acid and processed for scintillation counting as described in Method I. The efficiency of trapping of carbamyl phosphate by this procedure was about 40% of the enzymatic method.

The reaction products of the above methods were characterized as carbamyl aspartate and hydroxyurea, respectively, using ion exchange paper chromatography (8) and a conventional Whatman paper chromatographic system (7). Commercially available carbamyl aspartate and hydroxyurea were used as standards. With both methods, the reaction was linear with time for at least 30 min at 37°C and linear with extract up to 0.8 mg of protein. Activities are reported as nanomoles of product formed per minute.

Aspartate transcarbamylase and ornithine transcarbamylase assays. Aspartate transcarbamylase and ornithine transcarbamylase were assayed in 100 mM potassium phosphate (pH 7.6), 10 mM carbamyl phosphate, and either 50 mM potassium L-aspartate or 5 mM potassium L-ornithine, respectively. The amount of carbamyl aspartate and citrulline formed was quantitated colorimetrically by the method of Prescott and Jones (19); activities are re-

ported as nanomoles of product formed per minute.

Agarose gel filtration. A Bio-Gel A-1.5 m column (47 by 1.5 cm) was used. The column was equilibrated and eluted with 100 mM potassium phosphate, 10% glycerol, and 1 mM reduced glutathione (pH 7.6 at 4°C). Sample volumes of 1.5 ml were applied to the top of the column, and 1.0-ml fractions were collected at a flow rate of 6 ml/h.

Chemicals. Bio-Gel A-1.5 m (100 to 200 mesh) was purchased from Bio-Rad. Bovine serum albumin, ovalbumin, pyruvate kinase, and yeast alcohol dehydrogenase were purchased from Sigma Chemical Co. ATP was purchased from Sigma as the disodium salt; for most experiments it was converted to the potassium salt by treatment with Dowex 50X-8 (H^+) and neutralization with potassium hydroxide. [^{14}C]$KHCO_3$ was purchased from Amersham/Searle. All other chemicals were of reagent grade quality.

Protein measurement. Protein concentrations were determined by the method of Lowry et al. (14) after precipitation with 10% trichloroacetic acid and washing with ethanol. Bovine serum albumin was used as a standard.

RESULTS

Development of a reliable assay for *Bacillus* carbamyl phosphate synthetase. Initial attempts to assay carbamyl phosphate synthetase activity in sonic extracts or detergent-treated cells using the methods previously reported for the *B. subtilis* enzyme (17) were uniformly unsuccessful. Use of the assay conditions previously developed for the *Salmonella* enzyme (1) gave readily detectable activity, however. This led to the discovery that, contrary to previous reports (10, 17), *B. subtilis* carbamyl phosphate synthetase(s) absolutely requires potassium ions for activity. Inclusion of potassium ions and glycerol in buffers for preparation and storage of cell extracts and use of the assay conditions described in Materials and Methods enabled sensitive and reproducible assays to be made, which were linear with time of reaction and the amount of enzyme assayed.

Response of carbamyl phosphate synthetase activity to supplementation of the medium with uracil or arginine. The amount and properties of the carbamyl phosphate synthetase activity of *B. subtilis* were dramatically affected by growth of the cells in the presence of uracil or arginine (Table 1). Cells grown on basal medium contained the highest level of enzyme. The activity of the enzyme was strongly, but not completely, inhibited by 5 mM UMP and was stimulated by phosphoribosylpyrophosphate (PRPP). Both aspartate transcarbamylase and ornithine transcarbamylase activities were present in the cell extracts, which indicated that both pyrimidine and arginine biosynthetic pathways were derepressed. When arginine was added to the medium, the cells contained slightly less carbamyl phosphate synthetase activity, which was fully inhibited by 5 mM UMP. Ornithine transcarbamylase was repressed under these conditions. (The repressive control of ornithine transcarbamylase and the arginine-repressible carbamyl phosphate synthetase is complex. As long as cells were harvested in midlog phase, both enzymes were fully repressed by 50 μg of arginine per ml or higher. If the cells were harvested at the end of exponential growth or in early stationary phase, however, both of these activities were present in substantial levels even with 200 μg of arginine per ml. Legrain et al. [12] have also reported an apparent induction of the biosynthetic ornithine transcarbamylase in *B. subtilis* under conditions of oxygen limitation.) Supplementation of the medium with uracil yielded cells with sharply reduced carbamyl phosphate synthetase activity and repressed levels of aspartate transcarbamylase. The remaining carbamyl phosphate synthetase activity was, however, virtually insensitive to uridine nucleotides or PRPP. Arginine and ornithine did not have significant or reproducible effects on the carbamyl phosphate synthetase activity under any growth conditions. Growth of the cells in the presence of both uracil and arginine resulted

TABLE 1. *Dependence of the specific activity and sensitivity to effectors of carbamyl phosphate synthetase on growth conditions*

Growth condition	Sp act[a] of:			Relative enzyme activity[b] in presence of:				
	Carbamyl phosphate synthetase	Ornithine transcarbamylase	Aspartate transcarbamylase	Mg-UMP	Mg-UTP	Arginine	Mg-PRPP	Ornithine
Basal	4.75	256	60	0.08	0.25	0.90	1.58	1.00
+Arginine	4.55	3	64	0.01	0.16	1.00	1.73	1.00
+Uracil	0.93	289	7	0.89	0.99	0.97	1.00	1.00
+Arginine + uracil	0	3	8					

[a] Expressed as nanomoles per minute per milligram of protein.

[b] Carbamyl phosphate synthetase activity in the absence of effector is defined as 1.00. Effectors were used at 5 mM each.

in complete repression of carbamyl phosphate synthetase.

Gel filtration of carbamyl phosphate synthetase activity from cells grown with and without uracil and arginine supplementation. A sonic extract of *B. subtilis* cells grown in the presence of arginine was analyzed by agarose gel chromatography (Fig. 1A). A single peak of carbamyl phosphate synthetase activity emerged at a position corresponding to a molecular weight of about 100,000. This activity was completely inhibited by 5 mM UMP. When this experiment was repeated with an extract of cells grown in the presence of uracil, a single peak of carbamyl phosphate synthetase activity was again resolved, but in this case at a volume corresponding to a molecular weight of 200,000 (Fig. 1B). The activity was uniformly inhibited by about 10% by 5 mM UMP. Sonic extracts of cells grown on basal medium yielded a broad peak of carbamyl phosphate synthetase activity (Fig. 1C). Assay of the column fractions in the presence of 5 mM UMP demonstrated a peak of activity in a position corresponding to the activity in uracil-repressed cells. Thus, it appears that the carbamyl phosphate synthetase activity of cells grown on basal medium consists of two components, which are partially separated by gel filtration. The smaller of these components was completely inhibited by 5 mM UMP, as is the carbamyl phosphate synthetase activity of arginine-repressed cells.

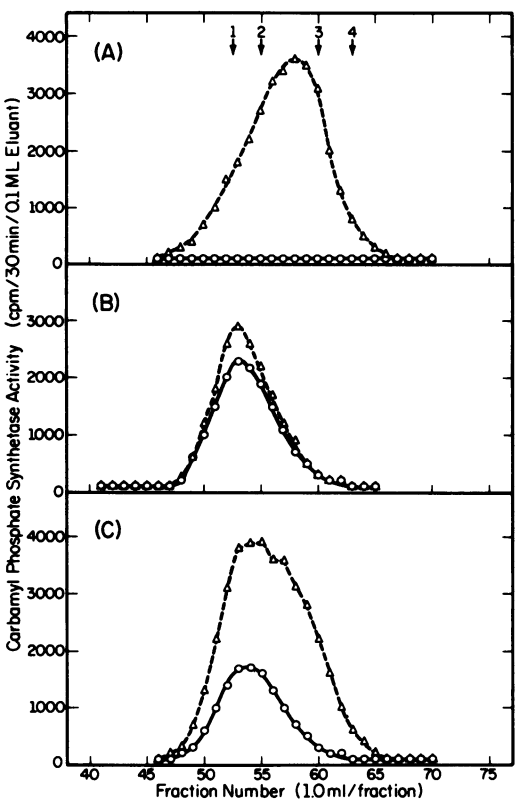

FIG. 1. *Gel filtration chromatography of carbamyl phosphate synthetase from cells grown with and without arginine and uracil.* (△) *Activity assayed without addition of effectors;* (○) *activity assayed in the presence of 5 mM UMP. One nanomole of carbamyl phosphate corresponds to 340 cpm. (A) Extract of arginine-repressed cells. (B) Extract of uracil-repressed cells. (C) Extract of cells grown on basal medium. Numbered arrows indicate the elution volume of the standard proteins, which have molecular weights as follows: 1, pyruvate kinase (237,000); 2, yeast alcohol dehydrogenase (151,000); 3, bovine serum albumin (68,000); 4, ovalbumin (45,000).*

Carbamyl phosphate synthetase activities in arginine-sensitive and uracil-sensitive mutants. It has been proposed that arginine-sensitive and uracil-sensitive phenotypes arise from the mutational loss of uracil-repressible and arginine-repressible carbamyl phosphate synthetases, respectively (9, 17). To test this proposal we examined the properties of the carbamyl phosphate synthetase activities of three such mutants, kindly provided by B. Potvin and J. Hoch. The mutants were grown on basal medium, and sonic extracts were assayed for carbamyl phosphate synthetase activity in the presence and absence of effectors (Table 2). The carbamyl phosphate synthetase activity of the uracil-sensitive strains JH861 and H59 responded to UMP and PRPP in the same way as did cells of the wild-type strain that were grown in the presence of arginine. The carbamyl phosphate synthetase activity of the arginine-sensitive strain H37 was almost insensitive to UMP and PRPP, as was the activity of uracil-repressed wild-type cells.

It is important to note that Potvin et al. (18) have found the H37 (arginine-sensitive) mutation to map adjacent to the other *pyr* genes of *B. subtilis*. They did not map the mutation in strain H59, but the uracil-sensitive mutation of strain JH861 has been mapped next to the *argC4* locus (9).

Gel filtration of the carbamyl phosphate synthetase activities from arginine-sensitive and uracil-sensitive mutants. Figure 2 shows the results of agarose gel chromatography of the carbamyl phosphate synthetase activities in extracts of strains H37 (arginine-sensitive) and JH861 (uracil-sensitive) grown on basal medium. The uracil-sensitive mutant produced a single peak of activity, which was completely inhibited by 5 mM UMP (Fig. 2A). The molecular weight of this activity was estimated to be 90,000. The arginine-sensitive mutant also produced a single peak of carbamyl phosphate syn-

TABLE 2. *Properties of carbamyl phosphate synthetase activity in mutant strains grown on basal medium*

Strain	Sp act[a] of:			Relative enzyme activity[b] in presence of:				
	Carbamyl phosphate synthetase	Ornithine transcarbamylase	Aspartate transcarbamylase	Mg-UMP	Mg-UTP	Arginine	Mg-PRPP	Ornithine
168[c]	4.75	256	60	0.08	0.25	0.90	1.58	1.00
JH862[c]	2.46	282	61	0.14	0.34	0.96	1.31	1.00
JH861 (uracil-sensitive)	2.05	394	73	<0.01	0.21	1.00	1.46	1.00
H59 (uracil-sensitive)	2.67	389	90	<0.01	0.26	0.98	1.66	1.00
H37 (arginine-sensitive)	0.69	340	299	0.82	1.00	0.99	1.16	1.06

[a] Expressed as nanomoles per minute per milligram of protein.

[b] Carbamyl phosphate synthetase activity in the absence of effector is defined as 1.00. Effectors were used at 5 mM each.

[c] Wild type with respect to carbamyl phosphate synthetase.

thetase activity, which had a molecular weight of 200,000 (Fig. 2B). This activity was only slightly inhibited by 5 mM UMP. The patterns with uracil-sensitive and arginine-sensitive mutants are strikingly similar to those seen with wild-type cells grown in the presence of arginine and uracil, respectively (Fig. 1A and B).

Heat stability of carbamyl phosphate synthetase activities from uracil-repressed and arginine-repressed cells. Sonic extracts from arginine-repressed and uracil-repressed cells were incubated at 44°C (Fig. 3). At 44°C the carbamyl phosphate synthetase activity of arginine-repressed cells declined with a half-life of 10.5 min, whereas the activity of uracil-repressed cells decayed with a half-life of 6 min. The carbamyl phosphate synthetase activity of uracil-repressed cells was also less stable during dialysis at 4°C and storage at −20°C.

Kinetic properties of the arginine-repressible and uracil-repressible carbamyl phosphate synthetases. The results presented above justify the conclusion that *B. subtilis* elaborates two carbamyl phosphate synthetases, which will be designated hereafter as carbamyl phosphate synthetase A and carbamyl phosphate synthetase P. Carbamyl phosphate synthetase A is produced by a gene mapping near *argC4*, has a molecular weight of about 200,000, is repressed by arginine, is insensitive to UMP and PRPP, and is more heat labile than carbamyl phosphate synthetase P. Carbamyl phosphate synthetase P is governed by a gene mapping near other *pyr* genes, has a molecular weight of 90,000 to 100,000, is repressed by uracil, is stimulated by PRPP and inhibited completely by 5 mM UMP, and is more stable than carbamyl phosphate synthetase A. In the foregoing studies the two activities were assayed under identical conditions. Having established the occurrence of the two species, we determined their individual kinetic properties more completely in studies with uracil-repressed (carbamyl phosphate synthetase A) and arginine-repressed (carbamyl phosphate synthetase P) cell extracts, using conditions optimal for each isozyme.

When Na^+ ions were substituted for K^+ ions, using either tris(hydroxymethyl)aminomethane-hydrochloride or triethanolamine hydrochloride as the buffer, no carbamyl phosphate synthetase activity was detectable. Figure 4 shows the K^+ ion activation curves for carbamyl phosphate synthetases A and P, using Na-HEPES as the buffer. The optimal K^+ concentrations were 100 mM and 250 mM for carbamyl phosphate synthetases A and P, respectively. Concentrations of Na^+ ions up to 460 mM failed to elicit activity. Other cations, such as Li^+, tris(hydroxymethyl)aminomethane, triethanolamine, tetramethylammonium, or tetraethylammonium, when added at 50 mM in the presence of 300 mM K^+, were inhibitory. The two carbamyl phosphate synthetases were quite similar in their sensitivities to cations, but the arginine-repressible enzyme was generally inhibited more by equivalent cation concentration.

Both carbamyl phosphate synthetases A and P have a requirement for free magnesium ions. When 18 mM magnesium ion was added in combination with 6 mM ATP, both enzymes were stimulated about twofold over the activity seen with 6 mM $MgCl_2$ plus 6 mM ATP. When excess ATP was added, the enzymes were almost totally inhibited, even though the concentration of Mg-ATP was still approximately 6 mM. We interpret this inhibition to be due to the removal of free magnesium ions by chelation. Mg-ATP concentrations as high as 40 mM were not inhibitory. A requirement for free magnesium of carbamyl phosphate synthetases from other sources has also been reported (21).

Other kinetic properties of the two carbamyl

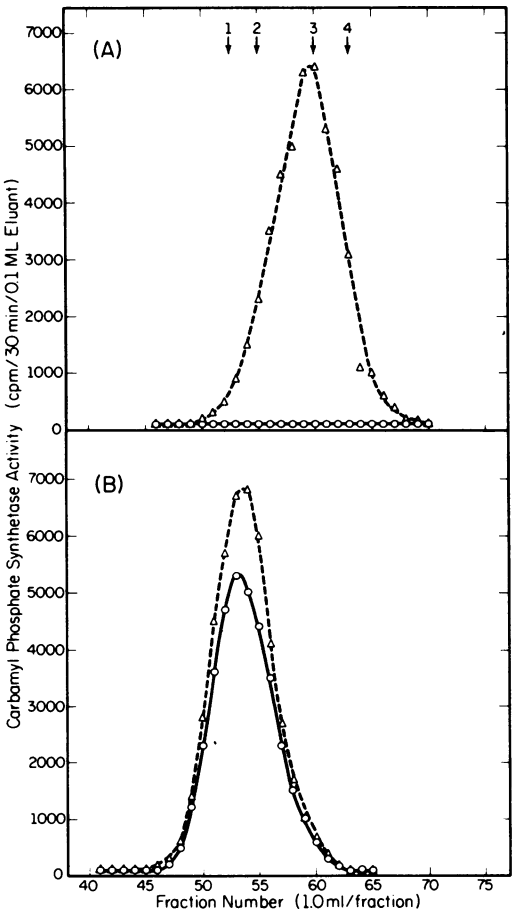

FIG. 2. *Gel filtration chromatography of carbamyl phosphate synthetase from mutant strains grown on basal medium.* (△) *Activity assayed without addition of effectors;* (○) *activity assayed in the presence of 5 mM UMP. One nanomole of carbamyl phosphate corresponds to 350 cpm. (A) Extract of strain JH861 trpC2 (uracil-sensitive). (B) Extract of strain H37 (arginine-sensitive). The standard proteins were as in Fig. 1.*

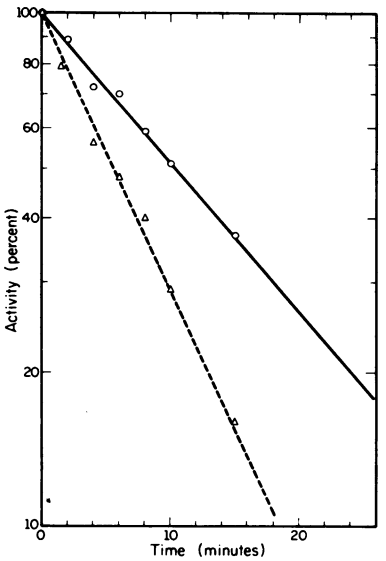

FIG. 3. *Inactivation of carbamyl phosphate synthetases A and P by heating. Extracts of arginine-repressed* (○) *and uracil-repressed* (△) *cells were each adjusted to 12 mg of protein per ml and incubated at 44°C. Samples were placed on ice at the times shown and assayed. For* (△) *100% is 0.83 nmol/min per mg of protein; for* (○) *100% is 3.53 nmol/min per mg of protein.*

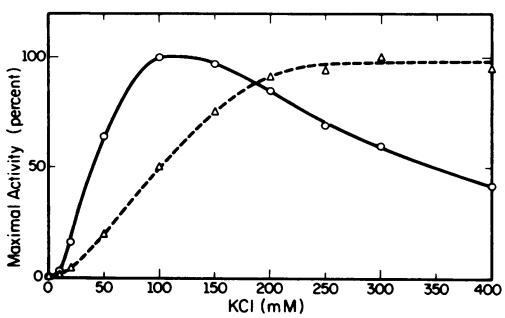

FIG. 4. *Activation of carbamyl phosphate synthetases A and P by potassium ions. Carbamyl phosphate synthetase A (uracil-repressed extract)* (○) *and carbamyl phosphate synthetase P (arginine-repressed extract)* (△) *were assayed in 25 mM Na-HEPES, 12 mM Na-ATP, 12 mM $MgCl_2$, 10 mM glutamine, 10 mM [^{14}C]$NaHCO_3$ (137 cpm/nmol), and 1 mM reduced glutathione. The final pH at 37°C was 7.6. The total sodium ion concentration was about 60 mM. Extracts were prepared by dialysis aginst 25 mM Na-HEPES, 10% (vol/vol) glycerol, and 1 mM reduced glutathione (pH 7.6) at 4°C. Carbamyl phosphate was assayed by Method II. For* (○) *100% is 2.4 nmol/min per mg; for* (△) *100% is 4.0 nmol/min per mg.*

phosphate synthetases are summarized in Table 3. Carbamyl phosphate synthetases A and P have very similar pH optima and K_m values for glutamine and bicarbonate. A major difference is in the optimal potassium ion concentration (see also Fig. 4). Both carbamyl phosphate synthetases were able to use NH_4^+ as an amide donor, although much less efficiently than glutamine. The activity of carbamyl phosphate synthetase P with 10 mM NH_4Cl was approximately 20% of the activity with 10 mM glutamine. For carbamyl phosphate synthetase A this ratio was 10%. $S_{0.5}$ values are reported for NH_4Cl because linear double reciprocal plots were not obtained. This may be due to a second effect of NH_4^+ as a monovalent cation. Cell extracts prepared from cultures grown in the presence of arginine plus uracil and harvested in log phase had no detectable carbamyl phosphate synthetase activity with either glutamine or NH_4Cl, which demon-

TABLE 3. *Some kinetic properties of carbamyl phosphate synthetases A and P*

Carbamyl phosphate synthetase	pH optimum[a]	$S_{0.5}$ (mM) for:		K_m (mM) for:	
		K^+[a]	NH_4^+[b]	$KHCO_3$[b]	Glutamine[b]
A	7.5	40	17	2.91 ± 0.46	0.60 ± 0.064
P	7.5	100	11	3.63 ± 0.046	0.35 ± 0.016

[a] Performed using method II for the determination of carbamyl phosphate.

[b] Assayed in standard assay mixture, substituting 50 mM potassium-HEPES and 50 mM KCl for 100 mM triethanolamine and 100 mM KCl.

strates that the potentially interfering enzymes acetate kinase or carbamate kinase (10) were not present under these conditions.

Effects on carbamyl phosphate synthetases A and P of potential metabolic effectors. Arginine, citrulline, glutamate, *N*-acetyl glutamate, *N*-acetyl ornithine, and ornithine, each at 5 mM, were examined as possible effectors of carbamyl phosphate synthetase A. All were tested at saturating (10 mM) and subsaturating (0.5 mM) glutamine and found to be without effect on the activity of the enzyme. PRPP, UMP, and UTP, each at 5 mM, were tested at 6 mM and 24 mM ATP and were also without effect. Finally, combinations of 5 mM arginine or 5 mM ornithine plus 5 mM UMP were tested and also found to have no effect on carbamyl phosphate synthetase A activity. The small effects of UMP seen previously were not evident when optimal assay conditions were used. Thus, the only potential regulation of this isozyme that we have observed is repression by arginine.

Table 4 shows the effects of a number of potential metabolic effectors on the activity of carbamyl phosphate synthetase P. Contrary to a previous report (17), neither ornithine nor dihydroorotate (up to 20 mM was tested) had effects on carbamyl phosphate synthetase P activity. The most potent inhibitor of this isozyme was UMP. The K_i for UMP was estimated to be ≤ 50 μM; an accurate determination was prevented by the apparent breakdown of UMP during the assay. Other uridine nucleotides were also inhibitory, but cytidine nucleotides had little effect. Guanine nucleotides and PRPP were found to be activators of PRPP synthetase. Both inhibition by uridine nucleotides and activation by GTP and PRPP appeared to result from altering the affinity of carbamyl phosphate synthetase P for ATP, so that much greater effects were seen at subsaturating ATP concentrations (Fig. 5). GTP and PRPP tended to decrease the Hill coefficient for ATP, whereas uridine nucleotides increased it. At 6 mM ATP, which is nearer the physiological concentration than the higher levels tested in Fig. 5, the activation by GTP and PRPP was quite marked (Fig. 6). The apparent activation constants for GTP and PRPP at 6 mM ATP and 12 mM $MgCl_2$ were 0.8 and 1.4 mM, respectively. Whereas the total activity of carbamyl phosphate synthetase P and the K_a for PRPP were constant during storage at −20°C for several days, the extent of activation by PRPP decreased during storage. These effects might be caused by desensitization of an allosteric site or by dissociation of a regulatory subunit. Sensitivity to UMP was not altered during storage at −20°C. The effects of GTP and PRPP on carbamyl phosphate synthetase P differed in one important respect: GTP was able to reverse inhibition by UMP, but PRPP was unable to do so. In Fig. 6 it is seen that inhibition by 50 μM UMP gradually decreased with increasing GTP concentrations, but this inhibition remained nearly constant with increasing PRPP until a 100-fold ratio of PRPP over UMP was obtained. It is reasonable to propose that activation of carbamyl phosphate synthetase P by PRPP and guanine nucleotides and inhibition by uridine nucleotides, together with repression

TABLE 4. *Inhibition and activation of carbamyl phosphate synthetase P under optimal assay conditions*[a]

Effector added (5 mM)[b]	Relative enzyme activity[c]
PRPP	1.42
K_2HPO_4	0.95
UMP	0.02
UDP	0.04
UTP	0.19
CMP	0.84
CTP	0.95
AMP	0.45
ADP	0.59
GMP	1.31
GDP	1.11
GTP	1.57
IMP	0.91
Arginine	0.95
Ornithine	0.96
Citrulline	1.00
N-Acetyl glutamate	1.01
N-Acetyl ornithine	1.00
Glutamate	1.01
Dihydroorotate	1.00
Arginine + UMP	0.02
Ornithine + UMP	0.02

[a] Assayed in standard assay mixture, substituting 50 mM potassium HEPES and 50 mM KCl for 100 mM triethanolamine and 100 mM KCl. Total potassium ion concentration was estimated at 250 nM.

[b] All phosphate compounds were added with equimolar additional $MgCl_2$.

[c] No addition is defined as 1.00.

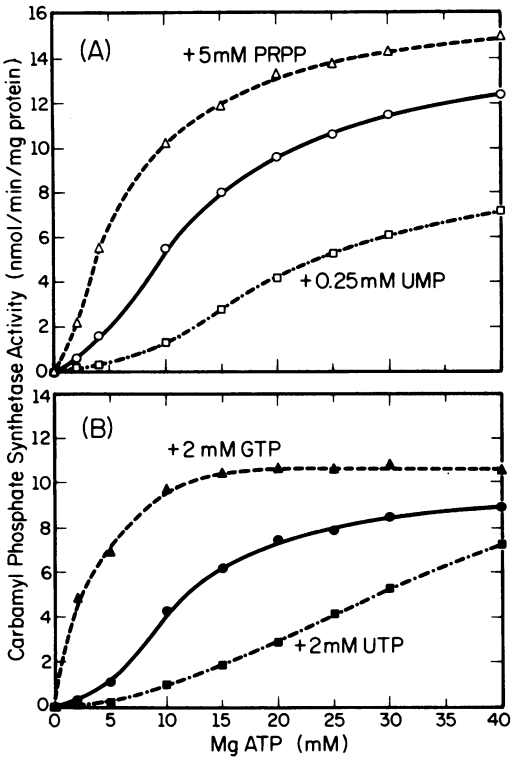

FIG. 5. *Carbamyl phosphate synthetase P activity as a function of Mg-ATP concentration (with 6 mM excess $MgCl_2$ added), with and without the addition of effectors. (A)* (○) *No effectors;* (△) *plus 5 mM PRPP;* (□) *plus 0.25 mM UMP. (B)* (●) *No effectors;* (▲) *plus 2 mM GTP;* (■) *plus 2 mM UTP. The standard assay system substituted 50 mM potassium HEPES for both 100 mM triethanolamine and 200 mM KCl. All effectors were added with equimolar $MgCl_2$. Potassium ion was maintained constant with KCl. KF (25 mM) was added to inhibit phosphatase activity.*

of this isozyme by pyrimidines, provide the basis for regulation of its activity in vivo.

DISCUSSION

The experiments reported in this paper demonstrate clearly that *B. subtilis* produces two carbamyl phosphate synthetases with different physical and catalytic properties and different sensitivities to metabolic effectors and repressors. The simplest view is that each carbamyl phosphate synthetase is the product of structural genes that are defective in strains H37, H59, and JH861. More complex alternative models, in which a single gene product undergoes an interconversion between the A and P forms that is catalyzed by an enzyme governed by one of the above loci, are not excluded, but seem unlikely to us. Potvin and Gooder (17) also concluded that *B. subtilis* produces two carbamyl phosphate synthetases, but for reasons pointed out in the Introduction their evidence was not conclusive without the supporting biochemical data in this paper. Still unexplained is the claim of Issaly et al. (10) to have isolated a point mutant lacking carbamyl phosphate synthetase that was a double arginine and uracil auxotroph. We can only suggest that this mutant did not in fact possess all of the properties attributed to it.

The difficulties in assaying *B. subtilis* carbamyl phosphate synthetase reported by previous workers (10, 17) prevented them from obtaining the kinds of data clearly demonstrating two isozymes reported here. These difficulties were largely due to the previously unrecognized absolute requirement of both isozymes for K^+ ions for both activity and stability. The small amounts of activity found by Potvin and Gooder (roughly 10 to 20% of the levels reported here) were probably detected because these workers used the K^+ salts of ATP and HCO_3^- (17), and

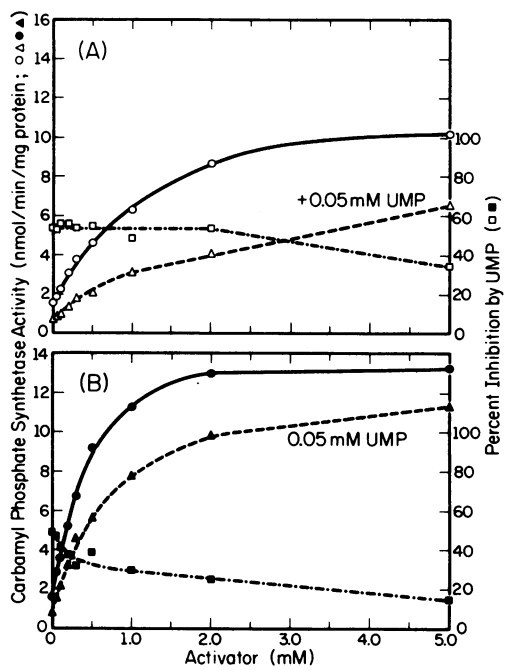

FIG. 6. *Carbamyl phosphate synthetase P activity at 6 mM ATP (12 mM $MgCl_2$) as a function of activator concentration. (A) Activator was PRPP:* (○) *no UMP;* (△) *plus 0.05 mM UMP;* (□) *percent inhibition by 0.05 mM UMP at the PRPP concentrations shown. (B) Activator was GTP:* (●) *no UMP;* (▲) *plus 0.05 mM UMP;* (■) *percent inhibition by 0.05 mM UMP at the GTP concentrations shown. The standard assay was as described in Fig. 5. All effectors were added with equimolar $MgCl_2$.*

the detergent-treated cells may well have contained some residual K^+ (the intracellular K^+ concentration of *B. subtilis* is about 100 to 200 mM, ref. 6). Furthermore, tris(hydroxymethyl)aminomethane buffers, which were used in previous studies (10, 17), are inhibitory to both isozymes. It is unlikely that Potvin and Gooder actually measured the activity of carbamyl phosphate synthetase A, because they report that this activity could not be detected when greater than 7.5 μg of uracil per ml was included in the growth medium, whereas in fact this activity is not repressed by as much as 100 μg of uracil per ml. Issaly et al. (10) grew their cells in a medium containing 0.1% Casamino Acids, which contains substantial levels of arginine, so it is probable that carbamyl phosphate synthetase A was repressed, at least during vegetative growth, in their experiments as well. Use of the chemical trapping assay of Levine and Kretchmer (13) also led to the false conclusion that carbamyl phosphate synthetase A is stimulated by ornithine. We also observed an apparent 2.5-fold activation by ornithine when this assay was used, but no activation was detected when the enzymatic trapping assay (Method I) was used. We propose that in the presence of ornithine and endogenous ornithine transcarbamylase the efficiency of trapping of carbamyl phosphate into an acid-stable form via citrulline formation is nearly 100% (as in Method I), whereas in the chemical trapping assay the efficiency of trapping is only about 40% (13). Thus, addition of ornithine gives an apparent stimulation that is not seen when the enzymatic trapping assay is used. It is also likely that the inhibition of carbamyl phosphate synthetase by dihydroorotate reported by Potvin and Gooder (17), which we were not able to reproduce, was the result of interference with the trapping assay by the very high level (>100 mM) of dihydroorotate used. In view of the many factors that could affect the efficiency of the chemical trapping assay, we suggest that an enzymatic procedure for trapping carbamyl phosphate is generally preferable for assaying carbamyl phosphate synthetase.

B. subtilis is unique among the procaryotic microbes so far examined in possessing two carbamyl phosphate synthetases. This species resembles such eucaryotic microorganisms as yeast (11) and *Neurospora* (24) and higher organisms rather than the enteric bacteria (16) in this respect. The properties of the arginine-sensitive and uracil-sensitive mutants indicate that, while the two carbamyl phosphate synthetases are under differential repressive control, either enzyme can supply carbamyl phosphate for both arginine and pyrimidine biosynthesis. The carbamyl phosphate pools are not compartmentalized, as they have been shown to be in *Neurospora* (23). Yeast also fails to show such compartmentalization (11). Neumann and Jones (15) have shown that *Streptococcus faecalis,* like *B. subtilis,* possesses an aspartate transcarbamylase that is not subject to inhibition by nucleotides. It would be interesting to examine the regulation of carbamyl phosphate synthesis in *S. faecalis.*

The present findings provide a more complete picture of the regulation of pyrimidine and arginine biosynthesis in *Bacillus.* Carbamyl phosphate synthetase P, like the other enzymes of pyrimidine biosynthesis (18), is strongly repressed by pyrimidines in the growth medium. Feedback inhibition of the pyrimidine pathway occurs primarily by the inhibitory action of uridine nucleotides. Activation of carbamyl phosphate synthetase by PRPP has been observed with this enzyme from mammals (22) and reflects an important role of this metabolite in stimulating nucleotide biosynthesis. Activation of enzymes of pyrimidine biosynthesis by purine nucleotides has been observed in several instances (16); activation of carbamyl phosphate synthetase P by guanine nucleotides is part of this pattern. It seems desirable to postpone further speculation about regulation of this isozyme until careful kinetic studies can be conducted with purified enzyme. Regulation of the arginine pathway by inhibition or activation is less clear. Our studies have identified only repression of carbamyl phosphate synthetase A as a potential regulatory mechanism. It may be noted that map positions of the two carbamyl phosphate synthetases are consistent with their coordinate repression with other enzymes of the arginine and pyrimidine biosynthetic pathways, but this has not been demonstrated.

Potvin et al. (18) have suggested that the first three enzymes of the pyrimidine biosynthetic pathway may be physically associated in a complex. The suggestion was based on the high incidence of pleiotropic *pyr* mutants and the cosedimentation of the three activities on sucrose gradients at a position corresponding to a molecular weight of 130,000. We have not examined this proposal thoroughly. However, the fact that the molecular weight of carbamyl phosphate synthetase P is about 100,000 and that of purified aspartate transcarbamylase is 100,000 (4) makes it likely that cosedimentation of these activities is coincidental rather than a consequence of complex formation.

In studies to be published later we have found that carbamyl phosphate synthetases A and P show very different patterns of appearance in and disappearance from cells during growth and sporulation on glucose-nutrient broth medium.

These findings may help to clarify the physiological roles of the two enzymes.

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LITERATURE CITED

1. **Abd-El-Al, A., and J. L. Ingraham.** 1969. Control of carbamyl phosphate synthesis in *Salmonella typhimurium*. J. Biol. Chem. **244**:4033–4038.
2. **Abd-El-Al, A., and J. L. Ingraham.** 1969. Cold sensitivity and other phenotypes resulting from mutation in *pyrA* gene. J. Biol. Chem. **244**:4039–4045.
3. **Anagnostopoulos, C., and J. Spizizen.** 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81**:741–746.
4. **Brabson, J. S., and R. L. Switzer.** 1975. Purification and properties of *Bacillus subtilis* aspartate transcarbamylase. J. Biol. Chem. **250**:8664–8669.
5. **Cox, D. P., and R. S. Hanson.** 1968. Catabolite repression of aconitate hydratase in *Bacillus subtilis*. Biochim. Biophys. Acta **158**:36–44.
6. **Eisenstadt, E.** 1972. Potassium content during growth and sporulation of *Bacillus subtilis*. J. Bacteriol. **112**: 264–267.
7. **Fishbein, L., and M. A. Cavanaugh.** 1965. Detection and paper chromatography of N-substituted hydroxy-, 2-hydroethyl-, 2-chloroethyl-, and N,N,-bis-(2-hydroxyethyl)- derivatives. J. Chromatogr. **20**:283–294.
8. **Herrmann, E. C., J. H. Dunn, and R. R. Schmidt.** 1973. DEAE paper chromatography to separate intermediates of the pyrimidine biosynthetic pathway and to assay aspartate transcarbamylase and dihydroorotase activities. Anal. Biochem. **53**:478–483.
9. **Hoch, J., and J. Mathews.** 1972. Genetic studies in *Bacillus subtilis*, p. 113–116. *In* H. O. Halvorson, R. Hanson, and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D.C.
10. **Issaly, I. M., A. S. Issaly, and J. L. Reissig.** 1970. Carbamyl phosphate biosynthesis in *Bacillus subtilis*. Biochim. Biophys. Acta **198**:482–494.
11. **Lacroute, F., A. Piérard, M. Grenson, and J.-M. Wiame.** 1965. The biosynthesis of carbamoyl phosphate in *Saccharomyces cerevisiae*. J. Gen. Microbiol. **40**:127–142.
12. **Legrain, C., V. Stalon, J.-P. Noullez, A. Mercenier, J. P. Simon, K. Broman, and J.-M. Wiame.** 1977. Structure and function of ornithine carbamoyltransferases. Eur. J. Biochem. **80**:401–409.
13. **Levine, R. L., and N. Kretchmer.** 1971. Conversion of carbamyl phosphate to hydroxyurea. An assay for carbamyl phosphate synthetase. Anal. Biochem. **42**: 324–337.
14. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
15. **Neumann, J., and M. E. Jones.** 1964. End product inhibition of aspartate transcarbamylase in various species. Arch. Biochem. Biophys. **104**:438–447.
16. **O'Donovan, G. A., and J. Neuhard.** 1970. Pyrimidine metabolism in microorganisms. Bacteriol. Rev. **34**: 278–343.
17. **Potvin, B., and H. Gooder.** 1975. Carbamyl phosphate synthesis in *Bacillus subtilis*. Biochem. Genet. **13**: 125–143.
18. **Potvin, B. W., R. J. Kelleher, Jr., and H. Gooder.** 1975. Pyrimidine biosynthetic pathway of *Bacillus subtilis*. J. Bacteriol. **123**:604–615.
19. **Prescott, L. M., and M. E. Jones.** 1969. Modified methods for the determination of carbamyl aspartate. Anal. Biochem. **32**:408–419.
20. **Switzer, R. L., C. L. Turnbough, Jr., J. S. Brabson, and L. M. Waindle.** 1975. Enzyme inactivation of de novo nucleotide biosynthesis in sporulating *Bacillus subtilis* cells, p. 327–334. *In* P. Gerhardt, R. N. Costilow, and H. L. Sadoff (ed.), Spores VI. American Society for Microbiology, Washington, D.C.
21. **Tatibana, M., and K. Shigesada.** 1972. Control of pyrimidine biosynthesis in mammalian tissues. IV. Requirements of a quantitative assay of glutamine-dependent carbamyl phosphate synthetase and effect of magnesium ion as essential activator. J. Biochem. (Tokyo) **72**: 537–547.
22. **Tatibana, M., and K. Shigesada.** 1972. Control of pyrimidine biosynthesis in mammalian tissues. V. Regulation of glutamine-dependent carbamyl phosphate synthetase: activation by 5-phosphoribosyl-1-pyrophosphate and inhibition by uridine triphosphate. J. Biochem. (Tokyo) **72**:549–560.
23. **Williams, L. G., S. A. Bernhardt, and R. H. Davis.** 1971. Evidence for two discrete carbamyl phosphate pools in *Neurospora*. J. Biol. Chem. **246**:933–978.
24. **Williams, L. G., and R. H. Davis.** 1970. Pyrimidine-specific carbamyl phosphate synthetase in *Neurospora crassa*. J. Bacteriol. **103**:335–341.