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## Carbamyl Phosphate Synthetase A of *Neurospora Crassa*

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Carbamyl phosphate synthetase A of *Neurospora crassa* was partially purified from mitochondrial extracts. It is an extremely unstable enzyme ( $t_{1/2} = 45$  min at 25°C) made up of two unequal subunits. The native enzyme has a molecular weight of approximately 175,000, and the large subunit has a molecular weight of about 125,000. Both the native enzyme and its large subunit are quite asymmetric, as revealed by slow sedimentation in sucrose gradients (7.3S and 6.6S, respectively). The small subunit has not been identified physically as a separate entity. The denaturation of the native, glutamine-dependent activity is correlated with dissociation of subunits, the larger of which retains a more stable, ammonia-dependent activity. Neither substrates nor any other agents except glycerol or polyethylene glycol appreciably stabilized the glutamine-dependent activity. Kinetic studies showed the native enzyme to have a  $K_m$  for glutamine of about 0.16 mM, and a  $K_m$  for  $\text{NH}_4\text{Cl}$  of about 16 mM, at the optimal pH, 8.0. The enzyme, using either N donor, has a  $\text{K}^+$  requirement for activity, for which  $\text{NH}_4^+$  can substitute. The glutamine  $\rightarrow$  glutamate reaction, which requires the small subunit, also requires the large subunit and all reaction substrates for optimal activity. Other evidences of subunit interaction are the greater activity of the native enzyme, as opposed to the large subunit, with low concentrations of adenosine 5'-triphosphate- $\text{Mg}^{2+}$ , and in the stimulation of the ammonia-dependent activity of the native enzyme by glycine. Curiously, although the enzyme's role in biosynthesis is confined to the arginine pathway, it is completely indifferent to arginine or its precursors as feedback effectors or activators. The enzyme is compared with carbamyl phosphate synthetases of other organisms.

The study of carbamyl phosphate synthetase (EC 2.7.2.9) in various organisms has revealed differences in the number of enzymes, their localization, and their regulation (11, 18, 22). In enteric bacteria, one enzyme serves both the arginine and pyrimidine pathways and is regulated by both end products (22, 25). In eucaryotes, two enzymes commonly exist, one specific for each pathway. In *Neurospora crassa*, carbamyl phosphate synthetase A serves the arginine pathway and is located in mitochondria, together with ornithine transcarbamylase and the enzymes of ornithine synthesis (7, 42). Another enzyme, carbamyl phosphate synthetase P, serves the pyrimidine pathway and lies in the nucleolus (3). Both enzymes use bicarbonate, ATP-Mg, and glutamine as substrates in vivo. The two carbamyl phosphate synthetases are specified by entirely different genes and are separately regulated (44). Moreover, carbamyl phosphate, the product of both enzymes, is maintained in separate pools, so that mutants lacking a given carbamyl phosphate synthetase

require the end product of the deficient pathway (45).

Carbamyl phosphate synthetase A is a two-component enzyme specified by two unlinked genes (10). The *arg-3* locus specifies a large component which catalyzes carbamyl phosphate synthesis, using ammonia as a nitrogen donor (9). The *arg-2*<sup>+</sup> locus specifies a smaller component which endows the native enzyme with the ability to use glutamine as a substrate in addition to ammonia (10). In this respect, carbamyl phosphate synthetase A is similar to the enzyme of enteric bacteria and to the carbamyl phosphate synthetase A of yeast (26), and different from the carbamyl phosphate synthetase I of mammals, which uses only ammonia as a nitrogen donor (14). Like the arginine-specific carbamyl phosphate synthetase of most eucaryotes, the *Neurospora* enzyme is mitochondrial, whereas in certain yeasts, including *Saccharomyces cerevisiae*, it is cytosolic (40).

The *Neurospora* enzyme, like that of yeast, is insensitive to feedback inhibition (4, 10, 37, 44). The enzyme, however, is almost completely absent in cultures grown in the presence of excess arginine. The regulation appears to be directed

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much more strongly to the glutamine-binding component than to the large component (7). Given the complex regulation of the enzyme, it was of interest to purify and to study the enzyme and its components from *Neurospora* as a prelude to studies of polypeptide aggregation, regulation, and the process of enzyme insertion into mitochondria. A study of the large component of the *Neurospora* enzyme was published some years ago, before the unstable small component was discovered (9). The problem of enzyme instability has severely hampered our efforts to purify the native enzyme and to characterize it in physical terms. Here, we report the characteristics of a partially purified preparation.

### MATERIALS AND METHODS

**Strains, media and growth conditions.** For the major work on purification of the native enzyme, *arg-5* (27947 and CD-6) and *pyr-3 arg-12* (DFC-3, UM-107) strains were used. The *arg-5* mutations block ornithine synthesis at the acetylornithine acetyltransferase step (EC 2.6.1.11), and they can be starved for arginine. The *pyr-3* mutation is blocked in the synthesis of carbamyl phosphate in the pyrimidine pathway, and the *arg-12* mutation imposes an ornithine transcarbamylase deficiency. For purification of the large component of carbamyl phosphate synthetase A, *arg-2* (MEP-7 and CD-80) or *pyr-3 arg-2* (DFC-3, MEP-7) strains were used; *arg-2* is deficient in the small component of the enzyme. For identification of the large subunit after polyacrylamide gel electrophoresis, an *arg-3* nonsense mutant, CD-186, was used (Davis, unpublished data). The medium used was that of Vogel (41), using L-arginine and uridine as supplements where necessary. Stocks and conidia for inoculation were grown in medium solidified with 1.5% agar and supplemented with 200  $\mu$ g of L-arginine and 100  $\mu$ g of uridine per ml.

Growth of strains for enzyme purification was initiated by introducing conidia at a final concentration of  $3 \times 10^6$  per ml into 700 ml of Vogel medium contained in 2,500-ml low-form culture flasks, supplemented with 100  $\mu$ g of arginine per ml and, if necessary, 300  $\mu$ g of uridine per ml. The flasks were maintained on a reciprocal shaker for 32 to 36 h at 27°C. The conditions were designed to allow growth to about 1.3 mg (dry weight) per ml within the first 24 h and enzyme derepression during a phase of arginine starvation thereafter.

**Localization of enzyme activity.** Isolation and fractionation of mitochondria were based on previous work by others (5, 12, 29). Cells from two or three shake-flasks were collected and washed in a buffer which included 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4), 0.3 M sucrose, 1 mM ethylene glycol-bis( $\beta$ -aminoethyl ether), and 3 mg of bovine serum albumin per ml. Cells were ground in a mortar in this buffer at 4°C with half their pressed wet weight of sand. Sand, unbroken cells, and debris were removed by centrifugation at 1,000  $\times$  *g* for 10 min. The supernatant was centrifuged at 9,000  $\times$  *g* for 15 min, and the pellet was suspended in 10 ml

of buffer, recentrifuged at 600  $\times$  *g* for 10 min (removing sediment), and centrifuged again at 9,000  $\times$  *g* to recover the washed mitochondrial pellet. This pellet was suspended in 5 ml of buffer containing 2 mM HEPES (pH 7.4), 0.3 M sucrose, and 0.5 mg of bovine serum albumin per ml. Digitonin (10 mg/ml) was added to a final concentration of 0.4 mg/mg of mitochondrial protein (5), and the suspension was stirred for 15 min, then diluted threefold with the suspension buffer and gently homogenized (three strokes) with a Teflon-glass homogenizer. The suspension was centrifuged at 45,000  $\times$  *g* for 15 min, and the supernatant was removed. The pellet was washed by suspension and centrifugation, and the wash was combined with the first supernatant. This supernatant was expected to have outer membrane and intermembrane components and was designated SI. The washed pellet was suspended in 1 to 2 ml of buffer, and Lubrol WX was added to give 0.5 mg/mg of mitochondrial protein (5). The suspension stood at 4°C for 15 min and was then centrifuged at 45,800  $\times$  *g* for 30 min. The supernatant (SII, representing the "matrix fraction") was removed, and the pellet (PII, representing inner membrane components) was suspended in 1 ml of buffer. Enzyme assays were done on the SI, SII, and PII fractions in parallel with whole mitochondria to which Lubrol WX (0.5 mg/mg of mitochondrial protein) had been added.

**Purification of the native enzyme.** Mycelia of *arg-5* or *pyr-3 arg-12* strains from 10 to 20 flasks were harvested in cheesecloth and washed with extraction buffer. This buffer contained 0.05 M potassium phosphate (pH 7.3), 1 mM EDTA, and 0.3 M sorbitol, the last being included to stabilize mitochondria upon cell breakage. The cells were squeezed to a wet pad. The pad was ground in the cold, with sand (2 g/g [dry weight] of mycelium) and extraction buffer, in a motor-driven porcelain mortar and pestle for 10 min. The resulting slurry was filtered through cheesecloth, and the material that did not pass through was washed three times in extraction buffer, with the filtrate being collected each time. The combined filtrates were centrifuged at 600  $\times$  *g* for 10 min at 0°C. The supernatant was centrifuged at 9,000  $\times$  *g* for 20 min to sediment mitochondria. The mitochondria were suspended in the extraction buffer.

Two methods of mitochondrial extraction were used. (i) In early work, Triton X-100 was added to a final concentration of 0.5%. The preparation was centrifuged at 9,000  $\times$  *g* for 20 min, and the supernatant was collected. This was passed through a column of Sephadex G-25 equilibrated with 0.05 M phosphate buffer (pH 7.8) with 1 mM EDTA to remove small molecules. (ii) In more recent work, used for purification and polyacrylamide gel electrophoresis, enzymes were extracted from mitochondria by shaking mitochondria with glass beads, without Triton X-100. To 1-ml lots of mitochondrial suspension, cold, 0.45-mm glass beads were added up to the meniscus in culture tubes (13 by 100 mm). They were shaken twice for 45 s each time on a Vortex mixer (Scientific Products Co.), with cooling between each treatment. The homogenate was drawn from the bottom of the tube with a Pasteur pipette. The beads were washed twice with 0.5 ml of extraction buffer, and the washes were combined with the original extract. The extract was cen-

trifuged at  $12,000 \times g$  for 20 min and desalted on a Sephadex G-25 column equilibrated with either 0.06 M potassium phosphate (native enzyme) or 0.015 M potassium phosphate (large subunit) (pH 7.8) with 1 mM EDTA. The eluate was centrifuged at  $45,000 \times g$  for 15 min to remove remaining sub-mitochondrial particulates. The supernatant was passed through Sephadex G-25 as in the Triton extraction method and was used for purification.

The extract was divided equally and added to small DEAE-cellulose columns (1.1 by 7.0 cm) equilibrated with the buffer used to desalt the extract. Each column was washed with 75 ml of 0.064 M potassium phosphate buffer (pH 7.8) with 1 mM EDTA. In this step, ornithine transcarbamylase and the large component of the enzyme were eluted. The columns were then washed with 5 ml, then 15 ml of the same buffer, containing 0.1 M NaCl. The second fraction contained the glutamine-dependent activity.

Fractions containing the native enzyme were pooled and concentrated with an Amicon ultrafiltration apparatus, using a PM10 filter. The concentrated enzyme was passed through a Sephadex G-25 column equilibrated with 0.06 M  $K^+$  phosphate (pH 7.3) with 1 mM EDTA.

The preparation was diluted with water to bring the phosphate concentration to 0.03 M. The preparation was absorbed to small lots of hydroxylapatite in centrifuge tubes (1.0 mg of protein to 2.0 ml of gel bed). The hydroxylapatite was centrifuged and washed twice with 5.2 ml of a buffer which was 0.045 M potassium phosphate (pH 7.3) and 1 mM EDTA. Any remaining large components of the enzyme, and an adventitious glutaminase activity, were removed in these fractions. The gel was then washed three times with 2.6 ml of 0.1 M phosphate (pH 7.3) with EDTA. These three supernatants were combined, concentrated by ultrafiltration, and diluted with water to bring the phosphate concentration to 0.05 M. The preparation was quickly frozen in small lots and stored at  $-80^\circ\text{C}$ . The preparation was used for all kinetic work. Purifications, starting from (frozen) mitochondria, could be done in a day; occasionally material was frozen at the stage of the concentrated DEAE-cellulose eluate.

**Purification of the large component.** Procedures for purification of the large component from *arg-2* mutants were the same as for the native enzyme until the DEAE-cellulose step. The enzyme preparation was added to DEAE-cellulose in 0.015 M phosphate (pH 7.8), and the column was eluted with a 0.015 to 0.10 M potassium phosphate gradient. Fractions containing enzyme (ca. 0.05 M phosphate) were pooled, concentrated by ultrafiltration, and passed through Sephadex G-25 equilibrated with 0.01 M potassium phosphate (pH 7.3). This preparation in certain cases was then added to hydroxylapatite columns (0.7 by 2.0 cm; 0.38 mg of protein per column). The hydroxylapatite was washed with increasing concentrations of phosphate: 4 ml of 0.01 M, 4 ml of 0.015 M, and 5 ml of 0.045 M. The large component of the enzyme was eluted in the last wash; ornithine transcarbamylase, if present, was eluted in the first. The last wash was concentrated by ultrafiltration, brought to 0.05 M phosphate (pH 7.3), and stored at  $-80^\circ\text{C}$ .

**Gel filtration.** Estimation of Stokes radius was done by gel filtration on a Sephacryl S-200 column (2.5 by 85 cm) equilibrated with 0.05 M potassium phosphate (pH 7.8). *Escherichia coli*  $\beta$ -galactosidase, catalase, *E. coli* alkaline phosphatase, ovalbumin, and cytochrome *c* were used as standards. Because blue dextran adsorbed somewhat to the column (even at pH 7.8), phage lambda was used to estimate void volume.

**Sucrose density-gradient centrifugation.** Estimation of *s* values by density-gradient centrifugation was done by the method of Martin and Ames (19).

**Polyacrylamide gel electrophoresis.** Protein preparations treated with sodium dodecyl sulfate were resolved on 10% polyacrylamide slab gels (1.5 mm; 13 by 16 cm) with sodium dodecyl sulfate (16, 33). A 5% stacking gel was used, and slabs were stained with Coomassie brilliant blue.

The gels were run for 50 V for 1 h (stacking), then at 150 V for 2 h more. The standards used were  $\beta$ -galactosidase, bovine serum albumin, lactate dehydrogenase, catalase, and ovalbumin.

**Assays.** Carbamyl phosphate synthetase was assayed in 0.5-ml reaction mixtures containing 100 mM Tris-hydrochloride (pH 8.0), 12 mM  $MgCl_2$ , 12 mM ATP, 20 mM  $KH^{14}CO_3$ , and either 12 mM L-glutamine or 120 mM  $NH_4Cl$ . The final pH was 8.0. They were incubated for 30 min at  $25^\circ\text{C}$  and were stopped by addition of 0.2 ml of 1.5 M  $NH_4Cl$  and boiling for 10 min. This step converts [ $^{14}C$ ]carbamyl phosphate to [ $^{14}C$ ]urea. Reactions were acidified with 0.1 ml of 1 N HCl to remove most of the  $^{14}CO_2$ . The reaction mixtures, after centrifugation, were passed through Dowex-1X8 columns ( $OH^-$  form, 0.4 by 3.5 cm, 200 to 400 mesh) standing in scintillation vials. The urea (not bound to the resin) was counted in a mixture of 1 part of Triton X-100 to 2 parts of toluene; the latter component contained 5 g of 2,5-diphenyloxazole per liter. Conversion of carbamyl phosphate to urea is approximately 90%, but the correction was applied only where stoichiometric considerations prevailed. Assays of the glutamine-dependent activity were slightly nonlinear due to the enzyme inactivation. Again, no correction was applied.

Succinate dehydrogenase was determined with an oxygen electrode according to Schnaitman and Greenawalt (29); malate dehydrogenase was determined by the method of Ochoa (21); kynurenine hydroxylase was determined according to Schott et al. (30), but without *p*-chloromercuribenzoate.

Glutaminase was assayed according to Prusiner and Stadtman (28), using [ $^{14}C$ ]glutamine purified on columns just before use. Ornithine transcarbamylase was assayed according to Davis (8) with reaction mixtures scaled down to 1.0 ml.

**Chemicals.** Most biochemical materials, including DEAE-cellulose, were purchased from Sigma. ATP,  $\beta$ -galactosidase, and catalase were purchased from Boehringer-Mannheim. Hydroxylapatite (Hypatite C) was purchased from Clarkson Chemical Co., Williamsport, Pa. Sephadex G-25 and Sephacryl S-200 were purchased from Pharmacia, Inc. Triton X-100 (reagent grade) was purchased from Research Products International. 2,5-Diphenyloxazole was purchased from Mallinkrodt Chemical Co. Lubrol WX was a gift of M.

E. Jones and J.-J. Chen. Phage lambda was a gift of Daniel Wulff.

**RESULTS**

**Localization of carbamyl phosphate synthetase.** To determine whether carbamyl phosphate synthetase A was located on the outer membrane of the mitochondria or in the inter-membrane space, the extent of its solubilization with digitonin was tested. The release of the enzyme activity from the sedimentable fraction was compared to that of kynurenine hydroxylase and malate dehydrogenase, marker enzymes for the outer membrane and the matrix, respectively (5). The data (Fig. 1) show that all kynurenine hydroxylase was released into the supernatant (SI) at a concentration of 0.4 mg of digitonin per mg of protein, whereas over 65% of malate dehydrogenase and carbamyl phosphate synthetase A activities remained sedimentable. This indicates that carbamyl phosphate synthetase A was associated with the inner membrane or matrix. To distinguish between the latter two possibilities, digitonin-treated mitochondria, lacking outer membrane, were treated with Lubrol WX. The soluble materials (SII fraction) were separated from the pellet (PII) and analyzed for marker enzymes. Table 1 shows that the majority of carbamyl phosphate synthetase A, malate dehydrogenase, and ornithine transcarbamylase was released by this detergent, while succinate dehydrogenase, an inner membrane marker, remained sedimentable. The results indicate that carbamyl phosphate synthetase A and ornithine transcarbamylase are matrix enzymes, or are only weakly bound to the inner membrane. The results are consistent with the finding that both enzymes can be released from whole mitochondria by shaking vigorously with glass beads (see Materials and Methods).

**Purification of the enzymes.** Attempts to stabilize the glutamine-dependent activity were unsuccessful and are discussed in a later section.

Partial purification was carried out by the procedures given in Materials and Methods. A summary of purification, using the glass-bead method of mitochondrial extraction, is reported in Table 2. A 9.5-fold purification over the mitochondrial preparation was achieved, with a yield of less than 1%. The purification factor was about 1,600 if referred to the specific activity of crude extracts of wild-type cultures grown in minimal medium. The extraordinary losses of glutamine-dependent activity during purification were associated with dissociation of the large and small subunits, as discussed in a later

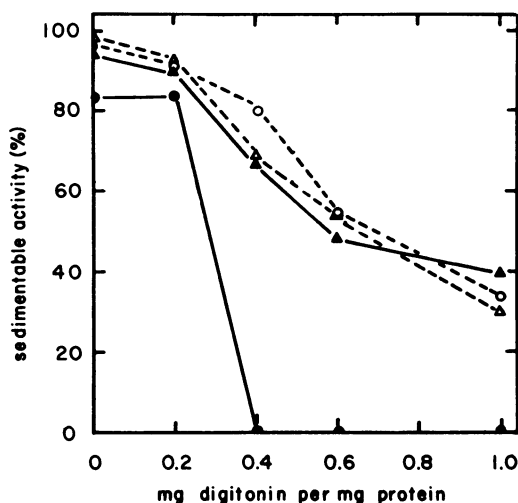


FIG. 1. Solubilization of kynurenine hydroxylase, malate dehydrogenase, and carbamyl phosphate synthetase A of mitochondria with increasing digitonin concentrations. The ordinate represents the amount of activity remaining in the sedimentable fraction as a percentage of the sum of activities recovered in sedimentable and supernatant fractions. Symbols: (●) kynurenine hydroxylase; (▲) malate dehydrogenase; (△) carbamyl phosphate synthetase A (ammonium-dependent activity); (○) carbamyl phosphate synthetase A (glutamine-dependent activity).

TABLE 1. Enzyme distribution in mitochondrial fractions

Enzyme	% Recovered activity in:			% Activity recovered	Units in original mitochondria
	SI	SII	PII		
Succinate dehydrogenase	2	22	76	66	2.78
Malate dehydrogenase	10	83	7	81	27.1 <sup>a</sup>
Ornithine transcarbamylase	4	83	13	81	7.0
Carbamyl phosphate synthetase A (ammonium-dependent)	9	83	8	113	0.008
Carbamyl phosphate synthetase A (glutamine-dependent)	0	94	6	82	0.002
Protein (mg)	1.3	0.66	0.9		

<sup>a</sup> 1 unit = Δ0.01 optical density unit per min.

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TABLE 2. Purification of carbamyl phosphate synthetase A (native enzyme)

Step	Vol (ml)	Protein (mg)	Carbamyl phosphate synthetase A					
			Glutamine-dependent (Gln)				Ammonium-dependent (Amm) (units: $\mu\text{mol/h}$ )	Ratio Gln/Amm
			Units ( $\mu\text{mol/min}$ )	Sp act (U/mg)	Yield (%)	Purification (fold)		
9,000 $\times$ g pellet	6.2	117	0.650	0.0056	100	1	0.778	0.84
Mitochondrial extract (glass beads)	11.0	27.3	0.178	0.0066	27	1.19	0.788	0.23
DEAE-cellulose eluate (concentrated)	2.8	1.7	0.042	0.0248	6.5	4.43	0.043	0.98
Hydroxylapatite eluate (concentrated)	1.9	0.20	0.006	0.0532	0.9	9.5	0.008	0.75

section. The initial evidence for this was that the glutamine-dependent activity, when rerun on a DEAE-cellulose column, lost much activity, with the concomitant appearance of the large subunit in its characteristic position. The ratio of glutamine- to ammonia-dependent activities in fresh preparations of the glutamine-dependent enzyme was about 1.3, a figure similar to that of derepressed, crude extracts. This suggests that the native enzyme, in its heteropolymeric form, catalyzes both reactions at similar rates when the N-donor is saturating. Purified preparations of the glutamine-dependent activity were free of ornithine transcarbamylase and a separable glutaminase activity found in crude extracts. Denaturing polyacrylamide gel electrophoresis showed more than the two bands expected (Fig. 2A), but the large subunit was identified clearly (see next section).

Purification of the large subunit (see Materials and Methods) yielded an enzyme that was 10- to 15-fold purified over the mitochondrial preparation, and a recovery of 50% after the DEAE-cellulose step. No further purification, and in fact serious loss, takes place with hydroxylapatite. This step was used only to remove ornithine transcarbamylase where that was necessary. If the mitochondria are broken with Triton instead of glass beads, the yield is similar (55% versus 50%), and the specific activity is approximately half that from glass-bead extracts. Gel electrophoresis demonstrates substantial impurity (Fig. 2A).

**Molecular weight determinations.** Gel filtration of the native enzyme and its large subunit was used to estimate their Stokes radii. DEAE-cellulose eluates prepared by the Triton extraction method were used. The native enzyme eluted just behind catalase, and the large subunit eluted somewhat later, but well ahead of *E. coli* alkaline phosphatase (Fig. 3). From these data, the Stokes radii were estimated at 5.1 nm for the

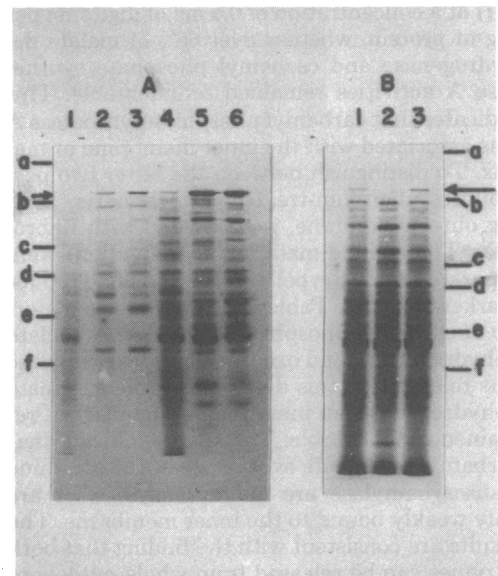


FIG. 2. Sodium dodecyl sulfate-polyacrylamide gels of mitochondrial and purified preparations. (A) Lanes 1, 2, and 3 are mitochondrial supernatant, DEAE-cellulose, and hydroxylapatite steps (all at 2  $\mu\text{g}$  of protein per gel) in native enzyme purification. Lanes 4, 5, and 6 are the same steps (all at 9  $\mu\text{g}$  of protein per gel) of the large subunit purification. (B) Lanes 1, 2, and 3 are mitochondrial supernatants of *arg-5* (used for native enzyme purification), *arg-3* (nonsense mutant lacking large subunit), and *arg-2* (used for large subunit purification), all at 14  $\mu\text{g}$  of protein per gel. The arrows point to the large subunit. The standards are: a, thyroglobulin (subunit molecular weight 335,000); b,  $\beta$ -galactosidase (116,000); c, bovine serum albumin (68,000); d, catalase (60,000); e, ovalbumin (43,000); f, lactate dehydrogenase (35,000).

native enzyme and 4.7 nm for the large subunit. Sucrose density-gradient centrifugation (Fig. 4) revealed that the native enzyme sedimented

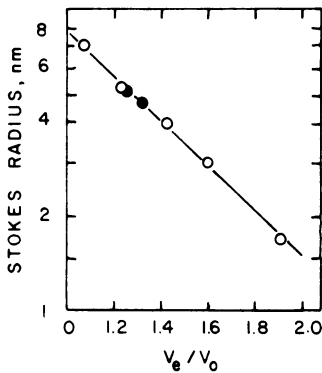


FIG. 3. Gel filtration estimate of Stokes radius of carbamyl phosphate synthetase A and its large subunit. Stokes radius (log scale) is plotted as a function of elution volume ( $V_e$ ) divided by the void volume ( $V_0$ ). Symbols: (●) native enzyme (upper) and large subunit (lower); (○) in descending order:  $\beta$ -galactosidase (molecular weight 540,000, 6.9 nm), catalase (232,000, 5.2 nm), *E. coli* alkaline phosphatase (80,000, 3.9 nm), ovalbumin (43,000, 3.0 nm), and equine cytochrome *c* (12,400, 1.7 nm). Estimated parameters are 5.1 nm for the native enzyme and 4.7 nm for the large subunit.

with lactate dehydrogenase, giving an  $s$  value of 7.3. The large subunit sedimented slightly faster than alkaline phosphatase, giving an  $s$  value of 6.6. The molecular weights and frictional ratios of the enzymes were calculated from the Stokes radii and  $s$  values by the method of Siegel and Monty (32). The native enzyme has an estimated molecular weight of 176,000 and an  $f/f_0$  of 1.38. The large subunit has an estimated molecular weight of 144,000 and an  $f/f_0$  of 1.32. The high frictional ratios reflect the substantially different relation of the two enzymes to the more globular alkaline phosphatase (and other standards) in gel filtration and sedimentation analysis. In this connection, no evidence for "monomer" formation was obtained by testing behavior of the large subunit in gel filtration in a large number of conditions, including column equilibration with and 20% sucrose, glycerol, and various buffers. Large-subunit preparations made by sucrose density-gradient centrifugation and by various cell extraction techniques were also tested for behavior in gel filtration; in no case was any lower-molecular-weight species found. In addition, large-subunit preparations from a Sephacryl column were subjected to sucrose density-gradient centrifugation. All activity behaved as a 6.6S species.

Denaturing polyacrylamide gel electrophoresis was used as another means of estimating the molecular weight of the large subunit. Because neither the native enzyme nor the large subunit has been purified, an *arg-3* nonsense mutant

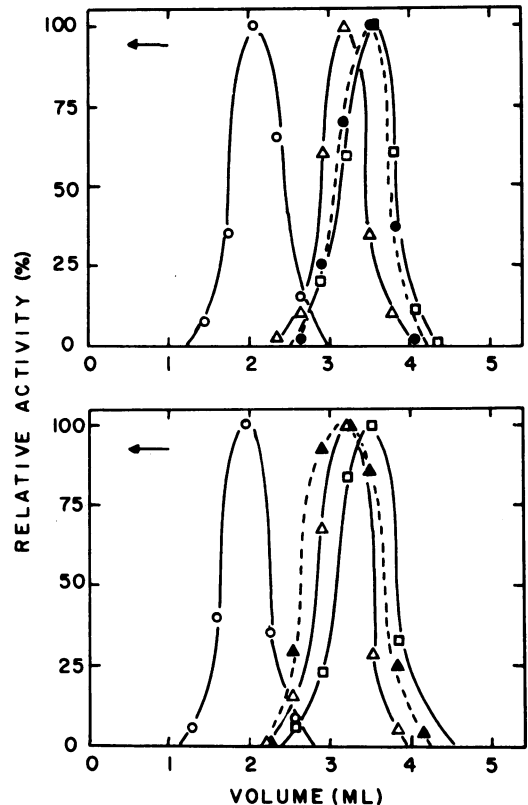


FIG. 4. Sucrose density-gradient centrifugation of carbamyl phosphate synthetase A, with standards; 5.4-ml gradients of 5 to 20% sucrose, buffered with 0.05 M potassium phosphate, were used. The gradients were spun for 10 h in a Beckman 50.1 rotor. Fractions were collected from a puncture in the bottom of the tube. The direction of sedimentation is shown by the arrows. Symbols: (○) catalase, 11.3S; (△) lactate dehydrogenase, 7.4S; (□) alkaline phosphatase, 6.3S; (●, upper panel) carbamyl phosphate synthetase, ammonia-dependent activity; (▲, lower panel) glutamine-dependent activity.

(CD-186; Davis, unpublished data) was used to identify the large subunit on gels. Mitochondrial extracts of this mutant made by the glass-bead method showed all but the largest of the proteins of the two *arg-3*<sup>+</sup> strains used for comparison (Fig. 2B). In addition, this protein was enriched in the partially purified preparations shown in Fig. 2A. Its molecular weight is 120,000 to 130,000, by reference to the standards. This is not greatly different from the molecular weight estimate of 144,000 for the large subunit derived above. Moreover, it establishes that there is only one polypeptide chain per large subunit. The small subunit has not been visualized with certainty on gels, but its molecular weight is expected to be in the neighborhood of 30,000 to

50,000 (or integral fraction thereof).

**Inactivation of the enzyme.** Both the crudest and the most highly purified preparations of the glutamine-dependent activity originating with Triton extraction of mitochondria had half-lives of 35 to 50 min at 25°C. The ammonia-dependent activity of such preparations was stable under these conditions, as was the activity of the large subunit in extracts of *arg-2* mutants. The indifference of the half-life of the glutamine-dependent activity to the stage of purification suggests that proteases were probably not responsible for the inactivation.

Many compounds were tested for their effects upon stability of the glutamine-dependent activity. The tests included substrates, Krebs cycle intermediates, amino acids, intermediates of the arginine pathway, bovine serum albumin, glycerol, sodium sulfate, aroclorin, polyethylene glycol,  $\beta$ -mercaptoethanol, dithiothreitol, EDTA, ethylene glycol-bis ( $\beta$ -aminoethyl ether) and the protease inhibitors, phenylmethylsulfonyl fluoride, aprotinin, and pepstatin. No compounds except glycerol and polyethylene glycol were effective in crude extracts. The latter two compounds had little or no stabilizing effect on purified preparations, and were not effective enough to be useful in purification. The glutamine-dependent enzyme was somewhat unstable even in a complete reaction mixture, and the 30-min reaction times yielded a slight underestimate of initial activity. This was probably not due to decay of carbamyl phosphate, since the ammonia-dependent activity is linear for 90 min or more in partially purified extracts.

Mitochondria extracted by glass-bead breakage yielded crude, glutamine-dependent activity having a 40-min half-life and DEAE-cellulose-purified preparations having half-lives as little as 15 min. Addition of aprotinin to the purified preparation extended its half-life to 40 min and had no effect on the crude preparation (not shown). The data suggest the existence of a protease liberated in glass-bead extracts which becomes effective after copurification with the glutamine-dependent activity. The experience with Triton extracts suggests that this protease is not liberated in an active form with detergent. The data further suggest that the 40-min half-life of the glutamine-dependent activity is intrinsic to the native enzyme itself, once it is liberated from the mitochondrial matrix. Concentration of inactivated preparations did not restore activity.

In the elution of the glutamine-dependent activity from Sephacryl S-200, the activity was always at the front of a broader peak of ammonia-dependent activity in which two components

were often discernible (Fig. 5A). The first component was the ammonia-dependent activity of the native heteropolymer. The second was in the position of the large subunit (Fig. 5C). When preparations of the glutamine-dependent activity were heated at 25°C for 90 min, all of the ammonia-dependent activity was recovered, but almost all of it was in a position corresponding to the purified large subunit (Fig. 5B). The results suggest that ammonia-dependent activity is a property of both the native enzyme and its large subunit. Furthermore, they show that inactivation of the glutamine-dependent activity is associated with a change of molecular weight of the remaining, ammonia-dependent activity.

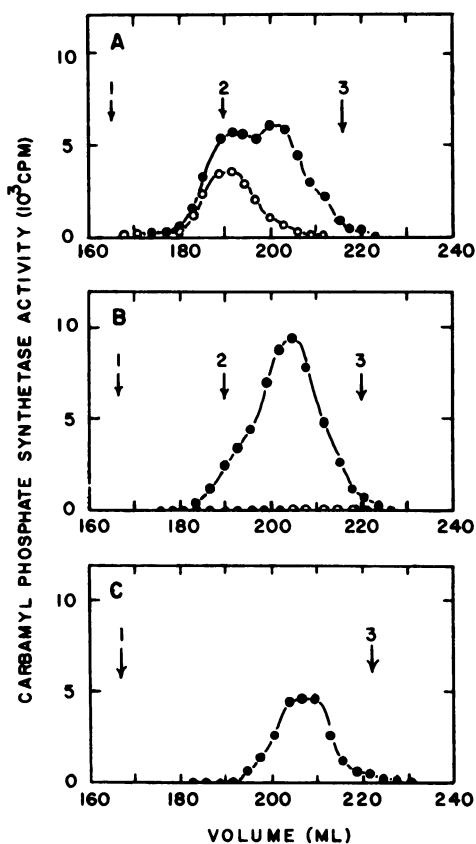


FIG. 5. Elution of carbamyl phosphate synthetase A from Sephacryl S-200 after heating. (A) Untreated, DEAE-cellulose-purified native enzyme. (B) Same, but treated at 25°C for 90 min. (C) DEAE-cellulose-purified large subunit from *arg-2* mutant. Markers: 1, void; 2, catalase; 3, alkaline phosphatase. The same amounts of activity (ammonia dependent) were used for (A) and (B). Recoveries of this activity were:  $5.0 \times 10^5$  cpm for column A and  $5.70 \times 10^5$  for column B. Symbols: (●) ammonia-dependent activity; (○) glutamine-dependent activity.



Thus inactivation is correlated with a dissociation of subunits. Whether inactivation of the glutamine-dependent activity precedes or coincides with this dissociation cannot be decided with this evidence, though it is noteworthy that a shoulder of higher-molecular-weight ammonia-dependent activity persisted after heat inactivation in the absence of glutamine-dependent activity (Fig. 5B).

**Kinetics. (i) N donor.** The native enzyme and its large subunit, both purified through the hydroxylapatite step after Triton extraction of mitochondria, were tested with respect to N donor in the carbamyl phosphate synthetase reaction. The native enzyme had a  $K_m$  (apparent) of 0.16 mM for glutamine. There were no indications of cooperative interactions at low substrate concentration. Both the native enzyme and its large subunit had a  $K_m$  (approximate) of 16.6 mM for  $\text{NH}_4\text{Cl}$ , indicating that the small subunit does not itself activate the ammonia-binding site. The preparation of the native enzyme had a ratio of activities for glutamine and  $\text{NH}_4\text{Cl}$  of 1.3.

**(ii) Bicarbonate.** The apparent  $K_m$  for bicarbonate, using the native enzyme with glutamine or  $\text{NH}_4\text{Cl}$  as N donors, or using the large subunit with  $\text{NH}_4\text{Cl}$ , was approximately 2.0 mM. Lineweaver-Burk plots were linear, with no indication of cooperative interaction. Care was taken to exclude dissolved bicarbonate from buffers and to maintain  $\text{K}^+$  at a constant level (20 mM) throughout (see below).

**(iii) ATP- $\text{Mg}^{2+}$ .** Using EDTA-free preparations of the native enzyme with glutamine or  $\text{NH}_4\text{Cl}$ , or of the large subunit with  $\text{NH}_4\text{Cl}$  as N-donor, the effect of varying ATP was tested. In this experiment, the  $\text{Mg}^{2+}$ -to-ATP ratio was maintained at 2. The results (Fig. 6) show that at low substrate concentration, the native enzyme with glutamine used ATP most efficiently ( $V_{0.5} = 0.9$  mM), and at high ATP concentration, it was inhibited. The large subunit displayed a definite sigmoid behavior in the low substrate range. The data indicate that the glutamine-binding polypeptide (small subunit), especially when it binds glutamine, activates the ATP-binding site at low ATP concentration. The data show clearly that the ammonia-dependent reaction of the native enzyme is not identical to that of the large subunit. This suggests that the native enzyme does not dissociate as a prelude to its use of ammonia in such reaction mixtures.

The ratio of ATP and  $\text{Mg}^{2+}$  was varied in a series of experiments (Fig. 7). The following points can be made. First, at 2 mM  $\text{Mg}^{2+}$ , the optimal ATP- $\text{Mg}^{2+}$  ratio was 2 for all three types of reaction (Fig. 7A, C, and E). As ATP was

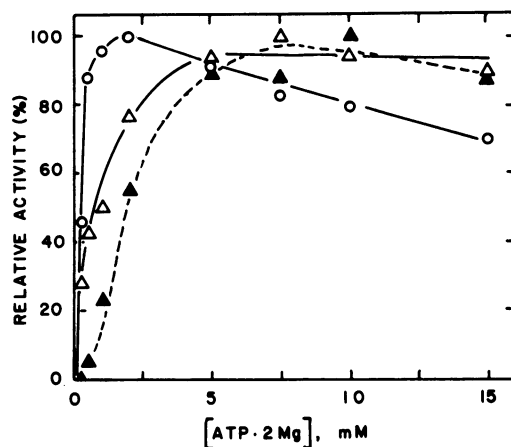


FIG. 6. Variation of carbamyl phosphate synthetase activity with  $\text{ATP} \cdot 2\text{Mg}^{2+}$  concentration. Symbols: (O) native enzyme, glutamine as N donor; ( $\Delta$ ) native enzyme,  $\text{NH}_4\text{Cl}$  as N donor; ( $\blacktriangle$ ) large subunit,  $\text{NH}_4\text{Cl}$  as N donor.

increased above 1 mM, it was severely inhibitory. At 12 mM  $\text{Mg}^{2+}$ , the reactions were less sensitive to excess ATP. (The ATP and  $\text{Mg}^{2+}$  concentrations of the standard reaction mixtures, 12 mM each, were in the optimal range.) The second point is that at fixed ATP concentrations (Fig. 7B, D, and F), there are in most cases threshold  $\text{Mg}^{2+}$  concentrations below which the enzymes are inactive. Optimum activity was found in most cases only where the  $\text{Mg}^{2+}$  concentration exceeded that of ATP; the excess was twofold at low ATP concentration. The data suggest that the enzymes have a requirement for free  $\text{Mg}^{2+}$ , as well as for ATP-Mg. This is the case with yeast carbamyl phosphate synthetase A (26) and the pyrimidine-specific enzyme of mouse spleen (35).

**(iv) Potassium.** The glutamine-dependent activity has an absolute  $\text{K}^+$  requirement, in reactions where the  $\text{Na}^+$  salt of bicarbonate was used. The optimal concentration of  $\text{K}^+$  was 20 mM; higher concentrations were somewhat inhibitory. The ammonia-dependent activity has a  $\text{K}^+$  requirement only at suboptimal  $\text{NH}_4\text{Cl}$  concentration. Evidently,  $\text{NH}_4^+$  substitutes well for  $\text{K}^+$ ; if the concentrations of the two ions are summed, the behavior of the ammonia-dependent activity becomes quite similar to that for the glutamine-dependent activity. In saturation curves in tests of  $\text{NH}_4\text{Cl}$  as a substrate, however,  $\text{NH}_4^+$  was not inhibitory at high concentration, and in fact it largely reversed the  $\text{K}^+$  inhibition at high concentration of  $\text{K}^+$  (not shown). The optimal  $\text{K}^+$  concentration is much lower than that of the yeast enzyme (100 mM) (26).

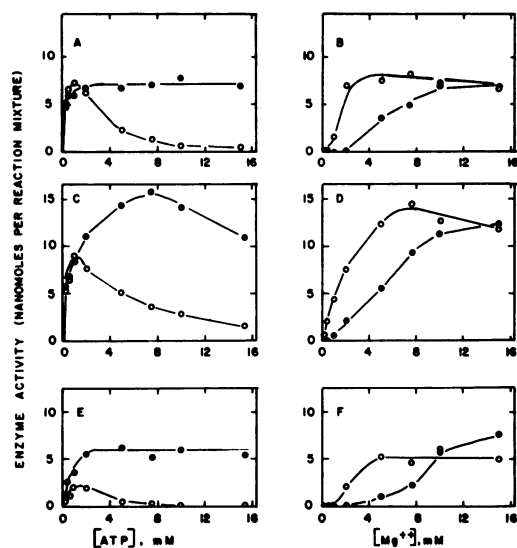


FIG. 7. Variation of carbamyl phosphate synthetase activity as ATP and  $Mg^{2+}$  are varied separately. Activities of the native enzyme with glutamine (A and B), of the native enzyme with  $NH_4Cl$  (C and D), and of the large subunit with  $NH_4Cl$  (E and F) are shown. (A, C, and E)  $Mg^{2+}$  was fixed at either 2 mM (○) or 12 mM (●). (B, D, and F) ATP was fixed at 2 mM (○) or 12 mM (●). Hydroxylapatite-purified preparations were used.

(v) **The glutamine-utilizing function.** The elimination of glutaminase activity during purification of the glutamine-dependent activity (Table 2) showed that the glutamine amidotransferase function of carbamyl phosphate synthetase A was not measurable as a glutaminase in a simple reaction mixture. An experiment designed to reveal the dependence of glutamate formation upon carbamyl phosphate synthetase substrates is shown in Table 3. No activity was seen with glutamine alone, as noted above; a very slight reaction (4% of maximal) was stimulated by bicarbonate alone. ATP-2 $Mg^{2+}$  yielded nearly 45% of maximal activity, whereas ATP and  $Mg^{2+}$  separately were ineffective.  $NH_4Cl$  was almost wholly ineffective and in fact inhibited the overall reaction with glutamine. Maximal glutamate formation was achieved only in complete synthetase reaction mixtures, and parallel reaction mixtures showed that carbamyl phosphate and glutamate were produced, as expected, in a 1:1 stoichiometric ratio (Table 3). These results are very similar to those from the mammalian cell enzyme of the pyrimidine pathway (20).

(vi) **pH optimum.** The enzyme has a pH optimum of pH 7.8 to 8.0 in Tris and phosphate buffers.

TABLE 3. Dependence of glutamate formation upon carbamyl phosphate synthetase A substrates

Reaction mixture <sup>a</sup>	Products formed (nmol)			
	Expt I		Expt II	
	Glutamate	Carbamyl phosphate	Glutamate	Carbamyl phosphate
Enzyme + [ <sup>14</sup> C]glutamine	0	0	0	0
+ $KHCO_3$	2		3	
+ ATP	0		0	
+ $Mg^{2+}$	0		0	
+ ATP, $Mg^{2+}$	11		29	
+ ATP, $Mg^{2+}$ , $KHCO_3$	15	13 <sup>b</sup>	68	64 <sup>b</sup>
+ $NH_4Cl$	0		2	
+ ATP, $Mg^{2+}$ , $KHCO_3$ , $NH_4Cl$	5		17	

<sup>a</sup> All reaction mixtures contained 100 mM Tris-hydrochloride (pH 8.0) and glutamine at 12.0 mM (experiment I) or 2.5 mM (experiment II). Different hydroxylapatite-purified preparations were used for the two experiments. Additions to reaction mixtures were at standard concentrations (see the text).

<sup>b</sup> Corrected for incomplete (90%) recovery of carbamyl phosphate.

(vii) **Potential effectors.** A systematic test of most of the intermediates of the arginine pathway, as well as of arginine itself, showed none to be specifically stimulatory nor inhibitory to carbamyl phosphate synthetase A (Table 4). This was true both of the native form using glutamine or  $NH_4Cl$  as N-donors, and of the large subunit using  $NH_4Cl$ . Putrescine and spermidine (derived from ornithine), aspartate (a substrate of argininosuccinate synthetase), UTP (an inhibitor of carbamyl phosphate synthetase P), and carbamyl phosphate had no pronounced effect. The data indicate that the enzyme is not modulated by any of the metabolites of the arginine pathway. The data are consistent with previous observations that the enzyme was not inhibited *in vivo* in conditions of arginine excess (4, 37, 45).

One effect of interest from the standpoint of polypeptide interaction is that of glycine (Table 4). Glycine had no effect upon the glutamine-dependent reaction nor upon the ammonia-dependent reaction of the large subunit. However, it activated the ammonia-dependent reaction of the native enzyme twofold. The data suggest, by analogy with the more detailed observations on the yeast enzyme (26), that glycine can bind to the small subunit and thereby activate the reactions of the large subunit. (It is curious that this experiment did not show the expected in-

TABLE 4. Test of potential effectors on carbamyl phosphate synthetase A activity

Addition to reaction mixture	Concn (mM)	Native enzyme		Large subunit (ammonia dependent) (%)
		Glutamine dependent (%)	Ammonia dependent (%)	
None		(100)	(100)	(100)
N-Acetyl-L-glutamate	10	119	118	110
N <sup>ε</sup> -Acetyl-L-ornithine	10	119	120	100
L-Ornithine	10	106	96	ND <sup>a</sup>
L-Citrulline	10	111	118	104
L-Argininosuccinate	10	94	105	86
L-Arginine	10	121	133	108
Carbamyl phosphate	10	104	97	89
UTP	5	86	92	86
L-Aspartate	5	115	115	100
Putrescine	5	109	113	98
Spermidine	5	107	97	94
Glycine	1	106	174	95
	5	111	209	106
	10	100	207	101

<sup>a</sup> ND, Not done.

hibition of the glutamine-dependent reaction.) Glycine activation is the most compelling indication that the ammonia-dependent reaction of the native enzyme is performed by the native enzyme rather than a dissociated large subunit.

## DISCUSSION

Enzymes of carbamyl phosphate synthesis vary greatly from one organism to the next. It is still too early (or, indeed, too late) to classify the modes of carbamyl phosphate synthesis, but the following patterns have been observed. (i) In the enteric bacteria, a single, two-polypeptide enzyme is found which serves arginine and pyrimidine synthesis (1, 39). It is glutamine dependent and is responsive to pyrimidine nucleotides (inhibitors) and ornithine (activator) (25). This pattern has also been found in certain higher plants (23). (ii) In *Bacillus subtilis*, two glutamine-dependent enzymes are found, one inhibitable and repressible by pyrimidine nucleotides, the other repressible by arginine (24). (iii) In yeast and *Neurospora*, two glutamine-dependent enzymes are found (10, 15). The arginine-specific carbamyl phosphate synthetase A is a two-polypeptide enzyme. The pyrimidine-specific carbamyl phosphate synthetase P is part of a complex or multifunctional protein with aspartate transcarbamylase (46). (iv) In ureotelic vertebrates, the hepatic enzyme, carbamyl phosphate synthetase I, is ammonia dependent and has only one polypeptide (17). It lacks the ability to use glutamine and requires acetylglutamate as a co-factor. Carbamyl phosphate synthetase II, a glutamine-dependent enzyme associated with py-

rimidine synthesis, is part of a multifunctional protein or aggregate with aspartate transcarbamylase and dihydroorotase (31). Enzymes which do not fall into these categories have been found in fish and snails (2, 38).

Carbamyl phosphate synthetase A of *Neurospora* has two polypeptides. This is indicated by the existence of two unlinked genes required for its structure and by the molecular weight change of the ammonia-dependent activity as the glutamine-dependent activity disappears in vitro. The glutamine-utilizing function is associated with the smaller of the two subunits. Extensive genetic studies (R. H. Davis, Genetics, in press) have shown no intragenic complementation in vivo among *arg-2* mutants (affecting the small subunit) or among *arg-3* mutants (affecting the large subunit). This is compatible with the existence of only one polypeptide of each kind. Complementation between *arg-2* and *arg-3* mutants is seen in vivo, and this can be reproduced in vitro, using purified large subunit and crude extracts of certain *arg-3* mutants (Davis, unpublished data). It is not yet known, however, whether the *arg-3* extract used in these tests contains free small subunit or merely a complex of the small subunit and a mutationally inactivated large subunit. The complementing activity so far resists any purification, being even more unstable than the native enzyme. In all the characteristics above, the *Neurospora* enzyme resembles that of *S. cerevisiae* (26).

Molecular weight estimates of carbamyl phosphate synthetase A by gel filtration and sucrose density-gradient centrifugation yield figures of 176,000 for the holoenzyme and 144,000 for the large subunit. The latter figure is similar to estimates (ca. 125,000) made by gel electrophoresis. The data for the yeast holoenzyme (26, 27) are quite inconsistent, but there is no good evidence that this species' enzyme differs substantially from that of *Neurospora*.

The kinetic properties of the *Neurospora* enzyme and its large subunit are not exceptional. Michaelis-Menten saturation curves were observed for bicarbonate, glutamine, and NH<sub>4</sub>Cl. There was no evidence for negative cooperative kinetics for glutamine, as reported for the yeast enzyme (27). The *Neurospora* enzyme, like all other carbamyl phosphate synthetases, has a requirement for K<sup>+</sup> which can be satisfied by NH<sub>4</sub><sup>+</sup>. It apparently has a free magnesium requirement. The ATP saturation curves vary according to the form of the enzyme and the N-donor (see below).

The ratio of activities with saturating glutamine versus saturating NH<sub>4</sub>Cl is difficult to estimate because of the instability of the gluta-

mine-dependent activity. The highest ratios observed, in freshly resolved preparations, are about 1.3. This is similar to the ratio for the *E. coli* enzyme (1.7) (43), that of pea plants (0.94) (23), or that of the mammalian pyrimidine-specific enzyme (1.3 to 1.8) (13, 34). It is however, far lower than the ratio of 8 to 10 observed for yeast carbamyl phosphate synthetase A (26) or for the unusual mitochondrial enzyme from teleost fish (12.5) (2). The different classes of ratio suggest a fundamental difference in the extent of activation by the two N-donors of the functions of the large subunit, or in the access of free ammonia to the active site.

Subunit interactions are seen with the *Neurospora* enzyme in three ways. First, ATP is used much more efficiently at low concentration by the native enzyme than the large subunit, even when  $\text{NH}_4\text{Cl}$  is the N-donor for the native enzyme. Second, glycine activates the native enzyme when  $\text{NH}_4\text{Cl}$  is the N-donor, but does not activate the ammonia-dependent large subunit. Third, the glutamate-forming function of the small subunit is activated significantly by  $\text{ATP-Mg}^{2+}$  and is maximal only in complete carbamyl phosphate synthetase A reaction mixtures. It is clear that activation of the glutamine amidotransferase activity is achieved via the large subunit, which has binding sites for all the other substrates. The subunit interactions are similar to those observed in other carbamyl phosphate synthetases. It should be noted that acetylglutamate has no stimulatory effect on either form of the *Neurospora* enzyme, as it does on mammalian carbamyl phosphate synthetase I and certain other synthetases.

The *Neurospora* enzyme is indifferent to arginine. The *Neurospora* enzyme, in fact, was not affected by any metabolite of the arginine path or related paths that was tested. One would expect that some feedback inhibition would be seen for this step, since it is a pace-setting enzyme. However, even tests in vivo revealed that the enzyme continues to function for some hours after arginine is added to cells (4). One of the peculiarities of carbamyl phosphate synthetase biochemistry is that no enzyme has yet been found that is sensitive to arginine. This is at least understandable in enteric bacteria, where one enzyme, responsive to the opposing effects of UMP inhibition and ornithine activation, serves both pathways (25). It is hard to understand in *Bacillus subtilis* (24) and the fungi, where specialized, arginine-specific enzymes are found. (In ureotelic vertebrates, arginine inhibition would be maladaptive in a pathway devoted to urea synthesis.) At one point, Cybis and Davis (7) claimed that the lack of feedback inhibition

by arginine may have evolved because the synthetase was mitochondrial and the metabolically significant arginine pool was cytosolic. This theory is untenable in view of the cytosolic locations of the yeast (40) and *B. subtilis* enzymes. However, carbamyl phosphate synthetase A of *Neurospora* is virtually absent in cells grown in arginine, and it may be that efficient repression has the role of the major control mechanism (7). Moreover, it is the glutamine-binding polypeptide or its effective association with the large subunit that responds so strongly to arginine. A special genetic circuit controls this polypeptide in yeast (36).

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