Copurification of Pyrimidine-Specific Carbamyl Phosphate Synthetase and Aspartate Transcarbamylase of *Neurospora crassa**

Larry G. Williams,† Sharon Bernhardt, and Rowland H. Davis

ABSTRACT: In *Neurospora* the initial enzymes of pyrimidine synthesis, pyrimidine-specific carbamyl phosphate synthetase and aspartate transcarbamylase, are specified by the same locus, *pyr-3*. A purification technique devised for the most labile of these two enzymes, the synthetase, resulted in the copurification of aspartate transcarbamylase. The two activities remained associated throughout $(NH_4)_2SO_4$ precipitation, calcium phosphate gel adsorption, gel filtration chromatography, DEAE-cellulose chromatography, and sucrose density gradient centrifugation. An enzyme preparation estimated to be 50% pure was obtained containing pyrimidine-specific carbamyl phosphate synthetase and aspartate transcarbamylase at specific activities 50 and 36 times that found in the original derepressed extract (250 and 180 times that found in wild type crude extracts). The molecular weight of the enzyme complex was estimated to be 650,000 g per mole on the basis of its elution pattern from agarose gel filtration columns and its sedimentation properties in sucrose density gradients. The synthetase activity of the purified complex is completely inhibited by the end product, UTP (uridine triphosphate), at 1×10^{-3} M. On sucrose density gradients the complex sediments at a value of 21 S in the absence of UTP and at 15 S in the presence of UTP. This suggests the dissociation of enzyme subunits in the presence of the feedback inhibitor; however no molecular weight change is observed, in the presence of UTP, in agarose gel filtration. Earlier studies from this laboratory have shown that under normal *in vivo* conditions carbamyl phosphate produced by the pyrimidine–enzyme complex is unavailable as a substrate for ornithine transcarbamylase in the arginine synthetic pathway. The observed confinement of carbamyl phosphate in pyrimidine synthesis could be explained if carbamyl phosphate existed as an enzyme-bound intermediate on the enzyme complex.

Neurospora has been shown to possess two enzymes which form carbamyl phosphate, an intermediate of both arginine and pyrimidine synthesis (Williams and Davis, 1970; Davis, 1965). The two carbamyl phosphate synthetases are under control of separate genetic loci and can be clearly distinguished on the basis of molecular weight, feedback inhibitors, derepression conditions, and stabilization factors. *In vivo* one enzyme produces carbamyl phosphate specifically for use in the arginine pathway while the second synthesizes carbamyl phosphate specifically for utilization *via* the pyrimidine pathway (Davis, 1967). The syntheses of CPS_{Pyr}[1] and ATC (carbamoylphosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2.) are coderepressed by pyrimidine starvation. The CPS_{Pyr} activity can be completely inhibited by $5\text{–}10 \times 10^{-4}$ M UTP (uridine triphosphate). ATC activity is not inhibited by UTP. CPS_{Pyr} is cold labile and has much greater affinity for L-glutamine than for ammonia as a nitrogen source (Williams and Davis, 1970).

CPS_{Pyr} and ATC catalyze the initial reactions of pyrimidine synthesis:[2]

$$2ATP + \text{L-glutamine} + CO_2 \longrightarrow \text{carbamyl phosphate} + \text{L-glutamate} + 2ADP + P_i$$

$$\text{carbamyl phosphate} + \text{L-aspartate} \longrightarrow \text{L-ureidosuccinate} + P_i$$

Several lines of evidence indicated that the CPS_{Pyr} and ATC activities might be associated in an enzyme complex: (1) single mutations at the *pyr-3* locus can eliminate either or both enzyme activities (Davis and Woodward, 1962; Williams and Davis, 1970), (2) the two activities appear to be translated from a common messenger RNA (Radford, 1969a,b), and (3) strains lacking CPS_{Pyr} activity have structurally altered ATC (Hill and Woodward, 1968).

We report here a purification procedure for CPS_{Pyr} and the observed association of CPS_{Pyr} and ATC activities at each step of the purification. Estimates of the molecular weight of the CPS_{Pyr}–ATC complex were made by comparing the behavior of the complex on gel filtration columns and sucrose gradients to that of proteins of known molecular weight.

* From the Department of Botany, The University of Michigan, Ann Arbor, Michigan 48104. *Received July 13, 1970.* This work was supported in part by a grant from the National Science Foundation (GB-6414) to R. H. Davis. The senior author acknowledges the support of a National Institutes of Health postdoctoral fellowship (No. 5-FO2-GM19703-02).

† Present address, Division of Biology, Kansas State University, Manhattan, Kansas 66502. To whom reprint requests should be directed.

[1] Abbreviations used are: CPS_{Pyr}, pyrimidine-specific carbamyl phosphate synthetase; ATC, aspartate transcarbamylase.

[2] The stoichiometry for the *Neurospora* CPS_{Pyr} has not been established; the equation represents the stoichiometry of *E. coli* carbamyl phosphate synthetase (Anderson and Meister, 1965).

Experimental Procedure

Materials. ATP, UTP, L-glutamine, L-aspartic acid, bovine thyroglobulin, and beef liver catalase were purchased from the Sigma Chemical Co. Jack bean urease was obtained from the Worthington Biochemical Corp. Dilithium carbamyl phosphate, Cleland's reagent (dithiothreitol), and Biogel agarose 0.5-m and 1.5-m gels were purchased from Calbiochem. The calcium phosphate gel adsorbent was prepared by a standard procedure (Keilin and Hartree, 1938). DEAE-cellulose, obtained from Bio-Rad, was washed sequentially with 0.5 N HCl, H_2O, 0.5 N NaOH, and H_2O after which it was stored at 4° in 0.03 M potassium phosphate buffer, pH 7.3.

Enzyme Preparation. An *arg-3, pyr-1* double mutant strain was used as the source of the enzyme complex. The *arg-3* mutation eliminates arginine-specific CPS activity, while the *pyr-1* mutation permits derepression of the pyrimidine synthetic enzymes under conditions of pyrimidine starvation. The conditions for mycelial growth, enzyme derepression, and stabilization of CPS_{Pyr} activity have been reported previously (Williams and Davis, 1970). The standard buffer system used in enzyme extraction and purification was 0.05 M potassium phosphate, pH 7.3, containing 1×10^{-3} M L-glutamine and 2×10^{-4} M Cleland's reagent. UTP, which retards the cold inactivation of CPS_{Pyr}, was added to the buffer in some steps. Unless otherwise noted, however, UTP can be assumed to be absent. Crude supernatants and $(NH_4)_2SO_4$ fractions were desalted by passage through short Sephadex G-25 columns before assay.

Enzyme Assays. The reaction mixture for CPS_{Pyr} contained 50 μmoles of Tris-acetate buffer (pH 7.5), 3 μmoles of L-glutamine, 15 μmoles of $KHCO_3$, 6 μmoles of ATP, 6 μmoles of $MgCl_2$, and enzyme preparation (0.10 ml) in a final volume of 0.50 ml. The labile product, carbamyl phosphate, was converted into urea (Williams and Davis, 1970), which in turn was measured colorimetrically by a modification of the method of Gerhart and Pardee (1962). Incubation conditions, controls, and the colorimetric procedure are discussed in more detail elsewhere (Williams and Davis, 1970). One unit of CPS_{Pyr} activity is defined as the formation of 1 μmole of carbamyl phosphate per hour at 25°.

The reaction mixture for ATC contained 150 μmoles of glycine buffer (pH 9.0), 10 μmoles of L-aspartic acid, 5 μmoles of freshly dissolved dilithium carbamyl phosphate, and enzyme preparation (0.20 ml) in a final volume of 1.00 ml. The ureidosuccinate produced was measured colorimetrically by the same procedure as urea in the CPS_{Pyr} assay. One unit of ATC activity is defined as the formation of 1 μmole of ureidosuccinate per hour at 25°. Specific activities for CPS_{Pyr} and ATC are expressed as enzyme units per mg of protein.

Urease activity was detected by the method of Sumner (1955). Catalase activity was measured as the time required for the optical density of an H_2O_2 solution in 0.05 M phosphate buffer, pH 7.0, to decrease from 0.45 to 0.40 at 240 nm. Protein determinations were made by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

Agarose Gel Filtration. Biogel agarose 1.5 columns (1.2 × 37.5 cm) were equilibrated at room temperature by the flow of 100 to 200 ml of standard buffer through the column. The protein solution, in a volume of 1.25 ml, was added to the top of the gel, followed by 0.50 ml of buffer. Following attachment of the buffer reservoir a constant flow rate of 0.25 ml/min was maintained by use of a Mariotte flask. Five-minute fractions of approximately 1.25-ml volume were collected. Void volumes as determined by the elution volume of high molecular weight protein complexes present in crude extracts were consistently in the range of 20.0 ± 0.5 ml.

Enzyme Purification. All purification steps were conducted at room temperature unless otherwise noted. *Neurospora* mycelia were harvested on cheesecloth and pressed between paper towels to form a damp pad. Approximately 80 g of damp mycelial pad, an equal volume of standard buffer containing 7.5×10^{-4} M UTP, and 16 g of fine sand were ground to a slurry with mortar and pestle at 0°. Sand and cell wall material was removed by 15-min centrifugation at 12,000*g* and 5°.

1. HIGH SPEED CENTRIFUGATION. The crude supernatant (110 ml) was centrifuged 30 min at 50,000*g* at 5°. After removal of the heavy lipid pellicle with a Pasteur pipet, the high speed supernatant, containing all the recoverable CPS_{Pyr} and ATC activity, was decanted from the residue and cooled at 0°.

2. $(NH_4)_2SO_4$ FRACTIONATION. I. Solid $(NH_4)_2SO_4$ was immediately dissolved in the high speed supernatant (2.5 g per 10 ml). Following 5-min stirring to dissolve the $(NH_4)_2SO_4$ and 5-min additional standing at 0° the protein precipitate was collected by 25-min centrifugation at 12,000*g* at 5°. The supernatant was discarded. The precipitate was dissolved in 8 ml of standard buffer (to about 15 mg of protein/ml) and desalted by passage of 4-ml portions through 3.3 cm i.d. × 4.5 cm tall Sephadex G-25 gel filtration columns, equilibrated with the same buffer, at room temperature.

3. CALCIUM PHOSPHATE GEL FRACTIONATION. The desalted protein solution (20 ml) was stirred for 3–5 min with 4.0–4.5 ml of calcium phosphate gel (21 mg dry weight/ml). The gel was sedimented by a brief centrifugation and discarded. To the supernatant was added an additional 13–14 ml of calcium phosphate gel. The second gel addition absorbed most of the CPS_{Pyr} and ATC activity and was collected by a brief low speed centrifugation. The supernatant was discarded. Protein was eluted from the gel by successive washes (suspension of the gel in buffer by 5-min stirring followed by centrifugation) with 10-ml portions of standard buffer (containing 1×10^{-4} M UTP) at pH 7.3, 7.8, and 8.1. The amount of calcium phosphate gel required to give optimum purification varied slightly from preparation to preparation. Therefore, a trial experiment was carried out on a small portion of each preparation to determine the volumes of gel needed.

4. $(NH_4)_2SO_4$ FRACTIONATION. II. The three washes of the calcium phosphate gel were combined and cooled to 0° and protein was precipitated by the addition of solid $(NH_4)_2SO_4$ (3 g/10 ml). Following 5 min of stirring and 5-min standing at 0° the precipitate was recovered by 15-min centrifugation at 50,000*g* at 5°.

5. AGAROSE GEL FILTRATION. The precipitate of the second $(NH_4)_2SO_4$ fractionation was immediately redissolved in 1.25 ml of standard buffer and applied to an agarose 1.5 gel filtration column as previously described. The column fractions between 21 and 29 ml, containing about 85% of the recoverable CPS_{Pyr} and ATC activity, were combined.

6. DEAE-CELLULOSE FRACTIONATION. The pooled CPS_{Pyr}–ATC fractions from step 5 were immediately applied to a

TABLE I: Purification of CPS_{Pyr} and ATC.[a]

Fraction	Vol (ml)	Protein (mg/ml)	Total Units		Specific Activity		Increase of Activity over Repressed Crude Extract	
			CPS_{Pyr}	ATC	CPS_{Pyr}	ATC	CPS_{Pyr}	ATC
Repressed crude extract					0.068	2.2	1	1
Derepressed crude extract	108.0	5.58	204	6610	0.34	10.9	5	5
High speed supernatant	88.0	3.90	140	4470	0.41	13.1	6	6
$(NH_4)_2SO_4$ I (desalted)	22.0	5.95	139	4810	1.06	36.6	16	17
Calcium phosphate gel	33.0	0.64	92	2040	4.40	97.0	65	44
$(NH_4)_2SO_4$ II	1.3	13.30	80	1660	4.60	96.0	67	44
Agarose 1.5 gel	8.4	0.90	55	1160	7.30	154.0	107	70
DEAE	11.4	0.15	30	472	17.20	272.0	253	124

[a] Details of the procedures are described in Materials and Methods. Units of activity = μmoles of product (carbamyl phosphate or ureidosuccinate) per hour. Specific activity = μmoles of product/mg of protein per hr.

DEAE-cellulose column (1.1 × 4.0 cm) equilibrated with 30 ml of standard buffer containing 1×10^{-4} M UTP. After the protein solution had entered the column bed the column was washed with 16 ml of standard buffer (1×10^{-4} M in UTP) and then eluted with a 100-ml exponential NaCl gradient of 0–0.5 M prepared in standard buffer (1×10^{-4} M in UTP). The flow rate was about 1 ml/min and fractions of 2.5 to 3.0 ml were collected. CPS_{Pyr} assays were run on a small portion of each fraction and those fractions having appreciable activity were pooled.

Enzyme Storage. Purified CPS_{Pyr}–ATC from step 6 was concentrated by $(NH_4)_2SO_4$ precipitation and centrifugation as in purification step 4. The precipitate was redissolved in 1.0–2.0 ml of standard buffer containing 5×10^{-4} M UTP, quickly frozen in a Dry Ice–methyl Cellosolve bath, and stored at −80°. For optimum recovery of CPS_{Pyr} activity the purification procedure is carried out in 2 days. The first day the preparation is taken through step II and the desalted, redissolved material is brought to 5×10^{-4} M in UTP and quickly frozen and stored at −80°. Activity of CPS_{Pyr} is rapidly lost at temperatures less than 10° in the absence of UTP, on freezing (even in the presence of UTP) at 0 to −20°, and on freezing at protein concentrations below 0.5 mg of protein per ml.

Sucrose Gradients. Exponential sucrose gradients, 0.15–0.64 M, of 4.7-ml volume were prepared. The sucrose solutions were made up in the standard buffer containing either no UTP or UTP at 1×10^{-4} M. A 0.20-ml portion of protein solution containing a maximum of 1.5 mg of protein was applied to the gradient. Centrifugation was at 38,500 rpm for 4.5 to 5.5 hr in a Beckman Model L ultracentrifuge at 15° using a SW-39 rotor. Fractions were collected at 20- or 30-sec time intervals. A flow rate of 0.50 ml/min was maintained by a pump.

Results

The purification procedure given under Experimental Methods was devised to give optimum yield and purity of CPS_{Pyr} activity while retaining its feedback inhibiting sensitivity. Using this procedure fractions were then assayed at all stages of purification for both CPS_{Pyr} and ATC activity. As shown in Table I, a purification of CPS_{Pyr} gave considerable purification of ATC activity. Five purification trials gave the following increases in CPS_{Pyr} and ATC activities of the DEAE-cellulose eluate over that found in crude repressed extracts: (1) 88- and 180-fold, (2) 126- and 125-fold, (3) 220- and 174-fold, (4) 246- and 181-fold, (5) 253- and 124-fold. CPS_{Pyr} activity is quite labile and a considerable variation in final activity between purification runs is not surprising. In those cases where lowered CPS_{Pyr} activity resulted the greater loss could be assigned to loss on storage between steps or to unusually long times in carrying out a purification step. In general the final purification factors for ATC activity were lower than for CPS_{Pyr}. In all cases this resulted from lowered recovery of ATC activity after its adsorption to calcium phosphate gel and DEAE-cellulose.

To test further the hypothesis that CPS_{Pyr} and ATC activities are associated, each purification step was examined under a number of fractionation conditions. Without exception, fractions found to contain CPS_{Pyr} activity also had ATC activity. Amounts of $(NH_4)_2SO_4$ or calcium phosphate gel required to recover various levels, 10, 25, 50% etc., of CPS_{Pyr} from solution also recovered or inactivated equal percentages of ATC.

Additional evidence that CPS_{Pyr} and ATC are associated is shown by the similar elution patterns of the two activities upon gel filtration chromatography (Figure 1). Almost identical results, both with respect to CPS_{Pyr}–ATC association

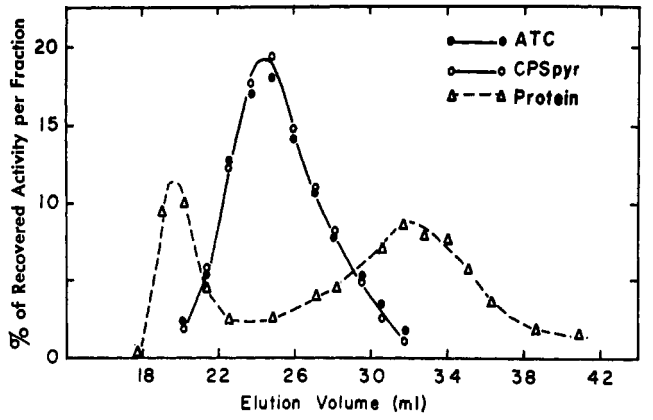

FIGURE 1: Distribution of CPS_{Pyr}, ATC, and protein from crude, derepressed extract of *Arg-3 Pyr-1* mycelium by gel filtration on Biogel agarose 1.5 (see Experimental Procedure). Recoveries of applied material were 100% for protein, 105% for ATC, and 75% for CPS_{Pyr}. Data are plotted as percentages of activity or protein recovered from the column.

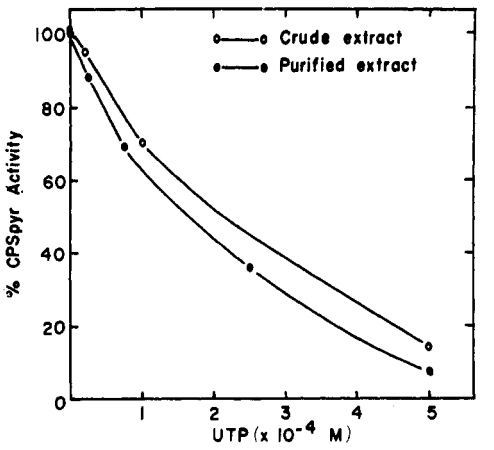

FIGURE 3: Feedback inhibition of crude and purified preparations of CPS_{Pyr} by UTP.

and to elution volume of the activities, were obtained whether the preparation added to the column came from a crude extract, an $(NH_4)_2SO_4$ fraction, or was eluted from calcium phosphate gel. In the course of the work enzyme preparations were subject to filtration chromatography on the following gels: Biogel P-300, A-0.5, A-1.5 and A-5.0. On all grades, the close correlation of the elution patterns of the two enzyme activities was maintained. On Biogel P-300 and A-0.5 columns (molecular weight exclusion roughly 400,000 and 500,000, respectively) the ATC and CPS_{Pyr} activities were eluted in the void volume.

The elution of protein, CPS_{Pyr}, and ATC from DEAE-cellulose by a NaCl gradient is shown in Figure 2. Two major points can be made with these data. First, the CPS_{Pyr} and ATC activity peaks are coincident. Second, the presence of a major protein peak associated with the enzyme activities suggests that the enzyme complex is approaching purity. The average specific activity of the two fractions from the DEAE column containing the highest enzyme activities was 200- and 174-fold that of CPS_{Pyr} and ATC found in crude repressed extracts.

Feedback inhibitability of CPS_{Pyr} by UTP was used to test for possible changes in the complex during purification, since in previous work, feedback sensitivity was shown to be a labile character of CPS_{Pyr}. A comparison of CPS_{Pyr} inhibition by UTP in a crude extract and a purified preparation is

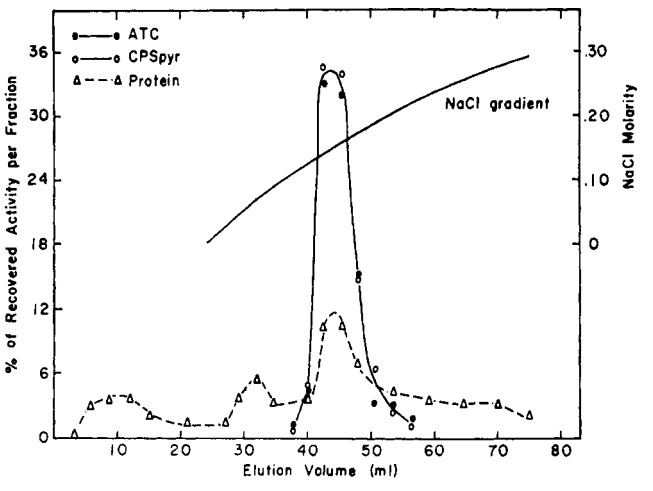

FIGURE 2: DEAE-cellulose chromatography of CPS_{Pyr} and ATC. The preparation applied to the column was purified through step 5 (see Experimental Procedure). Conditions of elution by NaCl gradient are described in Experimental Procedure. Recoveries were 58% of protein, 63% of CPS_{Pyr}, and 57% of ATC activity applied to the column. CPS_{Pyr} and ATC specific activities in two fractions of maximal activity were 15.0 and 383 units per mg.

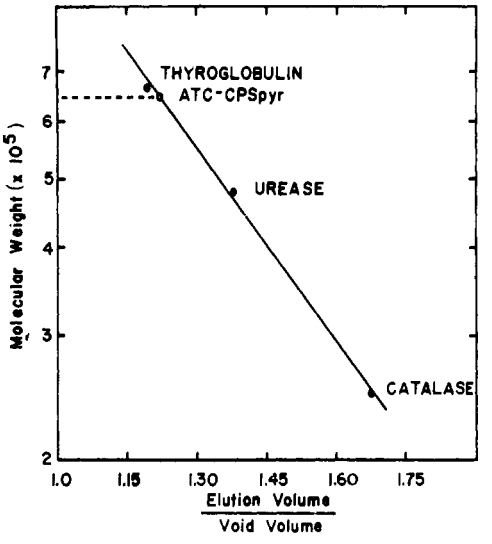

FIGURE 4: Molecular weight estimate of CPS_{Pyr}–ATC complex by agarose 1.5 gel chromatography: (●) standard protein markers catalase (248,000), urease (483,000), and thyroglobulin (669,000) (Klotz and Darnell, 1969); (○) CPS_{Pyr}–ATC complex. Each marker and a partially purified CPS_{Pyr}–ATC preparation were run through the column separately (two runs each). All determinations were made on the same column. The void volume and the elution volume of the CPS_{Pyr}–ATC complex were the same on the initial and last use of the column. Thyroglobulin was determined by the Lowry protein assay. Column methods and enzymatic assays as described in Experimental Procedure.

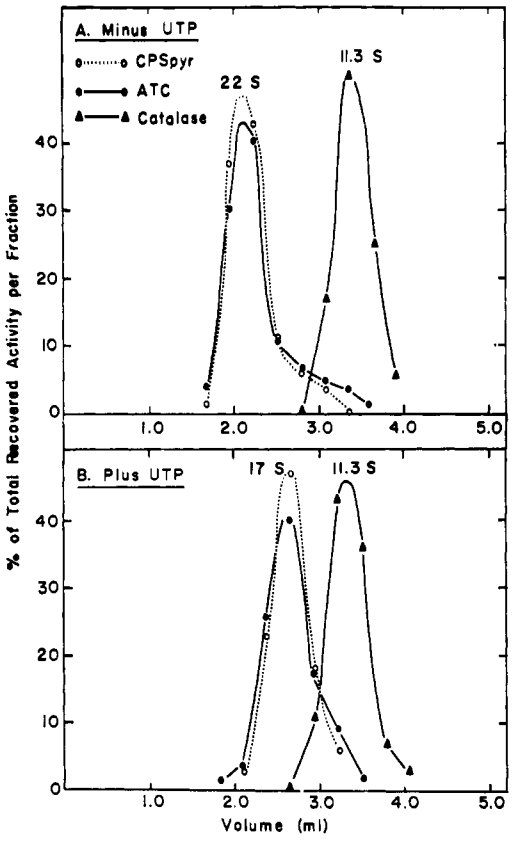

FIGURE 5: Distribution of CPS_{Pyr} and ATC activity on sucrose gradients. (A) Both enzyme preparation and gradient lack UTP. (B) Enzyme preparation and gradient contain 1×10^{-4} M UTP. Centrifugation was at 38,500 rpm for 5 hr; right side of figure = top of gradient. The final concentration of UTP in the CPS_{Pyr} incubation mixtures when enzyme came from the UTP gradient was 1×10^{-5} M, a level that inhibits CPS_{Pyr} by less than 5%. Other conditions described under Experimental Procedure.

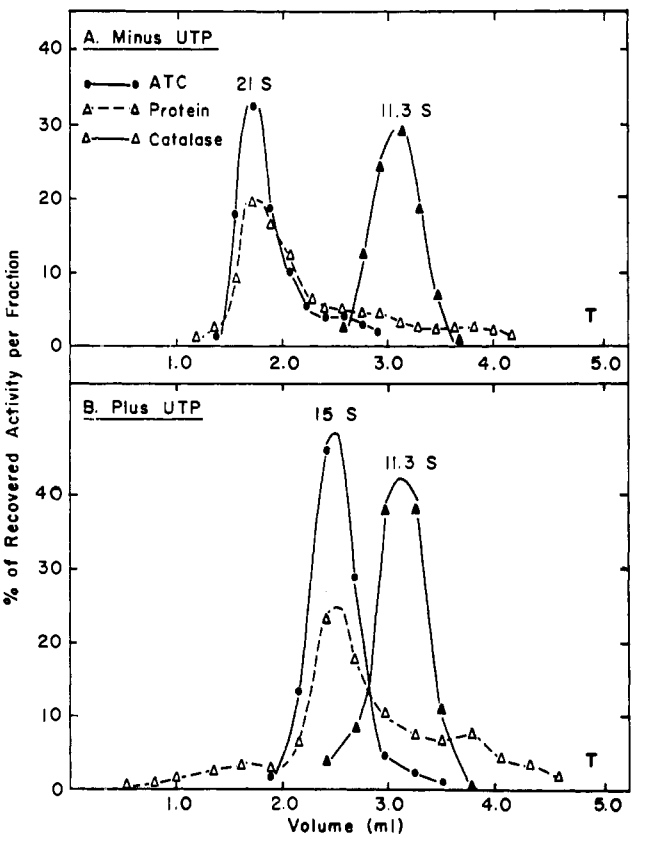

FIGURE 6: Distribution of ATC and protein on sucrose gradients. Centrifugation was at 38,500 rpm for 5.5 hr. Other conditions are described in the legend of Figure 5 and in the Experimental Procedure.

shown in Figure 3. The fact that feedback sensitivity of both preparations is quite similar supports the assumption that the enzyme complex is not greatly altered by the purification procedure.

Estimation of Molecular Weight by Gel Filtration. An agarose 1.5 gel column was calibrated by determining the elution volume of three proteins of known molecular weight. The relation of the molecular weight to the elution volume for those proteins is shown in Figure 4, together with the position of the CPS_{Pyr} and ATC activity peaks from the same column. Determinations of the void volume, marker protein, and CPS_{Pyr}–ATC elution volumes were highly reproducible and allowed a molecular weight estimate for the CPS_{Pyr}–ATC complex of 650,000 ± 50,000. The addition of UTP to the elution buffer did not alter the elution volume or shape of the CPS_{Pyr}–ATC peak.

Estimation of Molecular Weight by Sucrose Gradient Centrifugation. Preparations of CPS_{Pyr} and ATC from the purification steps outlined in Experimental Procedure were subjected to sucrose gradient centrifugation. The purposes of these experiments were (1) to determine if the two enzymatic activities cosedimented, (2) to determine the possible effect of UTP on the sedimentation behavior of the proposed complex, (3) to obtain an estimate of CPS_{Pyr}–ATC purity by comparing the distribution of protein throughout the gradient with that of enzymatic activity, and (4) to secure a second measure of CPS_{Pyr}–ATC molecular weight by comparison of its sedimentation rate to that of proteins of known molecular weight and sedimentation coefficient.

Figure 5 shows two main features: first, CPS_{Pyr} and ATC activity cosediment on the sucrose gradients; second, the enzyme complex sediments considerably slower in the presence of UTP, the CPS_{Pyr} feedback inhibitor, than in its absence. In the absence of UTP the majority of CPS_{Pyr}–ATC has a greater sedimentation coefficient although there remains a very definite and reproducible shoulder of the more slowly sedimenting form of the complex. Recoveries of CPS_{Pyr} and ATC activities from the gradient were 55 and 105% in the presence of UTP and 60–130% in the absence of UTP. (Other treatments, notably heat, which result in moderate to severe loss of CPS_{Pyr} activity often cause activation of ATC.)

Figure 6 again demonstrates the effect of UTP on the sedimentation rate of ATC. The fact that the major protein peak changes its position, with the activity peak, in the presence and absence of UTP, can be used as an indication of the purity of the enzyme preparation. Assuming that the protein which changes its sedimentation properties in response to UTP is CPS_{Pyr}–ATC the enzyme preparation applied to the gradient is approximately 50% pure.

Sedimentation coefficients of CPS_{Pyr}–ATC were approximated by the method of Martin and Ames (1961). Catalase

(11.3 S) was used as the standard protein. The sedimentation of catalase was not affected by UTP nor was the sedimentation of CPS_{Pyr}–ATC altered by catalase. Using catalase as a standard, the sedimentation coefficient of thyroglobulin, with a reported value of 19 S (Edelhoch and de Crombrugghe, 1966), was calculated to be 18.2 S. The sedimentation coefficients obtained for CPS_{Pyr}–ATC (average of four gradients) were 21.1 ± 0.3 in the absence of UTP and 15.3 ± 0.6 in the presence of UTP. Certain assumptions (Martin and Ames, 1961) allow estimates of molecular weight to be made from the sedimentation values. Assuming CPS_{Pyr}–ATC to have the sedimentation properties of catalase, the 21S and 15S components have calculated molecular weights of 630,000 and 385,000. Assuming ATC–CPS to have the properties of thyroglobulin, the 21S and 15S components have calculated molecular weights of 780,000 and 480,000.

Discussion

Work reported here demonstrates the copurification of *Neurospora* CPS_{Pyr} and ATC, the initial enzymes in the pyrimidine synthetic pathway. CPS_{Pyr} activity could not be separated from ATC by $(NH_4)_2SO_4$ fractionation, calcium phosphate gel adsorption, gel filtration chromatography, DEAE-cellulose chromatography, or sucrose density gradients. Enzyme preparations were obtained containing CPS_{Pyr} at a specific activity 250 times that found in wild type extracts (50 times that of the initial derepressed extract). These preparations were estimated to be about 50% pure on the basis of the amount of the preparation's protein shifted to a lower sedimentation coefficient by UTP.

The same preparations showed a lesser increase (125- to 180-fold) in ATC activity. The lower purification factor for ATC reflects a lesser recovery of ATC than of CPS_{Pyr} activity following calcium phosphate gel and DEAE-cellulose adsorption. The missing ATC activity could not be recovered by stronger elution techniques. A possible explanation of the lower ATC recoveries is that the ATC active site of the CPS_{Pyr}–ATC complex is more susceptible to inactivation by the adsorption steps. A second possibility is that in preparation of the crude extract, the CPS_{Pyr} site of some molecules could have been inactivated. The hypothesized monofunctional form of the enzyme could have copurified with the bifunctional forms as far as the adsorption steps, where the ATC site of the monofunctional form was inactivated or lost. The copurification of CPS_{Pyr} and ATC is consistent with genetic data (Williams and Davis, 1970; Davis and Woodward, 1962) in suggesting that in *Neurospora* the two enzymatic activities are carried by a bifunctional protein.

The production of carbamyl phosphate by CPS_{Pyr} is probably the rate-limiting step in pyrimidine synthesis. The specific activities of ATC and dihydroorotase, the next two enzymes in the pyrimidine pathway, are about 30 times that of CPS_{Pyr}. Conditions which extract the maximum amount of enzyme complex activity (Caroline, 1969), *i.e.*, an acetone-dried mycelial powder extracted with buffer containing Triton-X-100, yield a preparation capable of producing 300–500 nmoles of carbamyl phosphate per minute per gram of mycelium. To meet the pyrimidine requirements of *Neurospora* growing in log phase requires the production of 630 nmoles of carbamyl phosphate per minute per gram of mycelium. The extractable carbamyl phosphate synthesizing activity is therefore sufficient to meet the majority of the cell's needs. Conditions for extraction of maximal activity of the enzyme complex were not used in the purification scheme reported here as such treatment destroys the feedback inhibition site of CPS_{Pyr}.

The enzyme complex has a molecular weight of 650,000 (both in the presence and absence of UTP) as calculated from its distribution on gel filtration columns. On sucrose gradients the complex sediments at a value of 21 S in the absence of UTP and 15 S in its presence, corresponding to molecular weights of 630,000 and 385,000 (using a catalase standard). The significance of the apparent molecular weight changes due to UTP in relation to regulation mechanisms within the cell is uncertain. Since UTP causes both inhibition of CPS_{Pyr} activity and formation of a slower sedimenting form of the enzyme the possibility that feedback inhibition results from dissociation of enzyme subunits is suggested. The level of UTP required for formation of the 15S components (1×10^{-4} M or less) and for complete feedback inhibition of CPS_{Pyr} (5×10^{-4} M) are not directly comparable because ATP, a CPS_{Pyr} substrate used in feedback inhibition assays, sharply reduces the effectiveness of UTP as an inhibitor (Williams and Davis, 1970). Information is needed on the following points: (1) the molarity of UTP needed for the appearance of the 15S form in the presence of substrate levels of ATP; (2) the true molecular weights of the 15S and 21S forms; and (3) conditions required for further dissociation of the complex.

In yeast a similar bifunctional enzyme has been reported (Lue and Kaplan, 1969, 1970) in which both activities are subject to feedback inhibition by UTP. The effect of UTP on the CPS_{Pyr}–ATC complex of yeast is opposite to that found in *Neurospora*. On sucrose density gradients, the highest molecular weight form (600,000) is found in the presence of UTP. In the absence of UTP a 300,000 molecular weight form is found. In response to heat or passage through DEAE-Sephadex a 140,000 molecular weight class is formed which has only ATC activity and is not feedback inhibitable.

The enzymes involved in the production and utilization of carbamyl phosphate for arginine and pyrimidine synthesis have been studied to one degree or another in a wide variety of organisms. In *Escherichia coli* the presence of only one carbamyl phosphate synthetase has been shown and no association of this enzyme with ATC or ornithine transcarbamylase has been observed. The activity of the *Escherichia coli* carbamyl phosphate synthetase is under control of both pyrimidine and arginine metabolites (Piérard *et al.*, 1965). Anderson and Martin (1970) have provided evidence that a monomer–oligomer equilibrium occurs with *Escherichia coli* carbamyl phosphate synthetase. Formation of an enzyme state with a higher sedimentation coefficient is induced by inosine monophosphate and ornithine and its formation is prevented by uridine monophosphate.

Most, if not all, eucaryotic organisms, including fungi (LaCroute *et al.*, 1965; Hirsch and Gans, 1968; Davis, 1967), amphibia (Lan *et al.*, 1969), and mammals (Hager and Jones, 1967; Nakanishi *et al.*, 1968), have two carbamyl phosphate synthesizing enzymes. In most cases one of the enzymes can be shown to have properties of a pyrimidine-synthetic enzyme and the other of an arginine-synthetic enzyme. In *Neurospora* mutation of the CPS_{Pyr} site within the *Pyr-3* locus imposes a pyrimidine requirement on the cell, while mutation at the loci controlling the other CPS, *Arg-2* and *Arg-3*, causes an arginine

requirement (Davis, 1967). In *Neurospora*, *Coprinus*, and *Saccharomyces*, mutants which affect CPS_{Pyr} and ATC map at the same genetic locus. Further, in *Neurospora* and *Coprinus* the two activities appear to be translated from a single messenger RNA (Radford, 1969b; Gans and Masson, 1969).

The data regarding possible association of CPS_{Pyr} and ATC in *Coprinus* (Hirsch, 1968) are incomplete and subject to various interpretations. Crude *Coprinus* extracts subjected to gel filtration chromatography yielded a large peak of ATC activity with a shoulder extending into a small peak of CPS_{Pyr} having a heavier molecular weight. This can be interpreted as complete separability of ATC and CPS or as a small portion of CPS_{Pyr}–ATC complex remaining in the presence of dissociated enzyme which retains only ATC activity. The molecular weight estimates for the *Coprinus* enzyme based on Sephadex G-200 gel filtration were 370,000 for CPS_{Pyr} and 340,000 for ATC. Although possibly correct, these estimates are not justified because no protein marker above 250,000 molecular weight was used and because Sephadex G-200 gel effects little separation of molecules larger than 300,000 g per mole.

In view of the genetic and physical evidence for a bifunctional CPS_{Pyr}–ATC enzyme in the fungi, it is possible that such a complex might also be found in higher organisms. High-speed supernatants of rat liver contain ATC activity in 600,000 and 900,000 molecular weight forms (Oliver *et al.*, 1969), suggesting an organizational complex, perhaps with another pyrimidine enzyme.

A number of multifunctional complexes are known which catalyze two or more reactions of the same biosynthetic pathway. In *Neurospora* these include two complexes involved in aromatic synthesis (Gaertner and DeMoss, 1969; Giles *et al.*, 1967), a complex of three activities required in histidine biosynthesis (Minson and Creaser, 1969), and the ATC–CPS_{Pyr} complex reported here. Gaertner *et al.* (1970) have shown that the rates of the entire sequence of reactions catalyzed by the two aromatic enzyme complexes are greater when substrates for the initial enzyme activity of the complex are used than when reactions are initiated by substrates of enzymes later in the sequence.

In *Neurospora* carbamyl phosphate produced *in vivo* by the CPS_{Pyr}–ATC complex is unavailable as a substrate for ornithine transcarbamylase of the arginine synthetic pathway. The fact that an enzyme complex has been demonstrated in a situation where there is segregation of a substrate suggests that the complex could be responsible for the segregation phenomenon, possibly by retention of carbamyl phosphate as an enzyme-bound intermediate. Work to be reported elsewhere supports this possibility in that the molarity of CPS_{Pyr}–ATC in the cell is higher than the molarity of the pyrimidine-specific carbamyl phosphate pool.

References

Anderson, P. M., and Martin, S. V. (1970), *Biochemistry 9*, 171.

Anderson, P. M., and Meister, A. (1965), *Biochemistry 4*, 2803.

Caroline, D. F. (1969), *J. Bacteriol. 100*, 1371.

Davis, R. H. (1965), *Biochim. Biophys. Acta 107*, 54.

Davis, R. H. (1967), *in* Organizational Biosynthesis, Vogel, H. J., Lampen, J. O., and Bryson, V., Ed., New York, N. Y., Academic, p 302.

Davis, R. H., and Woodward, V. W. (1962), *Genetics 47*, 1075.

Edelhoch, H., and de Crombrugghe, B. (1966), *J. Biol. Chem. 241*, 4357.

Gaertner, F. H., and DeMoss, J. A. (1969), *J. Biol. Chem. 244*, 2716.

Gaertner, F. H., Ericson, M. C., and DeMoss, J. A. (1970), *J. Biol. Chem. 245*, 595.

Gans, M., and Masson, M. (1969), *Mol. Gen. Genetics 105*, 164.

Gerhart, J. C., and Pardee, A. B. (1962), *J. Biol. Chem. 237*, 891.

Giles, N. H., Case, M. E., Partridge, C. W. H., and Ahmed, S. I. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 1453.

Hager, S. E., and Jones, M. E. (1967), *J. Biol. Chem. 242*, 5074.

Hill, J. M., and Woodward, V. W. (1968), *Arch. Biochem. Biophys. 125*, 1.

Hirsch, M. (1968), *C. R. Acad. Sci. 267*, 1473.

Hirsch, M., and Gans, M. (1968), *C. R. Acad. Sci. 267*, 1511.

Keilin, D., and Hartree, E. F. (1938), *Proc. Roy. Soc., Ser. B 124*, 397.

Klotz, I. M., and Darnell, D. W. (1969), *Science 166*, 126.

LaCroute, F. A., Piérard, M., Grenson, M., and Wiame, J. M. (1965), *J. Gen. Microbiol. 40*, 127.

Lan, S. J., Sallach, H. J., and Cohen, P. P. (1969), *Biochemistry 8*, 3673.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Lue, P. F., and Kaplan, J. G. (1969), *Biochem. Biophys. Res. Commun. 34*, 426.

Lue, P. F., and Kaplan, J. G. (1970), *Can. J. Biochem. 48*, 155.

Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236*, 1372.

Minson, A. C., and Creaser, E. H. (1969), *Biochem. J. 114*, 49.

Nakanishi, S., Ito, K., and Tatibana, M. (1968), *Biochem. Biophys. Res. Commun. 33*, 774.

Oliver, I. T., Koskimies, O., Hurwitz, R., and Kretchmer, N. (1969), *Biochem. Biophys. Res. Commun. 37*, 505.

Piérard, A., Glansdorff, N., Mergeay, M., and Wiame, J. M. (1965), *J. Mol. Biol. 14*, 23.

Radford, A. (1969a), *Mol. Gen. Genetics 104*, 288.

Radford, A. (1969b), *Mutat. Res. 8*, 537.

Sumner, J. B. (1955), *Methods Enzymol. 2*, 378.

Williams, L. G., and Davis, R. H. (1970), *J. Bacteriol. 103*, 335.