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CARBAMYL PHOSPHATE SYNTHESIS IN *NEUROSPORA CRASSA*

II. GENETICS, METABOLIC POSITION, AND REGULATION OF ARGININE-SPECIFIC CARBAMYL PHOSPHOKINASE

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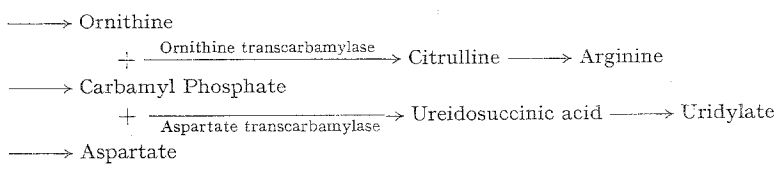
SUMMARY

The sole demonstrated enzyme of carbamyl phosphate synthesis in Neurospora appears to serve only the arginine pathway. The enzyme is completely absent in *arg-3* mutants, which require arginine. While pyrimidine synthesis requires carbamyl phosphate, pyrimidine synthesis is unimpaired in *arg-3* mutants. This suggests the existence of a pyrimidine-specific mode of carbamyl phosphate synthesis, and gene interaction experiments in support of this notion are presented. The regulatory behavior of arginine and pyrimidine enzymes in mutant strains also reinforces the hypothesis that dual modes of carbamyl phosphate synthesis prevail in this organism.

INTRODUCTION

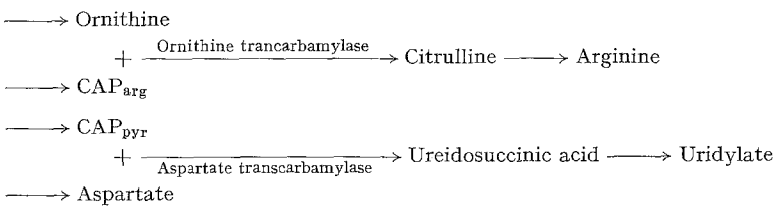
Arginine and pyrimidine synthesis in most organisms investigated is dependent upon a single precursor, carbamyl phosphate[1,2]. In the pathway of arginine synthesis, carbamyl phosphate and ornithine react to form citrulline, a reaction catalyzed by ornithine transcarbamylase (EC 2.1.3.3). In the pathway of pyrimidine synthesis, carbamyl phosphate and aspartate react to form ureidosuccinic acid, a reaction catalyzed by aspartate transcarbamylase. It has generally been assumed that the transcarbamylases compete for a single pool of carbamyl phosphate, which in turn is the product of a single enzyme. While the substrates and the enzyme of carbamyl phosphate synthesis may differ in various organisms, the diagrammatic representation below embraces the general scheme:



Abbreviations: CAP_{arg} and CAP_{pyr}, carbamyl phosphate pools specific for the arginine and pyrimidine pathways, respectively.

This scheme has in fact been shown to prevail in *Escherichia coli*,[3,4] although the substrates and the end-product regulation of the enzyme of carbamyl phosphate synthesis in this organism remain to be defined clearly[4].

The present paper will test the hypothesis, developed previously[5,6], that in Neurospora, there are two modes of carbamyl phosphate synthesis and two corresponding pools of carbamyl phosphate in the intact, normal cell. Each pool is specific for one of the two pathways dependent upon this compound, as indicated in the following diagram:



CAP_{arg} and CAP_{pyr} are symbols given to the two independently synthesized carbamyl phosphate pools. The previous data upon which this hypothesis is based are indirect, although extensive. The most important of these may be summarized as follows: The pyrimidine mutant *pyr-3a* is suspected of a CAP_{pyr} deficiency[5]. It will grow without uridine, however, if a second gene, *arg-12^s*, is introduced into the genome[7,8]. The latter gene has the effect of lowering ornithine transcarbamylase activity to a level of 3% normal[5]. This suggests that if CAP_{pyr} synthesis is prevented by the *pyr-3a* mutation, an ornithine transcarbamylase deficiency diverts CAP_{arg} to the pyrimidine pathway. In a completely symmetrical fashion, the mutant *arg-2*, suspected of a CAP_{arg} deficiency[5,9,10], will grow without arginine if another mutation, *pyr-3d*, is introduced[10]. The *pyr-3d* mutation eliminates aspartate transcarbamylase activity[11]. This suggests that if CAP_{arg} synthesis is disrupted by the *arg-2* mutation, an aspartate transcarbamylase deficiency diverts CAP_{pyr} to the arginine pathway. These conclusions were reinforced by the observation that the *pyr-3a*, *arg-12^s* double mutant regained a uridine requirement in the presence of arginine, as though pyrimidine synthesis here were dependent upon an arginine-inhibitable or -repressible step[5,8]. Exactly the same conclusions have been reached by CHARLES[12,13] through an analysis of similar mutants by patterns of carbon dioxide utilization for growth, and by FAIRLEY AND WAMPLER[14] through observations of α-aminobutyrate-supported growth of a mutant similar to *pyr-3a*. The same relationships between arginine and pyrimidine synthesis have recently been shown to prevail in yeast[15].

The results presented below will show that extracts of strains carrying the *arg-3* mutation (unlinked to *arg-2*, mentioned above) are completely deficient in carbamyl phosphokinase (EC 2.7.2.2) activity as measured previously[16]. Since this activity will be shown to be biologically relevant only to the arginine path (that is, it is formally equivalent to the enzyme of CAP_{arg} formation) a pyrimidine-specific mode of carbamyl phosphate synthesis, which is detectable *in vivo*, is clearly not being measured *in vitro*. A hypothesis regarding the segregation of the two modes of carbamyl phosphate synthesis in the intact normal cell is presented in the discussion. A preliminary note on this work has appeared previously[17].

MATERIALS AND METHODS

Materials

Neurospora crassa strains used in this work are described in Table I. All were from the author's stock collection, and they are available from the Fungal Genetics Stock Center, Dartmouth College, Hanover, N.H. (U.S.A.). Some multiple mutants were derived from crosses and are mentioned in the text. Stock cultures and conidial inocula were grown in VOGEL's[18] medium; 200 μg arginine·HCl/ml and 100 μg uridine/ml were added where necessary.

TABLE I

NEUROSPORA STRAINS USED IN THIS STUDY

Strain or locus number and isolation number[40]	*Requirement*	*Enzymic deficiency*	*Reference*
Wild type (73a)	None	None	39
Wild type (74A)	None	None	39
Arg-1 (UM-245)	Arginine	Argininosuccinate synthetase	25, 27
Arg-2 (33442)	Arginine	Unknown	
Arg-3 (30300)	Arginine	Carbamyl phosphokinase	This paper
Arg-12 (UM-3)	Arginine	Ornithine transcarbamylase	32
Arg-12^s (*s*)	None	Ornithine transcarbamylase (3% normal)	5, 19
Pyr-1 (263)	Uridine	Dihydroorotate dehydrogenase or dihydroorotase	Unpublished
Pyr-3a (37301)	Uridine	Possibly synthesis of CAP_{pyr}	5, 6, 11
Pyr-3d (45502)	Uridine	Aspartate transcarbamylase	11

L-Arginine·HCl and uridine were obtained from California Corporation for Biochemical Research, or from Sigma Chemical Co. L-Ornithine·HCl and ATP were obtained from Sigma Chemical Co. $KH^{14}CO_3$ and [$^{14}C_4$]aspartate were obtained from Volk Radiochemical Company. [^{14}C]Ureidosuccinate was made enzymatically from [^{14}C]aspartate, purified, and verified as such by cochromatography with pure, non-isotopic ureidosuccinic acid. Ornithine transcarbamylase was prepared by growing the *arg-3* strain on limiting arginine supplement, and by purification from acetone powders of the mycelium by the method of DAVIS[19].

METHODS

Mutant selection. Arginine-requiring mutants were isolated after ultraviolet irradiation of wild type 74A by the filtration concentration method of WOODWARD, DEZEEUW AND SRB[20]. Mutants relevant to this study were identified in regard to genetic constitution by crosses to known stock cultures (see RESULTS).

Growth and extraction of mycelia. Mycelia were grown from heavy conidial inocula for 18–24 h in 700 ml shaken cultures of VOGEL's medium at 28° as described by DAVIS AND HAROLD[21]. The inoculum was adjusted to give an approximately predetermined dry weight (0.4–1.2 g per flask depending upon the experiment) after overnight growth. Where transfers of mycelia to fresh media were done, the mycelia were

harvested in a Buchner funnel, washed thoroughly with water without being allowed to dry, and introduced into new flasks of medium. Because of the short period of growth following transfers, sterility was not a serious consideration during transfer.

All final harvests were done by drawing mycelia to moist pads on a Buchner funnel, and drying them by pouring acetone (reagent grade) over them. The pads were dried quickly in this way, and were used for both dry weight determinations and enzyme assays. None of the enzymes in this study were sensitive to this treatment. For extraction of enzymes, powders were prepared from the pads by grinding them in cold (—10°) acetone, followed by drying in air. The powders (100 mg) were then suspended in 4 ml 0.02 M potassium phosphate buffer (pH 7.0), centrifuged, and the supernatant saved. The residue was extracted again with 1 ml of buffer and the supernatant combined with the first. The extracts were dialyzed overnight against the same buffer; they could then be frozen indefinitely prior to assay. Protein was measured by the biuret method[22].

Aspartate transcarbamylase assay. Reaction mixtures[11] (3.25 ml) contained 500 μmoles glycine–NaOH buffer (pH 9.1); 40 μmoles L-aspartate; 20 μmoles freshly dissolved dilithium salt of carbamyl phosphate; and a rate-limiting amount of extract. After 15 min incubation at 25°, 0.5 ml 2 M $HClO_4$ was added. The tubes were centrifuged and 1.0 ml of the supernatant was used for ureidosuccinic acid determination by the method of KORITZ AND COHEN[23] as modified by DAVIS[19]. Specific activities are expressed as μmoles ureidosuccinic acid/mg protein/h.

Ornithine transcarbamylase assay. Reaction mixtures[19] (3.25 ml) contained 500 μmoles Tris–acetate buffer (pH 9.1); 20 μmoles L-ornithine·HCl; 10 μmoles freshly dissolved dilithium carbamyl phosphate; and a rate-limiting quantity of extract. After 5 min incubation at 25°, 0.5 ml 2 M $HClO_4$ was added. The tubes were centrifuged and the supernatants were used for citrulline determinations by the method used for ureidosuccinic acid. Specific activities are expressed as μmoles citrulline/mg protein/h.

Carbamyl phosphokinase assay. Reaction mixtures[16] (0.5 ml) contained 50 μmoles Tris–HCl buffer (pH 8.4); 8 μmoles $MgCl_2$; 30 μmoles NH_4Cl; 10 μmoles $KHCO_3$; 3 μmoles ATP; 2.5 μmoles L-ornithine·HCl; excess ornithine transcarbamylase; and a rate-limiting quantity of extract. After 30 min at 37°, 0.1 ml 2 M $HClO_4$ was added. After centrifugation, 0.4 ml of the supernatant was used to determine citrulline in one-half volume color reactions by the method used in the transcarbamylase assays. A large number of assays were done simultaneously by the above method and by the isotope method described in the previous paper[16], and the two determinations were found to give almost exactly the same ratio of μmoles citrulline to counts/min within any set of assays. Variations from day to day were ascribable to variations in the specific activity of the $KH^{14}CO_3$ substrate used in the isotope method. For this reason, the colorimetric method is used here: specific activities are μmoles citrulline/mg protein/h.

Ureidosuccinic acid determinations. Ureidosuccinic acid, accumulated by certain strains in the course of growth, was extracted from moist pads of mycelium grown in shaken cultures, the dry weights of which were determined with an acetone-dried replicate sample. The moist pads (the equivalent of about 100 mg dry wt.) were extracted successively with 2 ml 0.5 M trichloroacetic acid and two 2 ml volumes of water. The extracts were passed through a column of Dowex-50W (H^+ form, 200–400

mesh, 1.1 × 3 cm), and the eluate was made up to 10 ml. The eluate was tested for ureidosuccinic acid by the colorimetric method cited above.

The identity of ureidosuccinic acid was established by cochromatography on Dowex-1 columns of the extracted material with [^{14}C]ureidosuccinic acid. An extract to which [^{14}C]ureidosuccinic acid had been added was brought to pH 7.5 and applied to the surface of a Dowex-1 column (formate form, 200–400 mesh, 1.1 × 8 cm) previously equilibrated with 0.065 M sodium formate brought to pH 3.5 with formic acid. Elution followed with the same buffer; 10-ml fractions were collected.

Radioactivity was determined with a Nuclear-Chicago D-47 Gas-Flow counter, and ureidosuccinic acid was determined by the method of GERHART AND PARDEE[24]. Data related to identity of ureidosuccinic acid are given in the RESULTS.

RESULTS

Genetic control of carbamyl phosphokinase

Carbamyl phosphokinase may be detected in crude, dialyzed extracts of all strains tested except those carrying the *arg-3* mutation. Assays done by the colorimetric method or by the radioactive method give proportional results. Colorimetric data for several strains, including *arg-3*, are reported in a later section. The absence of detectable activity in *arg-3* is probably not the result of a free inhibitor, the lack of a cofactor, or a competing reaction, since when extracts of *arg-3* are mixed with those of *arg-12* (having carbamyl phosphokinase, but lacking ornithine transcarbamylase) in radioactive assays, the activity of the latter is unaffected (Table II).

TABLE II

EFFECT OF *arg-3* EXTRACT ON THE CARBAMYL PHOSPHOKINASE REACTION

Reaction mixtures (0.5 ml) were standard for the isotope assay. They contained crude dialyzed extracts of *arg-12* and *arg-3*. Ornithine transcarbamylase was used only as noted.

Extracts and enzymes added (ml)			*Counts/min per 0.4 ml per 30 min*
Arg-12	*Arg-3*	*Ornithine transcarbamylase*	
0.1		0.05	1260
	0.1	0.05	4
0.1	0.1	0.05	1337
0.1			11
	0.1		7
0.1	0.1		1252

Table II also gives data showing that when ornithine transcarbamylase is omitted from reaction mixtures neither *arg-12* nor *arg-3* alone can transform radioactive bicarbonate to citrulline, but when extracts are mixed, the carbamyl phosphokinase activity seen is characteristic of *arg-12*. This indicates that the ornithine transcarbamylase deficiency of *arg-12* is absolute, and that the *arg-3* extract may replace it.

The absence of carbamyl phosphokinase behaves as a single gene character in crosses, and is absolutely correlated with the *arg-3* nutritional phenotype. The isolate of *arg-3* used was verified as such by its close linkage[25] to the *arg-1* locus on chromo-

some I (2.6 map units, a value based upon recovery of wild types and double mutants in an *arg-3* × *arg-1* cross).

The work on the genetic control of carbamyl phosphokinase was based wholly on experiments with a single allele of *arg-3* (30300). Confirmation of the *arg-3* locus as a controlling genetic factor was obtained by selection of new mutants. Arginine-requiring mutants were obtained after ultraviolet treatment; the nutritional class which grew on citrulline but not on ornithine was investigated enzymatically and genetically. Four mutants, comprising one heterokaryon complementation group, were found to be completely deficient in carbamyl phosphokinase. Two remaining mutants of the same nutritional category which complemented with the first group but not with one another had normal carbamyl phosphokinase activity. Crosses of all mutants to both *arg-3* and *arg-2* strains were performed. Allelism was judged by the absence of prototrophic isolates in crosses of mutants, and non-allelism without linkage was inferred if one-quarter of the progeny were prototrophic (the double-mutants were not easily distinguishable from the parental classes). The results show that the enzyme-deficient class is allelic to *arg-3*; the other class is probably allelic to *arg-2* as judged by sterility in crosses, though genetic proof is lacking (Table III).

TABLE III

ANALYSIS OF NEWLY ISOLATED MUTANTS

New mutant (isolation number)	*Carbamyl phosphokinase: specific activity*	*Prototropic isolates when mated to:* % *found*	
		Arg-3 (30 300)	*Arg-2 (33 442)*
UM-602	0	<0.01	28
UM-603	0	<0.01	25
UM-604	0	<0.01	39
UM-605	0.19	13*	Sterile
UM-607	0	<0.01	22
UM-609	0.18	32	Sterile

* UM-605 was associated with a genetically complex morphological phenotype which influenced the segregation of the arginine genes.

These results are taken as proof that *arg-3* controls the formation of carbamyl phosphokinase, probably as a structural gene. Rigorous proof of *arg-3* as a structural locus would require the demonstration of *arg-3* alleles which impose structural or kinetic alterations of the enzyme. No partially-deficient strains of this locus have yet been studied to test this notion, however.

In previous experiments, strains carrying *arg-2*, a mutation unlinked to *arg-3* but virtually indistinguishable from it in any other way, were found to have normal carbamyl phosphokinase activity (see also Table III). Since *arg-2* strains also contain substantial ornithine transcarbamylase activity, the reason they cannot perform the ornithine to citrulline conversion *in vivo* remains quite obscure. This will be considered further below.

Metabolic position of carbamyl phosphokinase: nutritional evidence

The carbamyl phosphokinase activity studied here is apparently specific for the arginine pathway, since the *arg-3* mutant does not have a pyrimidine requirement

for growth. Furthermore, when uridine is used as a supplement in addition to arginine, the utilization of the latter is not spared (Fig. 1).

The hypothesis that two modes of carbamyl phosphate synthesis operate was based in part upon the effect of the *pyr-3d* mutation (deficient in aspartate transcarbamylase) upon the *arg-2* phenotype. The double mutant *pyr-3d, arg-2* behaves as though a pyrimidine precursor (CAP_{pyr}) were being diverted to citrulline synthesis.

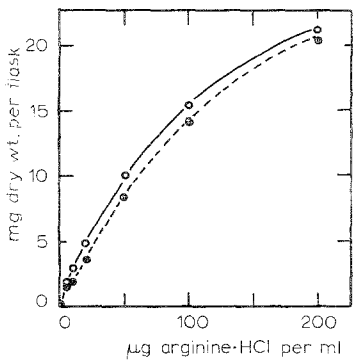


Fig. 1. Effect of uridine on the response of *arg-3* to arginine·HCl. Points represent the dry weights of *arg-3* mycelia in media containing various concentrations of arginine·HCl. Ten ml of medium was inoculated with conidia, and mycelia were harvested after 4 days stationary growth at 25°. O—O, no uridine added; ●– – –●, 100 μ uridine added/ml medium.

TABLE IV

INTERACTION OF *arg-3* AND ALLELES OF THE *pyr-3* LOCUS

Arg-3 and the double mutants *arg-3, pyr-3a* and *arg-3, pyr-3d* were grown in 10 ml stationary cultures for six days. The medium contained the concentrations of arginine·HCl and uridine indicated.

Arginine·HCl (μg/ml)	*Uridine* (μg/ml)	*Dry weight (mg/10 ml)*		
		Arg-3	*Arg-3, Pyr-3a*	*Arg-3, Pyr-3d*
0	0	0	0	0
0	10	—	0	6.5
5	10	—	1.1	9.1
0	100	0	0	10.8
5	100	1.6	1.1	13.5
200	100	20.4	23.8	31.7

thereby reducing the arginine requirement imposed by *arg-2* (see ref. 5). Precisely the same relationship between *pyr-3d* and *arg-3* should obtain; the prediction is all the more critical, since *arg-3* (in contrast to *arg-2*) has a demonstrable carbamyl phosphokinase deficiency. That *pyr-3d* does in fact reverse the nutritional effect of this deficiency is shown in Table IV, where *arg-3, pyr-3d* is compared to the single mutant *arg-3* in regard to arginine requirement. A parallel growth experiment was done with the double mutant *arg-3, pyr-3a*. This strain would not be expected to have a reduced arginine requirement since, according to the general hypothesis, both modes of carbamyl phosphate synthesis are missing (see INTRODUCTION). As shown by the data in Table IV, this strain does not, in fact, exhibit a reduced arginine requirement.

The exact parallel between these observations and those made by Reissig with *arg-2* reinforces the notion that *arg-2*, like *arg-3*, suffers a perturbation of carbamyl phosphate synthesis, at least *in vivo*.

Metabolic position of carbamyl phosphokinase: synthesis of citrulline and ureidosuccinic acid in vivo

The pyr-1 mutant is blocked in the synthesis of orotic acid from ureidosuccinic acid, and as a result, it accumulates large amounts of ureidosuccinic acid[26]. The *arg-1* mutant lacks argininosuccinate synthetase (EC 6.3.4.5), and thus accumulates citrulline[27]. These mutations provide an opportunity to demonstrate the metabolic position of carbamyl phosphokinase, since when the double mutants *arg-3*, *arg-1* and *arg-3*, *pyr-1* are studied, the effects of the carbamyl phosphokinase deficiency upon citrulline and ureidosuccinic acid accumulation may be observed. If the carbamyl phosphokinase missing in *arg-3* strains served both pathways, neither double mutant would accumulate a carbamyl compound. If, on the other hand, the enzyme served only the arginine pathway, citrulline would not accumulate in *arg-3*, *arg-1* and ureidosuccinic acid would accumulate in *arg-3*, *pyr-1*.

The double mutants were isolated from the appropriate crosses; the genetic constitution of *arg-3*, *arg-1* was confirmed by outcrossing to wild type, with the unambiguous detection of both mutations among the progeny. Unlike extracts of the *arg-1* parent, those of the *arg-3*, *arg-1* double mutant contained no accumulated citrulline. (In fact, the latter were initially identified in this way.) This demonstrates that carbamyl phosphokinase is indispensable for citrulline synthesis. This evidence is consistent with the inability of *arg-3* to grow on ornithine and its ability to grow on citrulline.

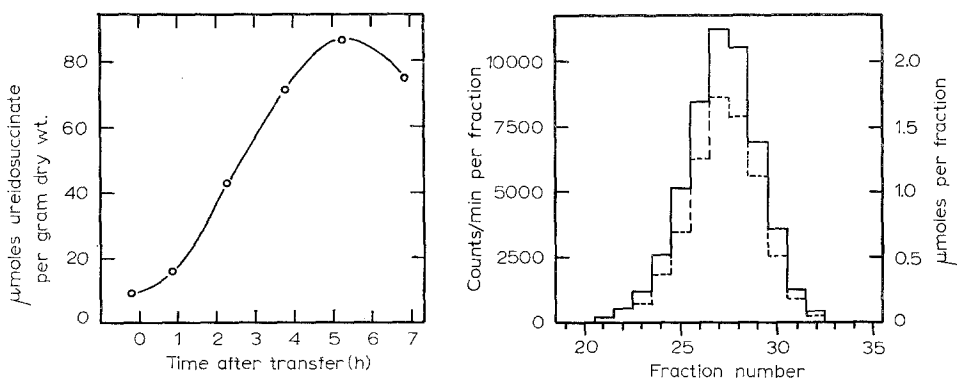


Fig. 2. Accumulation of ureidosuccinic acid by the double mutant, *arg-3*, *pyr-1*. A shaken culture of mycelium grown in 200 μg arginine + 100 μg uridine/ml was harvested and transferred to new medium containing 200 μg arginine · HCl at 0 h. The ureidosuccinic acid accumulated is measured subsequently in units of μmoles/g dry wt. Weight increased from 0.6 g/700 ml to about 0.95 g/700 ml during the experiment as endogenous pyrimidines were depleted.

Fig. 3. Cochromatography of 8 μmoles of ureidosuccinic acid accumulated by *arg-3*, *pyr-1* (last point in Fig. 2) with 0.2 μmole [^{14}C]ureidosuccinic acid (see METHODS). ———, radioactivity (left ordinate); – – – –, μmoles ureidosuccinic acid (right ordinate). Only a small additional amount (equivalent to 0.4 μmole ureidosuccinic acid) of colorigenic material was found in the extract used; this appeared at the solvent front (fraction 1, not shown).

The *arg-3, pyr-1* double mutant, however, is able to accumulate ureidosuccinic acid to the same extent as the *pyr-1* single mutant, if the two strains are compared on limiting concentrations of uridine: *pyr-1* accumulated 85 μmoles ureidosuccinic acid/g dry wt; *arg-3, pyr-1* accumulated 97 μmoles/g in one experiment; wild type contained at most 6 μmoles/g. The course of ureidosuccinic acid accumulation by *arg-3, pyr-1* is shown in Fig. 2, and resembles ureidosuccinic acid accumulation by *pyr-1* reported by DONACHIE[26]. A number of experiments showed that the extent of ureidosuccinic acid accumulation in the double mutant was inversely related to the uridine concentration. This suggests that the ureidosuccinic acid accumulated by this strain is not merely a metabolic product of the uridine added. Furthermore, sufficient arginine was required such that the uridine supplement was exhausted; only after growth ceased as a result of uridine depletion did ureidosuccinic acid accumulate. The ureidosuccinic acid accumulated by *arg-3, pyr-1* (a sample of 8 μmoles) was identified by column chromatography (Fig. 3) with [^{14}C]ureidosuccinic acid. Not only did the positions of the peaks of radioactivity and color coincide, but the specific radioactivity of the ureidosuccinic acid was essentially constant throughout the peak.

These results demonstrate in a direct fashion the existence of a system capable of ureidosuccinic acid synthesis in the absence of demonstrable carbamyl phosphokinase activity in extracts. They thereby reinforce the designation of this enzyme as "arginine-specific". The pyrimidine-specific system of carbamyl phosphate synthesis postulated above will be taken up in the discussion. At this point, it is sufficient to note that no net synthesis of ureidosuccinic acid has been achieved *in vitro* unless carbamyl phosphokinase is present[16].

Metabolic position of carbamyl phosphokinase: regulation of enzyme activities

Many enzymic mutants of Neurospora (like those of bacteria) which are starved for their growth-factor requirement respond with an elevation of the specific activity of some or all of the remaining enzymes in the deficient pathway. This response may be governed through a relief of enzyme repression imposed by the end product of the pathway, or through induction by elevated concentrations of non-metabolizable intermediates[28]. In either case, the response (in bacteria, at least) is usually specific, in the sense that high levels of enzymes are seen only in the synthetic pathway of the compound for which the organism is starved. That this is true of Neurospora is shown in a limited fashion in the experiments reported below, where the pathway specificity of carbamyl phosphokinase is tested.

The experiments reported in Table V involved transfers of mycelia to supplemented and unsupplemented media, and a measurement of the activities of ornithine transcarbamylase, aspartate transcarbamylase, and carbamyl phosphokinase. Table VI reports enzyme activities of strains grown without transfer in medium with limiting arginine or uridine. The experiments were designed to show whether the carbamyl phosphokinase observed in normal extracts and lacking in *arg-3* extracts served both the arginine and pyrimidine pathways, or whether it was relevant only to arginine synthesis. While the lack of a pyrimidine requirement in *arg-3* would in itself appear to rule out the former possibility, it might be argued that the arginine used to supplement *arg-3* is in some way utilized to satisfy a pyrimidine requirement. The most significant data related to this question are that when the *arg-3* mutant (lacking carbamyl phosphokinase) is deprived of arginine, only ornithine transcarbamylase

becomes elevated in its activity, while aspartate transcarbamylase remains at normal levels. The remainder of the data on the aspartate and ornithine transcarbamylases supports the specificity of their responses to starvation for uridine and arginine, respectively. The lack of response of aspartate transcarbamylase to arginine deprivation in *arg-3* suggests strongly that pyrimidine synthesis is not impeded by the lack of carbamyl phosphokinase when this strain is starved for arginine.

The specificity of carbamyl phosphokinase for the arginine pathway is also indicated by its response to the arginine concentration of the medium, and an indifference to uridine in the medium. Evidence of this sort is not as meaningful as the transcarbamylase data, since the activity of an enzyme shared by two pathways need not, a priori, be influenced by both corresponding end-products.

Two problems arose in experiments where arginine concentration was studied in its effect upon arginine enzymes. The first was that arginine in the medium induces arginase activity[29]*. Since this enzyme leads to a rapid destruction of arginine, the intramycelial concentrations of this compound are difficult to maintain at a known

TABLE V

SPECIFIC ACTIVITIES OF ASPARTATE TRANSCARBAMYLASE, ORNITHINE TRANSCARBAMYLASE, AND CARBAMYL PHOSPHOKINASE IN VARIOUS STRAINS

Mycelia were grown in and transferred to various media: MIN = unsupplemented; 200A = 200 μg arginine·HCl/ml; 100U = 100 μg uridine/ml.

Strain	*Original medium*	*Transfer medium*	*h after transfer*	*Aspartate transcarbamylase*	*Ornithine transcarbamylase*	*Carbamyl phosphokinase*
Wild	MIN		0	4.1	14	0.15
		MIN	5	2.8	18	0.18
		100U	5	2.8	21	0.13
		200A	5	3.2	21	0.13
Arg-1	200A		0	4.2	30	0.18
		MIN	5	1.9	53	0.26
		200A	5	1.6	30	0.09
Arg-3	200A		0	5.0	28	0
		MIN	5	4.5	52	0
		200A	5	3.8	25	0
Arg-12	200A		0	4.0	0	0.17
		MIN	4	4.5	0	0.18
		200A	4	3.2	0	0.05
Arg-12^s	MIN		0	2.8	0.64	0.42
		MIN	5	2.2	0.76	0.53
		200A	5	2.4	0.34	0.24
Arg-12^s	200A		0	3.9	0.37	0.25
		MIN	5	1.9	0.94	0.42
		200A	5	2.4	0.29	0.08
Pyr-1	100U		0	2.5	13	0.13
		MIN	4	10.4	16	0.16
		100U	4	4.2	18	0.20
Pyr-3a	100U		0	3.0	17	0.18
		MIN	5	12.0	13	0.19
		100U	5	3.7	17	0.16
Pyr-3d	100U		0	0	24	0.25
		MIN	4	0	20	0.24
		100U	4	0	26	0.25

* Personal communication, of W. M. THWAITES.

level where arginine is added. Any strain introduced as a conidial inoculum into media containing arginine will have disposed of a large part of it even by the earliest time (17 h) at which enzyme determinations are made. This accounts for the normal or high ornithine transcarbamylase and carbamyl phosphokinase levels found in most strains, in contrast to the low levels expected if a repression mechanism prevails. However, short term transfer experiments to arginine can be used to demonstrate "repression" of carbamyl phosphokinase.

TABLE VI

EFFECT OF LIMITING NUTRITIONAL SUPPLEMENTS ON ASPARTATE TRANSCARBAMYLASE, ORNITHINE TRANSCARBAMYLASE, AND CARBAMYL PHOSPHOKINASE

Strains were allowed to grow from the time of inoculation to terminal dry weight in the limiting concentrations of supplement indicated. Enzyme activities at the first (prior to terminal weight) and at the final harvest are given.

Strain	*Supplement concentration (μg/ml medium)*	*Harvest*	*Aspartate transcarbamylase*	*Ornithine transcarbamylase*	*Carbamyl phosphokinase*
Arg-1	50 arginine·HCl	First	2.4	60	0.31
		Last	2.4	76	0.44
Arg-3	50 arginine·HCl	First	4.0	43	0
		Last	3.3	85	0
Arg-12	50 arginine·HCl	First	2.7	0	0.26
		Last	2.3	0	0.56
Pyr-3d	25 uridine	First	0	25	0.25
		Last	0	17	0.15

A second problem was that transfers of absolute arginine mutants to fresh minimal medium did not result in large alterations of ornithine transcarbamylase and carbamyl phosphokinase activity, although the effects of this treatment upon the former are clear from Table V. This unresponsiveness to arginine starvation in absolute mutants may reflect a sudden impairment of protein synthesis imposed by deprivation of arginine. To circumvent this problem, mutants were grown on limiting arginine and were allowed to exhaust the supplement. In the process, activities of the arginine enzymes increased markedly. The problems associated with transfer experiments were also avoided by use of the partially mutant *arg-12*^{s} strain, since growth of this strain occurs in minimal medium. In this strain, both repression and increases of enzyme activity can be seen in transfer experiments.

Tables V and VI show the influence of arginine and uridine supplementation on carbamyl phosphokinase activity. First, the transfer experiments (Table V) show repression of carbamyl phosphokinase in mycelia of arginine-requiring strains transferred to arginine-containing medium. In contrast, carbamyl phosphokinase is not influenced in pyrimidine mutants transferred to uridine. Second, the experiments involving limiting supplements (Table VI), show that carbamyl phosphokinase activity becomes elevated in conditions of arginine starvation and not in conditions of uridine starvation. Elevation of this activity is also pronounced in transfers of *arg-12*^{s} to minimal medium (Table V). In numerous additional experiments not reported in the tables, no interaction of uridine and arginine in their effects upon the three enzyme activities could be detected.

The clear response of carbamyl phosphokinase to arginine and its lack of response to uridine are consistent with the previous conclusion that arginine synthesis depends upon this enzyme, but pyrimidine synthesis does not.

A last point is that enzymes of the wild type strain are relatively unresponsive to supplements in the medium. This suggests that internal intermediate concentrations are normally high enough to entail low enzyme activities, and additions to the medium have correspondingly little effect.

DISCUSSION

The evidence presented indicates in several ways that an arginine-specific system of carbamyl phosphate synthesis exists which is distinct from a pyrimidine-specific system. The data show that carbamyl phosphate formation *in vitro*, seen in wild type extracts under the conditions of assay used, is eliminated by mutant alleles of the *arg-3* locus. While the purification of the carbamyl phosphate-forming system has not been extensive, there is no reason at this point to feel that the enzyme activity seen *in vitro* is resolvable into two or more sequential steps. In fact, no other mutations affecting this activity have been detected. It may be concluded, therefore, that *arg-3* is the primary determinant of the activity *in vitro*. In the intact cell, however, the mode of carbamyl phosphate synthesis which involves this activity may also be dependent upon the product of the *arg-2* locus, since *arg-2* mutants have the same phenotype as *arg-3* mutants when judged on the basis of nutrition, response to CO_2 supplementation, and gene interactions. Until the action of the *arg-2* locus has been demonstrated, the biological steps of carbamyl phosphate synthesis for the arginine pathway will be incompletely known. As noted in the previous paper[16], the question of the true substrate of carbamyl phosphokinase has not been satisfactorily resolved in favor of carbamate. Possibly the formation of an unknown biological substrate depends upon the *arg-2* locus.

The evidence regarding the specificity of the carbamyl phosphokinase described, to be complete, must ultimately include the demonstration of a pyrimidine-specific system *in vitro* which is clearly separable from it. The data show that ureidosuccinate synthesis takes place in strains whose extracts lack the arginine-specific activity, or, in fact, any demonstrable capacity for carbamyl phosphate synthesis. While it is not certain that carbamyl phosphate is a biological intermediate in ureidosuccinic acid synthesis, the fact that aspartate transcarbamylase is found in Neurospora, that its absence leads to a pyrimidine requirement[11], and that its K_m for carbamyl phosphate is less than $2 \cdot 10^{-4}$ M (at pH 8.4)[26,*] encourages this view. This view is further reinforced by the gene interaction studies which show that *arg-3, pyr-3d* behaves as though CAP_{pyr} is diverted to ornithine transcarbamylase. The attempts to demonstrate the synthesis of CAP_{pyr} *in vitro* (the appearance of ureidosuccinic acid from [^{14}C]aspartate being taken as a criterion) have been made with a strain carrying simultaneously the *arg-3*, the *pyr-1* and the *arg-12* mutations. While this strain, like *arg-3, pyr-1*, accumulates large amounts of ureidosuccinic acid, even the crudest, undialyzed, cell-free preparations fail to engage in net synthesis of this compound under conditions used so far. Any transformation of [^{14}C]aspartate to [^{14}C]ureido-

* R. H. DAVIS, unpublished results.

succinic acid detected so far results from carbamyl exchange from accumulated ureidosuccinic acid. This work shows that conditions for CAP_{pyr} synthesis (*e.g.*, substrates, extract preparation, pH), as yet unknown, are quite different than those for CAP_{arg} synthesis.

If the theory of dual modes of carbamyl phosphate synthesis is accepted on the basis of the evidence presented, the segregation of the two pools of carbamyl phosphate in the normal cell requires explanation. That the sources of carbamyl phosphate are indeed segregated or channelled may be inferred from the fact that single nutritional requirements arise as a result of mutation of each enzyme of carbamyl phosphate synthesis. It appears, from the gene interaction studies, that where only one pool of carbamyl phosphate remains (as in *arg-3* or *pyr-3a*), there is a competitive relation between the two transcarbamylases, since the relationship may be altered by mutations affecting the transcarbamylases[5]. For channelling to occur however, it is also logically necessary to have a spatial segregation of at least one pool of carbamyl phosphate from the transcarbamylase of the other pathway.

Previous extensive work on the mutations of the *pyr-3* locus has led to the inference that the synthesis of CAP_{pyr} and its utilization via aspartate transcarbamylase are activities of a single protein molecule[6,30,31]. This accounts, among other things, for the allelism of *pyr-3a* and *pyr-3d*. Such a hypothesis provides a possible mechanism for the channelling of CAP_{pyr} in the normal cell, since this compound could be utilized for ureidosuccinic acid synthesis before entering a diffuse pool accessible to ornithine transcarbamylase[6]. This would not be true of mutants like *pyr-3d* (lacking aspartate transcarbamylase while retaining the ability to synthesize CAP_{pyr}), which act to reduce the arginine requirement of *arg-2* and *arg-3* (see ref. 10). The bifunctional protein catalyzing ureidosuccinic acid formation is inferred from an entirely different type of data obtained from yeast[15].

The channelling of CAP_{arg} to the synthesis of citrulline cannot be understood in a similar fashion, since ornithine transcarbamylase and carbamyl phosphokinase are already known to be separable activities, both by the locations of the corresponding genes[32], and by fractionation procedures. Channelling would take place, however, if the CAP_{arg} pool is an unlocalized one, and if ornithine transcarbamylase has a sufficient kinetic advantage over aspartate transcarbamylase in CAP_{arg} utilization. This advantage would be lost, however, in mutants carrying the *arg-12*^{s} mutation (low ornithine transcarbamylase), which is known to overcome the effects of *pyr-3a*. This hypothesis to account for channeling is a tentative one, since in limited experiments with wild type and *pyr-3a*, conditions *in vitro* which allow more than 70% utilization of the product of carbamyl phosphokinase by ornithine transcarbamylase in competition with aspartate transcarbamylase have not yet been found. This is accounted for by the greater affinity of the latter enzyme for carbamyl phosphate in comparison to the former, despite the 3- to 7-fold excess in specific activity of ornithine transcarbamylase over aspartate transcarbamylase. It is possible that the conditions which favor CAP_{pyr} formation will also favor the channelling of CAP_{arg}. A similar problem of channelling arises in the best-known case of biosynthetic enzyme duality, that of aspartokinase[33].

Carbamyl phosphate metabolism in Neurospora is complex, and elaborate genetic materials are required to display it fully. In view of this, many organisms which are less well-known in this regard may prove to be equally complex when

better known. A case in point is that of the pigeon and certain extrahepatic tissues of the rat[1,2,34] in which pyrimidine synthesis presumably prevails, yet where no enzyme catalyzing carbamyl phosphate formation has been demonstrated. It is possible that a pyrimidine-specific enzyme of carbamyl phosphate synthesis exists in these organisms and has so far escaped detection. Recent reports by LACROUTE *et al.*[15,35] indicate that yeast also has two activities for carbamyl phosphate synthesis.

The regulatory behavior of enzymes seen in these experiments suggests a control mechanism, although the significance and the mechanism of regulation may be different in Neurospora than in the bacteria[26,28]. The amplitude of variation in specific activity in these experiments is on the order of 5- to 10-fold in mutants. However, elevation of enzyme may be limited by starvation itself, and the conditions of starvation exert a profound effect (compare Tables V and VI). Without mutants which could be described as constitutive, therefore, it is impossible to judge the maximum inherent capacity of Neurospora for the synthesis of a given enzyme. The indications that there are two specific enzymes of carbamyl phosphate synthesis may be adaptively significant, in that independent end-product control of the two pathways may take place at this point, where ATP is used, rather than at the point of transcarbamylation. While no one enzyme may be thought of as consistently rate-limiting in a biosynthetic pathway[36], it appears that the rate of citrulline synthesis is far more sensitive to observed changes of carbamyl phosphokinase activity than to observed changes in ornithine transcarbamylase activity. This is shown by varying the ratio of the two enzymes in experiments performed *in vitro*. Furthermore, KACSER[36] has shown that a mutational reduction of argininosuccinase activity on the order of 20-fold has no effect upon the rate of arginine synthesis or the steady-state level of arginine in the mycelium. It is entirely possible that the flux through the entire arginine pathway is most sensitive to changes of carbamyl phosphokinase activity in comparison to normal variations of all other enzymes. In the pyrimidine pathway, the synthesis of CAP_{pyr} may be a similarly important control point, although this activity and aspartate transcarbamylase would be coordinately controlled if both were activities of a single protein. If only one protein were involved, it would be consistent with DONACHIE's[37,38] results, from which he inferred that aspartate transcarbamylase was the "pace-making" enzyme of pyrimidine synthesis.

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