Genes Influencing the Conversion of Citrulline to Argininosuccinate in *Neurospora crassa*

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SUMMARY

The enzymic conversion of citrulline $\rightarrow$ argininosuccinate $\rightarrow$ arginine in wild-type *Neurospora crassa* has been shown to be essentially like that in mammalian tissues in all respects tested. The enzymes responsible for the two reactions (condensing enzyme and argininosuccinase) have been partially separated. Five mutants at the *arg-1* locus have normal argininosuccinase and little or no condensing activity. The lack of condensing activity appears to be due to a simple absence of enzyme, alternatives such as inhibitor production or increased ATPase competition having been ruled out. Small amounts of apparent condensing activity, detected in the substrate-disappearance assay, were shown to be due to side reactions. When grown at the usual high arginine concentrations, three mutants at the *arg-10* locus, which are known to lack argininosuccinase, have normal condensing activity. However, when *arg-10* strains are grown at low arginine concentrations, the resulting extracts have very little condensing activity. This also appears to be due to a simple loss of condensing enzyme. It has not been determined whether the low activity is a specific secondary effect of *arg-10* on the condensing enzyme, or whether it is merely a non-specific effect of the inadequate supplementation, which might cause reductions in many enzyme activities. Despite its very low condensing activity *in vitro*, *arg-10* grown at low arginine concentrations must be active at some time *in vivo*, since its mycelium accumulates argininosuccinate but not citrulline. In contrast, an *arg-1 arg-10* double mutant, grown at low arginine concentrations, must be inactive *in vivo*, since it accumulates citrulline but not argininosuccinate. It is concluded that *arg-1* is probably the primary locus controlling the synthesis of condensing enzyme.

INTRODUCTION

Numerous studies of gene–enzyme relationships suggest that a single genetic locus determines the basic ability to form a given enzyme, but that the quantity of the enzyme produced can be modified by other genes and by varying environmental conditions (see review by Fincham, 1958; Yanofsky & St Lawrence, 1960). The present paper is an analysis of similar gene–enzyme relationships concerned with the conversion of citrulline to arginine in *Neurospora crassa*. The mechanism of this conversion in mammalian tissues has been studied extensively by Ratner and co-workers (Ratner & Petrack 1951, 1953*a, b*, 1956; Ratner, Petrack & Rochovansky, 1953). They found that two reactions were involved:

The condensation of citrulline and aspartate to form argininosuccinate (reaction 1) is greatly accelerated by the presence of inorganic pyrophosphatase (Ratner & Petrack, 1956).

Evidence is presented here that citrulline is converted to arginine in wild-type *Neurospora crassa* in essentially the same way as in mammalian tissues. Independently Boylen & Fincham (personal communication) have obtained preliminary evidence for the same reaction sequence in wild-type *Neurospora*, and have studied the argininosuccinase reaction in detail (Fincham & Boylen, 1957). Nine mutant strains blocked between citrulline and arginine have been classified genetically into two types, representing mutations at two unlinked loci (Newmeyer, 1957); these are called *arg-1* and *arg-10*. Fincham & Boylen (1957) have shown that *arg-10* mutants lack argininosuccinase. The present paper is concerned with the effects of the two loci on the condensation reaction.

METHODS

Strains. The origin and genetic analysis of the mutants were described previously (Newmeyer, 1957). The following stocks were used:

arg-1 (Linkage group I; uses arginine but not citrulline): alleles 36703T, 46004, H4250, B312, and B369.

arg-3 (Linkage group I; uses citrulline or arginine): allele 30300.

arg-10 (Linkage group VII; uses arginine but not citrulline): alleles B317, B368, and B370.

wild-type: ST-74A.

All mutants were inbred to the wild type.

The *arg-1 arg-10* double mutant was isolated from a non-parental ditype ascus from a cross of inbred lines of 36703T and B317. Its constitution was verified by crossing to wild type and recovering both single mutants from the same ascus.

Enzyme preparations. Cultures were grown in Erlenmeyer flasks at 30° with moderate shaking. Each flask contained 650 ml. Fries minimal medium (Beadle & Tatum, 1945) containing 20 g. sucrose per l., with or without arginine, as indicated in Results. The mycelia were harvested by filtration, washed with distilled water, sucked dry, and ground in a cold mortar with an equal weight of alumina powder (Buehler's Levigated, no. 1557 AB). The mixture was extracted with cold 0.01 M-phosphate buffer (pH 7.4), centrifuged at 21,600 g for 30 min. at 5°, and the extract stored at -15°. Dialyses were carried out against 0.01 M-phosphate buffer (pH 7.4) at 5° overnight. Crude extracts containing about 15 mg. protein/ml. were fractionated with solid ammonium sulphate at 0° as shown in Table 1. A sample of the 25-50% fraction was diluted to about 9 mg. protein/ml. and refractionated with ammonium sulphate as shown in Table 1. Precipitates were dissolved in 0.1 M 2-amino-2-hydroxymethylpropane-1:3diol (tris buffer; pH 7.4).

Chemicals and analytical methods. The colorimetric methods used for determining arginine, citrulline, urea and inorganic phosphate, as well as the preparation of reagents, substrates and supplementary enzymes, were all as described by Ratner (1955) and Ratner & Petrack (1951, 1953 b) except as noted. Extracts of urease (Nutritional Biochemicals Corporation) were made fresh each day, 50 mg. being extracted with 5 ml. water or 0.1 M-phosphate buffer (pH 7.4); these extracts did

not interfere in any of the assay procedures. Protein was estimated by the biuret method (Gornall, Bardawill & David, 1949) on samples clarified by repeated centrifugation.

Measurement of condensing activity. The complete assay system contained (in μ mole per 2 ml.): 15, L-citrulline; 15 L-aspartate; 13.2 MgSO_4 ; 100 tris or phosphate buffer (pH 7.4); 2.5 ATP; 25 to 75, D-3-phosphoglycerate (PGA). (The PGA was used to regenerate ATP. In *Neurospora* extracts, ATPase activity is much greater than condensing activity, so that 60 or more μ mole PGA were often necessary in order to achieve linear reaction rates. It was found unnecessary to add an outside source of the enzymes needed to regenerate ATP from PGA, even when fractions were being assayed.) The mixtures were incubated at 33° and the reactions stopped with trichloroacetic acid (TCA).

Table 1. *Separation of condensing and argininosuccinase activities in wild type Neurospora crassa*

Fraction no.	$(\text{NH}_4)_2\text{SO}_4$ saturation (%)	Protein (mg.)	Condensing activity		Argininosuccinase	
			Specific activity*	Total units†	Approximate specific activity*	Approximate total units†
Crude extract	—	3090	0.59	1823	0.76	2350
I	0–25	103	0	0	≤ 0.20	0–21
II	25–50	1740	0.86	1498	0.86	1498
III	50–85	842	≤ 0.035	0.29	≤ 0.03	0–25
II‡	25–50	739	0.86	635	0.86	635
IIa	0–30	214	≤ 0.05	0.11	0.13	28
IIb	30–44	284	1.01	287	2.03	577
IIc	44–70	187	1.68	314	0.01	2

* Units/mg. protein.

† One unit caused the disappearance of 1 μ mole citrulline or formation of 1 μ mole arginine in 1 hr. at 33°.

‡ The sample used for refractionation.

Condensation was assayed by measuring citrulline disappearance colorimetrically (Ratner, 1955). (*Neurospora* extracts contain too much urease to permit the assay of condensing activity by measuring urea production.) With the wild type, the citrulline disappearance method is reliable, since no side-reactions have been detected at the protein concentrations used for assay. With the mutants, however, this assay presents two difficulties.

(1) Citrulline disappearance might be due to side-reactions, because much larger amounts of protein must be tested in order to detect small amounts of condensing activity. Furthermore, citrulline accumulation resulting from a genetic block might induce the synthesis of enzymes catalysing such side-reactions. Therefore, the apparent small activity found in *arg-1* extracts was tested for its relevance to condensation by dependency tests, and by testing for argininosuccinate production by two-dimensional chromatography.

(2) With *arg-1* extracts it is hard to obtain accurate citrulline disappearance measurements without increasing the time and/or amount of enzyme so much that the linear range is exceeded; the enormous ATPase competition under these conditions often makes it impossible to add an excess of PGA. The *arg-1* 'specific

activity' values given under Results are therefore only best estimates, and are shown bracketed when the only assays were definitely in the non-linear range. In order to show that the difference between *arg-1*⁻ and *arg-1*⁺ is nevertheless real, typical raw data are also given which compare *arg-1*⁻ and *arg-1*⁺ extracts when both are assayed under similar conditions, at high time and enzyme levels.

All assays of citrulline disappearance were made on dialysed extracts, because undialysed extracts, particularly of *arg-1* mutants, contain material which gives colour in the citrulline assay; only part of this can be citrulline itself. Urea also interferes in the citrulline determination, but in crude extracts it is destroyed as fast as formed. In at least one assay on each extract, the enzymic reaction was stopped by heat and the mixture re-incubated with excess urease before citrulline was determined. This procedure always gave the same results as when urease was omitted.

Arginine desimidase (Oginsky & Gehrig, 1952) was not expected to interfere seriously because of its sensitivity to Mg^{++} and its low activity at pH 7.4; desimidase activity was specifically shown to be absent from wild-type fraction II under the conditions used here.

Measurement of argininosuccinase. An approximate assay was obtained by determining the amount of CO₂ formed in the presence of excess arginase and urease. Double-armed Warburg vessels were used; the main compartment contained 0.10 ml. arginase solution, 0.20 ml. urease solution (buffer extract), 0.20 ml. of 1.0 M-phosphate buffer (pH 7.4), and dialysed *Neurospora* extract in a total volume of 0.70 ml. The reaction was started by tipping in 0.30 ml. of a solution containing 6 μ mole argininosuccinate. Standard manometric procedures were used, with air as gas phase. Endogenous respiration was negligible. CO₂ production was linear with time so long as 25% of the substrate remained.

Measurement of ATPase. For estimating ATPase competition, crude assays were made by measuring the total inorganic phosphate liberation in the TCA filtrates used for measuring citrulline disappearance; the complete system was used except that ATP (20 μ mole/2 ml.) was used as sole phosphate donor. Most of the phosphate liberation was due to ATPase; approximate correction for the small amount that could be due to condensation (0-30% of the total) was based on the citrulline disappearance measurements. For measuring the ratio of phosphate liberation to citrulline disappearance, exact correction was made for ATPase, and also for a trace reaction involving aspartate, by subtracting the phosphate liberated in the absence of citrulline from the amount liberated when both amino acids were present.

Chromatography. Ascending paper chromatography was used to detect the formation of argininosuccinate and other products by the *Neurospora* extracts. Only dialysed extracts were used. Incubation mixtures were made with phosphate buffer since tris buffer altered the R_f values of several compounds. After incubation the mixtures were deproteinized by heating and chromatographed either with 4+1+2 by vol. butanol+acetic acid+water (run twice in the same direction) or with 3+1 by vol. phenol+water with or without ammonia atmosphere. For two-dimensional chromatograms, phenol without ammonia was used as the first solvent and butanol+acetic acid as the second; in this case it was not essential to develop twice with butanol+acetic acid. With the incubation mixtures used here, one-

dimensional phenol chromatograms rarely gave adequate separation of argininosuccinate from glutamate, and one-way butanol + acetic acid chromatograms failed to separate argininosuccinate from aspartate. Argininosuccinate was therefore identified by two-dimensional chromatography, and by elution and re-development, with similarly eluted authentic argininosuccinate as a standard. All critical identifications were checked by addition of known amino acids.

RESULTS

Neurospora crassa wild type

Extracts of wild-type *Neurospora* readily catalysed the disappearance of citrulline and the appearance of arginine in the presence of the complete system. The enzymes involved are quite stable to dialysis and storage. The rate of citrulline disappearance for various wild-type extracts is given in Table 2; this rate was affected very little by the presence or absence of arginine in the culture medium. The wild-type *Neurospora* system resembles the mammalian one in all respects tested. Specifically:

(1) In dialysed fractionated material, both citrulline disappearance and arginine formation were completely dependent on the presence of aspartate, citrulline, MgSO_4 and ATP. Aspartate could not be replaced by ammonium ion or glutamate.

(2) The rate of citrulline disappearance was greatest when a combination of D-3-phosphoglycerate + a catalytic amount of ATP was used as the source of high-energy phosphate, since high ATP concentrations inhibited the reaction. In the absence of D-3-phosphoglycerate, activity was decreased at ATP concentrations greater than $20\ \mu\text{mole}/2\ \text{ml.}$; this concentration gave only 50–80 % of the activity obtained with $2.5\ \mu\text{mole}$ ATP + optimal concentrations of D-3-phosphoglycerate (Table 2).

(3) The reaction is accompanied by a liberation of inorganic phosphate which depends on the simultaneous presence of citrulline and aspartate. The ratio of phosphate liberation to citrulline disappearance was determined using fraction II with $2.5\ \mu\text{mole}$ ATP + $45\ \mu\text{mole}$ D-3-phosphoglycerate; after correcting for phosphate liberated by ATPase, values of 1.53, 1.53, and 1.49 were obtained. In liver, the $\Delta\text{phosphate}/\Delta\text{citrulline}$ ratio is typically between 1.0 and 2.0, the exact value depending on the concentration of inorganic pyrophosphatase and on the source of ATP. When ATP was supplied from D-3-phosphoglycerate, values of about 1.5 were obtained only when the pyrophosphatase concentration was nearly optimal (Ratner & Petrack 1953*a*, 1956). The *Neurospora* results therefore suggest that pyrophosphatase is similarly involved in its condensing system, and is probably not seriously limiting. Pyrophosphatase is evidently not removed by the partial purification used here, since there is little loss of condensing activity during fractionation (Table 1).

(4) The reaction occurs in two steps with argininosuccinate as intermediate. The evidence for this is as follows. Citrulline disappearance always exceeded arginine formation. (No attempt was made to achieve equilibrium.) Partial purification gave two active fractions (Table 2). One (II*b*) caused a rapid disappearance of citrulline, of which 55 % was found as arginine; this fraction contained most of the argininosuccinase. The other active fraction (II*c*) caused a more rapid disappearance

Table 2. *Condensing activities of mutant and wild-type extracts*

Specific activities are based on measurements of citrulline disappearance in dialysed crude extracts. 'PGA system' indicates complete incubation mixture with 2.5 μ mole ATP + optimal concentrations of D-3-phosphoglycerate (PGA) as described in Methods. 'ATP system' indicates complete incubation mixture with 20 μ mole ATP and no PGA. For *arg-1*⁺ strains, incubation was for 1-4 hr., with 1-4 mg. protein. For *arg-1*⁻ it was necessary to use more protein. Where this resulted in non-linearity, the specific activity values are enclosed in brackets; in such cases the raw data indicate decidedly less activity than is given by *arg-1*⁺ under similar conditions. See Methods and Table 3.

Genotype	Allele	Extract no.	Growth conditions		Specific activity	
			No. days	Arginine (mg./ml.)	PGA system	ATP system
Wild type	ST-74A	5	3	0	0.59	—
	ST-74A	11	3	0.2	0.46	0.30
	ST-74A	13	3	0.2	0.63	—
	ST-74A	15	5	0	0.57	0.46
	ST-74A	17	5	0.2	0.79	0.39
	ST-74A	19	1.8	0.07	0.62	—
<i>arg-3</i>	30300a	26	5	0.2	0.71	—
<i>arg-10</i>	B317a	24	5	0.2	0.41	0.29
	B370a	30	5	0.2	0.46-0.53	0.16-0.18
	B368a	32	5	0.2	0.35-0.43	0.17-0.21
	B317a	38	3	0.5	0.48	0.28
	B317a	60	3	0.5	0.51	—
	B370a	64	3	0.5	0.60	—
<i>arg-1</i>	36703T-a	4	5	0.2	0.06*	—
	36703T-A	12C	5	0.2	—	0.07
	36703T-A	12D	5	0.2	—	0.11
	36703T-A	16C	5	0.2	0.08	0.10
	36703T-A	16D	5	0.2	—	0.04
	46004A	14	5	0.2	0.06	[0.03]
	46004a	44	5	0.2	[0.03]	—
	B369a	20	5	0.2	0.08	0.09
	H4250A	22C	5	0.2	0.10	0.13
	H4250A	22D	5	0.2	0.12	0.14
	H4250a	46	5	0.2	[0.06]	—
	B312a	10	4	0.2	0.04	[0.02]
<i>arg-1 arg-10</i> (double mutant)	36703T-B317	18	5	0.2	—	0.02*
	36703T-B317	42	3	0.5	[0.02]	0.01*

* Not significantly greater than zero.

of citrulline, of which not more than 5% was found as arginine; this fraction contained very little argininosuccinase and negligible arginase. It was shown by sequential incubations that fraction IIc converted the missing citrulline to an intermediate compound, which could be converted to arginine by an argininosuccinase preparation from yeast. (This preparation, the 35-50% ammonium sulphate fraction from a plasmolysed extract of baker's yeast, had negligible condensing activity.) The intermediate was identified as argininosuccinate by paper chromatography. When incubated with the complete system, the dialysed crude extract and fraction II each produced both arginine and argininosuccinate, while fraction IIc and a dialysed crude extract of *arg-10*(B317) each produced a strong argininosuccinate spot but never more than a dubious arginine spot. (Arginine was

detectable because, in the presence of the filtrates, it was displaced upward on phenol chromatograms, in the absence of ammonia, to R_f 0.70, and was thus separated from citrulline. Under the same conditions, ornithine was displaced downward, so as to be superimposed on slow-moving material present in all mixtures before incubation; the expected traces of ornithine were therefore not detectable.) No other products were found. By using fraction II, detectable argininosuccinate production was found to be completely dependent on the presence of citrulline, aspartate, MgSO_4 and ATP.

Neurospora crassa mutants

Condensing activity at standard growth conditions. Extracts of the *arg-1* and *arg-10* mutants (and also of the citrullineless mutant, *arg-3*) were ordinarily prepared from mycelia grown for 5 days on minimal medium + 0.2 mg. arginine/ml.; a few strains were also grown for 3 days at 0.5 mg./ml. The condensing activities of these preparations are summarized in Table 2 as specific activities, and typical raw data comparing *arg-1*⁺ with *arg-1*⁻ are given in Table 3. The *arg-1* extracts invariably caused much less citrulline disappearance than the wild-type controls, although they were not completely inactive. Extracts of *arg-10* and *arg-3*, on the other hand, were essentially as active as wild type. As expected, the *arg-1 arg-10* double mutant showed the reduced activity characteristic of *arg-1*.

The low activities of the *arg-1* extracts were not due merely to a deficiency in the enzymes which regenerate ATP from D-3-phosphoglycerate. This is shown, first, by the results with ATP as sole phosphate donor (Tables 2 and 3). The difference between *arg-1*⁻ and *arg-1*⁺ activities was smaller here than with the D-3-phosphoglycerate system, because the *arg-1*⁻ extracts did not show the ATP inhibition characteristic of liver and wild-type *Neurospora*. Even so, there was still no overlap in the activities of *arg-1*⁺ and *arg-1*⁻. Secondly, when extracts from three different *arg-1* mutants were assayed with the D-3-phosphoglycerate system, addition of a muscle preparation to supply the enzymes needed for regenerating ATP (Ratner, 1955) caused no increase in condensing activity.

Mixing crude extracts of *arg-1* with active control extracts caused no significant stimulation or inhibition (Table 4). As a further test, *arg-1* extract no. 4 was put through a single ammonium sulphate fractionation. The resulting fraction II, which should have contained all the condensing activity, was still quite inactive, and caused no stimulation or inhibition when combined with wild-type fraction II (Table 4). The mutant fraction II also caused no stimulation when combined with the inactive wild-type fractions I, IIa, and III. (A small amount of activity was found in mutant fraction III, however. The residual *arg-1* activity thus fractionated abnormally, suggesting that it might be due to a different enzyme.)

The low activity of the *arg-1* extracts does not appear to be ascribable merely to increased ATPase activity. ATPase assays on various extracts of the wild type, *arg-1*(36703T), *arg-10*(B317), and the *arg-1 arg-10* double mutant, showed that while this activity varied considerably, depending on the growth conditions, it was unaffected by the presence or absence of *arg-1*⁻. Excessive ATPase competition was further excluded by the lack of inhibition when *arg-1* extracts were combined with wild type (Table 4). The lack of inhibition also makes it unlikely that the low activity of the *arg-1* extracts was due to a large increase in arginine desamidase.

Table 3. *Typical raw data comparing arg-1⁺ and arg-1⁻ extracts of Neurospora crassa at high time and enzyme levels*
 See Methods for rationale. 'PGA system' indicates complete incubation mixture with 2.5 μ mole ATP + D-3-phosphoglycerate (PGA) as indicated. 'ATP system' indicates complete incubation mixture with 20 μ mole ATP and no PGA. Tests done under similar conditions are grouped together.

Strain	Extract no.	Assay conditions			μ mole citrulline disappearance				
		PGA (μ mole)	Protein (mg.)	Incubation time (hr.)	<i>arg-1</i> ⁺		<i>arg-1</i> ⁻		
					Wild type	<i>arg-10</i>	<i>arg-1</i>	<i>arg-1 arg-10</i> double mutant	
PGA system									
ST-74A	15	60	2.5	4.5	4.17	.	.	.	
B370a	64	35	2.4	4.2	.	4.45	.	.	
36703T-A	16C	50	2.9	4	.	.	0.95	.	
ST-74A	13	45	5.5	3	9.06	.	.	.	
B369a	20	45	5.6	3	.	.	1.35	.	
B317a	60	35	4.9	4.3	.	5.54	.	.	
46004a	44	35	6.3	4.5	.	.	0.97	.	
H4250a	46	35	5.6	4.5	.	.	1.57	.	
B370a	64	35	11.8	3.8	.	5.30	.	.	
36703T-B317	42	35	11.8	4.4	.	.	.	0.87	
ATP system									
B317a	38	—	8.6	1	.	2.42	.	.	
36703T-B317	42	—	8.6	1	.	.	.	0.10	
B317a	24	—	4.7	2	.	2.80	.	.	
36703T-A	12C	—	4.5	2	.	.	0.65	.	
36703T-A	16D	—	4.5	2	.	.	0.38	.	
ST-74A	11	—	2.5	2.5	1.84	.	.	.	
B369a	20	—	3.2	3	.	.	0.95	.	
H4250A	22C	—	3.2	3	.	.	1.27	.	

Table 4. *Mixtures of arg-1⁺ and arg-1⁻ extracts of Neurospora crassa*

Complete incubation mixture with 2.5 μ mole ATP and the indicated amounts of D-3-phosphoglycerate (PGA). Differences of less than 0.35 μ mole citrulline are within experimental error.

	<i>arg-1⁺</i>		<i>arg-1⁻</i>		Assay conditions		μ mole citrulline disappearance caused by		
					Incubation time (hr.)	PGA (μ mole)	<i>arg-1⁺</i>	<i>arg-1⁻</i>	Both together
	Strain	Protein (mg.)	Strain	Protein (mg.)					
Crude extracts	Wild type	0.60	H 4250	0.62	4.25	85	1.32	0.16-0.32	1.82
	Wild type	0.60	46004	0.63	4.25	85	1.32	0.09-0.16	1.40
	Wild type	0.60	36703 T-B 317	0.59	4.25	85	1.32	0.04-0.07	1.37
	Wild type 30300 (<i>arg-2</i>)	2.6 1.06	36703 T B 369	c. 3.1 4.0	2 3	45 45	2.43 2.28	0 1.07	2.60* 3.15*
Fractions	Wild type fraction II	3.57	36703 T fraction II	3.43	1	50	3.04	0	2.85

* In these two cases, it was impossible to add enough PGA to insure excess high energy phosphate when the two extracts were mixed; therefore a slight synergism might go undetected. All other tests contained at least twice the amount of PGA required by the *arg-1⁺* component alone.

This possibility was ruled out completely for the *arg-1 arg-10* double mutant, since the *arg-10* block prevents arginine formation.

Mixing extracts from two different *arg-1* mutants was tested in two cases (Table 5); neither mixture showed synergism. Furthermore, the five *arg-1* mutants do not complement in heterocaryons in any combination (Newmeyer, 1957). It is concluded that all five *arg-1* mutants involve a simple loss of the same enzyme.

The residual activity of the *arg-1* extracts cannot reasonably be ascribed to bacterial action during the rather long incubation periods because controls incubated without enzyme never showed any citrulline disappearance, and because of the reproducibility of the activity when any strain was grown repeatedly under the same conditions. The question remained whether the residual citrulline disappearance was due to condensation or to irrelevant side reactions, as discussed in Methods. To facilitate answering this question, growth conditions were found which gave increased residual activity. But the *arg-10* controls grown under these conditions gave unexpected results which should first be discussed.

Anomalous behaviour of arg-10 mutants. The results reported so far indicate that *arg-10* strains, grown as in Table 2, gave extracts with completely normal condensing activity. However, when grown for 3 days at low arginine concentrations (0.035 or 0.07 mg./ml.) both *arg-10* alleles tested gave extracts with very little condensing activity. Four extracts from mycelia grown at 0.07 mg./ml. had specific activities in the D-3-phosphoglycerate system of only 0.05–0.06; the one extract from mycelium grown at 0.035 mg./ml. had a specific activity of 0.15.

It was shown that the low activity of these extracts was not due to variations in the amount of grinding, or to inadequate regeneration of ATP, or to inhibitor production (Table 6, line 1), and that only a small part of the reduction in activity could be explained by increased ATPase. Since the defect thus seemed to be a simple loss, it was of interest to assay the defective *arg-10* extracts in admixture with *arg-1*, to test the possibility that one type lacked the condensing enzyme while the other had insufficient pyrophosphatase. Since no extract of an *arg-1* single mutant was available for these tests, an extract of the double mutant was used, which was made from mycelia grown under conditions which gave normal condensing activity for *arg-10* alone. No stimulation was found (Table 6, lines 2, 3, and 4); it appears therefore that both *arg-1* and defective *arg-10* extracts lack the same enzyme.

However, accumulation tests indicated that the low activity of the defective *arg-10* extracts did not accurately reflect the situation during growth of the cultures. These tests were made by chromatographing undialysed samples of extracts prepared as for enzyme assay. They showed clearly that the defective *arg-10* cultures accumulated large amounts of argininosuccinate and no or negligible amounts of citrulline. Therefore, even at low arginine concentrations, *arg-10* must have had condensing activity *in vivo*; furthermore, if this activity was subnormal, citrulline synthesis must have been subnormal also. In contrast, the *arg-1 arg-10* double mutant, grown under the same conditions, accumulated citrulline but not argininosuccinate; this would indicate that it had no condensing activity *in vivo*. A wild-type culture accumulated neither compound, as expected.

Since at these arginine concentrations *arg-10* gave only 0.35 and 0.60 times as much growth as comparable wild-type cultures, it is quite possible that its extracts

Table 5. *Mixtures of two Neurospora crassa arg-1- extracts*

Tested as in Table 4 (except as noted) with crude extracts in all cases.

1st component		2nd component		Assay conditions			μ mole citrulline disappearance caused by		
Allele	Protein (mg.)	Allele	Protein (mg.)	Incubation time (hr.)	PGA (μ mole)	ATP (μ mole)	1st component	2nd component	Both together
46004	2.5	B312	3.9	2	50	2.5	0.30	0.18-0.39	0.25
B369	3.2	H4250	2.4	3	0	20	0.95	1.05	1.40

Table 6. *Mixtures involving defective arg-10 extracts of Neurospora crassa*

Tests on dialysed crude extracts as in Table 4. Incubated 4.1 or 4.2 hr. with 71-75 μ mole D-3-phosphoglycerate (PGA), conditions being constant throughout a given experiment. Half the amount of PGA used was adequate for the active *arg-10* component of line 1. All extracts were made from mycelia grown for 3 days at the arginine concentrations indicated in Table.

Defective <i>arg-10</i>			Other component			μ mole citrulline disappearance caused by		
Allele	Arginine concentration of growth medium (mg./ml.)	Protein (mg.)	Type of extract	Arginine concentration of growth medium (mg./ml.)	Protein (mg.)	Defective <i>arg-10</i>	Other component	Both together
B317	0.07	1.30	active <i>arg-10</i>	0.5	2.36	0.36	4.29	4.05
B317	0.07	1.30	<i>arg-1 arg-10</i>	0.5	1.07	0.35	0.12	0.12
B370	0.07	1.55	double	0.5	1.07	0.35	0.10	0.35
B317	0.035	1.60	double	0.5	1.07	1.07	0.10	1.27
			<i>arg-1 arg-10</i>					
			double					

had low activity simply because the cultures were growing slowly, or had even stopped growing, at the time of harvesting. If so, many enzyme activities might be diminished, both inside and outside the arginine pathway; the effect would then be unspecific and unimportant. A specific effect has not been excluded however. Although it was not feasible in this case to run curves of growth *v.* time, such as were run for all other experimental conditions, the available data make it quite unlikely that the 0.07 mg./ml. cultures had actually stopped growing before they were harvested. Furthermore it appears that the poor growth was not simply due to arginine limitation, since at this same concentration the inbred *arg-1 arg-10* double mutant grew nearly as well as the wild type. It is therefore possible that the excessive accumulation of argininosuccinate may interfere with growth; it might also prevent further synthesis of condensing enzyme. It has been shown by accumulation tests that these effects could not occur through depletion of the free amino acid pool; but the effect on condensing enzyme could happen through a repression mechanism (Vogel, 1957), which could affect citrulline synthesis as well.

Nature of residual arg-1 activity. Both the abnormal fractionation behaviour and the lack of ATP inhibition suggested that the residual activity of the *arg-1* extracts was due to loss of citrulline through irrelevant side-reactions (e.g. via citrullinase). Furthermore, the *arg-1* activity, at least in two alleles, was found to be largely independent of aspartate, Mg^{++} and ATP, in contrast to the strong dependence of the controls (Table 7). Additional tests on extracts 16 and 40 indicated that no single constituent of the system was required, although multiple omissions decreased the activity somewhat. Adding acetyl glutamate, substituting ADP for ATP, and lowering the pH to 6.1, all gave slight increases in activity.

Direct evidence for side-reactions was sought by chromatographically testing whether the citrulline disappearance caused by *arg-1* (36703T) was accompanied by argininosuccinate formation (Table 8). In some cases the *arg-1 arg-10* double mutant was used (the *arg-10* block being included to prevent the removal, through argininosuccinase and arginase, of any argininosuccinate that might be formed). Of the double mutant extracts used, no. 78 was made from mycelium grown under the same conditions as the active *arg-10* control extract, so that the lack of argininosuccinate production could be ascribed to the *arg-1* block. The others (nos. 40 and 48) were prepared under the conditions which gave the defective *arg-10* extracts. They could therefore not be used as proof that *arg-1* prevents argininosuccinate formation, but, because of their higher activity, they provided the best evidence for extensive side-reactions, and facilitated identification of the side-products.

All the wild-type and active *arg-10* control filtrates gave the expected strong argininosuccinate spots. None of the *arg-1* or double mutant filtrates showed any argininosuccinate, although the sample sizes were such that if all the missing citrulline had been converted to argininosuccinate, it should have given more than 2.5 times the minimum detectable amount.

Instead of argininosuccinate, the *arg-1* and *arg-1 arg-10* filtrates each gave four spots detected in the *arg-1*⁺ controls; these were tentatively identified as glutamate, alanine, isoleucine and valine. In addition, a trace spot tentatively identified as carbamyl aspartate (detected by Ehrlich's reagent) was sometimes found, as well as dubious traces of ornithine. No arginine was detected, but it would not always have been detectable on these chromatograms. Its detection should be unnecessary,

Table 7. *Dependency tests on arg-1⁺ and arg-1⁻ extracts of Neurospora crassa*

All tests were made on dialysed crude extracts. 'Complete system' is as in Methods, with 2.5 μ mole ATP and the indicated amounts of D-3-phosphoglycerate (PGA). 'Minimal system' contains citrulline and buffer only, with no aspartate, Mg, PGA or ATP.

	Strain	Extract no.	Assay conditions			Δ citrulline (μ mole)		
			PGA (μ mole)	Protein (mg.)	Incubation time (hr.)	Complete system	Complete minus aspartate	Minimal system
High activity	Wild type	5	25	3.2	1	-1.88	+0.20	.
	Wild type	15	60	2.5	4.3	-4.17	.	.
	Wild type	15	0	10.0	4.7	.	.	+0.12
	Wild type	17	35	6.9	1.0	-4.55	.	.
	Wild type	17	0	9.2	4.7	.	.	-0.03
	<i>arg-10</i> (B317)	38 (active)	55	1.1	4.6	-2.51	.	.
	<i>arg-10</i> (B317)	38 (active)	35	8.6	1.0	-2.70	.	.
	<i>arg-10</i> (B317)	38 (active)	0	11.4	4.7	.	.	-0.03
Low activity	<i>arg-10</i> (B317)	34* (defective)	35	4.1	4.4	-0.70	.	.
	<i>arg-10</i> (B317)	34* (defective)	0	4.1	4.7	.	.	+0.22
	<i>arg-1</i> (36703T)	16C	40	4.2	4.0	-1.15	-1.15	.
	<i>arg-1</i> (36703T)	16C	0	c. 4.4	4.3	.	.	-0.77
	<i>arg-1</i> (36703T)	16C	0	c. 8.8	4.3	.	.	-1.07
	<i>arg-1</i> (46004)	44	35	7.0	4.4	-1.05	.	-0.30
	<i>arg-1</i> (46004)	44	0	7.0	4.7	.	.	.
	<i>arg-1</i> (H4250)	46	35	6.2	4.4	-1.50	.	.
	<i>arg-1</i> (H4250)	46	0	6.2	4.7	.	.	-1.08
	<i>arg-1 arg-10</i> (double mutant)	40*	20	10.1	4.6	-1.90	-1.75	.
		40*	0	10.1	4.6	.	.	-1.20

* From mycelia grown for 3 days at 0.07 mg. arginine/ml. Growth conditions for all other extracts are given in Table 2.

Table 8. *Chromatographic tests for argininosuccinate formation by arg-1 mutants of Neurospora crassa*

'Complete' indicates complete system, as described in Methods, with 35 μ mole PGA but only 10 μ mole aspartate, unless otherwise noted. 'Minimal system' indicates citrulline and buffer only. The incubation mixtures were deproteinized and developed on two-dimensional chromatograms. The estimated citrulline disappearance is based on assays performed under similar incubation conditions. For *arg-10* and the double mutant, the amount of citrulline disappearance per sample used on chromatograms should equal the amount of argininosuccinate found, if all activity is due to condensation. Under the conditions used, 0.14 μ mole argininosuccinate is readily detectable, as determined by adding authentic argininosuccinate to unincubated control filtrates on two-dimensional chromatograms. Results are reported only from runs in which all spots were well-separated, except for occasional superimposition of arginine and citrulline.

	Genotype	Extract no.*	Incubation conditions			Estimated amount of citrulline disappearance per chromatogram sample (μ mole)	Compounds detected†	
			Protein (μ g.)	Time (hr.)	Remarks		Arginino-succinate	Glutamate
<i>arg-1</i> ⁻ strains	<i>arg-1</i>	16C	c. 5.2	c. 5	Complete	c. 0.05	—	+
	<i>arg-1</i>	16C	c. 5.2	c. 5	Minimal system	0.045	—	+
	<i>arg-1 arg-10</i> (double mutant)	78	10.7	4	Complete (50 μ M-PGA)	0.035‡	—	+
	<i>arg-1 arg-10</i> (double mutant)	40	10.2	5	Complete	—§	—	+
	<i>arg-1 arg-10</i> (double mutant)	48	7.7	4	Complete	0.105	—	+
<i>arg-1</i> ⁺ controls	<i>arg-10</i>	60 (active)	1.94	4½	Complete	0.065	++	—
	Wild type	15	1.24	4½	Complete	0.07–0.09	++	—
	Wild type	15	1.24	4½	Complete (60 μ M-PGA)	0.057	++	—

* Extract 48 is from mycelium grown 3 days at 0.035 mg. arginine/ml.; extract 78, from mycelium grown 3 days at 0.5 mg./ml. Growth conditions for all other extracts are given in Tables 2 and 7.

† See text for additional products. Neither argininosuccinate nor any of the other compounds was found in unincubated controls, except for part of the glutamate.

‡ This extract not assayed under these conditions. Estimate based on activity of extract 42C, which was prepared from the same strain under the same conditions.

§ Not run on two-dimensional chromatograms. Tested by elution of the argininosuccinate glutamate region from a one-dimensional phenol chromatogram followed by development of the eluate with butanol+acetic acid+water.

however, because of the inclusion of the *arg-10* block. Actually, much of the glutamate and alanine was also formed in the absence of citrulline. The spots tentatively identified as isoleucine and valine, however, were strongly citrulline-dependent. These two spots were identified only by their failure to separate from the added authentic compounds; several other amino acids with similar R_f values were not excluded. How citrulline could assist in the formation of isoleucine, valine, or any of these alternative amino acids is not known.

The separation of argininosuccinate and glutamate on two-dimensional chromatograms was unequivocal. On such chromatograms the product in the *arg-1*⁻ and double mutant filtrates behaved exactly like glutamate. The possibility that *arg-1*⁻ extracts did form argininosuccinate, but immediately destroyed it, e.g. by cyclization (Ratner *et al.* 1953), was excluded by adding equal amounts of argininosuccinate to samples of a double mutant incubation mixture, before and after incubation. Chromatograms of these two were identical. It is concluded that most or all of the citrulline disappearance caused by *arg-1*(36703T) extracts was unrelated to condensation. The results of the dependency tests and the lack of ATP inhibition indicated that this was probably true also for alleles B369 and H4250. A trace of true condensing activity in mutant 46004 was not excluded.

Nature of residual activity in defective arg-10 extracts. A dependency test on a defective *arg-10* extract suggested that the remaining activity was really due to condensation, since it vanished completely in the absence of aspartate + Mg^{++} + ATP (Table 7). The low protein concentration of these extracts precluded the conversion of enough citrulline to argininosuccinate to test this chromatographically.

Argininosuccinase. Fincham & Boylen (1957) showed that the four *arg-10* mutants lacked detectable argininosuccinase; they mentioned that the *arg-1* allele 36703T had about the wild-type concentration of this enzyme. Results obtained here, with the extracts recorded in Table 2 confirmed their results and showed that the other four *arg-1* alleles were also highly active in this respect.

DISCUSSION

The enzyme missing from the *arg-1* extracts is almost certainly the condensing enzyme itself, rather than the stimulatory enzyme, inorganic pyrophosphatase. Inasmuch as inorganic pyrophosphate is involved in several essential reactions and inhibits several others, it seems most unlikely that a deficiency of pyrophosphatase would result in viable mutants with an essentially complete requirement for a single growth factor. This conclusion is strengthened by the essentially complete absence of condensing activity in the *arg-1* mutants. Since the *arg-1* mutants lacked activity under all conditions tested, and since insertion of this block into the double mutant prevented argininosuccinate accumulation *in vivo*, it seems likely that *arg-1* is the primary locus determining ability to synthesize the condensing enzyme. The defective *arg-10* extracts also appear to lack condensing enzyme, but the inadequate supplement used here may well cause drastic reductions in many enzyme activities. Therefore it is doubtful whether *arg-10* has any specific effect on the condensing enzyme. If there is such an effect, it must be a secondary one, since *arg-10* strains have normal activity when grown at high arginine concentrations.

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