Arginine Synthesis in *Neurospora crassa*; Genetic Studies

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SUMMARY: Nine mutants of *Neurospora crassa* which require arginine as a nutrient but cannot use citrulline were obtained from various sources. These fall into two classes, according to location in linkage group I or VII. (Enzymic tests, reported elsewhere, indicate that the two classes of mutants affect the enzymes which control the two reactions between citrulline and arginine.) Heterokaryon tests between mutants of the same group were negative, and crosses between mutants of the same group were semi-sterile, most of the ascospores being non-viable. Crosses between the five group I mutants produced no *arg*⁺ progeny, and separate mapping tests on four of them indicate that they are either allelic or closely linked. All crosses between the group VII mutants gave many *arg*⁺ progeny. For the one pair of mutants which was studied in detail, origin of the *arg*⁺ by means other than crossing-over (or gene conversion) has been virtually eliminated. However, mapping studies place the two mutants only 0-6 units apart. It is concluded that the high *arg*⁺ frequency is due to selection, and that the mutants might be pseudoalleles.

In recent years several enzymic studies have been made in order to test directly the hypothesis that there is a one-to-one relationship between genes and enzymes (Beadle, 1945). Unfortunately, relatively few of these studies have been made on organisms in which it could be established that the mutant phenotype was really due to a single gene, and in these few cases there has usually been only one mutant available for study. A more critical test is provided when a series of similar mutants is studied; such tests are also needed to settle the controversy about whether pseudoalleles control the same or different reactions. However, in the case of biosynthetic enzymes, material suitable for such a test is so rare that very few cases of this sort have been reported (Yanofsky & Bonner, 1955; Fincham, 1950, 1954). The present study is an attempt to provide two more such cases.

The conversion of citrulline to arginine in mammalian liver has been shown by Ratner and co-workers (Ratner, Anslow & Petrack, 1953; Ratner & Petrack, 1953) to consist of at least two reactions:

The first step requires two enzymes, both of which must be present to produce any detectable effect. An enzymic study of wild type *Neurospora crassa* (Newmeyer, unpublished) showed that it converted citrulline to arginine in the same way as liver, except that there is no evidence yet that two enzymes are required for the first reaction. Independently, Drs Fincham & Boylen (personal communication) have obtained preliminary evidence for the same reaction sequence in *Neurospora crassa*, and they have studied the second reaction in detail (Fincham & Boylen, 1957).

This system seemed ideal for the type of test described above, because nine mutants were available which might be expected to fall into two or three genetic groups corresponding to the two or three enzymes involved. Furthermore, if pseudoalleles ever do control sequential reactions requiring close proximity of two gene-products (as discussed by Pontecorvo, 1955, and by Lewis, 1955), the conversion of citrulline to argininosuccinate should be an excellent place to look for it.

The present paper reports a genetic study of the nine arginine-requiring mutants. Each segregates as though a single mutant gene is involved. Genetically they fall into two classes, located in linkage groups I and VII; preliminary enzyme tests on one member of each class indicated that the group I mutant affects the first reaction and the group VII mutant affects the second (Newmeyer, unpublished).

The group VII mutants were sent to Drs Fincham & Boylen for study; their results, reported in the accompanying paper (Fincham & Boylen, 1957), indicate that all four mutants lack the enzyme which catalyses the second reaction. For their results to be most meaningful, it is necessary to know whether these mutations involve the same or different genes. Much of the present paper is devoted to an attempt to answer this question. Because of sterility difficulties, it has been necessary to restrict this work to two of the mutants; these appear to be either pseudoallelic or closely linked.

A similar study of the group I mutants is still in progress. Enzymic tests (Newmeyer, D. & Högstöm, D. unpublished) indicate that all members studied thus far affect the first reaction. The genetic tests to date, which are reported here, indicate that all five mutants may be alleles.

METHODS

Arginine-requiring mutants

Origin. Only two mutants of *Neurospora crassa* which can use arginine but not citrulline have been reported previously; these are 36703T (*arg-1*) and 46004, described by Srb & Horowitz (1944) and Srb (1946). Seven others were obtained by screening new mutants from various sources. Mutant H 4250 was kindly provided by Dr F. P. Hungate, who isolated it after treatment with ³⁵S (Hungate & Mannell, 1952). Particular thanks are due to Dr V. W. Woodward, who supplied seventy-two unclassified arginine mutants for testing, of which six were the desired type. Of these, mutants B 312 and B 317 were isolated after ultraviolet treatment, and mutants B 362, B 368, B 369, and B 370 after gamma-irradiation. The method for obtaining the last four mutants was such that two or more of them might possibly have arisen from a single mutated nucleus; however, the data reported here show that mutant B 369 is genetically different, and suggest that the other three are independent also. Examination of a total of nineteen additional arginine mutants, kindly provided by Mr R. W. Colburn, by Dr C. M. Stevens, and by Dr R. W. Barratt, yielded none of the desired type.

Genetic basis. The evidence that the arginine requirement of a given mutant segregates as a single Mendelian factor is based on the ratio of *arg*⁺:*arg*⁻

random isolates in crosses to wild type (or to other *arg*⁺ strains). For each mutant, Table 1 gives the pooled results of all such crosses in which the degree of germination was high enough ($\geq 70\%$) to minimize skewing of the results. Eight of the mutants gave the expected 1:1 ratio. In the case of the ninth mutant (B312), it is concluded that the deviation is due only to differential viability, for the following reasons: (1) the number of non-germinants is sufficient to account for the deviation; (2) if the ratio obtained were due to the segregation of two or more genes, then the cross B312 $\times$ 30300 (Table 4) should give a minimum of 10% *arg*⁺ progeny; actually it gave 1.0–1.5% *arg*⁺.

Table 1. *Segregation ratios*

Mutant	No. of random isolates		
	<i>arg</i> ⁺	<i>arg</i> ⁻	Not germinated
B 312	106	72	30
H 4250	65	63	c. 20
46004	114	119	29
36703T	171	168	35
B 369	62	56	12
B 317	143	155	56
B 368	52	72	29
B 362	49	36	20
B 370	244	265	79

Pooled data from various crosses. *P* values for deviations from 1 *arg*⁺:1 *arg*⁻ are greater than 0.05, except for B312 where *P* = c. 0.01.

So far, all but two of the mutants appear to be point mutations. Mutant 36703T is associated with a translocation (Singleton, 1948), and shows linkage to groups I and V (Srb, cited in Singleton, 1948). There is also strong evidence that mutant H 4250 is associated with some kind of chromosomal aberration (Newmeyer, unpublished). There is no evidence that the arginine requirement of either strain is separable from the aberration. The initial stock of mutant B362 also behaved aberrantly, but this defect was evidently eliminated in the first generation of inbreeding; only apparently normal B362 stocks have been used for enzyme studies or for the crosses reported here.

Inbreeding. All nine mutants were repeatedly back-crossed to wild *Neurospora crassa*, so that any enzymic differences found could more validly be ascribed to the arginine loci under study, rather than to other differences between the stocks. The wild type *Neurospora crassa* used for this purpose was STA (also called ST-74A), which was kindly provided by Dr P. St Lawrence; this strain was also used as the wild type for enzyme experiments, and was the parent of the six mutants isolated by Dr V. W. Woodward. In each generation an *arg*⁻ *a* isolate was selected and crossed to STA; in the final generation of group I mutants, some *arg*⁻ *A* isolates were also chosen, in order to make more isogenic the short region between *arg* and *sex*. All group I mutants were back-crossed for four to seven generations, and all group VII mutants for two to four generations, before being used for enzyme studies.

Relation to other arginine mutants. It will be shown that the strict arginine mutants studied by Srb (1946) are both in group I; the group VII mutants

apparently represent a previously undescribed locus (or loci). The standard group VII mutant, B317, is not allelic with the unmapped arginine mutants 34105, 21502, 35401 and 44297 (kindly supplied by Mrs M. B. Mitchell). All crosses were highly fertile and produced many wild type progeny. These tests were made even though the unmapped mutants can use citrulline or other precursors, because Mitchell & Mitchell (1952) showed that the ability of arginine mutants to use precursors is subject to modification by other genes. For similar reasons, the group I mutant 46004 was tested for allelism with 27947 (ornithineless), which was known to be near centromere in either group I or group II (see Barrett, Newmeyer, Perkins & Garnjobst, 1954); they segregated independently.

Culture methods and markers

Most of the arginine mutants studied will not grow well on standard complete media unless extra arginine is added; ordinarily 0.5 mg. L-arginine HCl/ml. was added to medium No. 2 of Tatum, Barratt, Fries & Bonner (1950), or modifications thereof. For crosses carrying the marker *button* (see below), better germination was obtained on supplemented minimal medium. Crosses were made on 'synthetic crossing medium' (SC) of Westergaard & Mitchell (1947), usually containing 0.1 mg. L-arginine HCl/ml. Sorbose minimal medium (to induce colonial growth) was used as previously described (Newmeyer, 1954), except that it usually contained only 0.5% sorbose and 0.1% sucrose. The minimal medium and standard genetic methods are described by Beadle & Tatum (1945); special methods will be described in connexion with specific experiments.

Genetic nomenclature and references to previously described markers are given in Barratt *et al.* (1954). The new colonial marker, *button* (*bn*, isolation number B40), was kindly supplied by Dr D. D. Perkins, who located it in linkage group VII; it was initially isolated by Dr V. W. Woodward.

RESULTS

Separation of the mutants into two classes

Classification according to growth on argininosuccinate (the intermediate between citrulline and arginine) was not possible, because *Neurospora* is relatively impermeable to this compound (Newmeyer, unpublished), as is *Escherichia coli* (Walker & Myers, 1953). However, the nine mutants were readily separated into two classes by linkage, heterokaryon and fertility tests.

The first class, consisting of mutants 36703T, 46004, B312, B369 and H4250, shows linkage to *sex* (in group I), in agreement with Srb's data for mutant 36703T. Members of the second class (mutants B317, B362, B368, and B370) show no significant sex-linkage, but are all linked to markers in group VII (the latter class of mutants was first located by Dr D. D. Perkins). Linkage data for both classes are summarized in Table 2. Further evidence for location in group I or VII is given below under *Allelism tests* for all mutants except B362 and B368; these two were not studied in detail because of the possibility that they arose from the same mutation as B370.

Heterokaryon tests confirmed the above division of the mutants, and gave no evidence for further subdivision of either group. All possible combinations of either two group I mutants or two group VII mutants gave negligible growth on slopes of minimal medium, but the combination of mutant B317 (group VII) with any group I mutant gave a definite positive test, as did the combination of mutant B369 (group I) with any group VII mutant. In these tests it seems unlikely that the negative reactions were due to incompatible

Table 2. *Data locating arginine mutants in linkage groups I and VII*

	Arginine mutant	Group I markers			Group VII markers	
		<i>sex</i> *	36703T	46004	<i>bn</i>	<i>nt</i> †
Group I	36703T	76P:8R	—	—	—	—
	46004	232P:17R	—	—	—	—
	B369	22P:3R	—	—	—	—
	B312	39P:2R	—	—	—	—
		(5 not scored)				
	H4250	57P:10R	—	—	—	—
Group VII		(+ 9 bisexual)				
	B317	7P:111R	5PD:3T:3NPD (41 % recomb.)	36 <i>arg</i> ⁻ :17 <i>arg</i> ⁺ (64 % recomb.)	36P:16R	49P:3R
	B370	19P:14R	—	55 <i>arg</i> ⁻ :16 <i>arg</i> ⁺ (45 % recomb.)	43P:16R	67P:1R
	B368	21P:16R	—	—	46P:10R	—
	B362	8P:16R	—	—	21P:4R‡	—

P=Parental random isolates; R=recombinant random isolates; PD=parental ditype asci; T=tetrapype asci; NPD=non-parental ditype asci.

* Pooled data from various crosses. Data are not included if *sex* was scored only in a non-random sample of the isolates, in such a way as might introduce bias.

† Data of D. D. Perkins.

‡ Only 51 % germination. Germination in all other crosses was good.

combinations of heterokaryon-formation (*het*) genes, rather than to failure of the arginine mutants to complement each other. Garnjobst (1955) showed that, at least in the cases she studied, stable heterokaryons were formed only when the *het* genes of both strains were alike. On the assumption that all *het* genes involved here behave in this way, the isolates used for the present tests were all chosen from at least third generation backcrosses to wild type STA, to increase their chances of similarity, and a single isolate of each mutant was used for all tests. Since all mutants of one linkage group gave a positive test with the same isolate of the other group, it seems likely that they were all mutually compatible.

Failure to complement each other has been definitely demonstrated in the critical case of the group VII mutants B317 and B370, by isolation of the heterokaryon-compatible double mutants B370 *pan A* and B317 *nt A*. These were tested in growth tubes according to Ryan, Beadle & Tatum (1943). Neither strain alone grew in the absence of either of its required nutritional supplements; together they grew at wild type rate in each of three tests when only arginine was supplied, but failed to grow in each of three tests when only pantothenate and nicotinamide were present. The experiment was repeated on

slopes of solid media with similar results. Thus a clearly compatible combination of mutants B370 and B317 was unable to form an *arg*⁺ heterokaryon.

The classification of mutants described above is confirmed by fertility tests. Crosses between any two mutants of the same group have invariably been semi-sterile. On the best medium found (SC+0.1 mg. L-arginine HCl/ml.) they formed abundant black perithecia, but ascospore production was low and the vast majority of spores were white and did not germinate. On the other hand, each of the nine mutants was highly fertile when crossed to any other stock tested. Crosses of group I by group VII mutants were also highly fertile in the four combinations tested.

Allelism tests

From the heterokaryon tests it might be presumed that the members of each of the two groups of arginine mutants are alleles. The near-sterility in intra-group crosses would point to the same conclusion, since sterility in crosses between apparent alleles is a frequent occurrence (see, for example, Mitchell & Mitchell, 1956; Pritchard, 1955). However, it is probable that both these types of evidence are essentially indications of a biochemical similarity, and, in a study designed to test the one gene-one enzyme hypothesis, decisions on allelism must be based on purely genetic criteria.

Methods. The near-sterility of crosses between mutants of the same group creates a major obstacle to obtaining direct genetic evidence on allelism. Many attempts were made to overcome this problem, with some success. A major cause of the sterility is evidently that the high arginine concentration required for optimal growth (0.5–1.0 mg. L-arginine HCl/ml.) provides a higher concentration of available nitrogen than is compatible with the production of functional perithecia. The inhibition of perithecium formation by high nitrogen concentrations was described by Hirsch (1954). The best solution to this problem found so far is to make crosses in Petri plates on SC medium (Westergaard & Mitchell, 1947) containing a suboptimal concentration of arginine, and to add further arginine supplements after fertilization. In practice, 15 ml. of medium, usually containing 0.1 mg. arginine/ml., was used per plate. The cross was fertilized at 6–7 days, and six or more additions of sterile arginine solution (usually 0.15 ml. of a 10 mg./ml. solution) were made at a single point at the edge of the plate, usually at daily intervals, beginning 1 or 2 days after fertilization. Most of the ripe perithecia appeared in a zone $\frac{1}{2}$ –1 $\frac{1}{2}$ in. from the point of addition, and shot their spores on to the lid of the plate.

This procedure gave a great increase in ascospore production, but little or no improvement in the proportion of ripe spores. As many spores as possible were therefore scraped from the lid, freed from any mycelium growing there, heat-shocked, and plated in sorbose medium to screen for the few which were viable. Initial experiments were done by selective plating, i.e. spores were plated with and without arginine, and the % of *arg*⁺ mutants estimated from the numbers of colonies on the two types of media. To obtain more accurate values, some experiments were done by 'total isolation', i.e. all spores were plated with arginine, and all adequately separated colonies isolated and tested.

The same plates were sometimes used both for total isolation and for the estimated viable count on a selective plating.

Approximate information about allelism may also be obtained indirectly by mapping, to determine whether all mutants are in the same region. The results of both methods are given below.

Group I mutants. The direct-crossing method was less successful for group I mutants than for those in group VII. The sparse results for group I are given in Table 3; since no *arg*⁺ colonies were found, it is concluded that mutants 46004, H4250, B312, and B369 must at least be closely linked. Crosses B, C, D and F were analysed as described above. For cross A, the spores were instead heat-shocked on plates of complete medium without sorbose, and the few which germinated isolated and tested. For cross E, single ripe-looking spores were isolated directly. These two methods are too slow to be generally useful.

Table 3. *Crosses between group I arginine mutants*

Cross	Parents	No. of viable spores	No. of <i>arg</i> ⁺ spores	Method
A	46004 × H4250	176	0	Isolation of germinated ascospores
B	46004 × H4250	27*	0	Selective plating
C	46004 × 36703 T	31*	0	Selective plating
D	46004 × B312	452*	0	Selective plating
E	46004 × B312	38	0	Random isolates
F	46004 × B369	148*	0	Selective plating

* Estimated.

Because these mutants are located in a well-marked chromosome region, all further study of them was done by mapping. Various crosses of mutant 46004 to known markers showed that it is located between *sex* and *crisp*, and very close to *ad-5*. This location suggested close linkage to the marker *arg-3* (30300), which can use either citrulline or arginine. Ascospores from a cross of mutants 30300 × 46004, plated on sorbose medium, gave only one definite and one questionable *arg*⁺ colony out of approximately 665 viable spores. To eliminate spurious wild types arising by heterokaryon formation, ascospores from crosses of 30300 × 46004 and of 30300 by three other group I mutants were then plated on minimal agar and scored as *arg*⁺ or *arg*⁻ by the amount of growth produced shortly after germination, as described by Mitchell, Pittenger & Mitchell (1952). The rare apparent wild types, both in and out of the counted area, were isolated and retested, and those which proved to be really *arg*⁺ were scored for *sex* to obtain information on gene order. Of nineteen putative *arg*⁺ which were isolated and retested, two were actually *arg*⁻; therefore any *arg*⁺ which could not be isolated, because of closeness to other spores, were recorded as 'uncertain'. Results to date are summarized in Table 4. They indicate that all four arginine mutations are located within three map units of 30300, and probably between 30300 and *sex*. It is not yet known whether the lower *arg*⁺ frequency found for the 30300 × 46004 cross is valid. This cross is more difficult to score,

so that in this (but not in the other three crosses) occasional *arg*⁺ might go undetected. A cross of mutant 30300 by the fifth group I mutant, H4250, was not scorable by this method.

Table 4. *Crosses of 30300 (citrulline) × group I arginine mutants*

Ascospores classified by growth after germination on minimal agar plates.

Cross	No. of <i>arg</i> ⁺ spores in counted areas		Total germinated spores in counted area	% <i>arg</i> ⁺	Sex of certain <i>arg</i> ⁺ (not restricted to counted area)
	Certain	Uncertain			
30300a × 46004A	2	1*	c. 4300	0.05–0.07	2a, 0A
30300a × B312A†	4	2†	409	1.0–1.5	4a, 1A
30300a × B369A‡	3	5*	611	0.5–1.3	7a, 1A
30300a × 36703T A	2	1*	468	0.4–0.6	2a, 0A

* Could not be isolated and retested.

† Possibly due to heterokaryon formation.

‡ 5 certain *arg*⁺ from these crosses were crossed to wild type and found to breed true.

Group VII mutants. The first crosses between mutants in this group gave an unexpected result: many *arg*⁺ progeny were obtained from each combination tested; namely, B370 × B362, B370 × B368, and B370 × B317 (Table 6, first three lines). On the face of it, this would mean that the enzyme argininosuccinase was controlled by widely separated loci. However, in view of the data in Table 2 (which indicate rather close linkage), and also of the possible single origin of strains B362, B368 and B370, this explanation seemed so unlikely that it was suspected that the arginine-independent colonies arose from some cause other than crossing over, e.g. reversion or contamination, or that they were really only pseudo-wild types, as described by Mitchell *et al.* (1952).

Because of the great labour required to produce enough ripe spores to settle this question, further *arg*[−] × *arg*[−] crosses were confined to the single combination of strains B370 × B317. At the same time, careful mapping of each of these mutants was begun; the results showed clearly that B370 and B317 were at most a few units apart, and might well be alleles. The results of the *arg*[−] × *arg*[−] crosses showed almost equally clearly that the *arg*⁺ progeny were nevertheless true recombinants; their high frequency apparently is due to selective ripening of ascospores. The two mutants must therefore be either pseudoallelic or closely linked. The evidence is as follows.

The mapping consisted of a series of three-point crosses, each involving an arginine mutant plus the two closest markers, *bn* and *nt*. The results are given in Table 5. It is clear that for both arginine mutants the order is *bn* -- *arg* -- *nt*. In one cross the distance *arg* (B316) -- *nt* is somewhat greater than the average value for *arg* (B370) -- *nt*, but it is questionable whether this difference is real, considering the wide variation between crosses. Similar variations between crosses in different genetic backgrounds have been noted in *Neurospora* by Stadler (1956), and by F. de Serres, R. W. Barratt, and others (personal com-

munications). In any case B370 and B317 are most probably between 0 and 6 units apart.

Table 5. *Group VII three-point crosses*

Numbers of random isolates from crosses involving the markers button (B40), arginine (either B317 or B370), and nicotinic acid + tryptophan (65001).

Parent genotypes	Parental combinations	Recombinations		
		Singles region 1 (<i>bn-arg</i>)	Singles, region 2 (<i>arg-nt</i>)	Doubles, regions 1 and 2
$\frac{+ arg nt}{bn ++}$ (<i>arg</i> = B370)	43 + - -	7 + + +	2 + - +	0 + + -
	36 - + +	4 - - -	0 - + -	0 - - +
	79	11 (12.0 %)	2 (2.2 %)	
$\frac{+ arg nt}{bn ++}$ (<i>arg</i> = B317)	38 + - -	5 + + +	1 + - +	0 + + -
	37 - + +	6 - - -	0 - + -	0 - - +
	75	11 (12.6 %)	1 (1.2 %)	
$\frac{+ + +}{bn arg nt}$ (<i>arg</i> = B370)	79 + + +	22 + - -	1 + + -	1 + - +
	60 - - -	14 - + +	3 - - +	0 - + -
	139	36 (20.0 %)	4 (2.2 %)	1 (0.6 %)
$\frac{+ arg +}{bn + nt}$ (<i>arg</i> = B370)	36 + - +	15 + + -	0 + - -	0 + + +
	29 - + -	9 - - +	2 - + +	0 - - -
	65	24 (26.4 %)	2 (2.2 %)	
$\frac{+ arg +}{bn + nt}$ (<i>arg</i> = B317)	31 + - +	8 + + -	4 + - -	0 + + +
	31 - + -	11 - - +	3 - + +	0 - - -
	63	19 (21.6 %)	7 (8.0 %)	

Results of the *arg*⁻ × *arg*⁻ crosses are given in Tables 6 and 7. All crosses involving two different arginine mutants, i.e. B370 × B317, will be called intercrossoes (IC), and all crosses homozygous for one arginine mutant, e.g. B370 × B370, will be called selfings (S), regardless of the distribution of the marker *nt*. The results make highly improbable all obvious origins of the *arg*⁺ progeny other than crossing over.

A high rate of reversion (or suppressor mutation) is eliminated by the absence of *arg*⁺ progeny in the selfings. Selfings appear to be even less fertile than intercrossoes; nevertheless, the few data it has been possible to obtain are sufficient to eliminate sampling error. *P* for homogeneity of either selfing with pooled intercrossoes, by χ^2 test on the 'total isolation' figures, is < 0.001. All isolates from the selfings were tested twice to insure that the rare *arg*⁺ was not overlooked. It was also shown, by plating IC7 at different spore concentrations, that the absence of *arg*⁺ in the selfings was not due to any effect of population density on their survival. Tests for reversion during vegetative growth were also negative.

The results might still be explained by rare reversion, which by chance affected only the cultures used for the intercrossoes, followed by selection for the *arg*⁺ progeny. This explanation seems unlikely because several different isolates

Table 6. *Crosses between group VII arginine mutants*

Cross number	Parents	Total isolation				Selective plating		
		Total colonies	Total isolated	No. of <i>arg</i> ⁺ isolated	% <i>arg</i> ⁺ among those isolated	Estimated total viable	No. of <i>arg</i> ⁺	<i>arg</i> ⁺ isolate (c. %)
IC1	B370 × B362	—	—	—	—	400	37	9
IC2	B370 × B368	—	—	—	—	490	11	2
IC3	B370 × B317	—	—	—	—	196	25	13
IC4	B370 × B317	—	—	—	—	102	17	17
IC5	B317 × B370 <i>nt</i>	6	4	1	25.0	21	7	33
IC6	B370 × B317	497	212	93	43.8	—	—	—
IC7	B317 × B370 <i>nt</i>	62	41	17	41.5	—	—	—
IC8	B370 × B317 <i>nt</i>	29	27	17	63.0	29	11	38
IC9	B370 × B317 <i>nt</i>	10	10	4	40.0	8	6	75
S1	B370 × B370	9	9	0	0	—	—	—
S2	B370 × B370 <i>nt</i>	44	38	0	0	—	—	—
S3	B317 × B317 <i>nt</i>	36	32	0	0	18	0	0
S4	B317 <i>nt</i> × B317 <i>nt</i>	6	6	0	0	—	—	—

Selective plating gives only a rough estimate of the % *arg*⁺ isolates when the number of viable spores is so small, particularly since the viable count must be based on very few plates. Total isolation is not subject to these errors.

In IC7, 8 and 9, a dark ascospore could be seen in 52 of the 78 colonies isolated; 50.0 % of those with visible dark spores were *arg*⁺, compared to 48.7 % of the total.

For crosses carrying *nt* (65001), excess nicotinamide was added to all plates.

All spores were obtained by the plate-crossing method. The yield of viable spores per cross-plate varied from 5 to 22 for selfings and from 14 to 124 for intercrosses made under comparable conditions.

were used, and because both isolates used as parents for IC7 were also used as parents for S2 and S3.

Contamination during plating of ascospores is excluded because most colonies could be seen microscopically to have arisen from a dark ascospore. For IC7, 8, and 9, the numbers of colonies in which the spore could be seen were counted separately (Table 6); they yielded approximately the same *arg*⁺ frequency as did the total data.

Contamination during ascospore formation seems very unlikely, because of the high reproducibility of the results, and because intercrosses heterozygous for *nt* always gave some *arg*⁺*nt*[−] progeny (Table 7).

Tests for pseudo-wild types were carried out on eleven representative *arg*⁺ isolates from the B370 × B317 crosses, including two *arg*⁺*nt*[−] from colonies with visible dark ascospores. These were crossed to standard wild types and usually tested by isolating 50 spores at random, since the group VII arginine mutants are hard to score on minimal agar plates. No *arg*[−] progeny were found in any case. Therefore the 11 *arg*⁺ tested are not pseudo-wild types, and on statistical grounds it is likely that the same holds for most of those not tested.

Heterokaryon formation is eliminated by the double-mutant heterokaryon tests described earlier, and also by the pseudo-wild type tests. Non-genetic adaptation is excluded by the absence of *arg*⁺ progeny in the selfings, and by the pseudo-wild type tests.

Thus all obvious origins of the *arg*⁺ progeny other than recombination are virtually eliminated. Recombination is also indicated by the data on the segregation of *nt* (Table 7). However, the true recombination frequency can not be reflected by the frequency of the *arg*⁺ progeny, since this would require an unprecedented 90% recombination, which is in any case excluded by the three-point cross data. The only simple explanation would seem to be selection.

Table 7. Segregation of *nt* in group VII intercrossees

All adequately separated *arg*⁺ colonies from selective platings were isolated, and their results added to those from the total isolations; the results obtained by the two methods were homogeneous in all cases. The *arg*⁻ classes are included to show that the skewing in the *arg*⁺ classes is not due to selection for or against *nt*⁻.

	B370 × B317 <i>nt</i>			B370 <i>nt</i> × B317		
	IC8*	IC9*	Total	IC7*	IC5†	Total
<i>arg</i> ⁺ <i>nt</i> ⁺	20	4	24	3	2	5
<i>arg</i> ⁺ <i>nt</i> ⁻	4	6	10	14	6	20
<i>arg</i> ⁻ <i>nt</i> ⁺	4	2	6	16	0	16
<i>arg</i> ⁻ <i>nt</i> ⁻	6	4	10	8	3	11

* *nt*⁻ was carried by the fertilizing parent.

† *nt*⁻ was carried by the protoperithecial parent.

Indeed, there is reason to believe that the near-sterility of these crosses is largely due to a deficiency of arginine inside the perithecia. If, because of this deficit, only *arg*⁺ spores and their closest *arg*⁻ neighbours were able to ripen, there might be intense selection for *arg*⁺. It is hard to make an accurate estimate of ascospore viability because the unripe spores are nearly transparent; a very rough count for the cross IC6 indicated that only about 0.067% of the shot ascospores survived. If all the non-viable spores were *arg*⁻, the true *arg*⁺ frequency would be only 0.023%; i.e. within the range for crossing over between pseudoalleles.

Since the *arg*⁺ isolates evidently do arise by recombination, it is of interest to know whether the process results in restoration of the wild-type enzyme, and whether any trace of the arginine requirement remains. Drs Fincham & Boylen (personal communication) have tested six representative *arg*⁺ isolates from the B370 × B317 crosses, including two *arg*⁺*nt*⁻ which had been tested for pseudo-wild types. All of them had argininosuccinase activity like that of the standard wild type. Also, three *arg*⁺*nt*⁺ isolates were tested in flask cultures. Dry-weight determinations showed that arginine was not significantly more stimulatory for these isolates than for the standard wild-type controls.

DISCUSSION

The sterility problem has prevented localization of either group of mutants to an interval of less than a few map units. For the group VII mutants it obviously is not feasible to go further until better markers or a better crossing method become available; indeed, the analysis would never have been continued this far, had it not been necessary in order to give meaning to the

enzymic studies. The group I mutants, on the other hand, warrant further study, since an abundance of close markers partly compensates for the sterility of intra-group crosses.

Despite the technical difficulties, this investigation has been fruitful in two respects. First, the group VII mutants provide a new case in which major control of a biosynthetic enzyme is apparently restricted to a small chromosome region, and it seems likely that the group I mutants will add still another case when enzymic tests are completed. Previous work on biosynthetic enzymes suggests that, although changes at various loci can cause minor changes in activity of the same enzyme (Hogness & Mitchell, 1954; Yanofsky & Bonner, 1955), there is only one locus at which mutation can cause a deficiency so severe that it results in a nutrient requirement (tryptophan synthetase: Yanofsky & Bonner, 1955; and Weijer, 1954; glutamic dehydrogenase: Fincham, 1950, 1954). In the case of catabolic enzymes on the other hand, it is clear that severe enzymic deficiencies can be caused by mutations at numerous widely separated loci (lactase: Lederberg, 1951; O. Landman, cited in Bonner, 1951; tyrosinase: Markert, 1950). It appears that many of these loci act by controlling the production of other proteins needed for the induction of the enzyme itself (Monod, 1956; Landman, cited in Bonner, 1951), and it is perhaps reasonable that catabolic enzymes should be more subject than biosynthetic ones to such multi-gene control. The question remains whether the distinction between the two types of enzyme has any validity, or whether it merely results from the very small number of biosynthetic enzymes for which more than one mutant has been tested. The present two cases support the idea that the distinction is real, even though neither group of arginine mutants is localized to as small a chromosome region as would be desirable.

This investigation also provides a second case, in addition to tryptophan synthetase, in which mutants which cause the loss of the same biosynthetic enzyme are apparently separable by infrequent crossing over. However, the recent work of Lindegren (1955) and Mitchell (1955) raises the possibility that the apparent recombinants are really due to 'gene conversion'. The data in Table 7 indicate an excess of apparent double cross-overs like that found in several studies of complex loci (e.g. Pritchard, 1955; Mitchell, 1955; St Lawrence, 1956; Giles, 1956). In Mitchell's case (but not in Pritchard's) these proved to be due to conversion rather than recombination. With present methods it seems impossible to eliminate conversion in the case of either argininosuccinase or tryptophan synthetase. Until it is eliminated in a case which can be studied enzymically, direct evidence that pseudoalleles can govern the same enzyme will be lacking.

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