SUPPRESSION OF ARGININE AND PYRIMIDINE-REQUIRING MUTANTS OF *NEUROSPORA CRASSA*[1]

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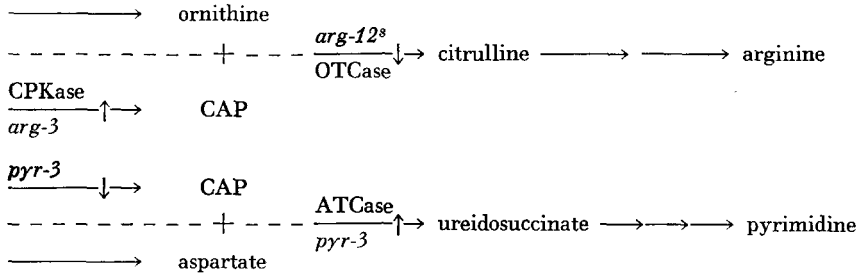
THE phenomenon of genetic suppression, whereby the effect of one mutation is circumvented by a second mutation, has been investigated in a number of organisms (YANOFSKY and ST. LAWRENCE 1960). Rather diverse mechanisms appear to be involved in suppression. Some apparently act at the level of protein synthesis, as in the system described by YANOFSKY, HELINSKI and MALING (1961). There the hypothesis was advanced that suppressor genes act by altering the amino acid-activating enzymes or the composition of the sRNA's, thus affecting their ability to combine with specific activating enzymes. The "nonsense to sense" theories (CRICK, BARNETT, BRENNER and WATTS-TOBIN 1961; GAREN and SIDDIQI 1962; BENZER and CHAMPE 1962) account for suppression by modifications in the DNA reading frame. More recently a case of suppression has been reported which is explicable in terms of changes in the code at the ribosomal level during translation from messenger to protein (GORINI and KATAJA 1964).

A number of cases of suppression, reported in Neurospora, apparently act indirectly, i.e., at the level of metabolic cross feeding. Shunting of a genetic block via an alternate pathway has been reported in the case of the acetate mutants (LEIN and LEIN 1952; STRAUSS and PIEROG 1954). Suppression resulting from relief of inhibition has also been reported (DOUDNEY and WAGNER 1955; SUSKIND and KUREK 1959).

The nature of the individual metabolic pathways of arginine and pyrimidine biosynthesis in Neurospora, and their interrelatedness, has been studied, in part, by means of suppression. For simplification the pathways as presently visualized are as follows:



HOULAHAN and MITCHELL (1947) first reported that the unlinked suppressor gene *s* (now *arg-12*^{s}) was capable of rendering certain *pyr-3* mutants independ-

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ent of their pyrimidine requirement. From present evidence the mechanism of suppression appears to revolve around the fact that these two metabolic pathways share a common substrate, carbamyl phosphate (CAP). The hypothesis has been advanced that there are two separate sources of CAP (DAVIS 1962; DAVIS and WOODWARD 1962; WOODWARD and DAVIS 1963); one is specific for arginine biosynthesis and the other for pyrimidine biosynthesis. Specificity is not absolute; however, CAP from one source may be used in the opposite pathway, and *vice versa*, under certain conditions. Thus, a block imposed by mutation at either of the transferase enzyme sites [ornithine transcarbamylase (OTCase) or aspartate transcarbamylase (ATCase)] may cause an accumulation of CAP in the cytoplasm. When in excess, the CAP from one source can "spill over" and be used in the other pathway. Mutations resulting in a block prior to CAP synthesis in one pathway are therefore capable of being suppressed by mutants blocked at the transferase step in the other pathway. This explanation is consistent with the data on the suppression of certain *pyr-3* mutants by *arg-12*^{s}, extracts of which are known to have only 2 to 3% as much OTCase activity as extracts of wild type (DAVIS 1961, 1962). It has been further established that only those *pyr-3* mutants possessing ATCase activity are capable of being suppressed by *arg-12*^{s} (DAVIS 1961; DAVIS and WOODWARD 1962). Reciprocally, DAVIS (1965) has shown that *pyr-3d*, which lacks functional ATCase, can suppress *arg-3*. The latter is blocked prior to CAP at the carbamyl-phosphokinase (CPKase) step (DAVIS 1963). REISSIG (1963) has shown also that certain ATC^{-} mutants suppress *arg-2*. Further evidence for reciprocal suppression will be presented here.

This paper describes four new suppressors of *pyr-3* mutants, whose mechanism of suppression is not mediated by a defective OTCase. These mutants represent at least two new distinct genetic loci which are in some way concerned with arginine biosynthesis. A preliminary report on this work has appeared (WOODWARD and MCDOUGALL 1964).

MATERIALS AND METHODS

Strains: The *pyr-3* mutants used in this study were derived from wild types 73a and 74A and have been described (SUYAMA, MUNKRES, and WOODWARD 1959; WOODWARD 1962). The arginine mutants used, *arg-3* (30300), *arg-4* (21502), *arg-5* (27947), *arg-6* (29997) and *arg-7* (34105) were from laboratory stocks, and are available from the Fungal Genetics Stock Center, Dartmouth College, Hanover, New Hampshire.

Media: VOGEL's Medium N was used for all cultures except for the crossing medium which included both cornmeal agar and WESTERGAARD and MITCHELL's medium.

Growth conditions: Growth experiments were carried out in both shaking and stationary flasks at 30°C. The mycelia were harvested and dried at 100°C for 24 hours, after which dry weights were taken. Qualitative growth responses of the isolates were made on various types of medium in the usual manner by inoculating conidia into liquid medium in culture tubes and scoring visually.

OTC assays: For the enzyme assays, mycelia were harvested after 48 hr growth in liquid medium aerated by shaking at a temperature of 30°C, washed, and freeze dried. Dried preparations were powdered in a Wiley mill, extracted 30 min in 0.05 M Tris buffer at pH 8.5 and the cell debris removed by centrifugation at 6000 rpm for 1 hour. The nucleoproteins were removed by precipitating with streptomycin sulfate (1/5 volume of 5% solution) (JONES 1962) followed

by centrifugation for 15 min at 6000 rpm. The resultant supernatant was used as crude extract for enzyme assays. The quantitative test used for OTCase activity has been described by DAVIS and WOODWARD (1962).

Isolation of suppressors: Suppressors of *pyr-3* ATC+ strains were obtained by irradiating conidia of KS20 (an ATC+ mutant) with ultraviolet light at the LD_{50}, and plating the conidia on minimal agar supplemented with 0.75% sorbose. Colonies from these plates were isolated on agar slants supplemented with arginine and uridine. Such isolates were tested for reversion *vs.* suppression by inoculating conidia into liquid minimal medium supplemented with arginine. (*pyr-3 arg-12*^{s} double mutants require pyrimidine for growth when arginine is added to the medium. See DAVIS and WOODWARD 1962.) Strains suspected of being suppressed *pyr-3* mutants were crossed to wild type as a check on the nutritional criterion.

RESULTS

Table 1 gives the number and types of tetrads obtained after crossing four arginine-sensitive isolates (suspected suppressed *pyr-3* mutants) to wild type 73a. The presence of three tetrads, from two of the crosses, showing 4 wild-type and 4 pyrimidine-requiring ascospores was not surprising, since the original isolates were very probably heterokaryotic. (Were it not for the other asci from the same cross, these three tetrads could possibly be nonparental ditypes of a *pyr-3, su* × wild-type cross, where the suppressor is not expressed in wild-type background.) The other tetrads seem straightforward.

An arginine-stimulated, single-spore isolate was taken from one of the asci showing 1:1:1:1 segregation from each cross. These isolates are the suppressor mutants described in the remainder of this paper and were designated RU1, RU3, RU12, and RU20, respectively. The arginine auxotrophs recovered from these double mutants are expected to be "leaky" since the method of selection from minimal medium would preclude the recovery of mutants with absolute requirements.

Genetic studies of the suppressor mutants: Each of the four suppressor mutants was crossed to marker stocks representing mutations on all seven of the linkage groups in Neurospora. Only two linkage groups are involved, i.e., linkage group I and V (Tables 2 and 3). RU3, 12 and 20 are located on the right arm of linkage group I, distal to *nic-1*, and RU1 is located on the right arm of linkage group V, between *inos* and *hist-1*. Since RU3, 12 and 20 are inter-sterile, it is impossible to

TABLE 1

Tetrad analysis of crosses between four suppressed pyr-3 *mutants and wild type 73a*

Cross	Kinds of tetrads and number of asci of each kind*			
	4++:4+b	4a+:4+b	4++:ab	2++:2a+:2+b:2ab
73a × RU1†	0	1	2	10
73a × RU3	1	3	5	14
73a × RU12	0	3	0	11
73a × RU20	2	1	4	6

* a = arginine phenotype; b = pyrimidine phenotype.
† RU is the prefix used for each isolation number. In this table, RU1, 3, 12 and 20 refer to the double mutants from which the arginine auxotrophs were isolated.

TABLE 2

Crosses between the pyr-3 *suppressor mutants RU3, RU12 and RU20 and linkage group I markers* nic-1 *and* al-1

Cross Reg. I	Reg. II	Recombination in progeny: Parental		Region I		Region II		Regions I and II		Germination
+	*nic-1* +	122	93	42	40	7	10	1	1	90.3%
RU3	+ *al-1*									
	26.6 6.0									
+	*nic-1* +	148	126	65	62	9	6	6	2	72.1%
RU12	+ *al-1*									
	31.8 5.4									
+	*nic-1* +	89	67	30	45	3	7	2	4	77.7%
RU20	+ *al-1*									
	32.8 6.5									

Recombination values (percent) are shown for each region under the cross genotype.

determine whether they are allelic. RU1 shows recombination with *arg-4*, *arg-7* and *arg-8*, all of which are located on the right arm of linkage group V. (Of 3,500 progeny analyzed from an *arg-4* × *arg-7* cross, no prototrophic progeny were recovered; these data support the conclusion that *arg-4* and *arg-7* are allelic (R. W. BARRATT, personal communication). It is also clear that these new suppressors are genetically distinct from *arg-12*^{s}, located on linkage group II (WOODWARD and SCHWARZ 1964).

Nutritional phenotypes of the RU-suppressor mutants: Table 4 shows the results of culturing the suppressor mutants in various of the intermediates of arginine and proline biosynthesis. The RU1 suppressor differs from the others in that it responds to glutamate almost to the same extent as it does to ornithine and arginine. Citrulline, for reasons unknown, is a poor provider for RU1. RU3, RU12 and RU20 respond to citrulline and arginine, and to a lesser extent to ornithine. Nutritionally these mutants appear similar, a fact that corroborates the genetic data. That all of the mutants are leaky was not surprising since the

TABLE 3

Crosses between the pyr-3 *suppressor, RU1, and various markers located on linkage group V*

Cross a × b	Isolation No. of b	Progeny: Parentals a+	+b	Recombinants ++	ab	Percent recombination	Percent germination
RU1 × *hist-1*	C91	81	72	6	10	9.5	90.4
RU1 × inos	37401	55	68	6	16	22.1	85.3
RU1 × *arg-4*	21502	428*		7	. .	3.3	85.8
RU1 × *arg-7*	34105	631*		9	. .	2.8	90.5
RU1 × *arg-8*	44207	155*		13	. .	14.4	81.2

* Nutritional tests do not readily permit an unambigous distinction between parental types, or between parentals and double mutants. The percent recombination is therefore only an estimate derived by doubling the number of wild-type recombinants and dividing that number by the total number of ascospores tested.

TABLE 4

Dry weights, in mg, of each suppressor mutant grown in 40 ml liquid medium supplemented with each amino acid at a concentration of 0.2 mg/ml of liquid. Mycelia were harvested after 24 and 48 hours growth from 125 ml flasks in stationary culture

Isolate	Time (hr)	Mininial	Glutamic acid	Proline	Ornithine	Citrulline	Arginine
RU1	24	0.2	3.6	1.7	2.6	0.6	1.0
	48	1.0	17.5	8.4	21.6	5.7	17.7
RU3	24	0.0	tr*	tr	0.8	3.6	3.3
	48	tr	0.3	0.4	18.9	46.0	45.2
RU12	24	tr	tr	tr	0.5	2.5	2.9
	48	0.3	0.2	0.1	9.9	47.1	37.0
RU20	24	tr	tr	tr	0.9	3.6	2.7
	48	2.9	3.7	3.4	16.7	49.1	38.2

* tr means trace amounts of growth too small to be weighed.

method used to isolate the mutants selects against completely blocked mutants; however, the leakiness of the RU-suppressors does not prevent their being distinguished from wild type in growth tests, which is a marked contrast to *arg-12*^{s}.

OTCase levels and the suppression of pyr-3 *mutants:* Extracts of the *arg-12*^{s} mutant are known to exhibit only 2 to 3% as much OTCase activity as extracts of wild-type Neurospora (DAVIS 1961, 1962) and this, coupled with derepression of CPKase, is thought to suppress *pyr-3* by providing excess endogenous CAP. It was therefore necessary to check the level of OTCase in the new suppressors. The OTCase activity of all four of the new suppressors is indistinguishable from that of wild type (less than 5% deviation of specific activity).

By genetic and enzymatic criteria the new suppressors have been shown to be distinct from *arg-12*^{s}. Like *arg-12*^{s}, however, the RU-suppressors will suppress only those *pyr-3* mutants with ATCase activity. The evidence for this is the fact that in combination with seven different *pyr-3* ATC$^+$ mutants suppression was observed, but with three *pyr-3* ATC$^-$ mutants suppression was not observed. In every instance, the presence of the suppressed mutant (*pyr-3* ATC$^+$) was verified by backcrossing the double mutant to wild type and isolating both single mutants.

Growth response of suppressed pyr-3 *mutants:* Table 5 illustrates the suppression of one particular *pyr-3* ATC$^+$ mutant, KS10, by each of the RU-suppressors. In addition to suppression, two other points are clear from the data: all four of the RU-suppressor mutants when in combination with a *pyr-3* ATC$^+$ mutant are sensitive to arginine, and ornithine promotes the growth of all double mutants with the exception of RU1 KS10. Citrulline inhibits growth of the double mutant to the same extent as arginine, and (Figure 1) sensitivity to arginine is overcome with time.

Suppression of arg-3 *by* pyr-3*:* While the synthesis of CAP and its subsequent utilization in arginine biosynthesis is controlled by at least two distinct genetic loci, *arg-3* and *arg-12* respectively, present evidence indicates that the two analogous steps in the pyrimidine pathway are under the control of only one locus

TABLE 5

Representative dry weights (in mg) from 125 ml stationary flask cultures at 30°C containing 40 ml of medium; supplements were used at a concentration of 0.2 mg/ml. The data illustrate suppression of a pyr-3 *mutant by each of the RU-suppressors*

Double mutant	Time (days)	Medium				
		Minimal	Arginine	Ornithine	Uridine	Arginine+Uridine
RU1 KS10	4	15.2	0.0	0.0	..	53.5
	8	45.9	7.8	2.6	..	77.4
RU3 KS10	4	6.7	0.0	31.0	..	73.5
	8	20.5	5.1	60.1	..	79.2
RU12 KS10	4	7.0	0.2	29.1	..	81.2
	8	21.6	8.5	60.6	..	74.8
RU20 KS10	4	10.2	0.0	38.5	..	80.0
	8	21.2	0.0	52.4	..	102.1
KS10	4	0	0	0	71.8	77.4
	8	0	0	0	89.6	103.3

(*pyr-3*) (DAVIS and WOODWARD 1962). Complementation studies have produced results in support of the bifunctional nature of the *pyr-3* locus (WOODWARD 1962; DAVIS and WOODWARD 1962). There are two complementation groups: one consists of the ATC$^+$ mutants (thought to be deficient at the CAP synthesizing site), and the second consists of ATC$^-$ mutants believed to possess a competent CAP synthesizing site on the ATC protein. A third group of mutants, also ATC$^-$, fails to complement with either of the first two groups. The ATC protein in these mutants is postulated to be defective at both sites.

The *arg-3* mutants possess defective CPKase (DAVIS 1963), the enzyme that

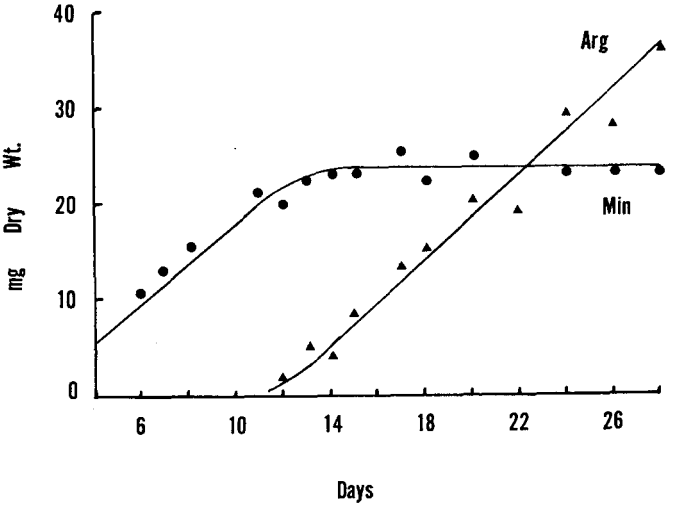

FIGURE 1.—Growth response of the double mutant RU3 KS-20 to minimal and arginine-supplemented medium at 30°C. The concentration of arginine was 0.2 mg/ml.

TABLE 6

Evidence for the suppression of arg-3 *by the* pyr-3 *mutant KS43*

Cross	Total number of asci	No. asci with double mutants	Phenotype of double mutant
Group I ATC+ Complementing			
KS16 × *arg-3*	22	16	arg, pyr
KS20 × *arg-3*	13	7	arg, pyr
Group II ATC- Complementing			
KS43 × *arg-3**	19	13	pyr
Group III ATC- Noncomplementing			
KS6 × *arg-3*	13	9	arg, pyr
KS36 × *arg-3*	24	12	arg, pyr
KS139 × *arg-3*	10	6	arg, pyr

* Backcrossing the double mutant *arg-3* KS43 to wild type and isolating both single mutants from the progeny furnished proof of the presence of the *arg-3* allele in the double mutant.

catalyzes the synthesis of CAP in the arginine pathway. These mutants are suppressed by *pyr-3* mutants described above as possessing ATC protein with a competent CAP synthesizing site and an incompetent transferase site. Other *pyr-3* mutants fail to suppress *arg-3* (Table 6).

Suppression of pyr-3 *mutants by* arg-7*:* Prior to the elucidation of the fine structure of the *pyr-3* locus and the enzymatic characterization of the various *pyr-3* mutants with respect to the presence or absence of ATC, MITCHELL and MITCHELL (1952) observed that *arg-7* suppressed *pyr-3a*. They showed also that to a lesser extent *arg-5* and *arg-6*, if supplied with ornithine or arginine, suppressed *pyr-3a*. These results have been confirmed, but again only ATC+ mutants are suppressible. Further, *arg-4* also suppresses *pyr-3* ATC+ mutants to the same extent as *arg-7*, a fact that supports the genetic evidence for allelism with *arg-7*.

This observation implicates another mechanism for the suppression of *pyr-3* mutants. It should be noted in this instance of suppression, as with the RU-suppressors of *pyr-3*, that neither of the transferase enzymes (ATCase or OTCase) is affected.

DISCUSSION

The data presented in this paper complicate, but do not negate the hypothesis offered above to explain genetic suppression in the arginine and pyrimidine biosynthetic pathways of Neurospora. According to the hypothesis there are two separate sources of CAP, each destined for one of the two (arginine or pyrimidine) pathways. Suppression of the *pyr-3* mutants by *arg-12*^{s} and reciprocally, *arg-3* by KS43, is explained on the basis of metabolic cross feeding. The fact that reciprocal suppression occurs lends credence to the bifunctional nature of the

pyr-3 locus. REISSIG (1963) has found that *arg-2* is also suppressible by *pyr-3* ATC$^-$ mutants. At present the position of *arg-2* in the biochemical pathway is unknown, but it is believed to be involved in the synthesis of arginine-specific CAP. Theoretically, excess CAP results from a reduction or loss of transcarbamylase activity following mutation. In the above instances of reciprocal suppression, one transcarbamylase enzyme is defective or absent while its counterpart is functional.

The RU-suppressors described here are stimulated by one or more precursors of arginine. Glutamate, ornithine and arginine stimulate RU1 about equally well, while RU3, RU12 and RU20 respond best to citrulline and arginine. A bit in contrast to expectations based on the nutritional data, all of the RU-suppressors exhibit wild-type OTC activity. These data argue against suppression resulting from CAP accumulation as a result of defective OTCase. They do not, however, prove that CAP from the arginine pathway is unavailable to the pyrimidine pathway.

Conceivably a reduction of ornithine in the cell could have the same effect on *pyr-3* mutants as an accumulation of CAP. In the case of the RU-suppressors, arginine-specific CAP could accumulate as the result of a genetic block which reduces the concentration of endogenous ornithine, thus making CAP available to the pyrimidine pathway. Suppression of *pyr-3* by *arg-7* may also be interpreted in this manner. CAP could also be made available to the pyrimidine pathway by derepression of CPKase following a block in the arginine pathway.

Although the point of metabolic block of the new suppressors has not been ascertained, some preliminary evidence indicates that at least one of the suppressors (RU3) may possess reduced amounts of ornithine in the mycelia. If suppression is promoted by a paucity of ornithine, it might be expected that the addition of ornithine would relieve suppression. This is the case with the double mutant RU1 *pyr-3*. Also, the MITCHELLS (1952) reported that *pyr-3a arg-7* grew slower on ornithine supplemented medium than it did on minimal. In contrast, double mutants consisting of a *pyr-3* ATC$^+$ mutant and either RU3, RU12 or RU20 are stimulated by exogenous ornithine, an observation that further complicates the rationalization that cross-feeding is the mechanism for suppression in these strains. If such is the case, exogenous ornithine apparently does not "use up" the CAP made available by the block in ornithine synthesis, nor is arginine produced rapidly enough from ornithine to repress CAP formation.

VOGEL (1955) has suggested that it is likely that some mutants express themselves through a disruption of enzyme organization rather than by alteration of enzymes relevant to a particular pathway. This possibility may exist here, if channeling of arginine intermediates occurs (VOGEL and KOPAC 1959). The concept of metabolic channeling affords an explanation for suppression if it can be envisioned that ornithine is preferentially shunted to pathways other than arginine as a result of mutation.

Addendum: Additional evidence supporting allelism between *arg-4* and *arg-7* was recently obtained by VOGEL and VOGEL (1965) who have found both mutants to be deficient for the enzyme ornithine-glutamate transacetylase.

SUMMARY

In a search for alleles of the *pyr-3* suppressor, *arg-12*^{s}, four new suppressor mutants have been isolated. These mutants represent at least two previously undescribed loci. Three of the mutants are located on linkage group I distal to *nic-1*; since they are inter-sterile it is impossible to determine if they represent one or more loci. The fourth suppressor maps on linkage group V near *arg-7*. Extracts of these mutants show ornithine transcarbamylase activity indistinguishable from that of wild type, a fact which also distinguishes them from *arg-12*^{s}. The precise metabolic position and mode of suppression of these mutants is unknown; however, a possible explanation for suppression is advanced.

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