A MUTATION REDUCING FEEDBACK REGULATION BY ARGININE IN SUPPRESSED *pyr-3* MUTANTS IN NEUROSPORA[1,2]

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DOUBLE mutants in Neurospora carrying a suppressible *pyr-3* mutation and the suppressor, *arg-12*^{s}, will grow on unsupplemented medium, but unlike wild type, display a profound sensitivity to the normal metabolite, L-arginine. Asexual spores, or conidia, of such double mutants will not grow when placed on minimal agar medium supplemented with as little as 0.5 μg/ml arginine (DAVIS 1961). If the pyrimidine riboside, uridine, is added to the medium along with arginine, normal growth takes place (HOULAHAN and MITCHELL 1947). Arginine has the effect of creating a pyrimidine requirement, but can also be thought of as negating the suppressor action.

Of particular concern in these studies are metabolic relationships shown in Figure 1 which were suggested principally by DAVIS (1962a,b). These include: (a) the duality of carbamyl phosphate (CAP) synthesis in wild type, i.e., a specific source for both arginine and pyrimidine biosynthesis, (b) a block in the synthesis of CAPpyr, here imposed by mutant KS-20, and (c) the less active form of ornithine transcarbamylase (OTCase) and the 80 to 90% reduction in mycelial arginine levels associated with *arg-12*^{s}. DAVIS hypothesized that CAParg accumu-

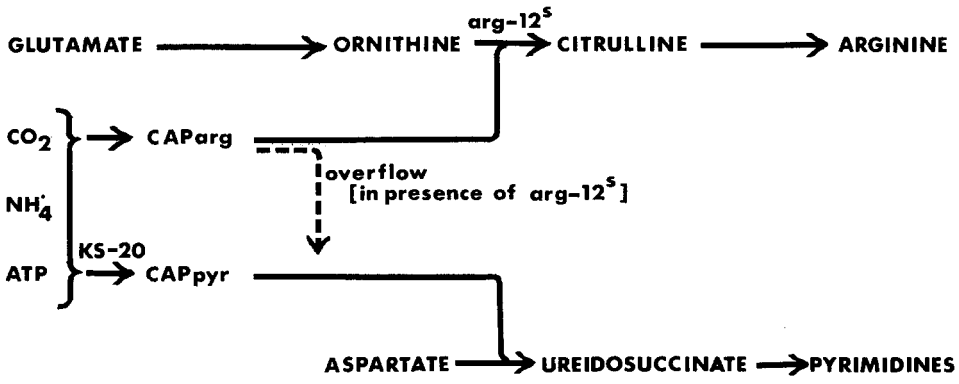

FIGURE 1.—Metabolic relationships proposed for arginine and pyrimidine synthesis in Neurospora.

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lates in the mycelia of *arg-12*^{s} and is able to overflow to a pyrimidine pathway deficient in CAPpyr, and the sensitivity to arginine seen in suppressed *pyr-3* double mutants reflects feedback control of the enzyme responsible for CAParg production.

This paper includes data which indicate a repression mechanism of control in arginine sensitivity of suppressed *pyr-3* double mutants, and reports the isolation and characterization of a modifying mutation which reduces both arginine sensitivity of the double mutant and its ability to concentrate arginine. The method employed in the isolation of the modifier can be used in attempts to obtain mutations altering other steps involved in feedback regulation of arginine synthesis.

MATERIALS AND METHODS

Strains: The strains used in these studies were derived from the collection of R. H. DAVIS at the University of Michigan. The *pyr-3* allele used was KS-20 (SUYAMA, MUNKRES and WOODWARD 1959). It is like other suppressible *pyr-3* mutants in that it has an active aspartic transcarbamylase (ATCase) and is susceptible to the suppressor, *arg-12*^{s} (DAVIS and WOODWARD 1962). As expected, it can be made to grow under air containing 30% of CO_2 (CHARLES 1964), and the CO_2-supported growth is inhibited by arginine. The *arg-12*^{s} gene has been characterized by DAVIS (1962b). Wild-type strains 73a and 74A were reisolated to eliminate a mutation found in our stocks (designated UM-300 by R. H. DAVIS) which indirectly influences the levels of OTCase. The *arg-1* mutation used was isolated from 74A after ultraviolet (UV) treatment and is designated UM-245.

Media: All growth media utilized the salt-biotin mixture (medium N) of VOGEL (1956) with appropriate modifications of carbon source, agar content, and supplementation. Two percent sucrose was used for liquid medium. One percent sorbose plus a 0.1% glucose-fructose mixture (0.05% each) was used to induce colonial growth on plates. Uridine, where used, was added at 100 μg/ml. The amount of arginine used varied widely; concentrations are given for each experiment. L-arginine-HCl (Sigma Chemical Company) was used throughout. Cornmeal agar (Difco) was used for a crossing medium.

Mutagenesis: Nitrous acid and UV were both employed. The nitrous acid treatment was carried out as described by MALLING and FREDERIKSEN (1962). For UV-irradiation, conidia were spread in a thin dry layer on the bottom of an open Petri dish and irradiated 1 min at 15 cm from an 8 watt germicidal lamp. The estimated survival for mutagenic treatment was approximately 50% in all cases.

Selection of modifying mutations: Originally it was assumed that mutations could be obtained which would lower the sensitivity to arginine seen in KS-20; *arg-12*^{s} double mutants by plating mutagen-treated conidia directly on medium containing arginine. However, no growth was detected when this procedure was followed. The method of selection was changed to allow the treated KS-20; *arg-12*^{s} condia a 5.5 hour period for germination on minimal plates at 35°C. After this interval, molten agar medium containing 100 μg arginine-HCl/ml was poured over the layer of conidia. The overlayered plates were incubated again at 35° for approximately 24 hours, then scanned for further mycelial growth through the arginine-containing layer.

Colonies were removed from the agar and placed on slants containing 100 μg/ml arginine. These strains were subsequently tested by streaking conidia on the surface of agar containing 5 μg/ml arginine. Those which grew quickly, and thus displayed an insensitivity to arginine, were saved for further analysis. Six isolates, when crossed to wild type, gave rise to KS-20 (pyrimidine-requiring) and KS-20; *arg-12*^{s} (arginine-sensitive) progeny showing that in each case the insensitivity was due to a modifying mutation not obviously linked to either *pyr-3* (linkage group IV) or to *arg-12* (linkage group II). Modifying mutations were obtained both with UV and with nitrous acid. One of the UV mutants, UM-535, was analyzed further.

CO_2 response: KS-20 strains will grow on minimal medium under air made to 30% CO_2,

but fail to grow if the medium is supplemented with arginine (5 μg/ml). KS-20; UM-535 double mutants do not show this sensitivity to arginine. Pyrimidine auxotrophs were tested for CO_2 + arginine response by the method of CHARLES (1964).

Growth conditions: Mycelia used in the measurement of enzymatic activity, arginine content, and dry weight were grown in 800 ml shaken cultures at 28° from large conidial inocula (DAVIS and HAROLD 1962). Initial attempts to show arginine-sensitivity of KS-20; *arg-12*^{s} cultures grown under these conditions gave no indication of growth retardation after the addition of arginine to the growing mycelia. It was discovered that such rapidly growing cultures have a capacity for a ten-fold increase in dry weight after the arginine is added. To demonstrate arginine-sensitivity in shaken cultures, arginine amounting to 200 μg/ml was added to 10 hour cultures which had attained a mycelial growth of about 5 mg dry weight per 50 ml medium.

Biochemical assays: The determination of mycelial arginine was made by drawing down a mycelial suspension to a moist pad on Whatman No. 1 filter paper in a Buchner funnel and washing briefly with distilled water. The moist pad was removed and immediately extracted at room temperature with 2 M perchloric acid (PCA). The insoluble material was washed by centrifugation three times and the washes were combined with the original extract. To this acid extract 4 N KOH sufficient to neutralize the PCA was added, and the $KClO_4$ precipitate removed by centrifugation. Arginine determinations of these extracts were then made by a modified Sakaguchi reaction (VAN PILSUM, MARTIN, KITO, and HESS 1956). By the addition of arginine to neutralized PCA extracts, it was determined that no compounds were present which interfered with the colorimetric test. As similar strains yielded arginine values very close to those determined by electrophoretic separation of arginine (DAVIS 1962a), it was concluded that no other compounds in the crude PCA extracts were being colorimetrically confused with arginine.

For determination of the arginine concentration in VOGEL'S medium, quantities of the minimal medium equal to those needed in the experimentals were added to the standards, since several ingredients of the medium, especially NH_4^+, interfere to a limited extent with the test for arginine.

Determination of carbamyl phosphokinase (CPKase) was made by the method of DAVIS (1965a). While DAVIS (1965b) was able to freeze dialyzed extracts, the procedure seemed to cause some loss of activity in my hands. DAVIS has shown the CPKase activity to be associated with the arginine pathway. No *in vitro* assay has yet been devised to show the synthesis of CAPpyr.

To distinguish qualitatively the OTCase activity of wild type from that of strains carrying the *arg-12*^{s} mutation, the simplified assay described in DAVIS and THWAITES (1963) was used.

Protein was measured by the Biuret method (LAYNE 1955).

Dry weight was determined from the acetone-dried pads before preparation of acetone powders.

RESULTS AND DISCUSSION

(1) *Physiological observations of arginine-sensitivity in KS-20;* arg-12^s: If in fact the rate of CAParg synthesis is regulated by the cellular concentration of arginine, it is understandable that a pyrimidine requirement might appear in the KS-20; *arg-12*^{s} double mutant when arginine is added to the medium. In this strain an accumulation of arginine in the mycelium would stop pyrimidine synthesis as well as arginine synthesis since the pyrimidine pathway is dependent on an overflow of CAParg. DAVIS (1963, 1965b) has demonstrated derepression of CPKase under conditions of arginine starvation in an arginine auxotroph and in *arg-12*^{s} mutants grown on minimal medium, and has shown this activity to be associated with the arginine pathway. However, direct evidence in support of the proposed metabolic relationships for suppressed *pyr-3* mutants would require a demonstration of (1) a relatively high level of mycelial arginine in the double

mutant during inhibited growth on arginine supplemented medium, and (2) coincidentally, a relatively low value in the specific activity of CPKase, serving as an indication of subnormal CAParg synthesis. Demonstrations of these two conditions are presented below.

It was noted that after the addition of arginine to rapidly growing cultures, a tenfold increase in dry weight occurs before growth plateaus (Figure 2A). This delayed inhibition of growing cultures by arginine is in marked contrast to the well known immediate inhibition of KS-20; *arg-12*^{s} conidia, which fail to germinate on arginine supplemented medium. As expected, inhibited cultures contained large quantities of extractible arginine, while uninhibited cultures of the KS-20;

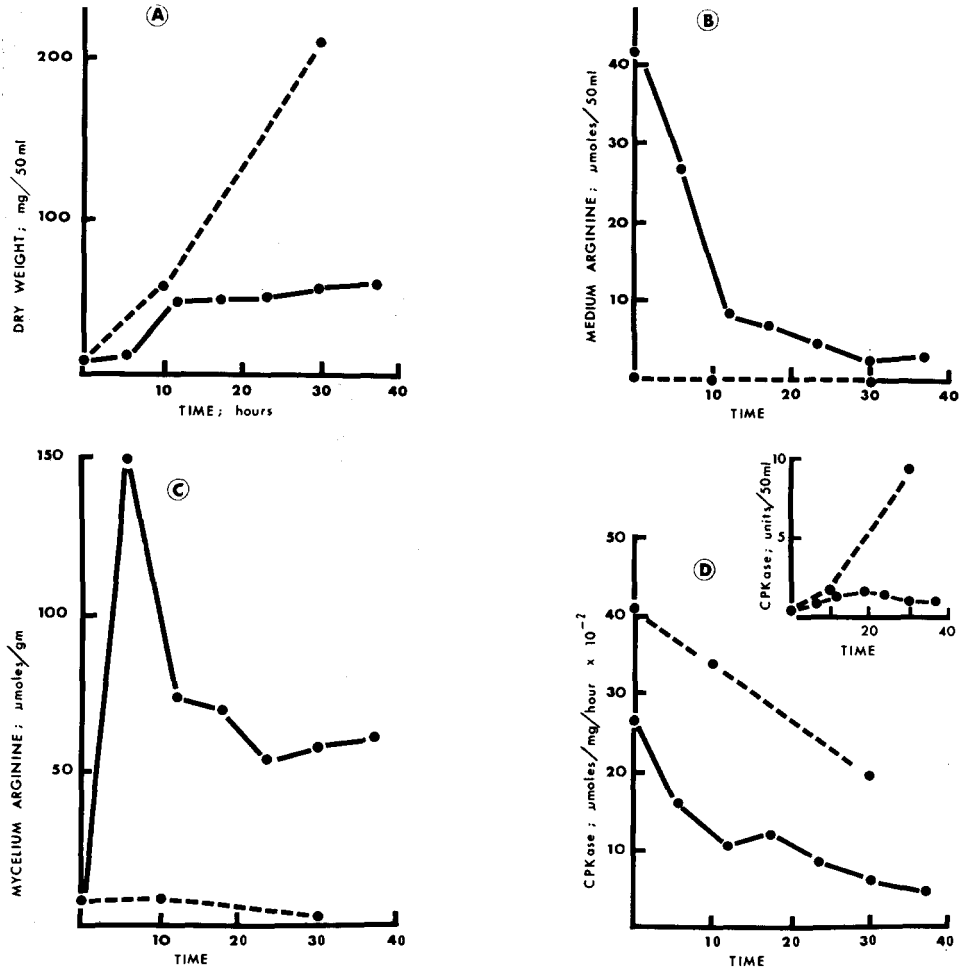

FIGURE 2.—The behavior of KS-20; *arg-12*^{s} on minimal (dashed lines) and on arginine supplemented media (solid lines). Arginine was added at time zero. (A), (B), and (C) as indicated. (D) Carbamyl phosphokinase—main graph is specific activity; insert is total activity per volume of culture.

arg-12^{s} double mutant, grown on minimal, had very small amounts of arginine typical of strains carrying *arg-12*^{s} (see DAVIS 1962a). Four or five hours after the addition of arginine to cultures of the sensitive strain, the mycelial arginine concentration rose sharply to a maximum. It then fell to a nearly constant value 5 to 10 hours after the maximum (Figure 2C). This steady state concentration of arginine in the inhibited double mutant grown on arginine supplemented medium was 5 to 20 times that of the uninhibited strain grown on minimal, and 2 or 3 times that of wild type grown on minimal medium.

In addition to measurements of mycelial arginine concentration, CPKase values for the double mutant were determined during growth in the presence and absence of arginine supplementation. The observed differences in enzyme specific activities between the two growth conditions (Figure 2D) can be partly ascribed to experimental error. It should be noted that there is a general downward trend in specific activity during growth under both conditions. The real difference in specific activity is probably not more than twofold at any stage of growth shown. The observed difference, however, was in the expected direction inasmuch as the arginine-inhibited mycelium apparently produced less, rather than more, CPKase. In addition, the initial rate of decrease observed for the mycelium exposed to arginine may be significantly greater than that observed for the mycelium grown on minimal medium.

In an experiment (Figure 3) in which the arginine-inhibited KS-20; *arg-12*^{s} mycelium was transferred back to minimal medium just after the growth plateau had been reached, it was observed that the inhibited cell mass had a capacity to reduce its internal arginine level when the outside source of the compound was removed. Only when the mycelial arginine level fell to a value less than that usually extracted from minimally grown wild-type cultures (DAVIS 1962a), did normal growth of the double mutant resume.

Of particular interest in this experiment was the apparent derepression of CPKase activity following the reduction of mycelial arginine levels. The specific activity was actually observed to go up after the depletion of mycelial arginine and before the resumption of growth (Figure 3). This is taken as confirmation that CPKase levels are increased in response to a shortage of mycelial arginine. It also seems probable that high CPKase levels may be necessary for the growth of KS-20; *arg-12*^{s} strains in the absence of pyrimidine supplementation (i.e., necessary for the suppression of the KS-20 phenotype).

(2) *Characterization of a mutation which reduces the arginine sensitivity of KS-20*; arg-12^{s}: It was assumed that KS-20; *arg-12*^{s} strains could lose arginine sensitivity either through loss of feedback control by arginine or through an inability to concentrate arginine because of a high destruction rate or a low rate of uptake. The modifier, UM-535, was shown to act through the reduction of arginine uptake in the following manner: The progeny of a cross of the triple mutant, KS-20; *arg-12*^{s}; UM-535, with wild-type 74A were analyzed nutritionally, genetically, and enzymatically to the extent that all eight expected genotypes could be identified and counted. The results of this analysis are presented in Table 1. The observed equal frequencies of the eight genotypes in the progeny

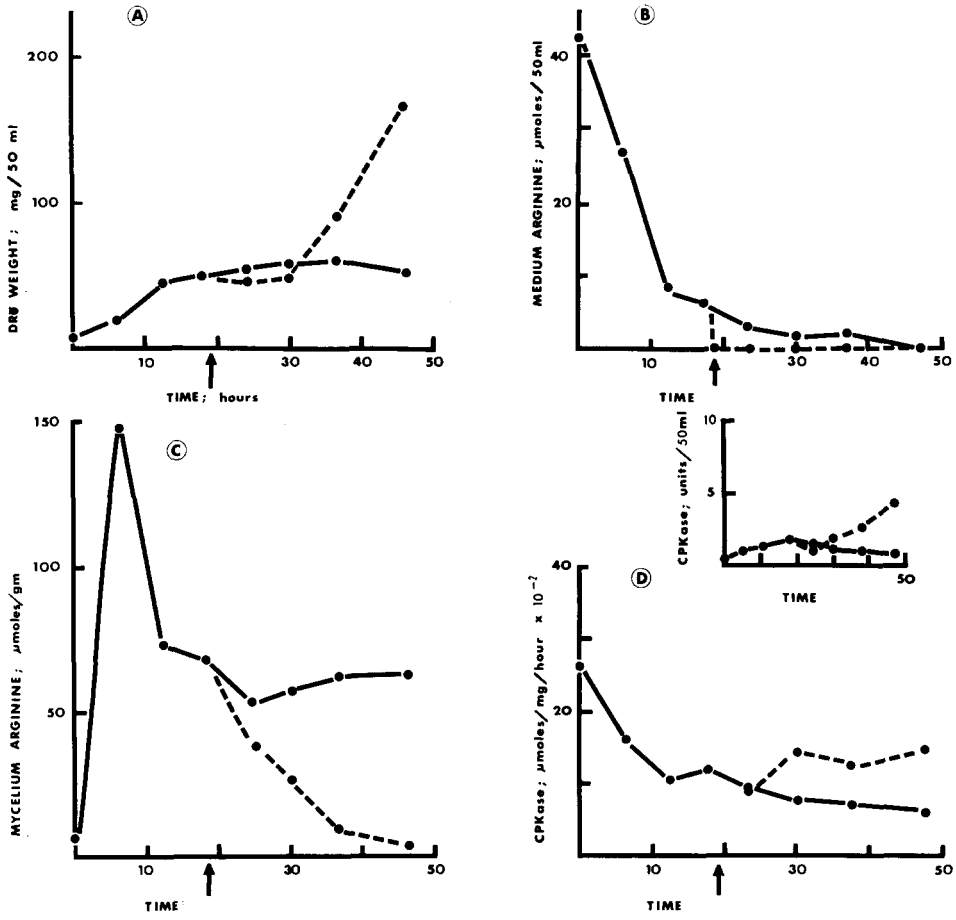

FIGURE 3.—The release of arginine inhibition from KS-20; *arg-12*[8]. Arginine was added at time zero. Mycelium was transferred back to minimal at 19 hours (arrows). Experimental (dashed lines), control (solid lines). The control in figure 2 is presented again for comparison. (A), (B), and (C) as indicated. (D) Carbamyl phosphokinase—main graph is specific activity; insert is total activity per volume of culture.

resulting from the cross of the triple mutant with wild type indicates that no linkage exists between the UM-535 locus and *pyr-3* (the KS-20 locus), or between UM-535 and *arg-12*. The mating type of 34 isolates containing UM-535 was determined and 14 of these (41%) were found to be mating type A, the non-parental mating type. Thus, it can be stated that UM-535 is no closer than 25 units from mating type with a 95% level of confidence. Since *arg-1* (argininosuccinate synthetase) and *arg-3* (CPKase) are both within 10 units of mating type and since *arg-2* (perhaps affecting another enzyme concerned with the synthesis of arginine-specific CAP) is within two units of *pyr-3*, it is very unlikely that UM-535 is allelic with *arg-1*, *2*, or *3*. The analysis of the cross also makes it clear that UM-535 itself does not act as a suppressor of KS-20.

TABLE 1

Results of a cross of KS-20, arg-12^s, *UM-535 and wild type*

Genotype			Phenotype						Frequency, %	
KS-20	*arg-12^s*	UM-535	Min	Arg	Arg+CO_2	Min+CO_2	OTCase	Other Criteria	Expected	Observed
+	+	+	+	+	..	..	high	.	12.5	11
+	+	—	+	+	..	..	high	*	12.5	9
+	—	+	+	+	..	..	low	.	25	29
+	—	—	+	+	..	..	low	*		
—	+	+	—	—	—	+	high	.	12.5	13
—	+	—	—	—	+	+	high	.	12.5	11
—	—	+	+	—	..	..	low	.	12.5	13
—	—	—	+	+	..	..	low	†	12.5	13

+ under *Genotype* signifies wild-type allele; + under *Phenotype* signifies growth.
* Will yield arginine-insensitive KS-20 when crossed with KS-20.
† Slightly inhibited by arginine, inhibited by lack of atmospheric CO_2

Thus, genetic evidence does not indicate the physiological mode of action of the UM-535 arginine-insensitivity mutation, and it was of interest to study the behavior of UM-535 directly by observing the response of the triple mutant to growth conditions known to halt the growth of the double mutant, KS-20; *arg-12^s*. It can be noted in Figure 4C that the triple mutant does show a sensitivity to arginine if the amount of arginine added to the medium equates with an initial concentration of 500 μg/ml. However, when arginine amounting to 200 μg/ml is added to cultures of both strains, a very pronounced growth difference is seen (Figure 4B). There is no difference in growth kinetics if the two strains are grown on minimal medium (Figure 4A).

In an experiment designed to show the maximum difference in growth response between the double mutant, KS-20; *arg-12^s*, and the triple mutant KS-20; *arg-12^s*; UM-535, measurements of the medium concentration of arginine, the mycelial arginine content, and the level of CPKase activity were made (Figure 5). The

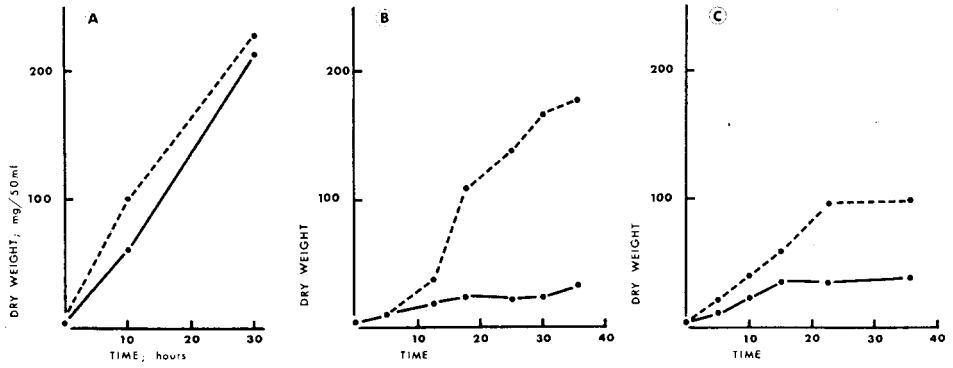

FIGURE 4.—Growth behavior of KS-20; *arg-12^s* (solid lines) and KS-20; *arg-12^s*; UM-535 (dashed lines) on varying concentrations of arginine added at time zero. (A) No arginine added (B) 200 μg/ml (C) 500 μg/ml.

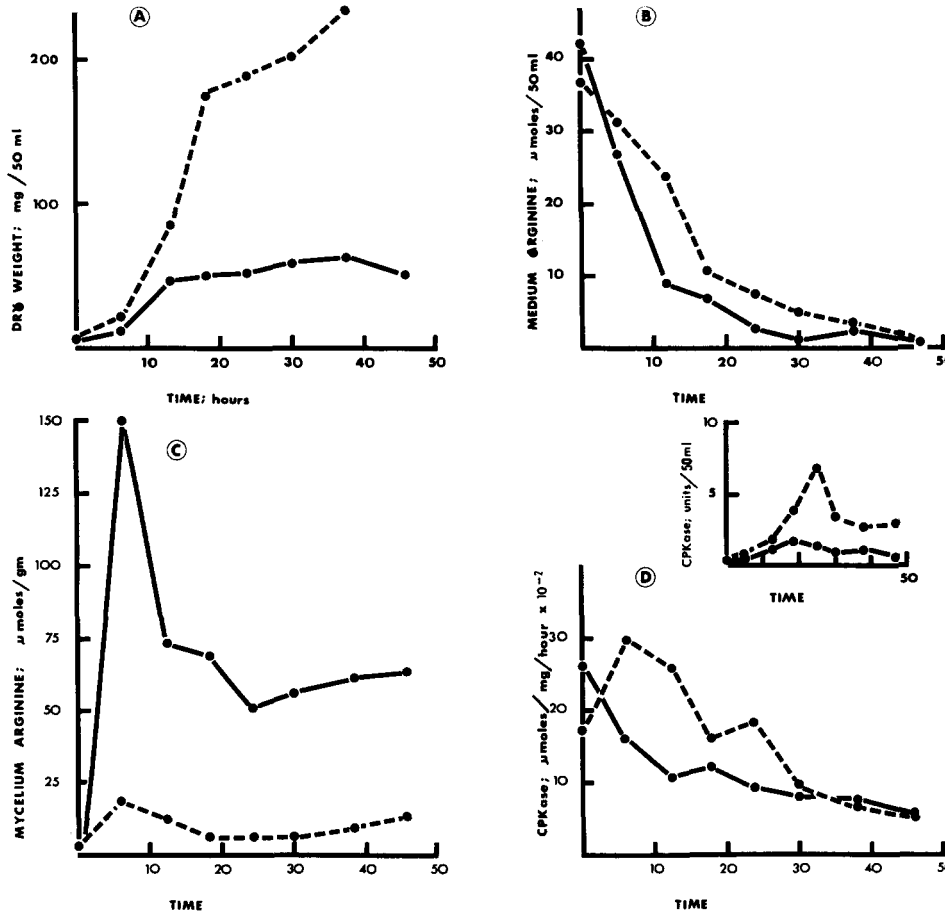

FIGURE 5.—The behavior of KS-20; *arg-12*^{s} (solid lines) and KS-20; *arg-12*^{s}; UM-535 (dashed lines) on medium supplemented with arginine at time zero. The KS-20; *arg-12*^{s} data are presented again for comparison. (A), (B), and (C) as indicated. (D) Carbamyl phosphokinase—main graph is specific activity; insert is total activity per volume of culture.

weight-specific rate of arginine removal is approximately five times higher in the arginine-sensitive strain, KS-20; *arg-12*^{s}, than in the strain having UM-535. Correlated with the observed difference in the ability to remove arginine from the medium the KS-20; *arg-12*^{s}; UM-535 strain was observed to have a smaller amount of mycelial arginine at all times after arginine supplementation (Figure 5C). In both strains the mycelial arginine per volume of culture was only a fraction of the amount of arginine lost from the same volume of medium, and the difference must be accounted for by utilization or destruction of arginine. The total arginine in the system would be shown in Figure 5B by curves slightly above the curves representing medium arginine.

It seems reasonable to assume that the low weight-specific removal rate is the cause of the low internal concentration of arginine in the strain carrying UM-535.

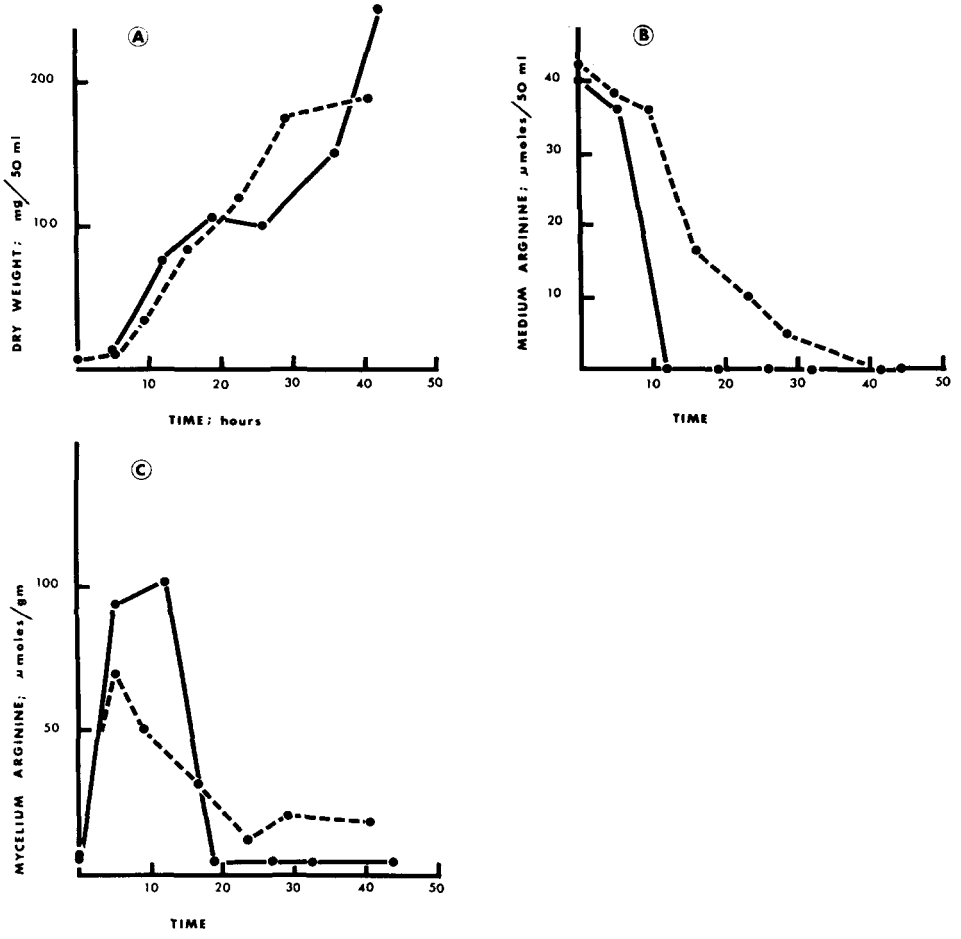

FIGURE 6.—The behavior of *arg-12*^{s} (solid lines) and *arg-12*^{s}; UM-535 (dashed lines) on medium supplemented with arginine at time zero.

If the reduced amounts of mycelial arginine were due to an abnormally high rate of arginine destruction, one might expect the assimilation rate to be normal or higher. The primary effect of UM-535 seems to be concerned with arginine uptake.

Previously published data on levels of CPKase (DAVIS 1965b) have indicated its activity in controlled by an arginine mediated repression mechanism. The comparison between CPKase in the sensitive strain, KS-20; *arg-12*^{s}, and the triple mutant reflects this phenomenon (Figure 5D). The difference between strains is not as great on a percentage basis as the differences in growth rate, arginine assimilation rate, or mycelium-arginine concentration. A calculation of total activities show that synthesis of CPKase is not completely repressed (i.e., stopped) in either strain for at least 20 hours after the addition of arginine. Nevertheless, the activity difference is maintained during the period when growth

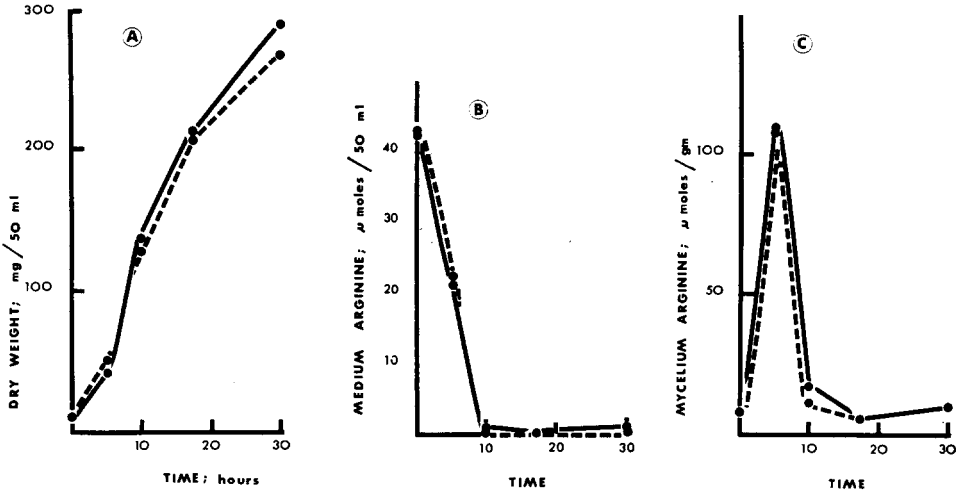

FIGURE 7.—The behavior of wild type (solid lines) and UM-535 (dashed lines) on medium supplemented with arginine at time zero.

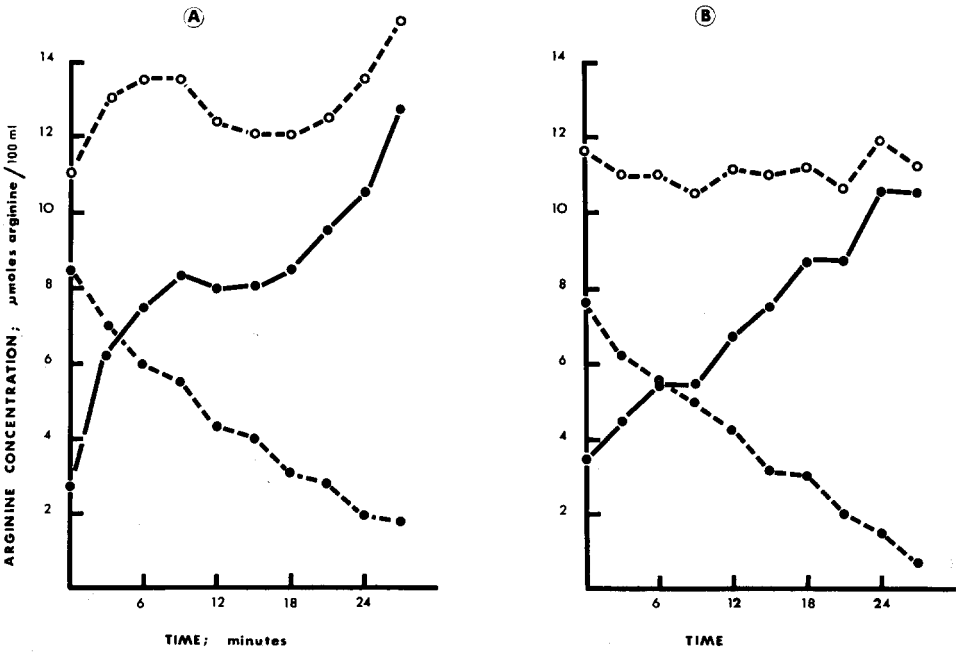

FIGURE 8.—Arginine assimilation kinetics of wild type (A) and UM-535 (B). Medium arginine (dashed lines with dots), mycelium arginine (solid lines), total arginine in system (dashed line with circles).

differences are observed and the difference is again in the expected direction in that the insensitive strain has the higher CPKase levels, and presumably can produce an overflow of the arginine-specific CAP pool to supply the needs for pyrimidine synthesis.

It could be argued that the assimilation rate difference is the *result* of growth inhibition rather than the *cause* of it. With this possibility in mind the strain, *arg-12*^{s} was compared to the doubly mutant strain, *arg-12*^{s}; UM-535 under the same conditions used to demonstrate the complete inhibition of the growth of the sensitive double mutant, KS-20; *arg-12*^{s}. Since pyrimidine synthesis is normal in both *arg-12*^{s} and *arg-12*^{s}; UM-535, neither strain shows any sensitivity to arginine, but there is a noticeable difference in the way the two strains assimilate arginine (Figure 6B). Whereas *arg-12*^{s} removed all detectable arginine from the medium within 10 hours, the *arg-12*^{s}; UM-535 strain took approximately 40 hours to do so. Thus, it seems quite clear that the reduced rate of arginine assimilation in strain carrying UM-535 is not the result of growth inhibition.

The reduction of arginine assimilation associated with UM-535 does seem to be dependent, however, on the reduced rate of endogenous arginine synthesis associated with *arg-12*^{s} and also on the external arginine concentration. In many experiments in which the rate of arginine assimilation was compared between wild type and the single UM-535 mutant it was found to be equal. A long-term growth experiment showed no difference in the rate at which arginine is removed from the medium or in the extent to which it is concentrated in the mycelium (Figure 7). In a short-term experiment, small quantities of mycelium were placed in a dilute arginine solution and a rapid assimilation of the arginine by the mycelial fraction of the mixture was observed. The arginine assimilated under these conditions was not metabolized to a detectable extent during the limited time in which the observations were made. Again no difference in the assimilation kinetics was seen between the wild-type and UM-535 strains (Figure 8).

When *arg-12*^{s} and *arg-12*^{s}; UM-535 were compared under growth conditions which produce a slight arginine inhibition of growth in the triple mutant (i.e., when arginine was added to growing cultures at 500 μg/ml), the differences in arginine assimilation largely disappeared (Figure 9). Only later, when levels of arginine had fallen below 300 μg/ml medium, was there a discernible difference in the ability of either strain to assimilate arginine. Again *arg-12*^{s}; UM-535 lagged behind *arg-12*^{s} in removing arginine from the medium.

It can be seen that two conditions are able to negate the UM-535 effect of reducing the assimilation rate of arginine—normal internal synthesis of arginine and a high concentration of external arginine. Some preliminary data suggest that if internal synthesis of arginine is completely absent, as in the case of an auxotrophic mutant, arginine uptake is completely absent in mutants also containing UM-535. Thus, when a singly mutant strain of UM-535 was crossed with *arg-1* (UM-245), a mutant blocked after the formation of citrulline (NEWMEYER 1962), the progeny could be separated easily into three categories: (1) that in which the ascospore germinated but failed to grow on 100 μg/ml arginine me-

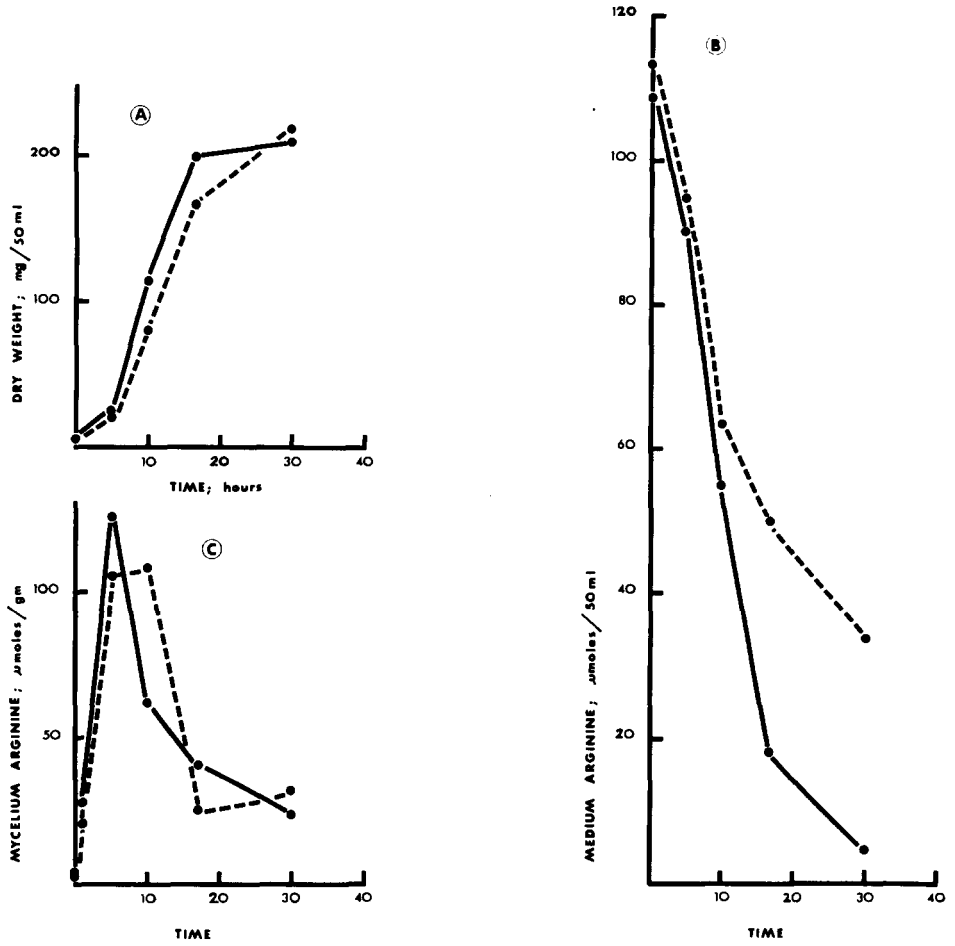

FIGURE 9.—The behavior of *arg-12*^{s} (solid lines) and *arg-12*^{s}; UM-535 (dashed lines) on medium supplemented with 500 μg/ml arginine at time zero.

dium, 22%; (2) that in which the isolate required arginine for growth, 27%; and (3) that in which the isolates were phenotypically wild type, 51%. It seems highly probable that the lethal class had an *arg-1*; UM-535 genotype.

The author is indebted to DR. R. H. DAVIS for his counsel and constructive suggestions during the course of this investigation.

SUMMARY

A simple selection procedure will yield mutations which confer a certain amount of arginine-insensitivity to the suppressed *pyr-3* strain, KS-20; *arg-12*^{s}. Such mutations are of interest since they can be expected to influence in some way the normal feedback control of carbamyl phosphate synthesis in the arginine pathway.—One mutation produced in this selection system, UM-535, has been

shown to reduce the rate of arginine assimilation from the medium. The expression of the UM-535 phenotype seems to depend on the reduced arginine synthesis associated with the *arg-12*^{s} suppressor mutation.

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