

COMPLEMENTATION ANALYSIS OF METABOLITE-RESISTANT
MUTATIONS WITH FORCED HETEROKARYONS OF
*NEUROSPORA CRASSA*¹

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Manuscript received February 28, 1973

ABSTRACT

The double mutant strain *pyr-3 arg-12^s* is a prototroph because a common precursor of arginine and pyrimidine is supplied by the arginine pathway. Growth of this strain is inhibited by exogenous citrulline or arginine. Citrulline-resistant mutants of this strain were selected, and they resulted from modifier mutations at other loci. Forced heterokaryons were used to study complementation among these modifiers. Since the complementation test requires the scoring of non-growth as the positive result, there was concern that variations in nuclear ratios could give erroneous results. This possibility does not seem significant, since groups of mutants established by complementation correspond with groups established by physiological, enzymatic, and recombinational measurements.—The technique has revealed that the most frequently mutated loci are *arg-1* and what is probably *un-3*. *Arg-1* mutations affect the conversion of citrulline to argininosuccinate, while *un-3* mutations reduce the citrulline uptake rate. Since most of these mutations are of the intracistronic complementing type, a complementation map was constructed for most of the affected loci. The high proportion of complementors in each map can be explained by assuming that partially functioning gene products are more likely to complement with each other than are those which are nonfunctional.

IN *Neurospora*, if not in all eukaryotes, both arginine and pyrimidine synthesis utilize carbamyl phosphate (CAP) as a precursor. Each biosynthetic pathway has its own source of CAP (Figure 1), with the pyrimidine source apparently being localized in the nucleus and the arginine source in the mitochondria (DAVIS 1972). The metabolic separation of the two paths in *Neurospora* is nearly complete, since mutations which specifically block the synthesis of pyrimidine-specific CAP (CAP_{pyr}) are auxotrophs under normal growth conditions. That the sequestering of CAP by the two pathways is not absolute, however, is demonstrated by the suppressor (*arg-12^s*) of CAP_{pyr}-deficient mutants. The *arg-12^s* mutation is a very leaky mutant affecting the utilization of CAP_{arg} but not causing an arginine requirement (DAVIS 1962). Insertion of the *arg-12^s* suppressor into a CAP_{pyr}-deficient mutant such as KS-20 (a *pyr-3* allele) results in the accumulation of CAP_{arg} and an overflow to the pyrimidine pathway. Thus the *pyr-3, arg-12^s* double mutant is a prototroph, but since CAP_{arg} production is subject to repression by arginine (WILLIAMS, BERNHARDT and DAVIS 1970),

¹ This study was supported by NSF grant GB 8639 and the California State University, San Diego Foundation.

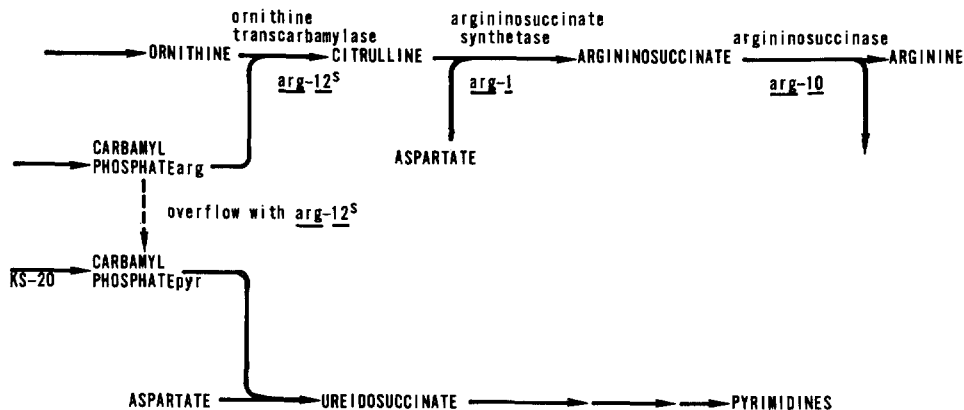


FIGURE 1.—Arginine and pyrimidine synthesis in *Neurospora*.

the double mutant is sensitive to the addition of arginine or its precursor citrulline if either or both are added to the growth medium without the simultaneous addition of a pyrimidine source such as uridine.

A number of systems have been employed for the purpose of selecting transport mutants in *Neurospora*. Apparently the most popular involves the use of toxic analogs of various biosynthetic end-products to select mutants which confer resistance (e.g. SANCHEZ, MARTINEZ and MORA 1972; STADLER 1966; KINSEY and STADLER 1969). The use of wild-type sensitivity to metabolic analogs is basically similar to using the double mutant's sensitivity to citrulline and arginine for the selection of resistant modifiers. The latter system has a theoretical advantage, however, in that it is based on a known sequence of cellular events. The ultimate site of damage with toxic analogs, on the other hand, usually involves some speculation.

Previously arginine sensitivity of the double mutant was used (THWAITES 1967) to select a resistance modifier, *bat* (THWAITES and PENDYALA 1969), which was found to lack the basic amino acid transport system described by PALL (1970).

Hypothetically, arginine-resistant modifications might affect many other functions in addition to arginine transport. Such a modification might involve various functions associated with arginine pool retention and the feedback control system, or could be a reversion of the *pyr-3* mutation.

Citrulline-resistant modifications, on the other hand, could involve all of the arginine-sensitivity functions plus those functions required for the transport of citrulline and its conversion to arginine. Competition studies (SANCHEZ, MARTINEZ and MORA 1972) show that citrulline is assimilated by at least two amino acid transport systems: the neutral and the general (see PALL 1969). The conversion of citrulline to arginine involves two enzymatic steps (see Figure 1) which have been studied by NEWMAYER (1962) and by WAMPLER and FAIRLEY (1967).

By selecting mutant modifiers which have gained citrulline resistance, but still

retain arginine sensitivity, presumably we can narrow the range of selected mutants to just those which affect citrulline accumulation or its conversion to arginine. The arginine sensitivity in such mutants gives some assurance that the arginine pool retention and feedback control functions are normal, and that *pyr-3* has not reverted.

To facilitate the genetic analysis of these citrulline-resistant strains we developed a system of complementation in which two complementing resistants will interact in a forced heterokaryon to give sensitivity. Traditionally complementation analysis has been performed between auxotrophs or host-range restricted mutants and complementation is manifested by growth. With resistant mutants the situation is reversed; complementation is seen as a failure to grow, i.e., sensitivity. The system is operationally similar to that used by LEWIS (1961) to analyze suppressors of a methionine requirement in *Coprinus*.

There was concern that in such a forced heterokaryon complementation system nuclear ratios could vary enough to give false complementation data while still satisfying the requirements of the forcing auxotrophies. In addition to this, there was a certain reluctance to base conclusions about complementation on negative data, i.e., failures of growth. The results reported here show that the concern was unjustified in this case since recombinational and physiological observations correlate well with complementation determinations.

MATERIALS AND METHODS

Strains: The *pyr-3^{KS-20}* and *arg-12^s* alleles were originally obtained from the collection of Dr. ROWLAND DAVIS at the University of Michigan, Ann Arbor, Michigan. Strains *arg-1* and 55701t were from the Fungal Genetics Stock Center, California State University, Arcata, California.

Chemicals: All C-14 compounds were from either Amersham-Searle or New England Nuclear. N-methyl-N'-nitro-nitrosoguanidine (MNNG) was obtained from the Aldrich Chemical Company. All amino acids were the "L" isomers except where noted.

Media: Medium N of VOGEL (1956) was used for vegetative growth and corn meal agar (Difco) was used as a crossing medium. Spreading growth on medium N was supported with 20 g/l sucrose while colonial growth on plates was induced with the substitution of 10 g/l L-sorbose plus 1 g/l sucrose for the energy source. Supplementations of the minimal medium usually consisted of 100 µg/ml of purine, pyrimidine nucleotide, or amino acid, and 2 µg/ml of vitamin. Exceptions are noted in the text. The following abbreviations will be used in referring to the supplements used: adenine (adn), thiamine (thi), citrulline (cit), uridine (uri), arginine (arg), 4-methyl-DL-tryptophan (4-mt), and DL-para-fluorophenyl-alanine (pfa).

Mutagenesis: UV irradiation was accomplished during a 3-minute exposure of a 3 mm deep water suspension of conidia (approximately 10⁵ spores/ml). A "Mineralight" lamp equipped with its shortwave filter gave a surface exposure of 80 ergs/mm²/sec when placed 15 cm above the suspension. This treatment resulted in the killing of approximately 50% of the spores, and an apparent mutational increase greater than 100 times the spontaneous rate.

Mutagenesis by MNNG typically consisted of exposure of conidia to a freshly prepared solution in water (10 µg/ml) for 20 minutes at 25°C. Exposed conidia were washed with liquid minimal medium on a Whatman #542 filter before plating. The treatment usually resulted in a kill of approximately 50%, and gave a mutational increase estimated to be more than 1000 times the spontaneous rate.

Auxotroph selection: Auxotrophs which would force the heterokaryons used in the resistance mutant complementation tests were selected in the citrulline-arginine sensitive double mutant *pyr-3, arg-12^s* by filtration enrichment after UV treatment in the manner of WOODWARD, DE-

ZEEUW and SRB (1954). From the filtration procedure we retained three strains which would form prototrophic, citrulline-arginine sensitive, stable heterokaryons in all three possible combinations. The strains are designated A-30, A-72, and T-40. The letters "T" and "A" refer to the respective thiamine or adenine requirement of each strain when grown as a homokaryon.

Citrulline-resistant selection: Citrulline-resistant mutations were induced and selected in each of the three auxotrophic citrulline-arginine-sensitive strains. The procedure was identical to that used for the production of the arginine-resistant mutant, *bat* (THWAITES 1967), except that citrulline (0.5 mM) was used in place of arginine in the overlayer. As with arginine resistance, we found it necessary to allow germination on unrestrictive medium (in this case *adn-thi*) before overlaying with the selection medium (*adn-thi-cit*). Without the germination step no resistant colonies were observed. Both UV and MNNG mutagenesis were employed.

Colonies which grew vigorously through the citrulline overlayer were isolated on *adn-thi-cit* slants and subsequently tested for growth on *adn-thi*, *adn-thi-cit*, and *adn-thi-arg* plates. Those which grew well on *adn-thi*, and *adn-thi-cit* media, but retained sensitivity to arginine on the *adn-thi-arg* plate, were saved for further testing. Approximately 90% of the mutants selected for citrulline resistance retained arginine sensitivity.

Mutation rate estimates: The effectiveness of UV and MNNG mutagenesis was judged by counting the number of citrulline-resistant colonies appearing on the overlaid selection plates after a specific mutagenic treatment, and comparing this value with the number of colonies appearing after no mutagenic treatment. Appropriate allowances were made for mutagen killing.

Recessiveness test: Each citrulline-resistant modification was tested for heterokaryon recessiveness by making a heterokaryotic strain composed of the resistant and an unmodified sensitive strain with a differing auxotrophy. If the resulting forced heterokaryon failed to grow on *cit* medium but did grow on minimal, the resistance modification was judged to be recessive to the wild-type sensitivity.

Complementation analysis: All possible forced heterokaryons were produced among the citrulline-resistant strains. Each of the resulting 1116 heterokaryons was scored for the extent of growth on minimal, *cit* and *arg* plates. All grew on minimal and failed to grow on arginine. If the extent of growth on the citrulline plate approximated that on the minimal plate, the heterokaryon was scored as noncomplementing ("0" on Figure 2). No growth on citrulline was scored as complementing ("+" on Figure 2). Occasionally the growth on citrulline was irregular or partially inhibited, and these determinations were scored as ambiguous (left blank in Figure 2). If two mutants had exactly the same complementation responses except for an occasional ambiguity, the two strains were grouped together for all subsequent complementation analysis required for map building.

It should be noted that not all pairs of mutants can be scored by this method. A given mutant can be compared with only approximately two-thirds of the remaining mutants, i.e. those mutants which were produced in strains with complementing auxotrophies of the mutant in question. We found that simulated grids with this type of informational deletion always were sufficient to reconstitute the major features of the map from which each partial grid was derived. For this reason we suspect that the map determined for this paper is similar to that which would have been obtained if all combinations could have been tested.

Transport assays: Short-term weight-specific transport rates were determined as described by WILEY and MATCHETT (1966). Concentrations of substrates are noted in the tables.

Whole-cell assay for argininosuccinate synthetase and argininosuccinase: Cells were grown from a heavy conidial inoculum approximately 12 hours with agitation at 30°C. The start of the assay consisted of adding C-14 citrulline to a concentration of 1 mM. The specific activity of the added citrulline was approximately 0.1 mCi/m mole. Since the control strain was sensitive to citrulline, uridine (100 µg/ml) was added along with the labeled citrulline.

Previous colorimetric determinations of arginine accumulation (SMITH 1970) had shown that a maximum level of arginine concentration is not reached for nearly 10 hours after the beginning of citrulline supplementation. Accordingly the uptake and conversion of citrulline was allowed to proceed for seven hours. A sample taken at 30 minutes showed that almost all extractable radioactivity was still in the citrulline form. After incubation the 100 ml cultures were

harvested on Buchner funnels. The cells were washed with cold water and then lyophilized. The dried cells were extracted with water containing 1 $\mu\text{g}/\text{ml}$ citrulline, argininosuccinate, and arginine carrier. The resulting 4-ml extracts were deproteinized with the addition of 6 ml of cold acetone. These extracts were concentrated by lyophilization to a volume of 250 μl for chromatography.

The concentrated extracts were subjected to paper chromatography with a phenol, water, ammonia solvent system in which undistilled stock phenol was dissolved in water (4 g phenol to 1 ml water) and the ammonia was supplied from a dilute solution of NH_4OH (5 ml water to 1 ml stock 30% NH_4OH) contained in a separate beaker and not contacting the paper.

The dried chromatograms were cut into strips and assayed for radioactivity in a liquid scintillation counter. Peaks of activity were chemically identified by comparison with parallel runs of unlabeled material stained with ninhydrin.

Cell-free assay of argininosuccinate synthetase and argininosuccinase: Cells were grown, harvested, and lyophilized as in the whole cell assay, but without the addition of C-14 citrulline or uridine during growth. Fifty mg of dried cells were extracted with 2 ml 0.01 M potassium phosphate buffer pH 7.3. The assay was essentially that of NEWMAYER (1962) and WAMPLER and FAIRLEY (1967). The reaction mixture contained in a volume of 2 ml: 40 μmoles L-aspartic acid (previously neutralized with NaOH); 20 μmoles MgSO_4 ; 1.5 μmoles L-citrulline-ureido C-14 (specific activity 0.03 $\mu\text{Ci}/\mu\text{mole}$); 2.0 μmoles ATP; 20 μmoles phospho (enol) pyruvate; 50 μmoles Tris-HCl buffer pH 8.2; and 0.2 ml mycelial extract containing 1.4 to 1.8 mg protein. Reaction mixtures were started with the addition of the protein extract and allowed to proceed at 37°C for 20 minutes. The reaction was terminated with an addition of 3 ml cold acetone. The protein precipitate was removed by centrifugation and the supernatant concentrated to a volume of 200 μl by lyophilization. The resulting concentrate was chromatographed and counted as in the whole-cell assay.

RESULTS

Recessiveness testing: Fifty-nine citrulline-resistant strains were finally saved for subsequent testing. Each of these was found to be a heterokaryon-recessive mutation.

Complementation analysis: Figure 2 represents a summation of complementation determinations for all 59 mutant strains. These data, representing the responses of 1116 heterokaryons on cit medium, can be reduced to the complementation maps shown in Figure 3. The figure shows three major groupings of mutants designated as "left- and right-hand segments" of a large complementation map, a "small map", and two "simple loci." Forty-seven of the mutants fall into either the right- or left-hand segments of the large map. The two segments of this map are joined by a single mutant A-30(89). Five of the mutants are classified as members of the "small map," while the remaining seven strains represent the two additional loci. The latter mutants are said to be members of "simple" loci since among them there is no evidence of intracistronic complementation.

An unusual result of this analysis is seen in the high proportion of complementing mutations. It is customary to find that complementing mutants are relatively infrequent among all mutants for a given locus (e.g., CATCHESIDE and OVERTON 1958). The usual mutant in the typical analysis maps as a continuous line from one end of the map to the other since it fails to complement with any other mutant at that locus.

In contrast, the typical citrulline-resistant mutant complements with at least one of the other mutants at the same genetic locus. Therefore very few mutants

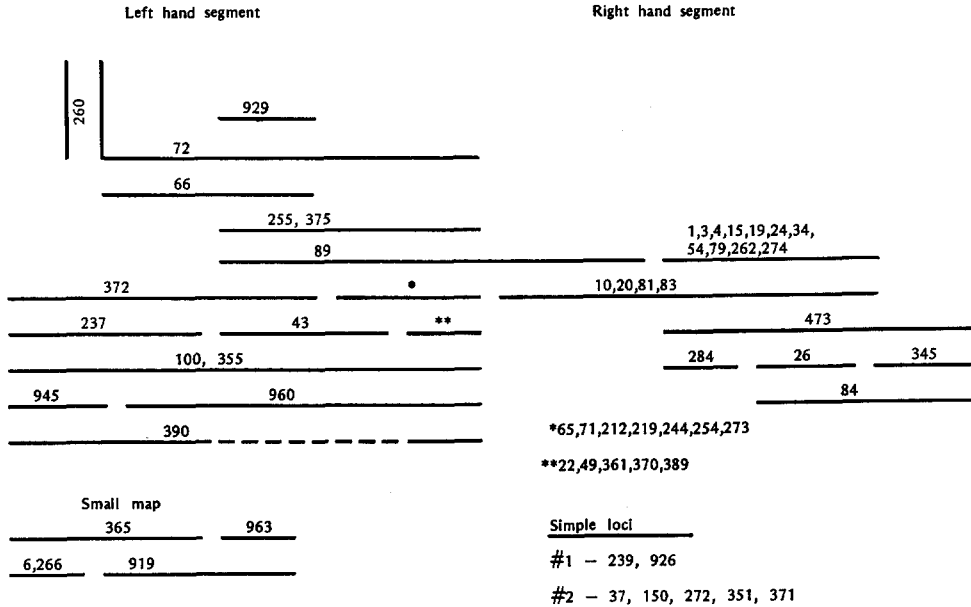


FIGURE 3.—Complementation maps derived from data shown in Figure 2. Mutant numbers are those in parentheses in the text.

MNNG, a mutagen known to induce multiple closely-linked mutations (MALLING and DESERRES 1970). We further assume that the “small map” is a third locus, and that the two “simple loci” constitute fourth and fifth loci.

Given these assumptions it can be seen that among the three loci showing intracistronic complementation, there are only two strains, T-40(100) and T-40(355), which are of the typical noncomplementing type. Both mutants are left-hand segment mutants.

Screening for ancillary phenotypes of citrulline resistance: To facilitate recombinational mapping of citrulline-resistant mutants we undertook a search for those which would have readily scorable phenotypes regardless of genetic background. Citrulline resistance cannot be used to follow the modifiers through a cross since this phenotype is dependent on the *pyr-3, arg-12^s* double-mutant background, and *pyr-3* cannot be made homozygous in such a cross due to its female sterility. Of a number of searches conducted for convenient mapping phenotypes, only those designed to find temperature-sensitive and analog-resistance phenotypes were successful.

Temperature-sensitivity tests: The search for temperature-sensitives yielded four such strains: T-40(272), T-40(237), T-40(260) and A-30(929). These strains not only provided the possibility of scoring a citrulline-resistant allele by its temperature sensitivity, but gave us further insight into the nature of these citrulline-resistant mutations. Further investigation showed that all four temperature-sensitives were sensitive by virtue of a lesion in arginine or pyrimidine synthesis, since each was able to grow at the elevated temperature if arginine and

uridine were added to the normal growth medium for these strains (adn-thi). We found that by outcrossing T-40(237) to an unrelated strain, we were able to separate the arginine and uridine requirements. Only the arginine requirement was temperature-sensitive in this strain.

Analog-resistance tests: The survey of mutant strains for possible resistance to 4-mt (150 $\mu\text{g/ml}$) or to pfa (10 $\mu\text{g/ml}$) showed that many citrulline-resistant mutants possess a limited ability to grow on one or both of these analogs. A completely resistant response to both analogs was seen only among right-hand mutants. Indeed, only two strains in this group—T-40(345) and A-72(34)—showed any sensitivity to either compound.

Functional identification of left-hand segment mutants: The existence of temperature-sensitive arginine auxotrophs in the left-hand segment of the large complementation map suggests that the locus involved may be concerned with the conversion of citrulline to arginine. The steps involved in the conversion are diagrammed in Figure 1 and reportedly involve the product of the *arg-1* gene and the product of the *arg-10* gene (NEWMAYER 1962).

The temperature-sensitive left-hand segment mutant T-40(237) was crossed with the temperature-sensitive mutant 55701t. All progeny from this cross can grow on thi-uri plates at 25°C. Only wild-type recombinants between 55701t and the citrulline-resistant component of T-40(237) can grow on the thi-uri plates incubated at 40°C. Since the number of colonies on the 40°C plates was 1.5% the number (>1000) seen on the 25°C plates, we can deduce that there is about 3% recombination separating the *un-3* locus (55701t) and the citrulline-resistance component of T-40(237). Since *arg-1* is about the same distance from *un-3*, it seems probable that the temperature-sensitive resistance component of T-40(237) is in fact an *arg-1* mutant.

Attempts to cross isolates of this presumed *arg-1* mutant with a standard *arg-1* strain have failed. The few spores finally expelled from the perithecia were poorly pigmented and inviable.

Since T-40(100) is a noncomplementing type of mutant, the standard interpretation of the complementation map would demand that all mutants failing to complement with it be allelic to it. With this in mind, the mutant was picked as a representative of the left-hand functional alteration and was used in a whole-cell assay for the conversion of citrulline to arginine. In this assay the resistant mutant was compared with the sensitive strain from which it had been selected, T-40. After 7 hours of incubation with C-14 citrulline the T-40 strain had large citrulline and arginine pools and a small, but detectable, argininosuccinate pool (Figure 4). T-40(100), on the other hand, had retained virtually all of its accumulated radioactivity as citrulline. The argininosuccinate pool was not detectable and only a trace of labeled arginine could be seen. Clearly T-40(100) has a block in the conversion of citrulline to argininosuccinate (the *arg-1* function). Had the block been between argininosuccinate and arginine we would have seen an accumulation of the former compound. The *arg-1* block is not complete, however, since T-40(100) does not have an arginine requirement.

In the course of screening mutants for possible uptake deficiencies we discov-

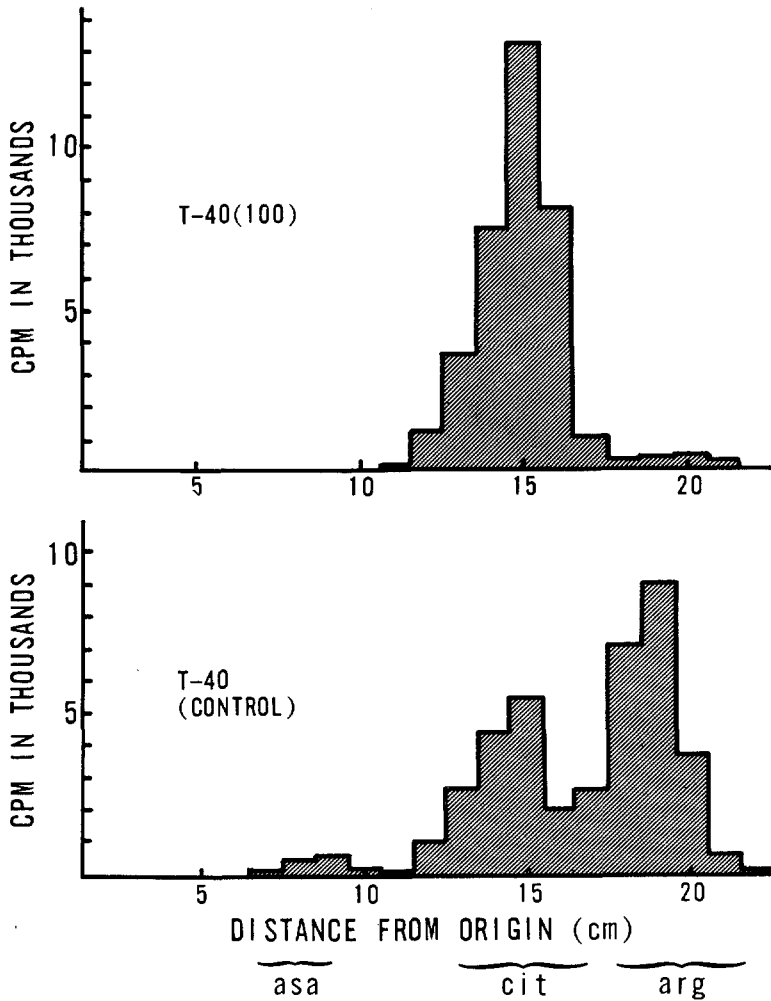


FIGURE 4.—Fate of C-14 citrulline in a left-hand mutant with nearly normal citrulline uptake.

ered that not all left-hand mutants are as proficient in the accumulation of citrulline as is T-40(100). While the initial transport rate of citrulline in T-40(100) is close to wild type, we found other left-hand strains with as little as 0.3% of wild-type activity (Table 1).

Nontransporting strains require the use of a cell-free citrulline conversion assay which eliminates the transport step which the whole-cell assay involves. Left-hand mutants A-30(43), A-30(89), and T-40(219) all showed a conversion pattern essentially identical to that shown by T-40(100) in the whole-cell assay (Figure 4). The transporting left-hand mutants T-40(100) and T-40(237) also gave the same argininosuccinate synthetase-deficient pattern with this test.

Using the cell-free assay, wild-type 74A, strain T-40, the mutant 55701t, and

TABLE 1

Citrulline transport rates in various left-hand mutants

Strain	Citrulline accumulation rate (μ moles/g/min)
T-40(100)	9.48
T-40(237)	9.15
T-40(219)	0.03
A-30(43)	0.23
A-30(89)*	0.79

* A-30(89) is both a left- and right-hand mutant. Initial concentration of citrulline = 1.0 mM.

right-hand mutants A-72(10), T-40(24), and T-40(54) all gave chromatogram profiles like that of the T40 control in Figure 4.

Functional identification of right-hand segment mutants: The observation that right-hand mutants are almost uniformly resistant to 4-mt and pfa suggested that these mutants are primarily concerned with uptake, as sensitivity to these analogs presumably does not involve the conversion of citrulline to arginine. Mutant A-30(89) which connects the right- and left-hand segments of the large complementation map implies a close linkage connection between the left-hand segment locus (*arg-1*) and the presumed transport locus represented by the right-hand mutants.

One transport deficiency locus which maps in the vicinity of *arg-1* is, of course, *un-3*, represented by the temperature-sensitive lethal mutant 55701t. Table 2

TABLE 2

Transport rates of various amino acids in wild-type, 55701t, and right-hand mutants

Strain	Amino acid	Initial concentration (mM)	Accumulation rate (μ moles/g/min)
wild type (74A)	L-citrulline	1.0	11.0
	L-aspartate	0.1	4.4
	L-phenylalanine	0.1	14.7
	L-arginine	0.1	10.8
55701t	L-citrulline	1.0	0.25
	L-aspartate
	L-phenylalanine
	L-arginine	0.1	0.28
A-30(473)	L-citrulline	1.0	0.07
	L-aspartate	0.1	0.30
	L-phenylalanine	0.1	0.35
	L-arginine	0.1	3.54
A-72(10)	L-citrulline	1.0	0.52
	L-aspartate	0.1	0.56
	L-phenylalanine	0.1	0.13
	L-arginine	0.1	3.38

shows that right-hand mutants have transport deficiencies which are quite similar to 55701t. A cross of the right-hand mutant A-30(473) with 55701t was fertile when the latter was used as the protoperithecial parent, but the viability among the resulting spores was only about 25%. Of 113 viable isolates none was clearly sensitive to 4-mt—an observation which suggests possible allelism between 55701t and the citrulline-resistant component of A-30(473). It seems probable that all right-segment mutants are *un-3* mutants or possibly mutants of some other transport locus in the vicinity of *arg-1* and mating type.

DISCUSSION

We conclude that our forced-heterokaryon system of complementation for citrulline-resistant mutants approaches a truly diploid system for reliability. We note that every mutant was judged to be recessive. In this test there is strong selection pressure on the cit medium for a nuclear ratio favoring the resistant component, but apparently no portions of the inocula have ever had a sufficiently high proportion of resistant nuclei to produce resistance and at the same time satisfy the auxotrophic requirements.

We can also note that the groupings which are made according to complementation behavior correspond to groupings which can be made on the basis of analog resistance. Here only the right-hand segment mutants show complete resistance to both 4-mt and pfa.

It is curious that all the right-hand segment mutants were derived from MNNG treatment, while every other complementation classification contains both MNNG and UV mutants. We are not sure of the molecular meaning of this observation, but we take note of the phenomenon.

The left-hand segment of the large complementation map is probably an extension of the map derived for the *arg-1* locus by CATCHESIDE and OVERTON (1958). Their *arg-1* mutants were all selected as auxotrophs following filtration enrichment. Of course our *arg-1* mutants were selected as bradytrophs which would not accumulate large amounts of arginine from an exogenous source of citrulline. If we assume that all of our left-hand segment mutants are *arg-1* mutants, we can make the comparison shown in Table 3. There is a striking difference in the proportion of noncomplementing mutants obtained by the two methods. It would seem that gene products which retain some portion of the wild-type activity are more likely to be those which can successfully engage in intracistronic complementation.

If we applied this same rule to the other complementation maps indicated in

TABLE 3

Proportion of noncomplementing mutants with two means of selection

Source	Total number of <i>arg-1</i> mutants	Noncomplementing <i>arg-1</i> mutants	Percent noncomplementing
This paper	27	2	7
CATCHESIDE and OVERTON	42	30	71

our analysis, we could predict from the lack of noncomplementors that the right-hand segment mutants and the small complementation map mutants are also of the partially functional type. They appear to be mutations at loci whose products can be reduced in function but not totally abolished. This seems to be the case for the *un-3* allele 55701t. At permissive temperatures the mutant is multiply transport-deficient (KAPPY and METZENBERG 1967). As the temperature is raised, the *un-3* gene product presumably becomes less functional until its activity falls below a life-sustaining value at about 35°C.

Another interesting aspect of this study is the transport deficiency seen in many of the *arg-1* mutants. One likely possibility is that the nontransporting strains accumulate large citrulline pools which inhibit amino acid transport. We could expect some sort of correlation between the size of the citrulline pool and the extent of the transport deficiency. Studies now in progress suggest that this is the case. It is interesting to speculate about how many other presumed transport-deficient mutants in the literature may have a similar explanation.

We are indebted to DR. CRAIG DAVIS and DR. JAN TURNER for their suggestions regarding interpretation of data and for their help with the manuscript.

LITERATURE CITED

- CATCHESIDE, D. G. and A. OVERTON, 1958 Complementation between alleles in heterocaryons. Cold Spring Harbor Symp. Quant. Biol. **23**: 137-140.
- DAVIS, R. H., 1962 A mutant form of ornithine transcarbamylase found in a strain of *Neurospora* carrying a pyrimidine-proline suppressor gene. Arch. Biochem. Biophys. **97**: 185-191.
- , 1972 Metabolite distribution in cells. Science **178**: 835-840.
- KAPPY, M. S. and R. L. METZENBERG, 1967 Multiple alterations in metabolite uptake in a mutant of *Neurospora crassa*. J. Bacteriol. **94**: 1629-1637.
- KINSEY, J. A. and D. R. STADLER, 1969 Interaction between analogue resistance and amino acid auxotrophy in *Neurospora*. J. Bacteriol. **97**: 1114-1117.
- LEWIS, D., 1961 Genetical analysis of methionine suppressors in *Coprinus*. Genet. Res. **2**: 141-155.
- MALLING, H. V. and F. J. DESERRES, 1970 Genetic effects of N-methyl-N'-nitro-N-nitrosoguanidine in *Neurospora crassa*. Molec. Gen. Genetics **106**: 195-207.
- NEWMAYER, D., 1962 Genes influencing the conversion of citrulline to argininosuccinate in *Neurospora crassa*. J. Gen. Microbiol. **28**: 215-230.
- PALL, M. L., 1969 Amino acid transport in *Neurospora crassa* I. Properties of two amino acid transport systems. Biochim. Biophys. Acta **173**: 113-122. ———, 1970 Amino acid transport in *Neurospora crassa* II. Properties of a basic amino acid transport system. Biochim. Biophys. Acta **203**: 139-149.
- SANCHEZ, S., L. MARTINEZ and J. MORA, 1972 Interactions between amino acid transport systems in *Neurospora crassa*. J. Bacteriol. **112**: 276-284.
- SMITH, S. I., 1970 Identification and description of a citrulline-insensitive mutant in *Neurospora crassa*. Masters thesis. California State University, San Diego, California.
- STADLER, D. R., 1966 Genetic control of the uptake of amino acids in *Neurospora*. Genetics **54**: 677-685.
- THWAITES, W. M., 1967 A mutation reducing feedback regulation by arginine in suppressed *pyr-3* mutants in *Neurospora*. Genetics **55**: 769-781.

- THWAITES, W. M. and L. PENDYALA, 1969 Regulation of amino acid assimilation in a strain of *Neurospora crassa* lacking basic amino acid transport. *Biochim. Biophys. Acta* **192**: 455-461.
- VOGEL, H. J., 1956 A convenient growth medium for *Neurospora* (Medium N). *Microbiol. Genet. Bull.* **13**: 42-43.
- WAMPLER, D. E. and J. L. FAIRLEY, 1967 Argininosuccinate synthetase of *Neurospora crassa*. *Arch. Biochem. Biophys.* **121**: 580-586.
- WILEY, W. R. and W. H. MATCHETT, 1966 Tryptophan transport in *Neurospora crassa*. I. Specificity and kinetics. *J. Bacteriol.* **92**: 1698-1705.
- WILLIAMS, L. G., S. BERNHARDT and R. H. DAVIS, 1970 Evidence for two discrete carbamyl phosphate pools in *Neurospora*. *J. Biol. Chem.* **246**: 973-978.
- WOODWARD, V. W., J. R. DEZEEUW and A. M. SRB, 1954 The separation and isolation of particular biochemical mutants of *Neurospora* by differential germination of conidia followed by filtration and selective plating. *Nat. Acad. Sci. U. S.* **40**: 192-200.