GENETIC CONTROL OF THE UPTAKE OF AMINO ACIDS IN NEUROSPORA[1]

DAVID R. STADLER

Department of Genetics, University of Washington, Seattle

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A number of amino acid analogs inhibit growth in microorganisms by repressing or inhibiting enzymes involved in the synthesis of the corresponding amino acids. The present work was begun in an attempt to study the regulation of tryptophan synthesis in Neurospora by examining mutants resistant to the analog 4-methyltryptophan (4MT). However, the mutants isolated in this study turned out to be of quite a different type—altered not in the regulation of tryptophan synthesis, but in the uptake of tryptophan and several other amino acids. Wild-type Neurospora has an effective system for taking up and concentrating these compounds from its environment. In the 4MT-resistant mutants this uptake system is greatly retarded or stopped. Thirty resistant mutants of this type have been examined genetically, and in every case the mutation maps in the same region of linkage group IV. This has been called the *mt* locus (LESTER 1966), and it may be the structural gene for a permease or some other essential component of the uptake system. A preliminary report of some of these observations was presented earlier (STADLER 1963).

MATERIALS AND METHODS

Selective media: Resistant mutants were selected on minimal sorbose medium supplemented with 110 mg/liter DL-4-methyltryptophan or with 10 mg/liter para-fluorophenylalanine. The same media were used in growth tests for the classification of segregants from crosses between sensitive and resistant strains.

Uptake measurements: Uptake was assayed in mycelial pads grown 48 hours in still culture in 20 ml liquid minimal medium in 125 ml flasks at 25°. The pads were washed in distilled water and resuspended in 50 ml of the uptake solution (10^{-4}M or 10^{-3}M L-amino acid labelled with 0.01 μc/ml of tritium) in 250 ml flasks and incubated with shaking. At the end of the incubation period (usually 10 minutes at 25°), each pad was quickly drained and rinsed using suction filtration. The pad was then extracted in 3 ml of water in a boiling water bath for 5 minutes.

The extracts were assayed for tritium in a Packard Tri-Carb liquid scintillation counter, using one-ml samples in 15 ml of Bray's solution (BRAY 1960). The uptake studies on 4MT (and some early studies on tryptophan) employed a colorimetric assay of the blue pigment produced in the reaction with dimethylaminobenzaldehyde (KOMM 1926).

The extracted pads were dried and weighed. The concentration of the uptake material which had been present in the pad at the conclusion of the uptake period was calculated by assuming a pad volume equal to the volume of water weighing five times the dry weight of the pad.

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RESULTS

Fourteen mutants resistant to 4MT were obtained from LESTER (1966). He had treated conidia of wild-type strain 74A with ultraviolet light and plated on minimal sorbose medium supplemented with 4MT. All the mutants were alike in showing vigorous growth on the selective medium. Tetrads from the crosses of these mutants to wild type consistently showed 2:2 segregation for resistance, demonstrating a single-gene difference. The resistance gene has been designated *mt* (LESTER 1966). Because this symbol has been used previously to designate mating type, we shall use the symbol *mtr* instead.

LESTER (1963) designed an *in vivo* demonstration of the inhibition by 4MT of early steps in tryptophan biosynthesis. He used a tryptophan mutant (*tryp-1*; isolation No. 10575) which accumulates fluorescent precursors of tryptophan (anthranilic acid and anthranilic ribonucleoside). The accumulation is inhibited by the presence of 4MT. In order to study the effect of the *mtr* mutation on this inhibition, the *mtr tryp-1* double mutant was obtained from a cross (see *Genetic studies*). The accumulation of fluorescent material by this strain was not inhibited by 4MT (LESTER 1966), indicating that the system was no longer sensitive to feedback inhibition. However, further observations of this strain, made independently by the present author and by LESTER (1966), demonstrated that this was an *indirect* result of the *mtr* mutation. The double mutant required higher concentrations of tryptophan for maximal growth than did the *tryp-1* strain. However, when indole (a precursor) replaced tryptophan as the growth supplement, both strains reached maximal growth on the same concentration. This suggested that the *mtr* mutation had resulted in an impaired capacity for uptake of tryptophan from the medium. If it were also impaired in the uptake of 4MT, this could account for the resistance. Direct tests of uptake of tryptophan and of 4MT by wild-type and *mtr* strains established that this was the case (Table 1, Figure 1).

The wild-type mycelium takes up tryptophan against a concentration gradient. The uptake rate is constant for 15 to 20 minutes. The internal concentration reaches a maximum of 45 to 60 minutes at a level over 100 times that which was present in the starting medium. The uptake is not dependent on an external energy source; it proceeds equally fast whether the tryptophan is suspended in

TABLE 1

Uptake of tryptophan and 4-methyltryptophan by wild type and by an mtr *mutant*

Uptake substrate	Strain	Concentration in pad
tryptophan	*74A*	47
	mtr6a	5.4
4-methyltryptophan	*74A*	56
	mtr6a	4.6

Pads were in 10^{-3}M DL-tryptophan or DL-4MT for 10 minutes at 25° before extraction. Uptake was assayed by the production of blue pigment in the reaction with dimethyl-amino-benzaldehyde. Concentrations are expressed as moles per liter $\times 10^4$.

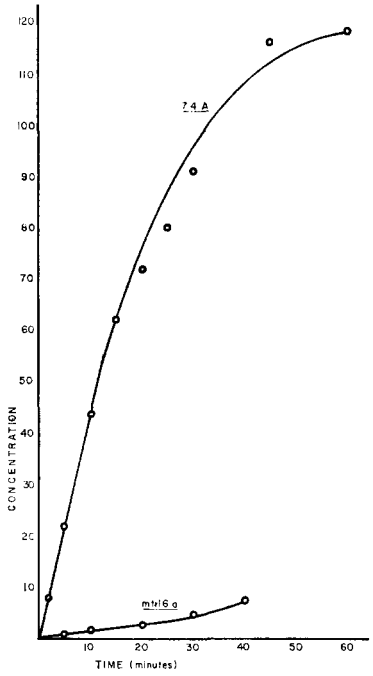

FIGURE 1.—Uptake of tryptophan by wild type (74A) and by an *mtr* mutant. Pads were in tritiated 10^{-4}M L-tryptophan at 25° for various times before extraction. Concentrations are expressed as moles per liter $\times 10^4$.

water or in minimal medium. (However, the presence of 10^{-3}M sodium azide in the medium lowers the rate of uptake by more than 80%.)

Tryptophan uptake by the mutant *mtr*16 proceeds at about 1/20 the rate of wild type. It is not known whether this represents a very retarded uptake system or merely passive accumulation. Six different *mtr* mutants were compared to wild type for tryptophan uptake (Table 2). All had rates less than 15% of that of wild type.

The same system which takes up tryptophan in Neurospora is also responsible for the uptake of the other aromatic amino acids. Wild-type mycelium is capable of active accumulation of tyrosine and phenylalanine from the medium, while the *mtr* mutants are very deficient in this uptake (Table 3). Thus it is not surprising to find that the *mtr* mutants are resistant to para-fluorophenylalanine (FPA), a potent inhibitor of wild type growth. Conidia of the mutants readily form colonies on minimal sorbose medium supplemented with 10 μg/ml FPA, a concentration which completely arrests the wild-type cells. A total of 30 mutants resistant to both 4MT and FPA have been studied. (These include 25 induced with UV, three induced with nitrous acid and two spontaneous mutants.) All 30 mutants map in the same genetic region. About half of them have been tested for tryptophan uptake and all were found to be deficient. Apparently the double resistance phenotype is diagnostic of mutation at the *mtr* locus. LESTER (1966) has observed that *mtr* mutants are also resistant to ethionine (an analog of methionine).

Several other amino acids appear to be controlled by the same uptake system

TABLE 2

Uptake of tryptophan by wild type and by mtr *mutants*

Strain	Concentration
74*A*	43.5
*mtr*6	0.92
*mtr*15	2.24
*mtr*16	1.6
*mtr*17	3.68
*mtr*18	0.32
*mtr*21	6.0

Pads were in 10^{-4}M L-tryptophan-H^3 for 10 minutes at 25° before extraction. Concentrations are expressed as moles per liter $\times 10^4$.

TABLE 3

Uptake of L*-amino acids by wild type and by an* mtr *mutant*

Amino acid	74A	mtr16a
tryptophan	43.5	1.6
phenylalanine	19.2	3.2
tyrosine	16.3	3.7
methionine	48.5	8.6
valine	82.0	8.1
leucine	29.3	13.3
histidine	51.9	23.3
arginine	54.1	43.1
lysine	80.3	89.6

Pads were in tritiated amino acid solutions for 10 minutes at 25° before extraction. Solutions of tryptophan, phenylalanine and tyrosine were 10^{-4} molar; all others were 10^{-3} molar. Concentrations are expressed as moles per liter $\times$ 10^4.

as the aromatic acids. The results in Table 3 show that mutation at the *mtr* locus distinctly retards the uptake of methionine, valine, and leucine and slows the uptake of histidine by about half. Uptake of arginine and lysine appear little affected by this mutation. Another method which has been employed to detect interactions in uptake involves the blocking of uptake of labelled tryptophan by wild type with a second amino acid which is unlabelled (Table 4). It may be seen that the most effective blocking amino acids are the same ones which depend on the presence of the mtr^+ allele for their own uptake.

The uptake system is specific for L-amino acids. D-phenylalanine is completely ineffective at blocking L-tryptophan uptake. Direct tests of uptake of D-tryptophan by mycelia gave the same very low rates for both wild type and an *mtr* mutant. The rate of uptake of indole was also found to be the same in both strains.

The *mtr* mutants are recessive to the wild-type allele. A heterocaryon was constructed between *mtr*6 *lys*-5 (lysine requiring) and *me*-4 (methionine requiring). Both component homocaryons had nutritional requirements, so only the heterocaryotic cells could grow when conidia from the heterocaryon were plated

TABLE 4

Effects of other amino acids in blocking the uptake of tryptophan by wild type

Blocking amino acid	Uptake of tryptophan	Blocking amino acid	Uptake of tryptophan
none	100%	isoleucine	80%
phenylalanine	33	glutamic acid	81
tyrosine	37	lysine	85
leucine	51	glutamine	86
methionine	57	glycine	88
cysteine	59	aspartic acid	93
alanine	73	threonine	94
asparagine	74	serine	98
valine	76	histidine	102
arginine	79	proline	119

Pads were in 10^{-4}M L-tryptophan-H^3 for 10 minutes at 25° before extraction. The concentration of the unlabelled blocking amino acid was 2×10^{-4}M.

on minimal medium. A duplicate plating on minimal plus 4MT gave no colonies, demonstrating that the heterocaryotic cells were sensitive. Similar tests were performed on three other *mtr* mutants. In each case *mtr* was segregating in a heterocaryon between two auxotrophic strains. In each case the heterocaryotic cells formed colonies on minimal medium but not on minimal plus 4MT.

Genetic studies: Before it was realized that the *mtr* gene was not directly involved in regulation, the mutant *mtr*6 was crossed to three different tryptophan-requiring mutants (*tryp-1*, *tryp-2* and *tryp-3*). The aim was to obtain the *mtr tryp* double mutants in order to study the regulation of specific steps in tryptophan synthesis. Direct identification of such double mutants was obscured by the fact that tryptophan requirement is epistatic to 4MT-resistance. That is, even the mtr^+ strains grow on a medium containing 4MT if tryptophan is added. Tryptophan-independent strains can be scored directly for resistance in minimal medium plus 4MT. The double mutant could be identified in a nonparental ditype ascus (one which contained two spore pairs of each recombinant type). In these crosses such an ascus would be made up of two wild-type spore pairs and two double mutant pairs. Both of the $tryp^+$ pairs could be tested on 4MT and shown to be sensitive, so both *tryp* pairs could be assumed to be resistant. Observations of such asci revealed that the double mutants (in the crosses to *tryp-1* and *tryp-2*) could be recognized directly, because they experienced a lag period of about one week after germination on minimal medium plus tryptophan before growing up and conidiating. The combination of *mtr*6 with *tryp-3* appears to be lethal. Spores which could be inferred to have this genotype did germinate but failed to grow further on either supplemented minimal medium or complete medium.

In a cross segregating at two linked loci, a nonparental ditype ascus can only be produced by a 4-strand double crossover (or a more complicated event) between the two loci. Although only a few asci were classified from each of these crosses, there were sufficient numbers of nonparental ditypes to show that *mtr* is not closely linked to any of these three tryptophan loci.

Pooled data from the tetrad analyses of the above crosses showed that *mtr* segregated in the first division in 32 out of 47 asci, thus the gene controlling this character is centromere-linked. The *mtr* mutant was crossed to centromere-linked markers in each of the seven linkage groups. Analysis of these crosses indicated linkage in only one case, the cross to *col-4*(70007), a morphological (colonial) mutant in linkage group IV. Random spores from this cross on minimal sorbose medium gave similar numbers of wild-type and colonial colonies. When these spores were plated on medium supplemented with 4MT, at least 99% of the colonies were wild-type. The infrequent resistant colonials on these plates were presumed to be the double mutants which resulted from a crossover between two closely-linked loci. This interpretation was confirmed by crossing the double mutant to wild type; when spores from this cross were plated on 4MT medium, nearly all the colonies were the colonial type. Subsequently the other 29 mutants with the *mtr* phenotype (resistant to both 4MT and FPA) were all crossed to *col-4* and each showed the above result, indicating close linkage.

Further crosses were studied to determine the location of the *mtr* locus more precisely. The *mtr*6 *col-4* double mutant was crossed to *tryp-4*, which was known to be in the same linkage group. However there was about 8% recombination between *mtr* and *tryp* in this cross, and *mtr* showed much closer linkage to *col-4*.

The *pdx-1* locus (pyridoxine requirement) is proximal to the *col-4* locus and closely linked to it (MITCHELL 1955). (In the present work, the classification of 670 germinated random spores from the cross *col-4* × *pdx-1* yielded 14 recombinants, a frequency of 2.1%.) Crosses were studied which were segregating for both *col-4* and *pdx-1* as well as *mtr*. Random spores were plated on minimal sorbose medium supplemented with 4MT and pyridoxine, and recombinants between *col-4* and *mtr* were picked out by visual examination. These were isolated and scored for the unselected marker *pdx-1* (first two crosses of Table 5). The results suggest that the *mtr* locus lies between the other markers, giving the following order, reading out from the centromere: *pdx-1-mtr-col-4*. However, the results are also compatible with *pdx-1* lying between the other two, if it is much closer to *mtr* than to *col-4*. The correct arrangement was determined by selecting *pdx*+ *mtr* recombinants on minimal sorbose plus 4MT and scoring them for the unselected colonial marker (last two crosses of Table 5). The only arrangement compatible with these results as well as the preceding ones is *pdx-1-mtr-col-4*.

TABLE 5

Linkage relations between pdx-1, col-4 *and mtr*

Cross	Total germinated spores	Selected recombinants	Classification for unselected marker
*col-4 mtr*18*A* × *pdx-1a*	1000	4 *mtr col*+	4 *pdx*+
74*A* × *pdx-1 mtr*19 *col-4a*	1400	7 *mtr col*+	6 *pdx*, 1 *pdx*+
74*A* × *pdx-1 mtr*19 *col-4a*	1500	8 *pdx*+ *mtr*	8 *col*
col-4A × *pdx-1 mtr*15*a*	2200	14 *pdx*+ *mtr*	14 *col*+

DISCUSSION

There are many reports of inhibition of amino acid-requiring mutants of Neurospora by other amino acids (see BROCKMAN 1964 for references). BROCKMAN studied a tryptophan mutant and observed that its growth on tryptophan was inhibited by any of 14 other amino acids. The most effective inhibitors were the same amino acids that have been shown to block the uptake of tryptophan by wild type in the present work. BROCKMAN noted that when one of these same inhibiting amino acids was added at much lower concentration to the tryptophan-supplemented medium, it *enhanced* the growth of the tryptophan mutant; he concluded that the other amino acid was slowing the uptake of tryptophan and thus permitting its more efficient utilization. The basis of this more efficient utilization with slowed uptake was revealed by the observation of MATCHETT and DEMOSS (1963) that Neurospora supplied with exogenous tryptophan returned much of it to the medium as anthranilic acid or formylanthranilic acid; if the strain was a mutant blocked between anthranilic acid and tryptophan, it wasted as much as 95% of the exogenous tryptophan by channeling it into this anthranilic acid cycle. The experiment reported in Figure 1 was performed with tryptophan labelled only on the side-chain, which remains inside the mycelium when anthranilate is split off and excreted. A parallel experiment using generally labelled tryptophan gave only about half as much accumulation of the labelled material in 10 minutes. This indicates that even in this short time, much of the newly entered tryptophan has gone into the anthranilic acid cycle.

One would like to know whether *mtr* is the structural gene for a permease, and, if not, what relation it bears to the uptake system. One kind of evidence which would favor the structural gene hypothesis would be *qualitative* alterations of the uptake system controlled by mutation at the *mtr* locus. Experiments now in progress were designed to select such qualitatively altered mutants. For example, starting with a tryptophan-requiring mutant, selection is made for a strain which can utilize exogenous tryptophan even in the presence of high concentrations of phenylalanine. Such "phenylalanine-resistant" mutants have been isolated and two have been found to map at or near the *mtr* locus. However, it is not yet clear whether the uptake system in these mutants has an altered substrate specificity, as opposed to a generally enhanced capacity for uptake.

Different *mtr* mutants show some differences in their rates of uptake of tryptophan (Table 2). These strains did not have identical genetic backgrounds, so the differences in uptake could have been controlled by genetic elements either at the *mtr* locus or elsewhere. In order to distinguish between these two possibilities, *mtr*18 and *mtr*21 were both crossed to the same mtr^+ strain and six *mtr* progeny from each cross were assayed for tryptophan uptake. There was some overlap in rates between the two sets, showing that at least part of the original uptake difference was *not* attributable to the *mtr* locus.

Several loci other than *mtr* have been shown to affect the uptake of some of the same amino acids. ST. LAWRENCE *et al.* (1964) studied a *tryp-3* mutant of Neurospora which was inhibited by the addition of yeast extract or peptone to

tryptophan-supplemented medium. They selected a mutant from this strain which was able to grow on the complex medium, and demonstrated that the escape from inhibition was controlled by a modifier (*mod-5*) unlinked to *tryp-3*. Seven other aromatic amino acid mutants were also released from the inhibitory effects of yeast extract or peptone by the action of *mod-5*. The authors demonstrated that leucine was a strong inhibitor of growth of the aromatic amino acid mutants and that *mod-5* relieved this inhibition. However, complications in the effects of *mod-5* on uptake of the aromatic amino acids, leucine and other related compounds led them to conclude that the primary action of *mod-5* was not on the uptake system itself but on a cell membrane barrier to permeability. Genetically *mod-5* is unlinked to *mtr*.

KAPPY and METZENBERG (1965) have shown that 55701, a mutant unlinked to either *mod-5* or *mtr*, is resistant to FPA and ethionine and has reduced uptake of ethionine.

An unlinked suppressor of *mtr* partially restores the uptake of the aromatic amino acids and the sensitivity to 4MT and FPA (STADLER 1965). A mutant resistant to FPA has been isolated in a strain carrying the suppressor; this mutant, R2, is at still another locus (unlinked to *mtr*, *mod-5*, and 55701) and mycelial pads of this strain show reduced uptake of the aromatic amino acids; however, germinating conidia of R2 appear to have normal uptake rates (while *mtr* strains have low uptake in either pads or conidia).

A study of the uptake of amino acids in yeast has led SURDIN *et al.* (1965) to conclude that a single permease controls the uptake of all the amino acids; these authors describe a mutant in which the rate of uptake of the various amino acids is lowered to about one-tenth that of wild type.

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SUMMARY

Thirty resistant mutants have been selected on media supplemented with inhibitory analogs of tryptophan and phenylalanine. All the mutants are alike in the following genetic and physiological characteristics: (1) resistance is controlled by a single-gene difference from wild type; (2) the mutant locus, *mtr*, is on linkage group IV between *pdx-1* and *col-4*; (3) the mutants are resistant to both 4-methyltryptophan and *p*-fluorophenylalanine; (4) wild-type strains have an active system for the uptake of tryptophan and phenylalanine and their analogs from the surrounding medium; in the resistant mutants this uptake system is largely or completely inoperative; the uptake of tyrosine, methionine, cysteine and leucine is also greatly depressed in these mutants.—The relationship of *mtr* to this uptake system is discussed.

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