MUTATIONAL ALTERATION OF PERMEABILITY IN NEUROSPORA: EFFECTS ON GROWTH AND THE UPTAKE OF CERTAIN AMINO ACIDS AND RELATED COMPOUNDS[1]

PATRICIA ST. LAWRENCE, BARBARA D. MALING,[2] LYNNE ALTWERGER, AND MARTIN RACHMELER[3]

Department of Genetics, University of California, Berkeley

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AROMATIC amino acid mutants of *Neurospora crassa* grow poorly on media supplemented with yeast extract or peptone unless the required amino acid or a metabolic precursor is added. However, when conidia of a tryptophan mutant were plated on media containing yeast extract or peptone, many colonies appeared after a few days. Most of these colonies consisted of organisms that still required tryptophan but had gained a capacity for prolific growth on media containing either supplement as the sole source of tryptophan. In the cases studied, the ability to grow well on complex media appeared to be determined by mutation of a single gene. The analysis of the genetics and physiology of one such modifying mutation, *mod-5*, is the subject of this report.

MATERIALS AND METHODS

Media: VOGEL's minimal medium N (VOGEL 1956) with 2 percent sucrose was used for assays of growth and the maintenance of cultures. This medium was supplemented as required; concentrations of metabolites and of Bacto yeast extract (Difco) or Bacto peptone (Difco) are given in the descriptions of individual experiments.

Crosses were made on synthetic cross medium (WESTERGAARD and MITCHELL 1947) with 2 percent agar and supplemented as required in particular crosses.

Standard growth assay: In general, conventional methods for the culture of Neurospora were used. Alteration of the procedure for quantitative growth assays described by TATUM and BEADLE (1942) was necessary to give reproducible results. There was considerable variability in the amount of growth obtained unless the number of conidia used to initiate growth was standardized. In all experiments, 10^4 conidia from 4 to 7 day old slants were inoculated into 20 ml of medium in a 125 ml Erlenmeyer flask and incubated at 34°C without agitation. The mycelium was harvested after 3 days, dried, and the average of the weight of mycelium from three flasks was recorded. For many supplements, it was observed that if the concentration of supplement is plotted on a logarithmic scale against the dry weight of mycelium in arithmetic units, a linear relation is observed at limiting concentrations of the growth factor.

Enzyme assays: The procedures of RACHMELER and YANOFSKY (1961) were followed for the extraction and partial purification of tryptophan synthetase, and enzyme activity was assayed by the methods of YANOFSKY (1955, 1956). The method of SUSKIND (1957) was used to assay material that cross-reacts with antibody to tryptophan synthetase.

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[2] Present address: Department of Bacteriology, University of California, Davis.

[3] Present address: Department of Microbiology, Northwestern University, School of Medicine, Chicago, Illinois.

Assays of peptidase activity were performed with crude extracts prepared by the methods used for tryptophan synthetase extraction.

The total amount of protein in extracts was determined by the method of LOWRY, ROSEBROUGH, FARR, and RANDALL (1951).

Uptake of metabolites: Mats produced by growth on 20 ml of medium at 34°C for 36 hours were washed with distilled water and placed in flasks containing the metabolite in 40 to 60 ml of minimal medium without added carbon source. The flasks were incubated on a reciprocating shaker in a 34°C water bath. Samples of the incubation fluid were removed at intervals and assayed for residual metabolite content. Attempts to assay the aromatic compounds by spectrophotometry at 270–280 mμ proved unsatisfactory because mycelial pads of all genotypes examined excreted material with appreciable absorption in this region of the spectrum. The interfering material, which was also produced by pads incubated in minimal medium or phosphate buffer, was not identified.

Measurement of metabolite concentrations: Indole was measured by the colorimetric procedure of YANOFSKY (1955). Free amino acids: leucine, phenylalanine, tyrosine, and tryptophan were determined by microbiological assay using *Leuconostoc mesenteroides* P-60. Glycyl-L-tryptophan was assayed with *Escherichia coli* td_4 and glycyl-L-tryrosine with *Bacillus subtilis* SB75.

The total leucine content of complex substrates was determined microbiologically after hydrolysis with hydrochloric acid or barium hydroxide. The total tryptophan content was assayed microbiologically after hydrolysis with barium hydroxide or by a modification of the Voisenet-Rhoade reaction (TOMIYAMA and SHIGEMATSU 1934–35).

Sephadex chromatography: Concentrated aqueous solutions of peptone (30 percent) or yeast extract (10 percent) were eluted from Sephadex G-50 columns with distilled water. All the ninhydrin positive material was collected in 4 ml fractions. Samples of the fractions were tested for growth stimulation or inhibition after sterilization. Other samples, after concentration *in vacuo* over NaOH, were chromatographed on paper and stained with ninhydrin and Ehrlich's reagent to identify tryptophan and peptides of tryptophan. One dimensional paper chromatography was performed with butanol-acetic acid as ascending solvent; in two dimensional chromatography butanol-pyridine was followed by phenol-ammonia (SMITH 1960).

Strains: The wild-type strains used were 74A, 74-OR8-1*a*, 74-OR23-1*A*. The aromatic amino acid mutant strains most extensively studied are listed below.

The designations *tryp-1*, *tryp-3*, and *tryp-4* describe different loci with a mutant phenotype of tryptophan requirement; *td* is the symbol for alleles of the *tryp-3* locus. *arom-1* mutants are

Designation in this paper	Isolation or allele number	Reference
tryp-1	10575	1
tryp-2	75001	1
tryp-3		
td-1	C83	1
td-2	S1952	1
td-3	td-3	2
td-16	td-16	2
td-24	td-24	2
td-201	A78	3
tryp-4	Y2198	1
arom-1	Y7655	1
tyr-1	Y6994	1
tyr-3	T-145	5
phen-tyr	NS 1 (symbol *pt*)	4

References: (1) BARRATT, NEWMEYER, PERKINS, and GARNJOBST 1954. (2) YANOFSKY and BONNER 1955. (3) AHMAD and CATCHESIDE 1960. (4) STRICKLAND 1961. (5) DEBUSK and WAGNER 1953.

blocked in an early step in aromatic amino acid biosynthesis and require shikimic acid or a mixture of tryptophan, phenylalanine, tyrosine, and *p*-aminobenzoic acid; *tyr-1* and *tyr-3* strains require tyrosine; *phen-tyr* strains require phenylalanine and tyrosine. The relationship of the mutants to each other is given in Figure 1.

The *tryp-3* and *tyr-1* mutants have been extensively backcrossed to the wild-type stocks. The other strains were obtained from DR. DAVID PERKINS or from the Fungal Genetics Stock Center and were backcrossed at least once to a wild-type stock. Segregants were selected for vigorous growth and are certainly diverse in genetic background. It was possible to obtain cultures of *tryp-4* that are not subject to the tryptophan inhibition reported to be characteristic of that mutant (NEWMEYER 1951), and our wild-type and *td*-1 stocks appear to be less sensitive to inhibition by tryptophan than those studied by SOBOREN and NYC (1961).

RESULTS

When conidia of a *tryp-3* mutant (with or without treatment with ultraviolet light (UV)) were plated on a medium containing 0.5 percent yeast extract as the only source of tryptophan and L-sorbose to restrict the mycelial growth, colonies of many sizes appeared after two or three days at 34°C. Additional colonies continued to appear on plates incubated for longer periods, and microscopic examination of the plates revealed still other centers of growth. Several of the large, fast-growing colonies were isolated from platings of UV irradiated conidia of strains *td*-1 and *td*-16. Extensive study of one isolate, designated *td*-16; *mod*-5, and preliminary observations on two others will be described.

Genetic studies: The *td*-16; *mod*-5 stock resembled the parental *td*-16 strain in its complete inability to grow on minimal medium but differed in its capacity to grow well on media supplemented with yeast extract or peptone. In progeny from crosses of the *td*-16; *mod*-5 strain to wild-type stocks, the modified phenotype could be recognized only in the auxotrophs; these were classified by testing on a medium containing yeast extract or peptone. Segregation of the ability to grow on complex media was evident among the tryptophan requiring members of asci from a cross of the *td*-16; *mod*-5 stock to wild type. Three asci were selected for further study: the presence of the *mod*-5 gene in the auxotrophic members

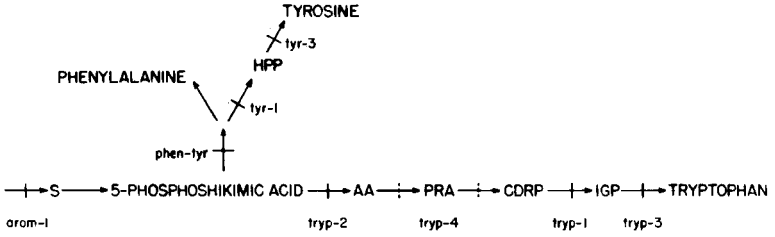

FIGURE 1.—A summary of the pathway of aromatic amino acid biosynthesis in *N. crassa*. S is shikimic acid; HPP, *p*-hydroxyphenylpyruvic acid; AA, anthranilic acid; PRA, N-(5′-phosphoribosyl) anthranilic acid; CDRP, 1-(*o*-carboxyphenylamino)-1-deoxyribulose-5-phosphate; IGP, indole glycerol phosphate. Positions of established metabolic blocks in the mutants are indicated by solid bars across the arrows; less well established blocks are indicated by broken bars. (See WEGMAN and DEMOSS 1964 for a discussion of the enzymes and intermediates between AA and IGP.)

of the asci was verified by the analysis of progeny from a backcross to wild type, and the presence of the *mod*-5 gene in the prototrophs was determined by examination of the progeny from a backcross to a culture of *td*-16. The *mod*-5 phenotype was associated with two spore pairs in each ascus and is, therefore, a gene-determined character. Additional analysis of approximately 25 asci has confirmed this conclusion.

The effect of the *mod*-5 gene on the growth of other alleles of the *tryp*-3 locus was studied. A prototrophic *mod*-5 strain was crossed to the five other *tryp*-3 mutants listed in MATERIALS AND METHODS; mutant progeny showing the modified growth potential were obtained from each cross. The genetic constitution of one or two asci from each cross was verified by the backcrossing procedures described in the preceding paragraph.

Crosses of stocks carrying the *mod*-5 gene to mutants blocked in earlier steps in the formation of tryptophan and to mutants with defects elsewhere in aromatic amino acid biosynthesis showed that, in every case, the *mod*-5 phenotype could be recognized in some of the auxotrophic progeny. The general scheme for the synthesis of aromatic amino acids in Neurospora and the position of the metabolic blocks associated with particular mutants studied is given in Figure 1. Concomitance of the modified phenotype and the presence of the *mod*-5 gene was established for each of the eight loci shown in Figure 1 by backcrossing.

When most of the genetic work had been completed, it was discovered that isolates carrying the *mod*-5 mutation were more sensitive than unmodified strains to certain analogues of aromatic amino acids (see the later section on: *The effect of the modifier on the uptake of metabolites*). This differential sensitivity provided a means of recognizing the *mod*-5 mutation in prototrophic and mutant progeny, and it was therefore possible to locate the *mod*-5 gene by linkage to the *tryp*-2 locus in linkage group VI. The *mod*-5 mutation shows about 3 percent second division segregation.

Preliminary observations on the mod-5 *mutation:* The results of quantitative growth assays of nine mutants, both modified and unmodified, on minimal, two concentrations of yeast extract, and peptone are recorded in Table 1. All the modified mutant strains grew better than their unmodified counterparts on the complex media although some unmodified mutants were capable of considerable growth (e.g., *tryp*-1). This growth of unmodified mutants on yeast extract or peptone appeared to be correlated with incomplete metabolic blocks in the mutants; leakage of the *tryp-1*, *tryp-2*, and *tryp-4* strains has been demonstrated (BONNER, YANOFSKY, and PARTRIDGE 1952), and both modified and unmodified cultures of the latter two produced traces of growth in minimal medium (column 3 in Table 1). The *tryp-3* strains appear to be much less leaky than the other tryptophan mutants; the *tryp-3* mutants described in Table 1 and the four others listed in MATERIALS AND METHODS all showed similar responses. It will be noted that the modified prototrophic strain grew less well than wild-type on all media. This difference in growth was observed with five other prototrophic pairs.

The effect of the modifier on tryptophan synthetase: The *mod*-5 mutation does not appear to act as a suppressor of any of the aromatic amino acid mutants:

TABLE 1

The growth of aromatic amino acid mutants on complex media

Strain	Presence (+) or absence (−) of *mod-5*	Dry weight in milligrams from minimal medium supplemented with: 0	0.5% Yeast extract	2% Yeast extract	2% Peptone
td-16	+	0	73.2	155.8	110.5
	−	0	0	0	7.3
td-201	+	0	42.9	124.0	63.1
	−	0	<1	1.5	2.1
tryp-1	+	0	85.8	163.9	106.6
	−	0	46.6	79.9	69.4
tryp-2	+	<1	80.4	177.1	132.1
	−	<1	5.0	21.2	26.3
tryp-4	+	<1	83.6	164.7	99.5
	−	<1	16.6	35.8	53.6
arom-1	+	0	67.9	116.9	132.5
	−	0	6.2	7.7	<1
tyr-1	+	0	28.5	29.0	68.0
	−	0	0	0	0
tyr-3	+	<1	138.4	201.3	143.8
	−	<1	59.6	57.6	0
phen-tyr	+	0	90.2	131.7	147.8
	−	0	22.0	29.1	12.2
wild-type	+	39.3	112.5	150.3	111.5
	−	52.0	137.5	193.3	136.0

none of the modified strains show an increased ability to grow on minimal medium. Furthermore, direct tests of *tryp-3* mutants for an effect of the *mod-5* mutation on the formation or activity of an enzyme involved in synthesis of one of the aromatic amino acids, tryptophan, were negative. The *tryp-3* mutants do not produce normal tryptophan synthetase, the enzyme that catalyzes the terminal step in tryptophan biosynthesis (MITCHELL and LEIN 1948). The *td*-16 mutant has no activity in any of the three reactions catalyzed by this enzyme; *td*-201 cultures convert indole to tryptophan (RACHMELER and YANOFSKY 1961); and *td*-24 produces a form of the enzyme more readily inhibited by heavy metals at temperatures below 30°C than normal tryptophan synthetase (SUSKIND and KUREK 1957). The enzymatic activity and immunological properties in extracts of strains carrying any one of these mutant sites were not altered by introducing the *mod-5* mutation into the genome. These results seem to preclude any effect of the *mod-5* mutation on the formation or activation of tryptophan synthetase.

The unaltered growth requirements and biosynthetic abilities of modified mutants indicated that the substances in complex media responsible for growth of these strains must be the aromatic amino acids. This conclusion was strengthened by the observation that the growth-promoting ability of yeast extract and peptone for all tryptophan mutants was abolished by prolonged acid hydrolysis. Two hypotheses were therefore considered. First, yeast extract and peptone might

contain insufficient amounts of free aromatic amino acids to support good growth of the unmodified mutants. If, however, considerable quantities of amino acid were bound in peptides that could be utilized only by *mod*-5 strains, the effects of the modifier would be explained. Second, it might be that the complex media do contain concentrations of free aromatic amino acids adequate for the growth of all strains but that some inhibitory substances prevent the growth of unmodified mutants. The action of the *mod*-5 mutation would therefore be to overcome an inhibition.

The effect of the modifier on the utilization of peptides: The capacity of modified and unmodified strains to utilize peptide-bound aromatic amino acids was tested with all the mutants using chromatographically pure compounds. The growth curves obtained with four mutants are given in Figure 2. The growth of *td*-16 and *td*-16;*mod*-5 on L-tryptophan and glycyl-L-tryptophan are shown in part A; essentially similar curves were obtained in assays of all the other *tryp-3* mutants. In Figure 2B, the results of assays of *tryp-1* and *tryp-1;mod*-5 on the same two supplements are given; comparable growth responses were obtained for modified and unmodified strains of *tryp-2* and *tryp-4*. Modified and unmodified strains responded equally to increasing concentrations of tryptophan, although some inhibition of the modified strains was usually observed at high levels of supplement. However, modified and unmodified mutants differed in ability to utilize limiting concentrations of the peptide. The poor response of the unmodified strains to low levels of glycyl-tryptophan could not be attributed to an inhibition by the glycine moiety of the peptide; assays at several concentrations of tryptophan with glycine added in equimolar proportions gave values equal to the growth on tryptophan alone.

Growth curves of modified and unmodified *tyr-1* strains on L-tyrosine and glycyl-L-tyrosine are shown in Figure 2C. In this case, the two strains responded differently to both the amino acid and the peptide. The unmodified *tyr-1* mutant produced only traces of growth on low concentrations of tyrosine and was unable to grow on any level of the 200-fold range of peptide concentrations used. The *tyr-1;mod*-5 culture utilized low concentrations of tyrosine and glycyl-tyrosine effectively but did not show a linear response to concentrations of tyrosine above 0.05μmole/ml. Both cultures described in Figure 2C were obtained from the same ascus, and assays of six other isolates gave almost identical results. It therefore appears that the particular pattern of responses to tyrosine and glycyl-tyrosine shown by the *tyr-1;mod*-5 strains is a property of the *mod*-5 mutation and not of other segregating genes.

Similar enchancement of growth by the modifier was observed with the *tyr-3* mutant, although the unmodified culture showed more growth on low levels of tyrosine than *tyr-1* strains and some growth on high concentrations of dipeptide. However, BROCKMAN, DEBUSK, and WAGNER (1959) reported that their strain did not grow on dipeptides. The extreme leakiness of all our *tyr-3* cultures examined may account for these growth characteristics.

The *phen-tyr* strain requires both phenylalanine and tyrosine, and growth of modified and unmodified mutants on equimolar concentrations of the two amino

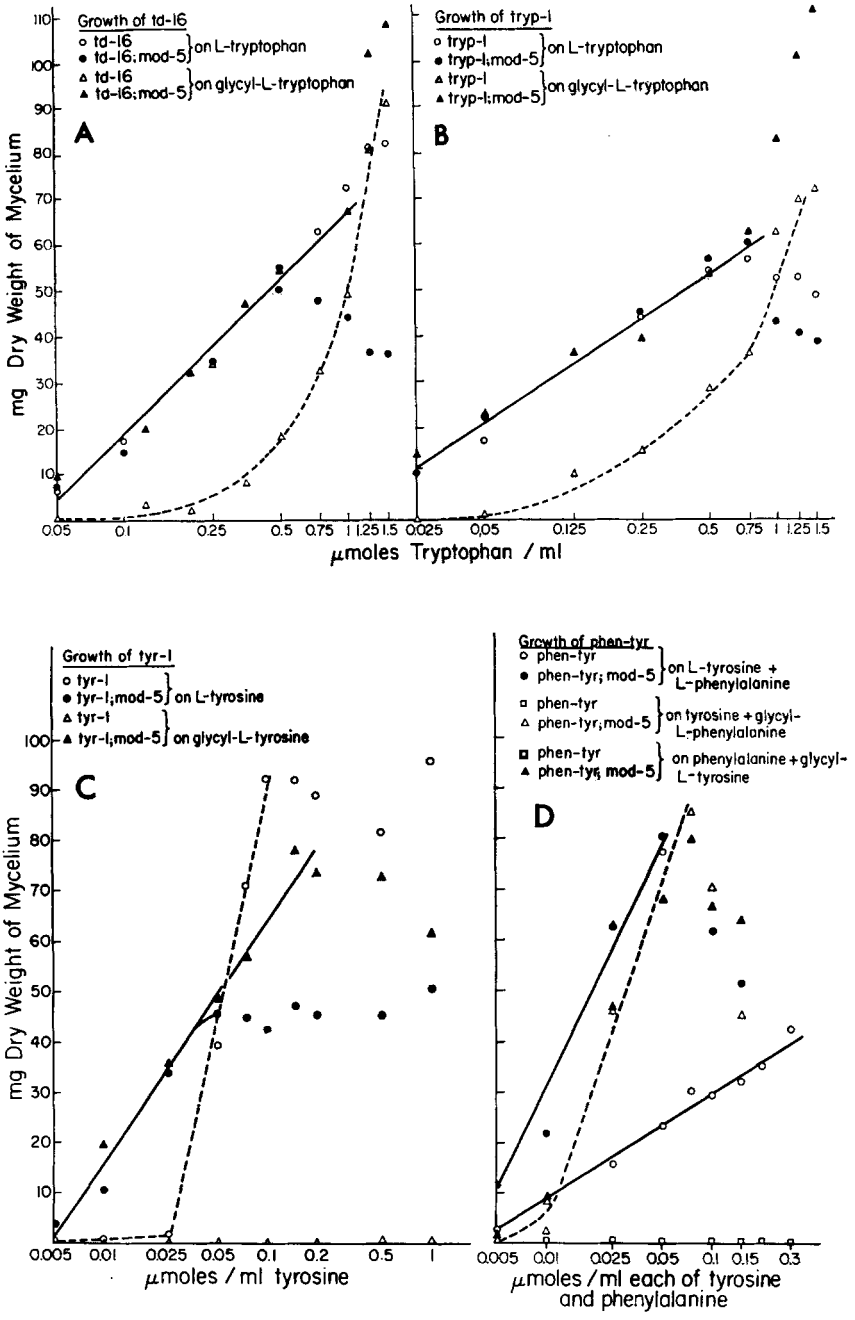

FIGURE 2.—The growth of aromatic amino acid mutants on aromatic amino acids and peptides. The concentration in the medium of the aromatic amino acid (whether free or peptide bound) is plotted on a logarithmic scale in the abscissa.

acids is shown in Figure 2D. The *phen-tyr;mod*-5 strain responded better to free amino acids than the unmodified *phen-tyr* culture. Growth on one aromatic dipeptide was assayed in the presence of an equimolar concentration of the other aromatic amino acid. The unmodified mutant was unable to grow on any concentration of either peptide, whereas the *phen-tyr;mod*-5 culture could use both peptides.

The *arom-1* mutant can grow on a combination of the three aromatic amino acids plus *p*-aminobenzoic acid. The ability of *arom-1* and *arom-1;mod*-5 strains to utilize glycyl-L-tryptophan instead of tryptophan (or glycyl-L-phenylalanine in place of phenylalanine) was determined. Although these tests were not extensive, the growth of the *mod*-5 culture on media containing low concentrations of a peptide was much better than that of the unmodified *arom-1* mutant.

The quantities of other aromatic amino acid peptides available for assay were limited. Modified strains of *td*-16 grew as well on tryptophanyl-L-phenylalanine or phenylalanyl-L-tryptophan as on free tryptophan, but the unmodified *td*-16 culture grew poorly on low concentrations of either peptide. Differential growth of modified and unmodified strains was not observed on leucine dipeptides: both *td*-16 strains grew equally, and well, on leucyl-L-tryptophan and tryptophanyl-L-leucine; *tyr-1* and *tyr-1;mod*-5 stocks responded equally to leucyl-L-tyrosine.

The increased ability of modified strains to grow on certain dipeptides could be due to alterations of specific peptidase activities as a result of the *mod*-5 mutation. However, even if increased peptidase activity could be demonstrated in modified strains, the differences in growth on free amino acids between the modified and unmodified stocks of the *tyr-1* and *phen-tyr* mutants would need additional explanation.

Direct analysis of extracts of modified and unmodified prototrophic, *td*-16, and *tyr-1* strains showed that the modifier does not increase the capacity to split glycine dipeptides of tryptophan and tyrosine. The results are given in Table 2. The differences in specific activity with glycyl-tyrosine as substrate are not considered significant. No peptidase activity was found in culture filtrates of these strains.

Investigations of yeast extract and peptone: The ability of modified strains to grow on peptides not active for the unmodified strains could be related to their increased growth on complex media. A search for peptides of tryptophan in yeast extract and peptone was therefore undertaken by examination of fractions eluted from a Sephadex G-50 column. No indication of any substantial amount of tryptophan peptides in any fraction from either yeast extract or peptone was obtained. Other samples of the eluted fractions were tested for the ability to stimulate growth of *td*-16 and *td*-16;*mod*-5 cultures by auxanography (RYAN 1950). Some of these fractions stimulated growth of both modified and unmodified strains. These active fractions all contained free tryptophan, and, when examined by the method of HIRS, MOORE, and STEIN (1956), proved to have essentially no peptide-bound amino acids. In fact, the component apparently responsible for most, if not all, of the growth of the modified mutant on complex media is free tryptophan.

TABLE 2

The specific activity of crude extracts for substrates A: glycyl-L-tryptophan, and B: glycyl-L-tyrosine

Strain and supplement in medium	Substrate and product assayed	Presence (+) or absence (−) of *mod*-5	Specific activity
td-16, tryptophan	A and tryptophan	−	1.5
		+	1.6
wild type, tryptophan	A and tryptophan	−	1.6
		+	1.4
td-16, tryptophan	B and tyrosine	−	0.4
		+	0.7
wild type, tryptophan	B and tyrosine	−	0.5
		+	0.8
tyr-1, tyrosine	B and tyrosine	−	0.5
		+	0.8
wild type, tyrosine	B and tyrosine	−	0.4
		+	0.4
tyr-1, glycyl-L-tyrosine	B and tyrosine	−	. .
		+	1.2
wild type, glycyl-L-tyrosine	B and tyrosine	−	0.4
		+	0.4

All strains were grown on minimal medium with: tryptophan, 0.2 mg/ml; or tyrosine, 0.02 mg/ml; or glycyl-L-tyrosine, 0.02 mg/ml. The specific activity is the μmoles of L-amino acid produced per mg of protein after incubation of crude extracts with excess dipeptide for 30 minutes at 37°C in 0.1M pH 7.0 phosphate buffer.

Some of the fractions obtained from peptone inhibited the growth of *td*-16 but not that of *td*-16;*mod*-5 cultures on tryptophan. An examination of these inhibitory fractions by two dimensional chromatography on paper, followed by a ninhydrin spray, revealed 11 spots each with the Rf of a known amino acid; the most intense spot corresponded in position to leucine. All of the common amino acids were tested for inhibition of the growth on tryptophan of modified and unmodified *td*-16 strains. Several amino acids were inhibitory, but only leucine affected the two strains differently: the growth of *td*-16;*mod*-5 cultures was not appreciably restricted at levels of leucine that completely suppressed growth of the *td*-16 stock.

The effect of leucine on the growth of modified and unmodified tryptophan mutants on minimal media supplemented with suboptimal concentrations of tryptophan and glycyl-L-tryptophan was assayed. The percent inhibition for two concentrations of leucine is given in Table 3. The variation between separate repetitions of these growth assays was approximately 15 percent, and it is considered that inhibitions of 15 percent or less are not significant. The inhibition of growth of the unmodified tryptophan mutants at both concentrations of leucine was, however, significant, particularly in comparison to the lack of inhibition of the *mod*-5 cultures. Similar results were obtained with modified and unmodified strains of the other aromatic amino acid mutants given in Figure 1. No inhibition of either modified or unmodified mutants was found when D-leucine was used.

TABLE 3

The inhibition of growth of tryptophan mutants by L-*leucine*

Strain	Presence (+) or absence (−) of *mod*-5	Percent inhibition of growth on media supplemented with: 0.25 μM/ml tryptophan + 2.5 μM/ml leucine	0.25 μM/ml tryptophan + 5 μM/ml leucine	0.25 μM/ml glycyl-L-tryptophan + 2.5 μM/ml leucine	0.25 μM/ml glycyl-L-tryptophan + 5 μM/ml leucine	0.25 μM/ml indole + 5 μM/ml leucine
td-16	+	0	0	4.2	0	. . .
	−	25.8	100	76.9	69.3	. . .
td-201	+	0	0	0	0	0
	−	60.7	100	71.8	58.8	6.3
tryp-1	+	0	0	21.0	14.4	0
	−	56.1	83	81.6	85.4	0
tryp-2	+	0	5	4.4	11.0	9.9
	−	42.0	100	61.4	60.9	0
tryp-4	+	0	0	4.0	0	7.1
	−	0	80.9	58.0	65.7	8.5
wild type	+	1.5	0	0	9.0	20.4
	−	0	0	0	5.3	1.0

If yeast extract and peptone have a sufficiently high concentration of leucine compared to tryptophan, the poor growth of unmodified mutants on these supplements would be accounted for. Therefore, the amounts of free L-tryptophan and L-leucine in unhydrolyzed yeast extract and peptone and the total amount of these amino acids released from peptide linkage by hydrolysis with hydrochloric acid or barium hydroxide were determined. The results of these determinations are given in Table 4. The analyses showed: (1) there is sufficient free tryptophan in the supplements at the concentrations used in growth assays to provide for more growth of unmodified tryptophan mutants than is actually observed; (2) the fraction of bound tryptophan is not sufficient to explain the difference in growth between modified and unmodified tryptophan mutants on yeast extract or pep-

TABLE 4

The approximate leucine and tryptophan concentration of yeast extract and peptone

	0.5 percent yeast extract		2 percent peptone	
	μmoles/ml of: L-leucine	L-tryptophan	μmoles/ml of: L-leucine	L-tryptophan
Free amino acid in untreated sample	1.01	0.13	1.92	0.22
Total amino acid in hydrolyzed sample	1.25	0.15	4.67	0.27
Percent bound amino acid in untreated sample	19	13	59	19
Molar ratio of leucine to tryptophan:				
in untreated sample	7.8		8.7	
in hydrolyzed sample	8.3		17.3	

Total leucine values represent an average of those obtained by $Ba(OH)_2$ hydrolysis corrected for racemization and those obtained by HCl hydrolysis. Total tryptophan is an average of the values obtained by $Ba(OH)_2$ hydrolysis (corrected for racemization) and by the Voisenet-Rhoade reaction on untreated material.

tone; (3) the molar ratio of free leucine to free tryptophan in unhydrolyzed yeast extract and peptone is approximately 8:1.

It is difficult to compare growth on minimal medium with defined supplements to growth on media containing complex undefined additions such as yeast extract or peptone, especially as the experiments in minimal medium required a higher concentration of tryptophan than that found in yeast extract and peptone to obtain a sufficient yield of mycelium for meaningful comparisons. However, it appears likely that the ratio of leucine to tryptophan in the complex supplements is unfavorable to the growth of unmodified mutants and partly accounts for the inability of these mutants to grow well. Accessory inhibitions by other amino acids cannot be disregarded.

The effect of the modifier on the uptake of metabolites: In the experiments demonstrating an inhibitory effect of leucine on the growth of tryptophan mutants, the prototrophic cultures seemed relatively unaffected (Table 3). Such strains must, of course, make aromatic amino acids. If leucine were interfering with some step in the synthesis or incorporation into protein of aromatic amino acids, prototrophs should be as readily inhibited as mutants. If, on the other hand, permeability of the aromatic amino acids were the process susceptible to leucine inhibition, then prototrophic strains would be immune. Those mutants capable of synthesizing the required aromatic amino acids from externally supplied precursors might likewise be resistant to inhibition by leucine provided, of course, that entry of the precursor was not prevented by leucine.

Certain tryptophan mutants are capable of forming tryptophan from the substitute, indole. The effect of adding excess leucine to media containing indole is shown in the last column of Table 3. Clearly, leucine did not inhibit the growth of either modified or unmodified mutants when supplied with indole. It is therefore concluded that excess leucine affects the permeation of tryptophan and not the endogenous formation or utilization of this amino acid.

The results of a comparable series of assays with *arom-1* and *tyr-1* cultures were more equivocal. When the medium was supplemented with the required aromatic amino acids, no inhibition of the growth of unmodified strains was observed in the presence of equimolar concentrations of leucine but complete inhibition was found at a ratio of 20 leucine to 1 aromatic amino acid. Modified strains were not inhibited by any concentration of leucine. When grown on the intermediary metabolite, shikimic acid, both modified and unmodified *arom-1* strains were partially inhibited by equimolar concentrations of leucine. Complete inhibition was never observed with either strain, even at a ratio of 20 leucine to 1 shikimic acid. Similar results were obtained with the *tyr-1* mutants grown on the tyrosine precursor, *p*-hydroxyphenylpyruvic acid (METZENBERG and MITCHELL 1956). Although the results with the *tyr-1* and *arom-1* mutants suggest that the role of leucine is more complex, an effect on the permeability of the aromatic amino acids is indicated.

A difference in permeation between modified and unmodified strains might be expressed as a difference in the rate of removal of metabolites from the medium. Therefore, young mycelial pads were incubated in the presence of a metabolite,

and the quantity of metabolite remaining in aliquots of the medium was determined at successive intervals by microbiological assay. The disappearance of an aromatic amino acid or indole from the incubation mixture was examined in the presence and absence of excess leucine; typical results are presented in Figure 3.

Figure 3A describes the removal of tryptophan by mycelia of genotypes representing all the four combinations of the *td*-16 and *mod*-5 genes. Studies on the removal of tyrosine by four cultures of differing genotype from an ascus segregating for the *tyr-1* and *mod*-5 genes are given in Figure 3B. Modified cultures removed tryptophan or tyrosine immediately and rapidly, unmodified pads appeared to have a lower initial rate, but the final rate was the same for all cultures. The addition of leucine to the incubation mixture delayed the initiation of tryptophan or tyrosine disappearance. Although modified cultures recovered from this inhibition more rapidly than unmodified cultures, the rates of removal never equalled those of pads incubated without leucine.

In contrast to all the other metabolites studied, phenylalanine was removed at a rate that decreased with time (Figure 3D). For both modified and unmodified prototrophic cultures, there was no demonstrable lag in the initiation of phenylalanine removal, but the unmodified mycelium showed a lower rate than the modified pads throughout the duration of the experiment. The decreasing rate of phenylalanine disappearance is not understood. Agreement between duplicate assays was often poor, particularly in studies of the effect of the addition of excess leucine to the incubation mixture. It was clear, however, that excess leucine completely suppressed the removal of phenylalanine by both modified and unmodified cultures for at least 3 hours.

The disappearance of indole from the medium was examined with pads of strains *td*-201 and *td*-201;*mod*-5 (Figure 3C). Indole removal in the absence of leucine was initially rapid and somewhat greater in the modified than in the unmodified strain. Leucine depressed the initial rate of both cultures, but to only about one half the final value. This partial inhibition was of much shorter duration than the inhibition of removal of the aromatic amino acids, and the final rate approximated that of the cultures incubated without leucine.

Mycelial mats of modified and unmodified prototrophic cultures were used to follow the disappearance of glycyl-L-tryptophan and glycyl-L-tyrosine from the medium. One experiment on glycyl-tryptophan removal is depicted in Figure 3D. In all experiments with this dipeptide, the unmodified strain showed a pronounced lag (from 2.5 to over 4 hours) before any uptake could be detected; the modified strain occasionally exhibited a short lag of ½ hour. The assays for glycyl-tyrosine disappearance were less satisfactory than those for glycyl-tryptophan. There was no lag before modified cultures began to remove glycyl-tyrosine, and a delay of 1.5 to 2 hours was evident with unmodified mycelium. The final rate of removal of these dipeptides was considerably less than the final rate of aromatic amino acid uptake and appeared to be independent of the genotype of the cultures.

The removal of L-leucine in the presence and absence of excess tryptophan was also studied with modified and unmodified prototrophic cultures. The course

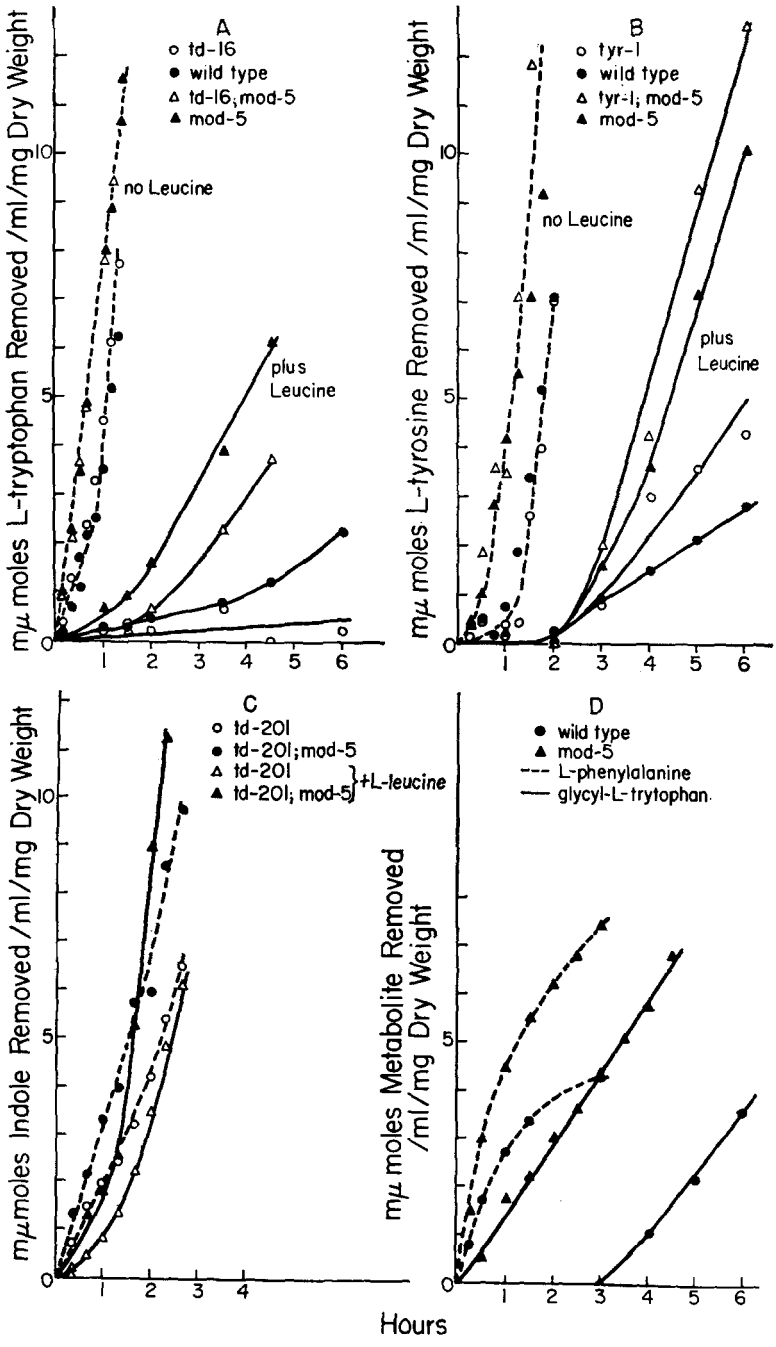

FIGURE 3.—The removal of metabolites from solution by various strains. The initial concentration of all metabolites was 0.15 μM/ml except for L-tyrosine: 0.22 μM/ml, and L-leucine: 3.0 μM/ml.

of these experiments closely resembled those for the reciprocal combinations of amino acids plotted in Figure 3A.

The addition of excess leucine to flasks of modified and unmodified mycelia actively removing tryptophan from the medium resulted in the abrupt cessation of tryptophan disappearance and the excretion of tryptophan for one hour. Tryptophan removal was again detectible after this time at a rate characteristic of pads incubated in the presence of excess leucine from the beginning (Figure 3A).

SOBOREN and NYC (1961) reported that excess leucine inhibits growth of a tryptophan mutant, *td*-1, on Fries minimal medium (BEADLE and TATUM 1945) supplemented with tryptophan. Therefore, several of the tryptophan mutants used in the present study were assayed with tryptophan and excess leucine using FRIES medium: no inhibition of growth of modified or unmodified cultures was observed. The substitution of FRIES for medium N did not, however, appreciably alter the characteristic responses of unmodified aromatic amino acid mutants to dipeptides and amino acids. FRIES medium and medium N differ in the type and quantity of various salts; the factors responsible for the manifestation of leucine inhibition were not explored. These observations suggest that immunity to leucine inhibition is a secondary consequence of the fundamental alteration occasioned by the *mod*-5 mutation.

If, as appears to be the case, the modifier increases the initial rate of entry of aromatic compounds into the mycelium, modified strains might be more sensitive than unmodified cultures to antimetabolites that interfere with the intracellular metabolism of particular aromatic amino acids. One such analogue is 4-methyltryptophan, reported to inhibit one or more steps in tryptophan biosynthesis (LESTER 1961). STADLER (personal communication) has studied the inhibition of growth of Neurospora by *p*-fluorophenylalanine, a compound shown to replace phenylalanine and tyrosine in the proteins of *E. coli* (MUNIER and COHEN 1956). Assays of modified and unmodified prototrophic strains on several concentrations of either 4-methyltryptophan or *p*-fluorophenylalanine demonstrated that growth of the modified strains was completely inhibited by concentrations of antimetabolites that had little or no effect on unmodified cultures.

This increased sensitivity of *mod*-5 strains was used to determine the dominance or recessivity of the *mod*-5 mutation in a heterocaryon. Conidia from two heterocaryons: *td*-16; *mod*-5$^+$ with *tyr-1*;*mod*-5 and *td*-16;*mod*-5$^+$ with *tyr-1*; *mod*-5$^+$, were plated in sorbose minimal medium with several concentrations of 4-methyltryptophan (.005, .01, .02 mg/ml). The appearance of colonies on plates containing the antimetabolite was delayed compared to the growth on the minimal plates, but there was no significant difference between the two types of heterocaryon in either the time of appearance or frequency of colonies. It was therefore concluded that the *mod*-5 mutation is recessive to its normal allele.

The effect of the modifier on other biochemical mutants: In order to examine the extent of action of the *mod*-5 mutation, it was introduced into the genome of three mutant strains biochemically unrelated to each other and to the aromatic amino acid mutants: *me-7* (methionine requiring, allele 4894), *lys-1* (lysine requiring, 33933), and *pyr-1* (pyrimidine requiring, H263). The genotype of

modified mutants was verified by backcrossing to strain *td*-16. This limited investigation of the spectrum of interaction of the *mod*-5 mutation suggested that the physiological changes associated with it can be detected whenever appropriate conditions for recognition are available.

The *me-7* strain was not inhibited by high concentrations of leucine and grew well on yeast extract and peptone. Growth assays of *me-7* and *me-7;mod*-5 cultures on glycyl-L-methionine gave curves similar to those obtained with the tryptophan mutants: better growth of the modified than the unmodified strain occurred at low concentrations of peptide.

The *lys-1* stock grew well on yeast extract but not at all on peptone. Leucine was not inhibitory. The *mod*-5 gene did not confer a capacity to grow on peptone when introduced into a *lys-1* stock, but the effect of the modifier was evident in the increased growth of *lys-1;mod*-5 cultures (compared to *lys-1* strains) on low concentrations of glycyl-L-lysine.

The *pyr-1* strain grew poorly on yeast extract and peptone; growth of *pyr-1*; *mod*-5 strains on complex media was excellent. The addition of excess leucine to minimal medium containing uridine did not inhibit the growth of either the modified or unmodified isolates.

Other modifiers of tryptophan mutants: One modifier, *mod*-2, originating in strain *td*-16, and one modifier, *mod*-26, induced in stock *td*-1, were studied. When the progeny of appropriate crosses were examined, both of these modifiers appeared to be mutations at loci different from each other and from the *mod*-5 gene. *tryp-3* stocks carrying *mod*-2 or *mod*-26 grow as well on yeast extract or peptone as do *mod*-5 cultures and are not inhibited by excess leucine. The modifiers differ, however, in their capacity to stimulate growth of *tryp-3* mutants on low concentrations of glycyl-L-tryptophan: *mod*-26 strains resemble *mod*-5 cultures in this property, but *mod*-2 has no effect on the utilization of the peptide. The *mod*-2 and *mod*-26 mutations have not been combined with other aromatic amino acid mutants.

DISCUSSION

A number of phenotypic changes are associated with the *mod*-5 mutation. Modified aromatic amino acids mutants show: (1) an increased capacity to grow on media supplemented with yeast extract or peptone; (2) an increased utilization of certain amino acids and peptides; (3) a decreased sensitivity to leucine inhibition. All modified cultures exhibit greater sensitivity than unmodified stocks to inhibition by analogues of the aromatic amino acids. In addition, the *mod*-5 mutation has a detectable effect on the growth of some mutants that require substances other than the aromatic amino acids. All of these phenotypic manifestations appear to be consequences of a single fundamental change in the permeability mechanism.

The relation of permeability to growth: The growth of unmodified aromatic amino acid mutants on limiting concentrations of various supplements indicates that these compounds do not enter the organism with equal efficiency. The effect of the modifier is not to alter the ultimate rate of removal of metabolites but to

decrease the lag before this final rate is established. If the removal of metabolites from the medium by conidia is governed by the same mechanism that operates in the mycelium, a correlation between the duration of the lag in the disappearance of metabolites and the growth responses is evident. The rapid establishment of the final rate of tryptophan uptake in unmodified strains (although increased by the modifier) presumably does not limit growth. On the other hand, the long lag preceding the disappearance of tyrosine from the incubation mixture may explain the poor growth responses of unmodified strains to low concentrations of tyrosine even though the molar concentration of amino acid required for maximal growth is one tenth that needed by tryptophan mutants. The lag in the uptake of glycyl-tyrosine is similar to that observed for tyrosine, but this lag combined with the low final rate for removal of the dipeptide appears to account for the failure of unmodified *tyr-1* cultures to grow on glycyl-tyrosine. The extremely long interval before glycyl-tryptophan is removed from the medium by unmodified cultures may be sufficient in itself to explain the nonlinear response of tryptophan mutants to low concentrations of this dipeptide even if the low final rate were not limiting.

Inhibition by leucine: The experiments with Fries medium suggest that the inhibitory effect of leucine on the uptake of aromatic amino acids is a secondary consequence of the *mod*-5 mutation. This conclusion is strengthened by the observation that the modifier can effect the growth of mutants (*lys-1*, *me-7*, and *pyr-1*) not subject to inhibition by leucine. Nevertheless, under some conditions, leucine does interfere in some manner with the entry of certain compounds into the mycelium. The mechanism for entry of aromatic amino acids in unmodified strains apparently operates at a level sufficient to allow growth in the absence of leucine but, in the presence of leucine, appears unable to accumulate enough of the required metabolite for growth. The modifier increases the initial rate of uptake of a variety of metabolites, including leucine itself. Presumably, the more rapid establishment of the rate of transport of required metabolites into the mycelium in modified mutant strains is adequate to counteract the interference of leucine. It is of interest that the slight initial depression by leucine of the rate of removal of indole from the medium does not cause indole to become growth limiting.

An interrelation between leucine and the aromatic amino acids has been described by BARRATT and OGATA (1954). Later experiments proved that these compounds share no common biosynthetic reactions (BARRATT, FULLER, and TANENBAUM 1956), and the metabolic relation of leucine to the aromatic amino acids therefore remains unexplained. SOBOREN and NYC (1963) have shown that leucine decreases the amount of anthranilic acid accumulated by their *td*-1 strain. The partial inhibition of both modified and unmodified *tyr-1* and *arom-1* mutants by leucine when these strains are grown on precursors of the aromatic amino acids may indicate that some reaction in aromatic biosynthesis is susceptible to interference by leucine. A number of amino acids, including leucine, were found to inhibit the growth of *tyr-3* (BROCKMAN *et al.*, 1959).

The nature of the permeability mutation: Although the general process of

amino acid permeability has received little study in Neurospora, MATHIESON and CATCHESIDE (1955) demonstrated that the uptake of histidine is inhibited by particular combinations of certain amino acids. BROCKMAN (1964) showed that any one of 14 amino acids (in the proper ratio) inhibited the growth of a *tryp-1* mutant on tryptophan but not on indole. He described inhibition of the uptake of tryptophan and suggested that the inhibitory amino acids competed with tryptophan for a common uptake site. There have also been reports of gene mutations leading to changes in the ability to concentrate specific metabolites. The ability to accumulate tryptophan from the medium is abolished in the mutant described by STADLER (1963). This gene is located in a different linkage group than the *mod*-5 mutation. The rate of arginine uptake is decreased by a modifier isolated from a wild-type strain by DAVIS (1963), but the pantothenate modifier studied by him increases the rate of entry of pantothenate into the mycelium (DAVIS 1960). The genetic relationship of these modifiers to the *mod*-5 mutation is unknown.

It appears that the permeability of Neurospora to aromatic amino acids, leucine, indole, and certain dipeptides is controlled by some process common to all these metabolites. Whatever the nature of this unknown mechanism, the differences in initial and final rates of entry among the compounds studied suggest that a degree of specificity is involved. This might be expected if penetration were mediated by a single permease with different affinities for the various metabolites or with different kinetics of induction for each compound. Implication of a single enzyme in the transport of such a variety of metabolites would, however, demand an enzyme with an unusually broad spectrum of action. Attempts to demonstrate that growth of unmodified tryptophan mutants of glycyl-tryptophan is adaptive or to show an effect of adaptation on the uptake of glycine dipeptides by unmodified prototrophic strains were unsuccessful. Furthermore, the immediate cessation of tryptophan removal upon the addition of excess leucine to pads actively removing tryptophan at maximal rate demonstrated that adaptation to the uptake of tryptophan afforded no protection against the inhibitory effects of leucine.

It appears more reasonable to suppose that the structure of the boundary membrane in the wild-type organism imposes a certain rate of entry upon different metabolites, each of which may be actively concentrated by a specific permease. A mutation altering the structure of the membrane could result in an increased flow of compounds into the mycelium. Understanding of the alteration in the permeability mechanism associated with the *mod*-5 mutation is hampered by lack of knowledge of the mode of entry of amino acids and related compounds in the normal organism. The *mod*-5 mutation increases rather than diminishes the efficiency of removal of metabolites from the medium; most mutations impede rather than stimulate gene function. The overall effect of the *mod*-5 mutation (measured in terms of the growth of modified and unmodified prototrophic cultures) is, however, deleterious. These observations can be reconciled if it is supposed that in normal Neurospora there is a "barrier" at the cell membrane limiting the permeability of some metabolites. By destroying or altering this "barrier", the *mod*-5 mutation may permit an increased flow of certain sub-

stances into the mycelium but might concomitantly upset the normal balance of metabolite entry. Speculation about the nature of the "barrier" is unwarranted. If such a structure or process exists, however, it could function as a mechanism for controlling the intracellular concentration of amino aids and other metabolites.

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SUMMARY

Mutants of Neurospora that require aromatic amino acids do not grow well on minimal media supplemented with yeast extract or peptone. Genetic analysis of a *tryp-3* strain capable of prolific growth on either complex supplement showed that the altered phenotype is associated with mutation of a gene located close to the centromere on linkage group VI. This mutation, *mod*-5, permits vigorous growth of eight different mutants blocked in the biosynthesis of the aromatic amino acids on yeast extract or peptone but does not suppress the metabolic defect of any of these strains. The growth of some of these mutants on glycine dipeptides of the required aromatic amino acid is stimuated if the *mod*-5 mutation is in the genome; similar effects were observed for the utilization of glycyl-lysine by a lysine mutant and of glycyl-methionine by a methionine mutant.

Growth of aromatic amino acid mutants on the required amino acid is inhibited by the addition of excess L-leucine. *mod*-5 strains are immune to this inhibition. The complex supplements contain sufficient tryptophan for good growth of tryptophan auxotrophs but quantities of leucine that, alone or in combination with other inhibitory substances, may account for the poor growth observed. Prototrophic strains and tryptophan mutants grown on indole are not inhibited by excess leucine, suggesting that leucine interferes with the permeability of tryptophan rather than its formation.

The final rates of removal of aromatic amino acids and glycine dipeptides from solution by mycelial mats were independent of the genotype of the cultures, but the initial lag before uptake began was decreased with *mod*-5 strains. Addition of excess leucine to the incubation mixture prolonged the lag period greatly; *mod*-5 cultures initiated aromatic amino acid removal more rapidly than *mod*-5^+ strains.

All the phenotypic manifestations of the *mod*-5 mutation can be rationalized as consequences of a change in permeability that facilitates the entry of a variety of metabolites into the organism. It is suggested that a structural alteration of the cell membrane rather than modification of an enzyme system may determine this increased permeation.

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