BIOCHEMICAL GENETICS OF NEUROSPORA PERTAINING TO VARIOUS AROMATIC METABOLITES

Thesis by

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ABSTRACT

The following nine mutant strains of Neurospora crassa were used in these studies: E5212, C86, B1312, 39401, 10575, C83, E5029, 4540, and 3416. Each of these nine mutants is able to utilize one or more of the following compounds: quinic acid, tyrosine, phenylalanine, anthranilic acid, indole, tryptophane, o-N-formyl-kynurenine, kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, and nicotinic acid.

Metabolic accumulations by four of the strains have been studied. The accumulation of anthranilic acid by mutant 10575 has been recognized for some time. It is now clear that this strain accumulates other highly fluorescent substances and at least one other biologically active substance. Mutant C83 accumulates relatively large quantities of anthranilic acid, and an unidentified derivative of indole. Strain E5029 accumulates a compound, apparently anthranilic acid, with biological activity for strain B1312; unidentified 39401-active and E5212-active substances; and kynurenic acid, which is biologically inactive. Mutant E5212 accumulates anthranilic acid, nicotinic acid, and also a substance which is active for E5212 itself.

Evidence has been obtained which indicates that in Neurospora anthranilic acid is a precursor of tryptophane, with indole as an intermediate between the two compounds; and also that tryptophane is degraded to anthranilic acid, with kynurenine as an intermediate. Thus a metabolic cycle involving anthranilic acid, indole, tryptophane, and kynurenine is clearly indicated.

Crosses involving mutants 39401 and C86 have furnished evidence that the mutation which prevents the growth of 39401 on minimal medium is the same as that which prevents the growth of C86 on minimal, and that a number of modifiers affect this primary mutation in such a way that offspring are produced which differ qualitatively in growth requirements from both C86 and 39401. Six modifiers have been postulated to explain the observed ascus segregations. In addition a suppressor of the primary mutation has been found. Possible mechanisms by which the modifiers exert their effects are considered briefly.

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I. BIOCHEMICAL STUDIES

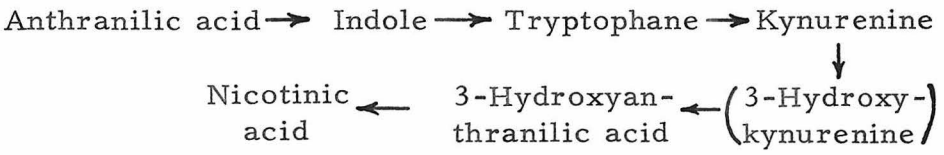
INTRODUCTION

The biosynthetic reactions leading to the formation of tryptophane constitute one of the first metabolic pathways to be investigated in the mold, Neurospora crassa. Tatum et al (1) in 1943 isolated anthranilic acid from the culture medium of a Neurospora mutant, strain 10575, which requires either indole or tryptophane for growth. The isolated compound functioned as a growth substance for a second mutant strain which could also utilize indole or tryptophane. This, incidentally, was the first demonstration in Neurospora of the association between a genetic block and the accumulation of a biologically active compound.

Soon after, it was found that the coupling of indole and serine to form tryptophane could be accomplished by washed mycelia of strain 10575 (2), and in 1946 Umbreit et al (3) succeeded in demonstrating this condensation using cell-free extracts of wild-type Neurospora, with pyridoxal phosphate as coenzyme. More recently (4) a mutant has been found which responds only to tryptophane and which yields cell-free extracts apparently incapable of coupling serine and indole. These observations indicate that indole and anthranilic acid function as precursors of tryptophane in Neurospora and that the order of the compounds in the biosynthetic pathway is

anthranilic acid ⟶ indole ⟶ tryptophane.

A metabolic relationship in Neurospora between tryptophane and nicotinic acid was suggested by the observation of Beadle, Mitchell, and Nyc (5) that the growth requirements of mutants 39401 and 65001 could be satisfied by either compound. These investigators found that kynurenine functions as an intermediate in the biosynthesis of the vitamin from the amino acid. In the course of attempts to discover other intermediates, Nyc and Mitchell (6) synthesized 3-hydroxyanthranilic acid and found it to be active for 39401 and 65001 but inactive for strains 4540 and 3416 which respond only to nicotinic acid or nicotinamide (7). Later, Bonner (8) found that a 39401-active substance which he had isolated from the culture fluid of strain 4540 was identical with the synthetic 3-hydroxyanthranilic acid of Nyc and Mitchell. In view of the activity of both kynurenine and 3-hydroxyanthranilic acid, Mitchell and Nyc (7) postulated 3-hydroxykynurenine as an intermediate between the two compounds, but no 3-hydroxykynurenine was available for testing at that time. Observations prior to the investigations to be reported here, then, indicated the following chain of reactions (7).



Recent reviews which deal in part with the metabolism of tryptophane, nicotinic acid, and other aromatic compounds by Neurospora

have been written by Horowitz (9), Mitchell (10), and Horowitz and Mitchell (11).

EXPERIMENTAL

MUTANT STRAINS

The mutant strains which were used in these studies are listed in Table I. Also listed are the types of mutagenic treatment following which the strains were isolated (in the cases where this information is available), the centromere distance for each mutant except B1312, and the linkage group in the cases where this has been determined. Asci of crosses between B1312 and wild type have not been dissected because in all the crosses tried thus far (at least ten reisolates of B1312 have been tried) there are very few asci with eight mature spores. In some of the crosses it was difficult to find a complete ascus of any kind, and in those crosses which had complete asci, most of the asci had at least one aborted spore pair as evidenced by the colorless spores. From one of the B1312 X wild crosses, approximately 10,000 ascospores were plated on minimal agar, and samples of the germinating spores were classified as wild or mutant according to hyphal length (12). From these counts it was evident that wild and mutant spores were present in approximately equal numbers, indicating that the B1312 phenotype depends primarily on a single gene.

Table I. Mutant strains.

Mutant	Treatment	No. of asci dissected	Centromere distance	Linkage group	References
E5212	Unknown[1]	22	18	Unknown	(13)
C86[2]	Ultraviolet	22	30	"	(12, 13)
B1312	Unknown[1]	--	Unknown	"	(13)
39401[2]	Ultraviolet	118	23.3	"	(14, 15)
10575	X-ray	59	15.2	C	(14, 15)
C83	Ultraviolet	30	29	Unknown	(12, 4)
E5029	Unknown[1]	22	23	"	(13)
4540	X-ray	18	0	A	(14, 15)
3416	"	23	6.5	A	(14, 15)

[1] Mutants E5212, B1312, and E5029 were isolated in the course of atomic radiation studies which were carried on at the California Institute of Technology under the sponsorship of the Office of Naval Research. Information is not available as to the type of radiation used on the cultures from which these particular mutants were isolated. It may have been atomic radiation or perhaps control irradiation with X-rays or ultraviolet.

[2] The genetic behavior of strains 39401 and C86 will be considered in more detail in Part II of this thesis.

COMPOUNDS UTILIZED BY THE MUTANTS

Unless otherwise specified, all the biological activity tests referred to in this thesis were carried out using 125-ml. Erlenmeyer flasks containing 20 ml. of minimal medium (14) supplemented with the various substances under test. Tests of this nature have disclosed that certain of the mutant strains are able to utilize substances other

than anthranilic acid, indole, tryptophane, kynurenine, 3-hydroxyanthranilic acid, and nicotinic acid. These other compounds are as follows:

1. 3-Hydroxykynurenine. The isolation of this compound from larvae of Calliphora erythrocephala was accomplished by Butenandt, Weidel, and Schlossberger (16) in 1949. Dr. Weidel generously furnished a sample of this compound for testing on Neurospora, and it was found to be active (13) as had been predicted (7). The activity observed was approximately equivalent, on a molar basis, to the activity of 3-hydroxyanthranilic acid, and was a little less than that of nicotinamide.

2. Phenylalanine and tyrosine. These compounds were found to have activity for strain C86 (17). Phenylalanine is more active than tyrosine, but considerably less active than tryptophane. Phenylalanine is also active for mutant E5212 but tyrosine is not (13, 17).

3. Quinic acid. Mutant C86 utilizes quinic acid less efficiently than tryptophane, for example, but the activity is sufficient to suggest that quinic acid is actually an early intermediate in the biosynthesis of tryptophane by Neurospora (18).

4. o-N-Formylkynurenine*. Since only a small sample of this

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* The o-N-formylkynurenine and ∝-hydroxytryptophane samples were kindly supplied by Dr. H. Kikkawa of the Sericultural Experiment Station, Taketoyo, Aichi, Japan. The compounds were synthesized by Dr. T. Sakan.

compound was available, its activity was tested on only four strains: C86, B1312, E5029, and the Nic. type which is described in another section of this thesis (p. 54). The compound is active for three of the four strains--for E5029 it appeared inactive. These results are in agreement with the observations of Amano et al (19) which indicate that formylkynurenine is an intermediate between tryptophane and kynurenine in Drosophila eye pigment formation and in Pseudomonas, and with the work of Knox and Mehler (20) and Mehler and Knox (21) which demonstrates the production of formylkynurenine during the breakdown of tryptophane by liver homogenates. It is probable that this compound is an intermediate in Neurospora also, but because only limited tests have thus far been made, it is not included in Table II.

A sample of formylanthranilic acid appeared inactive for C86. This result is not considered to be conclusive, however, for the identity of the compound was not established with certainty. In view of the apparent activity of formylkynurenine, it would not be surprising if formylanthranilic acid also were active for C86.

Four other compounds, trans-cinnamic acid (17), o-carboxy-N-phenylglycine (1), ∝-hydroxytryptophane*, and quinolinic acid proved slightly active for C86, but in all cases the activity was so low as to

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* See footnote, p. 5.

make it seem unlikely that these compounds are real intermediates. Quinolinic acid has been isolated from the culture fluid of strain 3416 by Henderson (22) and by Bonner and Yanofsky (23). Henderson reports that this compound is active for strain 4540 at concentrations of approximately 0.05 mg./ml., which is about fifty times the concentration of nicotinic acid required by this mutant. Bonner and Yanofsky have confirmed the slight activity of quinolinic acid for 4540 and have reported slight activity for 39401 also. In this laboratory, quinolinic acid was tested on strains C86 and 3416. The sample tested had some activity for both strains. After recrystallization from water only a trace of activity was left, suggesting that most if not all the original activity was due to an impurity, probably nicotinic acid. Henderson presumably has ruled out nicotinic acid as the cause of the activity he observed, but the fact remains that activity at best is slight and for this reason it is felt that quinolinic acid does not belong in the main chain of reactions between tryptophane and nicotinic acid.

The following compounds have been found inactive for strain C86 under the conditions used in testing (17 and unpublished results). These compounds were not autoclaved--they were either filter-sterilized or else added without sterilization to hot, freshly autoclaved minimal medium.

3,4-dihydroxyphenylalanine	salicylic acid
anthranil	coumarin
benzoic acid	coumaric acid
aniline	2-carboxyindole
p-aminobenzoic acid	3-carboxyindole
isatoic acid	cinnamaldehyde
cis-cinnamic acid	indole-3-aldehyde
phloroglucinol	2-carboxy-3-hydroxyindole
phenylacetic acid	o-aminocinnamic acid
p-aminophenylacetic acid	phenylserine
β-phenylethyl alcohol	N-phenylglycine
phenyl-DL-α-alanine	3,4-dihydroxybenzoic acid
β-phenylethylamine	shikimic acid

Table II lists the compounds utilized by the nine mutant strains used in these studies.

Table II. Compounds utilized by nine mutant strains of Neurospora.

Compound	Mutant strain								
	E5212	C86	B1312	10575	39401	C83	E5029	4540	3416
Tyrosine or Quinic acid	-	+	-	-	-	-	-	-	-
Phenylalanine	+	+	-	-	-	-	-	-	-
Anthranilic acid	-	+	+	-	-	-	-	-	-
Indole	-	+	+	+	+	-	-	-	-
Tryptophane	-	+	+	+	+	+	-	-	-
Kynurenine	-	+	+	-	+	-	-	-	-
3-Hydroxy-kynurenine	-	+	-	-	+	-	+	-	-
3-Hydroxyanthranilic acid	-	+	-	-	+	-	+	-	-
Nicotinic acid	-	+	-	-	+	-	+	+	+

METABOLIC ACCUMULATIONS

Reference has been made to the accumulation of anthranilic acid by strain 10575 (p. 1), of 3-hydroxyanthranilic acid by 4540 (p. 2), and of quinolinic acid by strain 3416 (p. 7). Several other interesting accumulations have now been found, and in some instances the accumulated compound has been identified. In testing for biologically active accumulations, the usual procedure was to filter the medium after removal of the mold, and then to pipet aliquots into flasks containing 20 ml. of hot, freshly autoclaved minimal. Similarly, when it was necessary to locate active substances on filter paper chromatograms, the chromatograms were cut into sections, the sections were eluted with water or phosphate buffer, the paper was centrifuged down and the eluates were poured into flasks of hot minimal. Although these methods might not be approved by one well versed in microbiological techniques, they avoided the breakdown of substances possibly unstable to autoclaving, and also the tedium of filter-sterilizing a large number of samples. Contamination was rare and thus did not present a serious problem.

Accumulations by strain 10575

In the original report (1) of the isolation of anthranilic acid from the culture medium of strain 10575, no data were given on the amount accumulated nor was there any mention of other accumulations by this strain. In an effort to learn something about these matters the

following work was done. Six flasks each containing 20 ml. of minimal medium supplemented with 400 γ of L-tryptophane were inoculated with 10575 conidia and were incubated at 25° for four days. The average dry weight of mold at the end of this time was 26 mg./flask. An 80-ml. portion of the medium was acidified to pH 2.5 and was then extracted with six 40-ml. portions of ether. Fluorescence measurements in 0.1 M KH_2PO_4, pH 3.5, were made on the original medium and on ether and aqueous phases using a Coleman photofluorometer; ultraviolet absorption spectra were determined in the pH 3.5 phosphate buffer using a Beckman Model DU spectrophotometer; and biological activities were determined with mutant C86 as the assay strain. Results of this experiment are shown in Table III. The calculations of concentration from the absorption spectra are based on optical densities at 330 mμ. At pH 3.5 anthranilic acid has an absorption peak between 325 and 330 mμ (fig. 1).

A sample of the ether phase was examined chromatographically using Whatman no. 1 filter paper with water-saturated butanol as descending solvent. In this system the ether phase gave two strong blue-fluorescent bands, the leading one of which corresponded in position with an anthranilic acid band (Rf = approx. 0.8). Behind the second strong blue band (Rf = approx. 0.5) were at least three very light blue-fluorescent bands. Biological assay on eluates from sectioned chromatograms disclosed that approximately 90% of the elutable

Table III. Concentration of anthranilic acid in 10575 medium as indicated by fluorescence, absorption spectrum, and biological activity.

	Concentration (γ/ml. of original medium) indicated by		
	Fluorescence*	Spectrum	Biological activity
Original medium	650	145	100
Aqueous phase	240	31	6
Ether phase	475	117	94

*The ether which was used had appreciable blue fluorescence. This may account for the rather large discrepancy between the fluorescence of the original medium and the sum of ether and aqueous phases.

activity came from the leading fluorescent band. The second strong fluorescent band had slight but definite activity for mutant C86, accounting for about 10% of the eluted activity. The slower-moving fluorescent bands had no activity under the conditions used in testing. It appears, therefore, that 10575 accumulates a number of blue-fluorescent compounds but that the principal biologically active compound is anthranilic acid.

The data shown in Table III indicate that the bulk of the fluorescence is produced by some compound(s) other than anthranilic acid. In a brief attempt to isolate the highly fluorescent material, a stage was reached at which the product was in the form of a barium salt with approximately twelve times the fluorescence of anthranilic acid, on a

weight basis. This barium salt had no biological activity and no further work was done with it.

Accumulations by strain C83

So far as is known, tryptophane is the only compound which satisfies the growth requirement of mutant C83. Cell-free extracts of this mutant appear to be incapable of coupling indole and serine to form tryptophane (4) while comparable preparations from wild type are capable of carrying out this condensation (3). It appears, therefore, that this mutant is blocked between indole and tryptophane. This being the case, one might expect C83 to accumulate indole or some closely related derivative. In tests of the culture fluid on which C83 was grown, activity was displayed for strains B1312 and C86 but not for strain 10575. Also, the medium had a strong blue fluorescence in ultraviolet light. These observations indicate that indole itself was not accumulated, but that something was present with a biological specificity and blue fluorescence reminiscent of anthranilic acid. As will be shown, an indole derivative is also accumulated by mutant C83.

Isolation of anthranilic acid--

Mutant C83 was inoculated into a five-gallon bottle containing 15 l. of minimal medium supplemented with 10 γ of L-tryptophane/ml. The culture was incubated at 25° with forced aeration. After five days the mold was removed and the medium was flash evaporated to about

one tenth of the original volume. The solids which separated were filtered off and the filtrate (vol. 1400 ml.) was continuously extracted with ether for 34 hours. The ether phase (vol. 310 ml.) was then extracted with five 30-ml. portions of 0.8% $NaHCO_3$. The first $NaHCO_3$ extract was used for other purposes. The other four were combined, acidified to pH 2.9, and extracted with three 60-ml. portions of ether. The ether phase was evaporated to dryness and the resulting solid was sublimed in vacuo at 100° C. The sublimate was dissolved in water, treated with decolorizing carbon, and allowed to recrystallize.

Melting points: (not corrected)	known anthranilic acid	143-143.5° C.
	isolated compound	144-144.5
	mixed melting point	144-144.5
acetyl derivative of		
	known anthranilic acid	184-186° C.
	isolated compound	184-186
	mixed melting point	184-186

Measurements of absorption spectra (fig. 1) and fluorescence furnished confirmation that the isolated compound was anthranilic acid.

Accumulation of an indole derivative--

In collaboration with Dr. D. E. Atkinson, a search was made for a Neurospora strain which accumulated indole-3-acetic acid. It was found that culture medium from mutant C83 gave a strong red color (characteristic of indole-3-acetic acid) with Salkowski's reagent (24). In this case, however, the red color must have been due to some other compound, for purified Salkowski-positive fractions from C83 medium

failed to show any activity in the Avena test. Although the compound is not indole-3-acetic acid, absorption spectra of eluates from paper chromatograms indicate that the Salkowski-positive substance does contain the indole nucleus. The substance was inactive for Neurospora. Since the compound proved to be inactive and since it was also somewhat unstable, no intensive effort was made to isolate and characterize it.

In experiments to determine the best conditions for accumulation, it was found that the greatest accumulations of Salkowski-positive material, fluorescence, and biological activity for strains C86 and B1312 were attained when C83 was grown on a concentration of tryptophane which gave approximately one-third maximal growth.

Accumulations by strain E5029

Biologically active accumulations--

The first indication of biologically active accumulations by mutant E5029 was the discovery that this mutant could cross-feed strain C86. It was found that the culture fluid had approximately five times as much activity for C86 when the E5029 was grown for four days on 10 γ of nicotinamide/20 ml. (dry weight of mold about 30 mg.) as when a concentration of 20 γ/20 ml. was used (dry weight approximately 60 mg.). In further tests, flasks with a nicotinamide concentration of 10 γ/20 ml. were inoculated with E5029 and at intervals of 2, 3, 4, and 5 days after inoculation pads were harvested (average dry weights in mg.: 2 days--3.5;

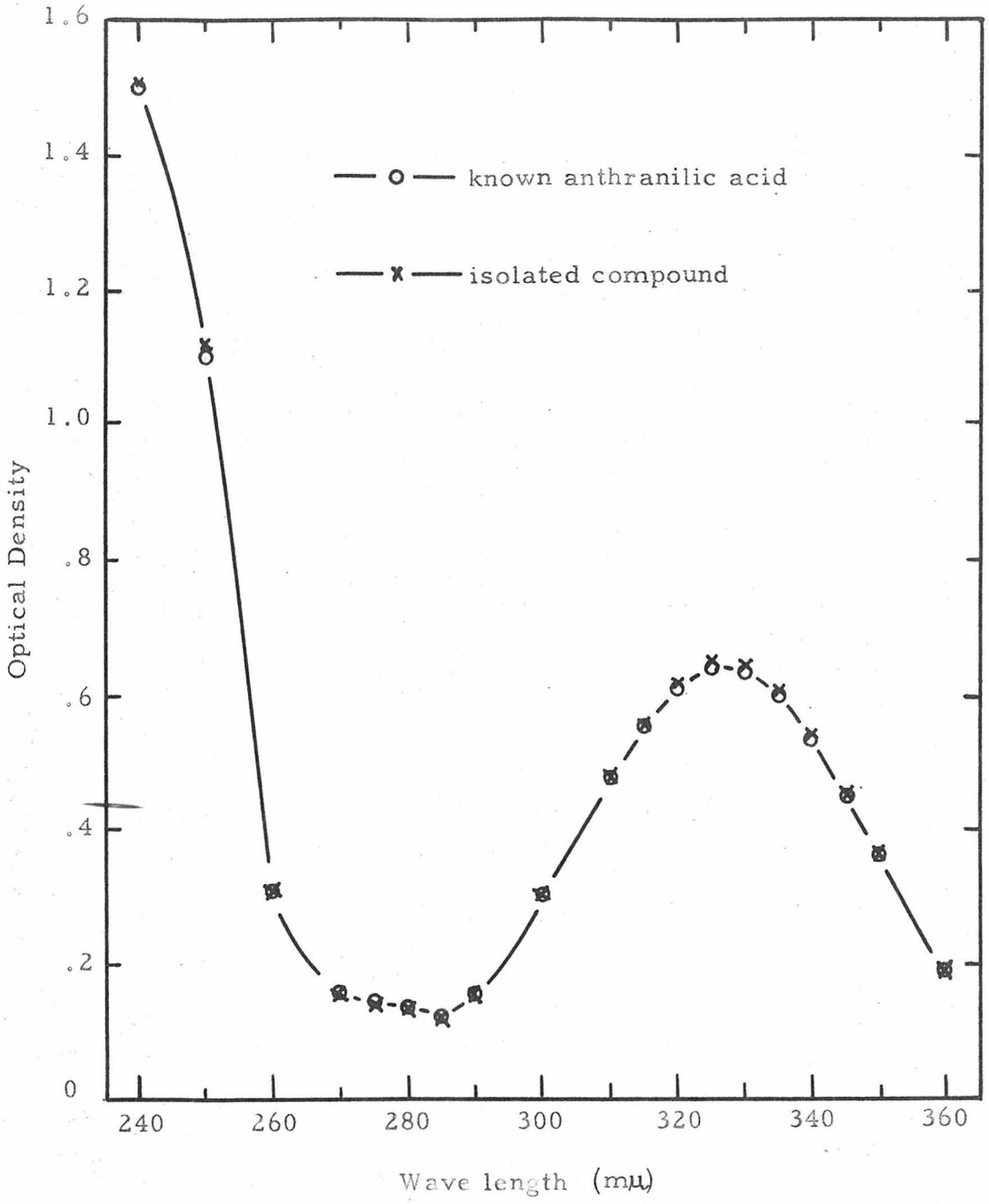


Figure 1. Ultraviolet absorption spectrum of anthranilic acid, 50γ/ml. in 0.1 M KH_2PO_4 pH 3.5.

3 days -- 17; 4 days -- 35; 5 days -- 47) and the medium was tested for activity on strains E5212, C86, B1312, 39401, 10575, 4540, and on E5029 itself. No activity was exhibited for the last three strains. For the others the activities of one ml. samples of the E5029 media are shown in Figure 2.. It is not known whether the decrease in activity for C86, 39401, and E5212 is the result of destruction of the growth substances or the production of inhibitory substances, or both.

In an effort to learn something of the properties of the active substances, the following work was done. Thirty-nine flasks, each containing 20 ml. of minimal medium supplemented with 10 γ of nicotinamide were inoculated with mutant E5029. After four days the mycelial pads were harvested (average dry weight per pad was 36 mg.) and the culture filtrates were combined and treated as follows:

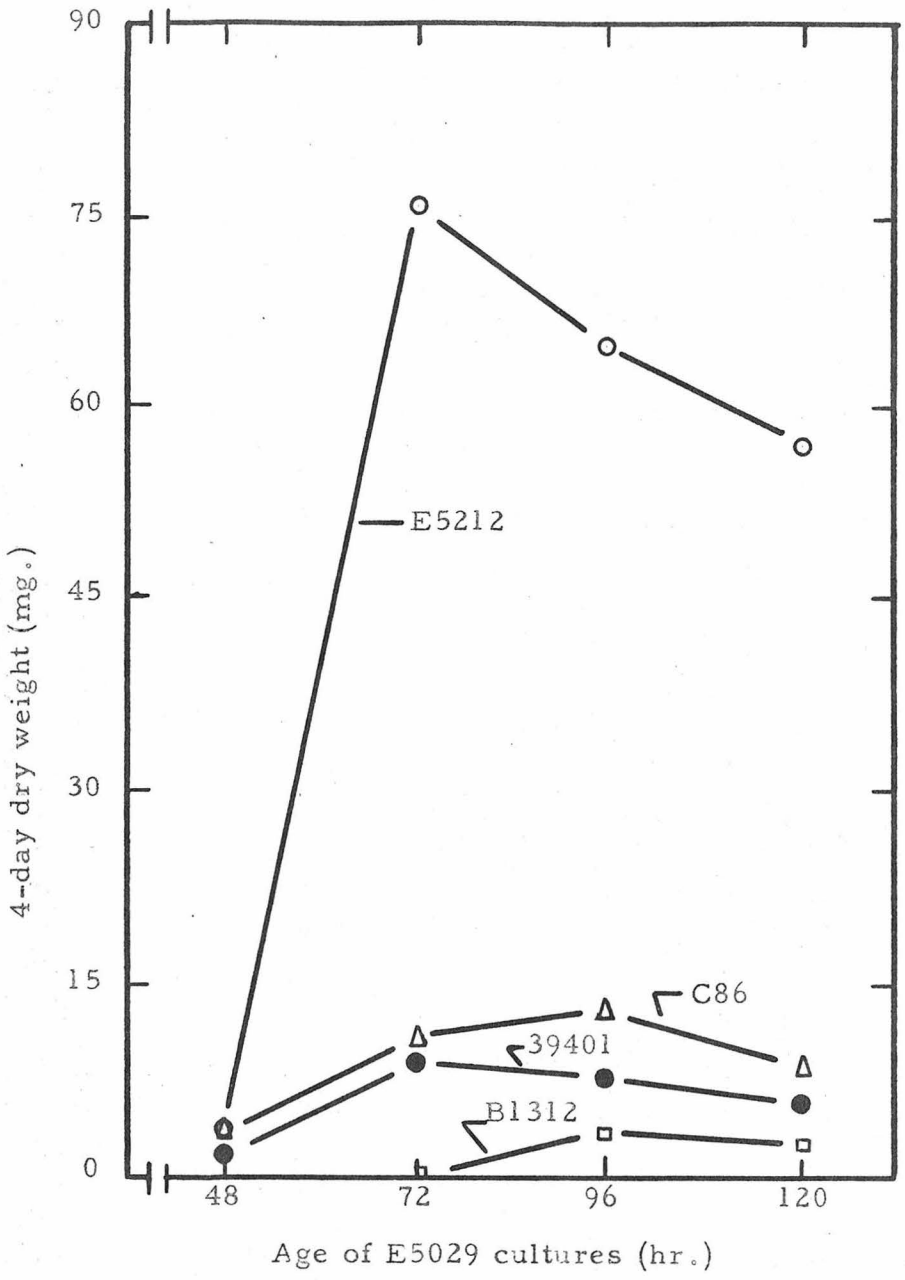
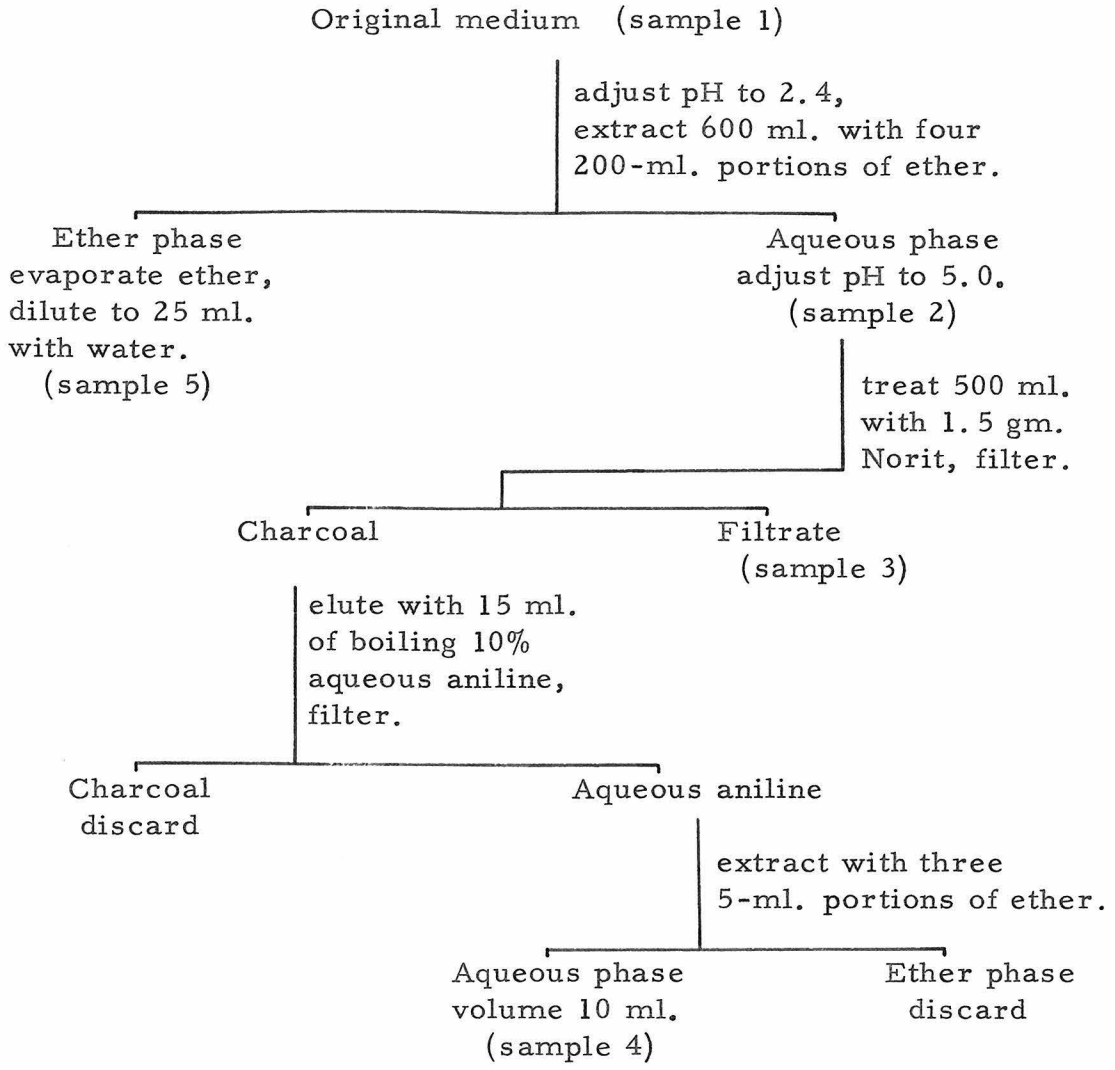


Figure 2. Biological activity of 1 ml. aliquots of E5209 media for four strains.



Activities of samples 1 to 5 for mutants E5212, B1312, and 39401 are shown in Table IV. Essentially no activity was shown for strains E5029 and 4540. Strains 10575 and C86 were not included in this test. From the results shown in Table IV, in conjunction with chromatographic work on samples 4 and 5, the following deductions may be made concerning the activities for E5212, B1312, and 39401.

Activity for E5212: The work thus far indicates only that the activity for E5212 divides between ether and water at pH 2.4, and that the part remaining in the water phase may be adsorbed, to a large

extent, on charcoal. Elution of the charcoal with aniline recovers only a small part of the activity.

Table IV. Biological activity of E5029 medium for strains E5212, B1312, and 39401.

Sample number	Volume added to 20 ml. of minimal medium (ml.)	Four-day dry weights (mg.)		
		E5212	B1312	39401
1	1	51	trace	4
	3	64	1	22
2	1	trace	trace	5
	3	26	"	20
3	1	trace	"	trace
	3	2	"	3
4	0.04	trace	"	3
	0.12	1	"	15
5	0.08	28	1	trace
	0.25	40	3	"
Minimal	-	trace	trace	trace

Activity for B1312: Strain E5029 accumulates only a small amount of activity for B1312, and this activity is extractable with ether at pH 2.4. On filter paper chromatograms with 0.1 N HCl-saturated butanol as solvent, the active band has an Rf identical with that of anthranilic acid (approximately 0.8) and it also displays blue fluorescence. It is probable, therefore, that the B1312-active substance is anthranilic acid.

Activity for 39401: The activity for 39401 is not extractable with ether at pH 2.4, and it can be adsorbed on charcoal and eluted with aniline. The aniline eluate was examined chromatographically. With 0.1 N HCl-saturated butanol as the solvent the active band had an Rf of 0.12, while with 1% NH_3-saturated butanol the Rf was 0.08. The somewhat higher value in the acid solvent suggests that the substance is acidic. Nicotinamide has an Rf of approximately 0.6 in both the acid- and ammonia-saturated butanol. Autoclaving (15 lbs./sq. in. for 15 min.) aliquots of sample 4 with the medium was found to reduce the activity by approximately 25%. Ascribing the activity for 39401 to residual nicotinamide is ruled out by chromatographic evidence, by the lack of activity for 4540 and E5029, and by the decrease in activity on autoclaving. The task of isolating the 39401-active substance has been started -- several carboys of mold have been grown -- but as yet not much progress has been made in the actual isolation.

The isolation of kynurenic acid --

Early in the course of the work on mutant E5029 it was noted that the absorption spectrum of culture medium from this mutant had a high plateau between 330 and 350 mμ. The high-spectrum material was not extractable with ether at pH 2.4, but it was adsorbed on charcoal and could be eluted from the charcoal with boiling 10% aqueous aniline. When the aniline eluates from the carboys referred to above

(for the attempted isolation of the 39401-active substance) were evaporated to a small volume, some solid material separated out. This material was found to have a strong absorption peak at 330 mμ, and the remaining filtrate had a peak at 350 mμ. The identity of this material with the 350 mμ peak is as yet undetermined. The solid material (330 mμ peak) was purified by two recrystallizations from 60% aqueous ethanol, and was eventually identified as kynurenic acid (2-carboxy-4-hydroxyquinoline). The criteria on which the identification was based are:

1. Melting points (not corrected)

Known kynurenic acid	283.5-284.5°C.
Isolated compound	283.5-284.5
Mixed melting point	283.5-284.5

2. Elementary analysis

	observed	calculated for $C_{10}H_7O_3N$
C	63.95%	63.49%
H	3.72	3.73
N	7.45	7.41

3. Ultraviolet absorption spectra for the isolated compound and for a known sample of kynurenic acid are identical (fig. 3).

4. Neutralization equivalent

observed	calculated for kynurenic acid
182	189

The recent isotopic work of Schayer (25) has demonstrated the formation of kynurenic acid from injected tryptophane in rats and rabbits, and the earlier work of Axelrod et al (26) demonstrated the

excretion of kynurenic acid in response to the feeding of tryptophane to dogs. In order to see whether mutant E5029 could convert exogenous tryptophane to kynurenic acid, the following experiment was done. Mutant E5029 was inoculated into the series of flasks shown in Table V. After four days the pads were removed and absorption spectra of the media were determined. The media were diluted 1 to 4 with minimal medium for the determination of spectra. Dry weights and optical densities at 330 mμ are shown in Table V. These results show that the growth of mutant E5029 is greatly inhibited by tryptophane. That inhibition by tryptophane at these concentration levels is not a general phenomenon is shown by the work of Cushing et al (27) which shows that wild-type Neurospora E5256A is partially inhibited by L-tryptophane in a concentration of 10^{-2} molar, and that a concentration of 10^{-3} molar is not inhibitory at all. The latter concentration amounts to approximately four mg./20 ml. It is worthy of note that the accumulation of high spectrum material per mg. of mold was roughly the same whether or not the mold was grown in the presence of tryptophane. It seems unlikely, therefore, that the added tryptophane was converted to kynurenic acid to any great extent.

Kynurenic acid is not biologically active for any of the strains used in these experiments. In this respect it is unlike the α-N-acetylkynurenine which Yanofsky and Bonner (28) have isolated from the culture filtrate of mutant Y31881, a mutant whose growth requirement

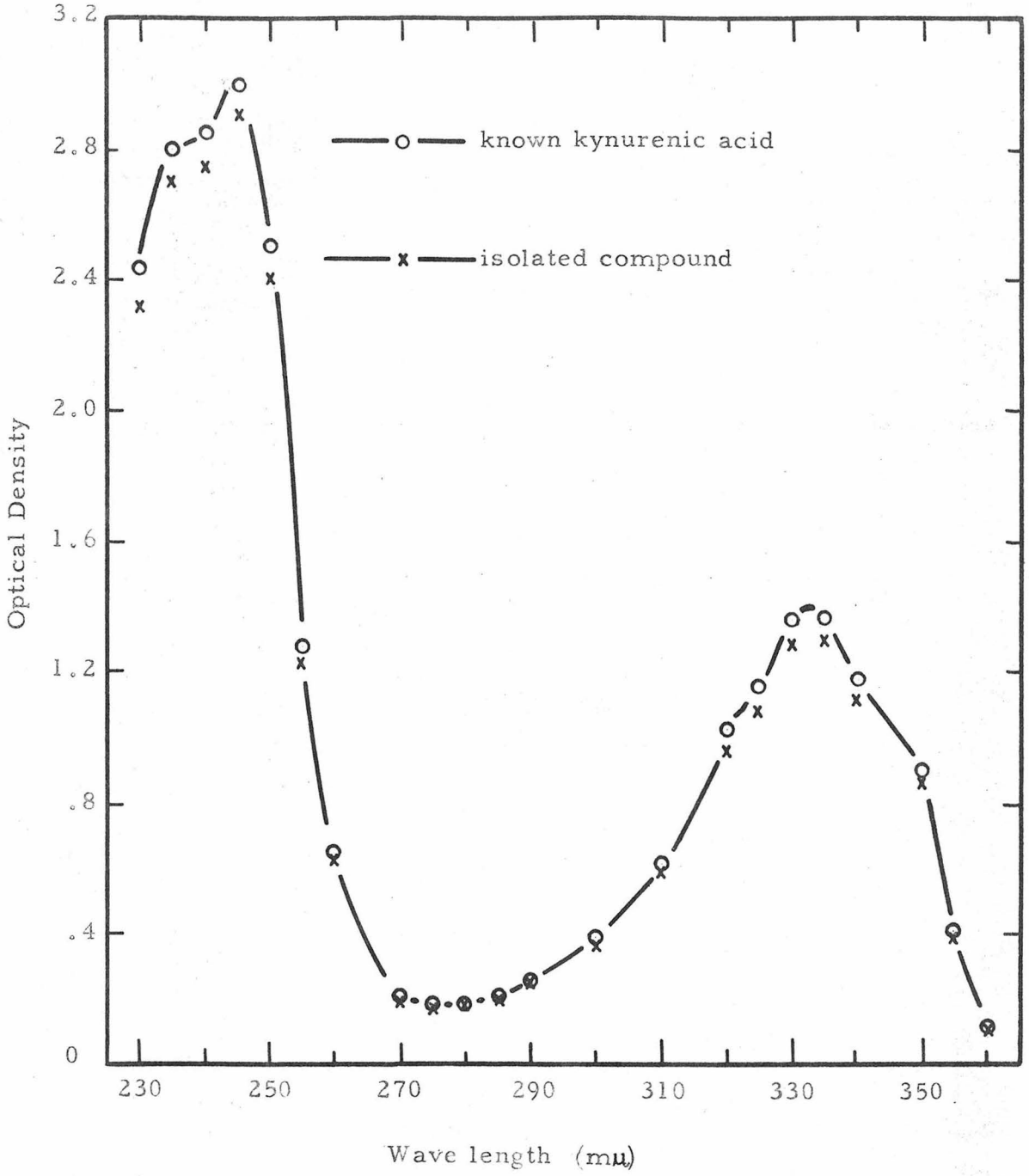


Figure 3. Ultraviolet absorption spectrum of kynurenic acid, 25γ/ml. in 0.1 M KH_2PO_4 pH 4.5.

Table V. Four-day dry weights of E5029 on mixtures of nicotinamide and tryptophane, and optical densities of the media at 330 mμ.

Supplement (per 20 ml.)		4-day dry wt. (mg.)	op. dens. at 330 mμ	op. dens. X 1000 / dry wt.
Nicotinamide	L-Tryptophane			
0	0	trace		
10 γ	0	29	0.91	31
"	0.2 mg.	15	0.41	27
"	0.5	12	0.295	25
"	1.0	10	0.31	31
"	2.0	7	0.233	33
"	5.0	3	0.106	35

appears similar to that of E5029. It is reported that α-N-acetylkynurenine is slightly active for mutant 39401.

Accumulations by strain E5212

Of the growth substances on which it has thus far been tested, mutant E5212 utilizes only phenylalanine. Tyrosine and tryptophane do not promote the growth of E5212 by themselves, and in combination with phenylalanine their effect, if any, is inhibitory rather than stimulatory. As amino acid requirements go, the requirement of E5212 is low, maximum growth being attained on a concentration of 100 γ of DL-phenylalanine/20 ml. This low requirement is very possibly related to the fact that E5212 grows slightly on minimal,

typically attaining a dry weight of 2 or 3 mg. in four days.

Mutant E5212 is like E5029 in that it accumulates biological activity for a number of different mutants. Unlike E5029, however, E5212 accumulates activity for itself also. Figure 4 shows the results of an experiment similar to that reported for E5029 in Figure 2. The E5212 was grown on a phenylalanine concentration of 50 γ/20 ml., and average dry weights attained were as follows: 2 days -- 2.5 mg.; 3 days -- 30 mg.; 4 days -- 64 mg.; 5 days -- 71 mg. It will be seen that the medium at one stage or another had considerable activity for strains E5212, C86, and 4540, and a small amount of activity for E5029 and B1312.

Activity for B1312 --

The medium in which mutant E5212 is grown typically displays intense blue fluorescence, most of which was found to be extractable with ether. The absorption spectrum of the ether extract corresponds very closely with that of anthranilic acid, having a peak at 320 mμ at pH 4.5. Chromatographically, with a mixture of two parts propanol and one part 1% aqueous ammonia as solvent, the ether extract gave two fluorescent spots -- an intense blue spot which corresponded in position with an anthranilic acid spot (Rf approximately 0.65) and a weak yellowish-blue spot (Rf approximately 0.8). The activity of the ether extract for strain B1312 was quantitatively equivalent, based on the optical density at 320 mμ, to that of anthranilic acid. It appears,

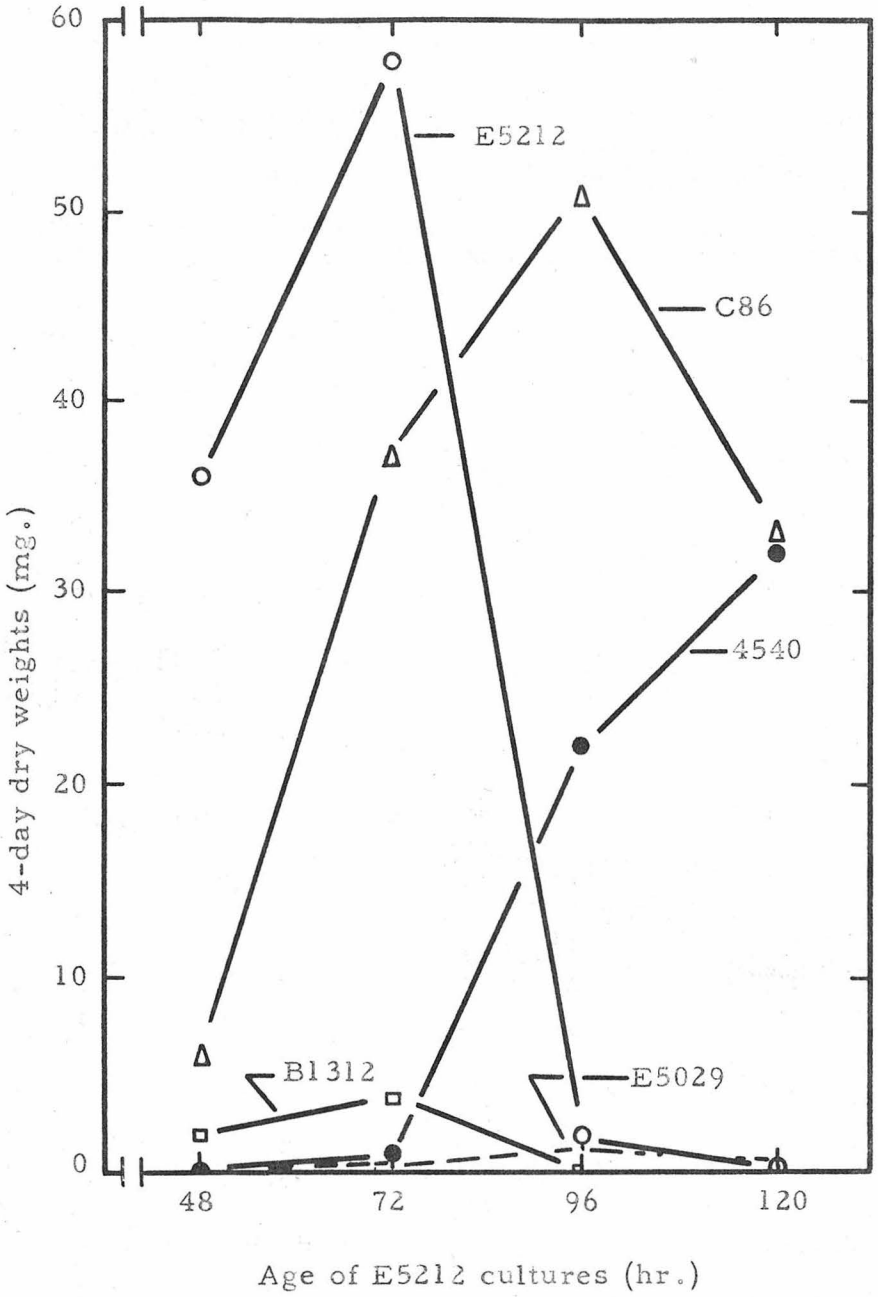


Figure 4. Biological activity of 1 ml. aliquots of E5212 media for five strains. (3 ml. aliquots for E5029.)

then, that E5212 is capable of accumulating anthranilic acid. The anthranilic acid, however, does not remain indefinitely in the culture fluid. Filtrates from four-day cultures of E5212 have been found to have no activity for B1312 (fig. 4), although they still display blue fluorescence. Possibly some of the anthranilic acid is converted into inactive blue-fluorescent compounds.

The accumulation by E5212 of activity for itself --

Horowitz (29) has found that a choline-requiring mutant of Neurospora, strain 47904, is actually able to synthesize choline, and Simonds (30) has reported that culture filtrates from a phenylalanineless strain of E. coli have a sparing action on the phenylalanine requirement of the same strain. Thus the accumulation by E5212 of activity for itself is not without precedent. Since E5209 also accumulates activity for E5212 (p. 17), one might ask whether this is an accumulation of which any actively growing Neurospora culture is capable. To answer this question, the nine mutants listed in Table I, 7A and 8a (wild types), 51602 (riboflavineless), and 1633, H193, 85465, 71301, 21850, and 38113 (p-aminobenzoicless) were cultured on minimal plus their respective requirements, and after four days of growth the culture medium from each was tested for its ability to support the growth of E5212. It was found that only the media from E5029 and E5212 had appreciable activity for E5212. The medium from B1312 was slightly active, and none of the other media exhibited any activity. Thus the accumulation seems

quite specific. It is apparent from the E5212 line in Figure 4 that the activity accumulated by E5212 fluctuates widely. In some cases it has been found that medium from four-day cultures had a considerable amount of activity, but in the case illustrated in Figure 4 very little activity was found after the third day. Age and size of inoculum seem to have great influences on the growth and accumulating ability of E5212.

The behavior of the E5212-active substance has been investigated briefly. Strain E5212 was grown for three days in 20 flasks each of which contained 50 γ of DL-phenylalanine in 20 ml. of medium. The average dry weight produced was 36 mg. After the mold was removed, the medium was treated with charcoal and then the charcoal was eluted with boiling 10% aqueous aniline. Bioassay of the various fractions with strain E5212 indicated that the charcoal removed the activity from the original medium and that aniline eluted approximately half the E5212 activity from the charcoal. Filter paper strips of the aniline eluate were run in butanol saturated with 0.1 N HCl and in butanol saturated with 1% ammonia (descending solvents). Recovery of activity from the filter paper was very poor, representing not more than 10% of the amount applied to the strips. Possibly the active substance was destroyed or was adsorbed on the paper so strongly that water would not elute it. Another possibility is that activity depends on the presence of two compounds and that the two were separated on the filter paper strips. The activity which was recovered had an Rf of 0.25 in the acid solvent and 0.19 in the basic solvent, indicating that an acidic substance was

involved. The E5212-active chromatogram zone had no activity for C86, so it seems unlikely that phenylalanine is responsible for the eluted activity although this possibility cannot yet be ruled out completely, in view of the fact that E5212 responds to lower levels of phenylalanine than does C86. Autoclaving for 15 minutes at 15 lbs./sq. in. was found to decrease the activity of the E5212 medium by 15-20%.

The activity which E5212 accumulates for itself appears to be somewhat more easily eluted from charcoal than the activity which E5029 accumulates. On this basis it may be that the two accumulations do not represent the same compound. As yet, however, the data are insufficient to permit any conclusions about the similarity or differences of the two E5212-active accumulations.

The isolation of nicotinic acid --

Although, as shown in Figure 4, E5212 medium has appreciable activity for C86 and 4540 and a slight activity for E5029 and B1312, strain C86 was the main assay strain used during the early part of the work on the E5212 accumulations. In a typical experiment, 20 flasks, each containing 20 ml. of minimal medium plus 100 γ of DL-phenylalanine were inoculated with E5212 and were incubated at 25° for four days. The average dry weight produced was 80 mg./flask. The culture filtrate was highly active for C86, very slightly active for E5029, and inactive for B1312 and 10575. A portion of the medium was acidified to pH 2.6

and extracted five times with a half volume of ether. Essentially all the activity for C86 stayed in the aqueous phase. A second portion of the original medium was treated with charcoal, and the charcoal was then eluted with boiling 10% aqueous aniline. This work disclosed that the activity could be removed from the medium with charcoal and could be recovered from the charcoal with aniline. The C86 activity in the aniline eluate moved as a single band in chromatograms using a solvent consisting of 2 parts propanol and 1 part water.

Following this preliminary work, a total of six carboys of E5212 were grown, each carboy initially containing 15 l. of minimal with a phenylalanine concentration of 5 γ/ml. As in the pilot run, after removal of the mold the medium was treated with charcoal and the charcoal was then treated with aqueous aniline. The eluted material was highly active for mutants C86, 39401, and 3416; slightly active for E5029; and inactive for B1312 and 10575. Based on the activity for C86, the combined aniline eluates contained the biological equivalent of approximately 220 mg. of nicotinamide in 135 ml. of solution. This solution was concentrated by evaporation at reduced pressure, and some of the inactive materials were removed by precipitation with ethanol. The evaporation and alcohol precipitation were repeated a total of three times after which bioassay with C86 indicated the presence, in the 25-ml. filtrate, of the biological equivalent of 125 mg. of nicotinamide.

At this stage a number of filter paper chromatograms were run, with butanol saturated with H_2O, 0.1 N HCl, and 1% NH_3 as solvents.

The solution was chromatogramed alone and in combination with nicotinic acid and nicotinamide. In no case was the activity from the solution separated from nicotinic acid. In the ammonia solvent, on the other hand, the separation of the unknown from nicotinamide was unequivocal. Thus it was evident that the unknown substance was not nicotinamide, but nicotinic acid was not ruled out as a possibility. A test of E5029 on concentration series of nicotinic acid and nicotinamide showed that this mutant utilized the amide much more efficiently than the acid at the pH of ordinary minimal medium, i.e., approximately 5.5. A nicotinamide concentration of 20γ/20 ml. gave maximum growth (four-day dry weight of 65 mg.), while 20γ of nicotinic acid gave only 12 mg., and the mold reached maximum growth only when the concentration of nicotinic acid was 50γ or more per 20 ml. Thus the low activity of the unknown for E5029 (p. 29) would not rule out nicotinic acid.

After the determination of biological activity and chromatographic behavior, the concentrated, alcohol-precipitated aniline eluate was chromatogramed on a chromatopile (31) consisting of nearly 700 sheets of 12.5-cm. Whatman no. 1 filter paper, with 1% NH_3-saturated butanol as the solvent. Doubtless because of overloading, the activity from the chromatopile was spread over about 180 sheets. These 180 sheets were removed and were incorporated into a short pile with a solvent consisting of equal volumes of ethanol and 0.1N HCl. By this means

the activity was concentrated into about 60 sheets. These sheets were used as the sample in a third pile, with butanol saturated with 0.1 N HCl as the solvent. The appropriate section was removed from the third pile and extracted in a Soxhlet with 95% ethanol. The resulting extract contained the biological equivalent of 20 mg. of nicotinic acid in a total dry weight of 240 mg.

The dried alcohol extract was sublimed in vacuo at 140-150° for 30 minutes. The sublimate, a yellow, oily material which contained the biological equivalent of 8 mg. of nicotinic acid, was then resublimed in a temperature gradient. This treatment gave a zone of white crystalline material which was surrounded by a brown oil. The crystals together with as little of the oil as possible were removed and were resublimed twice more in the temperature gradient apparatus. This treatment produced a material which could be recrystallized from 95% ethanol. After two recrystallizations, 1.5 mg. of crystals was recovered. The melting point determinations on this material were not well done -- the apparatus which was used was not very satisfactory in this particular temperature range, and sublimation of the compound also tended to complicate the procedure. Values obtained were:

Known nicotinic acid	223-228° (cor.)
Isolated compound	213-218 "
Mixed melting point	about 218 "

The value given in the literature for nicotinic acid is 235.2°.

Absorption spectra of the isolated compound and known nicotinic acid agree very well (fig. 5). The biological activities of known and

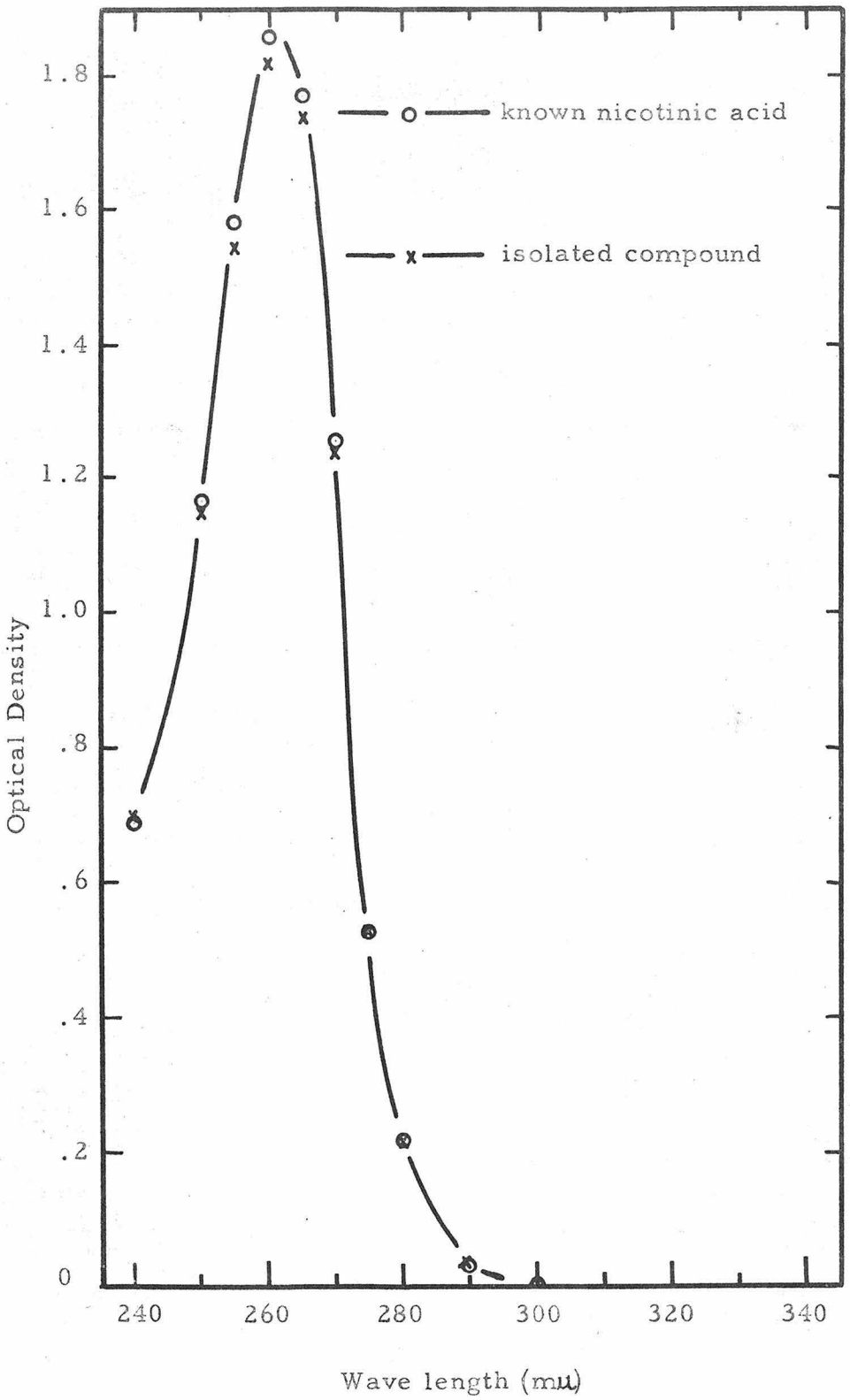


Figure 5. Ultraviolet absorption spectrum of nicotinic acid, 50γ/ml. in 0.1 M KH_2PO_4 pH 3.7

isolated compounds were compared for mutants C86, B1312, 10575, E5029, 4540, 39401, and E5212, and the agreement was excellent, both qualitatively and quantitatively. In view of the chromatographic, spectral, and biological evidence, there is little doubt that the isolated compound is nicotinic acid, despite the poor yield and the rather low melting point.

EVIDENCE FOR A TRYPTOPHANE CYCLE IN NEUROSPORA*

The work of Snell (32) on lactic acid bacteria provided the first evidence that anthranilic acid may act as a precursor to tryptophane in vivo. The evidence that anthranilic acid acts as a tryptophane precursor in Neurospora has already been considered (pp. 1, 2). Earlier work had demonstrated that anthranilic acid is a product of the degradation of tryptophane by bacteria (33, 34) and by the rat and other animals (35). In this laboratory it has been observed that when a medium containing tryptophane is inoculated with wild type or any one of a number of tryptophane-utilizing strains of Neurospora, a blue fluorescence resembling that of an anthranilic acid solution is produced. When an initial concentration of 400 γ of L-tryptophane/20 ml. was used, the fluorescence was detectable within a few hours after inoculation, reached maximum intensity at 24 to 30 hours, and then diminished until at 72 hours the medium displayed essentially no fluorescence except in

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*Most of the material in this section is from a publication, Evidence for a Tryptophane Cycle in Neurospora, reference 13.

the case of mutants C83 and 10575 which are known to accumulate anthranilic acid in considerable quantities. This appearance and subsequent disappearance of fluorescence in the medium, plus the identification of anthranilic acid (which fluoresces blue) as a precursor of tryptophane in some organisms and as a degradation product in other organisms suggested that in Neurospora some of the reactions by which tryptophane is synthesized and degraded may constitute a metabolic cycle. To investigate this possibility, the following work was done.

Disappearance of tryptophane from the culture medium

Flasks containing 20 ml. of minimal medium supplemented with 400 γ of L-tryptophane were inoculated with conidia from Neurospora strain B1312 and were incubated at 25°. Absorption spectra of the medium before inoculation and at 8-hour intervals thereafter were determined with a Beckman spectrophotometer (fig. 6). Dry weights of mold per flask at the different times were as follows: 16 hours, 0.6 mg.; 24 hours, 3.0 mg.; 32 hours, 9.1 mg. The subsequent dry weight attained at 96 hours was 55 mg. On the basis of the spectra at 280 mμ (the absorption maximum for the indole ring of tryptophane), it is obvious that at least 100 γ of tryptophane was gone when the growth of mold was only 0.6 mg. By the time 3 mg. of growth had been produced, at least half of the tryptophane had disappeared, and the shape of the absorption spectrum suggests that actually much more than half was gone.

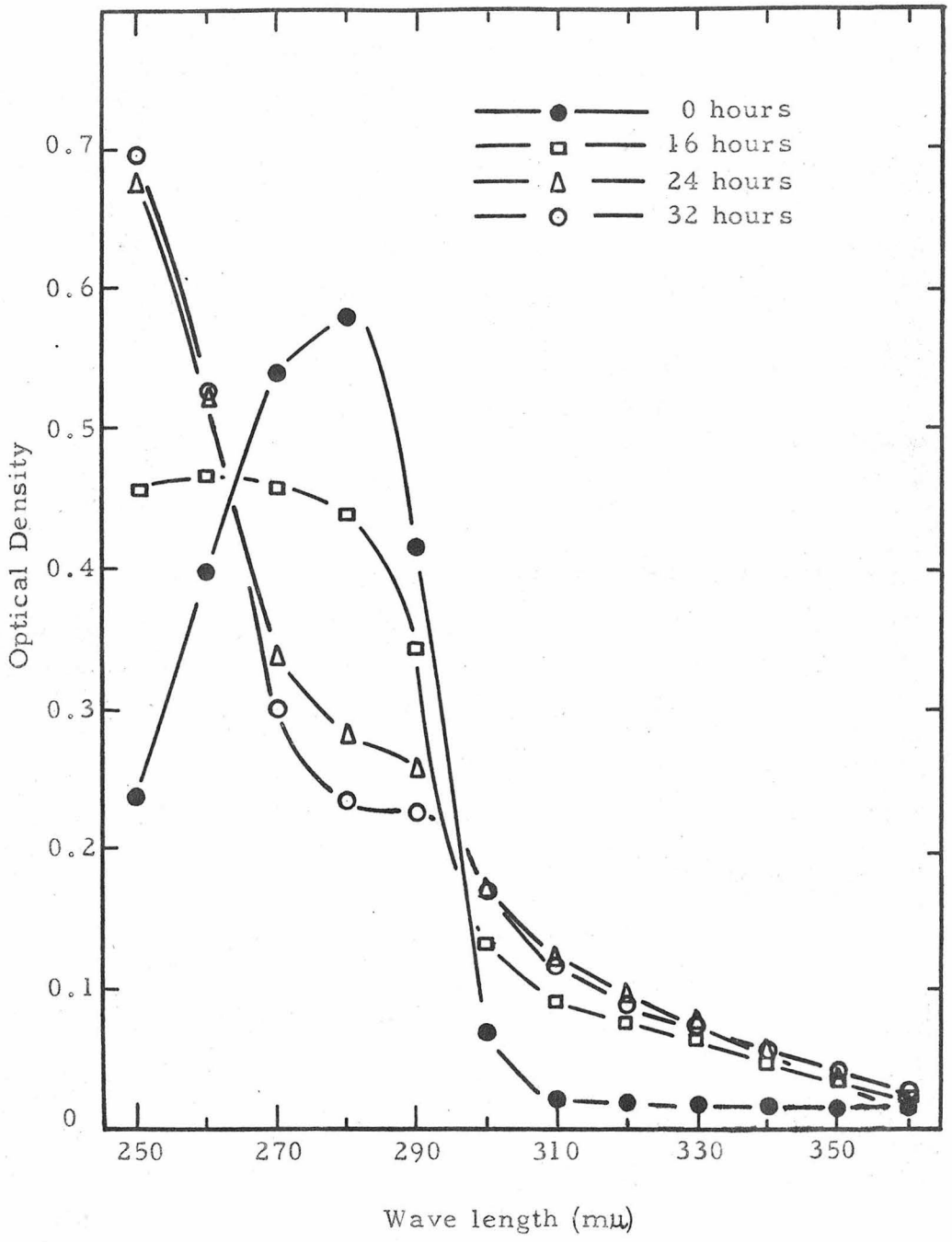


Figure 6. Ultraviolet absorption spectra of Neurospora media at various times after inoculation.

In order to determine whether or not the tryptophane was simply absorbed from the medium by the small amount of mold present in the early stages of growth, the mold was removed from the 32- and 96-hour cultures, hydrolyzed with 1 N NaOH, and the tryptophane content was determined by bioassay with S. lactis R. (32). A control sample of tryptophane was subjected to the same treatment. The tryptophane content of the 32-hour culture on a dry-weight basis was found to be 0.3% and that of the 96-hour culture 0.2%. After 32 hours the mold therefore contained approximately 27 γ of tryptophane as opposed to the disappearance of at least 200 γ. Thus it is clearly demonstrated that the tryptophane had not been taken up as such by the mold, but actually had been converted to other compounds.

Products of tryptophane degradation

Flasks of medium containing 400 γ of L-tryptophane/20 ml. were inoculated with conidia from wild-type strain 8a and from mutants B1312, C86, and 10575, respectively. After 32 hours' incubation at 25° the mold was removed from the flasks, the medium was filtered, the pH was adjusted to 2.5, and the medium was extracted four times with an equal volume of ether. The ether was evaporated on a warm water bath. With each strain the absorption spectrum of the material in the ether phase was found to be nearly the same as the absorption spectrum of the unextracted medium. Measurements of fluorescence using a Coleman photofluorometer disclosed that approximately 80% of the fluorescence originally present in the unextracted medium went to the ether phase.

The residual aqueous phase had an absorption spectrum corresponding to a maximum of 50γ of tryptophane. Bioassays using strains C86 and B1312 showed that from the original 400γ of tryptophane there remained in the medium after 32 hours' growth the biological equivalent of approximately 250γ. Of this, approximately 180γ was extracted with ether. With the unextracted medium strains 10575 and C83, which do not use anthranilic acid, gave growth which was equivalent to that obtained on about 50γ of tryptophane, but neither of these two strains utilized the concentrated ether extract. All four of the strains tested (C86, B1312, 10575, and 8a) gave the same general qualitative and quantitative results as determined by measurements of absorption spectra, fluorescence, and biological activity.

Filter paper strip chromatograms were run on the concentrated ether extract, with water-saturated butanol as the solvent. After development, the chromatograms were examined under ultraviolet light to detect fluorescent areas, and were then cut into sections, the sections were extracted with 0.1 M KH_2PO_4 (pH 3.5), and the biological activities of the resulting solutions were determined using B1312 for the assays. It was found that approximately 60% of the activity of the ether phase could be accounted for by the activity of a chromatogram band which resembled an anthranilic acid band in its position and fluorescence.

In order to obtain enough material for isolation of some of the degradation products, 8 l. of culture medium containing 8 gm. of

L-tryptophane was inoculated with a heavy inoculum of conidia from wild-type strain 8a. The culture was incubated with forced aeration in a 35° water bath for 19 hours. The mycelium was then filtered off, the pH of the filtrate was adjusted to 2.5, the filtrate was divided into two portions, and each was extracted with seven 500-ml. portions of ether. Following evaporation of the ether, the residual solution was absorbed on 29 sheets of filter paper and these sheets were incorporated into a chromatopile consisting of 600 sheets of 9-cm. Whatman no. 1 filter paper. Water-saturated butanol was used as the developer. After 15 hours, every twentieth sheet was removed from the pile and the location of anthranilic acid was determined by absorption spectra of extracts of the sample sheets. The desired section was then removed from the chromatopile, dried in a vacuum desiccator over $CaCl_2$, and extracted with ether in a Soxhlet. The residue from evaporation of the ether solution was sublimed at 100° in vacuo (20 microns Hg). The sublimate was crystallized from water and further purified by re-sublimation in a temperature gradient at 20 microns pressure. Melting points were as follows:

Known anthranilic acid	144-145° (cor.)
Isolated compound	143-144 "
Mixed melting point	144-145 "

The neutralization equivalent of the isolated compound was 137 (calculated for anthranilic acid -- 137). The absorption spectrum, fluorescence, and biological activity were identical with that of known anthranilic acid,

furnishing further confirmation that the isolated compound was indeed anthranilic acid.

Chromatographic examination of the products of tryptophane degradation by germinating Neurospora has demonstrated the existence of at least two C86-active substances in addition to anthranilic acid. Also, bioassay with L. arabinosus has shown the production of 4 γ of nicotinic acid from 400 γ of tryptophane in 32 hours.

A number of compounds other than tryptophane were tested to determine whether or not they were able to give rise to fluorescent degradation products in the medium. Strain C86 was used as the test strain. It was found that strain C86 produced the typical blue fluorescence in the medium when supplied with phenylalanine, indole, or kynurenine. No appreciable fluorescence was produced when the medium was supplemented with tyrosine, 3-hydroxykynurenine, or nicotinamide. A test on 3-hydroxyanthranilic acid appeared also negative but inasmuch as the compound itself had some blue fluorescence the test was not critical.

Pathway followed in the degradation of tryptophane to anthranilic acid

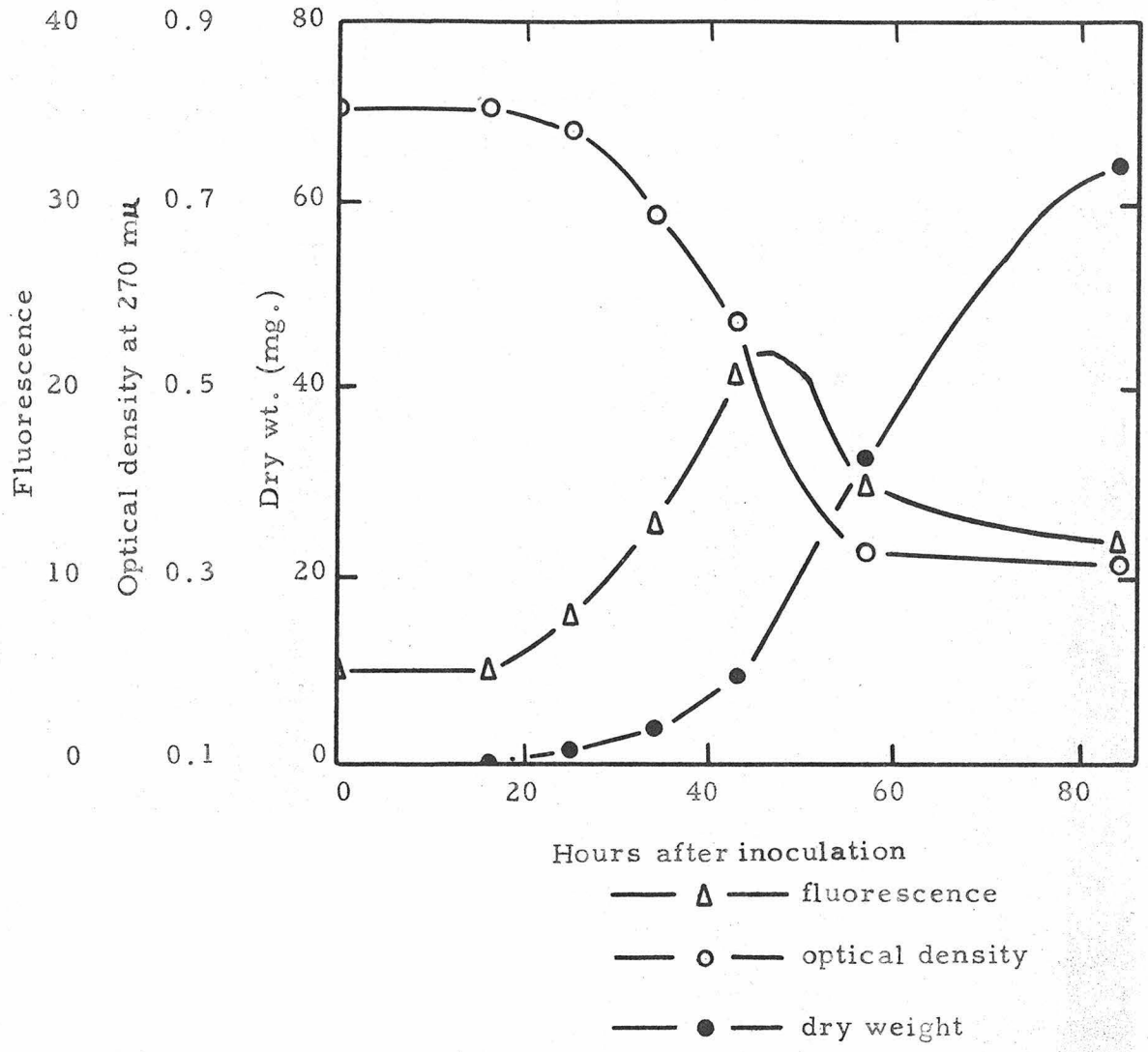
In seeking to establish the pathway of tryptophane breakdown one possibility which must be considered is that the degradation takes place by simple reversal of the biosynthetic reactions, i. e., that indole is an intermediate between tryptophane and anthranilic acid. A second possibility is that tryptophane is first oxidized to kynurenine and that the

side chain of kynurenine is then oxidized to a carboxyl group, giving anthranilic acid. The first of these two possibilities at once seems unlikely because of the fact that 10575, which is apparently blocked between anthranilic acid and indole, degrades tryptophane with about the same avidity as wild-type Neurospora. In addition, the failure of C83 and 10575 to utilize kynurenine for growth although this compound apparently gives rise to anthranilic acid (p. 46) indicates that kynurenine is an intermediate between tryptophane and anthranilic acid, rather than tryptophane being an intermediate between kynurenine and anthranilic acid. Further evidence that the degradation goes via kynurenine is furnished by the following experiment.

Seven flasks of each of four different media were inoculated with conidia from wild-type 7A. The four media used were:

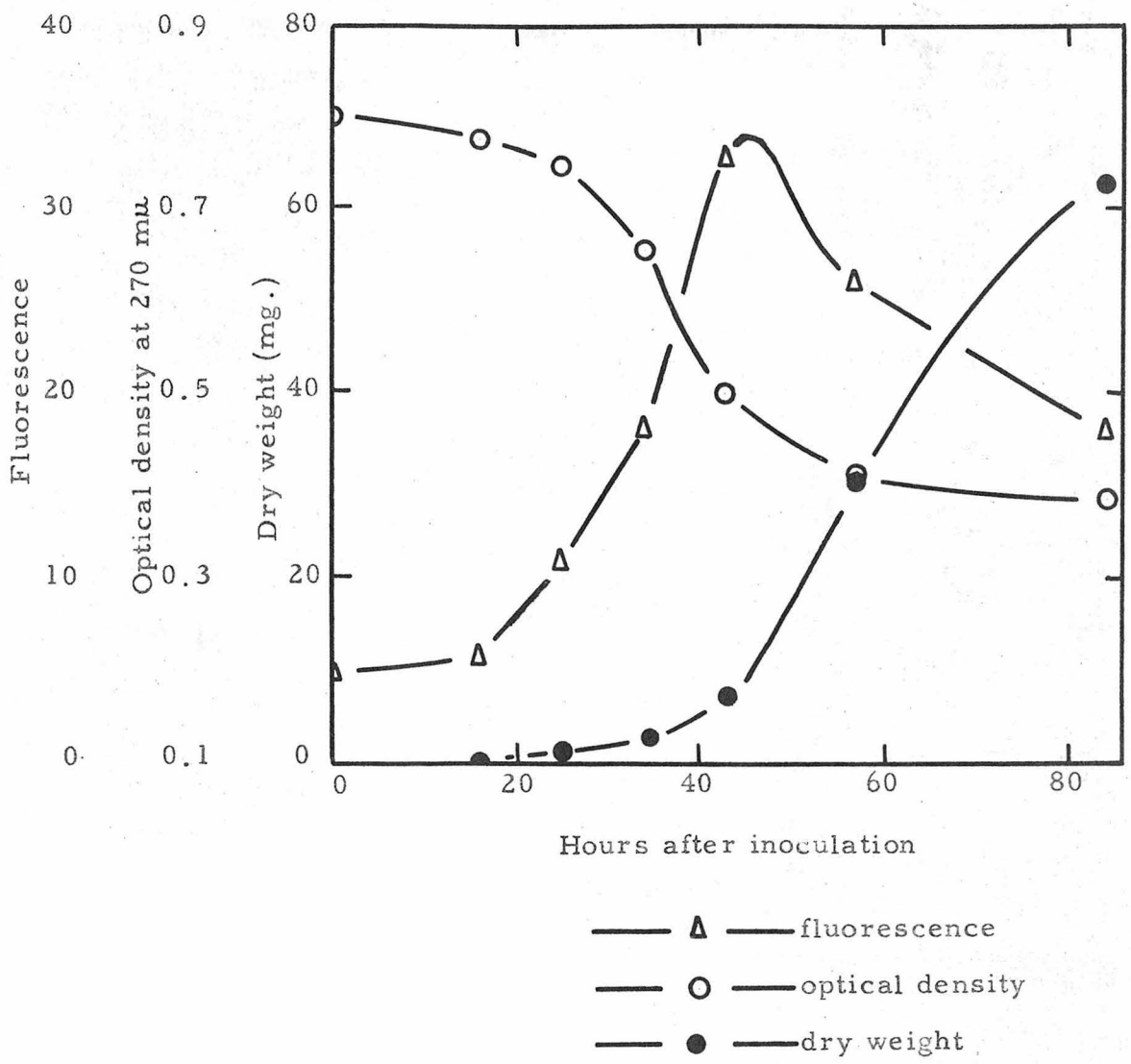
Minimal plus 400 γ indole/20 ml.
" " 400 γ indole and 2 mg. DL-serine/20 ml.
" " 400 γ L-tryptophane/20 ml.
" " 400 γ L-kynurenine/20 ml.

Flasks were harvested at intervals, and dry weight of mold, and absorption spectra and fluorescence of the media were determined. The results are shown graphically in Figure 7. The optical density values are given for wave lengths at which the different compounds show peaks in absorption. These values provide an indication of the concentration of the compound concerned which is quite reliable during the very early stages of growth, but as growth proceeds, the absorption background of the medium becomes so great that the spectrum does not provide a good

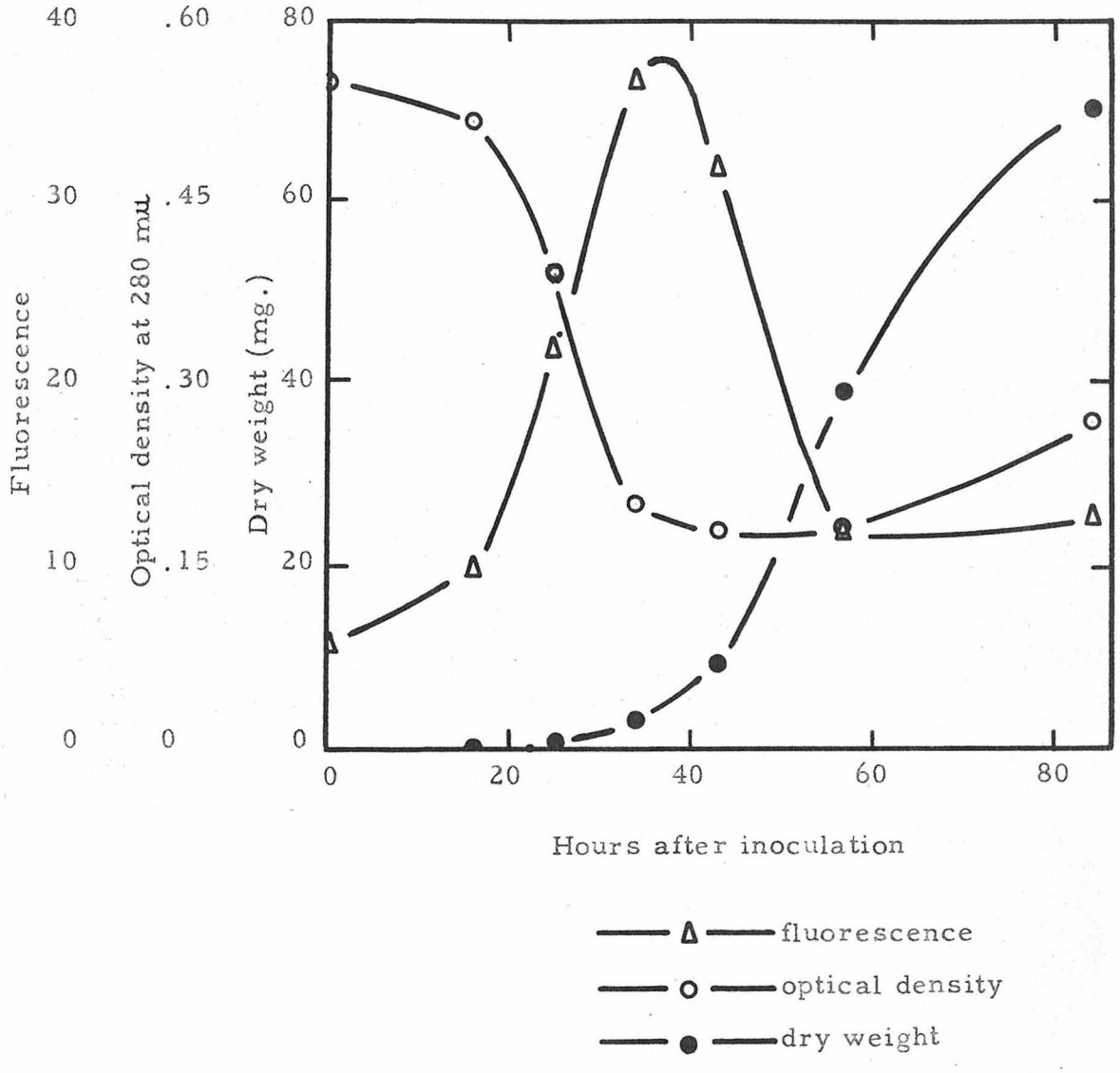


7a. Indole

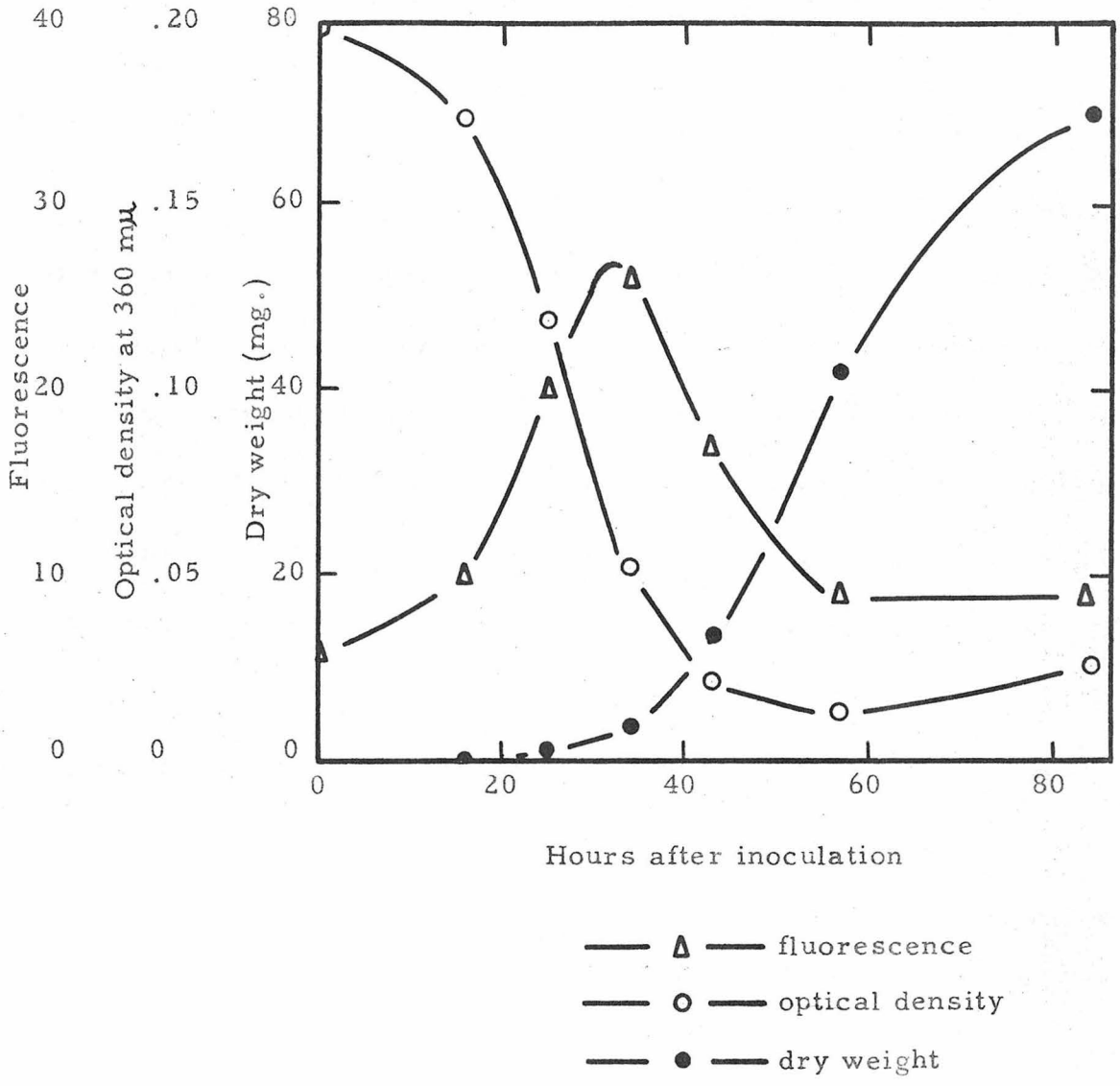
Figure 7a-d. Dry weight of mold, and optical density and fluorescence of four different media at various times after inoculation.



7b. Indole plus serine



7c. Tryptophane



7d. Kynurenine

index of the concentration of any single compound. This is clear from an examination of the spectra of a tryptophane-containing medium at various times after inoculation (fig. 6). Thus the compounds indole, tryptophane, and kynurenine actually disappear faster than the optical density values indicate.

The 25- and 34-hour flasks were combined for each of the four kinds of medium and 25-ml. portions were acidified to pH 2.5 and extracted with three 15-ml. portions of ether. The ether extracts were concentrated and chromatogramed on Whatman no. 1 filter paper strips using two solvents -- butanol saturated with 0.1 N HCl and butanol saturated with 1% NH_3. The chromatographic evidence thus obtained indicates that the principal blue-fluorescent compound produced when 7A was grown on indole, indole plus serine, tryptophane, or kynurenine was anthranilic acid. The data show that the disappearance of metabolite and the appearance of blue fluorescence, either as a function of time or of the dry weight of mold, was more rapid for tryptophane or kynurenine than for indole. The rates for tryptophane and kynurenine did not differ greatly -- if anything the metabolite disappeared and the fluorescence reached a peak a little faster in the kynurenine flasks than in the tryptophane flasks. The addition of serine seemed to accelerate the disappearance of indole to some extent. This is in agreement with Tatum and Bonner's observation that added serine would speed the disappearance of indole, presumably by helping along the indole $\longrightarrow$ tryptophane reaction (2). If indole were going to anthranilic acid by a more

direct route than via tryptophane, it seems unlikely that serine would speed the conversion. This evidence all points to the formation of anthranilic acid from tryptophane via kynurenine rather than indole. Thus, a metabolic cycle involving anthranilic acid, indole, tryptophane, and kynurenine is clearly indicated.

Recently Bonner and Partridge (36) have questioned the existence of such a cycle on the basis of their observation that N^{15}-labeled indole, when fed to a 10575-3416 double mutant, gave rise to N^{15}-labeled quinolinic acid, and that labeled anthranilic acid was produced only in what they considered to be insignificant quantities. It has been shown, however, that mutant 10575 under some conditions may accumulate approximately 100 γ of anthranilic acid/ml. of culture fluid (p. 11). When wild type was grown in the presence of 400 γ of indole/20 ml., on the other hand, the maximum fluorescence produced corresponded to about 3 γ of anthranilic acid/ml. Thus the anthranilic acid accumulated from sources other than the added indole might be expected to make up approximately 97% of the total anthranilic acid present, and the proportion of labeled anthranilic acid would almost certainly be very small. It follows that the argument advanced by Bonner and Partridge is not convincing, especially in the absence of more complete data.

The accumulation of acid-labile inorganic phosphate by mutant 65001 when grown on indole, tryptophane, or kynurenine but not on hydroxyanthranilic acid or nicotinamide (37) also suggests some special

metabolic relationship of the first three compounds and thus strengthens the evidence for the existence of the cycle. Mutant 65001 is genetically similar to mutant 39401 (p. 57).

DISCUSSION

The information thus far available regarding the metabolism of tryptophane, phenylalanine, nicotinic acid, and various other related compounds by Neurospora may be schematically summarized as shown in Figure 8. The positions of quinic acid and shikimic acid in this scheme are not clear. Tatum (38) has a Neurospora mutant, Y7655, which ordinarily requires the addition of four compounds, p-amino-benzoic acid, tyrosine, phenylalanine, and tryptophane to the culture medium, and he has found that shikimic acid alone can support the growth of this mutant (39). Davis (40) has found that shikimic acid also promotes the growth of an E. coli strain with the same quadruple requirement. Thus the evidence is accumulating in favor of a common precursor for a number of different aromatic compounds. If it were not for the inability of C86 to utilize shikimic acid, the available evidence would point to this compound as the common precursor. The reconciliation of the observations that Y7655 can utilize shikimic acid but not quinic acid while C86 responds to the second compound but not to the first awaits further knowledge of the metabolism of these two compounds.

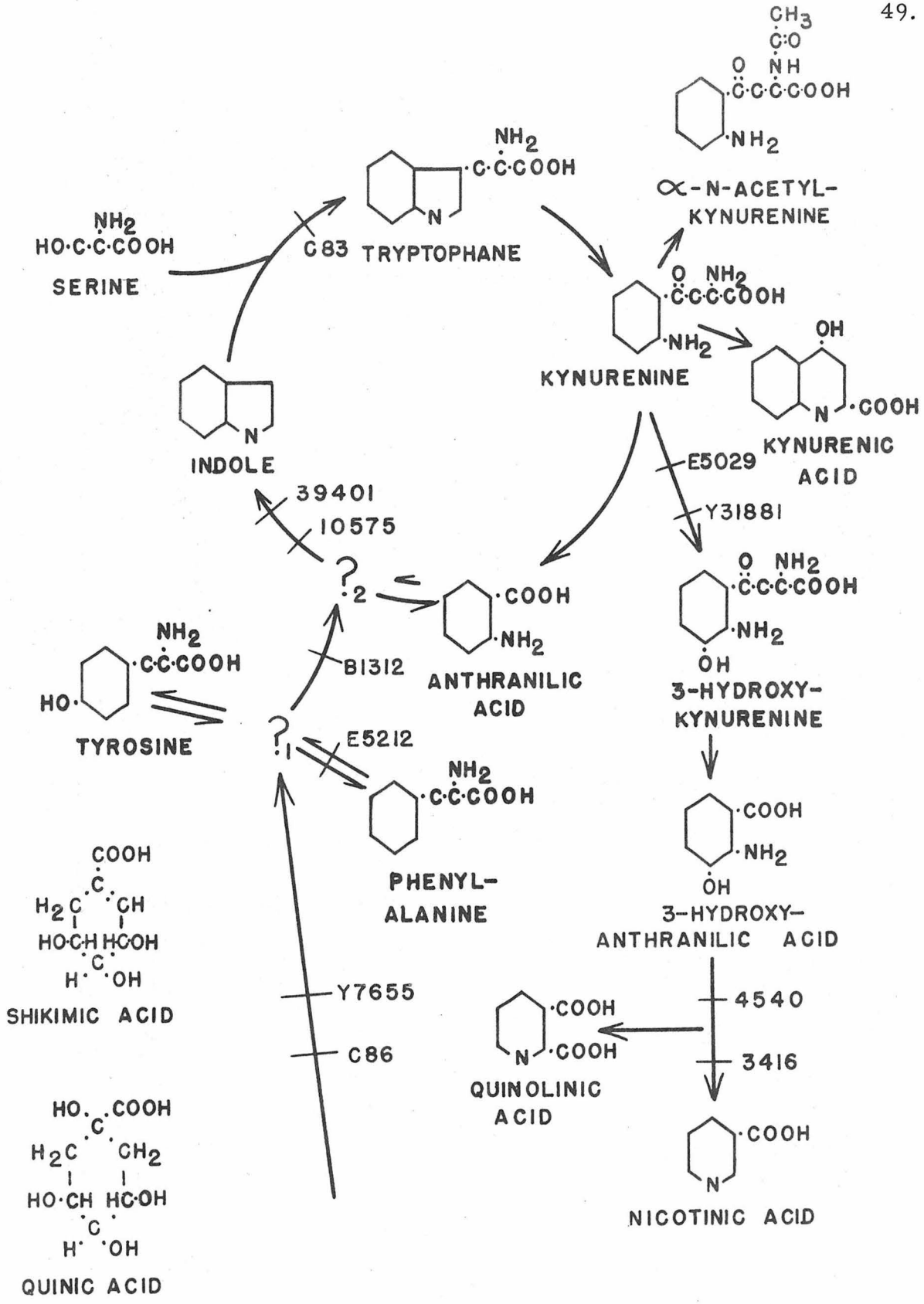


Figure 8. Schematic representation of nicotinic acid synthesis in Neurospora.

A compound, $?_2$, has been postulated between anthranilic acid and indole because, as reported by Nyc et al (41) the carboxyl carbon atom of anthranilic acid is lost as CO_2 during the metabolism of this substance by Neurospora. It seems most probable that this loss occurs during the conversion of anthranilic acid to indole since the work of Heidelberger et al (42, 43, 44) has traced the corresponding carbon atom of the indole ring of tryptophane to kynurenine and nicotinic acid in rats.

The fact that only two question marks are included in the scheme shown in Figure 8 certainly is not meant to imply that all but two intermediates are known. Strains E5212, 10575, and E5029 are known to accumulate biologically active compounds whose identity has not yet been established. These compounds obviously belong somewhere in the scheme. Some of the real intermediates will probably escape detection and identification for a long time to come, for there is little doubt that many of them are highly labile compounds.

Attention is directed to the fact that of the eleven mutant strains indicated in Figure 8, biological accumulations have been demonstrated in all but three. The mutants and the accumulated products which have been identified are:

E5212 - - - nicotinic acid and anthranilic acid

39401 - - - inorganic phosphate (37)

10575 - - - anthranilic acid (1)

C83 - - - - anthranilic acid

E5029 - - - kynurenic acid, anthranilic acid

Y31881 - - - ∝-N-acetylkynurenine (28)

4540 - - - 3-hydroxyanthranilic acid (8)

3416 - - - quinolinic acid (22, 23)

In addition these mutants accumulate a number of other compounds, some of which are biologically active and some inactive, which are still to be identified. It is of interest that four rather widely different mutants, E5212, C83, 10575, and E5029, all accumulate anthranilic acid. This accumulation, especially in the cases of strains E5212 and E5029, indicates that anthranilic is turned over more slowly than many of the related aromatic metabolites.

Accumulations are also common among mutant strains of Neurospora which require sulfur amino acids, purines, pyrimidines, choline, thiamine, or any one of numerous other compounds (9, 10). Thus it appears that biological accumulations are quite generally associated with genetic blocks. No generalization can be made, however, regarding the relation of the accumulated product to the apparent position of the block. The accumulation of anthranilic acid by 10575, of ∝-N-acetylkynurenine by Y31881, of kynurenic acid by E5029, of 3-hydroxyanthranilic acid by 4540, and of quinolinic acid by 3416 are examples in which

the accumulated product seems not far removed from the intermediate whose normal conversion has been blocked by the mutation -- indeed in the case of the 4540 accumulation, the 3-hydroxyanthranilic acid may be the actual intermediate which is just before the block. In other cases, the accumulated product seems rather far removed from the block itself -- this is especially true of the accumulations of nicotinic acid and anthranilic acid by E5212 and of inorganic phosphate by 39401. This matter of biological accumulations serves well to illustrate the great complexity of the metabolism of living things, but at the same time it provides a means by which this complex metabolism may be studied.

II. GENETIC STUDIES: AN EXAMPLE OF GENETIC MODIFICATION IN NEUROSPORA

INTRODUCTION

There are numerous cases on record in which a particular phenotypic character has been shown to depend on the action of more than one gene. Excellent examples of this type of phenomenon are found in the work on plant, aleurone, scutellum, and pericarp colors in maize (45), and on coat colors (46) and other characters (47) in guinea pigs. In Neurospora the effects of modifiers are suggested by quantitative differences in growth substance utilization among different reisolates of a mutant strain (48), and genetic suppression (49) could be regarded as an extreme type of modification. In addition, Srb (50) has found a case in which a single gene modifier appears to control the ability of a proline- or ornithine-utilizing strain of Neurospora to utilize arginine or citruline; and Mitchell and Mitchell (51) have found that some reisolates of a mutant which ordinarily requires uridine or cytidine can utilize orotic acid while others can not. The data to be presented here deal with an instance in which the modification is such that different reisolates from a cross of mutant by wild appear to be blocked at different steps in a biosynthetic pathway. The pathway concerned is that which leads to the formation of tryptophane and nicotinic acid by Neurospora.

EXPERIMENTAL

MUTANT STRAINS

Of the strains listed in Tables I and II, C86, 39401, E5029, and 10575 were used in this work. In addition, three other strain types which have been designated Int. (intermediate), Nic. (nicotinic), and S. M. (suppressed mutant) were used in the crosses to be discussed here. Int., Nic., and S. M. were derived from crosses involving C86, 39401, and wild-type strains. Int. types utilize anthranilic acid in addition to the compounds utilized by 39401; Nic. types differ from 39401 in being unable to utilize indole and tryptophane; and S. M. types are phenotypically wild.

ADAPTATION OF 39401 TO ANTHRANILIC ACID

As shown in Table II, mutant 39401 does not ordinarily use anthranilic acid. It was found, however, that a reisolate of this strain, 39401-7457-6A occasionally would produce growth in liquid culture on anthranilic acid in four days. This reisolate was cultured on slants of minimal agar supplemented with 5 γ of anthranilic acid/ ml., and although somewhat slow to start on this medium, the mutant eventually produced abundant growth. The response of this culture to phenylalanine, anthranilic acid, tryptophane, and nicotinamide appeared to be essentially the same as that of the parent strain before subculturing on anthranilic acid slants.

Transfers to anthranilic acid agar were continued, however, and after the seventh consecutive transfer the strain was again tested, this time on quinic acid, tyrosine, phenylalanine, anthranilic acid, indole, tryptophane, and nicotinamide. All of these substances were utilized, and it was found that within limits, increasing the concentration of any of them produced increased growth of the mold. The 39401-7457-6A which had been maintained on minimal agar plus tryptophane, and 39401-12A, another reisolate of the same mutant, were capable of efficient utilization of only indole, tryptophane, and nicotinamide; i.e., their growth requirements appeared unchanged in contrast to the culture that was maintained on anthranilic acid agar.

CROSSES OF 39401 WITH C86 AND WILD TYPE

Having acquired the ability to utilize quinic acid, tyrosine, phenylalanine, and anthranilic acid, the 39401-7457-6A from the anthranilic acid slants appeared to be very much like C86. It was of interest to determine whether or not these two strains might be genetically similar. To investigate this possibility, the following crosses were made:

1.	39401-7457-6A Anthran.	X	8a (wild type)
2.	"	X	C86a
3.	39401-7457-6A Trypt.	X	8a
4.	"	X	C86a
5.	39401-12A	X	8a
6.	"	X	C86a

Approximately 3000 ascospores from each of these crosses were allowed to germinate on plates of minimal agar. Microscopic examination of the

plates disclosed that germination percentage was 90% or more in each case. Certain of the germinated spores were selected on the basis of sprout length (see Table VII) and were transferred from the plates to 3" tubes of minimal agar supplemented with 2.5 γ of nicotinamide/ml. The resulting ascospore cultures were tested in 125-ml. Erlenmeyer flasks, and were classified according to their response to minimal; DL-phenylalanine, 500γ/20 ml.; anthranilic acid, 200γ/20 ml.; L-tryptophane, 500γ/20 ml.; and nicotinamide, 20γ/20 ml. Table VI lists the phenotypic classes into which the cultures were divided, together with the number of individuals, the mean dry weights attained, and the standard deviations for each class. Data from crosses 1-6 (p. 55) and a-k (p. 58) were combined in the preparation of this table. In most cases it was quite easy to assign a culture to one of the four large classes (C86, Int., 39401, or Nic.) but there were some borderline cases, particularly between C86 and Int. Some of the cultures which were classified as Int. produced between 20 and 30 mg. dry weight on phenylalanine, but in no case did they utilize the phenylalanine immediately -- there was a lag of 24 to 48 hours before growth became appreciable. The typical C86 cultures, on the other hand, grew immediately on phenylalanine.

The results of classifying the ascospore cultures from crosses 1-6 are shown in Table VII. Of the three crosses between different 39401 strains and C86a, the only one to give rise to phenotypically wild offspring was 39401-7457-6A Anthran. X C86a. Further testing

Table VI. Frequencies, means, and standard deviations (s) of the phenotypic classes from crosses 1-6 and a-k.

Phenotypic class	No. in class	4-day dry weights (mg.) produced on Minimal		Phenyl-alanine 500γ		Anthranilic acid 200γ		Tryptophane 500γ		Nicotinamide 20γ	
		Mean	s	Mean	s	Mean	s	Mean	s	Mean	s
C86h	7	38	13	58	6	65	7	76	3	70	3
C86	177	trace		43	8	65	8	76	11	77	6
Int. h	3	trace		20	5	50	5	64	9	67	6
Int.	108	trace		9	9	55	9	71	14	69	9
39401h	2	trace		10	10	17	0	61	4	68	10
39401	247	trace		trace		1	4	49	10	71	7
Nic.	34	trace		trace		1	2	4	4	74	7
	578										

showed that all five of these phenotypically wild offspring were suppressed mutants. This will be considered in a later section of this thesis.. It appears from these crosses, then, that C86 and 39401 are either allelic or else very closely linked. It is also worthy of note that it is possible to get the C86 phenotype from a 39401 X wild cross, and that each of the six crosses gave rise to the Int. type which is different from any of the parental strains*.

*Miss Dorothy Newmeyer, of Stanford University, has observed some of these same things in crosses involving C86, 65001, and 39401.

Table VII. Results of testing spores from crosses 1-6.

Cross no.	Basis of Spore Selection	Number tested	Types				
			Nic.	39401	Int.	C86	Wild
1	Short and medium sprouts	14	0	11	2	0	1
2	Short sprouts	5	1	2	0	2	0
	Medium sprouts	5	0	0	2	3	0
	Long sprouts	5	0	0	0	0	5
3	Short and medium sprouts	19	0	12	4	3	0
4	Longest sprouts possible	14	0	0	4	10	0
5	Short sprouts	10	0	8	2	0	0
	Medium sprouts	8	0	5	2	1	0
6	Longest sprouts possible	9	0	0	2	7	0

DISSECTION OF ASCI FROM CROSSES INVOLVING 39401, INT., C86, AND WILD TYPES

In order to study the inheritance of the three main mutant types (C86, Int., and 39401), asci from the following crosses were dissected.

a. 39401-12A X 39401-11a (from cross no. 5, p. 55)
b. " X Int. 2a (from cross no. 1, p. 55)
c. " X C86a
d. " X 8a
e. " X 25a } wild types
f. " X Em 8815-3a }
g. Int. 2a X Int. 1A (from cross no. 4, p. 55)
h. Int. 1A X C86a
i. " X 8a
j. C86A X C86a
k. " X 8a

Cultures from the dissected ascospores were tested in flasks in the same way as the ascospore cultures from crosses 1-6 (p. 55). In crosses

involving wild types, the dissected spore cultures were tested first on 3" tubes of minimal and minimal plus nicotinamide (2.5 γ/ml.) and only those which appeared to be mutant were tested in flasks. At the start both members of each mutant spore pair were tested. A total of 123 spore pairs were tested in this way, and it was found that agreement between the two members of any spore pair was excellent. Consequently, spores were dissected out and tested by pairs rather than individually.

Results of the crosses are shown in Table VIII. The "h" (high) strains from the cross of 39401-12A X Em 8815-3a require some explanation. It may be seen from Table VI that C86h includes those cultures which seemed much like C86 but which grew appreciably on minimal; in some cases the four-day growth was approximately half that of a normal wild strain. Int. h cultures include those which grew well on anthranilic acid, tryptophane, and nicotinamide; and poorly but appreciably on phenylalanine, i.e., around 20 mg. in four days. The 39401h cultures similarly had some growth on phenylalanine and anthranilic acid, but much less than the growth on tryptophane and nicotinamide.

No asci were dissected from crosses involving Nic.-type strains, but ascospores were plated at random from a cross of one such strain with C86a. Of approximately 25,000 spores which were plated on minimal agar, approximately 6,000 germinated. One of the germinated spores was phenotypically wild -- all the rest were mutant. It is probable that the phenotypically wild spore was the result of a suppressor

Table VIII. Ascus dissections from crosses a-k.

Cross	No. of asci tested	Constitution of asci: number of spore pairs of the indicated phenotypes						
		Wild	C86	Int.	39401	Nic.		
a.	19	0	0	0	4	0		
b.	3	0	0	1	2	1		
	10	0	0	0	2	2		
	1	0	0	2	2	0		
	1	0	0	0	3	1		
	15							
c.	7	0	1	1	2	0		
	3	0	2	0	2	0		
	1	0	0	2	2	0		
	11							
d.	4	2	0	0	2	0		
	8	2	0	1	1	0		
	2	2	1	0	1	0		
	14							
e.	10	2	0	0	2	0		
	1	2	0	1	1	0		
	2	2	1	0	1	0		
	13							
f.			C86h	C86	Int. h	Int.	39401h	39401	Nic.
	2	2	0	0	0	0	1	1	0
	1	2	0	0	2	0	0	0	0
	1	2	1	0	1	0	0	0	0
	1	2	2	0	0	0	0	0	0
	4	2	1	0	0	0	0	1	0
	4	2	0	0	0	0	0	2	0
	1	2	0	1	0	0	0	1	0
	14								

Table VIII (contd.). Ascus dissections from crosses a-k.

Cross	No. of asci tested	Constitution of asci: number of spore pairs of the indicated phenotypes				
		Wild	C86	Int.	39401	Nic.
g.	1	0	1	0	2	1
	2	0	2	0	0	2
	1	0	0	1	2	1
	2	0	1	1	1	1
	3	0	1	2	1	0
	2	0	1	2	0	1
	1	0	0	2	0	2
	3	0	0	1	3	0
	1	0	2	0	1	1
	2	0	0	2	1	1
	18					
h.	5	0	2	2	0	0
	4	0	3	1	0	0
	1	0	1	3	0	0
	1	0	4	0	0	0
	11					
i.	4	2	1	1	0	0
	1	2	2	0	0	0
	5	2	0	2	0	0
	10					
j.	11	0	4	0	0	0
	3	0	2	2	0	0
	14					
k.	18	2	2	0	0	0
	3	2	0	2	0	0
	1	2	1	1	0	0
	22					
Total	161					

mutation or of reversion, or possibly of contamination. Thus it appears that the Nic. type is prevented from growing on minimal by the same mutation which prevents the growth of C86, Int., and 39401 on minimal.

HETEROCARYON TESTS

The ability of different combinations of the various types to form phenotypically wild heterocaryons was investigated briefly. The combinations shown below were inoculated into flasks of liquid minimal. Eight flasks of each combination were prepared, and eight control flasks of each strain inoculated alone were also run.

39401-12A	plus	Nic. (from Int. 1A X Int. 2a)
"	"	Int. 1A
"	"	Int. 2a
"	"	C86A
Nic.	"	Int. 1A
"	"	Int. 2a
"	"	C86A
Int. 1A	"	Int. 2a
"	"	C86A
Int. 2a	"	"

There was no immediate growth in any of the flasks, but after three days some growth had started, especially in the flasks where C86A had been inoculated either alone or in combination. After seven days, only the 39401 controls were without significant growth. Among the other controls, one Nic., three Int. 1A, one Int. 2a, and six C86A had appreciable growth. None of the combinations had more growth than might have been anticipated from the growth of the controls. Thus there is no evidence for the formation of heterocaryons which can grow on minimal.

ACCUMULATION OF ANTHRANILIC ACID BY MINIMAL-ADAPTED C86A

It was noticed that in the flasks where C86A grew on minimal in the heterocaryon test, a strong blue fluorescence was produced. After the mold was removed from these flasks the medium was acidified to pH 3.3 and was extracted three times with a half volume of ether. The concentrated ether extract gave three blue fluorescent spots when chromatogramed on Whatman no. 1 filter paper with propanol and 1% NH_3 in a 2:1 mixture as the solvent. The middle one of the three spots was the most intense. Its Rf value in this system was the same as that of anthranilic acid (approximately 0.65). The absorption spectrum of the ether extract also exhibited a peak which corresponded closely with the anthranilic acid peak, and the ether extract was biologically active for mutant B1312, a strain which can use anthranilic acid, indole, tryptophane, or kynurenine (Table II). It seems highly probable, then, that the principal blue-fluorescent substance produced in the medium by the adapted C86A is anthranilic acid.

A GENETIC SUPPRESSOR

The cultures resulting from the five phenotypically wild spores from cross no. 2 (Table VII) were crossed to wild types 7A or 8a. Ascospores from these crosses were allowed to germinate on plates of minimal agar, and spores were selected for their mutant appearance, cultured on nicotinamide slants, and tested in flasks to determine whether they were actually mutant. By these means, mutant spores

were found among the progeny of each of the five crosses. It appeared likely, therefore, that genetic suppression of the type described by Houlahan and Mitchell (49) had occurred. Asci were dissected from the cross of 8a to one of the apparently suppressed mutants. Of the twelve asci tested, two had four wild spore pairs; eight had three wild and one mutant; and two had two wild and two mutant. The wild spores from this third type of ascus would be expected to carry the suppressor. One of these was selected and crosses were made as shown in Table IX. The

Table IX. Crosses involving the suppressor (S), suppressed mutant (SM), and other strains.

Cross	No. of asci tested	No. of asci with the indicated ratio of wild:mutant spore pairs		
		4:0	3:1	2:2
S X 8a	15	15	0	0
S X SMa	14	14	0	0
S X 39401-11a	15	0	5	10
S X Int. 2a	13	0	3	10
S X C86a	17	2	7	8
SM-a X 7A	14	2	12	0
SM-A X 8a	12	2	8	2
SM-A X C86a	16	0	0	16
S X 10575	22	0	0	22
S X E5029	24	0	0	24
Total	162			

classification of the offspring was based mainly on tests using 3" tubes of minimal and minimal plus nicotinamide (2.5γ/ml.), although some of the cultures were tested in flasks. Spores from the cross of S (suppressor) X 10575 were tested on tubes of minimal and minimal plus L-tryptophane (50γ/ml.).

In these crosses the suppressor exhibits the behavior of a single gene whose action repairs in some way the function of the mutant gene which C86, Int., and 39401 have in common, i.e., the mutation which distinguishes these strains from wild type. The influence of the suppressed mutant nuclei which are evidently present in the 39401-7457-6A Anthran. culture is not certainly known. As a first hypothesis, it seems that the presence of a few such nuclei changes the phenotype of the culture from 39401 to C86.

DISCUSSION

The results reported here indicate that C86, Int., 39401, and Nic. have in common a mutation which prevents their growth on minimal medium, and that this primary effect is modified by the action of a number of other genes so that the various strains differ qualitatively in their responses to phenylalanine, anthranilic acid, tryptophane, and nicotinamide. Actually there are quantitative differences as well, for there is considerable variation within each type in the amount of growth produced on any substrate, as shown by the standard deviations in

Table VI. It seems likely that if enough crosses were analyzed they would indicate a more or less continuous spectrum from the Nic. type to suppressed mutant or wild type. These quantitative differences have not been considered here -- only the qualitative differences have been taken into account. In the work done thus far these qualitative differences have, with few exceptions, been quite distinct.

In order to account for the observed segregations listed in Table VIII, in which 46 asci have spores of three different phenotypes and two have four different phenotypes, it is necessary to invoke the influence of at least seven genes on the utilization of phenylalanine, anthranilic acid, tryptophane, and nicotinamide. The postulated genes and their apparent effects are:

Gene	Effect
1	The mutation which is common to the four mutant phenotypes and which prevents their growth on minimal medium.
2	Makes Int. 1 from C86.
3	Makes 39401 from Int. 1 or C86.
4^+	Produces "h" types in cross f.
5^+	Suppresses gene 2.
6	Makes Int. 2.
7	Makes Nic. from 39401 except that the 39401 of the genotype 12^+3456^+ is not affected by 7.

It is also postulated that 6 combined with 2 gives the 39401 phenotype in the presence of 5, 4, and 7^+.

Genes 4^+ and 5^+ have been written with plus superscripts because these two genes seem to make the mold less specific in its requirements for growth substances, while the postulated genes 1, 2, 3, 6, and 7 appear to make the organism more specific in its requirements. Other than this, the plus superscripts are not intended to have any significance. Possible genotypes, with respect to the seven genes, of the strains used in crosses are as follows:

8a and 25a	$1^+2^+3^+4\ 5\ 6^+7^+$
Em 8815-3a	$1^+2^+3^+4^+5\ 6^+7^+$
C86A and C86a	$1\ 2^+3^+4\ 5^+6^+7^+$
Int. 1A	$1\ 2\ 3^+4\ 5\ 6^+7^+$
Int. 2a	$1\ 2^+3\ 4\ 5\ 6\ 7$
39401-12A	$1\ 2\ 3\ 4\ 5\ 6^+7^+$

Eight of the 161 asci listed in Table VIII cannot be accounted for by these parental genotypes. These eight include one ascus -- the only one obtained from this particular perithecium -- from cross h (Int. 1A X C86a) which had three Int. spore pairs and one C86 pair; and three asci from a single perithecium from cross j (C86A X C86a) and four asci from two perithecia from cross k (C86A X 8a) which contained Int. types. Such asci could be obtained if a few of the nuclei in the C86a and C86A cultures carried gene 6 or its equivalent in place of 6^+. Both C86a and C86A have been carried through many transfers, and it would be extremely surprising if they had not become heterocaryotic with respect to some loci. It is by no means certain, however, that the cultures

actually are heterocaryotic for gene 6 and its allele 6^+. It is quite possible that the eight segregations which cannot be accounted for by genes 1 to 7 are the manifestations of still other modifiers.

The genetic data are not sufficiently extensive to permit any conclusions regarding possible allelism among the modifiers. Explaining all the observed effects as resulting from a series of alleles of gene 1 is precluded, however, by the fact that many asci have spores of three or four different phenotypes.

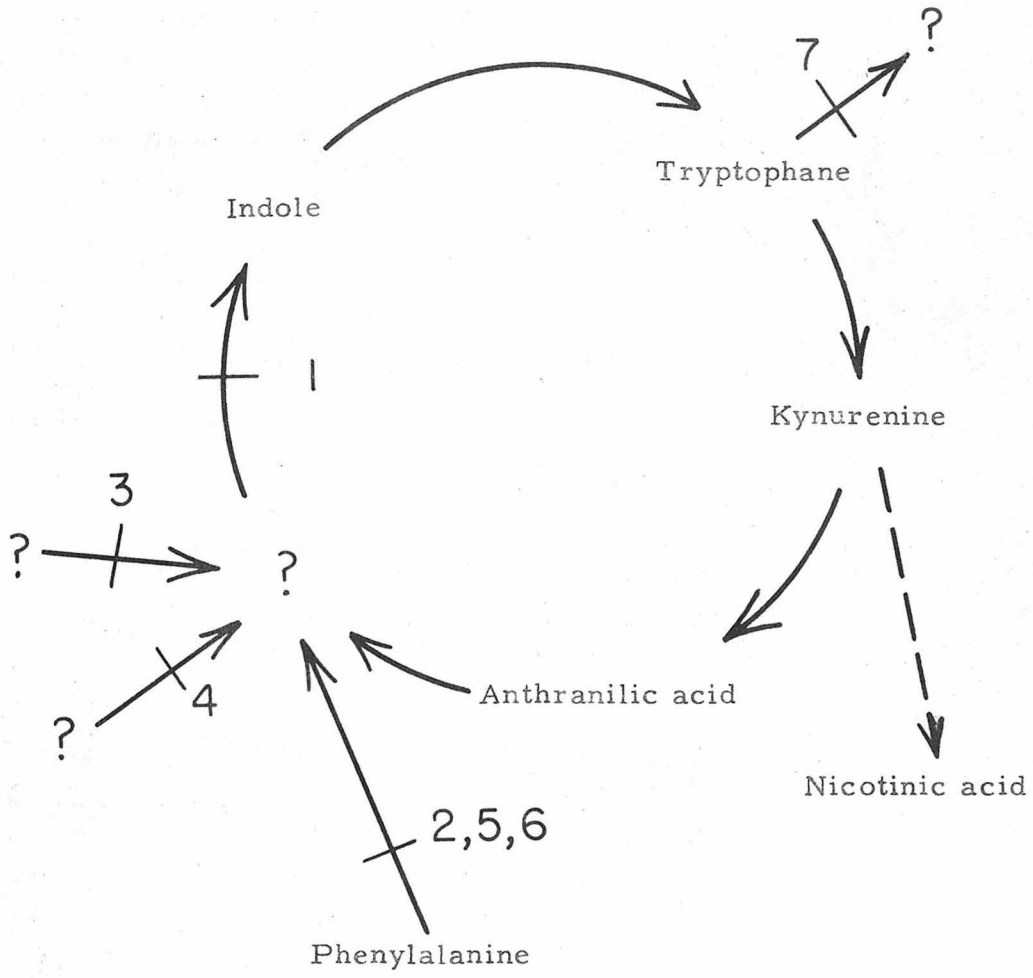
Although the evidence indicates clearly the influence of modifiers, the data are far too limited to warrant extensive speculation on the means by which the modifiers bring about their effects. Three possible mechanisms will be discussed briefly.

(1) There is some evidence which suggests that the primary mutation involves a step just before indole. This is indicated by the fact that 39401 types generally barely germinate on minimal while Nic., Int., and C86 strains usually produce a trace of growth in four days. The accumulation of what appears to be anthranilic acid by C86 as it adapts to minimal also suggests that the primary block is somewhere between anthranilic acid and indole. If it is assumed that gene 1 is concerned with a step between anthranilic acid and indole and that the block created by gene 1 is not completely effective, then genes 2, 3, 4, 5, and 6 could produce their effects by regulating the amounts of precursors which are formed before the block; and 7 could produce its effect by dissipating tryptophane. The evidence for such an effect of gene 7 is supported by

the observation that the Nic. type utilizes o-N-formylkynurenine very well while tryptophane is utilized extremely poorly. Thus gene 7 appears to be associated with a step in which tryptophane is converted to some other compound. This scheme might be illustrated as in Figure 9a.

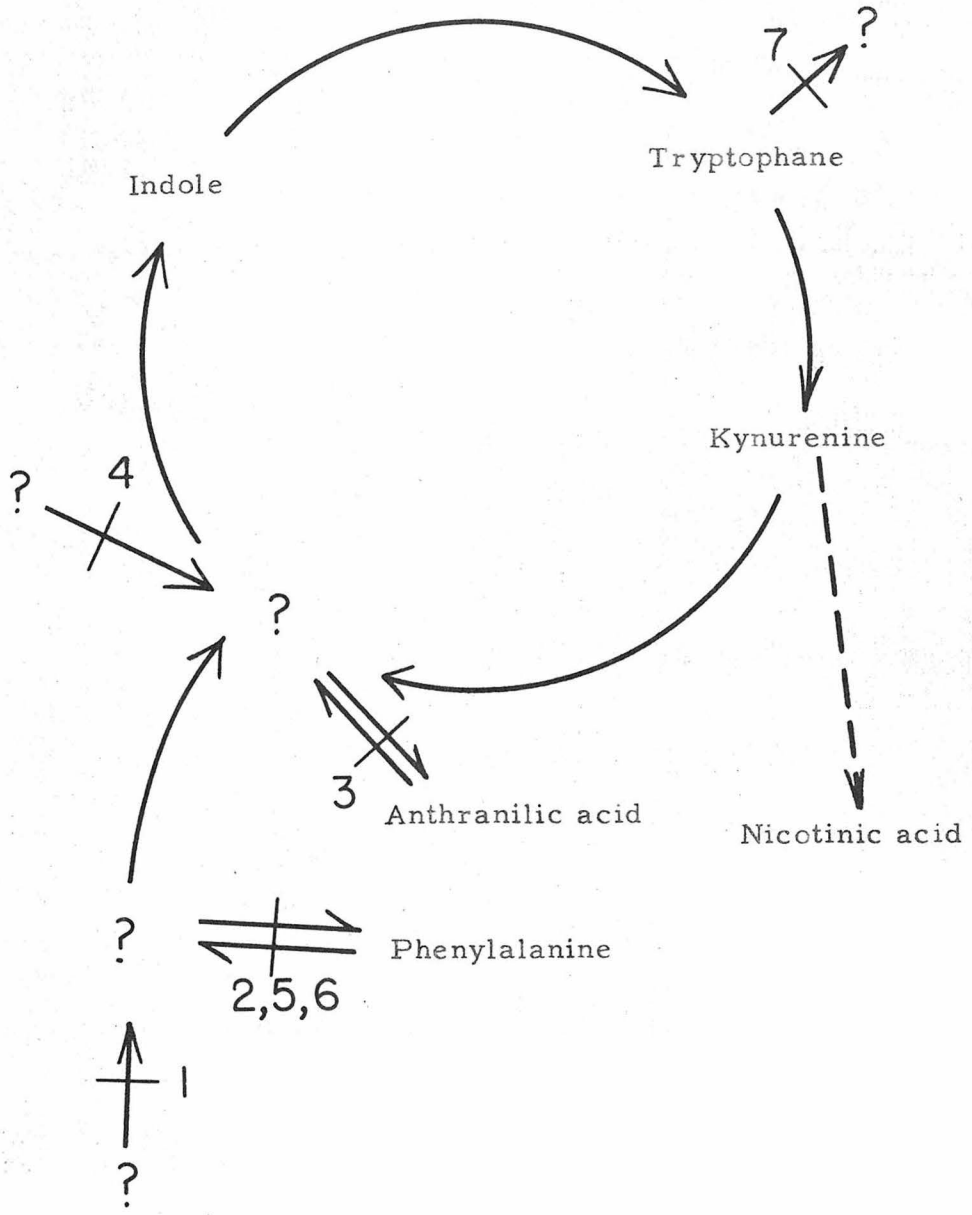
(2) If it is assumed that the primary mutation acts on some step in the reaction chain before phenylalanine, a scheme such as that shown in Figure 9b might be used to explain the observed results. In such a scheme, it will be noted, phenylalanine and anthranilic acid are not actual intermediates, but they may be transformed into the real intermediates. It is of course possible that many of the intermediates which have been postulated in the Neurospora work are of this type.

(3) A third possibility is that the enzymes which are required for the various reactions may be combined in some kind of aggregate -- each of the different strains, 39401, Int., C86, and Nic., may have all the component parts of the system, but the arrangement of the parts may be such as to make the strains appear to be blocked in different places. The modifiers might bring about their effects by somehow changing the arrangement of the parts of the aggregate with respect to each other, or perhaps by cementing together a loosely bound aggregate. A portion of such an aggregate might be pictured as in Figure 9c. The notches represent deficiencies in materials which bind together the parts of the aggregate, and the size of the notch in each case is an

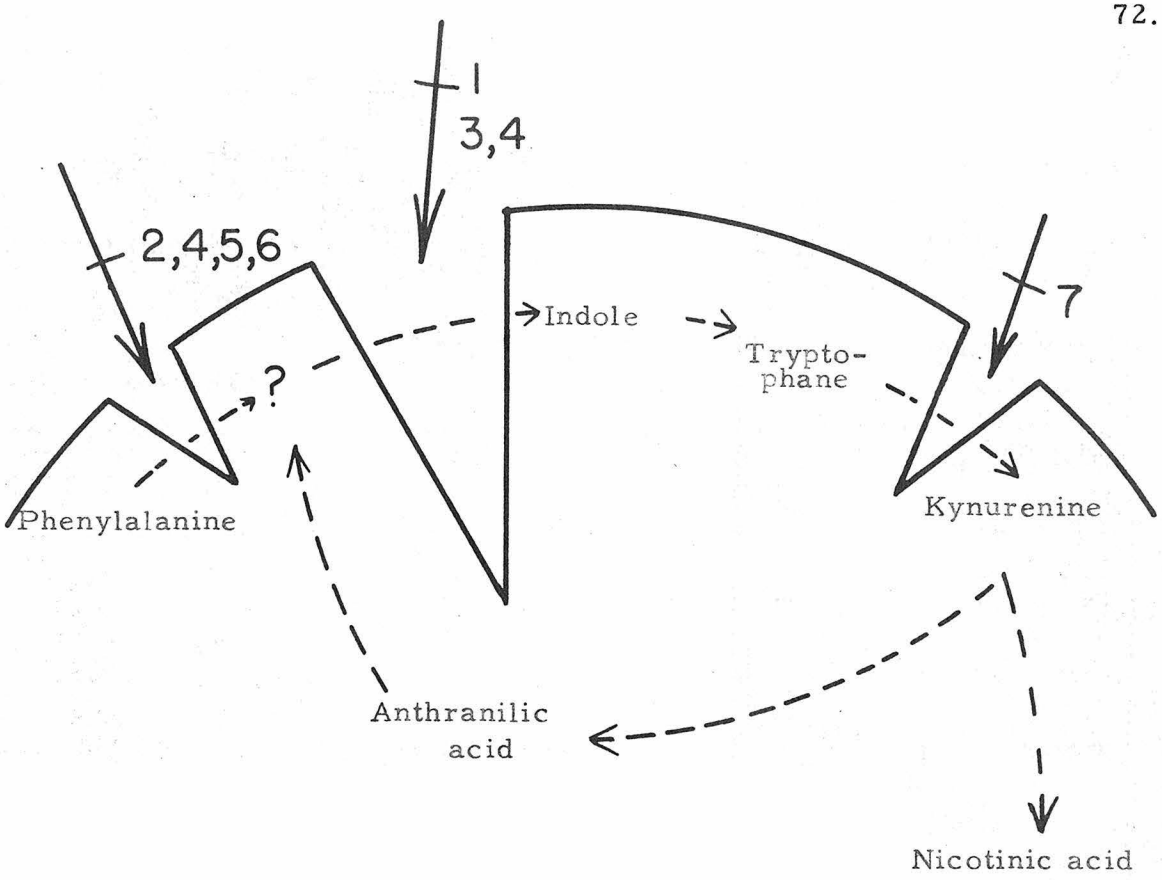


9a. Primary block before indole.

Figure 9a-c. Suggested means for the action of genes 1-7. Explanation in text.



9b. Primary block before phenylalanine.



9c. Enzyme aggregate.

indication of the seriousness of the deficiency. Thus the notch which is supposed to be filled by the products of gene 1^+ is a relatively large one, so large that genes 3^+ and 4^+ are not able to replace completely the material which is missing in the absence of 1^+. Hence none of the strains which have 1 in place of 1^+ can grow on minimal except the suppressed strains, in which S is able to fill the gap normally filled by 1^+. It is quite conceivable, then, that some biochemical mutants may actually be morphological mutants, the change in morphology being on a molecular aggregate scale. Such mutants would be in contrast to mutants like C83, in which experiments thus far have failed to demonstrate the activity of the enzyme which couples indole and serine to make tryptophane (4, 52). The effect of the mutation in C83 appears to be on the enzyme itself. Mutant 10575 and also a number of other mutants are probably like C83 in this respect.. One would expect suppressors or qualitative modifiers for such mutations to be quite rare, and this indeed appears to be the case.

Any of the three suggested schemes might be subjected to testing. Extensive genetic tests should furnish some information of the validity of the first two. The third might be approached by studying preparations of particulate material from Neurospora mycelia. If one were fortunate enough to find such a preparation which could convert phenylalanine or quinic acid to some compound later in the chain of reactions, e.g., kynurenine or kynurenic acid, it would indicate a considerable element of plausibility in the third hypothesis.

REFERENCES

1. Tatum, E. L., Bonner, D., and Beadle, G. W., Archiv. Biochem. (1943) 3, 477.

2. Tatum, E. L., and Bonner, D., Proc. Nat. Acad. Sci. (1944) 30, 30.

3. Umbreit, W. W., Wood, W. A., and Gunsalus, I. C., J. Biol. Chem. (1946) 165, 731.

4. Mitchell, H. K., and Lein, J., J. Biol. Chem. (1948) 175, 481.

5. Beadle, G. W., Mitchell, H. K., and Nyc, J. F., Proc. Nat. Acad. Sci. (1947) 33, 155.

6. Nyc, J. F., and Mitchell, H. K., J. Amer. Chem. Soc. (1948) 70, 1847.

7. Mitchell, H. K., and Nyc, J. F., Proc. Nat. Acad. Sci. (1948) 34, 1.

8. Bonner, D., Proc. Nat. Acad. Sci. (1948) 34, 5.

9. Horowitz, N. H., Advances in Genetics (1950) 3, 33.

10. Mitchell, H. K., Vitamins and Hormones (1950) 8, 127.

11. Horowitz, N. H., and Mitchell, H. K., Ann. Rev. Biochem. (in press).

12. Lein, J., Mitchell, H. K., and Houlahan, M. B., Proc. Nat. Acad. Sci. (1948) 34, 435.

13. Haskins, F. A., and Mitchell, H. K., Proc. Nat. Acad. Sci. (1949) 35, 500.

14. Beadle, G. W., and Tatum, E. L., Am. J. Bot. (1945) 32, 678.

15. Houlahan, M. B., Beadle, G. W., and Calhoun, H. G., Gen. (1949) 34, 493.

16. Butenandt, A., Weidel, W., and Schlossberger, H., Z. Naturforsch., (1949) 4b, 242.

17. Nyc., J. F., Haskins, F. A., and Mitchell, H. K., Archiv. Biochem. (1949) 23, 161.

18. Gordon, M., Haskins, F. A., and Mitchell, H. K., Proc. Nat. Acad. Sci. (1950) 36, 427.

19. Amano, T., Torii, M., and Iritani, H., Med. Jour. of Osaka University (1950) 2, 45.

20. Knox, W. E., and Mehler, A. H., J. Biol. Chem. (1950) 187, 419.

21. Mehler, A. H., and Knox, W. E., J. Biol. Chem. (1950) 187, 431.

22. Henderson, L. M., J. Biol. Chem. (1949) 181, 677.

23. Bonner, D., and Yanofsky, C., Proc. Nat. Acad. Sci. (1949) 35, 576.

24. Tang, Y. W., and Bonner, J., Archiv. Biochem. (1947) 13, 11.

25. Schayer, R. W., J. Biol. Chem., (1950) 187, 777.

26. Axelrod, H. E., Morgan, A. F., and Lepkovsky, S., J. Biol. Chem. (1945) 160, 155.

27. Cushing, J. E., Schwartz, M., and Bennett, R., J. Bact. (1949) 58, 433.

28. Yanofsky, C., and Bonner, D., Proc. Nat. Acad. Sci. (1950) 36, 167.

29. Horowitz, N. H., J. Biol. Chem. (1946) 162, 413.

30. Simmonds, S., J. Biol. Chem. (1950) 185, 755.

31. Mitchell, H. K., and Haskins, F. A., Science (1949) 110, 278.

32. Snell, E. E., Archiv. Biochem. (1943) 2, 389.

33. Sasaki, T., J. Biochem. (Japan) (1923) 2, 251; Chem. Abstracts (1923) 17, 1817.

34. Kotake, Y., and Otani, S., Z. Physiol. Chem. (1933) 214, 1.

35. Kotake, Y., J. Chem. Soc. Japan (1939) 60, 632; Chem. Abstracts (1942) 36, 4141.

36. Bonner, D., and Partridge, C. W. H., Fed. Proc. (1950) 9, 154.

37. Houlahan, M. B., and Mitchell, H. K., Archiv. Biochem. (1948) 19, 257.

38. Tatum, E. L., Fed. Proc. (1949) 8, 511.

39. Tatum, E. L., Private communication.

40. Davis, B. D., Experientia (1950) 6/2, 41.

41. Nyc, J. F., Mitchell, H. K., Leifer, E., and Langham, W. H., J. Biol. Chem. (1949) 179, 783.

42. Heidelberger, C., J. Biol. Chem. (1949) 179, 139.

43. Heidelberger, C., Gullberg, M. E., Morgan, A. F., and Lepkovsky, S., J. Biol. Chem. (1949) 179, 143.

44. Heidelberger, C., Abraham, E. P., and Lepkovsky, S., J. Biol. Chem. (1949) 179, 151.

45. Emerson, R. A., Beadle, G. W., and Fraser, A. C., Cornell Univ. Agr. Exp. Sta. Memoirs (1935) 180, 1.

46. Wright, S., Gen. (1949) 34, 245.

47. Ibsen, H. L., Proc. Sixth Int. Cong. of Gen. (1932) 2, 97.

48. Good, N., Heilbronner, R., and Mitchell, H. K., Archiv. Biochem. (1950) 28, 464.

49. Houlahan, M. B. and Mitchell, H. K., Proc. Nat. Acad. Sci. (1947) 33, 223.

50. Srb, A., Private communication.

51. Mitchell, M. B., and Mitchell, H. K., Unpublished results.

52. Mitchell, H. K., Unpublished results.