AMINO ACID INTERRELATIONSHIPS IN CERTAIN LEUCINE- AND AROMATIC-REQUIRING STRAINS OF *NEUROSPORA CRASSA*[1]

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Recently an unusual mutant strain of *Neurospora crassa* (H6196), utilizing several apparently unrelated amino acids, was described by Barratt and Ogata (1954). Any one of the following compounds supported essentially normal growth of this strain: phenylalanine, phenylpyruvic acid, tyrosine, ethyl acetoacetate, leucine, nor-leucine, and tryptophan and its related compounds β-indolepyruvic acid, indole and kynurenine. Still other compounds were shown to be less effective nutrilites. From the genetic analysis reported it appears that only one locus is responsible for these biochemical characteristics (Barratt and Ogata, 1954). In order to determine whether a single metabolic block is the basis for this unorthodox pattern of requirement, and to obtain some insight into the location of this presumptive block, an investigation was undertaken using isotopic carbon. Employing two-dimensional paper chromatography combined with radioautography, the labeling of metabolic products obtained when strain H6196 was grown in the presence of carbon-14 labeled phenylalanine was contrasted with the patterns obtained from another strain known to be blocked in aromatic biosynthesis, as well as with a wild type strain. Similarly, the distribution of radioactivity in the metabolic products of this anomalous mutant strain grown in the presence of carbon-14 labeled leucine was contrasted with that obtained from a strain known to be blocked in the biosynthesis of leucine, and again with a wild-type strain. The results to be presented here show that although this single-gene mutant strain utilizes either phenylalanine or leucine for its growth factor requirement, neither is converted to the other, nor do they appear to function through acetoacetate.

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MATERIALS AND METHODS

Organisms. The strains of *N. crassa* used in this investigation were H6196 (symbol: *phen*) (Barratt and Ogata, 1954), Y7655 (symbol: *arom*) (Tatum *et al.*, 1950), 33757 (symbol: *leu-1* and wild type SY7A. Macroconidial isolate H6196-1(11-3) was used throughout these experiments. *Arom* is blocked between dehydroshikimic and shikimic acid in the biosynthesis of the aromatic ring (Tatum *et al.*, 1954). *Leu-1* is blocked prior to ketoleucine in the biosynthesis of leucine (Regnery, 1944).

Preliminary C^{14} uptake experiments. Detailed dosage responses of the strains to phenylalanine and leucine, as well as time-response curves to four different concentrations of phenylalanine, are reported by Barratt and Ogata (1954). A preliminary experiment was conducted, in which both the disappearance of radioactivity from the culture medium and the increase in dry weight of the mycelium with time was followed. As seen from figure 1, at approximately 53 hr after inoculation maximum uptake of phenylalanine was found to have occurred. Concomitantly, the culture passed out of the log phase of growth. As an adjunct to this experiment, it was determined that 70 per cent of the isotopic phenylalanine was incorporated into the mycelium within 5 hr when added to the medium after 53 hr of growth (figure 1). Since maximal radioactivity was found in the mycelium when the tagged growth substrate was added initially, this procedure was followed in the ensuing experiments.

Growth conditions. Mycelia were grown at 30 C in 20 ml of standard Fries minimal medium

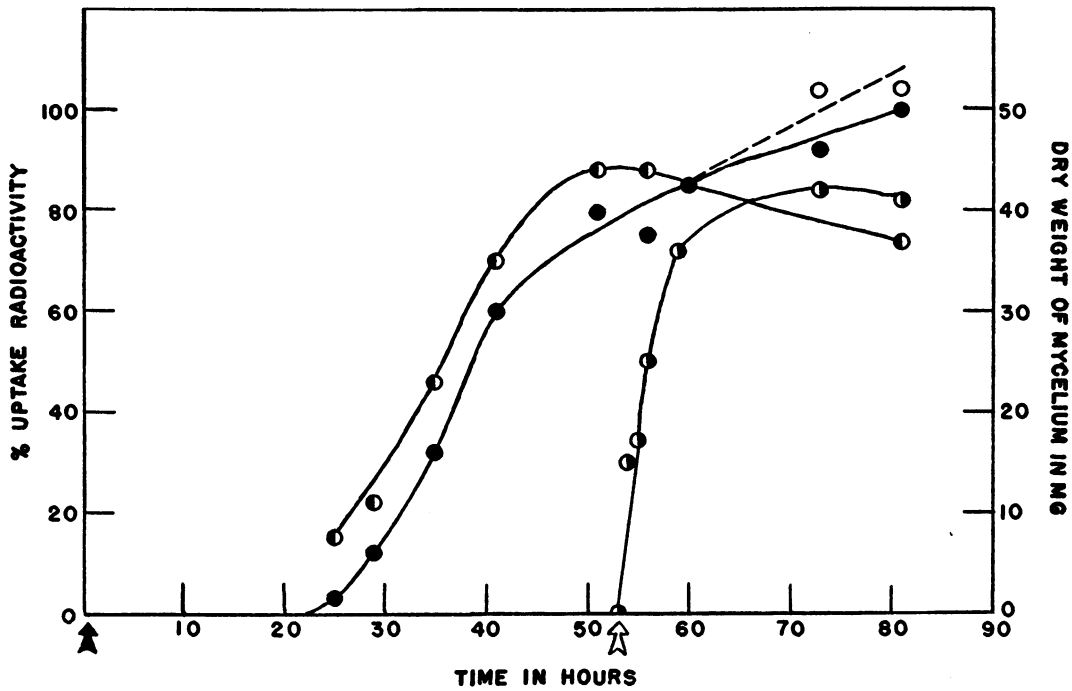

Figure 1. Relation of isotopic incorporation to growth of the *phen* strain on β-labeled DL-phenylalanine. Incorporation based on the disappearance of radioactivity from culture medium. Solid arrow: 0.5 mg substrate (1.67×10^6 disintegrations per min.) added at the initiation of the experiment. Open arrow: 0.25 mg labeled additional substrate added after growth for 53 hr on 0.5 mg unlabeled phenylalanine. ●, dry weight of mycelium with time when supplement added at initiation of experiment; ◐, per cent uptake of labeled phenylalanine with time (added initially); ○, dry weight of mycelium with time when additional supplement added after 53 hr; ◑, per cent uptake of labeled phenylalanine with time (added after 53 hr). Values plotted from the average of duplicate flasks. Medium: Fries minimal plus 2 per cent sucrose. Temperature: 30 C.

containing 2 per cent sucrose in 125-ml Erlenmeyer flasks. In one experiment β-labeled DL-phenylalanine was added to culture media in amounts which are known to elicit approximately equal growth responses in the *phen* and *arom* strains (Barratt and Ogata, 1954). Arbitrarily, the wild-type strain received the same amount of isotopic phenylalanine as did the *phen* strain. Similarly, in a second experiment, the quantity of β-labeled DL-leucine was the amount determined from the intersection of the dosage-response curves of the *leu-1* and *phen* strains (Barratt and Ogata, 1954). The wild type received an identical amount of isotopic substrate. In all cases the amount of tagged substrate added to the mutant strains was limiting. The tops of the culture flasks were fitted with a glass mantle whereby it was possible to sweep air continuously over the surface of the cotton plug without alteration of the normal carbon dioxide tension. Both the metabolic carbon dioxide diffusing through the plug and that in the air swept over the plug were trapped by bubbling through a saturated barium hydroxide solution.

Fractionation of mycelium. Mycelia were harvested 61 hr after inoculation by filtering the culture medium through a Buchner funnel. After washing with water, the damp mycelium was plunged immediately into boiling 80 per cent ethanol and refluxed for 1 hr. The filtered material was subsequently extracted by boiling with 20 per cent ethanol. These fractions will be referred to in tables 1 and 2 as "80 per cent ethanol" and "20 per cent ethanol," respectively. The alcohol-insoluble residue was hydrolyzed in 6 N hydrochloric acid by refluxing 15 hr, and the excess acid removed by thrice evaporating to dryness under reduced pressure followed by vacuum drying overnight over potassium hydroxide pellets. This fraction is referred to as

TABLE 1

Distribution of activity in various fractions from mycelium grown on β-labeled DL-*phenylalanine*

Fraction	*phen* Strain*		*arom* Strain†		Wild-Type Strain*	
	Total d/m	Per cent	Total d/m	Per cent	Total d/m	Per cent
Original culture medium	4.5×10^7	100	2.2×10^7	100	4.0×10^7	100
Carbon dioxide	6.4×10^5	1.4	2.3×10^5	1.1	9.7×10^5	2.4
Filtrate‡	8.8×10^6	19.7	3.8×10^6	17.6	4.8×10^6	12.2
20% ethanol	4.7×10^6	10.5	3.4×10^6	15.6	3.4×10^6	8.6
80% ethanol	7.0×10^5	1.6	5.7×10^5	2.6		
Protein hydrolyzate	2.5×10^7	55.5	1.3×10^7	59.5	2.4×10^7	61.4
Filter paper§	1.7×10^6	3.8	9.7×10^5	4.5	1.9×10^5	0.5
Per cent recovery		92.5		100.9		85.1

Cultures were grown in 20 ml of Fries minimal medium containing 2 per cent sucrose, and inoculated with a mycelium-free conidial suspension obtained from a 48-hr slant of the appropriate strain. Dry weights of the mycelia estimated from duplicate flasks grown on the same amount of unlabeled phenylalanine: *phen* 53.0; *arom* 37.6; wild type 77.6 mg.

* 0.85 mg of β-labeled DL-phenylalanine added.

† 0.43 mg of β-labeled DL-phenylalanine added. Also supplemented with 0.5 mg of tyrosine, 0.5 mg anthranilic acid, and 3.3 μg of *p*-aminobenzoic acid.

‡ Includes activity in the water used to wash the mycelial mat.

§ Primarily due to conidia embedded in the pores of the filter paper.

TABLE 2

Distribution of activity in various fractions from mycelium grown on β-labeled DL-*leucine*

Fraction	*phen* Strain		*leu-1* Strain		Wild-Type Strain	
	Total d/m	Per cent	Total d/m	Per cent	Total d/m	Per cent
Original culture medium	3.0×10^7	100	3.0×10^7	100	1.1×10^7	100
Carbon dioxide	1.4×10^6	4.7	9.7×10^5	3.2	3.7×10^5	3.4
Filtrate*	5.5×10^6	17.9	8.2×10^6	25.8	8.9×10^5	8.1
20% ethanol	1.3×10^6	4.3	6.3×10^5	2.1	1.5×10^6	13.9
80% ethanol	2.3×10^6	7.6	1.4×10^6	4.6		
Protein hydrolyzate	1.9×10^7	61.3	1.8×10^7	60.6	9.0×10^6	83.3
Filter paper†	1.3×10^6	4.1	3.7×10^5	1.2	7.3×10^4	0.7
Per cent recovery		99.9		97.5		109.4

Cultures were grown in 20 ml of Fries minimal medium containing 2 per cent sucrose, and inoculated with a mycelium-free conidial suspension obtained from a 48-hr slant of the appropriate strain. Dry weights of the mycelia estimated from duplicate flasks grown on the same amount of unlabeled leucine: *phen* 31.4; *leu-1* 22.6; wild-type 71.8 mg. To each flask 1.20 mg. of β-labeled DL-leucine was added.

* Includes activity in the water used to wash the mycelial mat.

† Primarily due to conidia embedded in the pores of the filter paper.

"protein hydrolyzate." For chromatographic examination these fractions were concentrated to a final volume of 1.0 ml.

Paper partition chromatography. Aliquots of the culture filtrates, 80 and 20 per cent ethanol extracts, and mycelial hydrolyzates were examined by 2-dimensional chromatography on Whatman no. 1 filter paper using phenol-water and butanol-propionic acid-water as solvents (Benson *et al.*, 1950). Although useful in demonstrating the over-all metabolic distribution of radioactivity, this method fails to separate phenylalanine, leucine and isoleucine from one another. Therefore, for these latter compounds, additional 1-dimensional methods were used sequentially for the specific separations. These

included the descending *t*-amyl alcohol-water-diethylamine method of Work (1949), ascending 77 per cent ethanol, and a descending methyl ethyl ketone-water-acetic acid system (Ehrensvard, *personal communication*). To visualize the amino acids, a 0.1 per cent ninhydrin solution in aqueous saturated butanol plus 5 per cent collidine was sprayed on the chromatogram which was then heated at approximately 100 C for 10 min. The chromatograms in which diethylamine was present were developed by spraying with a 1 per cent solution of isatin in aqueous saturated butanol followed by heating as above (Acher *et al.*, 1950). Radioautograms were prepared by exposing Kodak Noscreen X-ray film to each chromatogram according to the procedure of Benson *et al.* (1950).

Measurement of radioactivity. Samples were plated, dried, and counted with either a gasflow counter or a thin end-window GM tube. Suitable corrections to infinite thinness were applied. Aluminum planchets were used throughout except in the case of the protein hydrolyzates, which were plated on glass. In such cases a glass-to-aluminum self-absorption correction factor of 1.28 was determined and employed. All samples were counted to within 3 per cent standard error.

Specific activity determinations. Phenylalanine and leucine were isolated chromatographically from the mycelial hydrolyzates of each of the three strains grown on labeled phenylalanine and leucine, respectively. In order to isolate a sufficient quantity of amino acid for specific activity determinations, an aliquot of the hydrolyzate was streaked for several inches along the origin of the chromatographic paper. Two sequential 1-dimensional chromatograms, using different solvent systems with elution and concentration between steps, were necessary to effect complete separation. To avoid contamination from other amino acids, known mixtures were spotted at the origin on either side of the actual separation streak. The relevant isotopic area on each chromatogram was located by radioautography and carefully excised after lining up with ninhydrin-developed reference spots.

The isolated amino acids were determined quantitatively by a modified photometric ninhydrin method (Troll and Cannon, 1953) using an Evelyn colorimeter with a 580-mμ filter. A linear relation was found between per cent absorption and concentration from zero to 15 μg for each amino acid. All determinations were made in duplicate. Radioactivity was determined by counting triplicate planchets. The specific activities of the original phenylalanine and leucine used as substrates were also determined by these methods.

Phenylalanine. Descending chromatography of the hydrolyzate for 18 hr in a solvent mixture containing 9 parts methyl ethyl ketone and 1 part water, to which was added 1 per cent acetic acid, separated phenylalanine and the leucines from all the other amino acids. This leucine-isoleucine-phenylalanine area was cut from the chromatogram, eluted with water, concentrated and rechromatogrammed in ascending 77 per cent ethanol for 25 hr. The phenylalanine area was clearly separated from both leucine and isoleucine. This area was cut out, eluted, and concentrated to 1.0 ml for determination of the specific activity.

Leucine. Descending chromatography for 6 days in water saturated *t*-amyl alcohol, in a chamber containing a 1 per cent diethylamine solution in the bottom, separated leucine from phenylalanine and isoleucine. The leucine area was cut out from the chromatogram, eluted, concentrated, and rechromatogrammed in 77 per cent ethanol ascending for 20 hr in order to remove traces of residual diethylamine. The leucine area was again eluted and concentrated to 1.0 ml for specific activity determination.

RESULTS

The distribution of radioactivity in the various fractions and the recovery balances are summarized for three strains grown in the presence of isotopic phenylalanine in table 1 and for three strains grown in the presence of isotopic leucine in table 2. The percentage recovery of activity ranged from a low of 85 per cent to a high of 110 per cent. With either supplement only a very small percentage of the original activity appeared in the respiratory carbon dioxide. In every experiment over one half of the total activity appeared in the protein hydrolyzates.

Analysis of hydrolyzates. C^{14}-phenylalanine supplement. Examination of the alcohol-insoluble hydrolyzed portions of the mycelia showed the following results. In *phen* only two highly radioactive ninhydrin-positive spots appeared on the chromatograms and radioautograms. These were identified as phenylalanine and tyrosine. Identification of tyrosine was concluded on the basis

of color reaction of the radioactive spot with the ninhydrin reagent, with R_f values, and by elution followed by cochromatography with an authentic sample. Traces of radioactivity were also observed in aspartic and glutamic acids. None of the other amino acids of the cellular protein, including leucine, contained any isotope. Radioautograms of the *arom* strain also exhibited only two radioactive amino acids; these were again identified as phenylalanine and tyrosine. The wild-type strain protein contained, in descending order of activities: labeled phenylalanine, tyrosine, lysine, glutamic acid, serine, and glycine. These latter compounds were identified by their color reaction with the ninhydrin reagent and by their R_f's.

C^{14}-leucine supplement. The only highly radioactive spot on the radioautograms of the *phen* strain was identified as leucine. Distinctly less activity was found in glutamic and aspartic acids, proline, lysine, threonine (possibly serine), alanine, methionine, and valine in decreasing order. Both the wild-type and *leu-1* strains gave an isotope distribution pattern in their constituent amino acids identical to that obtained with the *phen* strain. No activity was detected either in phenylalanine or in tyrosine in the protein hydrolyzates of these auxotrophs.

TABLE 3

Specific activities of phenylalanine and leucine isolated from the mycelium

Strain Grown on Phenylalanine		Strain Grown on Leucine	
Source	Specific activity	Source	Specific activity
	μc/mg		μc/mg
Substrate phenylalanine	18.05	Substrate leucine	7.73
Phenylalanine isolated from *phen* mycelium	7.47	Leucine isolated from *phen* mycelium	4.34
Phenylalanine isolated from *arom* mycelium	7.61	Leucine isolated from *leu-1* mycelium	6.68
Phenylalanine isolated from wild type mycelium	12.14	Leucine isolated from wild-type mycelium	1.82
Leucine isolated from *phen* mycelium	0*	Phenylalanine isolated from *phen* mycelium	0*

* No detectable radioactivity on x-ray film after 1-month contact with leucine or phenylalanine area on chromatogram.

Analysis of the 80 per cent alcohol soluble fractions. Pool amino acids. Treatment of the mycelium with hot 80 per cent alcohol extracted the free or "pool" amino acids. With labeled phenylalanine as the nutrilite, the wild-type, *phen* and *arom* strains contained some labeling in aspartic and glutamic acids, threonine, alanine, tyrosine, lysine, asparagine, serine and glycine besides phenylalanine. These compounds were clearly demonstrable by their color reactions and R_f values. Other weakly radioactive unidentified ninhydrin-positive compounds were evident. The presence of asparagine in the free amino acid pool is noteworthy; this is the first demonstration of the existence of this amino acid amide in *Neurospora*, although a single gene mutant strain with a specific requirement for this substance has been reported (Tanenbaum *et al.*, 1954).

The only conspicuous labeled amino acids in the pools from the C^{14}-leucine experiments were, leucine, aspartic and glutamic acids. This pattern was consistent in the wild-type, *phen* and *leu-1* strains.

Ninhydrin-negative compounds. The 80 per cent alcoholic fraction contained several interesting radioactive compounds which failed to react with ninhydrin. One of these has an R_f of 0.50 in phenol-water and 0.35 in butanol-propionic acid water; the other has R_f's of 0.35 and 0.19, respectively. Visual inspection of the radioautograms showed that these areas contained about 10 per cent of the total activity of the ethanolic fraction. These were present on radioautograms of the *phen* strain grown on either phenylalanine or leucine, and were also evident, with diminished intensity, on radioautograms of the *arom* strain grown on phenylalanine. These two compounds were not present in extracts of the wild-type or of the *leu-1* strains. The areas in which these compounds were located are not those to which tricarboxylic acid cycle intermediates migrate in these solvents. These data suggest, therefore, that the radioactive ninhydrin-negative compounds are normal intermediates on the aromatic catabolic pathway.

The only highly radioactive area on the 2-

dimensional chromatograms of the 20 per cent ethanolic fraction was located at the origin. This area was found to be due to minute mycelial fragments and conidia in suspension. Chromatograms of the culture filtrates of all strains used in this investigation showed no significant differences.

Specific activities of reisolated compounds. The specific activity of the phenylalanines and leucines isolated from mycelia of the three strains grown with isotopic phenylalanine and isotopic leucine supplementation is summarized in table 3. These results demonstrate the non-interconversion of phenylalanine and leucine. The results further show that despite the absolute requirement there is considerable endogenous synthesis of both phenylalanine and leucine in the *phen* strain, as well as phenylalanine in the *arom* strain.

DISCUSSION

The most evident hypothesis to explain the growth of the *phen* strain on phenylalanine, leucine or acetoacetate is that the compounds are related through ketogenesis, leucine being transformed by way of a compound at the oxidation level of acetoacetate ultimately to the aromatic ring, or phenylalanine undergoing catabolism to fragments that can be incorporated into leucine. On the basis of the results reported here, these two compounds are not interconverted in the *phen* strain. This conclusion must be qualified, since the unlabeled portions of the phenylalanine and leucine molecules might have been exchanged. If, however, the usual degradative pathways for leucine and phenylalanine exist in *Neurospora*, the position of the isotope in the β carbon in both of these substances is such as to result in labeling it acetoacetate, subsequently in the 2-carbon "pool," and hence from leucine into the aromatic ring, or vice versa. The possibility that these two compounds are serving as acetoacetate precursors, supplying an alternate source of "C_2 fragments" because of a primary enzymatic block elsewhere in the formation of acetoacetate, appears unlikely for the following reasons: (1) homogentisic acid, the known intermediate between the aromatic amino acids and acetoacetate (Knox, 1955) and isovalerate, intermediate between leucine and acetoacetate (Coon *et al.*, 1955) are inactive as growth supplements for this strain. It has already been reported that β-hydroxybutyric and vinylacetic acids are also inactive (Barratt and Ogata, 1954). (2) Only a small percentage of the initial radioactivity appears in the respiratory carbon dioxide. Since Neurospora is a strict aerobe, and the labeled supplements were added at the time of inoculation, it would have been expected that a much larger percentage of the activity would have appeared in the carbon dioxide fraction via active acetate. (3) The majority of the added isotope appears in the mycelium as the compound supplied, with only slight activity in those amino acids known to arise from acetyl units in other organisms.

Two other explanations seem apparent to account for the nutritional result from this gene mutation. (1) Both phenylalanine and leucine might be converted to a mutual intermediate which remains undetected either because it is volatile, and thus lost during isolation, or because it exists only in trace quantities. The trace quantities would be acting as a "sparker" to overcome the metabolic block. That such a class of mutant strains does indeed exist is exemplified by the finding of a phenylalanine-requiring strain of Neurospora which grows normally with microgram quantities of nutrilite (E. L. Tatum, *personal communication*). An investigation of the labeled ninhydrin-negative areas in the 80 per cent ethanolic extract of the *phen* strain, when supplied either phenylalanine or leucine, may provide a clue as to the nature of such substances, if they exist. (2) The primary metabolic block in strain H6196 may result in the accumulation of a growth inhibitor, whose action is reversed by any one of the appropriate apparently unrelated supplements. In the final analysis the *phen* strain may turn out to be yet another case in which the phenotype is only circuitously related to the metabolic block.

Evidence exists for the incompleteness of the metabolic block in both the *arom* and *phen* strains. Inasmuch as the inoculum used in the isotope experiments failed to grow on minimal medium, the isotope dilution observed in our experiments cannot be accounted for on the basis of back-mutated conidia present in the inoculum; nor can it be accounted for on the basis of reversions during growth of the mycelium, since both strains routinely give growth responses proportional to the amount of supplement added. Thus the data support the contention of Bonner (1952) that limited endogenous synthesis of the required growth factor is a frequent property of biochem-

ical mutant strains. The data presented here further show that the *arom* strain synthesizes tyrosine from phenylalanine despite the fact that its growth requirement is specific for phenylalanine, tyrosine, tryptophan, and *p*-aminobenzoic acid. This finding indicates that the direct conversion of phenylalanine to tyrosine exists in Neurospora as an alternate to the route of tyrosine formation from shikimic acid.

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SUMMARY

Despite the alternative utilization of phenylalanine and leucine as growth factors in the *phen* strain of *Neurospora crassa*, the distribution of C^{14} when grown on β-labeled DL-phenylalanine and β-labeled DL-leucine showed that neither of these compounds is converted to the other. Only a small percentage of the initial radioactivity is found in the respiratory carbon dioxide. The interrelation of leucine, phenylalanine and tyrosine via acetoacetate and thus "C_2" units is ruled out as the basis for the *phen* mutation. There is no significant difference between the radioactive distribution pattern among the amino acids from the *phen* strain as compared to the *leu-1* and *arom* strains when grown on equivalent amounts of labeled supplements. Endogenous synthesis of both phenylalanine and leucine occurred irrespective of the compound supplied.

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