PHENYLALANINE-TYROSINE BIOSYNTHESIS IN *NEUROSPORA CRASSA*[1]

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BIOCHEMICAL investigations of the phenylalanine-tyrosine specific portion of the aromatic pathway reveal a reaction sequence depicted in Figure 1. Although the organisms studied use similar intermediates to accomplish phenylalanine and tyrosine synthesis, some important functional differences exist. *Aerobacter aerogenes* and *Escherichia coli* W possess two molecular forms of chorismate mutase separable by chromatography on DEAE-cellulose (COTTON and GIBSON 1965). One (CM-T) is associated with prephenate dehydrogenase activity and the other (CM-P) is associated with prephenate dehydratase activity. The activities of the CM-T aggregate are feedback-inhibited by tyrosine and activities of the CM-P aggregate are inhibited by phenylalanine. In each case the two associated activities can be affected simultaneously by one mutational event. Similar results have been reported for *E. coli* K12 (PITTARD and WALLACE 1966).

Bacillus subtilis produces three molecular species of chorismate mutase (LORENCE and NESTER 1967). No stable association with subsequent enzymes in phenylalanine or tyrosine synthesis occurs, but chorismate mutase activity and 3-deoxy-D-arabino-heptulosonic acid-7-phosphate (DAHP) synthetase activity does occur in a single aggregate in this organism (NESTER, LORENCE and NASSER

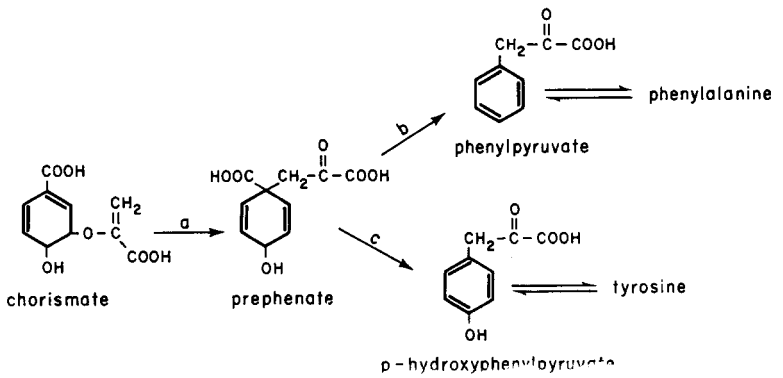

FIGURE 1.—Phenylalanine-tyrosine specific portion of the aromatic amino acid pathway. Enzyme activities investigated were a) chorismate mutase, b) prephenate dehydratase, c) prephenate dehydrogenase.

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1967). There is some evidence that shikimate kinase may also be involved in this aggregate. *B. subtilis* exhibits a sequential feedback mechanism (NESTER and JENSEN 1966) in which phenylalanine inhibits prephenate dehydratase and tyrosine inhibits prephenate dehydrogenase. Chorismate mutase activity is not affected by either amino acid. Both DAHP synthetase and shikimate kinase, however, are feedback-inhibited by chorismate and prephenate suggesting that the *B. subtilis* aggregate may have a functional significance involving aromatic pathway regulation (NESTER, LORENCE and NASSER 1967).

This report deals with genetic and biochemical studies of wild type (74A) and three mutants associated with phenylalanine and tyrosine biosynthesis in *Neurospora crassa.*

MATERIALS AND METHODS

Strains: Strains carrying the following mutations were employed in this work:

pt (S4342), requires phenylalanine (or phenylpyruvate) and tyrosine (or p-hydroxyphenylpruvate);

tyr-1 (T145), requires tyrosine (or p-hydroxyphenylpyruvate);

phen-2 (E5212), requires phenylalanine (or phenylpyruvate).

Strains carrying the following mutant genes were employed in recombination studies:

pdx-1 col-4, requires pyridoxine, exhibits colonial morphology;

pyr-3 col-4, requires uridine, exhibits colonial morphology;

arg-2 col-4, requires arginine, exhibits colonial morphology.

Reagents: Chorismate (essentially 100% pure) was obtained from the culture fluid of *A. aerogenes* 62–1 according to published procedure (GIBSON 1964; EDWARDS and JACKMAN 1965). Barium prephenate (80% pure) was a gift from E. W. NESTER (University of Washington). DEAE-cellulose (cellex-D) was obtained from Bio-Rad Laboratories and Sephadex from Pharmacia (Uppsala, Sweden). All other reagents were obtained from Calbiochem and used without further purification.

Growth of mycelia and preparation of extracts: Large numbers of conidia were obtained by growing stocks for 10 days at room temperature in a 250 ml Erlenmeyer flask containing 25 ml of minimal or supplemented minimal agar medium. The conidia were suspended in 25 ml of water and transferred to a Fernbach flask containing one liter of minimal or supplemented minimal medium. Cultures were grown with rotary shaking at 25°C for 18 hours. The mycelia were harvested by filtration through several layers of cheesecloth and washed with 0.025 M Tris-maleate buffer, pH 7.0, containing 10^{-4} M EDTA. The washed mycelia were then filtered over vacuum and ground in sand and a volume of buffer equal to 2.5 times the mycelial wet weight. This suspension was centrifuged twice at 15,000 $\times$ *g* for 10 minutes at 3°C followed by centrifugation at 20,000 $\times$ *g* for 30 minutes. The sediment was discarded after each centrifugation.

Ammonium sulfate fractionation: 85 ml of extract were treated with 3.4 ml of a 1.5% protamine sulfate solution. The protamine sulfate was warmed to 37°C and added at a rate of 1 drop per 3 seconds with continuous stirring. The stirring was continued for 10 minutes after addition of protamine sulfate and the suspension was centrifuged at 10,000 $\times$ *g* for 20 minutes. Solid ammonium sulfate (20.2g) was added slowly to the supernatant with continuous stirring. The stirring was discontinued 20 minutes after addition of ammonium sulfate and the suspension was kept in ice an additional 30 minutes before centrifuging at 20,000 $\times$ *g* for 20 minutes. The precipitate was discarded and the supernatant was treated with 4.76 g of solid ammonium sulfate and centrifuged as described above.

This precipitate was brought to 4.25 ml with 0.05 M Tris-maleate buffer, pH 7.0, and labeled 34–42% fraction. The supernatant was treated in a similar way with 5.95 g of solid ammonium sulfate. This precipitate was brought to 2.6 ml with buffer and labeled 42–52% fraction. The entire fractionation procedure was performed at temperatures of 3°C or lower.

Enzyme assays: Prephenate dehydratase was assayed by measuring the production of phenylpyruvate (GIBSON 1964). The enzyme was incubated with 0.1 μmoles of barium prephenate, 20 μmoles of Tris-maleate buffer at pH 6.6 (containing 0.02 μmoles EDTA), and water to a total volume of 0.4 ml. The reaction mixture was incubated for 5 minutes at 37°C and the reaction was terminated by adding 1.6 ml of 1 N NaOH. The absorbancy at 320 mμ was measured immediately. An extinction coefficient of 17,500 was used to convert absorbancy to μmoles of phenylpyruvate. Units of activity are presented as change in optical density at 320 mμ during 5 minutes incubation at 37°C. Specific activity is recorded in units per mg of protein.

Chorismate mutase was assayed by the procedure described by COTTON and GIBSON (1965). The reaction was carried out as described for prephenate dehydratase but the substrate in this case was 0.8 μmoles of chorismate. The reaction was stopped by adding 0.4 ml of 1.0 N HCl. After a second incubation of 5 minutes, in which the prephenate was chemically converted to phenylpyruvate, 2.2 ml of 1.0 N NaOH was added and absorbance was read immediately at 320 mμ. Units of activity and specific activity were calculated as described for prephenate dehydratase.

Prephenate dehydrogenase activity was measured by a modified Millon reaction (SCHWINCK and ADAMS 1959). Units of activity are presented as change in optical density at 490 mμ per hour at 37°C. Specific activity is equal to units per mg of protein.

Protein assay: Protein was measured by the method of LOWRY, *et al.*, using crystalline bovine plasma albumin as a standard (LOWRY, *et al.* 1951) or by absorbancy at 280 mμ when noted.

Gel filtration and column chromatography: A Sephadex G-200 column was prepared as outlined in technical data sheet number 6 (Pharmacia Fine Chemicals, Inc.) to a bed volume of 2.5 × 40 cm. The column was equilibrated with 0.05 M Tris-maleate buffer, pH 7.0, containing 10^{-4} M EDTA. The sample was applied to the surface of the column beneath a layer of buffer. The protein was eluted with equilibration buffer at the rate of 1 drop per 10 seconds. Three ml fractions were collected.

DEAE-cellulose was prepared for chromatography (PETERSON and SOBER 1962) and columns were packed to a bed size of 2.4 × 20 cm. The column was equilibrated with 0.01 M Tris-maleate buffer pH 7.8, containing 10^{-4} M EDTA. The protein was eluted with a linear gradient provided by 400 ml of equilibration buffer in the mixing chamber and 400 ml of this buffer containing 0.5 M NaCl in the reservoir. All column procedures were performed at 3°C.

RESULTS

Extracts of the mutants and wild type (74A) were prepared and assayed for chorismate mutase, prephenate dehydratase and prephenate dehydrogenase activities. The results, recorded in Table 1, indicate that the extract of mutant *pt* (S4342) lacks chorismate mutase, *phen*-2 (E5212) lacks prephenate dehydratase, and *tyr*-1 (T145) lacks prephenate dehydrogenase activities.

Since several molecular species of chorismate mutase have been separated from

TABLE 1

Enzyme activities in extracts of Neurospora crassa

Strain	Specific activity		
	Chorismate mutase	Prephenate dehydratase	Prephenate dehydrogenase
74A	1.5	0.9	0.3
phen-2	1.6	0	0.2
tyr-1	1.6	1.0	0
pt	0	0.9	0.15

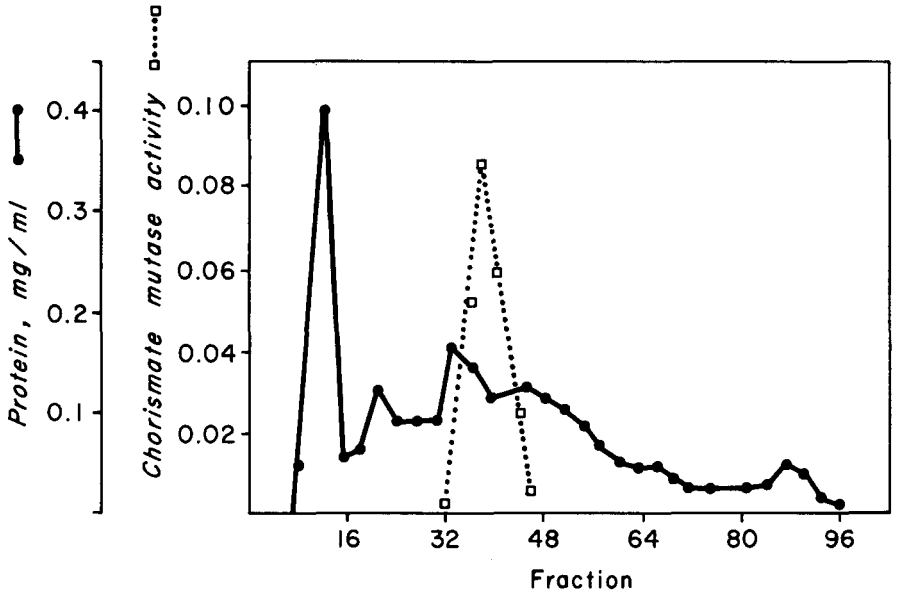

FIGURE 2.—DEAE-cellulose chromatography of chorismate mutase activity in Neurospora (74A) extract. Chorismate mutase activity represents units of activity in 0.1 ml of column fraction.

bacterial extracts by chromatography on DEAE-cellulose, this procedure was applied to extracts of the wild type Neurospora strain. Only one peak of chorismate mutase activity was eluted from these columns as shown in Figure 2. Prephenate dehydratase activity was detected in these same fractions but the elution profiles of the two enzymes were slightly different. Prephenate dehydrogenase activity was not recovered from the columns. Under the conditions utilized the dehydrogenase enzyme proved very unstable even in crude cell-free extracts.

Attempts were made to separate prephenate dehydratase and chorismate mutase to determine whether they are present as a stable aggregate in Neurospora extracts. Results show no evidence of aggregation. The two enzymes were separated either by gel filtration on Sephadex G-200 or by ammonium sulfate fractionation. A combination of these two procedures gave a clean separation as described in Table 2. A typical elution profile from a Sephadex column is shown in Figure 3.

Genetic analysis of pt *and* phen-*2 mutants:* The position of the *pt* locus has been previously established as centromere linked on chromosome IV (COLBURN and TATUM 1965). It was of interest to obtain a more definite location for *pt* on this well mapped linkage group. Three point crosses were prepared for this purpose utilizing the following double mutant strains: *pdx*-1 *col*-4, *pyr*-3 *col*-4, and *arg*-2 *col*-4. The results of these crosses, presented in Table 3, locate *pt* midway between *pdx*-1 and *col*-4 on linkage group IVR. A diagram of linkage group IVR showing the order of sites and map distances obtained in these crosses is presented in Figure 4.

TABLE 2

Separation of prephenate dehydratase and chorismate mutase activity

Fraction*	Prephenate dehydratase		Chorismate mutase	
	Total units	Specific activity	Total units	Specific activity
extract	850	1.2	890	1.2
protamine sulfate supernatant	765	0.9	830	1.0
34–42% ammonium sulfate	447	*2.3*	128	0.7
42–52% ammonium sulfate	156	0.8	416	*2.2*
Fractions 28–35 from gel filtration of 34–42%	149	*6.3*	0	0
Fractions 38–45 from gel filtration of 42–52%	0	0	243	*6.1*

* See Materials and Methods for fractionation procedure.

Crosses involving *phen*-2 were prepared in an attempt to establish its approximate location. Linkage was detected with *ad*-2 (20 map units) and *tyr*-1 (2 map units). These two loci are known to reside on chromosome IIIR, *tyr*-1 being the most distal marker on that linkage group. (BARRATT, *et al.* 1954). A three point cross between *tyr*-1 and an *ad*-2 *phen*-2 strain suggests that *phen*-2 lies between *ad*-2 and *tyr*-1. Results are presented in Table 4.

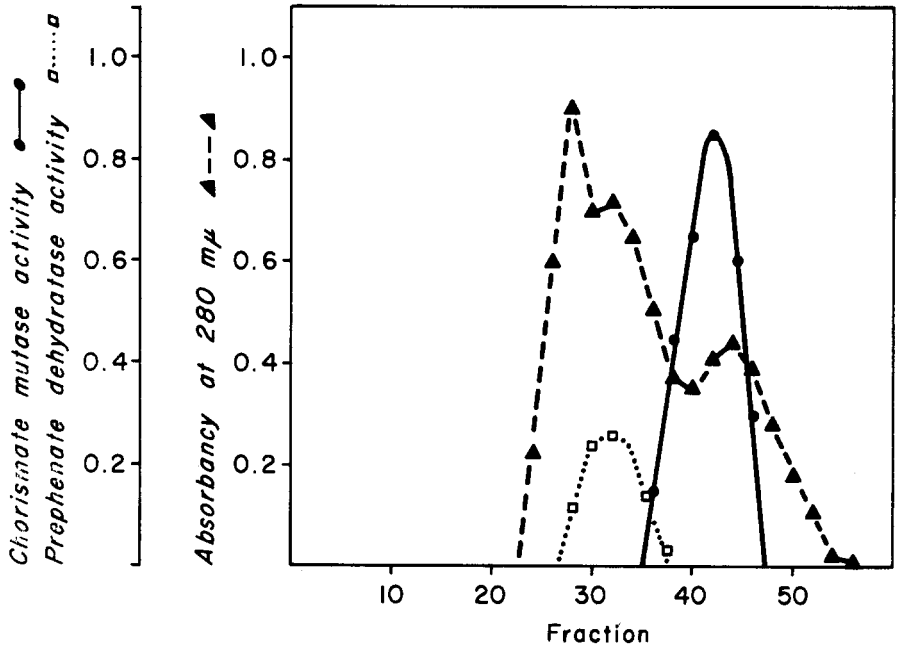

FIGURE 3.—Gel filtration of chorismate mutase and prephenate dehydratase activity in 42–52% ammonium sulfate fraction. The enzyme activity represents units of activity in 0.02 ml of column fraction.

TABLE 3

Results of crosses to determine position of the pt *locus on linkage group IV*

	Cross	Total germinating spores	Selected recombinants	Analysis of unselected marker	Suggested order
1.	*pdx*-1 *col*-4 *A* × *pt a*	3,400	34 *pdx*-1+ *pt*+	34 *col*-4	*pdx*-1 — *pt* — *col*-4
2.	*col*-4 *pyr*-3 *A* × *pt a*	4,500	130 *pt*+ *pyr*-3+	31 *col*-4+, 99 *col*-4	*pt* — *col*-4 — *pyr*-3
		4,534	70 *col*-4 *pyr*-3+	68 *pt*+, 2 *pt*	*pt* — *col*-4 — *pyr*-3
3.	*col*-4 *arg*-2 *a* × *pt A*	7,875	83 *pt*+ *arg*-2+	68 *col*-4+, 15 *col*-4	*pt* — *col*-4 — *arg*-2
		21,274	31 *col*-4 *arg*-2+	3 *pt*+, 28 *pt*	*col*-4 — *arg*-2 — *pt**

* See discussion of these results in text.

In all crosses prototrophs were selected by plating ascospores on minimal medium containing sorbose. In crosses 2 and 3 additional recombinants were selected by plating ascospores on minimal-sorbose medium containing phenylalanine and tyrosine and visually selecting colonies with colonial morphology.

DISCUSSION

The *Neurospora crassa* mutant *pt* (S4342), which requires both phenylalanine and tyrosine for growth, accumulates prephenate after 72 hours growth in supplemented minimal medium (COLBURN 1958). From this and other evidence (COLBURN and TATUM 1965) it has been suggested that this mutant lacks an enzyme which must function between prephenate and a yet undiscovered intermediate common to both phenylalanine and tyrosine biosynthesis. Our enzyme studies show that the *pt* extract lacks chorismate mutase but retains the ability to convert prephenate to phenylpyruvate and p-hydroxyphenylpyruvate (Table 1). One would expect chorismate rather than prephenate accumulation in this case. While we have no direct evidence for such accumulation by *pt*, the chemical conversion of chorismate to prephenate has been reported (GIBSON 1964). We find also that when chorismate (0.4 μmoles), is incubated at 37°C in Neurospora minimal medium (0.4 ml), 10 percent of the substrate is converted to prephenate in 2.5 hours. This should explain the presence of prephenate in the 72 hour *pt* culture medium. Thus the available evidence suggests that the *pt* locus is either the structural gene coding for chorismate mutase or a regulatory locus for this enzyme and there appears to be no reason to suspect an additional enzymatic step in the phenylalanine-tyrosine pathway in Neurospora.

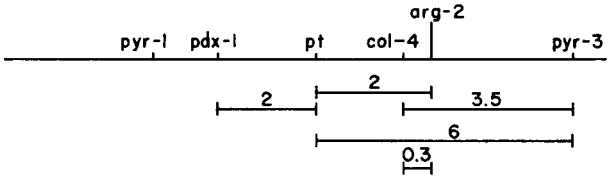

FIGURE 4.—Diagram of linkage group IV-R. Map units are based on recombinants obtained in crosses with *pt*. See Table 3 and text for details.

TABLE 4

Results of crosses to determine position of phen-*2 locus on linkage Group III*

Cross	Total germinating spores	Selected recombinants	Analysis of unselected marker
phen-2 *A* × *ad*-2 *a*	480	54 *ad*-2+ *phen*-2+	.....
phen-2 *A* × *tyr*-1 *a*	500	5 *phen*-2+ *tyr*-1+	.....
tyr-1 *A* × *ad*-2 *phen*-2 *a*	8,204	77 *phen*-2+ *tyr*-1+	62 *ad*-2+, 15 *ad*-2

Genetic and biochemical evidence suggest that the three enzymes studied may function in a different molecular form in Neurospora than in *A. aerogenes* or *B. subtilis*. Chorismate mutase activity has not been separated into more than one species by DEAE-cellulose chromatography or gel filtration and the easy separation of chorismate mutase and prephenate dehydratase activities indicates that no stable aggregate exists between these two enzymes. Although the number of mutations available at these loci is small, a mutation resulting in the loss of any one activity did not appear to affect the other two enzyme activities. It was shown earlier (BAKER 1966) that feedback regulation of this pathway in *Neurospora* is different from that found in bacteria. Neurospora chorismate mutase, unlike the bacterial enzyme, is activated by tryptophan and inhibited by phenylalanine and tyrosine. Recently we have found that prephenate dehydratase is also inhibited by phenylalanine, but unaffected by the other two amino acids. A report on the regulation of this pathway and the kinetics of these enzymes will appear elsewhere. An investigation of this pathway in peas by R. G. H. COTTON and F. GIBSON (personal communication) has demonstrated that chorismate mutase from that organism is also activated by tryptophan and only one peak of chorismate mutase activity is obtained on DEAE-cellulose chromatography. It appears that peas and Neurospora may utilize a similar mechanism for synthesis and regulation of these amino acids, but one quite different from either type described for bacteria.

The results of crosses presented in Table 3 are consistent with *pt* lying between *pdx*-1 and *col*-4, with one exception. When the cross *col*-4 *arg*-2 *a* × *pt A* was plated on arginine-deficient medium, 31 *col*-4 *arg*-2+ recombinants were visually selected from among 21,000 germinated ascospores. Most of these 31 would be expected to be pt+ on the assumption that the order of loci was *pt-col*-4-*arg*-2, but 28 of them were pt. This result may be spurious. It is known that *col*-4 is only one of a large number of loci in Neurospora at which mutation can result in a non-spreading habit. Some of these 28 presumed *pt col*-4 *arg*-2+ double crossover progeny could be *pt col*-4+ *arg*-2+ parental combinations with new colonial mutations at other loci. The *col*-4 *arg*-2 *a* parent will not grow on the arginine deficient medium (selection for *arg*+) but the *pt A* parent will grow. Thus, of the 21,274 spores that were able to germinate, approximately 11,000 were able to grow. Those that showed colonial morphology (selection for *col*-4) were analyzed for the unselected marker *pt*. If some of the 11,000 *col*-4+ *arg*-2+ *pt A* parents that were plated on minimal plus phenylalanine and tyrosine have mutated at one

of the hundred or more possible loci (other than *col*-4) that determine colonial growth, they would have been selected among the 28 *arg*-2+ *col*-4 *pt* strains that were thought to be new recombinants. This possibility is strengthened by the morphological variability which was noted among these strains. If this is true the distance between *col*-4 and *arg*-2 may be even less than 0.3 map units. The order of loci and map distances on linkage group IV reported in Figure 4 are in excellent agreement with those presented earlier (MITCHELL 1955).

During the preparation of this paper it was learned that several of the genetic and biochemical results reported here have been independently obtained by A. EL-ERYANI (personal communication) at Yale University.

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SUMMARY

Investigations of the phenylalanine-tyrosine portion of the aromatic amino acid biosynthetic pathway in Neurospora show that the reaction steps are similar to those formed in bacterial systems but important functional differences exist. Unlike the *A. aerogenes* and *E. coli* systems studied, Neurospora extracts yield one peak of chorismate mutase activity after chromatography on DEAE-cellulose and this enzyme does not appear to form a stable functional aggregate with the succeeding enzymes in the pathway. Three mutants affected in phenylalanine-tyrosine biosynthesis were studied. Extracts of *pt* (S4342), *phen*-2 (E5212), and *tyr*-1 (T145) lack chorismate mutase, prephenate dehydratase, and prephenate dehydrogenase activity respectively. The *pt* locus has been located midway between *pdx*-1 and *col*-4 on linkage group IVR. *phen*-2 lies approximately 2 map units proximal to *tyr*-1 on linkage group IIIR.

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