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Isolation and Preparation of Pretyrosine, Accumulated as a Dead-End Metabolite by *Neurospora crassa*

R. A. JENSEN,[1]* L. ZAMIR,[2] M. ST. PIERRE,[1] N. PATEL,[1] AND D. L. PIERSON[3]

Departments of Biological Sciences[1] and Chemistry,[2] State University of New York at Binghamton, Binghamton, New York 13901, and Department of Microbiology,[3] Baylor College Medicine, Houston, Texas 77025

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Pretyrosine is an amino acid intermediate of phenylalanine and/or tyrosine biosyntheses in a variety of organisms. A procedure for the isolation of high-quality pretyrosine as the barium salt is described. Stable solutions of ammonium pretyrosine that are suitable for use as substrate in enzyme assays can be prepared in good yield with relatively few purification steps. A triple mutant of *Neurospora crassa*, bearing genetic blocks corresponding to each initial enzyme step of the three pathway branchlets leading to the aromatic amino acids, accumulates prephenate and pretyrosine. Although the time courses of prephenate and pretyrosine accumulations were found to be parallel in any given experiment, the ratios of the two metabolites varied as much as 100-fold depending upon such variables as carbon source, temperature of growth, accumulation, and especially the presence of aromatic pathway metabolites. Under appropriate nutritional conditions of accumulation, pretyrosine concentrations in excess of 4 mM in culture supernatant fluids were obtained. Strains individually auxotrophic for phenylalanine or tyrosine accumulate lesser amounts of prephenate and pretyrosine. The metabolic blocks of the mutant result in high intracellular levels of prephenate, which is then partially transaminated to pretyrosine. In *N. crassa*, pretyrosine is a dead-end metabolite since it is not enzymatically converted to phenylalanine or tyrosine. At a mildly acidic pH, pretyrosine is quantitatively converted to phenylalanine in a nonenzymatic reaction.

Recently an amino acid, denoted pretyrosine and having the structure illustrated in Fig. 1, has been identified as an intermediate of tyrosine (8, 9, 13) and phenylalanine (12) biosyntheses. Comparative data suggest that the pretyrosine pathway of tyrosine and phenylalanine syntheses is widely distributed in nature among microorganisms and plants (8, 9, 12; unpublished data). In our initial report (13) pretyrosine was prepared enzymatically through the use of prephenate aminotransferase, which was partially purified from a species of blue-green bacteria (algae). This relatively laborious procedure limits considerably the scope and feasibility of experiments that depend upon the ready availability of high-quality pretyrosine. The development of a better procedure for preparing the larger amounts of pretyrosine needed for physiological and enzymological experiments was desirable.

Neurospora crassa appeared to be ideally suited as a candidate organism for pretyrosine accumulation since (i) we previously noted the presence of an aminotransferase capable of transaminating prephenate to form pretyrosine (8), yet (ii) *N. crassa* lacks the in vitro capability to metabolize pretyrosine further; i.e., no pretyrosine dehydratase or pretyrosine dehydrogenase activities were detected. The biochemical potential for the dead-end accumulation of pretyrosine in *N. crassa* is illustrated in Fig. 1. It should be noted that wild-type cultures of *N. crassa* do not accumulate detectable levels of pretyrosine, and presumably the prephenate-reactive aminotransferase activity identified in vitro merely reflects a breadth of substrate specificity that is not ordinarily realized in vivo. We thought that the in vitro potential for transamination of prephenate might be exploitable in vivo, provided that intracellular concentrations of prephenate could be elevated substantially. Since mutant strains of *N. crassa* are well known as a source of accumulated prephenate (11), we examined culture supernatants from these strains on the chance that pretyrosine might accumulate in addition to prephenate. Acceptable levels of pretyrosine were in fact observed, and a series of experiments was carried out to

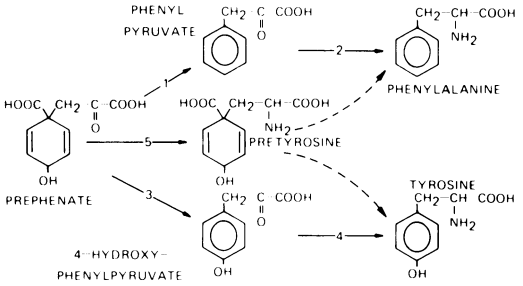

FIG. 1. *Dead-end formation of pretyrosine in N. crassa. The upper and lower sequences show the phenylpyruvate route to phenylalanine biosynthesis and the 4-hydroxyphenylpyruvate route to tyrosine biosynthesis, respectively. Pretyrosine is an amino acid formed by transamination of prephenate. The dotted lines indicate enzymatic reactions that exist in other organisms (12) for the conversion of pretyrosine to phenylalanine or tyrosine, but that are not present in N. crassa. Enzyme denotations: 1, prephenate dehydratase; 2, phenylpyruvate transaminase; 3, prephenate dehydrogenase (oxidized nicotinamide adenine dinucleotide dependent); 4, 4-hydroxyphenylpyruvate transaminase; and 5, prephenate transaminase. The triple mutant used for pretyrosine accumulation possesses mutant deficiencies in enzymes 1 and 3, as well as in anthranilate synthase (11).*

formulate a protocol for satisfactory accumulation during culture and for purification of pretyrosine as the barium salt.

MATERIALS AND METHODS

N. crassa. A multiply blocked auxotroph (75001/5212/C-167), phenylalanine auxotroph 5212, and tyrosine auxotroph C-167, originally described by Metzenberg and Mitchell (11), were obtained from R. L. Metzenberg. The triple mutant requires tryptophan, phenylalanine, and tyrosine for growth, owing to enzymatic deficiencies in anthranilate synthase, prephenate dehydratase, and prephenate dehydrogenase, respectively. Phenylalanine auxotroph E5212 (3) was obtained from A. G. DeBusk. Stock cultures were maintained on 2% agar (Difco) slants of Vogel minimal medium (14). For greater longevity, stocks were also maintained in silica gel.

Nutritional procedures. Unless otherwise indicated, the inoculum for the accumulation medium was prepared from three 50-ml cultures (each started from the conidial population of a Trypticase soy agar slant) in Trypticase soy broth (BBL) and incubated for 48 h at 37°C. The culture was filtered through sterile Miracloth (Chicopee Mills), and the entrapped mycelial material was maneuvered with sterile forceps into the accumulation medium. The accumulation medium was a modified version of Westergaard medium (15) containing (per liter): KNO_3, 1.0 g; K_2HPO_4, 0.7 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; NaCl, 0.1 g; biotin, 0.05 g; the indicated amount of sucrose (or other carbon source); L-tryptophan, 25 mg; L-tyrosine, 25 mg; L-phenylalanine, 5 mg; and 0.1 ml of trace element stock solution made by dissolving in 95 ml of water: citric acid monohydrate, 5.0 g; $ZnSO_4 \cdot 7H_2O$, 5.0 g; $Fe(NH_4)_2(SO_4)_2$, 1.0 g; $CuSO_4 \cdot 5H_2O$, 0.25 g; $MnSO_4 \cdot H_2O$, 0.5 g; H_3BO_3, 0.05 g; and $Na_2MoO_4 \cdot 2H_2O$, 0.05 g. The pH was adjusted to 7.2 with NaOH. Miracloth-covered beakers used for the aseptic collection of mycelial growth were sterilized by wrapping them with aluminum foil and autoclaving.

All data presented in this paper were obtained with the multiple aromatic auxotroph. Unless otherwise indicated, the three aromatic amino acids were present in the initial medium at the low concentrations cited above. The basic strategy (as employed by Metzenburg and Mitchell [11] to enhance prephenate accumulation) is to supply growth-limiting levels of aromatic end products to avoid regulatory effects (e.g., upon 3-deoxy-D-*arabino*-heptulosonate 7-phosphate [DAHP] synthetase and chorismate mutase) that would decrease precursor flow. Where high concentrations (usually 1 mg/ml) of particular metabolites are noted as experimental variables, the remaining amino acids were added at low concentrations.

Determinations of pretyrosine concentration. Pretyrosine is quantitatively converted to phenylalanine at acid pH (13), in exact analogy with the acid-labile conversion of prephenate to phenylpyruvate (6). Assay samples containing pretyrosine were acidified with an equal volume of 1 N HCl and incubated for 10 min at 37°C. The concentration of aromatized product, phenylalanine, was measured by the assay of a fluorescent derivative of phenylalanine (reference 10 or see Sigma Technical Bulletin 60-F). This procedure provides a sensitive assay, but it is important to include adequate controls to account for a variety of compounds that cause deviation from true values read from standard curves. At various steps, the determination of phenylalanine concentrations by the fluorometric assay was compared with several other procedures. (i) Samples were evaluated by amino acid analysis (courtesy of George Glover), or (ii) an alternative assay procedure for pretyrosine entails its enzyme-catalyzed conversion to tyrosine via pretyrosine dehydrogenase from *Agmenellum quadruplicatum*. The enzyme, partially purified as previously described (13), reacts with pretyrosine but not with prephenate. The reaction goes to completion, thus allowing a quantitative assay of pretyrosine. A reaction mixture containing 0.05 ml of 10 mM oxidized nicotinamide adenine dinucleotide (in 50 mM tris(hydroxymethyl)-aminomethane-hydrochloride, pH 7.8), 0.05 ml of partially purified enzyme, and 0.1 ml of a pretyrosine-containing sample (buffered at pH 7.8) was incubated for 90 min at 37°C. The nanomoles of reduced nicotinamide adenine dinucleotide formed equal the nanomoles of pretyrosine originally present.

Thin-layer chromatography of pretyrosine. Samples of 2 to 200 μl (depending upon pretyrosine concentrations) were applied to cellulose thin-layer plates (Eastman Chemical Products). Chromatograms were developed in a solvent system of propanol-water (70:30) and a trace of pyridine. The migration positions of amino acids were detected visually by the use of ninhydrin reagent. The R_f of pretyrosine was 0.31 and that of phenylalanine was 0.75.

Determination of prephenate concentrations. Prephenate concentrations were calculated from the molar extinction coefficient of 17,500 (5) for phenylpyruvate in 1 N NaOH at 320 nm after the acid-catalyzed conversion of prephenate to phenylpyruvate (5).

Chemicals and resins. Amino acids, shikimate, and other biochemicals were from Sigma Chemical Co. All other chemicals and reagents were the best grade commercially available. Dowex-hydroxide was prepared from Dowex-chloride-21K-anion exchange resin (Bio-Rad Laboratories) by treatment with 1 N NaOH.

RESULTS

Time course of metabolite accumulation. The triple aromatic mutant of *N. crassa* has been found (11) to accumulate prephenate into the growth medium when (i) the required end products, phenylalanine and tyrosine, are present at limiting concentrations, and (ii) neutral pH is maintained to prevent acid-catalyzed conversion of prephenate to phenylpyruvate. Using the above procedure as established for prephenate accumulation, we noted the presence of pretyrosine as well. The identity of pretyrosine was confirmed by several assays as detailed above. The single auxotrophs for phenylalanine and for tyrosine (carrying the same mutant marker as the triple auxotroph) were found to excrete 50 to 75% of the pretyrosine accumulated by the triple auxotroph.

Accumulations of prephenate and pretyrosine by the triple auxotroph were compared under one set of growth conditions as shown in Fig. 2. The upper pair of curves demonstrates that the time courses of prephenate and pretyrosine accumulations roughly parallel one another. Although the ratio of the two metabolites remains reasonably constant as a function of accumulation time, significant quantitative variation in accumulation and in the ratio of prephenate to pretyrosine may occur, depending upon such parameters as culture volume and aeration. When cultures are grown in 10-liter batches in a New Brunswick fermentor, mycelial clumps form and enlarge progressively around the metallic components of the fermentor interior. Cells located within the interior of such clumps are probably dead or senescent. In a carboy or flask that is vigorously charged with forced air, the growth mass is much more dispersed and viability is probably greater per mass unit of cells. Net growth ceases after 2 to 3 days, at which time accumulation persists at a constant rate for at least another 3 to 5 days. The duration of growth and accumulation was held to a 5-day interval as a satisfactory compromise between the labile nature of pretyrosine and the achievement of maximal yields. Experiments conducted in the fermentor produced the most consistent results, but yields of both prephenate and pretyrosine were distinctly greater in aerated carboys (as illustrated with pretyrosine in Fig. 2). In any of the procedures used, variation of accumulation yields in different experiments by a factor as great as three is to be expected, regardless of diligent duplication of the accumulation protocol.

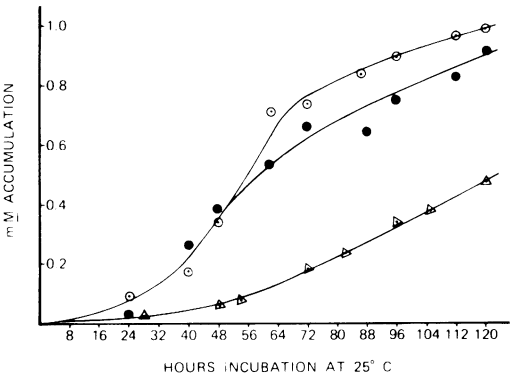

FIG. 2. *Accumulation of pretyrosine and prephenate in culture supernatants. In one experiment a New Brunswick fermentor was used to monitor pretyrosine accumulation at 25°C (△). A total of 10 liters of culture was inoculated and sampled for pretyrosine accumulation as described in the text. In another experiment (upper pair of curves), a 1,500-ml volume of culture was aerated vigorously at 25°C after inoculation with conidia recovered from 4-day cultures grown at 37°C in Trypticase soy broth. Periodically, samples were assayed for pretyrosine (⊙) or prephenate (●) concentrations; each is plotted on the same ordinate scale. In each case, Westergaard minimal medium, supplemented with 25 μg each of L-tryptophan and L-tyrosine per ml and 5 μg of L-phenylalanine per ml, was used. Samples of 50 μl were taken to measure pretyrosine concentrations after acid conversion to phenylalanine as described in the text. Samples of 100 μl were taken to measure prephenate concentrations after acid conversion to phenylpyruvate as described in the text. In all experiments, 0.75% sucrose was present as a carbon source.*

Nutritional manipulation of pretyrosine accumulation. Pretyrosine formation depends upon the fortuitous action of aromatic amino-transferase. It seemed feasible that the use of certain nutritional conditions might increase the intracellular concentrations of prephenate and/or potential amino-donor substrates for prephenate transaminase, thereby accentuating pretyrosine formation.

In one series of experiments, alternative sources of carbon during growth and accumulation were compared (Table 1). The total yield of pretyrosine increased in the following order

TABLE 1. *Effect of carbon source upon accumulation of pretyrosine and prephenate*[a]

Carbon source[b] (0.75%)	Accumulated metabolite concn[c]		Total PRT + PPA (mM)	Ratio of PPA to PRT
	PRT (mM)	PPA (mM)		
Sucrose	0.35	2.70	3.05	7.8
Fructose	0.30	3.43	3.73	11.4
Glucose	0.27	3.83	4.10	14.2

[a] Cultures of a 50-ml volume were grown in 250-ml flasks containing modified Westergaard medium at 25°C with vigorous shaking. Cultures were started from conidia under comparable conditions.

[b] Acetate, glycerol, ribose, and galactose were also tested; growth yields were poor, and accumulations were proportionally poor.

[c] Assays for accumulation were carried out after 5 days of incubation. Pretyrosine and prephenate concentrations were determined as described in the text. PRT, Pretyrosine; PPA, prephenate.

with respect to carbon source: sucrose, fructose, and glucose. The total yield of both metabolites in glucose was about 35% better than in sucrose, data that reflect primarily the differential effect of carbon source upon prephenate accumulation. Sucrose was the best carbon source for pretyrosine accumulation, whereas glucose favored prephenate accumulation. The ratio of prephenate to pretyrosine varied twofold, when accumulation in sucrose-containing medium was compared with that in glucose-containing medium. Table 2 shows that prephenate accumulation can be progressively increased through the use of greater sucrose concentrations in the culture medium. However, in contrast, pretyrosine accumulation decreases progressively at sucrose concentrations greater than 0.5 to 0.7%. The substantial differences in ratios of prephenate to pretyrosine noted in column 5 of Table 2 reflect this differential effect of initial carbon source upon the accumulation of the two metabolites. The pattern of data obtained with fructose and glucose at 0.75% concentrations (Table 1) would fit smoothly within the series shown in Table 2 between the 0.75 and 1.0% sucrose data lines.

Temperature optima for prephenate and pretyrosine accumulation were also not coincident (Table 3). Pretyrosine accumulation was optimal at 25°C, whereas prephenate accumulation was optimal at 30°C. Accumulation experiments carried out at 37°C temperatures led to very poor accumulations of both prephenate and pretyrosine.

Effect of shikimate and amino acids upon accumulation. The direct influence of a prephenate/pretyrosine precursor (shikimate) upon accumulation was tested (Table 4). Although a tendency to increased prephenate accumulation has been consistently noted in a large number of experiments, the effect is marginal. Furthermore, no significant increases in pretyrosine levels were observed. Since L-glutamate, L-leucine, and L-methionine serve as amino-donor sub-

TABLE 2. *Effect of sucrose concentration upon accumulation*[a]

Sucrose concn (%)	Accumulated metabolite concn		Total PRT + PPA (mM)	Ratio of PPA to PRT
	PRT (mM)	PPA (mM)		
0.25	0.33	0.94	1.27	2.9
0.50	0.48	2.03	2.51	4.2
0.75	0.35	2.70	3.05	7.8
1.00	0.22	3.95	4.17	18.0
1.50	0.11	4.72	4.83	43.0

[a] Abbreviations and experimental conditions of growth and assay for metabolite accumulation were those described in the footnotes to Table 1 (with sucrose).

TABLE 3. *Effect of growth temperature upon metabolite accumulation*[a]

Growth temp (C)	Accumulated metabolite concn		Total PRT + PPA (mM)	Ratio of PPA to PRT
	PRT (mM)	PPA (mM)		
20	0.23	1.88	2.11	8.1
25	0.47	3.82	4.29	8.1
30	0.34	4.00	4.34	11.8
37	0.14	1.04	1.18	7.4

[a] Abbreviations and experimental conditions of growth and assay for metabolite accumulation were those described in the footnotes to Table 1 (with sucrose).

TABLE 4. *Effect of shikimate and amino acids upon metabolite accumulation*[a]

Addition to medium	Accumulated metabolite concn		Total PRT + PPA	Ratio of PPA to PRT
	PRT (mM)	PPA (mM)		
None[b]	0.35	2.70	3.05	7.8
Shikimate[c]	0.34	3.30	3.64	9.7
Amino acids[d]	0.32	1.68	2.00	5.3

[a] Abbreviations and experimental conditions of growth and assay for metabolite accumulation were those described in the footnotes to Table 1 (with sucrose).

[b] Repetition of first data line from Table 1.

[c] Added to a final concentration of 1 mg/ml.

[d] Amino acids (L-glutamate, L-methionine, and L-leucine) were added to final concentrations of 200 μg/ml each.

strates in the prephenate aminotransferase reaction in vitro (N. Patel and R. Jensen, manuscript in preparation), the possible stimulatory effect of these supplements upon pretyrosine accumulation was also tested. Negative results were obtained.

By far the best amino donor for transamination with prephenate is L-phenylalanine; L-tyrosine is less effective (Patel and Jensen, manuscript in preparation). The potential for L-phenylalanine to enhance the intracellular transamination of prephenate is offset by the inhibitory action of L-phenylalanine upon a regulatory isoenzyme of DAHP synthetase (7). The latter regulatory effect accounts for the findings that the presence of L-phenylalanine in accumulation medium decreases prephenate and pretyrosine formation markedly (Fig. 3). However, when shikimate supplementation is used to bypass the diminution of aromatic precursors by L-phenylalanine (far right, Fig. 3), dramatic increases in pretyrosine accumulation were achieved.

Supplementation of accumulation medium with shikimate alone did not produce results significantly different from those obtained with unsupplemented cultures (Fig. 3 and Table 3). Apparently, shikimate kinase is already saturated with endogenously formed shikimate in accumulation medium that lacks sufficient levels

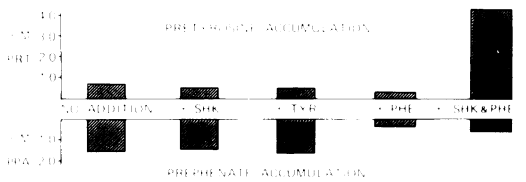

FIG. 3. *Improvement of pretyrosine accumulation in the presence of shikimate and phenylalanine. In this series of experiments, 200-ml volumes of minimal medium (5% sucrose) in 250-ml Erlenmeyer flasks were inoculated with conidia and force aerated vigorously for 5 days at 25°C. Final initial concentrations of shikimate (SHK), L-tyrosine (TYR), and L-phenylalanine (PHE), when present, were 1 mg/ml. The following extra step in the assay of pretyrosine was necessary because of the presence of residual phenylalanine in some flasks at the end of 5 days. Two 50-ml samples were spotted side by side on thin-layer chromatograms of cellulose and placed in a developing chamber, and the pretyrosine spots were allowed to migrate in 1-propanol–water (70:30) adjusted to pH 7.8 with pyridine. The position of pretyrosine from one sample was visualized with ninhydrin so the second sample could be scraped off the thin-layer matrix and dissolved in 200 μl of pyridine-water (pH 8.0). This was clarified by centrifugation, and 50 μl was used to measure the concentration of phenylalanine (10) after acidification.*

of aromatic amino acids to inhibit DAHP synthetase. This is consistent with our findings that substantial levels of shikimate (and other aromatic intermediates) are readily detected in 5-day culture supernatants.

The lack of L-tyrosine influence upon prephenate excretion (Fig. 3) was initially unexpected since L-tyrosine, like L-phenylalanine, inhibits activities of regulatory enzymes that precede prephenate formation. L-Tyrosine, but not L-phenylalanine, was found to be completely catabolized from the medium (based upon thin-layer chromatography) after 24 h of growth in accumulation medium. It seems likely that L-tyrosine is degraded rapidly by tyrosinase, an enzyme known to be present at 1 to 5% of total protein under certain conditions (4). Indeed, results similar to those obtained with L-phenylalanine or L-phenylalanine plus shikimate as shown in Fig. 3 could be mimicked by replacing L-phenylalanine with L-tyrosine, provided that L-tyrosine was added daily at 1 mg/ml (data not shown).

Enhancement of pretyrosine formation from prephenate by a transaminase substrate other than L-phenylalanine would be technically advantageous since pretyrosine levels are monitored by acid conversion to phenylalanine. Therefore, higher concentrations of the best nonaromatic substrates for prephenate transaminase were tested, in the absence and presence of shikimate (Table 5). Effects, if any, were slight.

Differential conditions favoring prephenate or pretyrosine accumulation. Different conditions of culture alter the absolute levels of prephenate/pretyrosine excreted into the accumulation medium. Representative accumulation results are shown for five different culture conditions: (i) 50 ml/250-ml flask with shaking (Tables 1 to 4), (ii) 10 liters in a New Brunswick

TABLE 5. *Effect of nonaromatic amino acids and shikimate upon pretyrosine accumulation*[a]

Addition[b]	Pretyrosine excreted (mM)
None	0.49
Shikimate	0.64
L-Glutamate	0.41
L-Leucine	0.65
L-Glutamate and shikimate	0.64
L-Leucine and shikimate	0.70

[a] Erlenmeyer flasks (250-ml) containing 200-ml volumes of minimal medium (0.75% sucrose) were inoculated with conidia and force aerated vigorously for 5 days at 25°C.

[b] Appropriate additions to the culture media were made to give final concentrations of 1 mg/ml.

fermentor (Fig. 2), (iii) 1,500 ml/2-liter flask with force aeration, (iv) 200 ml/250-ml flask with force aeration (Fig. 3 and Table 5), and (v) 15-liter carboy with force aeration.

The relative proportions of prephenate to pretyrosine (i.e., ratios) are quite responsive to such variables as temperature, carbon source, and presence of certain aromatic metabolites, as indicated in Tables 1 to 4. Thus, prephenate accumulation is favored at 30°C, using glucose (in excess of 1.5%) as the carbon source, and in the presence of limiting concentrations of the three aromatic amino acids. On the other hand, pretyrosine accumulation is favored at 25°C, using sucrose (at an optimal concentration of 0.50 to 0.75%) as the carbon source, and in the presence of high concentrations of shikimate and aromatic amino acids.

Standard protocol for routine accumulation of pretyrosine. The preceding experimental data plus many other unpublished experiments have led to adoption of the following procedure for the relatively large-scale accumulation of pretyrosine. Three 250-ml Erlenmeyer flasks containing 100 ml of tryptic soy broth (Difco) are inoculated with conidia of triple mutant 75001/5212/C-167 and grown at 37°C for 3 to 4 days. This strain often reverts, particularly for the phenylalanine requirement. Care must be taken to avoid overgrowth by such revertants to maximize the yield of pretyrosine (and prephenate). The mycelial growth is collected on the surface of a Miracloth-covered beaker. Sterile forceps are used to transfer the mycelia into a carboy sterilized with 15 liters of Westergaard synthetic medium containing sucrose at a final concentration of 1.0%. If the shikimate-phenylalanine combination is used to boost pretyrosine levels of accumulation, the following supplements are used. The final concentrations of L-phenylalanine, L-tryptophan, and L-tyrosine are 1 mg/ml, 100 μg/ml, and 25 μg/ml, respectively. The final concentration of shikimate is 500 μg/ml. In accumulation runs in which shikimate is omitted and L-phenylalanine concentrations are decreased, the sucrose concentration is decreased to 0.5% (Table 2). The pH of the original medium (about 6.6) is adjusted with 10 N NaOH (usually about 4 ml) to pH 7.6. A 20-mg amount of bromothymol blue is added so pH changes can be conveniently monitored visually throughout the 5-day accumulation period. A 1- to 2-ml volume of 10 N NaOH is added when the indicator color first begins to change from blue to yellow (usually 3- to 4-day range). The carboy is aerated vigorously from a filtered air source and growth/accumulation is carried out at room temperature.

For the isolation of pretyrosine from culture supernatants, the following steps are followed:

(1) The mycelium is separated from the medium by filtration through a Büchner funnel. Pretyrosine concentrations were estimated as described above.

(2) The filtrate is flash evaporated at 42°C to a 320-ml volume, and this solution is clarified by centrifugation.

(3) A 180-ml volume of 2 M barium acetate is added, followed by 1 liter of methanol and 1.5 liters of 1-propanol. The precipitate is allowed to settle overnight at 0°C.

(4) The mixture is centrifuged and the supernatant is discarded.

(5) The pellet is extracted five times with 200 ml of 1 mM potassium phosphate, pH 8.0. The five preparations are combined.

(6) The 1-liter solution containing prephenate and pretyrosine is diluted 14-fold with 1 mM potassium phosphate, pH 8.0.

(7) The green-colored supernatant fluid is passed over a Dowex-chloride column (50.0 by 6.0 cm) at a flow rate of 10 ml/min. The column is then washed with 2 liters of 1 mM potassium phosphate, pH 8.0. Prephenate and pretyrosine are retained in the Dowex-chloride resin.

(8) Pretyrosine is eluted batchwise from the Dowex-chloride column with a 2-liter volume of 0.3 M NH_4HCO_3 followed by 2 liters of 0.4 M NH_4HCO_3. The latter eluate is combined with the first, provided that sufficient pretyrosine is present. The elution band was identified visually with ninhydrin on thin-layer chromatograms after the application of 200-μl sample volumes as described above. (The prephenate bound to the Dowex-chloride is eluted with 2 liters of 1.0 M NH_4HCO_3, and the barium salt of prephenate is prepared as described by Metzenberg and Mitchell [11].

We find it convenient and less expensive to use the pooled and concentrated fractions of ammonium pretyrosine recovered in this procedural step as substrate for many enzymological experiments in which highly purified pretyrosine is not essential.

When greater purification is desirable, the following steps leading to the preparation of barium pretyrosine are employed:

(9) The ammonium salt of pretyrosine is flash evaporated at 42°C to a volume of 135 ml. Great care must be exercised to maintain basic pH; about 1.0 ml of 10 N NaOH per 75 mg of pretyrosine is required. A large white precipitate forms that usually contains about 25% of the total pretyrosine. The latter may be recovered separately.

(10) An excess of barium acetate (2 M) is

added to the concentrated solution of pretyrosine. Two volumes of methanol and 3 volumes of 1-propanol are added, and the precipitate is allowed to settle for 30 min at 4°C.

(11) The pellet is washed twice with 50 ml of pyridine-containing water (pH 7.3 to 7.7), and the combined solutions are again precipitated as in step 9 above.

(12) The pellet is redissolved in 20 ml of pyridine-water and precipitated once more. The resulting barium pretyrosine is stored in vacuo at 4°C in a dessicator with Drierite indicator.

The data summarized in Table 6 are representative of results obtained through application of the procedures detailed above.

The yield of ammonium pretyrosine through step 8 is about 50%. This preparation is free of shikimate and other pathway intermediates present in the accumulation medium. If barium prephenate is isolated (steps 9 to 12), the final yield is about 25%.

DISCUSSION

Pretyrosine, a dead-end metabolite. *N. crassa* appears to possess the phenylpyruvate and 4-hydroxyphenylpyruvate pathways of phenylalanine and tyrosine syntheses, respectively. Hence, prephenate dehydratase (2) and prephenate dehydrogenase (2, 4, 8) activities have been reported, but no pretyrosine dehydratase (Patel and Jensen, unpublished data) or pretyrosine dehydrogenase activities (8) have been found. In contrast to *N. crassa,* pretyrosine is a normal intermediate of phenylalanine and tyrosine biosyntheses in a number of microorganisms (8, 9, 12, 13). However, prephenate is transaminated to pretyrosine in *N. crassa* under intracellular conditions in which prephenate accumulates behind metabolic blocks. Since pretyrosine is formed enzymatically, but is not further metabolized in appropriate mutants of *N. crassa,* this organism can be used for the microbiological production of pretyrosine in good yield. The recovery of pretyrosine from *N. crassa* accumulation media represents a large improvement over enzymatic means for pretyrosine preparation. The scale of pretyrosine isolation is suitable for recovery of amounts sufficient for stable substrate preparations, for use in nutritional and physiological experiments, and for confirmatory proof-of-structure and characterization studies.

Optimal accumulation of pretyrosine. The ratio of pretyrosine to prephenate may vary more than 100-fold in response to variation in culture and accumulation conditions. Different cultural and nutritional conditions are necessary, depending upon whether optimization of prephenate or of pretyrosine accumulation is desired. Increased pretyrosine formation at the expense of prephenate depends upon the intracellular availability of a suitable amino-donor substrate for prephenate transamination. In vitro, L-phenylalanine is the best amino donor. However, L-phenylalanine inhibits two regulatory enzymes needed for maximum precursor flux to prephenate: an isoenzyme of DAHP synthetase (7) and chorismate mutase (1). The inhibitory effect of L-phenylalanine upon DAHP synthetase is successfully bypassed through the exogenous supply of shikimate in the accumulation medium (Fig. 3). The inhibitory effect of L-phenylalanine upon chorismate mutase is negated by the exogenous supply of L-tryptophan, an activator ligand for chorismate mutase that antagonizes inhibitory effects of both L-phenyl-

TABLE 6. *Purification of pretyrosine and prephenate from accumulation medium*[a]

Procedural step[b]	PRT		Recovery (%)	PPA (g)	Recovery (%)
	g	mg			
2. Supernatant concentrate	2.47			12.98	
5. Phosphate buffer extract	1.63		66	12.28	95
8. Dowex-chloride eluate (0.4 M)	1.30		53	0	
8. Dowex-chloride eluate (1.0 M)	0			10.83	83
8. Dowex-chloride eluate		124		A volume containing 124 mg of pretyrosine from step 8 was purified as described under steps 9 to 12 in the text.	
10. Barium pretyrosine precipitate		106	85		
12. Barium pretyrosine precipitate[c]		63	51		

[a] A 15-liter carboy supplemented with 0.7% sucrose and limiting concentrations of aromatic amino acids as described in the text was incubated at room temperature for 5 days. Abbreviations are the same as those given in footnote *c* of Table 1.

[b] The steps indicated correspond to the step numbers used in the text for the description of the purification procedure for pretyrosine.

[c] The purity of this preparation was calculated to be >87%.

alanine and L-tyrosine (1).

The capability of *N. crassa* to synthesize substantial amounts of pretyrosine under specialized conditions may reflect an evolutionary remnant of an ancient aromatic sequence to phenylalanine and tyrosine via a common pretyrosine intermediate (8).

Revised interpretation of *phen-2* auxotrophs. Brooks et al. (3) reported that several *phen-2* auxotrophs, known to lack prephenate dehydratase (2, 3), accumulate L-phenylalanine in the culture supernatant. The seemingly impossible observation that a phenylalanine-requiring mutant nevertheless excretes phenylalanine was explained as a compartmentation phenomenon. The mutant prephenate dehydratase of *phen-2* mutants was presumed to be active in vivo, but somehow disorganized spatially. This involved explanation can now be eliminated by our finding that the *phen-2* mutant, similar to the triple auxotroph, excretes substantial amounts of pretyrosine into the culture medium.

The mildly acidic pH of *Neurospora* culture medium routinely used tends to convert pretyrosine to L-phenylalanine nonenzymatically, thereby accounting for the slow growth of *phen-2* mutants. Nonenzymatic conversion of accumulating prephenate to phenylpyruvate probably contributes to leaky growth as well. The excreted pretyrosine was mistaken for phenylalanine (3) since the culture supernatant was analyzed by amino acid analysis, a procedure carried out at acidic pH. Since pretyrosine is quantitatively converted to phenylalanine at acid pH (13), an apparent accumulation of phenylalanine was recorded.

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