JOURNAL OF BACTERIOLOGY, June 1979, p. 805-815
0021-9193/79/06-0805/11$02.00/0

Vol. 138, No. 3

Obligatory Biosynthesis of L-Tyrosine via the Pretyrosine Branchlet in Coryneform Bacteria

AKRAM M. FAZEL[1] AND ROY A. JENSEN[1,2]*

Department of Biological Sciences[1] and Center for Somatic-Cell Genetics and Biochemistry,[2] State University of New York at Binghamton, Binghamton, New York 13901

Received for publication 2 February 1979

Species of coryneform bacteria (*Corynebacterium glutamicum*, *Brevibacterium flavum*, and *B. ammoniagenes*) utilize pretyrosine [β-(1-carboxy-4-hydroxy-2,5-cyclohexadien-1-yl) alanine] as an intermediate in L-tyrosine biosynthesis. Pretyrosine is formed from prephenate via the activity of at least one species of aromatic aminotransferase which is significantly greater with prephenate as substrate than with either phenylpyruvate or 4-hydroxyphenylpyruvate. Pretyrosine dehydrogenase, capable of converting pretyrosine to L-tyrosine, has been partially purified from all three species. Each of the three pretyrosine dehydrogenases is catalytically active with either nicotinamide adenine dinucleotide or nicotinamide adenine dinucleotide phosphate as cofactors. The K_m values for nicotinamide adenine dinucleotide phosphate in *C. glutamicum* and *B. flavum* are 55 μM and 14.2 μM, respectively, and corresponding K_m values for nicotinamide adenine dinucleotide are 350 μM and 625 μM, respectively. The molecular weights of pretyrosine dehydrogenase in *C. glutamicum* and in *B. flavum* are both about 158,000, compared with 68,000 molecular weight in *B. ammoniagenes*. In all three species the enzyme is not feedback inhibited by L-tyrosine. Results obtained with various auxotrophic mutants, which were used to manipulate internal concentrations of L-tyrosine, suggest that pretyrosine dehydrogenase is expressed constitutively. Pretyrosine dehydrogenase is quite sensitive to *p*-hydroxymercuribenzoic acid, complete inhibition being achieved at 10 to 25 μM concentrations. This inhibition is readily reversed by thiol reagents such as 2-mercaptoethanol. Coryneform organisms, like species of blue-green bacteria, appear to lack the 4-hydroxyphenylpyruvate pathway of L-tyrosine synthesis altogether. The loss of pretyrosine dehydrogenase in extracts prepared from a tyrosine auxotroph affirms the exclusive role of pretyrosine dehydrogenase in L-tyrosine biosynthesis. Other reports in the literature, in which the presence in these organisms of prephenate dehydrogenase is described, appear to be erroneous.

The biosynthesis of L-tyrosine is known to proceed via different immediate precursors in various organisms (15). The 4-hydroxyphenylpyruvate branchlet has been extensively documented in some of the best-known eubacteria (4, 6-8). The pretyrosine branchlet of L-tyrosine synthesis was initially described in species of blue-green (cyanobacteria) algae (24, 25). Coexisting, dual pathways to L-tyrosine (and to L-phenylalanine) were recently described in *Pseudomonas aeruginosa* (20, 21). The pretyrosine and 4-hydroxyphenylpyruvate biosynthetic routes to L-tyrosine are illustrated in Fig. 1. Both pathways comprise two steps which employ both an aminotransferase reaction and a dehydrogenase reaction.

Like blue-green bacteria and pseudomonad organisms, coryneform bacteria are an important and widely distributed group of microorganisms in which pretyrosine is a biosynthetic intermediate for L-tyrosine. This study shows that L-tyrosine biosynthesis in three species of coryneform bacteria (*Brevibacterium flavum*, *B. ammoniagenes*, and *Corynebacterium glutamicum*) is similar to that of blue-green bacteria, apparently relying exclusively upon the pretyrosine pathway for L-tyrosine biosynthesis.

MATERIALS AND METHODS

Microorganism. *B. flavum* (ATCC 14067), *B. ammoniagenes* (ATCC 6872), *B. ammoniagenes pheA1* (ATCC 19351), and *C. glutamicum* (ATCC 13032) were obtained from the American Type Culture Collection, Rockville, Md. *B. flavum pheA5*, a phenylalanine auxotroph, was derived from *B. flavum* (ATCC 14067). *B. flavum pheA5 shkA1* is a double mutant in

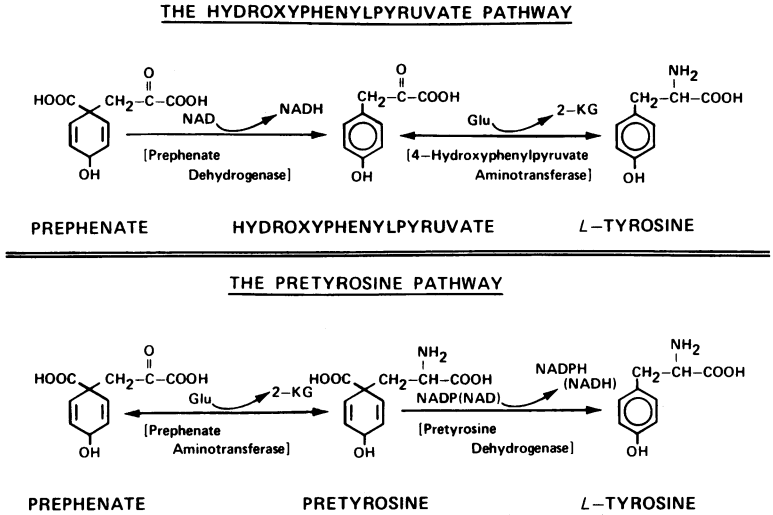

FIG. 1. *Biochemical pathways of L-tyrosine biosynthesis in nature. Abbreviations: Glu, glutamate; 2-KG, 2-ketoglutarate.*

which a mutant block prior to shikimate in the common aromatic pathway was induced in the genetic background of *B. flavum pheA5*. This strain grows at the wild-type rate in the presence of both shikimate and L-phenylalanine, or in the presence of all three aromatic amino acids.

Biochemicals and chemicals. Protein standards for molecular-weight estimation were obtained from Pharmacia Fine Chemicals. Amino acids, amino acid analogs, NAD, NADP, hydroxyphenylpyruvate, shikimate, and Tris-hydrochloride were obtained from Sigma Chemical Co.; DEAE-cellulose (DE-52), hydroxylapatite, and Ultrogel were obtained from Whatman, Bio-Rad Laboratories, and LKB Instruments, Inc., respectively. *p*-Hydroxymercuribenzoic acid was obtained from Mann Research Laboratories. Radioactive chemicals obtained from Amersham were further purified by organic solvent extraction (19). Liquifluor was purchased from New England Nuclear Corp. Tryptic soy broth and tryptic soy agar were purchased from Difco Laboratories.

Prephenate and pretyrosine were isolated from a multiple aromatic mutant of *Neurospora crassa* (17). Prephenate concentrations were estimated after quantitative conversion to phenylpyruvate at acid pH, using a molar extinction coefficient of 17,500 at 320 nm for phenylpyruvate (6). Pretyrosine was dansylated and converted nonenzymatically to dansyl-phenylalanine, and fluorescence was measured with an Aminco-Bowman spectrophotofluorometer as described later. The concentration of acid-converted dansyl-pretyrosine (i.e., dansyl-phenylalanine) was determined from a dansyl-phenylalanine standard curve. The prephenate preparation used was 79% pure by weight and was free of detectable pretyrosine and phenylpyruvate. The pretyrosine preparations used for most experiments were about 50% pure, contained no detectable prephenate or other keto acids, but were contaminated with about 10 to 20% phenylalanine, 20 to 25% shikimate, and several other unidentified compounds (as determined by high-pressure liquid chromatography).

All pH adjustments were made at room temperature with an electrode suitable for use with Tris-hydrochloride buffers as well. All spectrophotometric measurements were done with a light path of 1 cm.

Growth medium. The organisms were grown in minimal medium (26) at 32°C with vigorous shaking in a New Brunswick gyratory shaker. The growth medium (pH 7.1) contained (per liter volume): 10 g of urea, 1 g of KH_2PO_4, 0.4 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 2 mg of $MnSO_4 \cdot 7H_2O$, 100 μg of thiamine-HCl, 30 μg of D-biotin, and 7.0 ml of 6 N HCl. Glucose was sterilized separately and added to a final concentration of 3.6% (wt/vol). Solid media contained 1.5% (wt/vol) agar (Difco) sterilized separately. All amino acids used as growth supplements were autoclaved separately and later added to the growth medium. Shikimate was filter sterilized.

Starvation for L-phenylalanine in mutant *pheA1* and for L-tyrosine in *pheA5 shkA1* double mutants. Cells of *pheA5* were grown in minimal glucose medium supplemented with various concentrations of L-phenylalanine. Similarly, *pheA5 shkA1* cells were grown in the presence of 50 μg of L-phenylalanine per ml and different concentrations of shikimate. (The limiting concentrations for L-phenylalanine and shikimate were determined by a series of growth curves.) At a 5-μg/ml concentration (limiting) of L-phenylalanine, *pheA5* cells reached a final optical density of 4.5 at 600 nm. Growth of the double mutant, when cultured in limiting concentrations of shikimate, plateaued at turbidities of 2.3 and 5.1 at 600 nm in correspondence with initial shikimate concentrations of 5 μg/ml and 10 μg/ml, respectively.

Mutagenesis. Cell cultures of 10-ml volume in the late exponential phase of growth were exposed to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (75 μg/ml) in minimal glucose medium for 30 min at 30°C. Successive washes of centrifuged pellets were repeated five times with 10-ml volumes of prewarmed minimal glu-

cose medium to remove the mutagen. The final cell pellet was suspended in 10 ml of tryptic soy broth and incubated with shaking for 2 h. The culture was then serially diluted, and surviving cells were plated out on tryptic soy agar plates to obtain isolated colonies. (The unused portion of the original mutagenized culture was frozen and stored in the presence of 15% glycerol [vol/vol] at −80°C for use later as a source of mutant cells.) Survivors of mutagenesis which failed to replicate to minimal glucose agar plates were tested for positive growth response to aromatic amino acids to identify aromatic biosynthetic pathway auxotrophs.

Preparation of crude extracts. The cells were cultured in minimal glucose medium, unless otherwise stated. During late exponential phase of growth (absorbance of 10 to 15 at 600 nm, 1-cm light path), the cells were harvested by centrifugation at 4°C at 5,000 × *g* for 15 min. The cell pellets were washed twice in 0.05 M Tris-hydrochloride (pH 7.5) and then suspended in 4.0 ml of 0.05 M Tris-hydrochloride (pH 7.5) per gram of wet pellet. The cell suspensions were sonicated at 60 mV (Lab-Line Ultratip, Labsonic System) for four 30-s bursts and centrifuged at 27,000 × *g* for 60 min at 4°C. The supernatant was dialyzed overnight against 2,000 volumes of buffer, and this preparation was labeled as crude extract.

Extraction of free intracellular amino acids. Amino acids were extracted by a modified method described by Herbert et al. (11). Cells were washed five times with 50 mM Tris-hydrochloride (pH 7.5). One gram (wet weight) of each pellet was suspended in 25 ml of 0.25 N $HClO_4$ and incubated at 0°C for 30 min, and the suspensions were centrifuged at 5,000 × *g*. The pellet was extracted again in 10 ml of 0.25 N $HClO_4$ at 0°C for 30 min. After centrifugation, the supernatants were mixed and the pH was brought to 2.0 by addition of 1 N NaOH. The extracts were applied to a Dowex AG-50×-4 column (40 by 0.5 cm) and washed with 200 ml of water at pH 4.0. The ninhydrin-positive fractions were pooled and flash evaporated four times at 42°C and placed in a desiccator in the presence of concentrated H_2SO_4. The amino acids were dissolved in 10 ml of glass-distilled water and passed through a membrane filter (Millipore Corp., type GS, 0.22 μm pore size) and dried in a desiccator over concentrated H_2SO_4. This residue was dissolved in 20 ml of glass-distilled water. Aliquots were analyzed on a Glenco MM-70 amino acid analyzer.

Enzyme purification. DEAE-cellulose columns were equilibrated with 50 mM Tris-hydrochloride (pH 7.5), and 100 mg of crude extract protein was applied at 4°C to a 24- by 1.5-cm column. The column was then washed with 100 ml of 0.05 M Tris-hydrochloride buffer (pH 7.5), followed by application of a linear NaCl gradient (150 ml of 0.1 M NaCl in one reservoir and 150 ml of 1.0 M NaCl in the other, both in 50 mM Tris-hydrochloride [pH 7.5]). Fractions of 2.2-ml volume were collected. Appropriate enzyme fractions were pooled as indicated elsewhere and concentrated to a final volume of 5 ml by passage through an Amicon PM 10 membrane. The concentrate was applied to a Bio-Gel hydroxylapatite (Bio-Rad) column (1.5 by 20 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.03). The column was then washed with 100 ml of 10 mM phosphate buffer (pH 7.03), followed by a linear phosphate gradient (150 ml of 10 mM potassium phosphate buffer in one reservoir and 150 ml of 0.2 M potassium phosphate buffer [pH 7.03] in the second reservoir). Fractions of 2.2-ml volume were collected.

Analytical techniques. Protein concentrations were estimated by the method of Bradford (2) as described in Bio-Rad Technical Bulletin 1051.

Pretyrosine dehydrogenase. The reaction mixture (100 μl) contained 0.25 mM NADP or NAD, enzyme, and 0.1 mM pretyrosine in 50 mM Tris-hydrochloride (pH 7.5); the formation of NADPH or NADH was measured continuously with an Aminco-Bowman spectrophotofluorometer (excitation wavelength of 340 nm, emission wavelength of 460 nm). For qualitative determinations, the reaction mixture was spotted on cellulose thin-layer plates that had been equilibrated in 1-propanal–water (70:30 vol/vol; pH 8.5) adjusted by addition of pyridine. The chromatographs were developed in 1-propanol–water (70:30 vol/vol; pH 8.5), and the identity of the R_f of the reaction product with that of authentic L-tyrosine on the same plate was established.

Prephenate dehydrogenase. The reaction mixture (150 μl) contained 50 mM Tris-hydrochloride (pH 7.5), 0.25 mM NAD or NADP, 0.5 mM prephenate, and enzyme. The continuous formation of NADH or NADPH was determined spectrophotofluorometrically as above. The modified Millon assay (22) was used for certain experiments as described in the text.

Shikimate dehydrogenase. The reaction mixture (150 μl) contained 50 mM Tris-hydrochloride (pH 7.5), 0.25 mM NAD or NADP, 0.5 mM shikimate, and enzyme. The continuous formation of NADPH was measured spectrophotofluorometrically as above.

Prephenate dehydratase. The reaction mixture (100 μl) contained 1 mM prephenate, 50 mM Tris-hydrochloride (pH 7.5), and enzyme. After incubation at 37°C for 20 min, 0.9 ml of 2.5 N NaOH was added and phenylpyruvate was measured spectrophotometrically at 320 nm (6) using a Gilford spectrophotometer (model 250).

Prephenate aminotransferase. The method described by Miller and Thompson (19) was employed for prephenate aminotransferase. The reaction mixture (0.1 ml) contained 50 mM Tris-hydrochloride (pH 7.5), 25 μg of bovine serum albumin, 0.25 mM pyridoxal-5′-phosphate, 0.125 μCi of ^{14}C-amino acid (1 mM unlabeled amino acid), 5 mM of keto acid, and enzyme. After incubation for 20 min at 37°C, the reaction was stopped by addition of 0.3 ml of 1 N HCl. The radioactive keto acid product was extracted into 1.0 ml of ethyl-acetate–toluene (4:1 vol/vol). A 200-μl sample of the organic phase was transferred into scintillation vials containing 10 ml of New England Nuclear liquifluor, and the radioactivity was measured in a Packard scintillation counter.

Dansylation procedure for amino acids. In cases where pretyrosine or L-tyrosine was identified as the product of an enzyme reaction, the reaction was stopped with 100 μl of ice-cold acetone. The following procedure was utilized to dansylate the enzyme product. To 100 μl of sample, 10 μl of 4 M KH CO_2 (pH 9.8) and 50 μl of 1% dansylchloride in acetone were added.

The mixture was incubated at 37°C for 30 min. After incubation, the reaction vessels were cooled and 10 μl of 6 N $HClO_4$ and 100 μl of ice-cold acetone were added and rapidly mixed. A 1.0-μl portion of dansylated mixture was spotted on a micropolyamide plate (5 by 5 cm). The plates were dried and developed in solvent I (H_2O–NH_4OH, 4:2 [vol/vol]). The plates were dried again and exposed to acidic vapors (88% formic acid) for 5 min. Pretyrosine, positioned near the solvent front after development in solvent I, is converted to phenylalanine after acidification. The chromatograms are then developed in the second dimension with solvent II (benzene–glacial acetic acid–pyridine, 5:05: 0.1 [vol/vol/vol]). The dansylated compounds were located by their fluorescence under a short-wave UV lamp and were compared with authentic dansylated compound on the reverse side of the same plate.

Molecular weight estimation by gel filtration. Molecular weights were estimated by the method of Andrews (1) using LKB Ultrogel AcA34. Ultrogel columns were equilibrated with 50 mM Tris-hydrochloride (pH 7.5). The column (2.25 by 55 cm) was calibrated with the following protein standards: aldolase (158,000 molecular weight), ovalbumin (45,000 molecular weight), chymotrypsinogen A (25,000 molecular weight), and RNase A (13,700 molecular weight). The void volume was determined with Blue Dextran. Enzyme preparations (4.0 ml) were applied to the column separately, and 2.2-ml fractions were collected.

RESULTS

Prephenate aminotransferase. All three species of coryneform bacteria under study possessed at least one prephenate-reactive aminotransferase. The single aromatic aminotransferase of *B. ammoniagenes* was considerably more reactive with prephenate than with either phenylpyruvate or 4-hydroxyphenylpyruvate (Table 1). Both *C. glutamicum* and *B. flavum* possessed an aminotransferase (denoted aromatic aminotransferase II in Table 1) with similar properties. In addition, a second aminotransferase (denoted aromatic aminotransferase I) that exhibited a less pronounced preference for prephenate compared with the other aromatic keto acids than was observed with aromatic aminotransferase II was partially purified from the latter two species.

Identity of the product of the prephenate transamination reaction as pretyrosine was verified by the location of dansyl-phenylalanine formed from pretyrosine by acid conversion after thin-layer chromatography in an experiment in which the reaction mixture was acidified after the enzymatic formation and subsequent dansylation of pretyrosine.

Pretyrosine dehydrogenase. A single species of pretyrosine dehydrogenase was recovered from each of the three coryneform organisms under study (Fig. 2). Pretyrosine dehydrogenase utilizes either NAD or NADP, although NADP tends to be the preferred cofactor. The preference for NADP is more dramatic in *B. flavum* than in *C. glutamicum*. The K_m values for pretyrosine and for NAD or NADP are provided in Table 2. The kinetic data obtained (not shown) suggest an ordered, bi-bi-sequential mechanism, as characterizes many dehydrogenases.

A tyrosine auxotroph (*tyrB2*) was obtained, after nitrosoguanidine mutagenesis, which lacked pretyrosine dehydrogenase activity in crude extracts that were prepared identically to those of wild type. The possible enzymatic formation of tyrosine from pretyrosine was tested

TABLE 1. *Substrate specificities of partially purified aromatic aminotransferases from B. ammoniagenes and from C. glutamicum and B. flavum*

Source of aminotransferase	Sp act[a] of:		
	Prephenate[b]	Phenylpyruvate[b]	4-Hydroxyphenylpyruvate[b]
B. ammoniagenes[c]	66.0	4.8	3.6
C. glutamicum and *B. flavum*[d]	44.0/3.6 24.0/32.8	18.0/0.12 3.2/2.4	20.4/0.2 2.4/7.6

[a] Specific activities are expressed as nanomoles of product formed per minute per milligram of protein.

[b] Keto acid acceptor.

[c] Crude extract (100 mg) from *B. ammoniagenes* was loaded on a DEAE-cellulose column, and the aminotransferase fractions (15 mg of protein) were concentrated to 5 ml as described in the text. The concentrate was applied on a hydroxylapatite column, and the active fractions were used for determination of specific activities. At this stage of purification the aminotransferase was 115-fold pure and the yield was 54%. L-[^{14}C]glutamate was used as the amino donor substrate as described in the text. Specific activities are expressed as nanomoles of product formed per minute per milligram of protein.

[d] Specific activities for *C. glutamicum* are the leftward numbers, whereas the values given on the right are the specific activities for *B. flavum*. The aromatic aminotransferases from *C. glutamicum* and *B. flavum* were separable by ion-exchange chromatography into two peaks of activity designated as aminotransferase I and aminotransferase II. The two aminotransferase-active fractions were pooled and concentrated separately as described in the text. The concentrates were applied separately to a hydroxylapatite column, and this enzyme preparation was assayed using L-[^{14}C]glutamate as amino donor (see text). The purity and yield for aminotransferase II at this stage of purification for *C. glutamicum* were 45-fold and 62% whereas in *B. flavum* the purity and yield were 52-fold and 68%, respectively. The purity and yield for aminotransferase I were 81-fold and 52% for *C. glutamicum* and 69-fold and 60% for *B. flavum* at this stage of purification.

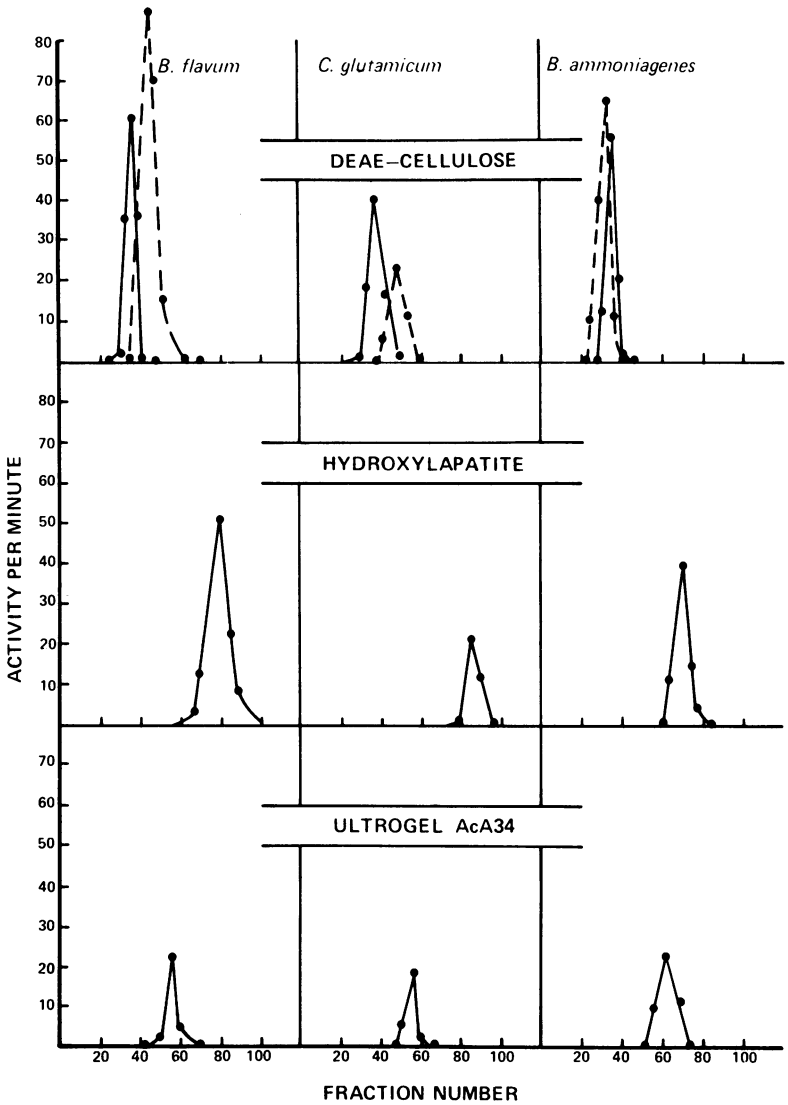

FIG. 2. *Fractionation of pretyrosine dehydrogenase. A DEAE-cellulose (DE-52) column was loaded with 100 mg of protein as described in the text. Fractions were assayed for pretyrosine dehydrogenase activity (solid lines) using NAD as the cofactor. Shikimate dehydrogenase activity is NADP-dependent (dotted lines) and exhibited an overlapping elution profile with pretyrosine dehydrogenase. Appropriate fractions eluting from DEAE-cellulose (fractions 30 to 37 with B. flavum, 35 to 45 with C. glutamicum, and 25 to 40 with B. ammoniagenes) were pooled, concentrated to a 5-ml volume by use of an Amicon PM 10 membrane, and applied to a hydroxylapatite column. Fractions possessing pretyrosine dehydrogenase activity were pooled, concentrated as before, and loaded on an ultrogel AcA34 column that had been equilibrated with 0.05 M Tris-hydrochloride (pH 7.5). Enzyme activity (left ordinate) is defined as fluorescence units (excitation at 340 nm, emission at 460 nm) where 20 fluorescence units equal 1.0 nmol of NADH (200 μl).*

by looking for NADPH formation and by thin-layer chromatography as described in Materials and Methods. The mutant excretes pretyrosine into the growth medium up to about 0.5 mM concentration after culture on growth-limiting concentrations of L-tyrosine. Excess L-tyrosine in the growth medium abolishes pretyrosine accumulation. This mutant will be described separately in more detail since it is superior to the mutant strain of *Neurospora crassa* previously employed for pretyrosine accumulation (17).

The initial fractionation after DEAE-cellulose chromatography did not completely resolve pretyrosine dehydrogenase from shikimate dehy-

drogenase. The latter enzyme may complicate pretyrosine dehydrogenase assays whenever pretyrosine preparations are contaminated with shikimate. Whenever this is a problem, pretyrosine dehydrogenase can be assayed reliably in the presence of NAD since shikimate dehydrogenase has an obligatory requirement for NADP in these organisms. Shikimate dehydrogenase was completely separated from pretyrosine dehydrogenase in the second purification step (hydroxylapatite). The purification data corresponding to Fig. 2 are given in Table 3. The enzyme was stable throughout partial purification and was purified about 56-fold with a yield of almost 60%.

The Ultrogel AcA34 purification step shown in Fig. 2 was used to estimate the molecular weights of the three pretyrosine dehydrogenases. The column was calibrated with the molecular-weight standards cited in Materials and Methods. In other experiments using enzyme preparations of varying purity, similar molecular-weight data were obtained. The enzyme sizes of both *B. flavum* and *C. glutamicum* were estimated at 158,000 molecular weight, whereas that of *B. ammoniagenes* was 68,000 molecular weight.

TABLE 2. *K_m values for pretyrosine dehydrogenase[a] from B. flavum and C. glutamicum*

Coryneform species	Substrate	K_m (mM)[b]
B. flavum	Pretyrosine (NAD)	0.122
	Pretyrosine (NADP)	0.020
	NAD	0.625
	NADP	0.014
C. glutamicum	Pretyrosine (NAD)	0.083
	Pretyrosine (NADP)	0.043
	NAD	0.353
	NADP	0.055

[a] Partially purified (21-fold and 46-fold purified from *B. flavum* and *C. glutamicum*, respectively) enzyme preparations were used for determination of K_m values. The purification procedure was described in the text.

[b] K_m values were obtained from secondary plots in which the appropriate intercepts of double reciprocal plots were plotted against the reciprocal of the fixed-substrate concentrations.

TABLE 3. *Purification of pretyrosine dehydrogenase from B. flavum*

Purification steps	Total protein (mg)	Sp act[a]	Total activity	Fold purification
Initial crude extract	100	6.5	650	1.0
DEAE-cellulose	13.8	39.5	545	6.1
Hydroxylapatite	3.1	140.0	434	21.5
Gel filtration	0.96	365.2	380	56.2

[a] Specific activity is defined as nanomoles of NADH per minute per milligram of protein.

The pH optimum for pretyrosine dehydrogenase activity was 7.8. Activity exhibited proportionality with respect to time and enzyme concentration under all conditions of assay used. Assays were routinely carried out at 37°C although the temperature optimum was about 33°C. *p*-Hydroxymercuribenzoate was an effective inhibitor of pretyrosine dehydrogenase, total inhibition being achieved in the 10 to 25 μM concentration range depending upon the microbial species. Thiol reagents such as dithiothreitol or 2-mercaptoethanol were capable of reversing *p*-hydroxymercuribenzoate inhibition.

Pretyrosine pathway of L-tyrosine synthesis. This pathway consists of prephenate aminotransferase and pretyrosine dehydrogenase, both stable and highly active enzymes in coryneform bacteria. Two-dimensional, thin-layer chromatography data are shown in Fig. 3 to illustrate the identity of the reaction product of prephenate aminotransferase as pretyrosine and the identity of the reaction product of pretyrosine dehydrogenase as L-tyrosine.

A previous report of prephenate dehydrogenase activity in certain coryneform organisms (9, 10, 26) raised the question of whether dual enzymatic pathways leading to L-tyrosine might exist as occurs in *P. aeruginosa* (20, 21). Dialyzed crude extracts of *C. glutamicum* or *B. flavum* lacked detectable prephenate dehydrogenase activity in our hands. Nor was prephenate dehydrogenase activity detected in crude undialyzed extracts in contrast to the report of Hagino and Nakayama (9).

The prephenate dehydrogenase activity reported (9) in undialyzed, crude extracts of *C. glutamicum* seems to be only apparent. The following observations support a rationale that apparent prephenate dehydrogenase activity in crude, undialyzed extracts is an artifact which can be accounted for by a multistep series of reactions. In crude extracts, prephenate is readily transaminated to pretyrosine via one or more very active aminotransferases which do not require added pyridoxal-5'-phosphate, using glutamate as the amino donor. (The glutamate content of the supernatant of a late-exponential-phase culture of *B. flavum* was 158 μmol per ml, whereas 67 nmol of intracellular glutamate per gram [wet weight] was measured [see Materials and Methods] after growth in minimal medium.) The reconstruction experiments in Table 4 show that loss of apparent prephenate dehydrogenase activity after dialysis is restored by addition of glutamate. Hence, it seems likely that removal of glutamate by dialysis prevents transamination

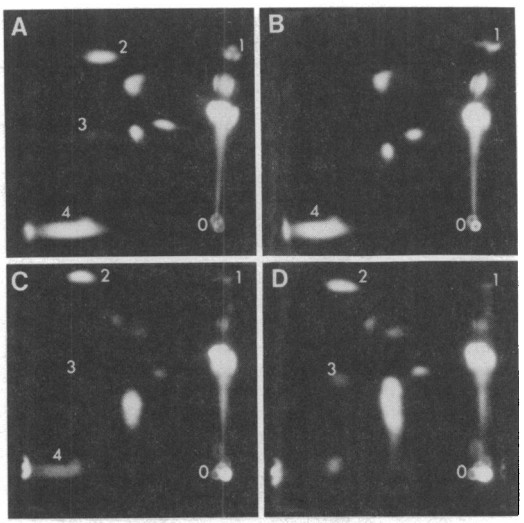

FIG. 3. *Confirmation of pretyrosine and tyrosine as reaction products of prephenate aminotransferase and pretyrosine dehydrogenase, respectively. Dansylated amino acids were separated by thin-layer chromatography in two dimensions as described in the text, and then photographed under UV light to visualize the fluorescent reaction products. (A and B) Prephenate aminotransferase. (A) The sample applied at the origin was a reaction mixture of 1.25 mM prephenate, 5 mM L-tyrosine, 0.25 mM pyridoxal-5'-phosphate, and prephenate aminotransferase II (37-fold purified) from B. flavum that was incubated 20 min at 37°C prior to termination of the reaction by addition of 100 μl of ice-cold acetone. Dansyl-pretyrosine migrated near the solvent front in the first direction of chromatography (bottom to top). Since dansyl-pretyrosine comigrates with dansyl-glutamate and dansyl-aspartate, a convenient routine employs the conversion of dansyl-pretyrosine to dansyl-phenylalanine prior to chromatography in the second direction. Dansyl-pretyrosine was converted to dansyl-phenylalanine on the polyamide plate after exposure to acidic vapors (see text), and the chromatogram was developed in the second direction (right to left). Dansyl-phenylalanine was mobile in the second solvent. Symbols: 0, origin; 1, marks the position of a bluish dansylation by-product which approximates the position of dansyl-pretyrosine after development in the first dimension; 2, dansyl-phenylalanine (from acidified dansyl-pretyrosine); 3, dansyl-phenylalanine (probably from a small amount of prephenate dehydratase activity contaminating the prephenate aminotransferase preparation); 4, dansyl-tyrosine. All other spots are dansyl derivatives. (B) The result obtained with an identical preparation that was not allowed to react enzymatically. (C) The sample applied at the origin was a pretyrosine dehydrogenase (22-fold purified) reaction mixture that had been incubated at 37°C for 20 min as described in the text. A sample from an unincubated control was applied at the origin. The dansyl-phenylalanine present in C and D originated from contamination of pretyrosine with small amounts of phenylalanine.*

of prephenate, thereby aborting formation of pretyrosine which is otherwise utilized by pretyrosine dehydrogenase. This explanation is consistent with inhibition (Table 4) of the apparent prephenate dehydrogenase by phenylhydrazine, a reagent that selectively inhibits aminotransferase activity (28). In contrast, the activity of pretyrosine dehydrogenase was not inhibited at all by phenylhydrazine. Thin-layer chromatography was used to verify the multistep formation of L-tyrosine in crude extracts incubated with

TABLE 4. *Prephenate dehydrogenase in C. glutamicum*

Substrate	Addition(s) (1 mM)	Sp act[a] Millon assay (22)	Sp act[b] (nmol of NADH/ min per mg)
Undialyzed[c]			
Prephenate/ NADP	0	0.7	
Prephenate/ NAD	0	0.7	
Prephenate/ NADP	Phenylhydrazine	0	
Dialyzed[c]			
Prephenate/ NADP	0	0	
Prephenate/ NAD	0	0	0
Prephenate/ NADP	Glutamate[d]	5.5	
Prephenate/ NAD	Glutamate[d]	5.0	4.5
Pretyrosine/ NADP	0	13.2	
Pretyrosine/ NAD	0	10.6	8.5
Prephenate/ NADP	Phenylhydrazine[e] + glutamte	1.3	
Prephenate/ NAD	Phenylhydrazine + glutamate	1.2	0.8
Pretyrosine/ NAD	Phenylhydrazine		8.9

[a] The reaction mixture contained 50 mM Tris-hydrochloride (pH 7.5), 2 mM prephenate, 0.1 mM pretyrosine, 1 mM NAD (NADP), and 250 μg of extract protein. The reaction time was 20 min at 37°C. Specific activity is defined as nanomoles per minute per milligram of protein.

[b] NADP-dependent reactions with prephenate or pretyrosine were complicated by the activity of NADP-dependent shikimate dehydrogenase (see text). In undialyzed crude extracts (top three lines), background is too high to permit assays based upon NADH or NADPH formation.

[c] A crude extract (12 mg/ml) was prepared from wild-type cells cultured on minimal glucose medium. The 4-ml extract was split into equal portions, one of which was dialyzed overnight at 4°C against 4 liters of Tris-hydrochloride buffer (pH 7.5).

[d] Incubation of crude extracts with glutamate and NAD or NADP did not result in Millon-positive product.

[e] Activities of prephenate aminotransferase (8.2 nmol of pretyrosine per min per mg of protein) and phenylpyruvate aminotransferase (21.0 nmol of phenylalanine per min per mg of protein) were inhibited 90% at 1 mM phenyhydrazine concentrations.

prephenate and NADP. Since the Millon assay (22) for 4-hydroxyphenylpyruvate was used in the assay of prephenate dehydrogenase by Hagino and Nakayama (9), the product measured in their studies was undoubtedly L-tyrosine. A positive Millon reaction was indeed obtained (Table 4) when pretyrosine was used as the substrate. Pretyrosine itself is not Millon positive. The involvement of two enzymatic steps as the basis for apparent prephenate dehydrogenase activity is further shown in Table 5. When prephenate was incubated with glutamate (thus permitting pretyrosine formation) prior to NADP addition (Table 5, lines 1 and 2), the rate of NADPH formation was increased about threefold. If phenylhydrazine was also present (inhibiting prephenate aminotransferase activity), then preincubation or prephenate and glutamate made little difference (Table 5, lines 3 and 4), and the overall specific activity was substantially reduced. The activity of pretyrosine dehydrogenase was not affected by phenylhydrazine (Table 5, lines 6 and 7). Control experiments established that no NADP-dependent glutamate dehydrogenase activity was present and that prephenate was not contaminated with any dehydrogenase substrates.

Regulation of L-tyrosine biosynthesis. L-Tyrosine was totally ineffective as an allosteric inhibitor of pretyrosine dehydrogenase, even at low substrate concentrations. A variety of other amino acids was tested as effector molecules, but none affected reaction rates. (A pretyrosine preparation, purified free of the L-phenylalanine usually present [see Materials and Methods], was used to confirm the absence of phenylalanine effects upon pretyrosine dehydrogenase activity.)

Pretyrosine dehydrogenase also does not appear to be subject to repression control, at least in *B. flavum* (Table 6). Exogenous L-tyrosine did not significantly repress the levels of pretyrosine dehydrogenase below the levels found in wild type growing on unsupplemented minimal medium. Phenylalanine auxotroph *pheA5* (lacking prephenate dehydratase) was grown under conditions of L-phenylalanine starvation in the presence of excess L-tyrosine. This would be expected to relieve allosteric control exerted upon 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthetase (23), increasing the flow of intermediates into the common branch of the aromatic pathway. Flow of carbon from prephenate into the L-phenylalanine branchlet would not occur because of the mutant block. If any pathway intermediates exerted regulatory roles in repression or induction, the perturbation of normal intermediate levels in the *pheA5* mutant under the nutritional conditions used might cause alteration of pretyrosine dehydrogenase levels. However, no significant difference from wild type was observed.

Since it is possible that endogenous levels of L-tyrosine formed in wild-type cells cultured on minimal glucose medium were sufficient to pro-

TABLE 5. *Multistep nature of apparent prephenate dehydrogenase activity*

Substrates incubated[a] prior to assay	Additions	Sp act[b] (nmol of NADPH/min per mg)
No preincubation	Prephenate + glutamate + NADP	0.90
Prephenate + glutamate	NADP	2.50
No preincubation	Prephenate + glutamate + NADP + phz	0.12
Prephenate + glutamate + phz	NADP	0.15
Prephenate + phz	Glutamate + NADP	0.05
No preincubation	Pretyrosine[c] + NADP	11.6
No preincubation	Pretyrosine + NADP + phz	12.1
No preincubation	Prephenate + glutamate	0
No preincubation	Glutamate + NADP	0
No preincubation	Prephenate + NADP	0

[a] Preincubation was 20 min at 37°C. Prephenate and glutamate were present at 2.5 mM; phenylhydrazine (phz) was present at 0.5 mM. NADP was added to a final concentration of 0.25 mM.

[b] NADPH formation was measured continuously as described under "Materials and Methods". The enzyme preparation used was the same as in Table 4.

[c] Pretyrosine used was free of shikimate.

TABLE 6. *Repression control of pretyrosine dehydrogenase in B. flavum*

B. flavum strain	Growth-limiting[a] supplement	Supplements present[b] in excess	Sp act[c]
Wild type			7.4
		L-Tyrosine	7.0
pheA5	L-Phenylalanine	L-Tyrosine	6.1
	L-Phenylalanine	L-Tyrosine + L-tryptophan	6.5
pheA5 shkA1		L-Phenylalanine + shikimate	8.2
	Shikimate, 20 μg/ml	L-Phenylalanine	8.5
	Shikimate, 10 μg/ml	L-Phenylalanine	9.6
	Shikimate, 5 μg/ml	L-Phenylalanine	10.0

[a] Limiting concentrations of L-phenylalanine and shikimate were determined by growth curves to establish limiting yields of cell mass as described in the text.

[b] Excess concentrations of shikimate, L-tyrosine, or L-phenylalanine were 100 μg/ml, amounts which are in excess of the requirements of appropriate auxotrophs to achieve maximal growth yield.

[c] Expressed as nanomoles of NADH per minute per milligram of crude extract protein.

duce maximal repression, an experiment was carried out in which cells were tested for possible derepression in response to tyrosine starvation. As shown in Table 6, a double mutant blocked prior to shikimate and lacking prephenate dehydratase was grown on excess L-phenylalanine and on various growth-limiting concentrations of shikimate. The exhaustion of shikimate results in L-tyrosine starvation (L-tryptophan requirements are quantitatively minor). The data suggest a trend to some derepression, but the range of variation, if significant, is less than a factor of two.

DISCUSSION

Synthesis of L-tyrosine via the pretyrosine branchlet in coryneform bacteria. The pretyrosine branchlet proceeds from prephenate and contains two enzymes, prephenate aminotransferase and pretyrosine dehydrogenase. All three species of coryneform bacteria studied exhibited pretyrosine dehydrogenase activity, but no prephenate dehydrogenase activity in crude extracts prepared from wild-type cultures. Although these organisms possess aromatic aminotransferases that will react with 4-hydroxyphenylpyruvate, prephenate was found to be the preferred substrate. The isolation of a tyrosine auxotroph (A. M. Fazel and R. A. Jensen, manuscript in preparation) which lacks pretyrosine dehydrogenase supports the exclusive reliance of this microbial group upon the pretyrosine branchlet for L-tyrosine biosynthesis. This contrasts with the dual presence of pretyrosine and 4-hydroxyphenylpyruvate branchlets in *P. aeruginosa* (20, 21). Pretyrosine dehydrogenase was not found to be regulated by allosteric or repression control. Each pretyrosine dehydrogenase examined was quite sensitive to *p*-hydroxymercuribenzoate and was capable of utilizing either NAD or NADP cofactors, although NADP was distinctly preferred. All of the above characteristics are strikingly reminiscent of pretyrosine dehydrogenase contained in species of blue-green bacteria.

Lack of prephenate dehydrogenase. The results summarized above indicate that the 4-hydroxyphenylpyruvate branchlet, a pathway that is well documented for L-tyrosine biosynthesis among microorganisms (4, 6–8, 18), is not present in the coryneform species studied. This conclusion is contrary to that of Sugimoto et al. (26) and Hagino and Nakayama (9) who believed that they were measuring prephenate dehydrogenase activity in crude extracts. Curiously enough, this activity was lost after dialysis. We were only able to repeat this result using the Millon assay and suspected that apparent prephenate dehydrogenase activity was somehow dependent upon a small molecule present before dialysis. These microorganisms accumulate glutamic acid, and it was found that addition of glutamate to inactive, dialyzed preparations restored activity. Hence, it appears that apparent prephenate dehydrogenase can be accounted for as a two-step reaction, prephenate being transaminated in the presence of glutamate to pretyrosine, followed by formation of tyrosine via pretyrosine dehydrogenase. The Millon assay used by the other workers (9, 26) to measure 4-hydroxyphenylpyruvate probably detected tyrosine instead, since either compound is equally reactive (22) with Millon reagents.

A second difficulty could have been an unappreciated contamination of the prephenate substrate used with pretyrosine. We have found that a number of mutant organisms used to accumulate prephenate also accumulate pretyrosine in small amounts (E. L. DeFuria, M. D. Brown, and R. A. Jensen, manuscript in preparation). This might be very pronounced when mutant coryneform bacteria are used to accumulate prephenate (9). The highly active prephenate aminotransferase capabilities of coryneform bacteria in conjunction with their overproduction of glutamate (27) suggest the likelihood that conversion of accumulated prephenate to pretyrosine in the mutant strains used might be substantial.

Regulation of L-tyrosine biosynthesis. In organisms containing the 4-hydroxyphenylpyruvate branchlet, prephenate dehydrogenase is positioned at a metabolic branch point. As such, generalized rules of allosteric regulation are consistent with the effective feedback inhibition by L-tyrosine that is usually observed (6, 7, 15). On the other hand, in organisms containing the pretyrosine branchlet, pretyrosine dehydrogenase is not positioned at a metabolic branch point, and there is accordingly no compelling rationale for allosteric control. Such lack of control is, in fact, observed for pretyrosine dehydrogenases of all coryneform and blue-green bacteria studied (although pretyrosine dehydrogenase in *P. aeruginosa* is feedback inhibited by L-tyrosine). The synthesis of pretyrosine dehydrogenase, at least in *B. flavum*, is not subject to repression control by L-tyrosine.

Prephenate dehydratase in coryneform bacteria, on the other hand, is quite sensitive to feedback inhibition by L-phenylalanine, as is appropriate to its branch-point position. L-Tyrosine activates the enzyme markedly (Fazel and Jensen, manuscript in preparation), such that the intracellular activity of prephenate dehydratase is probably influenced dramatically by the

ratio of L-phenylalanine to L-tyrosine. Apparently, coryneform bacteria are able to control tyrosine and phenylalanine biosynthesis through end product effects that impinge upon only the two control points, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthetase (subject to synergistic feedback control by the combined presence of phenylalanine and tyrosine [23]) and prephenate dehydratase (also controlled by both end products).

Biochemical variation in metabolic pathways. Although the biochemical unity of major pathways in nature is an established concept, an accumulating body of examples documenting alternative pathway sequences reveals more biochemical diversity then was previously anticipated (13). Alternative enzymological sequences to more than 6 amino acids are now known. The aromatic pathway branchlets to both phenylalanine and tyrosine are variable in nature. Either a phenylpyruvate or pretyrosine branchlet may be used for L-phenylalanine biosynthesis, whereas either a 4-hydroxyphenylpyruvate or a pretyrosine branchlet may exist for L-tyrosine biosynthesis. In some cases dual branchlets may exist in the same organism, e.g., dual branchlets to phenylalanine in coryneform bacteria (Fazel and Jensen, manuscript in preparation) or dual branchlets to tyrosine in Mung bean (J. L. Rubin and R. A. Jensen, manuscript in preparation). Even more complicated, *P. aeruginosa* possesses dual branchlets to both phenylalanine and tyrosine (20, 21). At the moment it is a toss-up as to which of these various branchlet patterns for aromatic amino acid biosynthesis will prove to be most common in nature.

Taxonomic applications. It seems likely that the pretyrosine pathway of tyrosine biosynthesis may typify *Corynebacterium* at the generic level although more species will have to be examined to establish this. Such enzymological approaches to biochemical taxonomy have been very successful at roughly the hierarchical level of genus (3, 5, 14, 16). Sometimes finer distinctions can be made (12). In our small sampling of species, *B. ammoniagenes* differs from *C. glutamicum* and *B. flavum* in several respects. *B. ammoniagenes* contains a single aromatic aminotransferase; the other species contain two aromatic aminotransferases. The molecular weight of *B. ammoniagenes* pretyrosine dehydrogenase is about half that of the other two species. Thus, *B. flavum* resembles *C. glutamicum* more than *B. ammoniagenes* on these criteria, a result which is consistent with the lack of any firm and credible basis for *Brevibacterium* as a meaningful taxon, e.g., it is considered *genus incertae sidis* in *Bergey's Manual of Determinative Bacteriology* (8th ed.)

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant AM-19447 and by Biomedical Research Support Grant Funds (RR07149-05) from the National Institutes of Health.

We acknowledge the help of J. R. Bowen, who developed the dansylation procedure in use (J. R. Bowen and R. A. Jensen, in preparation).

LITERATURE CITED

1. **Andrews, P.** 1964. Estimation of molecular weights of proteins by Sephadex gel-filtration. Biochem. J. **91:** 222-223.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248-254.
3. **Canovas, J. L., L. N. Ornston, and R. Y. Stanier.** 1967. Evolutionary significance of metabolic control systems. Science **156:**1695-1699.
4. **Champney, W. S., and R. A. Jensen.** 1970. The enzymology of prephenate dehydrogenase in *Bacillus subtilis*. J. Biol. Chem. **245:**3763-3770.
5. **Cohen, G. H., R. Y. Stanier, and G. Le Bras.** 1969. Regulation of the biosynthesis of amino acids of the aspartate family in coliform bacteria and pseudomonads. J. Bacteriol. **99:**791-801.
6. **Cotton, R. G. H., and F. Gibson.** 1965. The biosynthesis of phenylalanine and tyrosine: enzymes converting chorismic acid into prephenic acid and their relationship to prephenate dehydratase and prephenate dehydrogenase. Biochim. Biophys. Acta **100:**76-88.
7. **Friedrich, B., C. G. Friedrich, and H. G. Schlegel.** 1976. Purification and properties of chorismate mutase prephenate dehydratase and prephenate dehydrogenase from *Alcaligenes eutrophus*. J. Bacteriol. **126:**712-722.
8. **Friedrich, B., and H. G. Schlegel.** 1975. Aromatic amino acid biosynthesis in *Alcaligenes eutrophus* H16. Arch. Microbiol. **103:**141-149.
9. **Hagino, H., and K. Nakayama.** 1974. Regulatory properties of prephenate dehydrogenase and prephenate dehydratase from *Corynebacterium glutamicum*. Agric. Biol. Chem. **38:**2367-2376.
10. **Hagino, H., and K. Nakayama.** 1975. The biosynthetic control in aromatic amino acid producing mutants of *Corynebacterium glutamicum*. Agric. Biol. Chem. **39:** 351-356.
11. **Herbert, D., P. J. Phillips, and R. E. Strange.** 1971. Chemical analysis of microbial cells, p. 209-344. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 5B. Academic Press Inc., New York.
12. **Jensen, R. A.** 1970. Taxonomic implications of temperature dependence of the allosteric inhibition of 3-deoxy-D-*arabino* heptulosonate-7-phosphate synthetase in *Bacillus*. J. Bacteriol. **102:**489-497.
13. **Jensen, R. A.** 1976. Enzyme recruitment in evolution of new function. Annu. Rev. Microbiol. **30:**409-425.
14. **Jensen, R. A., D. S. Nasser, and E. W. Nester.** 1967. The comparative control of a branch-point enzyme in microorganisms. J. Bacteriol. **94:**1582-1593.
15. **Jensen, R. A., and D. L. Pierson.** 1975. Evolutionary implications of different types of microbial enzymology for *L*-tyrosine biosynthesis. Nature (London) **254:**667-671.
16. **Jensen, R. A., and J. L. Rebello.** 1970. Comparative allostery of microbial enzymes at metabolic branchpoints: evolutionary implications, p. 105-121. *In* C. J. Corum (ed.), Developments in industrial microbiology, vol. 11. Plenum Press, New York.
17. **Jensen, R. A., L. Zamir, M. St. Pierre, N. Patel, and D. L. Pierson.** 1977. The isolation and preparation of

pretyrosine, accumulated as a dead-end metabolite by *Neurospora crassa*. J. Bacteriol. **132**:896–903.

18. **Koch, C. E., D. C. Shaw, and F. Gibson.** 1971. The purification and characterization of chorismate mutase-prephenate dehydrogenase from *Escherichia coli* K-12. Biochim. Biophys. Acta **229**:795–804.
19. **Miller, J. V., and E. B. Thompson.** 1972. Radioactive assay for tyrosine aminotransferase. Anal. Biochem. **47**: 487–494.
20. **Patel, N., D. L. Pierson, and R. A. Jensen.** 1977. Dual enzymatic routes to *L*-tyrosine and *L*-phenylalanine via pretyrosine in *Pseudomonas aeruginosa*. J. Biol. Chem. **252**:5839–5846.
21. **Patel, N., S. L. Stenmark-Cox, and R. A. Jensen.** 1978. Enzymological basis of reluctant auxotrophy for phenylalanine and tyrosine in *Pseudomonas aeruginosa*. J. Biol. Chem. **253**:2972–2978.
22. **Schwinck, I., and E. Adams.** 1959. Aromatic biosynthesis. XVI. Aromatization of prephenic acid to *p*-hydroxyphenylpyruvic acid, a step in tyrosine biosynthesis in *Escherichia coli*. Biochim. Biophys. Acta **36**:102–116.
23. **Shiio, I., S. Sugimoto, and R. Miyajima.** 1974. Regulation of 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate synthetase in *Brevibacterium flavum*. J. Biochem. **75**:987–997.
24. **Stenmark, S. L., and R. A. Jensen.** 1975. The ancient origin of a second microbial pathway for *L*-tyrosine biosynthesis in prokaryotes. J. Mol. Evol. **4**:249–259.
25. **Stenmark, S. L., D. L. Pierson, R. A. Jensen, and G. I. Glover.** 1974. Blue-green bacteria synthesize *L*-tyrosine by the pretyrosine pathway. Nature (London) **247**:290–292.
26. **Sugimoto, S., M. Nakagawa, T. Tsuchida, and I. Shiio.** 1973. Regulation of aromatic amino acid biosynthesis and production of tyrosine and phenylalanine in *Brevibacterium flavum*. Agric. Biol. Chem. **37**:2327–2336.
27. **Veldkamp, H.** 1970. Saprophytic coryneform bacteria. Annu. Rev. Microbiol. **24**:209–240.
28. **Yonaha, K., S. Toyama, M. Yasuda, and K. Soda.** 1977. Properties of crystalline ω-amino acid: pyruvate aminotransferase of *Pseudomonas* sp. F-126. Agric. Biol. Chem. **41**:1701–1706.