JOURNAL OF BACTERIOLOGY, June 1981, p. 1013-1019
0021-9193/81/061013-07$02.00/0

Vol. 146, No. 3

Induction of L-Phenylalanine Ammonia-Lyase During Utilization of Phenylalanine as a Carbon or Nitrogen Source in *Rhodotorula glutinis*

WILLIAM C. MARUSICH,[1] ROY A. JENSEN,[1,2*] AND LOLITA O. ZAMIR[1,2,3]

Department of Biology,[1] Department of Chemistry,[3] and Center for Somatic-Cell Genetics and Biochemistry,[2] State University of New York at Binghamton, Binghamton, New York 13901

Received 12 January 1981/Accepted 16 March 1981

Rhodotorula glutinis is a convenient source of L-phenylalanine ammonia-lyase, an enzyme that is useful as a biochemical reagent in the assay of L-phenylalanine. There have been previous descriptions of induced lyase production in complex medium where induction occurs late in exponential growth, suggesting a role in secondary metabolism such as is the case in higher plants. A higher specific activity of L-phenylalanine ammonia-lyase (sixfold higher than in complex medium) can be obtained during midexponential growth in a defined medium containing L-phenylalanine as the sole source of carbon. L-Phenylalanine will also induce lyase synthesis during exponential growth in minimal medium in which L-phenylalanine is the sole source of nitrogen. The appearance of lyase in complex medium supplemented with L-phenylalanine is probably triggered fortuitously by exhaustion late in growth of a prime source of nitrogen. In this study, *R. glutinis* appeared to express a single lyase enzyme, regardless of whether induction was nitrogen signaled or carbon signaled. Thin-layer chromatographic analysis of ether extracts prepared from cultures induced with doubly labeled (U-^{14}C; ring-4-^{3}H) L-phenylalanine provided evidence of a catabolic sequence containing cinnamic acid, benzoic acid, and 4-hydroxybenzoic acid as degradative intermediates. 3,4-Dihydroxybenzoic acid was not identified as a catabolic intermediate.

L-Phenylalanine ammonia-lyase catalyzes the nonoxidative deamination of phenylalanine to yield *trans*-cinnamic acid and a free ammonium ion (10). Since the initial isolation and description of this enzyme in barley shoots (16), the presence of L-phenylalanine ammonia-lyase has been noted in a variety of botanical and microbial species. In plants, L-phenylalanine ammonia-lyase is a ubiquitous protein which constitutes the initial step of a highly branched and quantitatively significant pathway of secondary metabolism. The regulation of this enzyme in plants has been intensively studied in an attempt to gain understanding of developmental phenomena (6, 9, 12, 14, 20, 22).

Although L-phenylalanine ammonia-lyase is sometimes found in procaryotes (1), its distribution among microorganisms appears to be limited mainly to certain families of fungi and yeasts. Although the role of L-phenylalanine ammonia-lyase in particular microbial systems may on occasion equate with secondary metabolism (18), as it does in plants, its central role in microbes seems to be catabolic, i.e., serving as an initial degradative step to generate usable carbon and energy (17).

Partially purified preparations of L-phenylalanine ammonia-lyase can be used to assay for L-phenylalanine with high sensitivity, owing to the strong absorbance of cinnamate at 273 nm. An excellent source of enzyme is the red yeast *Rhodotorula glutinis*, which expresses substantial amounts of enzyme in complete medium supplemented with L-phenylalanine. Some enzymological characterizations have been carried out with enzyme that has been partially purified from *R. glutinis* (7, 13). The timing of the appearance of enzyme early in stationary-phase physiology mimics the timing of the events of secondary metabolism. In this paper we show the latter timing to be fortuitous and the synthesis of L-phenylalanine ammonia-lyase to be inducible in response either to a signal of nitrogen deprivation or to a signal of carbon limitation.

(This study represents part of a thesis presented by W.C.M. to the Graduate School of the State University of New York at Binghamton in partial fulfillment of the requirements for the M.A. degree, 1980.)

MATERIALS AND METHODS

Microbiological aspects. *R. glutinis* was obtained from the Institute for Fermentation, Osaka, Japan, as *R. glutinis* 0559. The recipe for complex medium was

1% malt extract (Difco Laboratories), 0.1% yeast extract (Difco), and supplements as indicated. Basal minimal salts medium contained 50 mM potassium phosphate buffer (pH 6.2), 100 μg of $MgSO_4$ per ml, 10 μg of biotin per ml, and the indicated sources of carbon and nitrogen. Yeast carbon base contains no source of nitrogen and dextrose (Difco) as the sole source of carbon, whereas yeast nitrogen base contains no source of carbon, and ammonium sulfate as the sole source of nitrogen (see p. 251 of *Difco Manual*, 9th ed., for complete composition of media). Biotin and amino acids were filter sterilized (Millipore HA filter, 0.45-μm pore size). Growth rates were established by following turbidity increases with a Klett-Summerson colorimeter equipped with a no. 66 filter. For routine conditions of growth, a New Brunswick fermentor was used to maintain 4 liters of culture at 25°C and mechanical agitation at 125 rpm. Stock cultures were maintained on complex medium solidified with 1.7% agar (Difco).

Cinnamic acid failed to support growth when initially tested as a sole source of carbon, but it was subsequently found that carbon source levels of buffered cinnamate inhibited growth when present in fructose-$(NH_4)_2SO_4$-minimal medium. Growth of a culture inoculated to a turbidity of 80 Klett units was inhibited 100% by 2 mM cinnamate. A 0.75 mM concentration caused a growth lag of 8 h, followed by a normal growth rate and mass yield. Similar complications were found with benzoate. Mutants were sought that might be resistant to cinnamate or benzoate or both and that might therefore serve as carbon source nutrients, but none were found.

Preparation of crude extracts. Cultures were harvested by centrifugation at 7,000 × *g* at 4°C. On occasion, when fresh cells were not used immediately, the pellet was quick-frozen and maintained at −40°C until extract preparation was done (within 48 h). Cells were suspended in 50 mM Tris-hydrochloride buffer at pH 8.5 and 4°C before ultrasonic disruption via three 30-s bursts of ultrasonic energy applied by use of a Lab-Line Ultratip Labsonic System. The resulting solution was clarified of debris that was sedimented at 25,000 × *g* and then was dialyzed overnight against 4,000 volumes of buffer at 4°C. The resulting crude extracts contained L-phenylalanine ammonia-lyase that maintained stability for at least 9 months of storage at −40°C.

Partial purification. A 50-mg amount of crude extract was layered on a column (1.5 by 25 cm) of DEAE-cellulose that had been equilibrated at 4°C with 50 mM Tris-hydrochloride buffer (pH 8.5). The column was washed with 66 ml of buffer before application of a linear gradient (300 ml) of NaCl (end gradient concentration, 0.5 M). Fractions of 2.2-ml volume were collected at 4°C and assayed for L-phenylalanine ammonia-lyase activity and for protein concentration (3, 5, 21).

Fractions containing 90% of the total enzyme activity were combined and concentrated by passage through an Amicon PM-10 membrane. This sample was layered on a hydroxylapatite column (1.5 by 10 cm) that had been equilibrated with 1 mM potassium phosphate buffer (pH 6.2). After the column was washed with 100 ml of phosphate buffer, enzyme was eluted with 300 ml of a linear salt gradient (1 to 250 mM potassium phosphate, pH 6.2). Fractions of 2.2-ml volume were collected at 4°C.

L-Phenylalanine ammonia-lyase preparations obtained after use of phenylalanine as the carbon source or as the nitrogen source were compared with respect to kinetic parameters. No significant differences were obtained. Double-reciprocal plots yielded an apparent K_m value of 0.57 mM for L-phenylalanine.

Enzyme assay. The activity of L-phenylalanine ammonia-lyase can be followed as a continuous function of time with a convenient and sensitive assay. The appearance of cinnamic acid was monitored at 290 nm (ϵ = 10,000 in 0.1 M borate buffer, pH 8.8) in a Gilford 250 spectrophotometer. Although the λ_{max} of *trans*-cinnamate is at 273 nm, assays were carried out at 290 nm (7) to minimize interference from compounds that absorb at 273 nm. The reaction mixture contained 5.0 mM L-phenylalanine, enzyme, and 50 mM Tris-hydrochloride (pH 8.5) in a total volume of 1.5 ml. The reaction was initiated with L-phenylalanine, and velocities lagged as much as 4 min before constant rates were achieved (then enduring for at least 30 min). Activities were proportional functions of enzyme concentration. Specific activities were calculated as nanomoles of cinnamate formed per minute per milligram of protein at 25°C.

TLC. Mixtures of compounds to be separated were prepared as ether extracts. The entire culture was acidified to pH 2 by addition of 6 N HCl before extraction with 3 volumes of ether. Neutral and acidic fractions were separated (15) and dried with anhydrous Na_2SO_4. The ether was evaporated with a stream of gaseous nitrogen in a ventilated hood to yield a residue which was suspended in 1.0 ml of methanol. Two different bidimensional thin-layer chromatography (TLC) systems were employed for analytical purposes. In system 1, glass-backed cellulose plates (20 by 20 cm; Comaz) with fluorescent indicator were used. Ether extract was spotted in the lower left corner, and the plate was developed in the first direction with formic acid-water (98:2). After drying, the plate was developed inthe second direction in benzene-acetic acid-water (10:7:3). In system 2, Eastman Silica Gel 60 F-254 plastic-backed plates (20 by 20 cm) were developed in the first direction with benzene-acetic acid-water (6:7:3). After drying, the plates were developed in the second direction with benzene-dioxane-acetic acid (90:25:4).

For preparative purposes, unidirectional TLC was used employing Analtech Silica Gel F-254 (1 mm thick) on glass plates (20 by 20 cm). After streak-application of the sample about 2 cm from the lower edge, development was carried out in benzene-acetic acid-water (10:7:3). The elution solvent was ether.

Tracing of radiolabeled compounds. A 1-liter culture was grown in minimal salts medium containing Difco nitrogen base and 15 mM L-phenylalanine (as a carbon source) and was harvested at the late exponential phase of growth. Cells were pelleted by centrifugation at 7,000 × *g* and then suspended in 75 ml of water. L-[U-^{14}C]phenylalanine (536 mCi/mmol) and L-[ring-4-^{3}H]phenylalanine (12 Ci/mmol) were mixed to yield a $^{3}H/^{14}C$ ratio of 10.5 (as established by counting). After the addition of doubly labeled L-phen-

ylalanine, 25-ml samples of cell suspensions were removed at postlabeling times of 4, 8, and 16 min; these were quick-frozen by immersion in liquid nitrogen. Each sample was thawed, acidified to pH 2.0 with N HCl, and then disrupted with three 30-s pulses of sonic energy applied at 80 W. The slurries thus obtained were extracted with ether and separated into acidic and neutral fractions as described earlier. Amounts of 20 mg of authentic and unlabeled cinnamate, benzoate, and 4-hydroxybenzoate, and 5 mg of 3,4-dihydroxybenzoate were added to each acidic residue. Each mixture was subjected to preparative TLC as described above, and the migration position of each compound was identified by monitoring fluorescence under UV light. The silica gel containing each compound was excised and suspended in ether. The silica gel particles were removed by filtration under vacuum, and the ether was then evaporated. Purification of each compound was carried out via recrystallization from appropriate solvents (11). Since cinnamate and benzoate migrated near one another in preparative TLC, they were further purified by additional TLC, using cellulose plates and a solvent system of formic acid-water (98:2).

Amounts of each compound recovered were calculated from spectrophotometric measurements, using the following molar extinction coefficients: cinnamate, 20,893 at 273 nm; benzoate, 11,900 at 228 nm; 4-hydroxybenzoate, 15,100 at 254 nm; and 3,4-dihydroxybenzoate, 3,890 at 290 nm.

Radioactive counting was done with a Beckman LS-7500 computerized scintillation counter. Quenched standard series for ^3H and ^{14}C were used to generate quench coefficients for counting efficiency and channel overlap. After suspension of each compound in methanol, a small sample was added to 15 ml of Aquasol and counted. Each compound was repurified until both of the radiospecific activities and the $^3H/^{14}C$ ratio obtained remained constant over at least three recrystallizations. The highest incorporation of isotopic L-phenylalanine into *trans*-cinnamic acid [(radioactivity of cinnamic acid × 100)/radioactivity of L-phenylalanine administered], i.e., about 10%, was obtained at the earliest sampling time of 4 min.

Biochemicals and chemicals. Tris-hydrochloride, amino acids, biotin, benzoate, 4-hydroxybenzoate, 3,4-dihydroxybenzoate, and cinnamate were obtained from Sigma Chemical Co. Yeast carbon base and yeast nitrogen base were purchased from Difco. Fisher Scientific Co. supplied D-fructose and all organic solvents. Aquasol, ^3H and ^{14}C quenched standard series, and L-[*U*-^{14}C]phenylalanine were obtained from New England Nuclear Corp.; L-[ring-4-^3H]-phenylalanine was obtained from Amerham Corp. We purchased DEAE-cellulose (DE-52) from Whatman, and both the hydroxylapatite (Bio-Gel HTP) and the dye reagent concentrate for protein assay were from Bio-Rad Laboratories. All other chemicals were of reagent grade.

RESULTS

Induction of L-phenylalanine ammonia-lyase by L-phenylalanine. In complex medium the expression of lyase activity required added L-phenylalanine in the medium (Table 1). In a defined minimal salts medium *R. glutinis* grew at a rate which was only 50% slower than in complex medium. L-Phenylalanine served as the sole source of nitrogen as well as did ammonium sulfate, each sustaining a doubling time of 9 h in fructose-minimal medium. In contrast, L-phenylalanine was a relatively poor carbon source (doubling time, 16 to 17 h) when substituted for fructose. The presence of L-phenylalanine was necessary, but not sufficient, for the induction of L-phenylalanine ammonia-lyase (Table 1). When used as the sole source of carbon, L-phenylalanine induced specific activity levels of 50 to 60. When L-phenylalanine was used instead as the sole source of nitrogen, with fructose as the carbon source, phenylalanine am-

TABLE 1. *Nutritional influences on expression of L-phenylalanine ammonia-lyase*

Basic medium[a]	Carbon source[b]	Nitrogen source[c]	Addition[d]	Doubling time[e]	Sp act[f]
Complex	Undefined	Undefined		6	<0.5
Complex	Undefined	Undefined	L-Phenylalanine	6	9
Minimal	Fructose	Ammonium sulfate		9	<0.5
Minimal	Fructose	Ammonium sulfate	L-Phenylalanine	9	<0.5
Minimal	Fructose	L-Phenylalanine		9	12
Minimal	L-Phenylalanine	Ammonium sulfate		16	59
Minimal	L-Phenylalanine	L-Phenylalanine		17	54

[a] See text for composition of media.

[b] Fructose was present at a final concentration of 1.0% (wt/vol); the final concentration of L-phenylalanine was 15 mM.

[c] Ammonium sulfate was present at 0.15% (wt/vol); the final concentration of L-phenylalanine was 15 mM.

[d] L-Phenylalanine was used at a final concentration of 15 mM.

[e] Doubling times are given to the nearest hour. Growth vessels contained 250-ml cultures shaken at 125 rpm at 25°C.

[f] Specific activities, expressed as nanomoles of cinnamate formed per minute per milligram of extract protein, were obtained through sacrifice of the cultures at the end of exponential growth and subsequent preparation of crude extracts (see text).

monia-lyase was present in a specific activity range of 9 to 12. Fructose and ammonium sulfate (if present as carbon and nitrogen sources, respectively) abolished the ability of L-phenylalanine to induce lyase. Thus, fructose and ammonium sulfate are carbon and nitrogen compounds which prevent the expression of phenylalanine ammonia-lyase that is potentially induced in the presence of L-phenylalanine, provided L-phenylalanine is not being utilized as the sole source of carbon or nitrogen.

Timing of physiological induction of L-phenylalanine ammonia-lyase. When L-phenylalanine is added as a supplement to complex medium, L-phenylalanine ammonia-lyase activity is not apparent until well into the exponential phase of growth, and peak activity is not achieved until the early senescent phase (7). This could reflect the delayed utilization of L-phenylalanine after depletion of some preferred carbon or nitrogen source. Depletion of nitrogen was implicated because the specific activity of lyase from complex medium-grown cultures was more similar to that obtained by using L-phenylalanine as a nitrogen source in minimal medium (Table 1) than to that obtained by using L-phenylalanine as a carbon source in minimal medium. The pattern of delayed lyase appearance in complex medium was mimicked in defined medium, where it was observed that the induction of lyase by L-phenylalanine was delayed for about 18 h until $(NH_4)_2SO_4$ was depleted from the medium (Fig. 1). When cells were required to shift to L-phenylalanine as a new source of nitrogen, a fairly lengthy lag time in growth correlated with the induction of lyase formation (Fig. 1).

Experiments in which a nutritional shift was made requiring the induction of lyase to utilize L-phenylalanine as the sole source of carbon are shown in Fig. 2. The increase in specific activity continued steadily throughout almost two generations, leveling off at a value of about 60 until the early stationary phase. We consistently noted declining specific activities during stationary-phase physiology.

Partial purification of L-phenylalanine ammonia-lyase. If carbon signal-induced lyase (i.e., conditions of line 6, Table 1) were a different protein from nitrogen signal-induced lyase (i.e., conditions of line 5, Table 1), the enzymes would probably show distinctive physical characteristics that would influence the elution position from chromatographic resins. A crude extract was prepared from a maximally induced culture, utilizing L-phenylalanine as the carbon source (specific activity, 50.6). The activity eluted from DEAE-cellulose at 0.28 M NaCl in a salt gradient as a single, sharp peak. The peak

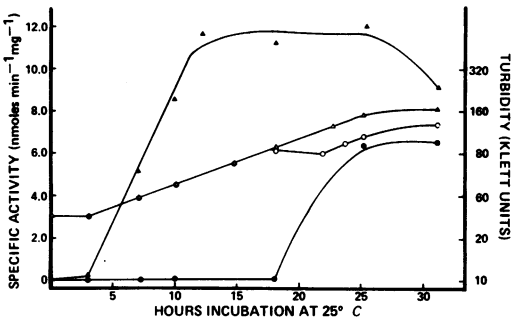

FIG. 1. *Response of L-phenylalanine ammonia-lyase levels to nitrogen deprivation in the presence of L-phenylalanine. A 1-liter culture grown in fructose-$(NH_4)_2SO_4$-minimal medium was harvested at a turbidity of 120 Klett units. Half of the pellet was suspended in 2 liters of minimal medium (Difco carbon base [see text]) containing 10 mM L-phenylalanine as the sole nitrogen source; samples were taken for enzyme assay (▲) and for turbidity measurements (△) at the indicated times. The second half of the pellet was suspended in identical medium which also contained 0.15% $(NH_4)_2SO_4$; samples were taken for enzyme assay (●) and for turbidity measurements (○).*

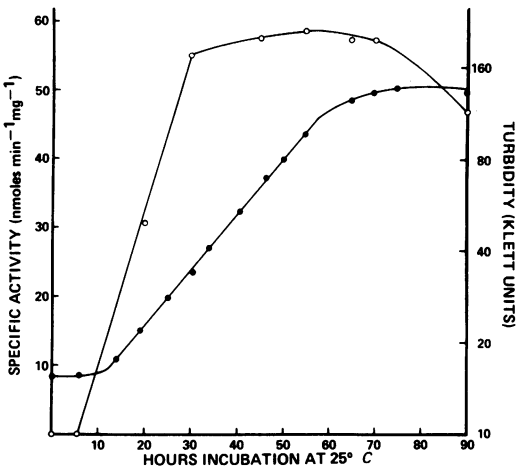

FIG. 2. *Induction of L-phenylalanine ammonia-lyase by L-phenylalanine (as the carbon source). An inoculum in late exponential-phase physiology was prepared in Difco nitrogen base minimal medium containing fructose as the carbon source. At zero time the sedimented cells were suspended at about a 10-fold dilution in 4 liters of Difco nitrogen base medium containing 15 mM L-phenylalanine as the sole source of carbon. Samples were taken for enzyme assay at the times indicated on the abscissa scale (hours). Symbols: ○, lyase activity (left ordinate); ●, turbidity (right ordinate).*

tube contained lyase having a specific activity of 168. In a second purification step of hydroxylapatite chromatography, activity was again recovered in a single, sharp peak at 91 mM in the

salt gradient (specific activity, 692). The purification thus obtained was about 14-fold. This same protocol was applied starting with a crude extract derived from cultures induced by L-phenylalanine under nitrogen signal conditions. Elution positions of the two enzyme preparations were concluded to be identical when single, sharp peaks of activity were recovered from DEAE-cellulose and hydroxylapatite after mixture of the two preparations in equal specific activity portions. In other experiments (data not shown), sedimentation of lyase through sucrose density gradients did not reveal detectable molecular weight differences. Kinetic parameters of activity were also very similar (K_m = 0.57 mM at 22°C).

Intermediates of L-phenylalanine catabolism. Cultures catabolizing L-phenylalanine as the sole source of carbon were harvested late in exponential growth for TLC analysis. Extraction with ether (see above) was carried out, and the acidic fraction was analyzed in TLC systems 1 and 2. Three distinct spots were seen which were not apparent in comparable preparations from cultures grown on fructose-ammonium sulfate. One compound migrated with the same R_f as 4-hydroxybenzoate, was faintly fluorescent under UV light, and turned light brown after spraying with diazotized *p*-nitroaniline. The remaining compounds comigrated with cinnamic acid and benzoic acid, were intensely fluorescent under UV illumination, and were unreactive with diazotized *p*-nitroaniline. *p*-Nitrodiazonium salts couple with phenols such as 4-hydroxybenzoate. The lack of a 4-hydroxy substituent in *trans*-cinnamic acid and in benzoic acid accounts for their nonreactivity with diazotized *p*-nitroaniline.

A small portion (about 10%) of the acidic fraction was developed in TLC system 2. The three metabolites (located by fluorescence detection) were scraped from the TLC plate and eluted into ether. The ether was evaporated, and the residue was taken up into 1.0 ml of methanol. The UV absorption profiles obtained were exact matches for profiles obtained with authentic samples of cinnamic acid, benzoic acid, and 4-hydroxybenzoic acid.

When the above procedures were carried out with extracts derived from cultures responding to L-phenylalanine as the sole nitrogen source, results were qualitatively the same except that 4-hydroxybenzoic acid was strikingly more prominent by visual inspection.

Catabolism of doubly labeled L-phenylalanine. L-Phenylalanine was catabolized as the sole source of carbon under conditions specified above. Each carbon atom carried a ^{14}C label, whereas the C-4 substituent of the L-phenylalanine ring was tritiated (Fig. 3). Samples were taken at 4, 8, and 17 min after the addition of the double label (4-^3H; *U*-^{14}C) to growing cells of *R. glutinis*. All three samples yielded evidence for incorporation of L-phenylalanine into cinnamic acid, benzoic acid, and 4-hydroxybenzoic acid. The best conversion (about 10%) was obtained with the 4-min sample. Therefore, samples were analyzed 4 min after the administration of doubly labeled L-phenylalanine, and repeated crystallizations were carried out. The data summarized in Fig. 3 cite $^3H/^{14}C$ ratios that were constant (±0.1) throughout the last three recrystallizations. The $^3H/^{14}C$ ratio of cinnamic acid was 10.5, exactly the double-label ratio of the parent L-phenylalanine molecule administered 4 min earlier. Oxidation of the double bond in the side chain of cinnamic acid, resulting in a product (benzoic acid) lacking two of the original

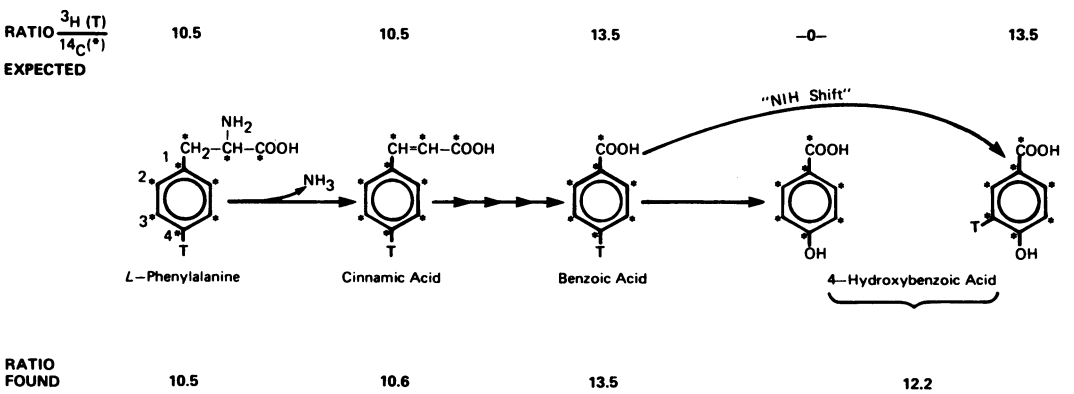

FIG. 3. *L-Phenylalanine was supplied to a 1-liter culture of R. glutinis growing in Difco nitrogen base medium which lacked carbon source molecules other than L-phenylalanine. L-Phenylalanine was doubly labeled as shown, i.e., tritiated at C-4 of the aromatic ring and uniformly labeled with ^{14}C. The $^3H/^{14}C$ ratio was initially set at 10.5 dpm. Observed ratios were determined from multiply purified samples (see text).*

carbon atoms, predicts a new ratio of 13.5, which is very near the number obtained. The hydroxylation step converting benzoic acid to 4-hydroxybenzoic acid occurs with retention of a large fraction (about 90%) of tritium labeling, a result that is consistent with an NIH shift mechanism (8).

We sought evidence for incorporation of labeled L-phenylalanine into 3,4-dihydroxybenzoic acid, the next metabolite expected in the catabolic sequence. However, labeled 3,4-dihydroxybenzoic acid was not detected.

DISCUSSION

Role of L-phenylalanine ammonia-lyase in *R. glutinis*. *R. glutinis* produces a lyase that can be conveniently purified for use as an assay reagent (7). The timing of its appearance in complex medium at the late exponential or early stationary phase might suggest its classification as an enzyme of secondary metabolism, an impression that is further prejudiced by the widely documented role of L-phenylalanine ammonia-lyase in secondary metabolism in plant eucaryotes. Our results show that L-phenylalanine ammonia-lyase of *R. glutinis* is induced by L-phenylalanine in response to carbon limitation or to nitrogen limitation. Formation of lyase enzyme late in growth in complex medium probably reflects the depletion of the nitrogen source and subsequent induction in a timing pattern that is a fortuitous accident of medium composition. Unlike typical enzymes of secondary metabolism, L-phenylalanine ammonia-lyase is readily demonstrated during exponential growth. Indeed, enzyme preparations can be obtained from exponential cultures grown in defined medium which have specific activities that are superior (five- to sevenfold) to extracts prepared from complex medium cultures.

Direction of L-phenylalanine catabolism. Pathways in fungi for degradation of L-phenylalanine include at least the cinnamate, the phenylpyruvate, and the 4-hydroxymandelate routes (15). In *R. glutinis*, TLC results implicated cinnamic acid, benzoic acid, and 4-hydroxybenzoic acid as L-phenylalanine catabolites. To obtain unambiguous data showing incorporation of L-phenylalanine label via intact molecules in the series, we employed an appropriate double-labeling protocol (Fig. 3). The expected $^{3}H/^{14}C$ ratios are compared with actual experimental values obtained.

The ratio of 12.2 obtained for 4-hydroxybenzoate is interpreted in terms of an NIH shift, a phenomenon whereby hydroxylation of aromatic compounds proceeds by a mechanism (8) that would translocate a (tritium) substituent at the hydroxylation site to the adjacent carbon. About 90% of the ^{3}H in benzoic acid was retained in 4-hydroxybenzoic acid.

It is probable that *R. glutinis* utilizes the same catabolic pathway for L-phenylalanine as is employed by another yeast, *Sporobolomyces roseus* (17). It seems likely that steps of hydration, formation of a coenzyme A derivative, aldol cleavage, and oxidation may intervene between cinnamic acid and benzoic acid if we consider the analogous steps between 4-hydroxycinnamic acid and 4-hydroxybenzoate proposed by Sparnins et al. (19) in tyrosine degradation by *Trichosporon cutaneum*. The absence of detectable radiolabeled 3,4-dihydroxybenzoate in our results was surprising. We suspect that the presence of a highly active dioxygenase may have prevented the accumulation of detectable amounts of 3,4-dihydroxybenzoate under our conditions of experimentation. Alternatively, 4-hydroxybenzoate may proceed to a different product. A precedent for this possibility is the conversion of 4-hydroxybenzoate to 2,5-dihydroxybenzoate (gentisate) in *Bacillus laterosporus* (4).

Regulation. L-Phenylalanine ammonia-lyase can serve two physiological functions: (i) liberation of ammonia as the sole source of nitrogen and (ii) catalysis of the first step in a pathway forming an ultimate usable source of carbon. A priori, one might expect two species of enzyme, one inducible (perhaps as part of an operon organization) during carbon limitation and another inducible during nitrogen limitation. (Different regulatory isoenzymes of L-phenylalanine ammonia-lyase have been characterized in one plant system [2].) The reproducible difference in specific activities obtained in *R. glutinis*, depending upon whether L-phenylalanine was primarily supplying nitrogen or carbon, did indeed suggest the possibility of separate enzyme proteins. However, no obvious differences in physical characteristics of nitrogen signal-induced lyase or carbon signal-induced lyase were found. Although the lyase enzyme alone would be sufficient to liberate inorganic nitrogen from L-phenylalanine in response to nitrogen limitation, it appears that a succeeding block of enzymes is also induced since benzoate and 4-hydroxybenzoate were detected by TLC. Since induced nitrogen-limited cultures appeared to accumulate more 4-hydroxybenzoate than did induced carbon-limited cultures, a distinct possibility is that the enzymes between L-phenylalanine and 4-hydroxybenzoate are induced en bloc in response to nitrogen limitation. Cinnamic acid cannot be tested as a carbon source nutrient because it is highly inhibitory to growth, as is benzoate. It is therefore possible that, if the multistep pathway

shown in Fig. 3 is induced by L-phenylalanine during nitrogen limitation, not only is usable nitrogen liberated by lyase action, but noxious coproducts such as cinnamate are transformed to 4-hydroxybenzoate, which is not growth inhibitory.

ACKNOWLEDGMENT

This work was supported in part by Department of Energy contract EP-78-S-02-4967.

LITERATURE CITED

1. **Bezanson, G. S., D. Desaty, A. V. Emes, and L. C. Vining.** 1970. Biosynthesis of cinnamide and detection of phenylalanine ammonia-lyase in *Streptomyces verticillatus*. Can. J. Microbiol. **16**:147-151.
2. **Boudet, A., R. Ranjeva, and P. Gadal.** 1971. Proprietes allosteriques specifiques des deux isoenzymes de la phenylalanine-ammoniaque lyase chez *Quercus pedunculata*. Phytochemistry **10**:997-1005.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins using the principle of protein-dye binding. Anal. Biochem. **72**:248-254.
4. **Crawford, R. L.** 1976. Pathways of 4-hydroxybenzoate degradation among species of *Bacillus*. J. Bacteriol. **127**:204-210.
5. **Dawson, R. M. C., D. Elliot, W. H. Elliot, and K. M. Jones.** 1969. Data for biochemical research, 2nd ed., p. 625-626. Oxford University Press, New York.
6. **Englesma, G.** 1967. Photoinduction of phenylalanine deaminase in gherkin seedlings. Planta **75**:207-219.
7. **Fritz, R. R., D. S. Hodgins, and C. W. Abell.** 1976. Phenylalanine ammonia-lyase: induction and purification from yeast and clearance in mammals. J. Biol. Chem. **251**:4646-4650.
8. **Guroff, G., J. Daly, D. Jerina, J. Renson, B. Witkop, and S. Udenfriend.** 1967. Hydroxylation-induced migration: the NIH shift. Science **157**:1524-1530.
9. **Halbrock, K., and E. Wellmann.** 1970. Light-induced flavone biosynthesis and activity of phenylalanine ammonia-lyase and UDP-apiose synthetase in cell suspension cultures of *Petroselinum hortense*. Planta **94**:236-239.
10. **Hanson, K. R., and E. A. Havir.** 1972. Mechanism and properties of phenylalanine ammonia-lyase from higher plants. Recent Adv. Phytochem. **4**:45-85.
11. **Herbron, I. H.** 1965. Dictionary of organic compounds. Eyre & Spottiswoode, Publishers, Ltd., London.
12. **Hiroshi, H., K. Hiroyuk, and S. F. Yang.** 1978. Induction of phenylalanine ammonia-lyase and increase in phenolics in lettuce leaves in relation to the development of russet spotting caused by ethylene. Plant Physiol. **62**:31-35.
13. **Hodgins, D. S.** 1971. Yeast phenylalanine ammonia-lyase: identification, properties and the identification of catalytically essential dehydroalanine. J. Biol. Chem. **246**: 2977-2985.
14. **Johnson, C., T. Attridge, and H. Smith.** 1975. Regulation of phenylalanine ammonia-lyase by cinnamic acid: its implication for the light mediated regulation of the enzyme. Biochim. Biophys. Acta **385**:11-19.
15. **Kishore, G., M. Sugumaran, and C. S. Vaidyanathan.** 1976. Metabolism of DL-(±)-phenylalanine by *Aspergillus niger*. J. Bacteriol. **128**:182-191.
16. **Koukol, J., and E. E. Conn.** 1961. The metabolism of aromatic compounds in higher plants. IV. Purification and properties of the phenylalanine deaminase of *Hordeum vulgare*. J. Biol. Chem. **236**:2692-2698.
17. **Moore, K., P. Y. Subba Rao, and G. H. N. Towers.** 1968. Degradation of phenylalanine and tyrosine by *Sporobolomyces roseus*. Biochem. J. **106**:507-514.
18. **Perrin, P. W., and G. H. N. Towers.** 1973. Hispidin biosynthesis in cultures of *Polyploues hispidus*. Phytochemistry **12**:589-592.
19. **Sparnins, V. L., D. G. Burbee, and S. Dagley.** 1979. Catabolism of L-tyrosine in *Trichosporon cutaneum*. J. Bacteriol. **138**:425-430.
20. **Sugano, N., K. Koidi, Y. Ogawa, Y. Moriya, and A. Nishi.** 1978. Increase in enzyme levels during the formation of phenolic acids in carrot cell cultures. Phytochemistry **17**:1235-1238.
21. **Warburg, O., and W. Christian.** 1971. Isolierung und Kristallisation des Gärungsferments Enolase. Biochem. Z. **310**:384-425.
22. **Zucker, M.** 1965. Induction of phenylalanine deaminase by light and its relation to chlorogenic acid synthesis in potato tuber tissue. Plant Physiol. **40**:779-782.