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Metabolism of Aromatic Amino Acids during the Growth Cycle of Batch Suspension Cultures of *Catharanthus roseus**

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Summary

Profiles of the levels and metabolism of aromatic compounds in suspension-cultured cells of *Catharanthus roseus* during the growth cycle were determined. The level of total protein-amino acids, i.e., sum of the amounts of amino acids in hydrolyzates of proteins, and the level of total phenolic acids increased after transfer of the cells in the stationary phase to fresh Murashige-Skoog medium. The maximum levels of the protein-amino acids and those of the phenolic acids were observed on days 3-5, and on days 6-8, respectively, when the levels were expressed per gram fresh weight. In contrast, the level of total free amino acids, i.e., the sum of the amounts of amino acids in the ethanol-soluble fraction, and the levels of phenylalanine and typosine in the cells decreased during the two days that followed transfer of the cells to fresh medium, reached a minimum during days 3-8, and then increased again. Tracer experiments using [U-^{14}C] L-phenylalanine and [U-^{14}C] L-tyrosine indicated that most of these amino acids was utilized for the synthesis of proteins and most of the remainder was used for the synthesis of phenolic acids and lignin. Phenylalanine was a much a better precursor for the biosynthesis of the phenolic compounds than was tyrosine at any stage of cell growth. Small but significant amounts of phenylalanine and tyrosine were degraded in the cells. The level of phenylalanine ammonia-lyase increased after the transfer of cells to fresh medium and its maximum level was observed on days 3-5. The activity of tyrosine ammonia-lyase was always lower than that of phenylalanine ammonia-lyase. The metabolism of phenylalanine and tyrosine in seedlings of *Catharanthus roseus* was similar to that in cultured cells. The role of phenylalanine ammonialyase in the biosynthesis of phenolic compounds is discussed.

Introduction

In higher plants the aromatic amino acids, such as phenylalanine and tyrosine, are starting materials for secondary metabolites that include

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alkaloids, cummarins, flavonoids, lignin, derivatives of indole and other phenolic compounds [1–3], as well as being precursors for protein synthesis.

These aromatic amino acids are synthesized from phosphoenolpyruvate and erythrose-4-phosphate via the shikimate pathway (Fig. 1). Recently, the metabolic sequence of biosynthetic events and the conversion of these aromatic amino acids to secondary metabolites has been established [4]. However, little information is available about the overall metabolism and the metabolic role of these compounds during the growth of plant cells.

As part of our extensive study of the metabolic regulation in *Cathranthus roseus* cells in batch suspension culture, the metabolic fate of radiolabelled phenylalanine and tyrosine was investigated in these cells at different growth stages. In addition, changes in the levels of phenylalanine ammonia-lyase and tyrosine ammonia-lyase, key enzymes for the biosynthesis of secondary metabolites, were monitored during growth of the cells. For comparison, similar experiments were also conducted using seedlings of *Catharanthus roseus*.

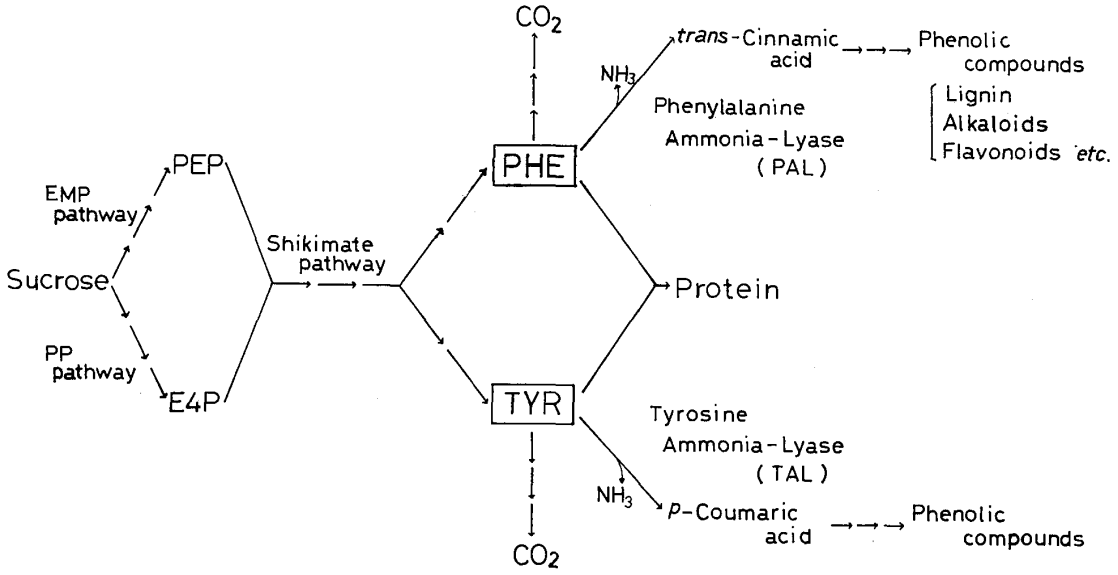

Fig. 1. Possible routes of biosynthesis and conversion of phenylalanine and tyrosine in plant cells. EMP pathway, Embden-Meyerhof-Parnus pathway; E4P, erythrose-4-phosphate; PEP, phosphoenolpyruvate; PHE, phenylalanine; PP pathway, pentose phosphate pathway; TYR, tyrosine.

Materials and Methods

Plant materials

Cell suspension cultures of *Catharanthus roseus* (L.) G. Don (strain A/MN) were maintained in Murashige-Skoog basal medium (Flow Laboratories, Irvine, Scotland) that was supplemented with 2.2 μM dichlorophenoxyacetic acid, 0.4 mg l^{-1} thiamine-HCl and 3% sucrose. Experimental

cultures were initiated by the transfer of a portion (5 ml) of 10-day-old stock cultures to 45 ml of fresh medium in 300-ml Erlenmeyer flasks. The cultures were grown at 27°C on a horizontal rotary shaker (90 strokes min^{-1}, 8 cm amplitude) in the dark. Cell numbers and fresh weights of cultures were determined as described in earlier papers [5, 6]. Seedlings of *Catharanthus roseus*, grown on 0.55% agar gel in the light (3000 lux), were also used.

Determination of levels of amino acids and phenolic compounds

Levels of free and protein-amino acids were determined with a fluorometric amino-acid analyzer as described elsewhere [7]. Levels of ethanol-soluble phenolic compounds were determined by the method of Swain and Hillis [8].

Metabolism of radiolabelled phenylanine and tyrosine

[U-^{14}C] L-Phenylalanine and [U-^{14}C] L-tyrosine were obtained from ICN Radiochemicals, Irvine, California, U. S. A. Aliquots (2 ml) of cell suspension, containing approximately 100 mg fresh weight of cells or segments of seedlings, plus 2 ml of 10 mM sucrose were placed in the main compart-

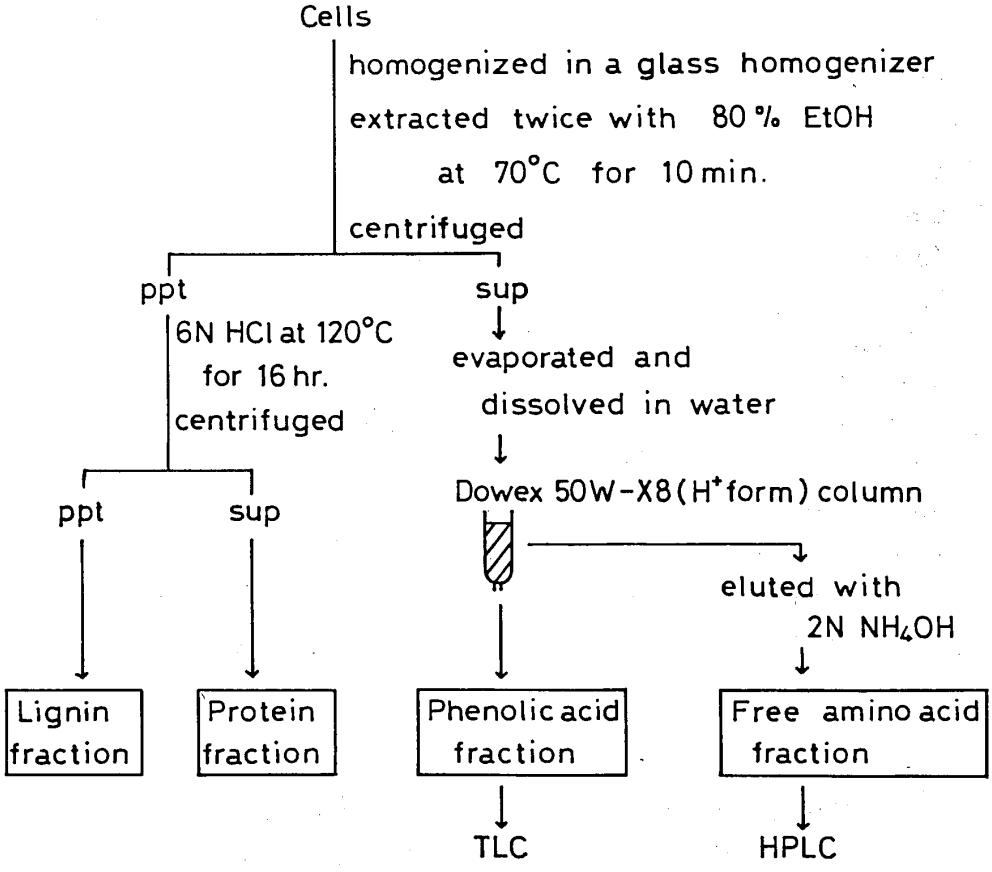

Fig. 2. Flow diagram of the separation of ^{14}C-labelled compounds.

ment of 30-ml Erlenmeyer flasks, fitted with a small glass tube that contained a piece of filter paper wetted with 0.1 ml of 20% KOH. Incubation was initiated by addition of 0.2 ml (37 kBq) of a solution of [U-^{14}C] phenylalanine (specific activity, 18.3 MBq μmol^{-1}) or [U-^{14}C] tyrosine (specific activity 14.8 MBq μmol^{-1}) to the main compartment of the flask. The flasks were incubated in an oscillating water bath operated at 120 strokes min^{-1}, 5 cm amplitude, at 27°C for 4 h. After the incubation period, the cells were collected on a sheet of Miracloth, washed with distilled water and frozen with liquid nitrogen. The frozen cells were homogenized in 80% ethanol and fractions that contained lignin, protein, phenolic acids, and free amino acids were prepared according to the scheme shown in Figure 2. Constituents of the phenolic acid fraction were further fractionated by thin layer chromatography on a cellulose plate with benzene-acetic acid-water (6 : 7 : 3) as the solvent system. Free and protein- amino acids were analyzed on an amino acid analyzer. The peaks of radioactive amino acids from acids from the analyzer were monitored with a radioanalyzer (Aloka, type RCL-551) and radioactive fractions were collected into vials for measurements. The radioactivity of each fraction was determined using a Packard Tri Carb liquid scintillation spectrophotometer, type 3255, as described previously [9].

Preparation of extracts for measurements of maximum catalytic activity of enzymes

The washed cells (approximately 2 g fresh weight) or seedlings (approximately 1 g fresh weight) were homogenized in 15 ml of 25 mM borate buffer (pH 8.8) which contained 5 mM 2-mercaptoethanol. The degree of disruption of cells was checked under a microscope. More than 90% of the cells or tissues were broken by this treatment. The homogenate was centrifuged at 25,000×g for 30 min at 2°C. The supernatant obtained was treated with finely ground, solid ammonium sulphate. The protein fraction precipitating at 80% saturation was collected by centrifugation and dissolved in 2.5 ml of the buffer used for extraction. The fraction was desalted on a column of Sephadex G-25 (bed volume 9 ml) which was pre-equilibrated with 25 ml of the same buffer. The eluted protein fraction (approximately 3.5 ml) was used immediately as the enzyme preparation for the assays.

Assays of enzymatic activities

The reaction mixture for the assay of phenylalanine ammonia-lyase (PAL) contained 30 mM borate buffer (pH 8.8), 5.0 mM L-phenylalanine and the enzyme preparation in a total volume of 3 ml. For the assay of

tyrosine ammonia-lyase (TAL), the reaction mixture was the same as for PAL, but L-phenylalanine was replaced by 2.7 mM L-tyrosine. The reaction was initiated by the addition of the enzyme preparation, and terminated by the addition of 0.5 ml of 2 N perchloric acid. Reactions were usually incubated for 0, 15, and 30 min, at 30°C. After termination of the reaction, the mixture was centrifuged at 25,000×g for 20 min, and absorbance of the supernatant (280 nm for PAL, 288 nM for TAL) was determined. Molecular extinction coefficients of *trans*-cinnamic acid and *p*-coumaric acid were obtained from the absorbance of known amounts of these compounds dissolved in borate buffer (pH 8.8).

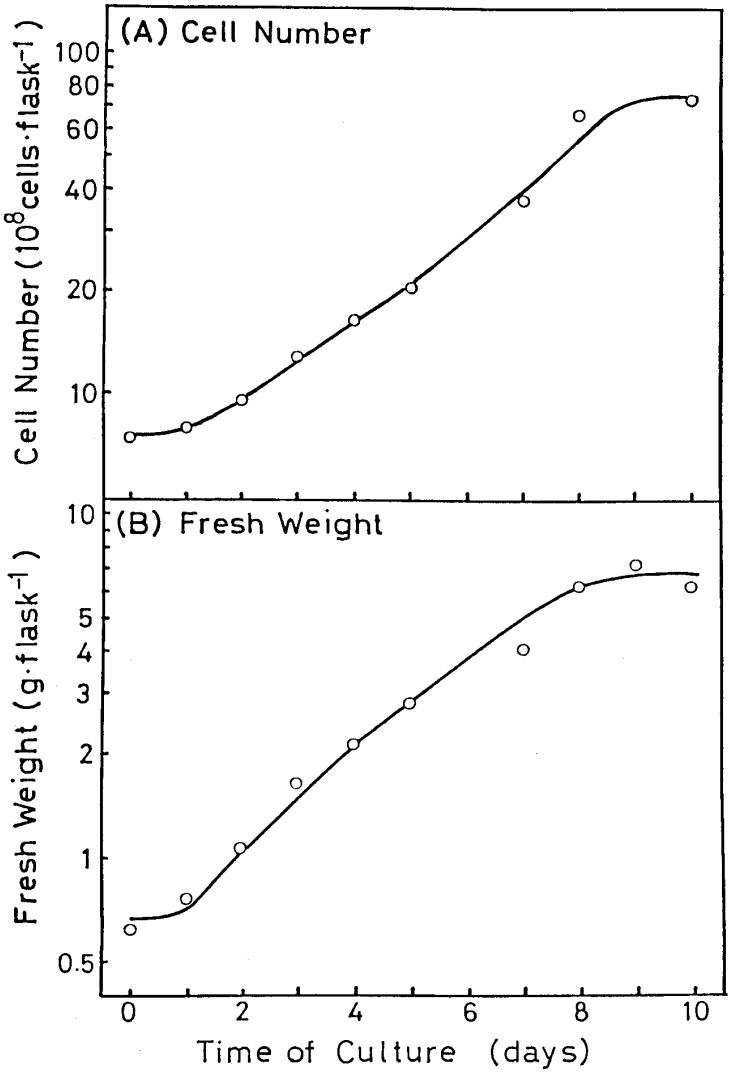

Fig. 3. Changes in numbers (A) and fresh weight (B) of cells in suspension cultures of *Catharanthus roseus*. Each point is the average of results from duplicate samples.

Results

Growth of cells in batch suspension culture

The pattern of growth of *Catharanthus roseus* cells in batch suspension culture is shown in Figure 3. Both the number of cells and fresh weight increased exponentially after a lag period of one day and the stationary phase was reached on day 8. An approximately 10-fold increase

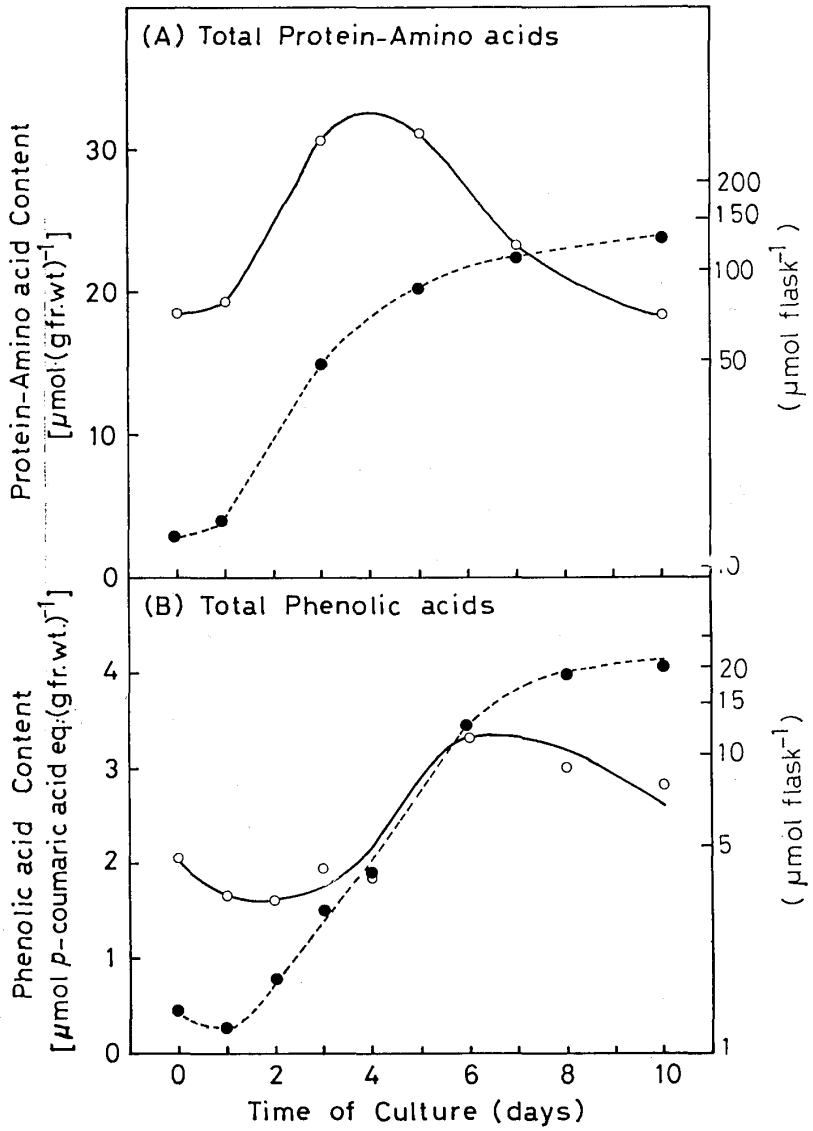

Fig. 4. Changes in levels of total protein-amino acids (A) and total phenolic acids (B) during growth of suspension cultures of *Catharanthus roseus*. The levels of amino acids and phenolic acids are expressed as μmol (g fresh weight)$^{-1}$ (○), or as μmol (flask)$^{-1}$ (●). The level of total protein-amino acids is the sum of the amounts of amino acids in protein hydrolyzates, and the level of total phenolic acids is expressed as equivalent of *p*-coumaric acid. Each point is the average of results from duplicate samples.

in cell number and fresh weight was observed in 10-day-old cultures.

Changes in levels of amino acids and phenolic acids

Changes in levels of total protein-amino acids and ethanol-soluble phenolic acids are shown in Figure 4. Although the levels of both components increased after a lag period of one day, the pattern of the changes in the levels of protein-amino acids and that of levels of phenolic acids are different. This difference is obvious when the levels are expressed per gram fresh weight: the maximum level of protein-amino acids was observed on days 2–4, while that of phenolic acids was observed on day 6.

Changes in levels of total amino acids, phenylalanine, and tyrosine in the ethanol-soluble fraction are shown in Figure 5. The level of free amino acids was high in the cells used as inoculum (day 0), dut decreased after cells were transferred into fresh medium. The levels of total amino acids and tyrosine decreased immediately after transfer of cells, but the level of phenylalanine increased slightly during the lag phase (day 0–1) of cell

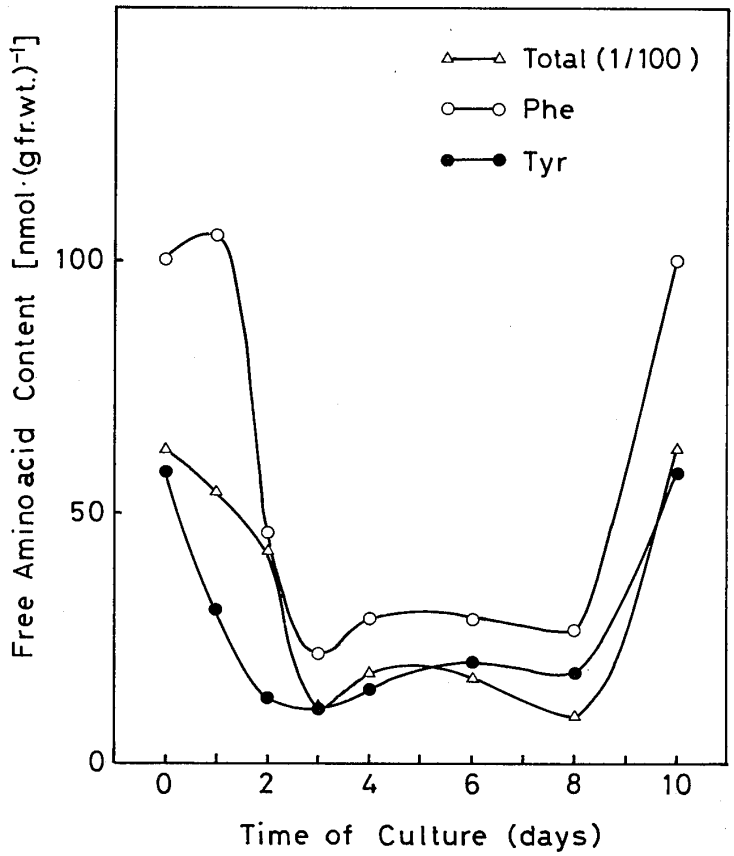

Fig. 5. Changes in levels of free amino acids during growth of suspension cultures of *Catharanthus roseus*. △, total free amino acids (the values reduced ×1/100); ○, phenylalanine; ●, tyrosine. Each points is the average of results from duplicate samples.

Table 1. Uptake and metabolism of [U-^{14}C] phenylalanine and [U-^{14}C]-tyrosine in suspension-cultured cells of *Catharanthus roseus*.

Time of culture days	[U-^{14}C] Phenylalanine			[U-^{14}C] Tyrosine		
	Uptake	Metabolized		Uptake	Metabolized	
	kBq	kBq	(%)	kBq	kBq	(%)
0	236.5	62.2	(26.3)	146.0	102.3	(70.1)
1	155.0	101.7	(65.6)	99.2	85.7	(86.4)
2	113.5	65.7	(57.9)	92.5	82.2	(88.9)
3	156.5	97.3	(62.2)	89.3	76.3	(85.4)
4	144.7	83.0	(57.4)	137.2	116.8	(85.1)
5	160.0	116.8	(73.0)	236.5	193.8	(81.9)
7	136.7	79.0	(57.8)	*	*	
8	158.5	113.3	(71.5)	261.3	208.7	(79.9)
10	139.3	39.8	(28.6)	258.5	178.0	(68.9)

* not determined

growth and then decreased. Minimum levels of free amino acids were noted on days 3–8 when the level of protein-amino acids was high. Subsequently, the level of free amino acids increased markedly again with the cessation of cell proliferation.

Uptake of [U-^{14}C] phenylalanine and [U-^{14}C] tyrosine by cultured cells

Table 1 shows the uptake of [U-^{14}C] phenylalanine and [U-^{14}C] tyrosine by cultured cells of *Catharanthus roseus* at various growth stages, during incubation for 4 h. The radioactivity in metabolized compounds was estimated from the difference between the total radioactivity taken up by the cells and the radioactivity in phenylalanine or tyrosine in the ethanol-soluble fraction. In general, the uptake of phenylalanine and tyrosine by the cells was relatively slower during exponential growth of the cells. In contrast, the proportion of the amino acids metabolized within the cells was usually higher in the exponential growth phase than in the lag or stationary phases.

Metabolism of [U-^{14}C] phenylalanine and [U-^{14}C] tyrosine in cultured cells

Figure 6 shows the incorporation of [U-^{14}C] phenylalanine and [U-^{14}C] tyrosine into the fractions that contain proteins, phenolic acids, lignin, and into CO_2 by cultured cells. More than 50% of metabolized phenylalanine and 80% of tyrosine were incorporated, as the corresponding amino acid residues, into proteins at any stage of culture. Approximately 20–50% of radioactivity from [U-^{14}C] phenylalanine was incorporated into phenolic acids and lignin. The rates of incorporation into these phenolic compounds were relatively higher on days 2–5. The radioactivity in the phenolic acid frac-

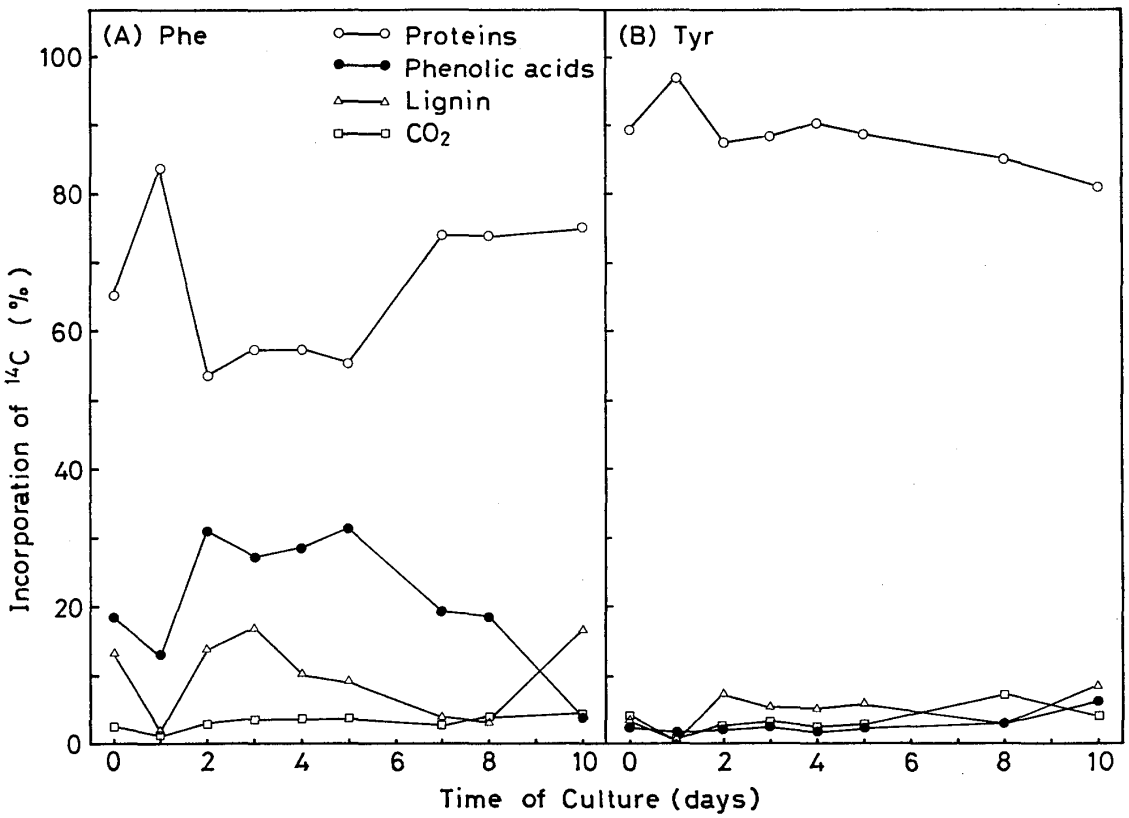

Fig. 6. Incorporation of [U-^{14}C] phenylalanine (A) and [U-^{14}C] tyrosine into proteins (○), phenolic acids (●), lignin (△) and CO_2 (□) in suspension-cultured cells of *Catharanthus roseus*. The values are expressed as % of the total radioactivity that was taken up by the cells in 4 hr. Each point is the average of results from duplicate samples.

tion was distributed in trans-cinnamic acid, *p*-coumaric acid, and in unidentified compounds retained at the origin after TLC (data not shown). [U-^{14}C] Tyrosine was also converted to phenolic acids and lignin, but the rate was very much lower than that of conversion from [U-^{14}C] phenylalanine. The rate of degradation of [U-^{14}C] phenylalanine and [U-^{14}C] tyrosine, which was indicated by the release of $^{14}CO_2$, was very low. No conversion of these amino acids into any other amino acids could be detected (data not shown).

Changes in levels of ammonia lyase of phenylalanine and tyrosine

Changes in the maximum catalytic activities of phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) are shown in Figure 7. The activity of PAL was much higher than that of TAL at any stage of the culture. The highest activity of PAL was found on day 3, when activity was expressed per gram fresh weight, and was found on days 3 and 4 when activity was expressed per mg protein. In contrast, a broad peak over time of TAL activity was found when the activity was expressed

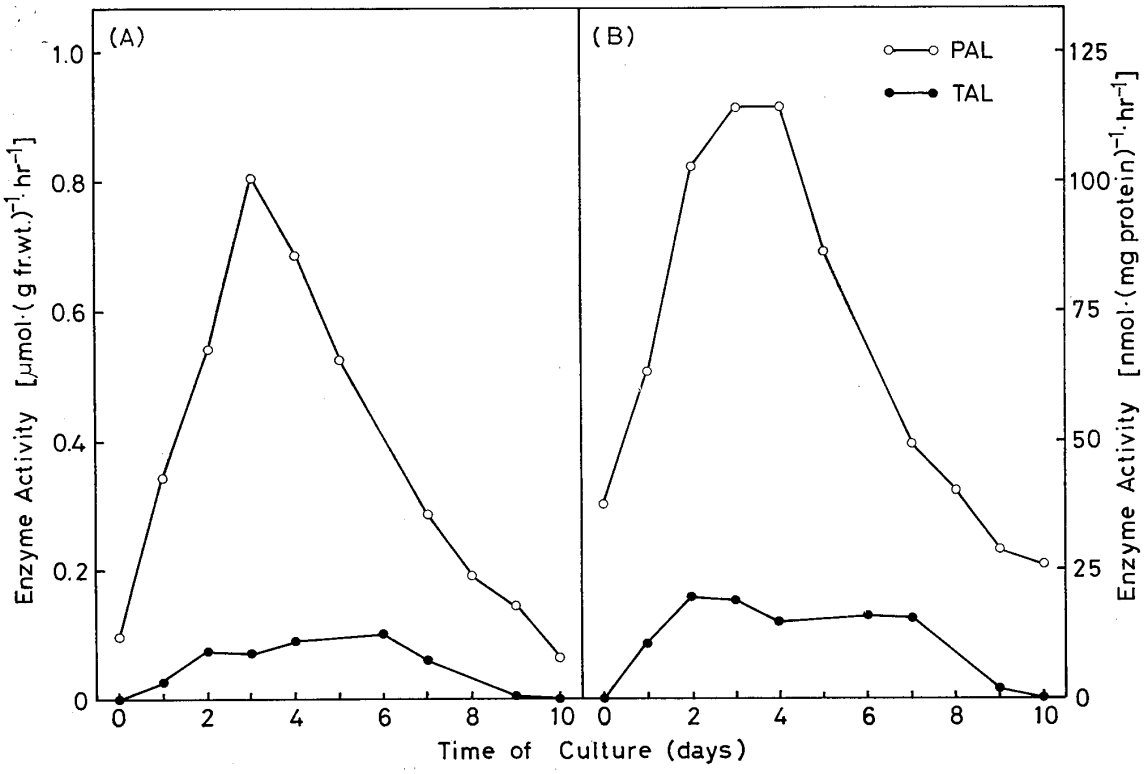

Fig. 7. Changes in the levels of activity of phenylalanine ammonia-lyase (○) and tyrosine ammonia-lyase (●) during growth of suspension cultures of *Catharanthus roseus*. The activities of the enzymes are expressed as as μmol (g fresh weight)$^{-1}$ (hr)$^{-1}$ (A) or nmol (mg protein)$^{-1}$ (hr)$^{-1}$ (B). Each point is the average of results from duplicate samples.

either per gram fresh weight or per mg protein. The minimum activity was found on the initial day (day 0) and during the stationary phase (days 9 and 10).

Metabolism of phenylalanine and tyrosine in seedlings

The metabolic fate of exogenously supplied [U-^{14}C] phenylalanine and [U-^{14}C] tyrosine was also examined in segments of 7-day-old seedlings of *Catharanthus roseus* (Fig. 8A). Approximately 70% of [U-^{14}C] phenylalanine and 95% of [U-^{14}C] tyrosine that was metabolized within the segments were incorporated into proteins. The extent of incorporation of radioactivity from [U-^{14}C] phenylalanine into phenolic acids and lignin was 6–8 times higher than that from [U-^{14}C] tyrosine. In contrast, release of $^{14}CO_2$ from [U-^{14}C] tyrosine was slightly higher than that from [U-^{14}C] phenylalanine.

The maximum catalytic activity of PAL and TAL was also determined in extracts of the seedlings. The activity of PAL was approximately 2 times higher than that of TAL (Fig. 8B).

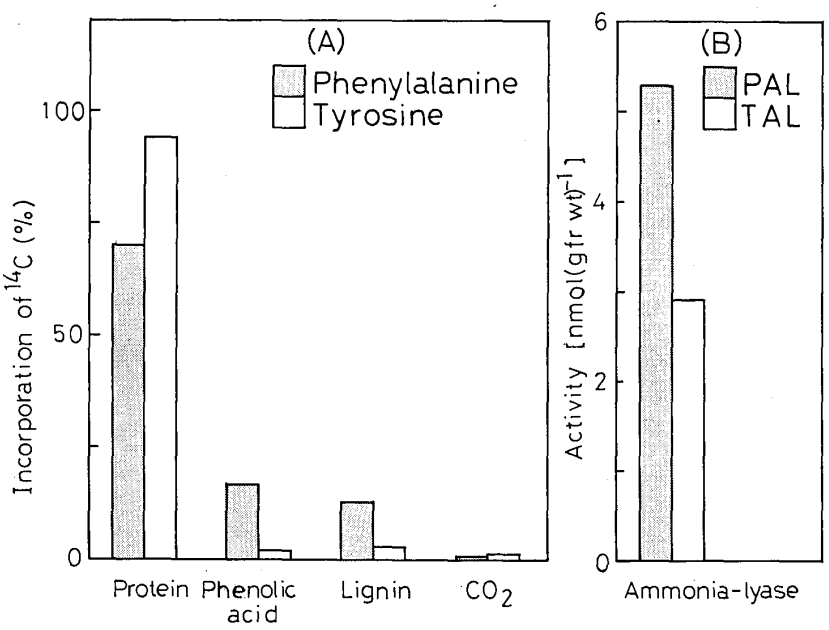

Fig. 8. Incorporation of [U-^{14}C] phenylalanine and [U-^{14}C] tyrosine into proteins, phenolic acids, lignin, and CO_2 (A) ; and levels of activity of phenylalanine ammonia-lyase and tyrosine ammonia-lyase (B) in 7-day-old seedlings of *Catharanthus roseus*. The rates of incorporation are express as % of the total radioactivity that was taken up by the segments of seedlings in 4 hr. The activities of enzymes are expressed as μmol (g fresh weight)$^{-1}$ (hr)$^{-1}$. Each value is the average of results from duplicate samples.

Discussion

The results of the present study indicate that the intracellular levels of total free amino acids, phenylalanine, and tyrosine decreased after *Catyaranthus* cells were transferred to fresh Murashige-Skoog medium. Their levels attained a minimum during the period when rapid proliferation of cells was observed. In contrast, the level of proteins was low during the initial lag phase of growth and increased with cell proliferation. These results suggest that free amino acids, which had been stored in the cells as inoculum, i. e., 10-day-old cells at stationary phase, were utilized for protein synthesis. Decrease in levels of free amino acids accompanied by the synthesis of proteins has often been observed in cultured plant cells [6, 10, 11].

More than 30% of the [U-^{14}C] phenylalanine taken up by the cells was not metabolized within the cells during the lag period (day 0) and during the stationary phase (day 10). This effect is probably the result of dilution of the labelled compounds with large amounts of endogenous phenylalanine. A similar situation was also observed when [U-^{14}C] tyrosine was

administered, but the amount of unmetabolized labelled tyrosine was much smaller. The reason for this difference is unclear, but it may be due to the difference in membrane transport systems for these two amino acids.

Most of radioactivity from [U-^{14}C] phenylalanine and [U-^{14}C] tyrosine was found in the protein fraction. Thus, the bulk of these aromatic amino acids seems to be utilized as building blocks for protein synthesis in the cells.

The levels of total phenolic acids decreased transiently after transfer of cells to fresh medium and then increased at the late exponential growth phase. Similar observations have been reported in cell cultures of Paul's Scarlet rose [12]. In *Catharanthus* cells, only 15% of the radioactivity from [U-^{14}C] phenylalanine was incorporated into phenolic acids and lignin by cells in the lag phase (day 1), but in cells from the subsequent exponential growth phase, more than 40% of the [U-^{14}C] phenylalanine that was metabolized in the cells was incorporated into these phenolic compounds. Similar findings have been reported in suspension cultures of carrot cells: the rate of incorporation of [U-^{14}C] phenylalanine into caffeic, ferulic and *p*-hydroxybenzoic acids was higher in cells in the early logarithmic phase than at any other phase and rapidly declined as cells reached the stationary phase [13].

Compared with phenylalanine, a smaller amount of ^{14}C from [U-^{14}C] tyrosine was incoporated into phenolic compounds. This finding seems to result from the fact that the level of activity of PAL is much higher than that of TAL in these cells as has been shown in many other plant species [4]. The level of activity of PAL was high during the exponential phase of growth. The peak of its activity coincided with the peak in levels of phenolic compounds, as well as the high rate of incorporation of radioactivity from [U-^{14}C] phenylalanine into phenolic acids and lignin. Therefore, the reaction catalyzed by PAL seems to be a major route for the synthesis of secondary metabolites in *Catharanthus roseus* cells. The role of PAL in the formation of phenolic acids and lignin in higher plants has been proposed by several investigators [15-18]. In cell cultures of carrot, Sugano et al. [15] suggested that the increase in the level of phenolic acids during the linear phase of growth may be due to enhancement of the activity of PAL and *o*-methyl transferase at this phase. Futhermore, Yoshida and Shimokoriyama [16] reported that nearly 80% of PAL activity in the buckwheat plant was distributed in the regions where secondary tissues had already developed. Since phenylalanine is a substrate for phenylalanyl-tRNA synthetase as well as for PAL, the rate of protein synthesis may exert a strong influence on the rate of synthesis of the secondary metabolites. Margna [18] has argued that accumulation

of phenylpropanoid in plant cells is controlled by the supply of phenylalanine.

Small but significant amounts of $^{14}CO_2$ were released from [U-^{14}C] tyrosine during every stage of culture. In contrast to what happens in microorganisms, the rate of degradation of these aromatic amino acids is generally low in higher plants, as shown in seedlings of *Pisum sativium* [19] and tissue cultures of several plant species, including *Ruta graveolens* and *Melilotus alba* [20, 21].

Metabolism of phenylanine and tyrosine in seedlings of *Catharanthus roseus* was similar to that in cultured cells. In several culture systems, the rate of biosynthesis of secondary compounds fell to zero or almost zero in cultured cells. However, our present results suggest that our cultures possess similar activities to those found in intact plants.

This study has outlined the uptake and metabolism of aromatic amino acids during the growth of plant cells in suspension culture. These data provide the basic information for further detailed investigations of primary and secondary metabolism in this culture system.

Acknowledgements

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