Changes in Levels and Rates of Biosynthesis of Amino Acids during the Growth of *Catharanthus roseus* Cells in Batch Suspension Culture

Miyuki Saito[1], Tetsuji Tokoro[2] and Hiroshi Ashihara[3]

Department of Biology [1,3] and Radioisotope Laboratory[2],
Faculty of Science, Ochanomizu University,
2-1-1, Otsuka, Bunkyo-ku, Tokyo, 112, Japan

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Summary

Changes in levels of individual amino acids and rates of the biosynthesis of these amino acids from sucrose were determined at various stages of growth of *Catharanthus roseus* in suspension culture. The levels of most of the free amino acids decreased gradually after the cells were transferred to fresh culture medium, while levels of some amino acids, i.e. glutamine, glutamic acid, aspertic acid and phenylalanine increased transiently during the lag or early logarithmic phase of cell growth. The levels of most amino acids increased again at stationary phase. In contrast, levels of protein amino acids increased during cell proliferation and decreased during stationary phase. The labelled carbon atoms from [U-^{14}C] sucrose were incorporated into glutamic acid, glutamine, glycine, phenylalaine, serine, arginine, aspartic acid, threonine, valine, isoleucine, leucine and lysine. Except for its incorporation into some free amino acids, such as glutamic acid and glutamine, most of the radioactivity was found in the protein fraction. The rate of biosynthesis of most amino acids increased during cell proliferation and decreased during the stationary phase.

Introduction

The suspension culture system is useful for studies of the correlation between metabolism and cell growth of higher plants. Amino acids are precursors for the biosynthesis of proteins, nucleotides, phenolic compounds and alkaloids as well as respiratory substrates, and their levels in the cell seem to fluctulate during the growth cycle.

It is important to know the sizes of the pools of individual amino acids, because some of them may be limiting factors in the biosynthesis of primary and secondary compounds in higher plants. Although there are several reports

[1] Present address, Yokohama Futaba High School, 88 Yamate-cho, Naka-ku, Yokohama, 231, Japan.
[3] To whom correspondence should be addressed.

on the amino acid composition of cultured plant tissues (Krikorian and Steward, 1969; Gamborg and Finlayson, 1969), only a little information is available on fluctuation of amino acid levels during the growth cycle of plant cells in suspension culture (Koiwai et al., 1971; Durzan and Chalupa, 1976; Bright et al., 1979; Sakano, 1981).

In this work, we have determined the levels of free and protein amino acids during growth of the suspension-cultured cells of *Catharanthus roseus*. Furthermore, the rate of biosynthesis of some amino acids was estimated by the incorporation of [U-^{14}C] sucrose into the free and protein amino acids.

Materials and Methods

Plant materials

Stock suspension cultures of *Catharanthus roseus* (L.) G. Don [= *Vinca rosea* L.] cells (strain MN-1) were subcultured every 7 days in 50 ml of Murashige-Skoog basal medium (Murashige and Skoog, 1962) that contained 2.2 μM 2, 4-D and 87.6 mM sucrose in 300 ml Erlenmeyer flasks. Cultures were maintained in the dark at 27°C on a horizontal rotary shaker (90 strokes·min^{-1} with 8 cm amplitude). Cell numbers and fresh weight were determined as described in a previous paper (Ukaji and Ashihara, 1986).

Experimental cultures were initiated from 10-day-old stock cultures. The original suspension (7 ml, approximately 7×10^8 cells) was transferred into 43 ml of fresh medium in a 300 ml-flask and maintained as described above.

Extraction of amino acids

The cultured cells were harvested by filtration through Miracloth (Calbiochem-Behring Corp. Ltd., La Jolla) and washed with distilled water. A portion of the cells (200 mg fresh weight) was homogenized in 80% ethanol using glass homozenizer. Soluble amino acids were extracted from the homogenate by boiling three times for 10 min at 70°C with the ethanol. After centrifugation, a known amount of carnosine was added to the supernatant as an internal standard for determination of recovery of free amino acids. The supernatant was evaporated to dryness, taken up in 2 ml of distilled water, washed with diethyl-ether, and evaporated again to dryness. The residue was dissolved in 0.25 ml of 0.1 N HCl, centrifuged at 20000 g for 20 min, and the resultant supernatant was used as the free amino acid fraction.

The ethanol-insoluble residue was hydrolyzed in 3 ml of 6 N HCl at 120°C for 16 hr, in a sealed screw cap tube, after a known amount of aminoadipic acid had been added as an internal standard for the recovery. The hydrolyzate was centrifuged at 2000 g for 10 min and the supernatant was evaporated to dryness. The residue was taken up in 0.25 ml of 0.1 N HCl. This fraction, which contained the amino acids that had been incorporated into protein, was recentrifuged at 20000 g for 10 min before amino acid analysis. The flow sheet is shown in Fig. 1.

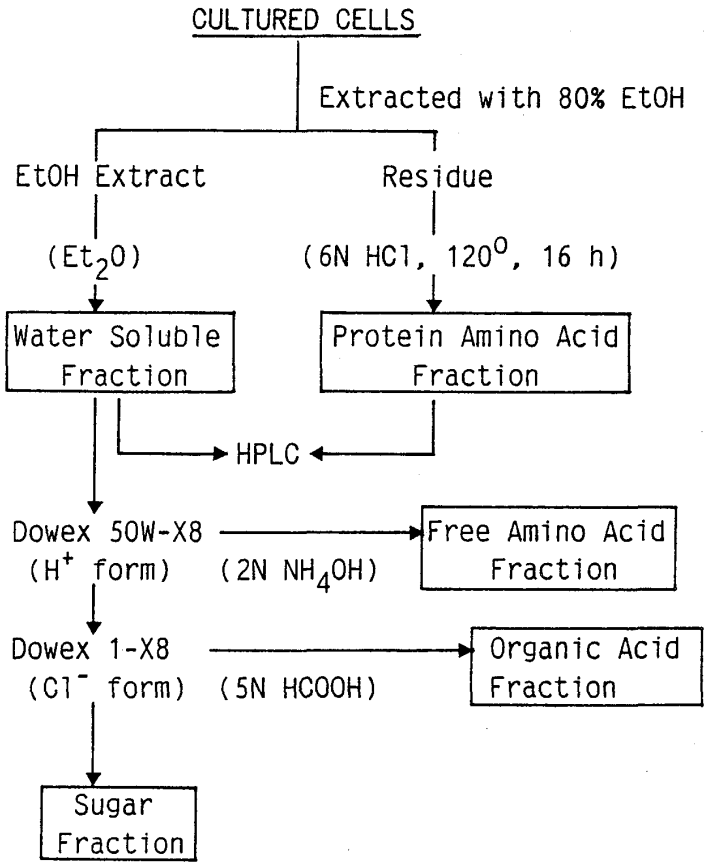

Fig. 1. Flow sheet of the separation of ^{14}C-labelled metabolites.

Analysis of amino acids

Amino acids were analyzed with a fluorometric amino acid analyzer, which consisted of a Shimadzu HPLC System type LC-4A connected with Shimpack ISC-07 (Li^+ type) and ammonia trap column (S-1504), a Shimadzu fluometric monitor (type 530) and a recorder (Shimazu Chromato-Pack, type C-R1B). For determination of radioactivity, an Aloka radioanalyzer (type RCL-551) was also connected. Three different solvents: 0.15 N lithium citrate, pH 2.65, which contained 7% methylcelosolve; 0.3 N lithium citrate, pH 10.0; and 0.2 N lithium hydroxide were used for the mobile phase. The detailed analytical conditions were as described in the manual for the Shimadzu fluorometric amino acid analysis system. The total running time was 180 min. The recovery of amino acids was corrected from measurements of the internal standard.

Metabolism of [^{14}C] sucrose

Washed cultured cells (200 mg fresh weight) were maintained in 2.0 ml Murashige-Skoog medium that included 2.2 μM 2, 4-D and 1 mM [U-^{14}C] sucrose (46.6 kBq, specific activity 23.3 kBq·μmol^{-1}) in a 30 ml Erlenmeyer flask fitted with a centre well that contained 0.2 ml of 20% KOH. The flasks were incubated in an oscillating water bath, 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. The soluble amino acids together with protein hydrolyzates were prepared as described above (Fig. 1). Aliquots of the water soluble fraction were fractionated into sugars, amino acids and organic acids on two types of ion-exchange resins, Dowex 50W-X8 (H^+ form) and Dowex 1-X8 (Cl^- form). Free amino acids were eluted with 15 ml of 2N NH_4OH from the Dowex 50W-X8 column, evaporated to dryness and the residue was taken up small amount of 0.1N HCl. Free and protein amino acids were analyzed using the amino acid analysis system described above. The peaks of radioactive amino acids from the amino acid analyzer were monitored with the radioanalyzer; radioactive fractions were collected into vials for measurements with the scintillation spectrophotometer.

Organic acids, eluted from the Dowex 1-X8 column with 15 ml 5N HCOOH, were also dried and dissolved in a small amount of distilled water and separated by thin layer chromatography on a cellulose plate with a solvent system of phenol: water: formic acid (75: 25: 1).

The radioactivity in CO_2, amino acids, organic acids and sugars was determined using a Packard Tri Carb liquid scintillation spectrophotometer, type 3255, as described previously (Nobusawa and Ashihara, 1983).

Results

Changes in cell number and fresh weight during growth of *Catharanthus roseus* cells are shown in Fig. 2. The rapid proliferation of cells was apparent after a lag period of 2 days and the stationary phase was reached on day 8.

Fig. 3 shows the level of free amino acids. The major free amino acids in *Catharanthus* cells were glycine, glutamine, serine, asparagine and phenylalanine. In contrast, pools of methionine, tyrosine, histidine and alanine were small throughout the culture period. In addition to the amino acids shown in Fig. 3, small or trace amounts of amino acids that do not become incorporated into proteins were detected in *Catharanthus* cells, namely: phosphoserine (the level changed from 0.04–0.05 μmol·g fresh weight^{-1} during culture); taurine (0.01–0.05 μmol); aminoadipic acid (0–0.01 μmol); citrulline (0–0.07 μmol); and γ-aminobutyric acid (0.05–0.26 μmol). Ornithine, an intermediate in the biosynthesis of arginine, could not be detected at any stage of culture.

The level of total free amino acids decreased after the cells were transferred to fresh culture medium; the level attained a minimum at day 7; but was increased by day 10 (Fig. 3a). The levels of most of the free amino acids fluctuated in a similar manner, but the levels of some amino acids, including glutamine, glutamic acid, aspartic acid and phenylalanine, increased during the lag (Fig. 3f, g and o) or the early logarithmic phase (Fig. 3b) of cell growth.

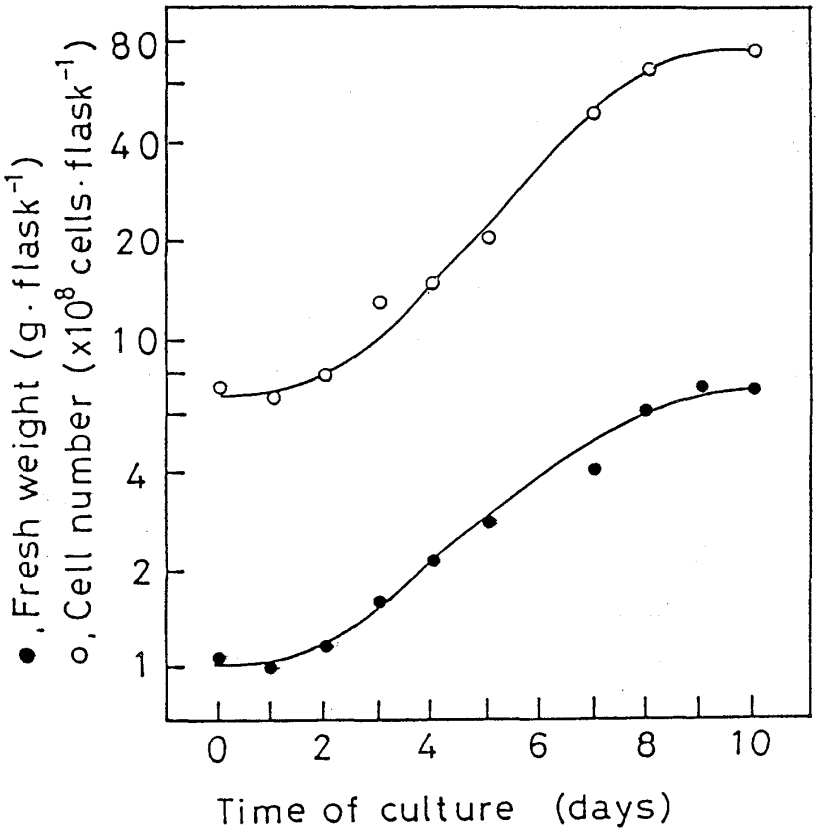

Fig. 2. Changes in fresh weight (●) and cell number (○) of the suspension culture of *Catharanthus roseus*. Each point is the average of duplicate samples.

The levels of amino acids in protein hydrolyzates increased in tandem with cell proliferation and decreased when the cells entered stationary phase (Fig. 4). A similar trend was observed for almost all the amino acids in protein, but the levels of proline, methionine, tyrosine and histidine were low through the culture period (Fig. 4).

Incorporation of [U-^{14}C] sucrose into CO_2, the amino acid and organic acid fractions is shown in Fig. 5. Although most of the radioactivity was released as $^{14}CO_2$, a significant amount of ^{14}C was incorporated into amino acids and organic acids. The peak of radioactivity in $^{14}CO_2$ was observed on day 1, during the lag phase of cell growth, while the peak of amino acids appeared on day 3, where amino acids incorporated into protein reached their highest level (Fig. 4a and Fig. 5). Incorporation of ^{14}C into organic acids was greater in the early phase (day 0–3) than in the later phase (day 5–10). The organic acid fraction obtained from 3-day-old cells was chromatographed, as described in Materials and Methods. The majority of the radioactivity was located in malic acid and citric acid, which contained approximately 60% and 30% of the radioactivity in this fraction, respectively.

Incorporation of [U-^{14}C] sucrose into the sugar fraction was 0.46, 0.38, 0.50,

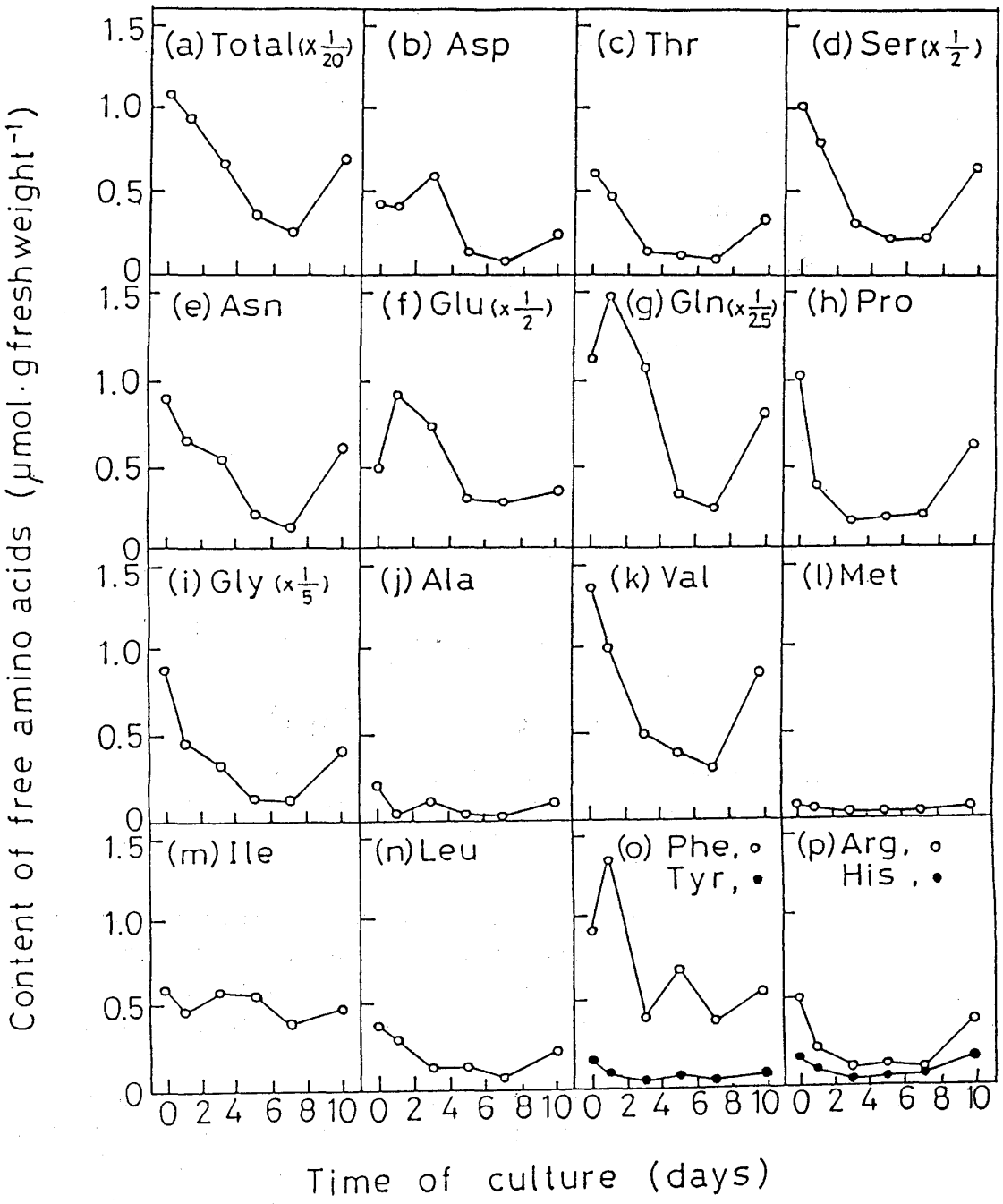

Fig. 3. Changes in levels of free amino acid levels during growth of the suspension culture of *Catharanthus roseus*. The content of amino acids are expressed as μmoles·(g fresh weight)$^{-1}$. Each point is the average of duplicate samples. (a) Total free amino acids (the values reduced×1/20); (b) aspartic acid; (c) threonine; (d) serine (reduced×1/2); (e) asparagine; (f) glutamic acid (reduced×1/2); (g) glutamine (reduced×1/2.5); (h) proline; (i) glycine (reduced×1/5); (j) alanine; (k) valine; (l) methionine; (m) isoleucine; (n) leucine; (o) phenylalanine (○) and tyrosine (●); (p) arginine (○) and histidine (●).

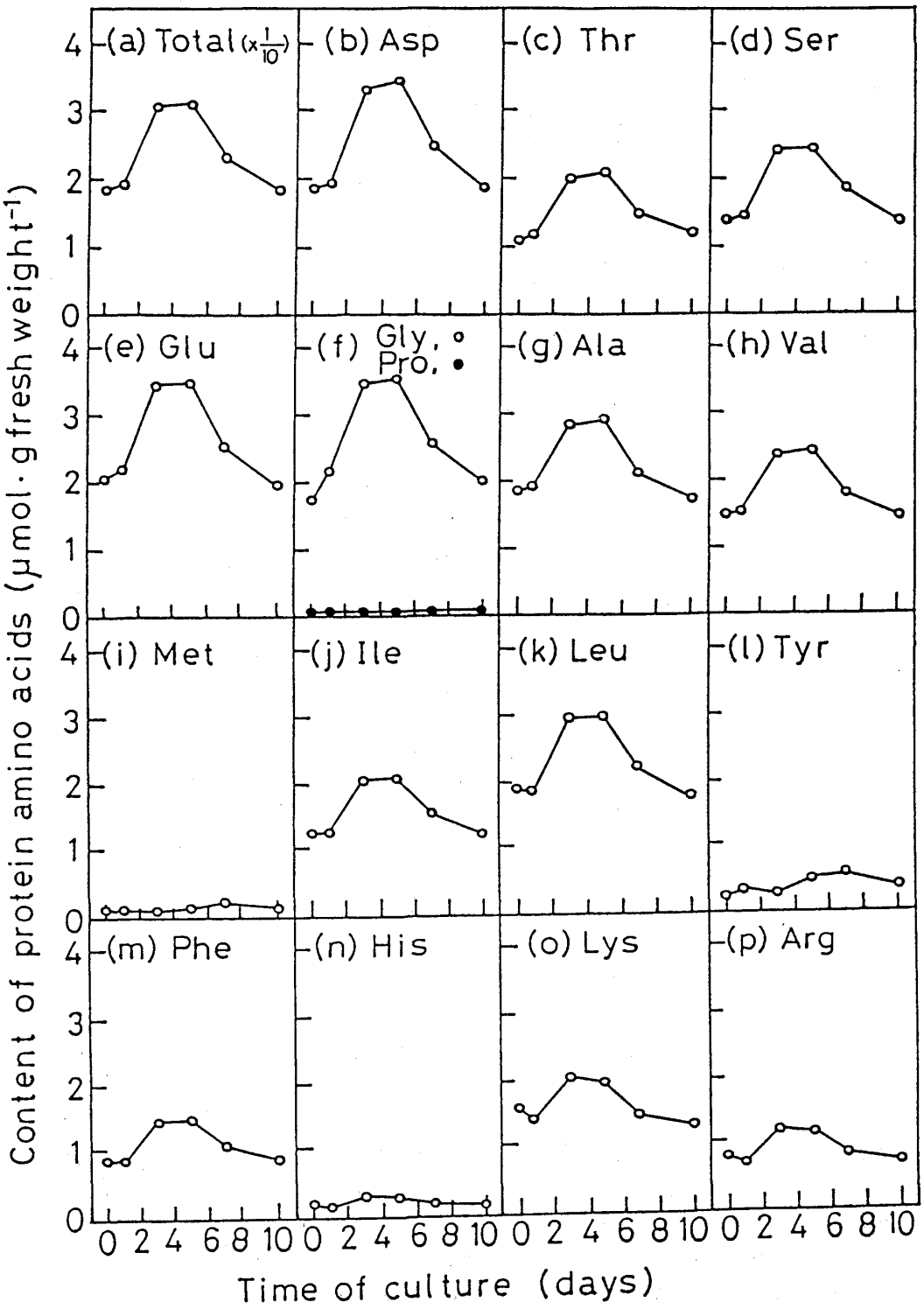

Fig. 4. Changes in levels of protein amino acids during growth of the suspension culture of *Catharanthus roseus*. The content of amino acids is expressed as μmoles·(g fresh weight)$^{-1}$. Each point is the average of duplicate samples. (a) Total free amino acids (the values reduced×1/10); (b) aspartic acid; (c) threonine; (d) serine; (e) glutamic acid (reduced×1/2); (f) glycine (○) and proline (●); (g) alanine; (h) valine; (i) methionine; (j) isoleucine; (k) leucine; (l) tyrosine; (m) phenylalanine; (n) histidine; (o) lysine and (p) arginine.

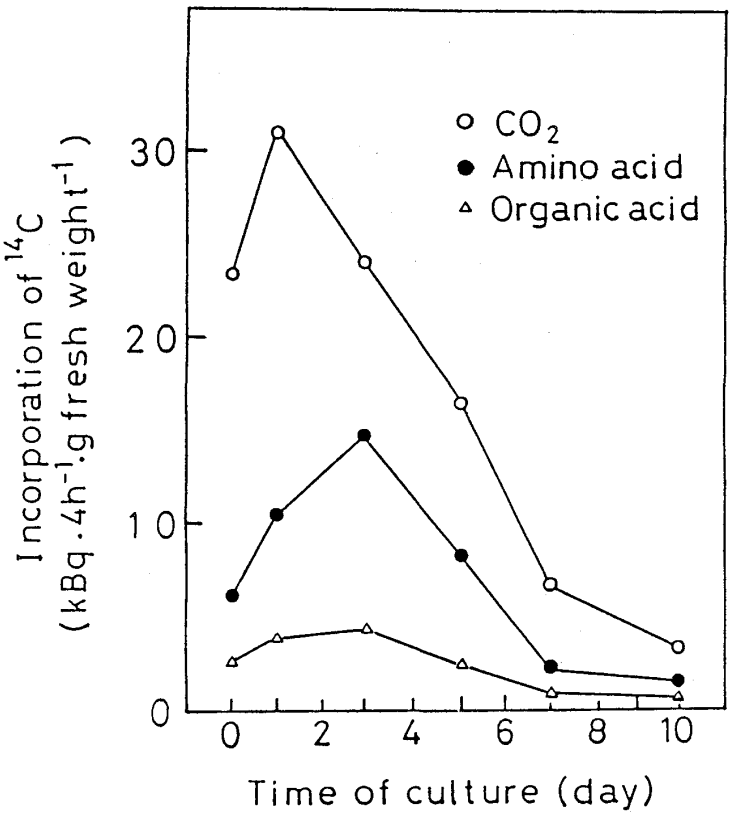

Fig. 5. Incorporation of ^{14}C atoms from [U-^{14}C] sucrose into CO_2 (○), amino acid (free plus incorporated into protein) (●), and organic acid (△) fractions of *Catharanthus roseus* cells. The values are expressed as incorporation of ^{14}C [KBq·(4 hr)$^{-1}$·(g fresh weight)$^{-1}$] Each point is the average of duplicate samples.

1.00, 0.35 and 0.42 μmol·g fresh weight^{-1} in 0-, 1-, 3-, 5-, 7- and 10-day-old cells, respectively.

Incorporation of radioactivity into individual amino acids is shown in Fig. 6. More than 30% of the radioactivity that appeared in the total amino acid fraction could be recovered in a glutamine plus glutamic acid fraction at any stage of the culture. Significant radioactivity was also detected in glycine plus alanine (most of the radioactivity was distributed in glycine), phenylalanine, serine, arginine, aspartic acid, threonine, valine, isoleucine, leucine and lysine. Possible synthetic routes of these amino acids from [U-^{14}C] sucrose are shown schematically in Fig. 7. Only a limited numbers of pools of free amino acid was labelled by [U-^{14}C] sucrose. These pools contained glutamine, glutamic acid, phenylalanine, serine and aspartic acid (Fig. 6). In contrast, less than 5% of the total radioactivity found in threonine, valine, isoleucine, leucine, lysine and arginine was found in the pools of free amino acids (Fig. 6). This results indicate that amino acids synthesized from sucrose are channelled through small and rapidly turned over pools, before being incorporated in protein.

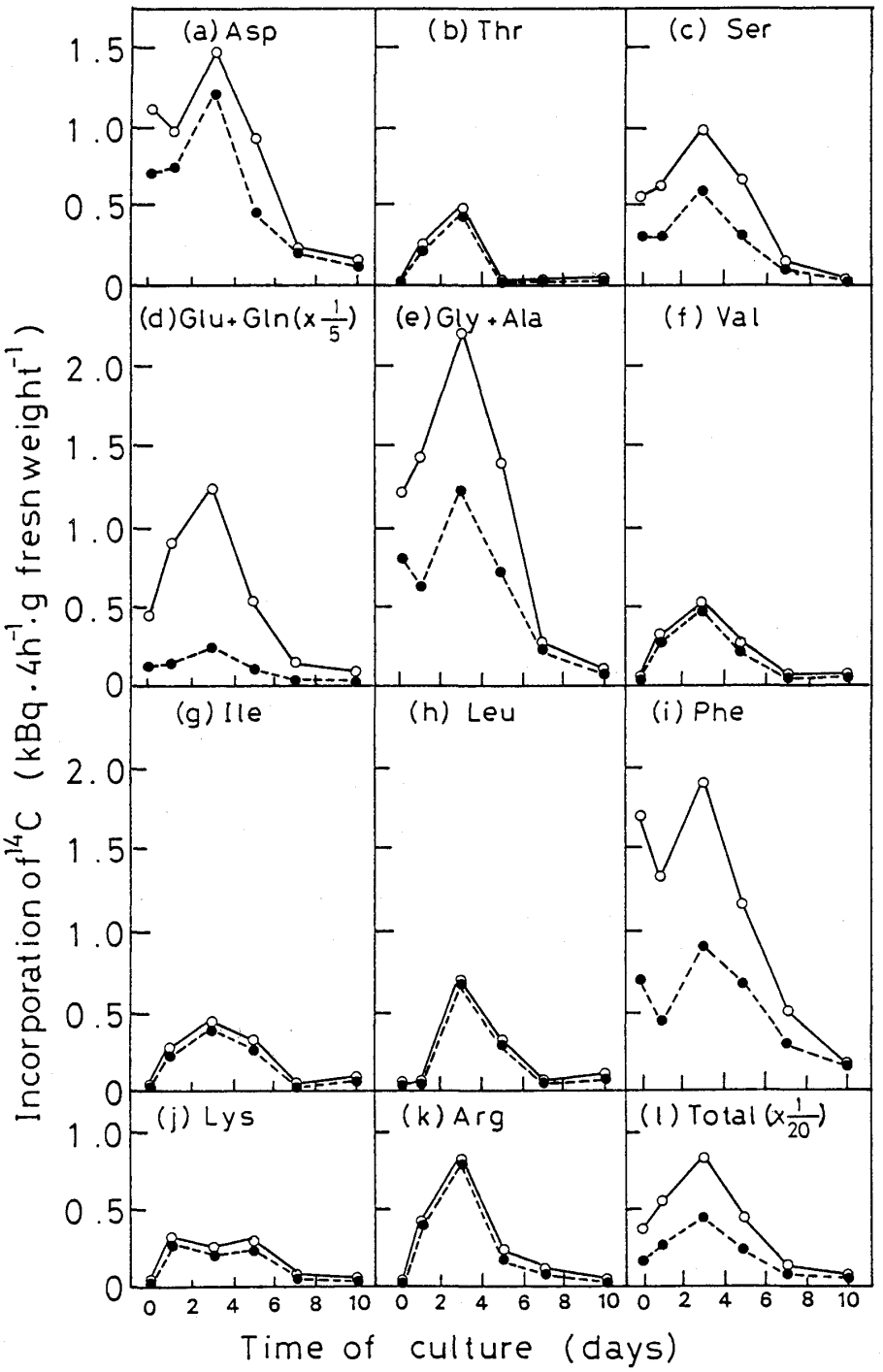

Fig. 6. Incorporation of [U-^{14}C] sucrose into (a) aspartic acid; (b) threonine; (c) serine; (d) glutamic acid plus glutamine (reduced×1/5); (e) glycine plus alanine; (f) valine; (g) isoleucine; (h) leucine; (i), phenylalanine; (j) lysine; and (k) arginine. Incorporation into total amino acids is also shown in (l) at reduced scale (×1/20). The values are incorporation of ^{14}C (Bq) into into individual amino acids in the protein-(●, broken lines) and the total (i.e., ethanol soluble plus protein) fractions (○, solid lines)·(4 hr)$^{-1}$·(g fresh weight)$^{-1}$.

Incorporation of [U-^{14}C] sucrose into almost all amino acids increased in tandem with cell proliferation and decreased during the stationary phase of growth (Fig. 6).

Discussion

The results obtained here show that pronounced changes in the levels of free and protein amino acids occur during culture of *Catharanthus roseus* cells. The level of total free amino acids decreased just after the cells were transferred to fresh medium and reached a minimum when rapid cell proliferation was observed, while the level of amino acids in protein was low during the lag phase of growth and increased during the logarithmic phase. This result indicates that free amino acids, which are stored in the inoculant cells, are utilized for protein synthesis during growth. This conclusion is in agreement with the concept, proposed by Steward et al. (1958), that the ratio of "alcohol-soluble" to "alcohol-insoluble nitrogen" is much greater in resting than in proliferating plant tissues.

Levels of free amino acids increased again at the stationary phase of growth of the *Catharanthus* cells, when their protein content decreased (Fig. 3). Although the increase was not so remarkable on day 10 of the cultures described here, more significant increases were observed in some other batches of cells (Ukaji and Ashihara, unpublished results; Nagaoka and Ashihara, 1988). This increase seems to be due in part to the degradation of proteins. The extent of the increase in the levels of free amino acid was variable in each culture. However, it is noteworthy that the level increased gradually in each case, until the cells were autolysed. A similar trend in changing patterns of free and protein amino acids has been reported by Sakano (1981), although he described only a limited variety of amino acids. In tobacco cells, levels of some amino acids changed during the growth cycle, but the degree of fluctuation was not as large as that seen in *Catharanthus* cells. The maximum level of free and protein amino acids was only 1.7 and 1.8 times respectively the minimum level for each amino acid (Koiwai et al., 1971). In contrast to our results, no correlation between levels of free and protein amino acids was reported by Bright et al. (1979), who found that the levels of both free and protein amino acids decreased during the exponential phase of growth of carrot cells. The discrepancy between the trends of changes in levels of amino acids during growth of cell cultures, may be due to the use of different plant species. However, culture conditions may also affect the difference; the cells in our batch suspention cultures are almost synchronised, in part as a result of the concentration of inorganic phosphate in the medium (Ashihara and Tokoro, 1985; Ukaji and Ashihara, 1986).

Results of tracer experiments with [U-^{14}C] sucrose suggest that active synthesis of amino acids occurs during the early exponential phase of cell growth where protein synthesis is activated. At least 12 amino acids were synthesized from [U-^{14}C] sucrose during 4 hr-incubation. Among them, glutamine, glutamic

acid, glycine, phenylalanine, aspartic acid and serine were heavily labelled with ^{14}C atoms from [U-^{14}C] sucrose (Fig. 6 and 7). Increases in free amino acid pools of glutamine, glutamic acid, aspartic acid and phenylalanine, observed in the early phase of cell growth (Fig. 3), seem to reflect a higher rate of biosynthesis of these amino acids.

The marked changes in levels of free and protein amino acids during the cell growth of *Catharanthus roseus*, as described above, provide us with a suitable experimental system for the study of the regulatory mechanisms of amino acid metabolism in plant cells. Further detailed studies on the control of the biosynthesis of individual amino acids, using this culture system, have been initiated

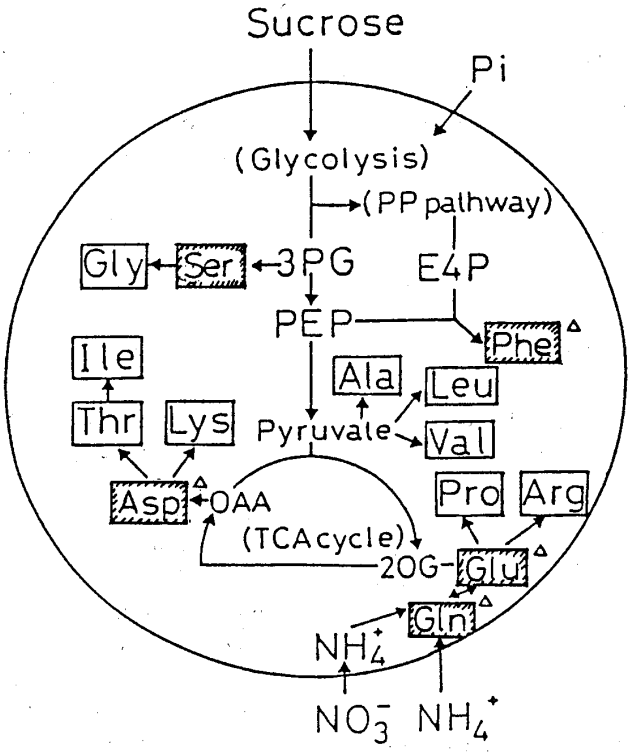

Fig. 7. Possible biosynthetic routes of amino acids from [U-^{14}C] sucrose in cultured *Catharanthus roseus* cells. Radioactivity was appeared in amino acids as indicated. Amino acids in which most of the ^{14}C appeared in the protein fraction are enclosed in rectangles, and amino acids in which ^{14}C was distrubuted in both the ethanol soluble and protein fractions are in hatched rectangles. △, amino acids whose levels were increased during the early phase of cell growth, E4P, erythrose-4-phosphate; OAA, oxaloacetate; 20G, 2-oxoglutarate; PEP, phosphoenolpyruvate; 3PG, 3-peosphoglycerate; Pi, inorganic phosphate; PP pathway, pentose phosphate pathway; TCA cycle, tricarboxylic acid cycle. Abbreviations of amino acids were indicated in legend for Fig. 3.

in our laboratory.

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References

Ashihara, H. and T. Tokoro: Metabolic fate of inorganic phosphate absorbed by suspension cultured cells of *Catharanthus roseus*. J. Plant Physiol. **118**, 227-235 (1985).

Bright, S.W.J., M.M. Leggatt and B.J. Miflin: Amino acid levels in carrot cell suspension culture: No correlation with aspartate kinase isoenzyme levels. Plant Physiol. **63**, 586-588 (1979).

Durzan, D.J. and V. Chalupa: Growth and metabolism of cell and tissue of jack pine (*Pinus banksiana*) 6. Free nitrogenous compounds in cell suspension cultures of jack pine as affected by light and darkness. Can. J. Bot. **54**, 496-506 (1976).

Gamborg, O.L. and A.J. Finlayson: The amino acid composition of TCA-precipitated proteins and total residues of plant cells grown in suspension culture. Can. J. Bot. **47**, 1857-1863 (1969).

Koiwai, A., M. Noguchi and E. Tamaki: Changes in the amino acid composition of tobacco cells in suspension culture. Phytochemistry, **10**, 561-566 (1971).

Krikorian, A.D. and F.C. Steward: Biosynthetic potentialities of tissue. In: Plant Physiology, A Treatise, Vol. VB, pp. 227-326, Academic Press, New York, 1969.

Murashige, T. and F. Skoog: A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15**, 473-497 (1962).

Nagaoka, N. and H. Ashihara: Metabolism of aromatic amino acids during the growth cycle of batch suspension cultures of *Catharanthus roseus*. Nat. Sci. Rep. Ochanomizu Univ. **39**, 1-14 (1988).

Nobusawa, E. and H. Ashihara: Purine metabolism in cotyledons and embryonic axes of black gram (*Phaseolus mungo*) seedlings. Int. J. Biochem. **15**, 1059-1065 (1983).

Sakano, K.: Regulation of aspartokinase isoenzyme levels in cultured cells of *Vinca rosea*. Plant Cell Physiol. **22**, 1343-1353 (1981).

Steward, F.C., J.F. Thompson and J.K. Pollard: Contrasts in the nitrogenous composition of rapidly growing and non-growing plant tissues. J. Exp. Bot. **9**, 1-10 (1959).

Ukaji, T. and H. Ashihara: Changes in levels of cellular constituents in suspension culture of *Catharanthus roseus* associated with inorganic phosphate depletion. Z. Naturforsch. **41c**, 1045-1051 (1986).