Changes in Levels of Cellular Constituents in Suspension Culture of *Catharanthus roseus* Associated with Inorganic Phosphate Depletion*

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Changes in growth and the level of various cellular constituents were monitored for 96 h after transfer of stationary phase cells of *Catharanthus roseus* to fresh complete ("+Pi") or phosphate deficient ("−Pi") Murashige-Skoog medium. In the cultures transferred to "+Pi" medium, cell number and fresh weight increased rapidly after an initial lag period, while little or no increase in cell number or fresh weight was observed in cultures transferred to "−Pi" medium. The levels of ATP, glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), RNA and protein, increased in the cultures in "+Pi" medium, while the levels of free amino acids decreased. In contrast, we found a decrease in the levels of ATP, G6P and F6P and an increase in the levels of free amino acids in the cultures in "−Pi" medium. Appreciable DNA synthesis was observed only in cells growing in "+Pi" medium, at 72 h after cell transfer. The rate of RNA synthesis in the "+Pi" culture was generally higher than that in the "−Pi" culture. The levels of ethanol soluble-phenolic compounds increased transiently in the cells grown in both media just after cell transfer, but the level in the "+Pi" culture decreased progressively with cell division.

The metabolic role of inorganic phosphate in metabolism of *Catharanthus roseus* cells is discussed.

Introduction

The suspension cell culture is a useful system for studying the role of nutrients in the metabolism and growth of plant cells, since the metabolism of cultured plant cells is highly dependent on the chemically defined composition of the culture medium.

Recently, it has been claimed that inorganic phosphate (Pi) influences significantly the growth and metabolism of cultured plant cells. MacCarthy *et al.* [1] reported that increments in cell numbers of *Catharanthus roseus* were related to the level of Pi in the medium. Furthermore, Amino *et al.* [2] argued that the factor that limits cell division of *Catharanthus* cells is the level of Pi in the medium. They postulate that Pi might be essential for the nucleic acid metabolism that is required for progression of the cell cycle. On the other hand, Knobloch and Berlin [3] observed rapid accumulation of secondary products such as tryptamine, indole alkaloids and phenolics, after *Catharanthus* cells were transferred to medium devoid of phosphate.

Thus, Pi is a very important moiety which affects many metabolic processes and, as a result, influences the rate of cell growth and the accumulation of secondary products. Until now, only a little information has been available about the effects of Pi on the metabolism of cultured plant cells, although the limitation of photosynthesis by decreased supply of Pi to the chloroplast has recently been claimed [4].

In this investigation, we examined the changes in the level of major cellular constituents and in the synthesis of DNA and RNA, that are associated with the presence or absence of Pi from the medium in which suspensions of *Catharanthus roseus* cells are cultured.

Materials and Methods

Materials

Cellulase "Onozuka" R-10 and Macerozyme R-10 were obtained from Yakult Pharmaceutical Industry

Co. Ltd., Nishinomiya, Japan. Luciferin and luciferase were purchased from Sigma Chemical Co., St. Louis, U.S.A. Glucose-6-phosphate dehydrogenase, phosphoglucose isomerase and other biochemicals were obtained from Boehringer Corp., Mannheim, Fed. Rep. Germany. [Methyl-^{3}H]thymidine and [2-^{14}C]uridine were obtained from Centre D'Études Nucleaires de Saclay, Gif-Sur-Yvette, France. Miracloth was from Calbiochem-Behring Corp., La Jolla, U.S.A.

Cells in suspension culture

Cell suspension cultures of *Catharanthus roseus* (L.) G. Don (Strain TU-1) were maintained and subcultured every 10 days in Murashige-Skoog basal medium [5] that contained 2.2 µM 2,4-dichlorophenoxyacetic acid and 3% sucrose ("+Pi" medium). After 10 days of incubation, a portion of the cells was washed once with the same medium but without Pi ("−Pi" medium) and then transferred to the "−Pi" medium. The control cells were transferred directly to the "+Pi" medium without washing. In both cases, 5 ml of the original suspension (approximately 3.0×10^8 cells) were transferred into 45 ml of the fresh "+Pi" or "−Pi" medium in 300 ml Erlenmeyer flasks. The cells were grown at 27 °C on a horizontal rotary shaker (90 strokes·min^{-1}, with 8 cm amplitude), in the dark.

Cells were harvested and washed with distilled water by vacuum filtration through a layer of Miracloth. After the cells had been placed briefly between several sheets of filter paper to remove any remaining water, fresh weight was measured. Cell numbers were estimated using a haemocytometer after the cell clusters had been enzymatically dispersed. The dispersion mixture contained 1.5 ml of the enzyme solution which contained 0.8% cellulase "Onozuka" R-10, 0.2% Macerozyme R-10 and 1% CaCl$_2$ in 0.35 M mannitol and 500−600 mg fresh weight of cells. The mixture was incubated for 1−2 h at 27 °C in a shaker (Toyo Type TC-1 Incubator; 100 strokes·min^{-1} with 2.6 cm amplitude).

Determination of Pi, ATP and sugar phosphates

Freshly harvested cells (approx. 600 mg for estimation of Pi, 300 mg for ATP and 1 g for sugar phosphates) were frozen with liquid nitrogen and stored at −80 °C until needed. The frozen cells were homogenized with a Potter-Elvehjum homogenizer

in ice cold 6% perchloric acid (PCA) for 30 sec. The degree of cell disruption was checked under a microscope. More than 90% of the cells were broken during this treatment. The homogenate was centrifuged at $20,000 \times g$ for 20 min. The supernatant was decanted and stored, and the precipitate was extracted again with 6% PCA. After recentrifugation, the second supernatant was combined with the first one and the pH was adjusted to between 6 and 7 with 3 N KOH to remove potassium perchlorate. The neutralized extract was centrifuged as before and the supernatant was used for the assay.

The Pi content was determined colorimetrically by the method of Fiske and Subbarow [6].

The ATP level was examined luminometrically with a Packard Model 6100 Picolite Luminometer as described in an earlier paper [7]. The reaction mixture contained 20 µl of the extract of plant cells and 80 µl of a luciferin and luciferase preparation (pH 7.4) that contained 50 mM potassium arsenate and MgSO$_4$. The reaction was started by the addition of the luciferin and luciferase preparation. The actual data points were taken from 15 to 45 sec after the start of the reaction. Data for each sample were adjusted against an internal standard (100 pmol ATP).

The levels of glucose-6-phosphate and fructose-6-phosphate were assayed by the method described by Lang and Michal with the slight modification [8], in a Hitachi double beam spectrophotometer, type U-3200, which was fitted with an accessory for enzymatic analysis. The reaction mixture contained 25 mM HEPES-NaOH buffer (pH 7.6), 0.2 mM NADP$^+$, 5 mM MgCl$_2$, plant extract, 1.5 U (10 µl) glucose-6-phosphate dehydrogenase and 7 U (10 µl) phosphoglucose isomerase. The reaction was initiated by the addition of glucose-6-phosphate dehydrogenase and increase in absorbance at 340 nm was monitored. After glucose-6-phosphate in the extracts was almost completely exhausted (usually after 5 min), phosphoglucose isomerase was added, and the increase in absorbance was measured again (usually after 15 min). The data were adjusted against the internal standards (10 nmol of each compounds).

Determination of nucleic acid levels and the rate of synthesis of DNA and RNA

Levels of DNA and RNA were determined by the methods of Schmidt and Thannhauser [9] as described in our earlier papers [10, 11].

Incorporation of [methyl-³H]thymidine and [2-¹⁴C]uridine into the nucleic acid fraction was determined by the method of Amino et al. [2]. The cell suspension (200–300 mg fresh weight) was incubated with 2.5 µCi [methyl-³H]thymidine (specific activity, 44 Ci·mmol⁻¹) or 0.25 µCi [2-¹⁴C]uridine (specific activity, 57.8 mCi·mmol⁻¹) for 60 min at 27 °C. The extraction procedure was exactly the same as described by Amino et al. [2]. The radioactivity was measured with a Packard Tri Carb, Type 3255, liquid scintillation spectrophotometer.

Determination of amino acids and phenolics

Total free amino acids and protein amino acids were determined by the standard ninhydrin reaction [12] with L-leucine as a standard.

The level of total phenolic compounds was estimated by the method of Swain and Hills [13]. The values were expressed as µmol of p-coumaric acid equivalent.

Results

Cell growth

The effects of Pi on the pattern of growth of Catharanthus roseus cells are shown in Fig. 1. The fresh weight of the culture in the "+Pi" medium increased and the rate of increase become more significant after 48 h. In contrast, the fresh weight of cells in the "−Pi" medium decreased for 72 h, although the weight increased slightly between 72 and 96 h. The cell numbers in the "+Pi" medium increased exponentially after an initial lag period. The number of cells doubled between 72 and 96 h. The number in the "−Pi" medium increased slightly, but the rate of increase was less than 20% of that observed for the culture in the "+Pi" medium.

When the fresh weight is expressed as ng per cell, the value per cell for the culture in "+Pi" medium decreases significantly between 72 and 96 h; i.e. the values are 3.5 and 1.9 for 72 h- and 96 h-cultures, respectively. This result suggests that active cell division occurred between 72 and 96 h in the culture.

Level of phosphorous metabolites

The intracellular levels of the major low molecular weight-phosphorous compounds, Pi, ATP, G6P and F6P, in the cells grown in the "+Pi" and the "−Pi"

media were compared (Fig. 2). The intracellular level of Pi in the cells grown in the "+Pi" medium increased 24 h after transfer to fresh medium and then decreased. In contrast, the level in the cells in the "−Pi" medium was low throughout the culture period (Fig. 2A).

Changes in the levels of ATP during growth of the cells in the "+Pi" and the "−Pi" media are shown in Fig. 2A. The level of ATP in the cells in the "+Pi"
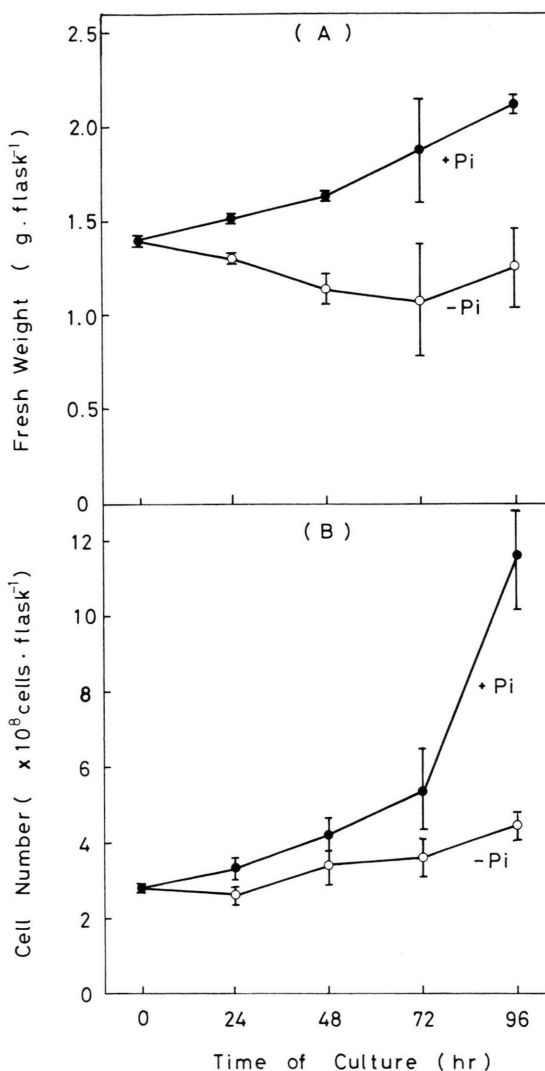

Fig. 1. Changes in fresh weight (A) of cells and cell number (B) of suspension cultures of Catharanthus roseus grown in the complete (●) and the Pi-deficient (○) Murashige-Skoog Media. The cells were grown in 50 ml of the medium in a 300 ml Erlenmeyer flask. Vertical lines represent standard deviations.
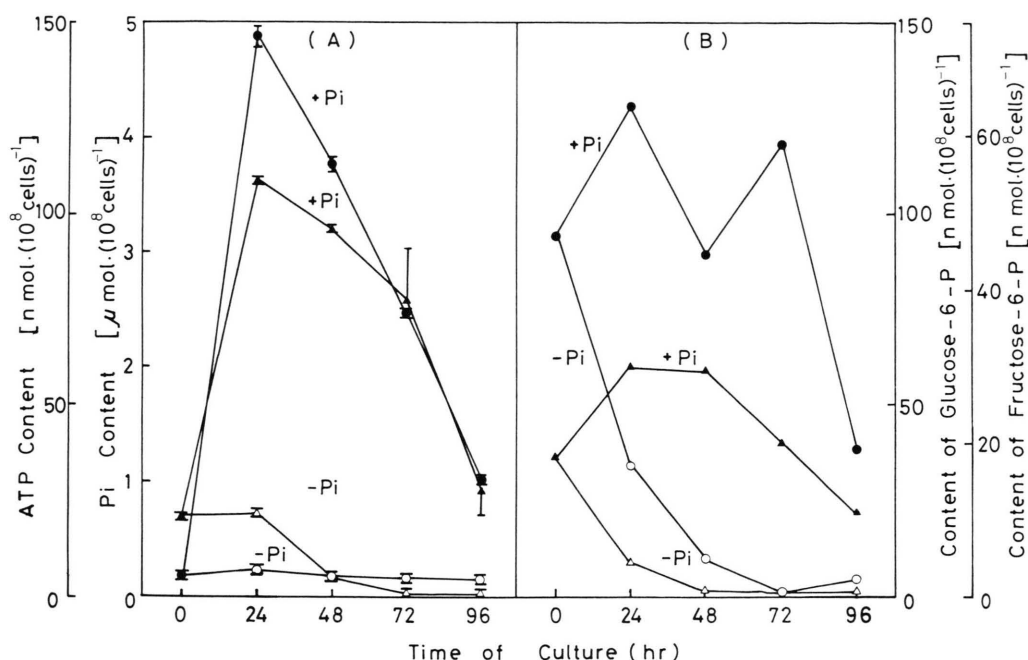
Fig. 2. (A) Changes in levels of Pi (circles) and ATP (triangles) in cells from suspension cultures of *Catharanthus roseus* grown in the complete (●, ▲) and the Pi-deficient (○, △) media. The contents Pi and ATP are expressed as μmol·(10^8cells)$^{-1}$ and nmol·(10^8cells)$^{-1}$. Vertical lines represent standard deviations.
(B) Changes in levels of glucose-6-phosphate (circles) and fructose-6-phosphate (triangles) in cells from suspension cultures of *Catharanthus roseus* grown in the complete (●, ▲) and the Pi-deficient (○, △) media. The levels are expressed as nmol·(10^8cells)$^{-1}$. Each point is the average of two samples.

medium increased more than 5-fold during first 24 h and then decreased. In the cells in the "−Pi" medium, the level was maintained for 24 h, but then decreased to the extreme low value of less than 1 nmol·(10^8cells)$^{-1}$.

The levels of G6P and F6P, both major intermediates in glycolysis, are shown in Fig. 2B. The levels in the cells in the "+Pi" medium were always higher than those in the "−Pi" cells. The increase in the levels of the sugar phosphates observed in cells in the "+Pi" medium during first 24 h (136% of initial value for G6P and 163% for F6P) were much smaller than the increase in the level of ATP (517%).

Level of nucleic acids

The RNA content of the cells grown in the "+Pi" medium increased rapidly after an initial lag period of 24 h and attained a maximum value at 72 h, subsequently the level decreased (Fig. 3A). As the values are expressed as μg per 10^8 cells, the decrease in

RNA content in individual cells at 96 h derives from the division of RNA into the newly formed cells. An increase in the DNA content of approximately 40% was observed in the cells in "+Pi" medium at 72 h, while a slight decrease in DNA content was observed in the cells grown in "−Pi" medium at this time.

DNA and RNA synthesis

Incorporation of [methyl-^{3}H]thymidine and [2-^{14}C]uridine into the nucleic acid fraction is shown in Fig. 3B. Significant incorporation of [methyl-^{3}H] was observed only in the cells grown in the "+Pi" medium. The maximum incorporation was observed at 72 h.

Increase in the incorporation of [2-^{14}C]uridine into the nucleic acid fraction was observed in the cells grown in both the "+Pi" and the "−Pi" media. [2-^{14}C]uridine was incorporated into RNA as well as into DNA. However, the rate of incorporation into DNA was extremely low in the *Catharanthus* cells
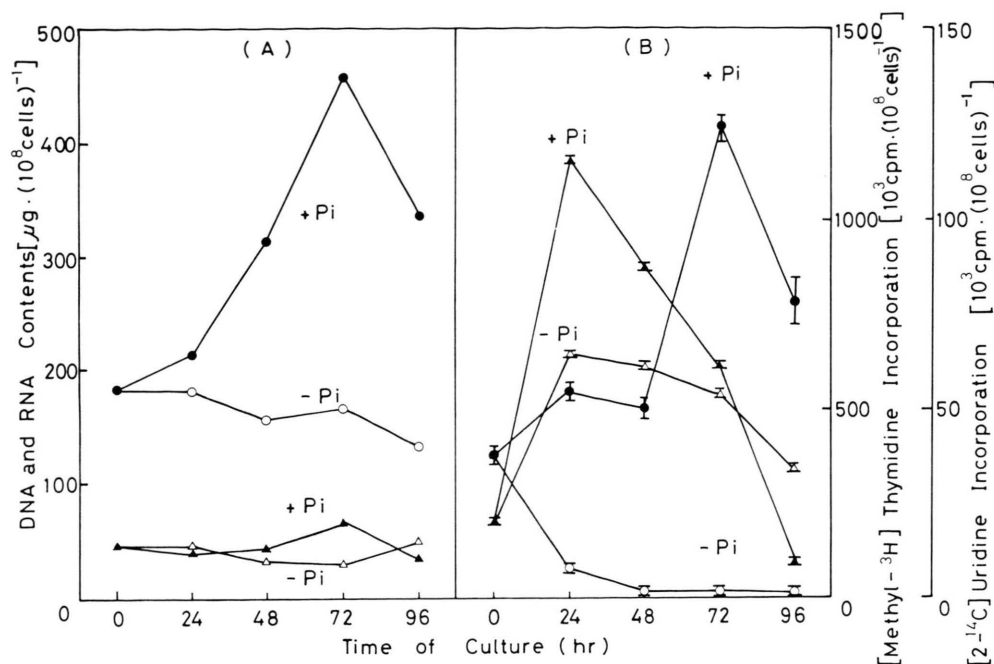
Fig. 3. (A) Changes in levels of RNA (circles) and DNA (triangles) in cells from suspension cultures of *Catharanthus roseus* grown in the complete (●, ▲) and the Pi-deficient (○, △) media. The levels are expressed as μg · (10⁸cells)⁻¹. Each point is the average of two samples.
(B) Changes in incorporation of [methyl-³H]thymidine (circles) and [2-¹⁴C]uridine (triangles) into the nucleic acid fraction of cells from suspension cultures of *Catharanthus roseus* grown in the complete (●, ▲) and Pi-deficient (○, △) media. The rates of incorporation are expressed as radioactivity(cpm) · h⁻¹ · (10⁸cells)⁻¹. Vertical lines indicate standard deviations.

[14]. Until 72 h, the rate of incorporation of [2-¹⁴C]uridine by the cells in "+Pi" medium exceeded that by the cells of "−Pi" medium. After 96 h in culture, the rate of incorporation in the cells in "−Pi" medium was higher than that in the "+Pi" medium cells. However, it may be necessary to take into account the effects of dilution of the radioactive compound. The uridine nucleotide pool in the cells grown in the "+Pi" medium may be much larger than that in the "−Pi" medium cells. Therefore, net synthesis of RNA in the "+Pi" culture may be much higher than that in the cells in the "−Pi" medium, at least during first 72 h.

Level of free and incorporated amino acids

The level of free amino acids decreased in the cells in "+Pi" medium during culture, while the level in the cells in "−Pi" medium increased in the early

phase of culture to a high level that was maintained throughout the experimental period (Fig. 4A).

In contrast, the level of amino acids incorporated into proteins increased in the cells in "+Pi" medium and decreased in the cells in "−Pi" medium. The decrease in the level of protein in the cells in "+Pi" medium after 96 h seems to be due to the partition of proteins into new daughter cells, as observed in the case of RNA (Fig. 4A).

Level of phenolic compounds

The level of low-molecular weight phenolic compounds increased transiently in the cells in both the "+Pi" and the "−Pi" media just after cell transfer. The free phenolic compounds remained at the initial level in the cells in "−Pi" medium at 72−96 h, but the level was clearly decreased in cells in the "+Pi" medium (Fig. 4B).
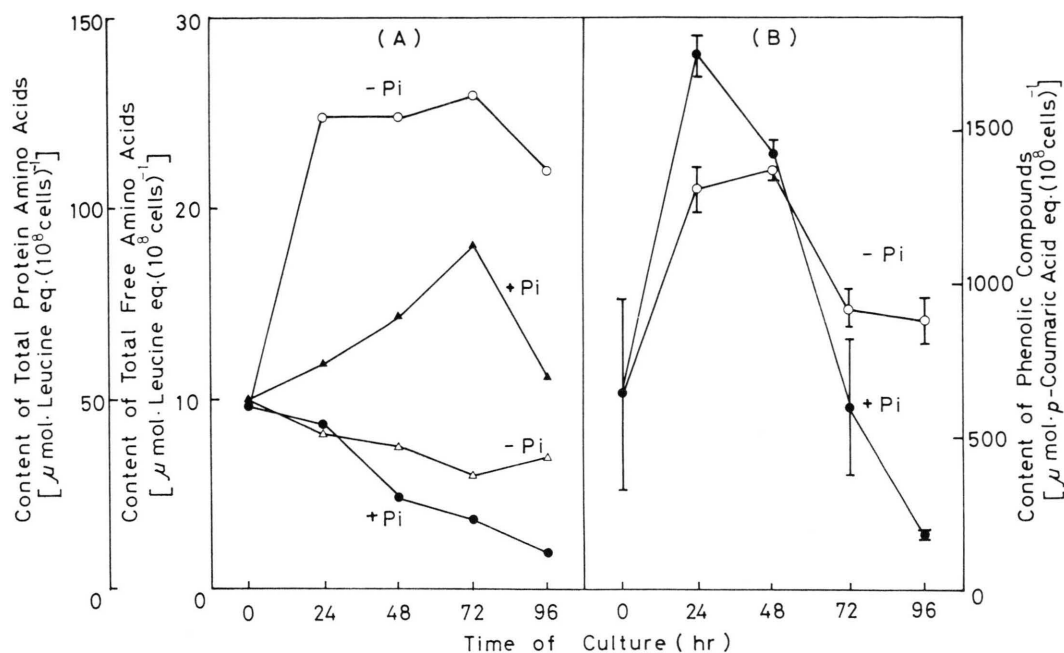
Fig. 4. (A) Changes in the levels of the total free amino acids (circles) and total protein amino acids (triangles) in cells from the suspension cultures of *Catharanthus roseus*, grown in the complete (●, ▲) and Pi-deficient (○, △) media. The content of amino acids is expressed as μmol leucine equivalent·$(10^8 cells)^{-1}$. Each point is the average of two samples.
(B) Changes in the level of the total ethanol-soluble phenolic compounds in cells from the suspension cultures of *Catharanthus roseus*, grown in the complete (●) and Pi-deficient (○) media. The levels are expressed as nmol *p*-coumaric acid equivalent·$(10^8 cells)^{-1}$. Vertical lines represent standard deviations.

Discussion

Studies on phosphorous deficiency in whole plants have been carried out extensively [15, 16], but the effects of Pi-deficiency on plant cell metabolism have not yet been clearly defined. Suspensions of cultured plant cells are very useful for studying plant nutrition at the biochemical level, since rapid metabolic changes caused by defined environmental factors can be easily observed. This type of study is also important for research in plant biotechnology. The concentration of Pi in the culture medium greatly influences the formation of a variety of useful, secondary plant products. Several reports have already published to support this point of view [3, 17–20].

Among the several biochemical changes observed in *Catharanthus* cells, the most remarkable change was a rapid increase in the level of ATP, just after the cells were transferred to the fresh "+Pi" medium (Fig. 2A). This kind of increase in the level of ATP was recently observed in suspensions of several types of cultured cells including *Catharanthus roseus* [21], soy bean [22] *Nicotiana tabacum* [23] and *Datura in-*

noxia [24]. Our results described here indicate that the increase is completely depressed by Pi-depletion. Therefore, a close relationship between intracellular levels of ATP and Pi concentration can be presumed.

Simultaneously, the levels of G6P and F6P, intermediates in the initial steps of the Embden-Meyerhof-Parnas pathway and/or the pentose phosphate pathway, were increased in the cells grown in "+Pi" medium (Fig. 2B). The synthesis of these compounds seems to be dependent on the level of ATP, which is elevated in the cells grown in "+Pi" medium. The activation of the glycolysis and TCA cycle by the supply of sugar phosphates may generate much more ATP which can, in turn, be utilized in the subsequent synthesis of nucleic acids and proteins.

Significant DNA synthesis was detected only in the cells in "+Pi" medium at 72 h (Fig. 3B) and frequent cell division was observed between 72 and 96 h (Fig. 1B). Although the culture methods were different, the lag period for DNA synthesis after cell transfer seemed to be slightly longer than that reported for *Catharanthus roseus* cells by Amino et al. [2]. The

major reason for the differences in the lag periods may be the difference in the age of the cells which were subcultured to the fresh medium. We used 10-day-old cells; Amino *et al.* used 7-day-old cells.

RNA synthesis was observed both in cells grown in "+Pi" and in "−Pi" medium (Fig. 3B). Comparison of metabolic activity between cells grown in the "+Pi" medium and those grown "−Pi" is very difficult, because endogenous pool sizes of intermediates (uridine nucleotides in this case) may be very different and, furthermore, exogenously administered precursors are not always mixed with the endogenous pool of intermediates. Nevertheless, the data obtained here indicate that RNA synthesis was stimulated by Pi in the medium, because it is easily assumed that large pools of uridine nucleotide develop in the "+Pi" cells. An increase in the level of uridine nucleotide just after transfer of cells to fresh medium has been reported for *Datura* cells [24].

The level of free amino acids decreased in the cells grown in "+Pi" medium; at the same time, the protein level was increased in the cells (Fig. 4A). This result suggests that free amino acids were utilized for the protein synthesis which was activated in the cells in the "+Pi" medium, probably mediated by the increasing level of ATP.

Converse changes were observed in the cells grown in the "−Pi" medium. Here, the level of free amino acid increased and the level of protein amino acids decreased (Fig. 4A). Increase in the level of free amino acids, associated with Pi-deficiency, has been reported for some whole plants [25, 26].

The level of soluble phenolic compounds increased when the cells were transferred to fresh medium (Fig. 4B). This increase was observed in cells in both "+Pi" and "−Pi" media. Therefore, this increase seems to be a "transfer effect". The level of soluble phenolics decreased progressively with cell division in the "+Pi" cells. Although detailed analysis of the phenolics was not carried out, it is obvious that level of the secondary compounds, including phenolics, are low in actively growing cells, as suggested by many researchers [17].

The data obtained here reveal the outlines of the metabolism of plant cells in suspension culture under conditions of Pi-deficiency. Further detailed studies of the influence of Pi on several pathways in the cells are in progress.

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