The Short-Term Effects of Inorganic Phosphate on the Levels of Metabolites in Suspension-Cultured *Catharanthus roseus* Cells*

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In order to analyze the effects of inorganic phosphate (Pi) on the energy metabolism of suspension-cultured cells of *Catharanthus roseus*, short-term changes in levels of glycolytic metabolites were monitored after the addition of 1.25 mM Pi to cultures of cells previously cultured in a Pi-free Murashige-Skoog medium for 24 h. The levels of all phosphorylated intermediates of glycolysis examined were found to increase after addition of Pi. The most striking increases were observed in levels of glucose-6-phosphate and fructose-6-phosphate which rose to approximately 8 (after 30 min) and 15 times (after 120 min) their initial values. The levels of ATP and pyrophosphate doubled during the first 30 min of incubation. In contrast, the level of pyruvate decreased significantly during the initial 30 min and then began to increase again for the next 90 min. The short-term effects of Pi on the rates of synthesis of proteins and RNA were estimated from the rates of incorporation of ^{14}C-labelled amino acids and uridine into the macromolecules. No appreciable stimulation of the synthesis of proteins and RNA was induced by Pi within 120 min after the addition of Pi. Possible mechanisms are discussed that may be involved in the changes in metabolism initiated by the addition of Pi to the cultures.

Introduction

The importance of inorganic phosphate (Pi) in the regulation of the growth and metabolism of cultured plant cells has frequently been emphasized. In general, Pi activates primary metabolism and, as a result, stimulates the proliferation of cells [1–2], while starvation of Pi induces the accumulation of secondary compounds, such as tryptamine, indole alkaloids and phenolics [3–5]. However, the controls of metabolism induced by the addition or depletion of Pi have not yet been fully elucidated. In our earlier studies [6–9], we monitored growth and metabolism of suspension-cultured *Catharanthus roseus* cells for 96 h, at 24 h intervals, after transfer of stationary-phase cells to fresh complete ("+ Pi") and phosphate deficient ("− Pi") medium. Our results indicated that growth and division of the cells were dependent on Pi in the medium, and marked increases in the rates of biosynthesis of nucleotides, nucleic acids and proteins were observed in the cells in the "+ Pi" medium. These metabolic changes seem to be closely correlated to the levels of ATP in the cells. Thus, in the present study, in order to examine the more immediate effects of the addition of Pi on the metabolism of the cells, we determined the levels of certain metabolites that are associated with energy metabolism, and measured the rates of synthesis of proteins and nucleic acids during the first 120 min after the addition of Pi to cultures of the cells.

Abbreviations: F6P, fructose-6-phosphate; F1,6BP, fructose-1,6-bisphosphate; G6P, glucose-6-phosphate; PEP, phosphoenolpyruvate; PFK, phosphofructokinase (EC 2.7.1.11); PFP, PPi:fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90); 3PGA, 3-phosphoglycerate; "+ Pi" medium, complete Murashige-Skoog medium; "− Pi" medium, Pi-deficent Murashige-Skoog medium; PK, pyruvate kinase (EC 2.7.1.40).

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Materials and Methods

Plant materials

Suspension cell cultures of *Catharanthus roseus* (L.) G. Don (strain A/L) were maintained and subcultured every 10 days in Murashige-Skoog basal medium [10] that contained 2.2 μM 2,4-dichlorophenoxyacetic acid and 3% sucrose ("+ Pi" medium) as described earlier [2]. Experimental cultures were initiated from 10 day old stock cultures.

The suspension of cells (approx. 800 mg cells in 2 ml) was transferred to 18 ml of Pi-deficient Murashige-Skoog ("− Pi") medium in 100 ml Erlenmeyer flasks. Aliquots of a sterilized solution of Pi were added to the cultures, to a final concentration of 1.25 mM, 24 h after inoculation. For control ("− Pi") cultures, the same amount of distilled water was added.

Determination of metabolites

Freshly harvested cells (approx. 1.6 g fresh weight) were washed with distilled water and immersed in liquid nitrogen in a chilled mortar. The frozen cells were immediately ground to a powder, dropped into 2.5 ml of 6% perchloric acid in a Potter-Elvehjum homogenizer, and homogenized for 30 sec. The homogenate was centrifuged at $15{,}000 \times g$ for 10 min at 2 °C; the resultant supernatant was collected and the pH was adjusted to 3.5–5.0 with KOH; and finally, potassium perchlorate was removed by centrifugation ($15{,}000 \times g$, 10 min). The supernatant obtained was used immediately for the assay of metabolites.

The level of ATP was determined luminometrically with a Packard Model 6100 Picolite Luminometer, as described earlier [11]. The levels of other metabolites were measured spectrophotometrically by following changes in absorbance at 340 nm. The measurements were made at 37 °C in a Hitachi double-beam spectrophotometer, type U-3200, which was fitted with accessories for enzymatic analysis. Analysis of levels of the following individual metabolites was carried out in essentially the same way as described in the references cited: glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) [12]; fructose-1,6-bisphosphate (F1,6BP) [13]; 3-phosphoglycerate (3PGA) [14]; phosphoenolpyruvate (PEP) [15]; pyruvate [16]; and pyrophosphate (PPi) [17].

In preliminary experiments, the methods for the extraction of metabolites described by others [18–20] were examined with our materials. The results were essentially the same as those obtained when our methods, as described above, were used. The degree of disruption of cells and the recovery of individual metabolites were checked as described an earlier paper from our laboratory [21]. More than 90% of the cells were disrupted by the extraction treatment and recovery of each metabolite was more than 80%.

Determination of the rates of synthesis of proteins and RNA

The rates of synthesis of proteins and RNA were estimated from the incorporation of radioactivity from a mixture of [U-^{14}C]L-amino acids (specific activity, 1.85 MBq·μmol^{-1}; ICN Radiochemicals, Irvine, C.A., U.S.A.) and from [2-^{14}C]uridine (specific activity, 2.14 Bq·μmol^{-1}; CEA, Gif-Sur-Yvette, France) into the ethanol-insoluble fraction, respectively. Total radioactivity taken up by the cells was estimated by summation of the radioactivity recovered as CO_2, ethanol-soluble, ethanol-insoluble components. Cells grown in fresh "− Pi" medium for 24 h (200 mg fresh weight) were collected and resuspended in the same "− Pi" medium (1.7 ml) in the main compartment of a 30 ml Erlenmeyer flask fitted with a centre well which contained 20% KOH. Aliquots (0.2 ml) of 12.5 mM Pi and labelled compounds (0.1 ml, 18.5 kBq) were added simultaneously to the main compartment of the flask. For the control ("− Pi") cells, distilled water (0.2 ml) was added instead of Pi. The flasks were incubated in an oscillating water bath, at 120 strokes·min^{-1}, 50 mm amplitude, for 30 or 120 min at 27 °C. After the incubation, the cells were collected on a sheet of Miracloth (Calbiochem, La Jolla, C.A., U.S.A.), washed with distilled water, frozen with liquid nitrogen, and stored at −20 °C. The frozen cells were homogenized with a Potter-Elvehjum homogenizer in 80% ethanol, and the homogenate was centrifuged at $3{,}000 \times g$ for 10 min. The ethanol extraction was repeated twice. Radioactivity in the ethanol-soluble and the ethanol-insoluble macromolecular fractions and in CO_2 was determined with a Packard Tri Carb liquid scintillation spectrophotometer, type 3255, in scintillation fluid, ACS-II (Amersham International plc, Amersham, U.K.).

Results and Discussion

Table I shows changes in the levels of some glycolytic metabolites in *Catharanthus* cells after addition of Pi. The levels of phosphorylated metabolites were initially very low, but a several-fold larger pool of pyruvate than of other metabolites was observed in the initial Pi-starved (0 min) cells. Approximately 8-fold (30 min) and 15-fold increases (120 min) in the levels of G6P and F6P were detected after incubation of the cells with 1.25 mM Pi. Levels of the

Table I. Changes in the levels of metabolites in suspension-cultured *Catharanthus roseus* cells after addition of 1.25 mM Pi.

Metabolites	Duration of incubation [min]		
	0	30	120
Glucose-6-phosphate	13.9 ± 0.7	115.8 ± 5.3	217.2 ± 26.8
Fructose-6-phosphate	3.5 ± 1.7	27.1 ± 1.3	49.1 ± 5.5
Fructose-1,6-bisphosphate	11.6 ± 2.1	12.2 ± 0.5	27.4 ± 1.2
3-Phosphoglycerate	12.1 ± 5.4	26.3 ± 5.5	26.8 ± 1.0
Phosphoenolpyruvate	17.5 ± 3.3	24.4 ± 0.9	20.4 ± 3.6
Pyruvate	158.3 ± 3.6	53.4 ± 9.2	106.8 ± 42.5
ATP	30.5 ± 0.2	68.8 ± 3.8	88.5 ± 11.3
PPi	19.4 ± 2.9	43.1 ± 5.6	88.6 ± 12.0

The levels of metabolites are expressed as nmol·g fr. wt.$^{-1}$. The averaged values and standard deviations represent results from more than four separate experiments.

other phosphorylated intermediates of glycolysis also increased, but the rates of increase were lower those of the hexose phosphates. The levels of ATP and PPi in the Pi-starved cells were approximately 30 and 20 nmol per g fresh weight, respectively. The levels doubled within 30 min after addition of Pi, and increased further over the next 90 min. No appreciable changes in the levels of any metabolites were observed during the course of 120 min in the cells in control ("− Pi") cultures (data not shown).

The crossover theorem proposed by Chance *et al.* [22] may be applicable to the identification of those steps of glycolysis which are regulated by the addition of Pi. Fig. 1 shows the crossover diagrams for glycolytic intermediates during the periods 0−30 min and 30−120 min after addition of Pi. A negative crossover between PEP and pyruvate during the 0−30 min period (Fig. 1A) may not be indicative of the inhibition of the step catalyzed by pyruvate kinase (PK), but may rather suggest the rapid consumption of pyruvate accompanied by the stimulation of respiration by Pi.

A positive crossover between PEP and pyruvate during the 30−120 min period (Fig. 1B) suggests that the step catalyzed by PK is stimulated during this period. Furthermore, the higher rate of increase in the level of F1,6BP compared to that of F6P during this latter period suggests that the step catalyzed by phosphofructokinase (PFK) and/or PPi:fructose-6-phosphate 1-phosphotransferase (PFP) may also be stimulated, although a positive crossover is not evident. Increases in the levels of all phosphorylated intermediates during the period may eliminate the definite crossover between F6P and F1,6BP. In *Catharanthus* cells, PFP seems to be functional as an alternative enzyme in glycolysis, because the maximum catalytic activity of PFP is always higher than

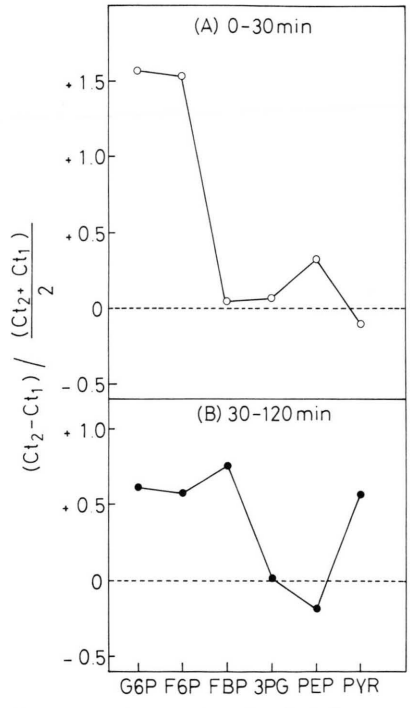

Fig. 1. Crossover diagrams for glycolytic intermediates during the periods 0−30 min (A) and 30−120 min (B) after the addition of 1.25 mM Pi to the Pi-starved *Catharanthus roseus* cells in suspension culture. C_{t1} and C_{t2} are concentrations of the metabolites at 0 and 30 min (A) or 30 and 120 min (B). G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; 3PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate. The values were calculated from the data shown in Table I.

that of PFK in the cells [17], and significant amounts of PPi and fructose-2,6-bisphosphate, a potent activator of PFP, are present in the cells (Table I and [17]). However, it is very difficult to estimate the respective contributions of PFK and PFP to the flux of glycolysis *in vivo*. Exogenously supplied Pi is taken up by the cells, and elevates the cytosolic concentration of Pi as well as of ATP and PPi [2]. Pi is a positive effector of PFK, *i.e.* stimulates the activity of PFK directly [23, 24] and reverses the inhibition of PFK by metabolites such as PEP [25], but Pi is a negative effector of PFP in glycolytic direction [26, 27]. Thus, activities of PFK and PFP in the cells, after Pi is taken up, may be controlled in a very complicated manner.

In our next experiments, in order to examine the short-term effects of Pi on the synthesis of proteins and RNA, rates of incorporation of a ^{14}C-labelled mixture of amino acids (a mixture of 15 pure L-amino acids in the same proportions as those in a typical hydrolysate of algal proteins) into proteins, and of [2-^{14}C]uridine into RNA were determined (Table II). Uptake of amino acids and uridine by the cells was significantly reduced when Pi was present in the medium. The mechanism of the reduction has not yet been clarified, but it is possible that competition for active transport between Pi and these molecules may occur. Incorporation of [U-^{14}C]amino acids into proteins was reduced by Pi even when the rate of incorporation was expressed as the percentage of total radioactivity taken up by the cells. Thus, stimulation of synthesis of proteins by Pi is unlikely during the first 120 min after addition of Pi. In contrast, the rate of incorporation of [2-^{14}C]uridine into RNA was slightly (approximately 20%) higher in the presence of Pi during both the 0–30 min and the 0–120 min periods, when the rate was expressed as a percentage of total uptake.

From these results, we propose the following scheme for the activation of metabolism by exogenously supplied Pi. First, the generation of ATP, *i.e.*, the oxidative phosphorylation of ADP to ATP, occurs shortly after the cells are incubated with Pi, during which time pre-existing adenylates and respiratory substrates, such as pyruvate, and externally supplied Pi are utilized. Subsequently, hexoses are phosphorylated in the reactions catalyzed by hexokinase and/or fructokinase. During the next 90 min, the levels of all intermediates of glycolysis increase, and, probably, reactions catalyzed by regulatory enzymes, such as PFK and PK, are stimulated. Accompanied by an increased supply of respiratory substrates generated by glycolysis, the rate of production of ATP increase still further. However, synthesis of proteins and nucleic acids does not occur at any significantly elevated rate during the 2 h period examined here. Many more biochemical processes, including the net synthesis of adenylates and formation of the various building blocks may be required for the synthesis of these macromolecules. Changes in the metabolism of proteins and nucleic acids at later stages of culture have been reported before [2, 6, 7].

Table II. Uptake and utilization of [U-^{14}C]L-amino acids and [2-^{14}C]uridine by the suspension-cultured *Catharanthus roseus* cells in the presence and absence of Pi.

Compounds added	Incubation time [min]	Pi	Uptake	Ethanol-insoluble material [%]
Amino acids	30	+	1199 ± 8	268 ± 14 (22.4)
		−	1484 ± 86	423 ± 45 (28.5)
	120	+	3109 ± 93	521 ± 2 (16.8)
		−	4784 ± 720	1229 ± 254 (25.7)
Uridine	30	+	9177 ± 899	2832 ± 95 (30.9)
		−	11393 ± 1135	2835 ± 167 (24.9)
	120	+	25504 ± 1974	5900 ± 286 (23.1)
		−	30945 ± 1167	5850 ± 66 (18.9)

The net of uptake, over the indicated period of time, of radioactivity by the cells was obtained from the summation of radioactivity observed in CO_2, and in the ethanol-soluble and ethanol-insoluble fractions; it is expressed as Bq·g fr. wt.$^{-1}$. The net incorporation of radioactivity into the ethanol-insoluble fractions is expressed as Bq·g fr. wt.$^{-1}$ and as a percentage of total uptake (in parentheses). The averaged values and standard deviations were obtained from duplicate samples in a typical experiment.

The present studies strongly suggest that one of the short-term effects of Pi on the metabolism of suspension-cultured cells is to increase the rate of oxidation of carbohydrates, which is a result of the phosphorylation of sugars. This process occurs before net synthesis of any proteins, even, probably, of the enzymes involved in glycolysis.

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[1] S.-I. Amino, T. Fujimura, and A. Komamine, Physiol. Plant. **59,** 393 (1983).
[2] T. Ukaji and H. Ashihara, Z. Naturforsch. **41c,** 1045 (1986).
[3] K.-H. Knobloch and J. Berlin, Plant Cell Tissue Organ Culture **2,** 333 (1983).
[4] K.-H. Knobloch and J. Berlin, Z. Naturforsch. **35c,** 551 (1980).
[5] T. Yamakawa, S. Kato, K. Ishida, T. Kodama, and Y. Minoda, Agric. Biol. Chem. **47,** 2185 (1983).
[6] T. Ukaji and H. Ashihara, Ann. Bot. **60,** 109 (1987).
[7] H. Ashihara, X.-N. Li, and T. Ukaji, Ann. Bot. **61,** 225 (1988).
[8] T. Ukaji and H. Ashihara, Int. J. Biochem. **19,** 1127 (1987).
[9] X.-N. Li and H. Ashihara, Ann. Bot. **64,** 33 (1989).
[10] T. Murashige and F. Skoog, Physiol. Plant. **15,** 473 (1962).
[11] H. Ashihara and T. Ukaji, J. Plant Physiol. **124,** 77 (1986).
[12] G. Michel, in: Methods in Enzymatic Analysis, 3rd ed. (H. U. Bergmeyer, ed.), **Vol. 6,** p. 191, Verlag Chemie, Weinheim 1984.
[13] G. Michel, in: Methods in Enzymatic Analysis, 3rd ed. (H. U. Bergmeyer, ed.), **Vol. 6,** p. 342, Verlag Chemie, Weinheim 1984.
[14] R. Czok, in: Methods in Enzymatic Analysis, 3rd ed. (H. U. Bergmeyer, ed.), **Vol. 6,** p. 537, Verlag Chemie, Weinheim 1984.
[15] W. Lamprecht and F. Heinz, in: Methods in Enzymatic Analysis, 3rd ed. (H. U. Bergmeyer, ed.), **Vol. 6,** p. 555, Verlag Chemie, Weinheim 1984.
[16] W. Lamprecht and F. Heinz, in: Methods in Enzymatic Analysis, 3rd ed. (H. U. Bergmeyer, ed.), **Vol. 6,** p. 570, Verlag Chemie, Weinheim 1984.
[17] H. Ashihara and T. Horikosi, Z. Naturforsch. **42c,** 1215 (1987).
[18] H. P. Ruffner and J. S. Hawker, Phytochemistry **16,** 1171 (1977).
[19] W. L. Dixson and T. ap Rees, Phytochemistry **19,** 1297 (1980).
[20] R. Leegood, in: Manual for the Advanced Course on Techniques in Photosynthesis, Research Institute for Photosynthesis, University of Sheffield, 1988.
[21] H. Ashihara, T. Horikosi, X.-N. Li, K. Sagishima, and Y. Yamashita, J. Plant Physiol. **133,** 38 (1988).
[22] B. Chance, W. Holmes, J. Higgins, and C. M. Connelly, Nature **182,** 1190 (1958).
[23] B. M. Radzali and C. V. Givan, Biochem. Physiol. Pflanzen **176,** 490 (1981).
[24] X.-N. Li and H. Ashihara, Asian J. Plant Sci. **1,** 23 (1989).
[25] J. F. Turner, J. D. Tomlinson, R. A. Caldwell, Plant Physiol. **66,** 973 (1980).
[26] E. Kombrink, N. J. Kruger, and H. Beevers, Plant Physiol. **74,** 395 (1984).
[27] H. Ashihara and S. Stupavska, J. Plant Physiol. **116,** 241 (1984).