Role of Pyrophosphate: Fructose-6-phosphate 1-Phosphotransferase in Glycolysis in Cultured *Catharanthus roseus* Cells*

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Dedicated to Professor Akira Tsukamoto on the occasion of his 65th birthday

Catharanthus roseus (= *Vinca rosea*), Pyrophosphate: fructose-6-phosphate 1-Phosphotransferase, Phosphofructokinase, Fructose-2,6-bisphosphate, Plant Cell Culture

The maximum catalytic activity of pyrophosphate: fructose-6-phosphate 1-phosphotransferase (PPi-PFK) was approximately three fold greater than that of ATP: fructose-6-phosphate 1-phosphotransferase (ATP-PFK) in *Catharanthus roseus* cells at any stage of culture. The levels of both enzymes increased after subculture of the cells, reached their maximum levels on day 3–4, and then decreased. PPi-PFK partially purified from *Catharanthus roseus* required fructose-2,6-bisphosphate (F2,6BP) for its activity. The Ka value of the enzyme for F2,6BP was 26 nM. The Km values for fructose-6-phosphate (F6P) and sodium pyrophosphate (PPi), at physiological pH (7.2) in the presence of 1 μM F2,6BP, were 0.59 mM and 48 μM, respectively. Intracellular levels of PPi and F6P varied from 17–71 nmol and from 37–65 nm per g fresh weight of the cells during culture. These results suggest that PPi-PFK is functional in *Catharanthus roseus* cells *in vivo*. The role of PPi-PFK in carbohydrate metabolism in heterotrophic, cultured plant cells is discussed.

Introduction

The pathway of glycolysis, the so-called Embden-Meyerhof-Parnas pathway, is widely distributed in living organisms. In many organisms, the first committed step in this pathway is catalyzed by ATP:fructose-6-phosphate 1-phosphotransferase (ATP-PFK, EC 2.7.1.11). However, in addition to ATP-PFK, higher plants possess the enzyme pyrophosphate: fructose-6-phosphate 1-phosphotransferase (PPi-PFK, EC 2.7.1.90), which utilizes pyrophosphate (PPi) and its activated by fructose-2,6-bisphosphate (F2,6BP) [1–5].

Recently, several reports on the properties of PPi-PFK from higher plants have been published [6–14], but the physiological role in glycolysis of this alternative enzyme has not yet been unequivocally established. Both kinetic data for this enzyme and also a physiological examination of the pathway are essential to reveal the real function of the alternative enzyme in plant metabolism.

In the present study, we first monitored the fluctuations in the maximum catalytic activities of PPi-PFK and ATP-PFK in cultures of *Catharanthus roseus* during hetrotrophic growth in batch suspension. Secondly, we determined some kinetic values of PPi-PFK which was partially purified from *Catharanthus roseus*. Finally, we compared the kinetic data of PPi-PFK with the estimated intracellular concentrations of substrates and of an activator, in order to ascertain whether the alternative enzyme is functional *in vivo* in *Catharanthus roseus*. From the results obtained in the present study and in previous studies, a possible role for PPi-PFK in the cells is suggested.

Materials and Methods

Plant materials

Stock suspension cultures of *Catharanthus roseus* (L.) G. Don [= *Vinca rosea* L.] were prepared from stem sections of intact plants in 1969. The cultures (strain TH-1) were maintained in 50 ml of Murashige-Skoog basal medium supplemented with 2.2 μM 2,4-dichlorophenoxyacetic acid and 3% sucrose, in 300 ml Erlenmeyer flasks. The cultures were incubated at 27 °C on a horizontal rotary shaker which was operated at 90 strokes min^{-1}, 8 cm amplitude, in the dark. The pattern of growth of the

Abbreviations: ATP-PFK, ATP:D-fructose-6-phosphate 1-phosphotransferase [EC 2.7.1.11]; F2,6BP, fructose-2,6-bisphosphate; F6P, fructose-6-phosphate; PPi, inorganic pyrophosphate; PPi-PFK, inorganic pyrophosphate:D-fructose-6-phosphate 1-phosphotransferase [EC 2.7.1.90].

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cells was essentially the same as described in an earlier paper [15]. Four growth phases: (i) the lag phase (days 0–1); (ii) the cell division phase (days 1–4); (iii) the cell expansion phase (days 4–7); and the stationary phase (days 7–10), were recognized.

Biochemicals

All biochemicals used in these experiments were obtained from Sigma Chemical Company, St. Louis, USA. Sulphate-free preparations of PPi-PFK (from potato tubers or from mung beans), aldolase, glycerophosphate dehydrogenase and triose phosphate dehydrogenase, which were supplied as lyophylized powders, were dissolved in 50% glycerol and stored at −20 °C. Fructose-6-phosphate and glucose-6-phosphate were treated with HCl and neutralized as described in ref. [16], in order to remove any F2,6BP present as a contaminant. The exact concentrations of substrates and F2,6BP were determined enzymatically.

Preparation of enzyme extracts for determination of maximum catalytic activity

Cells from several stages of culture were collected on a layer of Miracloth and washed with distilled water. The washed cells (approximately 1.5 g fresh weight) were homogenized immediately in 15 ml of 50 mM imidazole-HCl buffer (pH 7.6) which contained 2 mM $MgCl_2$, 1 mM sodium EDTA and 0.1% (v/v) 2-mercaptoethanol. The degree of cell disruption was checked under a microscope. More than 90% of the cells were broken by this treatment. The homogenate was centrifuged at $40{,}000 \times g$ for 30 min at 2 °C. The supernatant obtained was treated with finely ground, solid ammonium sulphate. The protein fraction precipitating at 70% saturation was collected by centrifugation, and dissolved in 2.5 ml of 200 mM HEPES-NaOH buffer (pH 7.2). The fraction was desalted on a column of Sephadex G-25 (bed volume 9 ml). The eluted protein fraction (approximately 3.5 ml) was used immediately for the assay.

Purification of PPi-PFK

PPi-PFK was partially purified from 6-day-old cultures of *Catharanthus roseus* by the method of Van Schaftingen *et al.* [6], except that the final step of purification was replaced by HPLC on an ion-exchange column. Washed cells (approximately 25 g fresh weight) were homogenized in a Potter-Elvehjum type glass homogenizer with 2–3 volumes of ice-cold 20 mM HEPES-NaOH buffer (pH 8.2) which contained 20 mM potassium acetate and 2 mM dithiothreitol. After centrifugation at $40{,}000 \times g$ for 20 min, finely ground, solid PPi and 1 M $MgCl_2$ were added to the supernatant to give a final concentration of 2 mM of each, and the pH was adjusted to 8.2 at 0 °C. The mixture was immediately brought to 59 °C and maintained at that temperature for 5 min. After cooling in an ice bath, the pH of the mixture was lowered to 7.1 and the mixture was centrifuged at $40{,}000 \times g$ for 10 min. Polyethylene glycol 6000 was added and the protein fraction which precipitated between 6% and 8% (w/v) was collected by centrifugation. The resulting precipitate was dissolved in 20 mM Tris-HCl buffer (pH 8.2) which contained 20 mM KCl and 20 mM dithiothreitol. The protein fraction was desalted on a Sephadex G-25 column, and then filtered through a cellulose nitrate membrane disc (Milipore, type HA, pore size 45 μm). The filtrate (3 ml) was loaded, for HPLC, onto a Shodex IEC QA-824 column (Showa Denko Co., Tokyo), equilibrated with 20 mM Tris-HCl buffer (pH 8.2) which contained 20 mM KCl and 20 mM dithiothreitol. After the column was washed with 10 ml of the equilibration buffer for 10 min, the enzyme was eluted over the course of 50 min with a linear gradient from 20 to 1000 mM KCl in the Tris-HCl buffer which contained 20 mM dithiothreitol. The flow rate was 1.0 ml per min, and fractions of 1.0 ml were collected. PPi-PFK was eluted as a single peak, as shown in Fig. 1. Active fractions (usually, fraction number 38) were pooled and used for assay of enzymatic activity.

Specific activity of PPi-PFK was 1.2–2.3 units per mg protein in the presence of 1 μM F2,6BP at pH 7.2. No activity of ATP-PFK was detected in the preparation, but glucose phosphate isomerase could not be removed completely: the pooled fraction contained less than 0.3 units per mg protein of the isomerase activity.

Assay of enzymatic activities

The activities of PPi-PFK and ATP-PFK were measured spectrophotometrically by following changes in absorbance at 340 nm at 30 °C with a Hitachi double beam spectrophotometer, type U-3200, which was fitted with an accessory for enzymatic analyses.

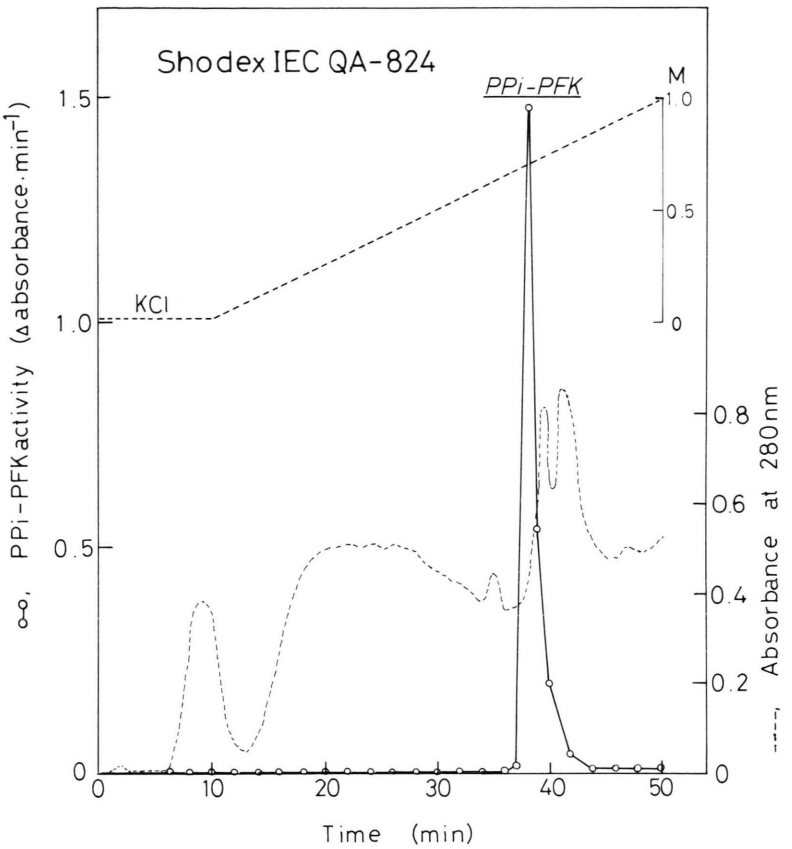

Fig. 1. Elution profiles of PPi-PFK chromatographed on the Shodex IEC QA-824 column. The experimental procedure is described in Materials and Methods. The enzymatic activity is expressed as Δ absorbance at 340 nm per min.

The reaction mixture for the assay of PPi-PFK in crude extracts contained 50 mM HEPES-NaOH buffer (pH 7.2), 17.5 mM glucose-6-phosphate, 10 mM fructose-6-phosphate, 1 mM PPi, 1 μM F2,6BP, 5 mM $MgCl_2$, 0.2 mM NADH, 1 U aldolase, 1 U triose phosphate isomerase, 1 U glycerophosphate dehydrogenase and the preparation of enzyme. In the mixture for determination of the kinetic parameters of PPi-PFK, the concentrations of substrates and of an effector were changed as indicated. The mixture for determination of the maximum catalytic activity of ATP-PFK was the same as for PPi-PFK, except that 1 mM ATP replaced PPi, and F2,6BP was omitted. In the reference cuvettes for the assay of PPi-PFK and ATP-PFK, PPi and ATP, respectively, were removed from the reaction mixtures.

In the case of determination of maximum catalytic activity, the reaction was started by the addition of the preparation of enzyme. The proportionality of the initial velocity of the reaction to the amounts of enzyme was checked for every assay by plotting the initial velocity against at least three different amount of enzymes. In preliminary experiments, the presence or absence of inhibitors of enzymes in the crude extracts from *Catharanthus roseus* was checked by the addition of the extracts to the reaction mixture which contained aliquots of commercial preparations of PPi-PFK and ATP-PFK. Little or no effect of the plant extracts on the activity of these enzymes was found in the range of amounts of extracts (25–100 μl) used for the enzyme assay. In the determination of the kinetic parameters of PPi-PFK, the reaction was started by the addition of PPi after a 5 min preincubation, as described by Van Schaftingen *et al.* [6].

Extraction and assay of PPi and fructose-6-phosphate

Washed cells (approximately 1 g fresh weight) were homogenized in 4 ml of 6% perchloric acid in

the same way as in the method for enzyme extraction. The homogenate was centrifuged at 40,000 × *g* for 20 min, and the supernatant was neutralized with 20% KOH. After any precipitated potassium perchlorate was removed by centrifugation, the extract was used immediately for the assay. The levels of PPi and fructose-6-phosphate were determined by enzymatic analysis. The mixture for determination of PPi (total volume 0.8 ml) contained 150 mM imidazole-HCl buffer (pH 7.4), 6 mM $MgCl_2$, 0.6 mM $MnCl_2$, 60 μM $CoCl_2$, 0.5 mM sodium citrate, 9 μM EDTA, 12 mM fructose-6-phosphate, 0.8 mM NADH, 4 mg bovine serum albumin, 0.1 U PPi-PFK (from *Propionibacterium freudenreichii*), 2 U aldolase, 12 U triosephosphate isomerase, 1.2 U glycerophosphate dehydrogenase and 100–250 μl of plant extracts. A linear relationship between the amount of PPi and absorbance was obtained at least up to a concentration of 20 nmol of PPi. The mixture for assay of fructose-6-phosphate was the same as described in an earlier paper [17].

Results

Fluctuation of activities of PPi-PFK and ATP-PFK during growth

Maximum catalytic activities of PPi-PFK and ATP-PFK were determined in the extracts from cultures of *Catharanthus roseus* grown in batch suspension. The activity of PPi-PFK was always approximately 3 times higher than that of ATP-PFK (Fig. 2). The levels of both enzymes increased just after transfer of the cells into fresh medium, reached their maximum at day 3–4, and then decreased gradually.

In the similar cultures, the rate of respiration, estimated from uptake of O_2 by the cells also increased after inoculation and maximum rates were observed at day 2–3 [15].

Properties of purified PPi-PFK

The effect F6P and PPi on the activity of PPi-PFK, partially purified from 6-day-old *Catharanthus roseus*

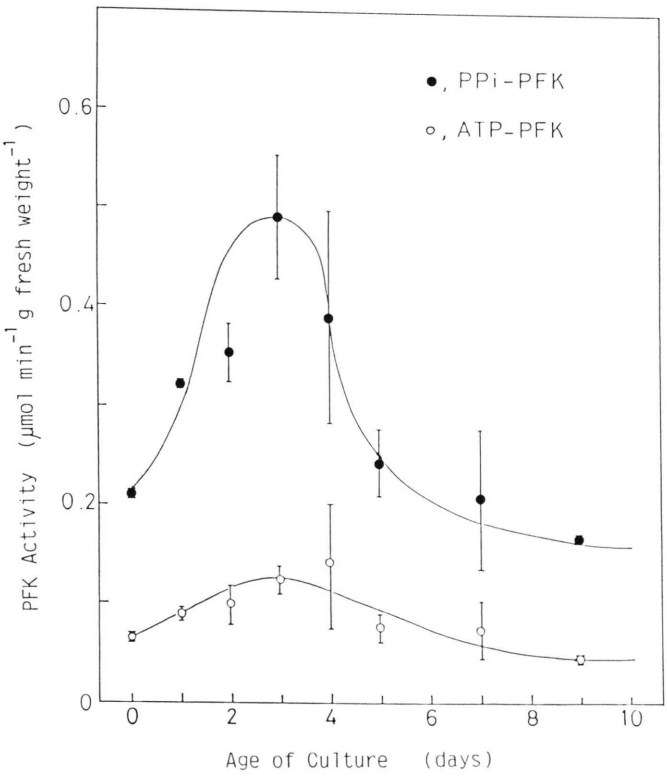

Fig. 2. Changes in activity of PPi-PFK (●) and ATP-PFK (○) during growth of *Catharanthus roseus* cells in batch suspension culture. The activity of enzymes is expressed as μmol of fructose-1,6-bisphosphate formed per min per g fresh weight. Vertical lines represent standard deviations of more than three determinations.

cells, is shown in Fig. 3. The PPi-PFK exhibited Michaelis-Menten type saturation curves with F6P and PPi. The apparent Km values for F6P and PPi were 0.59 mM and 48 μM, respectively, in the presence of 1 μM F2,6BP, at physiological pH (7.2). In the absence of F2,6BP, no activity of PPi-PFK was detected in the preparation of PPi-PFK. The effect of the concentration of F2,6BP on the activity of PPi-PFK is shown in Fig. 4. The saturation curve is hyperbolic, and the apparent K_a value of the enzyme for F2,6BP is 26 nM. The value was slightly higher than that of PPi-PFK from cultured soybean cells (17 nM), but the assay conditions were different in each case [18].

Intracellular levels of substrates for PPi-PFK

Levels of PPi and F6P, substrates of PPi-PFK, in cells at three different phases of growth were determined (Table I). Levels of F2,6BP, an activator of PPi-PFK, as reported in a previous paper [19], are also shown in Table I as reference. The levels of PPi and F6P varied from 17–71 and from 37–65 nmol per g fresh weight, respectively. The presence of PPi in plant cells was reported recently [20, 21], although it had been suggested that PPi is hydrolyzed immediately by pyrophosphorylase in plant cells. The level of PPi in *Catharanthus roseus* cells was almost the same as the level in maize seedlings [22], but slightly

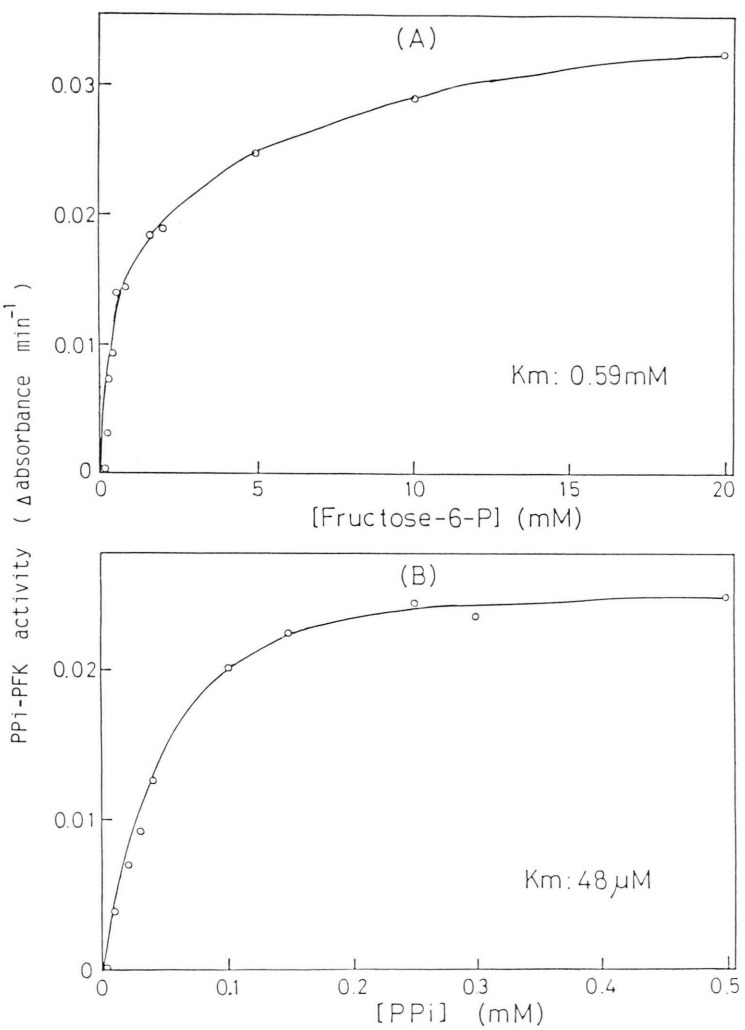

Fig. 3. Effect of F6P (A) and PPi (B) on the activity of PPi-PFK from *Catharanthus roseus* cells. The enzymatic activity was assayed as described in Materials and Methods, except that concentrations of F6P (A) and PPi (B) were varied as indicated. Initial velocity of the reaction was determined and is expressed as Δ absorbance at 340 nm per min.

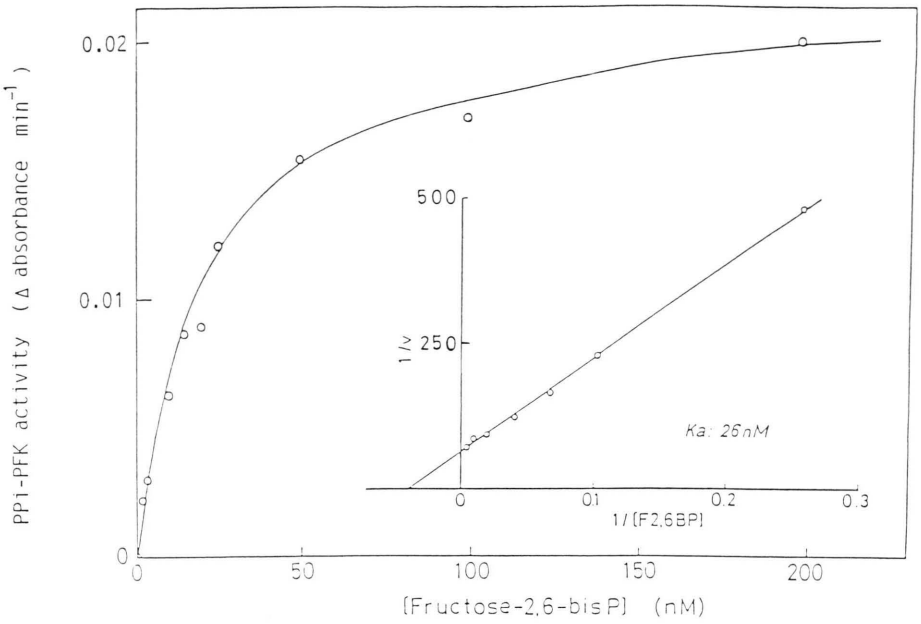

Fig. 4. Effect of F2,6BP on the activity of PPi-PFK from *Catharanthus roseus* cells. The enzymatic activity was assayed as described in Materials and Methods except that the concentration of F2,6BP was varied as indicated. Initial velocity of the reaction was determined and is expressed as Δ absorbance at 340 nm per min.

higher than that in pea seedlings [21, 22]. Intracellular levels of the substrates, as well as of F2,6BP, increased initially one day after transfer of cells in stationary phase (10-day-old cells) into fresh medium.

Table I. Levels of fructose-2,6-bisphosphate (F2,6BP), pyrophosphate (PPi) and fructose-6-phosphate (F6P) in *Catharanthus roseus* cells in batch suspension culture. The levels are expressed as nmol per g F. W. Estimated concentrations of these compounds were calculated with the assumption that cytoplasm comprises 5–10% of the volume of the cells. The Ka or Km values of PPi-phosphofructokinase for F2,6BP, PPi and F6P are also shown.

Compound	(day)	Content	Estimated Concentration [μM]	K_a or Km [μM]
F2,6BP	1	0.448 ± 0.109	4.48 – 8.96	0.026
	4	0.038 ± 0.016	0.38 – 0.76	
	10	0.145 ± 0.019	1.45 – 2.90	
PPi	1	70.8 ± 14.1	710 – 1420	48
	4	49.6 ± 1.4	500 – 1000	
	10	17.3 ± 12.7	170 – 340	
F6P	1	65.1 ± 16.8	650 – 1300	590
	4	62.4 ± 22.8	620 – 1240	
	10	36.7 ± 30.8	370 – 740	

The concentration of these compounds was calculated in accordance with the following assumptions. (i) The cytoplasm comprises 5–10% of the total volume of the cell as suggested by Meyer and Wagner [20] and by Edwards *et al.* [21]. (ii) These compounds are located in cytoplasm. Estimated concentrations of F2,6BP and PPi were much higher than these of the K_a value of PPi-PFK for F2,6BP and the Km value for PPi (Table I). The concentration of F6P was almost equivalent to the Km value of PPi-PFK for F6P (Table I).

Discussion

The present results indicate that PPi-PFK with significant activity is present in *Catharanthus roseus* cells at any stage of culture. Furthermore, the estimated concentrations of F2,6BP, of potent activator of PPi-PFK, and of substrates, seems to be sufficient for activity of PPi-PFK *in vivo*. Therefore, PPi-PFK is very probably functional as an alternative enzyme in glycolysis in cultured cells of *Catharanthus roseus in vivo*. The maximum catalytic activity of PPi-PFK was always higher than that of ATP-PFK during culture (Fig. 1). As is the case with our results, the level

of PPi-PFK was approximately 4-fold higher than that of ATP-PFK in cultured cells of sycamore [23]. A higher level of PPi-PFK has been observed in a variety of higher plants recently, but some exceptions have also reported. Ashihara and Stupavska [8] found that PPi-PFK was not the predominant PFK activity in cotyledons of *Phaseolus mungo* at the early phase of germination when active alcoholic fermentation occurs. ap Rees *et al.* [24] also obtained results which strongly suggested that PPi-PFK made little contribution to glycolysis in clubs of *Arum maculatum* during thermogenesis. These findings suggest that PPi-PFK is less important in cells and organs which function exclusively in the production of energy. ap Rees *et al.* [24] proposed the hypothesis that PPi-PFK participates in glycolysis in tissues where there is significant biosynthetic activity. In *Catharanthus roseus* cells, the maximum level of activity of PPi-PFK was observed in 3-day-old cells (Fig. 1) where active biosynthesis was occurring. In fact, the maximum rate of incorporation of radioactivity from [U-^{14}C]sucrose into organic acids, amino acids and proteins was observed in cells at this stage [25]. These findings support the suggestion of ap Rees *et al.* [24]. PPi-PFK may contribute to the supply of respiratory intermediates for the synthesis of cellular components. In contrast to ATP-PFK, the activity of which is controlled by intracellular level of adenylate, PPi-PFK is not controlled strictly by the level of adenylate but probably by the level of F2,6BP. These intrinsic regulatory properties may suggest the different role of these enzymes.

Recently, Edwards and ap Rees [26] and Huber and Akazawa [23] speculated that sucrose synthetase makes a major contribution to the breakdown of sucrose by generation of UDP-glucose (reaction (8) in Fig. 5), which is converted to glucose-1-phosphate by UDP-glucose pyrophosphorylase (reaction (9)) with the PPi generated by the back reaction of PPi-PFK (reaction (5)). These metabolic events may also occur in *Catharanthus roseus*, but other mechanism also can be considered in our system. In *Catharanthus roseus*, in batch suspension, the rapid biosynthesis of nucleotides including UDP-glucose occurred 24 h after transfer of cells in stationary phase (10-day-old cells) to fresh medium [27]. A scheme for the possible relationship between glycolysis and the biosynthesis of pyrimidine nucleotides in *Catharanthus roseus* cells is shown in Fig. 5. The pathways of nucleotide biosynthesis include many pyrophos-

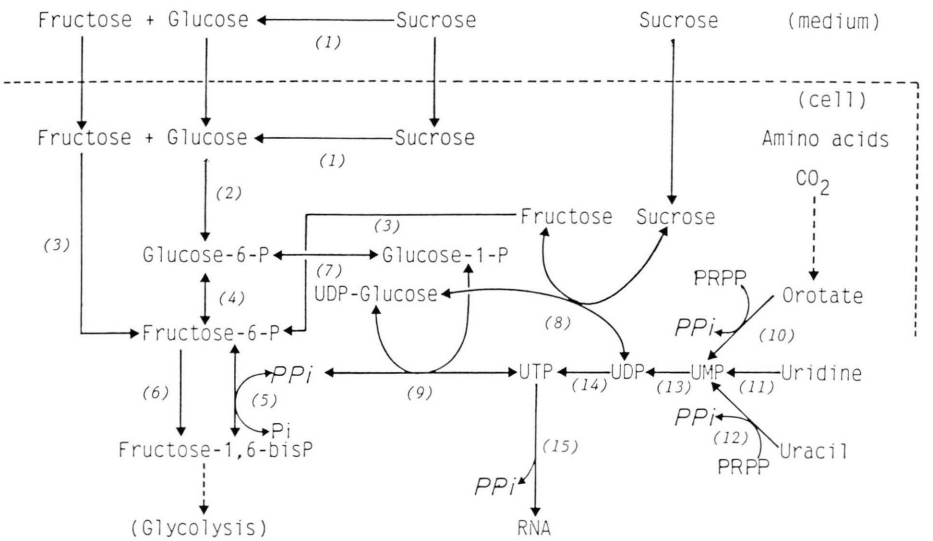

Fig. 5. Possible relationship between the alternative pathway of glycolysis and the biosynthesis of uracil nucleotides in cultured *Catharanthus roseus* cells. The numbers represent enzymes as indicated below. (1) invertase; (2) hexokinase (glucokinase); (3) fructokinase (hexokinase); (4) phosphoglucoisomerase; (5) PPi-PFK; (6) ATP-PFK; (7) phosphoglucomutase; (8) sucrose synthetase; (9) UDP-glucose pyrophosphorylase; (10) UMP synthetase (orotate phosphoribosyltransferase-orotidine-5-monophosphate decarboxylase complex); (11) uridine kinase; (12) uracil phosphoribosyltransferase; (13) nucleoside monophosphate kinase; (14) nucleoside diphosphate kinase; (15) RNA polymerase. PRPP: 5-phosphoribosyl-1-pyrophosphate.

phorylase reactions, *i.e.*, several phosphoribosyltransferases are involved in *de novo* or salvage pathways of purine and pyrimidine nucleotides (*e.g.*, reactions (10) and (12)) as well as UDP-glucose pyrophosphorylase (reaction (9)). The highest level of PPi, observed 24 h after inoculation of the cells (Table I), coincides with the highest level of UDPG-glucose in *Catharanthus roseus* cells [27]. Therefore, it seems plausible that PPi-PFK utilizes PPi which is produced as a byproduct of the rapid biosynthesis of nucleotides. Thus, this system seems to be reasonable in terms of conservation of energy by the cells.

In suspension cultures of carrot cells, sucrose in the culture medium was completely hydrolyzed by extracellular invertase to glucose and fructose, and these monosaccharides were taken up by the cells [28]. If the situation is the same in *Catharanthus roseus*, the role of PPi-PFK in the degradation of sucrose, which has been proposed for other tissues and cells [23, 26] may be less important in our system. Further studies to confirm our hypothesis are in progress.

Acknowledgements

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[1] N. W. Carnal and C. C. Black, Biochem. Biophys. Res. Commun. **86,** 20 (1979).
[2] D. C. Sabularse and R. L. Anderson, Biochem. Biophys. Res. Commun. **103,** 848 (1981).
[3] D. A. Smyth and C. C. Black, What's New Plant Physiol. **15,** 13 (1984).
[4] C. C. Black, D. A. Smyth, and M.-X. Wu, in: Nitrogen Fixation and CO_2 Metabolism (P. W. Ludden and J. E. Burris, eds.), p. 361, Elsevier, Amsterdam 1985.
[5] S. C. Huber, Annu. Rev. Plant Physiol. **37,** 233 (1986).
[6] E. Van Schaftingen, B. Lederer, R. Bartrons, and H.-G. Hers, Eur. J. Biochem. **129,** 191 (1982).
[7] E. Kombrink, N. J. Kruger, and H. Beevers, Plant Physiol. **74,** 395 (1984).
[8] H. Ashihara and S. Stupavska', J. Plant Physiol. **116,** 241 (1984).
[9] S. Kowalczyk, B. Januszewska, E. Cymerska, and P. Maslowski, Physiol. Plant. **60,** 31 (1984).
[10] M.-X. Wu, D. A. Smyth, and C. C. Black, Proc. Natl. Acad. Sci. USA **81,** 5051 (1984).
[11] T.-F. J. Yan and M. Tao, J. Biol. Chem. **259,** 5087 (1984).
[12] B. L. Bertagnolli, E. S. Younathan, R. J. Voll, C. E. Pittman, and P. F. Cook, Biochemistry **25,** 4674 (1986).
[13] B. L. Bertagnolli, E. S. Younathan, R. J. Voll, and P. F. Cook, Biochemistry **25,** 4682 (1986).
[14] F. C. Botha, J. G. C. Small, and C. de Vries, Plant Cell Physiol. **27,** 1285 (1986).
[15] I. Kanamori, H. Ashihara, and A. Komamine, Z. Pflanzenphysiol. **93,** 437 (1979).
[16] E. Van Schaftingen, in: Methods of Enzymatic Analysis 3rd ed., **Vol. 6,** (H. U. Bergmeyer, ed.), p. 335, Verlag Chemie, Weinheim 1984.
[17] T. Ukaji and H. Ashihara, Z. Naturforsch. **41c,** 1045 (1986).
[18] F. D. Macdonald and J. Preiss, Planta **167,** 240 (1986).
[19] H. Ashihara, Z. Naturforsch. **41c,** 529 (1986).
[20] R. Meyer and K. G. Wagner, Physiol. Plant. **65,** 439 (1985).
[21] J. Edwards, T. ap Rees, P. M. Wilson, and S. Morrell, Planta **162,** 188 (1984).
[22] D. A. Smyth and C. C. Black, Plant Physiol. **75,** 862 (1984).
[23] S. C. Huber and T. Akazawa, Plant Physiol. **81,** 1008 (1986).
[24] T. ap Rees, J. H. Green, and P. M. Wilson, Biochem. J. **227,** 299 (1985).
[25] M. Saito, T. Tokoro, and H. Ashihara, Beitr. Biol. Pflanzen, in press (1987).
[26] J. Edwards and T. ap Rees, Phytochemistry **25,** 2033 (1986).
[27] H. Sasamoto and H. Ashihara, Int. J. Biochem., in press (1987).
[28] J. Kanabus, R. A. Bressan, and N. C. Carpita, Plant Physiol. **82,** 363 (1986).