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Subcellular compartmentation of pyrophosphate and alkaline pyrophosphatase in leaves

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The subcellular localisation of pyrophosphate and alkaline pyrophosphatase in leaves has been studied using non-aqueous density gradient centrifugation of spinach leaves, and membrane filtration of wheat mesophyll protoplasts. The pyrophosphate was measured in extracts prepared in trichloroacetic acid, and could be quantitatively recovered from the leaf material. It was located predominantly in the cytosol, with a concentration of 0.2–0.3 mM. In contrast, the alkaline pyrophosphatase was largely, if not, exclusively, located in the chloroplast. By comparing the pyrophosphate levels in the cytosol with previously published data on the cytosolic levels of phosphate and metabolic intermediates, it is shown that the reactions catalysed by pyrophosphate: fructose-6-phosphate phosphotransferase and UDP-glucose pyrophosphorylase are close to the thermodynamic equilibrium and, thus, freely reversible in vivo. Comparison of the pyrophosphate levels with the reported electrical and pH gradient across the tonoplast membrane shows the free energy released during pyrophosphate hydrolysis is similar to that required to move a proton across the tonoplast membrane. It is suggested that pyrophosphate could operate as a secondary energy donor in the cytosol of plant cells.

Introduction

It has been widely accepted that hydrolysis of PP_i provides the driving force for a wide range of biosynthetic polymerisations, during which a precursor is activated by forming a nucleotide-diphospho derivative [1,2]. However, it is also clear that PP_i can act as an energy donor in some specialised microorganisms, including *Rhodospirillum rubrum,* where it is actively synthesised during photosynthetic electron transport [3,4]. The aim of the following article is to show that PP_i is present in the cytosol of plant cells at concentrations which would allow it to act as an energy donor.

It is known that plant cells contain at least three enzymes which, potentially, could use PP_i as an energy source. It has been proposed that UDP-glucose pyrophosphorylase operates the catalyse the formation of Glc1P and UTP from UDPGlc and PP_i in tissues which are degrading sucrose via sucrose synthase [4,5]. More recently it has been shown that the enzyme pyrophosphate–fructose 6-phosphate 1-phosphotransferase is widely present in plants [6,7]. This enzyme is activated by $Fru2,6P_2$, and catalyses the reversible

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Abbreviations: Fru6P, fructose 6-phosphate; $Fru1,6P_2$, fructose 1,6-bisphosphate; P_i, inorganic phosphate; PP_i pyrophosphate.

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phosphorylation of Fru6P using PP_i as a phosphoryl donor. It has also recently been shown that the bounding membrane of the vacuole, termed the tonoplast, not only possesses an ATP-dependent proton pump but also contains a PP_i-dependent electrogenic proton pump [8,9]. The potential significance of PP_i is also underlined by two recent reports which show that PP_i is present in a wide variety of plant tissues [10,11].

Although these findings are consistent with the idea that PP_i could be involved in energy metabolism in plants, they do not provide conclusive evidence. It has not yet been demonstrated that the PP_i concentration is high enough to allow it to act as an energy donor in vivo. An additional complication is introduced by the compartmentation of metabolism between the plastid and cytosol [12–14]. Although the reactions discussed above require a supply of PP_i in the cytosol, it was still unknown in which subcellular compartment the PP_i is localised. Further unresolved problems are posed by the very high activities of pyrophosphatases in plant tissues [12]. These will need to be regulated, or separated from PP_i by compartmentation, if PP_i is to act as an energy donor. Indeed, the alkaline pyrophosphatase activity is high in chloroplasts [12] and it has recently been shown that much, or even all, of the alkaline pyrophosphatase in non-photosynthetic soybean cell suspension cultures is present in the plastids [15]. These results are consistent with PP_i being present in the cytosol, rather than the plastid. However, other studies have suggested that there could be considerable pyrophosphatase activity outside the chloroplasts in spinach leaves [16].

We have therefore investigated the subcellular distribution of PP_i, using a recently developed method for the nonaqueous density gradient centrifugation of spinach leaves [17]. We have also studied how pyrophosphatase activities are distributed using the above method, and an independent technique in which protoplasts are fractionated by passing them through a nylon net and a series of membrane filters to absorb different organelles [18]. These studies were carried out in leaves, because more is known about the subcellular metabolite levels in leaves than in non-photosynthetic plant tissues, and this should make it easier to assess the potential contribution of PP_i.

Methods

Spinach was grown as in Ref. 17. Material for metabolite analysis was quenched by submerging it in liquid N_2 in an open mirrored container in the prevailing illumination conditions. Wheat was grown as in Ref. 18.

PP_i was extracted in trichloroacetic acid, using a method modified from Ref. 19. Spinach leaf material (0.3 g) was reduced to a fine powder in liquid N_2 in a mortar and pestle standing on dry ice in a polystyrene container, before 1.5 ml 16% (w/v) trichloroacetic acid in diethylether (precooled to the temperature of dry ice) was added and the homogenisation continued. After standing for 15 min on dry ice, the mortar was transferred onto ice at 4°C and 0.8 ml of a 16% (w/v) trichloroacetic acid solution in water containing 5 mM EGTA was added. The mixture was further homogenised, transferred to a 2 ml centrifuge tube, and held at 4°C for 3 h to achieve a complete denaturation of the pyrophosphatase. The extract was then centrifuged for 5 min and the lower (water) phase removed via a fine injection needle. The water phase was washed 3 times by shaking with 0.8 ml water-saturated ether and recentrifuging, neutralised with a small volume of a 5 M KOH/1 M triethanolamine mixture, treated with active charcoal to remove compounds which interfered with the assay, and then frozen in liquid N_2 until assay.

PP_i was assayed as described in Ref. 10, except that a ZFP 22 Sigma dual wavelength photometer was used to increase sensitivity (100-fold expansion), and the assay conditions were adjusted for this more sensitive apparatus and included 50 mM Tris/acetate (pH 8.0), 2 mM magnesium acetate, 1 mM Fru6P, 17 μM NADH, 0.45 $U \cdot ml^{-1}$ aldolase, 1.7 $U \cdot ml^{-1}$ glycerin-3-phosphate dehydrogenase, 5.0 $U \cdot ml^{-1}$ triose phosphate isomerase. The reaction was started by addition of 0.1 $U \cdot ml^{-1}$ pyrophosphate, fructose 6-phosphate 1-phosphotransferase (Sigma chemicals) from *Propionibacterium freudenreichii*. All cuvettes, glassware and active charcoal were washed with 0.1 M HCl before use, as preliminary experiments showed that all could be easily contaminated by PP_i which is not removed by washing in detergent. A control reaction was routinely carried out to correct for contamination.

Non-aqueous fractionation of spinach leaves was carried out as in Ref. 17. It is essential for the recovery of PP_i that water is totally excluded during such gradients. The contamination of gradients by water leads to the appearance of a green colouration in the upper fractions, which should be light yellow, and such gradients were discarded. Protoplasts were prepared from wheat leaves and fractionated using membrane filtration exactly as described in Ref. 18.

Pyrophosphatase was assayed as in Ref. 15. The identity of the PP_i was checked by showing that it could be removed by alkaline pyrophosphatase. Marker enzymes during nonaqueous fraction density gradient fractionation were assayed as in Ref. 17, and fumarase was assayed as in Ref. 18. The estimation of the subcellular distribution of PP_i and pyrophosphatase was carried out as in Refs. and 17 and 20. The partial purification of pyrophosphatase from spinach leaves by FPLC was carried out as in Ref. 21.

The activities of enzymes and content of PP_i are all expressed in terms of chlorophyll content. To do this, a conversion factor was used in which 1 mg chlorophyll corresponds to 27 mg protein. Protein and chlorophyll were measured as in Ref. 17.

Results

We first investigated the reliability of the methods for extracting PP_i. Previous studies have used $HClO_4$ to prepare extracts for plants for PP_i analysis [10,11], but preliminary experiments showed that this method was inadequate when used with spinach leaves. Even after treatment with 20% (v/v) $HCLO_4$ for 3 h, there was still enough pyrophosphatase activity to hydrolyse all the endogenous PP_i within minutes after reneutralising the extracts. In contrast, extraction with trichloroacetic acid led to a complete inactivation of the endogenous pyrophosphatase. To confirm the reliability of the extraction with trichloroacetic acid, experiments were carried out in which duplicate pairs of extracts were prepared, one of each pair receiving a small amount of PP_i (equivalent to 10 nmol per mg Chl) in the killing mixture of 16% trichloroacetic acid in diethylether. Following extraction and assay of the parallel samples, $93 \pm 4\%$ ($n = 5$) of the added PP_i was recovered. In comparison, a very variable recovery of between 5 and 80% was achieved when extracts were prepared using a killing treatment of 3 h in 10% (v/v) $HClO_4$. Experiments were also carried out to check that PP_i was not being generated by hydrolysis of nucleotide triphosphates during the acid extraction. To do this ATP was added at levels equivalent to 330 nmol per mg Chl in the killing mixture, which is a 5-fold excess over the endogenous content of leaves [18]. Comparison of the PP_i content of these extracts with those which received no ATP showed that no additional PP_i was generated by hydrolysis of ATP (data not shown).

The diurnal changes of PP_i in spinach leaves on plants in a 9 h light/15 h dark cycle are shown in Table I, revealing that the content of PP_i is constant at 4–5 nmol mg per Chl. The 10% increase after illumination and decrease during the photoperiod are not statistically significant and are much smaller than the changes of other metabolites in spinach leaves during these treatments [22,23].

The subcellular location of PP_i was studied in the experiments of Fig. 1 and Tables II and III. Spinach leaf material was quenched and powdered in liquid N_2, lyophilised at $-55\,^{\circ}C$, ultrasonicated in a heptane-carbontetrachloride mixture and subjected to centrifugation in a density gradient of hexane and carbon tetrachloride. The enzymes and metabolites in a given subcellular compartment precipitate together as the cell tissue is lyophilised, and the fragments generated by sonication differ in their density, depending upon the protein, lipid and salt constituents of the cell

TABLE I

LEVELS OF PP_i IN SPINACH LEAVES

The results are given as the mean ± SE ($n = 4$) of two separate experiments in each of which four extracts were prepared from separate plants. The spinach plants were grown in a 9 h light/15 h dark cycle.

	PP_i (nmol per mg Chl)			
	14.5 h dark	0.5 h light	8.5 h light	0.5 h dark
Experiment I	4.2 ± 0.3	4.5 ± 0.3	4.7 ± 0.5	4.7 ± 0.4
Experiment II	4.3 ± 0.2	5.0 ± 0.2	3.8 ± 0.2	4.0 ± 0.1

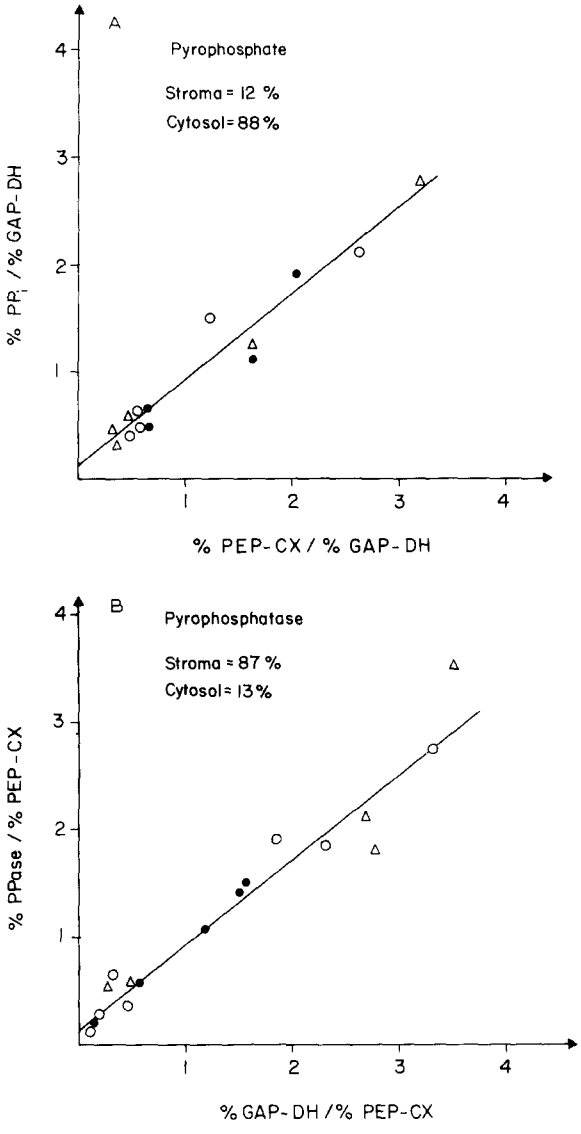

Fig. 1. Subcellular localisation of PP_i and alkaline pyrophosphatase in spinach leaves, studied by nonaqueous density gradient centrifugation. Spinach leaves were frozen in liquid N_2 and subjected to nonaqueous density gradient centrifugation. Six fractions were collected from the gradient and extracted for parallel measurements of marker enzymes and PP_i or pyrophosphatase. The distribution is shown in Tables II and III. (a) Estimation of regression coefficient between PP_i and phospho*enol*pyruvate carboxylase (PEP-CX). The calculation is described in Ref. 20. For each enzyme (or PP_i) the activity in each fraction is expressed as a percentage of that in the total gradient. The ratios of PEP-carboxylase : NADP-GAPDH and PP_i : NADP-GAPDH are then plotted for each fraction. NADP-GAP-DH, NADP-dependent glyceraldehyde 3-phosphate dehydrogenase). The plot shows the result of three separate gradients, and a regression line was fitted by computer (regression coefficient = 0.98). At the intercept on the *y*-axis, the ratio of PEP-carboxylase : NADP-GAPDH is zero, representing an extrapolation to uncontaminated chloroplast stroma. This intercept at the *y*-axis therefore yields the percentage of the PP_i which is in the stroma. (b) Estimation of regression coefficient between pyrophosphatase and NADP-GAPDH. The calculation is as above, except that the reverse ratios of NADP-GAPDH : PEP-carboxylase and pyrophosphatase : PEP-carboxylase are plotted. In this plot, the intercept at the *y*-axis yields the fraction of the pyrophosphatase activity in the cytosol, as at this point the ratio of NADP-GAPDH : PEP-carboxylase is zero, representing an extrapolation to pure cytosol. The regression coefficient was 0.98.

region from which they derive [17]. Following centrifugation, the gradient is divided into six fractions each having an approximately equal protein content. An aliquot of each fraction was used to measure the distribution of marker enzymes for the chloroplast stroma (NADP-dependent glyceraldehyde 3-phosphate), the cytosol (phospho*enol*-pyruvate carboxylase) and the vacuole (α-mannosidase). As shown in Table II, the chloroplasts are located at the top and in the middle of the gradient, the cytosol is particularly enriched in fraction 5, and the vacuoles in fraction 6. The distribution of PP_i resembled that of phospho*enol*-pyruvate carboxylase, suggesting that most of the PP_i is in the cytosol.

The reliability of this method depends upon the PP_i not having been lost during the processes of lyophilisation and centrifugation. The PP_i/protein ratio was therefore measured in a crude trichloroacetic acid extract of a leaf, in lyophilised material, and the summed fractions collected from the gradient. Values of 0.17, 0.17 and 0.18 nmol PP_i per mg protein were measured at each stage, showing that the PP_i content has been preserved throughout the lyophilisation and fractionation. The nonaqueous density gradient centrifugation also assumes that PP_i has not redistributed during the procedure. This seems unlikely, as any redistribution would be expected to lead to PP_i appearing in the top fractions of the gradient, which corresponds to the volume in which the extract is added to the preformed gradient. Fraction 1 contained only low levels of PP_i, except in experiments where the gradients were contaminated with H_2O (see Methods).

TABLE II

DISTRIBUTION OF PP_i AND MARKER ENZYMES DURING NONAQUEOUS GRADIENT CENTRIFUGATION OF LYOPHILISED SPINACH LEAVES

Spinach leaves were frozen in liquid N_2, homogenised in liquid N_2, lyophilised, ultrasonicated and subjected to nonaqueous gradient (CCl_4/hexane) centrifugation. Six fractions were collected from the gradient and extracted for parallel measurements of marker enzymes and PP_i. The results are given as a % of the sum of the total activity (or amount of PP_i) recovered, from the gradient. NADP-GAPDH, NADP-dependent glyceraldehyde 3-phosphate dehydrogenase; PEP, phospho*enol*pyruvate.

	% of total in each individual fraction					
	I	II	III	IV	V	VI
NADP-GAPDH (stroma)	13	18	19	22	15	13
PEP carboxylase (cytosol)	4	7	8	13	48	22
α-mannosidase (vacuole)	2	2	3	7	30	55
PP_i	8	6	9	15	44	18

TABLE III

DISTRIBUTION OF ALKALINE PYROPHOSPHATASE AND MARKER ENZYMES DURING NONAQUEOUS GRADIENT CENTRIFUGATION OF LYOPHILISED SPINACH LEAVES

Spinach leaves were quenched and subjected to nonaqueous density gradient centrifugation as in Table II. The distribution of marker enzymes and alkaline pyrophosphatase, assayed at pH 8.0 with 10 mM $MgCl_2$, are given as a percentage of the total activity recovered from the gradient. The distribution of enzyme activities between the fractions differs from Table II because the gradient was divided in a different way. NADP-GAPDH, NADP-dependent glyceraldehyde 3-phosphate dehydrogenase; PEP, phospho*enol*pyruvate.

	% of total in each individual fraction					
	I	II	III	IV	V	VI
NADP-GAPDH (stroma)	26	29	19	18	6	2
PEP carboxylase (cytosol)	14	9	9	28	28	10
α-mannosidase (vacuole)	2	3	4	18	24	51
citrate synthase (mitochondria)	16	15	12	31	18	9
Alkaline pyrophosphatase	27	26	16	19	10	2

In Fig. 1A, regression analysis [17,20] has been used to estimate how PP_i is distributed between the cytosol and stroma. To do this, it is assumed that negligible PP_i is present in the mitochondria and vacuoles. The results of Table II provide support for this assumption, as the PP_i present in the fractions containing the major peak of mitochondria (fraction 4, see Table III) and vacuoles (fraction 6, see Table II) can be accounted for by the material deriving from the cytosol in these fractions. The regression plot shown in Fig. 1A reveals that about 90% of the PP_i is in the cytosol, with a regression coefficient of 0.98 (see figure legend and Ref. 20 for details).

We then investigated the distribution of pyrophosphatase. At pH 8 in the presence of 10 mM Mg^{2+}, a total pyrophosphatase activity of 8–9 μmol PP_i per mg Chl per min was found at 25 °C. As shown in Table III, this pyrophosphatase activity shows a very different distribution to that of PP_i, following closely the activity of the chloroplast stroma marker NADP-dependent glyceraldehyde 3-phosphate. Estimation of the regression coefficient (Fig. 1B) suggests that at least 90% of the alkaline pyrophosphatase activity is in the chloroplast stroma. This method does not allow a clear conclusion about whether the remaining 10% is located outside the chloroplast, due to technical difficulties in measuring the low activities in some of the fractions, and also because of assumptions which are involved in studying whole leaves, which are a heterogenous collection of cells.

Two further approaches were taken to provide additional evidence that most, if not all, of the alkaline pyrophosphatase is located in the plastids. Firstly, unfractionated spinach leaf extract was fractionated by FPLC on a mono Q column to ascertain whether any minor peaks appeared which could be attributed to isoenzymes located in compartments outside the chloroplast. The entire applied activity eluted in one narrow band at about 20 mM NaCl (not shown). Secondly, wheat leaf mesophyll protoplasts were prepared and subjected to fractionation by membrane filtration [18]. This technique allows the preparation of a fraction containing almost all the cytosol with very little plastid contamination.

The distribution of marker enzymes and pyrophosphatase in different fractions obtained by

membrane filtration is shown in Table IV. The principle of this filtration method is as follows. Protoplasts are disrupted to release intact chloroplasts and mitochondria by passing them through a fine nylon net, and the different organelles are then selectively removed on membrane filters placed behind this nylon net to yield a series of filtrates from which different subcellular compartments have been removed. In one combination, the filters are omitted and the filtrate therefore corresponds to the entire protoplasts (designated F_{total}). In a second combination a 8 μm filter is included to remove the chloroplasts, yielding a filtrate which corresponds to the mitochondria, cytosol and vacuole, with about 10–20% chloroplast contamination (F_1). A third combination includes an 8 μm and a 0.3 μm filter, to remove most of the chloroplasts and mitochondria and yield a fraction corresponding to the cytosol and vacuole (F_2). A fourth filtration is also carried out in which the protoplasts are passed very slowly (1–2 min) through a filter holder containing a net. The intact protoplasts are retained with minimal breakage on the nylon net (F_{med}), and this filtration provides a control that there are not large amounts of broken protoplasts or other contaminants in the medium.

Following this treatment, the distribution of alkaline pyrophosphatase assayed at pH 8 was essentially identical with the distribution of the chloroplast marker, NADP-dependent glyceraldehyde 3-phosphate. Clearly, if activity is associated with the cytosol or mitochondria this cannot be more than a minute fraction of the total activity in the protoplast.

It was also investigated whether pyrophosphatase activity was differently distributed when assayed at lower pH. These measurements could be complicated by non-specific hydrolysis of PP_i by phosphatases from the vacuole which lead to an Mg^{2+}-independent PP_i hydrolysis with a pH optima at 5 or below (data not shown). A pH value of 7 was selected to search for further potentially cytosolic pyrophosphatases because this corresponds to the pH in the cytosol [23], and because the rate of non-specific PP_i hydrolysis is still relatively low at this pH. At pH 7, the rate of PP_i hydrolysis in the unfractionated extract was 3–4-fold lower than at pH 8, but still distributed in a very similar manner to the chloroplast marker enzyme (Table IV). At a maximum, about 6% of the total activity could be located elsewhere, equivalent to an activity of about 150 nmol per mg Chl per min, and this is likely to be at least

TABLE IV

DISTRIBUTION OF MARKER ENZYMES AND PYROPHOSPHATASE IN FRACTIONS PREPARED BY MEMBRANE FILTRATION OF WHEAT LEAF MESOPHYLL PROTOPLASTS

Protoplasts were forced through a nylon net to disrupt them, and organelles were then selectively removed on an 8 μm (chloroplast) and a 0.3 μm (mitochondria) filter (see Ref. 18 for details). The filtrates are designated as follows: F_{total}, the whole protoplast suspension; F_1, cytosol and mitochondria; F_2, cytosol. F_{medium} is a control in which low speeds are used to filter off intact protoplasts to assess what activities are present in the suspending medium. The enzyme activity is given as a % of that in the unfractionated protoplasts, and the results are the mean ± S.E. of three separate experiments. Pyrophosphatase activity was assayed at pH 8 and at pH 7, both in the presence of 5 mM Mg^{2+}. The total activity of pyrophosphatase was 5.1 and 2.7 μmol PP_i per mg Chl per min at pH 8 and pH 7, respectively. PEP, phospho*enol*pyruvate; NADP-GAPDH, NADP-dependent glyceraldehyde 3-phosphate dehydrogenase.

Designation of the filtrate	Filters included (μM)	Speed of closure (s)	Enzyme activity as % of that in unfractionated protoplasts				
			PEP-carboxylase (cytosol)	Fumarase (mitochondria)	NADP--GAPDH (stroma)	Pyrophosphatase assayed at	
						pH 8	pH 7
F_{total}	–	1	89±3	82±4	86±1	97±1	100±1
F_1	8	1	82±1	65±3	23±2	23±2	29±2
F_2	8; 0.3	1	80±3	14±1	15±1	15±1	21±1
F_{medium}	8; 0.3	100	5±2	6±2	6±2	3±2	2±1

partly due to the Mg^{2+}-independent activity which appears at low pH.

Discussion

Our results emphasise that special precautions are required during the preparation of extracts for the measurements of PP_i, especially in photosynthetic tissues. In spinach leaves, the alkaline pyrophosphatase activity is high enough (8 μmol per mg Chl per min) to hydrolyse the entire endogenous PP_i (4–5 nmol per mg Chl) in 30 μs, and is very resistant to treatment with $HClO_4$. Even in tissues where $HClO_4$ can be used to inactivate the pyrophosphatase [10] the trichloroacetic acid extraction may be preferable because the acid killing mixture can penetrate the tissue at below −60 °C, due to the trichloroacetic acid being introduced in diethylether in the presence of dry ice. This reduces the danger that local thawing of the tissue during the addition of the killing mixture may allow assess of the pyrophosphatase to the PP_i before the pH decreases sufficiently to inhibit its activity.

These measurements confirm that PP_i is present in significant levels in plant tissues. The levels (4–5 nmol per mg Chl) would be about 10-fold lower than the overall level of ATP in leaf material [18]. Given that leaves contain about 1.4 mg Chl/g fresh weight, the levels of PP_i found in spinach leaves resemble those found previously in non-photosynthetic plant tissues [10,11]. Our results show that most, if not all, of this PP_i is located in the cytosol. Assuming a cytosolic volume of 15–20 μl per mg Chl^{-1} [17] allows a cytosolic concentration of 0.2–0.3 mM to be estimated.

This PP_i concentration can be maintained in the cytosol because most, if not all, of the pyrophosphatase activity is located in the plastids. This conclusion confirms the recent investigation using soybean cell suspension culture [15] but conflicts with an earlier study with spinach leaves [16]. However, this earlier study was carried out before methods for the preparation of intact chloroplasts were widely available, and the apparent presence of pyrophosphatase activity outside the chloroplast could be due to breakage of chloroplasts during the fractionation procedure. In the present paper, two basically different techniques were used to show that the vast majority of the pyrophosphatase activity is located in the palstids. The presence of non-specific hydrolysis of PP_i at acid and neutral pH makes it difficult to exclude the possibility that a small fraction of the pyrophosphatase activity at pH 7 could be in the cytosol. However, the maximum activities which could be present are about 0.15 μmol per mg Chl per min. This can be compared with rates of sucrose synthesis during photosynthesis of about 0.3 μmol per mg Chl per min [23]. Since one PP_i is generated in the cytosol per molecule of sucrose generated, it is clear that the maximum activities of pyrophosphatase in the cytosol are barely adequate to cope with the rate at which PP_i is being generated in spinach leaves.

The question now arises, whether a PP_i concentration of 0.2–0.3 mM is high enough to allow it to act as an energy donor in the cytosol. Given that the cytosol contains 10–25 mM P_i [23], hydrolysis of PP_i would be accompanied by a free energy release of about 14–20 $kJ \cdot mol^{-1}$ ($K_{eq} = 10^3$, Ref. 24). For comparison, the hydrolysis of ATP to ADP and P_i in the cytosol will be accompanied by a free-energy decrease of about 50 $kJ \cdot mol^{-1}$ in the presence of cytosolic ATP and ADP concentrations of 2 and 0.2 mM [18], a P_i concentration of 10–25 mM, and a $\Delta G'_0$ for ATP hydrolysis of 32 $kJ \cdot mol^{-1}$. Thus, the hydrolysis of PP_i will release less energy than hydrolysis of ATP in the cytosol.

The potential significance of PP_i is revealed, however, by comparing it with the concentrations of the other substrates and products of reactions which utilise PP_i. Table V summarises previously published information [17,22,23] about the cytosolic metabolite concentrations in the cytosol of spinach leaves, and Table VI shows the estimated in vivo molar mass action ratio for these reactions when they proceed in a PP_i-hydrolysing direction. No information is available on the diurnal changes or compartmentation of UTP, but an overall content of 34 nmol per g fresh weight has been reported [25], and it is assumed that this is restricted to the cytosol (20 μl per g fresh weight) and does not change during the day. Comparison of the equilibrium constants [1,2,6] reveals that the reactions catalysed by pyrophosphate–fructose 6-phosphate 1-phosphotransferase and UDP-glucose

TABLE V

SUMMARY OF CYTOSOLIC CONCENTRATIONS OF METABOLITES IN LEAVES

The results for PP_i are take from Table I and Fig. 1. The cytosolic levels of Fru6P, Fru1,6P_2, UDPGlc and Glc1P were determined by nonaqueous density centrifugation [16]. The P_i concentration in the dark was measured by NMR [23] and the concentrations in the light were estimated from the appearance of phosphate in phosphorylated intermediates on the assumption that P_i did not redistribute across the tonoplast. In all the calculations a cytosolic volume of 20 μl per mg Chl was assumed.

Conditions	Metabolite concentrations (mM)					
	PP_i	Fru6P	Fru1,6P_2	P_i	UDPGlc	Glc1P
Dark	0.3	0.9	0.02	25	1.6	0.6
30 min Light	0.3	1.4	0.1	15	1.6	0.6
8 h Light	0.3	3.0	0.2	10	2.1	1.2

pyrophosphorylase are close to equilibrium. This implies that the flux direction will depend upon the concentrations of their substrate and products, including PP_i, and also, in the case of pyrophosphate–fructose 6-phosphate 1-phosphotransferase [6,7] on the Fru2,6P_2 concentration.

The presence of a PP_i-dependent proton pump in plant vacuoles [8,9] means it is also of interest to compare the free energy released during pP_i hydrolysis in the cytosol with the pH and electrical gradient across the tonoplast membrane. The pH gradient over the tonoplast in leaves has been estimated by ^{31}NMR to be about 1.5 pH units [23], and may be larger in other tissues [27], while the electrical ($\Delta\psi$) gradient of 10–15 mV (positive in the vacuole) is typical for the tonoplast [27]. The free energy associated with the transfer of one proton across the tonoplast can be calculated as $\Delta G = RT \ln(H^{+\,vac}/H^{+\,cyt}) - zF\Delta\psi$, yielding a value of about 10 kJ per mol H^+. This value is only an approximation, and the stoichiometry of the PP_i-dependent proton pump is not known. Nevertheless, these estimates suggest that the hydrolysis of PP_i ($\Delta G = 14$–20 kJ · mol^{-1}) may be in near-equilibrium with the tonoplast energisation. This underlines the need for regulation of the ATP- and PP_i-dependent proton-pumping proteins and raises the possibility that simultaneous activity of both could, in some conditions, lead to generation of PP_i in the cytosol. This might be

TABLE VI

COMPARISON OF MASS ACTION RATIOS IN SPINACH LEAF CYTOSOL AND THE THEORETICAL EQUILIBRIUM CONSTANT OF REACTIONS UTILISING PP_i

The mass action ratios were estimated from the data in Table V, using a UTP concentration of 1.7 mM [25]. The equilibrium constant for UDPglucose phyrophosphorylase and pyrophosphate–fructose 6-phosphate 1-phosphotransferase (PFP) are from Refs. 1 and 25. The free energy for PP_i hydrolysis depends on the ionic conditions [26] and an equilibrium constant is given which was evaluated for conditions though to be typical for the cytoplasm of a yeast cell [24].

Enzyme		Mass action ratio			Equilibrium constant
		dark	30 min light	8 h light	
UDPGlc pyrophosphorylase	$\frac{[UTP][Glc1P]}{[UDP][PP_i]}$	2.4	2.4	3.7	3.2
PFP	$\frac{[Fru1,6P_2][P_i]}{[Fru6P][PP_i]}$	1.2	3.6	2.2	3.2
Pyrophosphatase	$\frac{[P_i]^2}{[PP_i]}$	0.48	1.3	3.0	1000

especially so in tissues where the pH gradient over the tonoplast is larger as, for example, during malate turnover in crassulacean acid metabolism plants or stomati [27].

Finally, the more general question arises why PP_i is conserved during sucrose synthesis, but is hydrolysed during starch formation in the plastid. One reason may be the different structural requirements during synthesis of these two carbohydrates. Starch synthesis involves the polymerisation of additional hexose units. One hexose P is utilised per glycolysidic bond formed and, as both energy-rich bonds in ATP are used to drive this reaction, PP_i is not conserved. In contrast, during the synthesis of a sucrose molecule, two hexose P are utilised to synthesise one glycosidic linkage in which the reducing groups of the hexose units are linked. This additional energy input may make it possible to conserve one of the energy rich bonds of UTP as PP_i. The contrast between starch and sucrose metabolism also suggests an explanation for the compartmentation of starch and sucrose metabolism in higher plants [12–14]. Thus, starch is synthesised in the plastid where PP_i is maintained low by an active pyrophosphatase. The plastid is also the unique site of many other biosynthetic pathways, including fatty acids [12]. In contrast, sucrose metabolism in the cytosol is characterised by the maintenance of a substantial pool of PP_i, and the cytosol contains several reactions which utilise or generate PP_i. It is intriguing that these reactions are apparently all close to equilibrium. This raises the possibility that the mobilisation of sucrose via sucrose synthase and UDP-glucose pyrophosphorylase, the interconversion of triose P and hexose P via pyrophosphate–fructose 6-phosphate 1-phosphotransferase, and the control of the cytosolic pH and tonoplast energisation might all be interconnected via the pool of PP_i. Further studies are needed to establish if this is indeed the case and, if so, to elucidate how these interlinked reactions are regulated.

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