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Fluoride leads to an increase of inorganic pyrophosphate and an inhibition of photosynthetic sucrose synthesis in spinach leaves

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Spinach leaf discs were floated on fluoride-containing solutions, and then extracted in the dark, or after 12–15 min illumination in saturating CO_2. Fluoride led to a 4–5-fold increase of pyrophosphate, accumulation of hexose phosphates and UTP, depletion of UDP glucose, increased fructose 2,6-bisphosphate, and a selective restriction of photosynthetic sucrose synthesis. The effect of fluoride on the light-saturation curve and the induction kinetics of photosynthesis are consistent with the decreased rate of sucrose synthesis making a significant contribution to the inhibition of photosynthesis found in these conditions. The sensitivity of sucrose synthesis to fluoride was compared to the inhibition of glycolysis at the classic site at enolase, by measuring the change of the 3-phosphoglycerate-to-2-phosphoglycerate ratio. Similar concentrations of fluoride are needed for both effects. Fluoride also led to a selective net loss of the uridine nucleotides, but not of adenine nucleotides, and also decreased the activation of sucrose phosphate synthase. These results are discussed in terms of the significance of pyrophosphate for the control of cytosolic metabolism in higher plants.

Introduction

Recent research suggests PP_i could operate as an energy donor in the cytosol of higher plants. They maintain a considerable pool of PP_i [1,2] which is restricted to the cytosol [3]. This contrasts with the plastids, which contain very high alkaline pyrophosphatase activity [3,4] and negligible PP_i [3]. Three reactions could utilise PP_i in the cytosol, namely (a) UDPGlc pyrophosphorylase when it operates in combination with sucrose synthase to degrade sucrose, (b) PFP when it operates in the glycolytic direction, and (c) a PP_i-dependent proton pump on the tonoplast membrane between the cytosol and the vacuole [5–8]. The potential significance of PP_i is emphasised by the widespread occurrence of these three enzymes in plants [1,2,5–8], and by the fact that PFP is the only authenticated site at which the regulator metabolite $Fru2,6P_2$ is known to act during respiratory metabolism in plants [6,7].

The precise function of PP_i, however, remains unclear. Since the above three reactions are near to their thermodynamic equilibrium in vivo [4], it is difficult to decide whether a particular reaction is consuming or generating PP_i. The situation is made even more complex by the fact that each of these reactions can be replaced by an alternative which does not require PP_i [6–8], viz sucrose breakdown via invertase and hexokinase, glycolysis via the ATP-dependent PFK, and tonoplast energisation via the ATP-dependent proton pump. We do not know why this doubling up has occurred; and it is difficult to separate the contribution of the ATP- and the PP_i-dependent enzyme in any given case. It is also striking that the PP_i level is very constant [1,4]; this suggests the PP_i concentration is tightly controlled, but it is not known why this should be so, nor how it occurs.

Photosynthetic metabolism provides a useful system to study PP_i metabolism because there is a rapid and easily defined flux through the PP_i pool. For each molecule of sucrose formed one molecule of PP_i is produced in the reaction catalysed by UDPGlc pyrophosphorylase. Unless this PP_i is removed sucrose

Abbreviations: Chl, chlorophyll; Fru1,6Pase, fructose 1,6-bisphosphatase; $Fru1,6P_2$, fructose 1,6-bisphosphate; $Fru2,6P_2$, fructose 2,6-bisphosphate; Fru6P, fructose 6-phosphate; Glc6P, glucose 6-phosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate; PEP, phospho*enol*pyruvate; 2-PGA, glycerate 2-phosphate; 3-PGA, glycerate 3-phosphate; $Ru1,5P_2$, ribulose 1,5-bisphosphate; SPS, sucrose phosphate synthetase; triose P, the sum of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate.

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synthesis will be inhibited and, eventually, photosynthesis will decrease because too little P_i is returning from the cytosol. In this article we will show (a) that fluoride leads to increased levels of PP_i in spinach leaf discs, (b) that this increased PP_i leads, via displacement of the hexose P and UDPGlc levels, to a disruption of sucrose synthesis, and (c) that the accompanying inhibition of photosynthesis is associated with changes of the light-saturation curve and induction kinetics which are typical for a P_i limitation of photosynthesis. These results provide the first information about what happens when PP_i metabolism is disrupted in plants.

Methods

Spinach (*Spinacia oleracea* U.S. Hybrid 424) was grown in hydroponic culture as in Ref. 9. Leaf discs (diameter, 1 cm) were cut from mature leaves at the end of the photoperiod and were incubated overnight in the dark on solutions of 0–20 mM NaF, including NaCl as a compensatory osmoticum so the total osmolarity was 40 milliosmoles. Preliminary experiments showed overnight incubation of discs did not alter the rate of photosynthesis, partitioning of photosynthate, or metabolite levels compared to material taken directly from the plant in the morning.

O_2 evolution was measured in a leaf disc O_2 electrode as in Ref. 10 and the discs were quenched in liquid N_2 under continued illumination at the same light intensity within 2 s of opening the electrode. Extracts were prepared in trichloroacetic acid and assayed for PP_i as in Ref. 4, in $CHCl_3/CH_2OH$ and assayed for $Fru2,6P_2$ as in Ref. 10, and for SPS as in Ref. 9 using 3 mM UDPGlc, 2 mM Fru6P, 10 mM Glc6P and 5 mM P_i in the assay to provide selective conditions for active and inactive forms of SPS (see Ref. 9). Triose P, $Fru1,6P_2$, 2PGA, $Ru1,5P_2$, ATP, ADP and AMP were measured as in Ref. 10. Care was taken to use fresh NADH solutions and to include a blank measurement when assaying AMP. 2PGA and PEP were assayed as for 3PGA, adding 0.4 $U \cdot m^{-1}$ phosphoglycerate mutase and 0.4 $U \cdot m^{-1}$ enolase. All assays was carried out using a Sigma ZFP 22 dual-wavelength photometer.

Uridine nucleotides were assayed by a new method, adapted from Ref. 11 to allow sequential assay of UDPGlc, UTP, UDP and UMP. The assay included 200 mM glycine (pH 8.7), 0.8 mM EDTA, 60 μM NAD^+ and 0.25 mM Glc1P, and was started by adding 0.008 U of UDPGlc dehydrogenase. When the reaction was complete, further additions of 50 μM ATP, 25 μM ADP and 2.8 mM MgCl, were made, followed by 0.04 U of UDPGlc pyrophosphorylase, 0.4 U nucleoside diphosphate kinase, and 0.05 U of nucleoside monophosphate kinase to measure UTP, UDP and UMP, respectively. Precautions are essential in this assay because UDPGlc dehydrogenase can be contaminated by nucleoside diphosphate kinase. This contaminating activity was suppressed by omitting ATP and Mg^{2+} during the slow (5–10 min) determination of UDPGlc, which was usually present in large amounts. When Mg^{2+} was added to allow activity of the subsequent enzymes, ADP was also included to suppress activity of this low contaminating activity during the UTP determination. UDPGlc pyrophosphorylase was pretreated with active charcoal to remove contaminating UTP in some commercial preparations. Using this assay, all the uridine nucleotides could be quantitatively recovered when added individually at the start of the assay. The blank reactions were negligible. This assay was only applicable in trichloroacetic acid extracts.

The reliability of the extraction of the uridine nucleotides was checked by carrying out extractions in which small representative amounts of metabolites were included in the trichloroacetic acid. The recovery (as percentage of that added) was 80 ± 2, 86 ± 2, 115 ± 12, and 110 ± 13 (mean $\pm$ SE, $n = 4$) for UDPGlc, UTP, UDP and UMP, respectively. Similar experiments were carried out for 2PGA and PEP, because these had not previously been measured in our leaf material. The recoveries were 89 ± 3 and 89 ± 3, respectively.

Sucrose and starch synthesis rates were measured by supplying $[^{14}C]CO_2$ to leaf discs for 15 min in the electrode and separating the insoluble and the neutral, acidic and basic soluble components as in Ref. 10.

For enzyme assays, spinach leaf extracts (50 μg Chl per ml) were prepared in 100 mM Hepes-KOH (pH 7.5), 7 mM $MgCl_2$, 1 mM EDTA, 50 μg per ml phenylmethylsulphonylfluoride, and 0.5% (w/w) defatted bovine serum albumin. Enolase was assayed in 100 mM Tris HCl (pH 8.1), 5 mM $MgCl_2$, 0.2 mM NADH, 1.1 mM ADP, 19 $U \cdot ml^{-1}$ lactate dehydrogenase, 2.7 $U \cdot ml^{-1}$ lactate dehydrogenase, 2.7 $U \cdot ml^{-1}$ pyruvate kinase, 2 mM KH_2PO_4 and the reaction was started by adding 1 mM 2PGA. UDPGlc pyrophosphorylase was assayed in 100 mM Tris-HCl (pH 8.1), 5 mM $MgCl_2$, 1 mM UDPGlc, 1 mM $NADP^+$, 5 μM $Glc1,6P_2$, 6.6 $U \cdot ml^{-1}$ phosphoglucomutase, 9 $U \cdot ml^{-1}$ glucose-6-phosphate dehydrogenase, and the reaction was started by adding 1 mM PP_i. The influence of fluoride was checked with and without 2 mM KH_2PO_4, and at saturating and limiting concentrations of both substrates.

The mass action ratios of enolase and UDPGlc pyrophosphorylase were estimated as PEP/2PGA and $(PP_i \times UDPGlc)/(Glc1P \times UTP)$, respectively, where PEP, 2 PGA, UDPGlc, Glc1P and UTP are the measured levels of metabolites in nmol per mg Chl.

Results and Discussion

Increase of PP_i

Spinach leaf discs were floated for 15–20 h in the dark and then illuminated (500 $\mu mol \cdot m^{-2} \cdot s^{-1}$) in

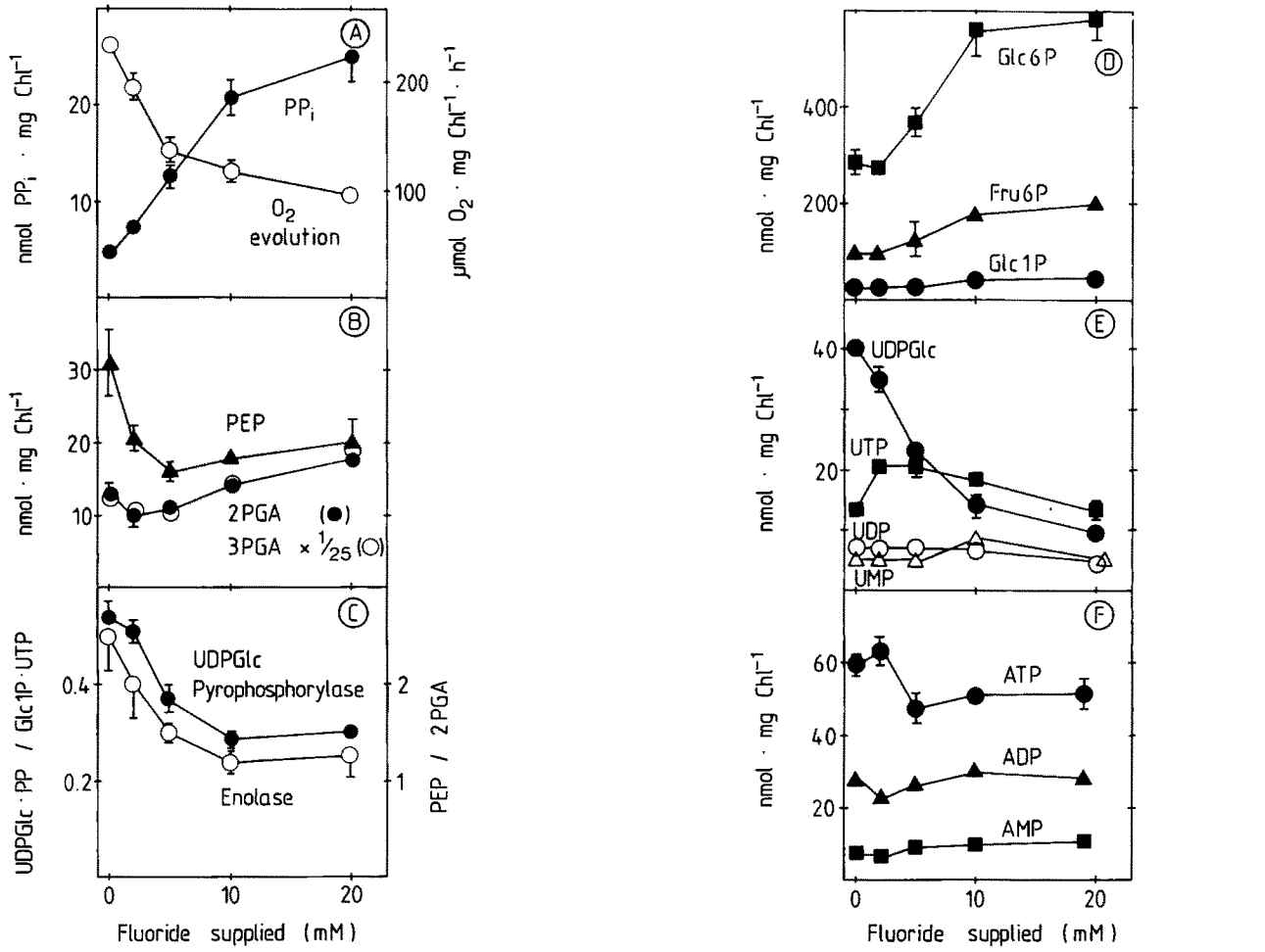

Fig. 1. Influence of fluoride on O_2 evolution, PP_i, uridine and adenine nucleotides, and other substrates of the reactions catalyzed by UDPGlc pyrophosphorylase and enolase in the light. (A) O_2 evolution (○) and Pp_i (●). (B) 2 PGA (●), 3 PGA (×1/25) (○) and PEP (▲). (C) Mass action ratio of enolase (○) and UDPGlc pyrophosphorylase (●). (D) Glc 1 P×20 (●), Glc 6 P (■) and Fru 6 P (▲). (E) UDPGlc (●), UTP (■), UDP (○) and UMP (△). (F) ATP (●), ADP (▲) and AMP (■). Results as mean ± S.E. ($n = 4$).

saturating CO_2 for 15 min before quenching metabolism in liquid N_2 (Fig. 1). Rising concentrations of fluoride led to a progressive inhibition of O_2 evolution. The leaf discs were then quenched in liquid N_2, and extracted and assayed for metabolites. Fluoride led to a 5-fold increase of PP_i (Fig. 1A). Parallel samples were quenched in the dark (Fig. 2). Again, fluoride led to increased PP_i, although the increase was only apparent at higher concentrations (Fig. 2A).

Mass action ratio of enolase

Before investigating the significance of the high PP_i levels, we investigated the effect of fluoride on enolase activity in these spinach leaves. We did this because fluoride is certainly a rather unspecific inhibitor, and an accumulation of PP_i would be of little interest if it only occurs at fluoride concentrations which are so high that other reactions have already been completely inhibited. Enolase was selected as a reference, because it is the classic site for inhibition of carbohydrate metabolism by fluoride [12]. To monitor the inhibition of enolase in vivo, we measured the changes of the substrate (2PGA) and product (PEP). Because 2PGA levels are low, we also measured 3PGA, which is in equilibrium with 2PGA via the phosphoglyceratemutase reaction. As expected, 3PGA and 2PGA changed in parallel when fluoride was supplied (Figs. 1B and 2B). In the light they remained low, while in the dark they increased to high levels, 3PGA reaching over 1000 nmol per mg Chl. Relative to PGA, there was a continuous decrease of PEP as fluoride increased. The resulting change of the mass action ratio (PEP/2PGA) for enolase is shown in Figs. 1C and 2C. Comparison with Figs. 1A and 2A shows that PP_i turnover and enolase activity have a comparable sensitivity to fluoride.

The concentrations of fluoride in the cytosol may be considerably lower than those which we supplied in the external medium. When enolase was assayed in an extract from spinach leaves, activity was 50% inhibited by 0.2 mM fluoride (data not shown). Enolase is inhibited by an $Mg^{2+} \cdot F^- \cdot F^- \cdot HPO_4^{2-}$ complex, and the sensitivity to fluoride depends on the Mg^{2+} and P_i

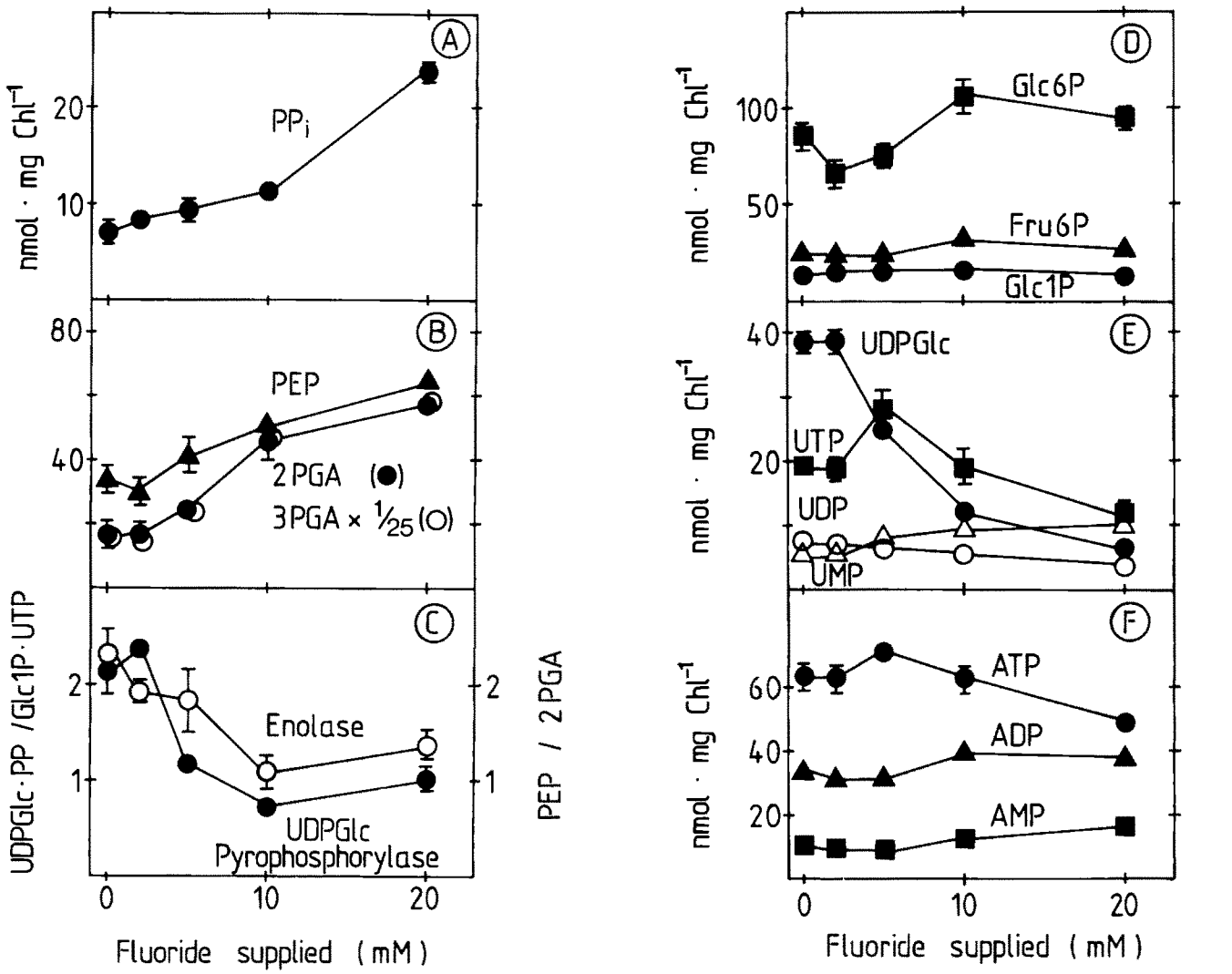

Fig. 2. Influence of fluoride on PP_i, uridine and adenine nucleotides, and other substrates of the reactions catalyzed by UDPGlc pyrophosphorylase and enolase in the dark. (A) PP_i. (B) 2 PGA (●), 3 PGA×1/25 (○) and PEP (▲). (C) Mass action ratio of enolase (○) and UDPGlc pyrophosphorylase (●). (D) Glc 1 P×20 (●), Glc6P (■) and Fru6P (▲). (E) UDPGlc (●), UTP (■), UDP (○) and UMP (△). (F) ATP (●), ADP (▲) and AMP (■). Results as mean ± S.E. ($n = 4$).

concentration [12]. Our assays were carried out with 5 mM Mg^{2+} and 2 mM P_i. Available measurement suggest the cytosolic P_i concentration might even be higher in leaves, especially in the dark [13]. No information is available on the Mg^{2+} concentration, but a concentration of 1–5 mM seems likely on comparative grounds [14,15]. Incidentally, spinach leaf enolase shows a typical sensitivity to fluoride. Sensitivity is expressed as the inhibition index i with $i = (\text{residual activity}/\text{inhibited activity}) \times [Mg^{2+}] \times [F]^2 \times [HPO_4^{2+}]$. Our results yield an inhibition index of $0.4 \cdot 10^{12}$ M^4, which compares well with the inhibition index for enolase from many other tissues [16].

Hexose phosphates and uridine nucleotides

Returning to the high concentrations of PP_i, we first investigated how they influence the reaction which produces PP_i during sucrose synthesis. Since the reaction catalysed by UDPGlc pyrophosphorylase is close to equilibrium [4], increased PP_i would be expected to lead to a decrease of the other product (UDPGlc) and an increase of the substrates Glc1P and UTP).

Hexose P increased 2–3-fold (Fig. 1D) in the light but by less in the dark (Fig. 2D). This can be compared with the large increase of PGA in the dark, rather than the light. These differences can be explained by the shifted relation between glycolysis and gluconcogenesis in the light and the dark. An inhibition of enolase is readily apparent during glycolysis in the dark, leading to a large accumulation of PGA. A restriction on hexose P removal is readily apparent during photosynthetic sucrose synthesis in the light.

There was a large decrease of UDPGlc in the light (Fig. 1E) and in the dark (Fig. 2E) and the UTP/UDPGlc ratio increased from 0.3 to 1.5. However, the results are complicated by a net decrease of the total

uridine nucleotides (see below). Although UTP increased at low fluoride concentrations, it decreased again at high fluoride concentrations (Figs. 1E and 2E).

The UTP/UDP ratios were generally somewhat higher than the ATP/ADP ratios, but both changed in parallel. In the dark (Fig. 3A) there was no evidence for a collapse of the energy status until over 10 mM fluoride was supplied. The ATP/ADP and UTP/UDP ratios even increased at 2 and 5 mM fluoride (Fig. 3A) and AMP decreases (Fig. 2F). This increase of the ratios at low fluoride cannot be explained at present, but it shows that there has not been a collapse of energy metabolism. The ATP/ADP and UTP/UDP ratios decreased at higher fluoride concentrations in the light (Fig. 3B); this will be discussed in more detail later. The high values for UTP/UDP ratios, compared to the ATP/ADP ratios, may be related to their subcellular location. The plastid and mitochondrial ATP/ADP ratios are much lower than the cytosolic ATP/ADP ratio [18]. These results could therefore be explained if a greater portion of the uridine nucleotides were in the cytosol.

Fig. 3C compares the summed totals of uridine nucleotides (UTP + UDP + UMP + UDPGlc) and adenine nucleotides (ATP + ADP + AMP) (see Figs. 1F and 2F for the original data about ATP. ADP and AMP). To estimate the total adenine nucleotides, the values in the dark were used when ADPGlc would be negligible [17]. There is no change in the total adenine nucleotide pool, but up to half of the uridine nucleotide pool is lost after supplying 20 mM fluoride (Fig. 3F). The adenine and uridine nucleotides represent two major energy-transfer systems in plants, with the uridine nucleotides being selectively associated with sucrose metabolism. It is intriguing that the total levels of both can be adjusted separately; further studies will be needed to establish whether fluoride acts directly on the metabolism of the uridine nucleotides, or whether it acts indirectly via changes of PP_i, or other perturbations in metabolism.

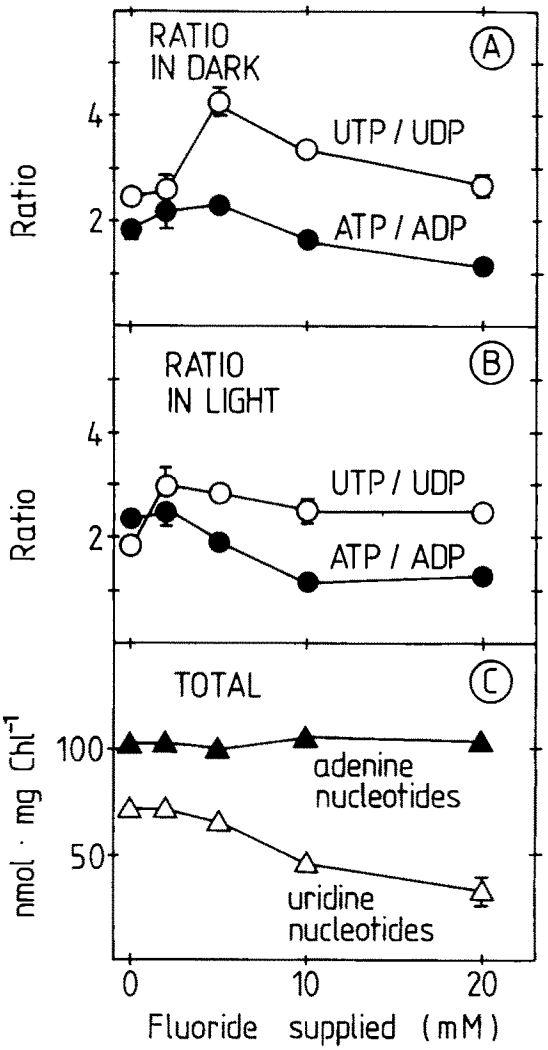

Fig. 3. Comparison of adenine and uridine nucleotides. (A) ATP/ADP (●) and UTP/UDP (○) ratios in the dark. (B) ATP/ADP (●) and UTP/UDP (○) ratios in the light. (C) Summed uridine nucleotides (UTP + UDP + UMD + UDPGlc, Δ) and adonine nucleotides (SATP + ADP + AMP, Δ). Results as mean ± S.E. ($n = 4$).

Mass action ratio of UDPGlc pyrophosphorylase

These results were used to estimate the mass action ratio for UDPGlc pyrophosphorylase (Fig. 1C), assuming the reactants are mainly restricted to the cytosol. As PP_i increases, the estimated mass action ratio decreased from 0.54 to 0.29. The estimated mass action ratios for UDPGlc pyrophosphorylase in the dark (Fig. 2C) were higher than in the light (note scale on Figs. 1C and 2C), but also decreased as PP_i accumulated.

Thus, a small displacement of the product/substrate relation may already be needed to drive the high flux across this enzyme during sucrose synthesis in the light. This displacement becomes even larger, after adding fluoride. This restriction is unlikely to be due to direct inhibition by fluoride. When UDPGlc pyrophosphorylase was assayed in extracts from spinach leaves, activity was not affected by 30 mM fluoride and was only 14% inhibited by 100 mM fluoride. Significantly, it is known that pyrophosphorylase from animals, fungi and plants is subject to non-competitive product-inhibition by PP_i [19]. Assuming a cytosolic volume of 20 μl per mg Chl [4], the PP_i concentration will be increasing from 0.3 to 1.3 mM. This compares with a typical Michaelis constant K_M (PP_i) of about 50 μM [19], and suggests that product inhibition and mass action may both contribute to the rise of hexose P and decrease of UDPGlc as PP_i accumulates. Depletion of uridine nucleotides will be an exacerbating factor at high fluoride concentrations. However, depletion of uridine nucleotides cannot be a major factor at 5 mM fluoride. At this concentration, PP_i doubled, hexose P and UTP both increased, and UDPGlc decreased, but the total uridine nucleotide pool was only marginally affected.

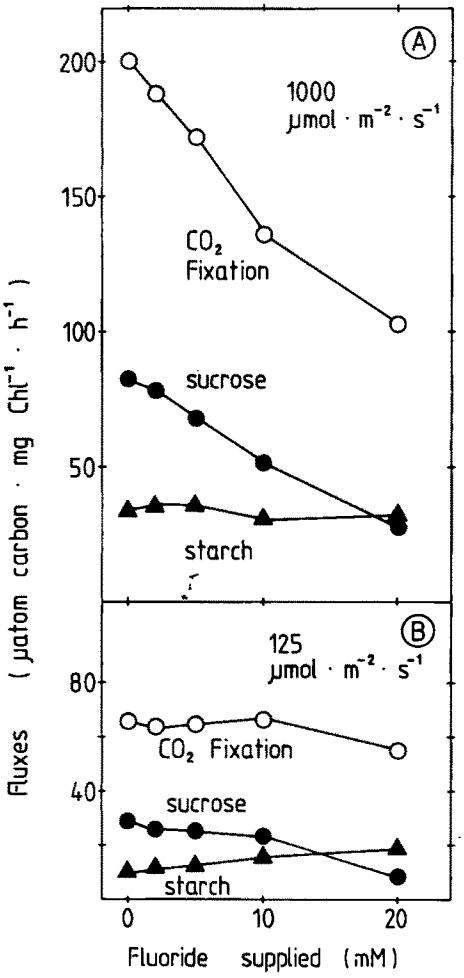

Fig. 4. Influence of fluoride on starch and sucrose synthesis. (A) Saturating light intensities (1000 μmol·m^{-2}·s^{-1}). (B) Limiting light intensities (125 μmol·m^{-1}·s^{-1}). ○, CO_2 fixation; ●, sucrose synthesis; ▲, starch synthesis. The S.E. was smaller than the symbol ($n = 4$).

Inhibition of sucrose synthesis

The question now arises, whether the increased PP_i and associated changes of UDPGlc and hexose P alter the rate of sucrose synthesis. Fig. 4 shows how sucrose and starch synthesis are modified by fluoride at high (1000 μmol · m^{-1} · s^{-1}) and low (125 μmol · m^{-2} · s^{-1}) light intensities. In both conditions, sucrose synthesis was decreased. Starch synthesis was unaffected in high light (Fig. 4A) and was even stimulated (Fig. 4B) in low light. This selective effect on sucrose synthesis is entirely consistent with an accumulation of PP_i in the cytosol.

Previous research has identified two important control points during photosynthetic sucrose synthesis [20], (a) the cytosolic Fru1,6Pase, which is controlled via metabolites and Fru2,6P_2, and (b) SPS, which is controlled via 'coarse' changes of the activity and via allosteric control by the Glc6P/Pi ratio. We investigated the response at these two control points, after supplying fluoride to spinach leaf discs. As photosynthesis is inhibited (Fig. 5A) there is the expected accumulation of hexose P and depletion of UDPGlc (Fig. 5B and C). This is accompanied by a decrease of SPS activity (Fig. 5D), an increase of Fru2,6P_2 (Fig. 5D) and a large increase of Fru1,6P_2 and triose P (Fig. 5E). PGA and Ru1,5P_2 decline slightly at 2 mM fluoride, and increase at higher fluoride concentrations (Fig. 5F). The amount of phosphate esterified in all these intermediates increased by over 800 nmol per mg Chl (Fig. 5G).

Three conclusions can be drawn about regulation of sucrose synthesis from these results. First, the precursors for sucrose synthesis (triose P and Fru1,6P_2) increase. This shows that sucrose synthesis is decreasing due to inhibition of reactions in the cytosol, rather than a decreased supply of photosynthate. Secondly, fluoride is not acting at the cytosolic Fru1,6Pase, because hexose P increase. Thirdly, fluoride is not just acting at SPS, because UDPGlc decreases 3–4-fold. It might also be noted that Fru1,6Pase and SPS are only marginally affected by 30 mM fluoride (Ref. 21; see also Stitt, M., unpublished data). We therefore suggest that SPS and the Fru1,6Pase are responding to charges in the concentrations of the substrates and products of the UDPGlc pyrophosphorylase reaction.

SPS has a relatively poor K_M for UDPGlc [9,21,22], and this affinity is not increased by the allosteric activator Glc6P [21], nor by the mechanisms involved in 'coarse' control of SPS [9,20]. Assuming a cytosolic volume of about 20 μl per mg Chl, the UDPGlc concentration will be decreasing from 2–3 mM down to 0.6–0.9 mM. This will restrict SPS activity, even though the second substrate (Fru6P) and an allosteric activator (Glc6P) both increase. It is also apparent that fluoride affects the 'coarse' control of SPS (Fig. 5F). The mechanisms involved in these changes are not known [9], so it is not possible to decide whether fluoride is acting directly or indirectly.

The flux at the cytosolic Fru1,6Pase will be decreased by the higher Fru2,6P_2 levels (Fig. 5G). The higher Fru2,6P_2 can be explained as a result of the high hexose P levels. It is known that Fru6P activates Fru6P,2-kinase and inhibits Fru2,6P_2ase [23]. Based on studies of Fru6P and Fru2,6P_2 levels during the photoperiod in spinach [24] and in *Clarkia xantiana* cytosolic phosphoglucose isomerase dosage mutants (Neuhaus, E., Kruckeberg, A.L., Feil, R. and Stitt, M., unpublished results), a 2.5-fold increase of Fru6P (Figs. 1D and 5C) would easily account for the 60% increase of Fru2,6P_2 (Fig. 5D).

Influence on photosynthesis

A decreased rate of sucrose synthesis will mean less P_i is recycled to the chloroplast. Recent research has shown that P_i-limitation of photosynthesis leads to characteristic changes in the light saturation curve and in

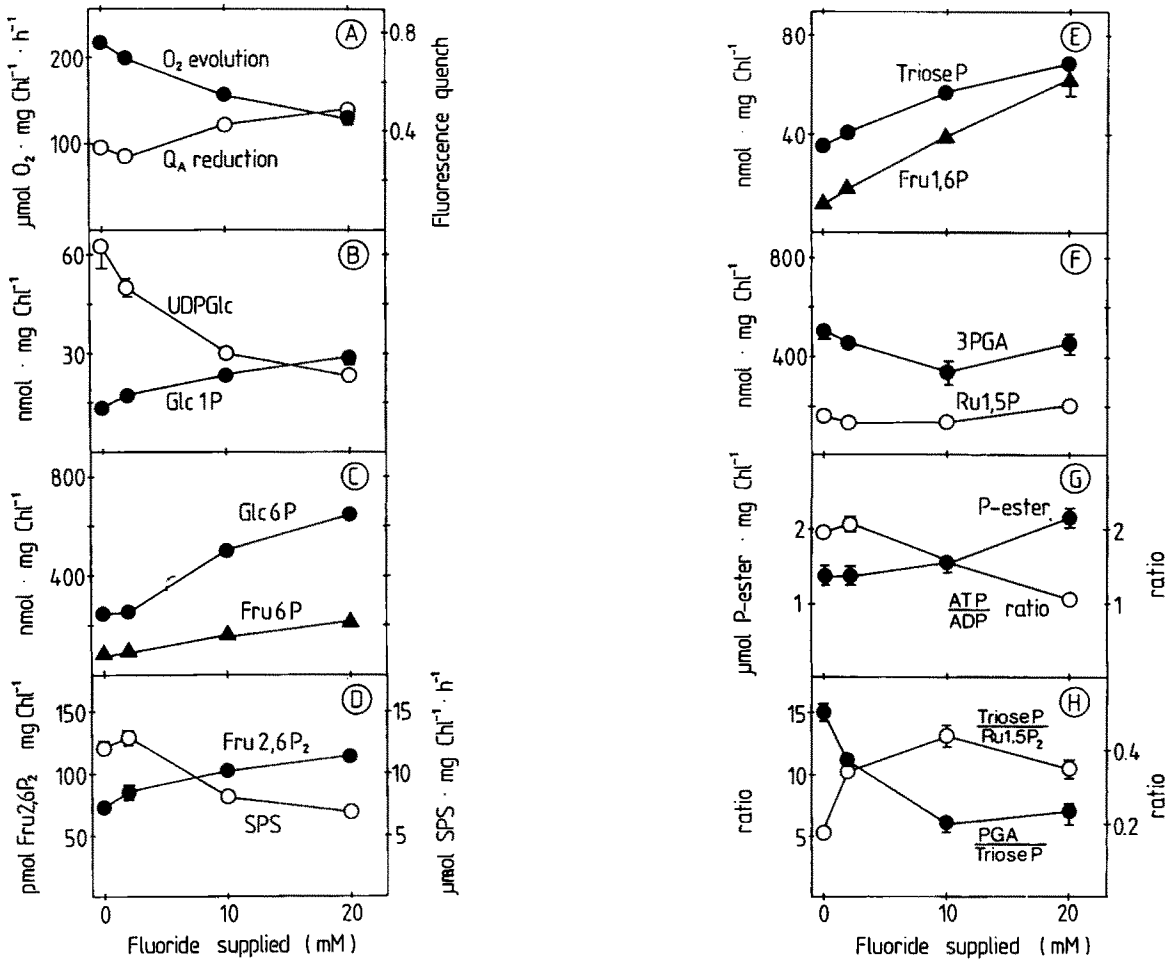

Fig. 5. Response of sucrose synthesis and photosynthesis to fluoride. (A) O_2 evolution. (B) UDPGlc (○) and Glc1P (●). (C) Glc6P (●) and Fru6P (▲), (D) Fru2,6P_2 (●) and SPS activity (○). (E) Fru1,6P_2 (▲) and triose P (●). (F) 3 PGA (●) and Ru1,5P_2 (○). (G) ATP/ADP ratio (○) and summed esterified phosphate (●). (H) 3PGA/triose P ratio (●) and triose P/Ru1,5P_2 ratio (○). Results as mean ± S.E. ($n = 4$).

the induction kinetics [25,26]. We therefore investigated how fluoride affected these parameters.

Moderate concentrations of fluoride inhibited photosynthesis at high light, but had little or no effect in low light (Fig. 6, see also Fig. 4). Thus, these concentrations of fluoride do not severely impair the efficiency of the light reactions, or ATP synthesis. This response is consistent with a P_i-limitation of photosynthesis, and resembles the effect of supplying mannose [25,26] or glycerol [27] to sequester P_i, or lithium [26] to inhibit the Fru1,6Pase. Fig. 4 illustrates why fluoride has little effect on the overall rate of photosynthesis in limiting light intensities. In low light (Fig. 4B), the inhibition of sucrose synthesis is accompanied by a stimulation of starch synthesis. This will allow recycling of P_i within the chloroplast, and photosynthesis is only slightly affected. In high light (Fig. 4A) starch synthesis is already occurring at high rates and cannot be increased to compensate for the inhibition of sucrose synthesis after

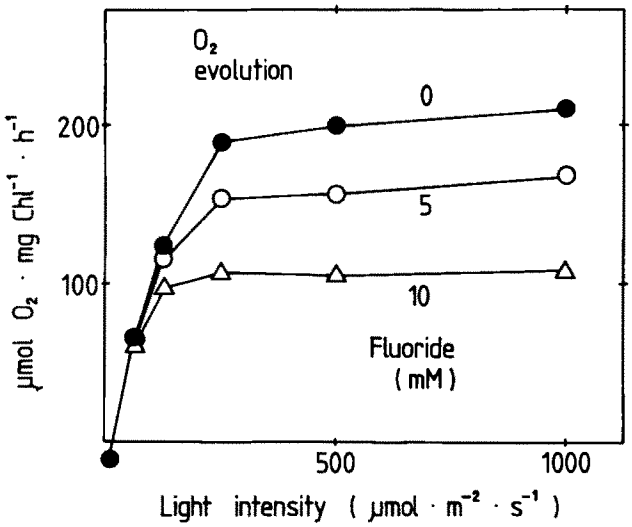

Fig. 6. Influence of fluoride on the light saturation response of photosynthesis. The leaf discs were pretreated with 10 mM NaCl (control), 5 mM NaF and 5 mM NaCl (○) or with 10 mM NaF (△). Photosynthesis was measured in saturating CO_2 at 15 °C.

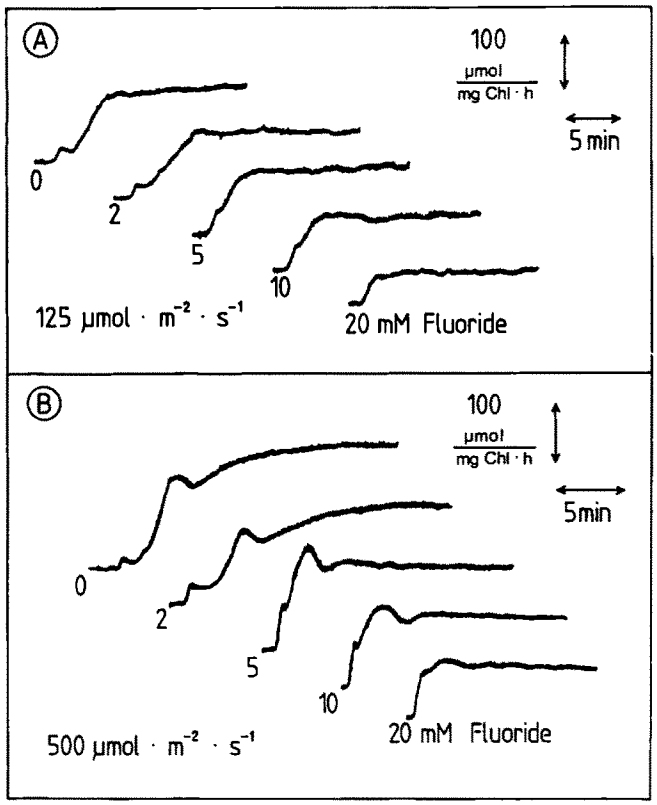

Fig. 7. Influence of fluoride on the induction of photosynthesis. (A) Limiting light (125 μmol·m^{-2}·s^{-1}). (B) Saturating light (1000 μmol·m^{-2}·s^{-1}). The leaf discs were predarkened for 15 h, before illumination at 15 °C in saturating CO_2.

supplying fluoride. Accordingly, the rate of CO_2 fixation falls when sucrose synthesis is inhibited.

The kinetics of O_2 evolution during induction are shown for limiting (Fig. 7A) and saturating (Fig. 7B) light. Fluoride shortens the induction period. This can be attributed to the increased 3PGA pool in the dark (Figs. 2E and 5E) which will shorten induction by decreasing the requirement for autocatalytic build-up of metabolic levels [28]. Fluoride also modifies the secondary kinetics which are found in high light and CO_2 [25–27]. In untreated leaves the rate of O_2 evolution falls after about $1\frac{1}{2}$ min of rapid photosynthesis and then gradually recovers over the next 5–10 min (Fig. 7B). This reinhibition and slow recovery of O_2 evolution is related to a slow activation of sucrose synthesis [10]. During the initial period, almost all of the fixed carbon is retained in pools of phosphorylated intermediates, because sucrose synthesis has not been activated. As the intermediates accumulate, a point is reached at which photosynthesis is inhibited, and recovery depends upon the activation of sucrose synthesis to allow recycling of P_i. This gradual recovery is selectively delayed or prevented after supplying fluoride (Fig. 7B). This is consistent with fluoride having a selective effect on sucrose synthesis.

Higher rates of photosynthesis are reached during the first 2 min than in the steady state after supplying 5 mM fluoride. This shows that electron transport, ATP synthesis and the Calvin cycle can all operate at higher rates than are required during steady-state photosynthesis, and is again in agreement with 5 mM fluoride inhibiting photosynthesis via a decreased rate of sucrose synthesis. However, 10 or 20 mM fluoride also decreases the initial rate, suggesting further sites are also being inhibited at these higher concentrations. A similar conclusion can be reached from the measurements of metabolites (Fig. 5). The inhibition of photosynthesis is accompanied by a net increase of esterified phosphate (Fig. 5G), suggesting P_i will have decreased, and the ATP/ADP ratio decreases (see Fig. 3), as expected if low P_i restricts ATP synthesis [29]. However, the increase of the triose P/Ru1,5P_2 ratio (Fig. 5H) suggests the regeneration of Ru1,5P_2 is also being inhibited by fluoride.

There is one difference to the results obtained after sequestering P_i with mannose or glycerol. The PGA/triose P ratio increases when mannose or glycerol are supplied [25,27,30], as expected of PGA reduction is restricted by a low ATP/ADP ratio, but the PGA/triose P ratio decreases after supplying lithium [25] or fluoride (Fig. 5H). This might reflect the differences between agents which act by sequestering P_i, leading to depletion of free P_i and of the phosphorylated metabolites, and agents which inhibit the enzymes involved in sucrose synthesis. The latter lead to an increased level of cytosolic metabolites, including triose P, and this will actually be one of the factors which restrict the rate of PGA reduction.

On PP_i metabolism in leaves

These results allow the following general conclusions. Firstly, fluoride acts on plant carbohydrate metabolism at a second site related to cytosolic PP_i metabolism, in addition to this classical site of inhibition at enolase. Secondly, they emphasise the importance of the mechanisms which regulate the concentration of PP_i. After supplying 5 mM fluoride, a doubling of the PP_i concentration leads to a marked perturbation of cytosolic metabolite levels, inhibits sucrose synthesis, and makes a significant contribution to the inhibition of photosynthesis which we find at this concentration of fluoride. Thirdly, we are reminded that, though the cytosolic Fru1,6Pase and SPS are certainly of major importance, other enzymes could interact with them to control the rate of sucrose synthesis. The effect of increased PP_i on the hexose P and UDPGlc concentrations, and their subsequent interactions with further enzymes in the cytosol provides an example of how an equilibrium reaction can contribute to control via mass action [31]. Fourthly, 10–20 mM fluoride leads to a selective loss of uridine nucleotides and interferes with the activation of SPS. More work is needed to clarify the relation between the increased levels of PP_i, the inhibition of

enolase, the perturbation of uridine nucleotide metabolism and the 'coarse' control of SPS.

Finally, although our results do not provide any direct information about the way in which PP_i is removed, they do suggest that fluoride interferes with PP_i turnover. Fluoride could obviously be acting directly or indirectly. It might be noted that PFP is relatively insensitive to fluoride [32], but the PP_i-dependent proton pump on the tonoplast is 50% inhibited by 1 mM fluoride [33]. Our results would be consistent with this membrane protein contributing to control of the cytosolic PP_i concentration by hydrolysing PP_i and pumping protons into the vacuole, but a final decision will require information about the H^+/PP_i stoichiometry and the use of the resulting energy gradient.

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