Observations on the phosphate status and intracellular pH of intact cells, protoplasts and chloroplasts from photosynthetic tissue using phosphorus-31 nuclear magnetic resonance

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Individual pools of intracellular inorganic phosphate (P_i) can be observed in the dark in intact cells, protoplasts and chloroplasts from photosynthetic tissue by using ^{31}P nuclear magnetic resonance (n.m.r.). Estimates for the pH of vacuolar and extravacuolar compartments are reported although it is shown that intracellular pH is determined by the pH of the suspending medium. Mannose treatment of asparagus (*Asparagus officinalis*) cells and spinach (*Spinacia oleracea*) protoplasts results in the inhibition of photosynthesis. The mechanism of mannose phosphate sequestration of free P_i is supported by the ^{31}P n.m.r. spectra of mannose-treated tissue. There is a fundamental difference in ^{31}P n.m.r. spectra of mannose-treated spinach protoplasts and asparagus cells, reflecting a difference in the availability of vacuolar P_i for cellular metabolism in these species. The ^{31}P n.m.r. spectrum of intact spinach chloroplasts is reported.

In recent years high-resolution ^{31}P n.m.r. has been successfully employed to provide detailed information on the metabolism of animal and microbial cells (Gorden *et al.*, 1980; Ogawa *et al.*, 1980; Burt & Wyrwicz, 1979). Roberts *et al.* (1980) have used this technique to estimate the intracellular pH of maize (*Zea mays*) root tips and have obtained not only clearly defined resonances for the vacuolar and cytoplasmic pools but also have identified several sugar phosphate resonances. The application of ^{31}P n.m.r. to leaves has proved rather more complicated. Mesophyll cells are interspersed with large air spaces, which cause excessive broadening of the signals. In addition, there are three major P_i -containing compartments in the leaf, i.e. the vacuole, the cytoplasm and the chloroplasts.

Phosphate metabolism is fundamentally important in the regulation of photosynthesis (Walker & Robinson, 1978). Orthophosphate regulates both the rate of photosynthesis and of the distribution of the newly fixed carbon between starch and sucrose (Heldt *et al.*, 1977; Walker & Herold, 1977; Steup *et al.*, 1977; Laing *et al.*, 1980). ^{31}P n.m.r. provides not only a non-destructive means of observing free P_i directly, but also allows an approximation of the pH of the environment from which a given phos-

phate signal emanates (Moon & Richards, 1973). We report here our observations on intracellular P_i in fully functional photosynthetic cells and organelles in an aerobic environment in the dark. We have employed ^{31}P n.m.r. to investigate the effect of the introduction of an intracellular phosphate sequestration reaction *in vivo*.

Materials and methods

Materials

Cellulase (Onozuka 3S) and pectinase (Macerozyme) were purchased from Yakult Biochemicals Co. Ltd., Nishinomiya, Japan. $^2\text{H}_2\text{O}$ was purchased from BDH Chemicals Ltd., Atherstone, Warwickshire, U.K. All other reagents were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Spinach (*Spinacia oleracea*) and asparagus (*Asparagus officinalis*) were grown in pots in our own greenhouses.

Isolation of intact asparagus cells

Intact asparagus cells were gently separated from the broken basal regions of excised cladophylls by scraping with the blunt end of a spatula, into 500 mM-sorbitol. Cells were filtered through two layers of muslin and centrifuged at 50 g for 2 min. The pellets were resuspended in 20 ml of 500 mM-sorbitol and re-centrifuged. The final pellets, con-

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sisting mainly of intact cells, were then resuspended in appropriate medium.

Isolation of spinach protoplasts

Spinach protoplasts were prepared from mature leaves by using the procedure described by Edwards *et al.* (1978), except that the tissue was sliced with a sharp razor into fine strips under 500 mM-sorbitol. For normal protoplasts the enzyme digestion medium contained 500 mM-sorbitol, 5 mM-Mes (4-morpholine-ethanesulphonic acid), pH 5.5, 1 mM-CaCl₂, 3% cellulase and 0.5% pectinase. For mannose treatments the incubation medium contained 250 mM-sorbitol and 250 mM-mannose in place of 500 mM-sorbitol (Herold *et al.*, 1981).

Isolation of intact chloroplasts

Intact chloroplast fractions were prepared mechanically from spinach leaves as described by Walker (1980) and Leegood & Walker (1981).

N.m.r. spectrometry

³¹P n.m.r. spectra were obtained at room temperature in the dark with a Bruker W.H. 400 spectrometer. The samples were spun with a constant stream of air blowing over the sample tubes to prevent anaerobiosis. Details relating to specific spectra are listed in the legends to the Figures. Individual P_i peaks in the spectra were distinct and clearly resolved. The pulse repetition time was 0.21 sec.

CO₂ fixation

CO₂-dependent oxygen evolution was measured polarographically at 20°C by using Hansatech electrodes illuminated by red light in the wavelength range 590–750 nm at an irradiance of 300 W·m⁻². The CO₂-fixation medium for intact asparagus cells consisted of 500 mM-sorbitol, 5 mM-NaHCO₃ and 50 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/KOH buffer, pH 7.5. The CO₂-fixation medium for intact spinach protoplasts was 500 mM-sorbitol, 1 mM-CaCl₂, 25 mM-Tricine {N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine}/HCl buffer, pH 7.6, and 5 mM-NaHCO₃. For intact chloroplasts the CO₂-fixation medium was 330 mM-sorbitol, 2 mM-EDTA, 50 mM-Hepes/KOH buffer, pH 7.6, 1 mM-MgCl₂, 1 mM-MnCl₂, 0.5 mM-P_i, 5.0 mM-PP_i, catalase (220 units/ml) and 5 mM-NaHCO₃. In each case the chlorophyll concentration was 50 µg/ml of reaction medium.

Chlorophyll determinations

Asparagus cells. Portions (100 µl) of intact cells were disrupted with 1 ml of pyridine. The insoluble material was pelleted and the remaining chlorophyll was extracted with 2 × 5 ml of 80% (v/v) acetone. The insoluble material was pelleted and all the soluble

fractions were pooled. Chlorophyll was determined as described by Mackinney (1941).

Spinach protoplasts and chloroplasts. Chlorophyll was determined by the method of Arnon (1949).

Determination of P_i

After 4 h incubation at room temperature in the dark the cells were killed by the addition of 1 ml of 30% (w/v) trichloroacetic acid and orthophosphate was measured as described by Tausssky *et al.* (1953).

Results and discussion

³¹P n.m.r. spectra obtained from whole leaves of spinach, wheat (*Triticum aestivum*), rye-grass (*Lolium perenne*) and *Kalanchoë daigremontiana* in air or in 20% ²H₂O were broad and difficult to interpret. The broadening of the ³¹P n.m.r. signals and the resultant lack of definition are primarily attributable to the large air spaces that occur between the mesophyll cells and by the presence of intracellular paramagnetic metal ions. Intact cells, protoplasts and chloroplasts isolated from photosynthetic tissue are more easily manipulated and were found to yield more defined spectra.

The position of a given phosphate resonance is predominantly determined by [H⁺] between pH 5.0 and pH 8.0 (Moon & Richards, 1973). The titration curve in Fig. 1 was obtained by using 5 mM-KH₂PO₄/KOH buffered solutions referenced to 85% aqueous orthophosphoric acid, and all determinations of intracellular pH were made by using the data from this curve. All our measurements were made

Fig. 1. Titration curve of the chemical shift (δ) (p.p.m.) of the ³¹P n.m.r. signal as a function of pH. The position of the ³¹P signal in 5 mM-KH₂PO₄ buffers was obtained at the given pH values. Shifts were referenced to orthophosphoric acid. Spectra were obtained at room temperature with ²H₂O as external lock.

within the range pH 5.5–7.5. Outside this range the margin of error is greatly increased.

Effect of extracellular pH

With intact cells, protoplasts and chloroplasts the position of the ^{31}P n.m.r. signals was directly influenced by the pH of the suspending medium, reflecting the changes exerted on the pH of the intracellular compartments by the external $[\text{H}^+]$. This property was used to distinguish the cellular compartments within the protoplasts (Fig. 1). These protoplasts maintained rates of CO_2 -dependent O_2 evolution of $50\text{--}200\ \mu\text{mol of O}_2\cdot\text{h}^{-1}\cdot(\text{mg of chlorophyll})^{-1}$ when illuminated, both before and after the spectra were taken. When the external medium was at pH 6.0 then a single broad inorganic-phosphorus signal was evident (Fig. 2) with a mean chemical shift (δ) of 2.07 p.p.m. By using the titration curve for the position of the free-inorganic-phosphorus n.m.r. peak as a function of pH (Fig. 1), a value of

pH 6.08 was obtained for the environment of the intracellular inorganic phosphorus. As the extracellular pH was increased, this single peak was resolved into two peaks at pH 6.5 and above (Fig. 2). At an external pH of 7.5 (Fig. 2), two pools of inorganic phosphorus could be clearly distinguished. One of these peaks was found to have a pH value close to the pH of the external medium and yielded a mean chemical shift of 3.35 p.p.m. (Table 1). By using the titration curve, a value of pH 7.34 was obtained for this peak which therefore probably originated from extracellular P_i present in the chloroplasts and cytoplasm. The second peak, at approx. 2.76 p.p.m. (Table 1), had an estimated pH of 6.67 and most probably originated from vacuolar phosphate. The total free P_i content of spinach protoplasts was between 8.5 and $13\ \mu\text{mol}\cdot(\text{mg of chlorophyll})^{-1}$. The pH of the external medium did not vary during the course of the experiments. The time taken from the start of each experiment, i.e. the suspension of the protoplasts in the appropriate buffered medium to the completion of the spectra, was approx. $1\frac{1}{2}$ h. The changes in the chemical shifts of the intracellular P_i peaks had therefore occurred during this time.

The ^{31}P n.m.r. spectrum produced by intact adult asparagus cells is shown in Fig. 3. The spectrum comprised three different regions; peaks (I) and (II) are P_i resonances and their position was again determined by the pH of the surrounding medium. If the spectrum was taken in water or in 0.5 M-sorbitol, then peak (I) has an estimated pH of 5.70 and probably represents vacuolar phosphate. Peak (II) has an estimated pH of 6.75 and probably represents extracellular phosphate. The region to the left of these peaks, designated (III) is generated by organic phosphate (Table 1).

Effect of mannose

When exogenous mannose is supplied to leaf discs or protoplasts it is converted by cytoplasmic hexokinase into mannose phosphate (Chen-She Sheu-Hwa *et al.*, 1975; Herold & Lewis, 1977). The reaction acts as a sink for cytoplasmic P_i and successfully competes with the phosphate translocator for the available phosphate in the cytoplasm. Many plants cannot rapidly metabolize mannose phosphate and thus, in these species, mannose feeding constitutes means of sequestering cytoplasmic orthophosphate (Herold *et al.*, 1980). Lowering the free P_i in the cytoplasm slows photosynthesis and favours starch at the expense of sucrose (Herold *et al.*, 1981). Table 2 shows that, in the present work, mannose feeding decreased both CO_2 -dependent O_2 evolution and total $[\text{P}_i]$ in spinach protoplasts and asparagus cells. Inhibition of photosynthesis was more severe in the spinach protoplasts than in asparagus cells. Mannose

Fig. 2. ^{31}P n.m.r. spectra of intact spinach protoplasts in media at various pH values

The protoplasts were suspended in 500 mM-sorbitol/1 mM- CaCl_2 /20% $^2\text{H}_2\text{O}$ and either 5 mM-Hepes/KOH or 5 mM-Mes/HCl. Spectrum (a) was obtained with 788 scans at pH 6.0, spectrum (b) was obtained with 953 scans at pH 7.5 and spectrum (c) with 959 scans at pH 8.0. In each case the chlorophyll concentration was 0.5 mg/ml.

Fig. 3. ^{31}P n.m.r. spectra of normal asparagus cells (a) and asparagus cells that had been treated with 250 mM-mannose (b)

For spectrum (a), asparagus cells were suspended in 500 mM-sorbitol/50 mM-Hepes/KOH buffer, pH 7.5, and 20% $^2\text{H}_2\text{O}$. The chlorophyll concentration was 0.45 mg/ml and the spectrum was obtained with 10000 scans. For spectrum (b) cells were suspended in 250 mM-sorbitol/250 mM-mannose/50 mM-Hepes/KOH buffer, pH 7.5, and 20% $^2\text{H}_2\text{O}$. The chlorophyll concentration was 0.50 mg/ml and the spectrum was obtained with 10000 scans. In each case newly prepared asparagus cells were used and incubated as above for 3 h in the dark before the spectra were taken.

lowered the total P_i in both, but the amount by which the total P_i was decreased in spinach was very low (0–5%), whereas the corresponding decrease in asparagus was approx. 20% over the same time period.

A comparison of ^{31}P n.m.r. spectra reveals a distinct difference in the distribution of P_i between the asparagus cells and spinach protoplasts after mannose feeding. Fig. 4 shows ^{31}P n.m.r. spectra of

Fig. 4. ^{31}P n.m.r. spectra of intact spinach protoplasts which were prepared (a) in the absence and (b) in the presence of 250 mM-mannose

The spectra of the protoplasts were taken immediately after isolation. The protoplasts were suspended in 500 mM-sorbitol/25 mM-Hepes/KOH buffer, pH 7.5, and 20% $^2\text{H}_2\text{O}$ at a chlorophyll concentration of 0.17 mg/ml. The spectra of the normal protoplasts (a) and protoplasts isolated in the presence of mannose (b) were each obtained with 12000 scans.

spinach protoplasts prepared in the absence (a) and presence (b) of mannose. It can be seen that, in the presence of mannose, peak (II) was decreased relative to peak (I) and a new peak in a mean position of 5.39 p.p.m. was present. This latter peak can be attributed to mannose phosphate, accumulated at the expense of the P_i in the cytoplasmic peak (II) while the vacuolar pool (I) was largely unchanged.

The ^{31}P n.m.r. spectrum of the asparagus cells after mannose feeding (Fig. 3b) shows a response different from that of the spinach protoplasts. Mannose caused a decrease in the acid peak (I) relative to the neutral peak (II). There was also a slight increase in the organic-phosphate resonance (III), presumably reflecting mannose phosphate

Table 1. *Position of the ³¹P n.m.r. signals in intact spinach protoplasts and isolated asparagus cells in media of different pH*

All solutions contained 0.5M-sorbitol and 20% ²H₂O as internal lock. The chemical-shift values are means $\pm$ s.d. calculated from data obtained in at least three separate experiments.

Peak ...	Chemical shift ($\pm$ s.d.) (p.p.m.)		
	(I)	(II)	(III)
Asparagus cells			
In water	1.75 $\pm$ 0.07	2.85 $\pm$ 0.18	†4.38 $\pm$ 0.78
In 50mM-Hepes/KOH, pH 7.5	1.97 $\pm$ 0.04	3.40 $\pm$ 0.15	†5.05 $\pm$ 0.36
Spinach protoplasts			
In 50mM-Hepes/KOH, pH 7.5	2.76 $\pm$ 0.32	3.35 $\pm$ 0.22	*5.39 $\pm$ 0.06
In 5mM-Mes/HCl, pH 6.0	2.07 $\pm$ 0.21	—	—
Peak ...	Corresponding pH		
	(I)	(II)	(III)
Asparagus cells			
In water	5.70	6.75	—
In 50mM-Hepes/KOH, pH 7.5	6.00	7.42	—
Spinach protoplasts			
In 50mM-Hepes/KOH, pH 7.5	6.67	7.34	—
In 5mM-Mes/HCl, pH 6.0	6.08	—	—

* Position of the peak produced by mannose feeding, which is presumably attributable to mannose phosphate.

† Organic phosphate.

Table 2. *Effect of mannose treatment on CO₂-dependent O₂ evolution and free P_i content in adult asparagus cells and spinach protoplasts*

	CO ₂ -dependent O ₂ evolution [μ mol of O ₂ · h ⁻¹ · (mg of chlorophyll) ⁻¹]		Free P _i [μ mol · (mg of chlorophyll) ⁻¹]	
	—Mannose	+Mannose	—Mannose	+Mannose
Adult asparagus cells				
Expt. (1)	76.0	52.5	3.79	3.21
Expt. (2)	92.0	70.0	3.98	3.18
Spinach protoplasts				
Expt. (3)	192.0	74.0	12.13	11.33
Expt. (4)	128.4	48.0	12.95	12.50

accumulation. Thus, in asparagus cells, the cytoplasmic P_i pool is virtually unchanged by mannose, while the vacuolar pool is decreased relative to it. Since the formation of mannose phosphate is believed to be predominantly a cytoplasmic event requiring ATP, it would seem that, in asparagus, the P_i content of the cytoplasm is largely maintained at the expense of the vacuolar pool. This might explain the fact that, although the total [P_i] is more severely depleted in asparagus than in spinach protoplasts, the inhibition of photosynthesis is less marked in asparagus. The effect of mannose feeding was most profound in young asparagus cells, where the vacuole was not fully expanded and total [P_i] is low [1–2 μ mol · (mg of chlorophyll)⁻¹]. In young cells the ³¹P n.m.r. spectrum indicated that the vacuolar P_i

pool could be largely depleted relative to the extravacuolar phosphate as a result of mannose feeding. The total free P_i content of adult asparagus cells used in these experiments was from 3–5 μ mol of P_i · (mg of chlorophyll)⁻¹.

Spinach chloroplasts

Fig. 5 shows a typical ³¹P n.m.r. spectrum of intact spinach chloroplasts. The spectrum shows a single narrow peak of P_i on the right of the spectrum, its position again dependent on the pH of the suspending medium. If the pH of the chloroplast medium was 7.6, as in Fig. 5, then the internal pH of the chloroplast stroma in the dark determined from the position of the phosphate peak (3.31 p.p.m.) was 7.28. To the left of the P_i peak there are three smaller

Fig. 5. ^{31}P n.m.r. spectrum of intact spinach chloroplasts. The chloroplasts were suspended in 330 mM-sorbitol / 1 mM- MgCl_2 / 1 mM- MnCl_2 / 2 mM-EDTA, 50 mM-Hepes/KOH buffer, pH 7.6, and 20% $^2\text{H}_2\text{O}$ at a chlorophyll concentration of 1.5 mg/ml. The spectrum was obtained with 44619 scans. The chloroplasts used in these experiments were from 70 to 80% intact and sustained rates of CO_2 -dependent O_2 evolution of $80\text{--}150\ \mu\text{mol}\cdot\text{h}^{-1}\cdot(\text{mg of chlorophyll})^{-1}$.

peaks of organic phosphate at 4.81, 4.90 and 5.42 p.p.m. As it is possible to determine the pH of the stroma from the position of the P_i peak, it is also possible to identify the sugar phosphates responsible for these resonances from titration curves similar to that for P_i . This is an area for future investigation.

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