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Observations on the cytoplasmic and vacuolar orthophosphate pools in leaf tissues using in vivo ^{31}P-NMR spectroscopy

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A full understanding of the central role of P_i in photosynthesis requires information on the size of the endogenous P_i pools and the extent to which they interact with each other under different physiological conditions. In vivo ^{31}P-NMR spectroscopy has the potential to assist in this objective but the early applications of this technique to leaf tissues were disappointing. Here we report ^{31}P-NMR data from maize and tomato leaf discs that: (i) cast doubt on earlier estimates of the cytoplasmic orthophosphate pool size; and (ii) prove that exogenously supplied D-mannose can reduce the cytoplasmic P_i level in some circumstances.

Compartmentation; Mannose, D-; Orthophosphate; NMR, ^{31}P-; Tonoplast transport

1. INTRODUCTION

The role of P_i in the regulation of photosynthesis can be investigated by manipulating the P_i supply to the chloroplast [1]. A useful experimental approach is to supply the tissue with a readily phosphorylated glucose analogue, such as D-mannose or 2-deoxy-D-glucose, and to exploit the fact that if the subsequent metabolism of the analogue is slow, then the accumulation of mannose 6-phosphate or 2-deoxyglucose 6-phosphate will cause a reduction in the endogenous P_i level [2]. This method has been useful in studies of the partitioning of photosynthate between starch and sucrose [2,3], and in demonstrating that the supply of P_i to the chloroplast can limit the photosynthetic rate in vivo [4].

The mannose experiment does not lead to a direct quantitative assessment of the endogenous P_i pools and in fact the interpretation of the physiological data depends on the reasonable, but unproven, assumption that mannose will deplete the cytoplasmic P_i pool despite the existence of other endogenous sources of P_i. To prove the validity of this assumption, and perhaps more importantly to assess the subcellular P_i distribution in vivo directly, it is necessary to use a non-invasive method and for this purpose the only available technique appears to be ^{31}P-NMR spectroscopy [5,6]. Unfortunately, the ^{31}P spectra of leaves are often disappointing, because of the line-broadening effects of intercellular air spaces [7] and endogenous paramagnetic ions [8], and early attempts to measure the cytoplasmic P_i level in mannose-treated leaf tissues gave contradictory results [7,9,10]. However, spectra suitable for quantitative analysis can be obtained from some species and here we report spectra that: (i) demonstrate the mannose-induced depletion of the

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Abbreviations: MDP, methylene diphosphonic acid; Mes, 2-(*N*-morpholino)ethane sulphonic acid; NMR, nuclear magnetic resonance; P_i, orthophosphate; ppm, parts per million; TEMDP, tetraethyl ester of methylene diphosphonic acid

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cytoplasmic P_i pool unequivocally; and (ii) suggest that previous work has overestimated the size of the cytoplasmic P_i pool.

2. MATERIALS AND METHODS

2.1. *Plant material*

Maize seeds (*Zea mays*, L. var. LG11) were washed with tapwater for several hours and germinated in the dark at 25°C between sheets of absorbent paper soaked in 0.1 mM $CaSO_4$. After 48 h, the seedlings were transferred to a solution culture system [11] and grown for several days under normal greenhouse conditions. Phosphate-deficient leaves were obtained by omitting P_i from the growth medium.

Two species of tomato, the normal cultivated species (*Lycopersicon esculentum* Mill. cv Alicante) and a highland variety of the Andean wild tomato (*L. hirsutum*) were grown to maturity under normal greenhouse conditions. Small leaves in good condition were selected for the NMR experiments.

2.2. *Sample preparation*

8 mm diameter leaf discs were cut with a cork borer and vacuum-infiltrated for 15 min in 10 mM Mes/0.1 mM $CaSO_4$, pH 6.0 (standard buffer). Approx. 50 tomato discs, or 80 maize discs, were stacked in a 10 mm diameter NMR tube and oxygenated standard buffer was circulated through the tube at 3 $ml \cdot min^{-1}$. The tissue was allowed to stabilise for 30 min before starting the NMR experiment.

2.3. *NMR spectroscopy*

^{31}P-NMR spectra were recorded at 121.49 MHz on a Bruker CXP 300 spectrometer using a double-tuned $^{13}C/^{31}P$-probe head. The sample was kept in the dark at 21–22°C and the spectra were accumulated using procedures described elsewhere [6,12,13]. A 5 s (*L. hirsutum*) or 10 s (*Z. mays*) recycle time was used to obtain fully relaxed spectra, but for qualitative purposes spectra were accumulated with a 90° pulse angle and a 0.8 s recycle time. The cytoplasmic and vacuolar P_i pools were quantified by changing the circulating medium to 1.5 mM MDP in the standard buffer and comparing the intensity of the MDP resonance (I_{sm}) and the appropriate tissue resonance (I_m) in spectra with a 20 s recycle time. The P_i concentration, averaged over the total volume of the tissue, is equal to $3.0\ I_m/(I_s - I_{sm})$ mM where I_s is the intensity of the MDP resonance in the absence of the tissue. Chemical shifts were measured relative to the signal from a capillary containing a 2% aqueous solution of TEMDP [12] and are quoted relative to the resonance from 85% H_3PO_4.

3. RESULTS AND DISCUSSION

In contrast to earlier work with leaf tissue [7,8,10,14], maize and tomato leaves gave good quality ^{31}P-NMR spectra in which the cytoplasmic and vacuolar P_i resonances were well resolved (fig.1). As expected from previous analyses of

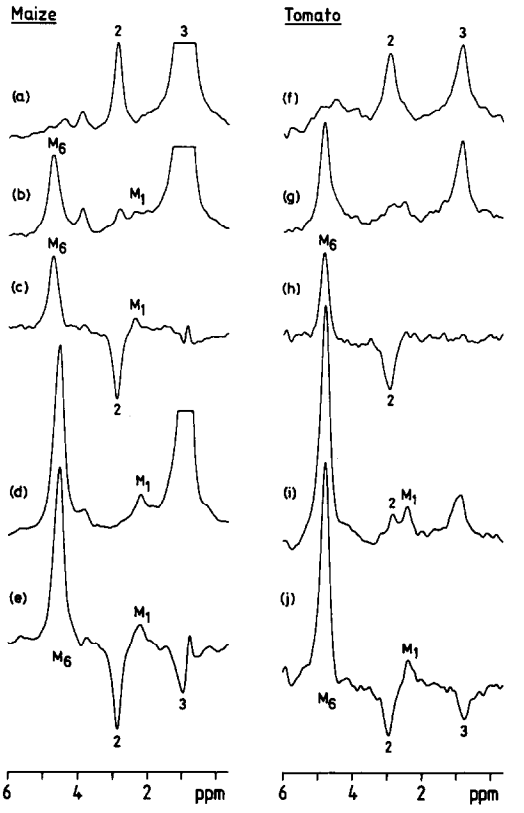

Fig.1. ^{31}P-NMR spectra of maize and tomato (*L. hirsutum*) leaf discs during mannose loading. After recording the starting spectra (a,f) in the standard buffer, the circulating medium was switched to 5 mM mannose in the same buffer and spectra were recorded after (b,g) 1.5 h and (d,i) 5.5 h exposure to mannose. The resonance assignments are: 2, cytoplasmic P_i; 3, vacuolar P_i; M_6, mannose 6-phosphate; M_1, mannose 1-phosphate. The difference spectra emphasise the spectral changes: (c) = (b) – (a); (e) = (d) – (a); (h) = (g) – (f); (j) = (i) – (f).

mannose-loaded maize leaf tissue extracts [15], phosphorylation of mannose led to the accumulation of mannose 6-phosphate and mannose 1-phosphate and to the detection of the corresponding resonances (fig.1). The difference spectra show that mannose caused an immediate fall in the cytoplasmic P_i signal (fig.1c,h), while having no effect on the vacuolar P_i, and that it was only later in the time course (fig.1e,j) that the vacuolar signal began to fall. Thus P_i transport across the tonoplast was slow relative to the demand for P_i by the cytoplasmic hexokinase (EC 2.7.1.1) and the cytoplasmic P_i level fell despite the existence of the vacuolar P_i pool. A similar sequence of events oc-

curs in maize root tissues [16,17] and may be presumed to occur whenever the endogenous phosphomannose isomerase (EC 5.3.1.8) activity is low in relation to the rate of accumulation of mannose 6-phosphate.

Table 1 summarises some quantitative measurements on the P_i fractions in maize and tomato leaves and compares them with the results of previous NMR investigations. Any attempt to convert the pool sizes represented by the intensities of peaks 2 and 3 (fig.1) into absolute concentrations requires an estimate of the corresponding compartmental volumes and this is complicated by the cellular heterogeneity of the leaves. The NMR approach has yet to be combined with a microscopic analysis of the leaf tissue used to record the spectra and for comparative purposes the concentrations in table 1 assume overall cytoplasmic and vacuolar fractions of 10% and 80%, respectively. The resulting concentrations are averaged over the whole of the cytoplasmic or vacuolar space and in the case of the cytoplasm no distinction is made between the cytosol and the chloroplast because the corresponding P_i

Table 1

Cytoplasmic and vacuolar P_i fractions in leaf tissues[a]

Tissue	P_i contents (mmol·l^{-1} tissue)		P_i concentrations (mM)		NMR data	
	Cytoplasm	Vacuole	Cytoplasm[b]	Vacuole[c]	$\Delta\delta$[d]	pH_{cyt}[e]
8 d maize (0 mM)[f]	1.15	4.1	11.5	5.1	2.00	~7.6
9 d maize (0 mM)[f]	1.05	4.3	10.5	5.4	1.98	~7.6
7 d maize (0.5 mM)[f]	2.1	22.4	21	28	2.02	~7.6
9 d maize (0.5 mM)[f]	2.15	30.3	21.5	38	1.95	~7.6
Tomato (*L. hirsutum*)	1.05	1.6	10.5	2.0	2.08	~7.6
Wheat [7]	–	–	(25.8)[g]	(13.7)[g]	(1.4)	6.9–7.2
Barley [8]	–	–	35[h]	(18.3–19.2)[i]	(1.06)[j]	7.0
Pea [8]	–	–	28	(8.6–11.5)[i]	(1.05)[j]	6.9
Wheat [8]	–	–	28	(11.2–13.7)[i]	(0.84)[j]	7.1
34 d barley (0 mM)[f] [14]	–	–	(20.8)[k]	(0)[l]	(1.0)	7.0
34 d barley (1 mM)[f] [14]	–	–	(48–53)[k]	(10–10.6)[l]	(1.0)	7.0
34 d barley (25 mM)[f] [14]	–	–	(61–78)[k]	(24.9–27)[l]	(1.0)	7.0
Barley [10]	–	–	(11.2)[m]	(8.0)[m]	(1.5)	7.2

[a] Values in parentheses were deduced from information in the cited papers
[b] Assuming a cytoplasmic fraction of 10% ≡ 40 μl·mg^{-1} chlorophyll
[c] Assuming a vacuolar fraction of 80% ≡ 320 μl·mg^{-1} chlorophyll
[d] The chemical shift difference in ppm between the cytoplasmic and vacuolar P_i resonances
[e] The cytoplasmic pH estimated from the chemical shift of the cytoplasmic P_i resonance
[f] External P_i concentration in the growth medium
[g] [7] assumed cytoplasmic and vacuolar volume fractions of 25% and 75%, respectively
[h] From table 7 of [8], but other information in the same table implies 47 mM
[i] Deduced from table 7 of [8]
[j] Deduced from table 6 of [8]
[k] Deduced from table 1 of [14]
[l] Deduced from tables 1 and 2 of [14]
[m] Personal communication from Dr C.H. Foyer based on fig.2 in [10]

resonances are superimposed in the cytoplasmic P_i signal.

Depriving the maize seedlings of an external P_i supply had a large effect on the vacuolar P_i content and a parallel can be drawn with earlier observations on the P_i distribution in root tissues [11]. The cytoplasmic P_i content was much less dependent on the P_i supply but the cytoplasmic P_i concentrations reported here are generally lower than those reported previously [7,8,14]. The earlier data are probably unreliable because: (i) the tissue in the NMR tube was not supplied with oxygen [7,8,14]; and (ii) the P_i fractions were determined indirectly, by dividing the total P_i in an extract between the cytoplasm and the vacuole in proportion to the relative intensities of the cytoplasmic and vacuolar resonances [8,10,14]. A shortage of oxygen is indicated [13,16,18] by the small values of $\Delta\delta$ and pH_{cyt} for the tissues that were not supplied with oxygen (table 1) and since hypoxia causes an increase in cytoplasmic P_i [13,19] it is important to avoid this problem when making accurate measurements of the cytoplasmic P_i level. The indirect calibration of the P_i intensities [8,10,14] is also suspect since it assumes: (i) that no P_i is produced by degradation during extraction; and (ii) that all of the tissue P_i is detectable in vivo. The latter assumption, which arises because NMR only detects the freely mobile metabolites in a tissue [12], is unlikely to be valid in view of the evidence that has been presented for the interaction of stromal metabolites with ribulose-1,5-bisphosphate carboxylase [20].

Overall, the quantitative data reported here do not support the view that the normal cytoplasmic P_i concentration in the dark is 15–40 mM [8] or that the P_i concentration in the vacuole is much lower than in the cytoplasm [10]. The low cytoplasmic P_i value, coupled with the inability of the vacuolar P_i fraction to meet the short term needs of the cytoplasm (fig.1), suggests that the cycling of P_i during sucrose and starch synthesis may be more important in determining the availability of cytoplasmic P_i than had been previously realised. This conclusion needs further investigation and the earlier attempt to correlate NMR data on the subcellular P_i distribution with information on the photosynthetic rate and the starch:sucrose ratio [14] needs to be repeated using leaf tissues that give high quality NMR spectra.

Quantitative measurements (fig.2) emphasize the differential effect of mannose on the two P_i fractions and provide strong support for the assumption that mannose reduces the cytoplasmic P_i in tissues that metabolise mannose-6-phosphate slowly [2]. NMR evidence to the contrary from asparagus cells [9] and wheat leaves [7] may reflect shortcomings in the way in which the tissue was handled in the NMR tube. In root tissues, early work with poorly aerated tissues suggested that mannose-6-phosphate accumulated only at the expense of the vacuolar P_i pool [21] and the effect on the cytoplasmic pool only became apparent as the NMR techniques were improved [16,17]. The limited amount of reliable leaf tissue NMR data available so far makes it difficult to generalise, but

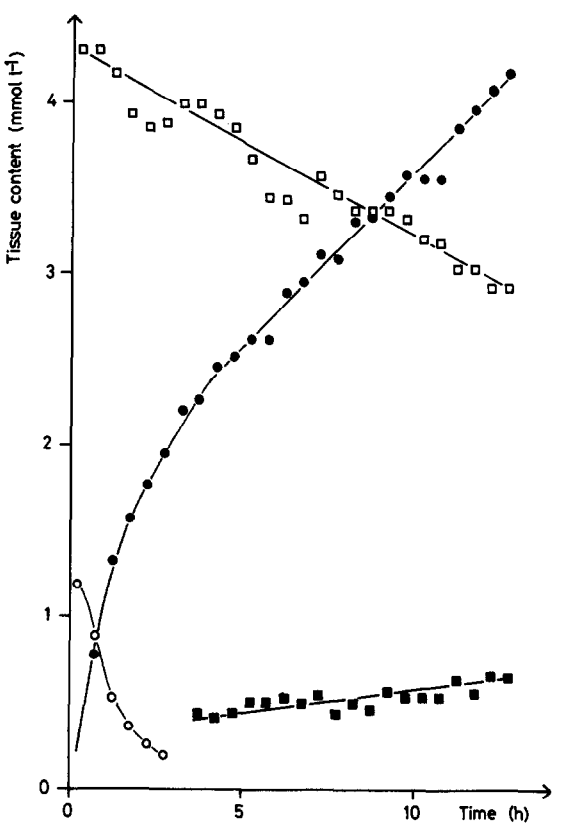

Fig.2. Time dependence of the cytoplasmic P_i (○), vacuolar P_i (□), mannose 6-phosphate (●) and mannose 1-phosphate (■) pools during a 5 mM loading experiment on maize leaf discs. The mannose 6-phosphate:mannose 1-phosphate ratio was constant at 6.6 indicating a steady state for the reaction catalysed by the mutase.

it is clear that the depletion of the cytoplasmic P_i during the accumulation of mannose-6-phosphate will depend in part on the transport of P_i across the tonoplast and future NMR work should focus on the factors that affect this process.

Figs 3 and 4 draw attention to two other important aspects of the mannose effect: (i) the fall in the NTP level that accompanies the depletion of the cytoplasmic P_i; and (ii) the reversibility of the effect. The fall in NTP, which presumably reflects the increasingly unfavourable free energy change for NTP synthesis [17], is generally ignored when mannose is used to reduce the cytoplasmic P_i level and the metabolic consequences of this change need to be investigated.

In conclusion, ^{31}P-NMR studies of suitable leaf tissue yield quantitative information about the cytoplasmic P_i level under both steady state and changing conditions and the application of this method should lead to further insights into the role of P_i in photosynthesis.

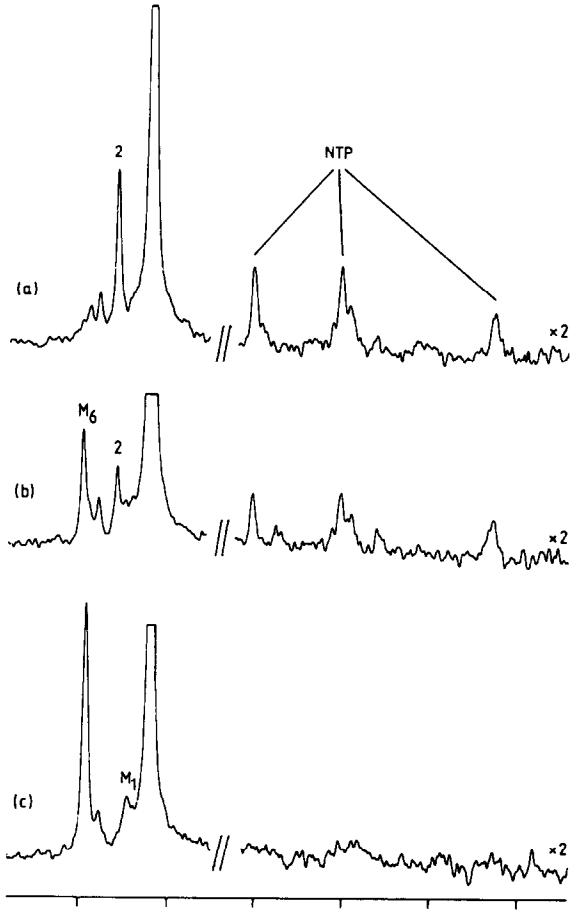

Fig.3. ^{31}P-NMR spectra of maize leaf discs showing the effect of mannose on the NTP resonance. Spectra were recorded after (a) 0 h, (b) 0.75 h and (c) 3.25 h exposure to 5 mM mannose.

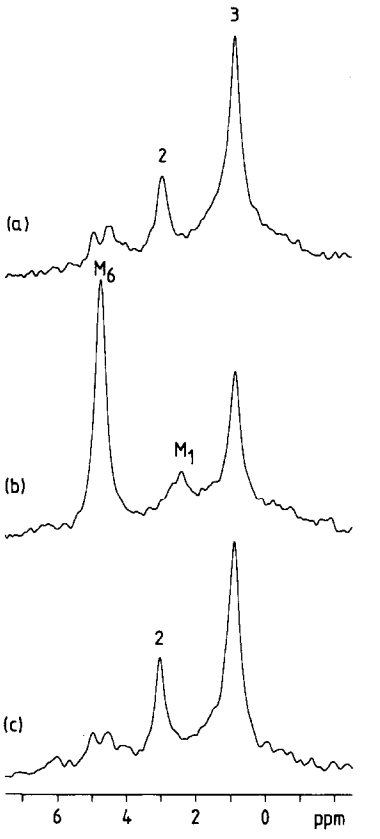

Fig.4. ^{31}P-NMR spectra of tomato (*L. esculentum*) leaf discs showing the reversibility of the mannose effect. Spectra were recorded: (a) before exposure to mannose; (b) after 7.5 h exposure to 5 mM mannose; and (c) after a 3.5 h recovery in the absence of mannose.

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