An experimental arrangement is described which enables high quality $^{31}$P NMR spectra of compressed spinach leaf pieces to be continuously recorded in which all the resonances observed (cytoplasmic and vacuolar $P_i$, glycerate-3- $P_i$, nucleotides) were sharp and well resolved. $^{31}$P NMR spectra obtained from intact chloroplasts showed a distinct peak of stromal $P_i$. An upfield shift of the stromal $P_i$ resonance was associated with a decrease in the external $P_i$ and vice versa. Nucleotides were largely invisible to NMR in intact chloroplasts, whereas the same nucleotides reappeared in a typical $^{31}$P NMR spectrum of an acid extract of intact chloroplasts. Perfusion of compressed spinach leaf pieces with a medium containing $P_i$ triggered a dramatic increase in the vacuolar $P_i$ over 12 h. Addition of choline to the $P_i$-free perfusate of compressed leaf pieces resulted in a steady accumulation of phosphorylcholine in the cytoplasmic compartment at the expense of cytoplasmic $P_i$. When a threshold of cytoplasmic $P_i$ concentration was attained, $P_i$ was drawn from the vacuole to sustain choline phosphorylation. In spinach leaves, the vacuole represents a potentially large $P_i$ reservoir, and cycling of $P_i$ through vacuolar influx (energy dependent) and efflux pathways is an efficient system that may provide for control over the cytosolic-free $P_i$ and phosphorylated intermediate concentrations. $^{31}$P NMR spectra of neutralized perchloric acid extracts of spinach leaves showed well defined multiplet resonances (quadruplet) of intracellular phyate. The question of cytosolic $P_i$ concentration in green cells is discussed.

In photosynthetic higher plant cells, cytosolic inorganic phosphate ($P_i$) plays a central role in the regulation of respiration (1) and photosynthesis (2). Unfortunately, by usual analytical techniques it is almost impossible to distinguish between the $P_i$ contents of the vacuolar and cytoplasmic compartments. This limitation has been partially overcome with the relatively recent expansion of nuclear magnetic resonance (NMR) spectroscopy to studies of intact nongreen plant tissues (3-5) and leaves (6, 7). However, even with this methodology, the investigation of the relative concentrations of ATP and cytosolic $P_i$ in photosynthetic cells, which are of great usefulness and importance in the study of the control of photosynthesis, have been hindered for two major reasons: (a) more than 80% of the volume of mature photosynthetic cells consists of a vacuole (8); and (b) the lacuna between upper and lower epidermis represents a large proportion of the total leaf volume.

Despite the aforementioned difficulties, in the present studies an experimental arrangement is described for monitoring by $^{31}$P NMR the behavior of spinach leaves over a long period of time under different conditions. Our data shed new light on the cytosolic $P_i$ status and on the controlled flux of $P_i$ into and out of the vacuole in photosynthetic tissues.
coated with 4.5 ml). To this end, small leaf pieces infiltrated with perfusion medium (7) were slightly compressed by hand between two circular polymer filters from a volume of 20 ml (150 μg of chlorophyll/ml) to a volume of 4.5 ml (80 μg of chlorophyll/ml) fitting exactly into a standard 10-mm NMR tube. Under our condition, 1 g (wet weight) of leaf tissue corresponded to 1 mg of chlorophyll. Details of this assembly and its operation have been described previously by Roby et al. (13). A reference capillary containing methylidinitrophosphate (pH 8.9 in 30 mM Tris) was inserted inside the inlet tube along the symmetry axis of the tissue sample. The perfusate consisted of the perfusion medium containing 20 mM succrose. Its pH was constantly monitored and adjusted to 6.5 using an automatic device. The flow rate routinely provided more than twice the measured oxygen consumption by 3 g (wet weight) of spinach leaves at 25 °C. Such a system prevented gas bubbles from collecting in the intercellular space sandwiched between upper and lower epidermis, which would have otherwise decreased the homogeneity of the magnetic field. For kinetic experiments on respiration, the gas flow (see Ref. 13) was switched alternately from O2 to N2. Furthermore, by modifying the composition of the circulating medium (introduction of P, choline) it was possible to perturb the leaf metabolism and to monitor the spectral changes simultaneously, obtaining several successive spectra from the same sample. For example, changes from aerobic to anaerobic conditions and vice versa could be performed at intervals as short as every 2 min.

The assignment of nucleotides and phosphate esters to specific peaks was carried out according to Navon et al. (14), Evans and Kaplan (15), and Roberts and Jardetzky (3) and from spectra of the perchloric extracts that contained the soluble low molecular weight components (13). A 5-s recycling time was used to obtain fully relaxed spectra (for P, determination), but for qualitative purposes, FIDs were accumulated using a 0.5-s repetition time and a 30° pulse angle.

Neutralized Perchloric Acid Extracts—For perchloric acid extracts, spinach leaves (5 g, wet weight) or intact chloroplasts (8 mg of chlorophyll) were quickly frozen at liquid nitrogen temperature to avoid ATP destruction and then finely ground with 1 ml of 70% (w/v) perchloric acid. The frozen powder was then placed at −10 °C and thawed. The thick suspension thus obtained was centrifuged at 10,000 × g for 10 min to remove particulate matter. The supernatant was neutralized with 2 mM KHCO3 to pH 6.0 and centrifuged at 10,000 × g for 10 min to remove precipitated KClO4. Next, 120 μmol of trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA), a strong chelating agent (16), was added. CDTA prevented the precipitation of specific Pi esters, such as phytate, as divalent metal ions and led to sharp lines for the phosphorus resonances of the standards (ATP, ADP, AMP, ATP, CTP, UTP, UMP, CMP, TMP, NAD, NADP, glucose-6-P, glucose-1-P, UDP-glucose, UDP-galactose, ATP-glucose, glycerate-3-P, glyceraldehyde-3-P, sn-glycerol-3-P, pyrophosphate, glyc erophosphorylcholine, phosphorylcholine, and phytate) were resolvable. Definitive attribution of resonances to specific compounds was made after running a series of spectra obtained by successive addition of the authentic compounds to the perchloric acid extracts. In the case of phosphorylated intermediates in leaf cells, the pH titrations were carried out to further substantiate the assignments.

**RESULTS**

Conditions for Incubation—Small leaf pieces were prepared in perfusion medium without sucrose and P, as described under "Experimental Procedures." The leaf pieces were then divided into four parts and incubated in the dark at room temperature with added 20 mM sucrose and 1 mM P, (Table I). At different times during incubation, the capacity to perform photosynthesis at saturating light and CO2 was measured. In order to prevent a possible inhibition of photosynthesis by the carbohydrate in leaf pieces (48), photosynthetic rates were measured in the absence of sucrose in the medium. When sucrose was omitted from the incubation medium, the leaf pieces rapidly lost the capacity to perform photosynthesis (Table I). When sucrose was added alone or together with P, a high rate of photosynthesis was observed even after 36 h at room temperature using a sterile perfusion medium. P, alone was, however, not sufficient to maintain a high photosynthetic activity. The initial response of leaf pieces to sucrose feeding varied depending on the size of the intracellular carbohydrate pools (starch, sucrose). For some leaves, the initial rate of photosynthesis was high in the absence of added sucrose; but to get good rates of photosynthesis after longer incubation times, addition of sucrose was always necessary. By using 31P NMR (see below) we have observed that the concentrations of the major intracellular metabolites (glucose-6-P, UDP-glucose, and glycerate-3-P) declined progressively once the sucrose pool had disappeared (not shown; for an explanation, see Ref. 13). In marked contrast, in the presence of sucrose in the perfusion medium, little change occurred. We have observed that a high rate of photosynthesis was always correlated with high intracellular metabolite levels.

Interestingly, Stitt et al. (17) have shown clearly that higher levels of phosphorylated intermediates in leaf cells shorten the length of the induction phase of photosynthesis. 31P NMR of Compressed Small Leaf Pieces—31P NMR spectra obtained from compressed leaf pieces supplied with oxygen and at pH 6.5 showed two distinct peaks of intracellular Pi (Cyt-Pi, and Vac-Pi, Fig. 1) at approximately 2.4 and 0.5 ppm. These positions correspond roughly to pH 7.5 and 5, respectively. These values reflect the presence of the vacuolar Pi pool at the acidic pH and the cytoplasmic Pi pool at the slightly alkaline pH (18). In the spectra obtained from spinach leaves, no other Pi peaks were identified unambiguously (see also Refs. 7–9). Such a result is surprising considering that in spinach mesophyll cells, plastids take up a much higher proportion of the chloroplastic volume than in nonchlorophyllian cells (6). This means that the dark, either the amount of P, present in the chloroplast stroma of spinach leaf is below the level of sensitivity of NMR and/or the pH difference between chloroplasts and the cytosol is too small for the signals to be discriminated. The first hypothesis is most...
unlikely because the concentrations of P\_i we have found in the stroma of freshly prepared chloroplasts was approximately 12 mM (we have verified that during the course of chloroplast preparation carried out at 0 °C, the slow leakage of P\_i from intact isolated chloroplasts was almost negligible (11); consequently, the P\_i content of intact chloroplasts reflected the *in vivo* situation). To determine accurately the cytoplasmic and vacuolar P\_i pools in leaf tissue, a calibration of the peak intensity of the P\_i resonance with known amounts of external P\_i, was first performed. In our experimental conditions, we have calculated that within the receiving coil the cell volume comprises approximately 66% of the total volume. The perfusion medium was pumped through the leaf pieces and was circulated via a 300-ml reservoir that was oxygenated by O\_2 bubbling. The FID recorded at 25 °C is the result of 96,000 transients obtained with a 30° pulse angle and 0.3-s repetition time. Data treatments include Lorentz-Gauss transformation and zero filling. Peak assignments: Glc-6-P, glucose-6-P; a, position of glycerate-3-P; b, position of phosphorylcholine; Cyd-P, cytoplasmic P\_i; Vac-P, vacuolar P\_i.}

However, meaningful interpretations of *in vivo* spectra require the rigorous identification of resonances corresponding to specific phosphorus-containing metabolites. Numerous studies have shown that the region of the 31P NMR spectrum, generally ascribed to sugar phosphates in previous investigations of a variety of plant tissues or cells (19), may contain other phosphomonoesters including lipid metabolites. These considerations point to the need for unambiguous determination of the original peaks. 31P NMR of Neutralized Perocholic Acid Extracts—Fig. 2 shows a typical undecoupled 31P NMR spectrum of a perchorlic acid extract of spinach leaf pieces (5 g, wet weight; 5 mg of chlorophyll) at pH 7.8 in the presence of a large excess of CDTA. The resonances that were observed were sharp insofar as the concentration of phosphorylated metabolites was not too high. By adding known compounds to the extract, the peaks obtained were identified as signals from phosphomonoesters including dihydroxyacetone phosphate, gluconate-6-P, glucose-6-P, glyceraldehyde-3-P, and phosphorylcholine; P\_i; γ-, α-, and β-ATP; γ-, α-, and β-UTP; β- and α-ADP; β- and α-UTP; β- and α-UTP; β- and α-UTP. As expected, ATP was the most abundant triphosphate. The NAD and NADP peaks, which yield a quadruplet centered at −10.7, −10.9, −11.1, and −11.2 ppm on the high-field side of α-UTP peak, were well identified in contrast with what was observed previously in nongreen tissues (19). However, NADH and NADPH are acid labile (20), and consequently, these reduced coenzymes are certainly destroyed during the course of perchorlic acid treatment of intact tissues. We observed large amounts of phytate in the perchorlic acid extracts from spinach leaves when they contained very high concentrations of CDTA (this chelating agent prevented the precipitation of phytate that is highly insoluble as magnesium salt). Phytate appeared as a complex multiplet resonance (quadruplet) showing absorption bands at approximately 1–2 ppm in our experimental conditions. The exact position of this quadruplet was obtained with a 30° pulse angle and 0.3-s repetition time. Data treatments include Lorentz-Gauss transformation and zero filling. Peak assignments: a, unidentified peak; b, position of phosphorylcholine; c, unidentified peak (phosphodiester?); DHAP, dihydroxyacetone phosphate; PGA, glycerate-3-P; OPC, glycerophosphorylcholine; PEP, phosphoenolpyruvate.
was highly sensitive to pH and to the chemical environment, making its correct identification difficult. Since this phosphorus compound is not easily NMR detectable in vivo (Mg²⁺ phytate is insoluble), it was not possible to determine in which compartment (vacuole or cytoplasm) it was accumulated. The peak at 1 ppm (peak c in Fig. 2) close to that of phytate was not assigned. Since the position of this peak did not change when pH was varied from near 6 to near 8, it was very likely a phosphodiester. Its characterization is under investigation in our laboratory. The peak at 0 ppm has been ascribed to glycerophosphorylcholine. An additional resonance was seen at 0.5 ppm and was tentatively identified as phosphoenolpyruvate. There are many metabolites that are known to be present in the NMR spectra of the extracts. Among these are fructose 1,6-bisphosphate, pyrophosphate, and glyceraldehyde-3-P. We estimate that in our conditions using 5 g of spinach leaves, the concentration of phosphorylated compounds lower than 100 μM in the cytoplasmic compartment was not distinguishable from the background noise.

The high NTP/NDP ratio as seen in vivo (Fig. 1) (the β-NDP peak is almost invisible) was not observed for cell perchloric acid extracts (Fig. 2). Likewise, in the case of maize (Zea mays L.) root tips, NTP/NDP ratios determined in vivo by ³¹P NMR were always higher than ratios observed in extracts (21). Since little if any hydrolysis of ATP had occurred during the extraction procedure, these results indicate that ADP became visible to NMR during the process of HClO₄ extraction and strongly suggest that ADP extracts (21). Since little if any hydrolysis of ATP had occurred during the extraction procedure, these results indicate that ADP became visible to NMR during the process of HClO₄ extraction and strongly suggest that ADP in vivo is invisible to NMR because it is either bound to some paramagnetic metals or sequestered in a large protein-rich compartment (e.g. chloroplast stroma). In order to confirm this hypothesis, a careful analysis of the ³¹P NMR spectra of spinach chloroplasts has been carried out.

³¹P NMR Studies of Spinach Chloroplasts—³¹P NMR spectra obtained from intact spinach chloroplasts (8 mg of chlorophyll, 200 mg of protein) (Fig. 3) suspended in the extraction medium (see "Experimental Procedures") containing 2 mM EDTA showed a distinct peak of stromal P₁ at 2.4 ppm equivalent to approximately pH 7.8. An upward shift of the stromal P₁ resonance was associated with a decrease in the external pH (not shown). Since chloroplasts were fully intact, such a phenomenon was probably attributable to a leakage of protons through the inner membrane of the chloroplast envelope. In support of this suggestion, when spinach leaves were perfused with a deoxygenated (N₂-bubbled) medium or if perfusion was stopped, the cytoplasmic P₁ peak, including P₁ sequestered in chloroplasts, quickly moved upfield, indicating a nearly concurrent acidification of both the cytosolic compartment and the stromal space (Fig. 4) (originally the cytoplasmic pH was 7.5, and during the first few min after anaerobiosis it acidified to pH 7.0). On reoxygenation, cytoplasmic pH returned to its original value (Fig. 4). Again, these results indicate that although the inner membrane of the

![Fig. 3. Representative ³¹P NMR spectra (162 MHz) of intact spinach chloroplasts (8 mg of chlorophyll/ml). Intact chloroplasts (4.5 ml) were maintained in the extraction medium, and the spectrum was run at 25 °C. The spectrum is the result of 12,000 transients (1 h). The percentage of intact chloroplasts present in the suspension at the end of the experiment measured by following the reduction of ferricyanide before and after an osmotic shock (2) was approximately 90%. Peak assignments: PGA, glycerate-3-P; a, position of phospholipids (phosphatidylglycerol, phosphatidylcholine).](http://www.jbc.org/)

![Fig. 4. Time course evolution of the most abundant ³¹P NMR-detectable phosphorus compounds in spinach leaf pieces subjected to a transient anoxia. The sample (3 g, wet weight) was first conditioned as described in Fig. 1. Each spectrum was obtained over 1 min. (Four successive experiments were run, separated by a delay of 5 min for full recovery in normoxia. Having checked their reproducibility, the corresponding spectra were then added to increase the signal-to-noise ratio. Hence, each spectrum represents the phosphorus status of the cells for a period of time of 1 min.) The top spectrum is of oxygenated leaf pieces. The N₂ spectra were obtained sequentially, top to bottom, after switching perfusion to nitrogen saturated. The bottom two spectra (O₂) were obtained sequentially, after switching perfusion to O₂-saturated. Note that hypoxia induced a strong decrease of NTP and that the released P₃ accumulates within the cytoplasmic compartment, not in the vacuole. In addition, the rightward shift of the cytoplasmic P₃ resonance including P₃ sequestered in chloroplasts indicates that cytosolic and stromal pH values fall when spinach leaves become hypoxic. Note the rapidity with which the cytoplasmic pH returns to its original value (7.5) during the course of a hypoxia/normoxia transition. Peak assignments: G6-P, glucose-6-P; a, position of glycerate-3-P; b, position of phosphatidylcholine; Cyt-P₃ cytoplasmic P₃; Vac-P₃ vacuolar P₃.](http://www.jbc.org/)
chloroplast envelope constitutes an "insulating barrier" for
the protons, the pH in the stroma can be varied by cytosolic
acidosis ascribed to a transient production of lactic acid in
the cytosolic compartment (22) and perhaps to a marked
decrease in cytosolic ATP concentration. It follows that
membranes in vivo can have an intrinsic permeability to protons.

There were also signals from stromal glyceraldehyde-3-P and
NADP including phosphate at the 2' position (Fig. 3). The
broad resonance at -1 ppm (peak a) could be attributable to
phospholipids (phosphatidylglycerol and phosphatidylincholine)
present in chloroplast membrane systems (thylakoids, envelope
membranes) (23). Nucleotides including NTP and NDP were
almost invisible, whereas the same nucleotides were
clearly observed, and therefore unmasked, in a typical under-
coupled 31P NMR spectrum of a perchloric acid extract of
intact spinach chloroplasts (Fig. 5). Under these conditions,
in the presence of a large excess of CDTA in the perchloric
acid extracts, the resonances of nucleotides (ATP, ADP,
AMP, UMP) were sharp, and the NADP peaks, including the
peak at 3.5 ppm (2'-P), were clearly seen. The stromal ATP/
ADP ratio was low and was much lower than that observed
in the perchloric acid extract of whole tissues (5). Such a
low ATP/ADP ratio value does not necessarily reflect the in
vivo situation because ATP hydrolysis might occur during the
course of chloroplast isolation. Additionally, signals from β-
and α-UDP-glucose, β- and α-UDP-galactose, and phytate
observed in the perchloric extract of leaves were not present
in the chloroplast extract, indicating that these compounds
are localized in the cytosolic compartment. In contrast, gly-
ceraldehyde-3-P involved in polar lipid synthesis, glyceraldehyde-3-P, and phospholipid were well identified in the perchloric acid
extract of intact spinach chloroplasts. These results indicate therefore that inside a leaf cell, large
amounts of nucleotides (ADP) sequestered in the chloroplast
compartments are largely invisible to NMR. The mobility of
nucleotides in the stroma which is more viscous (0.4 g of
protein/ml) (23) should be considered. It is also possible that
nucleotides may be bound to stromal proteins. There is also
some evidence that intramitochondrial ATP and ADP are
also invisible to NMR (24). This implies that in spinach leaf
pieces, only free nucleotides present in the cytosolic compart-
ment are observed in an NMR spectrum.

Relationships between the Cytoplasmic and the Vacuolar
Phosphate Pools in Spinach Leaves—During the course of our
experiments, we have observed that the total amount of free
Pi sequestered in the vacuolar compartment of spinach leaf
pieces infiltrated with perfusion medium without Pi was
highly variable from one experiment to another depending on
the prehistory of the leaves (9). Apparently, the vacuolar Pi
content of freshly harvested spinach leaves declines during
storage. Leaves held in storage for several days prior to
preparation in the laboratory were depleted in vacuolar Pi.
The decrease in the vacuolar Pi pool did not correlate with a
decrease in the cytoplasmic Pi pool unless the vacuolar Pi
pool was completely depleted (not shown). A parallel can be drawn
with earlier observations on the Pi distribution in nongreen
cells or tissues (19).

When spinach leaf pieces containing a low vacuolar Pi
concentration were perfused with a medium containing phys-
iological concentrations of Pi (400 μM), this anion entered the
cells and accumulated in the vacuole continuously (Fig. 6). It
is interesting to note that during the course of vacuolar Pi
refilling, which is a rather fast process, the concentrations of
cytoplasmic Pi and glucose-6-P increased slightly up to a new
steady-state level and then remained constant throughout
this period (Fig. 7). Once the demand for phosphorus in the
cytosolic compartment was met, Pi molecules that actively
entered the cytosolic compartment were continuously taken
into the vacuole (see also Rebeillé et al. (26), Mathieu et al.
(28), Brodelius and Vogel (27), and Lauer et al. (9)). Curiously,
the final vacuolar Pi concentration attained was considerable,
exceeding 50 mM. Under these conditions, the Pi concentra-
tion in the vacuole is much higher than in the cytoplasm.

FIG. 6. Representative 31P NMR spectra of spinach leaves
after addition of 400 μM Pi to the perfusing medium. Leaf
pieces (3 g, wet weight) were first maintained in a well aerated
perfusion medium containing 20 mM sucrose without Pi to increase
the intracellular phosphoesters pool at the expense of vacuolar Pi.
After 10 h, leaf pieces were compressed as indicated in (Ref. 13). A
spectral from leaf pieces at 10 h following Pi starvation. B–D, spectra
from leaf pieces recovering from Pi starvation 1, 3, and 5 h, respec-
tively, after the addition of 400 μM Pi to the circulating medium.
Each spectrum was the result of 12,000 transients with a repetition
time of 0.3 s (1 h) and a 30° pulse angle. Phosphate (400 μM) was
added to the perfusion medium at the end of the accumulation time
of the spectrum A. In spectra B, C, and D, note that P added to the
perfusion medium (pH 6.8) (ext-Pi) is clearly distinguished from
vacuolar (Vac-Pi) and cytoplasmic Pi (Cyt-Pi). Note the steady ac-
cumulation of vacuolar Pi, with time. For peak assignments, see Fig.
1.
pieces (3 g, wet weight) were compressed as indicated in Ref. 13 for 2 h, containing 50  

vacuole is an energy-dependent process. In support of this suggestion, we have observed that whenever the cytosolic ATP concentration is substantial, excess cytosolic Pi is preferentially expelled toward the vacuole (19). On the contrary, when aerobic to anaerobic conditions) (19) (Fig. 4), Pi derived from such a result indicates that the sequestration of Pi in the chloroplast compartment devoid of Pi. At first, compressed leaf pieces were maintained for 10 h in a continuously oxygenated circulating medium containing sucrose in order to fill the cytoplasmic compartment with phosphorylated compounds. Then, the compressed leaf pieces were carefully washed with a medium devoid of Pi. Addition of choline (50 μM) in the circulating medium led to a marked increase in the resonance at 3.3 ppm (Fig. 1, peak b), which was essentially attributable to phosphorylcholine as confirmed by perchloric acid extracts (see Fig. 2). The NMR titration curve of phosphorylcholine indicated that the position of the phosphorylcholine peak (Fig. 8) corresponded to phosphorylcholine above pH 7.0 (not shown). This result suggests that phosphorylcholine was derived from exogenously added choline and accumulated in the cytoplasmic compartment, pH 7.5, and not in the vacuole, pH 5.5. Identical results were obtained using nongreen material (sycamore cells) (29). A careful analysis of Figs. 8 and 9 indicates that after 2 h of choline perfusion, the cytoplasmic Pi concentration decreased from 8 to 5 mM. No further decrease was observed even when high concentrations of choline were supplied (not shown). Although the total amount of cytoplasmic Pi, declined noticeably, the N1P level remained almost constant (Figs. 8 and 9) (see, however, Ref. 8). In spinach leaves, when choline was used as a Pi-sequestering agent instead of mannose (8), only a part of the cytoplasmic Pi pool was used for choline phosphorylation because a large part of it remained sequestered in the chloroplast compartment devoid of choline kinase activity. The phosphorylcholine that appeared during the first 2 h after the addition of choline in the circulating system corresponded approximately to the total amount of cytoplasmic Pi, vacuolar Pi, and glucose-6-P which disappeared within the same period of time (Fig. 9). When a threshold of cytoplasmic Pi was attained, the phosphorylation of choline was sustained almost exclusively by the continuous release of Pi, from the vacuole (Fig. 9). In this particular experiment, when almost all of the vacuolar Pi had been utilized, the final cytoplasmic phosphorylcholine concentration attained was considerable, exceeding 40 mM (about six times the concentration of glucose-6-P, the most abundant phosphate ester measured in vivo by 31P NMR). We have also observed that phosphorylcholine remained in the cytoplasmic fraction and did not significantly leak out of the leaf pieces (not shown). On the other hand, when 100 μM Pi, was present

**FIG. 8.** Representative 31P NMR spectra of spinach leaves after addition of 50 μM choline in the perfusing medium. Leaf pieces (3 g, wet weight) were compressed as indicated in Ref. 13 for 2 h, standard spectrum, 2 h and 8 h, spectra obtained after 2 and 8 h, respectively, of perfusion of the compressed leaf pieces with a medium containing 50 μM choline. Each spectrum was the result of 12,000 transients with a repetition time of 0.3 s (1 h) and a 30° pulse angle. Note the steady accumulation of phosphorylcholine (P-choline) with time. Peak assignments are the same as in Fig. 1.

**FIG. 9.** Time course evolution of the most abundant 31P NMR-detectable phosphorus compounds in spinach leaves perfused with a medium containing 50 μM choline. The concentrations of mobile phosphorus compounds in the leaf sample were determined as in Fig. 7. Note that after the addition of choline there is a small lag phase followed by a steady decrease of the vacuolar Pi (Vac-Pi) to sustain phosphorylcholine (P-chol) synthesis in the cytoplasmic (Cyt) compartment.
in the perfusion medium at the beginning of the experiment, the addition of 50 μM choline led to the same initial velocity of phosphorylcholine synthesis which was observed in the absence of P_1 (not shown). However, under these conditions, the cytoplasmic P_1 concentration was maintained constant while the concentration of vacuolar P_1 increased steadily (see Figs. 6 and 7). These results together demonstrate that externally added P_1 was preferentially used to sustain P_1 consumption in the cytosolic compartment. These results also demonstrate that inside a green cell, the level of phosphorylated intermediates in the cytoplasm can depend upon the amount of P_1 accumulated in the vacuole.

**DISCUSSION**

Our results emphasize that special precautions are required for monitoring by $^{31}$P NMR the behavior of leaf tissues over a long period of time under different conditions. In mature spinach leaves, the lacuna between upper and lower epidermis represents a large proportion of the total leaf volume. More than 80% of the volume of a mature photosynthetic cell consists of a vacuole. Furthermore, the NMR resonances for the cytoplasmic metabolites are weak. Consequently, in order to increase both the homogeneity of the magnetic field and the signal-to-noise ratio for cytoplasmic metabolites in the NMR spectra, it is absolutely necessary to eliminate gas bubbles trapped in the intercellular space by vacuum infiltration as shown previously by Waterton et al. (6) and to compress small leaf pieces between two circular porous plates (13). Under these conditions, leaf pieces can survive at least 4 days as long as a well aerated nutrient medium is pumped through the system under slight pressure. Such a system enabled high quality $^{31}$P NMR spectra of leaf tissue to be continuously recorded in which all the resonances observed (cytoplasmic and vacuolar P_1, nucleotides) were sharp and well resolved. However, direct measurement of changes between light and dark were hampered by obvious technical difficulties, and future work should focus on this problem.

$^{31}$P NMR spectrum of spinach leaf pieces under dark conditions was almost identical to those of nongreen plant tissues or cells (for review, see Refs. 5 and 19). The same resonances were observed with varying intensities. For example, all plant materials examined so far contain high concentrations of UDP-glucose. As pointed out by Bielecki (30), UDP-glucose is frequently underestimated in plant tissues because it hydrolyzes rapidly under alkaline conditions. The presence of large amounts of UDP-glucose in plant cells is probably related to the initial breakdown of sucrose catalyzed by sucrose synthase (FC 2.4.1.13) to give UDP-glucose (31). In addition, the pH values of the vacuolar and cytoplasmic compartments of different plant materials are very similar. The pH is approximately 7.5 for the cytoplasmic compartment and 5.5 for the vacuole.

Although $^{31}$P NMR cannot discriminate among all the P_1 pools present in the cytoplasmic compartment, the results presented in this paper strongly suggest that oxygenated leaf tissues maintained in the dark contain a low cytosolic P_1 concentration and that the bulk of the cytoplasmic P_1 is sequestered in the chloroplasts. We appreciate that a portion of cytoplasmic P_1 is sequestered in the mitochondria but suggest that the small volume of the matrix (6) means that this portion is relatively small. The arguments that favor a low cytosolic P_1 concentration are numerous. First, the P_1 concentration found in isolated chloroplasts (12 mM) is higher than that measured in the cytoplasmic compartment (6–9 mM) using $^{31}$P NMR. Given that the chloroplast comprises more than 60% of the cytoplasmic volume, such a result strongly suggests that the cytosolic P_1 concentration is much lower than that of chloroplast stroma. Second, one of the most surprising findings in our studies was that the total vacuolar P_1 pool could fluctuate considerably, whereas the cytoplasmic pool remained almost constant (Figs. 6–9) (see also Refs. 25 and 27). It is difficult to understand how P_1 movement is regulated between the vacuole and the cytosol if there is a continuous high cytosolic P_1 concentration. Again, these observations are best explained if one assumes that the cytosolic P_1 concentration is low. Under these conditions, any small $^{31}$P NMR changes in the concentration of free cytosolic P_1 would directly or indirectly affect P_1 efflux and influx from or into the vacuole. Third, it has been shown clearly that external P_1 strongly influences the rate of photosynthesis by isolated chloroplasts (2). If P_1 concentrations are above 1 mM in the medium, photosynthesis is inhibited because triose phosphate molecules are withdrawn from the stroma too rapidly. This classical observation strongly suggests that in a leaf, the cytosolic P_1 concentration, a key factor determining the cellular partitioning of carbon between sucrose and starch synthesis (32), is low. In addition, a high cytosolic P_1 concentration would be utterly inappropriate to the precise regulation of sucrose functioning pathway in the cytosolic fraction (32–34). Fourth, in nongreen cells or tissues such as yucca root tips (35), calibration of the peak intensity of the cytoplasmic P_1 resonance with known amounts of external P_1 gave estimates of cytoplasmic P_1 levels of 0.6–1.2 mM including P_1 present in the cytosol and various cell organelles (mitochondria and amyloplasts). Thus, when organelles comprised a small part of the cytoplasmic volume, the cytoplasmic P_1 concentration measured using $^{31}$P NMR was low. This is another indirect proof that in leaf tissues the cytosolic P_1 concentration is much lower than the values that have been suggested previously (28, 36; see, however, Ref. 8).

The results presented in this paper indicated that owing to the utilization of choline, a new cytoplasmic P_1-sequestering reagent (29), vacuolar P_1, efflux can occur in response to the depletion of P_1 from the cytoplasmic compartment. Exogenously supplied d-mannose to maize and tomato leaves can also reduce the vacuolar P_1 level in some circumstances (8). These observations raise, therefore, the question of the problem of P_1 movement between the vacuole and the cytoplasm and vice versa. Although no clear answers are available to such questions at this time, the cycling of P_1 through vacuolar influx (energy dependent) and efflux (passive?) pathways is a good system that may provide (on the time scale of several min) for control over the cytosolic-free P_1, and phosphorylated intermediate concentrations and therefore may be involved in the regulation of photosynthesis. For example, according to Stitt et al. (17), the change of esterified phosphate during the day may represent a decrease of the cytosolic P_1 by about 10 mM. Conversely, the change of the esterified phosphate in the cytosol during the night may indicate an increase of the cytosolic P_1. Since our results suggest strongly that the concentration of P_1 in the cytosol is insufficient to sustain the accumulation of esterified phosphate observed during the day, this implies that part of the phosphorylated intermediates are synthesized at the expense of P_1 deriving either from the external medium or the vacuole.

In contrast to the cytosol, the present results demonstrate that the stroma of the chloroplasts contain a high concentration of free P_1. Such a high concentration of P_1 is required to sustain the rapid synthesis of the Benson-Calvin cycle intermediates, especially ribulose 1,5-bisphosphate after the onset of illumination (2). Our results strongly suggest that a one-way P_1 transport, distinct from the one-for-one exchange of
triose phosphate and free Pi, via the phosphate translocator, may be the means by which the total stromal Pi pool is normally maintained. In support of this suggestion, we have observed recently that pyrophosphate is transported through the chloroplast envelope leading to a marked increase in the stromal Pi concentration (37). Such a suggestion is attractive because the cytosolic fraction of all the plant cells examined so far contains a pyrophosphate:fructose-6-P 1-phosphotransferase capable of working in the reverse direction to produce PPi (38).

The results presented in this paper also demonstrate that intrachloroplasm ATP and ADP are invisible to NMR. Indeed, upon making a perchloric extract of intact chloroplasts and treating it with CDTA to chelate metal ions, all the resonance peaks of ADP and ATP reappeared in the extract medium. Thus, these data indicated that inside the chloroplasts the nucleotides may be bound to proteins or paramagnetic substances that render the nucleotides invisible to NMR (39). Under these conditions, the high ATP/ADP ratio measured in vivo should reflect the cytosolic status in good agreement with biochemical data (40, 41).

Our results demonstrate for the first time that leaf cells contain nonnegligible amounts of phytate. Its estimated cellular concentration was about 1 mM. The precise role of phytate in the cells remains unknown, but it may serve as a store of P, cations (Mg²⁺ and perhaps free iron), or of high energy phosphoryl groups that can be metabolized by phytate and phytate-nucleotide diphosphate transferase during cell metabolism (42). Thus, phytate may buffer the variations of free Mg²⁺ and P concentrations in the cytosol. It is also possible that phytate in the cytoplasmic compartment prevents the catalysis by iron of radical formation and subsequent oxidative damage. As a matter of fact, phytate by virtue of chelating free iron could be a potent inhibitor of iron-driven hydroxyl radical (OH) formation (43, 44).

Finally, the results presented here demonstrate that intact chloroplasts contain nonnegligible amounts of sn-glycerol-3-P. The estimated concentration of sn-glycerol-3-P was about 0.5 mM on the basis of the whole chloroplasts, an amount sufficient to sustain polar lipid synthesis (45). The presence of this metabolite in the chloroplast stroma is probably correlated with significant levels of dihydroxyacetone phosphate reductase activity in the chloroplast (46).

REFERENCES


31P NMR studies of spinach leaves and their chloroplasts.
R Bligny, P Gardestrom, C Roby and R Douce


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