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 \(P_i\), glyceraldehyde-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 the vacuole to sustain choline phosphorylation. The question of cytoplasmic \(P_i\) 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.

**EXPERIMENTAL PROCEDURES**

**Plant Material**—Mature spinach (Spinacia oleracea L.) leaves were obtained from the local market and cut into small pieces (approximately 1 mm\(^3\)) directly into ice-cold perfusion medium (0.2 M mannitol, 0.05% (w/v) insoluble polyvinylpyrrolidone (Kca 25, Serva), 0.05% bovine serum albumin, and 5 mM MES buffer (pH 6.5) containing 20 mM sucröse and 10 mM \(P_i\). The small leaf pieces were infiltrated with perfusion medium under slight vacuum (7-9) and were stored at 10 °C under dark conditions until use.

**Isolation of Intact Chloroplasts**—Intact chloroplasts were introduced into ice-cold chloroplast extraction medium (330 mM mannitol, 30 mM MOPS-NaOH (pH 7.8), 2 mM EDTA, and 0.15% bovine serum albumin) with a tissue/medium volume ratio of 1:3. Leaves were homogenized three times for 2 s each with a Waring blender, and intact chloroplasts were prepared as fast as possible according to the method of Nakatani and Barber (10). Chloroplasts thus obtained were purified by isopycnic centrifugation in a nontoxic silica sol (Percoll, Pharmacia LKB Biotechnology Inc.) gradient that maintained isosomatic conditions throughout the isolation procedure according to the method of Mourioux and Douce (11). The entire isolation procedure could be accomplished in less than 30 min. At this point in the procedure, the thick chloroplast suspension was used for the measurement of \(CO_2\)-dependent \(O_2\) evolution rates and \(P_i\) content of the stroma (11).

**P, Determination**—An aliquot (50 \(\mu\)l; 350-400 \(\mu\)g of chlorophyll) of the chloroplast suspension was suspended in 5 ml of extraction medium. The tube was centrifuged at 3 °C for 1 min at 1500 \(\times\) g (Sorvall SM 24 rotor). The supernatant was removed by aspiration, and the pellet was resuspended in a low osmolarity medium (10 mM MOPS-NaOH (pH 6.5), 2 mM EDTA; final volume, 1.1 ml). One hundred \(\mu\)l of this suspension was used for chlorophyll determination. The remaining suspension was centrifuged 10 min at 7000 \(\times\) g (Sorvall SM 24 rotor) after addition of 200 \(\mu\)l of 20% trichloroacetic acid. A fraction of the clear supernatant (1 ml) devoid of mannitol, which interferes with phosphomolybdate complex formation, was used for the colorimetric determination of \(P_i\) after isobutyl alcohol extraction (12).

**\(^{31}\)P NMR of Spinach Leaves**—A Bruker NMR spectrometer (AM 400) operating in the pulsed Fourier transform mode at 162 MHz was used to characterize the various intracellular pools of \(P_i\) and phosphorus compounds known to be present in leaves. In order to get a better signal-to-noise ratio, NMR experiments on leaves required small leaf pieces densely packed within a limited volume approximating that of the NMR receiver coil (volume within the detection

1. The abbreviations used are: MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinopropanesulfonic acid; FID(s), free induction decay; EDTA, trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; UDPG, uridine 5'-diphosphate glucose.
**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 glyceraldehyde-3-P) declined progressively once the intracellular 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 Studies of Spinach Leaves and Their Chloroplasts**

Leaves were cut into small pieces and infiltrated in the ice-cold perfusion medium (see "Plant Material"). At time 0, the leaf pieces were transferred to medium at 22 °C with additions as indicated. O₂ evolution was followed polarographically at 25 °C by using a Clark-type O₂ electrode system (47). The reaction medium devoid of sucrose and containing 5 mM HCO₃⁻ was gassed with argon before the addition of leaf pieces equivalent to 50 ± 100 µg of chlorophyll. Light was provided by a 150-watt xenon arc lamp source (Oriel corporation), giving an irradiance of 300 watts m⁻² at the surface of the vessel.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Time (h)</th>
<th>O₂ evolution (µmol O₂ h⁻¹ mg⁻¹ chlorophyll)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>1</td>
<td>51</td>
</tr>
<tr>
<td>20 mM sucrose</td>
<td>3</td>
<td>28</td>
</tr>
<tr>
<td>1 mM P,</td>
<td>24</td>
<td>8</td>
</tr>
<tr>
<td>20 mM sucrose + 1 mM P,</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>142</td>
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<tr>
<td></td>
<td>24</td>
<td>95</td>
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<td>24</td>
<td>120</td>
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</table>

Addition Time O₂ evolution h µmol O₂ h⁻¹ mg⁻¹ chlorophyll

**TABLE I**

Effect of sucrose feeding of spinach leaf pieces on the photosynthesis rate

- The 31P NMR spectra of neutralized perchloric acid extracts were obtained from compressed leaf pieces supplied with oxygen and at pH 7.8 showed two distinct peaks of intracellular P, (Cyt-P, and Vac-P, 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 P, pool at the acidic pH and the cytoplasmic P, pool at the slightly alkaline pH (18). In the spectra obtained from spinach leaves, no other P, 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 cytoplasmic volume than in nonchlorophyllian cells (6). This means that in 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
the chloroplast is approximately 12 mM, peaks resided underneath it. The resonance at 3.5 ppm was in vivo situation. To determine accurately the cytoplasmic have calculated that within the receiving coil the cell volume comprised approximately 66% of the total volume. By considering the ratio of vacuolar volume to cytoplasmic volume comprised approximately 66% of the total volume. The perfusion medium was pumped through the compressed leaf pieces and was circulated via a 300-ml reservoir that was oxygenated by O2 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; cyto-P, cytoplasmic P; vac-P, vacuolar P.

unlikely because the concentrations of P, 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, from intact isolated chloroplasts was almost negligible (11); consequently, the P, content of intact chloroplasts reflected the in vivo situation). To determine accurately the cytoplasmic and vacuolar P, pools in leaf tissue, a calibration of the peak intensity of the P, resonance with known amounts of external P, was first performed. In our experimental conditions, we have calculated that within the receiving coil cell volume comprised approximately 66% of the total volume. By considering the ratio of vacuolar volume to cytoplasmic volume (roughly 8.5), the curve thus generated gave estimates of cytoplasmic P, levels ranging from 6 to 9 mM for the average concentration in the chloroplast, cytosol, and other cell organelles in the dark. Assuming that the chloroplasts comprise more than 60% of the cytoplasmic volume (40 μl of chlorophyll) (17) and that the concentration of mobile P, in the chloroplast is approximately 12 mM, our results suggest strongly that in the dark the concentration of cytosolic P, is much lower than that of chloroplast stroma. Owing to obvious technical problems associated with the illumination of packed mesophyll cells, we were unable to follow the fluctuation of cytoplasmic P, concentration which might be expected in the dark/light transition and vice versa (2, 17).

In the representative 31P NMR spectrum of spinach leaves (Fig. 1), there were also signals from cytoplasmic glucose-6-P, β- and α-UDPG (with contribution of some NAD(P) to the β-peak of UDPG), γ-, α-, and β-phosphorus of NTP. The region of the spectrum to high field of glucose-6-P corresponded to glycerate-3-P (peak a). However, other minor peaks resided under it. The resonance at 3.5 ppm was attributable to NMP and phosphorylcholine (peak b). 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 Perchloric Acid Extracts—Fig. 2 shows a typical undecoupled 31P NMR spectrum of a perchoric acid extract of spinach leaf pieces (6 g, wet weight; 5 mg of chlorophyll) at pH 7.8 in the presence of a large excess of CD3. 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 phosphomonooesters including dihydroxyacetone phosphate, gluconate-6-P, glucose-6-P, glycerate-3-P, sn-glycerol-3-P, and phosphorylcholine; P, γ-, α-, and β-ATP; γ-, α-, and β-UTP; β-, α-ADP; β-, and α-UTP; β- and α-UTP-galactose. 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-NTF 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 perchoric acid treatment of intact tissues. We observed large amounts of phytate in the perchoric acid extracts from spinach leaves when they contained very high concentrations of CDTA (this chelating agent prevented the precipitation of phytate which is highly insoluble as magnesium salt). Phytate appeared as a complex multipeak resonance (quadruplet) showing absorption bands at approximately 1–2 ppm in our experimental conditions. The exact position of this quadruplet...
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 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 Pᵢ at 2.4 ppm equivalent to approximately pH 7.8. An upfield shift of the stromal Pᵢ 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 Pᵢ peak, including Pᵢ 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

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**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 hr). 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: PCA, glycerate-3-P; α, position of phospholipids (phosphatidylglycerol, phosphatidylcholine).
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 phosphatidylcholine) 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 uncoupled 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 (Fig. 1). 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, glyceraldehyde-3-P involved in polar lipid synthesis, glyceraldehyde-3-P, and phosphorylcholine 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 compartment are observed in an NMR spectrum.

Relationships between the Cytoplasmic and the Vacular 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 physiological 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 cytoplasmic compartment was met, Pi molecules that actively entered the cytosolic compartment were continuously taken into the vacuole (see also Rebillé et al. (25), Mathieu et al. (26), 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 concentration in the vacuole is much higher than in the cytoplasm.

**Fig. 5.** Proton-decoupled 31P NMR spectrum of perchloric acid extract of intact spinach chloroplasts. Perchloric acid extract was prepared from intact chloroplasts (5 mg of chlorophyll) (see "Experimental Procedures"). The acquisitions conditions and treatment of the FID were the same as that described in Fig. 2. All the interesting regions are shown on an expanded scale. FGA, glyceraldehyde-3-P; P-choline, phosphorylcholine. Note the absence of UDP-glucose in the chloroplasts (see Fig. 2).

**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 spectrum from leaf pieces at 10 h following Pi starvation. B-D, spectra from leaf pieces recovering from Pi starvation 1, 3, and 5 h, respectively, 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 (Cyto-Pi). Note the steady accumulation of vacuolar Pi with time. For peak assignments, see Fig. 1.
FIG. 7. Time course evolution of the most abundant 31P NMR-detectable phosphorus compounds in spinach leaves perfused with a medium containing 400 μM Pi. The concentrations of mobile phosphorus compounds in the leaf tissue (average over the total sample within the detector) were determined as described under “Experimental Procedures.” Note the dramatic accumulation of Pi in the vacuole (Vac-Pi), Cyt-Pi, cytoplasmic Pi.

Such a result indicates that the sequestration of Pi in the vacuole is an energy-dependent process. In support of this hypothesis, we have observed that whenever the cytosolic ATP concentration is substantial, excess cytosolic Pi was preferentially expelled toward the vacuole (19). On the contrary, when the ATP concentration is low (i.e. during transition from aerobic to anaerobic conditions) (19) (Fig. 4), Pi derived from nucleotide and glucose-6-P pools stays in the cytoplasmic compartment. It is clear therefore, that in the case of spinach leaf cells, the vacuole represents a potentially large Pi reservoir.

Conversely, we also studied the availability of vacuolar Pi for cellular metabolism (8, 19, 28) in spinach leaves. To do this, we examined the effects that choline, a powerful new Pi-sequestering agent (29), has on the fluctuation of the intracellular Pi pool in spinach leaves. This compound is phosphorylated by a cytosolic choline kinase to form phosphorylcholine, which is not subsequently metabolized. Fig. 8 illustrates the changes that occurred in the compressed leaf pieces when choline (50 μM) was added to the perfusion medium 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 NTP 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. 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 0.5 s (1 h) and a 90° 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_i (not shown). However, under these conditions, the cytoplasmic P_i concentration was maintained constant while the concentration of vacuolar P_i increased steadily (see Figs. 6 and 7). These results together demonstrate that externally added P_i was preferentially used to sustain P_i 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_i 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_i, 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 Bieleski (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 (RC 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_i 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_i concentration and that the bulk of the cytoplasmic P_i is sequestered in the chloroplasts. We appreciate that a portion of cytoplasmic P_i is sequestered in the mitochondria but suggest that the small volume of the matrix (6) means that this proportion is relatively small. The arguments that favor a low cytosolic P_i concentration are numerous. First, the P_i 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_i 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_i 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_i movement is regulated between the vacuole and the cytosol if there is a continuous high cytosolic P_i concentration. Again, these observations are best explained if one assumes that the cytosolic P_i concentration is low. Under these conditions, any small ^31^P NMR changes in the concentration of free cytosolic P_i would directly or indirectly affect P_i efflux and influx from or into the vacuole. Third, it has been shown clearly that external P_i strongly influences the rate of photosynthesis by isolated chloroplasts (2). If P_i 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_i concentration, a key factor determining the cellular partitioning of carbon between sucrose and starch synthesis (32), is low. In addition, a high cytosolic P_i 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 sycamore cells (13) or maize root tips (35), calibration of the peak intensity of the cytoplasmic P_i resonance with known amounts of external P_i gave estimates of cytoplasmic P_i levels of 0.6-1.2 mM including P_i 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_i concentration measured using ^31^P NMR was low. This is another indirect proof that in leaf tissues the cytosolic P_i 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_i-sequestering reagent (29), vacuolar P_i, efflux can occur in response to the depletion of P_i from the cytoplasmic compartment. Exogenously supplied D-mannose to maize and tomato leaves can also reduce the vacuolar P_i, level in some circumstances (8). These observations raise, therefore, the question of the problem of P_i 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_i 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_i 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_i 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_i. Since our results suggest strongly that the concentration of P_i 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_i 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_i. Such a high concentration of P_i 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_i 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 intrachloroplast 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 phytase and phosphate-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).

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31P NMR studies of spinach leaves and their chloroplasts.
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