Transport and Phosphorylation of Choline in Higher Plant Cells

PHOSPHORUS-31 NUCLEAR MAGNETIC RESONANCE STUDIES*

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When sycamore cells were suspended in basal medium containing choline, the latter was taken up by the cells very rapidly. A facilitated diffusion system appertained at low concentrations of choline and exhibited Michaelis-Menten kinetics. At higher choline concentrations simple diffusion appeared to be the principal mode of uptake. Addition of choline to the perfusate of compressed sycamore cells monitored by 31P NMR spectroscopy resulted in a dramatic accumulation of P-choline in the cytoplasmic compartment containing choline kinase and not in the vacuole. The total accumulation of P-choline over a 10-h period exhibited Michaelis-Menten kinetics. During this period, in the absence of Pi in the perfusion medium there was a marked depletion of glucose-6-P, and the cytoplasmic P1 resonance disappeared almost completely. When a threshold of cytoplasmic Pi was attained, the phosphorylation of choline was sustained by the continuous release of Pi from the vacuole although at a much lower rate. However, when 100 μM inorganic phosphate was present in the perfusion medium, externally added Pi was preferentially used to sustain P-choline synthesis. It is clear, therefore, that cytosolic choline kinase associated with a carrier-mediated transport system for choline uptake appeared as effective systems for continuously trapping cytoplasmic Pi including vacuolar Pi entering the cytoplasm.

The enzyme choline kinase (EC 2.7.1.32; ATP:choline phosphotransferase) which catalyses the phosphorylation of choline to form P-choline is the first step in the cytidine pathway originally described by Kennedy and Weiss (1) in which choline is incorporated into phosphatidylcholine. Evidence indicates that this is the major pathway for the incorporation of choline into phosphatidylcholine in plants (2, 3). The reaction catalyzed by the soluble choline kinase proceeds far towards completion (4). This observation suggests that choline synthesized in vivo would sequester cytosolic Pi as P-choline, a compound which has been characterized in plants for the first time by Maizel et al. (5). In the present experiments, we have used 31P NMR to investigate the changes in Pi and P-choline of sycamore cells following the introduction of choline into the medium. Our results shed new light on the controlled flux of Pi to and from the vacuole and on the accumulation of P-choline in the cytosolic compartment.

MATERIALS AND METHODS

Plant Material

Sycamore (Acer pseudoplatanus L.) cells were grown as a suspension in a liquid medium according to Biligny (6) except Mn2+ was excluded to prevent excessive broadening of the vacuolar 31P orthophosphate resonance. The cell suspensions were maintained in exponential growth by frequent subcultures.

31P NMR Experiments

31P NMR spectra of sycamore cells were obtained with a Bruker WM 200 WB spectrometer operating in the pulsed Fourier transform mode at 81 MHz. The spectra were obtained with compressed cells (4 cm in height; 3 × 106 cells; 9 g wet weight) placed in a 25-mm tube under constant perfusion. Details of this assembly and its operation have been described previously by Roby et al. (7). The perfusate consisted of the culture medium devoid of phosphate and manganese and was adjusted to pH 6.5 (7). We have demonstrated previously that sycamore cells slightly compressed between two circular polymer filters can survive several days as long as a well aerated nutrient medium is pumped through the system under slight pressure. Furthermore, such a system enables 31P NMR spectra of plant cells to be continuously recorded.

In vivo spectra were recorded after 12,000 accumulations with a repetition time of 0.6 s and a pulse angle of 45°. Doubling of the repetition time to 1.2 s showed no appreciable change in the relative intensities of the Pi1 to nucleotide resonances. Assignments of the nucleotide and phosphate ester peaks and Pi1 (cytoplasm and vacuole, respectively) were made in accordance with those given previously (7). Estimates of intracellular pH from cytoplasmic and vacuolar phosphate were made using the standard reference curve of pH versus 31P chemical shifts (6). A reference capillary approximately 0.8 mm in diameter containing 50 mM methylene diphosphonate (pH 8.9 in 30 mM Tris) was inserted inside the inner circulating tube (inlet tube) of the NMR assembly. The resonance of this reference was assigned a δ value of +6.38 ppm relative to the 85% H3PO4 resonance which has a chemical shift of 0.00 ppm.

Perchloric Extract

For perchloric acid extract, cells (9 g wet weight, corresponding to 3 × 108 cells) were quickly frozen at liquid nitrogen temperature to avoid ATP degradation and finely pestled with 1 ml of 70% (v/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, and the supernatant was neutralized with 2 M KHCO3 to about pH 6. Then 50 μl of 0.8 M CDTA was added in order to chelate Mg2+ (9) engaged with nystatin and hexakisphosphate (phytate) (below pH 4.5; CDTA precipitates, over pH 7 Mg2+-phytate precipitates). The supernatant was then centrifuged at 10,000 g for 10 min to remove KClO4; the resulting supernatant was lyophilized and stored at liquid nitrogen temperature. For the NMR measurements, this freeze-dried material was

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reresolved in 2.5 ml of 40 mM Hepes buffer, pH 7.8, containing 50 mM CDTA and 10% D2O (perchloric acid extract).

The 31P NMR spectra of neutralized perchloric acid extracts were measured on a Bruker NMR spectrometer (AM 400) equipped with a 10-mm multinuclear probe tuned at 162 MHz. The deuterium resonance of D2O was used as a lock signal. Each spectrum represents the accumulation of 2048 FID broadband-proton decoupled, recorded with a sweep width of 6,000 Hz, a 60° pulse angle, and a repetition time of 4 s. An exponential multiplicity (0.5 Hz line width) was used to increase the signal-to-noise ratio. The perchloric acid extract spectra are also referenced to the position of the 85% HPO4 resonance thanks to a sample of 180 mM methylene diphosphonic acid (MCDTA and 10% D2O (perchloric acid extract). Used to increase the signal-to-noise ratio. The perchloric acid extracts, In the case of P-choline and P-ethanolamine these spectra are also referenced to the position of the 85% HPO4 resonance.

Mid-log phase cells were harvested by filtration, resuspended in sterile fresh culture medium (final pH 6.0-6.2), and incubated at 25 °C with constant shaking. The cell density was 106 cells/ml. After preincubation for 5 h,[14C]choline and carrier choline were added. At time intervals, 400-μl portions were rapidly removed, strained on a fiberglass filter (15 s, pressure of suction 0.2 bar), and rapidly rinsed three times with 5- to 10-ml aliquots of fresh culture medium. Two rinses were sufficient to remove more than 98% of the extracellular isotope. For the [14C]choline measurements, cells suspended in 5 ml of water were mixed with 10 ml of aqueous counting scintillant (AC5, Amersham Corp.) and counted in an Intertechnique liquid scintillation counter (SL 4000). [14C]choline (specific radioactivity, 2 GBq/mmole) was obtained from Amersham Corp. Only the choline spot was radioactive when a portion of [methyl-14C]choline was subjected to high-voltage ionophoresis.

Measurement of Enzyme Activities

All enzyme activities were assayed at 25 °C. The buffer was used as either 50 mM Tricine-NaOH (pH value under 7.4) or 50 mM Mops-NaOH (for pH value under 7.4). Reference to the procedures are given together with any features of the reaction mixtures that differed from those in the references.

Choline kinase activity was assayed by a modification of the methods of Ansell and Spanner (10) and Burt and Brody (11) which measure the formation of [14C]phosphocholine from [14C]choline. Activity was found almost exclusively in the cytosolic fraction prepared as described below. Optimal conditions for the assay were found to be 50 mM Hepes, pH 7.5, 1 mM dithiothreitol, 20 mM MgCl2, 5 mM ATP, and 2 mM [methyl-14C]choline chloride (0.1 GBq/mmol). In addition, an ATP-generating system consisting of 20 mM P-enolpyruvate and 100 units/ml of pyruvate kinase was added to the incubation medium to prevent the buildup of ADP. The reaction was initiated by the addition of 2-10 μl of the cytosolic fraction for a total volume of 100 μl. Samples were incubated for 1 h at 37 °C, and the reaction was stopped by the addition of 200 μl of a solution of tetraphenylboron (reagent for the direct titration of univalent inorganic as well as organic cations; 50 mg/ml of butyronitrile). Samples were immediately microcentrifuged for 30 s, and the organic layer containing unreacted choline was aspirated. The extraction of unreacted choline was repeated three times. The aqueous layer containing the phosphocholine was counted by scintillation spectrometry.
RESULTS

Intracellular Location of Choline Kinase—This was investigated in protoplasts from suspension cultures of sycamore cells. The following marker enzymes were used: mitochondria, fumarase; plastids, ADP-glucose pyrophosphorylase; peroxisomes, catalase; cytosol, alcohol dehydrogenase. The gentle rupture of intact protoplasts passed through a fine nylon mesh followed by centrifugation carried out in three steps (see "Material and Methods") produced a supernatant that contained almost all the choline kinase activity corresponding to the phosphorylation of 30–35 nmol of choline/h/10⁶ cells. This was not due to massive mitochondrial and plastidial contaminations of the supernatant or to the location of the choline kinase in the peroxisomes as no more than 1% of the fumarase, 5% of the ADP-glucose pyrophosphorylase, and 8% of the catalase were in the supernatant (Table I). The observation that almost all the alcohol dehydrogenase activity was in the supernatant is consistent with the location of choline kinase in the cytosol. Further evidence that choline kinase in sycamore cells is not within a membrane-bound cell organelle is provided by the low latency of the enzyme in carefully prepared lysates (total extract) of protoplasts (Table II). These results demonstrated that choline kinase is readily released after the cell membrane is stripped and is present in ruptured preparation (100. ruptured preparation (100. ruptured preparation

Preparation of intact and broken protoplasts and centrifugation (12,000 X g for 10 min) of intact organelles were carried out as described under "Materials and Methods." These data are from a representative experiment and have been reproduced five times.

<table>
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<th>Enzyme</th>
<th>Total extract activity</th>
<th>Distribution</th>
<th>% total activity</th>
</tr>
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<tr>
<td></td>
<td>nmol/min/10⁶ cells</td>
<td>Supernatant</td>
<td>Pellet</td>
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<tr>
<td>Fumarase</td>
<td>25</td>
<td>1</td>
<td>97</td>
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<tr>
<td>ADP-glucose pyrophosphorylase</td>
<td>2</td>
<td>5</td>
<td>102</td>
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<tr>
<td>Catalase</td>
<td>550</td>
<td>8</td>
<td>82</td>
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<tr>
<td>Alcohol dehydrogenase</td>
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<td>92</td>
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</tr>
<tr>
<td>Choline kinase</td>
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<td>30</td>
<td>2</td>
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</table>

Enzymes activities of intact (in the presence of 0.5 mM sucrose) and ruptured (by adding 0.025% Triton X-100) cell fractions were measured as described under "Materials and Methods." The activity in the ruptured preparation (T) minus that in the intact preparation (U) is called latent and is expressed as a percentage of the activity of the ruptured preparation (100(T - U)/T) to give latency (10). These data are from a representative experiment and have been reproduced five times.

<table>
<thead>
<tr>
<th>Cell compartment</th>
<th>Total activity</th>
<th>Latency</th>
<th>Protein</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>nmol/h/10⁶ cells</td>
<td>%</td>
<td>mg</td>
</tr>
<tr>
<td>Total extract</td>
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<td>Pellet</td>
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Enzymes activities of intact (in the presence of 0.5 mM sucrose) and ruptured (by adding 0.025% Triton X-100) cell fractions were measured as described under "Materials and Methods." The activity in the ruptured preparation (T) minus that in the intact preparation (U) is called latent and is expressed as a percentage of the activity of the ruptured preparation (100(T - U)/T) to give latency (10). These data are from a representative experiment and have been reproduced five times.

Fig. 1. Effect of the concentration of choline on choline uptake by sycamore cells. The cells were incubated at 25 °C, with constant shaking in sterile fresh culture medium, pH 6.0, in which different concentrations of [methyl-¹⁴C]choline (specific radioactivity, 2 GBq/nmol) were added at time 0. Two-h kinetics were repeated five times, and the given values correspond to maximum rates of choline uptake at each concentration indicated on the graph. Cell rinsing and radioactivity counting were done as described under "Materials and Methods."
**$^{31}$P NMR of Cells**—$^{31}$P NMR spectra obtained from slightly compressed sycamore cells under aerobic conditions, at pH 6.5, showed two distinct peaks of intracellular Pi (cyt-P, and Vac-P, Fig. 2) at approximately 2.2 and 0.4 ppm equivalent to pH 7.5 and 5.9, 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 (8). In the spectra obtained from sycamore cells, no other Pi peaks can be identified unambiguously. As suggested in a previous paper (7), this means that the pH difference between organelles (mitochondria and amyloplasts) and the cytosol is too small for the signals to be discriminated. To determine accurately the internal cytoplasmic and vacuolar Pi concentration, a calibration of the peak intensity of the Pi resonance with known amounts of external Pi, was performed (see Ref. 7). The curve thus generated gave estimates of cytoplasmic Pi levels of 1.2 mM including Pi, present in cytosol and various organelles and approximately a 4 mM concentration of Pi, in the vacuole (to estimate intracellular Pi concentrations, we assumed that 6 g of wet cells contains 1 ml of cytoplasm). Interestingly, the Pi concentration observed in sycamore cells and in maize root tips (26) is much lower than that of green cells (approximately 10–15 mM) (27). There were also clear signals from cytoplasmic glucose-6-P, $\beta$- and $\alpha$-UDP-glucose, and $\gamma$-, $\alpha$-, and $\beta$-phosphorus of NTP. The low field region of the spectrum includes also signals from other phosphomonesters: peak a at 3.8 ppm which originates from several compounds including phosphoglyceric acid and fructose-6-P and peak b at 3.3 ppm which originates mainly from NMP and P-choline. Unambiguous determination of the origin of phosphate resonances in the spectra of *in vivo* plant cells were obtained from $^{31}$P NMR spectra of perchloric acid extracts (Fig. 3). The perchloric acid extracts were prepared from 9 g of sycamore cells (wet weight) and neutralized at pH 7.8 in the presence of about 60 mM CDTA. Under these conditions the observed resonance peaks were sharp. The major peaks were characterized by the addition of known amounts of P compounds to the extracts. The assignment for each resonance is given in the legend of Fig. 3. The peak at 3.3 ppm close to that of NADPH (2'-P) was assigned to P-choline. The complex multiplet centered at 2.3, 2.2, 2.1, and 1.9 ppm on the high-field side of the P, was attributable to $\gamma$-inositolhexakisphosphate (phytate). Phytate was $^{31}$P NMR-visible insofar as the medium contains a large excess of CDTA that chelates Mg$^{2+}$ engaged with phytate.

**Accumulation of Phosphorylcholine in Sycamore Cells**—Fig. 4 illustrates the changes that occur in the sycamore cell spectra when choline (10 $\mu$M) is added to the nutrient medium. In this experiment the cells were maintained for 36 h in a continuously oxygenated circulating medium (Pi-free nutrient medium) at pH 6.5. Total acquisition times of 120 min were used. Addition of choline in the circulating medium led to a marked increase of the resonance at 3.3 ppm (peak b) which was essentially attributable to P-choline as confirmed by perchloric acid extracts (Fig. 3, B and $B'$). The NMR titration curve of P-choline indicated that the position of peak b corresponds to P-choline above pH 7.0 (not shown). This result suggests that P-choline, derived from exogenously added choline, accumulated in the cytoplasmic compartment, pH 7.5, and not in the vacuole, pH 5.5. During this period, there was a marked depletion of glucose-6-P and the cytoplasmic Pi resonance disappeared almost completely (Figs. 4 and 5). A careful determination of P-choline made after a series of spectra obtained by addition of known amounts of P-choline to the perchloric acid extracts (see “Material and Methods”) demonstrated that the total amount of P-choline that appeared during the first 6 h after the addition of choline in the circulating system corresponded approximately to the total amount of cyt-P, and glucose-6-P that disappeared within the same period of time (Fig. 5). We calculate from Fig. 5 that the rate of net synthesis of P-choline during the first 2 h was 6–8 nmol of P-choline/h/10$^9$ cells at a fixed concentration of 10 $\mu$M added choline. Interestingly, although the total amount of cytoplasmic Pi dropped considerably, the NTP level remained high. Thus, after 4 h of choline perfusion, the cytoplasmic NTP concentration decreased from 1.2 to about 0.9 mM, whereas the NTP/NDP ratio was essentially unchanged (Fig. 3B). This observation indicates that the very low cytoplasmic P concentrations (less than 200 $\mu$M) attained after 4 h of choline perfusion do not significantly limit oxidative phosphorylation and confirms recent results showing that in *in vivo* the respiration rate of sycamore cells is not limited by the quantity of Pi, supplied to mitochondria (28). Since the perfusion medium does not contain Pi, it is clear, therefore, that during the first 6 h of choline perfusion, the phosphate necessary for P-choline synthesis is derived from cytoplasmic Pi, and Pi generated from phosphate esters and P-choline. When a threshold of cytoplasmic Pi was attained (in these series of experiments, not all of the cytoplasmic Pi pool could be used for choline phosphorylation because it is an obligatory anion necessary to sustain various metabolic sequences), the phosphorylation of choline was sustained by the continuous release of P, from the vacuole, at a rate that dropped from 5 to 1 nmol/h/10$^9$ cells (Fig. 5). The low rate of vacuolar P, efflux which occurs in response to the depletion of P, from the cytoplasmic compartment is sufficient to empty.

![Fig. 2. Representative $^{31}$P NMR spectrum (81 MHz) of compressed sycamore cells harvested at the end of the exponential phase of growth. Cells (9 g) are compressed by hand between two circular polymer filters in a 25-mm (diameter) NMR tube as described under "Materials and Methods" and maintained in a continuously aerated solution (Pi- and manganese-free culture medium). The spectrum was recorded after an acclimation time of 10 h, which is necessary for the compressed cells to remain physiologically stable. The spectrum recorded at 25 °C with a 45° pulse angle is the result of 12,000 transients with a repetition time of 0.6 s (2 h). Peak assignments: Glc-6-P, glucose-6-P; a, position of various P compounds including phosphoglyceric acid, fructose-6-P, ribose-5-P, P-ethanolamine, and NMP (nucleotides monophosphate); b, NADPH; and P-choline; cyt-P, cytoplasmic P; c, position of glucose-1-P; Vac-P, vacuolar P; d, position of different phosphodiesters and myo-inositol hexakisphosphate (phytate); NTP, nucleotides triphosphate (mainly ATP and UTP); NDP, nucleotides diphosphate (mainly ADP and UDP); UDPG, uridine-5′-diphosphate-α-D-glucose. Peak assignments were confirmed with the help of the spectra obtained from perchloric acid extracts (see Fig. 3).](image-url)


The vacuole of its Pi content (Fig. 5) within 20-30 h. When almost all the vacular P, has been utilized (not shown), the final cytoplasmic P-choline concentration attained is considerable, exceeding 10 mM (about four times the concentration of glucose-6-P which is the most abundant phosphate ester measured in vivo by $^{31}$P NMR). Addition of P, to the external medium (Fig. 5, arrow) led to a marked increase in the cytoplasmic P, and glucose-6-P resonances and stimulated the P-choline synthesis up to its original value of 5-6 nmol/h/10^6 cells. On the other hand, when 100 μM P, was present in the perfusion medium at the beginning of the experiment, the addition of 10 μM choline led to the same initial rate of P-choline synthesis that was observed in the absence of P,.

However, under these conditions, the cytosolic glucose-6-P and P, concentrations were maintained constant, demonstrating that externally added P, was preferentially used to sustain P-choline synthesis (unshown result). In this case, the cytoplasmic P-choline concentration reached higher values (15-20 mM in the cytoplasm) after 36 h of perfusion. Interestingly, such a high cytosolic P-choline concentration did not affect the survival of sycamore cells. Furthermore, we have observed that P-choline remained in the cytosolic fraction and did not significantly leak out of the cells.

When the initial velocity of P-choline synthesis was determined as a function of the choline concentration in the perfusion medium, the results could be fitted well by the simple Michaelis-Menten equation (Fig. 6). At pH 6, the mean estimated kinetic parameters of the system was $K_m = 1.5$ μM; $V_m = 6-7$ nmol of P-choline synthesized per h/10^6 cells.

Since the kinetic parameters of P-choline synthesis observed in vivo were almost identical to the kinetic parameters of choline transport across the plasmalemma membrane, at least at low choline concentrations, these results strongly suggest that the choline transported through the membrane was instantly converted into P-choline by the excess of choline kinase present within the cell cytosol. Bygrave and Dawson (29) have shown that a somewhat similar situation exists with the anaerobic prototaxan Entodinium, where choline kinase might possibly play an active role in the transport phenomenon. In support of this, using the tetraphenylboron reagent (see “Material and Methods”) to trap organic cations and the butyronitrile as solvent, no free choline could be detected within the cells at low external choline concentrations (up to 20 μM), after 4-h incubation. However, with higher concentrations of choline in the medium, the added choline was increasingly recovered as free choline probably sequestered in the vacuole (not shown).

Addition of limiting amounts of choline to the perfusion medium (10 μmol; volume of the perfusion medium: 10 liters; 3 x 10^6 cells) did not lead to the accumulation of identical amounts of P-choline in the cell cytosol (Fig. 7), because the synthesis of phosphatidylcholine with time (approximately 5 μmol) was apparent (Table III). For example, when all the added choline had been phosphorylated, the total amount of P-choline accumulated in the cytosol decreased slowly with time (approximately 0.5 nmol/h/10^6 cell). The linear decrease in cytosolic P-choline closely correlates with the linear increase in phosphatidylcholine amount (0.7 nmol/h/10^6 cells.
VlDP
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FIG. 4. Representative 31P NMR spectra (81 MHz) of sycamore cells after addition of 10 μM choline in the perfusing culture medium. Cells are prepared as indicated in Fig. 2. The spectra, recorded at 25 °C, with a 45° pulse angle were the result of 12,000 transients with a repetition time of 0.6 s (2 h). A, standard spectrum; B, C, D, and E, spectra obtained after 2, 6, 12, and 18 h, respectively, of perfusion of the compressed cells with a culture medium containing 10 μM choline. A 20-liter reservoir of perfusion medium was utilized in order to maintain a constant choline concentration. Note the steady accumulation of P-choline (phosphorylcholine) with time. Peak assignment are the same as in Fig. 2. The reference capillary contained approximately 1 nmol of methylene diphosphonate (MDP) at pH 8.9. From data of Table III. This result indicates that during the course of the perfusion with a well aerated nutrient medium the accumulated P-choline contributes to cell enlargement or multiplication which is not stopped. In support of this suggestion, the fresh wet weight increased from 9 g at the beginning of the experiment up to 13 g after 40 h of perfusion. Under these conditions, P-choline was slowly metabolized to sustain phosphatidylcholine synthesis, the major polar lipid of all the cell membrane systems.

DISCUSSION

The separation of the intact organelles from the lysed protoplasts obtained from sycamore cells indicates that choline kinase activity is essentially limited to the cytosolic fraction. Interestingly, Morré et al. (30), Warfe and Harwood (23), and Kinney et al. (31) have shown that in green onions, soja bean, and rye roots, the activity was found exclusively in the 100,000 g/60 min supernatant, suggesting a cytosolic localization. As in animal cells (25), the choline kinase of sycamore cells was inhibited by hemicholinium-3.

Transport systems for choline uptake have been described in various mammalian cells (32, 33) or tissues (34, 35) and in the anaerobic prototaxa Entodinium caudatum (29). The results presented in this paper strongly suggest that a carrier is also involved in the transport of choline into sycamore cells based on the observations that it was saturable and its activity was reduced by N-ethylmaleimide, a potent alkylating agent. However, although a facilitated diffusion system appears to low concentrations of choline (up to 20 μM) at

FIG. 5. Time course evolution of the most abundant 31P NMR-detectable phosphorous compounds in sycamore cells perfused with a culture medium containing 10 μM choline. The concentrations of mobile phosphorous compounds in the cell sample (average over the total sample within the detector) were determined either biochemically (18) or by comparing the area of the signals from the methylene diphosphonate capillary reference with its area relative to the area of the phosphorous resonances obtained from known concentrations standard solutions of glucose-6-P (Glc-6-P); P-choline (P-Chol); Pi; UDP-glucose (UDPG); and nucleotide triphosphate (NTP) (see Fig. 4). As formerly indicated (7), the ratio of vacuolar volume to cytoplasmic volume was roughly 5. After the addition of 200 μM Pi (arrow), note the almost immediate synthesis of glucose-6-P compared to the 2-h-delayed increase of the vacuolar and cytosolic Pi and of the NTP.

FIG. 6. Effect of the concentration of choline on P-choline synthesis by sycamore cells followed by NMR. Cells (3 x 10⁶) were prepared as indicated in Fig. 2. Different concentrations of choline were added to the culture medium. The rates of P-choline synthesis were calculated from kinetics performed as described in Figs. 4 and 5. The apparent Kₐ value for choline calculated from the Lineweaver-Burk representation was 2 μM. Higher concentrations (above 50 μM), simple diffusion appears to be the principle mode of uptake, because substrate saturation was no longer observed, and there was no indication of
specific inhibition.

The fact that sycamore cells exhibit a specific carrier driving choline inside the cell can be explained by the existence of large amounts of P-choline in the xylem vessels (5, 36). Indeed sycamore cells utilized in this work are derived from the cambium of Acer pseudoplatanus, a tissue which is in close contact with xylem vessels. It is possible, therefore, that P-choline molecules leaving the xylem vessels could be hydrolyzed in the cell wall by a specific acidic phosphatase (according to Martin and Tolbert (36), P-choline is a very stable molecule; it is resistant to strong acid hydrolysis and is hardly hydrolyzed by unspecified acidic phosphatases), and choline molecules thus formed could enter the cambium cells via a specific carrier. In support of this suggestion, preliminary experiments carried out in this laboratory indicated that perfusion of sycamore cells with their culture medium containing P-choline induced a rapid increase in cytosolic P-choline concentration and that P-choline was hydrolyzed outside the cell before its transport through the cell membrane. Since choline is not required for the normal growth of sycamore cells, the physiological significance of the presence of a carrier for choline entry into cell cytosol remains, however, an open question. This is in contrast with the situation observed in several animal tissues or cells which are dependent on a supply of choline from the blood stream or in the anaerobic protozoon E. caudatum (29) where growth of the organism depends on the availability of choline.

The data reported here also demonstrate that perfusion of slightly compressed sycamore cells with P-free culture medium containing small amounts of choline triggers a steady accumulation of P-choline in the cytoplasmic compartment at the expense of cytoplasmic P, including P, derived from glucose-6-P. When a threshold of cytoplasmic P, concentration is attained, P is drawn from the vacuole to sustain choline phosphorylation although at a much slower rate. These results in agreement with Roby et al. (7) emphasize, therefore, the slowness with which P, leaks out from the vacuole to refill the cytoplasmic P, pool. Guynn (4) has shown that the equilibrium constant under physiological conditions of the reaction of choline kinase is extremely high, that is the reaction proceeds far toward completion. Such an observation easily explains the reason why choline can sequester almost all the cytoplasmic P, and large amounts of the vacuolar P, as P-choline, which is not rapidly metabolized. In other words, choline kinase plays a crucial role in the rapid accumulation of choline either synthesized actively in vivo or deriving from the external medium, in the form of P-choline, and prevents its possible uptake into the acidic vacuole. It has been clearly shown by Chen-She et al. (37) and Herold et al. (38) that mannose and 2-deoxyglucose also sequester cytosolic P, as phosphorylated compounds which are not readily metabolized. Preliminary experiments carried out in this laboratory indicate that when sycamore cells are fed with mannose, only a small part of the intracellular P, is sequestered as mannose-P, that is the concentration of mannose-P attained at the equilibrium is much lower than that of P-choline. It is clear, therefore, that choline kinase appears as a more effective system than mannose/hexokinase for continuously trapping cytoplasmic P, including vacuolar P, entering the cytoplasm. Likewise, cytosolic glyceraldehyde-3-phosphate dehydrogenase appears as an effective system to trap intracellular pools of P (Bligny et al. (39)).

Our observations demonstrate that P-choline, which accumulates in the cell cytosol, is used only for phosphorylcholine synthesis. Interestingly, using sycamore cells Roby et al. (7) and Dorne et al. (40) have shown that after a long period of sucrose starvation, that is when almost all the intracellular carbohydrate pools have disappeared, the fatty acids derived from membrane polar lipids such as phosphatidylcholine are utilized for respiratory purposes, whereas cytoplasmic P-choline increased symmetrically. In addition they demonstrated that during the course of sucrose replenishment, P-choline previously accumulated was slowly re-used for phosphorylcholine synthesis. These results together demonstrate that P-choline exhibits a remarkable metabolic inertness (36) unless it is required for phosphorylcholine synthesis. However, in several plant tissues, choline can be oxidized to the quaternary ammonium compound betaine by a two-step oxidation (41). In our experimental conditions, the oxidation of choline to betaine aldehyde and betaine (41) seems unlikely since this process is the precisely controlled reaction of a few particular
groups of halophyte or semi-halophyte plants to water deficit or salt stress. In most plant cells, the concentration of P-choline observed in cell cytoplasm should reflect, therefore, a steady-state equilibrium in the production and utilization of P-choline.

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