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13C Nuclear Magnetic Resonance Studies of Malate and Citrate Synthesis and Compartmentation in Higher Plant Cells*

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The synthesis of malate and citrate by sycamore cells (Acer pseudoplatanus L.) perfused with KH13CO3 was analyzed using 13C NMR. To perform in vivo experiments, cells were compressed in a 25-mm tube and perfused with an arrangement enabling tight control of the circulating nutrient medium. An original method using paramagnetic Mn2+ that induced a complete loss of the vacuolar malate and citrate signals was developed to discriminate between cytoplasmic and vacuolar pools of malate and citrate. Our results indicated the following. (a) The accumulation of appreciable amounts of malate in sycamore cells required rather high (1 mM) concentrations of bicarbonate at all the pH values tested. (b) Malate was equally labeled at C-1 and C-4, suggesting that malate labeled at C-1 was produced by randomization of C-1 and C-4 by mitochondrial fumarase. Indeed, the separation of the intact organelles from the lysed protoplasts indicated the tonoplast in governing the vacuolar influx of citrate and the permanent exchange of malate between pools of malate and citrate. Our results indicated the mitochondria. Similarly, citrate was equally enriched at C-1 and C-5 + C-6 carboxyls. (c) Malate appeared first in the cytoplasmic compartment; and when a threshold of cytoplasmic malate concentration was attained, malate molecules were expelled into the vacuole, where they accumulated. On the other hand, citrate accumulated steadily in the vacuole. Pulse-chase experiments demonstrated the central role played by the tonoplast in governing the vacuolar influx of citrate and the permanent exchange of malate between the cytoplasm and the vacuole.

P-enolpyruvate carboxylase occurs in all plants and catalyzes the carboxylation of P-enolpyruvate to oxalacetate, which in turn can be reduced to malate by malate dehydrogenase. The reaction catalyzed by P-enolpyruvate carboxylase is highly exergonic (the ΔG for this reaction is in the vicinity of 30 kJ mol−1) (1, 2). Furthermore, Gout et al. (3) observed that any increase in cytoplasmic pH stimulates the synthesis of malate. Consequently, in vitro in the presence of 13CO2, malate labeled at C-4 should be the product of carboxylation via P-enolpyruvate carboxylase. Chang and Roberts (4) and Stidham et al. (5) used 13C NMR to observe the incorporation of 13CO2 into malate by maize root tips and intact leaves of Kalanchoë tubiflora. Surprisingly, both groups observed a significant incorporation of 13C label into C-1 of malate and suggested therefore that malate labeled at C-1 was produced by randomization of C-1 and C-4 by mitochondrial fumarase. However, reports by two independent groups showed that rat liver (6) and Saccharomyces cerevisiae (7) contain two isozymes of fumarase that are localized in different intracellular compartments, the mitochondria and the cytosol. Consequently, it is possible that the unexpected incorporation of 13CO2 into C-1 of malic acid was caused by equilibration with fumarate in the cytosol, where fumarase activity would exchange the label between C-1 and C-4.

In this investigation, we have used protoplasts from sycamore cells as a source of subcellular fractions and have concluded that in plant cells fumarase is confined to the mitochondria. In addition, 13C NMR spectroscopy of intact sycamore cells was used to follow malate and citrate accumulation in vivo. One great advantage of 13C NMR spectroscopy is that the resolving power of the technique allows for simultaneous identification and quantification of individual carbon atoms of the same molecule, in addition to distinguishing between molecules (8).

MATERIALS AND METHODS

Plant Material—The strain of sycamore (Acer pseudoplatanus L.) used in the study was grown as a suspension in a liquid nutrient medium containing 0.5 M mannitol, 10 mM KCl, 5 mM MgCl2, 1% (w/v) bovine serum albumin, and 0.1% (w/v) pectolyase Y-23 (Seishin Pharmaceutical Co., Nishinomiya, Japan) adjusted to pH 5.7. The cells were incubated with constant shaking (20 cycles/min) at 25°C. Digestion of young cells at 25°C for 45 min resulted in a high yield of protoplasts. After digestion, the suspension was filtered through one layer of Miracloth (Krantex, Alfortville, France), which retained any undigested cell aggregates; and protoplasts were collected by centrifugation at 150 X g for 10 min and then washed twice with 300 ml of suspension medium (0.5 M mannitol, 10 mM phosphate buffer (pH 7.5), 10 mM KC1, 5 mM MgCl2, 1% (w/v) polyvinylpyrrolidone (M, ~25,000, Serva), 0.1% (w/v) bovine serum albumin). Protoplasts were stored in suspension medium (600 ml) and were normally used within 1–2 h.

Gentle Rupture of Protoplasts and Separation of Organelles from Cytosolic Fraction—Since sycamore cell protoplasts have an average diameter of 20–30 μm, a rapid and effective procedure for the gentle rupture of intact protoplasts (i.e. for stripping the cell membrane) is to pass protoplasts through a fine nylon mesh (Nybolt PA, 20 μm).

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1 The abbreviations used are: Mops, 4-morpholino propane sulfonic acid; CDTA, trans-1,2-diaminocyclohexane-N,N,N’,N’-tetraacetic acid.
affixed to the cut end of a 100-ml disposable syringe (11). Thus, if protoplasts (equivalent to 10 g, wet weight, 50 ml) are taken up and expelled through the 20-μm nylon mesh, they will be completely ruptured. To separate the cytosolic fraction from the cell organelles, we subjected the broken protoplast fraction to centrifugation to yield a pellet largely free of cytosol and a supernatant enriched in cytosolic enzymes. Centrifugation was carried out in three steps (100,000 × g for 10 min, 400 × g for 5 min (RS-4 rotor, Kubota KN-70 centrifuge), and 20,000 × g for 20 min (SS-34 rotor, Sorval)). Each supernatant was centrifuged in a new tube, and the three successive pellets were combined together (cell organelles). This procedure ruptures all the protoplasts, leaving the intact mitochondria largely intact. We verified that, under these conditions, activities were linear with respect to time for at least 2 min and were proportional to the amount of protein. Fumarase (EC 4.2.1.2), ADP-glucose pyrophosphorylase (EC 2.7.7.27), catalase (EC 1.11.1.6), alcohol dehydrogenase (EC 1.1.1.2), and citrate synthase (EC 4.1.3.7) assays were performed according to previous publications (17, 18).

RESULTS

Intracellular Location of Fumarase—This was investigated in protoplasts from suspension cultures of sycamore cells. The following marker enzymes were used: mitochondria, citrate synthase; plastids, ADP-glucose pyrophosphorylase; peroxisomes, catalase; and cytosol, alcohol dehydrogenase. The gentle rupture of intact protoplasts passed through a fine nylon mesh followed by centrifugation carried out in three steps (see "Materials and Methods") produced a supernatant that did not contain fumarase activity (Table I). The observation that almost all of the alcohol dehydrogenase activity was in the supernatant is consistent with the absence of fumarase in the cytosolic compartment. Furthermore, very little latency was found for alcohol dehydrogenase in carefully prepared lysates of protoplasts (data not shown), indicating that almost all the protoplasts had been ruptured. These results demonstrate that fumarase is confined within a membrane-bound cell organelle. To localize fumarase activity more precisely in the pellet containing cell organelles, intact amyloplasts and mitochondria were isolated from sycamore cells. As expected (data not shown), fumarase was not associated with sycamore cell amyloplasts and was confined within the mitochondria (~110 nmol/min/mg of mitochondrial protein). These results together strongly support the unique location of the fumarase in mitochondria in plants in contrast to what was previously observed in yeast (7) and rat liver (6), where a substantial fraction of fumarase was found in the cytosol.

Accumulation of Citrate and Malate in Sycamore Cells during Utilization of [13C]Bicarbonate—In vivo 13C NMR spectra obtained under aerobic conditions at pH 6.5 (Fig. 1A) showed that the resonances of highest intensity corresponded to those

<table>
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</tr>
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<td>ADP-glucose pyrophosphorylase</td>
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<td>3</td>
</tr>
<tr>
<td>Catalase</td>
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<td>Citrate synthase</td>
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</table>
of the glucosyl and fructosyl moieties of sucrose and were estimated to correspond to an intracellular level of -70 pmol/g, wet weight, in good agreement with previous biochemical and growth data (19). Figs. 2 and 4 also indicate that, when sycamore cells were treated with [13C]bicarbonate for 1 h, the intensities of the signals from the C-1 and C-4 carbons of cytoplasmic malate were higher than those of vacuolar malate, whereas after a 3-h exposure with labeled bicarbonate, the intensities of the signals from carboxyls of vacuolar malate were much higher than those of cytoplasmic malate (Fig. 3). We have observed (data not shown) that the concentration of cytoplasmic malate attained at equilibrium (that is, when its rate of formation by the P-enolpyruvate carboxylase-malate dehydrogenase complex matches its rate of utilization) is strongly dependent on the activity of P-enolpyruvate carboxylase (18). Interesting, titration curves plotting chemical shift versus pH for citrate and malate in crude cell extracts (supernatant obtained after a 10-min centrifugation at 18,000 × g following disruption of cells by freeze-thawing) indicated that the in vivo position of carboxyl groups corresponds to citrate and malate at pH 5.8. This suggests that the bulk of citrate and malate molecules accumulated in the vacuolar compartment.

Fig. 2 illustrates the changes that occurred in the sycamore cell spectrum when [13C]bicarbonate (5 mM; isotopic enrichment of 99%) was added to the nutrient medium. In this experiment, the cells were maintained for >10 h in a continuously oxygenated circulating solution maintained at pH 7.5 (at this pH, the concentration of bicarbonate in the perfusion medium did not significantly decrease within 10 h). The major resonance at 161.2 ppm was attributable to bicarbonate present in the perfusion medium (pH 7.5) and the cytoplasmic compartment (pH 7.5), whereas the peak observed at 121.5 ppm was identified as the signal from free CO2 gas present in all the cell compartments and the perfusion medium. Important changes occurred during the first 3 h. During this time, the 13C resonances of C-1 and C-4 of malate increased considerably until a new steady state was obtained (Figs. 2–4). Since the chemical shifts of the carboxyl groups of malate are sensitive to pH <6.5 (5), 13C NMR can distinguish the vacuolar malate pool at acidic pH from the cytoplasmic malate pool at the slightly alkaline pH as previously shown by Chang and Roberts (4). The signals from C-4 of malate in the cytoplasm and vacuole were clearly discernible, whereas C-1 signals from the two pools of malate partially overlapped (Fig. 3A). A close examination of an expanded scale of the spectra between 174 and 184 ppm indicates in fact that malate appeared first in the cytoplasmic compartment (the position of both carboxyl groups (C-1 and C-4) corresponded to malate at pH >7).

When 0.5 mM Mn2+ was added to the perfusion medium, this paramagnetic ion accumulated specifically in the vacuole (see Ref. 19) and, as already observed for vacuolar phosphate (19), suppresses the peaks of vacuolar carboxylates. It was therefore possible to observe separately for the first time the cytoplasmic malate directly and the vacuolar malate by the difference in the corresponding spectra obtained in the absence of Mn2+ (Fig. 3B). When a threshold of cytoplasmic malate concentration was attained, malate molecules were slowly expelled into the vacuole, where they accumulated, reaching a constant level after ~5 h (Fig. 4). Thus, when sycamore cells were treated with [13C]bicarbonate for 1 h, the intensities of the signals from the C-1 and C-4 carbons of cytoplasmic malate were higher than those of vacuolar malate, whereas after a 3-h exposure with labeled bicarbonate, the intensities of the signals from carboxyls of vacuolar malate were much higher than those of cytoplasmic malate (Fig. 3).

We have observed (data not shown) that the concentration of cytoplasmic malate attained at equilibrium (that is, when its rate of formation by the P-enolpyruvate carboxylase-malate dehydrogenase complex matches its rate of utilization) is strongly dependent on the activity of P-enolpyruvate carboxylase, which exhibits a rather low affinity for bicarbonate (Refs. 1 and 2 and see below). Figs. 2 and 4 also indicate that, after a lag, vacuolar citrate increased throughout the experiment because the chemical shifts matched those of citrate added to crude cell extracts at pH 5.8. It is interesting to note...
13C NMR of Malate and Citrate Metabolism in Plant Cells

**Fig. 2.** Representative proton-decoupled 13C NMR spectra (100.6 MHz) of sycamore cells after addition of 5 mM H13CO3 in perfusion culture medium maintained at pH 7.5. Cells (9 g) packed in a 25-mm NMR tube as described for Fig. 1 and under "Materials and methods" were perfused with a well-aerated manganese-free culture medium containing 2.5 mM sucrose. At time 0, 5 mM H13CO3 was added to the perfusion culture medium. Spectra were obtained after 1, 2, 5, and 10 h. The pH of the culture medium was maintained constant at 7.5. The spectra were the result of 900 transients (1 h). Inset, perchloric extracts were prepared as described for Fig. 1 and in the text from cells incubated for 1 and 10 h in the presence of 5 mM HCO3 at pH 7.5. Spectra were obtained as described for Fig. 1.

**Fig. 3.** Representative proton-decoupled 13C NMR spectra (100.62 MHz) of sycamore cells showing labeling of carboxylates after addition of 5 mM H13CO3 in perfusion culture medium maintained at pH 7.5. A, for experimental conditions, see Fig. 2 legend. 0 h, standard spectrum; 1 h, 2 h, and 3 h, spectra obtained after the indicated times of perfusion of the compressed cells with culture medium containing 5 mM H13CO3. Note that it is possible to discriminate between the C-4 peak of malate present in the cytoplasm (cyl) (pH 7.5) and that present in the vacuole (vac) (pH 5.7) at least during the first 2 h of incubation in the presence of H13CO3 at pH 7.5. However, the C-1 peak of cytoplasmic and vacuolar malate and C-6 of citrate were not clearly separated. B, experimental conditions were as described for A, except that 0.5 mM Mn2+ was added to the perfusion medium. Spectra were obtained after 15 min and 1, 2, and 3 h of perfusion of the compressed cells with culture medium containing 5 mM H13CO3 and 0.5 mM Mn2+. The spectrum obtained at 15 min was the result of 225 transients; spectra obtained at 0, 1, 2, and 3 h were the result of 900 transients. Note that the addition of Mn2+ to the perfusion culture medium suppresses the peaks of vacuolar malate and citrate carboxylates (see Fig. 2B). The spectrum obtained at 2 h (A - B) is a difference spectrum of normal (without Mn2+) (A) and Mn2+-containing (B) cells obtained after 2 h in the presence of 5 mM H13CO3. Such a difference spectrum gives an accurate picture of the vacuolar citrate and malate carboxylates.

That when the accumulation of vacuolar malate ceased, signals from vacuolar citrate increased steadily. With time, other peaks appeared at 175.3, 175.2, and 175.5 ppm; these have been assigned to aspartate and glutamate carboxyl groups (the C-5 carboxyl of glutamate at 182 ppm was not labeled (Fig. 3B)). Titration curves plotting chemical shift versus pH for aspartate and glutamate in crude cell extracts indicated that the position of carboxyl groups corresponded to glutamate and aspartate at pH >7. This suggests that these amino acids accumulated in the cytoplasmic compartment. This was also confirmed by the fact that added Mn2+ in the perfusion medium did not eclipse the peaks corresponding to these...
carboxyls (resonances of aspartate and glutamate carboxyls are not detectable in the presence of Mn\textsuperscript{2+} ions).

When \textsuperscript{13}C bicarbonate was used as a precursor and after subtraction of the control spectrum obtained with unlabeled bicarbonate to correct for natural abundance (see Fig. 1), C-1 and C-4 of malate present either in the cytoplasmic compartment or in the vacuole were labeled with almost equal probability (Fig. 3). Comparison with experiments performed in the presence of unlabeled bicarbonate demonstrated (data not shown and Ref. 3) that there was no label in the methylene carbon and the carbon with the hydroxy group of malate (C-2 and C-3) (some experiments were conducted for >10 h). This is well exemplified in a typical decoupled \textsuperscript{13}C NMR spectrum of a perchloric acid extract of sycamore cells at pH 7.5 in the presence of 1 mM CDTA (Fig. 2). A similar pattern was observed for short-time exposures (10 min) with labeled bicarbonate (Fig. 3B). On the other hand, the citrate (C-1 + C-5) and C-6 signals increased with time and showed the same intensity, while under the same conditions, citrate C-2, C-3, and C-4 remained unlabeled.

Effect of Cytoplasmic pH and Bicarbonate Concentration on Rate of Malate and Citrate Synthesis by Sycamore Cells—

Since phosphoenolpyruvate carboxylase has a relatively low affinity for bicarbonate and since the pH of the cytosol is unlikely to be as high as the pH optimum of P-enolpyruvate carboxylase, we were prompted to examine the effect of cytoplasmic pH on the rate of malate accumulation in sycamore cells.

Table II indicates the effect of pH, on the intracellular pH values in sycamore cells. In agreement with Fox and Ratcliffe (20), the cytoplasmic pH (pHc) was independent of pHo, over the range 6–7.5 (see also Ref. 3). However, a loss of pH control was observed in response to the addition of 5 mM bicarbonate to the perfusion medium (Table II), especially when the external pH was acidic. Indeed, in the presence of 5 mM bicarbonate, pHc fell below its original value to reach a new steady state. The difference between the original pHc value and that attained after addition of 5 mM bicarbonate increased as the ΔpH across the plasma membrane increased (Table II). On the other hand, pHo, increased by up to 1 pH unit when external pH was increased from 7.5 to 9, irrespective of the presence of bicarbonate in the external medium (Table I). This observation strongly suggests that sycamore cells do not possess appropriate mechanisms to counteract the passive efflux of H\textsuperscript{+} and/or the passive influx of OH\textsuperscript{−} when the outwardly directed H\textsuperscript{+} gradient across the plasma membrane is reversed. Consequently, in the presence of 5 mM bicarbonate in the perfusion medium, pHo, increased progressively from 7.0 to 8.2 as pHc increased from 6 to 9. We have therefore studied the effect of pHc from 7 to 8.5 on the accumulation of intracellular malate and citrate in the presence of \textsuperscript{13}C bicarbonate.

Table II indicates that in the absence of bicarbonate, the rate of malate and citrate accumulation in sycamore cells is negligible up to pHc 7.5. Unexpectedly, at pHc >7.5, the accumulation of organic acids remained negligible. Indeed, the presence of higher amounts of bicarbonate in the cytosolic compartment (log([HCO\textsubscript{3}]/[CO\textsubscript{2}]) = pH – pK' (pK' = 6.4)) would be expected to stimulate the activity of the P-enolpyruvate carboxylase. It is therefore possible that the large volume of the alkaline perfusion medium, which facilitates the rapid diffusion of the respiratory CO\textsubscript{2} into the cells, leads to a continuous cell CO\textsubscript{2} content. In support of this suggestion, Table I indicates that the addition of 5 mM bicarbonate to the perfusion medium triggered a marked increase in the rate of malate and citrate synthesis at all of the pH values tested (the maximum rate of organic acid synthesis was already attained in the presence of 2 mM bicarbonate (data not shown)). It is noteworthy that the elimination of bicarbonate from the perfusion medium led to a progressive consumption of malate previously accumulated in the vacuole. Such a result strongly suggests that when the activity of P-enolpyruvate carboxylase declines owing to the collapse of cytosolic bicarbonate concentration, the net flux of malate toward the vacuole is stopped, and vacuolar malic acid diffuses across the tonoplast to be further metabolized in the cytoplasmic compartment.

In contrast, under the same conditions, citrate appeared to be very stable and was not further metabolized. To substantiate this, the flow of \textsuperscript{13}C label in these experiments was further studied in a chase experiment carried out at pHc 7.5, in which unlabeled bicarbonate was added to the sycamore cells after the elimination of \textsuperscript{13}C bicarbonate from the perfusion medium. The time courses of the various signals obtained in this experiment are shown in Fig. 5. The time course shows that the vacuolar malate C-1 and C-4 peaks, which had built up in the presence of \textsuperscript{13}C bicarbonate, decreased exponentially with a half-time of 3 h after the addition of unlabeled bicarbonate. Concurrent with these changes in the vacuolar malate peaks, \textsuperscript{13}C enrichment at C-6 and C-1 + C-5 of citrate increased progressively. Interestingly, during the course of this chase experiment, unlabeled malate increased steadily from its initial value of 3 μmol/g, wet weight (Fig. 5).
The cytoplasmic pH values ($pH_c$) were determined as described by Gout et al. (3). It was verified that the $pH_c$ values remained quite stable throughout the experiments. The total amounts of malate and citrate were measured from $^{13}$C NMR spectra after calibration with authentic compounds. The data are from a representative experiment and have been reproduced five times.

<table>
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<tr>
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<tr>
<td>Synthesized citrate ($\mu$mol/h/g cell, wet wt)</td>
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**Fig. 5.** Time course evolution of vacuolar malate and citrate in sycamore cells during pulse-chase experiment carried out with 5 mM H$^{13}$CO$_3$/H$^4$CO$_2$. Experimental conditions were as described for Fig. 4, except that after 3 h of experiment, H$^{13}$CO$_3$ was replaced by H$^{14}$CO$_2$. Note that during the chase, C-4 of malate (C$_4$-mal) steadily decreased, whereas the total amount of vacuolar malate continuously increased. cit, citrate.

**TABLE II**

$^{13}$C NMR of Malate and Citrate Metabolism in Plant Cells

<table>
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<td>4.0</td>
<td>3.6</td>
<td>3.3</td>
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</tbody>
</table>

**FIGURE 5**

- The results presented demonstrate that sycamore cells slightly compressed between two circular Teflon plates can survive for long periods as long as a well-aerated nutrient medium is pumped through the system under slight pressure. Such a system enables $^{13}$C NMR spectra of plant cells to be continuously recorded under various situations.

- In good agreement with Fox and Ratcliffe (20), our results indicate that the cytoplasmic pH was independent of the carefully controlled external pH over the range 5.5-7.5. If one assumes that all biological membranes systems, including the plasma membrane, exhibit an intrinsic permeability to protons, we are forced to conclude that the steady-state value of $pH$ across the plasma membrane reflects a balance between the outward proton flux driven by the plasma membrane ATPase and the inward proton flux due to passive proton leakage. This means that up to pH 5.5, the efficiency of the H$^+$ pump to react to back-leakage of protons was not limited. However, the loss of pH control observed in response to the addition of 5 mM HCO$_3^-$ to the perfusion medium is very likely attributable to the fact that the equilibrium pH gradient across the plasma membrane induces a rapid intake of CO$_2$ molecules (CO$_2$ diffuses very rapidly across all the biological membranes studied so far) into the cytoplasmic compartment, leading to a marked acidification of the cytoplasm ("acid load effect") (see Refs. 21 and 22). Under these conditions, the accumulation of HCO$_3^-$ in the cytoplasm is considerably enhanced, and the distribution of HCO$_3^-$ between the cytoplasm and the perfusion medium (which is predicted from the Henderson-Hasselbach equation) is inversely proportional to the distribution of protons. Under these conditions, the H$^+$ pump does not work fast enough to counterbalance the massive CO$_2$ intake linked to the rapid production of protons in the cytoplasm. Interestingly, the fact that alkaline conditions also cause loss of cytoplasmic pH control is very likely attributable to a net flux of hydroxyl ions across the plasma membrane.

- The response of intracellular pH to modifications of environmental factors has recently been reviewed by Kurkdjian and Guern (21). These authors consider the consumption (or synthesis) of organic acids such as malate, a process called biochemical pH-stat by Davies (22), as one of the mechanisms that allows the homeostasis of the cytoplasmic pH. Obviously, our results do not fit with this theory because the pool of malate attained at equilibrium in the cytosolic compartment is rather small, and, in addition, the bulk of this organic acid is excreted into the vacuolar space, where it accumulates. Furthermore, our results indicate that the synthesis of appreciable amounts of malate in the cytoplasmic and vacuolar compartments requires a rather high concentration of bicarbonate at all the external pH values tested. This raises the question of the regulation of cytoplasmic malate concentration in plant cells and, in particular, the problem of malate movement between the vacuole and the cytosol (and vice versa).

- Apparently, it is the concentration of malate on both sides of the tonoplast membrane that governs the efflux or influx of malate. When a threshold of cytosolic malate concentration is attained, malate molecules are slowly expelled into the vacuole, where they accumulate. Conversely, when malate is no longer synthesized, malate concentration declines in the cytosol, leading to a slow efflux of malate from the vacuole. According to Lüttege (24) and Smith (25), plant cells accumulate large amounts of malic acid in the vacuoles following fixation of bicarbonate in the cytosol. The process has an overall stoichiometry of 2 mol of H$^+$ accumulated per mol of malate and appears to be directly energized by the proton pumps of the tonoplast. This creates a potential gradient that is thought to drive the electrophoretic influx of malate$^-$ anions from the cytosol. Conversely, the efflux of the dissociated acid can be considered as a passive flux. Since during the course of the chase experiment unlabeled malate increased steadily, whereas the vacuolar $[^{13}$C]malate decreased exponentially, we are forced to conclude that both efflux (passive?) and influx (via a specific carrier; for example, see Marigo et al. (26)) of malate$^-$ ions occur simultaneously across the
tonoplast in vivo. In other words, the tendency of malic acid molecules to flow down their concentration gradient is balanced by the electrogenic influx of malate$^2$ ions via the carrier.

In marked contrast, citrate ions, also entering the vacuolar space via a specific carrier (27), behave differently because they remain sequestered in the vacule and removed from the equilibrium controlled by cytoplasmic enzymes. Indeed, the concentration of citrate and the labeled citrate carboxylate groups increased steadily throughout the experiment. These results indicate, in good agreement with a previous observation (14), that in these cells citrate exhibits a high metabolic inertness.

Our results demonstrate that C-1 and C-4 of malate become equally labeled when intact sycamore cells are treated with $^{13}$C bicarbonate. This concurs with the findings of Stidham et al. (5) and Chang and Roberts (4), who inspected the incorporation of $^{13}$C from $^{13}$C bicarbonate into malate in intact leaves of K. tibiflora and maize root tips. Likewise, the results of Osmond et al. (28) demonstrated that assimilation of $^{13}$CO$_2$ in the dark in a wide range of crassulacean acid metabolism plants leads to accumulation of a mixed population of [1-$^{13}$C] and [4-$^{13}$C] malic acid (see also Ref. 29). Since the cytosolic compartment of sycamore cells is devoid of fumarase activity, these results strongly suggest that malate labeled at C-1 is produced by randomization of C-1 and C-4 by mitochondrial fumarase because fumarate is a symmetrical molecule, and it is assumed that the two CH groups in it would react identically. We are therefore forced to consider that in vivo a permanent movement of malate molecules between the mitochondrial matrix and the cytosol occurs probably via the dicarboxylate carrier of the inner mitochondrial membrane (30), catalyzing a strict counter-exchange of only at C-1 and C-6 of citrate. On the other hand, if newly formed oxalacetate molecules labeled exclusively at C-4 in the mitochondrial matrix and is not necessarily "channeled" through a supramolecular complex bound to the mitochondrial fumarase because fumarate is a symmetrical molecule, and it is assumed that the two CH groups in it would react identically. We are therefore forced to consider that in vivo a permanent movement of malate molecules between the mitochondrial matrix and the cytosol occurs probably via the dicarboxylate carrier of the inner mitochondrial membrane (30), catalyzing a strict counter-exchange of only at C-1 and C-6 of citrate. This implies that most of the product of the reaction catalyzed by mitochondrial fumarase is released into the bulk phase and is not necessarily "channeled" through a supramolecular complex bound to the inner mitochondrial membrane (metabolon) containing all the tricarboxylic cycle enzymes (31, 32).

If malate equally labeled on both carboxyls (because of scrambling through fumarase) is metabolized by the citric acid cycle via citrate synthase, we should see a $^{13}$C enrichment only at C-1 and C-6 of citrate. On the other hand, if newly formed oxalacetate molecules labeled exclusively at C-4 in the cytosolic compartment enter the matrix space via the specific oxalacetate carrier reported in all the plant mitochondria isolated so far (30), we should see a preferential labeling at C-6 of citrate (for an explanation, see Ref. 33). Our results indicate that incorporation of $^{13}$C bicarbonate led to citrate highly labeled at C-1 and C-6, thereby confirming that in vivo labeled malate molecules synthesized by a sequence involving P-enolpyruvate carboxylase and malate dehydrogenase completely scrambled by mitochondrial fumarase are partly diverted into the tricarboxylic cycle to form citrate equally labeled at both C-1 and C-6. Such a situation is strongly favored because the cytoplasmic malate concentration attained at the equilibrium is rather high (15-20 mM) (Fig. 4), and, in addition, endogenous controls polarizing the malate/citrate exchange can facilitate citrate efflux as previously shown with intact isolated mitochondria (30). In addition, citrate is very actively incorporated into the vacuole since no labeled citrate was observed in the cytoplasm. Unfortunately, our results cannot discriminate between pyruvate formed by the glycolytic pathway and that produced by the mitochondrial NAD$^+$-linked malic enzyme (30) because both pathways lead to unlabeled acetyl-CoA, which condenses with oxalacetate to form citrate.

Finally, the results presented in this paper illustrate the resolving power of $^{13}$C NMR spectroscopy in distinguishing between individual carbon atoms of appropriate metabolites such as malate and citrate. This makes it possible to study intact cells as they respond to physiological perturbations.

REFERENCES