13C nuclear magnetic resonance studies of malate and citrate synthesis and compartmentation in higher plant cells

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To cite this version:
Elizabeth Gout, Richard Bligny, Nadine Pascal, Roland Douce. 13C nuclear magnetic resonance studies of malate and citrate synthesis and compartmentation in higher plant cells. Journal of Biological Chemistry, American Society for Biochemistry and Molecular Biology, 1993, 268 (6), pp.3986-3992. hal-02707364

HAL Id: hal-02707364
https://hal.inrae.fr/hal-02707364
Submitted on 1 Jun 2020

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The synthesis of malate and citrate by sycamore cells (Acer pseudoplatanus L.) perfused with KH$^{13}$CO$_3$ was analyzed using $^{13}$C 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 Mn$^{2+}$ 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 that fumarase activity was essentially limited to 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 $\Delta G$ 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 vivo in the presence of $^{13}$CO$_2$, 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 $^{13}$C NMR to observe the incorporation of $^{13}$CO$_2$ into malate by maize root tips and intact leaves of Kalanchoë tubiflora. Surprisingly, both groups observed a

significant incorporation of $^{13}$C 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 isoforms of fumarase that are localized in different intracellular compartments, the mitochondria and the cytosol. Consequently, it is possible that the unexpected incorporation of $^{13}$CO$_2$ 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, $^{13}$C NMR spectroscopy of intact sycamore cells was used to follow malate and citrate accumulation in vivo. One great advantage of $^{13}$C 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 according to the method of Bligny (9), except that Mn$^{2+}$ was excluded to prevent excessive broadening of the resonance of vacuolar compounds (10). The cell suspensions were maintained in exponential growth by frequent subcultures. The cell wet weight was measured after straining culture aliquots onto a glass-fiber filter.

Preparation of Protoplasts—Washed cells (130 g, wet weight) were suspended in their culture medium containing 0.5 mM mannitol, 10 mM Mops,$^1$ 1% (w/v) cellulase (Onozuka RS, Yakult Pharmaceutical Co., Nishinomiya, Japan), and 0.1% (w/v) pectolyase Y-23 (Seishin Pharmaceutical Co., Nishinomiya) 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 mM mannitol, 10 mM phosphate buffer (pH 7.5), 10 mM KCl, 5 mM MgCl$_2$, 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)
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. This extraction was carried out in three steps (100,000 × g for 5 min, 400,000 × g for 5 min (RS-4 rotor, Kubota KN-70 centrifuge), and 20,000,000 × g for 20 min (SS-34 rotor, Sorvall)). 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 mitochondrial pellet largely intact.

Perchloric Acid Extraction—The broken protoplasts were centrifuged for 10 min at 40,000 × g in a table-top Kubota KN-70 centrifuge (RS-4 rotor) to remove the bulk of amyloplasts containing big starch grains. The supernatant was centrifuged for 20 min at 20,000 × g (SS-34 rotor), and the mitochondrial pellet was resuspended in −0.5 ml of suspension medium.

Perchloric Acid Extract—For perchloric acid extraction, cells (9 g, wet weight) were quickly frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle with 1 ml of 70% (v/v) perchloric acid. The frozen powder was then placed at −10 °C and subsequently thawed. In this suspension was centrifuged at 10,000 × g for 10 min to remove particulate matter, and the supernatant was neutralized with 2 M KHC0₃ to pH 6.5. The supernatant was then centrifuged at 10,000 × g for 10 min to remove KClO₃; the resulting supernatant was lyophilized and stored in liquid nitrogen. For the NMR measurements, this freeze-dried material was redissolved in 2.5 ml of 0.5% D2O (perchloric acid extract).

The 13C NMR spectra of neutralized perchloric acid extracts were measured on a Bruker NMR spectrometer (AMX 400, narrow bore) equipped with a 10-mm multinuclear probe tuned at 100.62 MHz. Acquisition used 16-μs pulses (60°) at 4-s intervals (spectra of carboxyl groups were unchanged when a pulse interval of 10 s was employed). Two levels of proton decoupling were used: 2.5 watts during the data acquisition (0.54 s) and 0.4 watts during the delay period (3.46 s). Spectra were acquired over a period of 2 h (1800 scans). Free induction decays were accumulated using 16,000 data points and zero-filled to 32,000 prior to Fourier transformation. A 5-Hz line broadening was applied. Chemical shifts were obtained by reference to the hexamethyldisiloxane resonance at 2.7 ppm. Spectra of standard solutions of known carbon compounds at pH 7 were compared with the perchloric acid extracts.

Enzyme Total extract Supernatant Pellet

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<th>Pellet</th>
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<tr>
<td>Fumarase</td>
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<td>100</td>
</tr>
<tr>
<td>ADP-glucose pyrophosphorylase</td>
<td>18</td>
<td>0</td>
<td>100</td>
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<td>Catalase</td>
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</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>34</td>
<td>90</td>
<td>7</td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>18</td>
<td>0</td>
<td>100</td>
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RESULTS

In Vivo NMR Measurements—To get a better signal-to-noise ratio, an experimental arrangement was designed to analyze the maximum cell volume and to optimize the homogeneity of the cell incubation conditions. Cells (9 g, wet weight) were slightly compressed by hand and gently ruptured 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 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 (~1100 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 Maleate 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>
<thead>
<tr>
<th>Enzyme</th>
<th>Total extract</th>
<th>Supernatant</th>
<th>Pellet</th>
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<tr>
<td>Fumarase</td>
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<td>Citrate synthase</td>
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Fig. 1. Proton-decoupled 13C NMR spectra (100.62 MHz) of compressed sycamore cells (in vivo) (A) and of their perchloric extracts (B). A, cells (9 g, wet weight) were packed in a 25-mm NMR tube as described under “Materials and Methods” and continuously perfused with a well-aerated manganese-free culture medium maintained at pH 6.5. The cell volume composed ~90% of the total (cell + perfusion medium) volume. To reduce the intracellular concentration of sucrose, the circulating medium contained 2.5 mM instead of 50 mM sucrose (we have verified that cell respiration and growth were not modified by lowering the sucrose concentration of the nutrient medium). The spectrum is the result of 1800 transients (2 h). B, perchloric extracts were prepared from 9 g of oxygenated cells, wet weight, as described in the text. The resolution of the carboxyl groups was considerably improved by the addition of 1 mM NaCl to the extracts. The spectra (2.5 ml) containing 250 μl of 3H2O were analyzed for 2 h at 25 °C. The carboxyl group area (left) and part of the amino acid methylene groups (right) are shown on expanded scales. Peak assignments are as follows: suc, succinate; cit, citrate; iso, isocitrate; mal, malate; Asp, aspartate; Glu, glutamate; α, sucrose.

of the glucosyl and fructosyl moieties of sucrose and were estimated to correspond to an intracellular level of ~70 μmol g−1, wet weight, in good agreement with previous biochemical determination (14). The other major resonances in the chemical shift range of 50–40 ppm and those around 180 ppm arose from citrate and malate. The four resonances at 45.7, 76.3, 179.2, and 182.0 ppm coincided with those of C-2 + C-4, C-3, C-1 + C-5, and C-6 of citrate, respectively, whereas the two resonances at 43.2 and 71.1 ppm coincided with those of the methylene carbon (C-5) and the carbon atom with the hydroxy group (C-2) of malate, respectively. To obtain higher resolution and therefore more accurate quantification of the resonances, 13C NMR spectroscopy was performed on tissue extracts (Fig. 1B). The supernatants from two homogenates of compressed sycamore cells were much higher than those of cytoplasmic malate (Fig. 3). We have observed that, after a 3-h exposure with labeled bicarbonate, the intensities of the signals from the C-1 and C-4 carboxyls 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 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-malate dehydrogenase complex matches its rate of utilization) is strongly dependent on the activity of P-enolpyruvate carboxylase.
**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 (cyt) (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. 2.4). 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
plasma membrane is negligible, thus preventing the active transport probability (Fig. 4). This is well exemplified in a typical decoupled 13C NMR spectrum of a perchloric acid extract of sycamore cells at pH 7.5. On the other hand, the citrate C-1, C-2, C-3, and C-4 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 (pH$_c$) was independent of pH$_e$ 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, pH$_c$ fell below its original value to reach a new steady state. The difference between the original pH$_c$ 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, pH$_e$, 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$^+$ and/or the passive influx of OH$^-$ when the outwardly directed H$^+$ gradient across the plasma membrane is reversed. Consequently, in the presence of 5 mM bicarbonate in the perfusion medium, pH$_e$ increased progressively from 7.0 to 8.2 as pH$_c$ increased from 6 to 9. We have therefore studied the effect of pH$_c$ from 7 to 8.5 on the accumulation of intracellular malate and citrate in the presence of [13C]bicarbonate.

Table II indicates that in the absence of bicarbonate, the rate of malate and citrate accumulation in sycamore cells was negligible up to pH 7.5. Unexpectedly, at pH 7.5, the accumulation of organic acids remained negligible. Indeed, the presence of higher amounts of bicarbonate in the cytosolic compartment (log($[HCO_3^-]/[CO_2]$) = 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$_2$, drains continuously the cell CO$_2$ 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 [13C] label in these experiments was further studied in a "chase" experiment carried out at pH 7.5, in which unlabeled bicarbonate was added to the sycamore cells after the elimination of [13C]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 [13C]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, 13C 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,) were determined as described by Gout et al. (3). It was verified that the pH values remained quite stable throughout the experiments. The total amounts of malate and citrate were measured from 13C NMR spectra after calibration with authentic compounds. The data are from a representative experiment and have been reproduced five times.

**Table II**

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<td>7.0</td>
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<td>0</td>
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<tr>
<td>Synthesized citrate (µmol/h/g cell, wet wt)</td>
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<td>4.0</td>
<td>0</td>
<td>3.6</td>
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</table>

**DISCUSSION**

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 13C 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 ΔpH 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 HCO₃⁻ 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 CO₂ molecules (CO₂ 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 HCO₃⁻ in the cytoplasm is considerably enhanced, and the distribution of HCO₃⁻ 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 CO₂ 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 (23), 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 Lütge (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 ions from the cytosol. Conversely, the efflux of the undisassociated acid can be considered as a passive flux. Since during the course of the chase experiment unlabeled malate increased steadily, whereas the vacuolar 13C/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\textsuperscript{4\textsuperscript{-}} 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 vacuole 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 [\textsuperscript{13}\text{C}]{bicarbonate}. This concurs with the findings of Stidham et al. (5) and Chang and Roberts (4), who inspected the incorporation of [\textsuperscript{13}\text{C}]{bicarbonate} into malate in intact leaves of K. \textit{tubiflora} and maize root tips. Likewise, the results of Osmond et al. (28) demonstrated that assimilation of [\textsuperscript{13}CO\textsubscript{2}] in the dark in a wide range of crassulacean acid metabolism plants leads to accumulation of a mixed population of [1-\textsuperscript{13}\text{C}] and [4-\textsuperscript{13}\text{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 fumarase 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 \textit{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 labeled 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\textsuperscript{-}linked malic enzyme (30) because both pathways lead to unlabeled acetyl-CoA, which condenses with oxaloacetate to form citrate.

Finally, the results presented in this paper illustrate the resolving power of [\textsuperscript{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