Plant Physiol. (1990) 94, 826–832
0032-0889/90/94/0826/07/$01.00/0

Received for publication March 28, 1990
Accepted June 27, 1990

# Malate Metabolism in the Dark After $^{13}CO_2$ Fixation in the Crassulacean Plant *Kalanchoë tubiflora*[1]

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## ABSTRACT

**The metabolism of [$^{13}$C]malate was studied in the Crassulacean plant *Kalanchoë tubiflora* following exposure to $^{13}CO_2$ for 2 hour intervals during a 16 hour dark cycle. Nuclear magnetic resonance spectroscopy of [$^{13}$C]malate extracted from labeled tissue revealed that the transient flux of malate to the mitochondria, estimated by the randomization of [4-$^{13}$C]malate to [1-$^{13}$C]malate by fumarase, varied substantially during the dark period. At both 15 and 25°C, the extent of malate label randomization in the mitochondria was greatest during the early and late parts of the dark period and was least during the middle of the night, when the rate of $^{13}CO_2$ uptake was highest. Randomization of labeled malate continued for many hours after malate synthesis had initially occurred. Internally respired $^{12}CO_2$ also served as a source of carbon for malate formation. At 15°C, 15% of the total malate was formed from respired $^{12}CO_2$, while at 25°C, 49% of the accumulated malate was derived from respired $^{12}CO_2$. Some of the malate synthesized from external $^{13}CO_2$ was also respired during the night. The proportion of the total [$^{13}$C]malate respired during the dark period was similar at 15 and 25°C, and respiration of newly formed [$^{13}$C]malate increased as the night period progressed. These data are discussed with regard to the relative fluxes of malate to the mitochondria and the vacuole during dark $CO_2$ fixation.**

Many questions remain unanswered regarding the intracellular movement and metabolism of malate formed during dark periods in plants performing CAM (11, 12, 18). Of particular interest is the interaction between mitochondrial malate metabolism and the reactions associated with malate formation at night. In this regard, previous studies have revealed the following: (a) malate formed in the dark from isotopically labeled carbon dioxide is initially labeled in the 4-C position as a result of carboxylation of PEP[3] by PEP carboxylase (17); (b) after some time in the dark, a proportion of the labeled carbon atoms also appear in the 1-C position of malate—by the end of the night, the proportion of 4-C to 1-C labeled malate generally approaches a ratio of about 2:1 (1); (c) only singly labeled (*i.e.* [4-$^{13}$C] or [1-$^{13}$C]) malate appears. Mass spectrometry, which can differentiate between singly and doubly labeled malate, was used by Cockburn and McAuley (3) to demonstrate that only singly labeled malate can be isolated after $^{13}CO_2$ fixation in the dark, and this has been confirmed in a range of species (13). This result excludes the possibility of synthesis of doubly labeled malate ([1,4-$^{13}$C] malate) in the dark by the sequential fixation of $^{13}CO_2$ by ribulose 1, 5-bisphosphate carboxylase and PEP carboxylase (1). It has been demonstrated (14), however, that the double carboxylation pathway for malate synthesis can occur in the light. The only apparent explanation to account for the formation of [1-$^{13}$C]malate at night is the randomization of label in [4-$^{13}$C]malate by the mitochondrial enzyme fumarase.

Thus, the occurrence of a mixture of 4-$^{13}$C- and 1-$^{13}$C-labeled malate indicates that a proportion of the malate formed in the cytosol during the night enters the mitochondria, is equilibrated with the mitochondrial pool of fumarate, and then moves out of the mitochondria for subsequent storage in the vacuole. Recent comparisons of the extent of label randomization by fumarase in seven species of CAM plants suggest that, depending on the species and the temperature, 50 to 100% of the malate formed at night passes through the mitochondria (13).

Although the appearance of both 1-$^{13}$C- and 4-$^{13}$C-labeled malate indicates that mitochondrial malate uptake has occurred during the night, once in the mitochondria, malate could be subjected to a number of metabolic fates including: (a) label randomization via fumarase and exit from the mitochondria; (b) malate decarboxylation via NAD-malic enzyme to yield $CO_2$ and pyruvate; and (c) malate incorporation into the tricarboxylic acid pathway where both 1-C and 4-C carbonyls would be lost as $CO_2$ after one turn of the cycle. Another link between the reactions of nocturnal malate formation and mitochondrial respiratory metabolism is the refixation of respired $CO_2$ by PEP carboxylase to form malate (10, 19). Because the rate of malate accumulation declines late in the dark period in many CAM plants, this may imply an increased malate efflux from the vacuole at high malate concentrations and increased cytoplasmic malate, leading to feedback inhibition of PEP carboxylase and the possibility of increased respiration of malate and label randomization (7).

In the present study, the CAM plant *Kalanchoë tubiflora* was labeled using $^{13}CO_2$ for short intervals during an entire dark cycle. By harvesting plants immediately after labeling and at the end of the dark period, information was obtained on the temporal pattern of mitochondrial malate fluxes, the respiration of malate, and the formation of malate from respired $CO_2$ during the night. The effect of increased respi-

[1] This research was supported by a grant from the National Science Foundation to J.N.S. (CHE 86-07250).

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[3] Abbreviations: PEP, phosphoenolpyruvate; CER, net carbon exchange rate; $T_1$, spin-lattice relaxation time; IRGA, infrared gas analyzer.

ration on these processes was examined by conducting the experiment at two temperatures (15 and 25°C) during malate accumulation in the dark.

## MATERIALS AND METHODS

### Plant Culture

*Kalanchoë tubiflora* was vegetatively propagated from plants maintained in the greenhouse. Plantlets were transplanted at approximately 6 weeks of age into a combination of potting mix and gravel (2:1, v/v) in individual clay pots. Plants were grown in the Duke University Phytotron under conditions of a 16 h, 15°C dark period, and an 8 h, 30°C photoperiod, with 650 $\mu$mol photons $m^{-2}$ $s^{-1}$, PAR. RH in the chamber was maintained at 75%. Plants were watered daily and regularly supplemented with half-strength Hoagland solution. The experiment was conducted when plants were approximately 5 months old. Leaf positions 7 through 17 (acropetally numbered) were uniform in their nocturnal acid accumulation (135 $\mu$eq per g fresh weight ± 4.6) and were therefore used in the experiment. Leaves at positions below 17 were removed immediately prior to the experiment to minimize total $CO_2$ uptake.

### Dark $^{13}CO_2$ Fixation

Two intact *K. tubiflora* plants were supplied with $^{13}CO_2$ (99% enrichment, MSD Isotopes) for 2 h each during the 16 h dark period so that a total of 16 plants were labeled during eight 2 h sampling intervals. Plants were exposed to $^{13}CO_2$ in a 200 L Plexiglas chamber. The concentration of $CO_2$ in the chamber was maintained between 345 and 370 $\mu$L/L using an IRGA in line with a tank of $^{13}CO_2$. Net carbon exchange of plants in the chamber was estimated from a chart record of the carbon dioxide concentration measured by the IRGA. The chamber was maintained under positive pressure to minimize the entry of external $^{12}CO_2$ while plants were being moved in and out.

At the end of each 2 h labeling interval, the two plants were taken out of the Plexiglas chamber. Leaf positions 7 through 17 were removed from one plant and immediately frozen in liquid nitrogen. Frozen, intact leaves were pooled and separated into three aliquots which were stored at −80°C. The second $^{13}CO_2$-labeled plant remained in $^{12}CO_2$ under the same conditions for the remainder of the dark period, after which the equivalent leaves were removed, frozen, and stored as described above. The ambient temperature during the dark cycle was maintained at 15°C during one experiment and at 25°C during a second experiment.

### Extraction

Twenty grams of frozen leaf material was powdered in the presence of liquid nitrogen and boiled in 70 mL of 90% methanol/10% water for 10 min. The boiled extract was filtered through cheesecloth and spun at 20,000*g* for 15 min to remove particulates. Chloroform (25 mL) was added to the supernatant to remove lipids and pigments. The solution was mixed well and centrifuged at 20,000*g* for 15 min. After centrifugation, the methanol/water (upper) phase was removed, adjusted to pH 7.0, filtered, and evaporated to 8 mL under reduced pressure at 36°C. Deuterium oxide (2 mL) was added to the 8 mL extract.

### Nuclear Magnetic Resonance

NMR analysis of $^{13}C$-containing leaf extracts was conducted on a General Electric 300 MHz NMR spectrometer. The 8 mL leaf extract/2 mL $D_2O$ mixture was placed in a 20 mm (o.d.) NMR tube and maintained at 25°C during data acquisition. The deuterium oxide served as a lock signal. Proton decoupled spectra were obtained at 75 MHz using a spectral width of 1900 Hz, a 90° pulse angle, and a repetition time of 14.2 s. The longest $T_1$ for the $^{13}C$-labeled carbonyl groups of malate in the extract was determined by inversion recovery to be 4.1 s. Thus, 3.5 $T_1$s elapsed between successive pulses. A total of 272 scans were acquired.

The concentration of [$^{13}C$]malate in the extracts was determined by comparison of peak areas of the samples with peak areas of a range of natural abundance (1.1%) [$^{13}C$]malate concentrations. Spectra of samples and malate standards were plotted with identical gain and scaling factors and malate concentrations were calculated by cutting and weighing the 4-$^{13}C$ and 1-$^{13}C$ carbonyl peaks of malate. To calculate the ratio of [4-$^{13}C$]- to [1-$^{13}C$]malate, the relative peak intensities of the labeled carbonyl carbon atoms were determined using the curve deconvolution program (GEMCAP) of the General Electric 300 MHz spectrometer operating system (GEM). Relative peak intensities of 4-$^{13}C$ and 1-$^{13}C$ carbon atoms of malate were unchanged when a pulse sequence that eliminated the Nuclear Overhauser Effect was employed (9).

### Measurement of [$^{12}C$]- and [$^{13}C$]Malate

Total malate content of the extracts was measured enzymically using NADP-malic enzyme (4). Dilutions of extracts were made so that less than 100 nmol of malate was added to a 1.0 mL cuvette containing 0.2 M Hepes-NaOH (pH 7.5), 4 mM $MnCl_2$, 0.5 IU malic enzyme, and 1 mM NADP. The reaction was monitored to completion and malate content was calculated from the change in absorbance at 340 nm.

### Statistical Analysis

Three separate extracts were made from a mixture of whole leaves between leaf positions 7 and 17. Data points represent the mean value and standard error of the mean for the three extracts.

## RESULTS

### Carbon Exchange Rate

CER, estimated from the IRGA chart record obtained during the 16 h dark period, is shown in Figure 1 for plants labeled at 15 and 25°C. Although the basic features of their carbon exchange profiles were similar, net carbon gain at 15°C was greater than at 25°C for the entire dark cycle. During the first 2 h of the dark period, net CER was negative (*i.e.* the rate of respiratory release of $CO_2$ exceeded the rate of $CO_2$ fixation) at both 15 and 25°C. At 15°C, net carbon gain

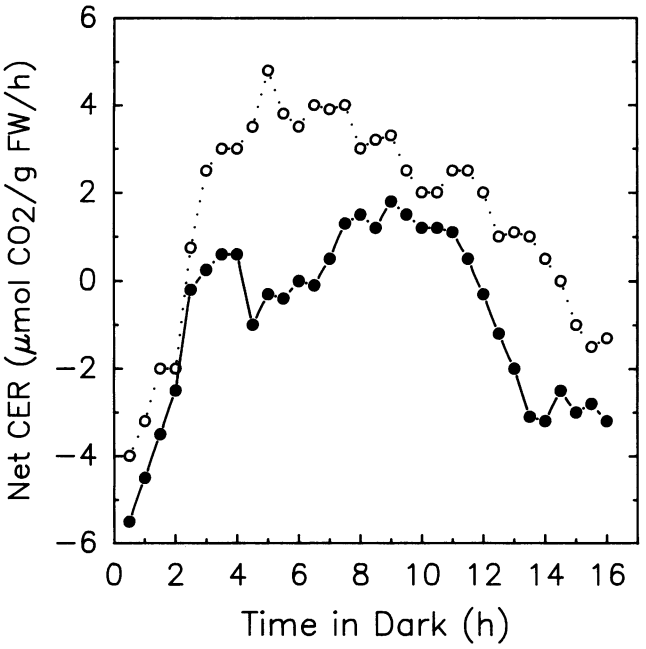


**Figure 1.** Net carbon exchange in *K. tubiflora* during the 16 h dark cycle at 15°C (○) and 25°C (●). Data shown are the results of a single representative experiment.

(positive CER) occurred during the middle 12 h of the 16 h night, and net carbon loss occurred during the last 2 h of the dark period. At 25°C, net carbon exchange was negative or close to zero during the first 6 h and the last 5 h of the dark period. Positive carbon gain only occurred for approximately 5 h during the middle of the night.

### Malate Accumulation

The accumulation of malate ([$^{12}$C]- plus [$^{13}$C]malate, measured enzymically) in leaves is shown in Figure 2 for plants fed $^{13}CO_2$ at 15 and 25°C. At 15°C, malate accumulated at a roughly linear rate during the entire dark period. At the beginning of the night, the level of malate was about 8 μmol per g fresh weight and increased to 61 μmol per g fresh weight by the end of the acidification phase.

Malate content of the tissue at the beginning of the 25°C dark period was the same as that at 15°C (approximately 8 μmol per g fresh weight). Early in the 25°C dark period (0–4 h), there was a small lag in the rate of malate accumulation, followed by an almost linear rate of malate formation until the end of the night. By the end of the 25°C night, the level of leaf malate had increased to about 56 μmol per g fresh weight.

### Malate Formation from $^{13}CO_2$

The [$^{13}$C]malate content of tissue harvested at the end of each 2 h labeling interval is shown in Figure 3 for plants fed $^{13}CO_2$ at 15 and 25°C. During the first 2 h of the 15°C dark cycle, the rate of $^{13}CO_2$ uptake was low (3.9 μmol per g fresh weight per 2 h) and increased to a maximum (9.3 μmol per g fresh weight per 2 h) after 6 h in the dark. The uptake of $^{13}CO_2$ remained fairly constant at almost 9 μmol per g fresh weight per 2 h between hours 6 and 10 of the dark cycle and by the end of the night, had declined to about the same level observed during the first 2 h of darkness.

During the entire 25°C dark cycle, the rate of [$^{13}$C]malate synthesis was substantially lower than the rate observed in plants labeled at 15°C. Greater variability is associated with these measurements because of the lower $^{13}$C enrichment of malate formed at the higher temperature. At 25°C, the rate of [$^{13}$C]malate synthesis was approximately 2.3 μmol per g fresh weight per 2 h for the first 4 h of the dark cycle, then reached a maximum of 5.4 μmol per g fresh weight per 2 h after 6 h in the dark. After this time, the rate declined gradually until the end of the dark period. The maximum rate of [$^{13}$C]malate synthesis at 25°C was only about 60% of the maximum rate measured for plants fed $^{13}CO_2$ at 15°C. The total amount of [$^{13}$C]malate formed at 25°C (28.5 μmol per g fresh weight) was slightly greater than half the amount of [$^{13}$C]malate formed at 15°C (51.6 μmol per g fresh weight).

Part of the [$^{13}$C]malate that was formed during the 2 h labeling interval was lost during the remainder of the dark period. This was evident in that the [$^{13}$C]malate content of plants harvested immediately after $^{13}CO_2$ feeding was higher than the [$^{13}$C]malate content of their counterparts that were harvested at the end of the night. Figure 4 shows the percentage of [$^{13}$C]malate respired during the hours after the 2 h $^{13}CO_2$ exposure for plants treated at 15 and 25°C. At 15°C, the percentage of [$^{13}$C]malate respired from the tissue increased as the night progressed. Thus, near the end of the dark period a greater percentage of the recently formed malate was respired compared to the percentage lost after labeling early in the night. Of the 51.6 μmol per g fresh weight [$^{13}$C] malate formed during the 15°C night, 80% was still present

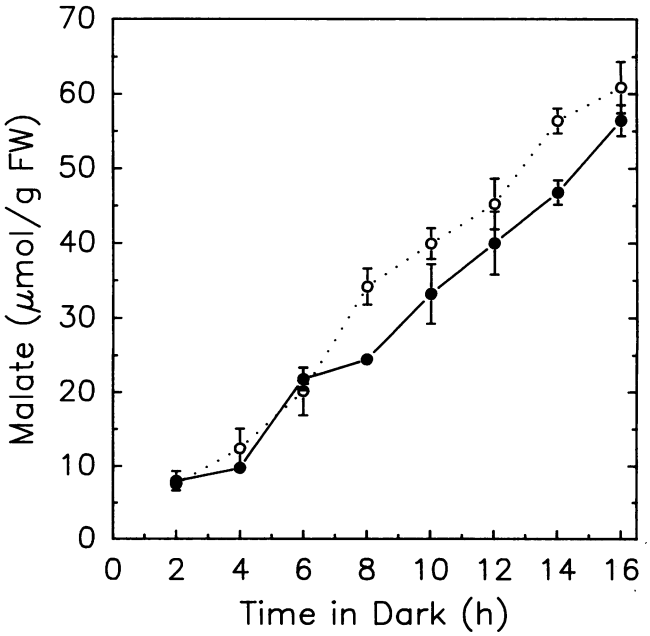


**Figure 2.** Total malate ($^{12}$C + $^{13}$C) accumulation in leaves during the 16 h dark cycle at 15°C (○) and 25°C (●). Total malate content was measured enzymically as outlined in "Materials and Methods."

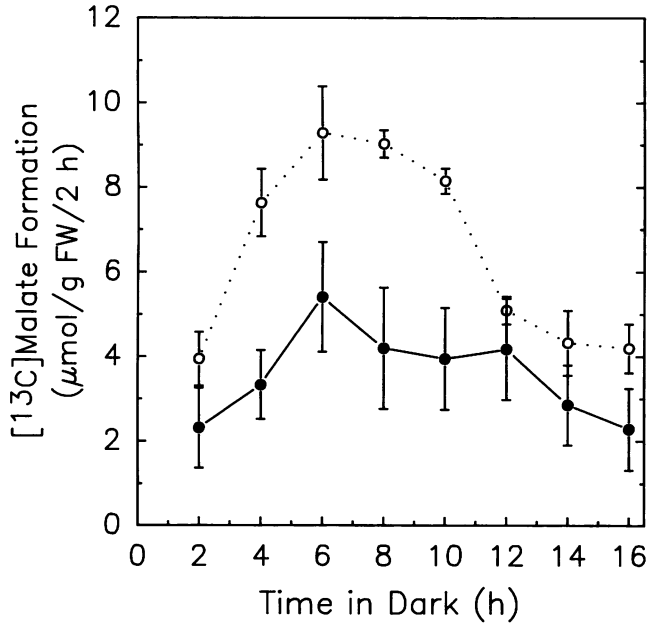


**Figure 3.** Rate of [$^{13}$C]malate synthesis during the 16 h dark cycle at 15°C (○) and 25°C (●) determined from NMR spectra.

in the tissue at the end of the night. The same pattern of loss of recently formed [$^{13}$C]malate was evident in plants labeled at 25°C. Of the 28.5 μmol per g fresh weight of [$^{13}$C]malate formed at 25°C, 76% was present in the tissue at the end of the night.

### Mitochondrial Malate Flux

The uptake of malate in the mitochondria is indicated by the formation [1−$^{13}$C]malate from [4−$^{13}$C]malate in the reaction catalyzed by the mitochondrial enzyme fumarase (3, 13). The probability of conversion of exclusively [4−$^{13}$C]malate to [1−$^{13}$C]malate by fumarase is 50%, so that a minimal estimate of the amount of malate which has access to fumarase in the mitochondria is two times the proportion of [$^{13}$C]malate that is labeled in the 1-C position. Considerations of concentration gradients as well as results based on direct measurements, indicate that most of the malate is in the cell vacuole of CAM plants (5). Based on this, the flux of malate through the mitochondria before transport to the vacuole can be estimated and is shown in Figure 5 for plants that were fed $^{13}CO_2$ in the dark at 15°C.

The extent of label randomization in recently formed malate varied significantly during the dark period. For the first 2 h of the 15°C night, essentially all of the malate that was formed passed through the mitochondria because 50% of the [$^{13}$C]malate pool was labeled in the 1-C position. The rate of malate flux to the mitochondria declined dramatically over the next 2 h such that about 20% of the labeled malate was randomized to [1−$^{13}$C]malate during $CO_2$ fixation between 2 and 4 h of darkness, indicating that 40% of the malate synthesized had been exchanged into the mitochondrial matrix enroute to the vacuole. During the subsequent 6 h period, the flux of malate through the mitochondria was maintained at this (40–50%) level. This period of minimum mitochondrial malate flux coincided with the period of maximum $^{13}CO_2$ uptake (Fig. 3). For the remaining 6 h of the dark period, malate exchange into mitochondria steadily increased, so that by the end of the night almost 100% of the [$^{13}$C]malate being formed had been exposed to fumarase.

Randomization of [4−$^{13}$C]malate continued to occur for some time after the initial synthesis of $^{13}$C-labeled malate. Evidence for this is found by comparing the 1-C to 4-C labeling ratio of malate in plants harvested immediately after $^{13}CO_2$ exposure, to that of plants labeled during the same interval, but harvested at the end of the 16 h night period. The continued randomization of malate after the 15°C labeling period is indicated in Figure 6. In general, the extent of further label randomization was related to the length of time the plants remained in $^{12}CO_2$ prior to harvesting at the end of the night. When plants were exposed to $^{12}CO_2$ for only 2 h before the end of the dark period, almost no further randomization was observed. However, when plants remained in $^{12}CO_2$ for up to 14 h after labeling, an additional 10–20% of the [$^{13}$C]malate was exposed to fumarase in the mitochondria.

The pattern of malate flux through the mitochondria in plants labeled in the dark at 25°C was similar, but not identical, to that observed at 15°C (Fig. 5). The flux of recently formed malate through the mitochondria decreased from approximately 100% during the first 2 h of darkness, to a minimum of about 60% after 6 h, and remained between 60 and 70% for the following 4 h. During the remaining 6 h of the night, the percentage of recently formed malate that moved through the mitochondria steadily increased from 70

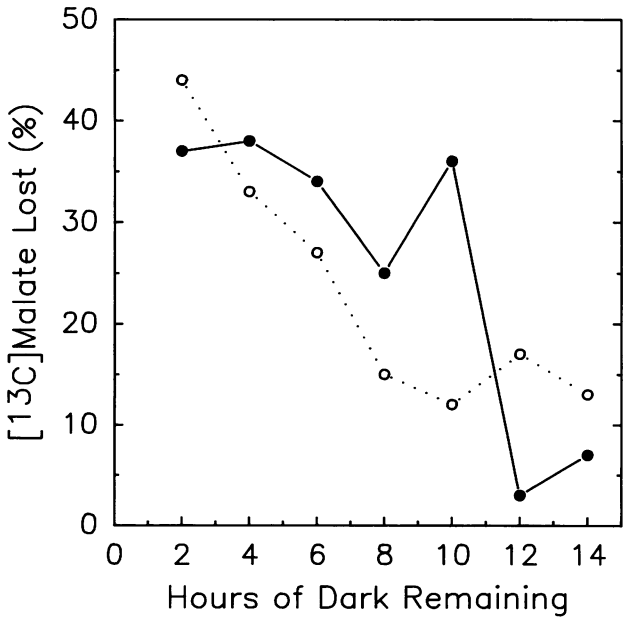


**Figure 4.** Percentage of [$^{13}$C]malate respired by the end of the night as a function of the hours plants remained in darkness following $^{13}CO_2$ fixation at 15°C (○) and 25°C (●). The percentage of [$^{13}$C]malate respired was calculated from the difference of the [$^{13}$C]malate content immediately after labeling and the [$^{13}$C]malate content at the end of the dark period.

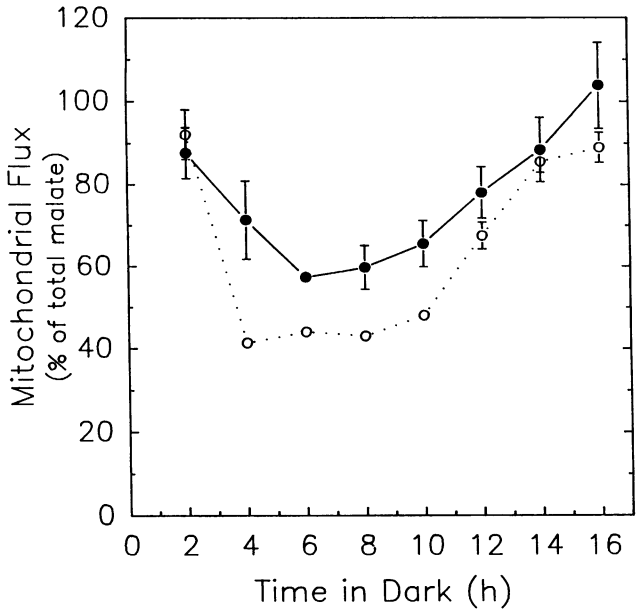


**Figure 5.** Flux of recently formed [$^{13}$C]malate through the mitochondria determined by label randomization following $^{13}CO_2$ labeling at 15°C (○) and 25°C (●).

to 100%. At 25°C, there was continued movement of malate through the mitochondria for some time after malate formation as evidenced by the greater proportion of [1−$^{13}$C]malate in plants kept in $^{12}CO_2$ until the end of the dark period compared with plants that were harvested immediately after the 2 h labeling period (Fig. 6).

## DISCUSSION

NMR is a useful technique to study [$^{13}$C]malate metabolism in plants because the 4−$^{13}$C and 1−$^{13}$C carbonyl groups of malate are spectrally well separated (2, 13, 16) and it has been established that only singly labeled malate is formed in the dark during CAM (3, 13). Thus, the sum of the 4-C and 1-C resonances represent the total pool of labeled malate. Quantitative information is obtained from peak intensities when spectra are acquired using a 90° pulse angle followed by a delay of at least three times the longest $T_1$ for the molecules of interest and then compared to a standard curve of signal intensities acquired under identical conditions (9).

### Carbon Exchange Rate and $^{12}CO_2$ Uptake

Atmospheric $^{13}CO_2$ was taken up by *K. tubiflora* at both 15 and 25°C during the entire dark period (Fig. 3) even though early and late in the dark cycle, net CER was negative (Fig. 1). Thus, the processes of dark respiration and carbon fixation resulted in simultaneous $^{12}CO_2$ evolution and $^{13}CO_2$ uptake. Comparison of the carbon exchange profile at 15 and 25°C (Fig. 1) indicates that the rate of respiration was significantly greater at the higher temperature. Some of the $^{12}CO_2$ evolved may be attributed to respiration at wound sites where lower leaves were removed to reduce the consumption of the limited amount of $^{13}CO_2$. Formation of $^{12}CO_2$ by respiration would also have led to some dilution of the 99% enriched $^{13}CO_2$ supplied to the chamber.

Respired $CO_2$ may be a source of carbon for malate formation in the dark (5, 10, 19). In the present study, a comparison of the total malate content ([$^{12}$C]- plus [$^{13}$C] malate) (Fig. 2) with the [$^{13}$C]malate content (Fig. 3) of the tissue integrated over the entire dark period provides an estimate of the extent to which respired $^{12}CO_2$ was a source of carbon for malate synthesis. At 15°C, of the 61 μmol per g fresh weight of malate accumulated during the dark period, 85% could be accounted for by the uptake of external $^{13}CO_2$, so that the remaining 9 μmol per g fresh weight of malate was formed from respired $^{12}CO_2$. When the temperature was held at 25°C, a much higher percentage of the total malate was formed from respired $^{12}CO_2$. Only 51% of the 56 μmol per g fresh weight of malate was formed from external $^{13}CO_2$, so that 27.5 μmol per g fresh weight of malate was synthesized from respired $^{12}CO_2$. Therefore, although the magnitude of malate accumulation was similar at 15 and 25°C, a higher percentage of the malate formed at 25°C was derived from respired $^{12}CO_2$.

The results presented here support those of Ritz *et al.* (15) who observed that 75% of the malate formed at 17°C was formed from externally supplied $^{13}CO_2$, while at 28°C, only 44% of the malate was synthesized from external, labeled $CO_2$. The fact that the accumulation of total malate was similar at 15 and 25°C, however, contrasts with their results (15) in which malate accumulation decreased linearly with increased night temperature. In our study, the relatively small decrease in malate accumulation observed at the higher temperature may be due to the fact that our plants had not been acclimated to the higher night temperature prior to the experiment.

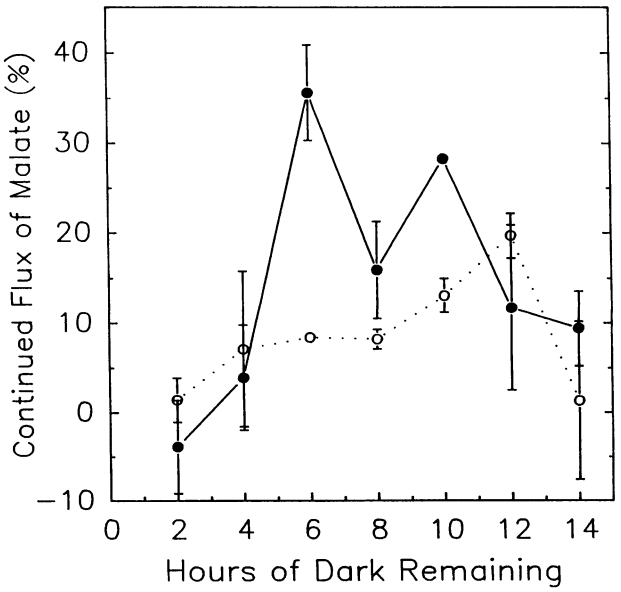


**Figure 6.** Continued flux of [$^{13}$C]malate through the mitochondria in the hours remaining until the end of the dark cycle for plants exposed to $^{13}CO_2$ at 15°C (○) and 25°C (●).

### Respiration of [$^{13}$C]Malate

The proportion of [$^{13}$C]malate respired in the hours following its synthesis increased as the night progressed (Fig. 4). Because the movement of malate into the vacuole requires a greater energy expenditure later in the acidification phase due to the increased electrochemical potential gradient resulting from malate accumulation and a limited vacuolar malate efflux (6, 7), an increase in the rate of [$^{13}$C]malate respiration could be expected. Calculations of ATP expenditure during CAM suggest that malate uptake into the vacuole represents a major respiratory cost (7, 8), and that increased respiration would be required to maintain a high vacuolar malate concentration concurrent with vacuolar efflux. The data suggest that it may be primarily newly formed malate, rather than malate previously stored in the vacuole that was lost.

### Mitochondrial Flux of [$^{13}$C]Malate

Significant changes were observed in the magnitude of label randomization in [$^{13}$C]malate during the night (Fig. 5). Two major factors may underlie these observations: (a) the balance of fluxes between the mitochondrial and vacuolar membranes determines the extent of mitochondrial malate uptake and (b) malate uptake into the mitochondria is under metabolic regulation.

If flux ratios between the mitochondria and vacuole determine the extent to which labeled malate is transported into the mitochondria, it may be expected that when the rate of malate transport to the vacuole is low, a high cytoplasmic and mitochondrial malate concentration would lead to equilibration of the [$^{13}$C]malate and fumarate pools and label exchange via fumarase. Although the rate of [$^{13}$C]malate synthesis was low early and late at night, essentially all of the labeled malate formed was exposed to fumarase in the mitochondria. In the present study, at 15°C, the rate of [$^{13}$C]malate formation (Fig. 3) was high during the middle of the night and, at this time, the extent of randomization, and hence the mitochondrial flux of malate, was at a minimum. This suggests that the flux of malate to the vacuole may be an important determinant in mitochondrial malate uptake. This suggestion is further supported by the opposite trend in the data obtained early and late in the dark period. The movement of malate into the mitochondria was insensitive to the concentration of vacuolar malate because the extent of randomization of malate was highest at times when the concentration of vacuolar malate was either low or high (*i.e.* early and late in the night, respectively). The high rate of respiratory $^{13}CO_2$ release at the end of the dark period suggests either that newly formed [$^{13}$C] malate is a preferred respiratory substrate or that a large proportion of the vacuolar malate pool is exchanging with the cytoplasm and mitochondria and some part of this pool is being respired. The extent to which these two alternative pathways for respiratory $^{13}CO_2$ formation occur late in the dark period could be resolved by direct measurements of $O_2$ uptake.

The randomization of label in [$^{13}$C]malate continued for many hours after the initial period of malate synthesis (Fig. 6). One implication of this result is that a significant pool of malate exists in the cytosol along with PEP carboxylase. This possibility seems unlikely, early in the dark period, given the sensitivity of PEP carboxylase to inhibition by malate, even at night (12). Rather, it may suggest that some vacuolar malate is released to the cytosol, passes through the mitochondria, and is transported back into the vacuole. Clearly this is wasteful, since no energy is derived from malate release from the vacuole, but ATP hydrolysis is required to transport malate back into the vacuole (7).

### ACKNOWLEDGMENTS

The authors acknowledge the service of Duke University Magnetic Resonance Center (DMRC). We are grateful for the invaluable assistance of Dr. A. Ribiero (DMRC Director) and Donald Mika (DMRC) and to Larry Giles of the Duke University Phytotron.

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