DIURNAL CHANGES IN THE MALIC ACID CONTENT OF VACUOLES ISOLATED FROM LEAVES OF THE CRASSULACEAN ACID METABOLISM PLANT, *SEDUM TELEPHIUM*

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1. Introduction

The accumulation of malic acid at night in green cells followed by malic acid depletion the next day is characteristic of Crassulacean acid metabolism (CAM) plants. The diurnal changes in malic acid content are accompanied by reciprocal changes in starch content with starch providing the carbon skeleton for phosphoenolpyruvate, the CO_2 acceptor at night. Malic acid is decarboxylated during the day to provide CO_2 for photosynthesis [1]. In contrast, other organic acids such as isocitric do not exhibit a daily fluctuation in levels [2]. With the realization that massive amounts of malic acid accumulate at night [1,2], it was widely postulated that malic acid is stored in the large vacuoles of green CAM cells. Indeed, calculations based on *Bryophyllum* leaf cell volumes and the amount of acid accumulated at night showed that 'if malic acid were confined to the cytoplasm, its concentration would be 7–8 M, but if it were in the vacuole also, its average concentration would be 0.25 M' [3]. Thus, to prevent a variety of deleterious effects of acidic conditions on cytoplasmic components, a green CAM cell would in theory temporarily sequester malic acid in its vacuole at night. However, data to test this hypothesis are not available.

Recently, techniques have been developed for isolating large quantities of vacuoles from plant tissues [4–7]. Isolated *Bryophyllum* leaf vacuoles [6] contained 0.002–0.02 nmol malic acid/vacuole which comprised the bulk of the malic acid present. However the daily malic acid content of vacuoles was not investigated. Using the CAM plant *Sedum telephium*, we have isolated intact vacuoles from leaf protoplasts with yields ~20%. In the present investigation we have used these isolated vacuoles to determine the cellular localization of malic and isocitric acids and to study the diurnal pattern of malic acid accumulation and depletion. These diurnal changes in malic acid are interpreted as showing that the vacuole is directly involved in the CO_2 assimilation pathway of green CAM plants.

2. Materials and methods

Sedum telephium L. was cultivated in growth chambers under 15 h day at 30°C and 9 h night at 15°C with an irradiation intensity of 150 μE $m^{-2}.s^{-1}$ at the upper left surface. Plants were well watered and fertilized under conditions which support active CAM [8]. Leaves were harvested throughout a day at the times indicated and protoplasts were isolated by digestion with commercial preparations of pectinase and cellulysin. Pure vacuoles were isolated within 2 h on Ficoll gradients using modifications of procedures in [7]. Protoplasts and vacuoles were counted and checked for purity microscopically. Further details of the isolation and purification of vacuoles and protoplasts will be presented separately (in preparation). Leaf titratable acidity was measured as in [8]. Malic acid was assayed using chicken liver malic enzyme purchased from Sigma Chem. Co. [9]. Isocitric acid was assayed with porcine heart isocitric dehydrogenase purchased from Calbiochem. Corp. [10]. The iso-

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citric lactone ring was opened prior to assay by adjusting the extracts to pH 12 and heating at 60–70°C for 30 min. The extracts were readjusted to pH ~10 and immediately assayed. $^{14}CO_2$ labeling experiments were performed by sealing leaves in Mylar bags, injecting 75 μCi $^{14}CO_2$ into the bags, and allowing the leaves to fix the $^{14}CO_2$ for 30 min. Dark $^{14}CO_2$ labeling was done at 21°C in complete darkness while light labeling was at 31°C and at 440 μE $m^{-2}.s^{-1}$. Vacuoles were isolated from these leaves immediately following these $^{14}CO_2$ incubation periods. These labeling times were selected so that acid was accumulating at night or the acid was at the lowest daily level [8].

3. Results and discussion

An experiment illustrating the data obtained on malic acid content of isolated vacuoles at timed intervals throughout a day is presented in fig.1. The malic acid content of isolated vacuoles reaches a low value each light period ~0.02–0.04 nmol malic acid/vacuole. Subsequently the vacuole malic acid content rises to peak values ~0.5–0.75 nmol/vacuole at the end of the night period. In the protoplasts used to isolate the vacuoles, the malic acid content was similar to the vacuole content except at the end of the night (fig.1). There we had an experimental problem of partial breakage of protoplasts and vacuoles, presumably due to their high acid content, so that accurate counts were difficult. Our vacuole isolation procedures were developed in low acid and we have not yet investigated the breakage problem when the acid content is high. Data are given in fig.1 on leaf titratable acidity showing that these *Sedum* leaves were in CAM [8]. All of these experiments have been repeated several times without discrepancies arising in the daily patterns.

Since CAM leaves are known to have high contents of isocitric acid [11], we assayed its daily pattern in intact leaves, in isolated protoplasts, and in isolated vacuoles. In contrast to malic acid, the vacuole content of isocitric acid showed little change throughout a day and no consistent pattern of change relative to night or day. The isocitric acid content of vacuoles was 0.12 ± 0.07 nmol/vacuole. Isocitric acid also did not show a pattern of diurnal change in leaves or in protoplasts. These studies confirm earlier work consistently showing that isocitric acid does not fluctuate in intact CAM leaves [11,12] and show that isocitric acid is also stored in the vacuoles of green CAM cells along with malic acid.

Since isocitric acid does not fluctuate in a diurnal fashion in leaves or isolated vacuoles we reasoned it could provide an internal standard on which to base

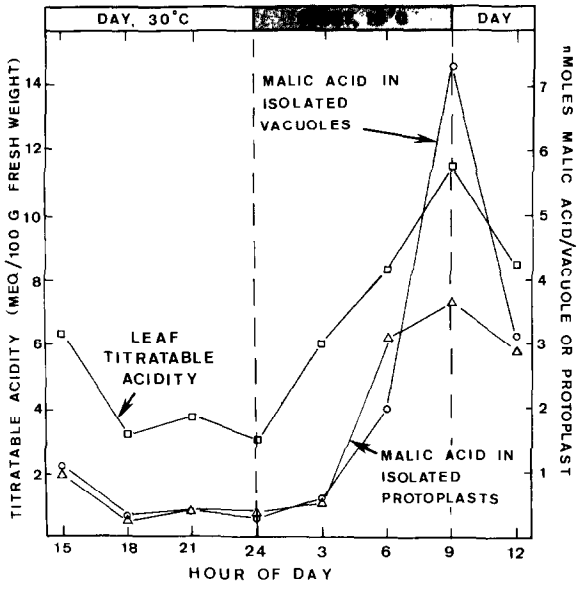

Fig.1. The diurnal pattern of leaf titratable acidity (□); and the malic acid content of isolated leaf vacuoles (○) and isolated leaf protoplasts (△) of *Sedum telephium*.

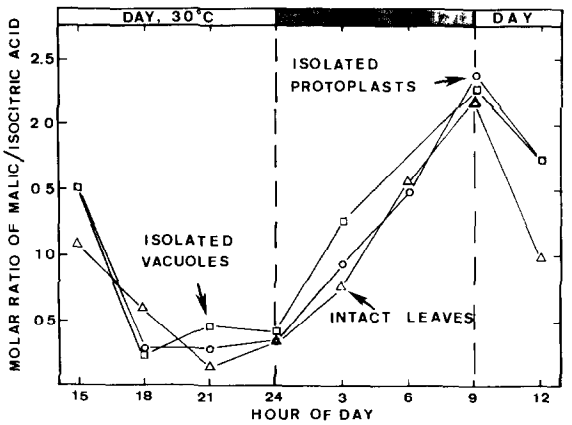

Fig.2. Diurnal changes in the molar ratio of malic acid to isocitric acids in isolated vacuoles (□); isolated protoplasts (○); and intact leaves (△) of *Sedum telephium*.

Table 1
Accumulation pattern of ^{14}C in cellular components isolated from *Sedum telephium* leaves fed $^{14}CO_2$ in the dark or light

Time of leaf $^{14}CO_2$ fixation	Recovery of ^{14}C from a vacuole purification		
	Purified vacuoles (% total ^{14}C in protoplasts)	Insolubles[a]	Solubles[b]
04.00–04.30 in dark	49	5	46
16.00–16.30 in light	~ 4	92	~ 4

[a] This purification fraction contains chloroplasts, starch and other insoluble cellular components. Over 99% of the chlorophyll is in this fraction

[b] This is a compilation of all purification fractions except the vacuoles or insolubles and is comprised of the soluble materials derived from broken protoplasts, vacuoles and other organelles

the malic acid content of vacuoles. We calculated the ratio of malic to isocitric acid, and fig.2 gives data for isolated vacuoles, isolated protoplasts, and intact *Sedum* leaves. The results show a similar diurnal pattern of malic acid accumulation and depletion among all three (fig.2). We interpret the data in fig.1,2 as showing that malic acid fluctuates in a diurnal fashion in CAM leaves and that the vacuole is its major site of accumulation at night.

In support of this hypothesis we also fed $^{14}CO_2$ to *Sedum* leaves in the light or in the dark and then immediately isolated the vacuoles. The Ficoll gradients were fractionated and individual fractions were assayed for both ^{14}C and malic acid. As shown in table 1, ^{14}C accumulated in the vacuoles at night while less than 5% of the ^{14}C was in the vacuole during the day. This vacuole ^{14}C was shown to be in malic acid. Little ^{14}C accumulated in insoluble materials during the night but 92% of the ^{14}C accumulated in this fraction during the day (table 1) showing the predominance of photosynthetic CO_2 fixation rather than β-carboxylation during the day.

4. Conclusion

At night CAM tissues primarily fix CO_2 via the β-carboxylation of PEP and subsequently store the malic acid in the vacuole of green cells. Malic acid is depleted during the next day via removal from the vacuole followed by a cytoplasmic decarboxylation of malic acid in *Sedum*, or of oxaloacetic acid in other CAM plants [13], with the CO_2 being used in photosynthesis. Isocitric acid also is stored in CAM leaf vacuoles but it does not show a constant pattern of daily change in concentration. These studies substantiate the long standing hypothesis [1,3] that malic acid in CAM plants is temporally stored in the vacuole and they clearly implicate the vacuole as a cellular organelle with an active role in the net fixation of CO_2 in CAM plants.

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References

[1] Ranson, S. L. and Thomas, M. (1960) Ann. Rev. Plant Physiol. 11, 81–110.
[2] Vickery, H. B. (1972) Ann. Rev. Plant Physiol. 23, 1–28.
[3] Stiller, M. L. (1959) PhD. Thesis, Purdue University, Lafayette, IN.
[4] Wagner, G. J. and Siegelman, H. W. (1975) Science 190, 1298–1299.
[5] Leigh, R. A. and Branton, D. (1976) Plant Physiol. 58, 656–662.
[6] Buser, C. and Matile, P. (1977) Z. Pflanzenphysiol. Bd. 82, 462–466.
[7] Saunders, J. A. and Conn, E. E. (1978) Plant Physiol. 61, 154–157.
[8] Crews, C. E., Vines, H. M. and Black, C. C. (1975) Plant Physiol. 55, 652–657.
[9] Ochoa, S. (1955) Methods Enzymol. 1, 739–753.
[10] Ochoa, S. (1955) Methods Enzymol. 1, 699–704.
[11] Vickery, H. B. (1952) Plant Physiol. 27, 9–17.
[12] Milburn, T. R., Pearson, D. J. and Ndegew, N. A. (1968) New Phytologist 67, 883–897.
[13] Dittrich, P., Campbell, W. H. and Black, C. C. (1973) Plant Physiol. 52, 357–361.