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CRASSULACEAN ACID METABOLISM AND PHOTOSYNTHESIS IN ALOE BARBADENSIS MILL*

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Aloe barbadensis Mill (Syn. *A. vera* L.) is a Crassulacean succulent and hence is of interest to investigate CO_2 photoassimilation. The leaf slices were exposed to $^{14}CO_2$ in light as well as in dark from three seconds to one hour. It appears that in light as well as in dark phosphoenol-pyruvate (PEP) incorporates CO_2 and yields oxaloacetate. The nature of resultant stable product depends on the pool saturation of malic or aspartic acids. It was observed that aspartate is the initial product of short term fixation in light while it is malate in the dark. Both these products are utilised for further synthesis of metabolites. The CO_2 assimilation pattern in the Crassulacean succulent and that of the C_4-plants appears similar as dicarboxylic acids are the first stable products. The results were further confirmed by enzyme studies which indicate that phosphoenol-pyruvate carboxylase is the main carbon assimilating enzyme in light as well as in dark. Ribulose-1-5-diphosphate carboxylase, malic dehydrogenase and pyruvate kinase are also present and possibly have some metabolic role to play. These studies indicate that *A. barbadensis* is possibly C_4-plant, rather than a C_3-one.

INTRODUCTION

Aloe-vera var. *indica* recently known as *A. barbadensis* is a Crassulacean succulent and exhibits diurnal fluctuations in acid contents of its chlorophyllous parts. Malic acid is the major acid involved in Crassulacean Acid Metabolism (CAM). The diurnal fluctuations in acid contents of the succulent have been reported by Bharucha and Joshi (1958). The fluctuations in malic acid can be correlated to phosphoenol-pyruvate carboxylase (PEP-Case) and malic dehydrogenase (MDH) activities (Ranson & Thomas, 1960). The malate formed by dark CO_2 assimilation is utilized during day or light as a source of carbon for photo-synthesis. Our earlier observations (Joshi & Bartakke, 1974) on light and dark $^{14}CO_2$ assimilation in the succulent indicate that aspartate and malate are the immediate products after 3 sec exposure to $^{14}Co_2$. At night it is malic acid which is predominantly synthesized while during day it is aspartate. As there are fluctuations in acid contents as well as in those of immediate products of carbon assimilation an attempt to study the nature of products of carbon assimilation at various periods during day/night i.e. 6 AM, 12 noon, 6 PM and 12 night has been made. The enzymes like PEP-Case, ribulose-1-5-diphosphate carboxylase (RuDP-Case), MDH and pyruvate-kinase

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were also studied. The possible mechanism of carbon economy in the light and dark in *A. barbadensis* is discussed in the present paper.

Materials and Methods

Plant material

Plantlets of *A. barbadensis* were raised in the garden in pot culture. The leaves of six months old plants were used for the experiments. The leaves were washed with tap water and then middle mucilagenous tissue was scrapped out and only chlorophyllous tissue was taken. It was washed with distilled water and then blotted dry and was taken for study of $^{14}CO_2$ assimilation. The time selected for $^{14}CO_2$ assimilation were 6 AM, 12 noon, 6 PM and 12 night. The leaf slices were exposed for one hour. In case of light feeding no extra light was provided but feeding was made in the garden (under natural) conditions but in a controlled chamber.

Methods

(i) $^{14}CO_2$ *assimilation*—The $^{14}CO_2$ assimilation studies were made by the method followed by Denius and Homann (1972). The method was modified as follows: 2.00 g of chlorophyllous tissues were cut into small discs of approximately 1 cm diameter. They were then transferred to stoppered Erlenmeyer flasks containing 0.05M tris-HCl buffer, *p*H 8.0. However, Denius and Homann had used standard buffer containing phosphate buffered (40 mM, *p*H 6.8) solution of 0.7 M mannitol. The leaf slices were fed with NaH $^{14}CO_3$ (radioactivity 48.3 mCi/m mole) obtained from BARC, Bombay. The leaf slices were exposed for 1 hr at 6 AM, 12 noon, 6 PM and 12 night respectively. The reaction was terminated by adding hot 80% ethanol. The material was homogenized in 80% ethanol to complete the extraction and filtered. The filtrate was condensed under reduced pressure to 3 ml. From the extracts 0.1 ml of aliquot was acidified and counted to determine the total activity incorporated.

The extracts were used for chromatographic separation of labelled compounds. Two dimentional paper chromatographic process using phenol-water (80:20 v/v) and *n*-butanol, acetic acid, water (80:20:50 v/v/v) was employed to separate and identify the compounds. The identity of each compound was confirmed by colour tests as well as co-chromatography with authentic samples. The radioactive compounds were detected by exposing the chromatograms to X-ray films (Kodak blue film). The radioactivity in each spot was counted on a Proportional Counting System (2π ECIL.).

(ii) *Enzyme assays*—The cell-free preparations and assay of the RuDP-Case were done by the method of Morris and Farrel (1971) while for assay of the PEP-Case the method used by Hatch and Slack (1967) was followed. The cell-free preparation and assay of MDH was made by the method of Ernest and Abood (1949) while pyruvate kinase was extracted and assayed by method of Weidner and Salisburry (1974). Proteins from enzyme extracts were determined by method of Lowry *et al.* (1951).

Results

Our earlier observations showed that aspartate and malate are the immediate products for short term $^{14}CO_2$ fixation in light and dark respectively. The carbon from

aspartate in light is transferred to malate and alanine. However, in dark maximum carbon is stored in malic acid.

Table I shows the distribution of radioactivity in individual components of ethanol soluble fraction at different light and dark periods.

TABLE I

Distribution of radioactivity in ethanol-soluble compounds formed during 1 *hr exposure of* A. barbadensis *leaf slices to NaH* $^{14}CO_3$

Compounds	Time			
	6 AM	12 N	6 PM	12 N
Citrate	5.37	6.53	—	—
Malate	38.60	9.19	43.61	43.42
Fumarate	11.70	—	—	6.28
Succinate	—	—	16.09	4.97
Total organic acids	55.67	15.72	59.70	54.67
Aspartate	8.90	29.81	Trace	17.10
Alanine	8.92	17.19	11.21	5.05
Glycine-serine	4.30	14.18	Trace	Trace
Phenylalanine	—	—	—	1.16
Valine	—	—	—	1.31
Leucine	—	—	—	0.90
Total amino acids	22.12	61.18	11.21	25.52
PEP + PGA	9.49	19.11	12.98	11.19
Sugar-mono-P	—	—	—	1.46?
Sugar-di-P	7.27	Trace	3.11	4.23?
Total sugar phosphates	16.76	19.11	16.09	16.88
Glucose	—	—	—	—
Fructose	—	—	—	—
Sucrose	5.50	4.11	12.98	2.86?
Total sugars	5.50	4.11	12.98	2.86?

*Values are expressed as percentage of radioactivity counted on chromatogram.

At 6 AM organic acids show much more label than amino acids, sugar phosphates and sugars. Malate accounts for 38% of the total radioactivity incorporated. At 12 noon amino acids are heavily labelled (61.18%) than organic acids, sugar phosphates and sugars. Aspartate, alanine and glycine-serine are the major amino acids which incorporate heavy label. Next to amino acids, PEP+PGA fraction has (19.11%) label and it is more than organic acid (15.72%) fraction. At 6 PM again organic acids (59.70%) are heavily labelled than amino acids (25.52%) sugar phosphates (16.09%) and sugars (12.98%). The lowest label in amino acids is seen at 6 PM. At 12 midnight organic acid fraction has more label (54.67%) than the amino acid (25.52%) (Fig. 1).

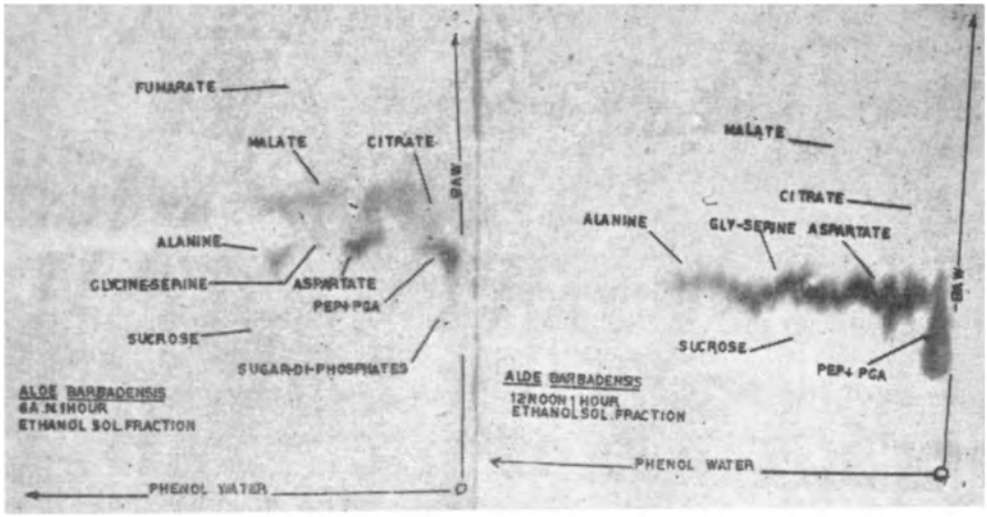

FIG. 1. Autoradiograms showing ethanol-soluble compounds formed at 6 AM and 12 noon for 1 hr exposure of chlorophyllous tissue of *A. barbadensis* to NaH $^{14}CO_3$.

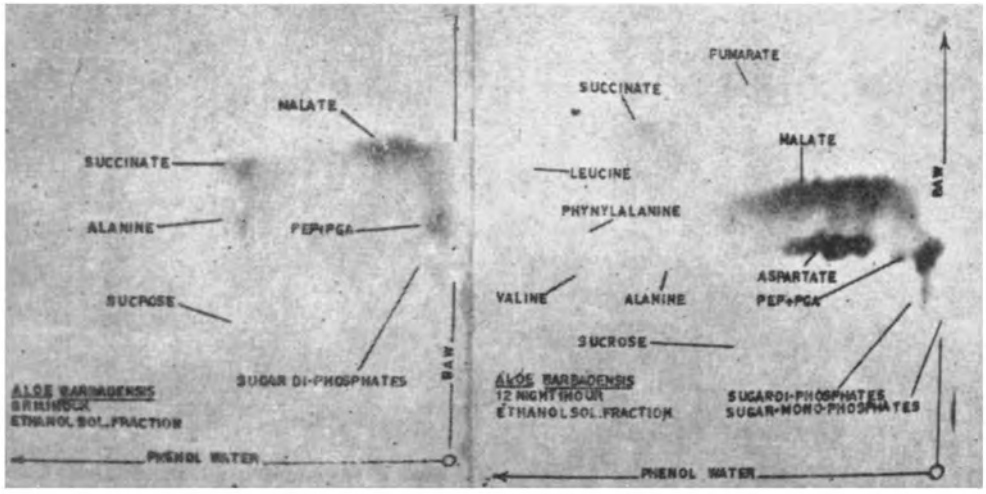

FIG. 2. Autoradiograms showing ethanol-soluble compounds formed at 6 PM and 12 night for 1 hr exposure of chlorophyllous tissue of *A. barbadensis* to NaH $^{14}CO_3$.

Malate, the major acid of dark fixation, has heavy label at 6 AM and it decreases by 12 noon and regains more label by 6 PM and maximum values are recorded at 12 night. Aspartate has little label at 6 AM which increases by 12 noon and decreases till 6 PM with a slight increase at 12 night. Alanine follows the same pattern like aspartate. Glycine-serine show label at 6 AM and 12 noon. PEP + PGA fraction has highest label at 12 noon (20%) which decreases at 6 PM and 12 night. Sugar di-phosphate has more label at 6 AM. Sucrose has more label at 6 PM rather than 6 AM or 12 noon. Citrate shows label at 6 AM and 12 noon only, whereas fumarate at 6 AM and 12 night and succinate at 6PM and 12 night. Thus highest incorporation is seen at 6PM in organic acids at 12 noon in amino acids, at 6 PM and 12 night in organic acids. Thus it appears that in light aspartate is the product of short term carbon fixation in *A.barbadensis*. At 12 noon amino acid pool shows saturation while organic acid pool saturations are noted at midnight.

The enzyme studies indicate that the PEP-Case and RuDP-Case are functioning in *A. barbadensis* and PEP-Case is found to be the major enzyme of carbon assimilation in light as well as in the dark. It has much more activity than RuDP-Case. The MDH and pyruvate-kinase are also found active in the succulent. The enzymes extracted at 10 AM to 12 noon period show that PEP-Case is 2–3 times more active than RuDP-Case while MDH is less active than pyruvate-kinase at same time.

DISCUSSION

A. barbadensis being a Crassulacean succulent synthesizes malate in dark. However in light aspartate, alanine, glycine and serine are the major products. In glycophytes as well as in *Bryophyllum tubiliflorum* during light $^{14}CO_2$ feeding radioactivity is mainly incorporated in sucrose in first hour while malate has less label (Kluge, 1969). It was further found that radioactivity in sucrose decreased, with more label in malate. Finally malate was found as the most heavily labelled product of $^{14}CO_2$ fixation during day. Kluge (1971) further reported that when tissue malate level was low, $^{14}CO_2$ fixation in the light into malate exceeded that in sugars. When tissue malate level was high, sugars were the predominant products of $^{14}CO_2$ fixation in light. However, carbon metabolism in *Aloe* species appears to be different. It appears sucrose is not the main product of carbon assimilation in the succulent. The results suggest that carbon is mainly fixed in amino acids and diverted towards insoluble compounds. Similarly carbon from sugar phosphates is stored in insoluble carbohydrate and not in sucrose. Avadhani, Osmond and Tan (1971) have shown that in the leaves of *Sedum prealtum* and *Bryophyllum calycanium* malate as the principal product of 5 sec. $^{14}CO_2$ fixation in light and this malate is largely labelled in the C_4-position in contrast to the "Crassulacean malate". The malate formation in light was prominent only in tissues with low acid content, however, when acid contents were high, *Sedum* leaves in light fixed $^{14}CO_2$ into sugars and phosphorylated compounds.

The label in sugar phosphates and little label in sucrose at night were confirmed repeatedly. The results do not indicate normal pathway and hence are being scrutinized further. It is possible that high generation of reducing power at night and energy from respiration may help in sucrose synthesis. However, the results require further confirmation before definite conclusion can be arrived at.

During day time PEP-Case and RuDP-Case are active at early hours of light. This results in massive synthesis of OAA which serves as precursor of malate. As MDH is active it results in more label in malate at 6 AM. PEP-Case shows 3 times more activity than RuDP-Case while MDH is found to be less active than pyruvate-kinase. The study of pyruvate-kinase shows that it may lead to resynthesis of PEP between 10 AM to noon. The high activity of PEP-Case, pyruvate-kinase and low activity of MDH during day may explain the amino acid pool saturation and less synthesis of organic acids. During this period CO_2 is mainly fixed by PEP-Case to form aspartate and malate and due to transaminases being active amino acid pools are saturated by 12 noon.

During dark there is predominant label in organic acids in CAM plants (Ranson & Thomas, 1960 & Black, 1973). These pools are possibly stored in vacuoles in these succulents and utilized during the light hours. Our results show that from 6 PM to night malate has heavy label than any other compound. The amino acids also have label next to organic acids. Label in sugar mono and diphosphates and sucrose is maximal at 6 PM. The results show that during light aspartate is the immediate product accompanying with malate and PEP + PGA while in dark malate is major product of $^{14}CO_2$ fixation for 3 sec. Saltman *et al.* (1956 & 1957) have reported in *Bryophyllum* that malate and aspartate as products of dark $^{14}CO_2$ fixation after 5 sec and they concluded malate and aspartate might well have originated from a common precursor. Since they were able to isolate labelled oxaloacetate from the products of longer periods of dark fixation and to detect PEP-Case in extracts from leaves they concluded that the "first product" of CO_2 fixation was OAA. Our enzyme studies show efficient activity of PEP-Case in *A. barbadensis* during light and dark periods. It is possible that reaction similar to *B. pinnatum* might be operative in *A. barbadensis*. The immediate products during light and dark may be different because of possibly the pool mechanism. As the malate contents are high at early hours of light that may inhibit malate synthesis due to feed back mechanism. Hence aspartate and other amino acids pools get operative and are saturated at 12 noon. The amino acids saturation also inhibits their further synthesis and it is possible that they are utilized for the synthesis of other metabolites. At night PEP-Case and MDH have been more active to form malate as malate pool is low.

Hatch, Slack and Johnson (1967) have suggested the C_4-pathway in some plants having C_4-acids as immediate products of photosynthesis and the role of PEP-Case, malic enzyme, pyruvate-pi-dikinase in C_4-plants. Laetsch (1974) have reported the C_3–C_4 intermediate in some CAM species. While Denius & Homann (1972) have shown that *Aloe arborescense* is a C_3–C_4 intermediate. Our observations also underline *A. barbadensis* as an intermediate of C_3–C_4 plants with more inclination towards C_4-type. Our studies on pyruvate-kinase show that it is functioning well from 10 AM to 12 noon. It is possible that this enzyme or pyruvate pi-dikinase may play an important role in regeneration of PEP from pyruvate. The studies on pyruvate-pi-dikinase and malic enzyme and stomatal behaviour in *A.barbadensis* are under progress and when the data are availed the possibility of CAM in *A.barbadensis* will be more clear.

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REFERENCES

Avadhani, P. N., Osmond, C. B. & Tan, K. K. (1971). Crassulacean acid metabolism and C_4 pathway of photosynthesis in succulent plants, pp. 288–293. In: *Photosynthesis and Photorespiration* (*Eds.* Hatch M. D., C. B., Osmond and R. O. Slatyer). Wiley-Interscience.

Bharucha, F. R., & Joshi, G. V. (1957). Studies in Crassulacean acid metabolism in *Aloe vera* Linn. *J. Univ. Bombay*, **15**, 23–28.

Black Jr., Clanton C. (1973). Photosynthetic carbon fixation in relation to net CO_2 uptake. *Ann. Rev. Pl. Physiol.*, **24**, 253–286.

Denius Jr. H. P., & Homann, P. H. (1972). The relation between photosynthesis respiration and Crassulacean acid metabolism in leaf slices of *Aloe arborescens* Mill. *Pl. Physiol.*, **49**, 873–880.

Ernest, K., & Abood, L. G. (1949). Colorimetric estimation of succinic dehydrogenase by T. T. C. *Science*, **109**, 144.

Hatch, M. D., Slack, C. R. & Johnson Hilary, S. (1967). Further studies on a new pathway of photosynthetic carbon-di-oxide fixation in sugarcane and its occurrence in the other plant species. *Biochem. J.*, **102**, 417–422.

Joshi, G. V., & Bartakke, S. P. (1974). Light and dark $^{14}CO_2$ fixation in *Aloe vera* var. *indica*. *Maharashtra Vidnyan Mandir Patrika* (*Sumati Patil Commemoration*), **9**, 22-28.

Kluge, M. (1969). Changes in the labelling patterns after feeding *Bryophyllum tubiliflorum* with $^{14}Co_2$ at different times during the light/dark period. I. $^{14}CO_2$ fixation in light. *Planta*, **88**, 113–129.

———(1971). Studies on CO_2 fixation by succulent plants in the light. pp. 283–287. In: *Photosynthesis and Photorespiration*. (Ed. Hatch, M. D., C. B. Osmond and R. O. Slatyer). Wiley–Interscience.

Laetsch, W. M. (1974). C_4-syndromes. *A., Rev. Pl. Physiol.* **25** pp.

Lowry, O. H., Rosenbough, N. J. Farr, A. L., & Randall, R. J., (1951). Protein measurement with folin-phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

Morris, I., & Farrell, K. (1971). Photosynthetic rates, gross patterns of carbon dioxide assimilation and activities of Ribulose-di-phosphate carboxylase in marine algae grown at different temperatures. *Physiol. Plant.*, **25** 372–377.

Ranson, S. L., & Thomas, M. (1960). Crassulacean Acid Metabolism. *A. Rev. Pl. Physiol.*, **11**, 81–110.

Saltman, P., Kunitake, G., Spolter, H., and Stitts, C. (1956). Dark fixation of CO_2 by succulent leaves. The first products. *Pl. Physiol.*, **31**, 464–468.

Saltman, P., Lynch, V. H., Kunitake, G. M., Stitts, C. & Spolter, H. (1957). The dark fixation of CO_2 by succulent leaves: Metabolic changes subsequent to initial fixation. *Pl. Physiol.*, **32**, 197–200.

Weidner, M., & Salisburry, F. B. (1974). The temperature charcteristics of RuDP-Carboxylase, nitrate reductase and pyruvate kinase from seedlings of two springer varieties. *Z. Pflanzenphysiol.*, **71**, 398–412.