

CO₂ Fixation in *Opuntia* Roots¹

Irwin P. Ting² and W. M. Dugger, Jr.

Department of Life Sciences and Dry-Lands Research Institute,
University of California, Riverside

Received September 2, 1965.

Summary. Nonautotrophic CO₂ metabolism in *Opuntia echinocarpa* roots was studied with techniques of manometry and radiometry. The roots were grown in a one-quarter strength nutrient solution for several days; the distal 2 cm was used for physiological studies. The roots assimilated significant quantities of ¹⁴CO₂ and appeared to show a crassulacean-type acid metabolism with respect to quality and quantity. Most of the ¹⁴C activity was associated with the distal portion of the elongating root indicating correlation with metabolic activity. The ¹⁴CO₂ assimilation was comparable to a crassulacean leaf succulent, but 3 times greater than that found for stem tissue of the same *Opuntia* species.

The rates of O₂ and CO₂ exchange and estimated CO₂ fixation were 180, 123, and 57 μl/g per hour. A respiratory quotient of 0.66 was found.

The products of ¹⁴CO₂ fixation were similar in most respects to reported experiments with leaf succulents. Equilibration of the predominant malic acid with isocitric, succinic, and fumaric acids was not evident. The latter observation was interpreted as metabolic isolation of the fixation products rather than poor citric acid cycle activity.

A rapid turnover of the fixed ¹⁴CO₂ was measured by following decarboxylation kinetics and by product analysis after a postincubation period. The first order rate constant for the steady state release was $4.4 \times 10^{-3} \text{ min}^{-1}$ with a half-time of 157.5 minutes. Amino acids decayed at a more rapid rate than organic acids.

Nonphotosynthetic incorporation of CO₂ with the subsequent formation of organic acids is widespread in living organisms and may very well be ubiquitous. Succulent plants, as is well known, are characterized by a high capacity for nonautotrophic CO₂ fixation concomitant with an accumulation of organic acids (10). For the most part, malic acid is the predominant diurnally fluctuating acid; however, certain halophytic succulents accumulate more amino acids (20). Data obtained in our laboratory indicated that stem tissue from members of the *Cactaceae* has the capacity for a true crassulacean-type acid metabolism, but to a much lesser degree than the crassulacean leaf succulents (15).

CO₂ fixation by root tissue has been demonstrated by several workers (see 6); however, based on RQ data, this does not appear to be quantitatively significant (3, 7, 17). It is of interest, therefore, to investigate the pattern of CO₂ fixation in roots of a plant capable of high levels of dark CO₂ fixation to determine if a true acid metabolism is operative.

The present study was initiated to determine the capacity for nonautotrophic CO₂ fixation by *Opuntia* roots and to evaluate the subsequent utilization of the fixed carbon. This research is a part of an extensive investigation in our laboratory concerning metabolism of desert plants.

Materials and Methods

Joints of *Opuntia echinocarpa* Engel. and Bigel. were collected from plants growing in the Anza-Borrego Desert of Southern California. In the laboratory, terminal joints were separated and dried in the dark at room temperature until visible root primordia had formed on the areoles (fig 1). The joints with visible primordia were placed in an aerated, one-quarter strength Hoagland's solution under continuous incandescent light (4.8×10^5 ergs/cm² per sec). The water bath temperature was maintained at about 28°. Within several days, uniform adventitious roots 10 to 15 cm in length had developed. These roots were removed from the joints and washed thoroughly with distilled water before subsequent treatments. Clonal roots were used within individual experiments. Respiratory quotients were determined at 30° in a Warburg apparatus. The

¹ Supported by a grant from the National Science Foundation (GB-2547).

² National Science Foundation Postdoctoral Fellow (1964-1965).

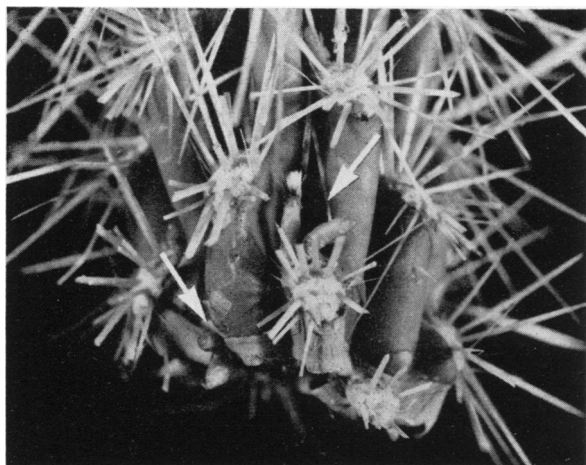


FIG. 1. Terminal joint of *Opuntia echinocarpa* showing root primordia on areoles after 3 weeks drying.

reaction flasks contained 2 ml of 0.01 M Tris buffer (pH 7.3) and about 0.1 g fresh weight of root tissue cut from the first 2 cm (see fig 3).

The longitudinal distribution of assimilated CO₂ was determined by incubating whole root sections in H¹⁴CO₃⁻, cutting the roots into 1 or 2-cm segments, and placing them directly into liquid scintillation counting vials for radioactivity analysis.

Organic acids were separated and determined by 1-dimensional thin-layer chromatography (16). Amino acids were eluted from a Dowex 50 column with 2 N NH₄OH (2) and separated on Silica gel H in a chloroform-methanol-ammonia developer made in the ratio of 25 to 25 to 7 (20% ammonium hydroxide). The neutral fraction referred to in the text was the material which passed through both Dowex 1 (formate form) and Dowex 50 (hydrogen form) columns. Other than those outlined above, all procedures and techniques of sample preparation, product separation and identification, and radioactivity counting were described earlier (16).

Exchange and decarboxylation of fixed ¹⁴CO₂ were followed in a closed system with a 1-liter Cary-Tolbert ion chamber and a Cary Model 31 vibrating reed electrometer. The ¹⁴C detection efficiency of the entire system (1.5 liters) was about 21% with a flow rate of 1 liter per minute. After a 2-hour ¹⁴CO₂ fixation period, the washed root sections were placed in a 100 ml round bottom flask containing 10 ml of M/15 phosphate buffer at pH 6.4. A continuous record of the released ¹⁴CO₂ was obtained by attaching the flask to the closed circulating system and bubbling the air stream through the buffer.

All incubations were conducted with ¹⁴CO₂ supplied as NaH¹⁴CO₃ in 1 N NaOH (2.2 μC/μmole). Longitudinal distribution analysis was conducted in Warburg vessels containing 1 μC of ¹⁴C (0.02 ml) in 2 ml of the phosphate buffer for 2 hours at 30°. All other incubations were conducted in 125 ml modified Erlenmeyer flasks with 50 μC (1.0 ml) in

10 ml of phosphate buffer at 25°. Sample sizes, other than those stated, were about 0.2 g fresh weight.

Results

Respiratory Quotient. Manometric analyses indicated that O₂ consumption was linear with time and comparable to active plant tissue (fig 2) (5). CO₂ evolution was somewhat erratic, but on the average linear. Assuming that the RQ would be 1 without CO₂ fixation, we have estimated that 57.0 μl/g per hour of CO₂ was fixed. The O₂ uptake and CO₂ evolution rates were 180 and 123 μl/g per hour respectively. The apparent respiratory quotient for the 8-hour period was 0.66.

Longitudinal Assimilation of CO₂. The longitudinal pattern of ¹⁴CO₂ assimilation was similar to typical inorganic cation or anion uptake (fig 3)

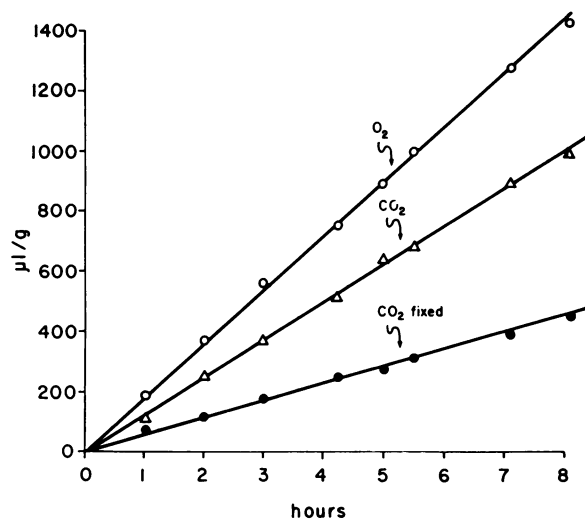


FIG. 2. Respiratory gas exchange by *Opuntia echinocarpa* roots. An apparent RQ of 0.66 is indicated.

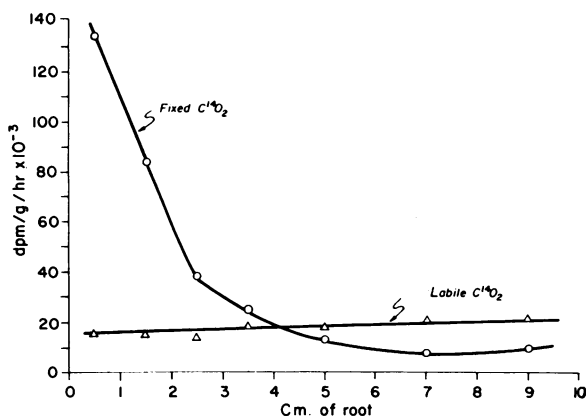


FIG. 3. Longitudinal distribution of fixed and labile ¹⁴C in *Opuntia echinocarpa* roots. DPM's are plotted against the mean length of segment.

(8, 13). In the experiment reported, there was more ^{14}C activity in the first 1 cm of root than in the medium (134,000 dpm in the root as compared to 109,500 dpm in the medium on a v/v basis). The upper curve of fig 3 depicts the stable fixation products expressed on a fresh weight basis. The lower curve represents radioactivity which was removed from the counting vials by lowering the pH to 2 and/or vigorous swirling. We have interpreted this loss as readily exchangeable CO_2 or possibly apparent free space CO_2 with respect to dark fixation. In this particular experiment (fig 3), the exchangeable CO_2 calculated to be 12.1 % of the total uptake (considering only the distal 2 cm of root tissue). Autoradiograms of whole root sections showed the same pattern of $^{14}\text{CO}_2$ fixation.

Products of CO_2 Fixation. The products of a 2-hour $^{14}\text{CO}_2$ fixation period were quite similar to those reported for crassulacean leaf succulents (11). In general, about 80 % of the ^{14}C activity was found in the organic acid fraction and about 20 % in the amino acid fraction. Little or no activity was detected as lipoidal compounds and no activity was ever detected in carbohydrates or other neutral compounds. From 0.5 to 2.0 % of the total uptake was detected as insoluble residues after complete extraction (chloroform-methanol-water extraction (16) followed by 100 % ethanol). Malic acid was the primary stable product of the fixation with lesser amounts of ^{14}C detected in citric acid, isocitric acid, aspartic acid, glutamic acid, and alanine. Only trace amounts of activity were detected as succinic or fumaric acids (table I). Similar products were obtained in the dark or in light of 1.35×10^5 ergs/cm² per sec. In the latter experiment, the roots were in continual light from time of development in the culture flasks to liquid nitrogen freezing at the end of the incubation. The above listing of products does not imply that these were the only labeled metabolites resulting from the fixation, but only that they were labeled sufficiently for detection with the chromatographic procedures.

Decay of Products. In order to follow the fate of the incorporated carbon, experiments were de-

signed in which the tissue was incubated in nonradioactive buffer for several hours after a 2-hour incubation period. Tissue was sampled after the initial incubation period and again at the end of the postincubation period. The time course of release was followed in the manner described above. The data (table II) indicated that at least 50 % of the stable products were lost during a 2-hour postincubation period. On a percentage basis, the organic acids increased and the amino acids decreased. In the experiment reported in table II, the water soluble compounds decreased in activity by 72 %. There was a 67 % and 90 % decrease in activity associated with organic acids and amino acids respectively. The insoluble residue decreased only 19 % in activity and accounted for a greater percentage of the total

Table II. *Products of 2-Hour Nonautotrophic $^{14}\text{CO}_2$ Incorporation by Opuntia Roots and Remaining Activity after a Subsequent 2-Hour Postincubation Period*

Product	Incubation		Postincubation	
	dpm/g	%	dpm/g	%
Lipid	20,000	0.7	3500	0.4
Water soluble	2,820,000	98.7	788,000	97.8
Anion	2,198,000	76.9	724,000	90.0
Neutral	0	0.0	0	0.0
Cation	622,000	21.8	64,000	7.9
Insoluble	17,200	0.6	14,000	1.7
Total	2,857,200	100.0	805,500	100.0

activity after the postincubation period than after the fixation period. In general, there was significant loss of activity from all fractions.

It was of interest to determine the changes in organic acids during a postincubation period. In 1 such experiment, with a 2-hour fixation period, 3,160,000 dpm/g fresh weight of ^{14}C were incorporated. After a 7.5-hour postincubation period, a similar sample contained 796,000 dpm/g. Of the total organic acids (75 % of total uptake in the reported experiment), 85.8 % or about 2,030,000 dpm/g were detected in malic acid and 14.2 % or about 340,000 dpm/g in citric acid. At the end of the postincubation period, the organic acids accounted for 88 % of the total activity or about 700,000 dpm/g. There was 77.2 % or about 540,000 dpm/g as malic acid and 22.8 % or about 159,000 dpm/g as citric acid. Whereas the total activity had decreased 74.8 %, malic had decreased 73.4 % and citric only 53.2 %. These data do not indicate whether or not malic acid was converted to citric acid, or if there was a different decay rate. Even after the postincubation of 7.5 hours, only trace amounts of activity could be detected in the other citric acid cycle acids.

A graphical analysis of the time course of the decrease in activity within the tissue showed a 2 phase reaction (fig 4). If graphed semilogarithmically (upper curve, fig 4), the data followed a curvi-

Table I. *Long-Term (2 hrs) Products of Nonautotrophic $^{14}\text{CO}_2$ Incorporation by Opuntia Roots*

Data are expressed as a percentage of total activity on chromatoplates. Raw data (dpm) are given in parentheses for the organic acid chromatoplate. Unaccounted for percentages represent activity at origins.

Organic acid*		Amino acid	
Fumaric	Trace (14)	Aspartic	2.9
Succinic	Trace (52)	Glutamic	7.4
Isocitric**	1.6 (140)	Alanine	4.6
Malic	66.4 (6260)	Unknown	3.9
Citric	11.4 (900)		

* 80 % of total activity.

** Estimated as the lactone (see 16).

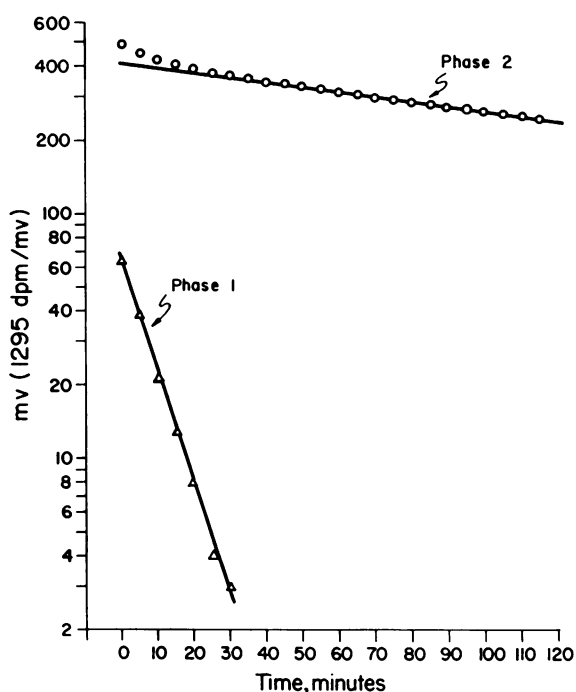


FIG. 4. Time course of decrease in ¹⁴C in root tissue of *Opuntia echinocarpa* roots after a 2-hr fixation period. The points of phase 1 were calculated from the difference between the actual datum points of the upper curve and the extrapolation of phase 2.

linear line. When the linear portion of the upper curve (fig 4) was extrapolated to the y-axis, the estimated activity could be subtracted from the actual datum points. A plot of the calculated values resulted in a second first order curve (phase 1, fig 4). The first order rate constants were $103 \times 10^{-3} \text{ min}^{-1}$ and $4.4 \times 10^{-3} \text{ min}^{-1}$ for phases 1 and 2 respectively. The half-times for the reactions were 6.72 minutes and 157.5 minutes for phases 1 and 2. The ratio of the rate constants (and the half-times) was 23.4.

Comparison between ¹⁴CO₂ Uptake in Roots and Stems. The magnitude of the ¹⁴CO₂ fixation in root tissue was assessed by comparing the root tissue with stem tissue from the same species and the leaf tissue from a typical crassulacean succulent, *Dudleya saxosa* (Jones) Britt. and Rose. Experimental plants of the latter 2 were kept for at least 3 weeks in a growth chamber on 8-hour days (light, 5×10^4 ergs/cm² per sec; day temperature, 29°; night temperature 18°; relative humidity both day and night 50%). The tissue for analysis (ca. 1 g of 1 mm thick discs of stem tissue sampled with a No. 5 cork borer and ca. 1 g of 1-mm thick sections of leaf tissue) was sampled at the end of the 8-hour light period and incubated in the same manner as the root tissue. Quantitatively, the root tissue was quite comparable to the crassulacean succulent (table III), whereas the stem tissue of *Opuntia echinocarpa* incorporated considerably less ¹⁴C into stable products.

Table III. Two-Hour Nonautotrophic ¹⁴CO₂ Fixation of a Leaf Succulent and a Stem Succulent

The leaf succulent was *Dudleya saxosa* (Jones) Britt. and Rose, and the stem succulent was *Opuntia echinocarpa* Engel. and Bigel.

	Leaf succulent Leaf	Stem succulent Stem	Root
dpm μ/g	2,892,000	852,000	2,456,000
% Anion	89.0	78.6	79.0
% Cation	11.0	21.4	21.0

Discussion

The CO₂ metabolism of *Opuntia* roots is similar in most respects to typical crassulacean-type acid metabolism. The high rate of CO₂ fixation and relatively low respiratory quotients attest to this conclusion. One might not expect a diurnal fluctuation such as was found in chlorophyllous tissue (9); however, experiments were not designed to test such a cycle. A dependence on the chlorophyllous stem tissue would certainly be predicted. Since the adventitious roots were rapidly elongating, high rates of metabolism (CO₂ fixation) would be expected.

Longitudinal Assimilation of CO₂. The gross pattern of CO₂ uptake by *Opuntia* roots was similar to typical inorganic cation or anion uptake (8, 13), i.e., more accumulated toward the distal end. The greater uptake toward the tip might have been due to greater CO₂ permeability; however, the free CO₂ toward the base was at least equal to, and probably greater than, the free CO₂ at the tip (fig 3). This suggested that permeability was not a significant factor with respect to uptake. These results were found with intact root sections and 1 to 2 cm segments. Fadeel (4) found, nevertheless, a measurable resistance to CO₂ diffusion by root epidermis. In our experiments, we found an accumulation of ¹⁴C at the root tip against a ¹⁴C concentration gradient. These findings were important since they indicated that ¹⁴CO₂ fixation was not simply an equilibration with free CO₂ in the root or in the medium. In chlorophyllous wheat roots which were in the dark, Fadeel (3) found an accumulation of free CO₂ as high as 10% by volume; the greatest concentration was toward the tip corresponding to the greatest O₂ consumption. Since more free CO₂ (endogenous) would be present in the tissue at the tip, and since the ¹⁴CO₂ will diffuse independent of ¹²CO₂, there would be greater dilution of the ¹⁴CO₂ by ¹²CO₂ at the tip than away from the tip. The result would be that the ¹²CO₂ would act as a competitive inhibitor with respect to ¹⁴CO₂ fixation, and proportionately less ¹⁴CO₂ would be fixed per unit of enzyme(s). For this reason considerably more enzymatic activity was probably present toward the tip.

Respiratory Quotient. The respiratory quotient of nonsucculent root tissue is seldom much less than one. Machlis (7) found average values ranging

from 0.93 to 0.99 for barley roots. In the case of added HCO_3^- , respiratory quotients decreased significantly (17). Fadeel (3) measured a respiratory quotient of 0.85 for flax roots, but he attributed the low value to the oxidation of lipids by the young root preparations. His data with wheat, on the other hand, showed a respiratory quotient of 1.0. The respiratory quotients of *Opuntia* roots, without added HCO_3^- , indicated a significant fixation of CO_2 which was greater than that for roots of nonsucculents (fig 2). Furthermore, the fixation was quantitatively comparable to leaf tissue of crassulacean succulents (table III).

Products of CO_2 fixation. In general, the products of the carboxylation reactions were predictable from the current literature (11); however, the lack of appreciably labeled isocitric, succinic, and fumaric acids may be significant. In *Opuntia* stem tissue, as much as 10% succinic acid was found in the organic acid fraction (15). The latter 2 observations might indicate low citric acid cycle activity in the roots (cf. 11, 14), but the respiratory data would not support such a conclusion. It seemed more probable that such observations reflected little equilibration of the primary products (malic, citric, aspartic, and glutamic) with other metabolites. A lack of equilibration was quite clear from our postincubation experiments. Varner and Burrell (18) were able to demonstrate some redistribution of the label in *Bryophyllum* leaves, nevertheless, their data indicated a loss of activity primarily by a reversal of the fixation reactions.

Decay of Products. A rapid turnover of the incorporated ^{14}C was indicated by our data. Under the conditions of our experiments, 50% or more of the $^{14}\text{CO}_2$ fixed into stable products was released in a subsequent 2-hour period. The kinetics of release were apparently biphasal (fig 4). One might assume that the first or rapid phase represented exchangeable CO_2 , i.e., free CO_2 or HCO_3^- salts, which was present in the tissue and in equilibrium with the labeled incubation atmosphere; however, in the experiment reported, 16.3% of the total fixed ^{14}C was lost by the rapid phase and 35.7% by the slower phase. Furthermore, the half-time for the latter was 2.63 hours, in poor agreement with the actual extraction of stable products from the tissue (table II). The latter indicated 50% or more of the fixed ^{14}C was lost in the 2-hour postincubation period. Both phases, therefore, probably represented actual decarboxylations. The rapid release (phase 1) may have been the result of the change in $p\text{CO}_2$ when the tissue was transferred from the incubation flasks to the postincubation flasks, i.e., phase 1 may be artifactual. Refixation of released $^{14}\text{CO}_2$ may have complicated our measurements; however, the very low specific activity resulting from the released $^{14}\text{CO}_2$ into a volume of 1.5 liters and the small quantity of root tissue (ca. 0.1 g) should preclude significant errors.

In the experiment reported in table II, there was a 67% decrease in organic acids and a 90% decrease in amino acids during the postincubation period. On

a percentage basis (after the postincubation), there was more activity associated with the organic acids than the amino acids. It was evident that the rate of amino acid decay was greater than that for organic acids. Although kinetic data would be necessary for a definite analysis, in all probability transaminase activity (19) accounted for much of the amino acid decay observed. We have evidence (to be reported later, and see ref. 15) that in stem tissue aspartic and glutamic acids decay at a greater rate than alanine. During the decay process, there was no evidence for equilibration of label with organic acids other than possibly citric acid. In sum, there was a relatively rapid turnover of the fixed $^{14}\text{CO}_2$ and little equilibration of the primary products with other constituents. We did, however, detect a small percentage of activity in insoluble components which did not decay as rapidly as the water soluble compounds.

Phosphoenolpyruvate carboxylase is implicated in the fixation of CO_2 by succulent plants and by root tissue (6, 12). Since fixation via this enzyme is not reversible (1), the mechanism of CO_2 release is an important question. The metabolic pathway concerning fixation and subsequent release is currently being investigated in our laboratory.

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