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Dark CO_2 Fixation and its Role in the Growth of Plant Tissue

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Summary. Experiments were designed to determine the significance of dark CO_2 fixation in excised maize roots, carrot slices and excised tomato roots grown in tissue culture. Bicarbonate-^{14}C was used to determine the pathway and amounts of CO_2 fixation, while leucine-^{14}C was used to estimate protein synthesis in tissues aerated with various levels of CO_2.

Organic acids were labeled from bicarbonate-^{14}C, with malate being the major labeled acid. Only glutamate and aspartate were labeled in the amino acid fraction and these 2 amino acids comprised over 90 % of the ^{14}C label in the ethanol-water insoluble residue.

Studies with leucine-^{14}C as an indicator of protein synthesis in carrot slices and tomato roots showed that those tissues aerated with air incorporated 33 % more leucine-^{14}C into protein than those aerated with CO_2-free air. Growth of excised tomato roots aerated with air was 50 % more than growth of tissue aerated with CO_2-free air. These studies are consistent with the suggestion that dark fixation of CO_2 is involved in the growth of plant tissues.

The ability of various plant tissues to assimilate CO_2 by nonphotosynthetic mechanisms has been recognized for some time. The best known example is the diurnal fluctuation of the organic acids in succulents (19). Although nonsucculents do not vary so greatly in their organic acid content, there is good evidence that leaves of many species assimilate CO_2 in the dark (25). Fixation of CO_2 by intact root tissue has also been reported with the primary labeled compound being malate (8, 18). Increased CO_2 concentrations have stimulated the growth of *Avena* mesocotyl and etiolated coleoptile (13, 14) and the root growth of cotton (11), tomatoes (5), and peas (6, 24). This CO_2 stimulation may be related to the biosynthetic role of the citric acid cycle (20).

For each turn of the citric acid cycle, one molecule of oxaloacetate is regenerated to initiate the succeeding turn of the cycle. However, for several intermediates of this cycle, particularly oxaloacetate, α-ketoglutarate and succinyl CoA, there are other metabolic fates, alternate to those of the citric acid cycle. Synthesis of aspartate from oxaloacetate and glutamate from α-ketoglutarate, would, inevitably, decrease the rate at which the cycle could operate unless these losses were offset by a renewal of the supply of oxaloacetate (28). In microorganisms, the oxaloacetate supply is replenished by CO_2 fixation with pyruvate or P-enolpyruvate (20, 28).

The physiological basis for the increased growth of nongreen plant tissues when grown in air rather than CO_2-free air has not been established. The present studies were conducted to gain a better understanding of this CO_2 stimulation. The results obtained are consistent with the suggestion that dark fixation of CO_2 is required to renew the carbon of the citric acid cycle when carbon from this cycle is involved in synthetic events.

Materials and Methods

Maize seeds (var. WR-9 × 38–11) were germinated on 0.5 % agar in petri dishes. Carrots and tomato seeds were purchased locally. Cylinders of carrot phloem tissue were removed with a cork borer (4 mm diameter) and cut into disks 0.5 mm thick. The carrot tissues were rinsed briefly in deionized water and lightly blotted dry. Tomato seeds were sterilized briefly in chlorox, rinsed and allowed to germinate for 4 days in sterilized petri dishes on moist filter paper at 25°. Tomato root tips were removed and placed in 50 ml of White's medium (27) and aerated with air or CO_2-free air (air passed through KOH). Care was taken to insure sterile conditions.

Maize root tips were placed in Warburg flasks with bicarbonate-^{14}C solutions whereas carrot tissue was placed in 125 ml Erlenmeyer flasks. These were then gently shaken. When using labeled leucine, the solutions and tissue samples were placed in No. 15 medium fritted glass filter funnels. Air was passed through a NaOH or water scrubber and then through the base of the filter to aerate the tissue suspended in solution. Respired CO_2 was collected as $BaCO_3$ (12).

At predetermined times, the tissues were removed, rinsed with deionized water, transferred into 75 ml of boiling 100 % ethanol and boiled for 3 minutes. The ethanol was decanted and the tissues were ground

with a mortar and pestle. The residues were successively extracted with boiling 80 % (v/v) ethanol, 50 % ethanol, water, and 80 % ethanol. The extracts were combined and taken to dryness at 40° under reduced pressure.

The dried ethanol extract was dissolved in water and fractionated sequentially on Dowex 50 × 8 (H^+) and Dowex 1 × 10 (formate) resins (3, 12). The basic or amino acid fraction was further fractionated (7) by passage through Dowex 1 × 10 (acetate). A dicarboxylic amino acid fraction containing free glutamic and aspartic acids was thus obtained. The organic acid fraction was separated by gradient elution from 1 × 11 cm columns of Dowex 1 (formate) (3, 17). The residue was hydrolyzed with 6 N HCl for 12 hours at 220° and then treated in the same manner as the ethanol extract.

The organic and amino acids were identified by co-chromatography with known acids on the column and on paper with butanol: propionic acid: water (623:310:437, v/v/v) (1) and water-saturated phenol as solvents.

A sample aliquot was dried on a nickel planchet and the ^{14}C activity determined in a gas flow GM counter. Leucine-1-^{14}C (21 mc/mmole), leucine-U.L.-^{14}C (246 mc/mmole) and $Ba^{14}CO_3$ (0.44 c/g) were obtained from commercial sources. The $Ba^{14}CO_3$ was converted to potassium bicarbonate-^{14}C before use.

Results and Discussion

Young rapidly growing excised maize root tips (incubated with bicarbonate-^{14}C) incorporated 25 % of the label into nonvolatile components (table I). As might be expected from dark CO_2 fixation, the labeled organic acids were acids of the citric acid cycle, with the expected heavy label in malate. Aconitate, a prominent acid in maize roots (12), had an equal label of ^{14}C which indicated that it, like malate, had a large exogenous pool in equilibrium with the acids of the citric acid cycle (12, 22). Glutamate and aspartate were the only labeled amino acids. These 2 amino acids were the only detected labeled components in the hydrolyzed residue.

Table I. *Incorporation of ^{14}C into Maize Root Tips*

Eight root tips, 68 hours old (5 cm length) were incubated in 2 ml water (pH 7.2) with 9 μmoles of bicarbonate-^{14}C at 25° for the times shown.

Fraction	Activity cpm		
	3 hr	4 hr	5 hr
Bathing solution	190,000	180,000	145,000
Organic acids			
Malate	18,000	20,000	29,000
Aconitate	14,000	17,000	24,000
Others	800	1000	3000
Amino acids			
Glutamate	10,000	11,000	17,000
Aspartate	20,000	24,000	34,000
Insoluble residue	700	800	1000
% Recovery of added ^{14}C	100	99	90

Table II. *Fixation of ^{14}C into Different Maize Root Segments*

Forty segments (1 cm length) were removed from 3 separate areas of maize roots and incubated in 2 ml of 0.1 M phosphate (pH 7.8) with 10 μmoles of bicarbonate-^{14}C for 3 hours. Original roots were 72 hours old and 8 cm long.

Segment	Activity in water-soluble fraction (cpm)	% of added ^{14}C
Tip cm	76,000	10.5
4 to 5 cm from tip	42,000	5.8
Cm closest to kernel	32,000	4.4

Table III. *Effects of Chloramphenicol Upon CO_2 Fixation in Maize Roots*

Roots were incubated with 9 μmoles of bicarbonate-^{14}C in 2.2 ml of 0.05 M phosphate (pH 7.4) for 6 hours. The 2 cm roots (20 in number) were 48 hours old, the 4 cm roots (10 in number) were 72 hours old.

Tissue	Chloramphenicol conc	cpm	
		Water-soluble	Insoluble residue
2 cm root tip	None	160,000	7000
	0.8 mg/ml	92,000	4000
4 cm root tip	None	135,000	2000
	0.8 mg/ml	78,000	1000

We supplied bicarbonate-^{14}C to various isolated 1-cm segments of maize roots and determined the assimilation of bicarbonate into the water-soluble components. The results are shown in table II. The tip cm incorporated the greatest activity of ^{14}C with decreased activity toward the kernel. Jensen (10) and Clowes (4) have shown that the rate of protein synthesis is highest in the root tip. Maize root tips synthesize many of the amino acids used in protein synthesis (16). If CO_2 is required to replenish the acids of the citric acid cycle when this cycle is acting in a biosynthetic role, then the highest CO_2 fixation would be expected to occur in tissue furthest from the endosperm (table II). Here the tissue is growing more rapidly and must synthesize more of the amino acids derived from the cycle. In all root segments a large proportion (53 %) of the assimilated ^{14}C was found in glutamate and aspartate, amino acids synthesized from citric acid cycle carbon.

The effect of chloramphenicol upon $^{14}CO_2$ fixation in maize roots was to reduce the amount of ^{14}C incorporated into both the ethanol-water soluble and insoluble fractions (table III). This reduction was greatest in the younger 2-cm root tips. Jacoby and Sutcliffe (9) showed chloramphenicol to inhibit the incorporation of ^{14}C-labeled glutamate, glycine and

proline into protein by carrot slices. At higher concentrations, chloramphenicol also reduces ion accumulation (26), and absorption of glutamate, glycine and proline by carrot tissue (9). However, the concentration required to reduce uptake was 5 times higher than that reported in table III.

We interpret our results to mean that in actively growing plant tissues, chloramphenicol reduces protein synthesis (15) with a concomitant decrease in CO_2 assimilation. When protein synthesis decreases, synthesis of amino acids whose carbon is derived from the citric acid cycle would also decrease. Therefore, assimilation of CO_2 would not be required for renewal of the cycle's carbon.

The effect of CO_2 on growth was assessed with excised tomato roots growing in tissue culture. Air or CO_2-free air was bubbled through cultures of excised tomato roots and it soon became obvious that growth was superior in the presence of CO_2. The effects of CO_2 upon dry weight and total length of tomato roots are recorded in table IV. The dry weights of roots grown in CO_2-free air were 40 % less than those of the controls grown in air at 5 days (expt 1). The corresponding figure in experiment 2 (15 days) was 45 % and in experiment 3 (22 days) 53 %. The diminished growth in CO_2-free air was also evident as decreased root length.

Table IV. *Effect of CO_2 Upon Growth of Excised Tomato Roots*

Tissues in experiments 1 and 2 were 2 cm root tips; experiment 3 was 1 cm root tip. All explants were grown on White's medium (27).

Treatment	Time (days)	Dry Wt (mg)	Total length (cm)
Expt 1			
Air	5	3.40	38.7
CO_2-free air	5	2.05	20.1
Expt 2			
Air	15	8.95	...
CO_2-free air	15	4.95	...
Expt 3			
Air	22	8.95	...
CO_2-free air	22	4.15	...

All roots grown in solution culture with air bubbling through exhibited abundant secondary branching while roots provided with CO_2-free air were characterized by a lack of branching. Many of these latter roots increased in thickness but increased only slightly in length. This increased growth of the roots aerated with air as compared with CO_2-free air is in agreement with the results of Stolwijk and Thimann (24). They found a 7.6 % acceleration of growth with pea roots and a 10 % acceleration with oat roots when these roots were aerated with 0.6 % CO_2 or less rather than CO_2-free air.

Incubation of bicarbonate-^{14}C with carrot slices for up to 30 minutes showed that aspartate was the first detectable compound labeled (1300 cpm). After 60 minutes, no additional label appeared in aspartate. The ^{14}C content of malate was lower than that in aspartate for the first 30 minutes (800 cpm), and then rose exponentially with time. Label appeared slowly into glutamate. This labeling pattern suggests that CO_2 was incorporated first into oxaloacetate, which is a precursor of both aspartate and malate.

The enzyme system responsible for fixation of CO_2 in the tissues used is presumably P-enolpyruvate carboxylase or P-carboxykinase rather than malic enzyme. The equilibrium of the malic enzyme system in isolation is unfavorable to malate synthesis at the low CO_2 concentrations used in this study (19). The labeling of aspartate before malate also points to an enzyme system different from malic enzyme.

The carboxylation of P-enolpyruvate to yield oxaloacetate by P-enolpyruvate carboxylase is essentially irreversible and in vivo oxaloacetate is converted into malate by malic dehydrogenase. P-enolpyruvate carboxylase has a high affinity for CO_2 which allows it to fix CO_2 even in CO_2 tensions less than 0.03 % (19). As the tissues used were not CO_2-free internally, respired CO_2 may have been fixed by this enzyme and partially offset any losses of citric acid

Table V. *Effect of CO_2 Upon Leucine Incorporation into Carrot and Tomato Root Tissue*

Carrot tissue (2 g) was incubated with 0.119 μmole (10^6 cpm) of leucine-1-^{14}C for 10 hours in experiment 1 and 36 hours in experiment 2. Tomato roots (2 cm explants) were incubated with 0.04 μmole (10^7 cpm) of leucine-U.L.-^{14}C for 48 hours. Tissues were placed in 4 ml of 0.1 M potassium phosphate (pH 7.4).

Root tissue	Treatment	cpm			
		Insoluble residue	Organic acids	Amino acids	Respired CO_2
	Expt 1				
Carrot	CO_2-free	8,000	...	...	...
Carrot	Air	9,000	...	...	...
Carrot	3 % CO_2	11,000	...	...	...
	Expt 2				
Carrot	CO_2-free	886,000	20,000	16,000	260,000
Carrot	Air	994,000	10,000	5000	156,000
Tomato	CO_2-free	320,000	32,000	228,000	100,000
Tomato	Air	467,000	24,000	131,000	65,000

cycle carbon when this carbon was used in synthetic events. Thus the effect on tissues grown in atmospheres lacking CO_2 may appear to be considerably less than if the tissues had been CO_2-free internally (tables IV, V).

Birt and Hird (2) have reported that a maximum of 7.5 % of the leucine absorbed by carrot slices was degraded. The remaining leucine was incorporated into protein. Leucine-^{14}C would therefore be a tool to determine the effect of CO_2 upon protein formation.

Excised tomato roots were grown in solution culture with air or CO_2-free air bubbling through them. After 5 days they were placed in fritted filters with leucine-^{14}C, still maintaining the tissues in their respective gaseous atmospheres. Fresh carrot disks were placed in additional fritted filters and treated in the same manner. Table V lists the ^{14}C activity incorporated into the various fractions.

In all trials, the ethanol-water insoluble residue was hydrolyzed and the ^{14}C label was found exclusively in leucine. Those tissues aerated with CO_2-free air contained more label in all fractions except the residue. Tissues aerated with air showed only labeled leucine in the amino acid fraction whereas tissues aerated with CO_2-free air contained a trace of other amino acids as well.

Carrot tissues (table V, expt 1) aerated with CO_2-free air incorporated less lecuine-^{14}C than those tissues aged in air, which in turn incorporated less leucine-^{14}C into protein than tissues aged in 3 % CO_2. Tissues grown in air incorporated 32 % more leucine-^{14}C into protein than similar tissues grown in CO_2-free air. These results are in disagreement with those of Steward's group (23). In potato tuber disks high CO_2 tensions completely suppressed the incorporation of soluble nitrogen compounds into protein. This response was attributed to a specific effect of CO_2 (23). At low CO_2 levels, carboxylation reactions necessary to mediate the entry of carbon from glycolysis into the citric acid cycle would be limited by a lack of CO_2. In their interpretation, however, this would lead to an increase in protein synthesis, the carbon for such synthesis being derived from sugars rather than amino acids (23). In our interpretation, protein synthesis would decrease as the citric acid cycle could not play a biosynthetic role (20, 28) and amino acids required for protein synthesis would be limiting. Indeed, our results suggest (table V) that leucine is incorporated into protein and that this incorporation is enhanced by elevated levels of CO_2 (table V, expt 1).

In these studies with leucine-^{14}C, 93 % of the ^{14}C label in the organic acid fraction was in 3 unidentified noncitric acid cycle acids. In addition, the respired CO_2 was heavily labeled (table V, expt 2). Animal tissues deaminate leucine to its corresponding keto acid. This keto acid is then oxidized to CO_2 (21). This route of degradation of leucine-^{14}C would account for the labeling patterns observed. Tissues aerated with CO_2-free air contained more free leucine-^{14}C and less leucine-^{14}C in protein (table V, expt 2). Thus, in these tissues, more leucine-^{14}C was degraded, resulting in 33 % more $^{14}CO_2$ being respired.

These studies with leucine-^{14}C as an indicator of protein synthesis and the similar studies reported in table III and IV are clearly consistent with the suggestion that dark fixation of CO_2 is required for growth of plant tissues. The manner in which this CO_2 is utilized by both microorganisms and plant tissues appears to be similar; CO_2 is required to renew the carbon of the citric acid cycle when this carbon is involved in synthetic events (20, 28).

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