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Dark CO_2 Fixation in Avocado Roots, Leaves, and Fruit[1]

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In a previous study avocado roots fixed $C^{14}O_2$ about as rapidly as orange and lemon roots but much slower than bean roots (2). However, homogenates of avocado roots or leaves did not fix CO_2. This study was made to explore reasons for the failure of homogenates of avocado to show CO_2 fixation, and also to understand more of the relatively low degree of CO_2 fixation by avocado roots in. case it may have a bearing on the susceptibility of the species to lime-induced chlorosis (6, 10).

MATERIALS AND METHODS

Avocado *(Persea americana* var. *drymifolia)* roots and leaves were tested for dark $C^{14}O_2$ fixation both as intact organs and as cell-free homogenates. Intact leaves and roots were exposed to a $C^{14}O_2$ atmosphere for 4 hours, then analyzed for organic acids to determine the extent of CO_2 fixation. The plant material was washed and then placed into a wide-mouth gallon jar used to maintain a $C^{14}O_2$ enriched atmosphere. Sufficient acid was introduced into the jar, under vacuum, to react with 3 ml of 0.05 N $KHC^{14}O_3$ (1.2 X 10 CPM/0.1 ml as $BaCO_3$ precipitate on a micromil window of a O-gas flow counter). This was accomplished by adding the acid through tubing which extended through the lid of the jar into a suspended beaker containing the $KHC^{14}O_3$. This was adequate for 3/4% CO_2. The jar was maintained at high humidity with a vial of water in the bottom of the jar. A portion of the leaves and roots, separately, were removed arid ground in a Waring blender in boiling 70% ethanol to determine the non-volatile organic acids. Another portion was ground in ice cold 5 N H_2SO_4 and the keto acids extracted as described by Kunitake, *et al.* (7). The C^{14} was counted by drying aliquots of the final solutions on glass planchets and determining C^{14} activity with a Q-gas flow counter.

The ethanol extract of organic acids was filtered, evaporated to dryness on a 40° C water bath under forced air, and separated into cation, anion, and non-charged fractions by passing the solutions through cation and anion exchange resins as previously described (3). The differently charged, fractions were evaporated to dryness as above and. counted for C^{14}. The anion fraction was also separated into its individual organic acids on a silicic acid column as described by DeKock and Morrison (5). Each fraction was titrated against 0.002 N NaOH, dried on. glass planchets and counted for C^{1}-^{4}.

In vitro reactions for $C^{14}O_2$ incorporation were as follows: a) endogenous, b) PEP + Mg (PEP carboxylase) (1), c) PEP + Mn (9) and d) R5P + Mg + ATP (a 3-enzyme sequence involving phos-phoriboisomerase, phosphoribulokinase, and carboxylation enzyme)

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[2]The abbreviations used in this report are: TRIS, tris(hydroxymethyl)amino-methane; PEP, phosphoenolpyruvate; ADP, adenosine diphosphate; ATP, ade-nosine triphosphate; R5P, ribose-5-phosphate; EDTA, ethylenediaminetetra-acetic acid.

(14). Cell-free homogenates were prepared by grinding 1 part fresh weight of leaf or root, respectively, with a mortar and pestle at 0° C in 5 parts of TRIS[2] (0.2 M, pH 8). The homogenate was strained through 2 layers of cheesecloth, centrifuged at 1000 X *g* for 10 minutes. An aliquot of the cell-free homogenate was added to the reaction mixtures and incubated 10 minutes at 37° C. The reactions were stopped and the excess $C^{14}O_2$ driven off by adding HC1 saturated with 2,4-dinitrophenylhydrazine (which fixed the keto acids as hydrazones) to the reaction mixture. The reaction mixtures were centrifuged to decant the protein; aliquots dried on glass planchets and counted for C^{14} activity.

With reactions using more than one plant homogenate, the orange leaf cell-free homogenates was prepared and procedures carried out as described.

RESULTS AND DISCUSSION

The amount of C^{14} in the differently charged fractions from the avocado roots and leaves supplied $C^{14}O_2$ in the dark are shown in Table 1. The greatest amount of C^{14} activity in both roots and leaves was in the anion fraction with relatively small amounts in the cation and non-charged fractions. The amount of C^{14} in the anion fraction of both roots and leaves was primarily in malic and citric acids (Table 2). The pattern was similar to other materials that fix CO_2 with PEP as a substrate (1, 2, 7, 9, 10), indicating the existence of the same enzymatic systems. Although avocado roots contained relatively the same quantities of malic and citric acids a larger amount of C^{14} was in malic acid. The total amount of malonic acid was small but the specific activity of C^{14} in this acid was greater than for any of the other acids measured in avocado roots. Of the C^{14} fixed into the keto acids, avocado leaves fixed more than roots, and in each case, oxaloacetic acid contained the highest C^{14} activity. Since previous studies had indicated no $C^{14}O_2$ fixation in systems with avocado leaf and root homogenates, the effect of avocado on orange leaf homogenates was studied. Data on the effect of avocado root homogenates on the inhibition of $C^{14}O_2$ fixation in the reaction systems from orange leaves are presented in Table 3. The PEP reaction with Mn was not affected as much as was the PEP reaction with Mg and the R5P system.

Table 1.—C^{14} activity of charged fractions[a] in avocado roots and leaves exposed to $C^{14}O_2$ in the dark.

Material	Fraction			
	Non-charged	Cation	Anion	Total
	cpm/g fresh weight			
Roots	70	350	4,400	4,820
Leaves	430	980	22,800	24,210

[a]Fractions were separated by ion exchange resins.

The effects of varied orange homogenate, varied PEP, and varied $HC^{14}O_3^-$ on the PEP carboxylase reaction system are given in Table 4. Double reciprocal graphs of these data indicated non-competitive inhibition for HCO_3^- and for PEP as substrates.

Table 2.—C^{14} activity and titratable organic acids of anion fraction of avocado roots and leaves.

Organic acid	Titratable acids	C^{14} activity	Specific activity
	Avocado roots		
	μe/g fresh weight	cpm/g fresh weight	cpm X 10^{-3}/me
Fumaric	0.73	negligible	0
Succinic	0.94	negligible	0
Malonic	0.10	40	417.0
Malic	3.96	930	234.0
Citric-isocitric	4.04	350	86.2
Total	9.77	1,320	135.0
	Avocado leaves		
Fumaric	3.36	120	347.0
Succinic	6.34	90	147.0
Malonic	5.40	1,200	224.0
Malic	4.48	2,680	598.0
Citric-isocitric	14.52	12,300	847.0
Total	34.10	16,390	480.0

Table 3.—Inhibition of $C^{14}O_2$ fixation in orange leaf homogenates by different concentrations of avocado root homogenates.

Reaction system	ml avocado			
	0.0	0.1	0.3	0.5
	cpm/.2 ml aliquot			
Endogenous	135	33	16	42
$HC^{14}O_3^-$ + PEP + Mg	1,230	449	96	8
$HC^{14}O_3^-$ + PEP + Mn	1,644	1,599	1,375	547
$HC^{14}O_3^-$ + R5P + ATP + Mg	1,122	677	81	22

Total volume was 1.4 ml with in μM 200 TRIS; 20 Mg; 2 R5P; 1 PEP; 2 Mn; 4 ATP; 5 $HC^{14}O_3^-$, and 0.2ml orange leaf homogenate.

To understand more about the nature of the inhibitor, a fraction separation was made of the crude cellular constituents. After the avocado root extract was centrifuged at 30,000 X g the precipitate was resuspended in 5 ml TRIS and used as the mitochondrial fraction. Five ml of supernatant was poured into each of 4 test tubes. One of the 5 ml fractions of supernatant was saved as the supernatant fraction. One 5 ml fraction was passed through a cation exchange resin without any washing and the resultant solution was used for the anion fraction. The cation fraction was obtained by passing another 5 ml fraction through an anion exchange resin. The remaining 5 ml portion was put through both cation and anion exchange resins without washing and the resultant liquid was used for the non-charged fraction. Each of the above fractions were run on the reaction PEP + Mg at different levels of avocado homogenates. The results in Table 5 indicated that the inhibitor may be in the non-charged fraction.

A series of studies were made to know if avocado preparations could be made that had active enzymes for CO_2 fixation, or could be prepared free of the inhibitor. A chelating agent in the grinding mixture or in the reaction mixture failed to overcome the inhibition. Also a reducing agent ($Na_2S_2O_4$), NH_4 OH infiltration, sucrose infiltration, cyanide, and albumin in grinding all failed to overcome the inhibition.

To determine if the inhibiting factor could be removed by sephadex the homogenate was passed through a column previously saturated with TRIS buffer of G-50 sephadex (Pharmacia, Uppsala, Sweden) to remove the low molecular weight substances in case the inhibitor was such a compound. The results (Table 6) indicated that sephadex did at least in part remove the inhibitor.

Polyethylene glycol (carbowax 4000) at 7% of buffer concentration was used in the grinding mixture since several workers (4, 8, 11, 12, 15) have shown that this will

inactivate tannins which inhibit at least some enzyme reactions or overcome other types of inhibition. The results (Table 7) indicated that a considerable amount of the inhibition was removed by the treatment and, hence, the inhibitor could be a tannin. This technique of overcoming tannin inhibition in woody plants may be very useful in studies of other woody plants.

Table 4.—Effect of varied PEP, HCO_3^-, orange, and avocado root preparations on $HC^{14}O_3^-$ fixation by orange leaf homogenates in the PEP carboxylase reaction.

Concentration or ml, substrate	ml, avocado root homogenate		
	0.0	0.1	0.3
	cpm/0.2 ml aliquot		
ml orange homogenate			
0.1	6,030	563	62
0.2	6,340	1,580	178
0.3	5,270	2,850	354
0.4	5,270	3,300	414
μM PEP			
0.5	3,590	2,180	323
1	5,060	2,550	492
2	7,460	4,200	720
5	7,940	5,500	1,300
μM $HC^{14}O_3^-$			
1	1,900	1,180	175
5	4,510	2,600	496
10	6,340	3,390	658
50	8,330	6,210	1,250

Reactants were the same concentrations as those in Table 3.

Table 5.—Inhibition by avocado root preparations of CO_2 fixation with the PEP reaction in orange leaf preparation.

Type of avocado preparation	Amount of avocado preparation, ml			
	0	.1	.3	.5
	cpm/0.2 ml aliquot			
1000 × *g* centrifugation homog.	3900	600	100	60
30,000 × *g* supernatant (A)	3900	600	100	70
Mitochondria in TRIS	3900	3200	3100	2500
(A) through cation exchange resin	3900	1000	110	60
(A) through anion exchange resin	3900	500	130	80
(A) through both resins	3900	1000	130	100
Minus orange preparation	148	328	62	37

Reactants otherwise were the same concentrations as those in Table 3.

EDTA increased the $C^{14}O_2$ fixation with R5P in preparations from avocado leaves. Even with the polyethylene glycol the inhibition of the avocado preparations on the orange leaf R5P system was not completely overcome. Addition of $FeSO_4$ caused even greater inhibition and combinations of $FeSO_4$ and EDTA indicated that an iron-tannin complex gave greater inhibition than tannin alone.

Cyanide has been used to stabilize the C^{14} oxaloacetate synthesized in the PEP reaction as used in this study (13). The use of this technique overcame some of the avocado inhibition on orange leaf preparations (Table 8). Either cyanide inactivated the inhibitor or it prevented the inhibitor from causing the product to be decarboxylated. In either case the effect of the inhibitor then may not be directly on the enzyme.

Table 6.—Effect of passing avocado through sephadex in overcoming its inhibitory effects.

ml avocado leaf preparation per reaction mixture	Reaction system					
	PEP + Mg		PEP + Mn		R5P + ATP + Mg	
	Without sephadex	With sephadex	Without sephadex	With sephadex	Without sephadex	With sephadex
	cpm/0.2 ml aliquot					
0	2800	3100	2090	2230	11,600	10,600
.1	112	2360	1310	1970	500	7,700
.3	38	690	220	1540	40	2,400
.5	30	230	70	1310	30	700

Reactants were the same concentrations as those in Table 3.

Table 7.—Effect of preparing avocado in polyethylene glycol in overcoming the inhibitory effect of avocado on $C^{14}O_2$ fixation in an orange preparation.

ml avocado prepared in polyethylene glycol	Assays with sweet orange leaf preparations		
	PEP + Mg	PEP + Mn	R5P + Mg + ATP
ml	cpm/0.2 ml aliquot avocado leaf as inhibitor		
0	2980	1500	9070
.1	2710	1490	5500
.3	2650	1440	4850
.5	2650	1110	4850
0.3 (no orange)	1200	360	1410
	avocado root as inhibitor		
.1	2600	1320	4500
.3	2390	1000	3200
.5	2030	870	2440
0.3 (no orange)	150	60	12

The reactants where appropriate were in μM/ml reaction mixture, 1 PEP, 10 Mg, 2 Mn, 2 R5P, 1 ATP, 5 $HC^{14}O_3^-$, 140 TRIS buffer of pH 8.0, and 0.2 ml orange leaf preparation.

The question that prompted this investigation was partially answered. Avocado roots appeared to have a very inactive or poorly active system for fixation of CO_2 with PEP as a substrate. Since the inhibition by avocado root preparations of the same system in orange leaves was overcome by polyethylene glycol, the data can be interpreted as meaning that the PEP carboxylase system was essentially absent or at least very low in the avocado root. The endogenous acids in roots were one-fourth those in leaves, *in vivo* $C^{14}O_2$ fixation was one-twelfth, and *in vitro* $C^{14}O_2$ fixation was one-ninth. A check of a reversal of the isocitric dehydrogenase system as a means of fixing CO_2 in avocado roots gave negative results. The method, therefore, with which CO_2 is fixed in avocado roots is as yet unknown.

Table 8.—Effect of cyanide in partially overcoming the effect of avocado root inhibition of $C^{14}O_2$ fixation in an orange leaf preparation.

ml avocado root/ml of reaction system	μM PEP/ml reaction mixture							
	½		1		2		5	
	−CN	+CN	−CN	+CN	−CN	+CN	−CN	+CN
	% inhibition							
.1	79	14	74	15	74	14	59	13
.3	97	75	97	73	96	74	95	62

Reaction mixture was 1.0 ml and contained in μM/ml where appropriate, 10 CN, 10 Mg, 5 $HC^{14}O_3^-$, 140 TRIS buffer, and 0.2 ml orange leaf preparation.

Since many active enzyme systems have been obtained from avocado fruit, portions taken 0.3 cm below the peel of 5- and 12-month-old Hass avocado fruits were used for

homogenate preparations (1 g fruit in 5 ml buffer) to test their ability to fix $C^{14}O_2$. The results in Table 9 indicate the presence of the PEP carboxylase system and also the complete sequence that synthesizes 3-phosphoglyceric acid from R5P and CO_2 (14). The latter reactions relate to photosynthesis. An important conclusion is that the fruit does not contain the inhibitor.

Table 9.—$C^{14}O_2$ fixation by avocado fruit homogenates.

Reaction system[a]	CPM/0.2 ml aliquot
Mg + $HC^{14}O_3^-$	60
PEP + Mg + $HC^{14}O_3^-$	4,000
PEP + Mn + $HC^{14}O_3^-$	2,070
PEP + Mn + $HC^{14}O_3^-$ + ADP	2,380
R5P + Mg + $HC^{14}O_3^-$ + ATP	1,300

[a]The reactants in μmoles per 1 ml reaction mixture were 1 PEP, 5 Mg, 2 Mn, 2 ADP, and 5 $HC^{14}O_3^-$. The procedures otherwise were like those for leaves.

SUMMARY

Avocado roots in an atmosphere containing $C^{14}O_2$ fixed CO_2 into organic acids to a slight extent, and the labeling pattern of products was similar to that of other materials that fix CO_2 into organic acids.

Without special techniques, homogenates of avocado roots and leaves not only failed to fix CO_2 even with partial purification of systems but greatly inhibited CO_2 fixation in preparations from orange leaves, a species having high ability to fix CO_2 in the dark.

The nature of the inhibition was non-competitive in regard to PEP arid HCO^- as substrates.

The inhibition caused by preparations from avocado roots was very marked with the PEP + Mg and the R5P + ATP + Mg reaction systems, but much less so with the PEP + Mn reaction system.

Fractionation of the avocado preparations indicated that the inhibiting substance was dialyzable and was possibly present in the non-charge fraction. It was not associated with the mitochondria nor was it hydronium ion.

The inhibitor of the PEP system was largely removed by grinding leaves or roots with polyethylene glycol and was partially removed by sephadex or by the addition of cyanide. Polyethylene glycol only partially removed the inhibitor of the R5P system. When polyethylene glycol was used it was possible to demonstrate the PEP and R5P systems in avocado leaves but the PEP system was very low in roots. This is significant since the effect of the inhibitor was completely overcome. The inhibitor was probably a tannin. With the R5P system EDTA resulted in greatly increased CO_2 fixation in homogenates prepared with polyethylene glycol. An iron-tannin complex was more inhibitory than tannin alone.

Avocado fruit preparations did not contain the inhibitor and fixed $C^{14}O_2$ with PEP and also with R5P as substrates.

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