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Some Aspects of Carnitine Metabolism in Avocado (*Persea americana*)

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The fact that palmitoyl-L-carnitine is oxidized by avocado mitochondria at a rate comparable with that of succinate oxidation suggests that there are at least two systems for β-oxidation in higher plants. The carnitine-associated system is located in a mitochondrial fraction, whereas the glyoxylate-cycle-associated system is located in the glyoxysomes.

In animals, carnitine is widely distributed (Fraenkel, 1953, 1954) and is important in the β-oxidation of fatty acids. The addition of carnitine generally increases the rate of fatty acid oxidation in animal systems, and the explanation widely accepted is that acylcarnitine derivatives, but not acyl-CoA, have access to the β-oxidation system of intact mitochondria (Fritz, 1963; see the review by Greville & Tubbs, 1968).

One of the aims of this laboratory has been to examine the possible role of carnitine in higher plants. Although β-oxidation has been studied in plants (Stumpf & Barber, 1956; Cooper & Beevers, 1969*b*; Laties & Hoelle, 1967), there appear to be no reports on the metabolism of carnitine in plants. Although Fraenkel (1953) found that only two out of six plants chosen contained carnitine, Panter & Mudd (1969) reported carnitine in several plant species, and of these avocado was the richest source.

Although it seemed likely from animal work that a carnitine-associated oxidation of fatty acids would be localized in the mitochondria, attention has been turned to glyoxysomes as the main, if not sole, location of β-oxidation in plant seedlings (Cooper & Beevers, 1969*b*; Hutton & Stumpf, 1969). Besides measuring the effect of carnitine on fatty acid oxidation by slices of avocado mesocarp, we have incubated avocado mitochondria with various acylcarnitine derivatives and measured the rate of oxidation polarographically. If the mitochondria contained no appropriate carnitine acyltransferase or acylcarnitine hydrolase, the plant would presumably have no other mechanism to utilize the acylcarnitine derivatives, and no oxidation would be observed.

Materials and methods

Materials. The following were bought from the Sigma Chemical Co. (St. Louis, Mo., U.S.A.): Tris, NAD^+, ADP, ATP, palmitoyl chloride, palmitoyl-CoA and polyvinylpyrrolidone. Calbiochem (Los Angeles, Calif., U.S.A.) supplied CoA, $NADP^+$, NADH, NADPH, palmitic acid and bovine serum albumin (fatty acid-poor). The L-carnitine and D-carnitine were obtained from Mann Research Laboratories (New York, N.Y., U.S.A.). The [1-^{14}C]hexanoic acid, [1-^{14}C]octanoic acid, [1-^{14}C]decanoic acid, [1-^{14}C]palmitic acid and [3-^{14}C]butyric acid were obtained from New England Nuclear Corp. (Boston, Mass., U.S.A.). The [1-^{14}C]stearic acid, DL-[*Me*-^{14}C]carnitine and [1-^{14}C]acetyl-CoA were supplied by the International Chemical and Nuclear Corp. (Irvine, Calif., U.S.A.).

Acetylcarnitine was prepared as described by Bremer (1962) and palmitoylcarnitine as described by Bremer (1968). The acylcarnitine derivatives recrystallized as white powders and showed only one spot on t.l.c. with chloroform–methanol–water (4:4:1, by vol.) as solvent and 1% I_2 in methanol as detector.

Plant material. Avocados (*Persea americana* var. Fuerte or Hass) were either harvested from university orchards or purchased at local markets. For tissue slices, cylinders were cut from avocado mesocarp with a 9mm-diam. cork-border, and discs approx. 1mm thick cut with a razor blade. These were used immediately.

Preparation of avocado mitochondria. Avocado mesocarp was grated with an Oster juice extractor (John Oster Manufacturing Co., Milwaukee, Wis., U.S.A.) kept at 0–4°C. During the grating of the tissue, buffer (1:1, v/w) consisting of 50mM-Tris–HCl buffer, pH 7.5, 0.5M-sucrose, 2% (w/v) polyvinylpyrrolidone, 1mM-dithiothreitol, 0.02% fatty acid-poor bovine serum albumin and 0.5mM-EDTA, pH 7.5, was admitted to the machine with a separating funnel. After centrifuging of the emergent liquid at 1000*g* for 10min in the SS34 rotor of a Sorvall RC2-B centrifuge, the pellet was discarded and the supernatant centrifuged at 10000*g* for 30min. The resulting pellet consisted of a light-green layer over a small dark pellet and variable amounts of starch. Only the light-green portion was used.

Counting of radioactivity. A Nuclear–Chicago 720 Series liquid-scintillation counter was used for all radioactive samples. By measuring the amount of

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quenching by the channels-ratio method, the efficiency of counting was shown to lie in the range 40–53%. For the counting of the radioactivity of papers and toluene-soluble samples, the scintillation fluid consisted of 0.5% 2,5-diphenyloxazole (PPO) and 0.03% 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP) in toluene. For water-soluble radioactive samples, Aquasol (New England Nuclear Corp.) was used as scintillation fluid.

Collection of $^{14}CO_2$. For the measurement of $^{14}CO_2$, Erlenmeyer flasks of 30ml capacity were fitted with a centre well approx. 2cm deep and 6mm internal diameter. The tissue slices were placed in the main compartment of the flasks, and a 2cm×2cm square of Whatman no. 4 filter paper saturated with 0.1ml of freshly made 10% (w/v) KOH was folded and placed into the centre well. The labelled substrate, cofactors etc. were then added to the main compartment and the top was sealed with a serum cap. The flasks were shaken at 80 strokes/min in a Dubnoff incubator at 30°C for the required time, and 0.2ml of 5M-H_2SO_4 was injected through the serum cap into the main compartment to displace the $^{14}CO_2$. Incubation with shaking was then continued for a further hour, after which the papers were carefully removed from the flasks and either their radioactivities were counted wet (Buhler, 1962) or they were dried in air before being placed in scintillation fluid.

For the separation of tissue slices into lipid and aqueous components the slices were rapidly rinsed in 500ml of water and then extracted by the method of Bligh & Dyer (1959), a Potter–Elvehjem homogenizer being used for grinding before a shaking with chloroform–methanol (1:2, v/v).

Oxygen electrode. For the O_2-uptake experiments, a Clark-type electrode was used at 0.8V. The chamber was maintained at 30°C, and contained 6.0ml when the electrode and injection cap were in place. It was estimated that there were 2.76μg-atoms of oxygen in the water-filled chamber (*Handbook of Chemistry and Physics*).

Protein assay. Protein was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard.

Results and discussion

The effect of L-carnitine (the naturally occurring isomer) and D-carnitine on the oxidation of a number of fatty acids of various chain lengths by avocado slices is shown in Table 1. The values for each acid in Table 1 represent a separate day's experiment and

Table 1. *Effects of L-carnitine and D-carnitine on the oxidation of fatty acids of various chain lengths by avocado slices*

Each flask contained mannitol (600μmol), dithiothreitol (0.4μmol), Tris–HCl buffer, pH 8.0 (100μmol), 100μmol of labelled acid in aqueous suspension and 40μmol of carnitine, where indicated, in a total of 2.0ml. Three slices were added per flask and incubations were at 30°C for 5h with shaking.

Fatty acid	Radioactivity added per flask (d.p.m.)	Carnitine added	Radioactivity as $^{14}CO_2$ (d.p.m.)	Average relative oxidation (%)
[3-^{14}C]Butyric acid	247000	None	3360± 590 (6)	100
	247000	L-Carnitine	7840±1070 (6)	234
	247000	D-Carnitine	3190± 360 (6)	95
[1-^{14}C]Hexanoic acid	167000	None	29100± 770 (6)	100
	167000	L-Carnitine	39400±1360 (6)	136
	167000	D-Carnitine	23600±1170 (6)	81
[1-^{14}C]Octanoic acid	189000	None	21800±1410 (6)	100
	189000	L-Carnitine	17000±2230 (6)	78
	189000	D-Carnitine	15100±1770 (6)	69
[1-^{14}C]Decanoic acid	276000	None	14300±4250 (6)	100
	276000	L-Carnitine	28600±1490 (6)	200
	276000	D-Carnitine	6470± 960 (6)	45
[1-^{14}C]Palmitic acid	238000	None	4010± 130 (6)	100
	238000	L-Carnitine	4950± 220 (6)	124
	238000	D-Carnitine	4380± 120 (6)	109
[1-^{14}C]Stearic acid	230000	None	10900 (1)	100
	230000	L-Carnitine	13700± 570 (6)	126
	230000	D-Carnitine	10300± 760 (5)	95

a fresh avocado, and so the oxidation rates of the different fatty acids are not strictly comparable. The addition of L-carnitine stimulated oxidation of all acids except octanoic acid, and D-carnitine inhibited oxidation in all but octanoic acid and palmitic acid. When the incubation period was completed and the KOH-soaked papers were removed, some of the slices were rinsed, ground, separated into lipid and aqueous components, and placed into vials for the counting of radioactivity. The sum of radioactivity (d.p.m.) in the lipid fraction plus that in the aqueous fraction plus that as $^{14}CO_2$ gave the proportion of the fatty acid added that diffused into the slices: butyric acid, 65%; hexanoic acid, 25%; octanoic acid, 24%; stearic acid, 46%. The results also gave the proportion of absorbed labelled fatty acid that was oxidized: butyric acid, 3%; hexanoic acid, 87%; octanoic acid, 54%; stearic acid, 21%.

The changes in O_2 uptake on adding palmitoyl-L-carnitine or other substrates to avocado mitochondria are indicated in Table 2. In this and similar experiments, a run with succinate for comparison was standard procedure. Also, the substrate of the first run of the day was used in the last run (about 2h later) to check that the mitochondria were not progressively inactivated. The most significant result from Table 2 is that palmitoyl-L-carnitine more than doubled the rate of O_2 uptake, whereas palmitoyl-D-carnitine had no effect on the rate. Palmitic acid was not oxidized unless ATP, CoA and $MgCl_2$ were added; although one or more of these was important for palmitic acid oxidation, palmitoyl-L-carnitine by contrast was oxidized more slowly in the presence of these cofactors. Palmitoyl-CoA was oxidized at about half the rate of palmitoyl-L-carnitine. The O_2 uptake due to endogenous substrates was considerable but fairly constant. It should be noted that the oxidation rate of palmitoyl-L-carnitine was greater than three-quarters of that of succinate at the same concentration.

Several cell-free systems of fatty acid oxidation in higher plants have been described, including α-oxidation (Martin & Stumpf, 1959) and β-oxidation (Stumpf & Barber, 1956) by peanut cotyledons, α-oxidation by pea and castor-bean leaves (Hitchcock & James, 1966) and β-oxidation by germinating castor bean (Cooper & Beevers, 1969*b*). In our experiments with avocado mitochondria, the cofactor requirements for palmitate oxidation have indicated that the β-oxidation mechanism is functioning.

Preliminary evidence that carnitine is involved in fatty acid oxidation by avocado mitochondria has been derived from the effects of the isomers of carnitine on oxidation by tissue slices. Octanoic acid was the only substrate whose oxidation was not stimulated by L-carnitine as compared with the value with no carnitine added; Fritz (1963) reported almost negligible carnitine stimulation with this acid in rat heart homogenates. The oxidation of short-chain fatty acids by avocado slices was stimulated by carnitine, in contrast with animal slices (Fritz & Kaplan, 1960). The possibility exists that L-carnitine stimulated the transport of fatty acid across the cell membrane rather than the mitochondrial membrane. In the incubation of hexanoic acid with slices, the sum of radioactivities (d.p.m.) as $^{14}CO_2$ plus lipid plus aqueous fraction was greater by 53% in the presence of L-carnitine compared with D-carnitine. The

Table 2. *Oxidation rates of various acyl compounds by avocado mitochondria*

At the start of each run with the oxygen electrode, the chamber contained 5.2mg of mitochondrial protein, sucrose (1.5mmol), potassium phosphate buffer, pH7.2 (60μmol), Tris–HCl buffer, pH7.2 (60μmol), EDTA, pH7.2, (3μmol), 4.5mg of bovine serum albumin, L-malate (0.1μmol), ADP (0.1μmol), dithiothreitol (6μmol), and, where indicated, $MgCl_2$ (6μmol), CoA (1.3μmol) and ATP (0.6μmol), in a total of 6.0ml at 30°C. When the recorder was showing a steady rate of O_2 uptake due to endogenous substrates, 1.0μmol of acyl substrate in 0.05ml of water was added, and the increase in respiration rate measured.

Substrate	Rate of O_2 uptake (nmol/min per mg of protein)	
	Before added substrate	After added substrate
Palmitoyl-L-carnitine	3.6	9.0
Palmitoyl-D-carnitine	3.8	3.8
Palmitoyl-CoA	2.6	5.3
Palmitate	3.2	3.2
Palmitate plus ATP, CoA, $MgCl_2$	3.7	10.1
Palmitoyl-L-carnitine plus ATP, CoA, $MgCl_2$	3.3	4.6
Acetyl-L-carnitine	3.0	3.0
Succinate	3.4	10.3

results for butyric acid and stearic acid, however, show that for these acids L-carnitine had no effect on transport greater than D-carnitine.

Further evidence of carnitine participation in plant metabolism was obtained from the studies of oxidation of acylcarnitine derivatives by isolated avocado mitochondria. The oxidation of palmitoyl-L-carnitine in this system means that an enzyme in the mitochondria was either converting palmitoylcarnitine into palmitoyl-CoA (carnitine palmitoyltransferase activity) or forming free palmitic acid plus carnitine (palmitoylcarnitine hydrolase activity). We consider that the second alternative is very unlikely, since palmitoyl-L-carnitine was oxidized faster than palmitic acid and since addition of ATP, CoA and $MgCl_2$ did not increase palmitoylcarnitine oxidation, though having a large stimulatory effect on palmitate oxidation. Since palmitoyl-D-carnitine was not oxidized by mitochondria, and D-carnitine failed to stimulate fatty acid oxidation by slices, the substrate stereospecificity of the carnitine palmitoyltransferase in the avocado is presumably similar to that of the animal enzyme (Fritz & Schultz, 1965).

The subcellular location of the enzymes of β-oxidation in germinating fatty seeds has been reported (Cooper & Beevers, 1969*b*; Hutton & Stumpf, 1969). For castor bean it has been found that β-oxidation takes place entirely within the glyoxysome fraction, and is not associated with mitochondria (Cooper & Beevers, 1969*b*). For peanut, some β-oxidative activity was found in the mitochondria, but here again the glyoxysomes were more active (Hutton & Stumpf, 1969).

Results with avocado indicate that there is a system of β-oxidation quite distinct from that of germinating castor bean. Carnitine is not a likely factor in β-oxidation in castor bean because no carnitine can be detected in the tissue (Panter & Mudd, 1969), and acylcarnitine derivatives are not oxidized (Cooper & Beevers, 1969*b*). The second distinction between β-oxidation in germinating castor bean and that in avocado is that the activity is probably situated in avocado mitochondria rather than in a glyoxysome fraction, since avocado contains neither observable glyoxysomes nor malate synthetase (Cooper & Beevers, 1969*a*). Since peanuts contain carnitine (Panter & Mudd, 1969), β-oxidation by peanut mitochondria may be similar to that of avocado.

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