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Is Acetylcarnitine a Substrate for Fatty Acid Synthesis in Plants?[1]

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Long-chain fatty acid synthesis from [1-^{14}C]acetylcarnitine by chloroplasts isolated from spinach (*Spinacia oleracea*), pea (*Pisum sativum*), amaranthus (*Amaranthus lividus*), or maize (*Zea mays*) occurred at less than 2% of the rate of fatty acid synthesis from [1-^{14}C]acetate irrespective of the maturity of the leaves or whether the plastids were purified using sucrose or Percoll medium. [1-^{14}C]-Acetylcarnitine was not significantly utilized by highly active chloroplasts rapidly prepared from pea and spinach using methods not involving density gradient centrifugation. [1-^{14}C]Acetylcarnitine was recovered quantitatively from chloroplast incubations following 10 min in the light. Unlabeled acetyl-L-carnitine (0.4 mM) did not compete with [1-^{14}C]acetate (0.2 mM) as a substrate for fatty acid synthesis by any of the more than 70 chloroplast preparations tested in this study. Carnitine acetyltransferase activity was not detected in any chloroplast preparation and was present in whole leaf homogenates at about 0.1% of the level of acetyl-coenzyme A synthetase activity. When supplied to detached pea shoots and detached spinach, amaranthus, and maize leaves via the transpiration stream, 1 to 4% of the [1-^{14}C]acetylcarnitine and 47 to 57% of the [1-^{14}C]acetate taken up was incorporated into lipids. Most (78–82%) of the [1-^{14}C]acetylcarnitine taken up was recovered intact. It is concluded that acetylcarnitine is not a major precursor for fatty acid synthesis in plants.

The origin of the acetyl-CoA used for the synthesis of fatty acids in chloroplasts has been the subject of much speculation (Murphy and Walker, 1982; Givan, 1983; Treede et al., 1986). Because exogenous acetyl-CoA is a poor substrate for fatty acid synthesis by intact plastids (Weaire and Kekwick, 1975; Roughan et al., 1979a), it probably does not cross the chloroplast envelope and is presumed to be synthesized in situ either from precursors derived directly from photosynthetic carbon fixation within the organelle or from precursors imported from the cytosol. Although acetate seems to be the precursor incorporated most efficiently into fatty acids of isolated chloroplasts (Roughan et al., 1979b; Murphy and Walker, 1982), and chloroplasts contain ACS activity that is more than sufficient to account for rates of fatty acid synthesis in vivo, a role for pyruvate, formed from 3-phosphoglycerate, and pyruvate dehydrogenase in generating acetyl-CoA within chloroplasts has appealed to many workers (e.g. Leidvogel, 1985).

More recently, it has been proposed (Masterson et al., 1990a, 1990b) that cytosolic acetylcarnitine is, in fact, the true substrate for fatty acid synthesis in vivo, because rates of fatty acid synthesis in isolated pea chloroplasts were 5-fold greater from acetylcarnitine than from acetate. Carnitine facilitates the movement of acyl groups across mitochondrial membranes (Pande and Parvin, 1980) and has rarely been implicated in chloroplast metabolism (Thomas et al., 1983; McLaren et al., 1985). However, if chloroplasts contain CAT activity, acetylcarnitine could be an alternative precursor of acetyl-CoA for chloroplast fatty acid synthesis. Because the maximum rates of fatty acid synthesis reported by Masterson et al. (1990a) were 4- to 5-fold greater than the best rates previously reported for isolated chloroplasts, the proposed role for acetylcarnitine warranted further study.

We demonstrate here, using a variety of established methods for chloroplast isolation, that the rate of acetylcarnitine utilization for fatty acid synthesis by isolated chloroplasts is less than 2% of the rate of acetate incorporation. Furthermore, CAT, which is required for the synthesis of acetyl-CoA from acetylcarnitine, appears not to be present in leaves in sufficient activity to account for rates of fatty acid synthesis in vivo. This enzyme deficiency probably also explains the very slow metabolism of acetylcarnitine compared to acetate by whole leaves and leaf homogenates.

MATERIALS AND METHODS

Chloroplasts were isolated from expanding leaves, and fatty acid synthesis was assayed as described by Roughan (1987), unless otherwise stated. Purification of the chloroplasts was normally by centrifugation in sorbitol/Percoll media, but Suc gradients (Douce and Joyard, 1982; Masterson et al., 1990a) were also used for comparison. Spinach and pea chloroplasts were in some cases pelleted directly from low ionic strength buffers (Nakatani and Barber, 1977; Cer-

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Abbreviations: ACS, acetyl-CoA synthetase; CAT, carnitine acetyltransferase; C/M, chloroform/methanol; C/M/W, chloroform/methanol/water.

novic and Plesnicar, 1984) without the use of density media. Both sorbitol and betaine (Larkum and Wyn Jones, 1979) were used as osmotica in separate experiments. To determine ratios of unsaturated to saturated fatty acids synthesized by isolated chloroplasts, total fatty acids recovered from saponified reactions were treated with ethereal diazomethane, and the resulting fatty acid methyl esters were separated on thin layers of 5% (w/w) $AgNO_3$ in silica gel G developed with petroleum ether:ether (9:1, v/v). Following detection using dichlorofluorescein, saturated, monoenoic, and dienoic fractions were transferred with the adsorbent to a detergent-based scintillation cocktail containing 10% (v/v) water, and the amount of radioactivity in each fraction was determined.

Homogenates were prepared by grinding spinach leaf laminae or pea shoots in a mortar with 2 to 4 volumes of 50 mM Hepes/K (pH 7.8), 5 mM $MgCl_2$, 5 mM EDTA, and 10 mM mercaptoethanol and filtering the slurry through two layers of Miracloth. One milliliter of the filtrate was immediately desalted on a 5-mL column of Sephadex G-25 equilibrated with 25 mM Hepes/K (pH 7.8), 2 mM $MgCl_2$, 1 mM EDTA, and 2 mM DTT. Concentrations of protein (Bradford, 1976) and Chl (Arnon, 1949) were determined both before and after the Sephadex step.

For in vivo labeling experiments, plants grown in a controlled environment were transferred to near darkness for 60 min before leaves were detached, and the cut ends or petioles were immediately transferred to water. The detached leaves were kept in near darkness for an additional 30 min before their transfer to full light (350–400 $\mu E\ s^{-1}\ m^{-2}$), and water uptake was monitored on individual leaves. Only those leaves transpiring rapidly were subsequently transferred to solutions containing the radioactive precursor.

ACS activity was measured in the direction of acetyl-CoA formation using a modification (G. Roughan and J. Ohlrogge, unpublished data) of the filter paper disc assay (Roughan et al., 1979a). CAT activity was also measured in the direction of acetyl-CoA formation by incubating 10 to 20 μL of chloroplasts or leaf extracts with 25 mM Hepes/K (pH 7.8), 0.2 mM CoA, and 0.4 mM [1-^{14}C]acetylcarnitine (10 Ci mol^{-1}) in a 50-μL final volume. TLC was performed on 0.25-mm layers of Sigmacell (type 20) using C/M/W (12:6:1, v/v/v) for separating labeled acetylcarnitine alone and butan-1-ol:acetic acid:water (5:2:4, v/v/v) for separating both labeled acetyl-CoA and labeled acetylcarnitine from reaction mixtures or leaf extracts. Separated compounds were detected by autoradiography and quantified by scintillation counting. L-Carnitine (3-hydroxy-4-trimethylamino-butyric acid, inner salt), CoA, ATP, NADH, NADPH, and Percoll were from Sigma (St. Louis, MO). Acetyl-L-carnitine was prepared according to the method of Fraenkel and Friedman (1975) and was also supplied by Serva (Heidelberg, Germany) and by Sigmatel (Palezia, Italy). [1-^{14}C]Acetylcarnitine (10 Ci mol^{-1}) was prepared from [1-^{14}C]acetate and L-carnitine using ACS and CAT and was purified by TLC (Roughan and Browse, 1992). [1-^{14}C]Acetate (58 Ci mol^{-1}) was from Amersham.

Pea (*Pisum sativum*) seed was obtained from Burpee Seeds ("Little Marvel"; Warminster, PA) and from Yates New Zealand Ltd. ("Greenfeast"; Auckland, New Zealand). Spinach (*Spinacia oleracea*) seed was from Sunseeds Genetics Ltd. (hybrid No. 7R) and from Watkins Seeds Ltd. ("Tyee Hybrid"; New Plymouth, New Zealand). Maize (*Zea mays*) seed (hybrid "P3551" and a hybrid sweetcorn "Miracle") were gifts from Dr. Ravi Bansal (Department of Scientific and Industrial Research, Auckland, New Zealand), and amaranthus (*Amaranthus lividus*) seed was gathered from the wild.

RESULTS

Chloroplast Isolation

Initial testing of the claim that acetylcarnitine was a superior substrate for fatty acid synthesis in isolated chloroplasts was performed with plastids isolated from expanding leaves of spinach and pea using methods that had been successful in retaining high rates of fatty acid synthesis from acetate. This involved following the recommendations of Cernovic and Plesnicar (1984) but including a cushion of 40% (v/v) Percoll in low ionic strength buffer in the initial centrifugation (Mills and Joy, 1980) to enhance further the proportion of intact plastids in the final preparation. In some instances intact chloroplasts were purified from "crude" pellets using linear gradients of Percoll in low ionic strength buffer and brief centrifugation (Heinz and Roughan, 1983).

Chloroplasts having high rates of photosynthesis and of fatty acid synthesis from acetate were also prepared from spinach and pea by direct sedimentation from low ionic strength buffers (Nakatani and Barber, 1977; Cernovic and Plesnicar, 1984), i.e. without using Percoll cushions. When acetylcarnitine was found to be inactive in such preparations (Table I), chloroplasts were isolated from spinach and pea using methods identical with or similar to those used by Masterson et al. (1990a; C. Wood, personal communication).

Table I. *Typical rates of fatty acid synthesis from acetate and acetylcarnitine by spinach and pea chloroplasts prepared by direct sedimentation from low ionic strength buffers*

Chloroplasts equivalent to 50 μg of Chl were incubated in 0.25 mL of the basal medium (Roughan, 1987) supplemented with 2 mM MgATP, 0.25 mM CoA, and 0.2 mM of the labeled substrate indicated. The effect of unlabeled acetylcarnitine on acetate incorporation into fatty acids was also tested. Chloroplasts were prepared using low ionic strength media (Cernovic and Plesnicar, 1984) containing either sorbitol or betaine as osmoticum.

Species	Osmoticum	Labeled Substrate	Acetylcarnitine	Fatty Acid Synthesis
			mM	*nmol of acetyl incorporated h^{-1} mg^{-1} of Chl*
Pea	Sorbitol	Acetate	0	2015
		Acetate	0.4	1980
		Acetylcarnitine	0	12
	Betaine	Acetate	0	1750
		Acetate	0.4	1755
		Acetylcarnitine	0	14
Spinach	Sorbitol	Acetate	0	1340
		Acetate	0.4	1330
		Acetylcarnitine	0	13
	Betaine	Acetate	0	1390
		Acetate	0.4	1330
		Acetylcarnitine	0	13

Spinach leaves and pea shoots were homogenized in 0.33 M sorbitol, and the crude chloroplast pellet was purified on a discontinuous gradient of increasing Suc concentration (Miflin and Beevers, 1974). However, the procedure was tedious, and separations of intact from broken chloroplasts were inferior to those achieved with Percoll gradients. A much more rapid and convenient alternative for purifying chloroplasts by centrifugation on Suc gradients was a scaled down version of the method of Douce and Joyard (1982). Suc was not used for the isolation of active chloroplasts from amaranthus or maize. Betaine has been used as an osmoticum in the isolation of chloroplasts from spinach (Larkum and Wyn Jones, 1979; Bertrams et al., 1981) and was shown to have some advantages over sorbitol (Larkum and Wyn Jones, 1979). It was also found to be a suitable alternative to sorbitol in the isolation of active chloroplasts from pea (Table I), amaranthus, and maize (results not shown).

Fatty Acid Synthesis from Acetate and Acetylcarnitine

Chloroplasts isolated using sorbitol/Percoll-based or betaine/Percoll-based media invariably exhibited higher rates of fatty acid synthesis from acetate than did those prepared using Suc (Table II). This was consistent with previous experience showing that, in contrast to those purified on Percoll gradients, chloroplasts purified on Suc gradients completely lost the ability to carry out bicarbonate-dependent oxygen evolution (Douce and Joyard, 1982; Leegood and Walker, 1983). Spinach and pea chloroplasts with high rates of fatty acid synthesis from acetate were also prepared by direct sedimentation from low ionic strength buffers (Cernovic and Plesnicar, 1984) without recourse to density gradients or cushions (Table I). However, irrespective of whether chloroplasts were from direct sedimentation or purified using Percoll or Suc density media, acetylcarnitine at up to 0.4 mM had no effect on the incorporation of 0.2 mM acetate into fatty acids (Table I). A concerted effort to demonstrate acetylcarnitine inhibition of fatty acid synthesis from acetate by spinach and pea chloroplasts, using a variety of isolation methods and incubation techniques, including duplication of the procedures used by Masterson et al. (1990a), was unsuccessful. Nor did acetylcarnitine inhibit acetate incorporation into the fatty acids of amaranthus and maize chloroplasts.

It is not surprising, therefore, that label from [1-^{14}C]-acetylcarnitine was not significantly incorporated into fatty acids of chloroplasts isolated from spinach, pea, amaranthus, or maize (Table II). The labeled acetylcarnitine was recovered quantitatively using cellulose TLC (Roughan and Browse, 1992) from chloroplast reactions after a 10-min incubation in the light. Including ATP, CoA, Triton X-100, and pyridine nucleotides in the assay medium did not stimulate the incorporation of [1-^{14}C]acetylcarnitine into fatty acids. On the other hand, [1-^{14}C]acetate released upon alkaline hydrolysis of the [1-^{14}C]acetylcarnitine preparations was readily incorporated into chloroplast fatty acids (results not shown).

Although the [1-^{14}C]acetylcarnitine used here was prepared enzymically and was fully active in the CAT assay with the commercial enzyme (Roughan and Browse, 1992), it seemed possible that some material copurifying with the putative substrate interfered with its utilization in the fatty acid synthesis assay. Indeed, earlier preparations that had been purified by paper chromatography inhibited fatty acid synthesis from acetate in both spinach and pea chloroplasts, although later preparations purified by TLC did not. Therefore, the suitability of acetylcarnitine for supporting chloroplast fatty acid synthesis was tested under conditions in which its purification was not required.

Reaction mixtures were preincubated with commercial ACS and CAT (desalted) to convert acetate to acetyl-CoA and acetylcarnitine before adding chloroplasts and measuring fatty acid synthesis (Table III). Immediately before the chloroplasts were added, 2-μL aliquots of the reactions were removed for analysis of the labeled products by TLC. In the absence of carnitine, virtually all of the acetate was converted by the ACS to acetyl-CoA, which was not subsequently utilized for fatty acid synthesis, whereas in the presence of carnitine and enzymes, 66% of the acetyl-CoA was converted to acetylcarnitine, which, however, was not utilized for fatty acid synthesis either (Table III). This lack of acetylcarnitine utilization cannot be attributed to the presence of an approximately equimolar concentration of L-carnitine in the reaction. If we assume that both compounds are taken up into chloroplasts equally, then carnitine may inhibit but cannot completely prevent acetyl transfer to CoA. In this instance, the equilibrium of the CAT reaction would more likely be controlled by the CoA:acetyl-CoA ratio. Furthermore, carnitine at concentrations up to 1 mM did not inhibit fatty acid synthesis from 0.2 mM acetate by chloroplasts from spinach, pea, maize, or amaranthus (data not shown).

Table II. *Fatty acid synthesis from acetate and acetylcarnitine by chloroplasts isolated from spinach leaves and pea shoots using sorbitol/Percoll or Suc media and from maize and amaranthus leaves using sorbitol/Percoll media*

Incubations contained 2 mM MgATP (pea and spinach only), 0.25 mM CoA, substrate, and chloroplasts (equivalent to 50 μg of Chl) in the basal medium buffered to pH 8.0 with Hepes/Na. Reactions (0.25 mL) were illuminated for 10 min. [1-^{14}C]Acetate (0.2 mM) was 8 Ci mol^{-1} and [1-^{14}C]acetylcarnitine (0.2 mM) was 10 Ci mol^{-1}. Added pyridine nucleotides had no effect on rates of fatty acid synthesis from acetylcarnitine. The range of values shown represents results from separate chloroplast isolations: 21 from spinach, 40 from pea, 10 from maize, and 15 from amaranthus. There was no significant inhibition of fatty acid synthesis from acetate by unlabeled acetylcarnitine (0.4 mM) in any experiment. FAS, Fatty acid synthesis.

Plant	Percoll	Suc
	nmol of acetyl incorporated h^{-1} mg^{-1} of Chl	
FAS from acetate		
Spinach	1000–2500	100–400
Pea	900–1500	100–500
Maize	670–770	n.d.[a]
Amaranthus	850–1200	n.d.
FAS from acetylcarnitine		
Spinach	4–12	5–11
Pea	2–4	3–6
Maize	4–5	n.d.
Amaranthus	5–8	n.d.

[a] n.d., Not determined.

Table III. *Fatty acid synthesis by spinach chloroplasts from acetate and from acetyl-CoA and acetylcarnitine generated enzymically in preincubated reaction mixtures*

The preincubation reactions (200 μL), containing 0.33 M sorbitol, 2.5 mM MgATP, 0.31 mM CoA, and 0.19 mM [1-^{14}C]acetate (1 μCi), were supplemented with 0.25 mM L-carnitine and desalted ACS and CAT (enzymes) as indicated. After 45 min at 25°C, 2 μL was removed for chromatographic identification (see "Materials and Methods") of the labeled products, and 50 μL of chloroplasts were added to the remaining solution. When the chloroplasts were added, the "control" and "+ carnitine" reactions contained 150 μM acetate, the "+ enzymes" reaction contained <1 μM acetate and 148 μM acetyl-CoA, and the "+ enzymes and carnitine" reaction contained 45 μM acetyl-CoA and 105 μM acetylcarnitine (and 95 μM carnitine). The chloroplasts were then incubated with these labeled substrates in the light for 10 min. Acetate incorporation was not affected by 0.2 mM AMP or PPi or by the enzymes alone in the absence of added ATP. The chloroplasts had been isolated by sedimenting through 40% Percoll in low ionic strength buffer, and the osmoticum was 0.33 M betaine.

Treatment	Fatty Acid Synthesis
	nmol of acetate incorporated h^{-1} mg^{-1} of Chl
Control	1450
+Carnitine	1440
+Enzymes	5
+Enzymes and carnitine	3

Because it seemed possible that acetylcarnitine could not penetrate the envelopes of our chloroplast preparations, intact chloroplasts (50 μL) were added to reaction mixtures (200 μL) containing CoA, ATP, NADH, and NADPH but no osmoticum. In this hypotonic medium (0.06 M sorbitol) chloroplasts swell and the inner membrane of the envelope either ruptures or becomes completely permeable to solutes, yet rates of fatty acid synthesis from acetate were 95 to 100% (n = 3) and 24 to 53% (n = 4) of those in isotonic media for spinach and pea chloroplasts, respectively. Even under these conditions, however, there was no inhibition of fatty acid synthesis from [1-^{14}C]acetate by acetylcarnitine and no fatty acid synthesis from [1-^{14}C]acetylcarnitine. Added pyridine nucleotides had no effect on fatty acid synthesis from either substrate in hypotonic or isotonic media.

The apparent K_m for acetate incorporation into fatty acids of pea chloroplasts in the present study was 50 μM, and the ratio of unsaturated to saturated fatty acids synthesized was 7 to 9, being highest at the highest rates of fatty acid synthesis. Ratios of unsaturated to saturated fatty acids synthesized by spinach, maize, and amaranthus chloroplasts were 3 to 4, 3 to 5, and 3 to 4, respectively.

CAT Activity in Isolated Chloroplasts

That carnitine at concentrations up to at least 1 mM had no effect on fatty acid synthesis from acetate suggested that CAT activity in the organelles was quite low, because high concentrations of carnitine would be expected to divert acetate into acetylcarnitine and away from fatty acid synthesis. Although the chloroplasts contained ACS activities 5- to 10-fold in excess of the rate of fatty acid synthesis from acetate, CAT activity was not detected in any chloroplast preparation in this study (Table IV and results not shown) whether isolated using Suc or Percoll media. CAT was routinely assayed in the direction of acetyl-CoA formation from [1-^{14}C]-acetylcarnitine using a sensitive trapping assay, but in some cases it was also assayed in the direction of CoA-sulphydryl formation. However, the result was the same in both instances. In previous reports of CAT activity in chloroplasts (Thomas et al., 1983; McLaren et al., 1985), it was not possible to calculate reaction rates.

CAT Activity in Whole Leaf Homogenates

Homogenates of expanding spinach and amaranthus leaves and of pea shoots contained no discernible CAT activity, whereas ACS activity (9–12 μmol h^{-1} mg^{-1} of Chl) was readily detected. Even when protein in the assays was concentrated 10-fold compared with that in assays for ACS activity, there was no significant acetylcarnitine utilization during 60 min of incubation at 25°C. However, the assay for CAT activity seemed to be valid, because equilibration of acetylcarnitine and acetyl-CoA was achieved in <2 min when commercial CAT was added to assays already containing leaf extracts.

Acetylcarnitine Metabolism in Vivo

[1-^{14}C]Acetylcarnitine (5 μCi, 0.5 μmol) was supplied in about 1 mL of water to a detached, expanding spinach leaf (2.0 g fresh weight) through the cut petiole and in the light. After 45 min, when 4.6 μCi had been taken up, the leaf was homogenized with 10 volumes of C/M (1:1, v/v). [1-^{14}C]-Acetate (5 μCi, 0.625 μmol) was similarly supplied to an equivalent leaf at the same time, and 4.9 μCi were taken up. The C/M extracts were partitioned into lipids and water-soluble fractions (Bligh and Dyer, 1959), and the radiocarbon content of both fractions was determined. Of the label taken up by the leaves, 57% of the acetate but <2% of the acetylcarnitine was incorporated into lipids (Table V).

In a similar experiment, 4.5 μCi of [1-^{14}C]acetylcarnitine (0.45 μmol) and 4.7 μCi of [1-^{14}C]acetate (0.58 μmol) were taken up separately into detached, expanding maize leaves (third leaves of 12-d-old plants) via the transpiration stream during 90 min in the light. The leaves (0.95 g wet weight) were extracted into C/M, and the extracts were partitioned as above. Of the label taken up by the leaves, 1% of the acetylcarnitine and 56% of the acetate was incorporated into lipid (Table V). By contrast, the aqueous fraction of the C/

Table IV. *Activities of ACS and CAT in chloroplasts isolated from spinach leaves and pea shoots*

Acetyl-CoA formation was followed using a filter paper disc assay (G. Roughan and J. Ohlrogge, unpublished data).

Enzyme	Percoll Purified	Suc Purified
	μmol of acetyl-CoA formed h^{-1} mg^{-1} of Chl	
ACS	8–10	4–6
CAT	<0.01	0

Table V. *In vivo incorporation of [1-^{14}C]acetylcarnitine and [1-^{14}C]-acetate into lipid and water-soluble fractions of expanding spinach and maize leaf, 8-d-old pea shoots, and mature amaranthus leaves*
See the text for experimental details.

Species	Precursor	μCi of ^{14}C			
		Taken up	In lipids	In aqueous phase	In acetyl-carnitine
Spinach	Acetate	4.90	2.81	0.80	0.10
	Acetylcarnitine	4.63	0.07	4.20	3.80
Pea	Acetate	3.10	1.47	0.56	0.07
	Acetylcarnitine	4.56	0.05	4.20	4.20
Maize	Acetate	4.68	2.60	0.40	0.08
	Acetylcarnitine	4.46	0.05	4.10	3.46
Amaranthus	Acetate	2.33	1.31	0.38	0.07
	Acetylcarnitine	2.24	0.09	1.91	1.80

M/W partitions contained 91 and 92% of the ^{14}C taken up into acetylcarnitine-labeled leaves of spinach and maize, respectively, and 16 and 9% in the acetate-labeled leaves. A combination of ion-exchange and TLC (Roughan and Browse, 1992) was used to show that 85 to 100% of the label in the aqueous fraction from acetylcarnitine-labeled leaves was unmetabolized [1-^{14}C]acetylcarnitine (Table V). About 2% of label from the acetate incorporated into spinach and maize leaf cochromatographed with acetylcarnitine (Table V). Most of the label unaccounted for in the [1-^{14}C]acetate-fed leaves in Table V was incorporated into the C/M-extracted solid residue.

Similar results were obtained using pea shoots (Table V) and the youngest mature leaves of 12- to 16-d-old day maize plants (results not shown). Results using the youngest mature amaranthus leaves supplied with 2.5 μCi of substrates (Table V) indicated a slightly higher incorporation of acetylcarnitine (4% of that taken up) into lipids, but an increased metabolism of acetylcarnitine was not confirmed using homogenates of amaranthus leaves. Because its metabolism in vivo was so insignificant, either acetylcarnitine could not readily gain entry into leaf cells or the cells do not possess the ability to metabolize acetylcarnitine readily in the amounts supplied, as suggested by the experiments with leaf homogenates (above).

DISCUSSION

In the present work, acetylcarnitine was not a significant substrate for fatty acid synthesis by chloroplasts isolated from pea, spinach, maize, and amaranthus; acetate was utilized at least 50 times more rapidly. Similarly, unlabeled acetylcarnitine had no effect on the incorporation of labeled acetate into long-chain fatty acids of isolated chloroplasts. Therefore, we believe it unlikely that acetylcarnitine is a significant substrate for fatty acid synthesis in chloroplasts. Indeed, acetylcarnitine may not be quantitatively important in leaf cell metabolism in the light, because not only was labeled acetylcarnitine a poor substrate compared with acetate for fatty acid synthesis by chloroplasts isolated from spinach, pea, maize, and amaranthus, but there was no evidence for significant utilization of labeled acetylcarnitine by homogenates of leaves from the above species and, when compared to acetate, acetylcarnitine was poorly utilized in vivo. Although it could be possible for acetylcarnitine to be excluded from the symplast when taken up into leaves, this lack of acetylcarnitine metabolism by chloroplasts, leaf homogenates, and whole leaves is in keeping with an observed deficiency of CAT activity relative to ACS activity both in isolated chloroplasts and in leaf homogenates.

Our inability to detect significant utilization of acetylcarnitine by chloroplasts, leaf homogenates, and intact leaves is unlikely to be due to some deficiency in the substrate itself. The labeled acetylcarnitine used in the present study was synthesized enzymically, purified by TLC, and standardized by an enzymic method (Roughan and Browse, 1992), so its biological activity seems assured. In addition, its specific radioactivity was 62 times greater than that used by Masterson et al. (1990a, 1990b), thus providing much higher sensitivities in assays of fatty acid synthesis and CAT activity. Yet this labeled acetylcarnitine was poorly utilized for chloroplast fatty acid synthesis in every instance in which it was tested and had no effect on fatty acid synthesis from acetate. Unlabeled acetyl-L-carnitine was either synthesized chemically and crystallized from organic solvent or obtained commercially. These preparations were found to be essentially free of carnitine by TLC. In more than 70 experiments in the present study, involving different chloroplast isolations and varied assay conditions, including duplicating as nearly as possible the conditions of Masterson et al. (1990a, 1990b), neither preparation of acetylcarnitine, when used at 2- to 4-molar excess, significantly inhibited acetate incorporation into fatty acids by chloroplasts isolated from spinach, pea, maize, or amaranthus. This contradicts the results previously reported for pea chloroplasts.

It is also unlikely that our chloroplast preparations had somehow lost an ability to metabolize acetylcarnitine. On the contrary, precedent suggests that chloroplasts purified on Suc gradients, such as those reported by Masterson et al. (1990a, 1990b), are biosynthetically less active than those purified on Percoll gradients (Leegood and Walker, 1983). Pea chloroplasts purified using Suc gradients were leaky and were presumed to have lost metabolites and cofactors during isolation; they were not "physiologically intact" (Bertrams et al., 1981). Similarly, spinach chloroplasts purified on Suc gradients, although morphologically intact, were incapable of bicarbonate-dependent oxygen evolution (Douce and Joyard, 1982), even when great care was taken to preserve integrity when diluting the Suc concentration of chloroplast suspensions. Percoll gradients or cushions, on the other hand, permit the very rapid (5–10 min) isolations that are normally recommended (Leegood and Walker, 1983; Roughan, 1987) for retaining highest rates of biosynthetic processes in isolated chloroplasts.

The experience of the present study supported previous findings that chloroplasts purified using Suc gradients are clearly biosynthetically inferior to those purified using Percoll. Other indicators of the "quality" of chloroplast fatty acid synthesis, namely, high proportions of unsaturated:saturated fatty acids synthesized and low K_m for acetate incorporation into fatty acids, independently suggest that the chloroplasts prepared for the present study were of the highest quality.

Nonetheless, we were unable to demonstrate significant utilization of acetylcarnitine by isolated chloroplasts even when varying isolation procedures, plant species, and leaf maturities.

The best estimates currently available for rates of fatty acid accumulation in vivo, and from "physiological" precursors, by spinach leaves (Browse et al., 1980; Murphy and Leech, 1981) are only slightly less than the best rate of fatty acid synthesis achieved using isolated, intact spinach chloroplasts (Roughan, 1987). This is to be expected if fatty acids synthesized by chloroplasts also provide the carbon chains for other alkyl compounds, e.g. fatty alcohols. However, the rate of fatty acids synthesis supported by acetylcarnitine in pea chloroplasts is suggested (Masterson et al., 1990a) to be 5- to 7-fold greater than rate of fatty acid synthesis in expanding spinach leaves. Also, it is commonly believed that pea shoots are at their optimum for chloroplast isolation at 6 to 10 d from planting, depending on the growing environment. At least under our growing conditions the expectation that more mature shoots would yield chloroplasts with inferior rates of fatty acid synthesis from acetate was confirmed (results not shown).

In conclusion, despite our efforts to encourage acetylcarnitine metabolism using a variety of experimental conditions, including attempts to duplicate those of Masterson et al. (1990a, 1990b), we could find no evidence that it was a significant substrate for fatty acid synthesis in isolated chloroplasts or in whole plants.

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