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Origin of Acetate in Spinach Leaf Cell[1]

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ABSTRACT

Mitochondria were isolated from spinach (*Spinacia oleracea* L.) leaves using a Percoll gradient step. The high purity of the organelle fraction is demonstrated by electron microscopy and biochemical parameters. In the matrix space of these mitochondria, a short-chain acyl-coenzyme A hydrolase is present that converts acetyl-coenzyme A to acetate and coenzyme A with reasonable rates (K_m, 150 micromolar; V_{max}, 140 nanomoles acetate formed milligram[1] protein hour^{-1}). The enzyme is product inhibited by coenzyme A-sulfhydryl, other thiols are ineffective; however, the disulfides 5,5'-dithio-bis-(2-nitrobenzoate) and cystamine stimulate the hydrolysis. The possible role of this mitochondrial enzyme as a means of generating free acetate from pyruvate via acetyl-coenzyme A in the mitochondria of mature spinach leaves is discussed. It is suggested that free acetate moves rapidly from the mitochondrion to the chloroplast where acetyl-coenzyme A synthetase, solely localized in this organelle, converts the metabolically inert free acetate to the highly active acetyl-coenzyme A.

Ever since it was reported in 1952 that free acetate was readily incorporated into fatty acids by plant tissues (29), this compound has served as the experimental substrate in studies on the synthesis of fatty acids by plants. Despite its widespread use during the past two decades, little if any information is available on the formation of free acetate in higher plants. In plants, free acetate is rapidly converted to acetyl-CoA by acetyl-CoA synthetase which was first described by Millerd and Bonner (22) and has been further examined by Hiatt (7) and Rebeiz *et al.* (31), and purified, and its properties studied by Huang and Stumpf (11). Very recently this enzyme has been found to be solely localized in the chloroplast stroma phase of the spinach leaf cell (16), which is also the organelle where fatty acid synthesis takes place (30). Thus, acetate when supplied to leaf tissue externally is not converted to the metabolically active acetyl-CoA until it reaches the chloroplast.

Two questions may now be raised: (a) is free acetate formed externally to or internally in the chloroplast; and (b) what is the function of acetyl-CoA synthetase, which is solely localized in the chloroplast, if acetyl-CoA is directly derived from the oxidative decarboxylation of pyruvate in this organelle? There is some evidence in pea chloroplast that the following sequence can occur: CO_2 → triosephosphate → phosphoenolpyruvate → pyruvate → acetyl-CoA. PDC[3] has been detected in pea chloroplasts (4, 41). Non-green plastids also appear to contain pyruvate dehydrogenase complex (17, 32, 33, 36). In spinach chloroplasts, however, conflicting results have been reported about the presence of PDC and, therefore, about the source of acetyl-CoA. Some investigators have suggested a direct flow from CO_2 via C_3 intermediates to pyruvate and hence to acetyl-CoA. Grumbach and Forn (6), Murphy and Leech (24), and Yamada and Nakamura (42) have provided evidence on the intraplastid presence of the necessary glycolytic enzymes and an active PDC. In contrast to these results, others have failed to demonstrate PDC in spinach chloroplasts or were able only to show low activities (25, 26, 34). In addition, Murphy and Stumpf (25) have shown a decrease in PDC activity dependent on (a) increasing age of plant material and (b) after Percoll gradient purification of plastids.

Since acetyl-CoA synthetase in the spinach leaf is specifically localized in the chloroplast, and since a measurable free acetate pool (range, ~1 mM) is present in green leaf tissue (16), it became important to determine the primary source of free acetate. Murphy and Stumpf (25) recently were able to demonstrate and to characterize an acetyl-CoA hydrolytic activity associated with mitochondria isolated from spinach leaves. This enzyme was absent in isolated chloroplasts. In this paper, we further document the role of the mitochondrial acetyl-CoA hydrolase as it relates to the generation of free acetate. Whether or not this mechanism is widespread in plant tissues will require further studies.

MATERIALS AND METHODS

Materials. Spinach (*Spinacia oleracea* L.) was purchased from the local supermarkets. [1-^{14}C]Acetyl-CoA (57 mCi/mmol), [1-^{14}C]acetate (58 mCi/mmol), and [2-^{14}C]pyruvate (16 mCi/mmol) were obtained from New England Nuclear; and [1,5-^{14}C]citrate (1.5 mCi/mmol) was obtained from California Corporation for Biochemical Research (Los Angeles, CA). All fine chemicals used were from Sigma Chemical Company.

Isolation of Cell Organelles. Isolation of chloroplasts and mitochondria from spinach leaves was carried out as described in Murphy and Stumpf (25).

Because of the localization of acetyl-CoA hydrolytic activity within the matrix space, the mitochondrial pellet was resuspended in 1.5 ml incubation buffer (see below), and sonicated for 10 s at 0°C with the aid of a microtip (20% of maximal intensity; Biosonik III; Brownwill Scientific, Rochester, NY). To remove the mitochondrial membranes, the sonicated fraction was centrifuged for 1 h at 100,000*g* in a Beckman type 40 rotor, using 1.5-ml microfuge tubes sunk into sand-filled ultracentrifuge tubes.

Isolation of microbodies from spinach leaf cells was carried out according to Huang and Beevers (9).

[1-^{14}C]Acetyl-CoA Assay. The standard incubation mixture consisted of [1-^{14}C]acetyl-CoA (0.08 μCi) adjusted to 0.2 mM concentration with unlabeled acetyl-CoA (if not otherwise indicated), 50 mM Tricine, and 0.5 mM $MgCl_2$ (pH 7.5). Other cofactors were usually added in 2- to 4-μl aliquots; the final reaction volume was 100 μl in a 1.5-ml capped microfuge tube. Normal incubation time was 30 min at 25°C. The reaction was stopped by addition of 10 μl glacial acetic acid.

Determination of Hydrolytic-Formed [1-^{14}C]Acetate. The assay

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[3] Abbreviations: PDC, pyruvate dehydrogenase complex, DTNB, 5,5'-dithio-bis-(2-nitrobenzoate); SH, sulfhydryl.

system is based on the volatility of acetate in acid solution and on the adsorption of unreacted acetyl-CoA by activated charcoal (16, 28). A 50-μl aliquot of the incubation mixture was mixed with 50 μl 0.01 N HCl and 10 μl of a suspension containing 15% (w/v) activated charcoal and 5% (v/v) Percoll (to stabilize the suspension) in 0.01 N HCl. After shaking in 1.5-ml microfuge tubes for 30 min, the unreacted [1-^{14}C]acetyl-CoA bound on the charcoal was removed by centrifugation (5 min; 500*g*). While one 40-μl aliquot of the clear supernatant was counted immediately (containing labeled acetate and citrate), another aliquot was transferred to an empty vial and, after addition of 10 μl glacial acetic acid as a carrier, was blown with the aid of a cold-air dryer (at least 45 min). The nonvolatile residue in the vial represented labeled citrate as was shown by TLC using authentic [1,5-^{14}C] citrate as a standard (8, 38). The difference in radioactivity gave the amount of free acetate. Further proof that the volatile radioactive substance actually corresponded to acetate was provided by employing a special acetate kit (Boehringer Mannheim GmbH, Mannheim, F.R.G.) that converted acetate to the nonvolatile citrate (see also Ref. 25). In addition, the decrease of labeled [1-^{14}C]acetyl-CoA was followed by the method of Huang (10) with the Gelman paper technique.

DTNB Assay. With this alternative method, the appearance of the free SH group of the released CoA-SH was followed spectrophotometrically. Ellman's reagent forms a yellow product when reacting with free CoA-SH; the reaction can be followed at 412 nm, where the mercaptide ion has a strong absorption (E = 13,600) (5, 37). The assay medium consisted of 50 mM Tricine (pH 7.5); 100 μM DTNB, 0.2 to 1.0 mM acetyl-CoA (or other CoA esters), and mitochondrial matrix protein corresponding to 100 to 150 μg.

Other Procedures. Incubation conditions for fatty-acid synthesis in chloroplasts, extraction and TLC of the lipids were exactly as in Kleinig and Liedvogel (14).

Microdialysis of mitochondrial matrix fractions were carried out according to Marusyk and Sergeant (21) (Millipore VMWP; pore size, 0.05 μm). Dialysis against incubation buffer took place in Petri dishes for 1 h in a cold room. Catalase activity was assayed from the method described by Luck (20), malate dehydrogenase according to Davies (3), and Cyt *c* oxidase according to Schnarrenberger *et al.* (35).

Protein was determined with the Lowry method with BSA as standard.

Samples for electron microscopy were essentially prepared as described previously (18). Electron micrographs were taken with a Zeiss electron microscope EM 10 CR (Zeiss, Oberkochen, F.R.G.).

RESULTS

Localization. Three organelles, namely chloroplasts, mitochondria, and microbodies, were isolated from spinach leaves and were assayed for acetyl-CoA hydrolase activity (Table I). Although intact mitochondria exhibited low activity, sonicated mitochondria had a 9-fold higher activity. When the matrix phase was separated from the mitochondrial membranes, major activity was associated with the soluble proteins of the matrix. In all experiments to be described, the matrix fraction, separated from the membranes by ultracentrifugation (100,000*g*; 1 h), served as a source of the enzyme. Inasmuch as the enzyme activity measured with intact mitochondria was very low, it suggests that acetyl-CoA is not freely permeable through at least the inner mitochondrial membrane. The hydrolytic activities found in both isolated chloroplasts and microbodies were rather low (Table I). While it is possible to purify chloroplasts (free of mitochondria) by Percoll gradients, the enzymic activity associated with microbodies purified by sucrose gradient centrifugation (9) may be due to the mitochondrial contamination of this fraction. The supernatant fraction was not tested since corrections for activities derived from disruptured mitochondria are imprecise. It could be suggested that the hydrolase does not occur in the cytosol (supernatant) since acetyl-CoA would not occur in this compartment. Acetyl-CoA would be generated only either in the mitochondrion via PDC or in the chloroplast via acetyl-CoA synthetase.

Table I. *Localization of Acetyl-CoA Hydrolase in the Spinach Leaf Cell*

Incubation conditions as described in the experimental part; acetyl-CoA concentration, 0.2 mM; assayed with the [1-^{14}C]acetyl-CoA method.

Fraction	Acetyl-CoA Hydrolase Activity
	nmol acetate formed mg^{-1} protein h^{-1}
Intact mitochondria	2.2
Whole mitochondria (sonicated)	19
Mitochondrial matrix fraction	43
Mitochondrial membrane fraction	17
Whole chloroplasts (sonicated)	2.5
Chloroplast stroma fraction	2
Chloroplast membrane fraction (envelopes and thylakoids)	1.5
Whole microbodies (sonicated)	4

Table II. *Purity Criteria for the Mitochondria Fraction Isolated from Spinach Leaves*

Morphometric Evaluation of electron micrographs (particle counting)	%	
Mitochondria	94	
Microbodies	4	
Thylakoid membranes	<1	
Free protein crystals (catalase)	<1	
Enzymic Activities		
	Mitochondria	Microbodies
	relative units	
Malate dehydrogenase (NAD-linked)	1.0	0.1
Cyt *c* oxidase	1.0	0.09
Catalase	0.13	1.0

Purity of the Mitochondria Fraction. The association of a certain enzymic activity to a specific organelle requires that the organelle fraction be free of any other organelle system. The criteria for purity and intactness of the mitochondria used in our experiments are listed in Table II. The electron micrographs of isolated mitochondria (Fig. 1) complement these data. Particle counting for intact mitochondria in a number of electron micrographs indicated that 94% of all particles were intact mitochondria, whereas a minor component of about 4% was accounted for as microbodies. Very few thylakoid membranes as well as free protein crystals, most likely representing catalase, were observed. Galactolipids, the marker lipids for plastid membranes, were not detected on 2-dimensional thin-layer; contamination by thylakoids (as evaluated by the presence of Chl) was less than 2% (19). The main phospholipids were phosphatidylcholine, phosphatidylethanolamine, and diphosphatidylglycerol, while phosphatidylglycerol was apparently absent from the fraction (23). Malate dehydrogenase and Cyt *c* oxidase showed highest activities in mitochondria, whereas catalase activity suggested low microbody con-

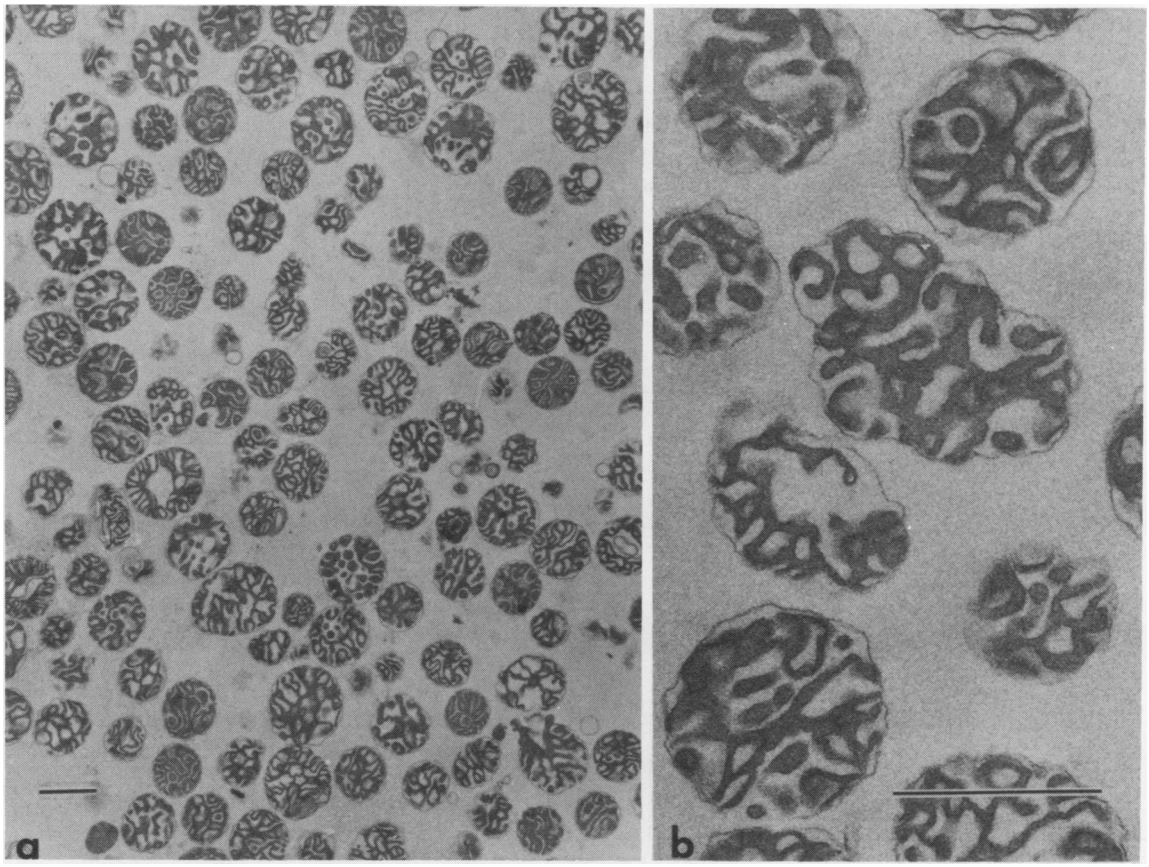

FIG. 1. Electron micrographs of ultrathin sections from a mitochondria fraction isolated from spinach leaves. A, Survey over the fraction containing intact mitochondria; B, detail with higher magnification (the bar represents 1 μm in both of the pictures. Magnification: A, × 9,000; B, × 32,000).

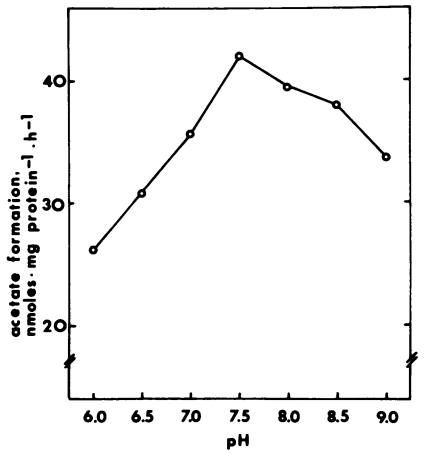

FIG. 2. Dependence of pH of acetyl-CoA hydrolytic activity from the matrix compartment of isolated spinach mitochondria. Buffer system, 200 mM Pipes/Tricine; [1-^{14}C]acetyl-CoA assay; protein content, 350 μg/sample.

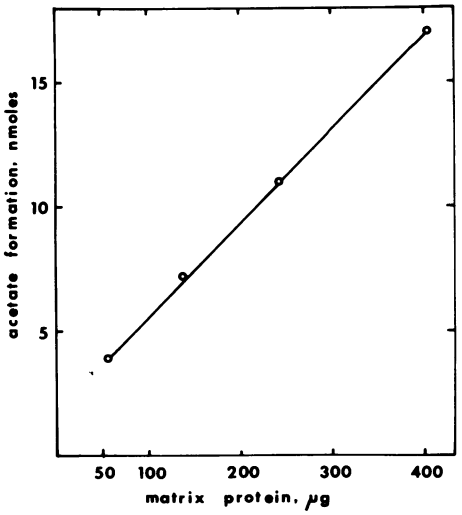

FIG. 3. Dependence of acetate formation from acetyl-CoA on the amount of mitochondrial matrix protein. [1-^{14}C]Acetyl-CoA assay; protein concentration of the original mitochondrial matrix fraction, 2.7 μg/μl; final volume, 200 μl/sample.

tamination. In addition, intact mitochondria were monitored by O_2 electrode for respiratory control. In the presence of either malate or pyruvate and ADP, the mitochondria actively reduced O_2. We therefore conclude that the isolated leaf mitochondria were intact and had physiological integrity.

Properties of Acetyl-CoA Hydrolase. The effect of pH on the enzyme activity is shown in Figure 2. The pH optimum ranges about 7.5; higher pH values result in an increase of nonenzymic hydrolysis (*i.e.* about 5% of substrate was nonenzymically hydrolyzed at pH 9). The values shown in Figure 2 were corrected for this effect. The release of free acetate from acetyl-CoA depends on the amount of mitochondrial matrix protein available in the incubation mixture (Fig. 3). The data for the heat stability of the acetyl-CoA hydrolytic activity are given in Table III. Soluble matrix extract was preincubated for 5 min at different temperatures and then assayed in the usual manner. Preincubation at

Table III. *Heat Stability of Acetyl-CoA Hydrolase Activity*

[1-^{14}C]Acetyl-CoA assay, but the acetyl-CoA concentration was 0.5 mM; protein content, 330 μg/sample; incubation time, 40 min.

Temperature of 5-min Preincubation before Assay		Acetyl-CoA Hydrolase Activity
	°C	*nmol acetate formed mg^{-1} matrix protein h^{-1}*
Control	25	42
	45	44
	50	49
	55	30
	60	13
	100	0

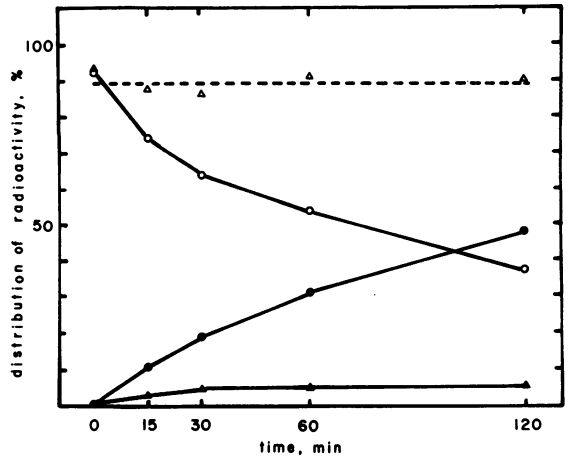

FIG. 4. Time course of [1-^{14}C]acetyl-CoA utilization by the mitochondrial matrix fraction from spinach leaf cells. Incubation took place in a total volume of 200 μl; protein content, 460 μg/sample; 100 nmol [1-^{14}C]acetyl-CoA corresponding to 175,000 cpm. (○), Acetyl-CoA; (●), acetate; (▲), citrate; (△), recovery of the radioactivity.

50°C increased in activity by about 16% when compared with the 25°C control. Higher preincubation temperatures led to a drastic decrease in activity. On the other hand, freezing of the matrix fraction at −70°C for 5 d did not affect the enzyme activity at all, whereas freezing at −20°C for the same time period led to a loss in activity of 40% in the absence and only 15% in the presence of 20% glycerol.

The substrate-product relation of a typical incubation is given in Figure 4. The production of free acetate increases with time. Citrate synthesis, catalyzed by endogenous citrate synthetase is negligible because of limiting amounts of endogenous oxaloacetate. With an acetyl-CoA concentration of 1 mM, the incorporated radioactivity into citrate was about 5 to 7% of the total radioactivity. Microdialysis of the matrix fraction for 1 h on millipore filters (21) diminished the label in citrate to less than 3% of the total radioactivity by removal of the endogenous oxaloacetate. Because there was also some loss in enzyme activity caused by this treatment, the additional dialysis step usually was omitted. The decrease in radioactivity in acetyl-CoA therefore reflected the increase in free acetate.

Regulatory Factors. Different patterns of substrate saturation curves were obtained depending on the assay system used (Fig. 5). The standard assay method measured the production of free [1-^{14}C]acetate (see experimental part). The alternative assay system was based on measuring the free -SH group of CoA with DTNB (see Refs. 5 and 37). A consistent stimulation of enzymic hydrolysis of acetyl-CoA was observed in the presence of DTNB. Since CoA released in the hydrolysis of acetyl-CoA is removed in reacting with DTNB, it is suggested that free CoA may inhibit the hydrolytic reaction. The explanation was confirmed, when it could be shown that the addition of exogenous CoA (0.5 mM) to the assay system resulted in a decrease of acetyl-CoA hydrolase activity (Fig. 5). Evaluation of four experiments in terms of Lineweaver-Burk plots suggested a noncompetitive manner of inhibition. In contrast to this, acetate, the other reaction product, had no effect on the enzyme activity. Possible effects of several other thiol and disulfide compounds were tested for their influence upon acetyl-CoA hydrolysis (Table IV).

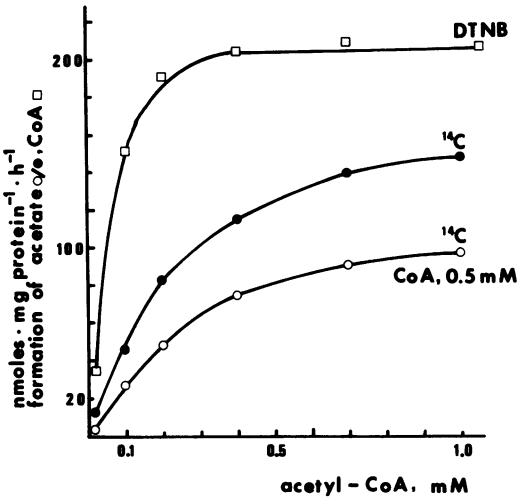

FIG. 5. Acetyl-CoA concentration *versus* rate of acetate and CoA-SH production, respectively. DTNB means rate of hydrolysis was determined with the DTNB assay; [^{14}C] means rate of hydrolysis was determined with the [1-^{14}C]acetyl-CoA assay. DTNB has the same stimulatory effect when used in the [1-^{14}C]acetyl-CoA assay (Table IV).

Table IV. *Effect of Various Thiol and Disulfide Compounds on Acetyl-CoA Hydrolysis*

[1-^{14}C]Acetyl-CoA assay; protein content, 195 μg/sample.

Incubation Conditions	Acetyl-CoA Hydrolysis
	nmol acetate formed mg^{-1} matrix protein h^{-1}
Control (acetyl-CoA, 0.2 mM)	51
Thiols	
+ CoA, 0.2 mM	21.4
+ CoA, 0.5 mM	15
+ CoA, 2.0 mM	9.3
+ DTT, 0.5 mM	48.5
+ Cys, 0.5 mM	50.7
+ GSH, 0.5 mM	55
+ β-Mercaptoethanol, 0.5 mM	51
Disulfides	
+ DTNB, 0.5 mM	77
+ Cystamine, 0.5 mM	65

A search of other positive or negative effectors on the enzyme was made. A series of compounds (NADH, NADPH, AMP, ADP, ATP, glycerate 3-P, P-enolpyruvate, pyruvate, citrate, fructose-1,6-di-P) as well as bivalent cations (Mg^{2+}, Mn^{2+}, Ca^{2+}) were added to the standard incubation mixture at 0.5 mM level without any significant effect.

Specificity. The acetyl-CoA hydrolytic enzyme possesses a rather broad specificity spectrum, as shown in Table V. Acetyl-CoA, butyryl-CoA, and propionyl-CoA were hydrolyzed with

Table V. *Efficiency of Mitochondrial Matrix Protein in Hydrolysis of Different CoA Thioesters*

Calculated by the DTNB assay: concentration of the CoA esters, 0.5 mM; protein content, 300 μg/sample; final volume, 200 μl.

Substrate	Rate of Hydrolysis
	nmol CoA-SH released mg^{-1} matrix protein 5 min^{-1}
Acetyl-CoA	16
Butyryl-CoA	14
Propionyl-CoA	15
Pentynyl-CoA	7
Hexanoyl-CoA	9
Palmitoyl-CoA	10

Table VI. *Co-incubations of Mitochondria and Chloroplasts Isolated from Spinach Leaves*

The protein content of the mitochondria fraction was 760 μg/150 μl suspension: Chl content of the chloroplasts, 122 μg/200 μl suspension; radioactivity precursors, 45 nmol corresponding to 700,000 cpm; final volume, 370 μl. The reaction mixtures were preincubated with mitochondrial fractions for 30 min, then the chloroplast suspension was added and incubated for a further 60 min in the light; 25°C.

Fractions	Radioactivity Incorporated into Fatty Acids when Different Carbon Precursors were Administered		
	[1-^{14}C]-Acetate	[1-^{14}C]-Acetyl-CoA	[2-^{14}C]-Pyruvate
	cpm		
Intact chloroplasts (control)	134,920	8,096	4,477
Intact mitochondria (control)	1,615	1,876	1,475
Intact chloroplasts + intact mitochondria	146,460	9,666	9,640
Intact chloroplasts + sonicated mitochondria	146,145	25,138	10,280

about the same efficiency. Even CoA esters with longer chain length were attacked with reasonable rates of hydrolysis. Nevertheless, the palmitoyl-CoA thiolysis observed in our incubation system was not due to the long-chain acyl-CoA thioesterase, recently described for the chloroplast envelope (13), because UDP-galactose diacylglycerol transferase, a well-known marker enzyme for the chloroplast envelope was totally absent from our mitochondria fraction. Furthermore, both acetyl-CoA hydrolysis as well as palmitoyl-CoA hydrolysis showed about the same inhibitory behavior when treated with CoA. While this hydrolase appeared to lack specificity, under normal *in vivo* conditions this enzyme would probably never be exposed to these substrates.

Physiological Aspects. From our point of view, the short-chain acyl-CoA hydrolase located in the mitochondrial matrix may provide free, permeable, metabolically inactive acetate that can readily penetrate into the chloroplasts to be converted to acetyl-CoA by the stroma enzyme, acetyl-CoA synthetase (16). To prove this possibility experimentally, model incubations were carried out combining intact Percoll-purified chloroplasts with both intact and ruptured mitochondria. Equimolar amounts of [1-^{14}C]-acetate, [2-^{14}C]pyruvate, and [1-^{14}C]acetyl-CoA adjusted to corresponding specific activities served as carbon precursors for the plastidal fatty-acid synthesizing system. After preincubation of the labeled compounds for 30 min with the mitochondrial fractions, chloroplasts were added for an additional h, and the radioactivity associated with the fatty-acid fraction was determined (Table VI). For intact chloroplasts [1-^{14}C]acetate was by far the best incorporated precursor, whereas [1-^{14}C]acetyl-CoA and [2-^{14}C]pyruvate were incorporated only to a very small extent. No incorporation occurred into fatty acids with isolated mitochondria alone because the fatty-acid synthesizing system is absent in these organelles. However, the co-incubations of intact chloroplasts and intact mitochondria brought out an increase of incorporation of ^{14}C label from [^{14}C]pyruvate, when compared with the chloroplast control. The combination of intact chloroplasts and sonicated mitochondria resulted in a further increase by either [^{14}C]acetyl-CoA or [^{14}C]pyruvate incorporation into the fatty-acid fraction.

DISCUSSION

In plants, little information is available concerning an acetyl-CoA hydrolytic enzyme. However, acetyl-CoA hydrolases from different animal systems have been described (*e.g.* 1, 27). In brown adipose tissue from hamster (1), the enzyme is localized in the mitochondrial matrix. Its presumed function there is to control the acetyl-CoA concentration in the cell. The hamster enzyme is strongly product-inhibited by CoA as well as by NADH, but noncompetively activated by ADP. In another animal system, the pineal gland of the rat, acetyl-CoA hydrolase was also inhibited by CoA but markedly stimulated by cystamine (27). While these authors apparently have evidence that the cystamine effect is caused by protein thiol-disulfide exchange, our interpretation for cystamine as well as for DTNB stimulatory effects on the spinach acetyl-CoA hydrolytic enzyme is that these compounds remove the inhibition effects of CoA-SH from the reaction by disulfide exchange. Although the specificity of the spinach enzyme for acetyl-CoA is not pronounced (Table V), its strict compartmentation together with the fact that, most likely, acetyl-CoA is formed by PDC and represents the main acyl-CoA ester in the mitochondrial matrix space would further define the specificity of the hydrolase.

Acetyl-CoA is the substrate for the transcarboxylase step of acetyl-CoA carboxylase supplying the fatty-acid synthesizing system in the chloroplast stroma phase with its cosubstrate, malonyl-CoA, and is also the substrate for the acetyl-CoA:ACP transacetylase, the starting reaction in the multistep sequence leading to the formation of acyl-ACP (39). The most direct way to provide the chloroplast stroma phase with acetyl-CoA via an intraplastid PDC seems to be limited in the spinach cell of mature leaves (25, 26, 34). In our experiments, [2-^{14}C]pyruvate was incorporated very poorly into fatty acids by isolated chloroplasts (Table VI). Furthermore, acetyl-CoA synthesized elsewhere in the cell apparently would not be able to enter the plastid envelope because of permeability barrier (2, 17; see also Table VI). On the other hand, since acetyl-CoA synthetase is specifically localized in the stroma phase of the spinach chloroplast (16), externally supplied acetate moves very rapidly into the chloroplast (12) and is then converted to acetyl-CoA in the stroma phase. If free acetate is a physiological substrate, its origin by known reactions which generate free acetate must be defined (for a review of this problem, see Ref. 40). A number of possible reactions including citrate-oxaloacetate lyase, *o*-acetyl serine sulfhydrolase, and oxalate synthesis from oxaloacetate, all of which provide free acetate as a reaction product, were checked as potential systems generating acetate for incorporation into fatty acids by spinach chloroplasts (15, 16). In all of these experiments, no evidence was found to implicate these systems as sources of acetate, although it has been shown that acetate is present in the spinach tissue in about 1 mM concentration (16). When Murphy and Stumpf (25) observed an acetyl-CoA hydrolytic activity associated with isolated spinach leaf mitochondria, further investigations were indicated. Our results now demonstrate the presence of a short-chain acyl-CoA hydrolase localized specifically in the matrix space of spinach mitochondria which attacks acetyl-CoA at a reasonable rate (maximal activity, about 140 nmol

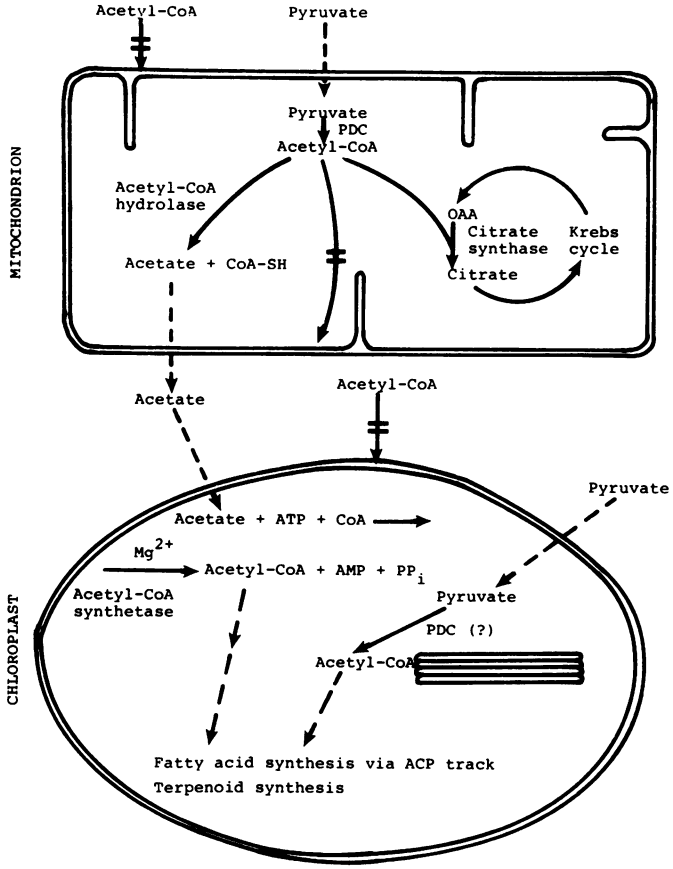

FIG. 6. Hypothetical scheme illustrating the cooperation between mitochondria and chloroplasts in providing the stroma enzyme acetyl-CoA synthetase with acetate. Acetyl-CoA formed by mitochondrial PDC is believed to be unable to leave the mitochondrion and reach the chloroplast stroma by vectorial diffusion.

acetate formed mg^{-1} matrix protein h^{-1}).

When model incubations combining isolated chloroplasts and mitochondria were designed to examine the incorporation rates of ^{14}C-labeled acetate, acetyl-CoA, and pyruvate into fatty acids, only the combination of ruptured mitochondria plus intact chloroplasts showed a significant increase of incorporated [1-^{14}C]acetyl-CoA (Table VI). We conclude, therefore, that acetyl-CoA, which is not freely permeable through both the mitochondrial inner membrane (see Table I) and the chloroplast envelope (2), is hydrolyzed to acetate and CoA. The free acetate anion is then able to enter the chloroplast stroma phase, where it is converted to an impermeable anion by acetyl-CoA synthetase (16). The interaction of two organelles in the spinach leaf cell, namely the mitochondrion which will provide acetate via PDC and an acetyl-CoA hydrolase and the chloroplast which utilized this acetate to form acetyl-CoA, the key substrate for fatty-acid synthesis, is summarized in Figure 6.

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