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Increased Fatty Acid β-Oxidation after Glucose Starvation in Maize Root Tips

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ABSTRACT

The effects of glucose starvation on the oxidation of fatty acids were studied in excised maize (*Zea mays* L.) root tips. After 24 hours of glucose starvation, the rate of oxidation of palmitic acid to CO_2 by the root tips was increased 2.5-fold. Different enzyme activities were tested in a crude particulate fraction from nonstarved root tips and those starved for 24 hours. The activities of the β-oxidation enzymes crotonase, hydroxyacyl-coenzyme A (CoA) dehydrogenase, and thiolase and those of catalase, malate synthase, and peroxisomal citrate synthase were higher after starvation. However, no isocitrate lyase activity was detected, thus suggesting that the glyoxylate cycle does not operate. The overall β-oxidation activity was assayed as the formation of [^{14}C]acetyl-CoA from [^{14}C]palmitic acid after high-performance liquid chromatography analysis of the CoA derivatives. An activity was detected in sugar-fed root tips, and it was increased by two- to fivefold in starved roots. Because the recovery of enzyme activities is only marginally better in starved roots compared with nonstarved roots, these results indicate that the β-oxidation activity in the tissues is increased during sugar starvation. This increase is probably an essential part of the response to a situation in which lipids and proteins replace carbohydrates as the major respiratory substrates. These results are discussed in relation to the metabolic changes observed in senescing plant tissues.

It is usually thought that carbohydrates are the major respiratory substrates of plant cells, whereas lipids and proteins make a negligible contribution to the respiratory carbon flux (1). However, there is growing evidence that the carbohydrate reserves quickly decrease to low levels in darkness (4, 20). This suggests that carbohydrates may become limiting, and the contribution of lipids and proteins to respiration increase, not only in senescing tissues (1) but also during the normal plant life.

The metabolic consequences of carbohydrate starvation have been studied in a number of plant species. The depletion of intracellular carbohydrate is associated with a decrease of the respiration rate and a decline in the respiratory quotient from 1 to 0.75. After a long period of starvation, a delay is needed for the recovery of respiration (27) and fermentation (28). These results suggested that cellular components were degraded to sustain respiration. More recently, the decrease of total proteins and fatty acids and increasing levels of phosphorylcholine (9) and asparagine (12) provided more direct evidence for phospholipid and protein breakdown during sugar starvation in sycamore cells in culture. Similar results were obtained with maize root tips, in which proteins and fatty acids were also found to decrease during starvation (5). These results suggested that, during starvation, fatty acids would be degraded by β-oxidation, thus producing acetyl-CoA necessary to the tricarboxylic acid cycle. However the role of β-oxidation is not well established. This pathway has been generally associated with neoglucogenesis, but it has been recently shown that, in lettuce embryos, the β-oxidation of fatty acids directly feeds the tricarboxylic acid cycle, providing the substrate for respiration during the early steps of germination (29). It can be hypothesized that β-oxidation could play a similar role in sugar-starved tissues.

The cellular localization of β-oxidation is still a matter of debate. It was considered as exclusively peroxisomal (14, 17), but previous works indicating a mitochondrial localization of β-oxidation (33) have been recently reinforced (24). In the roots of maize seedlings (13), the activities of the four β-oxidation enzymes have been previously detected in the peroxisomal, not in the mitochondrial, fraction.

The aim of the present work was to determine whether the overall β-oxidation pathway was functional in maize root tips and to compare the peroxisomal β-oxidation and glyoxylic cycle activities before and after a starvation treatment. Our results indicate that the β- oxidation of palmitic acid is active in maize root tips and that this activity increases during carbohydrate starvation.

MATERIALS AND METHODS

Plant Material

Maize seeds (*Zea mays* L. cv DEA, Pioneer France Maïs, France) were germinated for 3 d in the dark at 25°C, and the 3-mm-long primary root tips were excised as previously described (5).

Starved root tips were obtained after a 24-h incubation in the mineral solution described in ref. 27 supplemented with 0.1 M Mes (pH 6) and 1% (v/v) of an antibiotic-antimycotic solution (Sigma reference No. A7292) and aerated with a gas flow of N_2/O_2, 50:50 (v/v), to keep oxygen partial pressure >35 kPa, which is the critical oxygen pressure of maize root tips in aqueous solution (27). Nonstarved and control root tips were placed immediately after excision in the same solution supplemented with 0.2 M glucose. Nonstarved root tips were ground within 30 min after the first tips were excised. Control root tips were incubated for 4 or 24 h; after 24-h incubation, they were recut to 3 mm before use. Each treat-

ment usually involved a sample of 300 tips. When different treatments were to be compared, the corresponding samples were prepared from the same batch of germinated seeds.

Chemicals

All reagents and enzymes were obtained from Sigma. Labeled substrates were purchased from NEN (Paris, France): [U-^{14}C]palmitic acid, 800 mCi·mmol^{-1} (29 GBq·mmol^{-1}); [^{3}H]acetyl-CoA, 3.3 Ci·mmol^{-1} (121 GBq·mmol^{-1}).

Extraction and Determination of Substrates

Soluble sugars and fatty acids were extracted and analyzed as described in ref. 5.

In Vivo Assay of [^{14}C]Palmitic Acid Oxidation

Excised maize root tips were incubated in the presence (control root tips) or absence (starved root tips) of 0.2 M glucose for 0, 4, or 24 h as indicated above. Samples of 20 tips (recut to 3 mm after a 24-h incubation) were then incubated for 2 h in 1 mL of a solution containing 68.8 kBq (2 μM) of carrier-free [U-^{14}C]palmitic acid, 20 mM (pH 6) potassium phosphate, 10 μL of the antibiotic-antimycotic solution (Sigma reference No. A7292); for time zero and control tips, this medium also contained glucose (0.2 M final concentration). A piece of filter paper with 200 μL of 10% KOH was placed in the center well of the vial to collect CO_2. At the end of incubation, the paper wick was removed, the well was rinsed three times with 0.5 mL of water, and the radioactivity was counted. Counts were corrected for luminescence and for the radioactivity of blanks run in the absence of root tips.

Enzyme Extracts

A particulate fraction containing the peroxisomes was obtained essentially as described in ref. 15. All procedures were carried out at 0 to 4°C. Root tips (n = 300) were gently ground in a smooth mortar with pestle in 30 mL of a medium containing 1 M sucrose, 170 mM Tricine (pH 7.5), 10 mg·mL^{-1} fatty acid-free BSA, 5 mM EDTA, 1 mM $MgCl_2$, 10 mM KCl, and 10 mM β-mercaptoethanol. The homogenate was filtered through four layers of cheesecloth, and the filtrate (called "crude extract") was layered on the top of a one-step sucrose gradient (60 and 35%, w/w, sucrose in 5 mM EDTA adjusted to pH 7.5) and centrifuged for 90 min at 83,000g using a Beckman L565 ultracentrifuge and a SW 27 rotor. The interface between 35 and 60% sucrose (called "particulate fraction") was collected and kept in ice until assayed. The volume of the particulate fraction was about 7 mL.

Spectrophotometric Enzyme Assays

Enzymic assays were performed with 50 or 100 μL of extract in a final volume of 1 mL.

All the β-oxidation enzymes were assayed according to the method described in ref. 14 with some modifications. For the thiolase assay, 0.2 mM acetoacetyl-CoA, 3 mM $MnCl_2$, and 100 mM Tricine (pH 7.5) were used. For the acyl-CoA oxidase assay, the palmitoyl-CoA concentration was 50 μM.

Fumarase activity was determined as described by Hill and Bradshaw (18). Isocitrate lyase and malate synthase were assayed as described by Cooper and Beevers (6).

The peroxisomal and mitochondrial citrate synthase activities were measured as described by Schnarrenberger *et al.* (30) for the enzymes of castor bean endosperm. Whereas the mitochondrial enzyme is insensitive to DTNB[1], the glyoxysomal isozyme is inhibited after preincubation for 5 min with 1 mM DTNB; it is protected against this inactivation when oxaloacetate is present. Papke and Gerhardt (25) showed that the method applies to the peroxisomal citrate synthases of different tissues. The assay mixture contained 0.2 M Tris-HCl (pH 8), 1 mM oxaloacetate, 1 mM DTNB, 0.4 mM acetyl-CoA, and 50 or 100 μL of the particulate fraction. Total citrate synthase activity was first estimated by starting the reaction with the addition of the enzyme fraction. The mitochondrial (DTNB insensitive) activity was measured in a second assay in which the addition of oxaloacetate followed a 20-min preincubation of the enzymes in the assay mixture containing DTNB. This long incubation time was necessary to obtain full inhibition of the peroxisomal activity; the residual (mitochondrial) activity was not affected because it remained the same after a 30-min incubation.

Catalase was determined polarographically as described by del Rio *et al.* (8).

Radiochemical β-Oxidation Assay

Incubation

The particulate fraction (0.5 mL corresponding to 20 root tips) was incubated at 25°C in 5 mL of the incubation medium containing 175 mM Tris-HCl (pH 8.5), 0.1 mM NAD, 0.1 mM CoA, 1 mM ATP, 15 mM $MgCl_2$, 10 μg·mL^{-1} leupeptin, 10 μg·mL^{-1} chymostatin, 10 mM α-toluenesulfonyl fluoride, and [U-^{14}C]palmitic acid (73 kBq/assay). The reaction was started by the addition of the particulate fraction and stopped by acidification with 3 mL ice-cold 0.5 M potassium phosphate (pH 3.0), and 0.675 mL 1 mM HCl and setting on ice. Internal standards of palmitoyl-CoA (20 nmol/assay) and [^{3}H]acetyl-CoA (3 kBq/assay) were added to determine the yield of the purification of these acyl-CoAs. The complete medium was then stored at −20°C. Time zero assays were performed by adding the acellular fraction to the incubation medium after cooling and acidification.

Purification of the Acyl-CoAs

The acyl-CoAs were partially purified by solid phase extraction using C_{18} Sep-Pak cartridges (Millipore) as described in ref. 26: the acyl-CoAs were eluted with 4 mL of a solution of 0.01 M tetrabutylammonium phosphate in methanol:water (90:10, v/v), pH 5.3, by formation of an ion pair (3). The eluate was collected into a glass tube and concentrated to 0.1 mL under vacuum (Speed Vac). An aliquot (usually 40 μL) was applied to the HPLC column.

[1] Abbreviation: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

HPLC Analysis of the Acyl-CoAs

The acyl-CoAs were analyzed by HPLC as previously described (26): the acyl-CoAs with carbon atom numbers from 2 to 16 were well separated. Fractions of 0.5 mL were collected, and the radioactivity was counted in a Packard scintillation counter with the conventional $^3H/^{14}C$ dual-label protocol. The recovery of palmitoyl-CoA, calculated from the area of the palmitoyl-CoA peak on the HPLC chromatogram, was close to 80%. The recovery of acetyl-CoA, calculated from the recovery of ^3H radioactivity, was close to 55%.

Protein Assay

Protein was determined by the method of Lowry *et al.* (22) with BSA as standard.

RESULTS

The decrease in total fatty acids and soluble carbohydrates during glucose starvation is shown in Figure 1. After 24 h of starvation, the total fatty acid level had reached 60% of its initial value and was still decreasing, whereas the level of carbohydrates remained steady at 18% of its initial value.

Oxidation of [U-^{14}C]Palmitic Acid by Root Tips

As a preliminary test of β-oxidation activity in maize root tips, the oxidation of [U-^{14}C]palmitic acid to $^{14}CO_2$ by control or starved root tips during a 2-h incubation was compared. The results are shown in Figure 2. Labeled CO_2 was produced in all cases. The amount of $^{14}CO_2$ produced by control root tips (aged in the presence of glucose) was not changed after 4 h and then underwent a 30% decrease after 24 h compared with nonstarved tips. With starved root tips, the amount of $^{14}CO_2$ evolved showed a 64% increase above the initial value after 4 h of starvation and a 2.5-fold increase after 24 h of starvation.

Activity of Individual β-Oxidation Enzymes

The activities of β-oxidation enzymes in the crude extract and the particulate fraction of nonstarved root tips and those starved for 24 h were compared. The results are reported in Table I.

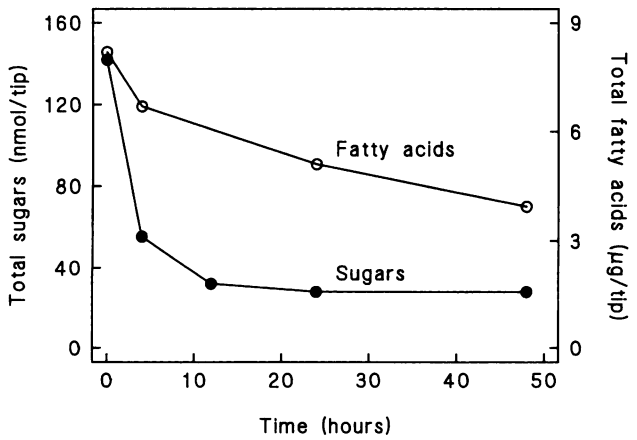

Figure 1. Changes in total fatty acids (○) and total sugars, expressed as hexose equivalent (●), in excised maize root tips during glucose starvation.

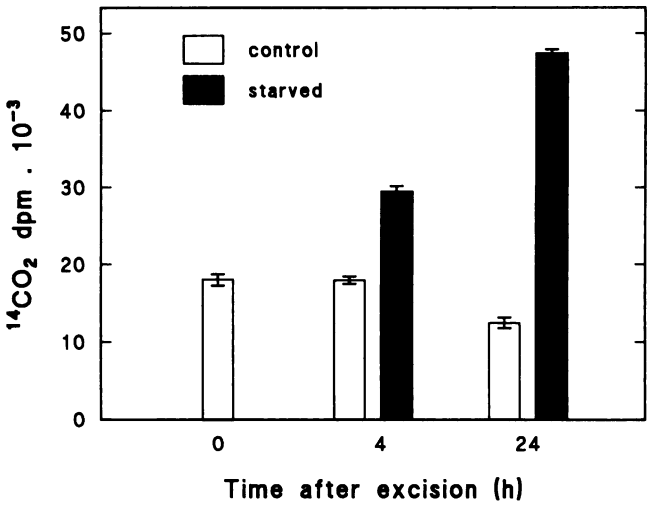

Figure 2. Production of $^{14}CO_2$ from [U-^{14}C]palmitic acid by maize root tips. Nonstarved, control, or starved 3-mm root tips were incubated for 2 h in the presence of [U-^{14}C]palmitic acid; $^{14}CO_2$ was collected on KOH and counted as indicated in "Materials and Methods." Results are the means and range of duplicate samples; similar results were obtained in two different experiments.

The activities of crotonase and 3-hydroxyacyl-CoA dehydrogenase were found to increase in both the crude extract and the particulate fraction after glucose starvation. The 3-oxoacyl-CoA thiolase activity of the particulate fraction also increased. However, the thiolase activity in the crude extract showed a different behavior: in nonstarved roots it was higher (528 nmol·min^{-1}·50 tips^{-1}) than that of any of the other β-oxidation enzymes, and it decreased after starvation. This activity may be due mainly to the cytosolic thiolase, which catalyzes the formation of acetoacetyl-CoA, the precursor of terpene synthesis (31). The acyl-CoA oxidase activity was at the detection limit in either the crude extract or the particulate fraction, even after glucose starvation.

Catalase and Glyoxylic Acid Cycle Activities

The activities of catalase and malate synthase in the particulate fractions of nonstarved root tips and those starved for 24 h are reported in Table I. Catalase activity increased from 1900 to 2600 nmol·min^{-1}·50 tips^{-1} during glucose starvation. Malate synthase activity was detectable in starved root tips but not in nonstarved material. No isocitrate lyase activity was detected in either the crude extract or the particulate fraction, even after glucose starvation (data not shown).

Citrate Synthase Activity

The total and DTNB-insensitive activities of citrate synthase were measured in the particulate fractions from nonstarved and starved root tips. Table II shows that total citrate synthase activity increased from 84 to 164 nmol·min^{-1}·50 tips^{-1} during glucose starvation, whereas the DTNB-insensitive (mitochondrial) activity decreased from 33 to 15 nmol·

$min^{-1} \cdot 50\ tips^{-1}$. These results indicated that the peroxisomal citrate synthase activity, which represented 50 to 70% of total citrate synthase activity in nonstarved root tips, was increased threefold and represented about 90% of the total activity after 24 h of glucose starvation.

Overall β-Oxidation Activity

The radiochemical assay of β-oxidation that has been used in the present work was based on the detection of labeled acetyl-CoA produced from [U-^{14}C]palmitic acid. In preliminary assays performed as described in ref. 26 in the absence of protease inhibitors, a β-oxidation activity was detected in both nonstarved and starved root tips, but the rate of labeled acetyl-CoA production decreased with time, precluding the interpretation of the higher rate observed with the particulate fraction from starved tips.

We found that the amount of palmitoyl-CoA in the experiment described above reached a maximum within 15 to 30 min of incubation and then remained constant (Fig. 3A) and that the recovery of [^{3}H]acetyl-CoA after a 1-h incubation under the conditions of the β-oxidation assay, but without palmitic acid, was 80 to 100% (result not shown). Thus, neither a limitation by the amount of substrate nor the degradation of the product of the reaction were sufficient to explain the decreasing rate of acetyl-CoA production with time. The two serine-protease inhibitors α-toluenesulfonyl fluoride and chymostatin (11) and the cathepsin inhibitor leupeptin (2) have been shown to inhibit efficiently the protease activities in extracts from maize roots and castor bean endosperm, respectively. In our β-oxidation assay, the association of all three inhibitors maintained the linearity for >20 min (Fig. 3B) and increased the production of acetyl-CoA in both starved and nonstarved tips (not shown). No such increase was observed when the protease inhibitors were added to the extraction medium (data not shown).

Table II. *Effect of Glucose Starvation on Citrate Synthase Activities*

Citrate synthase activities were determined on particulate fraction extracted from nonstarved or starved root tips. Total citrate synthase was measured by adding protein last. To measure the DTNB-insensitive activity, proteins were preincubated for 20 min with reaction medium in the presence of DTNB and the reaction was started by the addition of oxaloacetate. Results are the means ± SD of duplicate assays on two different preparations.

Activity	Control	Starved
	nmol/min/50 tips	
Total	84 ± 19	164 ± 20
DTNB insensitive	33 ± 7	15 ± 4
DTNB sensitive	51 ± 22	150 ± 16

In the experiment shown in Figure 3, the rate of acetyl-CoA production, measured during a 20-min period, was higher by a factor of 2.6 in the assay using starved root tips. Although the β-oxidation activity in nonstarved root tips varied considerably according to seed batches (Fig. 3, Table III), an increase of activity was constantly found after glucose starvation. In five experiments, the β-oxidation activity measured in starved root tips was two to five times higher than that in the corresponding nonstarved tips.

β-Oxidation measurements were performed in starved, nonstarved, and control root tips. The results (Table III) indicate that the activity of β-oxidation in control root tips is not significantly different from, or lower than, that of nonstarved root tips. Therefore, the observed increase of β-oxidation can

Table I. *Effects of Glucose Starvation on Enzyme Activities in the Crude Extract and the Particulate Fraction Prepared from Nonstarved or 24-h Glucose-Starved Maize Root Tips*

The amount of extract added to 1 mL of assay medium corresponded to one and two tips for the crude extract and to two and four tips for the particulate fraction. Results are the means ± SD of assays on three different preparations for crotonase, 3-hydroxyacyl-CoA dehydrogenase, and 3-oxoacyl-CoA thiolase and two preparations for catalase, fumarase, and malate synthase.

	Nonstarved	Starved	Starved to Nonstarved Ratio
	nmol/min/50 tips		
Crotonase			
Crude extract	310 ± 60	925 ± 145	2.98
Particulate fraction	140 ± 4	393 ± 63	2.8
3-Hydroxyacyl-CoA dehydrogenase			
Crude extract	120 ± 30	259 ± 25	2.16
Particulate fraction	54 ± 11	129 ± 22	2.39
3-Oxoacyl-CoA thiolase			
Crude extract	513 ± 14	337 ± 73	0.66
Particulate fraction	<2	21 ± 7	>10
Catalase			
Particulate fraction	1900 ± 150	2600 ± 110	1.37
Malate synthase			
Particulate fraction	<0.3	3.9 ± 0.8	>13
Fumarase			
Crude extract	446 ± 12	388 ± 2	0.87
Particulate fraction	226 ± 1	196 ± 4	0.87

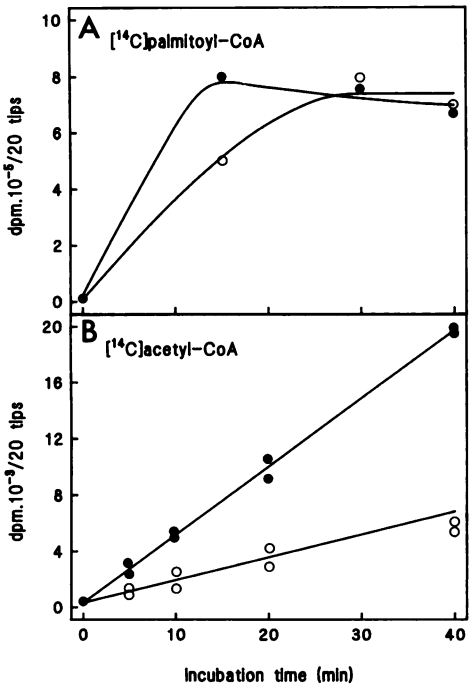

Figure 3. Time course of the formation of palmitoyl-CoA and acetyl-CoA from [U-^{14}C]palmitic acid by a particulate fraction from nonstarved (○) or starved (●) maize root tips. Radioactivities in palmitoyl-CoA (A) and acetyl-CoA (B) were calculated from the radioactivities in the HPLC palmitoyl-CoA and acetyl-CoA fractions, corrected for recovery.

be specifically ascribed to sugar starvation and is not due to the excision stress.

Yield of Organelle Extraction from Nonstarved and Starved Root Tips

Sugar starvation of the root tips could lead to an increased activity of β-oxidation in the particulate fraction either by increasing this activity in the root tips or by improving the yield of organelle extraction. Two methods were used to distinguish between these two possibilities. First, the yield of protein extraction from nonstarved and starved roots was determined by comparing the protein content of the filtrate and the total protein of root tips. The yield was 69 and 81%

Table III. *β-Oxidation Activity in Particulate Fractions from Root Tips*

The particulate fractions were prepared as described in "Materials and Methods" either immediately after excision of the root tips (nonstarved) or from root tips incubated for 24 h in the absence (starved) or in the presence (control) of glucose. Rates were determined from an experiment similar to that described in Figure 3.

	[U-^{14}C]Acetyl-CoA
	dpm/min/20 tips
Nonstarved	843
Starved	5493
Control	582

Table IV. *Yield of Protein Extraction from Root Tips*

Protein content was measured in root tips ground either in a rough mortar (total protein) or in a smooth mortar as described in "Materials and Methods" for the preparation of the particulate fraction (crude extract) except that BSA was omitted. Results are the means ± SD of two independent experiments with duplicate samples.

	Total Protein	Crude Extract	Yield
	mg/50 tips		%
Nonstarved	7.9 ± 1	5.4 ± 0.4	69 ± 4
Starved	5.8 ± 0.5	4.7 ± 0.5	81 ± 2

for nonstarved and starved root tips, respectively (Table IV). This small difference could not explain the two- to fivefold increase of the β-oxidation activity.

The recovery of crude extract enzyme activities in the particulate fraction can be calculated from the data given in Table I. For crotonase, 3-hydroxyacyl-CoA dehydrogenase, and fumarase activities, the recovery is close to 45% in both nonstarved and starved roots. Thus, the increased activity of these enzymes in the particulate fraction of starved maize root tips can be ascribed to an increase of activity in the tissue rather than to artifactual differences in the recovery of enzyme. This also applies to the activities of thiolase, catalase, and malate synthase (Table I). The fact that the activities of β-oxidation increase and that of fumarase decreases in both the crude extract and the particulate fraction as a result of starvation confirms this conclusion.

DISCUSSION

In this paper, we compared the activities of β-oxidation in maize root tips before and after 24-h glucose starvation using assays of individual enzymes and a radiochemical assay of the overall β-oxidation pathway.

The increase of the activities of the different enzymes of β-oxidation and of the overall β-oxidation that was found *in vitro* after 24 h of starvation can be ascribed to increased activity in the tissue rather than to higher yield of enzyme extraction, for the following reasons. (a) The recovery of enzyme activities is similar with nonstarved and starved root tips because (i) the efficiency of grinding, determined as the recovery of total protein, was only marginally better for starved (81%) than for nonstarved (69%) root tips (Table IV) and (ii) the recovery of 3-oxoacyl-CoA-dehydrogenase and crotonase activities in the particulate fraction was not different with nonstarved or starved root tips. (b) It explains the increased conversion of [U-^{14}C]palmitic acid to $^{14}CO_2$ in the *in vivo* assay and suggests that this oxidation occurred, at least in part, by β-oxidation.

Previous work (13) showed that the β-oxidation enzymes are localized in the peroxisomes of maize root cells. Although we do not exclude the possibility that a β-oxidation activity is also present in the mitochondria (23), it appears likely that the activities observed in the present work are peroxisomal because the assay conditions used (high CoA concentration and absence of carnitine in the overall β-oxidation assay, high osmotic pressure of the media in all enzyme assays) would not allow the detection of the mitochondrial β-oxidation

activities (32). Moreover, the simultaneous increase of catalase, an ancillary enzyme of β-oxidation that degrades the H_2O_2 formed at the oxidase step, and of malate synthase, a glyoxylate cycle enzyme, suggests that glucose starvation is associated with a general increase of peroxisomal activities.

It is noteworthy that the increase of the peroxisomal activities occurs at the same time as enzyme activities of carbohydrate metabolism decrease (5).

In anise cell cultures under sugar deprivation, the activities of the glyoxylate cycle enzymes increase but the levels of catalase and citrate synthase decrease or, if acetate is added, are maintained at their initial level (21). More recently, an increase of some peroxisomal activities was found in senescing leaves (7, 16) and carnation petals (10). Because sugar starvation occurs during the senescence of leaf tissues (19), it is possible that the stimulation of glyoxysomal or peroxisomal enzymes in senescing leaves or petals is specifically related to the situation of sugar starvation as it is in root tips. However, the response appears to differ according to tissues or species: an increased β-oxidation activity was observed in the case of rice leaves, as in maize root tips, but not in sugar beet leaves or pumpkin cotyledons (7). In all the senescing leaves examined (7, 16), an increase of both malate synthase and isocitrate lyase activities was observed, thus suggesting that fatty acids could be utilized for gluconeogenesis through the glyoxylate cycle. In maize roots, however, the absence of any detectable isocitrate lyase makes it unlikely that the glyoxylate cycle operates. Therefore, the role of β-oxidation in maize roots may be to provide acetyl-CoA directly for respiration. The carbon pathway into the tricarboxylic acid cycle during sugar starvation in maize root tips may resemble that physiologically present in germinating lettuce seeds (29). Because proteins are actively degraded in sugar-starved maize roots, it may be suggested that the malate synthase activity is involved in the catabolism of glycine by incorporating glyoxylate, the product of glycine transamination, into malate (24).

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