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Compartmental Utilization of Carboxyl-^{14}C-tripalmitin by Tissue Homogenate of Pine Seeds[1]

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TE MAY CHING
Seed Laboratory, Oregon State University, Corvallis, Oregon 97331

ABSTRACT

A tissue homogenate of megagemetophyte of germinating seeds of Jeffrey pine (*Pinus Jeffrii* Grev. and Balf.) was incubated with sonication-dispersed and albumin-carried ^{14}C-tripalmitin in order to elucidate the sequential and quantitative role of cellular organelles in utilizing lipid reserve in seeds. After 5 minutes at 30 C, 25% of the tracer was localized in the fat body fraction, 9% in the pellet containing mitochondria and glyoxysomes, 14% in the supernatant, and 2% was found as CO_2. Radioactivity increased with time of incubation in the latter three fractions indicating the forward direction of utilization. Fat bodies contained mainly lipases and hydrolyzed the tracer to palmitate with diglyceride and monoglyceride as intermediates. About two-thirds of the palmitate had left the fat bodies in 5 minutes and entered the pellet fraction within which the tracer was distributed 1:2 in mitochondria and glyoxysomes, respectively. Longer incubation reduced the ratio to 1:3 while both organelles acquired more radioactive intermediates. Labeled acetyl-CoA and intermediate of β-oxidation were found in both organelle-containing fractions. The supernatant fraction contained radioactive diglycerides, monoglycerides, palmitate, sterol esters, and phospholipids, indicating lipase activity and direct utilization of fatty acid for the synthesis of sterol esters and polar lipids.

Germinating fatty seeds hydrolyze reserve triglycerides to fatty acids and glycerol by lipases associated with fat bodies or spherosomes (4, 13, 15, 16, 23). Where and how the fatty acids are subsequently utilized have never been traced even though enzymes of the β-oxidation sequence were found to be mainly localized in glyoxysomes of castor bean endosperm (7, 11) and in mitochondria of pine seeds (21). This study uses labeled substrate to discern the sequential and quantitative role of cellular organelles in utilizing lipid reserve in megagametophyte of germinating seeds of Jeffrey pine.

Since the gametophytic tissue does not have a circulatory or vascular system for transporting the substrate as in animal organs or leaves, tissue homogenate was used in this study to facilitate the distribution of the tracer. Furthermore, tissue homogenates have been successfully used for the study of the oxidation of palmitate in heart (18), the formation of palmitylcarnitine in liver (9), and the hydrolysis of triglyceride in fir seed (4).

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MATERIALS AND METHODS

Materials. In a preliminary study it was observed that the megagametophyte, the storage tissue of the seeds in Jeffrey pine (*Pinus Jeffrii* Grev. and Balf.), contains on the average 50% lipids, 20% nitrogenous compounds, and 2% starch and sugars. During germination, lipids were utilized with a small increase of sugars in the tissue in a manner similar to what was observed in ponderosa pine seeds (6). Ultrastructurally, changes were found identical to those which occurred in germinating ponderosa pine (6). These changes included an increase in the number of mitochondria and glyoxysomes, a decrease in the size and number of fat bodies, and a fragmentation and solubilization of protein bodies. The chemical and structural changes indicated an active lipolysis and gluconeogenesis in this species, but the seeds are three to four times larger than ponderosa pine seeds and less time consuming in obtaining experimental material. Therefore, three lots of Jeffrey pine seeds with high germinability were chosen for the intended study.

Seeds were soaked in water for 2 hr, stratified or chilled at 4 C for 3 weeks to break their dormancy, and then germinated for 10 days at a daily cycle of 30 C for 8 hr in light and 20 C for 16 hr in the dark. Megagametophytes were dissected from the germinated seedlings, washed with 1% sodium hypochlorite, and chilled at 4 C.

Subsequent procedures were conducted at 0 to 4 C unless specified. Glassware was dry-sterilized at 150 C for 30 min. All solutions were filtered through a nitrocellulose membrane (0.45 μ in pore size, Brinkmann Instruments, Inc.). Polyethylene tubes and utensils were disinfected by wiping with 70% ethanol and air-drying prior to use.

Preparation and Incubation. Seven grams of megagametophytes were cut in to small pieces in 10 ml of homogenizing medium and gently ground to a smooth paste in a mortar with pestle. The homogenizing medium consisted of tris-HCl buffer, 50 mM, pH 7.5, 0.4 M sucrose, 10 mM dithiothreitol, 10 mM potassium chloride, 1 mM disodium EDTA, 0.5 mM magnesium chloride, and 0.1% bovine serum albumin. The homogenate was filtered through eight layers of cheesecloth which had been washed and saturated with the medium. The filtrate was centrifuged at 500*g* for 10 min to sediment nuclei, cell walls, debris, whole cells, and aggregates. The top fat layer was removed to reduce the endogenous substrate. The supernatant was mixed with 5 μc of carboxyl-^{14}C-tripalmitin (21 mc/mmole) which had been dispersed by sonication in 1 ml of homogenizing medium (14). One-tenth of the mixture (about 1.5 ml) was transferred to a 15 ml Warburg flask and incubated at 30 C with shaking. After incubation 1 ml of 2 N H_2SO_4 was added into the reaction mixture, and the $^{14}CO_2$ evolved was collected in 0.2 ml of 2 N KOH adsorbed on a paper wick in the center well, which was then washed free of radioactivity.

The washes were combined with the KOH and paper wick and made to a total volume of 5 ml. An aliquot was counted in the Bray's solution. The remaining nine-tenths of the mixture was incubated in a flask at 30 C with shaking for a designated time and fractionated.

Four samples for each seed lot were run simultaneously with one sample each incubated for 0, 5, 10, or 20 min. The total recovery of administered tracer was 15 to 22% (about 1.6–2.3 $\times$ 10^6 dpm) in each sample. The low recovery was due partly to the number of steps involved in the analytic procedures and partly to the tenaciously adhesive nature of the substrate that resulted in a substantial loss on the glassware.

Fractionation. After incubation, the reaction mixture was centrifuged at 10,000*g* for 20 min to sediment the pellet containing mainly mitochondria and glyoxysomes (10K-pellet). The fat layer was carefully removed by a spatula, the postpellet supernatant was collected by a syringe, and the pellet was suspended in 1 ml 32% sucrose containing 1 mM DTT and 1 mM disodium EDTA. The pellet suspension was layered over a discontinuous sucrose density gradient containing 9 ml each of 60, 55, 50, 45, 40, and 35% (w/w) sucrose. All the sucrose solutions contained 1 mM dithiothreitol and 1 mM Na_2 EDTA. The gradient tube was centrifuged at 105,000*g* for 5 hr, and 50 tubes of 1 ml each were collected from the top with a density gradient fractionator.

One hundred microliters of the contents of odd numbered tubes were counted in Bray's solution in a Packard liquid scintillation spectrometer Model 3375, and an internal standard was added to calculate the counting efficiency. A low efficiency of 25 to 39% was generally obtained in the concentrated sucrose density gradient fractions. The counting efficiency was improved to 42 to 55% when Triton X-100-Toluene was used as a counting solvent (17). Protein content, specific activity of fumarase (the marker enzyme for mitochondria), and isocitrate lyase (the marker enzyme for glyoxysomes) were also determined in these fractions (6). Based on density, protein content, and marker enzyme activities, the remaining contents of the tubes were pooled into four fractions: top layer, mitochondria, "proplastids" (mainly aggregates of mitochondria and glyoxysomes), and glyoxysomes (6). One volume of distilled water was added to each fraction to reduce the density, thus facilitating further extraction. Two extraction methods were applied. One was an extraction of the fractions with a two-phased system of 86% ethanol and petroleum ether (boiling point 30–60 C) to partition the polar and nonpolar compounds, respectively (10). The second method used dilute perchloric acid to extract acetyl-CoA and chloroform-methanol for lipids including acyl-CoA (22). The second method was used throughout the experiments since it was more reproducible and gave better partition.

Concentrated perchloric acid (70%) was added to the diluted fractions to a final concentration of 0.25 N to extract soluble metabolites and acetyl-CoA. The perchloric acid extract was separated by centrifugation from the insoluble residue containing acyl-CoA. The polar as well as the nonpolar lipids in the insoluble residue was extracted with 10 ml of chloroform-methanol (2.1 v/v). An aliquot of the acid and the lipid extract was counted in the Bray's solution. An average counting efficiency of 48% was obtained in the acid extracts and 70% in the lipid extracts.

The fat layer and postpellet supernatant were similarly extracted first with 10 to 15 ml of 0.25 N perchloric acid and then with 10 ml of chloroform-methanol. An aliquot of the extracts was counted in Bray's solution.

Identification of Reaction Products. An aliquot of chloroform-methanol extract, containing 2,000 to 4,000 dpm, was separated on a thin layer of Silica Gel G using a solvent system of hexane-diethylether-acetic acid (70.30.1 v/v 4, 5). Hydrocarbons, sterol esters and waxy esters, waxy alcohols, triglycerides, free fatty acids, diglycerides, sterols, monoglycerides, and polar lipids were resolved in descending order by this developing solvent. Standards were co-chromatographed with samples, and the separated spots were then stained with iodine vapor, scraped off the plate, and counted in toluene containing 0.4% 2,5-diphenyloxazole and 0.01% 2,2 paraphenylene-bis-5-phenyloxazole. The counting efficiency of these samples averaged about 85%.

The remaining chloroform-methanol extract was streaked on a Silica Gel G thin layer plate which was then developed in hexane-diethylether (1.1 v/v). Polar lipids, including acyl-CoA, remained at the origin which was scraped off and repeatedly extracted with 95% ethanol. The ethanol extract was saponified, and the fatty acids were recovered from the soap solution, methylated with diazomethane, and separated by the gas-liquid chromatographic technique (3) using a Beckman GC-4 and a Nuclear Chicago ionization detector Model 4998.

The perchloric acid extract was neutralized with KOH, and the precipitate was removed by centrifugation. The neutralized extract was lyophilized, dissolved in a small volume of water, and spotted on a cellulose thin layer plate (0.25 mm in thickness). The plate was developed in two solvent systems: 95% ethanol-1 M sodium acetate, pH 3.8 (7.3 v/v) and isobutyric acid-1 M NaOH-water (57.4.39 v/v) (19). The identification of acetyl-CoA was accomplished by co-chromatographing with a known standard (PL Biochemical, Inc.) and by the red color with the nitroprusside reagent (20). The thin layer chromatogram was scanned in a Packard Radiochromatogram scanner, Model 7201.

RESULTS AND DISCUSSION

Distribution of Radioactivity. The average distribution of total recovered radioactivity from three seed lots is summarized in Figure 1. The variation among samples was generally less than 20% of their mean in different fractions. Only one sample of 0 time homogenate was analyzed, as no radioactive intermediates were found in extracts of various fractions. A distribution of 89% radioactivity in fat layer, 1% in 10K-pellet, 10% in postpellet supernatant, however, was observed in 0 time homogenate. This distribution probably indicates the extent of nonspecific adsorption of the tracer on endogenous lipids, soluble proteins, and organelles.

After 5 min of incubation, as shown in Figure 1, 70% of the radioactivity was associated with the fat layer which contained mainly light fat bodies and their conjugated lipases (4). Of the 70% tracer found in this fraction, about 25% was being metabolized and 45% remained intact. The reaction products characteristic of lipases, such as labeled diglyceride, monoglyceride, and fatty acids, were observed. If this compartment had no outlet, fatty acid would be accumulated to at least 12% of the recovered radioactivity for the observed 3% radioactive monoglyceride and 12% radioactive diglyceride. Instead only 4% of the labeled fatty acid was found, indicating that at least two-thirds of the reaction product was transported to the next compartment of triglyceride utilization. The postpellet supernatant consisted of heavy fat bodies with their associated lipases (4), microsomes, ribosomes, and soluble enzymes. Approximately 19% of the tracer was located in this fraction, of which only 5% remained as the original substrate.

Utilization of fatty acid in this fraction and depletion from this fraction was much more rapid than the fat layer. Only 0.5% of the labeled fatty acid was detected instead of 4.5% based on the radioactivity of diglyceride and monoglyceride found in this fraction. It is, then, clearly shown that disap-

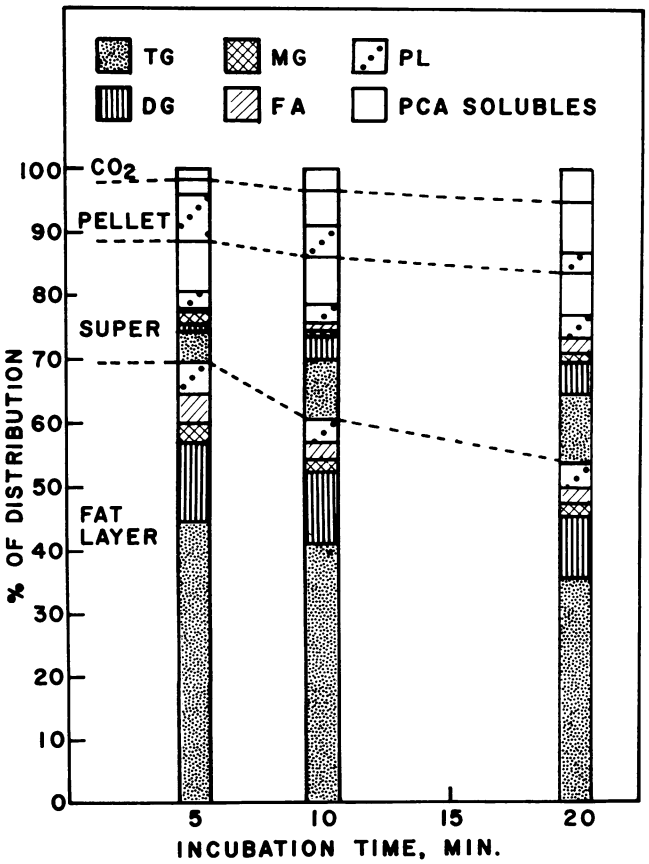

FIG. 1. Percentage distribution of radioactivity recovered in various fractions of tissue homogenate after 5, 10, and 20 min of incubation at 30 C with carboxyl-^{14}C-tripalmitin. TG: triglyceride; DG: diglyceride; MG: monoglyceride; FA: fatty acids; PA: polar lipids; PCA: perchloric acid; pellet: 10,000g pellet; super: postpellet supernatant; fat layer: top fatty layer after the homogenate had been centrifuged for 10 min at 10,000g.

pearance of at least nine-tenths of the hydrolyzed fatty acids was observed after 5 min in the postpellet supernatant. The 10K-pellet consisted mainly of mitochondria and glyoxysomes (6). In the 10% of the tracer associated with this fraction, about one-fifth was already metabolized to compounds soluble in perchloric acid, indicating the rapidity of fatty acid oxidation in this fraction. The fact that 2% radioactivity was found as CO_2 in 5 min further substantiates that conclusion. From all these observations it is shown that after 5 min of incubation at 30 C, of the 50% tracer entered into the metabolic stream, 39% was distributed in heavy and light fat bodies, 9% in 10K-pellet, and 2% in CO_2.

After 10 min of incubation, the proportion of tracer entering the metabolic stream had increased to 52% with a distribution of 37% in fat bodies, 11% in 10K-pellet and 4% as CO_2. Under the conditions applied, these findings indicate a direction of feeding forward of the utilization of triglyceride in this tissue homogenate. However, a slowdown of administered triglyceride entering into the metabolic pools is shown. An extension of this feeding forward was further illustrated in the material incubated for 20 min, with a division of 39% in the fat bodies, 12% in the 10K-pellet, and 5% in the CO_2. The rate of utilization, however, was slower than in the first 10 min. The $^{14}CO_2$ production rate in the first 5 min was 0.5% of the total radioactivity per min, the second 5 min was 0.2% per min, and during the second 10 min the rate dropped to 0.09% per min (Fig. 1). This reduction of $^{14}CO_2$ production rate could not happen if a steady state of metabolism had been maintained. Enzyme degradation, organelle dissociation, and limitation of coenzyme and energy supply might cause the reduction in this *in vitro* system.

Participation of Mitochondria and Glyoxysomes in Oxidation of Fatty Acids. Figure 2 summarizes the average percentage of radioactivity distributed in the various fractions of 10K-pellets separated in a discontinuous sucrose density gradient. After 5 min of incubation, 5.5% of the tracer was found in the mitochondrial fraction, 4% in the proplastids and aggregates, and 10.5% in the glyoxysomal fraction. The distribution in mitochondrial fraction was increased to 13% after 10 min of incubation and to 15% after 20 min. The percentage was raised, respectively, to 19% and 18.5% in aggregates fraction and 36.5% and 49.5% in glyoxysomal fraction. The ratio of the tracer found in mitochondrial to that of glyoxysomal fraction amounts to approximately 1:2 after 5 min of incubation and 1:3 after 10 and 20 min. The consistent ratio observed after 10 and 20 min of incubation probably is not indicative of an attainment of the steady state generally found in intact tissues, but rather it is partly because of a pile-up of labeled metabolites. The results observed after 5 min of incubation probably resembles that of *in vivo* conditions since the integrity of organelles remained (Fig. 3), and the specific activities of the respective marker enzymes of mitochondria and glyoxysomes did not change from that of 0 time (Figs. 4 and 5). Therefore, the best estimation of *in vivo* participation in oxidizing fatty acids by mitochondria and glyoxysomes would be 1:2 in this gametophytic tissue (Fig. 6). In the endosperm of germinating castor bean seeds, the total activity of β-oxidation of palmitic acid amounted to a ratio

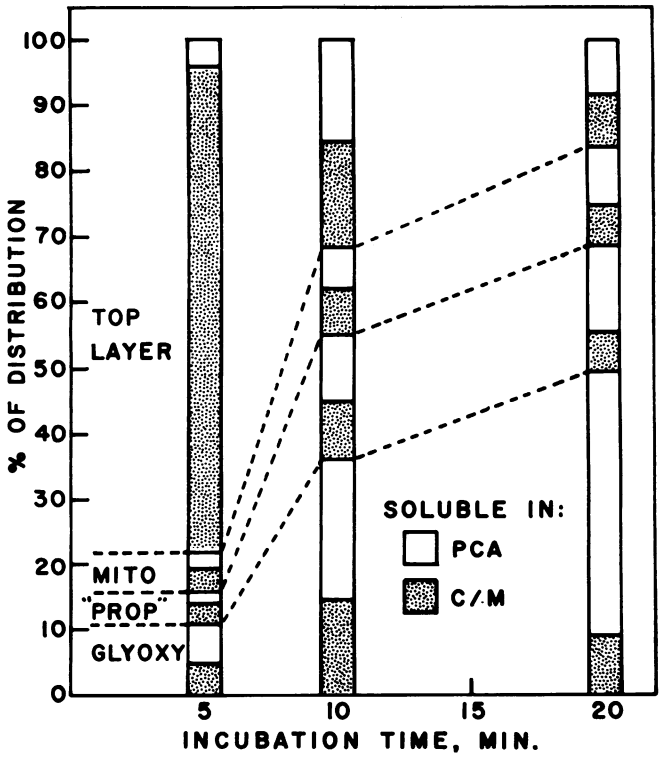

FIG. 2. Percentage distribution of radioactivity in extracts of different fractions separated by sucrose density gradient from 10K-pellet of tissue homogenate incubated with carboxyl-^{14}C-tripalmitin for 5, 10, and 20 min at 30 C. Top layer: the portion above mitochondrial band; Mito: mitochondrial fraction; "Prop": proplastids and aggregates; Glyoxy: glyoxysomal fraction; PCA: perchloric acid, 0.25 N; C/M: chloroform-methanol (2.1 v/v).

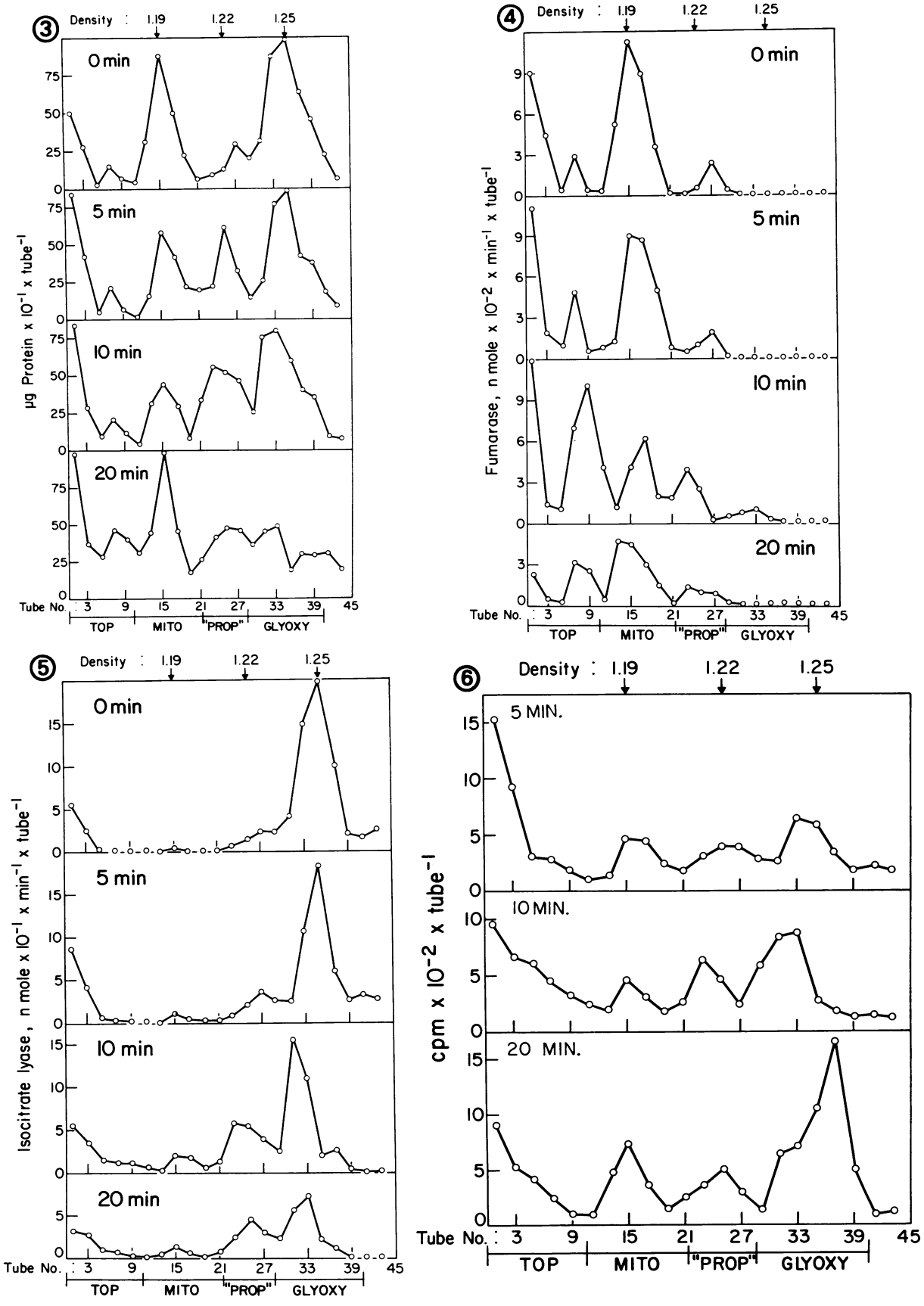

FIG. 3. Changes in protein distribution of 10K-pellet in a discontinuous sucrose density gradient after 5, 10, and 20 min of incubation of tissue homogenate with carboxyl-^{14}C-tripalmitin at 30 C.

FIG. 4. Changes in fumarase distribution of 10K-pellet in a discontinuous sucrose density gradient after 5, 10, and 20 min of incubation of tissue homogenate with carboxyl-^{14}C-tripalmitin at 30 C.

FIG. 5. Changes in isocitrate lyase distribution of 10K-pellet in a discontinuous sucrose density gradient after 5, 10, and 20 min of incubation of tissue homogenate with carboxyl-^{14}C-tripalmitin at 30 C.

FIG. 6. Changes in radioactivity distribution of 10K-pellet in a discontinuous sucrose density gradient after 5, 10, and 20 min of incubation of tissue homogenate with carboxyl-^{14}C-tripalmitin at 30 C.

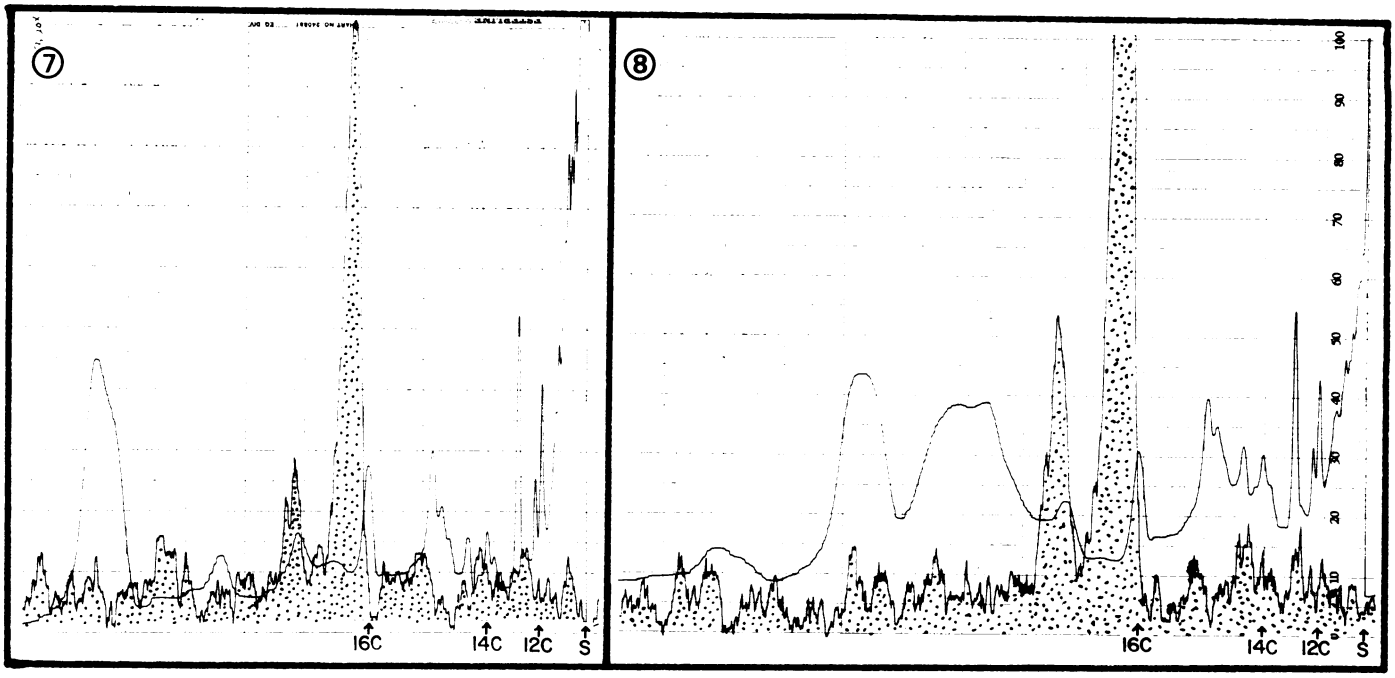

FIG. 7. Gas-liquid chromatogram of methyl ester of fatty acids extracted from mitochondrial fraction isolated from tissue homogenate incubated with carboxyl-^{14}C-tripalmitin for 10 min at 30 C. Arrows point at the retention time of saturated fatty acids of indicated carbon length; S-solvent front. The upper curve shows mass peaks, and the lower curve radioactivity. The mixture of fatty acids was separated by a 120 cm × 1.5 mm inner diameter glass column of 3% OV17 on gas-chrome Q, with a hydrogen flame ionization detector at a column temperature 180 C, inlet 260 C, and detector 220 C; flow rate of air 17 p.s.i., hydrogen 16 p.s.i., and argon 28 p.s.i.

FIG. 8. Gas-liquid chromatogram of methyl ester of fatty acids extracted from glyoxysomal fraction isolated from tissue homogenate incubated with carboxyl-^{14}C-tripalmitin for 10 min at 30 C. Conditions as in Figure 7.

of 1:2 in isolated mitochondrial and glyoxysomal fractions (11), even though purified mitochondria of the same tissue were incapable of oxidizing fatty acids (7) and did not have fatty acid activating enzymes (8). In our material, not only was the distribution of radioactivities consistent in different seed lots, but also the pattern of fatty acid intermediates was similar in mitochondria and glyoxysomes (Figs. 7 and 8). Furthermore, the contamination of glyoxysomes in the mitochondrial fraction was very low as indicated by the distribution of specific activity of isocitrate lyase (Fig. 5). Thus, the mitochondrion of pine seeds is capable of oxidizing fatty acids to the extent of about one-half of the glyoxysomal oxidation.

The regeneration rate of coenzyme A and ATP was probably also decreased after 5 min of incubation. Partial enzyme inactivation and gradual dissociation of organelles were most likely caused by proteases which are commonly present in seed storage tissue (1) and remain active in the tissue homogenate. These degradations are clearly shown in Figures 3 to 5. At 0 min of incubation, the profile of protein (Fig. 3) and marker enzymes (Figs. 4 and 5) demonstrates a typical composition of 10K-pellet either from castor bean endosperm (2) or pine megagametophyte (6). After 5 min of incubation, the profile of sucrose density gradient exhibited a large increase of soluble protein at the top of the sucrose density gradient, a slight increase of broken mitochondria at the first peak with a reduction of mitochondrial fraction at a peak density of 1.19 g/ml, a substantial increase of aggregates located in a band around a density of 1.22 g/ml and followed by a reduction of glyoxysomal fraction. After 10 min of incubation, the bands in the sucrose density gradient still remained; however, the band width increased, indicating a swelling of the organelles and a loosening of protein molecules. Some mitochondrial fumarase leached out, therefore a large amount was found in the soluble portion and even in the glyoxysomal fraction (Fig. 4). Mitochondrial fragments possessed the highest fumarase activity, implying some mitochondria broke down before the constituent enzymes (*e.g.*, fumarase) became degraded. Isocitrate lyase, on the other hand, seemed to disintegrate with the glyoxysome, as no major migration of enzyme activity was observed. Enzyme inactivation was definitely shown in materials incubated after 20 min as the average specific activity of fumarase in the mitochondrial fraction was 120% of that of 0 time material after 5 min of incubation, 105% after 10 min and 35 to 60% after 20 min. Isocitrate lyase exhibited a similar change in the time course of incubation except less reduction after 20 min of incubation (75–80% of the original specific activity).

In spite of the partial dissociation of enzymes and organelles, the uptake of tracer increased steadily with time into mitochondria and glyoxysomes (Fig. 6). This increase probably is attributable to the pile-up of intermediates.

Detection of Reaction Products. A typical thin layer chromatogram of lipids extracted from the postpellet supernatant after 5 min of incubation is shown in Figure 9. The presence of radioactive polar lipids (mainly phospholipids and glycolipids-P), monoglycerides, diglycerides, free fatty acids, triglycerides, and sterol esters indicates that enzymes for synthesis of phospholipids, glycolipids and sterol esters and lipases are active in this fraction. An average radioactivity distribution of 25, 19, 12, 4, 30, and 10%, respectively, was observed in the lipid extract of postpellet supernatant after 5 min of incubation. The radioactivity increased gradually with incubation time in all fractions as more tracer was associated with the supernatant. Relative distribution, however, was reduced in polar lipids and monoglycerides.

The lipids from the top layer of sucrose density gradient of the 10K-pellet showed similar lipid classes as the postpellet supernatant in thin layer chromatograms, except the distribu-

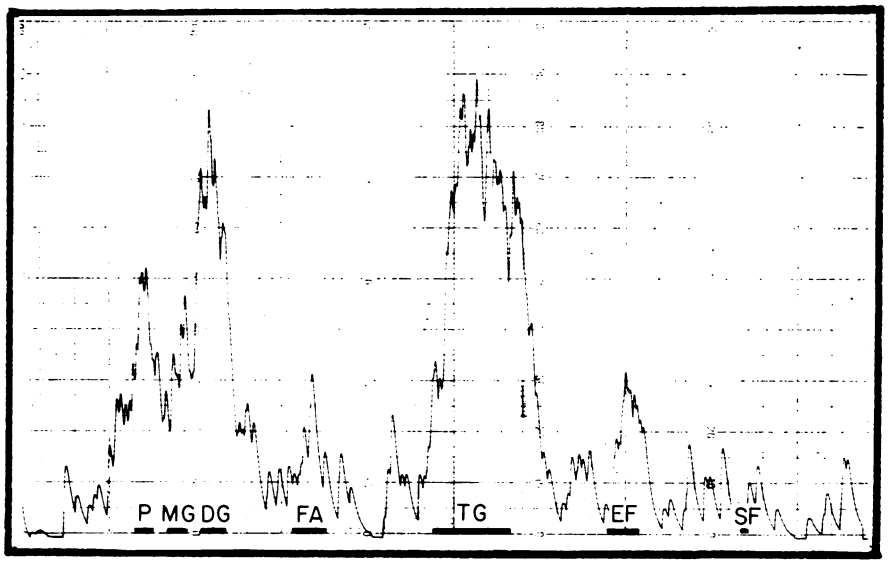

FIG. 9. Distribution of radioactivity in thin layer chromatogram of lipid extract of the supernatant incubated for 5 min with carboxyl-^{14}C-tripalmitin. SF: solvent front; EF: sterol esters; TG: triglycerides; FA: free fatty acids; DG: diglycerides; MG: monoglycerides; PL: polar lipids; solvent system: hexane-diethyl-ether-acetic acid (70.30.1 v/v).

tion was 42, 16, 14, 24, 3, and 1% for polar lipids, monoglycerides, diglycerides, fatty acids, triglycerides and sterol esters, respectively. The radioactivity decreased with incubation time in all fractions as the tracer enters into organelles for further utilization (Fig. 2).

A gas chromatogram of the fatty acids extracted from the mitochondrial fraction after 10 min of incubation is presented in Figure 7. The upper curve shows the mass peaks and the lower curve the radioactivity. Owing to the arrangement of detectors, a slight delay of timing in recording the radioactive peak is shown in the chromatogram. Nevertheless, one peak of 1-^{14}C-palmitate and one of its derivatives were observed in the radioactivity profile. At least 26 structural, as well as metabolic intermediates of fatty acid oxidation, were found in the mass profile of the mitochondria. The fatty acid composition in glyoxysomes again indicated the presence of 1-^{14}C-palmitate and one derivative in the radiochromatogram, and 22 fatty acids in the mass chromatogram (Fig. 8). Since carboxyl-^{14}C-tripalmitin was used as tracer, only 1-^{14}C-palmitate and its β-oxidation intermediates could be detected in the radiochromatograms. The two observed probably are 1-^{14}C-palmitate and 1-^{14}C-palmitoleate since β-hydroxylpalmitate and β-ketopalmitate would have different retention times. The component fatty acids showed some difference between mitochondria and glyoxysomes. Identification of these fatty acids awaits mass spectrometric analysis of gas-chromatographically separated components. A detailed study comparing their membranous and metabolic components would be of interest in characterizing these two organelles.

Acetyl-CoA was detected in the perchloric acid extracts of both mitochondria and glyoxysome on thin layer chromatograms by the reaction of nitroprusside and NaOH and by the R_F values of co-chromatographed acetyl-CoA in different solvent systems (Fig. 10 and 11). Radioactivity, however, was not exclusively associated with acetyl-CoA, and no quantitative trend was observed with the time of incubation. A more specific isolation procedure of acetyl-CoA, such as that used by Jones and Nelson (12), probably will eliminate the interference of other radioactive compounds in the perchloric acid extract. Other components in the perchloric acid extracts, such as the intermediates of glyoxylate and tricarboxylic acid cycles, should be identified (2, 7). Unfortunately, the extracts

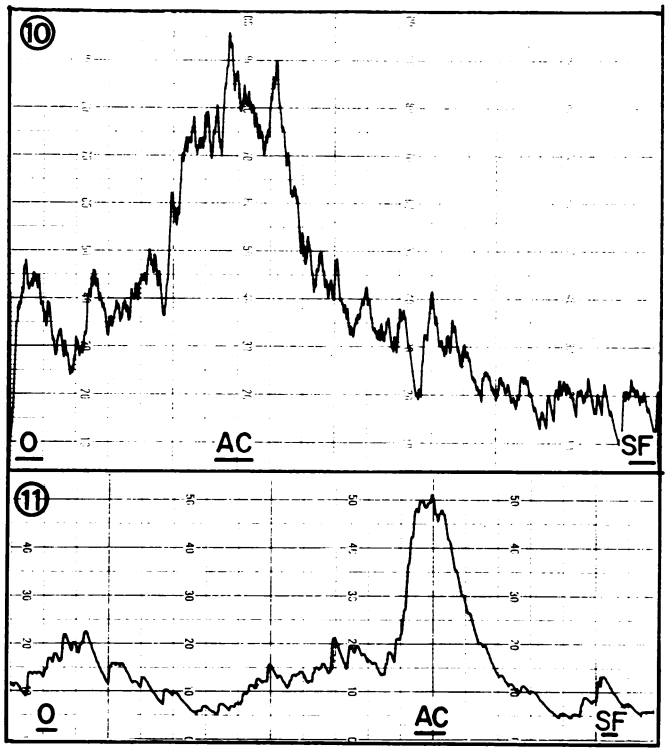

FIG. 10. Distribution of radioactivity on cellulose thin layer chromatogram of perchloric acid extract of mitochondrial fraction isolated from tissue homogenate incubated with carboxyl-^{14}C-tripalmitin for 5 min at 30 C. Solvent system: 95% ethanol-1 M sodium acetate, pH 3.8 (7.3 v/v). Scanning conditions: sensitivity range: 100 cpm; counting time constant: 30 sec; window opening: 10 mm; scanning speed: 1 cm/min. O: origin; AC: acetyl CoA; SF: solvent front.

FIG. 11. Distribution of radioactivity on cellulose thin layer chromatogram of perchloric acid extract of glyoxysomal fraction isolated from tissue homogenate incubated from carboxyl-^{14}C-tripalmitin for 5 min at 30 C. Solvent system: isobutyric acid-1 M NaOH-water (57.4.39 v/v). Scanning conditions: sensitivity range: 300 cpm; counting time constant: 30 sec. Window opening: 7.5 mm; scanning speed: 2 cm/min. O: origin; AC: acetyl CoA; SF: solvent front.

from this study were too low in radioactivity to be of value for further fractionation.

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