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Diacylglycerol Acyltransferase in Maturing Oil Seeds of Maize and Other Species[1]

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ABSTRACT

Diacylglycerol acyltransferase (EC 2.3.1.20) activity was detected in the microsomal fractions of maturing maize scutellum, soybean cotyledon, peanut cotyledon, and castor bean endosperm. The activity detected was high enough to account for the *in vivo* rate of triacylglycerol synthesis. The activity of the maize enzyme was characterized using diolein micelles prepared by sonication in Tween 20 as the substrate. The activity was highest at pH values of 6 to 7. The activity was proportional to the amount of enzyme added, and the reaction rate was linear for about 2 minutes. The enzyme was not inactivated by Tween 20, Zwitterion 3-08, Triton-X 100, and cholate, but was inactivated completely by sodium dodecyl sulfate. The enzyme was active on linoleoyl coenzyme A (CoA), palmitoyl CoA, and oleoyl CoA, although the activity was highest on linoleoyl CoA. Endogenous diacylglycerol was present in the microsomes, and the enzyme activity was only partially dependent on the addition of external diolein. Subcellular fractionation of the total scutellum extract in sucrose density gradients was performed. By comparing the migration of the enzyme between rate and equilibrium centrifugation, and between equilibrium centrifugation in the presence and absence of magnesium ions in the preparative media, the enzyme was shown to be associated with the rough endoplasmic reticulum. Some of the above findings on the maize enzyme were extended to the enzymes from castor bean, soybean, and peanuts.

Diacylglycerol acyltransferase (EC 2.3.1.20) catalyzes the final step in the synthesis of triacylglycerols in oil seeds (18, 22). It is also the only known enzyme unique to the long biosynthetic pathway of triacylglycerols, since the diacylglycerol produced could also be used to produce phospholipids or galactolipids. In spite of the importance of this enzyme in triacylglycerol biosynthesis in oil seeds, its properties have not been well studied.

Diacylglycerol acyltransferase in the microsomal fraction of developing seeds has been assayed directly or together with other enzymes (6, 10, 20, 21). In a recent, and so far the most detailed, study, the general properties of the enzyme in a safflower microsomal fraction were characterized (10). However, the detected level of activity was almost a magnitude lower than that required to catalyze the sequence of reaction from glycerol phosphate to triacylglycerol (21, 22). Using an enzyme assay which had been used successfully to study the enzyme in spinach leaves, Martin and Wilson (13) failed to detect enzyme activity in the cotyledon extract of developing soybean.

An important but unknown aspect of diacylglycerol acyltransferase is its subcellular location. In general, the microsomal fractions were used to study the enzyme activity, and they presumably contained vesicles of the ER as well as membranes of other subcellular particles, including broken plastids. In a recent detailed analysis of the subcellular location of the enzyme in spinach leaves (14), the enzyme was shown to be present in the outer membranes of the plastids. Whether or not this is also the case in the oil tissues of developing seeds is unknown.

We have developed an assay system with which we are able to detect the activities of diacylglycerol acyltransferase in the oil tissues of maize, peanut, soybean, and castor bean during seed maturation. These activities are high enough to account for the rate of *in vivo* oil synthesis. Some properties of the enzymes are studied. The enzyme in maize, and to a lesser extent in castor bean, has been shown to be localized in the RER and not in the plastid membranes. In this paper, we report our findings.

MATERIALS AND METHODS

Plant Materials. The oil storage tissues of maturing seeds, at a stage when the fresh weights were approximately half of the values in the mature seeds, were used. The scutella of inbred maize (*Zea mays* L.), Mo 17, the cotyledons of soybean (*Glycine max* L. Merr.) cv Coker 237, and the endosperm of castor bean (*Ricinus communis* L.) var. Hale were obtained from plants grown in the greenhouse. Peanuts (*Arachis hypogaea* L.) of unknown variety were obtained from a local farmer, and the cotyledons were used. Spinach (*Spinacia oleracea* L.) var. Bloomsdale long was grown in a growth chamber under a period of 24°C/9 h in light and 18°C/15 h in darkness. Mature leaves of 2-month-old plants were used.

Preparation of Microsomes. All operations were performed at 0 to 4°C. The tissues were chopped with a razor blade in grinding medium (4 ml/g tissue) in a Petri dish, and then homogenized gently with a mortar and pestle. The grinding medium contained 1 mM EDTA, 0.6 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 2 mM DTT, and 0.15 M Tricine-KOH buffer (pH 7.5). The homogenate was filtered through a Nitex cloth (20 × 20 μm). The filtrate was centrifuged at 10,000*g* for 15 min, and the supernatant was recentrifuged at 100,000*g* for 100 min. The pellet was resuspended in a small volume of grinding medium. This microsomal preparation was either used immediately or frozen at −70°C until use.

Sucrose Gradient Centrifugation (Rate and Equilibrium Centrifugation). The total homogenate of maize scutella or castor bean endosperm (1 g tissue/4 ml grinding medium) obtained after filtration was prepared as described above. Four ml of the homogenate were loaded onto each of 2 sucrose gradients. The gradient was composed of (starting from the bottom) 1 ml 60% (w/w) sucrose, 16 ml of a linear gradient from 56 to 30%, 4 ml 30%, 2 ml each of 27, 24, 21, and 19% sucrose. All the gradient solutions contained 1 mM EDTA-NaOH (pH 7.5). The gradients were centrifuged at 10,000 rpm for 10 min in a Beckman rotor SW 27. One gradient was removed (designated as the gradient

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obtained after 10 min centrifugation), and the other gradient was recentrifuged at 21,000 rpm for 4 h (designated as the gradient obtained after 4 h centrifugation). Both gradients were fractionated into 1-ml fractions.

Sucrose Gradient Centrifugation (with or without Mg^{2+}). The maize scutella were homogenized as described in a special grinding medium (1 g tissue/4 ml). The grinding medium contained 0.55 M sucrose, 1 mM EDTA, 10 mM KCl, 0.1 M Tricine-KOH buffer (pH 7.5), 0.1% heparin, 0.01% cycloheximide, and either 5 mM $MgCl_2$ ('plus Mg^{2+} treatment') or 0.1 mM $MgCl_2$ ('minus Mg^{2+} treatment'). After filtration as described earlier, 0.5 ml of the homogenate was loaded onto each gradient. The gradient was composed of (starting from the bottom) 1 ml 60% (w/w) sucrose, 1 ml 50% sucrose, 30 ml of a linear gradient from 50 to 20% sucrose, and 1 ml 20% sucrose. All the gradient solutions contained 1 mM EDTA, 10 mM Tricine-KOH buffer (pH 7.5), and either 3 mM $MgCl_2$ ('plus Mg^{2+}') or 0.1 mM $MgCl_2$ ('minus Mg^{2+}'). The gradients were centrifuged at 21,000 rpm for 4 h in a Beckman rotor SW 27, and fractionated.

Assays. Diacylglycerol acyltransferase activity was assayed by a procedure modified from those reported elsewhere (13, 25). For convenience, the assay was performed at room temp (24°C). Unless otherwise stated, the reaction mixture contained, in a final volume of 250 μl, 40 mM Tris-HCl buffer (pH 7.0), 6 mM $MgCl_2$, 4 mM DTT, 12 mM sucrose, 20 μM [1-^{14}C]oleoyl CoA (0.02 to 0.1 μCi, depending on the experiment), 0.4 mM 1,2-diolein in Tween-20, and enzyme. The diolein (2 mM) was emulsified with 10 mg Tween-20 in 5 ml of water, and 50 μl of the emulsified preparation were added to the reaction mixture to make a final volume of 250 μl. The reaction was initiated by the addition of enzyme. After 2 min (unless otherwise stated), the reaction was terminated by adding 1.5 ml heptane/isopropanol/0.5 M H_2SO_4 (10:40:1, v/v/v). After 30 min, 1 ml 0.1 M $NaHCO_3$, and 1 ml heptane containing 50 nmol triolein (as carrier) were added, and the mixture was shaken vigorously. The mixture was allowed to settle for 5 min. The upper phase was retained, and the lower phase was re-extracted with 1 ml heptane in the same manner. The combined upper phase was washed with 1.5 ml 1 M NaCl, and then evaporated to dryness under a stream of N_2. The residue was dissolved in heptane, and applied to a TLC plate (E. Merck, Silica Gel 60), and chromatographed in hexane/ether/acetic acid (50:50:1, v/v/v). The silica spot corresponding to standard triolein was visualized with iodine, and scraped into a scintillation vial and counted for ^{14}C.

In the assay of maize diacylglycerol acyltransferase, the addition and omission of triolein carrier generated equal amounts of ^{14}C in the triolein spots after TLC. Nevertheless, the carrier was added in all assays. Radioautography of the TLC plate showed that about 80% of the radioactivity was present in the triolein spot, and the remaining 20% was present in the spots of diolein, fatty acids, and the origin.

Diacylglycerol galactosyltransferase was assayed using UDP-[^{14}C]galactose (New England Nuclear Corp) according to the procedure described by Douce and Joyard (4). Triose-P isomerase was assayed by coupling with triose-P dehydrogenase (1). Catalase was assayed by monitoring the disappearance of H_2O_2 at 240 nm (12). NADH-Cyt reductase (all the Cyt reductase described in this report) was assayed by monitoring the appearance of reduced Cyt *c* at 550 nm (11). Cyt oxidase was assayed by monitoring the disappearance of reduced Cyt *c* at 550 nm (5). Sucrose concentration was assayed with a refractometer. Protein was assayed by the Coomassie blue method (3).

RESULTS

Diacylglycerol Acyltransferase in Developing Seeds. An assay procedure for the activity of diacylglycerol acyltransferase from the microsomes of maize scutella was developed. With this assay procedure (see "Materials and Methods" and next section), we were able to detect enzymic activities in the microsomes of oil tissues of all the four seed species examined (Table I). The four preparations had similar activity on a per g fresh weight basis. When the activity was expressed on the basis of the amount of microsomal protein, the enzyme activity varied substantially. It is likely that the microsomes contained different subcellular components (*e.g.* ER, plasmalemma, plastid membrane, and especially water-insoluble storage proteins) at diverse proportions which are seed-species dependent. As reported earlier (13), the enzyme activity was also present in the microsomes of spinach leaves. However, the activity in leaves was substantially lower than those in oil seeds on a per g fresh weight basis.

Using the scutella of maize as an example, we made an estimation to see if the detected enzyme activity is high enough to account for the rate of *in vivo* triacylglycerol synthesis. This comparison is generally used in metabolism studies to see if an enzyme may participate in a certain metabolic reaction; naturally, what is observed *in vitro* does not necessarily reflect what actually happens *in vivo*. The scutellum of the maize variety (inbreed line Mo 17) grown in the greenhouse accumulated triacylglycerol at a rate of about 10 to 12 mg triolein/g fresh weight·d during the period of linear oil accumulation (16). The recorded enzyme activity (Table I) is equivalent to approximately 8.3 mg triolein/g fresh weight·d, and this may be underestimated for the following reasons: (a) The enzyme assay was performed at 24°C, whereas the maize plants were grown under a cycle of 12 h light at 34°C and 12 h darkness at 28°C. (b) The microsome fraction (100,000*g* pellet obtained from the 10,000*g* supernatant) contained 70 to 80% of the enzyme activity; the rest of the activity was detected in the 10,000*g* pellet. (c) The gentle homogenization of the tissue in our subcellular fractionation of microsomes did not completely extract all the enzyme from the tissues. (d) The enzyme activity shown in Table I was measured using oleoyl CoA as the acyl group donor. However, the maize enzyme was twice as active with linoleoyl CoA (see next section). Thus, after these factors have been taken into consideration, diacylglycerol acyltransferase activity detected in maize microsomes is more than sufficient to account for the observed *in vivo* rate of triacylglycerol synthesis. Similar conclusion should also be extended to the other three seed species. In these other seed species, an extra factor should also be considered. The enzyme assay was generally optimized for the maize enzyme rather than for the enzymes from these other seed species.

Characterization of the Maize Diacylglycerol Acyltransferase. The activity of acyltransferase was assayed in a buffer solution containing diolein micelles prepared by sonication in the presence of Tween-20, and [^{14}C]oleoyl CoA. Most of the studies were done with maize microsomes, and the findings were occasionally extended to the microsomes of other seed species. The maize enzyme activity was highest at pH values of 6 to 7 (Fig. 1). The formation of [^{14}C]triacylglycerol was time-dependent (Fig. 2),

Table I. *Diacylglycerol Acyltransferase Activities in the Microsomes from Oil-Storing Tissues from Developing Seeds and from Spinach Leaves*

Plant Tissue	Diacylglycerol Acyltransferase	
	nmol/min·g fresh wt	*nmol/min·mg microsomal protein*
Maize scutella	6.52	3.10
Castor bean	2.79	0.76
Soybean	2.15	3.58
Peanut	4.43	0.75
Spinach leaves	0.26	0.76

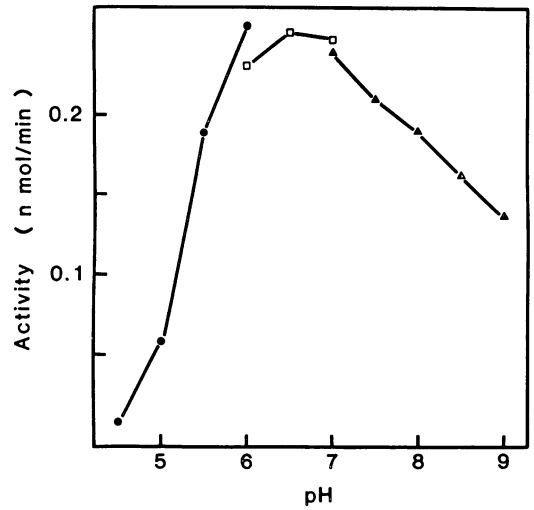

FIG. 1. Effect of pH on maize diacylglycerol acyltransferase. Buffers were succinate-NaOH (4.5–6), imidazole-HCl (6–7) and Tris-HCl (7–9).

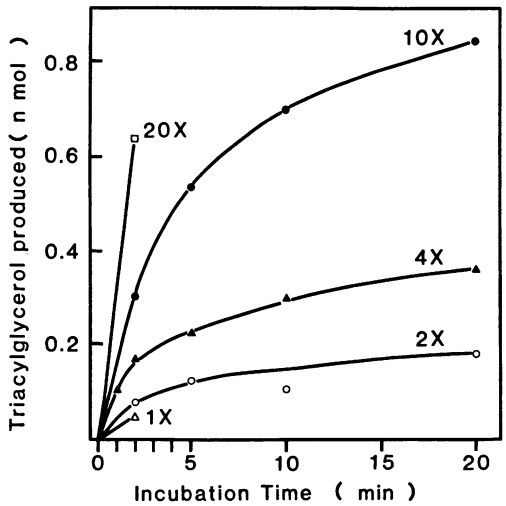

FIG. 2. A time course of the reaction catalyzed by different amounts of maize diacylglycerol acyltransferase. 1× represents 8.7 μg of microsomal proteins.

and it was fairly linear from 0 to 2 min. Based on this finding, an incubation time of 2 min was used in all other assays. Figure 2 also shows that the measured activity was dependent on the amount of added microsomes. In the presence of high amounts of enzyme and 5 min incubation, as much as 20% of the ^{14}C in [^{14}C]oleoyl CoA in the reaction mixture was converted to triolein.

The measured enzyme activity was proportional to the amounts of enzyme added when diolein was supplied in micelles emulsified with Tween-20 (Fig. 3). However, when diolein was supplied in micelles emulsified with Zwitterion 3-08, the proportionality of activities on the amount of enzyme added held only at low enzyme concentrations. Using small amounts of enzyme in the assay would hinder the accuracy of the assay or require the application of oleoyl CoA of a higher specific radioactivity. Thus, diolein emulsified in Tween-20 was used to do all other assays.

The leveling off of the reaction after 2 min (Fig. 2) and the lack of proportionality of activities *versus* high enzyme concentrations using Zwitterion micelles (Fig. 3) reflect the complexity of the assay conditions which involve insoluble substrates, membrane associated enzyme, insoluble reaction products, and other enzymic reactions competing for acyl CoA and diacylglycerol.

DTT, Mg^{2+}, and sucrose were included in the reaction mixture. However, omission of any one of these components did not substantially reduce the activity of the maize enzyme (Table II).

There were endogenous diacylglycerols in the maize microsomal preparation, such that omission of diolein in the assay only reduced the acyltransferase activity by one-third (Table II). The lipids in the maize microsomes were extracted with heptane/isopropanol/H_2SO_4 and with heptane, and separated by TLC. Iodine-reactive diacylglycerols were observed. The amount of diacylglycerols on the TLC plate was estimated by comparing the intensity of the spot with those of increasing amounts of diolein applied to the same plate. Diolein was used in this approximation since maize kernel triacylglycerols contain about 60% linoleic acid, 30% oleic acid, and 10% saturated fatty acids (7). In the maize microsomal preparation, there were approximately 23 nmol diacylglycerol (based on diolein) per mg protein. The amount of microsomal diacylglycerol was more than sufficient to serve as substrate in the current enzyme assay (3 nmol/min · mg protein). The origin of diacylglycerols in the microsomes is unclear. They might belong to the plastids, and the ER (as precursors of triacylglycerols present at the vicinity of budding, nascent lipid bodies).

Mild detergents, Tween-20 and Zwitterion 3-08, either applied

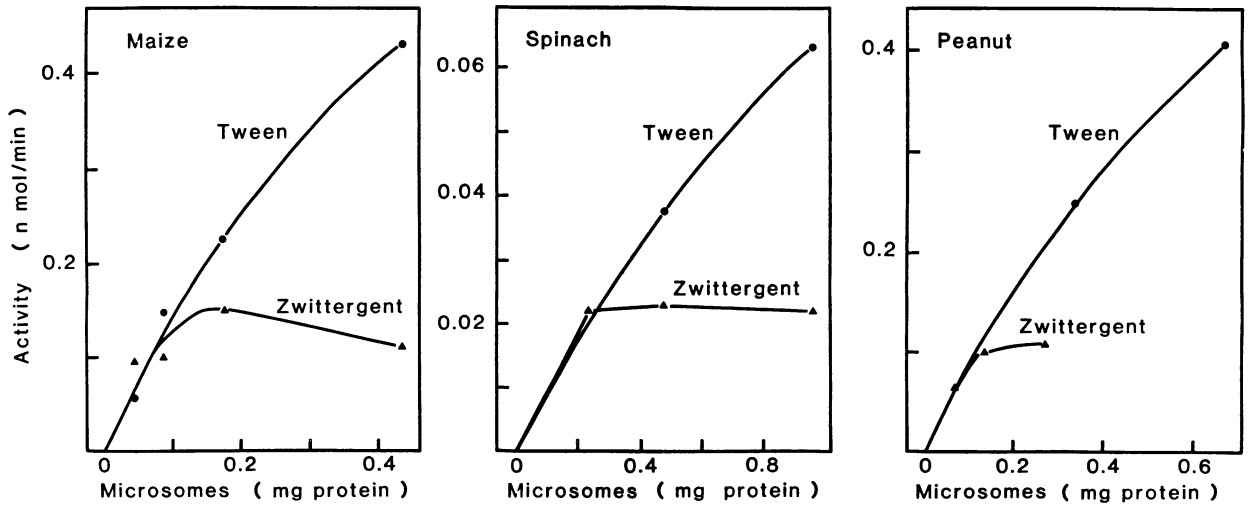

FIG. 3. Diacylglycerol acyltransferase activities measured in the presence of increasing amounts of microsomes. Diolein micelles were prepared by sonication with either Tween 20 or Zwitterion 3-08. Microsomes were prepared from maize, spinach, and peanuts.

to the assay mixture directly or used in a pretreatment of the microsomal preparation, did not substantially affect the maize enzyme activity (Table II). Triton-X 100 added to the reaction mixture (0.05%) reduced two-thirds of original enzyme activity. However, pretreatment of the microsomes with the same concentration of Triton-X 100 reduced only 17% of original enzyme activity (the reaction mixture contained 4 to 8% of the 0.05% Triton originated from the pretreated microsomal preparation). Thus, the enzyme apparently was not denatured by Triton-X 100 which merely reduced the activity when present in the reaction mixture. The effect of cholate was similar to that of Triton, except that a sizable amount of the enzyme activity was lost. The strong detergent, SDS, apparently denatured the enzyme when used in the assay mixture or in the pretreatment of the enzyme.

Table II. *Effects of Addition or Deletion of Components to or from the Assay Mixture for the Activity of Diacylglycerol Acyltransferase in Maize Scutellum Microsomes*

Two enzyme concentrations were used in each treatment and the average of the two activities was presented. Activity of 100% was 0.07 nmol/min. In the test of detergent effect, the detergent was added to the assay mixture (final concentration is shown). Alternatively, the enzyme preparation was preincubated with the detergent at the concentration indicated for 2 h in cold. After this pretreatment, the enzyme preparation containing the detergent was added to the assay mixture which contained no detergent. The final concentration of the detergent (originated from the enzyme preparation) in the assay mixture was 4% and 8% (two enzyme concentrations) of the concentration shown.

Mixture	Percent Activity
Complete	100
− Diolein	64
− Mg^{2+}	82
− Mg^{2+}, + Mn^{2+}	57
− DTT	94
− Sucrose	83
+ Tween 20, 0.04%	
(in assay)	92
(pretreatment)	93
+ Zwitterion 3-08, 10 mM	
(in assay)	89
(pretreatment)	100
+ Triton X-100, 0.05%	
(in assay)	34
(pretreatment)	83
+ SDS, 0.1%	
(in assay)	5
(pretreatment)	5
+ Cholate, 20 mM	
(in assay)	5
(pretreatment)	31

The maize enzyme was active on linoleoyl CoA, palmitoyl CoA, and oleoyl CoA (listed according to the order of activity, Fig. 4). These acyl groups are the major acyl constituents of the maize triacylglycerols. Similarly, the microsomal enzymes of castor bean, peanut, and soybean were also active on the same three acyl CoAs. The enzymes of maize and soybean, which have linoleic acid as the major acyl constituent in the 3-position of the triacylglycerols (51 and 45%, respectively) (7), exhibited highest activities on linoleoyl CoA. This correlation does not hold for the peanut enzyme which had almost equal activities with the three acyl CoAs. The 3-position of peanut triacylglycerols has 11% palmitoyl, 52% oleoyl, and 10% linoleoyl moieties (7). It is generally assumed that diacylglycerol acyltransferase has no acyl CoA specificity (10, 13, 24) and that the acyl moiety at the 3-position of triacylglycerol is determined by the pool of available acyl CoAs in the cell. Nevertheless, our comparative study of the enzyme from several seed species shows that the enzyme may also exert some selective effect in the determination of the acyl moiety at the 3-position of triacylglycerols.

Subcellular Localization of Diacylglycerol Acyltransferase. The subcellular location of acyltransferase in maize scutella was assessed by sucrose density gradient centrifugation. Two subcellular components, the ER and the plastids, are likely candidates. The former organelle is the generally assumed but not proven subcellular site of the enzyme, and the latter organelle (specifically, the outer membranes) is the demonstrated subcellular location of the enzyme in spinach leaves (14). The ER and the plastid outer membranes (from broken organelles) have similar if not identical equilibrium densities in sucrose density gradients (17). To make unambiguous interpretations of the experimental results, we performed gradient centrifugation of the total tissue extract for 10 min (rate centrifugation) and 4 h (equilibrium centrifugation). The slope of the gradients was designed to maximize the resolution of the ER at densities of 1.12 to 1.15 g/cm^3 at the expense of the resolution of mitochondria, intact plastids, and peroxisomes which occurred at densities 1.17 to 1.22 g/cm^3. These peroxisomes may represent pre-glyoxysomes (23).

The experimental results are shown in Figure 5. After 10 min of centrifugation, acyltransferase activity peaked together with a major peak of Cyt reductase (a marker of the ER and mitochon-

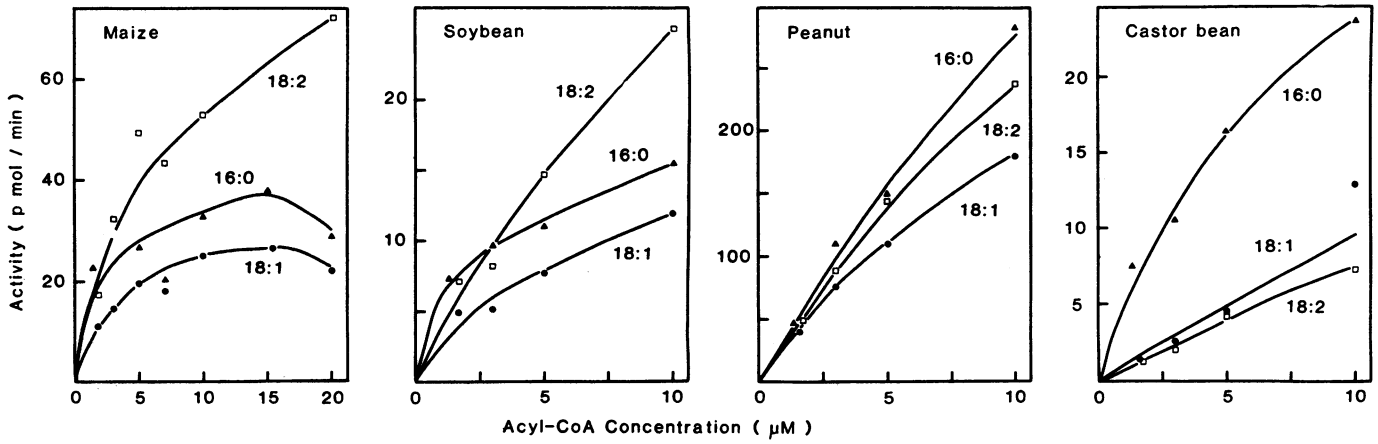

FIG. 4. Specificity of diacylglycerol acyltransferase on acyl donors. The microsomal enzymes were prepared from maize, peanut, soybean, and castor bean. Linoleoyl CoA (18:2), oleoyl CoA (18:1), and palmitoyl CoA (16:0) were used.

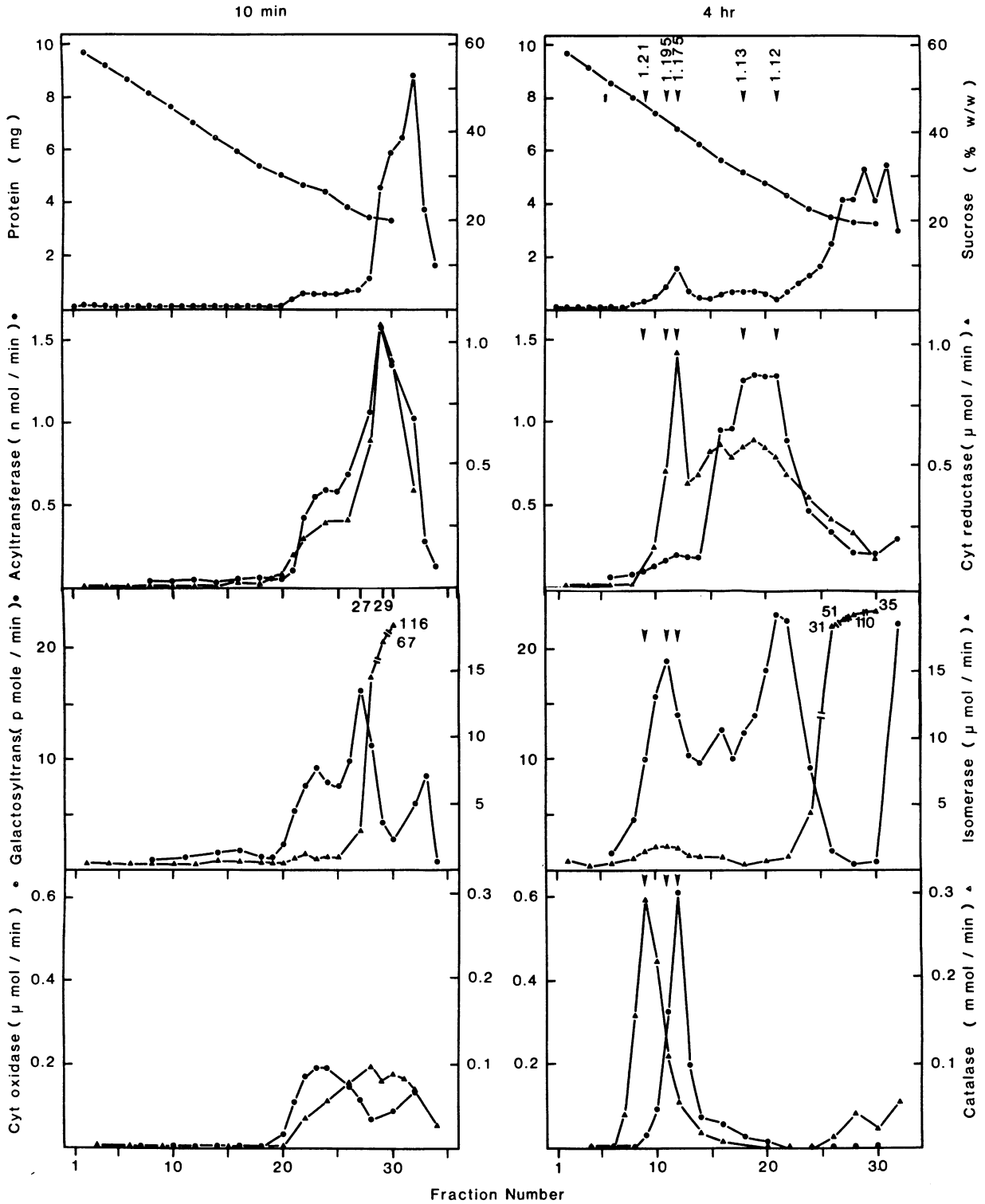

FIG. 5. Separation of organelles from maize scutellum extract by sucrose density gradient centrifugation. The total extract was applied directly onto sucrose gradients and centrifuged at 10,000 rpm for 10 min (rate centrifugation) or at 21,000 rpm for 4 h (equilibium centrifugation). The gradient medium contained no Mg^{2+}. Values of protein and enzyme activities are expressed on a per fraction basis. Arrows with numbers indicate densities in g/cm^3.

dria) (fraction 29 in Fig. 5). The activity of diacylglycerol galactosyltransferase, a marker of the inner and/or outer membrane of the plastids (for intact and broken plastids), occurred in three peaks. Of these peaks, the middle one (fraction 27) was closest to the acyltransferase peak but was at a region of slightly higher density (two fractions away from the acyltransferase peak). The fastest migrating galactosyltransferase peak represented that of the intact plastids since a small shoulder of triose-P isomerase activity (in plastid stroma and in cytosol) was also present in that region of the gradient. The peroxisomes (catalase as marker) and

mitochondria (Cyt oxidase and Cyt reductase as markers) were also present near the acyltransferase and galactosyltransferase peaks, and were not well separated from other organelles.

After a further 4 h of centrifugation (Fig. 5), the peroxisomes (catalase as marker, density 1.21 g/cm^3 at fraction 9), the intact plastids (galactosyltransferase and isomerase as markers, density 1.195 g/cm^3 at fraction 11), and the mitochondria (Cyt oxidase and Cyt reductase as markers, at density 1.175 g/cm^3 at fraction 12) were close to one another in the gradient, but were separated into distinct peaks. Acyltransferase was present together with a sizable percent of Cyt reductase at a density of 1.12 to 1.13 g/cm^3, and was absent in the intact plastids. Cyt reductase at this density in an equilibrium centrifugation should represent the ER (11, 17). At this region of the gradient, although one galactosyltransferase activity peak was also present, it did not match closely that of the Cyt reductase or acyltransferase activity. Overall, the data obtained from rate and equilibrium density gradient centrifugation were interpreted to show that acyltransferase migrated together with Cyt reductase in the ER and was not associated with the galactosyltransferase of the outer membrane of intact or broken plastids.

Corroborative evidence comes from experimental results of repeating the equilibrium sucrose gradient centrifugation but selectively adding or deleting Mg^{2+} in the isolation media. In addition, the experiment was designed to determine whether the enzyme is associated with the RER. In the presence of Mg^{2+} and other components, the RER would retain the polysomes attached to the membrane (11, 17). In the absence of Mg^{2+}, the RER

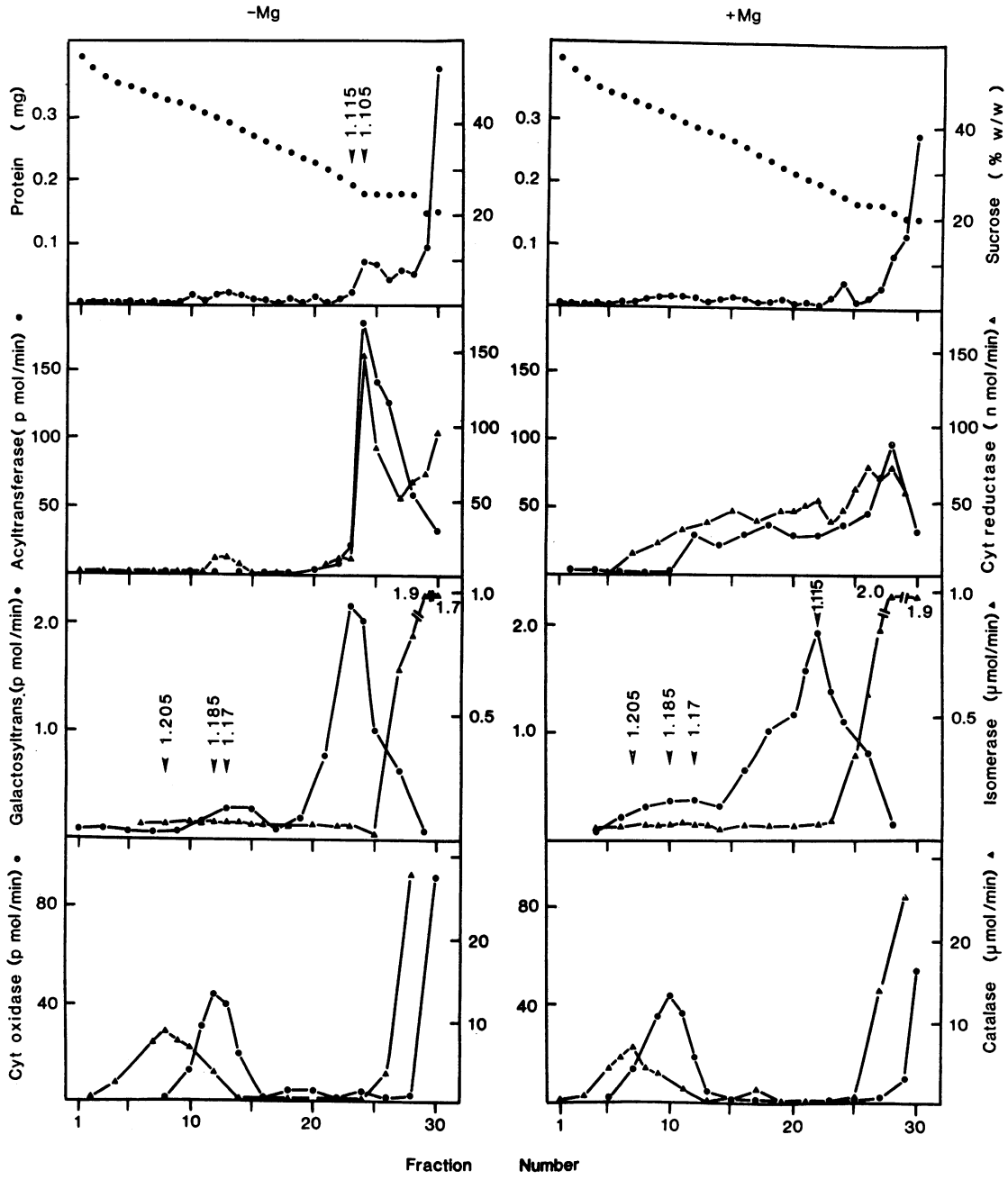

FIG. 6. Separation of organelles from maize scutellum extract by equilibrium sucrose gradient centrifugation. In the preparation, Mg^{2+} was either present or absent in the grinding medium and gradient medium. Values of protein and enzyme activities are expressed on a per fraction basis. Arrows with numbers indicate densities in g/cm^3. The gradients were centrifuged at 21,000 rpm for 4 h.

would loose the polysomes and become artifically smooth ER. In a sucrose gradient, an enzyme associated with the RER would be resolved with the organelles at a density higher than that associated with the smooth ER. The experimental results are shown in Figure 6.

Because of the differences in the grinding medium, the gradient medium, and the slope of the gradient, the separation of organelles in this experiment (Fig. 6) is not directly comparable with that shown in Figure 5. Nevertheless, the patterns are fairly similar. In the absence of Mg^{2+} in the isolation media, the peroxisomes (catalase as marker, density 1.205 g/cm^3), the mitochondria (Cyt oxidase and Cyt reductase as markers, density 1.185 g/cm^3), and the intact plastids (galactosyltransferase and isomerase as markers, density 1.17 g/cm^3) migrated to high densities, and were separated from one another. Acyltransferase activity peaked together with Cyt reductase activity at density 1.105 g/cm^3, and the peak was 1 fraction away from the major galactosyltransferase activity peak at density 1.115 g/cm^3. As judged from the distribution of galactosyltransferase activity along the gradient, it appears that most of the plastids had been broken during the preparation. In the presence of Mg^{2+} in the isolation media, the peroxisomes, the mitochondria, the intact plastids, and the plastid outer membrane (galactosyltransferase) remained at the same densities along the gradient. However, Cyt reductase and acyltransferase activities were much more disperse, and both had shifted to higher densities. The two enzymes followed each other closely along the gradient except at the region of the mitochondria. The mitochondrial region possessed a small fraction of the total Cyt reductase but no acyltransferase. Apparently, acyltransferase and the majority of Cyt reductase comigrated with the RER. The RER did not appear as a sharp peak but was distributed in many fractions of different densities, as expected (11, 17).

Similar subcellular localization of acyltransferase, although studied to a lesser extent, was also observed in castor bean endosperm (Fig. 7). In a 4 h centrifugation of the total tissue extract, the peroxisomes (catalase as marker, density 1.21 g/cm^3 at fraction 9), the intact plastids (galactosyltransferase and isomerase as markers, also density 1.21 g/cm^3 at fraction 9), the mitochondria (Cyt oxidase and Cyt reductase as markers, density 1.195 g/cm^3 at fraction 11) were separated. Acyltransferase was present together with a shoulder of Cyt reductase at density 1.12 to 1.13 g/cm^3. This fraction should represent the ER (11, 17). The outer plastid membrane (galactosyltransferase) derived from broken organelles peaked at a density of 1.15 g/cm^3, which was distinct from the ER fraction. About 20% of acyltransferase activity was associated with the Cyt reductase at the mitochondrial fraction at density 1.195 g/cm^3. Whether the mitochondria indeed contained some acyltransferase remains to be elucidated. The mitochondria from developing castor bean contained a large share of the total cellular Cyt reductase; this had been observed also in the mitochondria of germinated castor bean (8). As judged from the recovery of marker enzymes of intact organelles inside the gradient (in comparison with the solubilized enzymes at the top of the gradient), the castor bean organelles maintained their integrity during the preparation much better than those of maize scutella. The coappearance of the peroxisomes and intact plastids in the same region in the gradient may be due to the adhesion of the two organelles; this adhesion had been observed in preparations from the endosperm of germinated castor bean (9).

DISCUSSION

The assay system for diacylglycerol acyltransferase activity detected enzyme activities in extracts of all the four seed species examined. The activities are high enough to account for the *in vivo* rate of triacylglycerol synthesis. In earlier reports, the detected activities of the enzymes from developing seeds were quite low (10, 20, 22). In these reports, a time course of the reaction was not shown, and the reaction was terminated after 10 to 30 min. If the reactions in these assays also follow the kinetics of ours, a measurement of the reaction product at 20 min instead

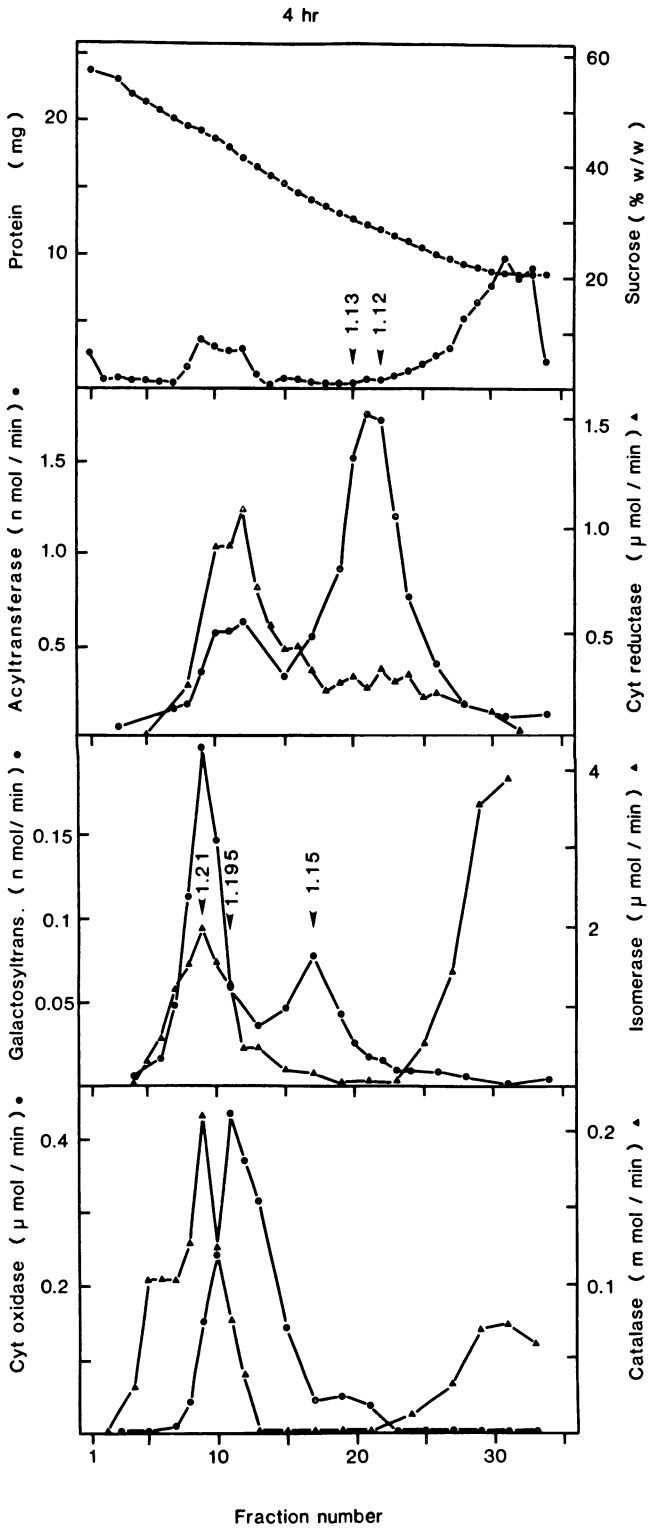

FIG. 7. Separation of organelles from castor bean endosperm extracts by equilibrium sucrose gradient centrifugation. The gradient medium contained no Mg^{2+}. Values of protein and enzyme activities are expressed on a per fraction basis. Arrows with numbers indicate densities in g/cm^3.

of 2 min would underestimate the activity by as much as 80% (Fig. 2). Also, in these earlier reports, the linear dependency of the activity on enzyme concentration was not shown. We have demonstrated that the enzyme dependency may not be linear, depending on how the diolein micelles were prepared and on the range of enzyme concentrations used. Finally, in the earlier work (10), the safflower microsomes were prepared by obtaining the pellet after centrifugation of the extract at 3,000*g* for 7 min. This centrifugation would only pellet a small percent of the total ER vesicles, and result in an underestimation of the enzyme activity.

The localization of the maize acyltransferase in the RER provides biochemical evidence that the lipid body is formed from the endoplasmic reticulum by vesiculation. In this model, the newly synthesized triacylglycerol is sequestered between the 2 phospholipid layers of the ER membrane (19, 23). Enlargement of the vesicle eventually produces a lipid body which is composed of a bag of triacylglycerol surrounded by a half-unit membrane of phospholipids and proteins. Earlier, we showed that the lipid body membrane proteins are synthesized on the RER (16). The localization of diacylglycerol acyltransferase, the last enzyme of triacylglycerol biosynthesis, in the ER further supports the ER vesiculation model. Thus, the three lipid body components, triacylglycerol (this report), membrane proteins (16), and membrane phospholipids (15), are all synthesized in the ER. An alternate model of lipid body biosynthesis states that the triacylglycerols are secreted into the cytosol as oil droplets first before they are encased by membrane materials (2, 22). We consider this model to be unlikely, since the cell would have to put in an extra effort to selectively assemble the membrane materials and since oil droplets in the cytosol have not been generally observed under the electron microscope (24). Nevertheless, the validity of this model cannot be ruled out.

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