Characteristics and biosynthesis of membrane proteins of lipid bodies in the scutella of maize (*Zea mays* L.)

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Storage lipid bodies, which are prominent organelles present in the storage tissues of most seeds, have not been subjected to intensive biochemical investigation. In the present studies the major proteins in lipid bodies isolated from eleven taxonomically diverse species were shown to be distinctly different, as revealed by SDS/polyacrylamide-gel electrophoresis. The lipid-body membrane of maize (*Zea mays* L.) contained three major proteins of low M_r (19500, 18000 and 16500), and they were chosen for further study. They all had alkaline pI values and behaved as hydrophobic integral proteins, as shown by their resistance to solubilization after repeated washing, amino acid composition and partitioning in a Triton X-114 system. Labelling *in vivo* with [35 S]methionine and translation *in vitro* using extracted RNA in a wheat-germ system showed that the proteins were synthesized during seed maturation and not germination. The proteins synthesized *in vivo* and *in vitro* exhibited no appreciable difference in their mobilities in two-dimensional gel electrophoresis (isoelectric focusing and molecular sieving). The most abundant protein, that of M_r 16500, was shown to be synthesized predominantly, if not exclusively, by RNA derived from bound polyribosomes and not from free polyribosomes. The implication of the results on the biosynthesis of the lipid bodies is discussed.

INTRODUCTION

Most seeds store triacylglycerols, which are synthesized in seed maturation and are used as food reserve for germination. The storage triacylglycerols are confined to organelles called lipid bodies (oleosomes, spherosomes, oil bodies) in the storage tissues [see reviews by Appelqvist (1975), Gurr (1980), Roughan & Slack (1982) and Huang (1983)]. The spherical organelles are roughly 0.1–1 μ m in diameter. They are essentially a package of triacylglycerols surrounded by a 'half-unit' membrane (Yatsu & Jack, 1972). In this membrane, there is only one layer of phospholipids in which the hydrophobic portion faces inside, interacting with the triacylglycerols, and the hydrophilic portion is exposed to the cytosol. The membrane contains several specific protein components which are different, as observed by SDS/polyacrylamide-gel electrophoresis, from those in the ER, glyoxysomes or mitochondria (Bergfeld *et al.*, 1978; Moreau *et al.*, 1980; Slack *et al.*, 1980). There are no unusual phospholipid components in the membrane, and the major components are phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol (Donaldson, 1976; Moreau *et al.*, 1980).

Lipid bodies are synthesized actively during seed maturation. The mode of biosynthesis has been pursued only by electron-microscopic observations. The lipid body may be formed by enlargement of a minute oil droplet to a mature size, followed by encasement of the droplet with a membrane-like structure (Bergfeld *et al.*, 1978). Alternatively, the lipid body may be formed by the addition of storage triacylglycerols to the space between the two phospholipid layers of a unit membrane of the

ER, such that vesiculation of the enlarged ER terminus would produce a lipid body of triacylglycerol surrounded by a half-unit membrane (Schwarzenbach, 1971; Wanner *et al.*, 1981). Triacylglycerol droplets in mammalian systems have also been proposed to be synthesized similarly from the ER (Coleman & Bell, 1983).

There are few major biochemical studies of the membrane proteins of lipid bodies in the literature. In view of this deficiency, we undertook an investigation of the membrane proteins of the lipid bodies in the scutella of maize (*Zea mays*). Here we present experimental results on the characteristics of the membrane proteins and their biosynthesis in the rough ER without any appreciable co- or post-translational processing.

MATERIALS AND METHODS

Plant materials

Kernels (termed 'seeds' in the present paper) of inbred maize (*Zea mays* L. cv. Mo 17) were obtained from the Illinois Foundation Seed Corporation (Champaign, IL, U.S.A.). The scutella of ungerminated seed (soaked for 3 h) were removed carefully from the endosperm and axis. In the use of germinated seed, the seed was soaked in running tap water for 16 h and allowed to germinate at 29 °C for 1 day. In the use of developing seed, the plants were grown in the field and the seeds were obtained at various time intervals after pollination.

The mature cotyledons of soybean (*Glycine max* L. Merr, Coker 237), cotton (*Gossypium hirsutum* L.), cucumber (*Cucumis sativus* L., cv. Ashley) seed, rapeseed (*Brassica napus* L.), jojoba (*Simmondsia chinensis* [link]

Abbreviations used: ER, endoplasmic reticulum; DTT, dithiothreitol.

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Scheider) seed, mustard (*Brassica juncea* L.) seed, sunflower (*Helianthus annuus* L., cv. 894), safflower (*Carthamus tinctorius* cv. S541) seed, and linseed (*Linus usitatissimum* cv. # 10, flax), and the mature endosperm of castor bean (*Ricinus communis* cv. Hale), were used.

Preparation of lipid-body membranes

All operations were performed at 0–4 °C. The scutella were chopped with a razor blade in grinding medium (10 g/30 ml) and then ground with a mortar and pestle. The grinding medium contained 0.6 M-sucrose, 1 mM-EDTA, 10 mM-KCl, 1 mM-MgCl₂, 2 mM-DTT and 0.15 M-Tricine buffer, adjusted to pH 7.5 with KOH. The homogenate was filtered through a piece of Nitex cloth (Petko, Elmsford, NY, U.S.A.) of pore size 20 µm × 20 µm. Each 15 ml filtrate was placed in one 40 ml centrifuge tube and 15 ml of grinding medium containing 0.5 M- instead of 0.6 M-sucrose was layered on top. After centrifugation at 10000 *g* for 10 min, the floated lipid bodies were removed with a spatula. The lipid bodies obtained from 10 g of scutella were washed by resuspending in 5 ml of grinding medium in a centrifuge tube. An overlay of grinding medium (0.5 M- instead of 0.6 M-sucrose) was introduced and the tube was centrifuged at 10000 *g* for 10 min. The floated lipid bodies were collected and washed once more.

The isolated lipid-body fraction was not appreciably contaminated by the ER. NADH: cytochrome reductase, which is a common marker of the ER (and mitochondria), was not detected in the lipid-body fraction [after the triacylglycerol had been removed with diethyl ether (as described below) so that it would not interfere with the spectrophotometric enzyme assay]. In addition, in SDS/polyacrylamide-gel electrophoresis the fraction did not contain any of the major protein bands that were unique to the microsome fraction (the 100000 *g*/90 min pellet obtained from the 10000 *g*/30 min supernatant of the homogenate).

The washed lipid bodies were extracted three times with equal volumes of diethyl ether to remove the triacylglycerols. The trace amount of diethyl ether remaining in the aqueous phase was evaporated under a stream of nitrogen. The remaining suspension contained the membranes of the lipid bodies and some solubilized membrane components (Moreau *et al.*, 1980; Lin *et al.*, 1983), and was used as the lipid-body membrane fraction.

SDS/polyacrylamide-gel electrophoresis

Protein samples were treated with SDS and β-mercaptoethanol before electrophoresis (Weber & Osborn, 1969). SDS/polyacrylamide-gel electrophoresis employing 12.5%, 15% and 20% (w/v) acrylamide was run and the proteins were stained with Coomassie Blue R (Weber & Osborn, 1969). Two-dimensional gel electrophoresis was performed with the first dimension by non-equilibrium isoelectric focusing and the second dimension by SDS/12.5%-polyacrylamide-gel electrophoresis (O'Farrell *et al.*, 1977).

Preparation of lipid-body L₃ protein from maize

Isolated lipid-body membrane fraction was subjected to 'one-well' SDS/15%-polyacrylamide-gel electrophoresis. After electrophoresis, the gel was stained for 0.5 h, rinsed with water, and the visible horizontal L₃ band was cut out with a razor blade. The gel strip was equilibrated in 20 ml of 0.125 M-Tris/HCl (pH 6.8)/0.1%

SDS/1 mM-EDTA (Cleveland *et al.*, 1977). It was subjected to further 'one-well' SDS/polyacrylamide-gel electrophoresis. The position of the unstained L₃ protein on the gel was located by cutting and staining a vertical strip of the gel. A horizontal gel strip containing the unstained L₃ band was cut out and ground with a glass homogenizer in the abovementioned buffer. The homogenate was centrifuged at 10000 *g* for 5 min. The supernatant was retained, and the pellet was washed with the same buffer and re-centrifuged. The two supernatants were combined and concentrated by ultrafiltration (membrane filter Type YM 10; Amicon Corp., Danvers, MA, U.S.A.).

Preparation of antibodies against L₃ protein

A sample of 0.2 mg of purified L₃ protein (1 ml) was emulsified with 1 ml of complete Freund's adjuvant and injected intradermally into a rabbit. After 2 weeks, another injection was performed by using 0.2 mg of L₃ protein (1 ml) mixed with 1 ml of incomplete Freund's adjuvant. The immunoglobulin G in the antiserum was obtained by DEAE Affi-Gel Blue column chromatography (Bio-Rad Corp., Richmond, CA, U.S.A.). The preparation was concentrated by ultrafiltration, freeze-dried and stored at –70 °C until use.

Amino acid analysis of L₃ protein

The purified L₃ protein was hydrolysed to amino acids with 4 M-methanesulphonic acid *in vacuo*. A procedure that preserved tryptophan was used (Simpson *et al.*, 1976). The protein was reduced with dithiothreitol for half-cysteine analysis. Amino acid analysis was performed on a Beckman 120-C instrument.

Phase separation in Triton X-114

The hydrophobicity of lipid-body membrane proteins was evaluated by the procedure of Bordier (1981). The protein samples were incubated in 0.2 ml of 10 mM-Tris/HCl buffer (pH 7.5)/150 mM-NaCl/1% Triton X-114 for 3 min at 0 °C. Each sample was layered on to 0.3 ml of 6% sucrose (w/v)/10 mM-Tris/HCl (pH 7.5)/150 mM-NaCl/0.06% Triton X-114 in a microcentrifuge tube. The tube was incubated at 30 °C for 3 min. After centrifugation at 15000 *g* for 1 min at room temperature, the upper aqueous supernatant phase and the lower detergent phase were obtained and analysed by SDS/polyacrylamide-gel electrophoresis.

Immunoblotting

After SDS/polyacrylamide-gel electrophoresis the protein bands were transferred electrophoretically from the gel to a nitrocellulose membrane (Towbin *et al.*, 1979). Success of blotting was confirmed by staining a strip of the membrane containing a duplicate lane with Amido Black. The unstained membrane was allowed to react with rabbit anti-L₃ IgG. Recognition of antibody–L₃ complexes was made with goat anti-(rabbit IgG) antibodies conjugated with horseradish peroxidase (Bio-Rad Corp., Richmond, CA, U.S.A.). Enzyme activity was revealed with Bio-Rad HRP colour reagent (Hawkes *et al.*, 1982).

Labelling of proteins with [³⁵S]methionine *in vivo*

Embryos (scutellum attached to axis) were separated from developing seeds and washed with distilled water. They were placed on a piece of moistened Whatman filter

paper in a Petri dish. A 2 μ l portion of [35 S]methionine (about 25 μ Ci of 20 nmol) were applied to the inner surface (originally facing the endosperm) of each scutellum. The scutella were incubated at 24 °C in darkness for 0.5–20 h. After incubation, the scutella were homogenized in grinding medium (four scutella/ml) in a glass homogenizer. The homogenates were subjected to SDS/polyacrylamide-gel electrophoresis directly.

Translation *in vitro* and immunoprecipitation

The scutella of developing or germinating seeds were homogenized in 7.5 M-guanidinium chloride with a Polytron instrument. The nucleic acids were precipitated with 0.7 vol. of pure ethanol and dissolved in buffer containing 0.1 M-Tris/HCl (pH 7.4)/0.3 M-NaCl/0.1% SDS, extracted with phenol/chloroform, then chloroform, and precipitated with ethanol. Nucleic acids were dissolved in water, and high- M_r RNA species were precipitated with 2 M-LiCl₂ (Palmiter, 1974) and were used to direct translation *in vitro* using [35 S]methionine and a wheat-germ system (BRL Corp., Gaithersburg, MD, U.S.A.). The procedure suggested by BRL Corp. was adhered to.

Immunoprecipitation was carried out as described by Dougherty & Hiebert (1980), with IgG-sorb (The Enzyme Center, Boston, MA, U.S.A.) as the immune absorbent.

Isolation of free and bound polyribosomes

The procedure used was that described by Larkins & Davis (1975), with modifications. All the buffers used in the preparation contained heparin (1 mg/ml) and cycloheximide (100 μ g/ml). A 2 g portion of scutella was collected from developing seeds 25 days after pollination. The tissue was chopped with a razor blade in 16 ml of buffer A and then ground with Polytron at speed 6 for 10 s. Buffer A was 200 mM-Tris/HCl (pH 8.5)/200 mM-sucrose/60 mM-KCl/50 mM-MgCl₂/5 mM-DTT/5 mM-EGTA. The homogenate was filtered through Miracloth and the filtrate was centrifuged at 500 g for 5 min. The supernatant between the lipid layer and the pellet was re-centrifuged at 37000 g for 10 min. The resulting supernatant and pellet were used to prepare free polyribosomes and bound polyribosomes respectively.

For isolation of free polyribosomes, the 37000 g supernatant was layered on a sucrose pad of 2.25 M-sucrose in Buffer B and centrifuged at 229000 g for 75 min in a Beckman type 65 rotor. Buffer B was 40 mM-Tris/HCl (pH 8.5)/200 mM-KCl/30 mM-MgCl₂/5 mM-EGTA. The pellet was resuspended gently in 250 μ l of Buffer B and layered on to a linear sucrose gradient of 12.5–60% (w/w) sucrose in Buffer C. Buffer C was 40 mM-Tris/HCl (pH 8.5)/20 mM-KCl/10 mM-MgCl₂. The gradient was centrifuged at 35000 rev./min for 1 h in a Beckman SW 41 Ti rotor. The gradient was fractionated and A_{254} was monitored with an ISCO UA-5 monitor. Fractions containing polyribosomes were collected and extracted with phenol/chloroform. Polyribosomal RNA species were ethanol-precipitated and treated with 2 M-LiCl₂ to remove low- M_r RNA species and heparin.

For isolation of bound polyribosomes, the 37000 g pellet was washed with Buffer A to remove contaminating free polyribosomes and re-pelleted at 37000 g for 10 min. The pellet was resuspended in 5 ml of buffer A, containing 1% Triton X-100, 0.5% Nonidet P40 and 0.5% Tween 20. After gentle shaking for 2 min, the

suspension was centrifuged at 37000 g for 10 min to remove membrane fragments. The supernatant, containing polyribosomes released from membranes, was subjected to sucrose-gradient centrifugation and the polyribosomal RNA was obtained as described in the above paragraph.

RESULTS

Lipid-body proteins from diverse species

Lipid bodies were isolated from ungerminated seeds of 11 taxonomically diverse species by repeated flotation centrifugation. After the triacylglycerols in isolated lipid bodies had been extracted with diethyl ether, the lipid-body membrane with its associated proteins was obtained, as revealed to sucrose-density-gradient centrifugation (Moreau & Huang, 1977; Moreau *et al.*, 1980; Lin *et al.*, 1983) and electron microscopy (Altschul *et al.*, 1963; Trelease, 1969; Yatsu & Jack, 1972). These proteins were analysed by SDS/polyacrylamide-gel electrophoresis (Fig. 1). All species possessed several major bands and numerous minor bands. Because of their high content, the major protein bands were most likely authentic lipid-body membrane proteins rather than minute contaminants. The minor protein bands may be either authentic lipid-body membrane proteins or contaminants. There were few similarities in the protein patterns between the species examined. The only exception was between the two closely related species, mustard and rapeseed (both of the same genus, *Brassica*), which yielded fairly similar protein patterns.

Previously it had been reported that two diverse species, linseed and safflower, appeared to contain major lipid-body proteins that were very similar on SDS/polyacrylamide-gel-electrophoretic analysis (Slack *et al.*, 1980). In our preparations, the general patterns of the lipid-body proteins from these two species were similar to those reported. However, the mobilities of, and thus the apparent M_r values for, the two major low- M_r proteins were detectably different between the two species when the lipid-body proteins were run separately or in combination in SDS/polyacrylamide-gel electrophoresis (Fig. 1). Thus the lipid-body membrane proteins of linseed and safflower are not identical as regards apparent M_r .

The lipid bodies from maize were selected for detailed study because their membrane protein pattern is relatively simple, and the most abundant, lowest- M_r , protein band could be isolated as a single protein free of contaminants. In addition, the membranes of the lipid bodies after diethyl ether extraction had been studied by sucrose-density-gradient centrifugation (Lin *et al.*, 1983) and by electron microscopy (Trelease, 1969).

General properties of maize lipid-body membrane proteins

In maize seed, about 90–95% of the lipid and lipid bodies are localized in the scutella, the remainder being present in the embryonic axis. For simplicity, we used only the scutella as the source of lipid bodies in all studies.

As shown in Fig. 1, the major membrane proteins of isolated lipid bodies were resolved by SDS/polyacrylamide-gel electrophoresis into two groups of proteins: a high- M_r (40000) band (H) and several lower- M_r bands (L_1 , 19500; L_2 , 18000; and L_3 , 16500). The simple protein pattern of the isolated lipid bodies (Fig. 1) was

Fig. 1. SDS/polyacrylamide-gel electrophoresis of lipid-body proteins isolated from the seeds of various plant species

(a) Lipid-body proteins from plant species indicated at top of Figure separated by SDS/polyacrylamide-gel electrophoresis (12.5% acrylamide). Positions of M_r markers are shown on the right. (b) Shows SDS/polyacrylamide-gel electrophoresis (20% acrylamide) of lipid-body proteins isolated from linseed and safflower seeds. A mixture of the two protein samples is shown in the middle lane. The two low- M_r bands of linseed had mobilities slightly different from those of the two major bands of safflower seeds. The gels were stained with Coomassie Brilliant Blue.

overshadowed by numerous other proteins in the total extract of mature or developing seed. The proteins in the total scutellum extract and in isolated lipid bodies were separated into many protein spots by two-dimensional gel electrophoresis, using non-equilibrium isoelectric focusing in the first dimension and SDS/polyacrylamide-gel electrophoresis in the second dimension (Fig. 2). In both cases, the L_3 protein could be identified. From isolated lipid bodies, the H-band was resolved into one protein spot having a relatively acidic pI, whereas the three L-bands were resolved into four spots (L_2 yielding two spots) having similar, but not identical, alkaline pI values.

In a biphasic partition of hydrophobic and hydrophilic proteins in the presence of Triton X-114 (Bordier, 1981), more L proteins were present in the hydrophobic than the hydrophilic fraction, whereas the H protein occurred only in the hydrophilic fraction (Fig. 3). The results suggest that the L proteins are moderate hydrophobic proteins, whereas the H protein is hydrophilic.

In subsequent studies we concentrated our attention on the L_3 protein, which, in isolated preparation or in the total extract, showed only one spot on two-dimensional gel electrophoresis. The 16500- M_r band in a one-dimensional SDS/polyacrylamide-gel electrophoresis gel of the total extract was represented mostly if not exclusively by the L_3 protein, as judged from the protein pattern in a two-dimensional gel (Fig. 2). The single-protein nature of L_3 protein allowed us to identify it in the total extract in a one-dimensional gel quickly and to

go to two-dimensional gel for confirmation occasionally. L_3 protein was also the most abundant protein among all the lipid-body membrane proteins.

Characteristics of L_3 protein

L_3 protein was obtained from isolated lipid bodies by preparative SDS/polyacrylamide-gel electrophoresis. Isolated L_3 showed one band in one-dimensional SDS/polyacrylamide-gel electrophoresis (Figs. 2 and 4) and one spot in two-dimensional gel electrophoresis (Fig. 2). Rabbit antibodies raised against L_3 protein were able to recognize specifically L_3 protein in the total scutellum extract, as shown by immunoblotting (Fig. 4). The antibodies did not recognize any protein of lipid bodies isolated from cottonseed, jojoba seed, castor bean and safflower seed in a similar immunoblotting procedure.

L_3 protein had the following amino acid composition (molar ratio): Cys₀, Asx_{8.1}, Thr_{4.8}, Ser_{7.3}, Glx_{9.9}, Pro_{2.8}, Gly_{15.2}, Ala_{13.4}, Val_{5.6}, Met_{2.2}, Ile_{3.2}, Leu_{9.0}, Tyr_{3.5}, Phe_{3.7}, Lys_{4.8}, Arg_{2.7}, His_{4.0}, Trp₀. The calculated hydrophobicity index (Capaldi & Vanderkooi, 1972) of the protein is 41.5, indicative of a moderately hydrophobic protein. This agrees with the hydrophobic behaviour of L_3 protein in the Triton X-114 partitioning (Fig. 3). Furthermore, we were unable to remove the L_3 protein from either isolated lipid bodies or the lipid-body membrane by repeated washing with buffer or high-salt solution.

Fig. 2. Two-dimensional gel electrophoresis of proteins of total homogenate (a), lipid-body proteins (b) and isolated L_3 protein (c) from maize scutella

The first dimension was non-equilibrium isoelectrofocusing, and the second, SDS/polyacrylamide-gel electrophoresis (12.5% acrylamide). In the second dimension a

Fig. 3. SDS/polyacrylamide-gel electrophoresis (15% acrylamide) of maize lipid-body proteins

A, lipid-body proteins; B, lipid-body proteins partitioned into the aqueous phase (hydrophilic) of a Triton X-114 system; C, lipid-body proteins partitioned into the detergent phase (hydrophobic) of a Triton X-114 system [controls were performed using cytochrome *c* (hydrophilic) and bacteriorhodopsin (hydrophobic)]; F, a mixture of the two proteins (the higher- M_r band was bacteriorhodopsin and the lower- M_r band was cytochrome *c*); D, the aqueous phase (containing cytochrome *c* only); E, the detergent phase (containing bacteriorhodopsin only). The gel was stained with Coomassie Brilliant Blue.

Biosynthesis of maize L_3 protein in seed maturation

About 20 days after pollination, the scutellum, as well as the whole seed, started to increase rapidly in size, fresh weight and the contents of lipid and protein (Fig. 5). When equal amounts of total scutellum extract (on a per-seed basis) from various maturation stages were subjected to SDS/polyacrylamide-gel electrophoresis, increases in L and H proteins were observed (Fig. 6).

Synthesis *in vivo* of L_3 protein was observed when the scutella were labeled with [35 S]methionine. After labelling for various periods, the scutella were homogenized and

slot was prepared on the right side of the gel on which a similar protein sample was applied and co-electrophoresed; this manipulation allowed the identification of the L_3 protein (shown by arrows) on the two-dimensional gels. The three two-dimensional gels were run in different experiments and the L_3 protein in each run did not migrate to the same position. The gels were stained with Coomassie Brilliant Blue.

Fig. 4. (a) SDS/polyacrylamide-gel electrophoresis (12.5% acrylamide) of total homogenate, isolated lipid bodies and isolated L_3 protein of maize scutella (the positions of M_r markers are shown on the left) and (b) blotting of SDS/polyacrylamide-gel electrophoresis of total scutellum homogenate

Total homogenate proteins were separated by SDS/polyacrylamide-gel electrophoresis and electrophoretically transferred to a nitrocellulose paper. One lane of transferred proteins was stained for protein by using Amido Black ('Protein'). A duplicate lane was allowed to react with rabbit antibodies ('+AB') raised against L_3 protein. The position of proteins recognized by L_3 antibodies was revealed by using a system of goat anti-(rabbit IgG) antiserum and peroxidase-dye reaction.

the total extracts were subjected to SDS/polyacrylamide-gel electrophoresis. The stained protein patterns showed approximately the same amount of each protein band in scutellum samples at each time point of labelling, indicating that the extent of extraction was roughly the same in all samples (Fig. 7). When the same gel was fluorographed, the amount of labelled methionine incorporated into the L proteins was observed to increase with time (Fig. 7). The labelling was maximal at about 3.5–7.5 h. Afterwards, the amount of incorporated label decreased, apparently due to turnover of the L proteins.

Synthesis *in vitro* of L_3 protein was studied. The total RNA extracted from either maturing or germinated seed was allowed to direct protein synthesis in a wheat-germ

Fig. 5. Changes in fresh weight, lipid content and protein content in the total homogenate of maize scutella during seed maturation

Fig. 6. SDS/polyacrylamide-gel electrophoresis (12.5% acrylamide) of total homogenate of maize scutella obtained from seed at various days after pollination

The H and L proteins are indicated. The gel was stained with Coomassie Brilliant Blue.

system using [35 S]methionine (Fig. 8). The products of translation *in vitro* were immunoprecipitated with L_3 -specific antiserum and the precipitated proteins analysed by SDS/polyacrylamide-gel electrophoresis followed by fluorography. The results (Fig. 8) showed that L_3 protein was synthesized by RNA from maturing, but not germinated, seed. Thus the mRNA for L_3 protein was present in maturing, but not germinated, seed.

Fig. 7. SDS/polyacrylamide-gel electrophoresis (12.5% acrylamide) of proteins labelled *in vivo* from total homogenates of maize scutella

[³⁵S]Methionine was applied to scutella of embryos which had been detached from seed 25 days after pollination. After 0, 0.5, 1.5, 3.5, 7.5 and 20 h of incubation, the tissues were homogenized and the homogenates were subjected to SDS/polyacrylamide-gel electrophoresis. For identification, membrane proteins of lipid bodies isolated from ungerminated maize were applied to the slot on the right labelled 'LB'. The gel was stained for proteins with Coomassie Brilliant Blue (a). After staining and photography, the same gel was subjected to fluorography (b).

As observed by SDS/polyacrylamide-gel electrophoresis (Figs. 7 and 8), there was no apparent difference in the M_r of the L_3 protein synthesized *in vivo* and *in vitro*. Our SDS/polyacrylamide-gel electrophoresis system is able to separate clearly protein bands of M_r difference 1000–2000 over the M_r range 14000–24000 (tested with lysozyme, haemoglobin, β -galactoglobulin, trypsin inhibitor and trypsinogen; results not shown). Therefore,

Fig. 8. Fluorography of SDS/polyacrylamide-gel electrophoresis of polypeptides synthesized *in vitro* using RNA extracted from scutella of (a) germinated (1-day-old) or (b) developing (25 days after pollination) maize

The translation system *in vitro* consisted of [³⁵S]methionine and a wheat-germ system. No protein was synthesized in the absence of added RNA (c). Antibodies (+AB) raised against the L_3 protein recognized the L_3 protein, which was synthesized by RNA from developing (e), but not germinated (d), maize.

if there is a difference in the M_r of the L_3 synthesized *in vivo* and *in vitro*, it should be less than 1000.

In addition, when the proteins synthesized *in vivo* and *in vitro* were separated alone and in combination by two-dimensional gel electrophoresis, L_3 protein remained at an identical position and appeared as one single spot (Fig. 9). Furthermore, this radioactive L_3 spot coincided with the Coomassie Brilliant Blue-stained protein spot of non-radioactive L_3 carrier which was added to the samples before electrophoresis. Therefore there was no appreciable difference in the M_r or the observed pI (the gel system was non-equilibrium isoelectric focusing, and the observed pI was close to, but not identical with, the actual pI) between the L_3 synthesized *in vivo* and *in vitro*.

L_3 protein was synthesized *in vitro* by RNA derived from bound polyribosomes but not from free polyribo-

somes. Highly intact polyribosomes derived from the cytosol and from the rough ER were isolated (Fig. 10). When the RNA species from the two populations of polyribosomes were used to direct protein synthesis *in vitro*, L_{43} protein was predominately, if not exclusively, synthesized by those from bound polyribosomes, as revealed by SDS/polyacrylamide-gel electrophoresis (Fig. 10).

Fig. 10. Profiles of A_{254} of polyribosomes separated by sucrose-density-gradient centrifugation (a,b) and fluorographs of their *in vitro* translation products after SDS/polyacrylamide-gel electrophoresis (c,d,e)

The polyribosomes were derived from the cytosol ('Free') or the ER ('Bound'). The RNA species were extracted from the separated polyribosomes and were used to direct translation *in vitro* in a wheatgerm system with [35 S]methionine.

Fig. 9. Fluorograph of two-dimensional gel electrophoresis of proteins synthesized *in vitro* using extracted RNA and a wheat-germ system (a) and *in vivo* by applying [35 S]methionine to intact scutella (b) [a mixture of the proteins synthesized *in vitro* and *in vivo* was also used (c)]

The first dimension was non-equilibrium isoelectrofocusing and the second dimension, SDS/polyacrylamide-gel electrophoresis (12.5% acrylamide). In the second dimension, a slot was prepared on the right side of the gel, on which a similar protein sample was applied and co-electrophoresed. The arrows indicate the L_3 protein.

DISCUSSION

Most seeds contain triacylglycerols as food reserve for gluconeogenesis in germination. Although the enzymes for the gluconeogenesis from fatty acid to sucrose, as well as the organelles (glyoxysomes and mitochondria) involved, have been studied extensively, the lipid bodies and their associated proteins have not been investigated to any great extent. Among diverse species, the gluconeogenic enzymes as well as the glyoxysomes and mitochondria share very similar properties (Beever, 1979; Huang *et al.*, 1983; Trelease, 1984). Some of these enzymes from diverse species are immunologically cross-reactive (Huang *et al.*, 1983). In contrast, the lipid bodies from different species are very different, as far as the major proteins are concerned (the present paper). In addition, the lipases (minor proteins) associated with the lipid-body membranes are also distinctly different among species (Huang, 1983). We conclude that, unlike the glyoxysomes, the lipid bodies from different species are quite distinct in their protein constituents. The applicability of our findings on the biosynthesis of the lipid-body protein in maize to other species remains to be seen. Nevertheless, our work represents the first detailed study of the ubiquitous organelle.

In maize, the L proteins are predominant proteins in the lipid bodies. They are apparently related in that they all have low M_r and alkaline pI values. However, they are not immunologically cross-reactive. L₃ protein (results are insufficient to assess L₁ and L₂ proteins) is synthesized in the rough ER and is not appreciably processed during or after translation. The physiological role of the L proteins is unknown. L₃ protein is a major protein, representing about half of the total protein in isolated lipid bodies. Even in the total extract it occupies a small percentage of the total protein. Because it is present in such large amounts, it is unlikely to be an enzyme. Rather, it may be a structural component of the lipid-body membrane, serving perhaps to stabilize the monophospholipid layer or to anchor the lipase in the mobilization of storage lipid after germination.

It is known that, in seeds, enzymes for fatty acid synthesis are localized in the plastids, whereas those for triacylglycerol as well as phospholipid synthesis are present in the microsomal fraction, which presumably represents the ER (Appelqvist, 1975; Gurr, 1980; Roughan & Slack, 1982). Thus our findings are in agreement with, although do not prove, the notion that the lipid body is formed by vesiculation of the ER. This formation of a lipid body should include: (a) the sequestration of newly synthesized triacylglycerols into the space between the two phospholipid layers; (b) the flow of newly synthesized phospholipids to the forming lipid body; and (c) the flow of newly synthesized proteins from the bound polyribosomes to the forming lipid body. Although our findings are not in favour of the proposal that the lipid body is formed by encasement of naked oil droplets in the cytosol with membrane materials (Bergfeld *et al.*, 1978), they do not rule out such possibility. A recent report shows that the last enzyme of triacylglycerol synthesis, diacylglycerol acyltransferase, is present in the chloroplasts of spinach (*Spinacia oleracea*) leaves (Martin & Wilson, 1984). This compartmentation

of the enzyme in leaves may be different from that in developing seeds, since leaves possess little triacylglycerol-containing lipid bodies. The chloroplast enzyme may well be used to synthesize plastid oil droplets which have been observed in the leaves of many species. The possibility still exists that the subcellular compartmentation of triacylglycerol biosynthetic enzymes is species-specific.

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