

Lipids, Proteins, and Structure of Seed Oil Bodies from Diverse Species¹

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Oil bodies isolated from the mature seeds of rape (*Brassica napus* L.), mustard (*Brassica juncea* L.), cotton (*Gossypium hirsutum* L.), flax (*Linus usitatissimum*), maize (*Zea mays* L.), peanut (*Arachis hypogaea* L.), and sesame (*Sesamum indicum* L.) had average diameters that were different but within a narrow range (0.6–2.0 μm), as measured from electron micrographs of serial sections. Their contents of triacylglycerols (TAG), phospholipids, and proteins (oleosins) were correlated with their sizes. The correlation fits a formula that describes a spherical particle surrounded by a shell of a monolayer of phospholipids embedded with oleosins. Oil bodies from the various species contained substantial amounts of the uncommon negatively charged phosphatidylserine and phosphatidylinositol, as well as small amounts of free fatty acids. These acidic lipids are assumed to interact with the basic amino acid residues of the oleosins on the surface of the phospholipid layer. Isoelectrofocusing revealed that the oil bodies from the various species had an isoelectric point of 5.7 to 6.6 and thus possessed a negatively charged surface at neutral pH. We conclude that seed oil bodies from diverse species are very similar in structure. In rapeseed during maturation, TAG and oleosins accumulated concomitantly. TAG-synthesizing acyltransferase activities appeared at an earlier stage and peaked during the active period of TAG accumulation. The concomitant accumulation of TAG and oleosins is similar to that reported earlier for maize and soybean, and the finding has an implication for the mode of oil body synthesis during seed maturation.

Seeds store TAG as food reserves for germination and postgerminative growth of the seedlings. The TAG are present in small, discrete intracellular organelles called oil bodies (Yatsu and Jacks, 1972; Appelquist, 1975; Stymne and Stobart, 1987; Huang, 1992). Isolated oil bodies have a spherical shape and possess diameters ranging from about 0.5 to 2.0 μm . They contain mostly TAG and small amounts of PL and proteins called oleosins. It is generally agreed that the oil body has a matrix of TAG surrounded by a layer of PL embedded with oleosins. The PL form a monolayer such that the acyl moieties of the molecules face inward to interact with the hydrophobic TAG in the matrix, and the hydrophilic PL head groups are exposed to the cytosol. The embedded oleosin molecule is composed of three structural domains: an N-terminal amphipathic domain, a central hydrophobic domain, and a C-terminal amphipathic α -helical domain (Vance and Huang, 1987; Qu and Huang, 1990; Murphy et al., 1991; Tzen et al., 1992). It is predicted that the hydrophobic portion

of the oleosin molecule penetrates the PL layer into the TAG matrix, and its amphipathic portion resides on the PL layer or protrudes to the exterior.

The structure of an oil body as described in the preceding paragraph implies that the relative contents of TAG, PL, and proteins in a pure preparation of oil bodies should be related to the size of the organelles. Recently, we used the maize oil body as a model system to correlate the relative contents of TAG, PL, and oleosins to the size of the organelle (Tzen and Huang, 1992). A formula was derived to describe a spherical mass of TAG of a certain diameter surrounded by a shell of one PL layer of 2.5 nm thickness. The layer contains 80% PL and 20% oleosin. Of each oleosin molecule, about 20% of the amino acid residues are embedded in the PL layer, 30% are located in the TAG matrix, and 50% are exposed to the exterior. The PL of the maize oil body include a substantial percentage of the negatively charged PI and PS (Tzen et al., 1992), which are unusual PL components in plant tissues (Mudd, 1980). It has been proposed that these negatively charged PL, together with a small amount of FAA, interact with the basic amino acid residues of the oleosins on the surface of the PL layer. Computation of the charges on the surface of an oil body reveals an isoelectric point of 6.2, which is in agreement with the value measured by pH-dependent aggregation and isoelectrofocusing (Tzen et al., 1992).

During seed maturation in maize (Qu et al., 1986), as well as soybean (Herman, 1981), the embryos accumulated TAG and oleosins concomitantly. This observation is in agreement with, although is not evidence in support of, a model of oil body synthesis in which TAG and oleosins are synthesized concomitantly in the ER, from which a nascent mature oil body is formed by budding (Huang, 1992).

It is unknown whether our knowledge on the structure of the maize oil bodies and their developmental pattern in maturing seed can be extended to the seed oil bodies of other species. The reported relative contents of TAG, PL, and proteins in seed oil body preparations from several species vary substantially (Appelquist, 1975; Murphy et al., 1989). The variation may indicate that our findings from the maize oil bodies are not valid for the seed organelles from other species. Alternatively, it may only reflect differences in the size of the oil bodies and the purity of the preparations. Another discrepancy is in the mode of accumulation of TAG and oleosins during seed maturation. It was reported that, in

¹ Supported by U.S. Department of Agriculture grant 91-01430 (A.H.C.H.).

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Abbreviations: FAA, free fatty acid(s); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid(s); PS, phosphatidylserine; TAG, triacylglycerol(s).

rapeseed (Murphy et al., 1989), unlike maize (Qu et al., 1986) and soybean (Herman, 1987) seeds, the accumulation of oleosins lagged temporally behind that of TAG. The data have been used to propose that the oil body is synthesized first with TAG, followed temporally by an encasement with subsequently synthesized oleosins. Whether or not this alternative model of oil body formation reflects a species-dependent difference in the organelle biogenesis is unknown.

To clarify these matters, we analyzed the seed oil bodies isolated from several species. We selected species that have seed oil bodies of diverse average diameters. Our findings show that the structural model of the oil body established with the maize organelle is applicable to those in other species. We also found that, in maturing rape as well as maize seeds, the accumulation of TAG and oleosins occurs concomitantly. Here, we present our findings and discuss the reported discrepancies concerning seed oil bodies from different species.

MATERIALS AND METHODS

Plant Materials

Embryos of maize (*Zea mays* L., inbred line MO 17), tripsacum (*Tripsacum dactyloides* L.), teosinte (*Z. mays* ssp. *mexicana*, var Chalco), yucca (*Yucca whipplei*), rape (*Brassica napus* L. var Westar), mustard (*Brassica juncea* L.), cotton (*Gossypium hirsutum* L.), jojoba (*Simmondsia chinensis* L.), sunflower (*Helianthus annuus* L.), soybean (*Glycine max* L.), flax (*Linum usitatissimum*), sesame (*Sesamum indicum* L.), and peanut (*Arachis hypogaea* L.), endosperm and embryo of palm (*Syagrus cocoides*, Martius), castor bean (*Ricinus communis* L.), Brazil nut (*Bertholletia excelsa*), and bran of oat (*Avena sativa* L.) and wheat (*Triticum aestivum* L.) were obtained from mature seeds. The mature seeds were either used directly (rape, mustard, flax, and sesame) or soaked in water for 1 h (soybean, peanut, oat, and wheat bran), 6 h (cotton and sunflower), 24 h (maize, teosinte, tripsacum, yucca, castor bean, Brazil nut, and jojoba), or 72 h (palm) before use.

Sampling of Maturing Seeds of Maize and Rape

Maize plants were grown at the university field station in summer 1990. The ear was bagged before the silk started to appear and was checked daily for the appearance of silk. When the silks were about 1.5 to 2 cm long, they were cut with a razor blade, and the ear was tagged. Fresh pollen was collected on the following day and used to pollinate the silk. Pollination was repeated on two consecutive days. The second day of pollination was designated as 0 d after pollination. Kernels were harvested every other day from 13 to 33 d after pollination. Embryos from 25 kernels of each maturing stage were used.

Rape plants were grown inside a greenhouse maintained at 26.5/18.5°C, about 14/10 h day/night cycle in fall 1990. As soon as the flowers opened, they were pollinated with pollen obtained from several plants. The pollinated flowers were tagged and the other open flowers without artificial pollination were removed. The pods were harvested every other day from 12 to 40 d after pollination. Two hundred seeds of each maturing stage were used.

Isolation of Oil Bodies

The tissue was homogenized at 4°C in grinding medium (5 g of tissue/20 mL) with a Polytron (Brinkmann, Westbury, NY) fitted with a PTA 10 generator at high speed for 40 s. 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 Nitex cloth having 20- × 20-μm pores (Tetko, Elmsford, NY). After filtration, each 15-mL portion of the homogenate was placed at the bottom of a 30-mL centrifuge tube, and 15 mL of flotation medium (grinding medium containing 0.4 instead of 0.6 M sucrose) was layered on top. The tube was centrifuged at 10,000g for 30 min in a swinging-bucket rotor. The oil bodies on top were collected and resuspended in 15 mL of grinding medium containing an additional 2 M NaCl. The resuspension material was placed at the bottom of a 30-mL centrifuge tube, 15 mL of floating medium (grinding medium containing 2 M NaCl and 0.25 M instead of 0.6 M sucrose) was layered on top, and the tube was centrifuged. The oil bodies on top were collected and resuspended in 15 mL of grinding medium. The resuspension material was placed at the bottom of a 30-mL centrifuge tube, 15 mL of floating medium (grinding medium containing 0.4 instead of 0.6 M sucrose) was layered on top, and the tube was centrifuged. This last step of flotation washing was repeated once. The oil bodies on top were collected and resuspended with grinding medium to give a concentration of about 100 mg of lipid/mL.

Measurement of Oil Body Diameter

In a preliminary study, seed oil bodies isolated from 18 species were observed under a light microscope with Nomarski optics. The oil bodies from seven species (rape, mustard, cotton, flax, maize, peanut, and sesame), which had a diverse range of sizes, were chosen for further studies. The oil body preparations were fixed in 2.5% glutaraldehyde in 50 mM potassium phosphate buffer, pH 7.5, for 3 h. After several rinses with the buffer, they were postfixed in 1% OsO₄ in the same buffer for 18 h. Dehydration was carried out in a graded acetone series. The samples were embedded in Spurr's resin. Serial sections 100 nm thick were stained with uranyl acetate and lead citrate and photographed under a Philips EM400 electron microscope. The photo of the middle section of the serial sections was used to mark all the oil bodies (150–500, depending on the species) within a defined area. Each of these marked oil bodies was traced throughout the serial sections to measure its largest diameter (R). The volume (V) of each oil body was calculated by the formula, $V = 4/3 \pi (R/2)^3$. The average volume (\bar{V}) of the oil bodies was obtained by dividing the sum of all the oil body volumes by the total oil body number. The average diameter (\bar{R}) of oil bodies was calculated from their average volume by the formula $\bar{V} = 4/3 \pi (\bar{R}/2)^3$.

IEF of Oil Bodies

IEF was performed in a Bio-Rad Rotofor Cell (Richmond, CA), which had the following design. The horizontal cylindrical focusing cell of 55 mL was divided vertically into 20

chambers by porous partitions to minimize diffusion during electrophoresis and disturbance of the gradient during fractionation. During electrofocusing, the Rotofor Cell rotated at 1 rpm and thus minimized the flotation of oil bodies. Fractionation of contents in every chamber was achieved simultaneously in 1 s by a vacuum harvesting system.

In the current study, we removed the partitions to allow free movement of the oil bodies during electrofocusing (Tzen et al., 1992). Because the gradient without the partitions could not be fractionated, the position of the oil bodies along the gradient after electrofocusing was photographed. The pH gradient, made from 1% ampholyte (0.5% Bio-Lyte 5–7 and 0.5% Bio-Lyte 7–9, both from Bio-Rad), was performed by applying an electric field at constant power (12 W) at 400 to 500 V for 10 min. Isolated oil bodies (about 20 mg of lipid) in 200 μ L of 10-mM KCl suspension were applied to the gradient at the high pH region. An electric field of the same wattage was applied for 10 min to focus the oil bodies. Movement of the oil bodies was observed visually. The pH gradient in the current setup was stable before and after sample application and electrofocusing, as well as among different runs. This was revealed by repeating the runs with the vertical partitions included in the chamber and then fractionating the gradient into 20 fractions and checking the pH in each fraction with a pH meter.

Quantitation of Constituents of Isolated Oil Bodies

Neutral lipids (mainly TAG), proteins, and PL of oil bodies were separated by the following procedure. A 500- μ L preparation of isolated oil bodies was extracted with 1 mL of diethyl ether in a 1.7-mL Eppendorf tube. After the preparation was centrifuged at 13,600g for 4 min, the upper ether layer was collected. The lower aqueous layer and the interfacial materials were extracted with 1 mL of diethyl ether two additional times. The ether fractions were pooled (3 mL), and the ether was evaporated under nitrogen gas. The aqueous layer, together with the interfacial materials, was allowed to evaporate under nitrogen for 1 h to remove the remaining ether. A volume of 750 μ L of chloroform:methanol (2:1, v/v) was added. After gentle shaking, the tube was centrifuged at 13,600g for 4 min. The lower chloroform layer and the interfacial materials were collected separately. The chloroform fraction (which contained PL) was washed three times, each time with 1 mL of methanol:water (1:1, v/v), followed by centrifugation. The interfacial materials (which were mostly proteins) were washed three times by the following procedure. They were mixed with 250 μ L of water and 750 μ L of chloroform:methanol (2:1, v/v), and the mixture was centrifuged. The interfacial materials were collected and resuspended in 0.5 mL of water. The resuspension material was sonicated with a 4-mm diameter probe in a Braun-Sonic 2000 ultrasonic generator (Freeport, IL) with a digital meter reading of 200 for 15 s.

The proteins in the interfacial materials were quantified by the Bradford method using BSA, fraction V, as standard (Smith et al., 1985). They were resolved into various constituents by SDS-PAGE with the separating gel and the stacking gel composed of 12.5 and 4.75% polyacrylamide, respectively

(Tzen et al., 1990). After electrophoresis, the gel was stained with Coomassie blue R-150 and destained.

The neutral lipids in the ether fraction were weighed directly (about 0.1 g from 1 mL of oil body suspension) after exhaustive evaporation of the ether. The chloroform fraction containing PL was spotted on a TLC plate (silica gel 60A from Whatman). The plate was developed in chloroform:acetic acid:methanol:water (70:25:5:2, v/v/v/v) and then allowed to react with iodine for color development. Standard PL (Sigma, St. Louis, MO) used for identification were α -PC (egg yolk type IX-E), α -PS (bovine brain), α -PI (bovine liver), α -PE (*Escherichia coli* type V), α -phosphatidylglycerol (dioleoyl), and α -phosphatidic acid (dioleoyl). After the different PL were identified, the TLC plate was placed in a vacuum evaporator to allow the iodine to evaporate. The gel corresponding to the different PL were scraped, and their phosphate contents were quantified by the Bartlett method using KH_2PO_4 as a reference (Dittmer and Wells, 1969). The PL content in the oil body fraction was measured directly by the same method. The identity of the PS from maize oil bodies was confirmed qualitatively by hydrolysis with phospholipase C (*Clostridium perfringens* type XIV; Sigma) followed by detection of the released phosphoserine (derivatized with phenyl isothiocyanate) by HPLC, using bovine brain α -PS as a control (courtesy of Dr. M. Madore).

FFA of the oil body fraction were determined by a colorimetric method (Nixon and Chan, 1979) using oleic acid and palmitic acid as standards (both gave an identical standard curve). We isolated oil bodies from freshly harvested mature seeds of rape, mustard, cotton, and maize to avoid the possibility of detecting FFA generated during aging.

Determination of Oil Body Constituents in Total Extracts of Maturing Seeds

Two hundred rape or 25 maize embryos were homogenized in 4 mL of grinding medium as described earlier, except that DTT was deleted from the grinding medium. After filtration, the homogenate was adjusted to 5 mL with grinding medium. The homogenate was either used directly or frozen at -80°C until use.

Total proteins in the homogenate were quantified by the Bradford method using BSA, fraction V, as the standard (Smith et al., 1985). The protein components were resolved by SDS-PAGE, using 12.5 and 4.75% polyacrylamide in the separating gel and the stacking gel, respectively. Two identical gels were run. One gel was stained with Coomassie blue R-150, and the other gel was subjected to an immunoblotting assay (Tzen et al., 1990). In both cases, the relative quantity of the 20-kD oleosins was obtained by the following method. The samples from the stage of 33 d (for maize) and 36 d (for rape) after pollination were chosen as standards (100%) for the calculation of the relative amounts from different stages. To estimate the relative amounts of TAG and major oleosins from different stages, a serial dilution (5, 10, 20 . . . 80, 90%) of the sample from the standard stage (33 or 36 d after pollination) plus the samples from other stages were resolved by TLC or SDS-PAGE. Visual comparison was used to determine the relative amounts of the TAG and oleosins.

For the analysis of total neutral lipid in the 33-d rape and

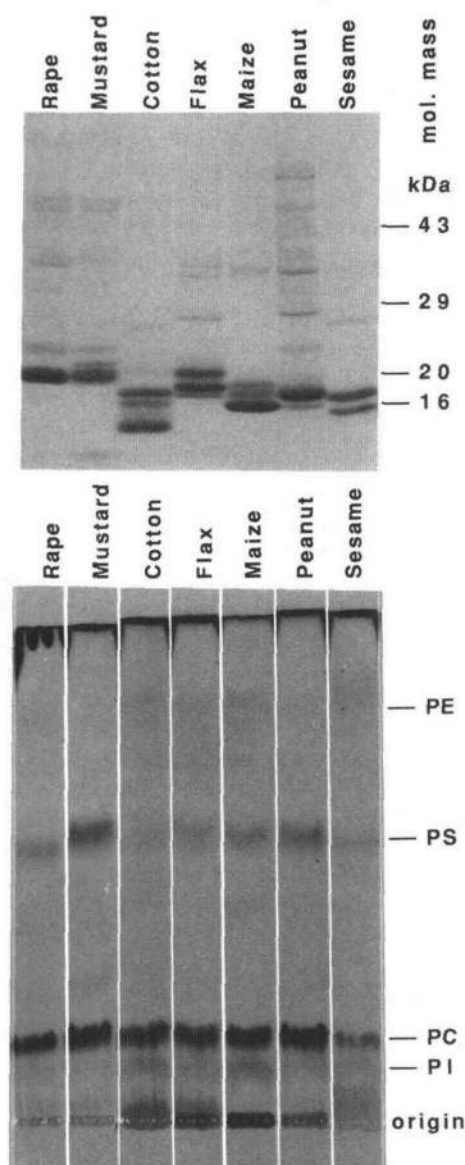


Figure 1. SDS-PAGE of proteins (top) and TLC of PL (bottom) of oil bodies isolated from the seeds of various species. Labels on the right indicate the molecular masses of proteins or the types of PL. The proteins were stained with Coomassie blue, and the PL were visualized with iodine.

36-d maize samples, the homogenate was extracted with an equal volume of diethyl ether three times. The combined ether fractions were subjected to exhaustive evaporation with nitrogen, and the residual neutral lipids were weighed. For the analysis of lipid constituents, the homogenate was extracted with an equal volume of chloroform:methanol (2:1, v/v). After the homogenate was centrifuged, the chloroform layer was collected and evaporated under nitrogen to about 50 μL . The sample was applied to a TLC plate (silica gel 60A; Whatman). The plate was developed in hexane:diethyl ether:acetic acid (80:20:2, v/v/v) and then allowed to react with iodine for color development.

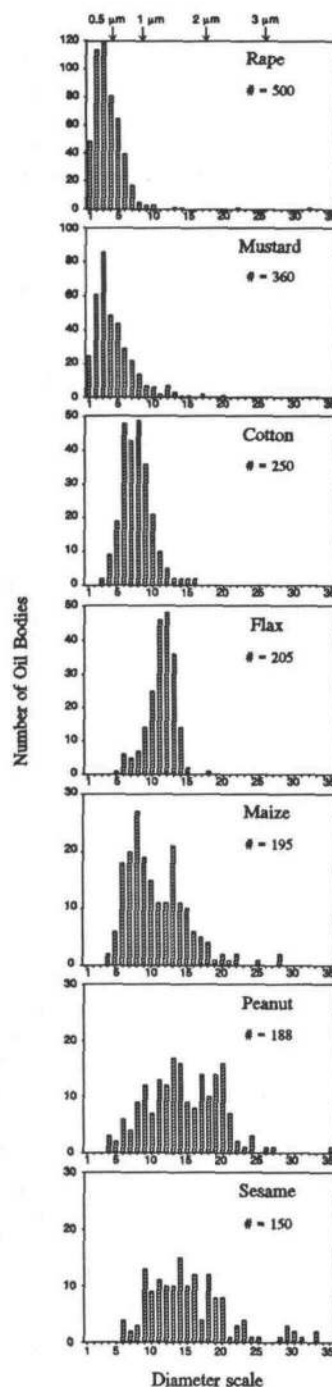


Figure 2. Size distribution of seed oil bodies isolated from seven species. The diameters of all the oil bodies in a defined area in an embedding Spurr block for EM were followed via serial sectioning. The total number of oil bodies measured for each species is indicated. One unit of diameter scale represents 0.116 μm (the departure from unity is due to the specific magnification of the electron micrographs). Each vertical bar shows the number of oil bodies having diameters of the indicated unit $\pm 50\%$ of 1 unit. The simplified units of diameter are shown at the top. Because the volume of a sphere is related to the diameter (R) by $V = 4/3 \pi (R/2)^3$ and not by linear proportionality, the average diameter of a population of oil bodies (Table I) is not at the peak of diameter distribution.

Enzyme Assays

The total homogenate was used directly. Glycerol-3-P acyltransferase activity was assayed using [^{14}C]glycerol-3-P and oleoyl CoA by monitoring the combined production of [^{14}C]lysophosphatidate and [^{14}C]phosphatidate (Sun et al., 1988). 1-Lysophosphatidate acyltransferase activity was assayed using 1-oleoyl-lysophosphatidate and [^{14}C]oleoyl CoA by monitoring the production of [^{14}C]phosphatidate (Oo and Huang, 1989). 1,2-Diacylglycerol acyltransferase activity was assayed using 1,2-dioleoyl-diacylglycerol and [^{14}C]oleoyl CoA by monitoring the production of [^{14}C]TAG (Cao and Huang, 1987).

Chemicals

[^{14}C]Glycerol-3-P (uniformly labeled, 151 mCi/mmol) and [^{14}C]oleoyl CoA (C-1 labeled, 58 mCi/mmol) were purchased from New England Nuclear (Boston, MA). 1,2-Dioleoyl diacylglycerol was obtained from NuChek (Elysian, MN). All other important chemicals of the highest grades were purchased from Sigma.

RESULTS

Seed Oil Bodies from Diverse Species Had Average Diameters That Were Different But within a Narrow Range

Preliminary experiments were performed to select species having seed oil bodies of a diverse size range. Seed oil bodies were isolated from 18 species, and their approximate sizes were observed under a light microscope with Nomarski optics. Seven species, which had seed oil bodies representing a diverse range of sizes, were chosen for detailed studies. Oil body preparations isolated from these species contained putative oleosins of low molecular masses of 16 to 24 kD and had few high molecular mass proteins, as revealed by SDS-PAGE (Fig. 1). Eleven other species also had oil bodies falling within the size range of the seven chosen species; they included oat, teosinte, tripsacum, wheat, palm, yucca, jojoba, Brazil nut, castor bean, sunflower, and soybean. The oil bodies from these 11 species were not studied further.

The diameters of seed oil bodies of several plant species in isolated preparations or in situ, as measured from electron micrographs, have been reported (Appelquist, 1975). However, they were all approximate or underestimated values because the oil bodies in an electron micrograph are usually not viewed in their actual equatorial planes but rather at randomly cut planes of the organelles. In addition, it is difficult to measure the diameters of oil bodies in mature seeds in situ because the organelles often assume irregular shapes due to their being pressed against other structures within the cell. Furthermore, the oil bodies within the same seed may vary in size, depending on the cell types in which they occur. In the current study, we overcame the above difficulties by doing serial sectioning of a preparation of isolated oil bodies from each of the seven species. We followed and measured the largest diameters of all 150 to 500 oil bodies within a defined area of the embedding block. As expected, oil bodies isolated from mature seeds resuspended in a dilute buffer at pH 7.5 assumed a spherical shape. Figure 2 shows the distribution of diameters of the oil bodies from the seven species. The average diameters ranged from 0.65 μm in rape to 2.0 μm in sesame. In each species, one to a few percent of oil bodies had sizes several times larger than the average values.

Contents of Neutral Lipids, Proteins, and PL in Oil Bodies from Diverse Species Were Correlated with the Sizes of the Organelles

The contents of the major components (neutral lipids, PL, FFA, and proteins) in the oil bodies isolated from the seven species are shown in Table I. Most of the neutral lipids are TAG (for convenience, we will use TAG to denote the neutral lipids in the subsequent portion of this report). The percent content of TAG increases, whereas the percent contents of PL, FFA (except in sesame, to be discussed), and proteins decrease with the increase in the diameter of the oil bodies (Table I). These correlations are consistent (Fig. 3) with a model of an oil body described earlier for the maize organelle (Tzen and Huang, 1992). The model describes a spherical particle of a TAG matrix surrounded by a shell of PL embedded with oleosins. The PL form a single layer with the

Table I. Average diameters and the amounts of various chemical constituents of oil bodies isolated from the seeds of various species

	Rape	Mustard	Cotton	Flax	Maize	Peanut	Sesame
Average diameter (μm)	0.65	0.73	0.97	1.34	1.45	1.95	2.00
Major constituents (% w/w)							
Neutral lipids	94.21	94.64	96.99	97.65	97.58	98.17	97.37
Proteins	3.46	3.25	1.70	1.34	1.43	0.94	0.59
PL	1.97	1.60	1.18	0.90	0.91	0.80	0.57
FFA	0.36	0.17	0.13	0.11	0.09	0.09	0.13/1.47 ^a
PL composition (% w/w)							
PC	59.9	53.1	58.6	57.2	64.1	61.6	41.2
PE	5.9	15.5	4.6	2.8	8.1	5.0	15.8
PI	14.0	13.1	18.1	6.9	7.6	8.4	20.9
PS	20.2	18.3	18.7	33.1	20.2	25.0	22.1

^a 0.13 and 1.47% were obtained from oil bodies isolated from seed freshly harvested and seed harvested and stored for several months, respectively. See "Results" for explanation.

hydrophobic acyl moieties facing the matrix and the hydrophilic head groups being exposed to the exterior. Eighty percent of this layer is filled with PL, and the remaining 20% is occupied by oleosins. Of each oleosin molecule, about 20% of the amino acid residues are embedded in the PL layer, 30% are located in the TAG matrix, and 50% are exposed to the exterior. The observed correlations are not consistent with an oil body having half, two, or three layers of PL embedded with oleosins (Fig. 3).

Seed Oil Bodies from Diverse Species Had an Isoelectric Point of 5.7 to 6.6 and thus Possessed a Negatively Charged Surface at Neutral pH

Earlier, we showed the presence of negatively charged PS, PI, and FFA in the oil bodies isolated from maize embryos (Tzen et al., 1992). We have proposed that these negatively charged components interact with the positively charged amino acid residues of the oleosins on the surface of the organelle. Here, we report that PS, PI, and FFA were also found in the organelles from the other six species (Fig. 1). The relative percentage of the various PL components in the oil bodies among the seven species were quite similar (Table I). PC was the most abundant component. PS and PI represented about 30 to 40% of the total PL. Also, FFA were present in small amounts in the oil bodies from the seven species, and their percent contents were correlated with the average diameters of the oil bodies (Table I). The only exception was the very high amount of FFA in the sesame oil body preparation; it may be the product of lipid degradation in aged seed. This suggestion was partly supported by a follow-up experiment. Plants from the same sesame seed lot were grown to maturity in a greenhouse. Oil bodies were isolated from freshly harvested mature seed, and their percentage of FFA was measured. A value of 0.13%, instead of 1.47% (Table I), was obtained. The new oil body sample was not subjected to further analyses.

Computation of the charges associated with PS, PI, and FFA, as well as basic and acidic amino acid residues of the oleosins, in a maize oil body suggests that the organelle has an isoelectric point of about 6.2 (Tzen et al., 1992). This value is consistent with that measured by pH-dependent aggregation and IEF (Tzen et al., 1992). In the current study, we also observed similar proportions of the negatively charged lipids in the oil bodies isolated from the other species (Table I). However, we are unable to compute the charges on these oil bodies because the complete amino acid sequences of the different oleosin isoforms of each species (Fig. 1) are not available. To determine whether the oil bodies from these other species also had a negatively charged surface at neutral pH, we subjected the oil bodies to IEF (Fig. 4). The oil bodies from each of the seven species were isoelectrofocussed at pH 5.7 to 6.6. Thus, they were negatively charged at the physiological pH of 7.

Embryos of Maize and Rape Accumulated TAG and Oleosins Concomitantly during Seed Maturation

In the embryo of maize during seed maturation, the accumulation of total proteins slightly preceded that of TAG and

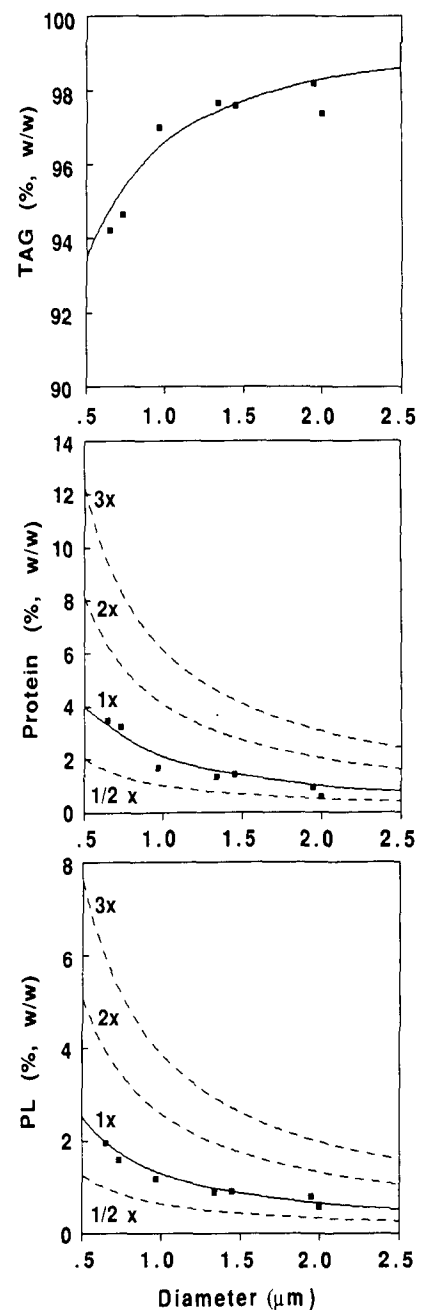


Figure 3. A comparison between experimentally determined and theoretically calculated values of oil body contents in relation to the average diameters of the organelles from seven species. The closed squares represent the experimentally determined values from the seven species (see Table I for the seven species and their corresponding oil body diameters). The curves show the theoretical values of the percentage contents of TAG, proteins, and PL calculated according to a formula derived from an oil body model (Tzen and Huang, 1992). The model postulates a spherical TAG matrix surrounded by a shell of one PL layer. The layer contains 80% PL and 20% oleosins. In each oleosin molecule, about 20% of the amino acid residues are embedded in the PL layer, 30% are located in the TAG matrix, and 50% are exposed to the exterior. The theoretical values representing half, two, and three layers of PL plus proteins in an oil body are shown by broken lines.

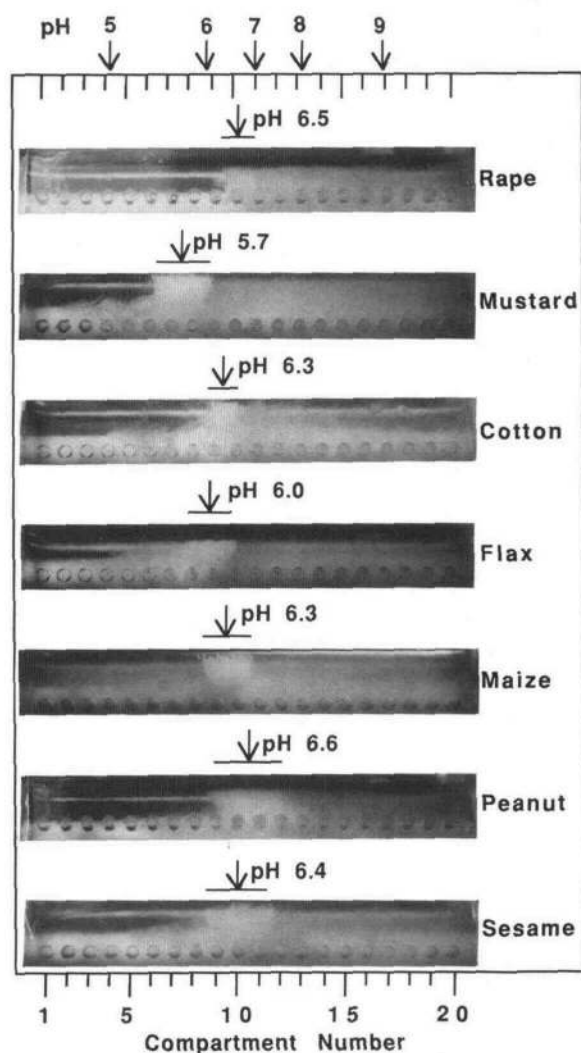


Figure 4. IEF of oil bodies from different species. IEF was performed in a horizontal Bio-Rad Rotofor Cell. The pH gradient was preformed. The oil bodies were loaded at the pH 9 region (compartment No. 18). The vertical porous partitions in the Rotofor Cell were removed before electrofocusing so that the oil bodies could move freely. Without the vertical partitions, the gradient could not be fractionated. The position of the isoelectrofocussed oil bodies along the gradient was recorded in a photograph, which is shown. Each of the 20 plugged holes of the Rotofor Cell represented the center of each of the 20 compartments. The pH along the gradient, indicated at the top of the figure, was determined experimentally in a separate run in which the vertical partitions were included and fractionation was performed. The horizontal line above each photograph indicates the region of the isoelectrofocussed oil bodies, and the pH at the center of that region is shown.

oleosins (Fig. 5). The accumulation of oleosins and TAG occurred concomitantly, as reported earlier but in a less detailed analysis (Qu et al., 1986). Similarly, in the embryo of rape during seed maturation, the accumulation of total proteins preceded that of TAG and oleosins (Fig. 6). Again, the accumulation of oleosins and TAG occurred concomitantly. The accumulation of different proteins and lipids during rapeseed maturation was followed by SDS-PAGE and

TLC, respectively (Fig. 7). Many, presumably housekeeping, proteins of molecular masses in the range of 40 to 120 kD appeared before the oleosins (the dominant protein band at the 20-kD region in the gel) and the bulk of the two classes of storage proteins, napin (4 and 9 kD, indistinguishable in the current gel system) and cruciferin (22–35 kD). The accumulation of the 20-kD oleosins, as revealed in the SDS-PAGE gel, was further confirmed by immunoblotting (Fig. 7). The accumulation of the oleosins was clearly concomitant with that of the TAG.

During the maturation of rapeseed, the activities of the three major acyltransferases for the synthesis of TAG (glycerol-3-P acyltransferase, lysophosphatidate acyltransferase, and diacylglycerol acyltransferase) appeared slightly earlier than the amount of TAG. They peaked at day 27 when the rate of TAG accumulation was at its maximum (Fig. 6).

DISCUSSION

Earlier studies have shown that within the same seed, oil bodies may be of different sizes in different cells or tissues (Trelease, 1969). For example, in maize embryo, the oil bodies in the scutellum are larger than those in the embryonic axis. It is likely that the size of the oil bodies is also dependent on environmental factors such as the nutritional status of the plants (e.g. available nitrogen for oleosins and phosphate for PL). Notwithstanding, the current study reveals that the sizes of seed oil bodies from diverse species are rather species dependent and fall within a narrow range. The oil bodies from the seven species with which we did detailed analyses have average diameters ranging from 0.65 to 2.0 μm . In addition, oil bodies falling within this size range are present

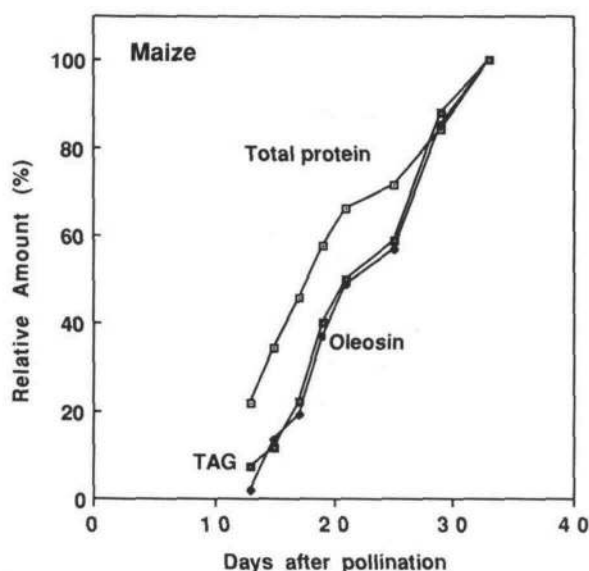


Figure 5. Accumulation of total proteins, TAG, and oleosins in the embryo of maize during seed maturation. The contents at day 33 after pollination were used as standards of 100% to calculate the relative contents in other maturation stages. The contents at day 33 were 50 mg fresh weight, 5.04 mg of total proteins, and 10.9 mg of neutral lipids per embryo.

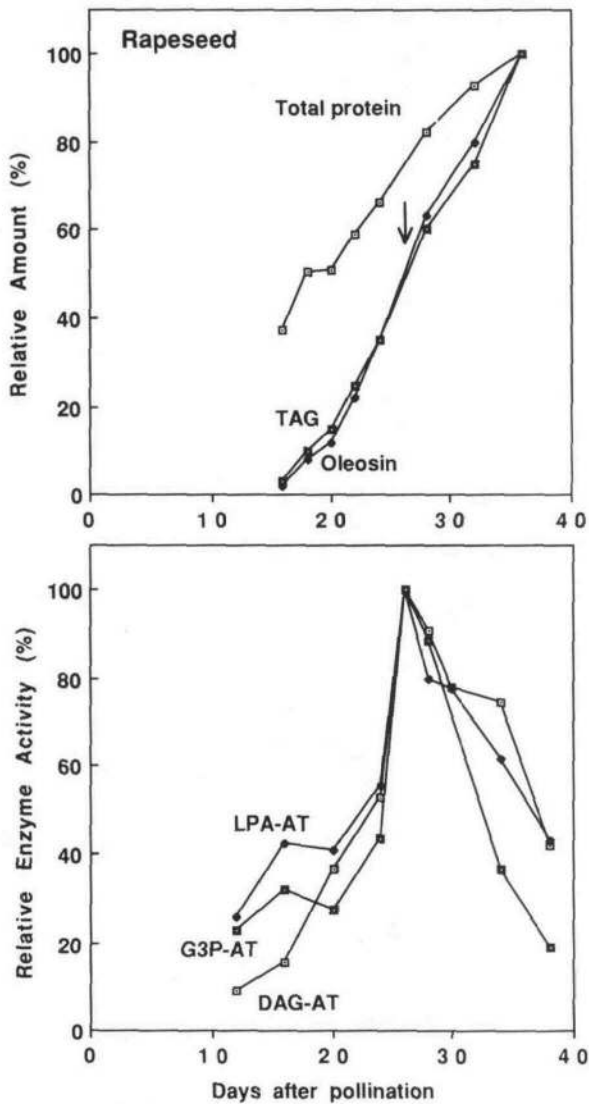


Figure 6. Accumulation of total proteins, TAG, oleosins, and acyltransferase activities in the embryo of rape (*B. napus*) during seed maturation. The contents at day 36 after pollination were used as standards of 100% to calculate the relative contents in other maturation stages. The contents at day 36 were 7.41 mg fresh weight, 1.85 mg of total proteins, 3.7 mg of neutral lipids, and 15.7 pmol/min of glycerol-3-P acyltransferase (G3P-AT), 85.6 pmol/min of lysophosphatidate acyltransferase (LPA-AT), and 27.5 pmol/min of diacylglycerol acyltransferase (DAG-AT) per embryo. The arrow in the top panel shows the time when maximal acyltransferase activities occurred; this time point occurred when the rate of lipid accumulation was at its maximum.

in 11 other species (first section in "Results"). Furthermore, oil bodies of this size range were reported to be present in several other species (Appelquist, 1975), as measured from individual electron micrographs (i.e. not by the more accurate serial sectioning used in the current study). The narrow size range of seed oil bodies may represent a compromise between providing a maximal surface area per unit TAG for lipase binding during germination (Wang and Huang, 1987) and

utilizing minimal amounts of oleosins and PL. The relatively small seed oil bodies are in great contrast to the large oil particles in fruit mesocarp of some species such as avocado (Platt-Aloia and Thomson, 1981) and oil palm (Mohankumar et al., 1990). The fruit mesocarp oils are not mobilized by the plants and are present in much larger intracellular oil particles of diameters $>10 \mu\text{m}$.

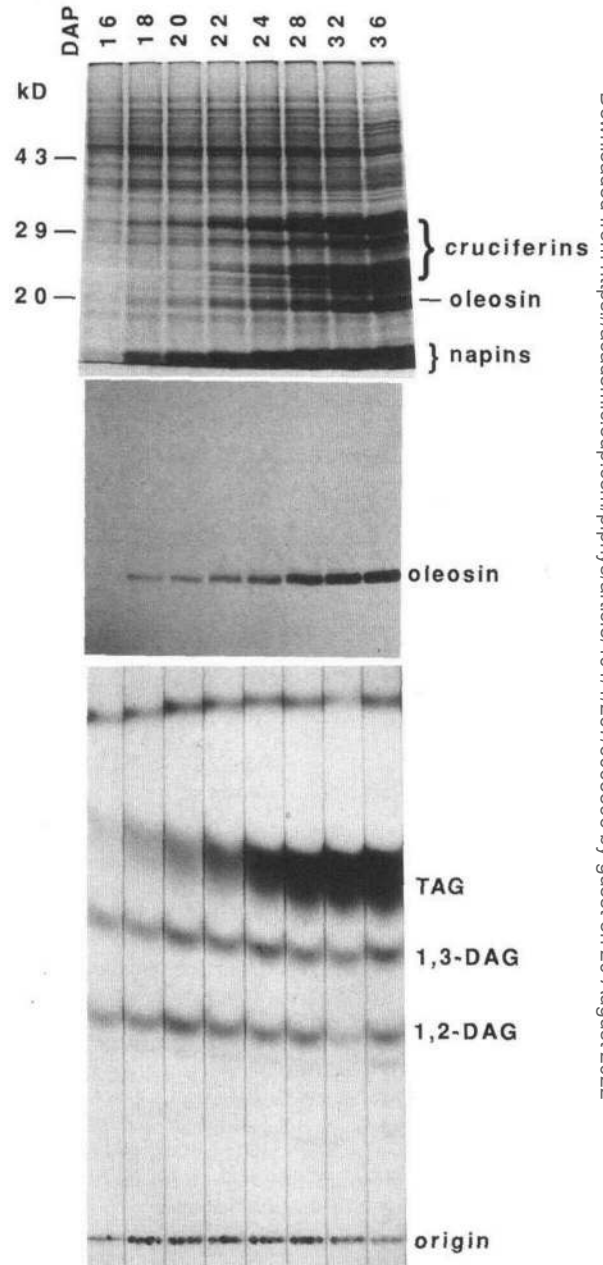


Figure 7. Accumulation of various proteins and lipids in the embryo of rapeseed (*B. napus*) during seed maturation. The various proteins were resolved by SDS-PAGE and either stained with Coomassie blue (top) or further treated for immunoblotting using antibodies raised against the *B. napus* 20-kD oleosins (middle). The various lipids were resolved by TLC (bottom). Molecular masses of proteins are marked in the left margin. The various known proteins and lipids are indicated in the right margin.

Earlier, we did extensive studies of the structure of oil bodies from mature maize embryo (Tzen and Huang, 1992; Tzen et al., 1992). Our findings from the maize oil bodies have now been extended to the seed oil bodies from six other species. The oil bodies from diverse species share the following structural similarities: (a) the relative amounts of TAG, oleosins, and PL are related to the size of the oil bodies according to a model of a spherical TAG particle surrounded by a shell of one layer of PL embedded with oleosins; (b) the oil bodies have a narrow size range, which may represent a compromise between providing a maximal surface area for active lipolysis during seed germination and utilizing minimal amounts of oleosins and PL; (c) oil bodies have the unusual negatively charged PL components (PS and PI) and FFA, which presumably interact with the positively charged amino acid residues of the oleosin, resulting in a negatively charged organelle surface at neutral pH. Thus, the earlier conclusion that maize oil bodies maintain their stability and individual entity by virtue of the steric hindrance provided by the abundant oleosins (Tzen and Huang, 1992) and the negatively charged surface (Tzen et al., 1992) can also be applied to the oil bodies in other species.

Although our results concerning the oil bodies of rape are similar to those of maize and other species, they differ drastically from those of the same rape species (*B. napus*) reported by another laboratory. The major differences are as follows: (a) The other laboratory reported that oleosins represented almost 12% (w/w) of the oil bodies (Cummins and Murphy, 1990) and 20 to 25% of the total proteins in the mature embryo (Murphy et al., 1989). However, we found that the oleosins occupied 3.5% of the oil bodies (Table I) and about 7 to 8% of the total embryo proteins (Tzen et al., 1990). In spite of this major difference, both laboratories reported that the oil bodies had similar sizes of 0.65 μm average diameter measured from serial sections (Table I) and 0.4 μm measured using individual in situ electron micrographs (Cummins and Murphy, 1990), that the mature embryo contained about 45% oils and 25% proteins (Murphy et al., 1989; see legend of Fig. 6), and that the oleosin molecule, having about one-third hydrophobic and two-thirds amphipathic or hydrophilic amino acid residues, resides on the surface of the oil body of one PL layer (Murphy et al., 1991; Tzen and Huang, 1992). By calculation, the theoretical amount of oleosins in an oil body of about 0.4 to 0.65 μm (Fig. 3) is approximately 4% (w/w) protein. It follows that the amount of oleosins in the seed is (4% oleosin/unit oil) \times (45% oil/seed weight), or 1.8% (w/w) oleosin in the seed. Thus, oleosins represent 1.8/25% protein/seed weight, or 7.2% oleosin in the total seed protein.

(b) It was reported that during seed maturation, the accumulation of oleosins lagged substantially behind that of TAG (Murphy et al., 1989). This set of data was used to propose that the oil body is synthesized first with the TAG, followed temporally by an encasement with subsequently synthesized oleosins. Our current results show that oleosins and TAG accumulated via indistinguishable developmental patterns during seed maturation.

(c) It was reported that during seed maturation a water-soluble glycerol-3-P acyltransferase (detected by immunoblotting) peaked after most of the TAG had been accumulated

(Fawcett et al., 1990). Our present findings reveal that the detected in vitro activities of the ER-associated glycerol-3-P, lysophosphatidate, and diacylglycerol acyltransferases peak during the active period of TAG accumulation. In an overall assessment, we do not offer an explanation for the discrepancies between the findings from the two laboratories. However, we do not believe that the differences in the rape (*B. napus*) varieties used and the growth conditions of the plants can account for such drastic variations in the contents of the oleosins and the relative developmental patterns of the oleosins, TAG, and the acyltransferases.

ACKNOWLEDGMENTS

We thank Dr. K. Platt for assistance with EM and Dr. E. Herman for a loan of the Bio-Rad Rotofor Cell.

Received June 26, 1992; accepted September 24, 1992.
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LITERATURE CITED

- Appelquist L-Å** (1975) Biochemical and structural aspects of storage and membrane lipids in developing oil seeds. In T Galliard, El Mercer, eds, Recent Advances in the Chemistry and Biochemistry of Plant Lipids. Academic Press, London, UK, pp 247–286
- Cao YZ, Huang AHC** (1987) Acyl CoA preference of diacylglycerol acyltransferase from the maturing seeds of *Cuphea*, maize, rapeseed, and canola. *Plant Physiol* **84**: 762–765
- Cummins I, Murphy DJ** (1990) Mechanism of oil body synthesis and maturation in developing seeds. In PJ Quinn, JL Harwood, eds, Plant Lipid Biochemistry, Structure, and Utilization. Portland Press, London, UK, pp 231–233
- Dittmer JC, Wells MA** (1969) Quantitative and qualitative analysis of lipids and lipid components. *Methods Enzymol* **14**: 482–530
- Fawcett T, Whitfield HV, Edwards E, Murphy DJ** (1990) Biochemical and molecular biological characterisation of acyltransferases in oilseeds. In PJ Quinn, JL Harwood, eds, Plant Lipid Biochemistry, Structure, and Utilization. Portland Press, London, UK, pp 172–174
- Herman EM** (1987) Immunogold-localization and synthesis of an oil-body membrane protein in developing soybean seeds. *Planta* **172**: 336–345
- Huang AHC** (1992) Oil bodies and oleosins in seeds. *Annu Rev Plant Physiol* **43**: 177–200
- Mohankumar C, Arumughan C, Kalesya raj R** (1990) Histological localization of oil palm fruit lipase. *J Am Oil Chem Soc* **10**: 665–669
- Mudd JB** (1980) Phospholipid biosynthesis. In PK Stumpf, EE Conn, eds, The Biochemistry of Plants, Vol 4. Academic Press, New York, pp 249–282
- Murphy DJ, Cummins I, Kang AS** (1989) Synthesis of the major oil-body membrane protein in developing rapeseed (*Brassica napus*) embryos. *Biochem J* **258**: 285–293
- Murphy DJ, Keen JN, O'Sullivan JN, Au DMY, Edwards EW, Jackson PJ, Cummins I, Gibbons T, Shaw CH, Ryan AJ** (1991) A class of amphipathic proteins associated with lipid storage bodies plants. Possible similarities with animal serum apolipoproteins. *Biochim Biophys Acta* **1088**: 86–94
- Nixon M, Chan SHP** (1979) A simple and sensitive colorimetric method for the determination of long-chain free fatty acids in subcellular organelles. *Anal Biochem* **97**: 403–409
- Oo KC, Huang AHC** (1989) Lysophosphatidate acyltransferase activities in the microsomes from palm endosperm, maize scutellum, and rapeseed cotyledon of maturing seeds. *Plant Physiol* **91**: 1288–1295
- Platt-Aloia KA, Thomson WW** (1981) Ultrastructure of the mesocarp of mature avocado fruit and changes associated with ripening. *Ann Bot* **48**: 451–465

- Qu R, Huang AHC** (1990) Oleosin KD 18 on the surface of oil bodies in maize: genomic and cDNA sequences, and the deduced protein structure. *J Biol Chem* **265**: 2238–2243
- Qu R, Wang SM, Lin YH, Vance VB, Huang AHC** (1986) Characteristics and biosynthesis of membrane proteins of lipid bodies in the scutella of maize (*Zea mays* L.). *Biochem J* **234**: 57–65
- Smith PK, Krohn RI, Hermanson GT, Malia AK, Gartner FH, Proverzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC** (1985) Measurement of protein using bicinchoninic acid. *Anal Biochem* **150**: 76–85
- Stymne S, Stobart AK** (1987) Triacylglycerol biosynthesis. In PK Stumpf, EE Conn, eds, *The Biochemistry of Plants*. Academic Press, New York, pp 175–214
- Sun C, Cao YZ, Huang AHC** (1988) Acyl coenzyme A preference of the glycerol phosphate pathway in the microsomes from the maturing seeds of palm, maize, and rapeseed. *Plant Physiol* **88**: 56–60
- Trelease RN** (1969) Changes and characteristics of lipid bodies during development. PhD thesis, University of Texas, Austin
- Tzen JTC, Huang AHC** (1992) Surface structure and properties of plant seed oil bodies. *J Cell Biol* **117**: 327–335
- Tzen JTC, Lai YK, Chan KL, Huang AHC** (1990) Oleosin isoforms of high and low molecular weights are present in the oil bodies of diverse seed species. *Plant Physiol* **94**: 1282–1289
- Tzen JTC, Lie GC, Huang AHC** (1992) Characterization of the charged components and their topology on the surface of plant seed oil bodies. *J Biol Chem* **267**: 15626–15634
- Vance VB, Huang AHC** (1987) The major protein from lipid bodies of maize. Characterization and structure based on cDNA cloning. *J Biol Chem* **262**: 11275–11279
- Wang SM, Huang AHC** (1987) Biosynthesis of lipase in the scutellum of maize kernel. *J Biol Chem* **262**: 2270–2274
- Yatsu LY, Jacks TJ** (1972) Spherosome membranes. Half unit-membranes. *Plant Physiol* **49**: 937–943