Some studies on the composition and surface properties of oil bodies from the seed cotyledons of safflower (*Carthamus tinctorius*) and linseed (*Linum usitatissimum*)

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1. The average oil-body diameter in intact cells of developing linseed (*Linum usitatissimum*) and safflower (*Carthamus tinctorius*) cotyledons was similar (about $1.4\mu\text{m}$), and there was little change in size after oil bodies were isolated and repeatedly washed. 2. The glycerolipid composition of washed oil bodies from both developing and mature cotyledons of the two species was similar; oil bodies from ten different batches of cotyledons contained $4.3 \pm 0.16\mu\text{mol}$ of 3-*sn*-phosphatidylcholine and $25.2 \pm 1.7\mu\text{mol}$ of diacylglycerol per $1000\mu\text{mol}$ of triacylglycerol. During four successive washings of a once-washed oil-body preparation, the proportion of diacylglycerol to triacylglycerol remained constant and that of 3-*sn*-phosphatidylcholine to triacylglycerol decreased by only 20%. 3. The protein content of thrice-washed oil bodies from the two species was similar, about 2.4% of the weight of glycerolipids, and appeared to be independent of the stage of cotyledon maturity. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis indicated that the protein of purified oil bodies from the two species consisted mainly of only four polypeptides and that two of the polypeptides from each species had apparent mol.wts. of 17 500 and 15 500. Similar patterns of polypeptides were obtained after the hydrolysis of the 15 500-mol.wt. polypeptides from linseed and safflower oil bodies by *Staphylococcus aureus* V8 proteinase, whereas the proteolysis of the 17 500-mol.wt. polypeptides from the two species produced different patterns of polypeptides. 4. The 3-*sn*-phosphatidylcholine in oil-body preparations was hydrolysed about 85% by bee-venom phospholipase A_2 without any apparent coalescence of the oil bodies. Incubation with lipase from *Rhizopus arrhizus* caused rapid coalescence of the oil bodies, and this lipase appeared to initially hydrolyse diacylglycerols in preference to triacylglycerol. 5. Oil bodies from both species were almost completely dispersed in suspensions of pH between 7.1 and 8.3, but formed large aggregates at pH values between 6.7 and 3.9; pH-induced aggregation caused no coalescence. Aggregates formed under acidic conditions were dispersed by re-adjusting the pH of suspensions to 8.3. 6. A freeze-etch electron-microscopic examination of isolated oil bodies indicated that these organelles were bounded by some form of membrane with a particle-free outer surface.

In plant cells and particularly in the cotyledons of oilseeds, triacylglycerols accumulate in spherical structures with a mean diameter of about $1\mu\text{m}$ that have been variously named spherosomes, oleosomes, lipid bodies, oil bodies, lipid droplets and lipid vesicles (Frey-Wyssling *et al.*, 1963; Yatsu, 1965; Yatsu *et al.*, 1971; Yatsu & Jacks, 1972; Gurr *et al.*, 1974; Appelqvist, 1975; Bergfeld *et al.*, 1978;

Kleinig *et al.*, 1978). Transmission electron microscopy of seed cotyledons and oil-body preparations have invariably shown in osmium-fixed sections that oil bodies possess an electron-dense boundary, and Frey-Wyssling *et al.* (1963) originally suggested that they were bounded by a normal unit membrane. More recent work, however, (Trelease, 1969; Yatsu & Jacks, 1972; Bergfeld *et al.*, 1978), has

suggested that oil bodies may be surrounded by a half unit membrane, the hydrophobic face being orientated inwards towards the triacylglycerol of the interior. Kleinig *et al.* (1978) have argued, however, that the amount of phospholipid in oil bodies from carrot (*Daucus carota*) suspension cells was inadequate to account for even a half unit membrane.

Since oil bodies from oilseed cotyledons are similar both in size and appearance, it might be argued that their composition with respect to triacylglycerol and any protein and phospholipid that might be associated with a bounding membrane should also be similar. However, although protein and phospholipid have been found in isolated oil bodies, the amounts of these components that have been reported vary considerably. The protein content of oil bodies that have been reported range from 0.2% by weight for oil bodies from peanut (*Arachis hypogaea*) (Yatsu & Jacks, 1972) to 20% for *Crambe abyssinica* (crambe) (Gurr *et al.*, 1974), and the molar ratios of triacylglycerol to 3-*sn*-phosphatidylcholine, the predominant phospholipid, range from about 100:5 for oil bodies of crambe to 100:1.5 for oil bodies of *Phaseolus vulgaris* (red kidney bean) (Allen *et al.*, 1971). In the present paper we report some details of the composition, properties *in vitro* and surface structure of oil bodies from developing and mature cotyledons of safflower and linseed that indicate that the bounding membrane of the oil bodies from these species may be similar.

Experimental

Materials

Plants of safflower (*Carthamus tinctorius*, cultivar 0-22) and linseed (*Linum usitatissimum*, cultivar Punjab) were grown in a heated glasshouse as described previously (Slack *et al.*, 1978). Lipase from *Rhizopus arrhizus* was obtained from Boehringer Mannheim Australia Pty., Melbourne, Vic., Australia, bee-venom phospholipase A₂ from Sigma Chemical Co., St. Louis, MO, U.S.A. and *Staphylococcus aureus* V8 proteinase from Miles Laboratories, Elkhart, IN, U.S.A. Sodium [1-¹⁴C]acetate was from The Radiochemical Centre, Amersham, Bucks., U.K. Sodium methoxide was prepared as previously described (Slack *et al.*, 1976).

Methods

Isolation of oil bodies and cell fractionation. Oil bodies were isolated from developing and mature cotyledons by the same procedure. Dry mature seed was soaked in tap water for 12 h at 12°C, then the cotyledons removed and stored at 0°C. Cotyledons, between one- and two-thirds fully expanded, were isolated from developing seeds as previously described (Slack *et al.*, 1978) and either used imme-

diately or incubated with sodium [1-¹⁴C]acetate as described previously (Slack *et al.*, 1978). Cotyledons (1–2 g) were homogenized in 8.0 ml of 50 mM-Tris/HCl buffer, pH 7.5, containing 500 mM-sucrose, 0.5 mM-EDTA and 0.5 mM-EGTA with a Polytron PT20 homogenizer (Kinematica, Lucerne, Switzerland) at half maximum speed for 2 s and the homogenate filtered through three layers of Miracloth. The filtrate (7.0 ml) was transferred to a 15 ml centrifuge tube and overlaid with two 3.0 ml layers containing 250 mM- and 125 mM-sucrose in the above buffer mixture and centrifuged at 18 000 g for 20 min to give a pellicle of oil bodies at the top of liquid and a pellet below. The 500 mM-sucrose layer was diluted with an equal volume of buffer mixture without sucrose and centrifuged at 105 000 g to pellet a microsomal preparation; the 250 mM- and 125 mM-sucrose layers were pooled and lipids extracted directly as described below.

The oil-body pellicle was readily lifted off with a spatula and, unless stated otherwise, was washed twice. The pellicle was dispersed in 0.5 ml of the 500 mM-sucrose buffer mixture first by squashing the pellicle against the side of a centrifuge tube with a glass rod, then after addition of 1.0 ml of the buffer mixture by repeatedly squirting the suspension through a Pasteur pipette. The suspension in 500 mM-sucrose was adjusted to 7.0 ml and overlaid with layers of more dilute sucrose solutions and centrifuged as above. This process was repeated.

Lipid extraction and analysis. Lipids were extracted from aqueous suspensions of oil bodies, 18 000 g and 105 000 g pellets and the pooled 125 mM- and 250 mM-sucrose layers by the method of Folch *et al.* (1957), the chloroform layer dried under reduced pressure and finally under N₂ and the lipids redissolved in chloroform. Samples of the chloroform solutions were chromatographed on silica-gel G in light petroleum (b.p. 60–80°C)/diethyl ether/acetic acid (75:25:1, by vol.) to separate di- and tri-acylglycerols and in chloroform/methanol/15 M-NH₃ (65:25:2, by vol.) to separate individual phospholipids, the lipids were stained lightly in I₂ vapour, and individual lipids transmethyated with sodium methoxide as described previously (Slack *et al.*, 1977). The amounts of fatty acid methyl esters formed from each lipid were measured by g.l.c. on a 2 m × 2 mm column of 15% (w/w) ethylene glycol succinate on Chromazorb W. Where appropriate, the specific radioactivity of individual lipids was measured from the quantities of fatty acids determined as above and by counting portions of the methyl ester preparations for radioactivity at 80% efficiency in a scintillation 'cocktail' of 0.5% *p*-terphenyl in xylene (w/v).

Protein measurement and polyacrylamide-gel/sodium dodecyl sulphate electrophoresis of polypeptides. To isolate protein from oil bodies, the

oil-body pellicle was removed from the top of the sucrose gradient after the final wash and transferred directly to 10 ml of chloroform/methanol (2:1, v/v) and the material dispersed and extracted overnight at 4°C. The insoluble material settled to the bottom of the solvent but could not be satisfactorily pelleted by centrifugation. Consequently most of this solvent was removed, then 5 ml of methanol added and the material pelleted by low-speed centrifugation, the pellet washed twice with 5 ml of diethyl ether, and the solvent removed with a stream of air. All solvent extracts were pooled. Alternatively, oil bodies suspended in 3 ml of the above buffer mixture without sucrose were homogenized with a Polytron homogenizer at low speed with 3 ml of diethyl ether at 0°C for 2 min to remove neutral lipids, essentially as described by Yatsu & Jacks (1972). The pellet recovered from the extracted oil bodies by centrifugation at 18000g for 10 min was resuspended in the buffer.

For protein measurements the material was dissolved in 2% sodium dodecyl sulphate and protein was measured by a modified Lowry method (Schacterle & Pollack, 1973), with bovine serum albumin as a standard and with a final concentration of 0.2% sodium dodecyl sulphate in both standards and unknowns. The pooled solvent extracts were dried, lipids dissolved in chloroform and samples transmethylated with sodium methoxide. The quantity of glycerolipids in the oil bodies was determined from g.l.c. analysis of the fatty acid methyl esters as described above, and the protein content of the oil bodies expressed as a percentage by weight of the glycerolipid.

The proteinaceous material from oil bodies was solubilized for polyacrylamide-gel electrophoresis in 5% glycerol, 1% sodium dodecyl sulphate and 0.125M-dithiothreitol at 100°C for 5 min. Soluble plus microsomal protein was precipitated from samples of the 0.5M-sucrose layer after the initial centrifugation of cotyledon homogenates at 18000g as described above by the addition of solid trichloroacetic acid to 10% (w/v), then pelleted by low-speed centrifugation, washed twice with 5 ml of diethyl ether to remove the trichloroacetic acid, extracted successively with chloroform/methanol, methanol and diethyl ether, dried and solubilized as described above. Electrophoresis was performed in a water-cooled apparatus with a 5% stacking gel and a 20% running gel by using the discontinuous buffer system described by Laemmli (1970). Protein (approx. 100µg) and Bromophenol Blue were introduced into 8mm slots and electrophoresed at 150 V until the marker dye had reached the bottom of the running gel. Gels were stained overnight with Coomassie Brilliant Blue R and destained as described by Cashmore (1976), except that polymeric-adsorbent beads of Amberlite XAD-2 were

included in the elutant to absorb the dye. To estimate the relative proportions of polypeptide bands in each gel lane, transparent positive photographs of the gels were scanned with a recording densitometer and the information processed by using the computer program of Schumaker (1978).

Partial proteolysis of particular polypeptide bands by various amounts of *S. aureus* V8 proteinase was performed during electrophoresis as described by Cleveland *et al.* (1977). The required bands were cut from 20% running gels in which about 150µg of protein had been electrophoresed in each lane, and, after the lightly-stained-band material had been incubated in the buffer mixture, each 'band' was cut in half transversely and these placed one above the other in a slot of the stacking gel above a 25% polyacrylamide running gel and overlaid with solutions of the proteinase. Electrophoresis was stopped for 1 h when the dye had reached the bottom of the stacking gel to allow the proteolysis to proceed.

Incubation of oil bodies with lipases. Oil bodies isolated from cotyledons that had been incubated with sodium [$1\text{-}^{14}\text{C}$]acetate were washed as described above and suspended in either 50mM-Tris/HCl buffer, pH 7.5, or 50mM-Bicine [N,N-bis-(2-hydroxyethyl)glycine]/NaOH buffer, pH 8.3, at a concentration equivalent to about 6mg of lipid/ml. Samples (0.3ml) of the suspensions in Tris buffer were transferred to tubes each containing 0.1 ml of the Tris buffer, 8mM-CaCl₂ and 20µg of *Rhizopus arrhizus* lipase and incubated at 0°C. At intervals, 10 ml of chloroform/methanol (2:1, v/v) were added and the tubes stored overnight, then 1.6 ml of water were added, the chloroform layer dried, and the lipids dissolved in chloroform. The distribution of radioactivity in individual lipids separated by t.l.c. as above was determined by counting for radioactivity the individual lipid zones as described previously (Slack & Roughan, 1975). The specific radioactivity of these lipids was measured as described above. Samples (0.3ml) of oil-body suspensions in the Bicine buffer were incubated with 0.1 ml of this buffer containing 8mM-CaCl₂ and 25µg of phospholipase A₂ at 30°C and the reaction stopped at intervals and the lipids analysed as outlined for the incubation with *Rhizopus* lipase.

Light-microscopic examination of oil-body suspensions and the estimation of oil-body size. Suspensions of oil bodies were examined by Nomarski differential-interference/contrast microscopy. To determine oil-droplet size, at least two fields on each slide were photographed and the photographs enlarged to give a total magnification of the oil bodies of $\times 3600$ and the diameter of about 100 measured for each suspension.

Freeze-etch electron microscopy of oil bodies. Droplets of a suspension of oil bodies from mature

safflower cotyledons in distilled water were rapidly frozen by plunging into liquid Freon-22 at -150°C and stored under liquid N_2 until required. These were freeze-fractured in a Balzers BA 500 freeze-etch unit (Balzers, Liechtenstein) at -100°C . In order to expose the bounding surface of the oil bodies, the fracture surface was etched by placing the knife, at -190°C , above the specimen table and permitting water to sublime from the specimen for 2 min. The etched fracture surface was shadowed with a mixture of carbon and platinum, the lipid dissolved with a mixture of chloroform/methanol (2:1, v/v) and the replica examined in a Philips 400 electron microscope (Philips, Eindhoven, The Netherlands).

Results

The size of oil bodies in vivo and in vitro

Spherical bodies with a mean diameter of about $1.4\mu\text{m}$ were apparent in intact cells viewed in hand-cut sections of developing linseed and safflower cotyledons by Nomarski differential-interference/contrast microscopy. These bodies were identified as oil bodies by staining sections with the lipid-specific stain, Nile Blue. Oil bodies of similar size distribution were isolated from developing cotyledons and from mature cotyledons (Table 1), indicating that only a minimum of coalescence occurred during isolation.

Distribution of lipids between oil bodies and other cell fractions and the lipid composition of the oil bodies

Fractionation of homogenates of developing cotyledons indicated, as expected, that essentially all

of the triacylglycerol was located in the oil-body fraction (Table 2). Triacylglycerol in the 125 mM- and 250 mM-sucrose layers probably represents oil bodies dislodged from the pellicle. Much of the 3-*sn*-phosphatidylcholine was isolated with particulate fractions, but some remained associated with the oil bodies, washed twice after the initial separation. To our knowledge, diacylglycerols have not been reported as constituents of the oil bodies from oilseed cotyledons, although Christiansen (1978) found that this lipid was 2% of the total lipid in oil bodies isolated from baker's yeast. In our experiments we consistently found that the bulk of the tissue's complement of diacylglycerol was isolated with the oil bodies (Table 2). It might be argued that diacylglycerols isolated with oil bodies could be an artefact derived from triacylglycerol by the action of lipase during isolation. This possibility seems highly unlikely, since when oil bodies were prepared from [^{14}C]acetate-labelled cotyledons, the specific radioactivity of the diacylglycerols varied between 500 and 1500 times that of the triacylglycerols in different experiments. Had the former lipid been derived from the latter, then the specific radioactivity of the fatty acids of the two lipids should have been similar.

If 3-*sn*-phosphatidylcholine and diacylglycerols are true components of oil bodies, then one would expect that the amount of these lipids relative to that of triacylglycerol should approach constant values with increasing purification of the oil bodies. During four successive washings of oil bodies from developing linseed cotyledons the relative amounts of di- and tri-acylglycerols remained essentially constant, and the proportion of 3-*sn*-phosphatidylcholine to

Table 1. *Oil-body size in vivo and after isolation from linseed and safflower cotyledons*

Hand-cut sections of developing seed cotyledons were examined by Nomarski optical microscopy, photographed, and the diameters of about 100 oil bodies measured. The diameter of oil bodies isolated and washed as described in the Experimental section was measured as above.

Source	Oil-body diameter (μm)		
	Mean	S.D.	S.E.M.
Linseed			
Developing cotyledons			
<i>In vivo</i>	1.46	0.29	0.03
<i>In vitro</i>	1.36	0.46	0.05
Mature cotyledons			
<i>In vitro</i>	1.49	0.50	0.06
Safflower			
Developing cotyledons			
<i>In vivo</i>	1.44	0.32	0.03
<i>In vitro</i>	1.45	0.46	0.05
Mature cotyledons			
<i>In vitro</i>	1.46	0.40	0.05

Table 2. *Distribution of the major glycerolipids of developing linseed and safflower cotyledons between cell fractions separated by differential centrifugation*

Developing linseed and safflower cotyledons (4 g each) were homogenized separately then fractionated and analysed as described in the Experimental section. The 105 000 g pellets were resuspended once, then re-pelleted and the oil-body pellicles washed thrice by resuspension and centrifugation before analysis. The intermediate fraction is material recovered from 0.25 M- and 0.125 M-sucrose layers after the initial centrifugation.

Total amounts (μ mol) of individual glycerolipids in homogenates of linseed and safflower cotyledons, and distribution of each lipid between cell fractions (%) (data for safflower cotyledons in parentheses)						
Amounts Fraction	3- <i>sn</i> -Phospha- tidylcholine		Diacylglycerol		Triacylglycerol	
	1.14	(1.38)	1.19	(1.25)	38.82	(34.32)
Oil bodies	12.6	(11.6)	74.1	(75.0)	92.5	(89.5)
Intermediate	18.0	(18.4)	8.1	(12.0)	6.9	(8.9)
15 000 g pellet	27.9	(36.9)	9.8	(8.1)	0	(1.3)
105 000 g pellet	41.4	(33.1)	7.9	(4.9)	0.5	(0.3)

Table 3. *Effect of repeatedly washing isolated oil bodies on the relative amounts of the major glycerolipids*
Oil bodies were isolated from developing linseed cotyledons (5 g) and washed five times by centrifuging the resuspended oil bodies through a sucrose gradient. Samples of the oil-body preparation were removed after the first, third and fifth wash and analysed as described in the Experimental section.

Amounts (mol) of individual glycerolipids relative to triacylglycerol (= 1000)			
No. of washings	3- <i>sn</i> -Phosphatidylcholine	Diacylglycerol	Triacylglycerol
1	5.0	30.0	1000
3	4.1	31.1	1000
5	3.8	29.7	1000

triacylglycerol fell by only 20% (Table 3), suggesting that these lipids are tightly associated with oil bodies.

The amount of 3-*sn*-phosphatidylcholine found in the oil bodies from developing linseed cotyledons (Table 3) was appreciably less than that reported previously for oil bodies of other species (Allen *et al.*, 1971; Gurr *et al.*, 1974). However, we have found that the proportions of 3-*sn*-phosphatidylcholine, and di- and tri-acylglycerols were similar in several different washed oil-body preparations from linseed and safflower cotyledons (Table 4). In oil bodies from both species, 3-*sn*-phosphatidylcholine represented about 65% of the polar lipids present.

Protein complement of oil bodies

In eight different oil-body preparations from linseed and safflower cotyledons, protein represented $2.46 \pm 0.13\%$ by weight of the glycerolipids present

and the amount appeared to be independent of both species and the stage of cotyledon development.

Kleinig *et al.* (1978) reported that they could find no polypeptides characteristic of the oil bodies from carrot suspension-culture cells by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, whereas Bergfeld *et al.* (1978), by using the same technique, found that protein from oil bodies of developing mustard (*Sinapis alba*) cotyledons gave a relatively simple pattern of nine dominant polypeptides that was distinctly different from that derived from a microsomal preparation. In view of these conflicting findings we decided to determine whether oil bodies from either safflower or linseed cotyledons possessed characteristic polypeptides. Consequently, we compared the polypeptide patterns derived from oil bodies at different stages of purification with that from the 500 mM-sucrose layer of the original centrifugation step, which contained both soluble and microsomal protein (Fig. 1). Preliminary

Table 4. *Relative amounts of the major glycerolipids of oil-body preparations from different batches of cotyledons*
Oil bodies were isolated, washed three times, then the amounts of individual lipids measured as described in the Experimental section.

		Amounts of individual glycerolipids relative to triacylglycerol (= 1000)		
Source		3- <i>sn</i> -Phosphatidylcholine	Diacylglycerol	Triacylglycerol
Linseed	Developing cotyledons	3.4	31.1	1000
		4.0	34.0	1000
		4.6	24.5	1000
		3.8	22.5	1000
	Mature cotyledons	4.0	24.0	1000
	Safflower	Developing cotyledons	5.2	30.4
4.5			27.7	1000
4.5			19.5	1000
Mature cotyledons		4.2	19.7	1000
		4.5	18.7	1000

Fig. 1. *Effect of washing on the polypeptides present in an oil-body preparation from mature safflower cotyledons and a comparison of the polypeptides of washed oil-body preparations from developing and mature safflower cotyledons and mature linseed cotyledons*

Oil bodies were isolated from mature safflower cotyledons and protein isolated from samples of the oil-body preparation taken after the initial centrifugation, after the oil bodies had been washed two and four times and from the 0.5 M-sucrose layer after the initial centrifugation. Protein was also obtained from thrice-washed oil-body preparations from developing safflower and from mature linseed cotyledons. The protein was solubilized in dithiothreitol and sodium dodecyl sulphate and the constituent polypeptides were separated by electrophoresis in a 20% (w/v) polyacrylamide slab gel. Details of the procedures are described in the Experimental section. (a)–(d) and (f), mature safflower cotyledon. (a) 15 000 g supernatant; (b) unwashed oil bodies; (c) twice-washed oil bodies; (d) and (f) four-times-washed oil bodies; (e) developing safflower oil bodies; (g) mature safflower + mature linseed oil bodies; (h) mature linseed oil bodies.

experiments had indicated that the major protein contaminants of oil-body preparations were from this fraction and not the 15 000 g pellet. It was apparent that, with purification of the oil bodies from mature

safflower cotyledons, four polypeptides became predominant (Fig. 1, lanes b to d). Whether or not all four of these polypeptides were also present in the soluble plus microsomal protein (Fig. 1, lane a) was

not clear; certainly the most mobile of these major oil-body polypeptides was not apparent. Densitometer measurements of the intensity of staining as described under 'Methods' indicated that the four polypeptides represented about 90–95% of the protein from oil bodies washed two and four times respectively. The same four polypeptides were also predominant in oil bodies from developing safflower cotyledons (Fig. 1, lane *e*). The oil bodies from

developing and mature linseed cotyledons (Fig. 1, lane *h*) also contained only four major polypeptides, and the two most predominant bands from the linseed oil bodies had the same mobilities as the second and third most mobile of the safflower oil-body polypeptides, with apparent mol.wts. of 17 500 and 15 500 respectively. Polypeptides from oil bodies of safflower and linseed were run together in lane (*g*) (Fig. 1). Oil bodies extracted with either

Fig. 2. *A comparison of the polypeptides produced from the 17 500-mol.wt. polypeptides (a) and 15 500-mol.wt. polypeptides (b) of linseed and safflower oil bodies by incubation with S. aureus V8 proteinase*

Four samples of oil-body polypeptides from thrice-washed oil bodies from mature linseed cotyledons and four similar samples of safflower polypeptides were electrophoresed in 20% (w/v) polyacrylamide slab gels as in Fig. 1. The gels were briefly stained, the 17 500-mol.wt. and 15 500-mol.wt. band material removed, incubated with buffered sodium dodecyl sulphate and stored at -15°C overnight. The linseed 17 500-mol.wt. material was placed in alternate slots (i, iii, v and vii) in a stacking gel (*a*) above a 25%-polyacrylamide running gel and the safflower 17 500-mol.wt. material in the remaining slots (ii, iv, vi and viii). The 15 500-mol.wt. material was arranged similarly in a second gel (*b*). The polypeptides were then electrophoresed through the stacking gels with different amounts of the *S. aureus* proteinase: (i and ii) 0 μg ; (iii and iv) 0.1 μg ; (v and vi) 0.5 μg ; (vii and viii) 2.5 μg , as described in the Experimental section. The slowest moving band visible in (v)–(viii) is the added proteinase.

diethyl ether or with chloroform/methanol yielded the same polypeptide pattern.

To provide some indication of the degree of similarity between the 17500- and 15500-mol.wt. polypeptides from the linseed and safflower oil bodies, these polypeptides were subjected to proteolysis with various amounts of *S. aureus* V8 proteinase, which hydrolyses peptide bonds only at the C-terminal side of glutamic acid residues (Houmard & Drapeau, 1972) and the products separated by polyacrylamide-gel electrophoresis. There was a clear difference between the proteolysis products of the 17500-mol.wt. polypeptides from linseed and safflower (Fig. 2a), but the products from the 15500-mol.wt. polypeptide from the two species were very similar (Fig. 2b).

Effects of pH and the action of exogenous lipases on oil-body aggregation and coalescence

We have routinely suspended oil bodies at pH 7.5 and have found the suspensions to be relatively stable; little change occurred in oil-body size or the extent of aggregation during storage at 4°C for 24 h. The appearance of oil-body suspensions was rapidly changed, however, when the pH was lowered to 6, and within minutes all the oil bodies had floated to the top of the medium. Examination of this floating material by optical microscopy revealed that it consisted of large aggregated clumps. A more precise study of the effect of pH was made by lowering the pH of stirred oil-body suspensions, initially at pH 8.3 in 10 mM-Mes (4-morpholine-ethanesulphonic acid)/10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/10 mM-Bicine/NaOH, by increments of 0.4 pH unit and examining the suspension microscopically after each pH change. With both linseed and safflower oil bodies aggregation occurred between pH 7.1 and 6.7 and the oil bodies, remained aggregated at all pH values down to 3.9. The aggregates were readily dispersed by raising the pH back to 8.3, and there was no indication of extensive coalescence of the oil bodies induced by this treatment.

We have examined the possibility that the 3-*sn*-phosphatidylcholine and diacylglycerol, isolated with oil bodies, are located at the oil-body surface by measuring their susceptibility to hydrolysis by added lipases. Since lipases function at a lipid/water interface (Dawson, 1973), it was argued that rapid deacylation of these lipids could only occur if they were suitably orientated on the oil-body surface. Incubation of oil bodies, in which the acyl moieties of the lipids had been labelled by incubating cotyledons with [¹⁴C]acetate before isolating the oil bodies, with bee-venom phospholipase A₂, resulted in essentially complete hydrolysis of the 3-*sn*-phosphatidylcholine. For instance, with safflower oil bodies the radioactivity in this phos-

pholipid represented initially 60% of that in all phospholipids plus fatty acids, but only 8 and 5% after 20 and 40 min incubation with the enzyme respectively.

The hydrolysis of 3-*sn*-phosphatidylcholine to lysophosphatidylcholine and fatty acid by phospholipase A₂ caused no apparent change in the oil-body suspension. In contrast, incubation with *Rhizopus arrhizus* lipase resulted in an extremely rapid coalescence of the oil bodies, and when viewed microscopically, the slide appeared covered by large slicks of oil. During the early stages of incubation with the enzyme there was a rapid loss of radioactivity from diacylglycerols and a concomitant increase in radioactivity in monoacylglycerols and fatty acids (Fig. 3). Some hydrolysis of triacylglycerol also occurred during the first minute, and this was reflected in a 4-fold increase in the amount of diacylglycerol in the oil bodies. Initially the specific radioactivity of the acyl groups of the diacylglycerols was 100 times that of the acyl groups of the triacylglycerol, and on a molar basis there was

Fig. 3. Changes in the distribution of radioactivity among different lipids during the incubation of oil bodies with lipase

Oil bodies, isolated from developing linseed cotyledons that had been incubated previously with [¹⁴C]acetate, were washed thrice, then incubated at 0°C with *Rhizopus arrhizus* lipase and samples taken at intervals and the distribution of radioactivity between triacylglycerol (○), diacylglycerol (●), monoacylglycerol (■) and fatty acid (□) determined as described in the Experimental section.

EXPLANATION OF PLATE 1

Freeze-etch electron micrograph of a section through a suspension of oil bodies from mature safflower cotyledons
A droplet from a suspension of thrice-washed oil bodies isolated from mature safflower cotyledons was frozen then fractured and the fracture face etched. This was shadowed and the replica viewed as described in the Experimental section. Magnification $\times 75\,000$; the bar represents $0.5\,\mu\text{m}$.

EXPLANATION OF PLATE 2

Freeze-etch electron micrograph of fused safflower oil bodies
Oil bodies were prepared as described in Plate 1. Magnification $\times 75\,000$.

40 times more triacylglycerol than diacylglycerol. Consequently, if the diacylglycerols had been dissolved in the triacylglycerol and uniformly distributed throughout the interior of the oil bodies, then the initial rates of loss of radioactivity from the two lipids would have been relatively similar. Since the initial rate of loss of radioactivity was considerably greater from the diacylglycerol, we suggest that this lipid is also located at the oil-body surface. The incubation of oil bodies with the *Rhizopus* lipase was carried out at 0°C to decrease the rate of lipid hydrolysis, and since there was a delay of about 3 min at a higher temperature before samples could be viewed microscopically, it was not possible to relate the onset of coalescence with the changes shown in Fig. 3. Samples taken after 1 min were coalesced when examined, and, after 10 min incubation, the assay mixtures had changed from a milky suspension of oil bodies to a relatively clear solution containing large oil droplets.

Examination of the oil-body surface by a freeze-etch technique

In an attempt to provide information about the oil-body surface additional to that obtained by transmission electron microscopy (Yatsu & Jacks, 1972), we examined the surface of isolated oil bodies by a freeze-etch technique without prior chemical fixation. In oil bodies from which only a small segment had been removed by the freeze-fracture it was possible to observe both the fracture facet formed on the lipid interior and part of the outer spherical surface exposed by the subsequent sublimation of some of the surrounding ice (Plate 1). The outer surface so exposed was typically rather smooth and without notable morphological characteristics. A feature common to all oil bodies observed, but most pronounced in those from which only a small segment had been fractured, was the presence of a raised rim at the junction of the fracture face and the outer surface. Small irregular projections of varying heights occurred along this rim that appeared to constitute an extension of the outer surface above the mean level of the fracture facet. This feature was not seen when droplets of an emulsion of soya-bean oil in water were examined by the same procedure.

Several of the oil bodies examined by the freeze-etch technique were partially fused. Where the fracture passed through a pair of fused oil bodies in such a way as to include a section of the fusion zone in the fracture facet (Plate 2), a line of small projections was invariably seen that followed the interface between the merging oil bodies. These projections resembled in size and general appearance those seen on the raised rim of the fracture facets in unfused regions (Plates 1 and 2). We interpret the above observations as indicating that oil

bodies are bounded by some form of membrane which, in view of the persistence of particulate material along the zone of fusion, is probably composed in part of non-lipid material.

Discussion

The present studies indicated that oil-body preparations from the cotyledons of two unrelated species, linseed and safflower, had a very similar composition that was in all probability largely independent of the stage of maturity of the cotyledons from which they were isolated. Assuming that the preparations contained essentially only glycerolipids and protein, the average values for all analyses were 2.5% protein, 96.8% neutral lipid and 0.7% phospholipid, by weight. Similar results have been obtained for an oil-body preparation from cotyledons of germinating peanut seeds, i.e. 1.3% protein, 97.3% neutral lipid and 0.8% phospholipid (Jacks *et al.*, 1967), but considerably different results were reported for oil bodies from mature peanut cotyledons, which contained appreciably less protein and phospholipid (Yatsu *et al.*, 1971) and particularly from crambe cotyledons (Gurr *et al.*, 1974), which contained 19% protein, 64% neutral lipids, 11% phospholipid and 6% water. As Gurr *et al.* (1974) have emphasized, washed isolated oil bodies from seed cotyledons of different species are remarkably similar in size and apparent purity when examined microscopically, and it is difficult consequently to explain the large differences in composition that have been reported, particularly since it is generally assumed (see below) that components other than triacylglycerols are localized entirely on the oil-body surface.

As yet it is not possible to use enzyme markers to measure the purity of oil-body preparations, since the enzyme complement of this organelle is not known, and hence one is obliged to adopt different criteria of purity. The similarity in the amounts of 3-*sn*-phosphatidylcholine, diacylglycerol and protein relative to that of triacylglycerol found in several different preparations suggests that these compounds are real components of oil bodies, since it is reasonable to assume that triacylglycerols do occur in this organelle. This view is supported by the observation that the proportions of these compounds remained relatively constant during repeated washing of the oil bodies. It might be argued, however, since oil-body size was relatively constant between different preparations, that the area available for adsorption of membrane material and soluble protein at the oil-body surface would also be constant and therefore that one might expect, as contaminants, relatively constant amounts of phospholipid and protein in oil-body preparations. This explanation seems unlikely for two reasons. Firstly,

if membrane material were adsorbed on to oil bodies, one would expect that the lipid complement of the oil bodies would resemble that of other cell fractions. This was not so; there was a very much higher ratio of diacylglycerol to 3-*sn*-phosphatidylcholine in oil bodies compared with other particulate fractions. Secondly, if membrane material and soluble protein were adsorbed during isolation, then the polypeptides of oil-body preparations would be expected to have some similarity to those of other cell fractions. In fact, washed oil-body preparations had a very simple and characteristic pattern of polypeptides and, furthermore, the polypeptides from the oil bodies of the two species examined were similar. We suggest, therefore, that these observations do indicate that the 3-*sn*-phosphatidylcholine, small amounts of other phospholipids, diacylglycerol and protein are true constituents of oil bodies.

It has been inferred (Jacks *et al.*, 1967; Yatsu & Jacks, 1972; Allen *et al.*, 1971; Bergfeld *et al.*, 1978) that protein and phospholipid are localized in a bounding oil-body membrane. The present finding, that the 3-*sn*-phosphatidylcholine in oil-body preparations was almost completely hydrolysed by phospholipase A₂ and was located, therefore, at a lipid/water interface, supports this view. No oil-body coalescence occurred as a result of the 3-*sn*-phosphatidylcholine hydrolysis, in contrast with the phospholipase A₂-induced lysis of erythrocytes (Greig & Gibbons, 1956). However, the rupture of the erythrocyte membrane appears to be an osmotic effect resulting from a lysophospholipid-induced increase in the membrane's permeability to Na²⁺ (Bierbaum *et al.*, 1979). The preferential hydrolysis of an oil-body pool of diacylglycerol with a high specific radioactivity compared with that of triacylglycerol by *Rhizopus* lipase suggests that this diacylglycerol is also localized at the lipid/water interface of the oil-body surface. However, we are uncertain at present as to whether the large change in the surface properties of the oil bodies as a result of *Rhizopus* lipase activity (which allowed coalescence of the oil-body lipid) was the result of the hydrolysis of this diacylglycerol *per se* or resulted from the increase in the amounts of diacylglycerol, monoacylglycerol and non-esterified fatty acid that were produced from triacylglycerol.

The influence of pH on the aggregation of isolated oil bodies indicated that there are exposed dissociatable groups on the oil-body surface that are protonated below neutrality. Since the dissociation constants of the phosphate and basic groups of phospholipids are several pH units away from neutrality (Garvin & Karnovsky, 1956), it seems unlikely that these groups could have been responsible for the appreciable change in electrostatic charge between pH 7.1 and 6.7 that resulted in aggregation. Presumably, therefore, some of the

protein associated with oil bodies must be exposed at the oil-body surface. It is an appealing possibility, in view of the marked similarity in the amount of protein and the size of the major polypeptides from the oil bodies of linseed and safflower, that these polypeptides have some particular function in a bounding oil-body membrane. Certainly the freeze-etch electron micrographs indicated that non-lipid material was located in the membrane, but that protein particles did not protrude outwards from the surface. Some information about the size of the native protein from which the polypeptides were derived, together with freeze-fracture electron microscopy of bilayer structures, produced from oil bodies by the method of Yatsu & Jacks (1972) might provide some indication as to how protein is arranged within the membrane. It is noteworthy that the same four polypeptides constituted the bulk of the protein from both developing and mature cotyledons. Since the metabolic states of the two tissues would have been very different, the former actively synthesizing triacylglycerol and the latter in a quiescent state, it seems unlikely that these polypeptides could have been derived from proteins involved directly in triacylglycerol metabolism. A more extensive survey of the polypeptides of oil bodies from other species would obviously be useful in determining whether the similarity between oil bodies of linseed and safflower can be extended. It is noteworthy in this regard that two of the major polypeptides from mustard-seed oil bodies had apparent molecular weights of about 15000 (Bergfeld *et al.*, 1978).

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