LONG CHAIN FATTY ACID SYNTHESIS IN RAPESEED COTYLEDONS

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Heather Carolyn Wright

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ABSTRACT

When developing rapeseed cotyledons were incubated with [^{14}C] acetate, approximately 70% of the label was found in triacylglycerol, in which erucate was the most heavily labelled fatty acid. Oxidative degradation studies to determine the distribution of radioactivity in oleate, eicosenoate and erucate of this labelled triacylglycerol showed that (a) the specific radioactivity of oleate, and of the oleoyl portions of eicosenoate and erucate were similar. Since the masses of these three fatty acids in the triacylglycerol of the cotyledons used were different, this suggests that a particular fatty acid is incorporated into triacylglycerol in proportion to the amount of each fatty acid already present in the oil. (b) the specific radioactivities of the oleoyl portions of eicosenoate and erucate were much lower than those of the carboxyl terminal carbons added by chain elongation, indicating that the specific radioactivities of the acetate utilized for *de novo* synthesis and that used for chain elongation were different; this suggests that there are distinct pools of acetate for these two processes.

In *in vitro* assays, rapeseed oil body preparations incorporated label from [^{14}C] malonyl CoA mainly into eicosenoate and erucate, whereas crude homogenates utilized the [^{14}C] malonyl CoA mainly for *de novo* synthesis of palmitate and stearate. In assays containing oil bodies, incorporation was dependent on the presence of freshly prepared dithiothreitol; NADPH was the most efficient reductant, and ATP was required for maximum incorporation. The addition of oleoyl or eicosenoyl CoA to assays did not stimulate incorporation but markedly affected the amounts of radioactive eicosenoate and erucate synthesized, providing evidence that long chain CoAs are substrates for the chain

elongation reaction. The lack of any dependence on acyl CoAs suggests that they were present in oil body preparations or synthesized during the assays. Generally the level of labelled long chain acyl CoAs was low and most of the radioactive fatty acids synthesized *in vitro* were found in triacylglycerol and phosphatidic acid.

From *in vivo* and *in vitro* studies it is suggested that the synthesis of eicosenoate and erucate involves the formation of the corresponding CoAs by elongation of oleoyl (or eicosenoyl) CoA with malonyl CoA and NADPH in a manner analogous to malonate-dependent elongation in mammalian microsomes, and that the synthesis of oleate and its subsequent elongation to eicosenoate and erucate occur at different sites in the cell utilizing different acetate pools. The intracellular location of chain elongation and the mechanism by which the fatty acyl CoA products of chain elongation are incorporated into triacylglycerol are discussed.

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ABBREVIATIONS

ADP	-	adenosine diphosphate
ATP	-	adenosine triphosphate
CoA	-	coenzyme A
dpm	-	disintegrations per minute
DSIR	-	Department of Scientific and Industrial Research, Palmerston North, New Zealand.
DW	-	dry weight
EDTA	-	ethylenediaminetetraacetic acid
EGTA	-	1,2-di (2-aminoethoxy) ethane-N,N,N^1,N^1-tetraacetic acid
FW	-	fresh weight
g.l.c.	-	gas liquid chromatography
Hepes	-	N-2-hydroxyethylpiperazine-N^1-2-ethanesulphonic acid
Mes	-	2-(N-morpholino) ethanesulphonic acid
NAD(H)	-	(reduced) nicotinamide adenine dinucleotide
NADP(H)	-	(reduced) nicotinamide adenine dinucleotide phosphate
rac	-	racemic
t.l.c.	-	thin layer chromatography
Tris	-	Tris (hydroxymethyl) aminoethane

Standard S.I. unit abbreviations are used.

INTRODUCTION

In higher plants, reserves are accumulated in the seed which are utilized during the initial growth of the seedling after germination. In oil seeds, triacylglycerol is the major storage compound and it can be extracted by man for food and for a wide range of other purposes. In seeds of certain species, triacylglycerol contains "unusual" fatty acids which are synthesized only for a brief period during seed development, concomitant with the accumulation of triacylglycerol. The ω-9 monoenoic fatty acids eicos-11-enoate, $C_{(20:1)}$ and erucate, $C_{(22:1)}$ are two such "unusual" fatty acids (Fig. 1). Erucate is thought to be a major component of seed triacylglycerol in most Cruciferous plants, and together with eicosenoate, represents a high proportion of the fatty acids present in triacylglycerols of seed cotyledons of rapeseed, Brassica napus and Crambé abyssinca. High levels of these two fatty acids also occur in the liquid seed wax of jojoba, Simmondsia chinensis (Hilditch and Williams, 1964).

Rapeseed oil contains erucate, eicosenoate, oleate $C_{(18:1)}$ and linolenate $C_{(18:2)}$, as its major fatty acid constituents and the proportion of $C_{(20:1)}$ and $C_{(22:1)}$ present is controlled by 2 gene loci. (Downey and Craig, 1965). It has been suggested that erucate acid can cause cardiopathogenic changes in various animals fed on diets rich in rapeseed (Rocquelin, G. et al., 1971) and for this reason non-erucate acid containing strains of rape have been successfully selected which are now in widespread commercial use. Rape varieties are therefore available in which the amount of $C_{20:1}$ and $C_{22:1}$ in their oil varies, ranging from 0-50% by weight (Downey and Craig, 1965). Both the studies of Gurr et al. (1974) with crambé cotyledons and those

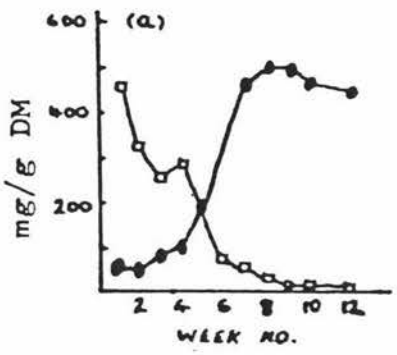
		Products of oxidative fission	
		Monocarboxylic fragment	Dicarboxylic fragment
Oleate (18:1)	$H_3C-(CH_2)_7-HC=CH-(CH_2)_7COOH \longrightarrow$	$H_3C(CH_2)_7COOH$ C_9	$HOOC-(CH_2)_7-COOH$ C_9
Eicosenoate (20:1)	$H_3C-(CH_2)_7-HC=CH-(CH_2)_9COOH \longrightarrow$	C_9	C_{11}
Erucate (22:1)	$H_3C-(CH_2)_7-HC=CH-(CH_2)_{11}COOH \longrightarrow$	C_9	C_{13}

FIG 1. Chemical structures of oleate, eicosenoate and erucate and the products formed by oxidative fission of these fatty acids.

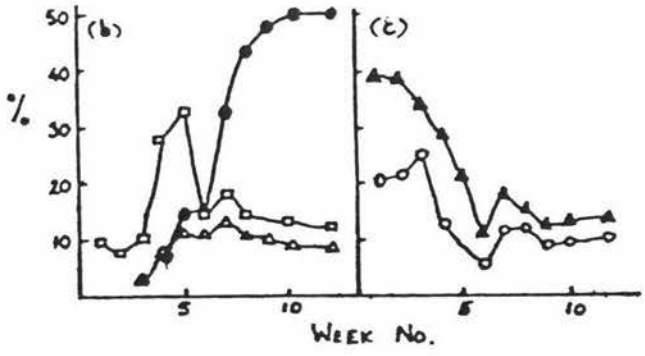
of Appelqvist (1975) with rape cotyledons, indicate that these long chain fatty acids are absent from all cotyledon lipids other than triacylglycerol. Consequently there must be fine metabolic control to regulate the rates of formation of the different constituent fatty acids and also to channel different fatty acids into specific lipids. Little is known about these processes or indeed about the mechanism and site of synthesis of eicosenoate and erucate in oil seeds, and these problems offer an interesting challenge to the lipid biochemist.

During rape seed development, total lipid content increases dramatically, commencing about 5 weeks after the first flowers appear (Norton and Harris, 1975). This rise is accompanied by a distinct change in the average fatty acid composition, from initially having high levels of palmitate ($C_{(16:0)}$), linoleate ($C_{(18:2)}$) and linolenate $C_{(18:3)}$, typical of structural galacto- and phospholipids in the immature seed, to containing high proportions of eicosenoate ($C_{(20:1)}$) and erucate ($C_{(22:1)}$), characteristic of storage lipid (Norton and Harris, 1975 and Fig. (2)). After this transition in fatty acid composition has taken place, storage of neutral lipid continues to be rapidly accumulated until, at seed maturity, it represents (by weight) 93% of the total lipids and 40% of the dry matter content of the seed.

In cotyledons of oil seeds of different species triacylglycerol accumulates in spherical structures called oil bodies that are similar in size (1 μm in diameter) (Slack et al., 1980). Controversy exists, resulting mainly from lack of information, as to both the origin and structure of these oil bodies, and also concerning the nature and chemical composition of



(a) Lipid and starch content in seed of Brassica napus during development. •, lipid; starch.



Fatty acid composition of the seed of Brassica napus during development. (b) •, $C_{(22:1)}$; □, $C_{(18:1)}$; Δ, $C_{(20:1)}$; (c) ▲, $C_{(18:2)}$; O, $C_{(18:3)}$.

FIG 2. Changes in rapeseed lipid content and composition during development, from Norton and Harris (1975).

their bounding membrane. It has been suggested that a half unit-membrane surrounds the triacylglycerol with hydrophilic moieties orientated into the cytoplasm and hydrophobic species directed towards the lipophilic interior (Yatsu and Jacks, 1972). Investigations of the chemical composition of oil bodies have shown that in addition to lipid, protein is also present (Yatsu and Jacks, 1972; Gurr et al., 1974; Slack et al., 1980), however, the function of this protein is unknown.

In initial investigations into the mechanism of synthesis of eicosenoate ($C_{(20:1)}$) and erucate ($C_{(22:1)}$), Downey and Craig (1965) noted that the strains of rapeseed with low concentrations of erucate in their triacylglycerol had oil contents similar to those of high erucate varieties, the deficit in erucate being compensated for by high levels of oleic acid. They suggested therefore that perhaps oleate was a precursor for the synthesis of eicosenoate and erucate. To examine this possibility they injected [^{14}C] acetate into developing pods of a high erucate variety of rape seed and after 24h, isolated [^{14}C]-labelled $C_{(18:1)}$, $C_{(20:1)}$ and $C_{(22:1)}$ from the cotyledons, which they subjected to oxidative fission. This process resulted in cleavage of the double bond to give a C_9 monocarboxylic fragment (a common product from $C_{(18:1)}$, $C_{(20:1)}$ and $C_{(22:1)}$) and C_9, C_{11}, C_{13} dicarboxylic fragments from the breakdown of $C_{(18:1)}$, $C_{(20:1)}$ and $C_{(22:1)}$ respectively (see Fig (1)). They concluded from the specific radioactivities of these fragments obtained (Table (1)) that $C_{(20:1)}$ and $C_{(22:1)}$ were synthesized by the addition of one 2-carbon unit (derived from acetate) to the carboxyl end of oleic acid to form $C_{(20:1)}$, and by the addition of two C_2 units to form $C_{(22:1)}$. Subsequent studies in which crambé seeds were labelled with [^{14}C]-oleate

TABLE 1. Specific radioactivities and chain length of mono- and dicarboxylic acids produced by oxidation of monoene esters (from Downey and Craig, 1965).

Fatty acid	Specific activity nCi/mmole			
	Monocarboxylic		Dicarboxylic	
oleate	C_9	26.9	C_9	20.7
eicosenoate	C_9	33.2	C_{11}	101.5
erucate	C_9	26.9	C_{13}	388.0

(Gurr et al., 1974) supported this hypothesis.

It is noteworthy that the specific radioactivities of the dicarboxylic oxidation fragments obtained by Downey and Craig (1965 and Table (1)) were in the ratio 1:5:19 (for C_9, C_{11}, C_{13} fragments respectively). Although Downey and Craig did not discuss the significance of this observation, it would appear to have some implications concerning the mechanism of formation of these long chain fatty acids. If the oleate acceptor for chain elongation were synthesized from the same acetate pool as the C_2 unit added subsequently to form eicosenoate then erucate, one would have expected the specific radioactivities (dpm/unit weight) of the dicarboxylic fragments from $C_{(18:1)}$, $C_{(20:1)}$ and $C_{(22:1)}$ to have been similar. Since this was not found, it would appear either that there was a large pool of unlabelled C_{18} acceptor within the cotyledons, or that de novo synthesis and chain elongation utilised different pools of [^{14}C]-acetate, and that the specific radioactivity of the [^{14}C]-acetate pool used for de novo synthesis was much lower than that used for chain elongation.

In an attempt to obtain more information concerning the mechanism and intracellular location of erucate synthesis in oil seeds, Gurr prepared cell fractions from a homogenate of crambé cotyledons, which he incubated with [^{14}C] malonyl CoA. Not only was the oil body fraction found to actively incorporate [^{14}C] malonyl CoA into fatty acids, but eicosenoate and erucate were the only fatty acids synthesized by this fraction. Labelled eicosenoate and erucate were also synthesized by the 800 x g and 23, 500 x g pelleted fractions but in these fractions incorporation into these long chain fatty acids represented a much smaller proportion of the total incorporation as fatty

acids with the whole range of chain lengths (C_{16} to C_{22}) were synthesized.

It has been shown that malonyl CoA, and not acetyl CoA, is the C_2 unit derived from acetate, that is involved in the elongation of oleate to form eicosenoate and erucate (Appelqvist, 1973; Pollard et al., 1979). However, controversy exists as to the identity of the 18-C compound which reacts with malonyl CoA in the elongation process. Obviously sufficient levels of the C_{18} acceptor compound were present in the oil body preparations from crambé cotyledons to enable high levels of ^{14}[C]-malonyl CoA to be incorporated into long chain fatty acids by this fraction in the in vitro studies of Gurr, as no C_{18} acceptor compound was added to the reaction mix. When acyl carrier protein (ACP) was added to the reaction mix, a 3.5 fold stimulation of [^{14}C]-malonyl CoA incorporation into eicosenoate and erucate, by an oil body fraction from crambé cotyledons, was observed (Gurr et al. 1974) and it was suggested that perhaps the unidentified C_{18} acceptor compound was the ACP thioester of oleate. However Ohlrogge et al. (1978) obtained [^{14}C]-labelled eicosenoate and erucate when a wax pad prepared from jojoba cotyledons (the equivalent of an oil body pellicle for this species) was incubated with [^{14}C] oleoyl CoA and [^{14}C] eicosenoyl CoA. Furthermore elongation of these substrates was inhibited by the presence of ACP in the reaction mix. It was therefore proposed , from these observations, that the acceptor compounds reacting with malonyl CoA might be the CoA derivatives of oleate and eicosenoate. The recent discovery that all the ACP of a leaf cell can be attributed to the chloroplasts (Ohlrogge et al., 1979) and the implication that this may also be the case regarding ACP localisation in the plastids of oil seeds

(Stumpf, pers. comm.) adds support to this hypothesis.

Similarities can be seen between the mechanism of eicosenoate and erucate synthesis in oil seeds, proposed on the basis of current evidence, and the malonate-dependent fatty acid elongation system operative in mammalian microsomes (Fig. 3) which involves the condensation of long chain acyl CoA with malonyl CoA in a manner exactly comparable to de novo synthesis. It is not known, however, whether this analogy can be extended to include the intracellular site of fatty acid elongation in seed and animal tissues. From studies using cell fractions prepared from crambe cotyledons, in which the oil body fraction was found to incorporate [^{14}C]-malonyl CoA predominantly into $C_{(20:1)}$ and $C_{(22:1)}$, Gurr et al. (1974) suggested that the oil bodies were the site of erucate synthesis. However, although [^{14}C]-labelled long chain acyl CoA substrates were elongated when incubated with a wax pad prepared from jojoba cotyledons, Pollard et al. (1979) concluded that the ability of the wax pad to synthesize long chain fatty acids was due to the contamination of the wax pad by membranous material and that elongation actually occurred on the microsomes (Stumpf, pers. comm.).

The aim of this work was to obtain more information on, which would hopefully lead to a greater understanding of, the mechanism of synthesis of eicosenoate (20:1) and erucate (22:1) in developing rape cotyledons; initially by repeating and then extending the preliminary studies of Downey and Craig (1965), and of Gurr et al. (1974), outlined above. It was hoped that by isolating an oil-body containing fraction from rape cotyledons and by defining an in vitro system which could incorporate high levels of [^{14}C]-malonyl CoA into elongation products, that the substrates and

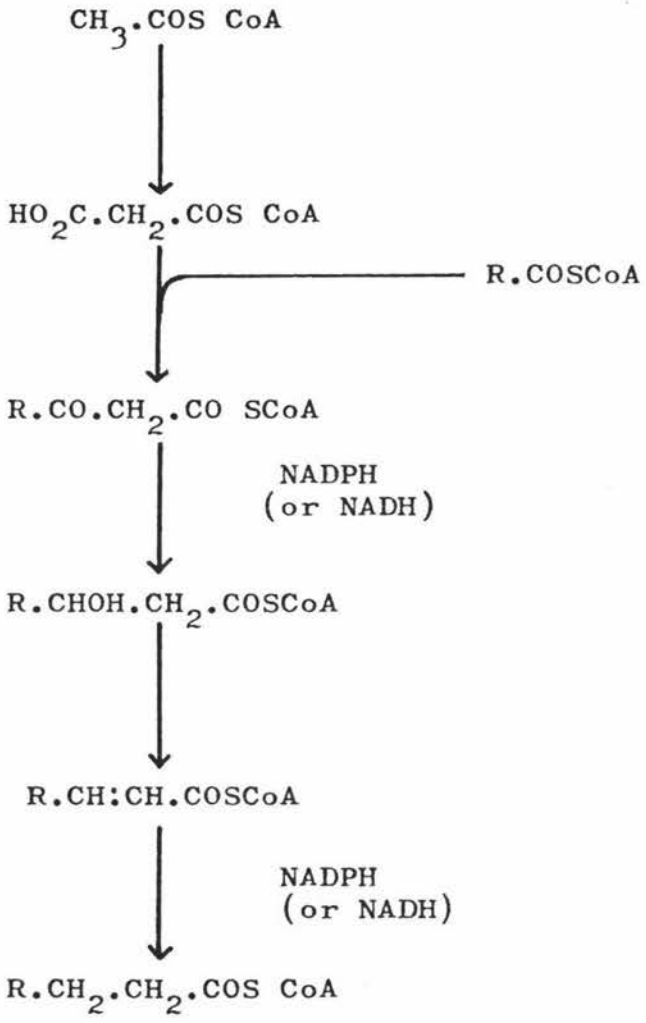


FIG. 3. Malonate dependent elongation in mammalian microsomes (Hitchcock and Nichols, 1971)

cofactors required for elongation and the nature of the elongation products themselves could be determined. By carrying out *in vivo* labelling and cell fractionation experiments, the intracellular location of the elongation system and the lipids involved in the synthesis of triacylglycerol rich in eicosenoate and erucate, were investigated.

MATERIALS AND METHODS

MATERIALS

Plant material.

Plants of rapeseed, *Brassica napus* (cultivars Target (high erucate) and Tower (low erucate)) from seeds supplied by Crop Research Division, DSIR, Palmerston North, New Zealand, were grown in the field or in North Carolina mix (70% gravel, 15% peat, 15% vermiculite) in a heated glasshouse and watered daily with nutrient solution (Hoagland & Arnon, 1938). The minimum night temperature was 15°C and the maximum day temperature was 25°C. Plants received natural lighting except during the winter when the daylength was artificially extended to 16 hours.

Chemical reagents.

A solution of sodium methoxide was prepared according to the method of Slack (Slack *et al.*, 1976). Diazomethane was synthesized by the method of Schlenk & Gellerman (1960). Fatty acid methyl ester standards for g.l.c. studies were purchased from Nu-check (Nu Check Prep, Elysian, MN, USA) and dicarboxylic and short chain fatty acid methyl esters were a gift from Dr Denis Body, Applied Biochemistry Division, DSIR.

Preparation of long chain acyl CoA.

Eicosenoyl CoA thioester was prepared by treating Coenzyme A (Lithium salt, Sigma Chem. Co.) with the mixed anhydride of eicosenoic acid according to the method of Sanchez *et al.* (1973), [11-eicosenoate was prepared by the saponification of methyl-11-eicosenoate (Nu-check)] and stored in 50 mM sodium acetate buffer, pH 4.5, at -20°C. The concentration

of the eicosenoyl CoA solution was determined both spectrophotometrically, from the amount of CoASH liberated by a thiohydrolase, according to the method of Barber and Lands (1971) and by a g.l.c. method (Slack et al., 1979) from the amount of methyleicosenoate formed by transmethylation with sodium methoxide. Good agreement was obtained between the results from the two methods. Oleoyl CoA synthesized by the same method, was a gift from Dr Claude Willemot, Plant Physiology Division, DSIR.

Synthesis of [2-^{14}C] malonyl CoA.

[2-^{14}C] malonyl CoA (5 mCi/mmol) was prepared essentially as described by Rutkoski and Jaworski (1978). However it was necessary to first convert the sodium [2-^{14}C] malonate supplied by the Radiochemical Centre, Amersham, Bucks, U.K. to the free acid by shaking an aqueous solution of the salt with an excess of Dowex 50 ion exchange resin, H^+ form. The acid was recovered in the supernatant, the resin washed twice with absolute ethanol, the bulked solutions dried under nitrogen, and the acid dissolved in tetrahydrofuran. The CoA ester was purified from a solution of the reaction products in tetrahydrofuran / 50 mM acetic acid (pH 4) 50:50 (v/v) by first extracting thiophenol and thiophenylmalonate with diethyl ether (Trams & Brady, 1960). The solution was then reduced in volume under nitrogen and chromatographed on Whatman No 1 paper in pentanol saturated with 5 M formic acid (Aronoff, 1960). Two main radioactive zones were obtained, one at the origin and the other with an R_f of 0.5 (Fig. (4)). The mobile compound co-chromatographed with malonate. Radioactive material was eluted from the origin with 50 mM sodium acetate buffer, pH 4.5 and was identified as the

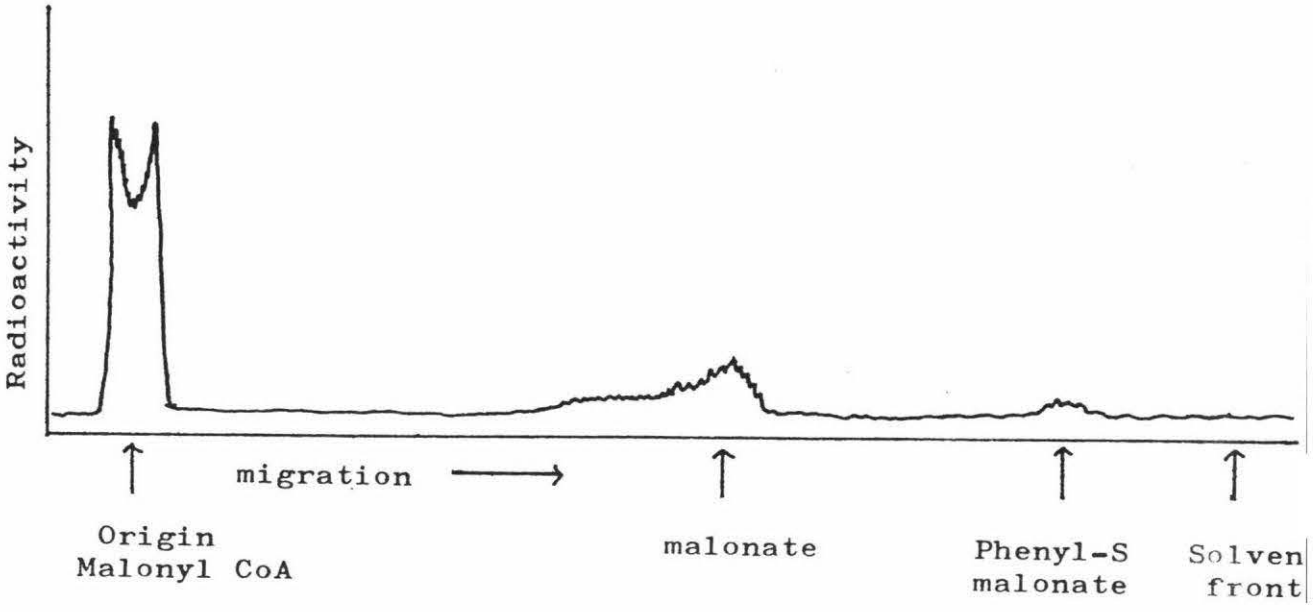


FIG. 4. Radioactivity scan of paper chromatogram of a solution containing [^{14}C] malonyl CoA.

The final mixture in [^{14}C] malonyl CoA synthesis, which had been purified by removal of thiophenol, and thiophenyl malonate into diethyl ether was streaked onto Whatman No 1 paper and the paper developed 31 cm from the origin with pentanol saturated with 5M formic acid.

CoA ester of malonate by its lability under alkaline conditions, yielding free malonic acid, and by adsorption peaks at 232 and 260 nm. The ester was stored at -20°C.

METHODS

Labelling of cotyledons and lipid extraction

Cotyledons were dissected from seeds of 17-week old rape plants (in which the cotyledons occupied most of the seed, but dehydration had not yet commenced) and placed in ice-cold water until the required number had been obtained. Batches of cotyledons (0.45 g fresh weight) were added individually to 2 ml of 50 mM Mes/KOH buffer, pH 5.0, containing 0.13 mM Sodium [1-^{14}C] acetate (57.2 mCi/mmol) in a 25 ml conical flask, covered with silver foil, and incubated at 25°C while shaking (70 shakes/min). At intervals, a flask was removed, ice cold water added to reduce further metabolism, the cotyledons removed from the incubation medium and washed. After being steam heated for 7 min., the cotyledons were homogenized with 3 ml chloroform/methanol (2:1 v/v) in a TenBroek homogeniser which was then washed with a further 10 mls of chloroform/methanol (2:1 v/v). A Folch partition (Folch *et al.*, 1957) was carried out and after centrifugation, the chloroform phase was transferred to a small screw-cap tube, dried under nitrogen and the lipid residue redissolved in 1 ml of chloroform.

Cotyledon fractionation and lipid extraction

Batches of rapeseed cotyledons (0.6 g fresh weight) were incubated as described above in 50 mM Mes/KOH

buffer, pH 5.0, containing 1.0 mM sodium [1-^{14}C] acetate (57.2 mCi/mmol) for periods of 15 and 180 min. The washed tissue was homogenized in 6 ml of a homogenisation buffer (50 mM Tris-HCl buffer, pH 5.0, 0.5 mM EGTA, 0.5 mM EDTA) containing 0.5 M sucrose in a polytron PT 20 homogeniser (Kinematica, Lucerne, Switzerland) by three bursts at half maximum speed for 1 s. After filtration through 3 layers of Miracloth, the homogenate was transferred to a 15 ml Corex tube, overlaid with 0.25 M and 0.1 M buffered sucrose (Fig. (5)) and the tube was centrifuged at 20,000 x g at 0°C for 15 min. Four fractions were isolated - a floating oil pellicle, an intermediate or 0.25 M sucrose layer, and a 20,000 x g pellet containing mitochondria and chloroplasts. The 0.5 M sucrose layer was centrifuged at 106,000 x g for 1 h to obtain a microsomal pellet; the supernatant was discarded. The oil body pellicle, the 20,000 x g, and 106,000 x g pellets were resuspended in 2 ml of 50 mM Tris-HCl buffer, pH 5.0, and extracted with 10 ml chloroform/methanol (2:1 v/v). The intermediate fraction was extracted by the method of Bligh and Dyer (1959) with chloroform/methanol/water (1:1:0.9 by volume). After centrifugation the chloroform layers were transferred to small screw cap tubes, dried down under nitrogen, and the lipid residue redissolved in 1 ml of chloroform.

Preparation of a homogenate and oil body suspension for incubation with [^{14}C] malonyl CoA.

Cotyledons were isolated as above and batches (1 g fresh weight) were homogenised in 6 ml of chilled 100 mM Hepes buffer pH 7.8 containing 1 mM EDTA, 1 mM EGTA and dithiothreitol (DTT) at concentrations specified in the results section, in a polytron PT 20

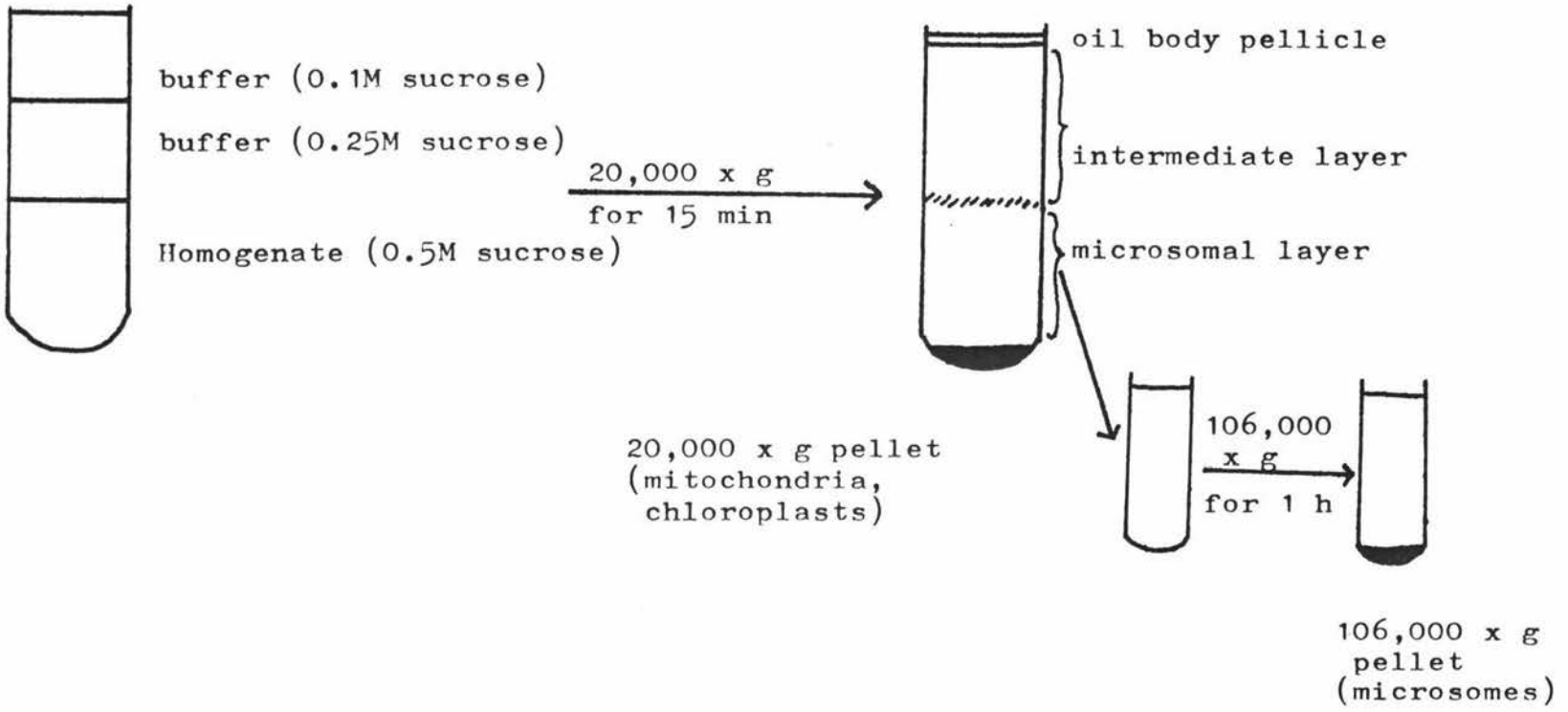


FIG 5. Preparation of the sucrose density gradient and the centrifugation procedure for isolation of subcellular fractions from rapeseed cotyledons.

homogeniser for 3 bursts of 1 s at half maximum speed. The homogenate was filtered through three layers of Miracloth, the filtrate volume adjusted to 7 ml with homogenisation buffer, and 1 ml of this homogenate solution removed and kept on ice until the oil body suspension had been prepared. The remaining 6 ml of homogenate was made 0.25 M with respect to sucrose, poured into a 15 ml Corex tube, and homogenisation buffer was gently layered on top to fill the tube, which was then centrifuged for 15 min at 20,000 x g at 0°C. The floating oil body pellicle was removed with a spatula, and, using a glass rod and a pasteur pipette, was fully resuspended in either 1 or 2 ml of homogenisation buffer. This resulted in oil body preparations of concentration [OB] and $[\frac{OB}{2}]$ respectively. Using the same weight of cotyledons, it was found that oil body preparations of similar concentration were obtained and [OB] was equivalent to approximately 30 mg triacylglycerol per ml of oil body suspension. Aliquots of the oil body preparation were taken for immediate use in [^{14}C] malonyl CoA incubation experiments, and for triacylglycerol analysis. For further purification, the oil body preparation was made 0.5 M with respect to sucrose, and centrifuged up (20,000 x g for 15 min at 0°C) through a discontinuous sucrose gradient (prepared with 4 ml, 3 ml and 3 ml of homogenisation buffer containing 0.25 M, 0.1 M and no sucrose respectively. This procedure was repeated several times and the washed oil body pellicle resuspended as before.

All the above procedures were carried out at between 0°-4°C.

Incubations of homogenate and oil body preparations with [^{14}C] malonyl CoA.

In a total reaction volume of 0.5 ml, 0.25 ml of

homogenate or oil body preparation were added to a reaction mix containing 0.56 mM [2-^{14}C] malonyl CoA, 2 mM ADP, 0.1 mM NADH, 0.1 mM NADP, 0.1 mM Glucose-6-phosphate, 0.8 mM rac glycerol-3-phosphate, 0.5 mM $MnSO_4.4H_2O$, 5 mM $KHCO_3$, 0.5 mM $MgSO_4.7H_2O$ according to the method of Gurr et al. (1974). This mixture was later modified to ultimately contain 0.56 mM [2-^{14}C] Malonyl CoA, 2 mM ATP, 0.1 mM NADPH, 0.8 mM rac glycerol-3-phosphate. The reaction mixture used and the concentrations of any other chemicals added e.g. oleoyl CoA, eicosenoyl CoA, CoA are given with the tables of results. Incubations were carried out in 15 ml screw cap tubes which were shaken (150 shakes/min) at 25°C generally for 1 h. The reaction was stopped by addition of 1 ml of 10% KOH in methanol (w/v) and the sealed tubes were heated at 80°C for 1 h. A non-saponifiable fraction was extracted with 2 x 5 ml petroleum ether (b.p. 40-60°C). The aqueous phase was acidified with H_2SO_4 and a saponifiable fraction extracted with petroleum ether as above. Both organic fractions were dried down under a nitrogen stream and the residue redissolved in 500 µl of petroleum ether (b.p. 60-80°C).

In order to determine the distribution of labelled fatty acids amongst individual lipids, and between lipids and acyl CoA esters, 10 ml of chloroform/methanol (2:1 v/v) was added to the reaction mixture, and after 2 h, 1.5 ml of H_2O was added. After shaking, and centrifugation, the chloroform phase was removed, dried down under nitrogen and redissolved in 800 µl of chloroform for determination of radioactivity content and lipid analysis. 3 ml of the aqueous-methanol phase was saponified by heating at 80°C for 1 h with 0.6 ml 40% aqueous KOH (w/v), the solution

acidified and extracted with petroleum ether as above.

To determine total radioactivity in chloroform extracts and saponifiable and non-saponifiable fractions in petroleum ether, samples of these solutions were counted in a scintillation fluid containing 0.5% p-terphenyl in xylene (w/v) and the counting efficiency determined by the channels ratio method.

Lipid and fatty acid analysis

Individual lipids were separated from aliquots of the chloroform extract by t.l.c. chromatography on silica gel G in petroleum ether (b.p. 60°-80°C)/ diethyl ether/acetic acid (75:25:1 by volume), solvent I, to separate individual neutral lipids, and in chloroform/methanol/15M-ammonia (65:25:2 by volume), solvent II, for separation of polar lipids. The plates were lightly stained with iodine vapour and the required lipid-containing zones were scraped into vials each containing 10 ml of a scintillation fluid containing 0.5% p-terphenyl in xylene-Triton X-114-water (6:2:1 by volume) and counted.

To prepare fatty acid methyl esters, the triacylglycerol, the diacylglycerol and the polar lipid zones were separated by t.l.c. in solvent I and lightly stained with iodine vapour. After destaining under nitrogen, the triacylglycerol and diacylglycerol-containing regions were scraped off and transmethylated directly by addition of 1 ml of sodium methoxide solution. After 20 min, 2.5 ml of light petroleum (b.p. 40°-60°), followed by 1.0 ml of water were added and the methyl esters immediately extracted into the organic phase. The polar lipids that

remained at the origin of the chromatogram in Solvent I were eluted from the silica gel by shaking with 1 ml water, 1.7 ml methanol, and 3.3 ml chloroform. The chloroform layer was dried under nitrogen and the individual lipids separated by t.l.c. in solvent II as above. The silica zone containing phosphatidyl choline was identified, transmethylated and extracted as described for the neutral lipids above.

The distribution of radioactivity amongst the fatty acid methyl esters of each lipid was investigated by two alternative methods. Fatty acid methyl esters were separated by argentation chromatography (see below) and visualized under ultraviolet light after spraying the plates with a 0.02% solution of dichlorofluorescein (w/v) in 85% ethanol containing 0.06% sodium acetate. The ester-containing zones were scraped into scintillation vials to which the water-containing scintillant (described above) was added. Argentation chromatography was also used to determine the distribution of incorporated radioactivity in the fatty acid methyl esters of saponifiable fractions that had been extracted into petroleum ether after [^{14}C]-malonyl CoA incubation experiments, having first methylated the saponifiable material using diazomethane.

Alternatively, the fatty acid methyl ester preparations from individual lipid species were separated by g.l.c. at 180^{o}C on a column (200 cm x 0.3 cm) of chromasorb W coated with 15% ethylene glycol succinate. The column was fitted with an effluent stream splitter so that 30% entered the flame-ionisation detector and the remainder was collected and counted for radioactivity in 0.5% p-terphenyl in xylene as described by Slack and Roughan (1975).

Quantitative lipid and fatty acid determinations

To determine the total lipid content and analyse the fatty acid composition of cotyledons from rapeseeds at different stages of maturity, ten cotyledons from each stage were homogenised with 4 ml of chloroform/methanol (1:1 v/v) with a TenBroek homogeniser, and the final volume made up to 5 ml. An aliquot was removed, transmethylated with sodium methoxide, the methyl ester extracted into petroleum ether and measured by g.l.c. as above. Triacylglycerol was the predominant lipid in rapeseed cotyledons examined, and to quantitate the amount of tissue used in *in vitro* studies of the metabolism of [^{14}C] malonyl CoA by total homogenate and oil body preparations, the lipid content of the homogenate and oil body preparations was determined by carrying out a Folch extraction, removing the chloroform layer, drying it under nitrogen and redissolving in chloroform. An aliquot was transmethylated and analysed as above.

Analytical and preparative argentation chromatography.

When fatty acid methyl ester preparations from lipid extracts of rapeseed cotyledons or oil body preparations were chromatographed in benzene at room temperature on activated t.l.c. plates (0.25 mm thick) coated with silica gel G containing 5% $AgNO_3$ (w/w), the fatty acid methyl esters separated out according to their degree of unsaturation, saturated compounds being the most, and trienoic compounds being the least mobile. However, methyl oleate, methyl eicosenoate and methyl erucate ran as three distinct bands in the monoenoic region of the chromatogram and complete separation of the three methyl esters was invariably

obtained at a total loading of less than 200 μg methyl ester/cm (Fig. (6)). Separation of these particular fatty acid methyl esters by argentation chromatography has been reported (Kirk et al. 1978) but at -23°C using toluene/hexane (95:5 v/v) as the developing solvent. Using argentation chromatography it was possible therefore to determine the amounts of radioactivity in the individual monoenoic acids (as previously described) and also to purify sufficient quantities of the methyl esters of these acids for the degradation studies described below. Individual esters were eluted from the silica gel with water/methanol/chloroform (2:3.3:6.6 v/v) separated from dichlorofluorescein by t.l.c. on silica gel G in benzene, and extracted with 2 x 2 ml of diethyl ether. (It was necessary to repeat this whole purification procedure several times to obtain sufficient quantities of the required fatty acid methyl esters). G.l.c. analysis of samples of these methyl esters indicated that methyl oleate and methyl erucate were greater than 90% pure. The purity of methyl eicosenoate ranged from 75-88%, and contained methyl erucate as the major contaminant.

Oxidative fission of [^{14}C] labelled methyl oleate, methyl eicosenoate and methyl erucate

Approximately 1.5 mg of each of the 18:1 Me, 20:1 Me and 22:1 Me, prepared by argentation chromatography as described above, was subjected to oxidative fission across the double bond essentially according to the method of Body and Shorland (1965). (The weights and volumes of all reactants used were scaled down to allow for 1.5 mg of sample). The oxidation products were dissolved in 0.4 ml of 10% methanol/diethyl ether (v/v),

$C_{(16:0)}$, $C_{(18:0)}$

$C_{(22:1)}$

$C_{(20:1)}$

$C_{(18:1)}$

$C_{(18:2)}$

origin

FIG. 6. Autoradiograph of $AgNO_3$ t.l.c. separation of fatty acid methyl esters prepared from triacylglycerol of rapeseed cotyledons fed with [^{14}C] acetate.

An aliquot of a fatty acid methyl ester preparation from triacylglycerol of rapeseed cotyledons fed with [^{14}C] acetate for 160 min was chromatographed in benzene on t.l.c. plates coated with silica gel G containing 5% $AgNO_3$ (w/w) as described in the Methods section. The t.l.c. plate was then autoradiographed. Linolenate, $C_{(18:3)}$ was not labelled, but was shown to be located about half-way between the origin and linoleate, by staining the plate with dichlorofluorescein.

methylated with diazomethane and a sample injected immediately into the g.l.c. at 140°C fitted with a column as above, but with a modified collector tube containing a U-bend immersed in an acetone-dry ice bath. Under these conditions, the C_9 monocarboxylic methyl ester, a common product of the oxidation of 18:1 Me, 20:1 Me and 22:1 Me (as described in the Introduction) was well separated from the solvent peak, and the sample passing into the collector tube was collected at low temperature. The oven temperature of the g.l.c. was raised to 190°C and other samples of the oxidation products injected to purify the C_9, C_{11} or C_{13} dicarboxylic methyl ester.

Oxidative degradation of [^{14}C] labelled methyl oleate, methyl eicosenoate and methyl erucate

The labelled long chain methyl esters obtained from preparative argentation chromatography were hydrogenated as described by Appelqvist (1972), and the complete hydrogenation to 18:0 Me, 20:0 Me, 22:0 Me was confirmed by g.l.c. The saturated methyl esters were saponified, subjected to α-oxidation by $KMnO_4$ (reaction period of 2 h) as described by Harris (1965), the products extracted into petroleum ether and methylated with diazomethane. The specific activities of the products were determined by g.l.c. analysis at 160°C as previously described.

Chlorophyll determination

0.2 ml samples of homogenate or oil body preparations were added to 0.8 ml acetone, the suspension centrifuged and the chlorophyll content of the acetone extract measured as described by Arnon (1949).

Protein measurement.

To isolate protein from oil bodies, the oil body pellicle was removed from the top of the sucrose gradient after the final wash, transferred directly to 10 ml of chloroform/methanol (2:1 v/v), 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 was washed twice with 5 ml diethylether, and the solvent removed with a stream of air. All solvent extracts were bulked.

For protein measurements, the material was dissolved in 2% sodium dodecylsulphate and protein measured by a modified Lowry method (Shacterle and Pollack, 1973) using bovine serum albumin as a standard and with a final concentration of 0.2% sodium dodecyl sulphate in both standards and unknowns. The bulked 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 and the protein content of the oil bodies expressed as a percent by weight of the glycerolipid.

RESULTS

I. Selection of seed tissue suitable for biosynthetic studies.

In rapeseed, the fatty acid composition and the isomeric forms of the monounsaturated fatty acids of the seed coat lipids have been found to differ from those of the cotyledon lipids (Appelqvist, 1975). Consequently for studies of eicosenoate and erucate biosynthesis in cotyledons, it was necessary to remove the seed coats.

It was found that there was only a short period (which occurred 16-18 weeks after seed planting) during which the cotyledons were easily removed from the seed coats, and it was therefore important to establish whether this stage of cotyledon development corresponded with the period of rapid erucate biosynthesis.

Rapeseed flowers arise on a raceme along which a developmental sequence exists. It was possible therefore, to obtain relatively immature and mature seeds from the same flowering head. The total content and fatty acid composition of lipid present in the cotyledons of both immature and mature seeds from 16, 17 and 18 week-old plants, were determined (Table 2) and a comparison made between these biochemical characteristics and the general anatomical appearance of the seeds and cotyledons from each stage (Fig. 7). In the immature seeds of 16 week-old plants, which contained liquid endosperm and small cotyledons, the lipid content was low and contained high amounts of $C_{(18:1)}$, $C_{(18:2)}$ and $C_{(18:3)}$, typical of leaf tissue. However cotyledons from mature seeds on 17 week-old plants, which had expanded to occupy most of the seed volume, contained large amounts of lipid (almost 40%

TABLE 2. Fresh weight, dry weight, total lipid content and lipid fatty acid composition of cotyledons prepared from immature and mature seeds of rapeseed plants of different ages.

Fresh and dry weight data given are the mean of 20 cotyledons from each stage. For determination of total lipid content and fatty acid composition, 10 cotyledons from each stage were homogenised with chloroform/methanol (1:1 v/v), and the volume adjusted to 5 ml. An aliquot was removed, transmethylated with sodium methoxide, the methyl ester extracted into petroleum ether and analysed by g.l.c. as described in the Methods section.

Age of plant (wk)	Developmental Stage	FW	DW	Total Lipid	Fatty acid composition (% total)						
		per cotyledon (mg)			16:0	18:0	18:1	18:2	18:3	20:1	22:1
16	immature	1.0	0.4	0.09	8	5	38	22	9	10	8
16	mature	3.2	1.5	0.54	4	2	31	11	6	13	33
17	immature	3.6	1.9	0.58	4	2	26	12	7	11	38
17	mature	4.1	2.5	0.97	3	1	22	11	6	11	46
18	mature	3.7	2.0	0.63	3	1	21	13	8	10	44

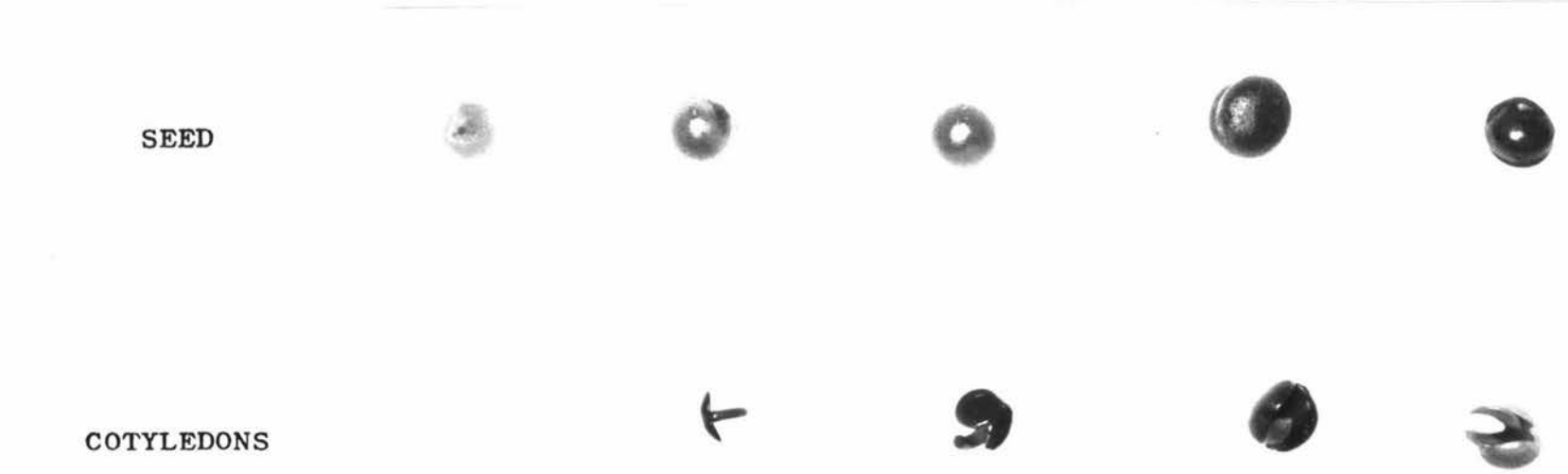


FIG. 7. Cotyledon size relative to seed size of immature and mature seeds taken from plants of different ages

I, immature; M, mature.

of dry weight of the cotyledons) rich in erucate.

Norton and Harris (1975) found that the commencement of rapid lipid accumulation occurred at the same time as the onset of $C_{(20:1)}$ and $C_{(22:1)}$ synthesis (Fig. 2). Consequently, fully expanded cotyledons that had not commenced dessication, were chosen for future studies.

II. In vivo labelling of rapeseed cotyledons with [^{14}C]-acetate.

Lipid composition.

Analysis of lipids present in the cotyledons showed that more than 90% was triacylglycerol, the other major lipid classes present being diacylglycerol and phosphatidylcholine. Approximately 50% of the fatty acids present in the triacylglycerol were the "unusual" fatty acids, eicosenoate and erucate (Table 3). Diacylglycerol and phosphatidylcholine contained high proportions of the C_{18} fatty acids but, in contrast to the findings of Gurr *et al.* (1974) with crambé cotyledons, these lipid classes also contained $C_{(20:1)}$ and $C_{(22:1)}$.

Time course of incorporation of [1-^{14}C]-acetate into rapeseed cotyledons.

In a time course labelling experiment, [1-^{14}C] acetate was rapidly incorporated into lipids of rapeseed cotyledons (Fig. 8). Incorporation into total lipid increased linearly for 20 min, then declined and ceased after 80 min. Triacylglycerol was found to contain the bulk of the incorporated radioactivity at all times (Table 4). The majority of the remaining incorporated label was located in diacylglycerol and phosphatidyl choline, and to a lesser extent free fatty acids. Very small amounts of label were present also in other phospholipids, monoacylglycerol and a hydrocarbon compound.

The three major lipids exhibited very different labelling patterns (Fig. 9). Incorporation of label into triacylglycerol occurred preferentially into erucate

TABLE 3. Fatty acid composition of rapeseed cotyledon lipids

Lipids were extracted from detached cotyledons which had been incubated with [^{14}C]-acetate as described in Fig. 8, and separated by t.l.c. on silica gel G. The lipid-containing zones were transmethylated with sodium methoxide and the fatty acid methyl esters analysed by g.l.c.

Lipid	Relative amounts (mass) of individual fatty acids (%)						
	16:0	18:0	18:1	18:2	18:3	20:1	22:1
Phosphatidyl choline	8	2	40	30	9	6	5
Diacylglycerol	6	3	30	19	7	10	25
Triacylglycerol	4	2	23	17	7	12	35

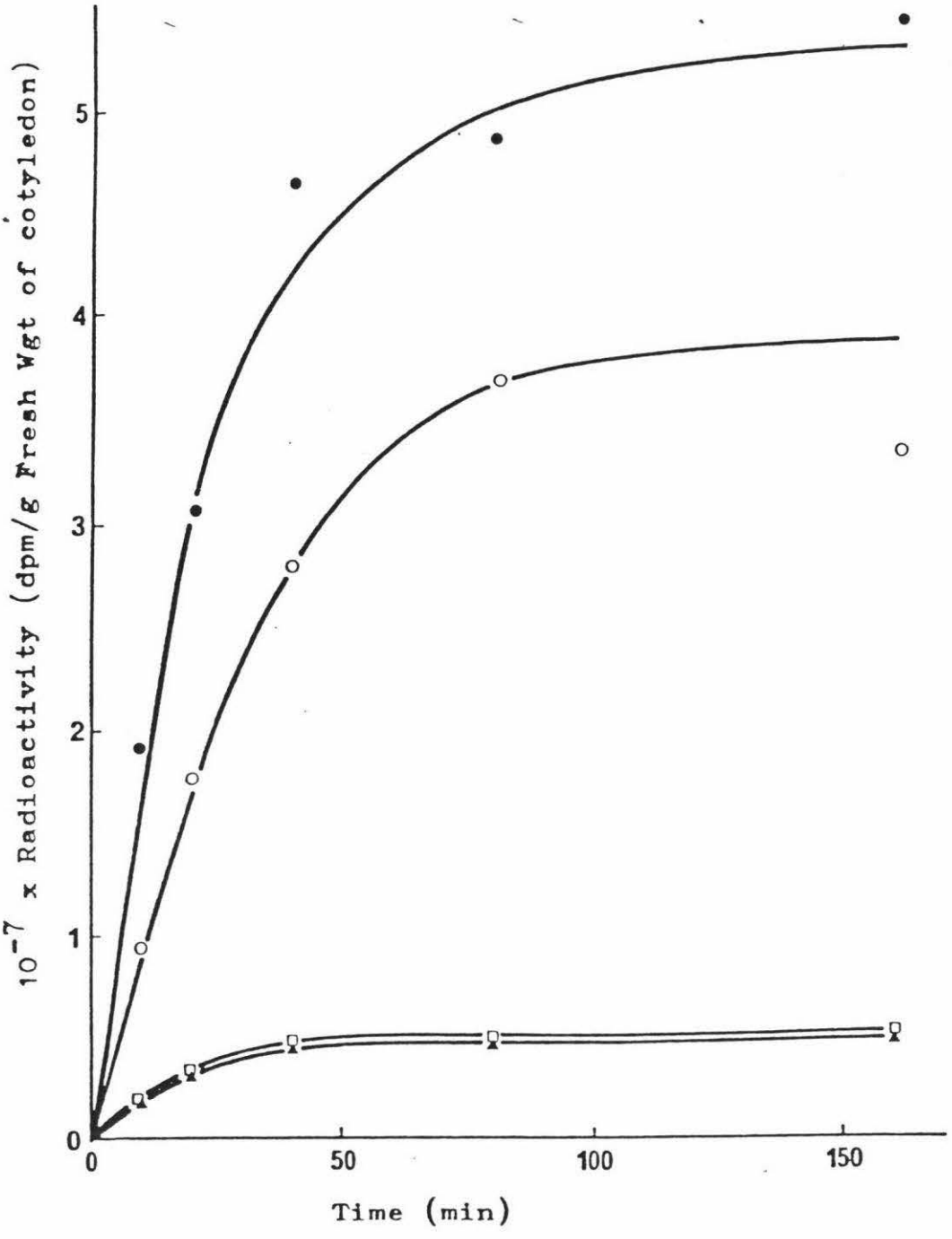


FIG. 8. Time course of incorporation of [1-^{14}C] acetate into lipids of developing rapeseed cotyledons.

Batches of rapeseed cotyledons (0.45 g fresh weight) were incubated with [1-^{14}C] acetate at 25°C as described in the Methods section. Iced water was added after the incubation times shown above, the cotyledons steam heated, and the lipids extracted into chloroform. The chloroform sample extracts were analysed for radioactivity and subjected to t.l.c. on silica gel G to separate individual lipids. Lipid containing zones were scraped into vials and counted.
Total radioactivity (o), Radioactivity in triacylglycerol (o), diacylglycerol (▲) and phosphatidyl choline (□).

TABLE 4. Effect of incubation time on the distribution of radioactivity amongst individual lipids.

Lipids were extracted from cotyledons labelled with [^{14}C] acetate in the experiment described in Fig. 8 and samples were separated by t.l.c. on silica gel G, the lipid containing zones scraped into vials and counted as described in the Methods section.

Lipid	Distribution of radioactivity (%) Time (min)				
	10	20	40	80	160
Phosphatidyl choline	12.4	12.2	11.7	8.8	11.2
Diacylglycerol	14.0	12.4	10.3	9.4	9.8
Free fatty acid	5.0	4.4	3.9	3.5	2.6
Triacylglycerol	58.0	62.8	64.4	74.9	67.0
Other compounds+	10.6	8.2	9.7	3.4	9.4

+ See text for details

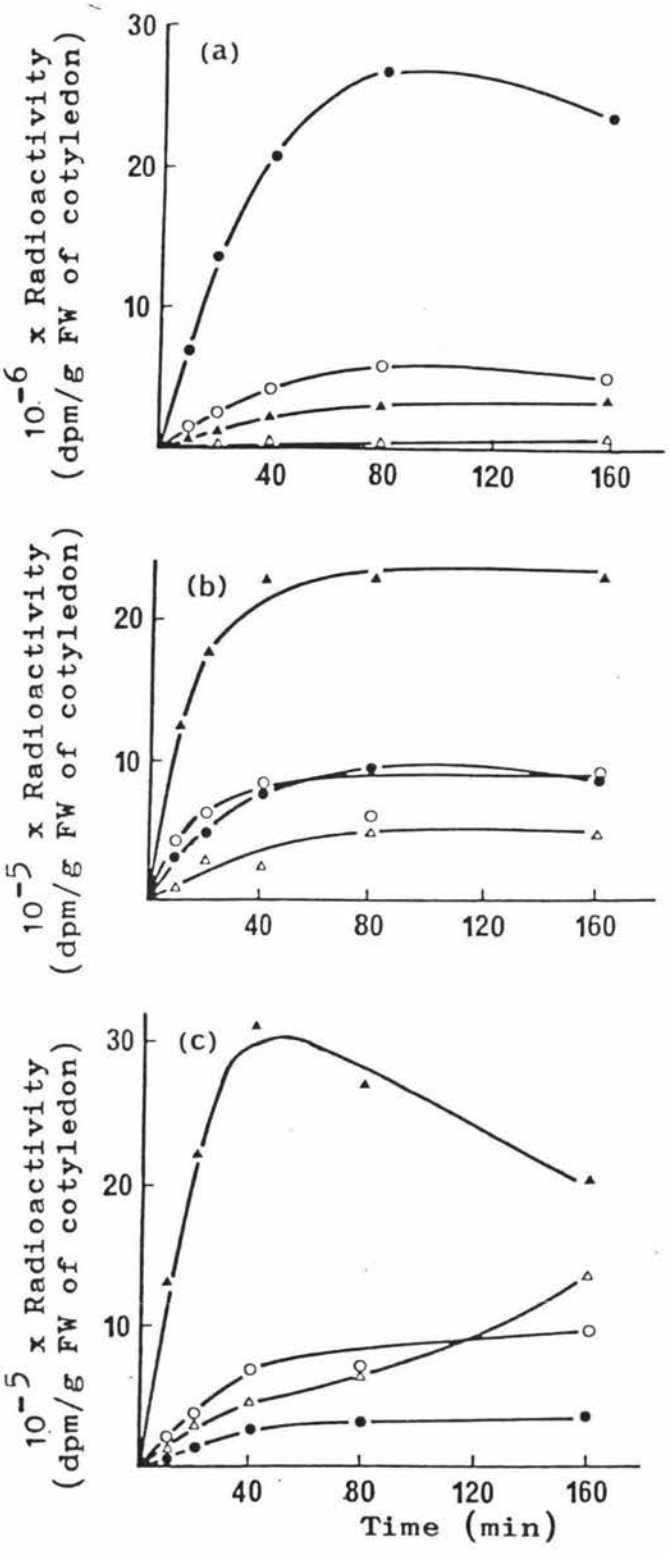


FIG 9. Time course of labelling of fatty acids of certain glycerolipids in developing rapeseed cotyledons.

The fatty acids of triacylglycerol (a), diacylglycerol (b), and phosphatidyl choline (c) labelled in the experiment described in Fig. 8 were analysed.

▲, oleate; △, linolenate; o, eicosenoate; ● erucate.

as this fatty acid contained more than 70% of the incorporated radioactivity and yet it represented only 35% of total fatty acids present (by mass). In diacylglycerol and phosphatidyl choline, however, oleate was the most highly labelled fatty acid. Although diacylglycerol contains more than twice as much eicosenoate than erucate (by mass), these two long chain fatty acids have incorporated the same amount of label in this lipid.

The kinetics of labelling of oleate and linoleate in phosphatidyl choline are consistent with the view that oleoyl phosphatidyl choline is a precursor of linoleoyl phosphatidyl choline (Slack et al., 1978). but there were no other obvious precursor-product relationships.

Cell fractionation.

In order to determine the sub-cellular location of the rapidly labelled triacylglycerol, rapeseed cotyledons were again incubated with [1-^{14}C] acetate for 15 and 180 min, then homogenised, and the homogenate subjected to sucrose density centrifugation. Four fractions were isolated from the gradient, a floating oil body fraction, a 20,000 x g pellet (nuclei, mitochondria and chloroplasts), a 106,000 x g pellet (microsomes) and the 0.25 M sucrose layer or "intermediate" fraction (material with a high membrane to oil content, perhaps small oil bodies).

The distribution of label amongst the fractions was essentially the same for both 15 and 180 min incubations (Table 5) and showed that, although all fractions contained label, most radioactivity was located in the oil body fraction.

Labelled phospholipid, diacylglycerol and triacylglycerol were found in all four sub-cellular fractions (Fig. 10). Over 90% of the radioactivity located in the heavily labelled oil body fraction, isolated from cotyledons which had been incubated with [^{14}C] acetate for 15 min, had been incorporated into neutral lipids (triacylglycerol and diacylglycerol) (Table 6). In both the intermediate and 20,000 x g pellet fractions neutral lipid was again heavily labelled, but a high proportion of the radioactivity was present in phospholipids. In the 106,000 x g pellet fraction, however, phosphatidyl choline contained 50% of the radioactivity incorporated into the lipids of this fraction.

The distribution of incorporated radioactivity

TABLE 5. Distribution of incorporated radioactivity between subcellular fractions prepared from rapeseed cotyledons labelled with [^{14}C] acetate for 15 and 180 min.

Batches of rapeseed cotyledons (0.6 g fresh weight) were incubated at 25°C with [^{14}C] acetate for 15 and 180 min, homogenised, filtered and the homogenate subjected to sucrose density centrifugation, as described in the Methods section, permitting the isolation of a floating oil body layer, 20,000 x g and 106,000 x g pellets and an intermediate fraction. The fractions were extracted into chloroform and samples assayed for radioactivity. The proportions of the [^{14}C] acetate supplied which were incorporated into lipids were 9 and 18% for the 15 and 180 min incubation respectively.

Subcellular fraction	Distribution of radioactivity amongst cell fractions (%)	
	15 min	180 min
20,000 x g	6	6
106,000 x g	12	10
Intermediate	8	5
Oil body	74	78

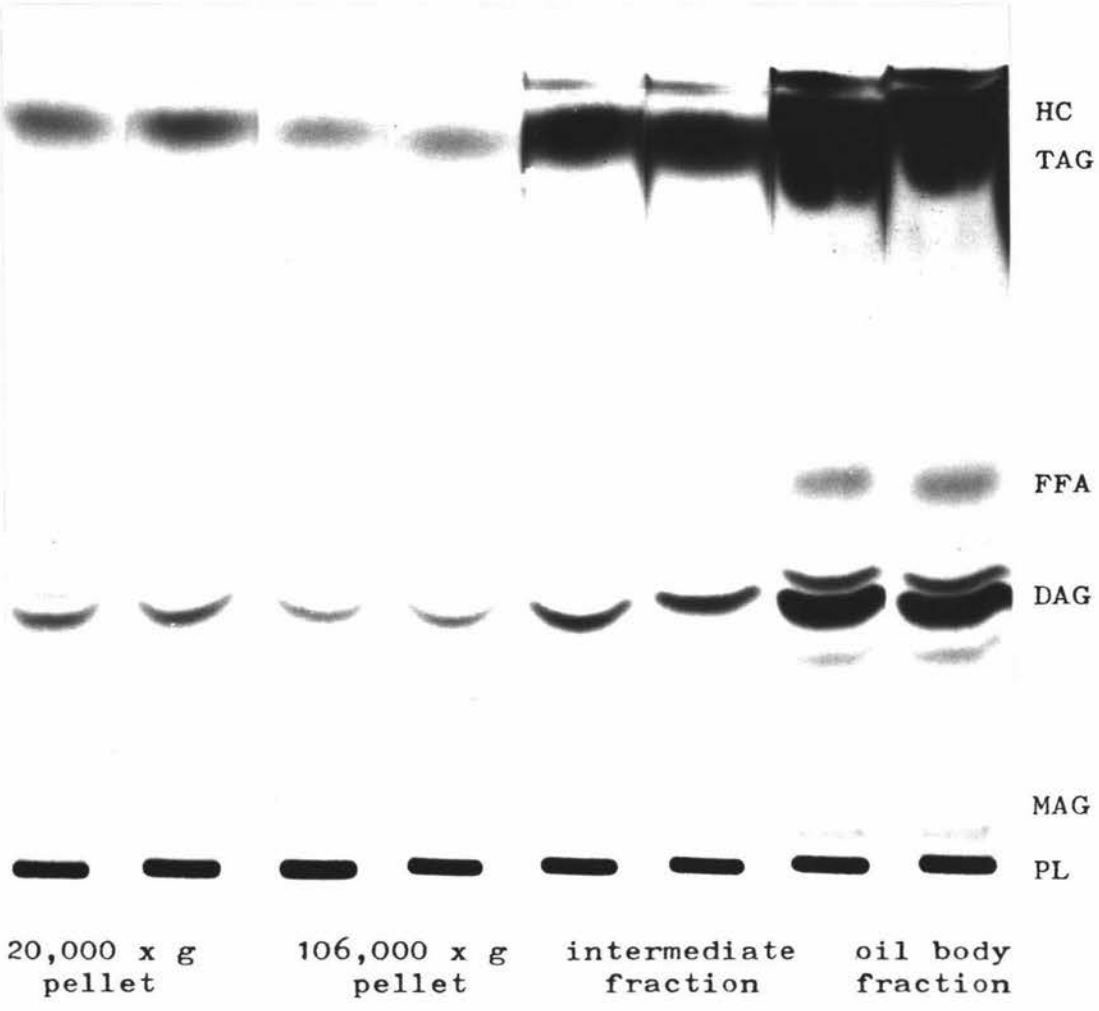


FIG. 10. Autoradiograph of t.l.c. separation of neutral lipids of subcellular fractions obtained from rapeseed cotyledons fed with [^{14}C] acetate for 180 min.

Samples of the chloroform extracts prepared from cotyledons which had been incubated with [^{14}C] acetate for 180 min, in the experiment described in Table 5, were analysed by t.l.c. on silica gel G as described in the Methods section. The small amount of labelled 1,3-diacylglycerol, an isomer of 1,2-diacylglycerol formed by acyl migration (Christie, 1973), is thought to have been produced during lipid extraction. PL, polar lipid; MAG, monoacylglycerol; DAG, diacylglycerol; FFA, free fatty acid; TAG, triacylglycerol; HC, unidentified hydrocarbon compound.

TABLE 6. Distribution of radioactivity between lipids present in different fractions prepared from rapeseed cotyledons labelled with [^{14}C] acetate for 15 and 180 min.

Individual lipids in chloroform extracts of the cell fractions obtained in the experiment described in Table 5, were separated by t.l.c. and analysed as described in the Methods section.

Lipid	Distribution of radioactivity among individual lipids of each fraction (%) prepared from cotyledons labelled with [^{14}C] acetate for 15 min and for 180 min (given in parentheses).			
	20,000 x g pellet	106,000 x g pellet	intermediate	oil body
Triacylglycerol	40 (27)	26 (17)	59 (58)	73 (81)
Diacylglycerol	25 (20)	20 (14)	19 (13)	20 (14)
Phosphatidylcholine	29 (32)	49 (59)	29 (26)	6 (4)
Phosphatidylethanolamine	6 (21)	5 (10)	2 (3)	1 (1)

amongst the lipids of the four fractions prepared from cotyledons which had been incubated with [^{14}C] acetate for 180 min resembled overall that obtained for the 15 min incubation, although some differences between the 2 sets of data were apparent. It is thought that these discrepancies may have arisen due to the isolation of non-identical fractions from the two cotyledon homogenates, caused by a variation in the degree of cross contamination by organelles during sucrose density centrifugation and for this reason they probably do not have any metabolic significance.

Analysis of the masses of each lipid found in the four sub-cellular fractions, showed that 88% of the triacylglycerol and 78% of the diacylglycerol in the cell were contained in the oil body fraction (Table 7) and that phosphatidyl choline was almost equally distributed between all four fractions. The observation that the intermediate fraction contained both a high proportion of the remaining neutral lipid of the cell, not found in the oil body fraction, together with high levels of phosphatidyl choline, is consistent with the hypothesis that this fraction perhaps contained organelles with a high membrane to oil content.

The specific radioactivities of each lipid, in the 106,000 x g pellet, the intermediate and the oil body fractions (Table 8), were very similar, and, in general, higher than in the 20,000 x g pellet fraction, perhaps suggesting the presence of unlabelled phosphatidyl choline, diacylglycerol and triacylglycerol in the organelles contained in this fraction.

The specific radioactivity of the phosphatidyl-choline, in each fraction, was found to be higher than

TABLE 7. Distribution of triacylglycerol, diacylglycerol and phosphatidylcholine among subcellular fractions prepared from rapeseed cotyledons.

Triacylglycerol, diacylglycerol and phosphatidylcholine were prepared from samples of chloroform extracts of cell fractions obtained from rapeseed cotyledons by t.l.c. on silica gel G as described in the Methods section. Zones containing the required lipids were scraped off, transmethylated with sodium methoxide and analysed by g.l.c.

Lipid	Proportion of the total amount of individual lipid present in each cell fraction (%).			
	20,000 x g pellet	106,000 x g pellet	intermediate fraction	oil body fraction
Triacylglycerol	3	1	8	88
Diacylglycerol	9	3	9	78
Phosphatidylcholine	23	26	20	30

TABLE 8. Specific radioactivities of triacylglycerol, diacylglycerol and phosphatidylcholine, present in subcellular fractions obtained from rapeseed cotyledons labelled with [^{14}C] acetate for 180 min.

Triacylglycerol, diacylglycerol and phosphatidyl choline were separated from the chloroform extracts of the different subcellular fractions isolated from cotyledons which had been incubated with [^{14}C]-acetate for 3 h, by t.l.c., transmethylated and extracted into petroleum ether. Masses of the lipids were then determined by g.l.c. and samples assayed for radioactivity by scintillation counting as described in the Methods section.

	Specific radioactivity $\left(\frac{dpm}{\mu g}\right)$ of lipids present in the different cell fractions			
Lipid	20,000 x g pellet	106,000 x g pellet	intermediate	oil body
Triacylglycerol	213	468	493	478
Diacylglycerol	2380	3389	3453	4317
Phosphatidylcholine	5914	7675	8583	7572

that of the diacylglycerol, which was approximately 10-fold greater than that of the triacylglycerol, presumably reflecting the small amounts of phosphatidylcholine and diacylglycerol, compared with that of triacylglycerol, present in the cotyledons and perhaps also the relative rate of turnover of each lipid. (It has been suggested that phospholipids in developing seeds are subject to substantial turnover whereas triacylglycerol is metabolically inert (Appelqvist, 1975).

On the basis of enzyme marker studies, it has invariably been shown that differential centrifugation as a means of preparing cell fractions, leads to some cross contamination of the different fractions. (Weaire and Kekwick, 1975). Consequently, the similar specific radioactivities of each lipid, in the different cell fractions, is probably indicative of contamination rather than a precise metabolic control that regulates the rates of labelling of each lipid in the different cell organelles in proportion to the amounts of lipid present. The observation that both the distribution of incorporated radioactivity among the fatty acids of each lipid and the fatty acid composition of a particular lipid is very similar in all sub-cellular fractions (Table 9) adds support to the hypothesis that cross contamination of the fractions probably occurred. In all fractions, as previously observed, erucate contained most of the radioactivity in the triacylglycerol, whereas in diacylglycerol and phosphatidylcholine, oleate was the most heavily labelled fatty acid.

The specific radioactivities of the fatty acids of each lipid (Table 10), in general reflected the trends seen above for the specific radioactivity data for the lipids themselves. It is noteworthy that the

specific radioactivity of eicosenoate of the diacylglycerol and phosphatidylcholine, but not of the triacylglycerol, was higher than that of the erucate.

TABLE 9. Distribution of radioactivity among fatty acids and the fatty acid composition of lipids in fractions prepared from rapeseed cotyledons labelled with [^{14}C] acetate for 180 min.

Triacylglycerol, diacylglycerol and phosphatidyl choline prepared as described in Table 8, were transmethylated with sodium methoxide. Individual fatty acid methyl esters were separated by $AgNO_3$ t.l.c. and the required zones scraped into vials and counted. Fatty acid composition of the lipids was determined by g.l.c.

Distribution of [^{14}C] acetate among fatty acids (%) and relative fatty acid composition (%) of lipids of cell fractions (given in parentheses)

Subcellular fraction	Triacylglycerol						Diacylglycerol						Phosphatidyl choline					
	16:0, 18:0	18:1	18:2	18:3	20:1	22:1	16:0, 18:0	18:1	18:2	18:3	20:1	22:1	16:0, 18:0	18:1	18:2	18:3	20:1	22:1
20,000 x g	3	15	3	1	14	64	3	49	16	2	18	11	3	51	22	2	17	5
	(6)	(30)	(13)	(5)	(12)	(33)	(11)	(39)	(20)	(11)	(7)	(11)	(9)	(36)	(31)	(12)	(6)	(5)
106,000 x g	3	16	4	0.4	14	62	5	47	17	3	16	12	3	50	22	3	17	5
	(8)	(29)	(14)	(5)	(11)	(32)	(15)	(32)	(29)	(9)	(6)	(12)	(9)	(37)	(31)	(8)	(7)	(8)
Intermediate	3	12	2	0.3	15	68	5	46	13	2	14	19	3	50	23	2	16	6
	(7)	(28)	(14)	(5)	(11)	(35)	(9)	(36)	(26)	(7)	(7)	(14)	(8)	(38)	(33)	(8)	(7)	(6)
Oil body	3	13	2	0.4	17	64	3	54	16	2	17	9	3	50	24	2	15	7
	(10)	(27)	(12)	(5)	(11)	(35)	(7)	(41)	(23)	(8)	(9)	(11)	(10)	(37)	(33)	(9)	(7)	(5)

TABLE 10. Specific radioactivity of fatty acids of triacylglycerol, diacylglycerol and phosphatidyl choline in subcellular fractions prepared from rapeseed cotyledons labelled with [^{14}C] acetate.

The specific radioactivities of the individual fatty acids of the lipids in the four subcellular fractions were calculated from the % distribution of radioactivity among the fatty acids of each lipid (Table 9), the masses of the individual fatty acids determined by g.l.c., and the total radioactivity incorporated into the lipid samples assayed by scintillation counting.

10^{-1} x specific radioactivity (dpm/μg) of each fatty acid

Fraction	Triacylglycerol						Diacylglycerol						Phosphatidyl choline					
	16:0, 18:0	18:1	18:2	18:3	20:1	22:1	16:0, 18:0	18:1	18:2	18:3	20:1	22:1	16:0, 18:0	18:1	18:2	18:3	20:1	22:1
20,000 x g	10	11	5	4	26	41	64	296	188	42	635	233	191	836	421	96	1625	574
106,000 x g.	18	26	13	3	59	90	119	507	202	119	949	356	256	1040	551	284	1810	473
Intermediate	22	21	7	3	66	96	184	436	172	95	663	485	313	1140	600	209	1946	876
Oil body	24	22	7	4	68	86	177	570	296	105	830	342	242	1031	554	162	1615	1131

Degradation studies

When Downey and Craig (1965) subjected labelled $C_{(18:1)}$, $C_{(20:1)}$ and $C_{(22:1)}$, prepared from triacylglycerol of rapeseed cotyledons that had been incubated with [^{14}C]-acetate for 24 h, to oxidative fission - a degradative process which results in the cleavage of monounsaturated fatty acids across the double bond (see p. 5), they found that the dicarboxylic fragments derived from $C_{(20:1)}$ and $C_{(22:1)}$ had specific radioactivities which were higher than those of the monocarboxylic fragments from the respective acids, and of the dicarboxylic fragment from $C_{(18:1)}$ (Table 1). They concluded that these results provided evidence for their proposal that eicosenoate and erucate are synthesized by chain elongation of oleate and this hypothesis has been supported by studies using crambé (Gurr et al., 1974) and jojoba (Pollard et al., 1979).

Analysis of the specific radioactivities of the fragments produced by oxidative fission of $C_{(18:1)}$, $C_{(20:1)}$ and $C_{(22:1)}$ obtained from the triacylglycerol of rapeseed cotyledons that had been labelled with [^{14}C]-acetate for 2 h (Table 11) produced results which were very similar to those obtained by Downey and Craig (1965), and supported their hypothesis that eicosenoate and erucate are synthesized by elongation of oleate. The unequal distribution of radioactivity in [^{14}C] labelled $C_{(20:1)}$ and $C_{(22:1)}$ suggested that the specific radioactivity of the carboxy terminal carbons added to oleate by chain elongation was greater than that of the oleoyl portion of the chains. This assumption was confirmed by complete α-oxidation of saturated fatty acids derived from oleate, eicosenoate and erucate of the triacylglycerol (Fig. 11 and Table 12).

TABLE 11. Specific radioactivities of the products of oxidative fission of [^{14}C]-labelled methyl oleate, methyl eicosenoate and methyl erucate.

Methyl oleate, methyl eicosenoate and methyl erucate, prepared by preparative $AgNO_3$ t.l.c. of triacylglycerol extracted from cotyledons which had been labelled with [^{14}C]-acetate for 40 min and 80 min (experiment 2), in the experiment described in Fig. 8, were subjected to oxidative fission across the double bond as described in the Methods section. The oxidation products were dissolved in 10% methanol/diethyl ether (v/v), methylated with diazomethane and the specific radioactivities determined by g.l.c. (using a column fitted with an effluent stream splitter). The oven temperature of the g.l.c. was 140°C and 190°C to obtain monocarboxylic and dicarboxylic fragments respectively.

Specific radioactivity (dpm/ μg)

	Experiment (1)				Experiment (2)			
Fatty acid	Monocarboxylic		Dicarboxylic		Monocarboxylic		Dicarboxylic	
oleate	C_9	104	C_9	86	C_9	92	C_9	106
eicosenoate	C_9	129	C_{11}	529	C_9	106	C_{11}	501
erucate	C_9	107	C_{13}	1177	C_9	141	C_{13}	1093

TABLE 12. Specific radioactivities of the products of α-oxidation of [^{14}C]-labelled $C_{(18:0)}$, $C_{(20:0)}$ and $C_{(22:0)}$

Labelled long chain methyl esters, obtained from preparative $AgNO_3$ t.l.c. of triacylglycerol extracted from cotyledons which had been labelled with [^{14}C]-acetate for 40 min, (in the experiment described in Fig. 8), were hydrogenated, saponified and subjected to α-oxidation by $KMnO_4$ as described in the Methods section. The products were extracted into petroleum ether, methylated with diazomethane and the specific radioactivities determined by g.l.c. (the column was fitted with an effluent stream-splitter). Radioactivity was measured by scintillation counting and masses of the products calculated from the area under the peaks of the g.l.c. trace determined by planimetry. Oxidation products with less than 14 carbon atoms were not analysed.

Substrate for oxidation	Specific radioactivity (dpm/μg) of oxidation fragments of chain length								
	14	15	16	17	18	19	20	21	22
C_{18}	57	64	63	79	69				
C_{20}	49	74	73	103	120	137	371		
C_{22}	54	94	66	189	120	267	551	664	960

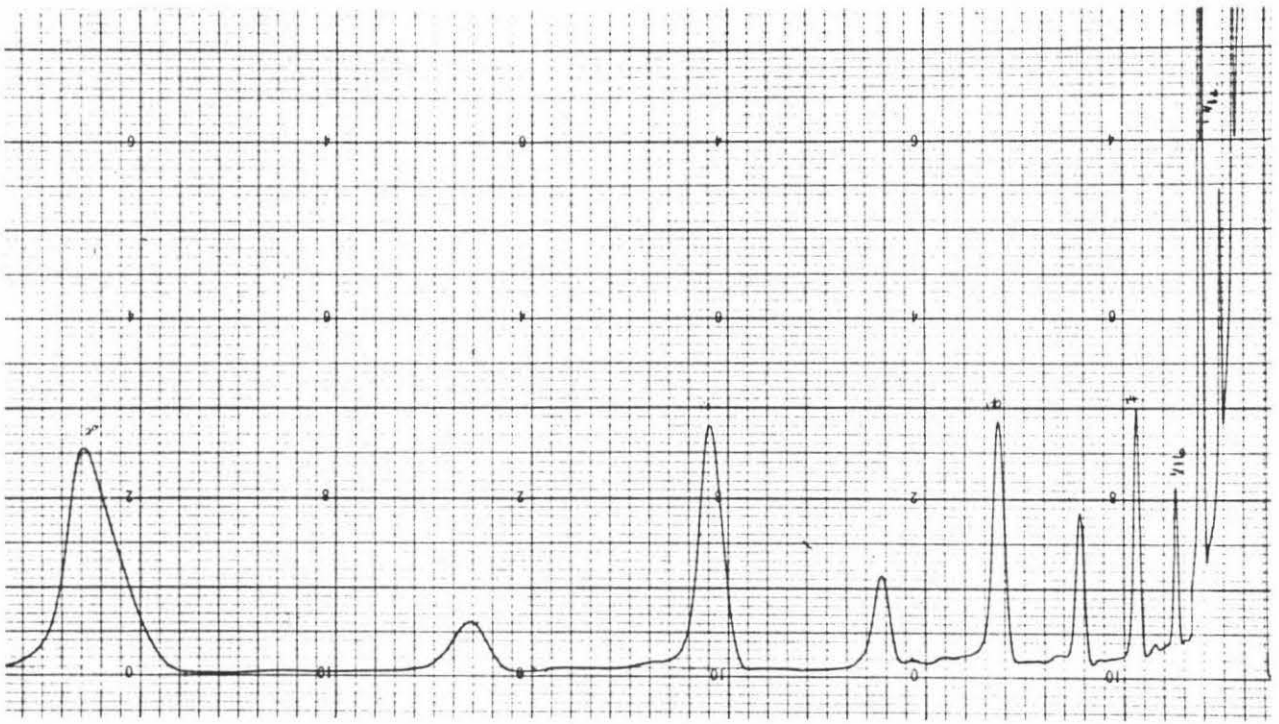


FIG. 11. G.l.c. separation of products of α-oxidation of $C_{(20:0)}$

$C_{(20:0)}$, derived from eicosenoate of triacylglycerol, extracted from rapeseed cotyledons incubated with [^{14}C] acetate for 40 min, was subjected to α-oxidation as described in the Methods section. The products were extracted into petroleum ether, methylated with diazomethane and analysed by g.l.c. at 160°C.

It was also found, as in the studies of Downey and Craig, that the C_9 monocarboxylic fragments derived by oxidative fission from $C_{(18:1)}$, $C_{(20:1)}$ and $C_{(22:1)}$ of the triacylglycerol had very similar specific radioactivities, which suggested that the specific radioactivities of the portions of these acids were probably similar. This was also confirmed by α-oxidation (Table 12).

These observations, on which Downey and Craig failed to comment, have interesting implications with regard to two problems. They provide information on the partitioning of oleate, or a derivative, between incorporation into triacylglycerol and its utilisation in chain elongation prior to incorporation into triacylglycerol. They also raise the possibility that either a large endogenous pool of cold oleoyl CoA exists in the cells of rapeseed cotyledons or alternatively that the specific radioactivity of the acetate used in de novo synthesis, and in chain elongation was different. The significance of these observations will be discussed in more detail in the Discussion section.

III. *In vitro* labelling of rapeseed homogenate and oil body preparations with [^{14}C] malonyl CoA.

General study of rapeseed oil bodies

The cells of expanded rapeseed cotyledons contain large numbers of oil bodies, which are spherical in shape and have a mean diameter of 1.5 μm (Fig. 12a). Suspensions of oil bodies resembling those seen *in vivo*, in size and appearance (Fig. 12b) were isolated from cotyledons by resuspending the floating oil body pellicle, obtained by centrifuging rapeseed cotyledon homogenates upwards through a discontinuous sucrose gradient, as described in the Methods section.

Purified oil body preparations, obtained by repeated resuspension and centrifugation of the floating pellicle, contained approximately 97% lipid and 3% protein (by mass). The lipid component was mainly triacylglycerol, and diacylglycerol and phosphatidyl choline were the other major lipids present (Table 13). The amounts of protein and phospholipid reported to exist in oil bodies from different oil seeds varies considerably (Gurr *et al.*, 1974; Slack *et al.*, 1980). The composition of rapeseed oil bodies was found to be similar to that recently reported for oil bodies from linseed and safflower cotyledons (Slack *et al.*, 1980) and supports the hypothesis (Slack *et al.*, 1980) that diacylglycerol is a constituent of oil bodies.

The fatty acid compositions of these oil body lipids were very similar to those obtained for whole cotyledons, and eicosenoate and erucate were present in all three lipid species of the oil bodies (Table 14).

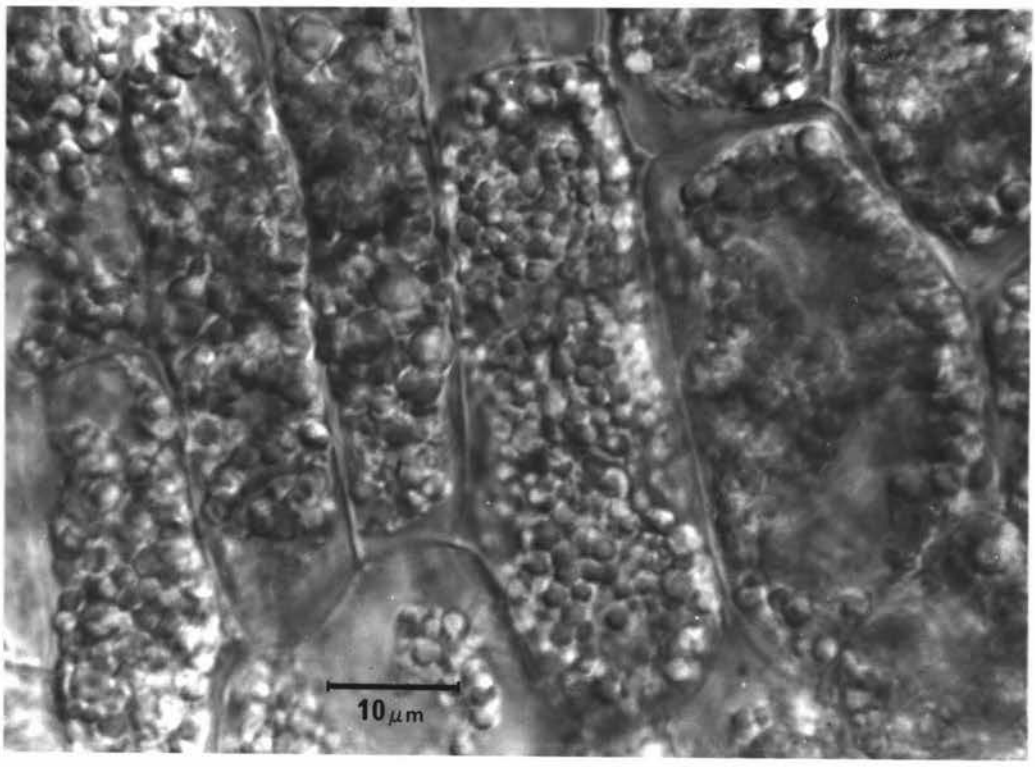


FIG. 12a. Transverse section of a developing rapeseed cotyledon.

Hand-cut sections of developing rapeseed cotyledons were examined by Nomarski light microscopy.

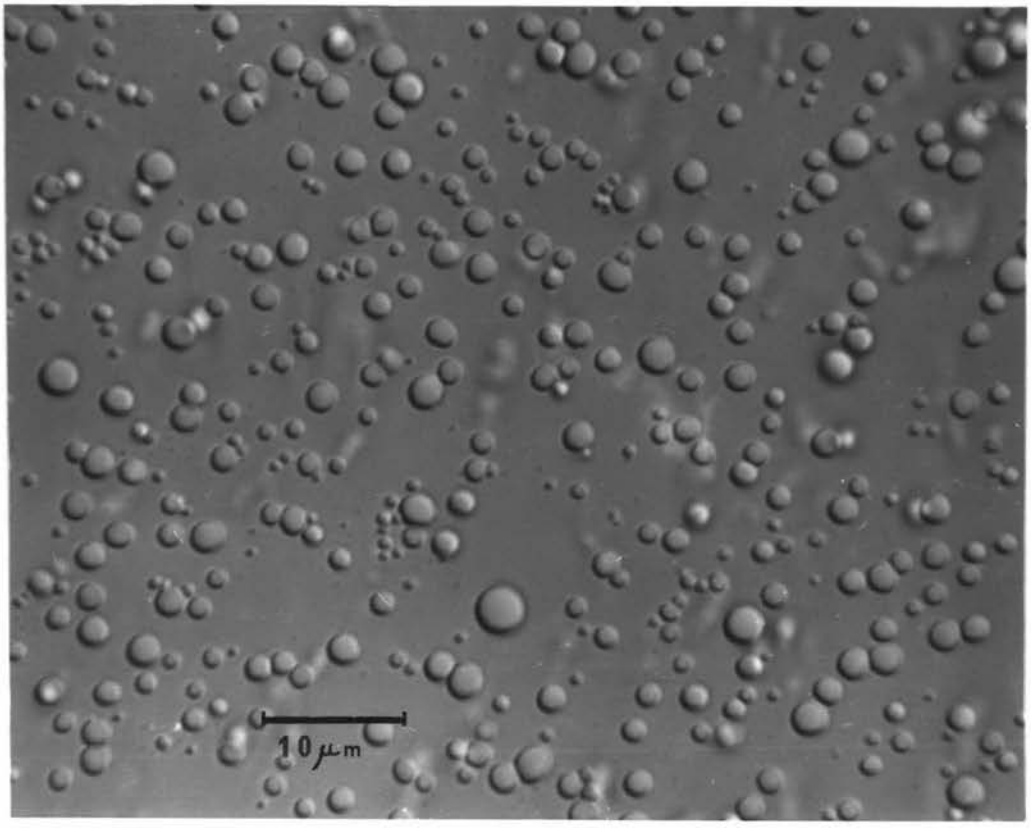


FIG. 12b. Oil body preparation from developing rapeseed cotyledons.

The floating oil body pellicle , washed three times by resuspension and centrifugation upwards through a discontinuous sucrose density gradient, was finally resuspended in buffer as described in Methods section and viewed by Nomarski light microscopy.

TABLE 13. Relative amounts of the major glycerolipids present in isolated rapeseed oil bodies.

In two separate experiments, oil bodies were isolated from rapeseed cotyledons (1 g fresh weight) and washed three times by centrifuging the resuspended oil bodies through a sucrose gradient. After the final wash, lipids were extracted from the oil body preparation into chloroform. Individual lipids were separated by t.l.c. on silica gel G and analysed by g.l.c. as described in the Methods section.

Oil body preparation	Relative amounts of individual glycerolipids (μmol)		
	Phosphatidyl choline	Diacylglycerol	Triacylglycerol
a	16.0	22.2	1000
b	11.4	33.5	1000

TABLE 14. Fatty acid composition of rapeseed oil body lipids

Aliquots of the individual lipid preparations obtained in the experiment described in Table 13, were transmethylated with sodium methoxide, and the fatty acid methyl esters analysed by g.l.c. as described in the Methods section.

Lipid	Relative amounts (mass) of individual fatty acids (%)						
	16:0	18:0	18:1	18:2	18:3	20:1	22:1
Phosphatidyl choline	9	2	42	25	11	6	5
Diacylglycerol	3	1	40	23	7	9	17
Triacylglycerol	3	1	23	11	5	12	45

Incorporation of [2-^{14}C] malonyl CoA by homogenate and oil body preparations

The ability of an oil body fraction, from crambé cotyledons, to incorporate [^{14}C] malonyl CoA into fatty acids, including eicosenoate and erucate, was observed by Gurr *et al.* (1974). Using Gurr's reaction mixture, [^{14}C] malonyl CoA incorporation by both homogenate and oil body preparations from rapeseed cotyledons was obtained (Table 15).

Excising cotyledons from whole rape seeds is a slow process, but the level of incorporation achieved by an oil body fraction prepared from whole seeds was only 15% of that obtained using an oil body fraction isolated from the same number of excised cotyledons (seed minus seed coat). This suggests that the enzymes necessary for [^{14}C] malonyl CoA incorporation were either present in smaller amounts in the oil body fractions isolated from intact seeds, or were inhibited, perhaps by certain seed coat constituents, such as polyphenoloxidases. Consequently for all *in vitro* studies, homogenate and oil body fractions were prepared from rapeseed cotyledons.

[^{14}C] malonyl CoA incorporation was measured initially into saponifiable and non-saponifiable compounds. Most of the radioactivity was incorporated into the saponifiable fraction, and 95% of the label in this fraction was contained in free fatty acids.

The incorporation of radioactivity into the non-saponifiable fraction was low but variable, and appeared to be unrelated to the amount of [^{14}C] malonyl CoA incorporated into the saponifiable compounds. The presence of radioactivity in the non-saponifiable fraction may perhaps reflect the synthesis of sterols

TABLE 15. Incorporation of [2-^{14}C] malonyl CoA into saponifiable and non-saponifiable products by homogenate and oil body preparations obtained from rapeseed cotyledons.

Rapeseed cotyledons (1 g fresh weight) were homogenized, and the cell free homogenate adjusted to 7 ml with buffer. 1 ml was removed and stored on ice (standard homogenate preparation). The remaining 6 ml were made 0.25 M with respect to sucrose, overlaid with buffer, and centrifuged at 20,000 x g for 15 min. The floating oil body pellicle was resuspended in 1 ml of buffer (standard oil body preparation). 0.25 ml of homogenate and oil body preparations were incubated at 25°C for 1 h with 0.25 ml of Gurr's reaction mix: (0.03 mM [^{14}C] malonyl CoA, 2 mM ADP, 0.1 mM NADH, 0.1 mM NADP, 0.1 mM glucose-6-phosphate, 0.8 mM rac glycerol-3-phosphate, 0.5 mM $MnSO_4.4H_2O$, 5 mM $KHCO_3$, 0.5 mM $MgSO_4.7H_2O$). Each reaction was stopped by the addition of 10% KOH in methanol, and the saponifiable and non-saponifiable compounds extracted, and assayed for radioactivity, as described in the Methods section. Results are shown for reactions carried out in duplicate.

Enzyme preparation	Proportion incorporated (%) of the [^{14}C] malonyl CoA supplied.	
	non-saponifiable compounds	saponifiable compounds
Homogenate	1.1	21.5
	3.4	30.3
Oil body	4.0	19.7
	6.5	17.5

or long chain hydrocarbon compounds, but it was not investigated further.

Analysis by $AgNO_3$ t.l.c. revealed a striking difference in the patterns of distribution of incorporated radioactivity among the various fatty acids in the saponifiable fractions extracted from incubation mixtures that had contained either homogenate or oil body preparations (Fig. 13). Over 70% of the [^{14}C] malonyl CoA incorporated by the homogenate was in palmitate, stearate and oleate and only 15% in eicosenoate and erucate. In contrast, approximately 60% of the radioactivity incorporated by the oil body preparation was in eicosenoate and erucate, and less than 10% was located in the saturated fatty acids $C_{(16:0)}$ and $C_{(18:0)}$. Those results suggest that both de novo fatty acid synthesis, and chain elongation to form $C_{(20:1)}$ and $C_{(22:1)}$ occurred in the homogenate and oil body preparations, but that competition between these two processes for [^{14}C] malonyl CoA was different in the two preparations. As the synthesis of labelled eicosenoate and erucate was greatly increased by using an oil body preparation, rather than a homogenate, oil body preparations were mainly used in subsequent experiments to study the elongation system of rapeseed cotyledons in vitro.

Substrate and cofactor requirements for the elongation reactions.

(i) Cofactor requirements.

Although [^{14}C] malonyl CoA had been incorporated into fatty acids by both homogenate and oil body preparations using Gurr's reaction mix, the composition of the reaction mix seemed to be perhaps unnecessarily complex as it contained two metal ions,

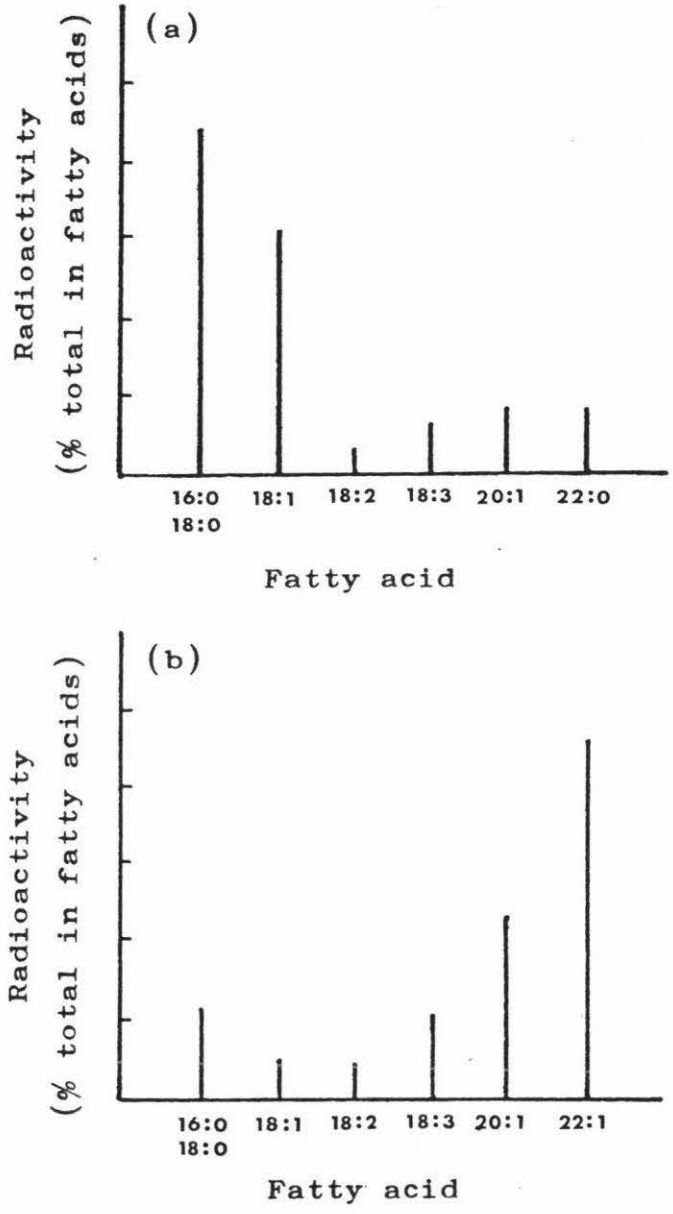


FIG. 13. Synthesis of fatty acids by homogenate and oil body fractions.

Saponifiable material extracted from assays in which (a) homogenate or (b) oil body preparation was incubated with [^{14}C] malonyl CoA, in the experiment outlined in Table 15, was methylated using diazomethane, and the fatty acid methyl esters separated by $AgNO_3$ t.l.c. The required zones were scraped into scintillation vials and counted, as described in the Methods section.

Mn^{++} and Mg^{++}, two non-reactants $KHCO_3$ and glycerol-3-phosphate, in addition to ADP, a mixture of oxidized and reduced pyridine nucleotides and glucose-6-phosphate. Consequently the effect of omitting each of these constituents on the extent of [^{14}C] malonyl CoA incorporation, by homogenate and oil body preparations, was investigated (Table 16a). Neither HCO_3^-, Mg^{++}, Mn^{++} or glycerol-3-phosphate was necessary for optimal incorporation and were not included (with the exception of glycerol-3-phosphate) in subsequent reaction mixtures. ATP, however, appeared to be essential for high levels of incorporation (although some incorporation did occur in the absence of ATP), and of the reductants supplied, NADPH was the most effective (Table 16b). Increasing the concentrations of both ATP and NADPH two-fold, did not lead to an increase in [^{14}C] malonyl CoA incorporation (Table 16b).

(ii) Substrate requirements

It has previously been shown that malonyl CoA, and not acetyl CoA, is the C_2 unit involved in the elongation reactions during synthesis of eicosenoate and erucate in developing oil-seed cotyledons (Gurr et al., 1973; Appelqvist, 1973). The identities of the other substrates, the C_{18} and C_{20} compounds which react with malonyl CoA, are unknown, but by analogy with both the mechanisms of fatty acid synthesis de novo and the malonate-dependent elongation system in mammals (Hitchcock and Nichols, 1971 and p 10), the hypothesis that they are also the long chain acyl CoA species, oleoyl- and eicosenoyl CoA, is a very likely one.

In all the above experiments, approximately 50% of the [^{14}C] malonyl CoA supplied was incorporated by oil body preparations, and most of the radioactivity

TABLE 16a. Effect of omitting various constituents from the reaction mixture on [^{14}C] malonyl CoA incorporation by oil body preparations.

Oil body preparations were incubated with 0.25 ml of reaction mixture for 1 h at 25°C. In experiment (1), the complete reaction mixture was Gurr's reaction mixture (see Table (15) plus 0.1 mM NADPH. In experiment (2), the reaction mix was as described for experiment (1), but no Mn^{++} or glycerol-3-phosphate was included. The reaction was stopped with 10% KOH in methanol and the saponifiable fraction assayed for radioactivity.

	Reaction mix	Incorporation of [^{14}C] malonyl CoA into fatty acids. (dpm)	(%)
(1)	complete	47775	41
	minus Mn^{++}	52165	45
	minus glycerol-3-phosphate	47700	41
(2)	complete	57175	49
	minus ATP	18340	16
	minus $KHCO_3$	53090	45
	minus Mg^{++}	58570	50

TABLE 16b. The effect of omitting NADH, NADPH, or NADP plus glucose-6-phosphate from the reaction mixture, on [^{14}C] malonyl CoA incorporation by an oil body preparation.

0.25 ml of an oil body preparation were incubated for 1 h at 25°C with 0.25 ml of a reaction mixture which in experiment (a) was the same as described for experiment (1) in Table 16a, and in experiment (b), contained 0.02 mM [^{14}C] malonyl CoA and 2 mM ATP. The concentration of all additional constituents was 0.1 mM unless otherwise stated. The reaction was stopped with 10% KOH in methanol and incorporation into the saponifiable fraction determined.

	Reaction mix	Incorporation of [^{14}C] malonyl CoA into fatty acids. (dpm)	(%)
(a)	complete	57175	49
	minus NADP, glucose-6-phosphate	63675	54
	minus NADH	63995	55
	minus NADPH	40360	34
(b)	complete plus NADP, glucose-6-phosphate, NADH and NADPH	49200	42
	plus NADP, glucose-6-phosphate	27605	24
	plus NADH	11075	9
	plus NADPH	46570	40
	plus 0.2 mM NADPH	44645	38
	plus NADPH, and 2 mM ATP	44525	38

was found in eicosenoate and erucate, although no exogenous oleoyl- or eicosenoyl CoA were supplied.

The inclusion of these compounds in reaction mixtures did not stimulate incorporation of malonyl CoA by oil body preparations capable of a high rate of eicosenoate and erucate formation (Table 17). It was noteworthy however, that exogenous acyl CoAs had a pronounced effect on the proportion of radioactive malonyl CoA incorporated into particular fatty acids. In the absence of added acyl CoA, 85% of the radioactivity was located in eicosenoate and erucate and was distributed almost equally between them. On addition of oleoyl CoA to the reaction mix, 75% of the incorporated radioactivity was located in eicosenoate, and conversely, on addition of eicosenoyl CoA, 67% of the radioactivity incorporated was found in erucate (Fig. 14). A similar effect of the addition of the acyl CoAs, on the relative amount of the two long chain fatty acids formed was also observed with homogenate preparations. The presence of exogenous oleoyl and eicosenoyl CoA however had not changed the extent to which elongation occurred relative to _de novo_ synthesis, suggesting that elongation in homogenate preparations is not limited by the presence of only small amounts of these long chain acyl CoAs.

The ability of oleoyl and eicosenoyl CoA to alter the proportions of the elongation products synthesized by homogenate and oil body preparations, provides evidence that these long chain acyl CoAs are involved in the elongation reactions _in vitro_. However, as no stimulation of incorporation by a washed oil body preparation occurred when these compounds were added to the reaction mixture, the endogenous concentrations

TABLE 17. Incorporation of [^{14}C] malonyl CoA into, and distribution of radioactivity among, fatty acids of homogenate and oil body preparations obtained from rapeseed cotyledons.

Homogenate and oil body preparations were incubated with Gurr's reaction mix plus 0.1 mM NADPH for 1 h at 25°C. When added, the concentrations of CoA and long chain acyl CoAs were 1 mM and 0.10 mM respectively. The reactions were stopped with 10% KOH in methanol, and samples of the saponifiable fractions assayed for radioactivity. Aliquots were also methylated with diazomethane and the individual fatty acids separated by $AgNO_3$ t.l.c. and analysed as described in Methods section.

Enzyme preparation	Additions to Reaction mix	Incorporation into fatty acids (dpm)	Distribution of radioactivity among fatty acids (%)					
			16:0,18:0	18:1	18:2	18:3	20:1	22:1
Homogenate	-	49525	54	1	1	2	14	28
Homogenate	C(18:1)CoA	44255	62	2	-	1	24	11
Homogenate	C(20:1)CoA	45335	64	3	-	1	9	23
Oil body	-	69415	7	3	4	1	45	40
Oil body	CoA	62125	9	2	2	1	62	24
Oil body	$C_{(18:1)}$CoA	67895	5	4	1	-	75	15
Oil body	$C_{(20:1)}$CoA	57220	5	2	2	1	23	67
Oil body	$C_{(18:1)}$CoA+CoA	52365	5	3	1	1	76	14
Oil body	$C_{(20:1)}$CoA+CoA	51575	6	3	1	2	42	46

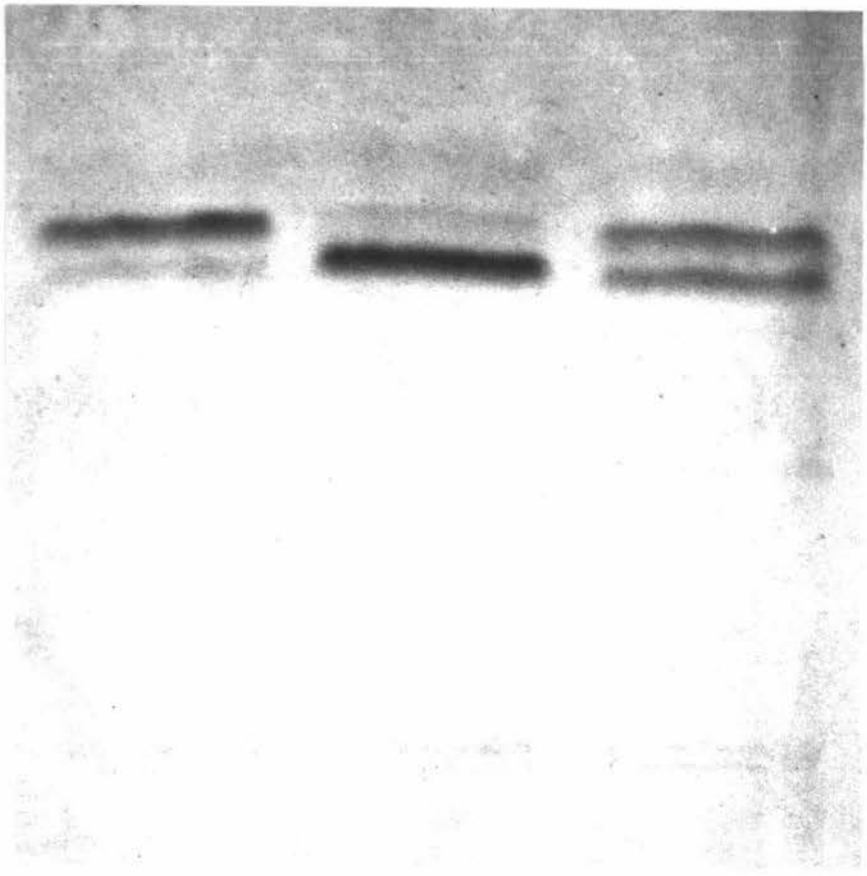


FIG. 14. Autoradiograph of $AgNO_3$ t.l.c. separation of fatty acids labelled during incubation of oil body preparations with [^{14}C] malonyl CoA when oleoyl or eicosenoyl CoA was included in the reaction mixture.

Samples of the saponifiable fractions obtained in the experiment described in Table 18, were methylated with diazomethane and separated into individual fatty acid methyl esters by $AgNO_3$ t.l.c. as described in the Methods section. The t.l.c. plate was then autoradiographed. Eicosenoate and erucate were the only fatty acids which were heavily labelled; the relative positions of the other fatty acids were indicated by spraying the $AgNO_3$ plate with dichlorofluorescein. (a), 0.1 mM eicosenoyl CoA added; (b) 0.1 mM oleoyl CoA added; (c) no acyl CoAs added to the reaction mix.

of oleoyl and eicosenoyl CoA in this *in vitro* system, do not appear to have been rate-limiting. This implies that either the long chain acyl CoAs were adsorbed to the oil body preparations, or that they were synthesised during the assays; these possibilities will be discussed in more detail in the Discussion section.

Inhibition of [^{14}C] malonyl CoA incorporation by long chain acyl CoAs.

Addition of oleoyl CoA at concentrations greater than 0.025 mM to assays containing oil body preparations of half the standard concentration (see p 18), markedly reduced the incorporation of radioactivity into chloroform soluble compounds (Table 18). This inhibitory effect was not due to a change in pH as a consequence of the acetate buffer in which the CoA ester was dissolved, since a final concentration of 8 mM acetate buffer, the highest concentration used, had no effect on incorporation. It was thought that this inhibition might be due to the detergent properties of long chain acyl CoA species (see p 104).

TABLE 18. Inhibition of [^{14}C] malonyl CoA incorporation by oil body preparations by oleoyl CoA.

An oil body pellicle was isolated from rapeseed cotyledons (1 g fresh weight) and resuspended in 2 ml of buffer (i.e. the oil body preparation concentration was half that of standard oil body preparation). Incubation mixtures were set up containing 0.25 ml of oil body preparation and 0.25 ml of reaction mix (0.033 mM [^{14}C] malonyl CoA, 2 mM ATP, 0.1 mM NADPH, 0.8 mM glycerol-3-phosphate and oleoyl CoA at the concentrations shown), incubated at 25°C, and the reactions stopped, at the times shown, with 10 ml chloroform/methanol (1:1 v/v). Oleoyl CoA was dissolved in 50 mM sodium acetate buffer pH 4.5 and for the maximum concentration of oleoyl CoA added, this was equivalent to 8 mM acetate buffer in the total reaction mix. Radioactivity into chloroform-soluble products was determined as described in Methods section.

Concentration of oleoyl CoA added to reaction mix (mM)	Incorporation of [^{14}C] malonyl CoA into chloroform soluble compounds (%)	
	15 min	75 min
0	18	37
0, 8 mM acetate buffer (pH 4.5)	19	39
0.025	16	38
0.1	2	14
0.4	0.4	1

Limitations to the incorporation of [^{14}C] malonyl CoA into fatty acids in vitro

In the experiments described so far, incorporation of [^{14}C] malonyl CoA into fatty acids by oil body preparations after a 1 h incubation period, did not exceed 50% of that supplied, even when oleoyl CoA and eicosenoyl CoA were added to the reaction mix. Consequently it was of interest to try and determine why the utilisation of malonyl CoA was incomplete. Time course assays of malonyl CoA incorporation by both homogenate and oil body preparations showed that incorporation was essentially complete after approximately 20 min, even when long chain acyl CoA was supplied (Fig. 15). It has already been concluded that the amounts of long chain acyl CoA in the oil body preparations are not rate-limiting. There are however, several other possible explanations for the cessation of incorporation:

(a) Competition between [^{14}C] malonyl CoA incorporation into free fatty acids and hydrolysis of [^{14}C] malonyl CoA by a hydrolase during the assays.
(b) Instability of the [^{14}C] malonyl CoA metabolising enzymes.
(c) Accumulation of an inhibitor.

To check out the possibility that incorporation might be limited by the availability of [^{14}C] malonyl CoA, the concentration of this substrate in the reaction mix was varied (Table 19). The proportion of malonyl CoA incorporated into fatty acids was constant over about a 3-fold concentration range from 0.013 to 0.033 mM, suggesting perhaps that malonyl CoA was being utilised by reactions other than fatty acid labelling. Further support for this hypothesis was

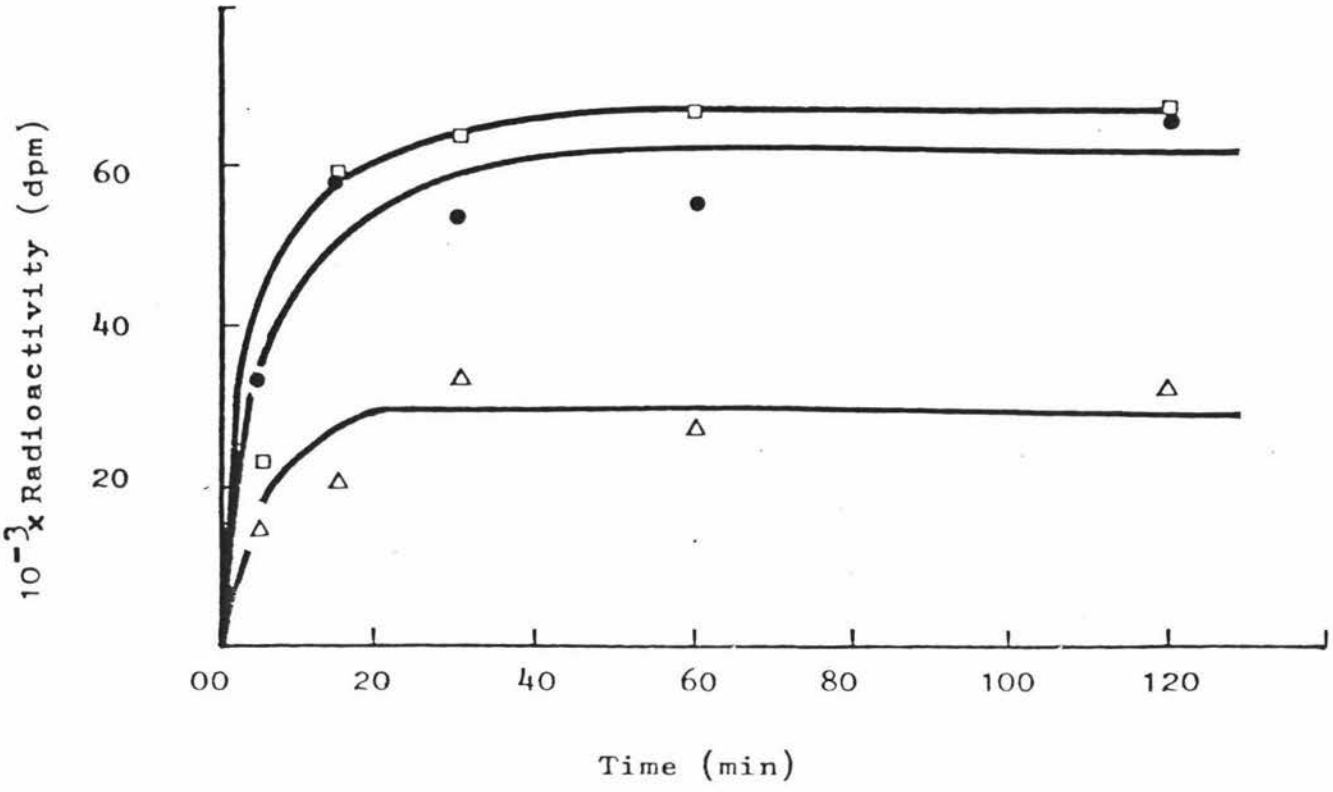


FIG. 15. Time course of [^{14}C] malonyl CoA incorporation by homogenate and oil body preparations.

Homogenate and oil body fractions were prepared from cotyledons as described in Methods section. 0.25 ml of homogenate or oil body preparation was incubated at 25°C with 0.25 ml of a reaction mix containing 0.02 mM [^{14}C] malonyl CoA, 2 mM ATP, 0.1 mM NADPH, 0.8 mM glycerol-3-phosphate for the time periods shown. Each reaction was stopped with 10% KOH in methanol and the saponifiable fraction assayed for radioactivity.
•, oil body preparation; , oil body preparation + 0.1 mM oleoyl CoA; Δ, homogenate preparation.

TABLE 19. Effect of varying [^{14}C] malonyl CoA concentration on incorporation of [^{14}C] malonyl CoA by oil body preparations.

0.25 ml oil body preparations were incubated for 1 h at 25°C with 0.25 ml of a reaction mix containing [^{14}C] malonyl CoA (concentrations shown below), 2 mM ATP, 0.1 mM NADPH and 0.8 mM glycerol-3-phosphate. The reaction was stopped with 10% KOH in methanol and radioactivity incorporated into the saponifiable fraction determined.

	[^{14}C] malonyl CoA concentration (mM)	Incorporation of [^{14}C] malonyl CoA into fatty acids	
		dpm	% (of [^{14}C] malonyl CoA supplied)
Experiment 1	0.033	82912	46
	0.013	32128	45
Experiment 2	0.033	71248	39
	0.066	67714	19

obtained, when it was found that if additional [^{14}C] malonyl CoA was added to a standard *in vitro* incubation, which had proceeded for 40 min (by which time incorporation would have ceased), further incorporation occurred (Fig. 16), (although an increase in the initial malonyl CoA concentration, from 0.033 to 0.066 mM did not increase the amount of radioactivity incorporated). However, when the radioactive compounds in six incubation mixtures, which had incorporated approximately 40% of the [^{14}C] malonyl CoA into fatty acids after 1 h, were analysed by the filter paper disc method[+] of Roughan and Slack (1976), it was found that approximately 40% of the radioactivity initially supplied, remained adhered to the disc, suggesting that most of the malonyl CoA not incorporated into fatty acids, had remained as malonyl CoA in the assays. Furthermore, when an extra 0.125 ml of oil body preparation was added to an incubation mixture which had reacted for 40 min, further incorporation occurred and overall, 65% of the radioactivity originally supplied, was incorporated (Fig. 16). These observations suggest, contrary to the previous experiments, that the concentration of [^{14}C] malonyl CoA in the reaction mix was not rate-limiting.

At present I can offer no explanation for these apparently conflicting observations, however it is possible that the balance of [^{14}C] malonyl CoA, long chain acyl CoA and active [^{14}C] malonyl CoA incorporating enzymes, is important in determining the level of incorporation by oil body preparations.

+ A sample of the mixture was spotted onto a filter paper disc, which was then immersed in 0.25% (w/v) trichloroacetic acid in ethanol/diethyl ether (1:4, v/v). This washing was repeated, and after a final wash in diethyl ether the disc was dried and assayed for radioactivity.

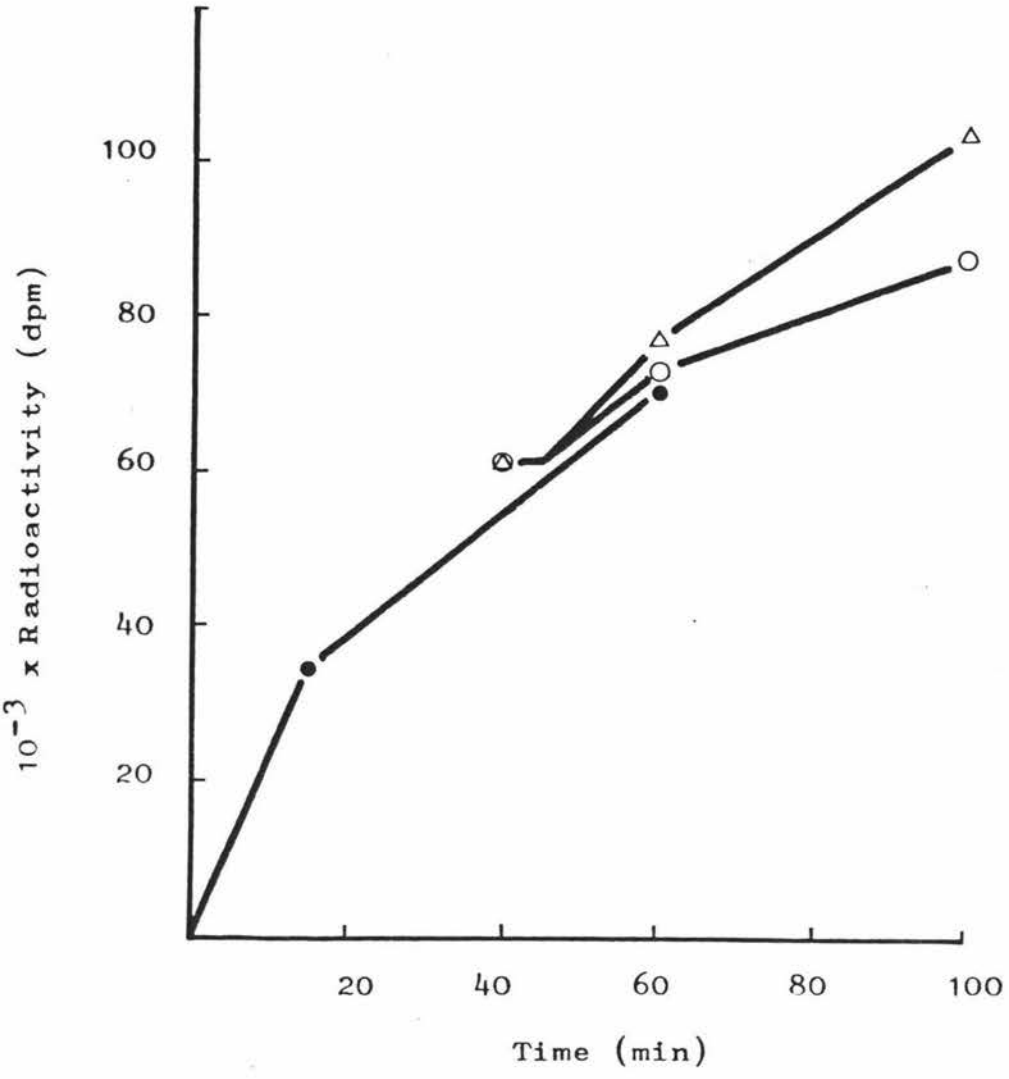


FIG. 16. Effect on [^{14}C] malonyl CoA incorporation by oil body preparations of supplying either additional [^{14}C] malonyl CoA or oil body preparation to assays during the incubation.

8 individual reactions were set up containing 0.25 ml of standard oil body preparation and 0.25 ml of a reaction mixture (2 mM ATP, 0.1 mM NADPH, 0.8 mM glycerol-3-phosphate and 0.016 μmoles of [^{14}C] malonyl CoA), incubated at 25°C, and at the times shown, the reactions were stopped with 10 ml chloroform/methanol (1:1 v/v). At 45 min a further 0.016 μmoles of malonyl CoA was added to 2 assays and 0.125 ml of oil body preparation to two other incubations. After a further 15 min and 40 min these reactions were stopped as above. Radioactivity incorporated into chloroform-soluble products was determined as described in the Methods section. •, standard *in vitro* system; o, additional [^{14}C] malonyl CoA; Δ, additional oil body preparation.

Effect of reduced sulphydryls on enzyme activity and stability.

In a series of early experiments, it was found that although the homogenate preparations incorporated [^{14}C] malonyl CoA into fatty acids in acceptable amounts, oil body preparations did not. It was subsequently discovered that malonyl CoA incorporation into fatty acids by oil body preparations, but not by homogenates, required the presence of freshly-prepared dithiothreitol (DTT) in both the buffer used for homogenisation of the cotyledons, and that in which the oil bodies were resuspended (Table 20). DTT was used in the early experiments in which incorporation by oil body preparations was very low, but it was taken from a stock solution, frozen between experiments. Since the ineffectiveness of this solution may have been due to partially oxidised sulphydryl reagent, fresh dithiothreitol was prepared before every *in vitro* experiment. Most of the malonyl CoA is incorporated into eicosenoate and erucate by oil body preparations but is utilized mainly for *de novo* fatty acid synthesis by the homogenate (p 60). It was of interest therefore, to determine whether the 30% reduction in malonyl CoA incorporation by homogenates without DTT was due to a selective inhibition of chain elongation in these preparations. However, this was not found to be the case, as the proportion of radioactivity in eicosenoate and erucate was in fact greater when DTT was not present in the homogenate preparation (Table 21), suggesting perhaps that the chain elongating enzymes may be more protected from oxidation in the homogenate preparation than in oil body preparations. It was

TABLE 20. Effect of omitting dithiothreitol from the buffer used both for homogenisation of cotyledons, and for resuspension of the oil body pellicle, on incorporation of [^{14}C] malonyl CoA by homogenate and oil body preparations.

Two buffers were prepared: the complete buffer contained 100 mM Hepes pH 7.8, 1 mM EGTA, 1 mM EDTA and 2 mM DTT and another buffer containing no DTT. Homogenate and oil body fractions were prepared using either the complete buffer or the buffer minus DTT. 0.25 ml of homogenate or oil body preparation were incubated with 0.25 ml of Gurr's reaction mix (0.03 mM [^{14}C] malonyl CoA) plus 0.1 mM NADPH for 1 h at 25°C. The reaction was stopped with 10% KOH in methanol, and the saponifiable fraction assayed for radioactivity.

Buffer	Enzyme preparation	Incorporation of [^{14}C] malonyl CoA into fatty acids (dpm)
Complete	Homogenate	29035
	Oil body	30445
Minus DTT	Homogenate	20570
	Oil body	1990

TABLE 21. Effect of omitting dithiothreitol from the buffer used for preparation of homogenate fractions, on the distribution of radioactivity among the fatty acids after incubation of homogenates with [^{14}C] malonyl CoA.

Samples of the saponifiable fractions extracted from those reactions containing homogenate preparations in the experiment described in Table 20, were methylated with diazomethane, the fatty acid methyl esters separated by $AgNO_3$ t.l.c. and analysed for radioactivity as described in the Methods section.

Buffer	Percentage distribution of radioactivity in fatty acids					
	16:0, 18:0	18:1	18:2	18:3	20:1	22:1
Complete	42	40	1	1	5	11
Minus DTT	40	12	1	3	21	23

noted that the proportion of radioactivity in oleate decreased when DTT was absent but the significance of this observation was not investigated.

As has been reported above (p 65), the high rates of malonyl CoA incorporation into eicosenoate and erucate by oil body fractions prepared with fresh dithiolthreitol, were not stimulated by the inclusion of oleoyl CoA or eicosenoyl CoA to assay mixtures. In the course of studies with oil body preparations made with aged DTT, that had an inherently low ability to incorporate malonyl CoA into long chain fatty acids, the interesting observation was made that malonyl CoA incorporation was stimulated by exogenously supplied long chain acyl CoA (Table 22). The significance of this observation with respect to the DTT-dependent oil body enzymes and the origin of long chain acyl CoAs in oil body preparations with high chain elongating activity, will be discussed in more detail later (p 103).

Both the initial rate and extent of malonyl CoA incorporation by oil body preparations was found to be increased by increasing the dithiothreitol concentration from 2 to 10 mM (Fig. 17). High rates of incorporation were obtained when 10 mM DTT was present either only in the homogenisation buffer, or only in the resuspension buffer, but the greatest incorporation was obtained when DTT was present at a concentration of 10 mM in both buffers (Table 23).

The ability of an oil body preparation to incorporate [^{14}C] malonyl CoA decreased with the 'age' of the oil body preparation. An oil body preparation which had been left to stand on ice (aged) for 40 min before being incubated with a reaction mix, produced both a slower initial rate of [^{14}C] malonyl CoA incorporation, and a lower final incorporation after 1 h

TABLE 22. Effect of addition of varying concentrations of $C_{(20:1)}$ CoA on [^{14}C] malonyl CoA incorporation by homogenate and oil body fractions, prepared using a buffer which contained ineffective dithiothreitol.

0.25 ml oil body and homogenate preparations were incubated with 0.25 ml Gurr's reaction mix (0.03 mM [^{14}C] malonyl CoA) for 1 hour at 25°C. Eicosenoyl CoA was added to the reaction mix at the concentrations shown. The reaction was stopped with 10% KOH in methanol and the saponifiable fraction assayed for radioactivity.

Enzyme preparation.	Concentration of eicosenoyl CoA added (mM)	Incorporation of [^{14}C] malonyl CoA into fatty acids (dpm)
Homogenate	-	57859
	0.001	57838
Oil body	-	200
	0.001	2447
	0.01	6301
	0.1	15474

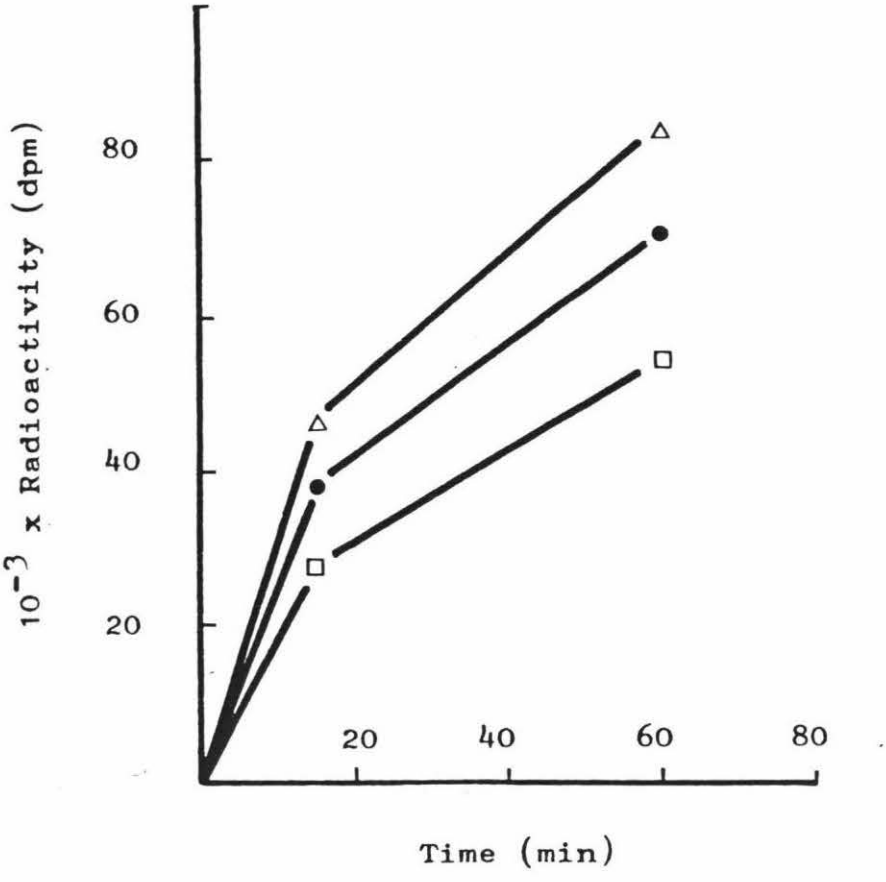


FIG. 17. Effect of increasing the dithiothreitol (DTT) concentration in the buffers used for preparation of an oil body fraction, and the effect of ageing, on incorporation of [^{14}C] malonyl CoA by oil body preparations.

Standard oil body fractions were prepared using buffers that contained 100 mM Hepes pH 7.8, 1 mM EDTA, 1 mM EGTA., and either 2 mM or 10 mM dithiothreitol. 0.25 ml of both oil body preparations were incubated with a reaction mix which contained 0.033 mM [^{14}C] malonyl CoA, 2 mM ATP, 0.1 mM NADPH, 0.8 mM glycerol-3-phosphate in a total reaction volume of 0.5 ml. In addition, the oil body fraction prepared using the 2 mM DTT buffer was left to stand on ice for 40 min, and then incubated for 1 h as above. The reactions were stopped with 10 ml $CHCl_3$/MeOH (1:1 v/v) and radioactivity into chloroform-soluble products determined as described in the Methods section. •, oil body preparation (2 mM DTT); Δ, oil body preparation (10 mM DTT); □, oil body preparation (2 mM DTT) aged for 40 min.

TABLE 23. Effect of omitting or changing the concentration of dithiothreitol in the homogenisation and resuspension buffers used during the preparation of oil body fractions, on the incorporation of [^{14}C] malonyl CoA by oil body preparations.

Homogenisation buffers containing 100 mM Hepes (pH 7.8), 1 mM EDTA, 1 mM EGTA, and either 0, 2, or 10 mM dithiothreitol were used to homogenise batches of cotyledons (0.5 g fresh weight) from which oil body pellicles were isolated as previously described in Methods section. The pellicles were resuspended in 1 ml of one of three resuspension buffers (composition identical to the homogenisation buffers above). These oil body preparations were incubated with [^{14}C] malonyl CoA and the products analysed as described in Fig. 17. The final DTT concentrations in the assay mixtures were 1 and 5 mM for assays containing oil bodies suspended in buffer containing 2 and 10 mM DTT respectively.

Concentration of DTT (mM)		Incorporation of [^{14}C] malonyl CoA into chloroform-soluble products(dpm)	% control
Homogenisation buffer	Resuspension buffer		
2	2	59920	1
10	10	83769	140
10	0	65854	110
0	10	69506	116
0	0	9805	

(Fig. 17). It was necessary in certain experiments to use oil body preparations that had been 'washed', by spinning them up through sucrose density gradients several times. As this washing procedure was lengthy, it was important to determine how the loss of [^{14}C] malonyl CoA incorporation activity with age of the oil body preparation could be minimized. It was found that the presence of high levels of dithiothreitol in both the homogenisation and resuspension buffers reduced the rate of loss of enzyme activity with ageing. After standing on ice for 6 h, an oil body fraction which had been prepared using buffers which contained 10 mM DTT, retained 91% of its ability to incorporate [^{14}C] malonyl CoA. Only 59% of the initial incorporating ability was retained if buffers containing only 2 mM DTT were used.

The detrimental effect of ageing on the ability of an oil body preparation resuspended in a buffer containing 2 mM DTT to incorporate [^{14}C] malonyl CoA appeared to be reversed if dithiothreitol was added so that it was present at 5 mM in the final incubation mixture (Table 24), implying that the effect of DTT on the enzymes was a rapid one. However for maximum retention of enzyme activity, it was concluded that high concentrations of DTT should be present both during the ageing period of the preparation, and the incorporation reactions.

TABLE 24. Effect of increasing the dithiothreitol concentration in oil body preparations that have been aged for 6 h in buffers containing low DTT concentrations, on incorporation of [^{14}C] malonyl CoA.

Oil body pellicles were prepared (from 0.5 g fresh weight of cotyledons), resuspended in 1 ml of resuspension buffers containing 100 mM Hepes pH 7.8, 1 mM EDTA, 1 mM EGTA and either 2 mM or 10 mM dithiothreitol, and either incubated immediately with a reaction mix containing 0.033 mM [^{14}C] malonyl CoA, 2 mM ATP, 0.1 mM NADPH and 0.8 mM glycerol-3-phosphate in a total volume of 0.5 ml, for 15 mins at 25°C or left to stand on ice for 6 h before incubation with [^{14}C] malonyl CoA. In one treatment, the DTT concentration was increased from 2 to 10 mM at the end of the ageing period. The reaction was stopped with 10 ml $CHCl_3$/MeOH (1:1 v/v) and incorporation into chloroform-soluble products determined as described in the Methods section.

Concentration of DTT (mM) in resuspension buffer and 6 h ageing period	Final concentration of DTT in incubation mix (mM)	[^{14}C] malonyl CoA incorporation into chloroform-soluble products (dpm)
2 mM (not aged)	1	41880
2	1	26685
2	5	34635
10	5	33165

Products of chain elongation and their incorporation into lipids.

If as the above results suggest, oleoyl and eicosenoyl CoAs are substrates for chain elongation, then by analogy with the malonate-dependent mammalian microsome system, eicosenoyl and erucyl CoAs would be expected to be the products of the reactions. To identify the products of chain elongation in oil body preparations, reactions were stopped and products extracted by the method of Bligh and Dyer (1959). By this procedure glycerolipids and fatty acids were partitioned into the chloroform phase and malonyl CoA, malonate and long chain acyl CoAs into the aqueous phase (Baker and Lynen, 1971; Stymne and Appelqvist, 1978).

In assays containing no added long chain acyl CoAs, most of the radioactive products were invariably chloroform soluble. Between 1 and 6% of the radioactivity supplied that was soluble in the aqueous methanol phase, was extracted into petroleum ether after saponification and acidification, suggesting that this small proportion of the radioactivity was in long chain acyl CoAs. However at high concentration of added oleoyl CoA about 50% of the radioactive products were present as long chain acyl CoAs (Table 25). It is noteworthy that in the assay containing 0.1 mM oleoyl CoA and therefore 0.05 μmoles of this compound, that about 0.004 μmoles of long chain acyl CoAs were formed over a 75 min incubation. Presumably if the added oleoyl CoA competed with the incorporation of radioactive long chain acyl CoA into glycerolipid then the observed accumulation of long chain acyl CoAs might be expected.

Analysis of the distribution of radioactivity among the chloroform-soluble products (Table 26) showed that

TABLE 25. Effect on the distribution of radioactivity between chloroform-soluble products and acyl CoA species, of the addition of oleoyl CoA to the reaction mix when an oil body preparation was incubated with [^{14}C] malonyl CoA.

The chloroform, and aqueous methanol fractions extracted from incubation mixtures in the experiment described in Table 18, were assayed for radioactivity as described in the Methods section.

Concentration of oleoyl CoA added (mM)	[^{14}C] malonyl CoA incorporation % (of that supplied) into	
	chloroform-soluble products	acyl CoA species
0	37.2	0.4
0.025	38.4	2.4
0.1	14.0	14.2
0.4	1.4	1.8

TABLE 26. Distribution of label among chloroform soluble products and the effect of concentration of the oil body preparation, or incubation time of [^{14}C] malonyl CoA with the oil body preparation, on this distribution.

Oil body pellicles were isolated from cotyledons (1 g fresh weight) and resuspended in 1 or 2 ml of buffer producing concentrations of [OB] or $\frac{[OB]}{2}$ as explained in the Methods section. 0.25 ml of oil body preparation was incubated with 0.25 ml of a reaction mix containing 0.033 mM [^{14}C] malonyl CoA, 2 mM ATP, 0.1 mM NADPH, 0.8 mM glycerol-3-phosphate for 15 and 75 min at 25°C. The reactions were stopped by addition of chloroform/methanol (1:1 v/v). The chloroform soluble products were separated by t.l.c. on silica gel G, and the lipid containing zones scraped into vials and assayed for radioactivity as described in the Methods section.

Concentration of oil body preparation	Distribution (%) of incorporated radioactivity after 15 min (in parentheses) and 75 min.			
	Origin	Diacylglycerol	Free fatty acid	Triacyl-glycerol
[OB]	(35) 32	(3) 3	(7) 11	(55) 54
$\frac{[OB]}{2}$	(26) 31	(3) 4	(5) 12	(66) 53

more than 50% of the radioactivity was present in triacylglycerol, between 30-40% in a compound which remained at the origin, subsequently identified as phosphatidic acid by 2-dimensional t.l.c., and the remainder in free fatty acids and diacylglycerol. Reducing the concentration of the oil body preparation or increasing the incubation period with [^{14}C] malonyl CoA did not change this distribution (Table 26).

Synthesis of labelled triacylglycerol by oil body preparations could occur "de novo" via the classical Kennedy pathway (Hitchcock and Nichols, 1971) since the reaction mix contained glycerol-3-phosphate. The observation that a high proportion of label was found in phosphatidic acid supports this hypothesis. However labelled triacylglycerol could also be synthesized by the esterification of long chain acyl CoAs to pre-existing diacylglycerol.

Subcellular location of the elongation system.

Large scale accumulation of triacylglycerol occurs in the cotyledons of developing rapeseed, and although 60% of the fatty acids present in rapeseed oil are the "elongated" fatty acids, eicosenoate and erucate, it is not known where in the cell, the massive synthesis of these unusual fatty acids takes place.

Oil body preparations from rapeseed, crambé (Gurr et al., 1974) and jojoba wax pad (Pollard et al., 1979) incorporate [^{14}C] malonyl CoA predominantly into the products of chain elongation, eicosenoate and erucate. Pollard et al. (1979) suggested that the jojoba wax pad contained not only oil bodies, but was contaminated by membrane material. They therefore proposed, in analogy with the location of malonate-dependent elongation in mammalian tissues (Hitchcock and Nichols, 1971; p.10), that elongation actually occurred on the endoplasmic reticulum. In contrast, by isolating various subcellular fractions from crambé cotyledons and determining the radioactivity incorporated into eicosenoate and erucate when these fractions were incubated with [^{14}C] malonyl CoA, Gurr et al. (1974) concluded that elongation was associated with the oil bodies and that the microsomal fraction did not incorporate [^{14}C] malonyl CoA into $C_{(20:1)}$ and $C_{(22:1)}$.

In order to express elongation in homogenate and oil body preparations, on the basis of the amounts of oil bodies present in each preparation, incorporation was expressed per μg triacylglycerol; the only means presently available to estimate oil body concentration. On this basis, an oil body preparation incorporated [^{14}C] malonyl CoA into $C_{(20:1)}$ and $C_{(22:1)}$ to the same extent as a total homogenate, as the incubation period was varied (Fig. 18). This observation supports the

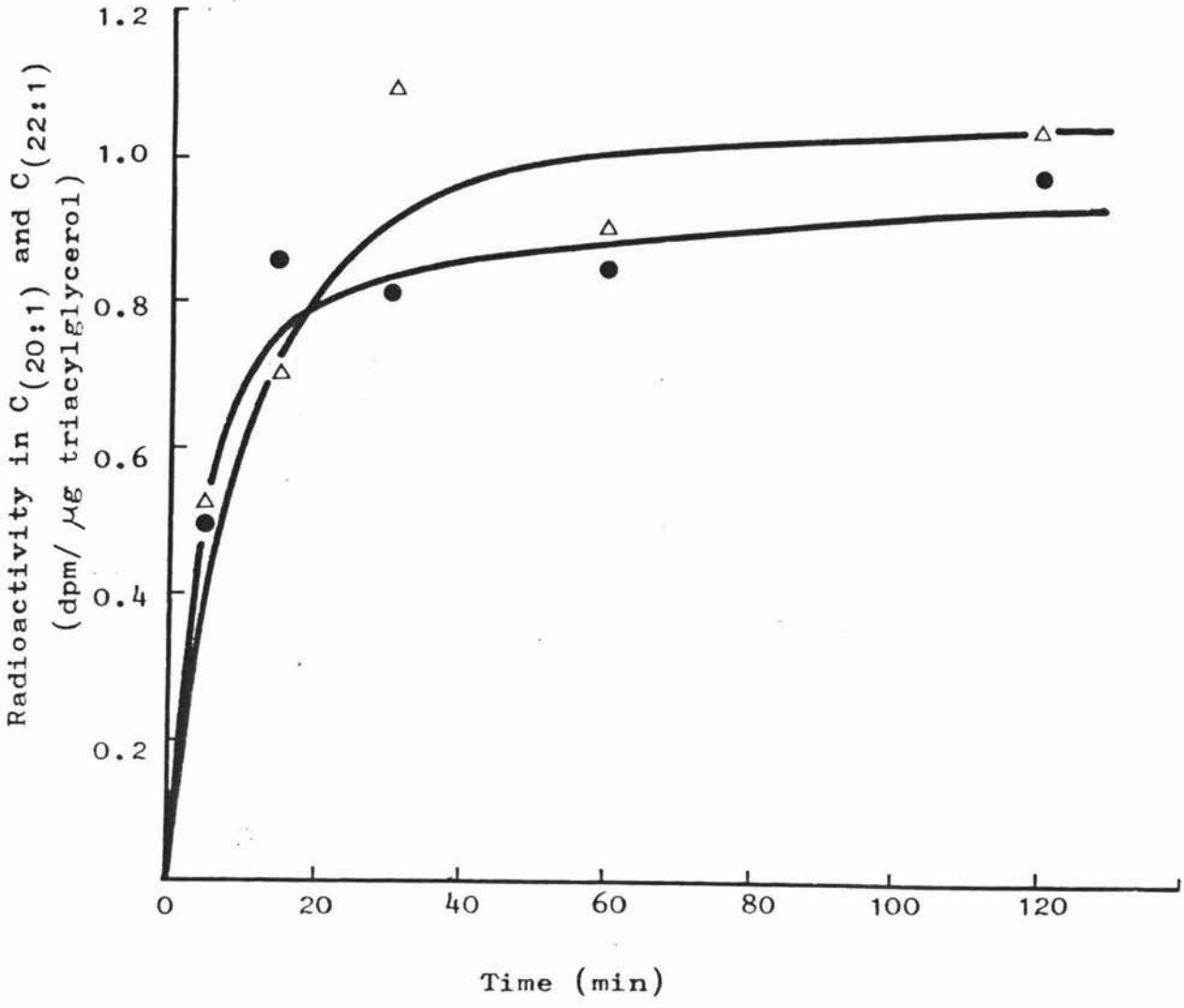


FIG. 18. Time course of [^{14}C] malonyl CoA incorporation into eicosenoate and erucate, expressed per μg of triacylglycerol, by homogenate and oil body preparations.

Incorporation of [^{14}C] malonyl CoA into eicosenoate and erucate was calculated from the data obtained in the experiment described in Table 15. Argentation chromatography was used to determine the proportion of radioactivity incorporated into eicosenoate and erucate. The amount of triacylglycerol present in the homogenate and oil body preparations was estimated by carrying out a Folch extraction on aliquots of the preparations. Aliquots of the chloroform layer were transmethylated and analysed by g.l.c. as described in the Methods section.

hypothesis that the elongation system may be associated with the oil bodies. However, the exact organelle composition of the oil body fraction is not known and there was an indication from cell fractionation studies that the oil body fraction was contaminated by membrane material, as it contained 30% of the phosphatidyl choline in the cell (p. 40). In addition it is possible that the extent of elongation may be limited in homogenate preparations, due to competition with de novo fatty acid synthesis for [^{14}C] malonyl CoA.

In order to investigate to what extent the membranous contaminants were involved in [^{14}C] malonyl CoA incorporation into eicosenoate and erucate, oil body preparations from rapeseed cotyledons were "purified" by repeated resuspension and centrifugation through sucrose density gradients. After each of three washes, aliquots of the oil body suspension were removed and their ability to incorporate [^{14}C] malonyl CoA was determined. In addition the triacylglycerol, and, as an estimate of membrane contamination, the phospholipid and chlorophyll content were measured (Table 27). Incorporation per μg of triacylglycerol by the oil body preparation which had been washed three times was only 50-60% of that obtained for the unwashed oil body preparation, although high levels of dithiothreitol were present (which had been shown to help maintain enzyme activity with time). However, during the washing process, the amount of membrane contamination, as indicated by the chlorophyll and phospholipid measurements, had fallen to 28% and 41% of the original values, respectively. These results tentatively support the hypothesis that the elongation system, and therefore synthesis of eicosenoate and erucate, may be associated with the oil bodies themselves, rather than with contaminating membrane material.

TABLE 27. Effect of repeatedly washing an oil body preparation on chlorophyll and phospholipid content, and incorporation of [^{14}C] malonyl CoA.

Oil bodies were isolated from rapeseed cotyledons (4 g) and washed 3 x by centrifuging the resuspended oil bodies through a sucrose gradient. All buffers used contained 10 mM dithiothreitol. Samples of the oil body preparation were removed for assay after each wash. [^{14}C] malonyl CoA incorporation was determined in duplicate, by incubating 0.25 ml of oil body preparation with 0.25 ml of reaction mix (0.033 mM [^{14}C] malonyl CoA, 2 mM ATP, 0.1 mM NADPH, 0.8 mM glycerol-3-phosphate) for 15 min at 25°C, stopping the reaction with 10 ml chloroform/methanol (1:1 v/v) and determining the radioactivity present in the chloroform phase. Samples of these chloroform extracts were separated into individual lipids by t.l.c. and the phospholipid and triacylglycerol (TAG) zones removed, transmethylated with sodium methoxide, extracted into petroleum ether and analysed by g.l.c. Chlorophyll content was determined spectrophotometrically, as described in the Methods section.

Oil body preparation	[^{14}C] malonyl CoA incorporation dpm/μg TAG	Chlorophyll μg/μg TAG	10^{-2} x phospholipid μg/μg TAG
Unwashed	5.96	2.68	2.9
wash 1	4.48	1.50	2.7
wash 2	2.88	1.05	1.0
wash 3	3.76	0.76	1.2

DISCUSSION

When the long chain fatty acids of triacylglycerol from rapeseed cotyledons, labelled with [^{14}C] acetate for 2 h, were subjected to oxidative fission, the specific radioactivities of the carboxyl ends of the eicosenoate and erucate were very much higher than those of the methyl ends of these fatty acids. These findings agree closely with the results of Downey and Craig (1965) and support their conclusion that eicosenoate and erucate are formed from oleate by chain elongation.

The results of oxidation fission experiments, both from the present studies and those of Downey and Craig, suggest that the specific radioactivity of oleate, and the oleoyl portions of eicosenoate and erucate were similar, and that the specific radioactivities of the oleoyl portions of $C_{(20:1)}$ and $C_{(22:1)}$ differed greatly from those of the carboxyl terminal carbons added by chain elongation. Both these assumptions were verified by complete α-oxidation of the saturated fatty acids derived from oleate, eicosenoate and erucate of the labelled triacylglycerol.

In the triacylglycerol extracted from the cotyledons used, the ratio of oleate, eicosenoate and erucate, according to mass, was 2:1:3.5 respectively. The simplest explanation for the observation that the specific radioactivities of the oleoyl portions of these acids were similar (based on the partitioning of oleate into triacylglycerol and into chain elongation) is that $\frac{2}{6.5}$ of the radioactive oleate synthesised was incorporated directly into triacylglycerol, $\frac{1.0}{6.5}$ received one, and $\frac{3.5}{6.5}$ received two additional acetate-derived units by chain elongation. That is, the radioactive fatty acids were being incorporated into triacylglycerol in proportion to the

amount of each individual fatty acid already in the oil. This conclusion is supported by the observation that the specific ratioactivity of the C_{13} dicarboxylic fragment derived from erucate was about twice that of the C_{11} dicarboxylic fragment derived from eicosenoate.

The observation that the specific radioactivities of oleate and of the oleoyl moieties of erucate and eicosenoate of the triacylglycerol were similar, suggests in addition, that the pool of [^{14}C] oleate, or its derivatives, incorporated into triacylglycerol, was the same as that used for chain elongation. When similar degradation studies were carried out on fatty acids obtained from jojoba cotyledons fed with [^{14}C] acetate, no radioactivity was detected along the oleoyl portion of eicosenoate and erucate, although oleate was labelled (Ohlrogge et al., 1978). It was concluded that elongation had occurred by addition of labelled C_2 units onto oleate from an unlabelled endogenous pool. This suggestion implies that two pools of oleate, or its derivatives, exist in the cells of jojoba cotyledons; one pool which is rapidly labelled and incorporated directly into wax, and a cold endogenous pool which is utilized for chain elongation. The results of the present study, which agree with those obtained by Downey and Craig (1965), suggest that there is only one C_{18} pool utilised for triacylglycerol synthesis and chain elongation. It therefore appears that the system in jojoba cotyledons may be different to that in rapeseed, although it is noteworthy that the technique used to assay radioactivity in the fragments of α-oxidation of labelled jojoba fatty acids was less sensitive than that used for similar analyses with rapeseed fatty acids.

The α-oxidation degradation studies of the labelled fatty acids of rapeseed triacylglycerol, also revealed

that the specific radioactivities of the terminal carbons of eicosenoate and erucate, added by chain elongation to oleate, were much higher than the specific radioactivities of the oleoyl portions of these fatty acids. It is possible to propose two different theories to explain this observation: (1) the specific radioactivity of the acetate used in chain elongation was much greater than that used for de novo synthesis. (2) a large pool of endogenous oleate, or its derivatives, existed in the cells of the cotyledons with which labelled oleate equilibrated. These alternatives could be rigorously tested by measuring the kinetics of labelling of the oleoyl portions and terminal acetate units of eicosenoate and erucate during a time course experiment. If radioactive oleoyl CoA entered a large cold oleoyl CoA pool prior to chain elongation, then with time, the proportion of counts in the terminal acetate units would decrease relative to that in the oleoyl portions of those long chain fatty acids. However if the radioactive oleoyl CoA equilibrates rapidly with a very small cold oleoyl CoA pool, then the proportion of counts in the terminal acetate units relative to that in the oleoyl portions would remain approximately constant with time. This type of experiment was not performed but indirect evidence suggests that the existence of a large endogenous oleoyl CoA pool is unlikely. In vitro studies (described below) suggest that the relative amounts of eicosenoate and erucate synthesised were determined by the relative concentrations of oleoyl and eicosenoyl CoAs present in the reaction mix. If this control operates in vivo, then since more erucate was synthesised than eicosenoate, one would expect the eicosenoyl CoA pool to be larger than the oleoyl CoA pool. The proposal that a large pool of oleoyl CoA is present in the cells of rapeseed cotyledons, implies that an even larger pool

of eicosenoyl CoA exists. However the specific radioactivities of oleate, and of the oleoyl portions of eicosenoate and erucate were approximately the same. It is difficult to envisage how this could have been achieved if labelled eicosenoyl CoA derived from a large oleoyl CoA pool equilibrated with an even larger eicosenoyl CoA pool, before chain elongation and subsequent incorporation of eicosenoate and erucate into triacylglycerol. If this were the case, one would expect the specific radioactivity of the oleoyl portion of these fatty acids to have been less than that of oleate.

An alternative explanation to account for the unequal distribution of label in eicosenoate and erucate when rapeseed cotyledons were incubated with [^{14}C] acetate, is that the specific radioactivity of the acetate used for de novo fatty acid synthesis, and chain elongation were different. Using the specific radioactivity data from the -oxidation degradation studies, estimates of the specific radioactivities of the C_2 units of elongation in eicosenoate and erucate were obtained by applying the relationship:-

$$\beta = \frac{n}{n+2}\alpha + \frac{(n+2)-n}{n+2}\gamma$$

where (n+2) : compound of n carbon atoms which has been elongated by the addition of a C_2 unit.

α : specific radioactivity of a chain with n carbon atoms, calculated as the average of the specific radioactivities of all the oxidation fragments of chain length $\leqslant$ n.

β : specific radioactivity of (n+2)

γ : specific radioactivity of C_2 elongation unit.

The two C_2 units added to oleate to synthesise erucate had the same specific radioactivity, which was similar to that of the terminal acetate unit in eicosenoate (Fig. 19),

of the terminal acetate unit in eicosenoate (Fig. 19), suggesting that both chain elongation steps utilised acetate of the same specific radioactivity which was therefore from the same pool.

It is therefore proposed that two pools of acetate exist in the cells of rapeseed cotyledons, one utilised for de novo fatty acid synthesis, and one used for chain elongation. Using radioimmunological techniques Ohlrogge et al. (1979) showed that all the ACP in a spinach leaf cell could be attributed to the chloroplasts, and consequently concluded that in leaves, de novo fatty acid synthesis occurs only in the chloroplasts. Similar unpublished results indicate that plastids are the only site of de novo synthesis in seed cotyledons (Stumpf, pers. comm.). The in vitro studies of Gurr et al. (1974) with crambé, Ohlrogge et al., (1978) and Pollard et al. (1979) with jojoba and those reported here with rapeseed, all indicate that the chain elongating enzymes are extra chloroplastic. It is therefore further proposed that the two acetate pools have different intracellular locations, and, on the basis of the results of the degradation studies, that when the cotyledons are fed with [^{14}C] acetate, the acetate pool in the chloroplast, used for de novo synthesis, has a much lower specific radioactivity than the extrachloroplastic pool, used for chain elongation. Malonyl CoA is the substrate for both these processes, and as it has been shown that the chloroplast membrane is not freely permeable to acetyl CoA or malonyl CoA, it is implied that an acetyl CoA synthetase (EC 6.2.1.1) and an acetyl CoA carboxylase (EC 6.4.1.2), exist both outside as well as inside the chloroplasts.

Starch granules are commonly seen in the chloroplasts of oil seed cotyledons (Rest and Vaughan, 1972) and starch degradation accompanies lipid accumulation in

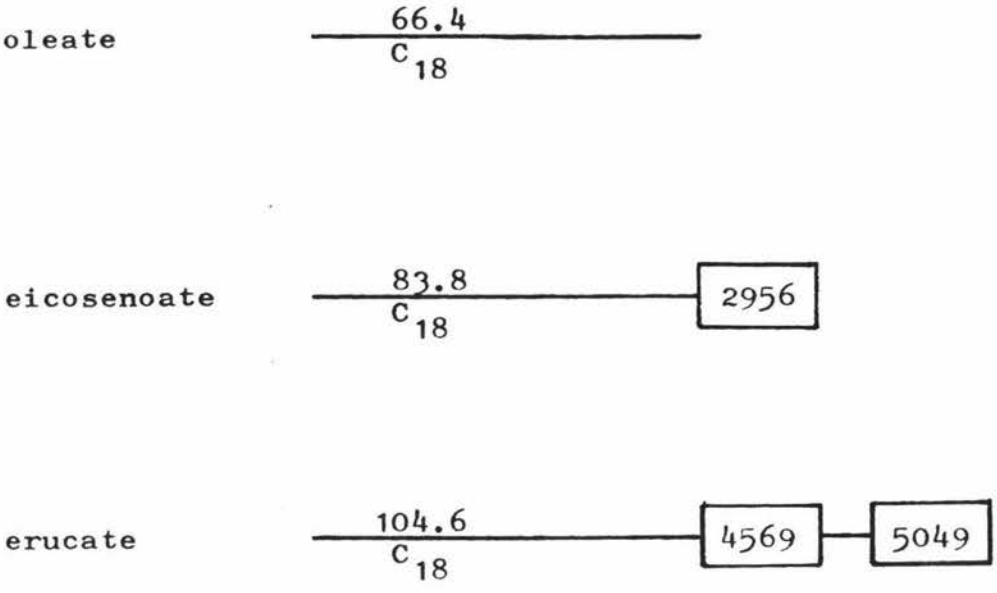


FIG. 19. Calculated specific radioactivities of the oleoyl portions and the C_2 elongation units of oleate, eicosenoate and erucate.

Specific radioactivities of the C_2 units added by elongation were calculated using the equation on p 95 , and the specific radioactivity data from the oxidation experiment shown in Table 12. The specific radioactivities of the C_{18} chains are the average of the specific radioactivities obtained for oxidation fragments with 14 to 18 carbons.

rapeseed cotyledons (Norton and Harris, 1975). It could therefore be proposed that cold acetate derived from starch breakdown could dilute the radioactive pool of acetate within the chloroplasts producing a labelled acetate pool of relatively low specific radioactivity. Inherent in this explanation is that acetate can be synthesised within the chloroplasts, however nothing is known concerning the pathway of acetate formation in oil seed cotyledons and the information regarding acetate synthesis in leaf chloroplasts is confused. In spinach leaf chloroplasts, Murphy and Leech (1978) have suggested that acetate can be derived from 3-phosphoglycerate by the route shown in Fig. 20 , and that all the necessary enzymes can be found in the chloroplasts. However, Stitt and Rees (1979) reported that pea chloroplasts do not contain phosphoglyceromutase (EC 2.7.5.3) and Roughan et al. (1979) did not detect pyruvate dehydrogenase (EC 1.2.2.2) in spinach leaf chloroplasts, suggesting that the chloroplasts are not autonomous with regard to their acetate supply. In view of the present lack of knowledge concerning the acetate source for de novo synthesis, any suggestion as to how starch-derived acetate could dilute the specific radioactivity of the chloroplast acetate pool and not that of the non-plastid pool used for chain elongation would be purely speculative.

As mentioned above, the chloroplast envelope is impermeable to malonyl CoA, therefore malonyl CoA synthesised inside the chloroplasts is unlikely to mix with any extrachloroplastic malonyl CoA pool. Spinach chloroplasts do contain however, an acetyl CoA synthetase thought to be located between the two membranes of the chloroplast envelope, which could synthesise acetyl CoA using ATP and CoA from outside the chloroplast (Roughan et al., 1980). If such an enzyme exists in seed cotyledons, it may supply acetyl CoA,

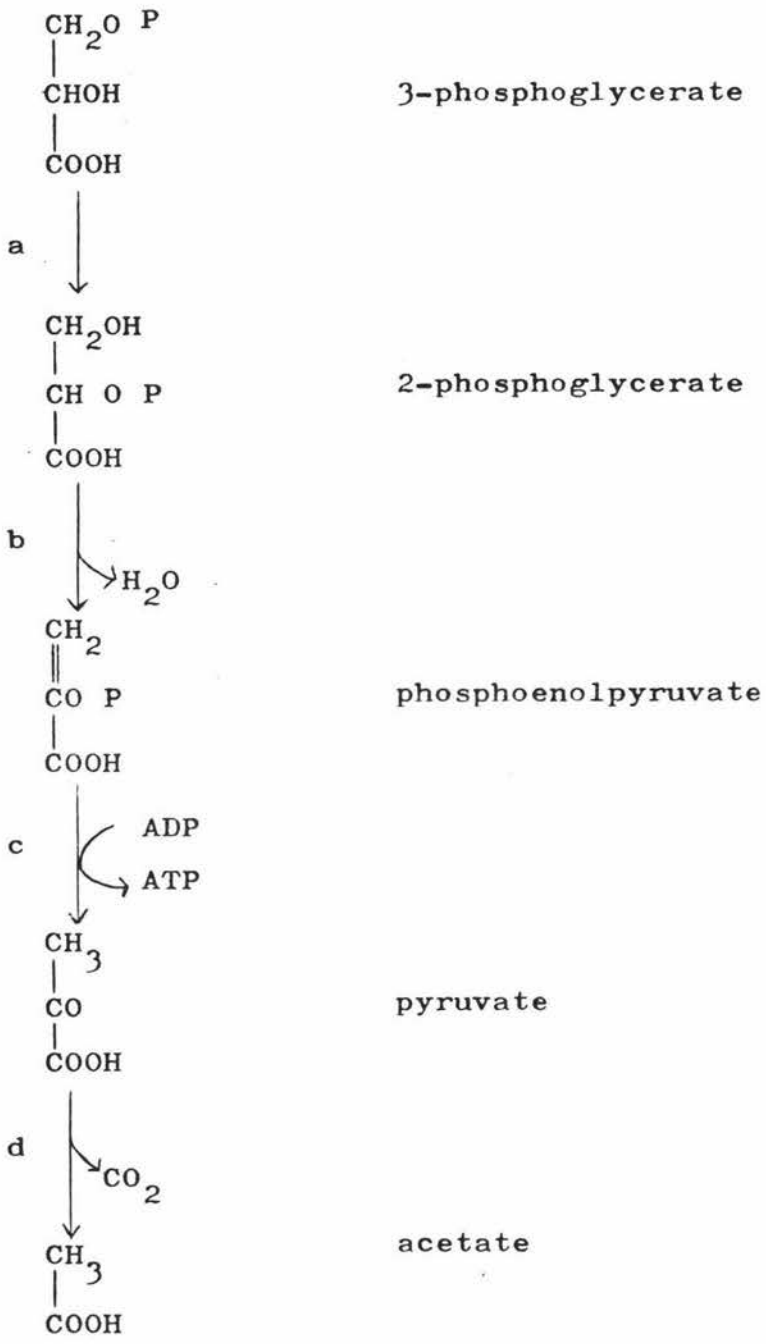


FIG. 20. Conversion of 3-phosphoglycerate to acetate in spinach leaf chloroplasts according to Murphy and Leech (1978)

a, phosphoglyceromutase; b, phosphopyruvate hydratase; c, pyruvate kinase; d, pyruvate dehydrogenase.

from which the malonyl CoA used in chain elongation is derived.

The above discussion as to the source of acetate units used for de novo synthesis and chain elongation is pertinent not only to the possible existence of two different acetate pools supplying these two processes, but also to a recent proposal of Stumpf (pers. comm.) to explain the apparent absence of label in the oleoyl portions of eicosenoate and erucate of jojoba wax (see above). Stumpf suggested that an acetyl CoA synthetase could exist in the cytosol of cells of jojoba cotyledons which converted [^{14}C] acetate entering the cells into acetyl CoA, and that since, by analogy with leaf chloroplasts, the jojoba plastids might be expected to be impermeable to acetyl CoA, the enzyme would effectively prevent radioactive acetate entering the chloroplast pool used for de novo synthesis. As has been discussed above, the source of acetate for de novo synthesis in the chloroplasts of oil seed cotyledons has not been established and there appears to be evidence both for and against the proposal that acetate is synthesised within the chloroplasts. Stumpf's suggestion fails to acknowledge the possible extrachloroplast origin of acetate, for if this were so, one would expect the proposed acetyl CoA synthetase to convert both the labelled and unlabelled acetate into acetyl CoA and hence starve the chloroplast of any supply of acetate for de novo synthesis.

In in vitro assays, rapeseed oil body preparations incorporated label from [^{14}C] malonyl CoA mainly into eicosenoate and erucate, as initially reported with crambé cotyledons (Gurr et al., 1974), but crude homogenates of the rapeseed cotyledons utilised the [^{14}C] malonyl CoA

mainly for *de novo* fatty acid synthesis, forming palmitate and stearate. In assays containing oil bodies, NADPH was the most efficient reductant and added ATP was necessary for maximum incorporation. The addition of oleoyl CoA or eicosenoyl CoA to assays did not stimulate incorporation. However inclusion of these compounds markedly affected the amounts of radioactive long chain fatty acids synthesised; addition of oleoyl CoA increased the formation of labelled eicosenoate at the expense of erucate and eicosenoyl CoA had the reverse effect, which was taken as indirect evidence that these long chain acyl CoAs were substrates for chain elongation. Radioactive long chain acyl CoAs only accumulated as the products of the chain elongation reactions, in certain assays, in which added oleoyl CoA competitively inhibited the incorporation of these products into lipids (see below). Generally the level of labelled long chain acyl CoAs was low (1-6%) and most of the radioactive fatty acids were found in triacylglycerol and phosphatidic acid.

In recent studies on the formation of long chain fatty acids and fatty alcohols by a jojoba cell free homogenate, Pollard *et al.* (1979) using an alternative *in vitro* system containing [^{14}C]-oleoyl CoA or [^{14}C] eicosenoyl CoA and unlabelled malonyl CoA, obtained incorporation of label into the products of chain elongation. This finding is more direct evidence for the belief that, in plants, long chain acyl CoAs are the acyl donors which react with malonyl CoA in the chain elongation process. Using this system, as for rapeseed oil bodies, NADPH was the most effective reductant but ATP was not required.

The lack of any dependence on added acyl CoAs in *in vitro* studies, despite the strong evidence that

these compounds are substrates for the elongation reaction, suggests that they were present in the oil body preparation or that they were synthesised during the assays. Acyl CoAs bind to microsomal membranes (Baker and Lynen, 1971), however if bound acyl CoAs were the substrates for elongation in vitro, then one must envisage some means by which tightly bound acyl CoAs, which were not removed by repeated washing of the oil body preparation, could be made available to the elongating enzymes in amounts which were not rate-limiting. At present I have no explanation as to how this might be achieved.

If acyl CoAs were being synthesised during the assays, then they must have been produced not only in sufficient amounts to saturate the enzyme since no stimulation of incorporation was obtained by adding these compounds, but also relatively quickly as no indication of a lag in [^{14}C] malonyl incorporation was observed. Although it could be suggested that long chain acyl CoAs were synthesised de novo during the assays, the proposal that fatty acid synthesis is localised in the chloroplasts in oil seeds (Stumpf, pers. comm.) would imply that synthesis of these compounds, and [^{14}C] malonyl CoA incorporation, was dependent on the presence of fragmented chloroplasts in the oil body preparation. However, as thoroughly washed oil body preparations still incorporated high levels of [^{14}C] malonyl CoA, this hypothesis is unlikely.

The marked stimulation of incorporation of label from [^{14}C] malonyl CoA into long chain fatty acids by added ATP, which is not a known reactant in other chain elongation reactions, suggests that the ATP may be required for acyl CoA synthesis by activation of free fatty acids by an acyl CoA synthetase (EC 6.2.1.3). Free

fatty acids could conceivably arise from lipid breakdown during isolation of the oil body preparation, and CoA, also required for this reaction, would probably have been present as a contaminant in the [^{14}C] malonyl CoA solution. This hypothesis is consistent with the finding that elongation in the jojoba *in vitro* system (see above) which contained [^{14}C] labelled long chain acyl CoAs and cold malonyl CoA, did not require added ATP (Pollard *et al.*, 1979). It might be possible to test this proposal by incubating an oil body preparation with [^{14}C]-labelled oleate in the presence of ATP and CoA - incorporation of label into lipids would imply that an acyl CoA synthetase was present in the oil body preparation. If the acyl CoA substrates were synthesised *in vitro* by fatty acid activation, stimulation of [^{14}C] malonyl CoA incorporation into eicosenoate and erucate might have been observed when long chain acyl CoAs were added to the reaction mix in the absence of ATP and CoA. These assays were unfortunately not performed. A small amount of [^{14}C] malonyl CoA incorporation did occur in the absence of ATP, and it would have been of interest to have determined whether any of the label was present in eicosenoate or erucate, suggesting that some endogenous long chain acyl CoAs were present in the oil body preparation.

The only assays in which added long chain acyl CoA did have a stimulatory effect, were those containing oil bodies which had been prepared using a buffer which contained partially oxidized dithiothreitol, which, even in the presence of ATP, incorporated only very low levels of [^{14}C] malonyl CoA into long chain fatty acids. This observation suggests that at least part of the requirement for dithiothreitol in the assays, could be to maintain an acetyl CoA synthetase in an active form.

Addition of long chain acyl CoAs at "high"

concentrations to the reaction mix, inhibited [^{14}C] malonyl CoA incorporation by oil body preparations. Substrate-induced inhibition by fatty acyl CoAs has previously been reported for microsomal chain elongation enzymes in mammalian tissues (Brophy et al., 1975; Bourré et al., 1976; Bernert and Sprecher, 1977). The suggested explanation for this inhibitory effect is that chain elongation enzymes utilise acyl CoAs in the monomeric state and at concentrations above the critical micelle concentration, when most of the acyl CoA exists as micelles rather than in a monomeric form, the enzymes would be effectively deprived of this substrate. The critical micelle concentration for fatty acid CoAs is 2-10 μM (Barden and Cleland, 1969). However, chain elongation by rapeseed oil body preparations was not inhibited at 0.1 mM oleoyl CoA. Although this is well above the critical micelle concentration, it has been shown that acyl CoAs bind to membranes (Baker and Lynen, 1971; Lamb et al., 1973) and it is conceivable that considerable binding of fatty acyl CoAs could have occurred to rapeseed oil body preparations, thus reducing the amount of acyl CoA available in solution for micelle formation.

In studies on long chain fatty acid and fatty alcohol formation by a jojoba cell free homogenate in vitro, Pollard et al. (1979) measured the incorporation of radioactivity from [^{14}C]-labelled long chain acyl CoAs at a concentration of 0.03 mM in the presence of unlabelled malonyl CoA. If acyl CoAs are inhibitory in this system, then it is possible that they may have underestimated the activity of the chain elongation enzymes. That this was so, is suggested by the stimulatory effect of supplying acyl CoAs with bovine serum albumin, since the adsorption of acyl CoAs by BSA has been shown to reduce the inhibitory effect of acyl CoAs on elongation by mouse brain microsomes

(Brophy and Vance, 1976), by maintaining the acyl CoAs in their monomeric form.

In an attempt to summarise the information obtained from the present study, and to highlight problems which require further research, a hypothetical schema has been drawn (Fig. 21) showing the chain elongation reactions in relation to the synthesis of oleate and triacylglycerol in developing rapeseed cotyledons. It has been reported that *in vivo* the starch reserves in rapeseed cotyledons are not sufficient to supply all the acetate required for the rapid triacylglycerol synthesis which occurs and that additional substrates are derived from photosynthates imported into the seeds from the pods (Norton and Harris, 1975). It is proposed that the synthesis of eicosenoate and erucate involves utilisation of two pools of acetate which are located at different sites in the cell. In the chloroplast, acetate is used for *de novo* synthesis of stearate, which is desaturated to oleate, and converted to oleoyl CoA in the chloroplast envelope (Roughan *et al.*, 1977). In the cytosol, oleoyl CoA may be incorporated directly into triacylglycerol, or, at the site of elongation, perhaps the oil body membrane, oleoyl CoA may be elongated to eicosenoyl CoA using malonyl CoA derived from the extra chloroplastic acetate pool and NADPH, in a manner exactly analagous to chain elongation on mammalian microsomes. Eicosenoyl CoA can either be incorporated into triacylglycerol, or further elongated to erucyl CoA prior to incorporation into triacylglycerol.

A two compartment system for the synthesis of eicosenoate and erucate has also been put forward by Pollard *et al.* (1979) from studies on jojoba cotyledons, however to lend credance to this proposal, it must be

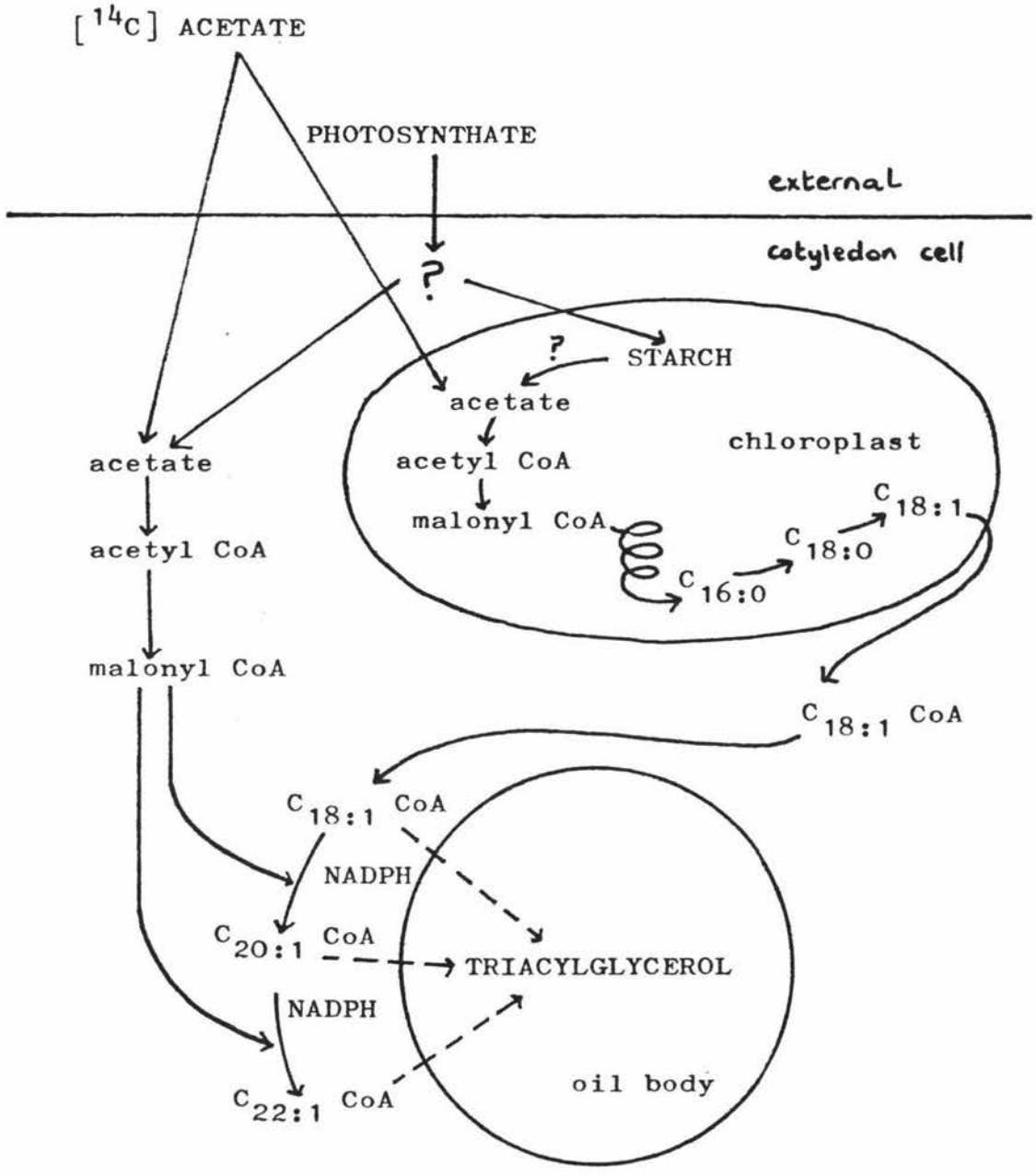


FIG 21. Hypothetical schema showing the chain elongation reactions in relation to the synthesis of oleate and triacylglycerol.

demonstrated that fatty acid synthetase is localised in the chloroplasts in this tissue, and that acetyl CoA synthetase, and acetyl CoA carboxylase activities can be detected outside, as well as inside, the chloroplasts.

The schema is incomplete in several aspects:

(a) As mentioned above, the pathway of acetate formation from starch and photosynthates in developing oil seeds has not been elucidated. This information is needed to explain why most of the radioactivity in eicosenoate and erucate is located in the carbons added by chain elongation, when cotyledons are incubated with [^{14}C] acetate. It is noteworthy that when jojoba cotyledons were fed with [U-^{14}C] glucose, these long chain fatty acids were uniformly labelled. Although interpretation is difficult until acetate metabolism is more fully understood, it would be interesting to determine if similar results could be obtained if rapeseed cotyledons were labelled with [U-^{14}C] glucose.

(b) The intracellular location of the chain elongation enzymes has not been determined. The most definitive method currently available for determining the intracellular location of an enzyme is to compare the distribution of the enzyme and of specific marker enzymes for each organelle in the various subcellular fractions, isolated along a continuous sucrose-density gradient. However no enzyme marker has been identified for oil bodies, and furthermore, although their origin remains unknown, it has been proposed that oil bodies may arise from endoplasmic reticulum (Frey-Wyssling *et al.*, 1963), and it is conceivable that the enzyme complements of both these organelles could be similar. The application of enzyme marker techniques to the problem of locating the intracellular site of chain elongation is severely limited and a solution may depend on evidence obtained using less direct methods.

When subcellular fractions, prepared by differential centrifugation of cotyledon homogenates, were incubated with [^{14}C]-labelled substrates, maximum elongation activity occurred in the oil body fraction of crambé (Gurr et al., 1974) and in the wax pad of jojoba (Pollard et al., 1979). Controversy arose as to whether elongation was associated with the oil bodies (Gurr et al., 1974) or with endoplasmic reticulum (Stumpf, pers. comm.) thought to have adhered to the wax pad during preparation (Pollard et al., 1979). It is noteworthy however that neither the microsomal fractions from crambé or jojoba cotyledons, incorporated label into long chain fatty acids, suggesting that endoplasmic reticulum may not be involved in chain elongation. It would be interesting to carry out similar cell fractionation experiments using rapeseed cotyledons.

When rapeseed oil body preparations were washed, [^{14}C] malonyl CoA incorporation did not decrease as rapidly as membrane contamination, estimated by chlorophyll and phospholipid measurements, supporting the hypothesis that elongation is associated with the oil bodies.

These experiments involving assay of cell fractions produced by differential centrifugation for certain metabolic activities, and purification of oil body preparations by washing, are more applicable to the problem of determining the intracellular site of the chain elongation enzymes than attempting to isolate and assay cell fractions on the basis of enzyme markers. The results obtained would be more meaningful if a non-enzymic method of estimating endoplasmic reticulum (or microsomes) directly, could be found. At present such a technique is not available, however preliminary

studies with linseed and safflower cotyledons (Slack, 1980) have shown that the structural protein complement of oil bodies and endoplasmic reticulum may be different. It is conceivable that immunological techniques, such as preparation of labelled antibodies to microsomal proteins, could be used to quantify the contamination of the oil body fraction by endoplasmic reticulum.

(c) The mechanism by which the long chain fatty acyl CoA products of chain elongation are incorporated into triacylglycerol and the intracellular location of triacylglycerol synthesis have not yet been determined. It has been proposed by Gurr et al., (1974) from kinetic labelling studies using [^{14}C]-glycerol that triacylglycerol in crambé cotyledons was synthesised via the classical Kennedy pathway (Hitchcock and Nichols, 1971), which involves acylation of positions 1 and 2 of sn-glycerol-3-phosphate to form phosphatidic acid, followed by dephosphorylation to a 1,2-diacylglycerol and finally acylation at position 3 to yield a triacylglycerol. Gurr further suggested that the oil body fraction contained all the enzymic apparatus necessary for triacylglycerol synthesis, but it is noteworthy that no diacylglycerol was reported to be present in crambé oil body preparations. Diacylglycerol was a component of rapeseed oil bodies and it contained eicosenoate and erucate. Furthermore, when rapeseed oil body preparations were incubated with [^{14}C] malonyl CoA in the presence of glycerol-3-phosphate, although triacylglycerol was the most heavily labelled product, a high proportion of the radioactivity was also found in phosphatidic acid. These observations appear to support the proposal that triacylglycerol synthesis in oil seeds occurs via the Kennedy pathway, however the following findings require explanation. Approximately 60% of the fatty acids in rapeseed triacylglycerol are eicosenoate and erucate, if diacylglycerol is a

precursor in triacylglycerol synthesis, then it follows that approximately 50% of the fatty acids in the diacylglycerol must be elongated fatty acids. However analysis of the fatty acid composition of diacylglycerol obtained from the whole cotyledon, or from the oil body fraction, revealed that only 20-30% of the fatty acids were eicosenoate or erucate. Secondly, it appeared, from *in vivo* labelling of rapeseed cotyledons, with [^{14}C] acetate, that triacylglycerol was being rapidly synthesised, perhaps by acylation of a pre-existing diacylglycerol pool. However, if diacylglycerol was synthesized to replenish this pool, utilising eicosenoate and erucate from the same pools which supply triacylglycerol synthesis, one would expect, as found for triacylglycerol, that erucate would be more heavily labelled than oleate in diacylglycerol, but the reverse was found. An explanation consistent with these observations is that two distinct pools of diacylglycerol are present in these cells. One relatively small pool, associated with the oil bodies involved in triacylglycerol synthesis, in which 50% of the fatty acids are eicosenoate and erucate, and in which erucate becomes more highly labelled than oleate when the cotyledons are fed [^{14}C] acetate. In addition, a large pool of diacylglycerol may also be present, containing mainly C_{18} fatty acids, which is not involved directly in triacylglycerol synthesis, and is located elsewhere in the cell. This theory could be investigated by analysing the fatty acid composition and incorporation of label into these fatty acids of diacylglycerol associated with purified oil body fractions prepared after incubation of rapeseed cotyledons with [^{14}C] acetate.

CONCLUDING REMARKS

The aim of the present study was to obtain more information that would hopefully lead to a greater understanding of long chain fatty acid synthesis in rapeseed cotyledons. Developing rapeseed cotyledons provided a good biosynthetic system for both *in vivo* and *in vitro* studies, from which it was concluded that the mechanism of eicosenoate and erucate synthesis involved the formation of the corresponding acyl CoAs by elongation of oleoyl (or eicosenoyl) CoA with malonyl CoA and NADPH. During the course of this study, a similar proposal for the mechanism of long chain fatty acid synthesis in jojoba cotyledons was put forward (Pollard *et al.*, 1979). However, some of the experimental data and hypotheses suggested from studies on jojoba were not in agreement with those recorded here for rapeseed. Controversy exists, as to whether the intracellular site of chain elongation is the oil bodies or the endoplasmic reticulum. This problem merits detailed investigation, as the implications of demonstrating that oil bodies are not simply storage organelles, but have a metabolic role in the synthesis of the storage compounds, are enormous.

Chain elongation is part of the complex series of synthetic reactions, leading to the formation of the seed oil and in order to fully interpret experimental data obtained, it was necessary to consider both the synthesis of oleate, in particular the source of acetate from which oleate was synthesised, and the subsequent incorporation of long chain acyl CoAs, produced by chain elongation, into triacylglycerol. However, as was shown in the schema above, knowledge is severely lacking in these areas, and although many interesting observations were made, it was difficult,

without this information, to assess their significance.

As perhaps would be expected with any brief period of research, many problems were raised and relatively few conclusions were made. However such a preliminary investigation serves to point out that further research is required before the pathways of triacylglycerol synthesis in seeds can be elucidated, and the opportunity arises to determine what factors firstly control the onset of oil synthesis, and secondly control the final composition of the oil.

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