LONG CHAIN FATTY ACID SYNTHESIS IN RAPESEED COTYLEDONS

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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

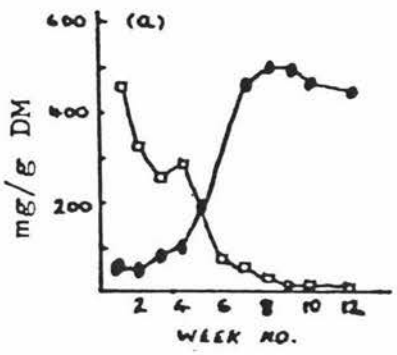
		Products of oxidative fission	
		Monocarboxylic fragment	Dicarboxylic fragment
Oleate (18:1)	$H_3C-(CH_2)_7-HC=CH-(CH_2)_7COOH \longrightarrow$	$H_3C(CH_2)_7COOH$ C_9	$HOOC-(CH_2)_7-COOH$ C_9
Eicosenoate (20:1)	$H_3C-(CH_2)_7-HC=CH-(CH_2)_9COOH \longrightarrow$	C_9	C_{11}
Erucate (22:1)	$H_3C-(CH_2)_7-HC=CH-(CH_2)_{11}COOH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

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

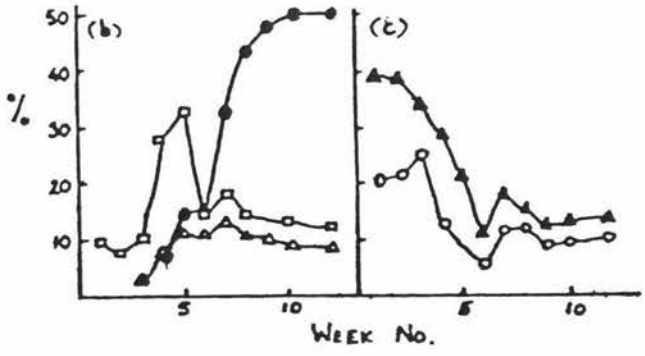
of Appelqvist (1975) with rape cotyledons, indicate that these long chain fatty acids are absent from all cotyledon lipids other than triacylglycerol. Consequently there must be fine metabolic control to regulate the rates of formation of the different constituent fatty acids and also to channel different 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

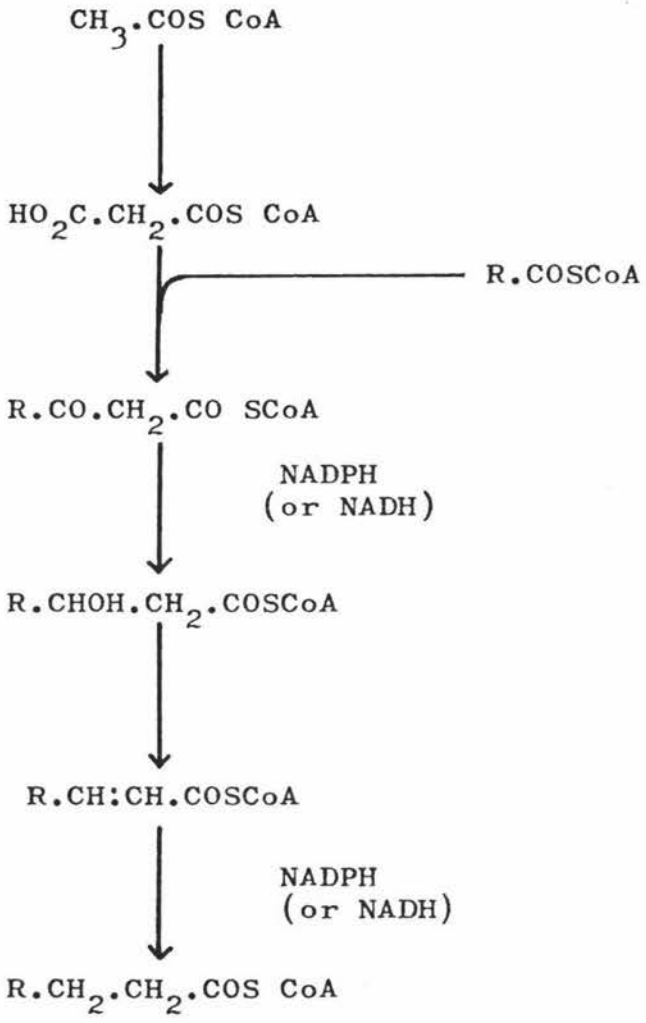


FIG. 3. Malonate dependent elongation in mammalian microsomes (Hitchcock and Nichols, 1971)

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.