Storage oil breakdown during embryo development of *Brassica napus* (L.)

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**Abstract**

In this study it is shown that at least 10% of the major storage product of developing embryos of *Brassica napus* (L.), triacylglycerol, is lost during the desiccation phase of seed development. The metabolism of this lipid was studied by measurements of the fate of label from [1-14C]decanoate supplied to isolated embryos, and by measurements of the activities of enzymes of fatty acid catabolism. Measurements on desiccating embryos have been compared with those made on embryos during lipid accumulation and on germinating seedlings. Enzymes of β-oxidation and the glyoxylate cycle, and phosphoenolpyruvate carboxykinase were present in embryos during oil accumulation, and increased in activity and abundance as the seeds matured and became desiccated. Although the activities were less than those measured during germination, they were at least comparable to the *in vivo* rate of fatty acid synthesis in the embryo during development. The pattern of labelling, following metabolism of decanoate by isolated embryos, indicated a much greater involvement of the glyoxylate cycle during desiccation than earlier in oil accumulation, and showed that much of the 14C-label from decanoate was released as CO₂ at both stages. Sucrose was not a product of decanoate metabolism during embryo development, and therefore lipid degradation was not associated with net gluconeogenic activity. These observations are discussed in the context of seed development, oil yield, and the synthesis of novel fatty acids in plants.

**Introduction**

Storage oil, in the form of triacylglycerol (TAG), is synthesized during the growth of embryos of oilseeds, and then degraded to provide carbon and energy during germination and early seedling growth via the successive operation of β-oxidation, the glyoxylate cycle, partial tricarboxylic acid (TCA) cycle, and gluconeogenesis (Eastmond and Graham, 2001). While the linkage between these metabolic processes, and embryo and seedling development is clear, it is evident, from data presented in previous studies, that lipid content may actually decrease during maturation of oilseeds such as *B. napus*, arabidopsis, *Crambe abyssinica*, and *Nicotiana tabacum* (McKillikan, 1966; Gurr *et al.*, 1972; Norton and Harris, 1975; Murphy and Cummins, 1989; Baud *et al.*, 2002; Tomlinson *et al.*, 2004). A decrease in seed oil content also occurs during maturation of linseed (*Linum usitatissimum*) (S Troufflard, JC Portais and S Rawsthorne, unpublished results). Surprisingly, in all but one of these reports the loss of lipid is not recognized. This loss therefore appears to be a common, but not understood, feature of oilseed development and requires further study. Moreover, the degradation of lipids and fatty acids during embryo development could have important consequences for oil yield and for attempts to introduce novel fatty acids into seed lipids through genetic manipulation.

Consistent with these observations of lipid breakdown during seed maturation, a number of studies have indicated that embryos have the potential to degrade fatty acids during growth, as well as during seed germination and seedling establishment. First, the activities of enzymes of β-oxidation and the glyoxylate cycle have been detected in developing seeds of cotton (Miernyk and Trelease, 1981), castor bean (Hutton and Stumpf, 1969), and cucumber (Köller *et al.*, 1979; Frevert *et al.*, 1980), in *Brassica napus* (L.), the mRNA transcripts, protein, and activity of malate synthase (MS) and isocitrate lyase (ICL) are present during the late stages of embryo development, but the activity of...
the glyoxylate cycle has not been investigated (Comai et al., 1989; Ettinger and Harada, 1990). Second, novel fatty acids, produced in seeds as a consequence of the expression of transgenes designed to alter fatty acid metabolism, may be degraded during seed development (e.g. in B. napus; Eccleston and Ohlrogge, 1998). Third, the activity of the β-oxidation pathway in developing arabidopsis seeds is revealed by the analysis of polyhydroxyalkanoates (PHA) in seeds expressing PHA synthase, an enzyme which synthesizes PHA from intermediates of β-oxidation (Poirier et al., 1999; Moire et al., 2004).

The extent and nature of fatty acid degradation during seed development and maturation in the commercially important oilseed species B. napus has been investigated here. Total fatty acid and triacylglycerol content has been measured, the activities of representative enzymes of the complete metabolic sequence of fatty acid catabolism has been assayed, and the fate of 14C-labelled decanoic acid fed to isolated developing embryos has been followed. It is revealed that developing B. napus embryos actively degrade fatty acids and that the complete pathway of catabolism is active in embryos during and after the main period of oil accumulation. Loss of CO2 is a substantial fate of the 14C-label in decanoic acid.

Materials and methods

Chemicals and radiochemicals

Substrates, coupling enzymes, and cofactors were purchased from Sigma Chemical Co. (Poole, Dorset, UK). Radiolabelled isotopes D-[U-14C]glucose, D-[U-14C]fructose, D-[U-14C]sucrose, and L-[1,4(2,3)-14C]malate were from Amersham Pharmacia Biotech (Little Chalfont, UK) and [1-14C]decanoic acid was from American Radiochemicals (St Louis, Missouri, USA).

Plant material and growth conditions

Brassica napus (L.) cultivar Topas was used for all experiments. Embryos from five distinct developmental stages (pre-, early-, mid-oil synthesis, desiccating, and mature stages: Chia and Rawsthorne, 2000; Eastmond and Rawsthorne, 2000) were harvested from plants grown in a glasshouse with 18/12 °C day/night temperatures and with 16 h of supplementary illumination to cover the natural photoperiod between October and March. Embryos were removed from their testas and placed into incubation medium without labelled substrate (see below). For germination studies, seeds were sterilized by soaking in 70% ethanol for 2 min, followed by 10 min in 5% sodium hypochlorite solution and then several washes with sterile water. Seedlings were obtained by germinating sterile seeds on wet filter paper for up to 7 d at 4 °C, and used directly for enzyme assays. (i) ICL and MS activities were measured according to Cooper and Beever (1969). Increased sensitivity of the MS assay was achieved by lowering the concentration of DTNB to 0.2 mM. (ii) PEPC activity was assayed according to Cooper et al. (1968) with modifications as described by Walker et al. (1999). (iii) The multifunctional protein (MFP) activity was determined as described by Binstock and Schulz (1981) using 50 μM crotonyl-CoA to give enoyl hydratase (EH) activity and 100 μM acetoacetoyl-CoA to give L-hydroxyacyl-CoA dehydratase (HD) activity. (iv) 3-ketoacyl-CoA thiolase (KAT) activity was determined essentially as described by Rybolt et al. (2001), which was adapted from Gerhardt (1983). The reaction volume of 0.75 ml contained 100 mM potassium phosphate buffer pH 7.5, 250 μM MnCl2, 2 mM DTT, 140 μM acetyl-pyridine-NAD (APAD), 3 mM sodium-malate, 0.2% (w/v) sodium azide, 0.05% w/v Triton X-100, 2 mM CoA, 5.5 units citrate synthase, 1.2 units malate dehydrogenase, and 0.4 mM acetoacetoyl-CoA. The appearance of reduced APAD was monitored at A363 with an extinction coefficient of 9100 M-1 cm-1 (Lizcano et al., 2000). All assays were carried out at 25 °C.

Western blotting analysis

About 20 mg of developing embryos were ground with an all-glass homogenizer in 1 ml of protein extraction buffer, according to Kim and Smith (1994). The extracts were centrifuged at 13 000 rpm in a bench top centrifuge for 5 min at 4 °C. The supernatants were denatured and the total soluble proteins resolved by electrophoresis (2000; Eastmond and Rawsthorne, 2000) operating at a proton frequency of 20 MHz (0.47 Tesla). Developing embryos and seeds (with intact testa) were harvested, oven-dried at 80 °C for 1 h in glass Petri dishes and stored in a sealed vessel containing dried silica gel for 30 min. Up to 15 samples of 200 mg dried embryos per developmental stage were quantified to obtain an average value for oil content.

Spectrophotometric enzyme assays

Protein extracts for enzyme assays were prepared from developing embryos, cotyledons, and radicles from isolated seedlings by homogenizing the tissues in 1 ml extraction buffer (150 mM TRIS-HCl pH 7.5, 10 mM KCl, 10 μM FAD, 10% v/v glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA, and 0.01% v/v Triton X-100). The supernatant was clarified by centrifugation at 13 000 rpm for 5 min at 4 °C, and used directly for enzyme assays. (i) ICL and MS activities were measured according to Cooper and Beever (1969). Increased sensitivity of the MS assay was achieved by lowering the concentration of DTNB to 0.2 mM. (ii) PEPC activity was assayed according to Cooper et al. (1968) with modifications as described by Walker et al. (1999). (iii) The multifunctional protein (MFP) activity was determined as described by Binstock and Schulz (1981) using 50 μM crotonyl-CoA to give enoyl hydratase (EH) activity and 100 μM acetoacetoyl-CoA to give L-hydroxyacyl-CoA dehydratase (HD) activity. (iv) 3-ketoacyl-CoA thiolase (KAT) activity was determined essentially as described by Rybolt et al. (2001), which was adapted from Gerhardt (1983). The reaction volume of 0.75 ml contained 100 mM potassium phosphate buffer pH 7.5, 250 μM MnCl2, 2 mM DTT, 140 μM acetyl-pyridine-NAD (APAD), 3 mM sodium-malate, 0.2% (w/v) sodium azide, 0.05% w/v Triton X-100, 2 mM CoA, 5.5 units citrate synthase, 1.2 units malate dehydrogenase, and 0.4 mM acetoacetoyl-CoA. The appearance of reduced APAD was monitored at A363 with an extinction coefficient of 9100 M-1 cm-1 (Lizcano et al., 2000). All assays were carried out at 25 °C.

Metabolism of [1-14C]decanoate by isolated embryos

Ten whole isolated embryos were incubated in 100 μl of the tissue culture medium adapted from Nitsch and Nitsch (1967) adjusted to pH 7.2 and containing 0.1 mM [1-14C]decanoic acid (2055 kBq μmol−1). All incubations were carried out at room temperature in preweighed un capped 2 ml Eppendorf tubes for up to 6 h with frequent gentle agitation. Reactions were stopped by removing the
medium and washing the embryos with four 200 µl washes of incubation medium over 10 s. The washed embryos were immediately frozen in liquid nitrogen prior to extraction.

Extraction of ethanol-soluble material was carried out by incubating the washed embryos in 200 µl of 100% ethanol, followed by 80% and then 50% ethanol at 80 °C for 20 min each (Method A). The supernatant was clarified by centrifugation at each step and the ethanol fractions were pooled to yield a total extraction volume of 600 µl. The ethanol-extracted embryos were then homogenized and the remaining lipids and aqueous metabolites were extracted as described in Kang et al. (1994) (Method B). When measured, $^{14}$CO$_2$ evolution was determined by carrying out incubations as described by Kang and Rawsthorne (1996) for isolated plastids, and trapping the $^{14}$CO$_2$ that was released into 15% w/v KOH.

Calculation of incorporation of $^{14}$C into lipids and aqueous metabolites

Incorporation of $^{14}$C into aqueous metabolites was calculated by subtracting the amount of neutral and polar lipids (quantified from TLC plates) from the total label recovered in the ethanolic extract (Method A) and summing this with that obtained from the aqueous fraction of the fatty acid re-extraction (Method B). Incorporation of $^{14}$C into lipids was calculated by subtracting the amount of free fatty acids (assuming it was unmetabolized decanoate) from the total lipid as quantified on the TLC plate (from Method A) and summing this subtotal with that obtained from the organic fraction of the fatty acid re-extraction (from Method B).

TLC analysis of aqueous extracts

Aliquots of the ethanolic extract were further analysed on TLC plates by using three separate solvent systems. (i) Labelled sugars and organic acids were resolved on a CEL 400 microcrystalline cellulose TLC plate (Camlab Limited, Cambridge, UK) by using the upper phase of ethyl acetate:acetic acid:water (6:2:4, by vol.) (Canvin and Beevers, 1961). Clear separation of standards was achieved by running the plate three times, with a drying period in between. Organic acids were visualized by spraying the plates with 70% ethanol for 80°C for 5 min to develop the colour. (ii) Glycolytic intermediates were resolved on CEL 400 microcrystalline cellulose TLC plates using methanol:ammonium hydroxide:water (60:10:30, by vol.) (Bandurski and Axelrod, 1951), and were visualized using bromocresol green. (iii) Neutral lipids were separated on a 250 µm, 20×20 cm Silica Gel-G plate (Anachem, Luton, Beds, UK) using hexane:diethyl ether:glacial acetic acid (80:20:2, by vol.) (Henderson and Tocher, 1992).

Separated samples were visualized by exposing the plates to a bio-imaging plate (Raytek Scientific Ltd, Sheffield, UK) overnight. The image was developed using a Fuji Bio-imaging analysis system 1000 (Fuji, Photofilm UK Ltd, London) and compounds were identified by co-migration with radiolabelled or unlabelled standards. The proportion of $^{14}$C incorporated into labelled bands within a lane was also quantified and used to calculate actual incorporation into metabolites using the total $^{14}$C that was applied to that lane.

Quantification of neutral sugars

Total sugars were extracted using the ethanol method as described above. The quantity of Glc, Fru, and Suc in extracts was determined according to Hill et al. (2003).

Results

Total fatty acid and TAG content in developing embryos

To quantify changes in lipid content during seed maturation in oilseed rape (B. napus L.), a combination of fatty acid methyl ester (FAMES) and non-invasive, nuclear-magnetic resonance (NMR) analyses from the early-oil to mature-seed stages has been used (Fig. 1). Measurements of lipid content estimated by FAMES and NMR were in close agreement. Values per embryo increased steadily from the early-oil stage to peak at the desiccating stage and then decreased by 10–14%, depending on the analytical method, to reach a final level of about 1.9 mg embryo$^{-1}$ in the mature seed, representing about 48% of total fresh weight. Because the NMR measurements reflect liquid oil (i.e. TAG), and the proportion of lipid that was extracted as TAG was unchanged between the desiccating and mature stages (data not shown), the vast majority of the decrease in seed lipid content is accounted for by a decrease in TAG.

Activity and protein abundance of enzymes of fatty acid catabolism

To study changes in the capacity for catabolism and turnover of fatty acids during embryo development and seedling germination, the activities and protein abundance of the enzymes of β-oxidation, the glyoxylate cycle, and gluconeogenesis were measured (Fig. 2). Activities of PEPCK, MS, ICL, 3-ketoacyl-CoA thiolase (KAT), and the multi-functional enzyme (MFP) of β-oxidation were detectable at all stages of embryo development with maximum activities at the desiccating- or mature-embryo stages (Fig. 2a–e).
activities of the two enzymes of the MFP—L3 hydroxyacyl-CoA dehydrogenase (HD) and enoyl hydratase (EH)—peaked at the desiccating and mature stages, respectively, with 5–20-fold higher activities than those of MS, ICL, KAT, or PEPCK during embryo development. These enzyme activities were compared with those in germinating cotyledons at 1–7 d after imbibition (DAI), in which fatty acids are being degraded. The activities of PEPCK, MS, ICL, and KAT rose from mature seed values, peaked at 3 DAI, and then declined steadily. By contrast, the EH activity
was highest in the mature seed and then dropped by 62% in 1 DAI cotyledons to a stable level for the remaining germination period. The activity of HD was relatively unchanged during germination.

To examine whether changes in enzyme activities during development were reflected in the abundance of the enzyme proteins, specific antibodies raised against MS, ICL, PEPCK, and KAT were used to probe immunoblots of embryo proteins (Fig. 2f). For the glyoxylate cycle enzymes, MS and ICL, the proteins were first detected at desiccating and mid-oil stages, respectively, and their abundance was maintained through to maturation. The KAT and PEPCK proteins were present throughout embryo development from the pre-oil stage to the mature embryo stage and although the abundance of both increased as the embryos progressed from oil synthesis towards maturity, for KAT there was a decrease from pre- to mid-oil stages. Therefore, with KAT as an exception to the pattern, there was a positive correlation between protein abundance and activity for ICL, MS, and PEPCK during embryo development.

The developing embryo consists of cotyledons, which are the main storage tissues and therefore the major site of TAG accumulation on a whole embryo basis, and a hypocotyl. To determine whether the activities of enzymes associated with fatty acid degradation were attributable to one or both tissues, the distribution of MS, ICL, and PEPCK activities between cotyledons and hypocotyls of desiccating and mature seeds was determined (Table 1). The cotyledons of these embryos contained 72–92% of the whole embryo activity of each of the three enzymes, colo-locating the majority of the activity of these fatty acid-degrading enzymes with the majority of TAG storage. For desiccating embryos, the cotyledons represented 82±2% (mean ± SE, n=3) of the embryo fresh weight and there was therefore no enrichment of enzyme activity in either part of the embryo on a per unit tissue basis. The enzyme activity distributions for developing and mature embryos were comparable to those during early seedling growth where the enzyme activities in 3 DAI cotyledons represented at least 94% of that in whole seedlings.

### Fatty acid catabolism in desiccating embryos

To determine the metabolic fates of fatty acids in embryos, [1-14C]decanoic acid was supplied to isolated embryos at the desiccating stage, where the activities of β-oxidation and glyoxylate cycle enzymes and PEPCK were relatively high (Fig. 2) and embryos were still easily dissected from the seeds. Decanoic acid is water-soluble and permeates intact tissues, enabling its use as a tracer of fatty acid metabolism. The partitioning of radioactivity into lipids and aqueous metabolites, identified by TLC, was measured over 6 h. The radioactivity in lipids excludes 14C in free fatty acids which is presumed to be largely as decanoate. The radioactivity in the aqueous fraction is that in ethanolic extracts less that in fatty acids. After 1 h, 90% of the decanoate had been taken up by the embryos and by 6 h virtually none was present in the incubation medium (Fig. 3a). The 14C content of the free

### Table 1. Distribution of enzyme activity between cotyledons and radicles

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Proportion of enzyme activity in the cotyledons (% of whole embryo/seedling)</th>
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</thead>
<tbody>
<tr>
<td>Desiccating embryo</td>
<td>MS 92 ICL 82 PEPCK 80</td>
</tr>
<tr>
<td>Mature embryo</td>
<td>89 72 81</td>
</tr>
<tr>
<td>3 DAI seedling</td>
<td>95 94 94</td>
</tr>
</tbody>
</table>

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Fig. 3. Metabolism of [1-14C]decanoic acid by desiccating embryos. Embryos were incubated with 0.1 mM [1-14C]decanoic acid for up to 6 h. (a) Utilization of decanoate from the incubation medium. (b) Distribution of 14C-label into the aqueous (Aq), organic (lipids less free fatty acids) (Lipid less FFA), and free fatty acid (FFA) fractions (see Materials and methods) and the estimated CO2 release (see Results). Values are expressed as nmol of decanoate (C10:0) equivalent embryo⁻¹ and are the mean ± SE of measurements made on three separate incubations.
fatty acid fraction changed little up to 1 h, but declined thereafter (Fig. 3b). Most of the decanoate was metabolized into compounds that appeared in the aqueous fraction, for which the $^{14}$C content increased steadily up to 1 h, remained constant until 3 h and then declined. The increase in $^{14}$C content of the aqueous fraction up to 1 h was largely accounted for by increases in organic and amino acids, principally in Glu, malate, and Asp (Fig. 4). Label was also incorporated into citrate an unidentified compound that co-migrated with a band that stained as an amino acid (Figs 4, 5a, Unk aa). Based upon the migration of unlabelled standards on the TLCs, the unassigned bands were not isocitrate, 2-oxoglutarate, fumarate, oxaloacetate, glyoxylate, lactate, pyruvate, phosphoenolpyruvate, Glc-6-P, Fru-6-P, or 2- or 3-phosphoglycerate. The $^{14}$C in Glu represented about 40% of the total label in the aqueous fraction between 1 h and 6 h. In contrast to the steady rise in the $^{14}$C content of malate, the label in citrate increased up to 10 min, changed little up to 40 min, and then declined thereafter. Of all of the metabolites, the label in citrate was the most closely correlated ($r^2=0.88$) with that in the unmetabolized decanoate (free fatty acid fraction) in the embryos during the incubation, suggesting a close metabolic relationship between this metabolite and the decanoate carbon source (Fig. 6). A small proportion (<12%) of the $^{14}$C in the aqueous fraction was attributable to a band that co-migrated with succinate close to the solvent front (and was not clearly resolvable in all chromatograms) and to two unidentified bands (Fig. 5a). There was no measurable incorporation of $^{14}$C into sucrose, hexoses or the raffinose family of oligosaccharides (RFOs) at any stage of the incubations. The incorporation of $^{14}$C into the lipid fraction also increased during the first 40 min but then declined and remained at a low value (Fig. 3b). Loss of $^{14}$CO$_2$ was routinely determined, indirectly, as the difference between the label added to an incubation, and the total label recovered in the incubation medium plus that in the extracted fractions. In separate experiments it was shown that this estimated CO$_2$ release was the same as directly-measured $^{14}$CO$_2$ release.

![Graph showing distributions of label in various metabolites over time](image)

**Fig. 4.** Aqueous metabolites formed following incubation of desiccating-stage embryos with [1-$^{14}$C]decanoic acid. Metabolites in the aqueous fraction (Fig. 2) were resolved by TLC and the $^{14}$C content was determined as described in the Materials and methods. Incorporation of label into malate (Mal), citrate (Cit), Glu, Asp, an unidentified amino acid (Unk aa), and two other unidentified compounds (Unk 1 and 2) was recorded. Values are expressed as nmol of decanoate (C10:0) equivalent embryo$^{-1}$ and are the mean ±SE of measurements made on three separate incubations.

![TLC separation of metabolites](image)

**Fig. 5.** Metabolism of decanoate into aqueous metabolites and complex lipids by embryos differs with developmental stage. Ethanollic extracts were prepared from (E) early-oil and (D) desiccating stage embryos that had been incubated with [1-$^{14}$C]decanoate for 10 min and subjected to TLC. (a) Separation of metabolites using a cellulose TLC plate (with the upper phase of 6:2:4 (v/v) ethyl acetate:acetic acid:water). Labelled components were identified by co-migration with standards that were radiolabelled or using unlabelled standards in combination with stains for organic and amino acids. Abbreviations for metabolites are as described in Fig. 3. The asterisked arrow indicates the position of Unk 2 which appears after more than 10 min. The lipid fraction runs with the solvent front in this system. (b) Separation of complex lipids using a silica TLC plate. TAG, triacylglycerol; FFA, free fatty acid; DAG, diacylglycerol (two bands); MAG, monoacylglycerol; and two asterisks, a band migrating in the position of fatty alcohols. The origin consisted of metabolites that did not resolve on this solvent system.
over a 6 h incubation (data not shown). The estimated total release of $^{14}\text{CO}_2$ increased throughout the incubation and accounted for 43% of the total $^{14}$C metabolized after 6 h (Fig. 3b). The rate of production of $^{14}\text{CO}_2$ decreased after the first hour of incubation. Between 1 h and 6 h, the estimated total $^{14}$CO$_2$ released was equal to the sum of the $^{14}$C-decanoate metabolized from the medium (Fig. 3a) plus the $^{14}$C lost from Glu, Asp, and malate (Fig. 4).

**Decanoate metabolism changes during embryo development**

To assess whether the nature of fatty acid catabolism changes during embryo development, metabolism of $^{14}$C-decanoate was compared in embryos at the early-oil and desiccating stages. Incubations were carried out for 10 min, which was within the linear phase of increase in $^{14}$C content of the aqueous fraction (Fig. 3b, data not shown for early-oil stage embryos). The rate of incorporation of $^{14}$C from decanoate into the lipid fraction was 75% lower in desiccating embryos than in embryos at the early-oil stage, while incorporation into aqueous metabolites was 20% higher in embryos at the desiccating stage (Table 2). These changes are similar to those reported for acetate metabolism by developing *B. napus* embryos (Chia and Rawsthorne, 2000) and represent a shift of over 5-fold in the partitioning of decanoate carbon from lipid synthesis to degradation as the embryo matured (A/O ratio, Table 2). The increase in relative and absolute fluxes of carbon from decanoate into the aqueous fraction was accompanied by a change in the metabolites labelled in this fraction (Fig. 5a). Whereas in early-oil stage embryos most of the label was in Glu, citrate, and an unidentified metabolite, in desiccating embryos Glu, malate, citrate, and Asp were the major labelled compounds (Table 3). There was no evidence of incorporation of carbon from decanoate into sucrose or hexoses by embryos at the early-oil stage. The loss of $^{14}$C as CO$_2$ was very similar at both developmental stages (Table 2).

To identify the neutral lipid classes that contained carbon from decanoate, a sample of the ethanolic fraction, which contained both aqueous compounds and lipids, was separated on a silica TLC plate (Fig. 5b). Extracts from both early-oil and desiccating stage embryos contained an average of 20% of the $^{14}$C-label as free fatty acids, most of which is assumed to be unmetabolized decanoate based upon the rate of increase in label in this fraction (Fig. 3). Both extracts also contained smaller proportions of label in TAG and a band that co-migrated with fatty alcohols. The major difference in the lipid fraction between the two embryo stages was the presence of labelled diacylglycerol (DAG) (sn-1, 3) at the early-oil but not the desiccating stage, and more label in monoacylglycerol (MAG) at the desiccating than the early-oil stage.

**Table 2. Decanoate metabolism by isolated embryos at pre-oil and desiccating stages**

<table>
<thead>
<tr>
<th>Embryo stage</th>
<th>Incorporation of carbon from decanoate (nmol embryo$^{-1}$ min$^{-1}$)</th>
<th>CO$_2$</th>
<th>Aquous (A)</th>
<th>Organic (O)</th>
<th>A/O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early-oil</td>
<td>2.66±0.17</td>
<td>12.3±1.53</td>
<td>16.8±0.66</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Desiccating</td>
<td>2.66±0.17</td>
<td>15.7±1.16</td>
<td>4.17±0.83</td>
<td>3.8</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3. Incorporation of carbon from decanoate into aqueous metabolites by isolated embryos at early-oil and desiccating stages**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Relative $^{14}$C content (% of total aqueous counts)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Early-oil embryo stage</td>
</tr>
<tr>
<td>Citrate</td>
<td>27±6</td>
</tr>
<tr>
<td>Malate</td>
<td>nd</td>
</tr>
<tr>
<td>Aspartate</td>
<td>nd</td>
</tr>
<tr>
<td>Glutamate</td>
<td>42±8</td>
</tr>
<tr>
<td>Unknown amino acid</td>
<td>nd</td>
</tr>
<tr>
<td>Unknown 1</td>
<td>31±5</td>
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</tbody>
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Fig. 6. Label in citrate is correlated with that in the free fatty acid fraction during incubation of isolated embryos with [1-$^{14}$C]decanoic acid. The $^{14}$C content of citrate (closed circles) or malate (open circles) (data from Fig. 3) is compared with that in the free fatty acid (FFA) fraction (data from Fig. 2b). The values were subjected to linear regression analysis. Citrate and FFA were positively correlated ($r^2=0.88$) while there was no correlation between malate and FFA.
Sugar content of developing embryos

To examine whether the change in carbon flux from lipid synthesis to breakdown during development was related to a change in the internal soluble sugar pool, the concentration of neutral sugars in the embryos was measured throughout development and at maturity. The total content of Suc, Glc, and Fru increased from 538±65 nmol hexose equivalents embryo \(^{-1}\) at the early-oil stage to an average of 783±120 nmol hexose embryo \(^{-1}\) at the mid-oil stage. There was no significant change from the mid-oil stage to maturity. When expressed as hexose equivalents, the content of Glc plus Fru was 5% of the Suc content at the early-oil stage and remained at 1% thereafter.

Discussion

The results show unambiguously that there is a loss of at least 10% of storage lipid from *B. napus* embryos during the desiccation stage of development. It is also shown that the activities of enzymes of the glyoxylate cycle (MS and ICL), \(\beta\)-oxidation (KAT, HD, and EH), and gluconeogenesis (PEPCK), are detectable during the development of the *B. napus* embryo, including the period of oil accumulation, and that they increase as the embryo desiccates and matures. The increases in MS, ICL, and PEPCK activity were correlated with increases in the abundance of their respective proteins. These activities were localized predominantly in the cotyledons, the major site of fatty acid synthesis and lipid accumulation in the embryo. Unlike those of EH and HD, the activities of MS, ICL, KAT, and PEPCK were much lower during embryonic development than during germination and early seedling growth (Fig. 2). However, they were within exactly the same range as those of acetyl-CoA carboxylase and plastidial pyruvate dehydrogenase, which are involved in the supply of carbon for fatty acid synthesis during embryonic development (Eastmond and Rawsthorne, 2000). Moreover, they were comparable to, or much greater than, the maximum rate of oil accumulation in vivo (1.3 nmol acetate min \(^{-1}\) embryo \(^{-1}\); Eastmond and Rawsthorne, 2000). The capacity for glyoxylate cycle, \(\beta\)-oxidation and gluconeogenesis activities is therefore sufficient for these pathways to play important roles in fatty acid metabolism in developing embryos. These results extend the significance of previous observations of the presence, at much lower levels than during germination, of glyoxylate cycle enzyme activities and mRNAs in developing *B. napus* embryos (Comai et al., 1989; Ettinger and Harada, 1990; Eccleston and Ohrogge, 1998), and the indirect evidence for the presence of the \(\beta\)-oxidation pathway in developing arabidopsis seeds (Poirier et al., 1999; Moire et al., 2004).

To discover the extent and nature of fatty acid degradation during development, the metabolism of \([1\text{-}^{14}\text{C}]\)decanoate by isolated embryos was studied at two developmental stages. The use of \([1\text{-}^{14}\text{C}]\)decanoate rather than \(^{14}\text{C}\)-acetate ensured that label entered embryo metabolism as acetyl-CoA that was generated in the peroxisome. The metabolism that was traced is therefore fully representative of the fate of acetate units that would be derived from endogenous C-18 acyl precursors that enter peroxisomal \(\beta\)-oxidation. Over a 6 h period up to 41% of the label in decanoate was released as CO\(_2\). It is proposed that a peroxisomal \(\beta\)-oxidation/mitochondrial partial TCA cycle pathway, operating as part of normal metabolism in the developing *B. napus* embryo, could account for this CO\(_2\) release. The reasons are as follows. First, degradation of fatty acids to acetyl-CoA is highly likely to be in the peroxisome: the enzymes of \(\beta\)-oxidation encoded in the arabidopsis genome are predicted to be exclusively located in the peroxisome and mutant analysis shows that peroxisomal KAT is essential for fatty acid degradation (Germain et al., 2001). Second, citrate is labelled early during \([1\text{-}^{14}\text{C}]\)decanoate metabolism. This is consistent with the rapid conversion of acetyl-CoA to citrate in the peroxisome. Third, about 40% of the label metabolized by the isolated embryos was released as CO\(_2\) during the first hour of incubation. This is not consistent with the much smaller proportion (approximately 20%) that would be expected for metabolism of \([1\text{-}^{14}\text{C}]\)labelled acetate units derived from decanoate if metabolism were predominantly through PEPCK (Canvin and Beevers, 1961). Moreover, when isolated embryos from either developmental stage were incubated with \([1\text{-}^{14}\text{C}]\)acetate, at least 53% of the label was recovered in CO\(_2\) after 1 h (data not shown). The figure of 40% that has been obtained with decanoate is, however, very similar to the value for CO\(_2\) release by germinating *icl* mutant arabidopsis seedlings (Eastmond et al., 2000), in which a cycle that links \(\beta\)-oxidation to the TCA cycle is proposed as the major metabolic fate of lipid degradation (Eastmond and Graham, 2001). This pathway (Fig. 7) has also been proposed to operate during lipid catabolism in germinating lettuce and sunflower seedlings (Salon et al., 1988; Raymond et al., 1992). Some flux to PEP through PEPCK might also occur and contribute to the CO\(_2\) release from decanoate by developing *B. napus* embryos. While this cannot be ruled out, it is unlikely in the early-oil stage embryos because of the very low activities of MS and ICL (see below), enzymes that would be required to sustain the supply of oxaloacetate for peroxisomal citrate synthesis.

In this proposed pathway, malate from the oxidation of citrate in the mitochondrion is returned to the peroxisome (although it is possible that this may occur as oxaloacetate) to produce oxaloacetate as an acceptor for the acetyl-CoA produced by \(\beta\)-oxidation (Fig. 7; solid lines). This may happen in developing *B. napus* embryos, but oxaloacetate could also be derived from citrate via the glyoxylate cycle (Fig. 7; dotted lines). Given these two possibilities, it is proposed, based on two lines of evidence, that the main route for the generation of peroxisomal oxaloacetate may change during development. First, the activities of ICL and
MS are very low during the early stages of oil accumulation but then rise, notably during desiccation. Second, labelling of malate and Asp (derived from oxaloacetate) is undetectable at the early-oil stage, but is significant during desiccation, consistent with the idea that the location and pathway of their labelling may change.

The origin of label in Glu during incubations of early-oil and desiccating embryos is unclear. It may come from 2-oxoglutarate in the mitochondria. However, in early-oil stage embryos, the kinetics of labelling of Glu were similar to those of citrate, while malate (downstream of 2-oxoglutarate in the mitochondrial TCA cycle) did not become labelled. This suggests that Glu may be labelled from citrate metabolism in the cytosol via cytosolic aconitase (Courtois-Verniquet and Douce, 1993; Hayashi et al., 1995) and cytosolic NADP-isocitrate dehydrogenase (Galvez and Gadal, 1995) to yield 2-oxoglutarate for transamination to Glu (Fig. 7; dashed lines). Interestingly, Glu and CO2 are major metabolic products during the metabolism of labelled fatty acids by cotyledons of germinating lettuce (Salon et al., 1988). This suggests that these organs have a metabolism that is similar to that of developing B. napus embryos, in which lipid breakdown is linked to respiration/TCA cycle activity rather than gluconeogenesis (see below). Cornah and Smith (2002) have argued that, during storage lipid breakdown, the partitioning of carbon from fatty acids to either respiration or to the anaplerotic/gluconeogenic pathways reflects the metabolic demands of the tissue. For example, in the endosperm of germinating castor seeds, anaplerotic metabolism of fatty acids to sucrose predominates, with little respiration occurring (Beevers, 1961; Canvin and Beevers, 1961). This reflects the role that the endosperm plays in the metabolism of stored lipid to sucrose, which is then transported and utilized elsewhere. However, in developing B. napus embryos and germinating lettuce and sunflower seedlings the carbon from fatty acid degradation is clearly used within the same tissue, removing the need for gluconeogenic metabolism and net sucrose synthesis.

The presence of PEPCK could indicate a capacity for gluconeogenesis. However, no evidence of gluconeogenic metabolism was found in the B. napus embryos (Fig. 7; grey pathway). There was no incorporation of 14C from decanoate into sucrose or hexoses at either the early-oil or the desiccating embryo stages, or into the RFOs that are synthesized in embryos of the Brassicaceae during desiccation (Leprince et al., 1990; Bentsink et al., 2000). As suggested by Leegood and Walker (2003), PEPCK may be involved in amino acid catabolism and dissimilation of organic acids in developing seeds and in other organs rather

![Fig. 7. Proposed pathways of fatty acid catabolism during embryo development in B. napus. The pathway of fatty acid oxidation via a peroxisomal/mitochondrial tricarboxylic acid cycle that occurs at the early-oil synthesis stage is shown by solid lines. Movement of carbon as malate from the mitochondrion to the peroxisome could also occur via oxaloacetate. The synthesis of glutamate by cytosolic aconitase (3) and isocitrate dehydrogenase (4) is shown in dashed lines. During embryo desiccation, the glyoxylate cycle (dotted lines), involving ICL (5) and MS (6), also operates. The presence of PEPCK (8) provides the potential for gluconeogenic metabolism of carbon derived from fatty acid oxidation to glycolytic intermediates and potentially sugars (grey pathway). However, metabolite labelling patterns, when embryos were supplied 14C-decanoate sugars, did not reveal the synthesis of sugars at either early-oil or desiccating stages of embryo development. Other numbered metabolic steps are: 1, fatty acid β-oxidation; 2, peroxisomal citrate synthase.](image-url)
than in gluconeogenesis to form sucrose. It has been reported that gluconeogenic flux from acetate to sucrose occurs in embryos of transgenic lines of B. napus expressing a medium chain thioesterase (MCTE) enzyme that leads to the synthesis of lauric acid (Eccleston and Ohlrogge, 1998). In these embryos, the incorporation of label from 14C-acetate was predominantly into malate and sucrose. This result differs from our own data, and may be explained by elevated activities of MS and ICL, and hence a greater capacity for the glyoxylate cycle in the MCTE-expressing embryos (Eccleston and Ohlrogge, 1998).

These studies have provided two independent lines of evidence that the turnover of fatty acids is a normal part of embryo metabolism in developing B. napus embryos. The first is that TAG is degraded during seed desiccation and maturation (Fig. 1; and see data presented in Norton and Harris, 1975; Murphy and Cummins, 1989; Kang et al., 1994; Eastmond and Rawsthorne, 2000). Second, during the time-course of 14C-decanoate metabolism by desiccating embryos, label accumulated transiently in complex lipids (i.e. MAG, DAG, TAG), suggesting that they were being turned over. Degradation of TAG during desiccation of the seed may be required to support continued metabolism after the import of carbon from the maternal tissues ceases. During the maturation of arabidopsis embryos, trophic connections between the seed and mother plant are lost before the end of metabolic activity (Baud et al., 2002). However, the synthesis of storage proteins, late embryogenesis abundant proteins (Cuming, 1999), and other metabolisms associated with seed maturation continues into desiccation. These processes require carbon sources for the production of energy and for carbon skeletons. The oilseed embryo does not have a starch reserve by this stage of development (da Silva et al., 1997; Baud et al., 2002) and it is shown here that the sucrose content remains unchanged from mid-oil synthesis onwards. Preserving the sucrose content may be important for supporting metabolism in the embryo prior to the breakdown of fatty acids during germination. Metabolism late in embryo development therefore resembles that of a carbon-starved tissue in which fatty acid turnover can become activated (Eastmond and Graham, 2001). The loss of lipid reported here for maturing B. napus embryos is also evident in other oilseed species (McKillikan, 1966; Gurr et al., 1972; Norton and Harris, 1975; Murphy and Cummins, 1989; Baud et al., 2002; Tomlinson et al., 2004). Whether the larger decreases are a direct result of growth in controlled conditions and/or cultivar/species differences requires further study.

Fatty acid degradation during the main period of oil accumulation in the developing embryo may play a ‘housekeeping’ function in the turnover of membrane lipids or in the regulation of cellular acyl-CoA levels. However, it is surprising that a major fate of decanoate entering fatty acid degradation in the oil-accumulating B. napus embryo was CO2 loss, probably as respiration, rather than entry into an anaplerotic pathway in order to conserve carbon. The measurements from this study illustrate what may happen if endogenous acyl chains enter peroxisomal β-oxidation. Others have shown that β-oxidation does occur during seed development, and more widely in plant tissues, implying a more general role than in lipid degradation during germination and seedling establishment (Mittendorf et al., 1999; Poirier et al., 1999; Richmond and Bleecker, 1999; Moire et al., 2004). The importance of β-oxidation during seed development is revealed by the kat2 mutation in arabidopsis which causes loss of KAT activity and approximately 30% less lipid in the seeds (Germain et al., 2001). The kat2 and WT lines have similar overall plant growth and morphology (Germain et al., 2001), and the decreased oil phenotype therefore appears to be determined by changes in the developing seeds rather than by changes in plant growth.

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