Plant Physiology, March 2000, Vol. 122, pp. 767–774, www.plantphysiol.org © 2000 American Society of Plant Physiologists

Coordinate Changes in Carbon Partitioning and Plastidial Metabolism during the Development of Oilseed Rape Embryos[1]

Peter J. Eastmond[2] and Stephen Rawsthorne*

John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, United Kingdom

Measurements of metabolic fluxes in whole embryos and isolated plastids have revealed major changes in the pathways of carbon utilization during cotyledon filling by oilseed rape (*Brassica napus* L.) embryos. In the early cotyledon stage (stage A), embryos used sucrose (Suc) predominantly for starch synthesis. Plastids isolated from these embryos imported glucose-6-phosphate (Glc-6-P) and partitioned it to starch and fatty acids synthesis and to the oxidative pentose phosphate pathway in the ratio of 2:1:1 on a hexose basis. Of the substrates tested, Glc-6-P gave the highest rates of fatty acid synthesis by the plastids and pyruvate was used weakly. By the mid- to late-cotyledon stage (stage C), oil accumulation by the embryos was rapid, as was their utilization of Suc for oil synthesis in vitro. Plastids from C-stage embryos differed markedly from those of stage-A embryos: (a) pyruvate uptake and utilization for fatty acid synthesis increased by respectively 18- and 25-fold; (b) Glc-6-P partitioning was predominantly to the oxidative pentose phosphate pathway (respective ratios of 1:1:3); and (c) the rate of plastidial fatty acid synthesis more than doubled. This increased rate of fatty synthesis was dependent upon the increase in pyruvate uptake and was mediated through the induction of a saturable transporter activity.

The temporal separation of storage product accumulation (starch, oil, and protein) has been reported previously for developing embryos of oilseed rape (*Brassica napus* L.) (Murphy and Cummins, 1989; da Silva et al., 1997). In particular, there is a shift from starch to oil accumulation during the early stages of cotyledon filling (da Silva et al., 1997). The factors that contribute to such changes in metabolism and the consequent partitioning of imported carbon in the developing embryo are not understood. Previous studies of plastids isolated from developing embryos of oilseed rape that accumulate both starch and storage oil have revealed that Glc-6-P is imported by the plastids for starch synthesis, and that Glc-6-P and pyruvate support the highest rates of fatty acid synthesis (Kang and Rawsthorne, 1994). When both Glc-6-P and pyruvate were supplied together, the in vitro rate of fatty acid synthesis by the plastid preparations increased as a result of the direct effect of increased carbon incorporation from both substrates and of the indirect effect of a Glc-6-P-dependent stimulation of pyruvate incorporation, presumably through the activity of the oxidative pentose phosphate pathway (OPPP) (Kang and Rawsthorne, 1996).

The ability of plastids from heterotrophic tissues to utilize exogenous substrates to support their biosynthetic and catabolic pathways is dependent on the activities of plastidial enzymes and, more importantly, on the presence of specific transporters on the plastid envelope (Emes and Neuhaus, 1997; Joyard et al., 1998; Rawsthorne et al., 1999). The study of metabolite uptake by plastids from storage tissues has focused on those from starch-accumulating organs, where it is believed that the import of carbon is facilitated by specific transporters for Glc-6-P, Glc-1-P, or ADP-Glc, depending upon the plant species and organ. In contrast, relatively little is known about the uptake of metabolites to support fatty acid synthesis. The activities of a Glc-6-P/inorganic phosphate (Pi) and a malate/Pi counter-exchange transporter have been reported for plastids isolated from oilseed rape embryos and castor endosperm, respectively (Eastmond et al., 1997; Eastmond and Rawsthorne, 1998).

Given that plastids from oilseed rape embryos utilize Glc-6-P and pyruvate, and that these substrates differentially supply starch and fatty acid synthesis, it is possible that changes in transporters or in intraplastidial metabolism could contribute to changes in the overall partitioning of carbon to starch and oil. To investigate this, we compared three stages of embryo development at which Suc entering the embryo goes (a) primarily to starch, (b) equally to starch and lipids, and (c) primarily into lipids. For each stage we have characterized the uptake and subsequent metabolism of Glc-6-P and pyruvate by isolated intact plastids.

MATERIALS AND METHODS

Chemicals, Radiochemicals, and Plant Material

Substrates, coupling enzymes, cofactors, and radiochemicals were as described in Kang and Rawsthorne (1994). Additional isotopes were from Amersham International (Bucks, UK) ([^{3}H]H_2O, D-[U-^{14}C]Suc) or NEN (DuPont, Herts, UK) (D-[U-^{14}C]sorbitol). Growth of oilseed rape (*Brassica napus* L. cv Topas) plants and harvesting of

[1] This work was supported by the Biotechnology and Biological Sciences Research Council through a Competitive Strategic Grant to the John Innes Centre and a postgraduate studentship to P.J.E.

[2] Present address: Department of Biology, University of York, P.O. Box 373, York YO10 5YW, UK.

* Corresponding author; e-mail steve.rawsthorne@bbsrc.ac.uk; fax 44–1–603–259882/456844.

developing embryos from siliques were as described by Kang and Rawsthorne (1994).

Determination of Starch, Oil, and Protein Content in Whole Embryos

Starch and total lipids were extracted from embryos and the amounts determined as described in Smith (1988) and Kang et al. (1994). To measure protein content, five embryos were homogenized in 1 mL of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)/NaOH, pH 7.4, using an all-glass homogenizer. The protein concentration in the homogenate was determined using the dye-binding assay (Bradford, 1976) with bovine serum albumin as the standard.

Incorporation of Suc into Starch, Lipids, and Protein by Isolated Embryos

Twenty embryos were incubated at 25°C in 100 μL of an incubation medium adapted from the tissue culture medium of Lichter (1982) in that hormones were excluded. The incubation was started by the addition of ^{14}C-Suc (50 MBq mmol^{-1}), and the contents were mixed by gentle agitation at regular intervals. Reactions were stopped by rapidly removing the incubation medium with three 1-mL washes of water and freezing the embryos in liquid nitrogen.

Starch

Starch was extracted from the frozen embryos and pelleted by centrifugation (Smith, 1988). Incorporation into starch was determined by measuring the ^{14}C in pellets that was labile to α-amylase and amyloglucosidase digestion (Smith, 1988).

Lipids

Saponified total lipid was extracted from frozen embryos (Kang et al., 1994) and the ^{14}C in fatty acids determined (Kang and Rawsthorne, 1994).

Protein

Frozen embryos were homogenized as described above for protein content. Protein was extracted by trichloroacetic acid precipitation (Hames, 1981), the pellets re-suspended in 50 μL of 0.1 M NaOH, and the ^{14}C content determined.

Preparation of Isolated Plastids, Enzyme Assays and Subcellular Distribution, and Uptake and Utilization of Metabolites

Preparation of isolated plastids from embryos and the measurement of enzyme activities were according to the methods described in da Silva et al. (1997) and Kang and Rawsthorne (1994), respectively. Determination of the activities of plastidial isoforms of enzymes was as described previously (Denyer and Smith, 1988; da Silva et al., 1997).

Feeding of ^{14}C-labeled metabolites, determination of the incorporation of carbon into starch and fatty acids, and measurement of the activity of the OPPP were all according to the method of Kang and Rawsthorne (1996). The uptake of metabolites was determined using the silicone oil centrifugation technique (Heldt and Sauer, 1971) as described by Eastmond and Rawsthorne (1998). Rates of uptake and utilization through the various pathways were linear within the time period of measurement. Uptake attributable to mitochondrial contamination of plastid preparations was measured using the procedures described above for the determination of isoform distribution. Unless otherwise stated, all rates were determined at a saturating substrate concentration. Rates of uptake or incorporation of carbon by lysed plastids were less than 5% of those obtained using intact plastids.

Activities are expressed per unit (micromoles per minute) of NADP-GAPDH activity in the same extract/preparation. Where appropriate, rates of incorporation of carbon into starch or fatty acids by isolated plastids have been converted from a per unit NADP-GAPDH basis to a per embryo basis (or vice versa) using the respective measurements of the activity of NADP-GAPDH per embryo and the intactness of the plastid preparations at each stage. Rates of metabolite uptake were corrected using the same principle, except the recovery of plastid marker enzyme in the post-centrifugation pellet was substituted for the intactness of the plastids. For embryos at stages A, B, and C, the activity of NADP-GAPDH was 11, 19, and 20 nmol embryo^{-1} min^{-1}. For plastid preparations from embryos at stages A, B, and C, the proportions of the organelles that were intact at the end of a 1-h incubation at 25°C were 51%, 48%, and 42%, respectively. The proportions of NADP-GAPDH activity present in the pellet following silicone oil centrifugation were 48%, 44%, and 41% for plastids from stages A, B, and C, respectively.

Determination of Metabolite Contents in Whole Embryos

All metabolites were extracted from 20 embryos according to the method of Jelitto et al. (1992), and their amounts were measured enzymatically following standard methods (acetate: Beutler, 1984; Glc-6-P and dihydroxyacetone phosphate [DHAP]: Stitt et al., 1989; malate and pyruvate: Smith et al., 1992).

RESULTS

Characterization of Carbon Partitioning in Developing Embryos

The total lipid, starch, and protein content of developing embryos was measured in embryos harvested from a single group of plants (Fig. 1). The patterns of deposition resembled those published previously for the individually measured products in oilseed rape (Murphy and Cummins, 1989; Kang et al., 1994; da Silva et al., 1997). From these measurements, three stages of development were chosen for further study. At the first stage (stage A), embryos were accumulating primarily starch and oil accumulation was

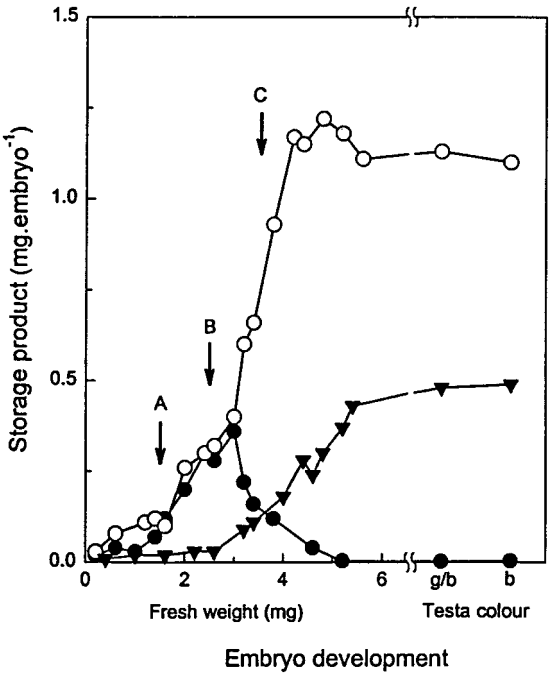

Figure 1. Accumulation of starch, oil, and protein during development of embryos of oilseed rape. Embryos were removed from testas and the content of each product determined. Oil was measured as total lipid classes. Development is expressed on the basis of increasing fresh weight of the embryo until the onset of seed desiccation. After this, testa color was used to provide a phenotypic scale (g/b, testa green with some browning; b, testa dark brown). Each data point represents a value obtained from a batch of five embryos. Developmental stages (A, B, and C) selected for detailed analysis are indicated by arrows. ○, Lipid; ●, starch; ▼, protein.

starting. At the second stage (stage B), the starch content was reaching its maximum and the oil content was rising rapidly. At the third stage (stage C), the oil content was still rising rapidly and protein accumulation had started, but now starch degradation was occurring. Stages A, B, and C correspond to early, early-to-mid, and mid-to-late cotyledon filling, respectively, approximating to 23, 32, and 41 d after anthesis (da Silva et al., 1997).

To define the short-term patterns of carbon partitioning at the three stages, ^{14}C-Suc was supplied to whole embryos dissected from their testas. The uptake of Suc was saturated at a concentration of 250 to 300 mM, and the rate of uptake increased from 35 to 70 nmol Suc embryo^{-1} h^{-1} between stages A and C (data not shown). The incorporation of carbon from Suc into total lipids, starch, and protein was saturated by a concentration of between 15 and 30 mM, and the rates were linear for 4 h following a short lag phase (data not shown). The rate of incorporation of carbon into starch was 4 to 5 nmol Suc embryo^{-1} h^{-1} at stages A and B, but declined by more than 55% by stage C (Fig. 2). In contrast, the rate of incorporation of carbon into fatty acids increased progressively from 2.4 to 12.1 nmol Suc embryo^{-1} h^{-1} over the three stages (Fig. 2). Incorporation of carbon into protein was relatively low in stages A and B, but increased to 4.9 nmol Suc embryo^{-1} h^{-1} by stage C (Fig. 2).

Substrate Utilization by Isolated Plastids

To determine whether the utilization of exogenous metabolites for fatty acid synthesis changed during development, plastids were isolated from A, B, and C stage embryos. The suitability of plastids isolated from these embryo stages for in vitro studies has been described previously (da Silva et al., 1997). Isolated plastids were incubated with ^{14}C-labeled Glc-6-P, DHAP, malate, pyruvate, or acetate, and the rate of incorporation of carbon into fatty acids determined (Fig. 3). For plastids from embryos at stages A and B, the highest rates of incorporation of carbon into fatty acids were from Glc-6-P and were approximately 0.45 μmol acetate unit^{-1} GAPDH h^{-1}. However, by stage C the rate of incorporation of carbon from pyruvate had increased markedly to about three times this value. Indeed, at stage C the rate of incorporation of carbon from pyruvate into fatty acids was more than 4-fold greater than that from Glc-6-P, DHAP, malate, or acetate. The rates of incorporation of carbon from DHAP, malate, and acetate into fatty acids were consistently lower than those from Glc-6-P and pyruvate during the period of oil accumulation (i.e. stages B and C) (Fig. 3).

To investigate whether there was interaction between Glc-6-P and pyruvate metabolism during embryo development, the two metabolites were supplied individually or together at a 1 mM concentration to plastids prepared from embryos at all three stages. These substrate concentrations were saturating for fatty acid synthesis by the isolated plastids when supplied alone (not shown). When substrates were supplied together, either both were ^{14}C-labeled or ^{14}C-labeled Glc-6-P was supplied with unlabeled pyruvate and vice versa. The total rates of incorporation of carbon into fatty acids increased by 2.5-fold between stages A and C, and at all stages the total rates when the two substrates were fed together were greater than the rates obtained when either substrate was fed individually (Fig. 4). The rates of carbon incorporation from labeled Glc-6-P

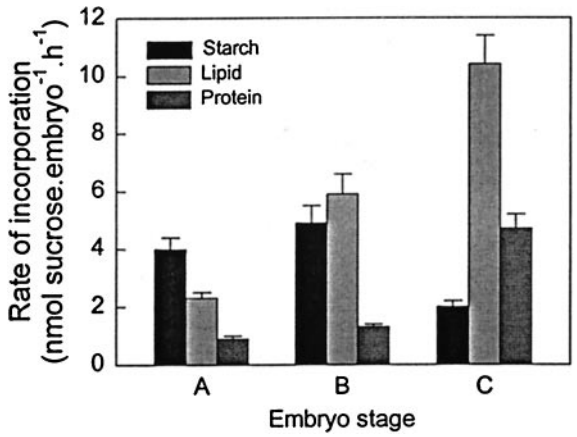

Figure 2. Incorporation of carbon from Suc into starch, lipids, and protein by developing embryos. The rates of incorporation of carbon from Suc (60 mM) into starch, total lipids, and protein were measured over the course of 4 h using embryos from stages A, B, and C. Each value represents the mean ± SE of measurements made on three separate batches of 20 embryos.

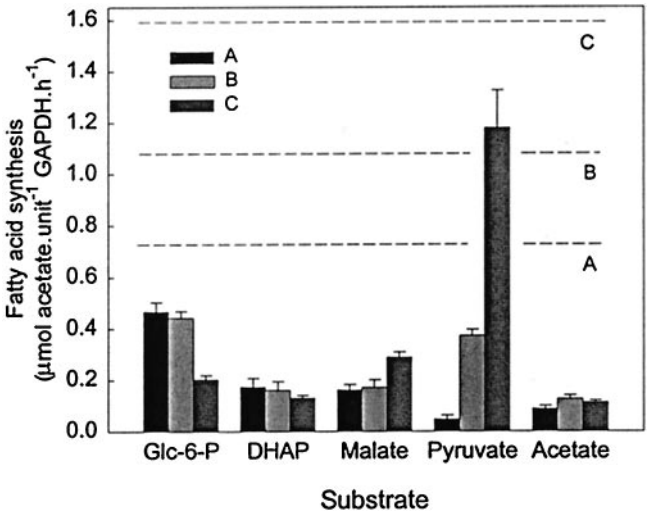

Figure 3. Incorporation of carbon from individual substrates into fatty acids by plastids from developing embryos. Plastids were incubated with each ^{14}C-labeled substrate (1 mM), and the incorporation into total saponifiable fatty acids was determined. Incorporation is expressed on an acetate-equivalent basis. Each value represents the mean ± SE of measurements made on three separate plastid preparations from embryos at stages A, B, and C. Horizontal dashed lines represent the rates of plastidial fatty acid synthesis that would be required to account for the measured in vivo rate of oil accumulation at stages A to C. These net rates of accumulation are 17, 44, and 78 nmol acetate embryo^{-1} h^{-1} at stages A, B, and C, respectively (Kang et al., 1994).

or pyruvate in the presence or absence of the other unlabeled substrate were not significantly different ($P > 0.05$). Therefore, there is little or no competition between Glc-6-P and pyruvate for incorporation into fatty acids throughout most of development. When converted to an embryo basis, the total rate of incorporation from Glc-6-P and pyruvate into fatty acids at all three stages was 70% to 89% of the activity required to account for the in vivo rates of oil accumulation (see Fig. 3).

In addition to providing carbon for fatty acid synthesis, Glc-6-P is also utilized for starch synthesis and the OPPP within the plastid (Kang and Rawsthorne, 1996). To study the partitioning of carbon from Glc-6-P during development, plastids from all three stages were incubated with 1 mM Glc-6-P and the simultaneous rates of incorporation of carbon into starch and fatty acids and the flux of carbon through the OPPP were measured (Fig. 5). Carbon was incorporated from Glc-6-P primarily into starch by plastids prepared from embryos at stages A and B. The absolute rates of incorporation of carbon into starch and fatty acids and CO_2 release from [1-^{14}C]Glc-6-P were similar at stages A and B, giving relative rates of approximately 2:1:1, respectively, when expressed on a hexose basis (Fig. 5). By stage C, the utilization via the OPPP had increased by 52%, while the rates of incorporation of carbon from Glc-6-P into starch and fatty acids had declined by 70% and 56%, respectively (Fig. 5). The relative rates of Glc-6-P supported starch synthesis, fatty acid synthesis, and OPPP activity therefore changed to approximately 1:1:3.

The partitioning of carbon from Glc-6-P between starch, fatty acids, and the OPPP by the plastids was also examined in the presence of 1 mM pyruvate. The addition of pyruvate to incubations made no difference in the rates of incorporation of carbon from Glc-6-P into starch and fatty acids in plastids at any stage, and no difference in the Glc-6-P flux through the OPPP at stage A (data not shown). However, at stages B and C, the addition of pyruvate stimulated OPPP activity by 12% and 24% ($P < 0.05$) (Fig. 5, stage C only).

Activities of Plastidial Transporters and Enzymes

The developmental changes in the utilization of substrates for fatty acids by the isolated plastids could be caused by changes in the activity of membrane transporters or plastidial enzymes, or by a combination of both. These plastidial activities were therefore measured at the three developmental stages. Between stages A and C, the activity of the plastidial Glc-6-P transporter decreased by 36% (Fig. 6a). In contrast, uptake of pyruvate at (1 mM) increased 19-fold during the same period (Fig. 6b). At stage A, pyruvate uptake increased linearly with respect to the substrate concentration, while at stages B and C the rate of uptake was saturated at 1 mM, with K_m values of about 0.20 mM. The proportion of pyruvate uptake that was attributable to mitochondrial contamination of the plastid preparations was 16% and 4% at stages B and C, respectively. When expressed on an embryo basis, the activities of the Glc-6-P and pyruvate transporters in stage C embryos were 39 and 77 nmol substrate embryo^{-1} h^{-1}, respectively, the latter

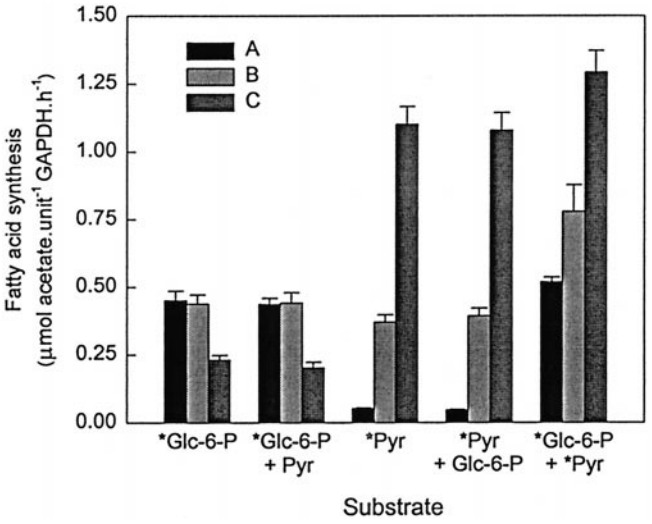

Figure 4. Incorporation of carbon from Glc-6-P and pyruvate into fatty acids by plastids from developing embryos. Plastids from embryos at stages A, B, and C were incubated with Glc-6-P and pyruvate (both at 1 mM) either individually or together. When both substrates were supplied simultaneously, either [^{14}C]Glc-6-P and ^{14}C-pyruvate were supplied together or [^{14}C]Glc-6-P was supplied with unlabeled pyruvate and vice versa. Labeled substrates are denoted by an asterisk. Incorporation is expressed on an acetate-equivalent basis. Each value represents the mean ± SE of measurements made on three separate plastid preparations.

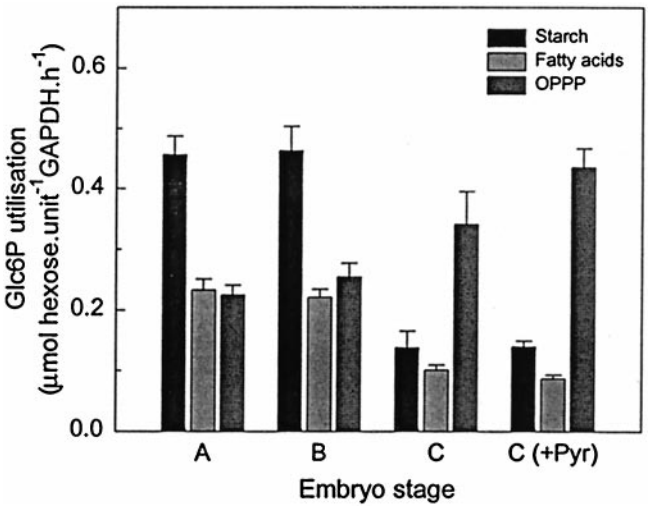

Figure 5. Utilization of Glc-6-P for starch and fatty acid synthesis and the OPPP by plastids from developing embryos. Plastids from embryos at stages A, B, and C were incubated with [1-^{14}C]Glc-6-P (1 mM). The effect of addition of unlabeled pyruvate (1 mM) at stage C is also shown. Metabolism of Glc-6-P is expressed on a hexose-equivalent basis. The activity of the OPPP was measured as release of $^{14}CO_2$. Net starch and fatty acid synthesis were measured as methanol/KCl-insoluble material and total saponifiable fatty acids, respectively. Each value represents the mean ± SE of measurements made on three separate plastid preparations.

rate being comparable to the net rate of oil synthesis in vivo.

Activities of all of the glycolytic enzymes were sufficient in both the cytosol and plastid to account for the net rates of oil synthesis (Table I). Between developmental stages A and B, the activities of the plastidial and cytosolic glycolytic enzymes increased by an average of 50% and 82%, respectively. Thereafter, the cytosolic activities and the majority of the plastidial activities remained relatively constant. However, the plastidial activities of aldolase, phosphoglycerate mutase, and enolase, which were low compared with those of the other glycolytic enzymes, decreased by 67% to 75% between stages B and C. The plastidial and mitochondrial activities of the pyruvate dehydrogenase complex changed relatively little during development, the former remaining well in excess of the net rate of oil synthesis in vivo. The total activity of acetyl-CoA carboxylase (ACCase) also changed relatively little during development (Table I). Initial studies revealed that approximately 90% of this ACCase activity was associated with the plastid at all three stages. At stages A and B, the estimated plastidial activity of ACCase is 1.5- to 2-fold greater than the net rates of oil synthesis in the developing embryo. However, at stage C, the plastidial activity of ACCase is only comparable to the net rate of oil synthesis.

Metabolite Content of the Developing Embryos

To provide an estimate of the concentrations of substrates for plastid uptake and metabolism in the embryo, the amounts of the metabolites used in the plastid feeding experiments were determined. The amount of each metabolite varied little during development and across all stages were 3.5 ± 0.2, 3.0 ± 0.3, 4.3 ± 0.3, 2.7 ± 0.3, and 1.0 ± 0.1 nmol per embryo for Glc-6-P, DHAP, malate, pyruvate, and acetate, respectively (mean ± SE of five replicate measurements at each stage). Assuming that a uniform distribution of these metabolites throughout the aqueous phase in the embryo would present the most conservative value, the estimated concentrations were all greater than 1 mM with the exception of acetate.

DISCUSSION

The changes in the profiles of starch and lipid accumulation in the developing embryo of oilseed rape are the result of a net increase in carbon flux to lipid and a net decrease into starch. These substantial changes in carbon flux are accompanied by changes in the metabolism of the plastids with respect to their ability to import metabolites through transporter proteins on the plastid envelope and in their complement of enzymes. Over the developmental

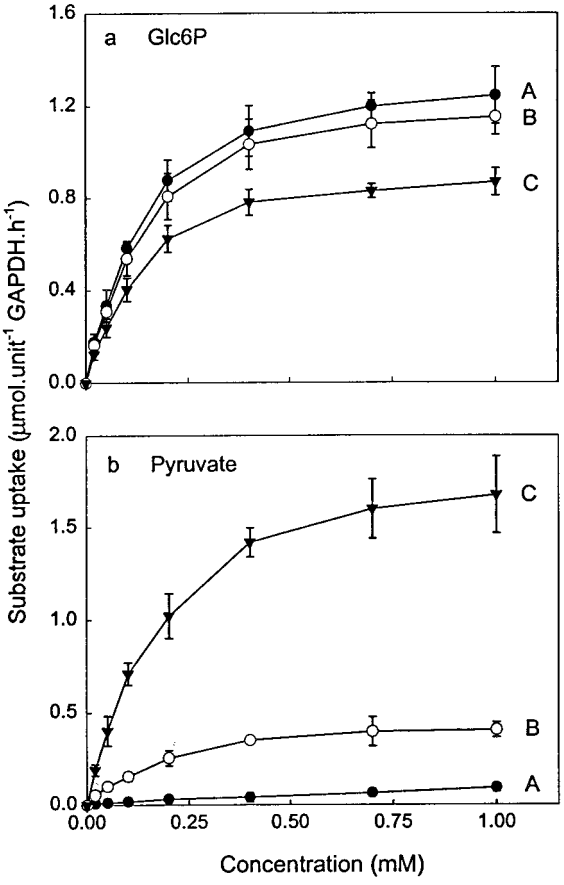

Figure 6. Concentration dependence of Glc-6-P and pyruvate uptake by plastids from developing embryos. Uptake of Glc-6-P (a) and pyruvate (b) by plastids isolated from embryos at stages A, B, and C was measured at a range of substrate concentrations. Uptake was measured using the silicone oil centrifugation technique. Each value represents the mean ± SE of measurements made on three separate plastid preparations.

Table I. *Activities of glycolytic enzymes, the pyruvate dehydrogenase complex, and ACCase in embryos at three stages in development*

The activities of enzymes were measured in total homogenates from embryos at stages A, B, and C. The amount of each enzyme activity in the plastid and the cytosol (or mitochondria in the case of pyruvate dehydrogenase) was determined using crude plastid preparations containing increasing amounts of the other subcellular compartment (da Silva et al., 1997). Each value is the mean ± SE of data from three separate plastid preparations, except for ACCase, which are data from two individual experiments.

Enzyme	Enzyme Activity					
	Stage A		Stage B		Stage C	
	Plastid	Cytosol	Plastid	Cytosol	Plastid	Cytosol
	nmol embryo^{-1} min^{-1}					
Phospho-Glc isomerase	19.7 ± 2.7	18.6 ± 1.4	32.0 ± 4.6	42.4 ± 3.9	32.5 ± 5.1	49.1 ± 1.9
ATP-phosphofructokinase	1.2 ± 0.2	2.2 ± 0.1	1.8 ± 0.4	2.8 ± 0.3	1.5 ± 0.2	1.4 ± 0.2
Aldolase	1.8 ± 0.3	0.9 ± 0.2	2.4 ± 0.4	1.6 ± 0.2	0.7 ± 0.5	1.4 ± 0.2
Triose-P isomerase	3.2 ± 0.3	6.2 ± 1.4	4.4 ± 0.7	11.4 ± 2.4	4.1 ± 0.6	30.6 ± 4.7
NAD-GAPDH	10.6 ± 1.1	78.2 ± 5.3	13.3 ± 1.2	151.1 ± 18.9	14.8 ± 2.5	134.7 ± 8.8
NADP-GAPDH	11.2 ± 1.8	0	18.5 ± 1.7	0	20.3 ± 1.2	0
Phosphoglycerate kinase	36.9 ± 3.7	56.1 ± 2.2	65.1 ± 4.4	142.1 ± 9.8	77.0 ± 4.9	94.9 ± 3.4
Phosphoglycerate mutase	4.3 ± 0.7	65.5 ± 1.2	5.3 ± 0.4	84.5 ± 7.4	1.3 ± 0.4	44.6 ± 3.0
Enolase	3.9 ± 0.5	18.7 ± 2.7	5.1 ± 1.0	34.1 ± 4.1	1.7 ± 0.4	31.1 ± 1.3
Pyruvate kinase	8.7 ± 1.2	8.3 ± 1.2	16.5 ± 1.5	12.8 ± 1.0	14.9 ± 2.3	10.0 ± 1.0
Pyruvate dehydrogenase	2.4 ± 0.2	1.6 ± 0.3	3.2 ± 0.4	1.9 ± 0.3	4.3 ± 0.3	2.2 ± 0.1
Acetyl-CoA carboxylase	0.74, 0.78	0.07, 0.06	1.08, 1.02	0.12, 0.08	0.94, 0.98	0.06, 0.12

stages studied, there was a progressive 19-fold increase in the activity of a plastidial pyruvate transporter that was coincident with a 25-fold increase in the utilization of pyruvate for fatty acid synthesis by plastids in vitro when expressed on the same basis. Over the same period the ability of the isolated plastids to utilize Glc-6-P as a substrate for fatty acid synthesis decreased. Despite this decrease, either pyruvate or Glc-6-P gave the highest rates of incorporation of carbon into fatty acids at all stages compared with the other substrates tested (DHAP, malate, and acetate).

These studies clearly reveal that the developmental increase in pyruvate utilization was absolutely required to enable the synthesis of fatty acids in vitro at rates that were comparable to the in vivo rate of oil accumulation, which in turn increased during development (Fig. 3). This suggests that increasing the supply of carbon to fatty acid synthesis by the induction of a specific transporter could be important in determining the amount of oil. A developmental increase in the ability to take up and use pyruvate in vitro will only determine significant changes in utilization in vivo if the pyruvate concentration in vivo is at least of the same order of magnitude as the K_m of the transporter. The derived concentrations of pyruvate and Glc-6-P in the developing oilseed rape embryos were in excess of 1 mM, and are likely to be underestimates of the true cytosolic concentration because no compartmentation of these metabolites was assumed. Given this caveat, these estimates were still at least 25-fold greater than the K_m of their respective transporters and varied little during development. It is therefore very likely that the increase in activity of the pyruvate transporter would lead to increased utilization of this metabolite during development of the embryo.

Others have also demonstrated that carbon supply to lipid synthesis can contribute to the determination of the amount of oil laid down in a seed. The regulatory properties of ACCase are increasingly evident (Post-Beittenmiller et al., 1992; Hunter and Ohlrogge, 1998; Kozaki and Sasaki, 1999; Savage and Ohlrogge, 1999), and the role of this enzyme in determining oil content has been demonstrated by increasing the plastidial ACCase activity in embryos of transgenic rapeseed to gain an increase in oil content under certain growth conditions (Roesler et al., 1997). Recently, Bao and Ohlrogge (1999) have shown that the rate of oil synthesis by embryos of oilseed species can be stimulated in vitro by the supply of free fatty acids, providing evidence that the supply of de novo-synthesized fatty acyl groups to the lipid biosynthetic pathway can be a limiting step.

While the developmental change in activity of the pyruvate transporter was the most marked change affecting fatty acid synthesis in the oilseed rape embryo, other important changes in plastidial metabolism were revealed. In particular, the notable decreases in activity of the glycolytic enzymes aldolase, phosphoglyceromutase, and enolase were interesting. The latter pair has been widely reported to be low in activity relative to other plastidial glycolytic enzymes (Rawsthorne et al., 1999), and aldolase activity markedly increases in the transition between chloroplast and chromoplast in the developing sweet pepper (*Capsicum annum* L.) fruit (Thom et al., 1998). The significance of the decreases in these glycolytic enzymes in the developing oilseed rape embryo are difficult to establish because even the lowest of these enzyme activities was well in excess of the rate of fatty acid synthesis by the isolated plastids. The decline in the activities of the Glc-6-P transporter, aldolase, phosphoglyceromutase, and enolase therefore correlates with, rather than explains, the decrease in utilization of Glc-6-P for fatty acid synthesis.

The respective increase and decrease in the uptake and utilization of pyruvate and Glc-6-P for fatty acid synthesis by the embryo plastids is the first demonstration (to our knowledge) of a developmental change in the utilization of metabolites by plastids from a storage organ. The majority of studies of metabolite uptake by plastids from storage

tissues have focused on those from starch-accumulating organs, where the import of carbon is facilitated by specific transporters for Glc-6-P, Glc-1-P, and ADP-Glc, depending upon the plant species and organ (Emes and Neuhaus, 1997). In these cases the nature of the metabolite that is imported is typically deduced from experiments with plastids isolated from a single developmental stage. To date, only very limited data have been available on the nature of the plastidial transporters that are involved in the supply of carbon substrates for fatty acid synthesis. In castor endosperm, the predominant activity is that of malate/Pi exchange (Eastmond et al., 1997). For other organs/tissues from other species the transporter activities can only be inferred from the ability of isolated plastids to utilize a supplied metabolite. The roles of the pyruvate and Glc-6-P/Pi transporters in fatty acid synthesis are now clearly established.

The Glc-6-P transporter facilitates not only the supply of carbon skeletons for fatty acid synthesis, but also the activity of the plastidial OPPP. Two lines of evidence presented here support the hypothesis that the plastidial OPPP could provide reducing power for fatty acid synthesis. First, within the plastid, both the activity of the OPPP and the partitioning of Glc-6-P toward the pathway increased as the rate of storage oil accumulation in the embryo increased. Second, the plastidial OPPP activity was stimulated by the presence of pyruvate in Glc-6-P-containing incubations with plastids that were able to import and utilize pyruvate for fatty acid synthesis (i.e. from B- and C-stage embryos). In contrast, there was no effect on plastidial OPPP activity in the A stage plastids. Evidence for interaction between fatty acid synthesis and the OPPP has been reported previously for plastids isolated from B-stage oilseed rape embryos and from developing *Cuphea* seeds (Heise and Fuhrmann, 1994; Kang and Rawsthorne, 1996).

In conclusion, as the developing oilseed rape embryo progresses from a starch- to an oil-accumulating organ, there are coordinate changes in the metabolism of the plastids. The present data show that a new path of carbon flow and an increase in flux into fatty acid synthesis is facilitated by a marked increase in the activity of a pyruvate transporter, and that the partitioning and flux of Glc-6-P through the plastid OPPP also increases, probably to provide reducing power for fatty acid synthetase. Concomitant with these increases, the activities of enzymes of starch synthesis in the plastid decrease (da Silva et al., 1997). We are currently investigating the extent to which the pyruvate transporter contributes in vivo to the flux of carbon into oil in the developing embryos of oilseed rape.

ACKNOWLEDGMENTS

The authors thank Profs. Alison Smith and Denis Murphy, and Drs. Lionel Hill, Matthew Hills, Kay Denyer, and Philip Johnson for their comments on the manuscript and their advice and encouragement during this study. Ian Hagon and his team are thanked for horticultural support. The seed of *B. napus* cv Topas was a kind gift of Dalgety Agriculture (Essex, UK).

Received September 7, 1999; accepted November 5, 1999.

LITERATURE CITED

Bao X, Ohlrogge J (1999) Supply of fatty acid is one limiting factor in the accumulation of triacylglycerol in developing embryos. Plant Physiol **120:** 1057–1062

Beutler H-O (1984) Acetate: determination with acetyl-CoA synthetase. *In* HU Bergmeyer, ed, Methods of Enzymatic Analysis, Vol VI. Verlag Chemie, Weinheim, Germany, pp 639–645

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

da Silva PMFR, Eastmond PJ, Hill LM, Smith AM, Rawsthorne S (1997) Starch metabolism in developing embryos of oilseed rape. Planta **203:** 480–487

Denyer K, Smith AM (1988) The capacity of plastids from developing pea cotyledons to synthesise acetyl-CoA. Planta **173:** 172–182

Eastmond PJ, Dennis DT, Rawsthorne S (1997) Evidence that a malate/inorganic phosphate exchange translocator imports carbon across the leucoplast envelope for fatty acid synthesis in developing castor seed endosperm. Plant Physiol **114:** 851–856

Eastmond PJ, Rawsthorne S (1998) Comparison of the metabolic properties of plastids isolated from developing leaves and embryos of *Brassica napus* L. J Exp Bot **49:** 1105–1111

Emes MJ, Neuhaus HE (1997) Metabolism and transport in non-photosynthetic plastids. J Exp Bot **48:** 1995–2005

Hames BD (1990) *In* BD Hames, D Rickwood, eds, Gel Electrophoresis of Proteins: A Practical Approach. IRL Press Limited, London, p 45

Heise KP, Fuhrmann J (1994) Factors controlling medium-chain fatty-acid synthesis in plastids from Cuphea embryos. Prog Lipid Res **33:** 87–95

Heldt HW, Sauer F (1971) The inner membrane of the chloroplast envelope as the site of specific metabolite transport. Biochim Biophys Acta **234:** 83–91

Hunter SC, Ohlrogge JB (1998) Regulation of spinach chloroplast acetyl-CoA carboxylase. Arch Biochem Biophys **15:** 170–178

Jelitto T, Sonnewald U, Willmitzer L, Hajirezeai M, Stitt M (1992) Inorganic phosphate content and metabolites in potato and tobacco plants expressing *E. coli* pyrophosphatase in their cytosol. Planta **188:** 238–244

Joyard J, Teyssier E, Miege C, Berny-Seigneurin D, Marechal E, Block MA, Dorne A-J, Rolland N, Aljani G, Douce R (1998) The biochemical machinery of plastid envelope membranes. Plant Physiol **118:** 715–723

Kang F, Rawsthorne S (1994) Starch and fatty acid synthesis in plastids from developing embryos of oilseed rape (*Brassica napus* L.). Plant J **6:** 795–805

Kang F, Rawsthorne S (1996) Metabolism of glucose 6-phosphate and utilization of multiple metabolites for fatty acid synthesis by plastids from developing oilseed rape embryos. Planta **199:** 321–327

Kang F, Ridout CJ, Morgan CL, Rawsthorne S (1994) The activity of acetyl-CoA carboxylase is not correlated with the rate of lipid synthesis during development of oilseed rape (*Brassica napus* L.) embryos. Planta **193:** 320–325

Kozaki A, Sasaki Y (1999) Light-dependent changes in redox status of the plastidic acetyl-CoA carboxylase and its regulatory component. Biochem J **339:** 541–546

Lichter R (1982) Induction of haploid plants from isolated pollen of *Brassica napus*. Z Pflanzenphysiol **105:** 427–434

Murphy DJ, Cummins I (1989) Biosynthesis of seed storage products during embryogenesis in rapeseed, *Brassica napus*. J Plant Physiol **135:** 63–69

Post-Beittenmiller D, Roughan G, Ohlrogge J (1992) Regulation of plant fatty acid biosynthesis. Plant Physiol **100:** 923–930

Rawsthorne S, Kang F, Eastmond PJ (1999) Carbon flux to fatty acids in oilseeds. *In* NJ Kruger, SA Hill, RG Ratcliffe, eds, Regulation of Primary Metabolic Pathways in Plants. Proceed-

ings of the Phytochemical Society of Europe, Vol 42. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 137–157

Roesler K, Shintani D, Savage L, Boddupalli S, Ohlrogge J (1997) Targeting of the Arabidopsis homomeric acetyl-CoA carboxylase to plastids of rapeseeds. Plant Physiol **113:** 75–81

Savage LJ, Ohlrogge JB (1999) Phosphorylation of pea chloroplast acetyl-CoA carboxylase. Plant J **18:** 521–527

Smith AM (1988) Major differences in isoforms of starch-branching enzyme between developing embryos of round-seeded and wrinkled-seeded peas (*Pisum sativum* L.). Planta **175:** 270–279

Smith RG, Gauthier DA, Dennis DT, Turpin DH (1992) Malate and pyruvate dependent fatty acid synthesis in leucoplasts from developing castor endosperm. Plant Physiol **98:** 1233–1238

Stitt M, McLilley R, Gerhardt R, Heldt, HW (1989) Metabolite levels in specific cells and subcellular compartments of plant leaves. Methods Enzymol **174:** 518–552

Thom E, Möhlmann T, Quick WP, Camara B, Neuhaus HE (1998) Sweet pepper plastids: enzymic equipment, characterisation of the plastidic oxidative pentose-phosphate pathway, and transport of phosphorylated intermediates across the envelope membrane. Planta **204:** 226–233