Fatty Acid Synthesis in Pea Root Plastids Is Inhibited by the Action of Long-Chain Acyl-Coenzyme A on Metabolite Transporters

Simon R. Fox, Stephen Rawsthorne, and Matthew J. Hills*

The Department of Brassica and Oilseeds Research, John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, United Kingdom

The uptake in vitro of glucose (Glc)-6-phosphate (Glc-6-P) intooplastids from the roots of 10- to 14-d-old pea (Pisum sativum L. cv Puget) plants was inhibited by oleoyl-coenzyme A (CoA) concentrations in the low micromolar range (1–2 μM). The \( IC_{50} \) (the concentration of inhibitor that reduces enzyme activity by 50%) for the inhibition of Glc-6-P uptake was approximately 750 nM; inhibition was reversed by recombinant rapeseed (Brassica napus) acyl-CoA binding protein. In the presence of ATP (3 mM) and CoASH (coenzyme A; 0.3 mM), Glc-6-P uptake was inhibited by 60%, due to long-chain acyl-CoA synthesis, presumably from endogenous sources of fatty acids present in the preparations. Addition of oleoyl-CoA (1 μM) decreased carbon flux from Glc-6-P into the synthesis of starch and through the oxidative pentose phosphate (OPP) pathway by up to 73% and 40%, respectively. The incorporation of carbon from Glc-6-P into fatty acids was not detected under any conditions. Oleoyl-CoA inhibited the incorporation of acetate into fatty acids by 67%, a decrease similar to that when ATP was excluded from incubations. The oleoyl-CoA-dependent inhibition of fatty acid synthesis was attributable to a direct inhibition of the adenine nucleotide translocator by oleoyl-CoA, which indirectly reduced fatty acid synthesis by ATP deprivation. The Glc-6-P-dependent stimulation of acetate incorporation into fatty acids was reversed by the addition of oleoyl-CoA.

In plants, metabolic pathways are often compartmentalized within the cell to a greater extent than in the animal kingdom (Dennis and Emes, 1990). This places importance on factors that affect the regulation of proteins that transport metabolites or cofactors across the intracellular membranes (Barbier-Brygoo et al., 1997; Flügge, 2000). The transport proteins effectively supply the various anabolic or catabolic pathways of the cell compartments with substrates and because their function may affect the flux of carbon through a metabolic pathway potentially they represent targets for genetic manipulation (Eastmond and Rawsthorne, 2000). To this aim, increasing our knowledge of how transporter proteins are metabolically regulated may be beneficial when it comes to altering gene expression.

As an example of such metabolic regulation, in oilseed rape (Brassica napus) embryos the Glc-6-phosphate (Glc-6-P) transporter (GPT), located on the plastid envelope, was found to be directly inhibited by low concentrations of long-chain acyl-coenzyme A (CoA) thioesters (acyl-CoAs; Fox et al., 2000; Johnson et al., 2000). Acyl-CoAs are activated forms of the acyl molecule by virtue of the carbon-sulfur bond and are the substrates for the acyltransferase enzymes of the Kennedy pathway that forms the main structural and storage lipids of the cell. The consequences of the inhibition of the GPT manifested themselves in a significantly lowered flux of carbon through certain metabolic pathways, namely fatty acid and starch synthesis and the oxidative reactions of the oxidative pentose phosphate (OPP) pathway (Fox et al., 2000; Johnson et al., 2000). The inhibition by acyl-CoAs had a similar effect on the flux of carbon from Glc-6-P for each of these pathways, around 70% inhibition for starch synthesis and the OPP pathway and up to 74% inhibition for carbon flux through fatty acid synthesis (Johnson et al., 2000). The inhibition of the transporter was specific to acyl-CoAs with a chain length of or greater than C12 (lauryl-CoA; Fox et al., 2000) and was reversed by proteins that bind acyl-CoAs.

Plastids have evolved into specialist compartments whose function largely reflects the overall metabolic requirements of the cell. Pea (Pisum sativum L. cv Puget) root plastids, sometimes referred to as leucoplasts, are from non-photosynthetic tissue; the metabolism and transport properties of these organelles have been reviewed by Emes and Neuhaus (1997).

The demonstration of the inhibition of the GPT in oilseed rape by acyl-CoAs led us to investigate whether other GPTs in different plants were similarly affected. Pea root plastids differ from oilseed rape...
RESULTS AND DISCUSSION

Effect of Acyl-CoAs on Glc-6-P Uptake by Plastids

The uptake of Glc-6-P by isolated pea root plastids was significantly (P < 0.02) inhibited by pre-incubation of the plastids with concentrations of oleoyl-CoA (C\textsubscript{18:1}-CoA) = 1.0 \, \mu M (Table I). At a concentration of 2.0 \, \mu M, Glc-6-P uptake was inhibited by 68%. The uptake of pyruvate or Glc (2.3 and 10.5 nmol units\textsuperscript{-1} glyceraldehyde-3-phosphate dehydrogenase [GAPDH] min\textsuperscript{-1}, respectively) was unaffected by 2 \, \mu M oleoyl-CoA. The inhibitory effect of the added acyl-CoA on Glc-6-P uptake was dependent upon the acyl chain length. Acetyl-, malonyl-, or propionyl-CoA (at 5 \, \mu M) had no effect (data not shown). Glc-6-P uptake in the presence of 2 \, \mu M lauryle-CoA (C\textsubscript{12:0}) or myristoyl-CoA (C\textsubscript{14:0}) was 13 (45% inhibition) and 18 nmol units\textsuperscript{-1}GAPDH min\textsuperscript{-1} (64% inhibition), respectively. Malonyl-CoA (5 \, \mu M) did not reduce the inhibitory effect of oleoyl-CoA, suggesting that there was no competition between these acyl derivatives, and CoASH (0.3 \, \mu M) alone did not inhibit Glc-6-P uptake (results not shown). The inhibition of Glc-6-P uptake by oleoyl-CoA is not attributable to detergent effects for two reasons. First, the added concentration is well below the critical micelle concentration for palmitoyl-CoA.

Table I. The uptake characteristics of Glc-6-P by pea root plastids

<table>
<thead>
<tr>
<th>Oleoyl-CoA (\mu M)</th>
<th>Rate of Glc-6-P Uptake (nmol units\textsuperscript{-1} GAPDH min\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>28.0 ± 2.6</td>
</tr>
<tr>
<td>0.5</td>
<td>24.4 ± 2.6</td>
</tr>
<tr>
<td>1.0</td>
<td>17.2 ± 3.0</td>
</tr>
<tr>
<td>2.0</td>
<td>9.1 ± 1.8</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Indicates significant difference (Student’s t test) from the control (0 \, \mu M) where P < 0.02.

Figure 1. The rate of Glc-6-P (100 \, \mu M) uptake by pea root plastids incubated in the presence of 5 \, \mu M oleoyl-CoA and increasing concentrations of rACBP. Silicone oil centrifugation was used to measure uptake of Glc-6-P. Values represent the average of three experiments with SEs.

Figure 1: Graph showing the rate of Glc-6-P uptake at different concentrations of rACBP.

The uptake characteristics of Glc-6-P by pea root plastids under similar conditions to those used in the present experiments is estimated to be 50 \, \mu M (Constantinides and Stein, 1985, 1988). Second, a range of molecules, including detergents (Tween 40 and Triton X-100) did not inhibit Glc-6-P uptake when used at substantially higher concentrations (5–10 \, \mu M) than the oleoyl-CoA (data not shown).

The K\textsubscript{m}(apparent) of Glc-6-P uptake was 0.2 \, \mu M and the IC\textsubscript{50} (concentration of inhibitor that reduces enzyme activity by 50%) of the inhibition by oleoyl-CoA was 750 \, \text{nm}. The form of the inhibition was difficult to assess but from reciprocal plots of 1/V against [S] (1/ enzyme activity against substrate concentration), for a range of concentrations of Glc-6-P, appeared to be nonlinear (Cornish-Bowden, 1995). The oleoyl-CoA inhibition of Glc-6-P uptake by the pea root plastids is explained by the action of this molecule on the Glc-6-P translocator (GPT) protein (Fox et al., 2000). Addition of recombinant acyl-CoA binding protein (rACBP; Fig. 1) at 10 \, \mu M or bovine serum albumin (BSA) at 5 \, \mu M (data not shown) to the pre-incubations containing oleoyl-CoA completely abolished the inhibition of the pea root plastid GAP activity by binding out oleoyl-CoA from the medium. rACBP binds acyl-CoAs with 1:1 stoichiometry (Knudsen et al., 1989); progressively less inhibition of Glc-6-P uptake by 5 \, \mu M oleoyl-CoA was observed as the rACBP was increased to 10 and 15 \, \mu M. BSA possesses six high-affinity fatty acid binding sites and two with high affinity for long-chain acyl-CoAs (Richards et al., 1990), explaining why lower concentrations of BSA overcame oleoyl-CoA inhibition of Glc-6-P uptake.

In a separate series of experiments, neither ATP nor CoASH significantly inhibited (P > 0.02) Glc-6-P uptake when added to pre-incubations separately being 17.8 and 18.6 nmol Glc-6-P units\textsuperscript{-1}GAPDH min\textsuperscript{-1}, respectively; the rate in these experiments for the control without ATP or CoASH was 20.8 nmol Glc-6-P units\textsuperscript{-1}GAPDH min\textsuperscript{-1}. However, when added together Glc-6-P uptake was reduced by approximately 60%, falling to 8.4 ± 1.1 nmol Glc-6-P...
Table II. The concentration of acyl-CoAs in pea root plastid incubations

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>Acyl-CoA Concentrations</th>
<th>μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CoASH</td>
<td>0.2</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>CoASH + ATP</td>
<td>1.5 ± 0.5</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>CoASH + ATP + 18:1</td>
<td>0.7 ± 0.4</td>
<td>4.1 ± 0.7</td>
</tr>
</tbody>
</table>

units$^{-1}$GAPDH min$^{-1}$. This inhibition is explained by the ATP- and CoASH-dependent synthesis of long-chain acyl-CoAs in the plastid pre-incubation (Table II), probably arising as a result of the acyl-CoA synthetase activity localized to the plastid outer envelope (Joyard and Stumpf, 1981; Pongdontri and Hills, 1997). When oleic acid (ammonium salt) was added to the pre-incubations there was a significant increase in the content of extracted oleoyl-CoA (Table II). The source of endogenous unesterified fatty acids for the synthesis of the acyl-CoAs is not clear at present.

**Effect of Acyl-CoAs on Glc-6-P Metabolism**

**Starch**

Pea root plastids synthesized starch from Glc-6-P. Starch synthesis was significantly inhibited by CoASH (0.3 mM) in the presence of 3 mM ATP (Fig. 2). In the presence of these cofactors starch synthesis fell by 73% after 40 min in comparison with other treatments, but recovered to be 33% lower after 60 min. The inhibition was alleviated by the inclusion of micromolar concentrations of rACBP (5 μM), further evidence for the direct inhibition of the GPT by acyl-CoAs. Following the demonstration that CoASH and ATP induced the synthesis of acyl-CoAs from endogenous sources of fatty acids, these experiments showed that it was this synthesis that led to the significant decrease in flux from Glc-6-P into the synthesis of starch.

**OPP Pathway**

In the absence of additions to the isolation medium (control experiment) the rate of $^{14}$CO$_2$ release from plastids incubated with Glc-6-P was 8.0 ± 0.3 nmol $^{14}$CO$_2$ units$^{-1}$GAPDH h$^{-1}$. That the plastids possessed an active OPP pathway agreed with the studies of Bowsher et al. (1993) and Emes and England (1986). Addition of ATP and CoASH decreased $^{14}$CO$_2$ release by 40% to 4.8 ± 0.2 nmol $^{14}$CO$_2$ units$^{-1}$GAPDH h$^{-1}$, presumably by the inhibitory effect of acyl-CoA (viz acetyl-CoA synthetase activity) on the GPT. The addition of rACBP or BSA (both 5 μM) in the presence of either ATP and CoASH, or oleoyl-CoA (2 μM) alleviated the inhibition on the GPT, restoring carbon flux through the OPP pathway to control values. In the presence of octanoyl-CoA (C$_{8:0}$; 3 μM) and absence of BSA, there was no significant decrease in carbon flux through the OPP pathway, as predicted from the earlier findings that acyl chain lengths of CoA derivatives <C$_{12}$ did not inhibit Glc-6-P transport.

**Fatty Acid Synthesis**

Fatty acid synthesis was observed from acetate (Fig. 3), as had previously been established by Kleppinger-Sparace et al. (1992). The rate of synthesis from 1 mM acetate (with 3 mM ATP) was 200 to 250 nmol units$^{-1}$GAPDH h$^{-1}$. Omission of ATP lead to a 70% inhibition of the rate of synthesis from acetate. It is evident that the process is highly dependent on
a supply of exogenous ATP, presumably part due to the energy requirements of the reactions catalyzed by acetyl-CoA synthetase (EC 6.2.1.1; acetate-CoA ligase) and acetyl-CoA carboxylase (EC 6.4.1.2).

We were unable to demonstrate fatty acid synthesis from carbon derived from Glc-6-P, so it was not possible to assess whether acyl-CoAs had any effect on the pathway, as described for the synthesis of starch and the inhibition of the OPP pathway. Borchert et al. (1993) have reported that pea root plastids lack or have very low activities for the enzymes phosphoglyceromutase (EC 5.4.2.1) and enolase (EC 4.2.1.11; phosphopyruvate hydratase), which would explain the lack of incorporation of carbon from Glc-6-P into fatty acids.

Addition of 1 mM Glc-6-P significantly increased the rate of plastidial fatty acid synthesis from 1 mM acetate by 35% to 40% (Student’s t test, \( P = 0.02, n = 3 \); Fig. 4, incubations included 3 mM ATP). The effect was reversed by the inclusion of oleoyl-CoA (2 \( \mu \)M), which inhibited fatty acid synthesis by 80%. The effect of oleoyl-CoA was alleviated by the addition of BSA (5 \( \mu \)M), restoring the stimulation by Glc-6-P. These results show that addition of Glc-6-P increased the flux of carbon through the OPP pathway, thereby increasing the supply of NADPH. Increased reductant supply may have increased the activity of the enzymes catalyzing condensing reactions of fatty acid synthesis that utilize NADPH. This same effect, where the addition of one substrate stimulated the incorporation of carbon from another, has previously been reported by Kang and Rawsthorne (1996); the addition of Glc-6-P was found to stimulate fatty acid synthesis from acetate in oilseed rape embryo plastids.

However, when fatty acid synthesis from acetate was measured in the presence of ATP and CoASH, unexpectedly, there was a 61% inhibition in synthesis in comparison to plastids incubated solely with ATP (Fig. 3). Measuring the same process in the presence of micro-molar concentrations of oleoyl-CoA again yielded significant inhibition of fatty acid synthesis from carbon derived from acetate. In this case the rates of fatty acid synthesis were reduced by 67% in the presence of 5 \( \mu \)M oleoyl-CoA. As with the inhibition of the GPT, this inhibition was alleviated by rACBP (5 \( \mu \)M) and/or BSA. Since acetate presumably diffused into the plastids, the inhibition recorded in either the absence of ATP or the presence of long-chain acyl-CoAs seemed to implicate that ATP transport into the stroma may have been affected by acyl-CoAs.

**Fatty Acid Synthesis and ATP Uptake**

The uptake of ATP by plastids was inhibited by progressively higher concentrations of oleoyl-CoA (Fig. 5). Inhibition saturated after 2 min with 2 to 4 \( \mu \)M oleoyl-CoA, resulting in 63% inhibition. The \( K_m \) of the adenylate transporter (in some instances the plastidial ATP/ADP transporter is referred to as AATP; see Winkler and Neuhaus, 1999) for ATP (presumably exchanging with ADP or Pi) was 30 \( \mu \)M. The \( IC_{50} \) (concentration of inhibitor that reduces enzymes activity by 50%) for the inhibition of the adenylate transporter was 2 to 4 \( \mu \)M; the nature of inhibition appeared to be nonlinear (results not shown). In a further series of experiments, incubation of plastids with either acetyl-CoA (C_{2:0}) or octanoyl-CoA (C_{8:0}), both at 2 \( \mu \)M, did not cause significant inhibition of ATP uptake (results not shown). However, the uptake of ATP after 2 min into plastids incubated with 2 \( \mu \)M lauryl-CoA (C_{12:0}) or myristoyl-CoA (C_{14:0}) was inhibited by 45% and 62%, respectively, in comparison with the control (no acyl-CoA added). Thus, the chain length of the acyl moiety, as demonstrated for control of the GPT, significantly effects the degree of inhibition of ATP uptake. In addition, inhibition of ATP uptake by plastids in the presence of 2 \( \mu \)M oleoyl-CoA and either BSA (10–20 \( \mu \)M) or acyl-CoA binding protein (ACBP; 5 \( \mu \)M), both of which bind long-chain acyl-CoAs, was completely alleviated.

**Figure 4.** The effect of Glc-6-P (1 mM) and oleoyl-CoA (2 \( \mu \)M 18:1-CoA) on the synthesis of fatty acids from [1-\(^{14}\)C]acetate by pea root plastids; no additions were made to the control. Incubations were of 60 min. Fatty acid synthesis is expressed as nanomoles acetate per unit glyceraldehyde 3-phosphate dehydrogenase. Values represent the average of three experiments with SEs.

**Figure 5.** The effect of oleoyl-CoA (18:1-CoA) on the uptake of ATP by pea root plastids. The process was determined using the silicone oil centrifugation technique and is described as nanomoles ATP per unit glyceraldehyde 3-phosphate dehydrogenase. Values represent the average of three experiments with SEs.
The inhibition of mitochondrial adenylate transporters by long-chain acyl-CoAs from mammalian tissues was demonstrated independently by Pande and Blancaer (1971) and Shug et al. (1971); both found that the activity of the rat mitochondrial adenylate transporter was sensitive to the presence of low concentrations of long-chain acyl-CoAs. It is interesting, however, that at the amino acid level in Arabidopsis, the sequence of the plastidial adenylate transporter and the mitochondrial adenylate transporter bear little if any similarity (Kampfenkel et al., 1995). This may reflect their differing functions; the mitochondrial transporter primarily pumps ATP into the cytosol, i.e. there is asymmetric uptake of ADP in favor of ATP during oxidative phosphorylation. In contrast, in heterotrophic plastids the adenylate transporter mainly supplies ATP to the stroma (Winkler and Neuhaus, 1999), even during periods of high photosynthetic activity (Schünemann et al., 1993).

Implications for Hexose and Energy Metabolism in Pea Root Plastids

In a previous study in oilseed rape it was found that in the late stages of embryo development the concentrations of long-chain acyl-CoAs and rACBP were similar (Fox et al., 2000). This, in association with the estimations of flux through fatty acid synthesis show that it is possible for rapid (<1 s) changes to occur in the relative concentration of acyl-CoAs and rACBP. Rapid changes in the balance of the concentrations of acyl-CoA and rACBP would allow for a sensitive regulation of flux through this pathway. Therefore, any event that affects the utilization of acyl-CoAs, such as a reduction in the activity of the acyltransferase enzymes on the endoplasmic reticulum, could lead to a rapid excess of such compounds over rACBP and potentially inhibit plastidial fatty acid synthesis. Because we do not have antibodies against the pea ACBP or cloned and expressed pea ACBP as standard, at present we cannot estimate the concentration of ACBP in pea roots. However, it is interesting to note that the concentration of the long-chain acyl-CoAs in pea roots was similar to that of oilseed rape embryos; the predominant acyl-CoA species found in the roots of 10- to 14-d-old plants was linoleoyl-CoA, which accumulated to approximately 0.5 to 1 pmol mg⁻¹ (fresh weight; S.R. Fox, unpublished results). This raises the possibility that, as shown by the effects on acetate utilization, carbon flux through fatty acid synthesis may in part be regulated by the effect of long-chain acyl-CoAs on the GPTs and adenylate transporters.

In vitro experiments with isolated plant organelles generally use a large excess of an acyl-CoA binding protein, usually BSA. The incubation medium often contains up to 1% (w/v) BSA, equivalent to 150 μM. It is evident that where such high concentrations of BSA are used, the effects demonstrated here on either the Glc-6-P or the adenylate transporter will not be encountered. In planta, however, the balance between the concentration of ACBP and acyl-CoAs may be an important mechanism for regulating the flux of carbon through pathways in plastids and contribute to the coordination of acyl-group metabolism in the cell.

MATERIALS AND METHODS

Preparation of Pea Root Plastids

Pea (*Pisum sativum* L. cv Puget) seeds were imbibed overnight and grown for 10 to 14 d in vermiculite. The roots were homogenized and the plastids isolated essentially as described by Kleppinger-Sparace et al. (1992). In brief, approximately 60 g of roots were thoroughly washed with water, chilled for 30 min at 4°C, and homogenized in a Waring blender (Waring Corp., Hartford, CT) for three bursts of 5 s in 200 mL 0.33 M sorbitol, 1 mM EDTA (disodium salt), 2 mM MgCl₂, and 50 mM Tricine (pH 7.9; isolation medium). The homogenate was filtered through two layers of Miracloth (22–25 μm mesh; Calbiochem-Novabiochem Ltd., Beeston, Nottinghamshire, UK) and centrifuged at 1,500 g for 2 min. The plastids which sedimented were resuspended in 1 to 2 mL of isolation medium and were purified by centrifugation through 10% (w/v) Percoll (Emes and England, 1986) and resuspended in 15 to 20 mL of isolation medium. After centrifugation at 1,500 g for 2 min the washed pea plastids were resuspended in a final volume of 2 to 3 mL of isolation medium and maintained on ice prior to incubations.

Silicone Oil Centrifugation

Plastids were separated from the incubation medium using the silicone oil method (Heldt, 1980) in 400-μL Microcentrifuge tubes (Elkay Products Inc., Shrewsbury, MA). The plastids were centrifuged at 12,500g through 55 μL of silicone oil (Wacker Silicone Fluid AP100/DEL AR200 [1:2; v/v], Wacker-Chemie, GmbH, Munich) into 30 μL 0.7 M Suc and 1% (v/v) trichloroacetic acid after various times listed in the text. Intact plastids passed through the oil into the Suc. The Suc phase was subjected to scintillation counting in 10 mL Optiphase HiSafe 3 (Fisher Scientific, Loughborough, Leicestershire, UK). The sorbitol impermeable space of the plastids, calculated as described by Kang and Rawsthorne (1996), was 5% to 10% of the volume of plastids which passed through the oil.

Measurement of Glc-6-P and ATP Uptake of Glc-6-P and ATP Uptake into Pea Plastids

Plastids (155 μL) were pre-incubated for 2 min with 10 μL of isolation medium. In some experiments additions were made to the isolation medium before addition of the plastids; additions included acyl-CoA thioesters, ACBP, BSA, and the detergents Tween 40 (polyoxyethylene-sorbitan monolaurate), Triton X-100 (t-octylphenoxypoly-ethoxyethanol), and fatty acid ammonium salts (concentra-
assess effects on the flux of [1-14C]Glc-6-P through the OPP pathway. Additions were made to the incubations (listed in text) to maintain in Micro tubes (1.5 mL; Sarstedt, Aktiengesellschaft and Co., Numbrecht, Germany), were placed in a 155-mL solution of plastids was mixed after a further 30 s with 10 μL isolation medium containing, on average, either 5.9 kBq [1-14C]-d-Glc-6-P (specific activity [sp. act.] 540 kBq mmol⁻¹) or 4.7 kBq [8-14C]ATP (sp. act. 1.85–2.29 GBq mmol⁻¹). Upon mixing, the plastids were immediately removed (155 μL) and subjected to silicone oil centrifugation after various times up to 120 s of incubation with the [14C] substrate.

The uptake of [1-14C]Glc-6-P or [8-14C]ATP and the measurement of starch synthesis, the OPP pathway, or the rate of fatty acid synthesis were determined as nanomoles of substrate per unit NADP⁺-GAPDH. The intactness of plastid preparations was measured by performing latency assays of GAPDH (Kang and Rawsthorne, 1996). In a further series of experiments plastids were mixed, separately with 4.3 kBq [2-14C]pyruvate (sp. act. 0.37–1.1 GBq mmol⁻¹) or 4.5 kBq [1-14C]Glc (sp. act. 1.48–2.22 GBq mmol⁻¹) after the pre-incubation and uptake measured over 60 s.

**Measurement of Starch Synthesis**

Plastids were incubated as described in the previous section (for 80 min) with [1-14C]-d-Glc-6-P. Additions made to the initial incubation medium at the start of the experiments included CoASH (0.3 mm), ACBP (5 μm), BSA (5 μm), and acyl-CoAs at concentrations listed in the text. Measurement of carbon incorporation from [1-14C]Glc-6-P (1 mm) into starch was based on Kang and Rawsthorne (1994). In brief, the lower fraction from the silicone oil centrifugation (plastids) was transferred to 500 μL of 75% (v/v) aqueous methanol containing 0.1% (w/v) KCl. Commercial potato starch (4–5 mg) was added to facilitate sedimentation. These solutions were centrifuged at 12,000 g for 2 min and the methanolic phase discarded. The process was repeated five times. The remaining pellet was subjected to scintillation counting.

**Measurement of the OPP Pathway**

Plastid incubations, in 155 μL of isolation medium, maintained in Micro tubes (1.5 mL; Sarstedt, Aktiengesellschaft and Co., Numbrecht, Germany), were placed in 20-mL glass vials; these contained a smaller Micro tube (0.6 mL) that contained 100 μL of 15% (w/v) KOH. Various additions were made to the incubations (listed in text) to assess effects on the flux of [1-14C]Glc-6-P through the OPP pathway. The pathway contains decarboxylation reactions that generate 14CO₂; hence, measurement of the latter is often used to determine activity. Air-tight seals (Suba-Seal stoppers, Fisher Scientific UK) were fitted and after 60 min of incubation, 50 μL of formic acid was syringed into the Micro tube containing the plastids. The acidification released 14CO₂, which was trapped in the KOH containing Micro tube. After 60 min, 5 μL was used for scintillation counting.

**Measurement of Fatty Acid Synthesis**

After incubation with either [1-14C]Glc-6-P or [1-14C]acetate, plastid solutions were quenched with 500 μL of 15% (w/v) KOH in methanol after 60 min. Samples were heated for 45 min at 80°C before solutions were acidified (pH 1) with 6 m HCl (200 μL) and an equal volume of chloroform added. After vortexing the chloroform phase was removed and the organic extraction repeated. The combined organic phases were concentrated and fatty acids purified by thin-layer chromatography on Silica Gel G, 20-cm × 20-cm, 250-micron plates (Analtech, Inc., Newark, DE) developed with iso-hexane/diethyl ether/acetic acid (70:30:1; v/v).

**Analysis of Acyl-CoAs**

Acyl-CoAs, synthesized by plastids during incubations with various substrates, were extracted and quantified by HPLC. The method was based on that described by Fox et al. (2000) with modifications from Larson and Graham (2001). After extraction of pigments with diethyl ether (Fox et al., 2000), samples were derivatized to chloroacetaldehyde derivatives (Larson and Graham, 2001), achieved by addition of 200 μL 0.5 m chloroacetaldehyde in 0.15 m citric acid buffer (trisodium citrate/citric acid; pH 4.0), and 0.5% (w/v) SDS. Samples were heated at 80°C for 30 min and purified on DEAE Sephacel columns (Pharmacia Biotech, Uppsala). The resin (0.5 cm³, acetate form) was washed with 4 to 5 mL water. The chloroacetaldehyde derivatives were applied (400 μL) and a further 200 μL of water added. Columns were washed with 4 to 5 mL of 80% (v/v) aqueous methanol before the acyl-CoA derivatives were eluted with 500 to 600 μL of 80% (v/v) methanol containing 0.6 m ammonium acetate and 10 mM acetic acid. Methanol was removed under N₂ before HPLC separation. HPLC conditions comprised a flow rate of 1 mL min⁻¹ with a gradient of 10 mM KH₂PO₄, pH 7 (99% [v/v]; solvent A)/3 mM KH₂PO₄ (pH 7), acetonitrile (30:70 [v/v]; 1%; solvent B), altered to 1% (v/v) solvent A/99% (v/v) solvent B after 23 min before returning to starting conditions. Acyl-CoAs were separated on a phenyl-hexyl 25-cm × 4.6-mm (5-micron particle size) Luna column (Phenomenex, Macclesfield, Cheshire, UK) and the compounds detected by fluorescence (λ_exc 340 nm/λ_em 402 nm) by a Perkin-Elmer LS-4 fluorescence spectrometer.

**ACKNOWLEDGMENTS**

We thank Ian Hagon and his team for supply of the pea plants and also Wacker-Chemie GmbH (Munich, Germany) for the silicone oil.

Received February 9, 2001; returned for revision March 14, 2001; accepted April 6, 2001.

**LITERATURE CITED**


Inhibition of Plastidial Transport Proteins by Acyl-Coenzyme As


