Factors Controlling Medium-Chain Fatty Acid Synthesis in Plastids from Maturing *Cuphea* Embryos

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The colorless embryos of *Cuphea wrightii* A. Gray accumulate capric (about 30%) and lauric acid (about 50%) in their storage lipids. Fractionation studies show that the capacities for the synthesis of these medium-chain fatty acids (MCFA) from [1-^{14}C]acetate were strictly bound to intact plastids. These, in turn, obligately required the addition of ATP. ATP could partially be substituted by ADP. Reduction of the pyridine nucleotide pool, required for optimum MCFA formation within the plastids, was driven by glucose 6-phosphate. Under these conditions the plastids were capable of synthesizing MCFA like the intact tissue. The presence of CoA in the incubation medium induced acyl-CoA formation.

The observed accumulation of unesterified capric and lauric acid in the absence of CoA suggests that acyl-ACP thioesterase activity is involved in the chain termination. Treatment with cerulenin led to an unexpectedly small reduction of total fatty acid synthesis while the chain elongation of capric acid was clearly inhibited. A similar accumulation of capric acid at the expense of longer chain fatty acids has been observed after replacing ATP by ADP. These findings implicate that even the condensing enzymes are involved in the control of chain termination.

Introduction

Biosynthesis of medium-chain fatty acids in *Cuphea wrightii* seeds occurs during a short and intense period of maturation [1]. This MCFA synthesis can be attributed to plastids from developing colorless cotyledons, enclosed by a green seed coat. As in safflower cotyledons [2], the plastid ultrastructure showed a densely staining matrix with enclosed membrane vesicles and starch grains. In addition, *Cuphea* plastids were found to have a polymorphic structure (unpublished results), which greatly increases their surface area and, thus, may facilitate the CoA-mediated transacylation of freshly synthesized fatty acids on to storage lipids (triacylglycerols) by intimate contact with the endoplasmic reticulum [3]. Important criteria for a production of MCFAs in cell-free preparations, characteristic of the intact tissue, appeared to be either the choice of the tissue investigated [4] or, in the case of *Cuphea,* the integrity of the plastid [3]. Otherwise, MCFA formation was significantly reduced in favor of long-chain products [5]. We have focussed our interest, therefore, on an improvement of the incubation conditions of intact plastid preparations from developing *Cuphea* embryos [3], required for optimum MCFA production. The main question was how to provide these energy-heterotrophic organelles sufficiently with reducing equivalents for fatty acid synthesis.

Sensitivity of fatty acid biosynthesis to cerulenin, an antibiotic produced by *Cephalosporium caerulens,* is mainly caused by the inhibition of β-ketoacyl-ACP synthase I (KAS I) and β-ketoacyl-ACP synthase II (KAS II) [6]. Recently, a third condensing enzyme called acetoacetyl-ACP synthetase (KAS III) has been reported in *E. coli* [7] and plant tissues [8, 9]. This enzyme is completely insensitive to cerulenin, it uses acetyl-CoA instead of acetyl-ACP as the primer substrate for fatty acid synthesis, and it produces short-chain acyl-ACPs. In this paper we describe the effect of cerulenin on MCFA synthesis by *Cuphea* plastids.

Abbreviations: ACP, acyl carrier protein; DAG, diacylglycerol; DHAP, dihydroxyacetone phosphate; FAS, fatty acid synthase; Fru6P, fructose 6-phosphate; FruBP, fructose 1,6-bisphosphate; Glc6P, glucose 6-phosphate; KAS, β-ketoacyl-ACP synthase; LCFA, long-chain fatty acids; MCFA, medium-chain fatty acids; OAA, oxaloacetate; PA, phosphatidic acid; PC, phosphatidylcholine; PEP, phosphoenolpyruvate; 3-PGA, 3-phosphoglycerate; TAG, triacylglycerol; TCA, trichloroacetic acid; UFA, unesterified fatty acids.

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Materials and Methods

Seed material. Seeds of *Cuphea wrightii* A. Gray were harvested and embryos prepared as described previously [3].

Preparation of plastids. The preparation of intact plastids was as described previously [3]. Modifications were as follows: The Percoll centrifugation was carried out at 5500 × *g*. The plastid pellet was resuspended in a medium containing 50 mM Tricine-KOH (pH 8.2); 330 mM sorbitol; 1 mM $MgCl_2$ (medium B). Osmotically shocked plastids were resuspended in 50 mM Tricine-KOH (pH 8.2); 1 mM $MgCl_2$. The first microsomal pellet (85,000 × *g*) was resuspended in medium B and centrifuged again (200,000 × *g* for 30 min). This pellet was resuspended in medium B (microsomes). The 85,000 × *g* supernatant was concentrated by ultrafiltration (Amicon YM 10 membrane) to yield the soluble fraction.

Latency and marker enzyme measurements. Latency measurements of the plastid pellet was as described [3]. As additional marker enzyme for plastids, ADP-glucose-pyrophosphorylase has been measured according to [10].

Incorporation experiments with subcellular fractions. Unless otherwise described, incubations were performed in a medium containing 50 mM Tricine-KOH (pH 8.0); 330 mM sorbitol; 2.5 mM $MgCl_2$; 1 mM $MnCl_2$; 10 mM $NaHCO_3$; 0.25 mM CoA; 1 mM cysteine and 0.2 mM [1-^{14}C]acetate (1.93–2.04 GBq·mmol^{-1}) for 30 min at 28 °C. ATP, Glc6P, other metabolites or nucleotides, and cerulenin were added as mentioned in the figures and tables. Cerulenin was used freshly or maintained as 1 mM stock solution (pH 4.0) at 4 °C [11]. The plastids were preincubated with the antibiotic for 15 min.

Extraction, separation and saponification of lipids and CoA and ACP thioesters was carried out as described [3, 12]. In some cases an aliquot (100 µl) of the reaction mixture was tested for its content of acyl-ACPs. The proteins were solubilized by addition of 0.8% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propansulfonate (CHAPS) and precipitated by addition of an equal volume of 10% (w/v) TCA, mixing, storing on ice for 10 min, and centrifugation. The resulting pellet was washed twice with 0.5 ml 5% TCA and redissolved in 0.5 ml MES (pH 6.0). Radioactivity was determined by liquid scintillation counting. It never exceeded 10% of that obtained after saponification of the aqueous phase. The label in this aqueous phase was therefore attributed to acyl-CoAs.

Prior to methylation of lipid-bound and unesterified fatty acids a mixture of the following triacylglycerols (20 µg each) was added as tracer: tricaprin, trilaurin, trimyristin, tripalmitin, tristearin and triolein. The extracted methyl esters were evaporated to a final volume of 20–50 µl in a mixture of *n*-heptane and *n*-octane. The *n*-octane was used to avoid the loss of methyl esters of medium chain length by evaporation to dryness. As a control for complete recovery of radioactivity in medium-chain methyl esters, the recovery of the unlabeled tracers was analyzed in the following GLC step. Additional fatty acids contained in the sample could be ignored. For GLC analysis and detection of labeled fatty acids we used a gas chromatograph (Perkin-Elmer, model 8600) with a glass column (2 m × 4 mm) containing 10% diethyleneglycolsuccinate (60–80 mesh) connected with a gas proportional counter (Canberra-Packard, model GCR).

Reproducibility. Due to difficulties in obtaining sufficient embryo material of optimum development, the reported rates of acetate incorporation are the result of a single experiment. When this experiment was repeated with different batches of plant material qualitatively similar results were obtained. The rate of acetate incorporation differed by a factor of up to 3, but metabolite and cerulenin effects in percent of the control were found to be very similar.

Results

Requirement for intact plastids. After Percoll-fractionation of homogenates from developing embryos of *Cuphea wrightii* according to [3], fatty acid synthesizing activities from [1-^{14}C]acetate on the gradient coincided with that of the (soluble) marker enzymes for plastids (Table I). But only the activities which were included in intact plastids synthesized medium-chain fatty acids in a manner that is characteristic of the intact tissue (more than 80% of total fatty acid biosynthesis [5]). Osmotically shocked plastids showed a substantially lower capacity for fatty acid synthesis accom-

Table I. Distribution of plastid enzymes in subcellular fractions derived from *Cuphea wrightii* embryos. The percentage recovery of the given activities is related to the total activity contained in the embryo homogenate of 400 embryos (~50 µg protein).

	Fatty acid synthesis[a]		Fatty acid synthesis + 80 µM cerulenin[a]		6-Phosphogluconate dehydrogenase		ADP-glucose pyrophosphorylase	
	Activity [nmol/h]	Recovery [%]	Activity [nmol/h]	(% of control)	Activity [nmol/min]	Recovery [%]	Activity [nmol/min]	Recovery [%]
Plastids:								
– Intact	23.9	7.5	21.5	(90)				
– Osmotically shocked	4.2	1.3	2.7	(64)	38	6.0	11	6.8
Microsomes	15.9	5.0	11.8	(74)	16	2.6	0.2	0.1
Soluble fraction	128.8	40	24.4	(19)	272	43	74	47

[a] Measured as [1-^{14}C]acetate incorporation under respective optimized conditions (all fractions: 2.5 mM ATP; 0.5 mM CoA; plastids: 0.5 mM glucose 6-phosphate; osmotically shocked plastids and microsomes: 1 mM NADH, 1 mM NADPH; soluble fraction: 1 mM NADH, 1 mM NADPH, 80 µg/ml ACP).

panied by a reduced MCFA-formation as consequence of plastid disintegration (Table I).

Requirement for energy and reducing equivalents. Fatty acid synthesis from [1-^{14}C]acetate by *Cuphea* plastids is obligately dependent on exogenous ATP as energy source (Table II). The response of the plastids to ATP showed typical saturation kinetics, saturation was reached at about 2.5 mM (Fig. 1). A small effect is seen with ADP, but this effect is presumably due to the operation of adenylate kinase [13]. The optimum concentration required was 5-fold higher than for ATP (about 12.5 mM). Nucleotides other than ATP and ADP (GTP, CTP, AMP) had no effect.

ATP could not be replaced by an indirect ATP transfer *via* substrate phosphorylation of glycolytic intermediates (Table II), including hexose phosphates (Glc6P), triose phosphates (DHAP,

Table II. Dependence of fatty acid synthesis from [1-^{14}C]acetate by *Cuphea* plastids upon exogenous sources for energy and reducing equivalents. The plastids were incubated for 30 min in the standard incubation medium (a: in the presence of Glc6P; b: in the presence of ATP) supplemented with nucleotides or metabolites. MCFA/LCFA ratio of mature seeds: 9.0; C_{12}/C_{10} ratio: 1.9 (C_{10} = 29%; C_{12} = 56%; C_{14} = 5%; C_{16} + C_{18} = 10%).

Additions [mM]	nmol acetate incorp. mg protein × h	MCFA/LCFA-ratio	C_{12}/C_{10}-ratio
a) Glc6P [0.5]	≤0.01	–	–
+ DHAP [1], PEP [1], or 3PGA [1]	≤0.03	–	–
+ AMP [2.5], CTP [2.5], or GTP [2.5]	≤0.03	–	–
+ DHAP [2] + OAA [2] + P_i [6]	≤0.03	–	–
+ ADP [2.5]	1.02	18.8	0.8
+ ADP [2.5] + PEP [1]	0.37	n.d.[a]	n.d.[a]
+ ATP [2.5]	2.54	3.1	1.3
b) ATP [2.5]	0.95	0.9	3.5
+ NADH [1] + NADPH [1]	1.42	1.2	3.7
+ Glc6P [0.5]	2.54	3.1	1.3
+ Fru6P [0.5]	2.27	2.1	2.2
+ FruBP [0.5]	1.48	2.2	1.8
+ DHAP [1]	1.36	1.8	3.7
+ 3PGA [1]	0.52	1.3	5.1
+ PEP [1]	0.36	n.d.[a]	n.d.[a]
+ Pyruvate [1]	0.70	1.4	8.6

[a] Not determined.

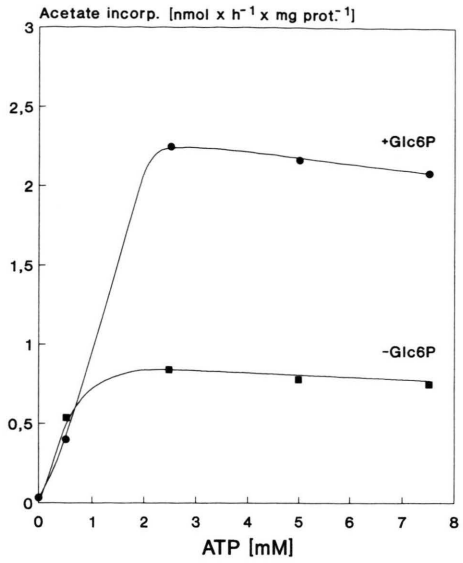

Fig. 1. Dependence of fatty acid synthesis from [1-^{14}C]acetate by *Cuphea* plastids on ATP in the presence and absence of 0.5 mM glucose 6-phosphate. The plastids were incubated in the standard incubation medium.

3-PGA) and PEP. Even the addition of the DHAP-shuttle components (DHAP, OAA, and P_i), which promoted intraplastidic ATP synthesis in spinach chloroplasts [14] and pea root plastids [15] could not replace ATP (Table II).

Addition of reduced pyridine nucleotides (NADH and NADPH) stimulated ATP-driven fatty acid synthesis from acetate by *Cuphea* plastids to only a low degree. However, addition of glucose 6-phosphate (Glc6P) and fructose 6-phosphate (Fru6P) increased the rate of fatty acid synthesis two- to three-fold. Even some other intermediates of glycolysis like FruBP and DHAP stimulated plastid fatty acid biosynthesis. Glucose 1-phosphate and 6-phosphogluconate showed only a very slight effect (about 1.1 pmol·mg^{-1} protein·h^{-1}) whereas 3-PGA, PEP and pyruvate considerably depressed [1-^{14}C]acetate incorporation into fatty acids (Table II).

Fatty acid synthesis was maximal with Glc6P at 0.5–1 mM (Fig. 2). The decrease in stimulation by Glc6P at concentrations exceeding 0.5 mM, as well as the inhibition by 3-PGA and PEP may be due to the competition of unlabeled degradation products with radioactive acetate as carbon source for fatty acid synthesis. This assumption was supported by experiments with [U-^{14}C]Glc6P and [2-^{14}C]-pyruvate. In any case, an incorporation of label into fatty acids could be measured. However, both substrates, especially [U-^{14}C]Glc6P, were less efficient precursors than [1-^{14}C]acetate (data not shown).

The importance of CoA. Like other plant materials [16], plastids of *Cuphea* seeds [1, 3] produce fatty acids which are converted to acyl-CoAs in the envelope [17] and transferred to extraplastid lipid biosynthesis. In absence of CoA during plastid incubation, radioactive unesterified fatty acids accumulated (>90% of total incorporation; Table III) with capric (~40%) and lauric acid (~50%) predominating. The labeled fatty acids could be converted substantially to acyl-CoAs when the addition of CoA was sufficient to saturate acyl-CoA synthetase (Table III). In this case the label in the acyl-CoA-pool (~60–70%) exceeded by far the 30% that were found in comparable experiments with spinach chloroplasts [16]. This observation points to the strict predominance of extraplastidic lipid biosynthesis in *Cuphea* embryos. As a consequence of acyl-CoA-formation, increasing amounts of label in PA, DAG and TAG were found (Table III). Especially the appearance of TAG points to microsomal contamination of the plastid sediment [3].

Labeling pattern of fatty acids. As a rule, stimulation of fatty acid synthesis by *Cuphea* plastids was paralleled by an increased MCFA/LCFA-

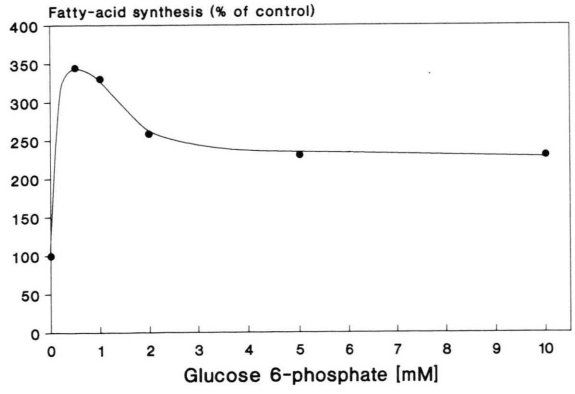

Fig. 2. Dependence of fatty acid synthesis from [1-^{14}C]acetate on the glucose 6-phosphate concentration. The standard incubation medium was supplemented with 2.5 mM ATP.

Table III. Effect of CoA on the distribution of [1-^{14}C]acetate label among products of fatty acid synthesis by *Cuphea* plastids. The standard incubation medium was supplemented with 2.5 mM ATP and 0.5 mM Glc6P. 100% represent the respective total [1-^{14}C]acetate incorporation (2.5–3 nmol × h^{-1} × mg^{-1} protein in this experiment).

CoA [mM]	UFA [%]	Acyl-CoA [%]	PC [%]	PA [%]	DAG [%]	TAG [%]
0	94	3.6	0.3	1.0	0.6	0.6
5	76	14	4.7	1.6	0.7	3.4
20	34	51	5.4	1.9	2.2	5.5
50	21	65	5.2	2.0	0.9	5.0
250	15	74	4.1	1.7	0.8	4.2

ratio (Table II). Thus, after an exogenous supply with Glc6P, formation of MCFAs (70–90%) in general and of capric acid (~30%) in particular reached values comparable to those observed *in vivo*. The data presented in our recent paper (about 50% MCFA production [3]) were achieved with NADH/NADPH as source for reducing power.

An unphysiological increase in the synthesis of capric acid at the expense of lauric acid (shown as low C_{12}/C_{10}- and very high MCFA/LCFA-ratio) was found when ATP was replaced by ADP as energy source (Table II).

Effect of cerulenin. As shown in Table I, cerulenin inhibited [1-^{14}C]acetate incorporation into fatty acids by *Cuphea* plastids to only a small extent (5–10%). It could be excluded that the low inhibition is caused by an impermeability of the envelope membrane for cerulenin, because the antibiotic significantly affected the labeling pattern of fatty acids (Fig. 3). On the other hand, the fatty acid synthase activities in a soluble fraction showed a marked sensitivity toward cerulenin (Table I). This fraction is derived from the same plant material and it produces only long-chain fatty acids from acetate [5].

In *Cuphea* plastids, cerulenin caused an accumulation of radioactive capric at the expense of lauric and longer-chain fatty acids. The synthesis of caproyl-ACP appears to be insensitive to cerulenin. Obviously, the caproyl-ACP is cleaved by a thioesterase. Unesterified capric acid or caproyl-CoA (in the presence of CoA) are the predominating products of fatty acid synthesis in the presence of cerulenin. In analogy to the results with the β-ketoacyl-ACP synthase III [7–9], label was expected in short-chain acyl-ACPs. However, in our experiments with *Cuphea* plastids no substantial

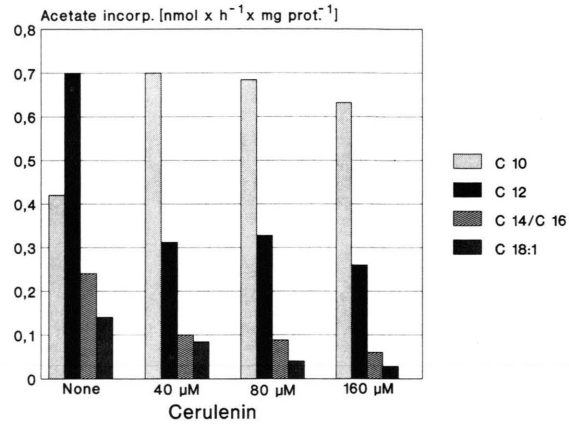

Fig. 3. Effect of cerulenin on the chain length of fatty acids synthesized from [1-^{14}C]acetate by *Cuphea* plastids. The standard incubation medium was supplemented with 2.5 mM ATP and 0.5 mM Glc6P. (C 10 = capric acid; C 12 = lauric acid; C 14 = myristic acid; C 16 = palmitic acid; C 18:1 = oleic acid.)

TCA-precipitable radioactivity was found (data not shown).

Discussion

Intact plastids isolated from the colorless embryos of *Cuphea wrightii* are capable of synthesizing MCFAs like the intact tissue. The carbon- and energy heterotrophic organelles require a supply of both energy and reducing equivalents for fatty acid synthesis from acetate. A direct uptake of ATP on the one hand and an indirect one in the case of reducing equivalents (Glc6P, Fru6P) on the other gave the best results. The strict dependence on exogenous ATP (or ADP) suggests an effective uptake mechanism like the adenylate translocator [18].

In contrast to chromoplasts [19] and pea root plastids [15], C_3-intermediates of the glycolytic pathway could not provide energy for fatty acid synthesis by substrate phosphorylation of ADP (Table II). The observed inhibition of [^{14}C]acetate incorporation by 3-PGA and PEP implies an uninterrupted glycolytic pathway from 3-PGA to pyruvate. Such a metabolic sequence was found in several non-green plastids [20]. The differential effect of 3-PGA and DHAP (Table II) may be due to a lack of an active glyceraldehyde 3-phosphate dehydrogenase. This assumption might also explain why the DHAP-shuttle components are unable to replace ATP as energy source.

In non-green tissues a deficient conversion of triose- into hexose monophosphates by lack of fructose 1,6-bisphosphatase has been observed, but cannot be generalized [21]. In plastids, hexose monophosphates are required both for starch synthesis and as substrates for the pentose phosphate pathway. Our results concerning the stimulation of ATP-driven fatty acid synthesis by sugar phosphates (Table II) suggest that Glc6P and Fru6P are capable of maintaining the plastidial pyridine-nucleotide complement in their reduced forms. The lower effect of DHAP and FruBP suggests an effective uptake of Glc6P and Fru6P which might be mediated by a phosphate translocator capable of transporting hexose monophosphates [22].

Termination in the chain extension of fatty acids in developing *Cuphea* embryos depends crucially on the arrangement (assembly) and the specific metabolic situation of the FAS-enzymes within this type of plastid. Thus, the coincidence of the recently shown *Cuphea*-specific high malonyl-CoA- and fatty acid turnover during the rapid maturation period [3] suggests a particular involvement of the condensing enzymes in the control of MCFA-accumulation.

Energy supply achieved by substitution of ATP by ADP promoted condensation reactions up to capric acid only (Table II), whereas the synthesis of longer chain fatty acids is largely inhibited. In safflower plastids long-chain fatty acid synthesis was not stimulated by ADP as well [2]. These results may be due to a reduced malonyl-CoA formation as a consequence of its inhibition by ADP [6]. Our results implicate the existence of different condensing enzymes involved in fatty acid synthesis in *Cuphea* seeds.

A short-chain condensing enzyme is further characterized by the cerulenin treatment. The inhibition of total fatty acid synthesis in *Cuphea wrightii* plastids is negligible (Table I), but a significant accumulation of capric at the expense of longer chain fatty acids is observed (Fig. 3). The only known condensing enzyme which is insensitive to cerulenin is the KAS III [7–9]. However, the purified KAS III from spinach is highly specific for the first condensation reaction [23].

Unfortunately, the non-involvement of acetyl-ACP as alternative primer for fatty acid condensation could not be demonstrated, since the envelope membrane is presumably impermeable for acetyl-ACP.

In cells of the lactating mammary gland the chain termination of fatty acids has been attributed to both chain length-specific thioesterases [24] and transacylases [25] in the cytosolic compartment, which either hydrolyze acyl-ACPs or convert them directly to acyl-CoAs. In plant cells, however, the stromal ACP-thioesterase and the envelope-bound acyl-CoA synthetase are separated by a membrane barrier. Therefore, without acyl-CoA-synthesizing conditions, fatty acid synthesis by *Cuphea* plastids ended with the accumulation of unesterified capric (~40%) and lauric acid (~50%). In the presence of cerulenin, capric acid (70–80%) predominated (Fig. 3). These unesterified fatty acids are converted into acyl-CoAs, when sufficient CoA is present (Table III).

The release of unesterified fatty acids from ACP points to thioesterase activities towards medium-chain acyl-ACPs. In contrast to the widespread occurrence of oleoyl-ACP-hydrolyzing activities [26], MCFA-specific thioesterase activities (specific for lauroyl-ACP) have been shown in *Umbellularia californica* cotyledons [4] only. In *Cuphea* seeds, thioesterases appear to hydrolyze medium-chain acyl-ACPs in a more unspecific manner [27]. The importance of chain length-specific thioesterases for MCFA production has recently been shown in transgenic *Arabidopsis* seeds [28]. The *Umbellularia californica* thioesterase, overexpressed in *Arabidopsis* seeds, led to an accumulation of up to 24% lauric acid at the expense of long-chain fatty acids.

From our results we conclude that in *Cuphea* seed plastids the chain length of fatty acids is controlled by the activities both of different condens-

ing enzymes and of unspecific thioesterase reactions. The cerulenin-induced accumulation of capric at the expense of lauric acid cannot be explained by changed thioesterase specifities. Therefore, in *Cuphea,* the acyl-ACP equilibrium may be primarily controlled by a cerulenin-insensitive condensing enzyme. This enzyme is capable of producing caproyl-ACP. This substrate is also the first FAS-product to be hydrolyzed by the *Cuphea* thioesterase [27]. In *Cuphea wrightii,* a cerulenin-sensitive elongation of caproyl-ACP occurs, and lauric acid is the main product of fatty acid synthesis and hydrolysis by a thioesterase. It seems likely that in other *Cuphea* species, like *Cuphea lanceolata,* a capric acid containing crop, no substantial elongation of caproyl- to lauroyl-ACP occurs. We could further show that both capric and lauric acid are immediately converted into acyl-CoAs (Table III). Finally, the medium-chain fatty acids are incorporated selectively into triacylglycerols. Their incorporation has been found to be controlled by the different acyltransferases involved in the triacylglycerol (Kennedy) pathway [29].

Acknowledgements

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