Planta (1994) 194:193–199

Planta

The role of the triose-phosphate shuttle and glycolytic intermediates in fatty-acid and glycerolipid biosynthesis in pea root plastids

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Received: 11 October 1993 / Accepted: 29 November 1993

Abstract. The capacity of the triose-phosphate shuttle and various combinations of glycolytic intermediates to substitute for the ATP requirement for fatty-acid and glycerolipid biosynthesis in pea (*Pisum sativum* L.) root plastids was assessed. In all cases, ATP gave the greatest rates of fatty-acid and glycerolipid biosynthesis. Rates of up to 66 and 27 nmol·(mg protein)$^{-1}$·h^{-1} were observed for the incorporation of acetate and glycerol-3-phosphate into lipids in the presence of ATP. In the absence of exogenously supplied ATP, the triose-phosphate shuttle gave up to 44 and 33% of the ATP-control activity in promoting fatty-acid and glycerolipid biosynthesis from acetate and glycerol-3-phosphate, respectively. The optimum shuttle components were 2 mM dihydroxyacetonephosphate (DHAP), 2 mM oxaloacetic acid and 4 mM inorganic phosphate (referred to as the DHAP shuttle). Glyceraldehyde-3-phosphate, as a shuttle triose, was approximately 82% as effective as DHAP in promoting fatty-acid synthesis while 2-phosphoglycerate, 3-phosphoglycerate, and phosphoenolpyruvate were only 27–37% as effective as DHAP. When glycolytic intermediates were used as energy sources for fatty-acid synthesis, in the absence of both exogenously supplied ATP and the triose-phosphate shuttle, phosphoenolpyruvate, 2-phosphoglycerate, fructose-6-phosphate and glucose-6-phosphate each gave 48%, 17%, 23% and 17%, respectively, of the ATP-control activity. Other triose phosphates tested were much less effective in promoting fatty-acid synthesis. When exogenously supplied ATP was supplemented with the DHAP shuttle or glycolytic intermediates, the complete shuttle increased fatty-acid biosynthesis by 37% while DHAP alone resulted in 24% stimulation. Glucose-6-phosphate, fructose-6-phosphate and glycerol-3-phosphate similarly all improved the rates of fatty-acid synthesis by 20–30%. In contrast, 3-phosphoglycerate, 2-phosphoglycerate and phosphoenolpyruvate all inhibited fatty-acid synthesis by approximately 10% each. The addition of the DHAP shuttle and glycolytic intermediates with or without exogenously supplied ATP caused an increase in the proportion of radioactive oleate and a decrease in the proportion of radioactive palmitate synthesized. The use of these alternative energy sources resulted in higher amounts of free fatty acids and triacylglycerol, and lower amounts of diacylglycerol and phosphatidic acid. The data presented here indicate that ATP is superior in promoting in-vitro fatty-acid biosynthesis in pea root plastids; however, both the triose-phosphate shuttle and glycolytic metabolism can produce some of the ATP required for fatty-acid biosynthesis in these plastids.

Key words: Dihydroxyacetonephosphate shuttle – Fatty-acid biosynthesis – Glycolytic metabolism –Nonphotosynthetic plastids – *Pisum* – Root

Abbreviations: DHAP = dihydroxyacetonephosphate; Fru6P = fructose-6-phosphate; G3P = glycerol-3-phosphate; Glc6P = glucose-6-phosphate; OAA = oxaloacetate; PEP = phosphoenolpyruvate; 2PGA = 2-phosphoglycerate; 3PGA = 3-phosphoglycerate; 3PGalde = glyceraldehyde-3-phosphate

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Introduction

Both ATP and reduced nucleotides (NAD(P)H) are essential cofactors for de-novo biosynthesis of fatty acids from acetate. While ATP is required for both the activation of acetate to acetyl-CoA and the acetyl-CoA carboxylase reaction, the reduced nucleotides are required in the reduction reactions of the fatty-acid synthetase system (Stumpf 1984). Nonphotosynthetic plastids are thought to derive these high-energy cofactors from their own in-situ enzymes of glycolytic and oxidative pentose-phosphate metabolism (Emes and Tobin 1993; Sparace and Kleppinger-Sparace 1993). Pea root plastids and daffodil chromoplasts are capable of some fatty-acid biosynthesis in the absence of exogenously supplied NADH or NADPH (Kleinig and Leidvogel 1978; Stahl and Sparace 1991). This suggests that a limited amount of internal reductant is available, presumably from endogenous gly-

colytic and pentose-phosphate metabolism. In contrast, ATP must be exogenously supplied in order for any fatty-acid biosynthesis to proceed in these plastids, indicating that the process is most limited by ATP.

A number of studies with nonphotosynthetic plastids from different sources have shown that the activities of one or more of the enzymes of glycolytic or pentose-phosphate metabolism are either absent or very low. Those enzymes most commonly not detected include phosphoglyceromutase and NAD-glyceraldehyde-3-phosphate (3PGalde) dehydrogenase in pea root plastids (Borchert et al. 1993; Trimming and Emes 1993) and sycamore amyloplasts (Frehner et al. 1990). Similarly, glucose-6-phosphate dehydrogenase is absent in sycamore amyloplasts and plastids from castor-bean endosperm (Simcox et al. 1977; Nishimura and Beevers 1979; Frehner et al. 1990). However, this latter enzyme has considerable activity in pea root plastids (Borchert et al. 1993; Trimming and Emes 1993). In any case, the low activities or absences of one or more of these enzymes can prevent efficient carbon flow completely through the glycolytic or oxidative pentose-phosphate pathways, suggesting that nonphotosynthetic plastids must interact with the extraplastidic compartment to maximize their rates of carbon metabolism by these pathways.

Borchert et al. (1993) have suggested that glycolytic carbon flow in pea root plastids is restricted by the absence of phosphoglyceromutase. Thus, hexose phosphates can be metabolized only as far as 3-phosphoglycerate (3PGA), and subsequently 2-phosphoglycerate (2PGA) can be converted to pyruvate. These same workers characterized the triose-phosphate translocator in these plastids and indicated that this translocator could compensate for the absence of phosphoglyceromutase in pea root plastids by allowing for the export of 3PGA and uptake of 2PGA. Kleppinger-Sparace et al. (1992) used the phosphate translocator as part of the dihydroxyacetonephosphate (DHAP)-shuttle mechanism to support fatty-acid biosynthesis in pea root plastids in the absence of exogenously supplied ATP. As originally described for chloroplasts (Werdan et al. 1975) this shuttle requires, in addition to the phosphate translocator, plastidic glycolytic carbon flow from DHAP to 3PGA via NADP-3PGalde dehydrogenase and 3PGA kinase, the latter being responsible for the generation of intraplastidic ATP. The shuttle also requires an exogenous supply of oxaloacetate (OAA), the dicarboxylate translocator, and malate dehydrogenase operating in reverse, in order to regenerate NADP for the dehydrogenase reaction. It is interesting to note that the successful operation of the DHAP-shuttle mechanism relies on the presence of a malate dehydrogenase, or similar mechanism, which has not yet been characterized in pea root plastids. In any case, Kleppinger-Sparace et al. (1992) found that the DHAP shuttle could promote up to 40% of the ATP-control activity for fatty-acid biosynthesis which was improved to the level of the ATP-control when supplemented with ADP. Castor-bean leucoplasts and daffodil chromoplasts are also capable of sufficient intraplastidic ATP generation for fatty-acid synthesis by metabolizing various glycolytic intermediates such as phosphoenolpyruvate (PEP) and 2PGA (Kleinig and Liedvogel 1980; Boyle et al. 1990). The purpose of the present investigation was to characterize the capacity of internal ATP-generating systems (the DHAP-shuttle mechanism and the metabolism of various glycolytic intermediates) of pea root plastids for supplying the ATP required for fatty-acid and glycerolipid biosynthesis from [^{14}C]acetate as a substitute for exogenously added ATP.

Materials and methods

All materials and methods for the isolation of pea root plastids and the assay and analysis of fatty-acid and glycerolipid biosynthesis were as described earlier (Kleppinger-Sparace et al. 1992). Reagent-grade organic acids, inorganic salts and bases were obtained from Anachemia Canada, Inc. (Montreal, Quebec). Organic solvents of reagent grade or better were obtained from Caledon Laboratories (Georgetown, Ontario, Canada). All organic cofactors and metabolites were from the Sigma Chemical Co. (St. Louis, Mo., USA). Sodium-[1-^{14}C]acetate was purchased from ICN Biochemicals Canada (St. Laurent, Quebec) and [U-^{14}C] glycerol-3-phosphate (G3P) was from Amersham Canada (Oakville, Ontario). Pea root plastids purified through 10% Percoll (Pharmacia Canada Inc., Montreal, Quebec) (Trimming and Emes 1993) were washed and resuspended without bovine serum albumin in 1.0 mM Bis-tris-propane (pH 7.5), 0.33 M sorbitol (Kleppinger-Sparace et al. 1992) to give approximately 1.0 mg·mL^{-1} plastid protein. Plastids equivalent to 40–60 μg protein were incubated for 1 h at 25°C in 0.5 mL of a standard reaction medium containing 0.25 M Bis-tris-propane buffer (pH 7.9), 0.33 M sorbitol, 0.2 mM Na-[1-^{14}C]-acetate (740–774 kBq·μmol^{-1}), 0.5 mM each of NADH, NADPH and CoA, 1 mM $MnCl_2$, 6 mM each of $MgCl_2$ and ATP, and 15 mM $KHCO_3$. Where indicated, ATP was ommitted, replaced or supplemented with the components of the DHAP shuttle or other potential energy-producing metabolites. Unless otherwise stated, the concentrations of the DHAP-shuttle components [DHAP, OAA, inorganic phosphate (Pi)] were 2 mM, 2 mM, and 4 mM, respectively, and the concentrations of glycolytic intermediates tested were 2 mM. Each experiment routinely had + and − ATP 6 mM control treatments. Reactions were initiated by addition of plastids and terminated upon addition of 3 mL chloroform:methanol:glacial acetic acid (1:2:0.1, by vol.) (Kleppinger-Sparace et al. 1992) and lipids extracted as described by Mudd and DeZacks (1981). Rates of fatty-acid biosynthesis in control treatments generally ranged from 40 to 60 nmol acetate·(mg protein)$^{-1}$·h^{-1}. All experiments were performed two to four times with duplicate analyses for each treatment within each experiment. Data shown are the averages of four corresponding observations derived from two replicate experiments (duplicate analyses from two separate experiments with identical treatments all performed within the same experiment) and are representative of each experiment type.

Results and discussion

Shuttle-stimulated lipid biosynthesis. Pea root plastids require ATP for both fatty-acid biosynthesis from acetate (Stahl and Sparace 1991) and glycerolipid biosynthesis from G3P (Xue 1993, pp. 62–66). The DHAP shuttle can partly satisfy this ATP requirement. This ATP dependency and the ability of the DHAP shuttle to substitute for ATP in fatty-acid and glycerolipid biosynthesis are illustrated in Table 1. In the absence of exogenously supplied ATP, the DHAP shuttle can restore approximately 40% of the ATP-dependent lipid biosynthesis. These observa-

Table 1. Stimulation of fatty-acid and glycerolipid biosynthesis in pea root plastids by the triose-phosphate shuttle

Treatment	Fatty-acid biosynthesis from [^{14}C]acetate	glycerolipid biosynthesis from [^{14}C]G3P
	(nmol·(mg protein)$^{-1}$·h^{-1})	
ATP control	47.0	27.5
−ATP	0.71	1.04
−ATP, +Shuttle[a]	17.9	8.97
+ATP, +Shuttle	64.5	n.d.[b]

[a] Shuttle components consisted of 2 mM DHAP, 2 mM OAA and 4 mM KH_2PO_4
[b] Not determined

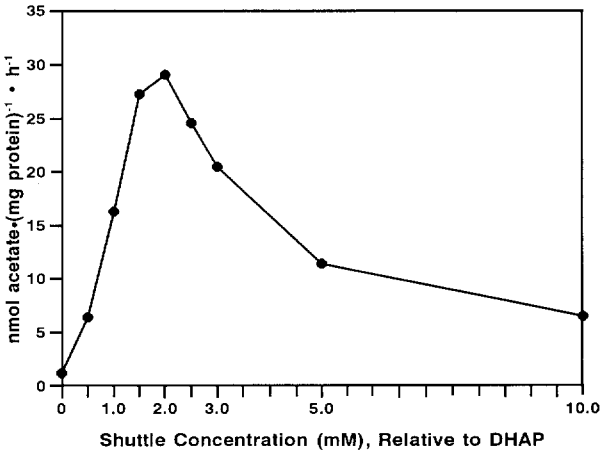

Fig. 1. Effect of increasing concentrations of the DHAP shuttle on fatty-acid synthesis by pea root plastids. A DHAP:OAA:Pi ratio of 1:1:2 was maintained in the reaction mixture. The rate of fatty-acid synthesis of the ATP control was 66.7 nmol·(mg protein)$^{-1}$·h^{-1}

tions are in agreement with our previous studies (Kleppinger-Sparace et al. 1992) and similar studies that use this shuttle with dark-incubated chloroplasts to promote CO_2 fixation (Werdan et al. 1975), fatty-acid biosynthesis (Sauer and Heise 1983), and sulfolipid biosynthesis (Kleppinger-Sparace and Mudd 1987). For pea root plastids, the similarity in response with each precusor for pea root plastids indicates that the processes of fatty-acid biosynthesis and acylation of G3P for glycerolipid biosynthesis are tightly coordinated. It is also interesting to note that exogenously supplied ATP and shuttle-generated ATP are apparently additive in terms of their effects on the rate of fatty-acid biosynthesis. These observations suggest that the rates of both ATP uptake and shuttle-promoted intraplastidic ATP synthesis are low enough to limit fatty-acid biosynthesis. However, it is also possible that intraplastidic levels of carbon available for fatty-acid and glycerolipid biosynthesis may be limiting and that some of the carbon that enters the plastid via the shuttle may be re-routed towards lipid biosynthesis via DHAP reductase and/or plastidic glycolysis.

The shuttle is designed to promote ATP synthesis by phosphoglycerate kinase. This is achieved by maintaining a low ratio of NAD(P)H/NAD(P) in order to favor the oxidation of 3PGalde to 1,3-diphosphoglycerate by 3PGalde dehydrogenase and thus insuring relatively high levels of substrate for phosphoglycerate kinase (Werdan et al. 1975). Recently, Borchert et al. (1993) and Trimming and Emes (1993) have independently shown that phosphoglycerate kinase is potentially the second or third most active glycolytic enzyme of pea root plastids. Thus, as pointed out earlier (Kleppinger-Sparace et al. 1992), in the absence of both externally available ATP and complete plastidic glycolysis, phosphoglycerate kinase is likely to be an important source of intraplastidic ATP in pea root plastids.

In an earlier study (Kleppinger-Sparace et al., 1992), all three components of the DHAP shuttle mechanism (DHAP, OAA, Pi) were required to promote fatty-acid biosynthesis. However, no attempt was made to determine the optimum ratio or concentrations of the shuttle components, or if other trioses or related compounds could be used in place of DHAP. In a series of preliminary experiments where each of the shuttle components were individually varied, the optimum molar ratio of DHAP:OAA:Pi was found to be 1:1:2 (data not shown), which is in agreement with Werdan et al. (1975). When this ratio was held constant and the absolute concentrations of shuttle components varied from 0 to 10 mM relative to DHAP, the optimum concentrations of shuttle components were 2 mM DHAP, 2 mM OAA and 4 mM Pi (Fig. 1). Under these conditions, fatty-acid biosynthesis from acetate was approximately 30 nmol·(mg protein)$^{-1}$·h^{-1}, which decreased relatively sharply to 8 nmol·(mg protein)$^{-1}$·h^{-1} at 10 mM relative to DHAP. The decline in fatty-acid synthesis at higher shuttle concentrations is likely due to the inhibitory effects of excess extraplastidic phosphate on the uptake of DHAP. Under such conditions of high external phosphate, the transport of DHAP would thus be reversed from that required by the shuttle (Flügge and Heldt 1984). Similarly, internal G3P required for glycerolipid biosynthesis could also leak out of the plastid by the same mechanism, and ultimately contribute to reduced rates of fatty-acid biosynthesis. Alternatively, the phosphorylation and inhibition of regulatory enzymes of the plastid might also lead to decreased fatty-acid biosynthesis (Emes and Tobin 1993). Our observed optimum concentrations of the shuttle components are the same as those obtained for sulfolipid biosynthesis in dark-incubated chloroplasts (Kleppinger-Sparace and Mudd 1987) and 1 mM less than that for CO_2 fixation in dark-incubated chloroplasts (Werdan et al. 1975). With pea root plastids, the optimum concentration of shuttle components gave only 42% of the ATP control activity for fatty-acid biosynthesis.

The greatest shuttle-stimulated rates of fatty-acid biosynthesis were obtained when DHAP was used as the shuttle triose (Table 2). Glyceraldehyde-3-phosphate was almost as effective, with approximately 82% of the DHAP-stimulated activity. Phosphoenolpyruvate, 2PGA and 3PGA were less effective as the shuttle triose, giving only 27–37% of the DHAP-stimulated shuttle ac-

Table 2. Ability of other triose phosphates to substitute for DHAP in triose-phosphate shuttle-stimulated fatty acid synthesis in pea root plastids

Treatment	Fatty-acid synthesis (nmol·(mg protein)$^{-1}$·h^{-1})
ATP control	40.0
−ATP	0.56
Shuttle Triose[a]	
DHAP	17.6
3PGalde	14.5
3PGA	5.60
2PGA	6.52
PEP	4.88
G3P	1.96

[a] The DHAP-shuttle components were 2 mM DHAP, 2 mM OAA, 4 mM of KH_2PO_4. All shuttle triose substitutions were 2 mM

Table 3. Effects of hexose and triose-phosphates on fatty-acid biosynthesis in pea root plastids in the presence and absence of ATP

Treatment[a]	Fatty-acid synthesis (nmol·(mg protein)$^{-1}$·h^{-1})	
ATP control[b]	50.2	55.4
−ATP	0.70	0.89
	−ATP	+ATP
+Glc6P	8.53	73.1
+Fru6p	12.7	69.3
+DHAP	3.03	70.9
+3PGalde	3.43	69.7
+3PGA	3.01	51.0
+2PGA	8.79	52.6
+PEP	24.6	49.9
+G3P	1.26	63.2

[a] All compounds were tested at a concentration of 2 mM
[b] Data shown for the − ATP and + ATP treatment groups are from two separate sets of experiments

tivity. These results largely reflect the specificity of the triose-phosphate translocator for the transport of these metabolites (Borchert et al. 1993). However, in this case the relatively low activities observed with 3PGA, 2PGA and PEP might also be expected because their utilization represents a break in the shuttle mechanism and they must eventually be metabolized by pyruvate kinase to provide ATP. This latter enzyme has a 10- to 20-fold lower activity as compared to phosphoglycerate kinase (Borchert et al. 1993; Trimming and Emes 1993). As observed before, no triose-shuttle combination was as effective as the ATP control in promoting fatty-acid biosynthesis.

Effects of glycolytic intermediates. Pea root plastids, like many other nonphotosynthetic plastids, possess almost a complete set of glycolytic enzymes for the conversion of hexoses to pyruvate (Borchert et al. 1993; Trimmings and Emes 1993). It is also apparent that glycolytic metabolism in these plastids interacts considerably with cytosolic glycolysis through the exhange of a number of hexose and triose intermediates (Dennis 1989; Borchert et al. 1993). The results shown earlier for pea root plastids indicate that under ideal shuttle conditions, complete glycolytic metabolism is not essential to provide intraplastidic ATP for fatty-acid biosynthesis. However, in order to fully understand the role of plastidic glycolytic metabolism in providing energy for fatty-acid biosynthesis in pea root plastids, it is important to also examine them under conditions where the DHAP-shuttle mechanism is not operating. We thus determined the capacities of several glycolytic intermediates to support fatty-acid biosynthesis in the presence and absence of exogenously supplied ATP (Table 3). In the absence of both exogenously supplied ATP and the shuttle components, there was virtually no fatty-acid biosynthesis unless hexose- or triose-phosphate compounds were provided. In contrast to the shuttle experiments, DHAP, 3PGalde and 3PGA added alone were relatively ineffective as an energy source for fatty-acid biosynthesis, providing only 6–7% of the ATP-control activity. These observations, in view of the results shown in Table 2, confirm that all components of the shuttle mechanism are required for the efficient uptake and metabolism of these compounds. The greatest stimulation of fatty-acid biosynthesis in the absence of exogenously supplied ATP and the shuttle was obtained when PEP was used as the energy source, resulting in almost 50% of the control activity. This was approximately 4–5 times greater than the stimulation observed when PEP was supplied as part of the shuttle mechanism (Table 2). These observations imply that the shuttle components are either sub-optimal or inhibitory for PEP uptake. Glucose-6-phosphate (Glc6P), Fructose-6-phosphate (Fru6P) and 2PGA were intermediate in their effects, all giving approximately 16–20% of the ATP control. In complementary experiments where exogenous ATP was supplied, Glc6P, Fru6P, DHAP and 3PGalde improved fatty-acid biosynthesis by 20–30% of the controls, while 3PGA, 2PGA and PEP each inhibited fatty-acid biosynthesis by approximately 10%.

Interpretation of the results shown in Table 3 is complicated by the fact that the glycolytic intermediates tested can exert their effects on fatty-acid biosynthesis in a number of ways. First, since ATP is the most limiting factor for fatty-acid synthesis in pea root plastids (Stahl and Sparace 1991), these intermediates are most likely metabolized to provide an intraplastidic source of ATP as previously discussed. However, it is also possible that the metabolism of these intermediates by either plastidic glycolysis or the oxidative pentose-phosphate pathway can provide a supply of reduced nucleotides (NADH and NADPH) which are also required in, and thus might promote fatty-acid biosynthesis. This is apparently particularly important for the high rates of Glc6P- and Fru6P-dependent fatty-acid synthesis in *Cuphea* plastids (Fuhrmann and Heise 1993) and nitrogen assimilation in pea root plastids (Bowsher et al. 1992). This possibility seems unlikely for the pea root plastids used in this investigation since exogenously supplied reduced nucleotides can promote fatty-acid biosynthesis, and are provided in saturating amounts in the studies described here (Stahl and Sparace 1991). However, the possibility that these

metabolites provide a more accessible intraplastidic supply of reduced nucleotides can not be ruled out.

It is also very important to point out that these metabolites can ultimately serve as a source of carbon for fatty-acid biosynthesis in non-photosynthetic plastids. The end-product of glycolytic metabolism, pyruvate can be an effective substrate for fatty-acid biosynthesis in non-photosynthetic plastids via the action of pyruvate dehydrogenase (Harwood 1988; Sparace and Kleppinger-Sparace 1993). These metabolites would thus "appear" to inhibit fatty-acid biosynthesis from acetate as they diluted the pool of labeled acetyl-CoA. This could explain the decrease in fatty-acid biosynthesis observed with 3PGA, 2PGA and DHAP in the presence of ATP (Table 3) as discussed earlier. However, these latter observations, particularly with PEP, also suggest that these compounds may reduce ATP uptake by the ADP/ATP translocator as recently demonstrated by Schünemann et al. (1993) in pea root plastids.

In the presence of ATP, DHAP and 3PGalde each improved fatty-acid synthesis by 20–25% (Table 3). In addition to mechanisms already discussed, these metabolites might also exert their effects by providing G3P for acylation via the combined action of triose-phosphate isomerase and DHAP reductase as in chloroplasts (Gee et al. 1988; Frentzen 1993). In some cases, Glc6P and Fru6P, in the absence or presence of ATP, were more effective in stimulating fatty-acid biosynthesis than some of the other trioses. These observations could be explained on the basis that the glycolytic oxidation of each hexose will yield two molecules of triose. However, as discussed earlier, these hexoses could also exert their effects by providing more accessible reduced nucleotides via the oxidative pentose-phosphate pathway.

Our results compare variously with two related studies. With daffodil chromoplasts, DHAP, 3PGalde and 3PGA were all almost as effective as ATP (Kleinig and Liedvogel 1980). However, these same glycolytic intermediates were essentially ineffective in promoting fatty-acid synthesis in castor-bean leucoplasts (Boyle et al. 1990). As with our pea root plastids, PEP gave the greatest stimulation of fatty-acid biosynthesis in daffodil and castor-bean plastids in the absence of ATP, however in the latter cases, PEP gave two- to threefold greater activity than the ATP controls. A likely explanation for the observations that PEP can easily replace exogenously supplied ATP in all these plastids is that it is very efficiently translocated into the plastid (Borchert et al. 1993) where only one metabolic reaction catalyzed by pyruvate kinase is required in order to produce the ATP required for fatty-acid biosynthesis. Other metabolites are less efficiently translocated and require multiple enzymic steps before ATP can be synthesized.

In order to better understand how PEP, Glc6P and Fru6P might be able to substitute for the ATP required for fatty-acid biosynthesis in pea root plastids, a range of concentrations of each metabolite up to 10 mM was tested in comparision to ATP (Fig. 2). As before, at all equivalent concentrations, ATP was the most effective in promoting fatty-acid biosynthesis. However, at 1.5 mM, PEP was almost 80% as effective as an equivalent concentration of ATP. More importantly, these data suggest that when cytoplasmic levels of ATP are low (less than 1 mM), transport of phosphorylated intermediates of carbohydrate metabolism and subsequent glycolytic metabolism within pea root plastids can compensate and may thus be essential for high rates of fatty-acid biosynthesis.

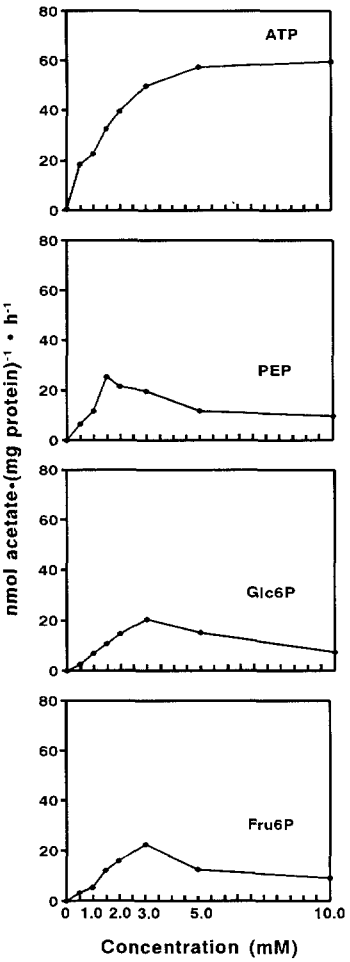

Fig. 2. Capacity of different concentrations of PEP, Glc6P and Fru6P to substitute for ATP in fatty acid synthesis by pea root plastids

Effects on fatty-acid and glycerolipid distribution patterns. Altered carbon metabolism within the plastid can also affect the profile of fatty-acids and glycerolipids accumulated. Changes in fatty-acid distributions are thought to occur by the provision of additional carbon, ATP and reduced nucleotides to favor fatty-acid elongation and desaturation (Harwood 1988). The effects of such carbon metabolism on glycerolipid biosynthesis are not well understood. We therefore determined the effects of a number of previously described treatments on the proportion of fatty-acids and glycerolipids accumulated by pea root plastids. As shown in previous studies (Stahl and Sparace 1991; Kleppinger-Sparace et al. 1992), isolated pea root plastids synthesized exclusively palmitate, stearate and oleate from [^{14}C]acetate (Table 4). In the absence or presence of exogenously supplied ATP, the triose-phosphate

Table 4. The effects of various treatments on the composition of radioactive fatty acids synthesized by pea root plastids. All data shown except that for the DHAP shuttle are from analyses of total fatty acids synthesized in treatments from previous experiments summarized in Table 3. Data for the DHAP shuttle are from experiments from Table 1. 16:0, 18:0, 18:1, palmitic, stearic and oleic acids, respectively

Treatment[a]	Distribution of radioactivity among fatty acids (%)		
	16:0	18:0	18:1
ATP control	38.0	11.8	50.2
−ATP	−[b]	−	−
Without ATP			
Complete DHAP shuttle	28.3	11.2	60.5
PEP	21.3	6.9	71.8
Glc6P	26.1	6.4	67.5
Fru6P	28.8	3.7	67.5
With ATP			
Complete DHAP shuttle	20.6	7.2	72.2
PEP	24.7	8.6	66.7
Glc6P	27.3	5.4	67.3
Fru6P	25.5	4.5	70.0

[a] The concentrations of each compound tested, including components of the DHAP shuttle, were all 2 mM except for shuttle KH_2PO_4 which was 4 mM

[b] Insufficient radioactivity for radio-gas liquid chromatographic analysis

Table 5. Effects of substituting various combinations of glycolytic compounds for ATP on the distribution of radioactivity among lipids synthesized by pea root plastids. Data shown are derived from analyses of chloroform extraction phases of replicate "without ATP" treatments shown in Table 4. MAG, DAG, TAG, mono-, di-, and triacylglycerol, respectively; FFA, free fatty acid; PA, phosphatidic acid, PC, phosphatidylcholine, PG, phosphatidylglycerol; PI, phophatidylinositol

Lipid	% Distribution				
	ATP control	DHAP shuttle	PEP	Glc6P	Fru6P
TAG	7.5	5.1	6.8	10.9	12.8
DAG	15.3	9.5	9.8	8.7	7.8
MAG	1.8	2.6	2.1	3.7	4.1
FFA	7.2	34.4	31.9	30.3	29.0
PA	29.4	10.2	15.3	14.0	14.9
PG	10.5	5.4	8.3	8.1	8.9
PC	21.1	18.7	16.9	16.5	14.8
PI	3.2	3.8	2.2	3.1	2.6
Unid[a]	3.9	9.8	6.7	4.7	5.1

[a] Unidentified material corresponding to radioactivity at solvent fronts and origins of TLC plates

shuttle, PEP, Glc6P and Fru6P all caused a marked increase in the proportion of radioactive oleate accumulated (from 50% up to 72%). The increase in oleate accumulation was accompanied by an almost corresponding decrease in the amount of palmitate accumulated, suggesting that the elongation of palmitate was promoted.

Radioactive fatty acids synthesized by pea root plastids are accumulated in a variety of glycerolipids and related intermediates relatively typical for plastids under similar in-vitro conditions (Sparace and Kleppinger-Sparace 1993). Phosphatidic acid, phosphatidylcholine, phosphatidylglycerol and diacylglycerol together represented greater than 75% of the total lipid radioactivity in the ATP controls (Table 5). The profile of glycerolipid labelling is similar to that observed with daffodil chromoplasts (Kleining and Liedvogel 1980). The substitution of any combination of metabolites which promoted intraplastidic ATP synthesis (via the shuttle or glycolytic metabolism) caused a four- to fivefold increase in the levels of free fatty acids with corresponding decreases in, primarily, phosphatidic acid and diacylglycerol. Similar observations were made with dark-incubated chloroplasts (Kleinig and Liedvogel 1979), and daffodil chromoplasts (Kleinig and Liedvogel 1980), except that with the latter, the increase in free fatty acids was accompanied by decreases in the amount of phosphatidylcholine. Further, with pea root plastids, when Glc6P or Fru6P was used as the energy source, the proportion of triacylglycerol (TAG) almost doubled from 7% to nearly 13% of the radioactivity. Although TAG biosynthesis typically occurs in microsomal fractions (endoplasmic reticulum) of developing oilseeds (Frentzen 1993), we have observed the accumulation of radiolabeled TAG by pea root plastids in a number of earlier studies (Stahl 1990, pp. 71, 91) and have ruled out the possibility of microsomal or other organellar contamination. This conclusion is based primarily on marker-enzyme studies in our laboratory (Xue 1993, p. 49) and by other workers using similar plastid preparations (Trimming and Emes 1993).

From the data shown in Table 5, it is tempting to speculate that both ATP levels and glycolytic metabolism can modulate fatty-acid and glycerolipid metabolism in nonphotosynthetic plastids. This is particularly important in view of the fact that these plastids, including pea root plastids, also have their own pyruvate dehydrogenase (Sparace and Kleppinger-Sparace 1993). Thus, the end-product of glycolytic metabolism can readily be converted to acetyl-CoA which can then be used for fatty-acid synthesis (Harwood 1988). Further, as discussed earlier and suggested by other workers (Kleining and Liedvogel 1980), in some situations the amount of stimulation of fatty-acid biosynthesis may be underestimated due to the eventual entry of exogenously added carbon into the acetyl-CoA pool and dilution of the [^{14}C]acetate tracer used in this and similar studies.

The results discussed here are also consistent with the idea that high extraplastidic ATP may promote fatty-acid export from the plastid through the increased conversion of free fatty acids to acyl-CoA's at the plastid envelope (Roughan and Slack 1977). In the presence of exogenously supplied ATP, the acyl-CoA fraction accounts for approximately 15% of the total radioactivity. However, this is generally reduced to less than 2% when any treatment leading to intraplastidic ATP generation is used in place of exogenous ATP (data not shown). Thus, it would be expected that conditions of relatively low external ATP could result in the accumulation of free fatty acids. Similarly, when glycolytic carbon flow is high (as would be expected in the presence of hexoses and trioses), an abundance of carbon (in the form of acetyl-CoA or malonyl-CoA) would favour the elongation of

16-carbon fatty acids to 18-carbon fatty-acids by fatty-acid synthetase II. Further, in pea root plastids, the activity of phosphatidic-acid phosphatase appears to be enhanced in the absence of exogenously supplied ATP. Thus, when glycolytic metabolism is promoted under these conditions, diacylglycerols are apparently routed towards endogenous, plastidic TAG which may serve as a carbon sink and to perhaps avoid the potentially toxic effects of high levels of free fatty acids. The complete physiological significance of TAG formation in these plastids, however, remains to be determined. Perhaps more importantly, because the plastid is the source of fatty-acids for glycerolipid biosynthesis in all compartments of plant cells (Stumpf 1984), the results presented here suggest ultimately that increased fatty-acid availability due to increased glycolytic carbon flow can lead to the stimulation of extraplastidic glycerolipid biosynthesis. This could be particularly important for TAG biosynthesis in the endoplasmic reticulum of developing oilseed cells (Dennis 1989).

The results presented here are in general agreement with similar studies of other nonphotosynthetic plastids. Pea root plastids, like daffodil chromoplasts (Kleinig and Liedvogel 1980) and castor-bean leucoplasts (Boyle et al. 1990), can utilize both 2PGA and PEP via glycolytic metabolism in place of exogenous ATP to support fatty-acid biosynthesis. Similarly, pea root plastids, like daffodil chromoplasts, can utilize DHAP and 3PGalde as alternative energy sources, particularly when their uptake and metabolism is facilitated as part of the triose-phosphate shuttle. Pea root plastids can also utilize Glc6P and Fru6P as an energy source to promote fatty-acid biosyntheis, but not nearly as well as exogenously supplied ATP. Thus, although pea root plastids can derive a portion of their energy requirements for fatty-acid biosynthesis from their own glycolytic metabolism, they must also rely on extraplastidic sources of ATP for maximum rates of fatty-acid synthesis.

This research was supported by grants from the Natural Sciences and Engineering Research Council of Canada.

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