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Characterization of Fatty Acid Biosynthesis in Isolated Pea Root Plastids[1]

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ABSTRACT

Fatty acid biosynthesis from Na[1-^{14}C]acetate was characterized in plastids isolated from primary roots of 7-day-old germinating pea (*Pisum sativum* L.) seeds. Fatty acid synthesis was maximum at 82 nanomoles per hour per milligram protein in the presence of 200 micromolar acetate, 0.5 millimolar each of NADH, NADPH, and coenzyme A, 6 millimolar each of ATP and $MgCl_2$, 1 millimolar each of $MnCl_2$ and glycerol-3-phosphate, 15 millimolar $KHCO_3$, 0.31 molar sucrose, and 0.1 molar Bis-Tris-propane, pH 8.0, incubated at 35°C. At the standard incubation temperature of 25°C, fatty acid synthesis was essentially linear for up to 6 hours with 80 to 120 micrograms per milliliter plastid protein. ATP and coenzyme A were absolute requirements, whereas divalent cations, potassium bicarbonate, and reduced nucleotides all variously improved activity two- to 10-fold. Mg^{2+} and NADH were the preferred cation and nucleotide, respectively. Glycerol-3-phosphate had little effect, whereas dithiothreitol and detergents generally inhibited the incorporation of [^{14}C]acetate into fatty acids. On the average, the principal radioactive products of fatty acid biosynthesis were approximately 39% palmitic, 9% stearic, and 52% oleic acid. The proportions of these fatty acids synthesized depended on the experimental conditions.

Fatty acid biosynthesis in higher plants is generally thought to occur in a variety of plastids (13). Among those studied are the chloroplasts of leaves (6, 8–10), chromoplasts of daffodil (4), leucoplasts of cauliflower buds (3), developing (2, 5) and germinating (14) castor bean seed, plastids of soybean suspension cells (7), and most recently, the leucoplasts of germinating pea (*Pisum sativum* L.) roots (12). All of these plastids have been variously characterized for their capacities for fatty acid biosynthesis. However, except for only a preliminary study (12), fatty acid synthesis by pea root plastids has not been fully characterized. The purpose of the present study was to define the optimum *in vitro* conditions for fatty acid biosynthesis by isolated pea root plastids.

MATERIALS AND METHODS

Plant Material and Plastid Isolation

Seeds of pea (*Pisum sativum* L. cv Improved Laxton's Progress) were germinated under sterile conditions (12). After 7 d, 6 to 10 g of root tissue (2–4 cm lengths) per experiment were homogenized in 2 mL of homogenization medium (12) per g of root tissue. The homogenate was successively filtered through two layers each of 250 and 50 μm nylon mesh to remove tissue fragments and whole cells. The filtrate was centrifuged at 500 *g* for 5 min to yield the plastid fraction (12). Plastids were resuspended in one-tenth the original homogenate volume in a medium containing 1.0 mM Bis-Tris-propane[2] buffer (pH 7.5) and 0.46 M sucrose to yield a suspension of plastid protein concentration between 1.0 and 1.5 mg/mL.

In Vitro Conditions for Fatty Acid Biosynthesis

Under standard reaction conditions, plastids equivalent to 40 to 60 μg protein were incubated in a total reaction volume of 0.5 mL containing 0.1 M Bis-Tris-propane buffer (pH 8.0), 0.31 M sucrose, 0.2 mM Na[1-^{14}C]acetate (11–14 μCi/μmol), 0.5 mM each of NADH, NADPH, and CoA, 1.0 mM each of G3P and $MnCl_2$, 6 mM each of ATP and $MgCl_2$, and 15 mM $KHCO_3$. Reactions were initiated by the addition of plastids, incubated at 25°C, and terminated after 1 h by the addition of 0.1 mL 8 N NaOH. Total fatty acids were hydrolyzed, extracted, and analyzed by liquid scintillation counting and radio-GLC as described previously (12).

All experiments were performed twice, and data shown are representative of each experiment. Data points represent averages of duplicate analyses within an experiment. Standard deviation of replicates was less than 10% of the means at least 90% of the time. Replicate values obtained in the fatty acid distribution analysis never deviated by more than 5% from the means. All other methods and reagents were as specified earlier (12).

RESULTS

Effects of Incubation Time and Plastid Concentration

Under optimum *in vitro* conditions, total fatty acid biosynthesis from [^{14}C]acetate was linear for 4 h and still increasing at 6 h (Fig. 1). Over this time period, palmitic acid was the major radioactive fatty acid accumulated at the shortest incubation period—57% at 0.25 h—which gradually decreased to 36% at 6 h while oleic acid increased from 31 to 56%.

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[2] Abbreviations: Bis-Tris-propane, 1,3-bis[tris(hydroxymethyl)-methylamino]-propane; G3P, glycerol-3-phosphate; 16:0, 18:0, and 18:1 correspond to palmitic, stearic, and oleic acids, respectively.

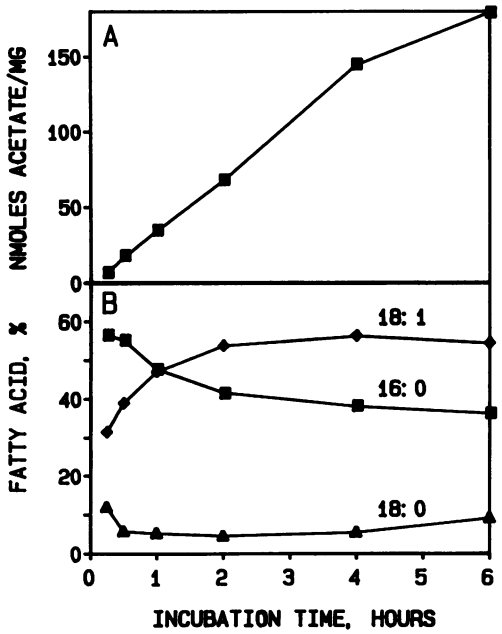

Figure 1. The effects of incubation time on fatty acid synthesis by pea root plastids. Plastids equivalent to 91 μg protein were incubated under standard incubation conditions for the indicated time periods. Panel A shows the total activity for fatty acid biosynthesis, and panel B shows the distribution of radioactivity among palmitic, stearic, and oleic acids.

After a decrease from 12 to 5%, the levels of stearic acid generally remained low.

Total fatty acid synthesis increased linearly with increasing concentrations of plastids up to 70 μg/mL plastid protein (Fig. 2). Beyond this point, fatty acid synthesis was still increasing with up to 240 μg/mL plastid protein, although the linearity of increase was declining. Plastid concentration had no effect on the proportions of radioactive fatty acids synthesized. On the average, the levels of palmitic, stearic, and oleic acids remained at 31, 12, and 57%, respectively.

Effects of Acetate and Bicarbonate Concentration

The rate of fatty acid synthesis by pea root plastids was saturated at 200 μM acetate (Fig. 3). However, concentrations of acetate ranging from 25 to 600 μM had no effect on the proportions of palmitic, stearic, and oleic acids synthesized. These remained at 29, 14, and 57% of the fatty acid radioactivity, respectively, over the range of acetate concentrations tested.

The addition of increasing amounts of $KHCO_3$ stimulated fatty acid biosynthesis up to 3.6-fold, which was achieved at 15 mM $KHCO_3$ (Fig. 4). Over the range from 0 to 10 mM $KHCO_3$, the proportion of palmitic acid accumulated decreased from 70 to 55% while oleic acid increased from 20 to 40%. These observations suggest that fatty acid synthetase II may be more limited than fatty acid synthetase I at $KHCO_3$ concentrations less than 10 mM. Concentrations of $KHCO_3$ above 10 mM had no further effect on the proportions of fatty acids synthesized.

Effects of pH, Buffer, and Temperature

Pea root plastids showed a relatively broad pH optimum for fatty acid biosynthesis between pH 7.5 and 8.5 using either Bis-Tris-propane or Tricine buffers (Fig. 5A). However, Bis-Tris-propane gave approximately 40% greater activity than Tricine. Hepes, Tes, and piperazine-*N*, *N'*-bis[2-hydroxypropanesulfonic acid] buffers were also tested, but they gave maximum activities of approximately 44, 71, and 64%, respectively, of that for Bis-Tris-propane (data not shown).

In contrast to the variations in incubation conditions described earlier, pH had a marked effect on the proportions of fatty acids synthesized. Bis-Tris-propane and Tricine showed virtually identical trends (Fig. 5B and C). With either buffer, as the incubation pH was raised from 6 to 10, the proportion of oleic acid steadily increased to a maximum of approximately 60% at pH 7.5 in Bis-Tris-propane or pH 8.0 in Tricine and then gradually decreased. This was accompanied by a corresponding decrease and then increase in the proportion of stearic acid synthesized. The proportions of palmitic acid accumulated steadily declined over the entire pH range tested.

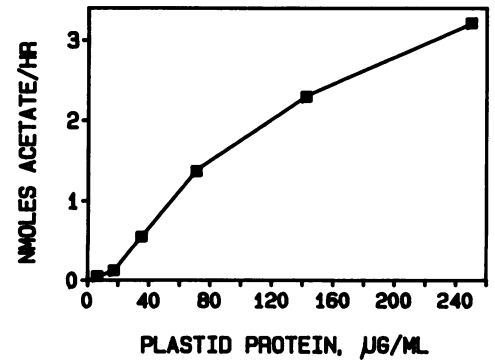

Figure 2. The effects of plastid protein concentration on fatty acid synthesis by pea root plastids. Plastids equivalent to the amounts of protein indicated were incubated under standard incubation conditions for 1 h. The proportions of palmitic, stearic, and oleic acids synthesized remained constant at 31, 12, and 57%, respectively.

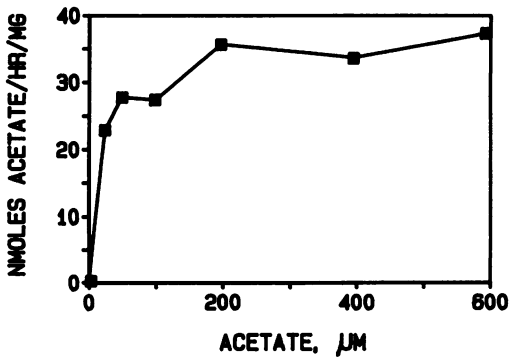

Figure 3. The effects of acetate concentration on fatty acid synthesis by pea root plastids. Plastids equivalent to 72 μg protein were incubated under standard incubation conditions. The proportions of palmitic, stearic, and oleic acids synthesized remained constant at 29, 14, and 57%, respectively.

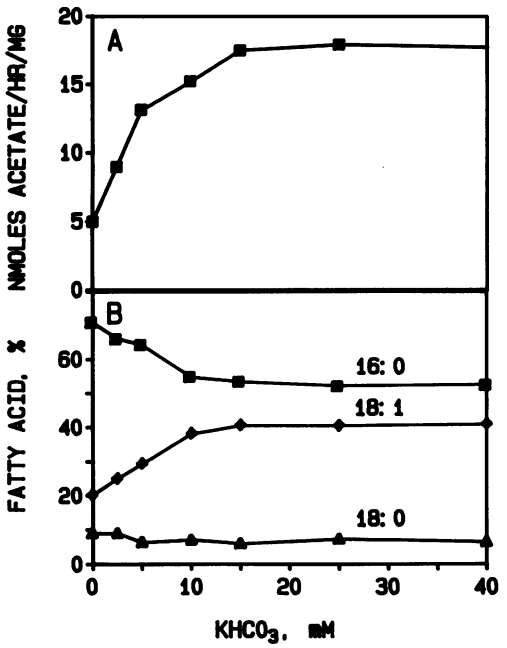

Figure 4. The effects of $KHCO_3$ concentration on fatty acid synthesis by pea root plastids. Plastids equivalent to 69 μg protein were incubated under standard reaction conditions. Panel A shows the specific activity for total fatty acid biosynthesis, and panel B shows the distribution of radioactivity among palmitic, stearic, and oleic acids.

These observations strongly suggest that the effects of incubation pH on total fatty acid biosynthesis are mediated at the level of stearate desaturase and that this enzyme is the rate-limiting step in *de novo* fatty acid biosynthesis by isolated pea root plastids.

When the effects of temperature were investigated, fatty acid synthesis showed a typical enzymic response (Fig. 6). The rate of acetate incorporation into fatty acid increased linearly from about 10 nmol/h·mg protein at 15°C to a maximum of 82 nmol/h·mg protein at 35°C before rapidly falling off beyond 40°C. The greatest changes in the proportions of each fatty acid synthesized were observed above 25°C, where the levels of oleic acid gradually decreased as palmitic and stearic acids increased.

Effects of Divalent Cations and ATP

Fatty acid biosynthesis by pea root plastids was completely dependent on exogenously supplied divalent cations and ATP (Figs. 7 and 8). In their absence, fatty acid synthesis was abolished. When the concentrations of either Mg^{2+} or Mn^{2+} were varied in the absence of the other cation, the optimum concentrations of these cations were 4 mM for Mg^{2+} and 2 mM for Mn^{2+} (Fig. 7). Mn^{2+} gave the greatest total activity below 2 mM while Mg^{2+} gave the greatest activity above 2 mM. Mn^{2+} became increasingly inhibitory at concentrations higher than 2 mM. These cations had different effects on the proportions of radioactive fatty acids synthesized. Mg^{2+} increased the amount of oleic acid synthesized from 36 to 66%, while it correspondingly decreased the levels of palmitic acid from 51 to 21%, without any significant effect on the proportions of stearic acid accumulated (Fig. 7B). Mn^{2+}, especially at concentrations above 6 mM, decreased the amount of oleic acid accumulated from approximately 57 to 10%, while simultaneously increasing the amount of stearic acid accumulated from 20 to 60%. These effects were also accompanied by a smaller increase from 20 to 35% palmitic acid accumulated. The cation effects suggest that Mg^{2+} may be a required cofactor or regulator of fatty acid synthetase II for the elongation of palmitic acid, and that Mn^{2+} may specifically inhibit stearate desaturase.

In the presence of 1 mM $MnCl_2$ and 6 mM ATP, the optimum concentration of Mg^{2+} was 6 mM (data not shown). When ATP and Mg^{2+} were simultaneously increased at equimolar concentrations, fatty acid biosynthesis was maximum at 6 mM (Fig. 8). However, no effects were observed on the proportions of palmitic, stearic and oleic acids synthesized, which remained essentially constant at 32, 11, and 57%, respectively. When concentrations of up to 10 mM Mn^{2+} were

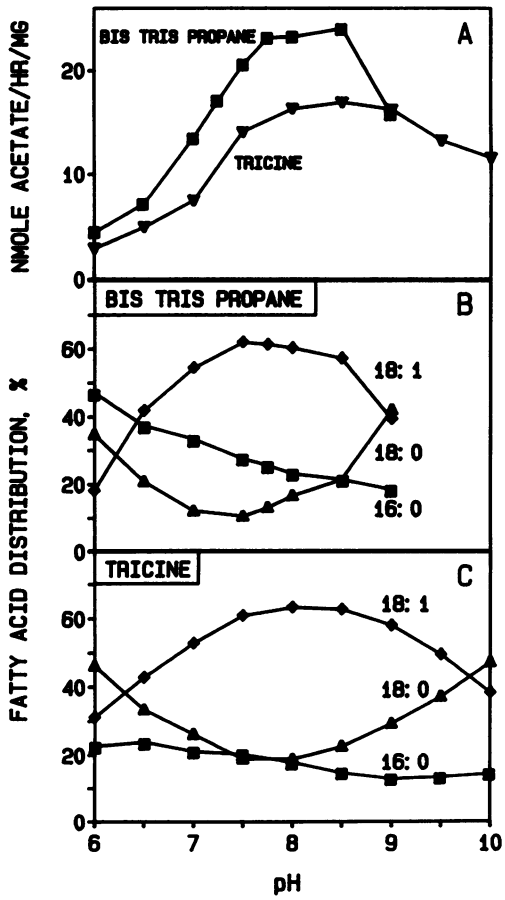

Figure 5. The effects of incubation pH and buffer on fatty acid synthesis by pea root plastids. Plastids equivalent to 98 μg protein were incubated in the presence of 0.1 M of either Bis-Tris-propane or Tricine at the indicated pHs. Panel A shows the specific activities for total fatty acid biosynthesis in the presence of either Bis-Tris-propane or Tricine buffers. Panels B and C show the distributions of radioactivity among palmitic, stearic, and oleic acids synthesized in the presence of each of these buffers.

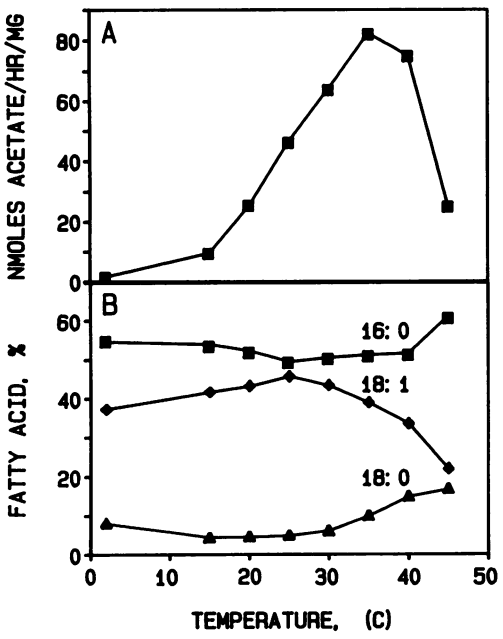

Figure 6. The effects of incubation temperature on fatty acid biosynthesis by pea root plastids. Plastids equivalent to 82 μg protein were incubated at the indicated temperatures under standard incubation conditions. Panel A shows the specific activity for total fatty acid biosynthesis, and panel B shows the distribution of radioactivity among palmitic, stearic, and oleic acids at each temperature tested.

used in the presence of 6 mM ATP and 6 mM Mg^{2+}, total fatty acid biosynthesis was gradually inhibited by greater than 50% and the proportions of stearate accumulated from 2 to 25% while oleate decreased from 45 to 27% (data not shown).

Effects of Reduced Nucleotides, CoA, and G3P

Exogenously supplied NADH and/or NADPH stimulated fatty acid biosynthesis by pea root plastids but were not essential. The addition of optimum concentrations of these nucleotides either separately or in equimolar amounts resulted in approximately a twofold increase in the total rate of fatty acid synthesis (Fig. 9). When supplied separately, each was optimum at 1 mM, with NADH generally resulting in higher activities especially at concentrations less than 1 mM. However, the greatest activities were observed when NADH and NADPH were used in combination at equimolar concentrations from 0.12 to 0.5 mM. The addition or omission of these reduced nucleotides had no effect on the proportions of radioactive fatty acids synthesized, which remained at 42, 5, and 53% palmitic, stearic, and oleic acids, respectively.

CoA was essential for fatty acid biosynthesis with approximately only 5% of the maximum activity obtained in its absence (Fig. 10). The greatest increase in activity caused by CoA was observed at the lowest concentration tested (approximately 75% of maximum activity achieved with 0.025 mM). Total activity was further increased by an additional 25% with up to 0.5 mM CoA. The addition of the lowest concentration (0.025 mM) CoA had a marked effect on the proportions of palmitic and oleic acids synthesized, causing approx-

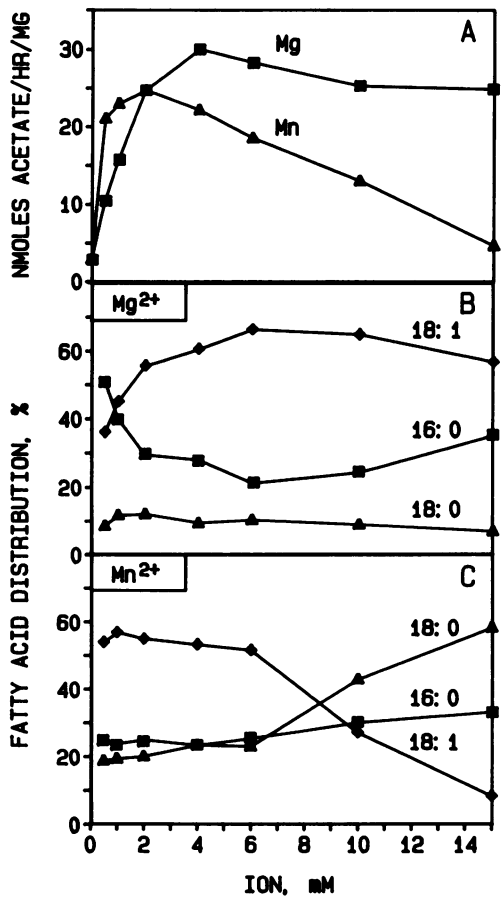

Figure 7. The effects of Mg^{2+} and Mn^{2+} concentrations on fatty acid synthesis by pea root plastids. Plastids equivalent to 59 μg protein were incubated in the presence of each cation individually. Cations were added as the Cl^- salts. All other cofactors and conditions were as indicated for the standard incubation conditions. Panel A shows the specific activities for total fatty acid biosynthesis in the presence of either Mg^{2+} or Mn^{2+} at the indicated concentrations, and panels B and C show the distributions of radioactivity among palmitic, stearic, and oleic acids for each cation concentration tested.

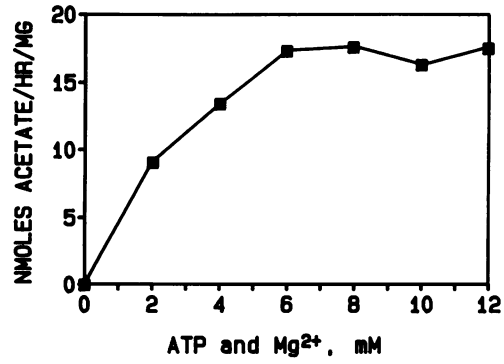

Figure 8. The effects of equimolar amounts of ATP and Mg^{2+} on fatty acid synthesis by pea root plastids. Plastids equivalent to 82 μg protein were incubated under standard incubation conditions. The proportions of palmitic, stearic, and oleic acids synthesized remained constant at 32, 11, and 57%, respectively.

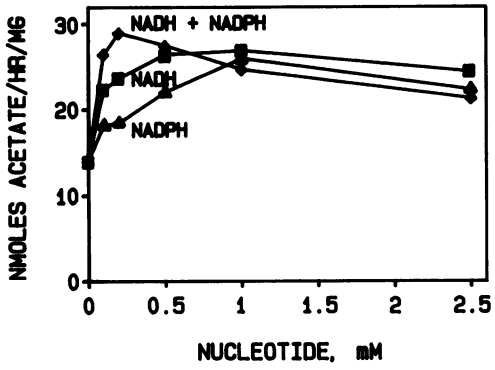

Figure 9. The effects of NADH and NADPH on fatty acid synthesis by pea root plastids. Plastids equivalent to 70 μg protein were incubated in the presence of NADH or NADPH either alone or in equimolar concentrations as indicated. The proportions of palmitic, stearic, and oleic acids synthesized remained constant at 42, 5, and 53%, respectively.

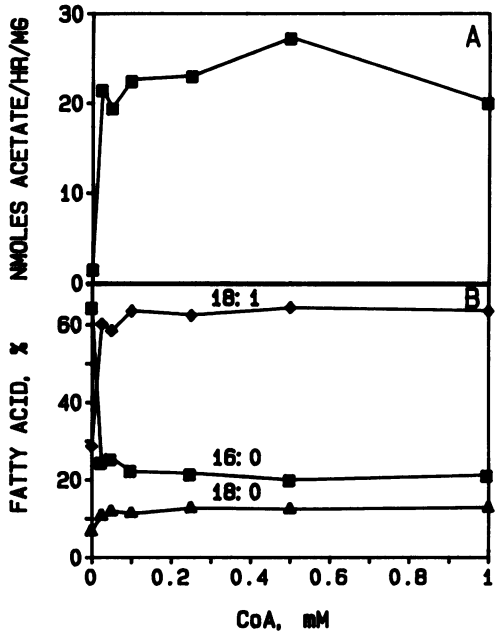

Figure 10. The effects of CoA concentration on fatty acid synthesis by pea root plastids. Plastids equivalent to 95 μg protein were incubated under standard incubation conditions with the indicated concentration of CoA. Panel A shows the specific activity for total fatty acid synthesis, and panel B shows the distribution of radioactivity among palmitic, stearic, and oleic acids.

imately a 40% decrease in palmitic acid with a 30% increase in the amount of oleic acid accumulated. Beyond 0.025 mM CoA, little or no effect was observed on the proportions of fatty acids synthesized.

G3P was not a requirement for fatty acid biosynthesis; however, activity was stimulated by approximately 42% by the addition of up to 0.5 mM G3P (Fig. 11). Within this range the amount of palmitic acid accumulated was slightly increased by 10% while oleic acid was decreased by a similar amount.

The Effects of DTT and Detergents

Increasing concentrations of DTT up to 0.5 mM gradually inhibited total fatty acid synthesis by approximately 50% (Fig. 12). Over this range, the proportion of stearate rose from about 10 to 40% while the amounts of oleate and palmitate were reduced by approximately equal amounts. However, at higher concentrations of DTT, essentially no further inhibition or change in the radioactive fatty acid composition was observed. These observations suggest that DTT may specifically inhibit stearate desaturase and provide further evidence that this enzyme may be the rate-limiting step for fatty acid biosynthesis in this system. Other sulfhydryl reagents (except CoA) were not tested.

The addition of 0.0012% Triton X-100 or 62.5 μM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate stimulated total fatty acid biosynthesis by approximately 15 and 13%, respectively (data not shown). However, higher concentrations of these detergents (up to 0.01% and 1 mM,

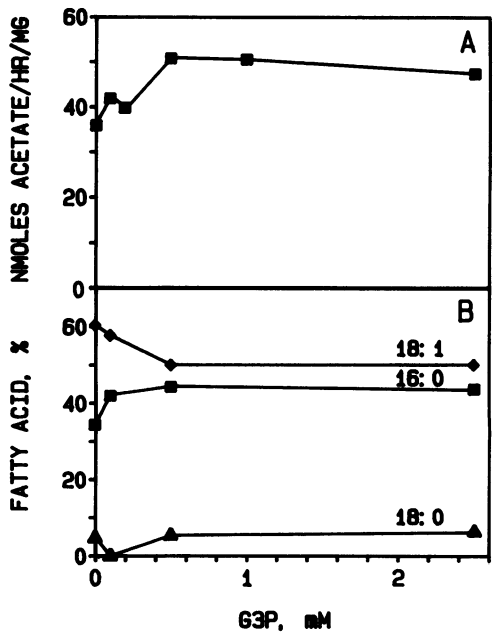

Figure 11. The effects of G3P concentration on fatty acid synthesis by pea root plastids. Plastids equivalent to 89 μg protein were incubated under standard incubation conditions with the indicated concentrations of G3P. Panel A shows the specific activity for total fatty acid synthesis, and panel B shows the distribution of radioactivity among palmitic, stearic, and oleic acids.

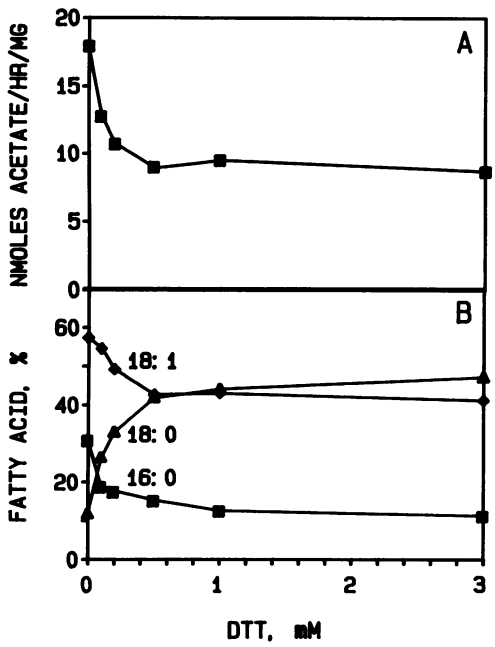

Figure 12. The effects of DTT on fatty acid synthesis by pea root plastids. Plastids equivalent to 82 μg protein were incubated under standard incubation conditions with the indicated concentrations of DTT. Panel A shows the specific activity for total fatty acid synthesis, and panel B shows the distribution of radioactivity among palmitic, stearic, and oleic acids.

respectively) gradually inhibited or abolished activity. Both detergents increased the proportion of oleic acid accumulated while the proportions of palmitic acid decreased and stearic acid remained the same.

DISCUSSION

The present study represents the first comprehensive characterization of fatty acid biosynthesis in plastids isolated from rapidly growing root tissues. In general, fatty acid synthesis by isolated pea root plastids closely resembles that in other nonphotosynthetic plastids (1, 3–5, 7, 14) and chloroplasts (6, 8–10). With little exception, pea root plastids synthesize exclusively palmitic, stearic, and oleic acids when [^{14}C]acetate is the labeled substrate. As with other systems, this is presumably because these fatty acids are the products released to the fatty acid pool from the fatty acid synthetase complex, which results in a marked decrease in their specific radioactivity. Consequently, it becomes difficult to monitor small amounts of subsequent metabolism or desaturation which normally occurs in another cellular compartment (the endoplasmic reticulum) (13).

In the process of fatty acid biosynthesis from acetate, ATP is normally required in reactions catalyzed by acetyl-CoA synthetase and acetyl-CoA carboxylase (13). For pea root plastids, exogenously supplied ATP was an absolute requirement for fatty acid biosynthesis. Similar results were observed with safflower cotyleons (1), cauliflower bud plastids (3), and daffodil chromoplasts (4). In contrast, the addition of ATP caused four- and twofold improvements in fatty acid biosynthesis in plastids from soybean suspension cells (7) and leucoplasts from the endosperm of developing castor bean seeds (5), respectively. Fatty acid synthesis in photosynthetically active spinach chloroplasts showed little or no stimulation with exogenously supplied ATP (8, 10). The lack of dependency of chloroplasts on externally supplied ATP is presumably due to the internal plastidic source of this cofactor during photosynthesis. The optimum ATP concentration of 6 mM reported here is comparable to the 3, 4, and 8 mM optima reported for soybean suspension plastids, daffodil chromoplasts, and safflower cotyledon plastids, respectively (1, 4, 7).

A divalent cation was also essential for fatty acid biosynthesis, with Mg^{2+} overall giving greater activity than Mn^{2+}. Similar requirements for divalent cations have been demonstrated for developing castor bean plastids (5) and chloroplasts (6). In the presence of 1 mM $MnCl_2$, the greatest activity for fatty acid biosynthesis was obtained when equimolar concentrations of ATP and Mg^{2+} were used. This suggests the involvement of an Mg-ATP complex in reactions involving ATP.

Pea root plastids also showed an absolute requirement for CoA. Fatty acid biosynthesis was only 5% of controls in the absence of this cofactor. A similar dependency was observed for soybean suspension plastids (7). However, daffodil chromoplasts, cauliflower plastids, and developing castor bean plastids were, respectively, only 50, 15, and 10% dependent on exogenously supplied CoA for maximum activity (3–5). Chloroplasts were intermediate with approximately 33% dependency on exogenously supplied CoA (8). The variability in these findings suggests that these different plastids all have different residual pool sizes of CoA when they are isolated.

The reduced nucleotides NADH and NADPH are required in the reduction reactions in *de novo* fatty acid biosynthesis and stearate desaturation (13). These nucleotides were not essential but gave approximately a 2.5-fold stimulation of fatty acid biosynthesis when both were provided. NADH was preferred at concentrations less than 1 mM. Similar observations have been made with plastids from developing castor beans (5), daffodil petals (4), and soybean suspension cells (7). In contrast, however, plastids from germinating castor beans required both reduced nucleotides for maximum activity (14), whereas safflower cotyledon plastids had no apparent requirement for either nucleotide (1).

When acetate is used as a substrate for fatty acid biosynthesis, bicarbonate generally must also be used as a co-substrate for acetyl-CoA carboxylase (13). Fatty acid biosynthesis from [^{14}C]acetate in pea root plastids was dependent on the co-incubation of $KHCO_3$ in the standard reaction medium. The addition of $KHCO_3$ resulted in greater than a threefold stimulation of activity. Maximum rates of fatty acid synthesis were achieved in the presence 200 μM acetate and 15 mM $KHCO_3$. These results are in close agreement with other studies using nonphotosynthetic plastids (1, 4) and chloroplasts (8, 9) but differ from the 2.5 mM acetate reported for plastids from soybean cell suspensions (7).

G3P is not required for fatty acid biosynthesis, but rather acts as the final acyl acceptor, thereby preventing the buildup of intermediates of fatty acid biosynthesis and their gradual feedback inhibition of *de novo* fatty acid synthesis. The addition of G3P to the standard pea root plastid reaction mixture

resulted in almost a 40% increase in total fatty acid biosynthesis. In contrast, Roughan *et al.* (8, 9), using spinach chloroplasts, found that concentrations of G3P similar to those used in this investigation had no effect on the rates of total fatty acid biosynthesis, whereas concentrations of G3P above 2 mM inhibited fatty acid biosynthesis. They also found that up to 5 mM G3P reduced the ratio of [^{14}C]oleate to [^{14}C] palmitate synthesized from 3.75 to 0.58. A similar, although not as marked, response was observed using pea root plastids in the present study.

Under the optimum concentrations of all cofactors, fatty acid biosynthesis by pea root plastids had a relatively broad pH optimum between 7.5 and 8.5 in either Bis-Tris-propane or Tricine buffer. Maximum activity was routinely achieved at pH 8.0 with Bis-Tris-propane. These observations are in agreement with those of other workers (4, 6, 7, 14). Over the range tested, pH had a striking effect on the proportions of radioactive fatty acids synthesized. With both buffers, the optimum pH for maximum oleate accumulation was virtually identical to that for total fatty acid biosynthesis. Any changes in the proportion of oleate accumulated was accompanied by corresponding opposite changes in the proportion of stearate accumulated. Thus, stearate accumulation predominated at the extreme pHs, while it was minimal at the pH optimum for total fatty acid synthesis and oleate accumulation. As indicated earlier, these observations suggest that stearate desaturase is the rate-limiting step in *de novo* fatty acid biosynthesis and that the effects of pH are manifested at the level of this enzyme. These observations also suggest that both buffers used in this investigation can readily penetrate the envelope of pea root plastids. Other cofactors had little or no effect on the proportions of palmitate, stearate, and oleate synthesized. Preliminary (12) and ongoing studies indicate that radioactive fatty acids are recovered in phosphatidic acid, diacylglycerol, triacylglycerol, phosphatidylglycerol, and phosphatidylcholine, which is, in part, in agreement with related studies (3, 4).

The present study raises some important questions concerning lipid metabolism in pea root plastids. The extent to which these plastids must rely on exogenously supplied ATP or their capacity to synthesize ATP internally for fatty acid biosynthesis is not known. However, there is considerable evidence indicating that nonphotosynthetic plastids can synthesize ATP via glycolytic pyruvate kinase and/or phosphoglycerate kinase (2–4, 11). Similarly, glycerolipid biosynthesis utilizing the products of *de novo* fatty acid biosynthesis in pea root plastids remains to be completely defined. The energy requirements for fatty acid biosynthesis by pea root plastids and the characterization of their capacity for glycerolipid biosynthesis are subjects of continuing investigations.

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