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On the Control of Long-Chain-Fatty Acid Synthesis in Isolated Intact Spinach (*Spinacia oleracea*) Chloroplasts

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1. Chloroplasts isolated from spinach leaves by using the low-ionic-strength buffers of Nakatani & Barber [(1977) *Biochim. Biophys. Acta.* **461**, 510–512] had higher rates of HCO_3^--dependent oxygen evolution (up to 369 μmol/h per mg of chlorophyll) and higher rates of [1-^{14}C]acetate incorporation into long-chain fatty acids (up to 1500 nmol/h per mg of chlorophyll) than chloroplasts isolated by using alternative procedures. 2. Acetate appeared to be the preferred substrate for fatty acid synthesis by isolated chloroplasts, although high rates of synthesis were also measured from $H^{14}CO_3^-$ in assays permitting high rates of photosynthesis. Incorporation of $H^{14}CO_3^-$ into fatty acids was decreased by relatively low concentrations of unlabelled acetate. Acetyl-CoA synthetase activity was present 3–4 times in excess of that required to account for rates of [1-^{14}C]acetate incorporation into fatty acids, but pyruvate dehydrogenase was either absent or present in very low activity in spinach chloroplasts. 3. Rates of long-chain-fatty acid synthesis from [1-^{14}C]acetate in the highly active chloroplast preparations, compared with those used previously, were less dependent on added cofactors, but showed a greater response to light. The effects of added CoA plus ATP, Triton X-100 and *sn*-glycerol 3-phosphate on the products of [1-^{14}C]acetate incorporation were similar to those reported for less active chloroplast preparations. 4. Endogenous [^{14}C]acetyl-CoA plus [^{14}C]malonyl-CoA was maintained at a constant low level even when fatty acid synthesis was limited by low HCO_3^- concentrations. Endogenous [^{14}C]acyl-(acyl-carrier protein) concentrations increased with increasing HCO_3^- concentration and higher rates of fatty acid synthesis, but were slightly lower in the presence of Triton X-100. It is proposed that rates of long-chain-fatty acid synthesis in isolated chloroplasts at saturating [1-^{14}C]acetate concentrations and optimal HCO_3^- concentrations may be primarily controlled by rates of removal of the products of the fatty acid synthetase.

Plastids are possibly the sole sites of long-chain-fatty acid synthesis *de novo* in plant cells (Stumpf & James, 1963; Zilkey & Canvin, 1972; Nakamura & Yamada, 1974; Weaire & Kekwick, 1975*a*; Nothelfer *et al.*, 1977; Vick & Beevers, 1978), and yet a number of polar lipids are apparently synthesized in other cellular compartments. Phosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol were all synthesized primarily within a microsomal fraction of leaf homogenates (Kates & Marshall, 1975), phosphatidic acid synthesis from *sn*-glycerol 3-phosphate and acyl-CoA was localized in the endoplasmic reticulum of castor-bean (*Ricinus communis*) endosperm (Vick & Beevers, 1977) and CDP-diacylglycerol was synthesized exclusively outside the plastids of a number of plant tissues (Bahl *et al.*, 1970). Even diacylgalactosylglycerol may be assembled in the chloroplast envelope (Douce, 1974) from 1,2-diacylglycerols orginating in the endoplasmic reticulum (Slack *et al.*, 1977). However, purified chloroplast envelopes, when supplemented with stromal protein, incorporated [^{14}C]glycerol 3-phosphate into phosphatidic acid, 1,2-diacylglycerol and diacylgalactosylglycerol (Joyard & Douce, 1977). Since fatty acids must be exported from plastids in some form to permit the synthesis of these polar lipids, it seemed possible that other cell compartments may exert some control over rates of long-chain-fatty acid synthesis within the plastids. There are at least three ways in which this might be accomplished: (*a*) by a modulation of the substrate supply; (*b*) by the action of external affectors and (*c*) by the rate of removal of the products of the synthesis. All of these possibilities should be amenable to test by using isolated, intact chloroplasts.

The biosynthesis of fatty acids from [^{14}C]acetate in isolated chloroplasts has been extensively studied in Stumpf's laboratory and factors affecting rates of acetate incorporation have been reported (Stumpf & Boardman, 1970; Givan & Stumpf, 1971; Kannangara & Stumpf, 1972). It would appear, however, that rates measured in those studies were substrate-

limited and could have been 2–3-fold greater had higher concentrations of [^{14}C]acetate been used (Nakamura & Yamada, 1975; Roughan *et al.*, 1976). Yamada & Nakamura (1975) reported that pyruvate was incorporated into fatty acids of isolated spinach chloroplasts at higher rates than was acetate and suggested that pyruvate may be the more physiological precursor for fatty acid synthesis. Although not confirming a more effective utilization of [2-^{14}C]-pyruvate compared with [1-^{14}C]acetate, Murphy & Leech (1978) supported the proposal of Yamada & Nakamura (1975) of a flow of carbon from CO_2 → phosphoglyceric acid → phosphoenolpyruvate → pyruvate acetyl-CoA → fatty acids, thus making the chloroplast self-sufficient for fatty acid synthesis. Nevertheless, acetate at low concentrations (20–66 μM) provided 4-fold as much carbon for fatty acid synthesis as did CO_2, and the possibility remains that acetate (or pyruvate) supplied from the outside could significantly affect rates of fatty acid synthesis within the organelle.

Improved isolation techniques and greater attention to the condition of the original plant material has resulted in a steady increase over recent years in the capacity of isolated chloroplasts to synthesize long-chain fatty acids (Kannangara & Stumpf, 1972; Nakamura & Yamada, 1975; Roughan *et al.*, 1976; Murphy & Leech, 1978), and in studies on factors affecting rates of fatty acid synthesis, a knowledge of the ultimate potential rate of the biosynthesis within the system is obviously desirable. We report here a further improvement in rates of long-chain-fatty acid synthesis from both [1-^{14}C]acetate and $H^{14}CO_3^-$ in chloroplasts isolated from expanding spinach leaves. We have also attempted to ascertain how these rates might be affected by different treatments.

Experimental

Materials

The sodium salts of [1-^{14}C]acetate (58.1mCi/mmol), [1-^{14}C]pyruvate (10.7mCi/mmol), [2-^{14}C]-pyruvate (8.2mCi/mmol), [2-^{14}C]malonate (17mCi/mmol), [1-^{14}C]acetyl-CoA (4.99mCi/mmol) and [^{14}C]bicarbonate (38mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Sodium [^{14}C]pyruvate was dissolved in a stoicheiometric amount of HCl solution (von Korff, 1964), diluted as required with unlabelled sodium pyruvate and standardized with lactate dehydrogenase (von Korff, 1969). Acetyl-CoA synthetase was from Boehringer, Mannheim, Germany, whereas all other biochemicals used were from Sigma, St. Louis, MO, U.S.A. The buffering compounds Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] and Mes (4-morpholine-ethanesulphonic acid) were also from Sigma. Oleic acid was prepared from methyl oleate supplied by Nu Chek Prep, Elysian, MN, U.S.A., and 1,2-diacylglycerol was prepared by hydrolysis of egg-yolk phosphatidylcholine with phospholipase C (Gurr & Brawn, 1970).

Methods

Spinach (*Spinacia oleracea*) plants (hybrid 102, Arthur Yates Ltd., Auckland, New Zealand) were grown under a controlled artificial environment as described previously (Slack & Roughan, 1975) and provided expanding leaves 8–12cm long in 3–4 weeks after transfer of the seedlings to water culture. Leaves (25–30g fresh weight) were collected about 90min after the beginning of the artificial day and floated on ice/water under a mercury-vapour lamp for 10min before chloroplasts were isolated by the procedure of Nakatani & Barber (1977). In comparing biosynthetic activities of chloroplasts isolated with different buffer mixtures, leaf laminae were halved by removing the midrib and the halves from each leaf were homogenized in separate lots in two different media. Four different isolating media were compared in three experiments. Washed chloroplasts isolated by the procedure of Nakatani & Barber (1977) were resuspended in 4vol. of 10mM-Mes/NaOH, 2mM-EDTA, 1mM-$MgCl_2$, 1mM-$MnCl_2$ and 0.3mM-KH_2PO_4 with a final pH of 6.0–6.1 at 0°C.

Photosynthetic O_2 evolution was measured (Delieu & Walker, 1972) at 25°C in 2ml of a buffer mixture containing 0.33M-sorbitol, 25mM-Hepes/NaOH, pH 7.9, 10mM-$NaHCO_3$, 2mM-EDTA, 1mM-$MgCl_2$, 1mM-$MnCl_2$, 0.3mM-K_2HPO_4, 4000 units of catalase (Kaiser, 1976) and chloroplasts equivalent to 50μg of chlorophyll. The reaction was light-saturated and was linear with respect to chloroplast concentration. The standard assay for fatty acid synthesis contained, in a final volume of 0.25ml: 0.33M-sorbitol, 25mM-Hepes/NaOH, pH7.9, 10mM-$NaHCO_3$, 2mM-EDTA, 1mM-$MgCl_2$, 1mM-$MnCl_2$, 0.5mM-dithiothreitol, 0.3mM-K_2HPO_4, 0.16mM-sodium [1-^{14}C]acetate (1μCi/100nmol of acetate) and chloroplasts equivalent to 50μg of chlorophyll. In hypo-osmotic reaction media the only sorbitol present was that added with the chloroplasts to give a final concentration of 0.06M. The final pH was 7.9 at 25°C. Reactions were carried out in culture tubes (125mm × 20mm) that could be closed with Teflon-lined screw caps and were stopped after 15min incubation at 25°C in an illuminated Warburg apparatus (shaking rate 130 strokes/min and stroke length of 5.5cm) by one of the three following methods. (1) KOH [10% (w/v) in methanol; 1ml] was added to each tube, which was then flushed with N_2, capped and heated at 80°C for 90min (Nakamura & Yamada, 1975). After cooling and acidification, fatty acids were extracted into light

petroleum (b.p. 40–60°C) and the combined extracts finally concentrated to 0.5ml in light petroleum (b.p. 60–80°C). (2) Acetic acid [2.5% (v/v) in propan-2-ol; 2vol.] was added, followed by 0.25ml of water, and non-polar lipids were extracted into light petroleum (b.p. 40–60°C) (Mancha *et al.*, 1975). The aqueous layer was treated with methanolic KOH (see above) to recover any ^{14}C-labelled fatty acids remaining in that fraction. (3) Chloroform/methanol [2:1 (v/v); 5ml] was added to produce a single phase and total lipids were recovered in the chloroform layer after adding 0.75ml of 0.9% (w/v) NaCl (Folch *et al.*, 1957) and shaking. For recovery of any [^{14}C]-fatty acids in the aqueous methanol layer 0.5ml of 40% (w/v) KOH was added followed by heating to 80°C for 90min (see above).

Portions (50–100μl) of the extracts were streaked across 1cm in 2cm lanes on thin layers of 5% (w/w) H_3BO_3 in silica-gel G and chromatograms were developed with 4% (v/v) acetone in chloroform (Thomas *et al.*, 1965). Fatty acids from saponifications were detected by lightly staining in I_2 vapour, and the appropriate zones on chromatograms were scraped up into 0.5% (w/v) *p*-terphenyl in xylene/methanol (9:1, v/v) for liquid-scintillation counting of radioactivity. Lipid extracts were resolved into 1,2-diacylglycerols, free fatty acids and polar lipids (origins) by using the same chromatographic system; radioautography showed that these were the only significantly radioactive zones on chromatograms. Free fatty acids and 1,2-diacylglycerols were located by co-chromatographing 10μg each of oleic acid and of 1,2-diacylglycerol with each sample and lightly staining the developed chromatograms with I_2 vapour. Radiocarbon in these zones was determined by liquid-scintillation counting as described above, whereas that on the origin was measured by using an emulsion of 10% (v/v) water in xylene/Triton X-100 (2:1, v/v) containing 0.35% *p*-terphenyl. A number of ^{14}C-labelled compounds were substituted for [1-^{14}C]acetate in this system to test the effectiveness of their incorporation into long-chain fatty acids. When chloroplasts were incubated in the presence of $NaH^{14}CO_3$, the acidified aqueous phases from which ^{14}C-labelled fatty acids had been extracted were diluted to 10ml with water and acid-stable radiocarbon was measured by liquid-scintillation counting. Fatty acid synthesis from $H^{14}CO_3^-$ was measured both in the high-light/low-chlorophyll system used for measuring photosynthetic O_2 evolution and in the low-light/high-chlorophyll system used for measuring fatty acid synthesis from [1-^{14}C]acetate.

Fatty acids were converted with diazomethane into their methyl esters, which were separated into saturated, monoenoic and dienoic fractions by $AgNO_3$-t.l.c. The methyl esters were also purified by t.l.c. on plain silica gel G prior to analysis by radio-g.l.c. As a check for long-chain fatty acid synthesis *de novo* from [1-^{14}C]acetate, 2–3mg of methyl oleate was added to the [^{14}C]fatty acid methyl esters and [^{14}C]oleate was isolated by $AgNO_3$-t.l.c. A portion of this purified [^{14}C]oleate was oxidatively cleaved at the double bond (Downing & Greene, 1968) and the two C_9 fragments were recovered for chromatographic separation as the free acids on H_3BO_3-t.l.c. as above. Nonanoic acid ($R_F = 0.43$) and C_9 dicarboxylic acid half-ester ($R_F = 0.29$) were localized by radioautography and the radiocarbon content was determined by liquid-scintillation counting.

Acetyl-CoA synthetase was measured by incubating 10μl of chloroplasts (about 10μg of chlorophyll) at 25°C in 50μl final volume of 50mM-Hepes/NaOH, pH7.9, 5mM-$MgCl_2$, 2mM-dithiothreitol, 2mM-ATP, 0.5mM-CoA and 0.4mM-[1-^{14}C]acetate (100nmol/μCi). Portions (20μl) of this reaction mixture were transferred at 2.5 and 5min to 25mm (diam.) discs of Whatman no. 1 filter paper, which were then washed twice for 5min each in 5ml of ethanol/ether (3:1, v/v) containing 0.25% (w/v) trichloroacetic acid and once with 5ml of ether. Blanks contained no CoA or ATP, both of which were essential for high rates of [1-^{14}C]acetate fixation to the discs. Pyruvate dehydrogenase was measured by the ATP-independent synthesis of [^{14}C]acetyl-CoA from [2-^{14}C]pyruvate in a reaction mixture containing 25mM-Hepes/NaOH, pH7.9, 2.5mM-$MgCl_2$, 1.75mM-NAD^+, 0.2mM-thiamin pyrophosphate, 0.5mM-CoA, 2mM-dithiothreitol, 1.2mM-[2-^{14}C]pyruvate (2μCi/μmol) and chloroplasts equivalent to 10μg of chlorophyll in a final volume of 50μl. The formation of [^{14}C]acetyl-CoA was monitored as described above and blanks contained no NAD^+ or thiamin pyrophosphate. In some experiments $NADP^+$ was substituted for NAD^+, and in others both were included. Pyruvate dehydrogenase was also measured in broken chloroplasts and in a chloroplast stromal preparation by spectrophotometric methods (Schwartz *et al.*, 1968).

Pyruvate decarboxylase was measured by the release of $^{14}CO_2$ from [1-^{14}C]pyruvate in a reaction mixture identical with that used for measuring acetyl-CoA synthetase activity, except that [1-^{14}C]-pyruvate was used and blanks contained no enzyme. Incubations were carried out at 25°C in a final volume of 50μl in 13mm×100mm screw-capped tubes into which were fitted 10mm×15mm strips of filter paper moistened with 10μl of 40% (w/v) KOH. After 5 and 10min, reactions were stopped by injecting 10μl of 9M-H_2SO_4 through a septum and the tubes were shaken for 30min at 25°C. The $^{14}CO_2$ adsorbed by the KOH was measured by liquid-scintillation counting. Acetyl-CoA carboxylase was measured as an acetyl-CoA-dependent fixation of $H^{14}CO_3^-$ into acid-stable radioactivity (Reitzel & Nielsen, 1976) or by the conversion of [1-^{14}C]acetyl-CoA into

[2-^{14}C]malonate (Kannangara *et al.*, 1973). Activities of the above enzymes were measured in darkness or at light intensities less than that of the ambient room lighting.

Concentrations of [^{14}C]acetyl-CoA plus [^{14}C]-malonyl-CoA and [^{14}C]acyl-(acyl-carrier protein) during fatty acid synthesis were measured by spotting 25μl portions of reaction mixture on to filter-paper discs and washing the discs in ethanol/ether/trichloroacetic acid (as described above). Radioactivity on the discs was measured by liquid-scintillation counting in 0.5% (w/v) *p*-terphenyl in xylene, after which the discs were washed twice with 5ml of acetone and twice with 5ml of ice-cold 5% (w/v) trichloroacetic acid. After another wash in acetone the discs were air-dried and re-counted for radioactivity. The difference between the first and second measurements represented radiocarbon in acetyl-CoA plus malonyl-CoA, whereas radioactivity remaining on the discs after washing in trichloroacetic acid represented radioactivity in acyl-(acyl-carrier protein). Alternatively, reactions were stopped after 15min with propan-2-ol/acetic acid (as described above), 0.25ml of water was added and 50μl was spotted on to filter-paper discs, which were treated as described above for analysis of [^{14}C]acetyl-CoA plus [^{14}C]malonyl-CoA and [^{14}C]acyl-(acyl-carrier protein). When incubated in the presence of both CoA and ATP, chloroplasts synthesized ^{14}C-labelled fatty acyl-CoA, which remained adsorbed to the discs throughout the entire washing procedure. Chlorophylls were measured by the method of Arnon (1949), and protein was determined by dye-binding (Bradford, 1976) or by a modified Lowry procedure (Schacterle & Pollack, 1973).

Results

Isolation buffers and biosynthetic activities

In a preliminary study with the half-leaf technique, CO_2-dependent O_2 evolution and long-chain-fatty acid synthesis from [1-^{14}C]acetate were compared in chloroplasts that had been isolated by using a number of different buffers. The low-ionic-strength buffers of Nakatani & Barber (1977) consistently yielded preparations with higher activities than did the more complex buffers previously used in this laboratory (Roughan *et al.*, 1976), and elsewhere (Cockburn *et al.*, 1968; Kannangara & Stumpf, 1972). A more equitable evaluation of some of these buffers (Table 1) showed that washing and resuspending isolated chloroplasts in the low-ionic-strength medium (Nakatani & Barber, 1977) produced preparations with significantly higher activities than did washing and resuspending in more complex mixtures.

In the present study the highest rate of photosynthesis measured *in vitro* was 369μmol of O_2 evolved/h per mg of chlorophyll and rates lower than 250μmol/h per mg of chlorophyll have been obtained only rarely. No O_2 was evolved from chloroplasts incubated in hypo-osmotic media (0.06–0.2M-sorbitol). The high rates of O_2 evolution were matched very closely in the early stages of illumination and were subsequently exceeded by rates of $^{14}CO_2$ fixation, which were measured concurrently (Walker *et al.*, 1968). Nevertheless, addition of catalase (Kaiser, 1976) was required for maximum rates of O_2 evolution by washed chloroplasts, rates being 20–30% lower in the absence of the enzyme. By contrast, $^{14}CO_2$ fixation and $^{14}CO_2$ incorporation into long-chain fatty acids were unaffected, and [1-^{14}C]-acetate incorporation into fatty acids was slightly decreased by the inclusion of catalase in the incubation system routinely used to measure fatty acid synthesis.

Table 1. *Photosynthetic and fatty acid-synthetic activities in spinach chloroplasts washed and resuspended in different media*

Leaves were homogenized in the low-ionic-strength buffer as described under 'Methods' and the homogenate was divided into four tubes. Pelleted chloroplasts were resuspended in 10ml of (1) 0.33M-sorbitol plus Tris to give pH7.5 at 20°C (Nakatani & Barber, 1977); (2) 0.33M-sorbitol, 25mM-Mes, 2mM-EDTA, 1mM-$MgCl_2$, 1mM-$MnCl_2$, 0.3mM-KH_2PO_4, final pH6.5 with NaOH; (3) 0.33M-sorbitol, 25mM-Hepes, 2mM-EDTA, 1mM-$MgCl_2$, 1mM-$MnCl_2$, 0.3mM-K_2HPO_4, final pH7.9 with NaOH; (4) 0.6M-sorbitol, 0.1M-Tricine {*N*-[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]glycine}, 0.1M-K_2HPO_4, 60mM-$NaHCO_3$, 1mM-dithiothreitol, final pH7.9, with NaOH (Kannangara & Stumpf, 1972). After recentrifuging, the chloroplast pellets were resuspended in 4vol. of the washing buffer. Buffer (3) was supplemented with 10mM-$NaHCO_3$ for measurement of O_2 evolution and with 10mM-$NaHCO_3$, 0.16mM-[1-^{14}C]acetate and 0.5mM-CoA for measurement of fatty acid synthesis.

Buffer mixture	Photosynthesis (μmol of O_2 evolved/h per mg of chlorophyll)	Fatty acid synthesis (nmol of [1-^{14}C]-acetate incorporated/h per mg of chlorophyll)
1	288	1120
2	250	972
3	230	890
4	0	620

Stability of activities

Chloroplasts washed in the low-ionic-strength medium were resuspended in 4–6vol. of a buffered salts mixture (see under 'Methods') at pH6.0–6.1 to prevent the serious losses of both photosynthetic and fatty acid-synthetic activities that occurred after resuspension in the wash medium.

Fatty acid synthesis by isolated chloroplasts with different labelled precursors

Only Murphy & Leech (1978) have previously reported significant rates of fatty acid synthesis from $H^{14}CO_3^-$ in isolated chloroplasts. However, since acetate was used at subsaturating concentrations in their work, it was not possible to determine rates of potential fatty acid synthesis from $H^{14}CO_3$ relative to that from [1-^{14}C]acetate. In the present study $H^{14}CO_3^-$ and [1-^{14}C]acetate incorporations were optimized in separate assay systems and acetate was found to provide 3–4 times as much carbon for fat synthesis as did $H^{14}CO_3^-$ (Table 2). In addition, low concentrations of exogenous acetate severely decreased rates of fatty acid synthesis from $H^{14}CO_3^-$ without affecting total $^{14}CO_2$ fixation (Table 3).

Added [1-^{14}C]acetyl-CoA was poorly incorporated into the fatty acids of intact chloroplasts (Table 2), as has previously been reported (Stumpf *et al.*, 1967; Weaire & Kekwick, 1975*a*). It has been assumed that this was because acetyl-CoA was unable to penetrate the chloroplasts envelope and reach the site of fatty acid synthesis, but, even when incubated in hypo-osmotic media (0.06M-sorbitol), chloroplasts incorporated [1-^{14}C]acetyl-CoA into fatty acids at only 4–6% of the rate for [1-^{14}C]acetate. Since these broken chloroplasts should be freely permeable to the [1-^{14}C]acetyl-CoA, it would appear that the material generated *in situ* was much more efficiently utilized than that which was added. This is consistent with the finding by Weaire & Kekwick (1975*b*) that neither [^{14}C]acetyl-CoA nor [^{14}C]malonyl-CoA was a better substrate than [^{14}C]acetate for fatty acid synthesis by sonicated plastids from avocado (*Persea americana*) fruit. Attempts to measure fatty acid synthesis from [2-^{14}C]pyruvate and from [2-^{14}C]-malonate were complicated by the presence of various amounts of [^{14}C]acetate in all preparations tested. However, alternative methods for assessing fatty acid synthesis by using either ^3H_2O (Yamada & Nakamura, 1975) or *sn*-[^{14}C]glycerol 3-phosphate together with non-radioactive fatty acid precursors indicated that acetate was the preferred substrate (Roughan *et al.*, 1979).

Table 2. *Rates of long-chain-fatty acid synthesis from different ^{14}C-labelled substrates by isolated spinach chloroplasts*

Incubations contained 50–55 μg of chlorophyll in 0.25ml of the standard assay medium with [1-^{14}C]-acetate omitted, but containing 0.5mM-CoA and the radioactive precursors shown. Incubation was for 15min at 25°C and reactions were stopped by adding 1ml of 10% KOH (w/v) in methanol or 1ml of 2.5% (v/v) acetic acid in propan-2-ol.

Substrate	Final concn. (mM)	Fatty acid synthesis (nmol of substrate incorporated/h per mg of chlorophyll)
$H^{14}CO_3^-$, high light*	10	600–750
$H^{14}CO_3^-$, low light*	10	289–355
[1-^{14}C]Acetyl-CoA	0.4	16–26
[1-^{14}C]Acetate	0.16	926–1355
[1-^{14}C]Acetate (dark)	0.16	2–4

* See under 'Methods'.

Table 3. *Inhibition by acetate of $H^{14}CO_3^-$ incorporation into fatty acids of isolated chloroplasts*

Chloroplasts equivalent to 50 μg of chlorophyll were incubated in 0.25ml of the standard assay medium with [1-^{14}C]acetate omitted, but containing 0.5mM-CoA and the additions shown. Incubation was for 15min at 25°C and reactions were stopped by adding 1ml of 10% KOH (w/v) in methanol. Total photosynthetic $^{14}CO_2$ fixation was unaffected by the presence of sodium acetate.

Additions	Fatty acid synthesis (ng-atoms of ^{14}C incorporated/h per mg of chlorophyll)	Inhibition (%)
$H^{14}CO_3^-$ (4 μCi)	342	0
$H^{14}CO_3^-$ + 0.16mM-acetate	198	42
$H^{14}CO_3^-$ + 0.4mM-acetate	113	66
0.16mM-[1-^{14}C]-Acetate (0.4 μCi)	1211	—

Activities of some enzymes involved in the early stages of fatty acid synthesis

Acetyl-CoA synthetase activity was present in isolated chloroplasts in amounts 3–4 times greater than required to account for maximum observed rates of acetate incorporation, but pyruvate dehydrogenase activity was not detected in these preparations (Table 4). Pyruvate decarboxylase activity was detected in amounts just sufficient to account for maximum rates of $H^{14}CO_3^-$ incorporation into fatty

Table 4. *Activities of some chloroplast enzymes involved in the early stages of fatty acid synthesis*

Intact chloroplasts were added to a hypo-osmotic reaction mixture (see under 'Methods'), which was subsampled for determination of products. The incubation temperature was 25°C.

Enzyme	Activity (μmol of substrate converted/h per mg of chlorophyll)
Acetyl-CoA synthetase	3.7–5.1
Pyruvate dehydrogenase	0
Pyruvate decarboxylase	0.8–1.0
Acetyl-CoA carboxylase	0.15–0.25

acids measured in the present study (Table 2). Only low acetyl-CoA carboxylase activities were detected in osmotically ruptured chloroplasts, although much higher activities must be inferred from the rates (0.4–0.6 μmol of acetate incorporated/h per mg of chlorophyll) of long-chain-fatty acid synthesis in broken chloroplasts supplied with [1-^{14}C]acetate, ATP, CoA, HCO_3^- and light. This was consistent with the observation (see above) that [1-^{14}C]acetyl-CoA was a poor precursor for fatty acid synthesis in broken chloroplasts, and may explain in part the difficulties others have had in detecting the carboxylase in preparations from chloroplasts (Burton & Stumpf, 1966; Kannangara *et al.*, 1973).

Factors affecting rates of [1-^{14}C]acetate incorporation into long-chain fatty acids and classes of lipids synthesized in isolated intact chloroplasts

Additions and conditions known to influence rates of fatty acid synthesis from [1-^{14}C]acetate were re-tested by using the highly active chloroplast preparations. Possibly as a result of the much higher activities, the well-known light requirement was even more pronounced in the present (Table 2) compared with previous work (Smirnov, 1960; Stumpf & James, 1963; Sherrat & Givan, 1973; Nakamura & Yamada, 1975). In contrast, stimulations of fatty acid synthesis by added Triton X-100 and CoA were considerably less than in previous reports (Stumpf & Boardman, 1970; Kannangara & Stumpf, 1972), and there was no dependence on added ATP. Stimulation by added CoA did not reflect a non-specific thiol requirement, since other thiol compounds did not enhance fatty acid synthesis (Table 5), and dithiothreitol at 4mM was inhibitory. The single most important factor after light was $NaHCO_3$ concentration, which was optimal at 10mM (Table 6). Although acetate incorporation is known to be dependent on added HCO_3^- (Stumpf *et al.*, 1967), inhibition of fatty acid synthesis at high $NaHCO_3$ concentrations (15 and 20mM) has not previously been reported. Indeed, Kannangara & Stumpf (1972) used incubation media containing 60mM-$NaHCO_3$. Whether the effect we observed was due to the Na^+ or HCO_3^- species was not determined, although the medium pH shifted from 7.9 to 8.05 at 20mM-$NaHCO_3$ and $^{14}CO_2$ fixation was also inhibited (Table 6).

With both CoA and ATP added to incubation media, long-chain [^{14}C]acyl-CoA and [^{14}C]acetyl-CoA accumulated (Tables 5 and 7). Since there was a frequent, but not inevitable, reversal of CoA-stimulated fatty acid synthesis by added ATP (Table 5), it seemed possible that exogenous oleoyl-CoA or acetyl-CoA might exert some regulatory effect on fatty acid synthesis within the intact organelle. However, neither acetyl-CoA (40–120 μM) nor oleoyl-CoA (1–5 μM) had any effect on rates of [1-^{14}C]acetate incorporation into fatty acids of isolated chloroplasts.

To determine whether acetate-incorporating activity was light-activated, chloroplasts were isolated from one half of each of 12 leaves, 10min before the end of the artificial night and then again 90min after the beginning of the artificial day. Chloroplasts from the dark leaves showed a longer lag before resuming photosynthesis compared with chloroplasts from the illuminated half-leaves, but within 3–5min had achieved a rate of O_2 evolution comparable with that achieved within 1min by the latter. Similarly, [1-^{14}C]acetate was incorporated into long-chain fatty acids at an accelerating rate in 'dark' chloroplasts

Table 5. *Specificity of coenzyme A stimulation of fatty acid synthesis in isolated chloroplasts*

Incubations were in the standard medium supplemented with the additions shown, and were stopped after 15min at 25°C by adding 1ml of 10% (w/v) KOH in methanol.

Thiol reagent added	Acetate incorporation (nmol/h per mg of chlorophyll)	Stimulation (%)
None	605	—
1mM-Mercaptoethanol	600	−1
1mM-Glutathione (reduced)	587	−3
1mM-Dithiothreitol	601	−1
4mM-Dithiothreitol	434	−28
1mM-CoA	916	+51
1mM-CoA+4mM-ATP	678	+12

Table 6. *Effect of bicarbonate concentration on [1-^{14}C]-acetate incorporation into chloroplast long-chain fatty acid*

Incubations were in the standard assay system, but containing the amounts of HCO_3^- shown, and were stopped by adding 1ml of 10% (w/v) KOH in methanol. Photosynthetic $^{14}CO_2$ fixation was maximal at 10mM-HCO_3^- and was 30% inhibited at 20mM-HCO_3, whereas incorporation of $H^{14}CO_3^-$ into long-chain fatty acids was 40% inhibited at 20mM compared with 10mM-HCO_3^-.

[Bicarbonate] added (mM)	Fatty acid synthesis (nmol of acetate incorporated/h per mg of chlorophyll)
0	262
5	655
10	1220
15	678
20	514

Table 7. *Effects of ATP and Triton X-100 on rates of [1-^{14}C]acetate incorporation and classes of lipids synthesized by isolated chloroplasts*

Chloroplasts equivalent to 50μg of chlorophyll were incubated in the standard assay system containing 0.16mM-[1-^{14}C]acetate, 0.5mM-CoA and the additions shown. Reactions were stopped by the addition of 0.5ml of 2.5% (v/v) acetic acid in propan-2-ol.

Additions	Incorporation rate*	Percentage distribution of incorporated substrate: Free fatty acid	Diacylglycerol	Polar lipid†	Ratio [^{14}C]oleate/[^{14}C]palmitate	Percentage distribution of ^{14}C within oleate: C-1–C-9	C-10–C-18
None	1355	70	14	16	3.8	59	41
+2mM-ATP	1355	38	18	44	3.7	60	40
+0.13mM-Triton X-100	1467	44	40	16	3.9	59	41

* nmol of [1-^{14}C]acetate incorporated/h per mg of chlorophyll.

† Includes acyl-CoA and acyl-(acyl-carrier protein); in the control incubations, radioactivity in the polar lipid fraction was distributed among thioesters (50%), sulpholipid (20%), phosphatidylglycerol (10%), phosphatidic acid (9%), phosphatidylcholine (5%) and diacylgalactosylglycerol (5%).

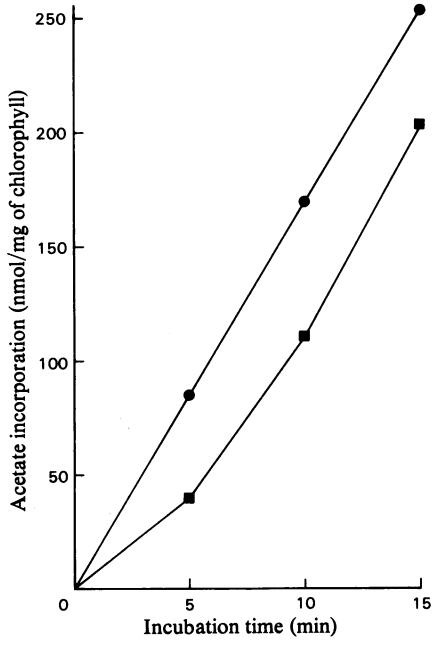

Fig. 1. *Fatty acid synthesis in chloroplasts isolated from leaves that had been in the dark for 14h (■) compared with chloroplasts from leaves that had been illuminated for 90min after 14h of darkness (●)*

Incorporation of [1-^{14}C]acetate was measured in the standard assay medium containing 0.5mM-CoA, and reactions were stopped with methanolic KOH.

and at a linear rate in 'illuminated' chloroplasts (Fig. 1). Acetyl-CoA synthetase activity was identical in both chloroplast preparations, so the enzyme for activating acetate was maintained at a constant activity, but fatty acid synthesis in the 'dark' chloroplasts may have been limited initially by a deficiency of ATP and NADPH.

Glycerophosphate had previously been shown (Roughan *et al.*, 1976) to stimulate 1,2-diacylglycerol synthesis from newly synthesized long-chain fatty acids, but, unlike Triton X-100, it had no effect on rates of acetate incorporation. We have now examined the effects of much lower concentrations of *sn*-glycerol 3-phosphate on rates of fatty acid synthesis, on the end products of fatty acid synthesis and on the relative amounts of oleate and palmitate synthesized. Low concentrations (50–250 μM) had no effect on [1-^{14}C]acetate incorporation, but

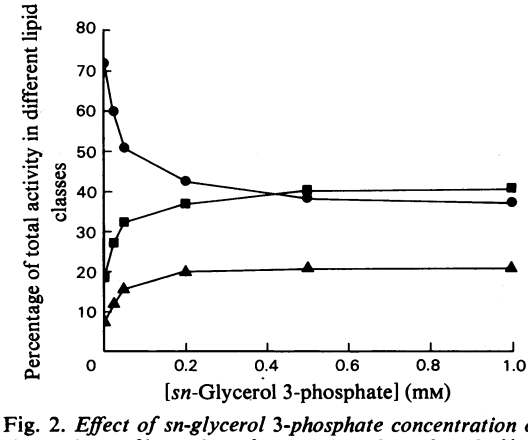

Fig. 2. *Effect of sn-glycerol 3-phosphate concentration on the products of long-chain-fatty acid synthesis from [1-^{14}C]-acetate in isolated chloroplasts*

●, Non-esterified fatty acids; ■, 1,2-diacylglycerols; ▲, polar lipids.

Table 8. *Effect of sn-glycerol 3-phosphate on the ratio of oleate to palmitate synthesized from [1-^{14}C]acetate by isolated chloroplasts*

Chloroplasts equivalent to 55 μg of chlorophyll were incubated in the standard assay medium containing 0.5 mM-CoA and the additions shown. Reactions were stopped by adding 1 ml of 10% (w/v) KOH in methanol and total fatty acids were recovered after saponification. Methyl esters were separated by $AgNO_3$-t.l.c. Incorporation rate of [1-^{14}C]acetate was 1115 nmol/h per mg of chlorophyll.

sn-Glycerol 3-phosphate added (mM)	0	0.05	0.10	0.25	0.50	1.0	2.5	5.0
Ratio $\frac{[^{14}C]\text{oleate}}{[^{14}C]\text{palmitate}}$	3.75	1.95	1.62	1.37	0.92	0.81	0.60	0.58

Table 9. *Accumulation of [^{14}C]acetyl-CoA plus [^{14}C]malonyl-CoA, [^{14}C]acyl-(acyl-carrier protein) and ^{14}C-labelled fatty acids during fatty acid synthesis in isolated chloroplasts*

Chloroplasts equivalent to 50 μg of chlorophyll were incubated in 0.25 ml of the standard assay medium with $NaHCO_3$ omitted, but containing 0.5 mM-CoA and the additions shown. Reactions were incubated for 15 min at 25°C and were stopped by adding 0.5 ml of 2.5% acetic acid in propan-2-ol followed by 0.25 ml of water. Portions (50 μl) of this solution were spotted on to filter-paper discs for analysis of [^{14}C]acetyl-CoA plus malonyl-CoA and [^{14}C]acyl-(acyl-carrier protein) and the remainder was saponified for determination of total ^{14}C-labelled fatty acids. Abbreviation used: n.m., not measured.

Additions	[1-^{14}C]Acetate incorporated (nmol/h per mg of chlorophyll)		
	Acetyl-CoA + malonyl-CoA	Acyl-(acyl-carrier protein)	Fatty acids
None	35.0	15.5	260
5 mM-HCO_3^-	41.5	30.4	655
10 mM-HCO_3^-	39.3	44.8	1021
10 mM-HCO_3^- + 0.13 mM-Triton X-100	41.5	34.6	1211
10 mM-HCO_3^- + 2 mM-ATP	360.0	n.m.	795

stimulated 1,2-diacylglycerol synthesis and also markedly decreased the ratio of oleic to palmitic acids synthesized (Fig. 2 and Table 8). Diacylglycerol synthesis was apparently saturated at about 0.3 mM-*sn*-glycerol 3-phosphate, and higher concentrations (2.5–5 mM) inhibited acetate incorporation. The changing ratio of oleic to palmitic acids synthesized in the presence of increasing concentrations of *sn*-glycerol 3-phosphate (Table 8) indicated that acyl transfer from palmitoyl-(acyl-carrier protein) to glycerophosphate occurred in preference to stearoyl-(acyl-carrier protein) synthesis under these conditions and suggested that this may be one of the important control points in leaf glycerolipid synthesis. Dihydroxyacetone phosphate (0.2–1.0 mM) did not stimulate 1,2-diacylglycerol synthesis in isolated chloroplasts. When present at the correct concentration (Stumpf & Boardman, 1970) in the reaction media, Triton X-100 consistently stimulated 1,2-diacylglycerol synthesis at the expense of free fatty acid accumulation (Table 7). An explanation for the effect of Triton X-100, therefore, might be that the detergent specifically stimulated the acyl-(acyl-carrier protein)–*sn*-glycerol 3-phosphate acyltransferase in the chloroplasts envelope, thus recycling acyl-carrier protein and allowing fatty acid synthesis to proceed faster.

Endogenous concentrations of [1-^{14}C]acetyl-CoA plus [1-^{14}C]malonyl-CoA and of [1-^{14}C]acyl-(acyl-carrier protein)

Both [-^{14}C]acetyl-CoA plus [1-^{14}C]malonyl-CoA and [1-^{14}C]acyl-(acyl-carrier protein) concentrations increased linearly with time so that it was not possible to obtain an estimate of endogenous concentrations of CoA and acyl-carrier protein during [1-^{14}C]acetate incorporation. However, it was shown that [1-^{14}C]-acetyl-CoA plus [1-^{14}C]malonyl-CoA accumulated to about the same extent under a number of different treatments and even when rates of long-chain-fatty acid synthesis were limited by low HCO_3^- concentrations (Table 9). Apparently, acetyl-CoA synthetase activity was regulated in the intact organelle, so that acetyl-CoA did not accumulate beyond a certain concentration. Rates of [1-^{14}C]acyl-(acyl-acyl-carrier protein) synthesis increased with increasing HCO_3^- concentration, probably reflecting the CO_2 requirement of the acetyl-CoA carboxylase reaction. Triton X-100 consistently decreased the rate of [1-^{14}C]acyl-(acyl-acyl-carrier protein) accumulation, which was in agreement with the proposal that the detergent stimulated acyl-(acyl-carrier protein)-*sn*-glycerol 3-phosphate acyltransferase activity. These results suggested that fatty acid synthetase activity was present in excess of acetyl-CoA synthetase and car-

boxylase activities, but that rates of [1-^{14}C]acetate incorporation into fatty acids by isolated chloroplasts were limited more by the rate of removal of products from the synthetase rather than by the rates of synthesis of acetyl-CoA and malonyl-CoA.

Discussion

Recent improvements in techniques for isolating chloroplasts from pea (*Pisum sativum*) and spinach leaves (Nakatani & Barber, 1977) have resulted in further increases in rates of chloroplast fatty acid synthesis *in vitro* over those reported previously (Roughan *et al.*, 1976). We have attempted to maximize rates of long-chain-fatty acid synthesis in these highly active preparations, since this should lead to a better understanding both of the control processes within the organelle and of the enzymology of the system. Murphy & Leech (1978) have indicated that rates of $^{14}CO_2$ incorporation into the acyl moieties of lipids in intact spinach leaves may be as high as 1.7μmol/h per mg of chlorophyll, and on a carbon basis the results reported here for [1-^{14}C]-acetate incorporation into isolated chloroplasts are 1.5 times higher than that. This close agreement suggests that the results obtained by using isolated chloroplasts with [1-^{14}C]acetate as a substrate may be interpreted as applying to the situation *in vivo* more confidently than would otherwise have been possible.

The evidence indicated that acetate was the preferred substrate for chloroplast fatty acid synthesis *in vitro* and that the potential for fatty acid synthesis was 3–4 times higher than endogenously produced substrates could support. Hence rates of chloroplast fatty acid synthesis *in vivo* could well be influenced by variations in the amount of acetate supplied from other cellular compartments.

The high rate of [1-^{14}C]acetate incorporation into fatty acids of freshly isolated chloroplasts were strongly affected by the $H^{14}CO_3^-$ concentration in the medium. This may simply reflect the CO_2 requirement for carboxylation of acetyl-CoA, or it is possible that simultaneous photosynthetic CO_2 fixation was somehow required to support high rates of acetate incorporation. It is noteworthy in this context that rates of both CO_2 fixation and acetate incorporation were maximal at 10mM-$H^{14}CO_3^-$ and were inhibited at higher concentrations. A partial explanation for higher rates of acetate incorporation in the present compared with previous studies could then be that the preparations used here were capable of higher rates of photosynthesis *in vitro*. CoA has been shown to stimulate fatty acid synthesis in plastid preparations (Kannangara & Stumpf, 1972; Weaire & Kekwick, 1975*a*; Nothelfer *et al.*, 1977; Kleinig & Liedvogel, 1978), and it has been assumed that the stimulation was connected with acetate activation within the organelles. However, Weaire & Kekwick (1975*a*) opined that, since exogenous acetyl-CoA was a poor substrate for fatty acid synthesis in intact avocado plastids, it was improbable that either acetyl-CoA or CoA entered the organelles. We found that [1-^{14}C]acetyl-CoA was a poor substrate both in intact and broken chloroplasts and therefore cannot use that argument. However, CoA probably did not enter intact chloroplasts, since added CoA did not stimulate acetyl-CoA formation in illuminated chloroplasts, but, when added along with ATP, resulted in the synthesis of acetyl-CoA, possibly in the space between the envelope membranes. How then could CoA stimulate rates of acetate incorporation if it is unable to enter the chloroplast? By analogy with the effect of Triton X-100 (see below), it seems possible that CoA interacted with the chloroplast envelope to stimulate an enzyme activity that exerted some control over rates of fatty acid synthesis. An attractive hypothesis is that CoA stimulated oleoyl-(acyl-carrier protein) thioesterase (Shine *et al.*, 1977) to enhance the production of free fatty acids, which in turn were the substrates for oleoyl-CoA synthesis in the chloroplast envelope (Roughan & Slack, 1977). The enhanced hydrolysis of oleoyl-(acyl-carrier protein) could also stimulate acetate incorporation through a more rapid recycling of acyl-carrier protein. Thus the rates of chloroplast fatty acid synthesis *in vivo* could be influenced by rates of acyl-CoA utilization in glycerolipid biosynthesis in other cell compartments. The hydrolysis of acyl-(acyl-carrier protein) is one of the committed steps in fatty acid metabolism (Shine *et al.*, 1977) and is therefore likely to be under allosteric control.

The detergent Triton X-100 also stimulated acetate incorporation into chloroplast fatty acids when added to reaction media at very low concentrations (Stumpf & Boardman, 1970). Maximum stimulation occurred when molar ratios of detergent to chlorophyll approached unity, and higher concentrations became inhibitory. Amounts of Triton X-100 producing maximum stimulation of fatty acid synthesis had no effect on photosynthetic oxygen evolution (Roughan *et al.*, 1976) and therefore did not significantly alter the permeability of the envelope membranes. These membranes have a high lipid content (Joyard & Douce, 1976), resulting in a high affinity for the detergent, and it seems reasonable to suppose that only the envelope membranes were affected by the small quantities of Triton X-100 added. In addition to stimulating fatty acid synthesis, Triton X-100 enhanced 1,2-diacylglycerol synthesis without affecting ratios of oleate to palmitate synthesized. One interpretation of these results is that the detergent specifically stimulated oleoyl-(acyl-carrier protein)–monoacyl-*sn*-glycerol 3-phosphate acyltransferase, which is probably localized in the chloroplast envelope (Joyard & Douce, 1977).

Alternatively, the detergent may simply have allowed the substrate more ready access to the enzyme site and stimulated the reaction by increasing the effective substrate concentration. In either case, a branch point in fatty acid metabolism was indicated; the acyl group of oleoyl-(acyl-carrier protein) was either released from acyl-carrier protein by the thioesterase or transferred to monoacyl-*sn*-glycerol 3-phosphate to eventually become part of the chloroplast 1,2-diacylglycerol. One of the effects of the detergent, therefore, would be to hasten the recycling of acyl-carrier protein and to enhance acetate incorporation as suggested above. By contrast, *sn*-glycerol 3-phosphate stimulated 1,2-diacylglycerol synthesis from [1-^{14}C]acetate and encouraged the synthesis of palmitate relative to oleate, but did not enhance rates of [1-^{14}C]acetate incorporation. The effect of glycerophosphate was probably, therefore, expressed at a different site from that affected by Triton X-100 and possibly indicated another branch point in fatty acid metabolism. Palmitoyl-(acyl-carrier protein) could either be converted into stearoyl-(acyl-carrier protein) and thence to oleoyl-(acyl-carrier protein) (Jacobson *et al.*, 1974), or the acyl group could be transferred to *sn*-glycerol 3-phosphate, probably by a stromal enzyme (Joyard & Douce, 1976), as the first step in the synthesis of chloroplast 1,2-diacylglycerol. Hence, other cell compartments might control the nature of the end products of fatty acid synthesis by varying the supply of *sn*-glycerol 3-phosphate available to the chloroplasts.

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