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High Rates of [1-^{14}C]Acetate Incorporation into the Lipid of Isolated Spinach Chloroplasts

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Spinach chloroplasts, isolated by techniques yielding preparations with high O_2-evolving activity, showed rates of light-dependent acetate incorporation into lipids 3–4-fold higher than any previously reported. Incorporation rates as high as 500 nmol of acetate/h per mg of chlorophyll were measured in buffered sorbitol solutions containing only $NaHCO_3$ and [1-^{14}C]acetate, and as high as 800 nmol/h per mg of chlorophyll when 0.13 mM-Triton X-100 was also included in the reaction media. The fatty acids synthesized were predominantly oleic (70–80% of the total fatty acid radioactivity) and palmitic (20–25%) with only minor amounts (1–5%) of linoleic acid. Linolenic acid synthesis was not detected in the system *in vitro*. Free fatty acids accounted for 70–90% of the radioactivity incorporated and the remainder was shared fairly evenly between 1,2-diacylglycerols and polar lipids. Oleic acid constituted 80–90% of the free fatty acids synthesized, but the diacylglycerols and polar lipids contained slightly more palmitic acid than oleic acid. Triton X-100 stimulated the synthesis of diacylglycerols 3–6-fold, but stimulated free fatty acid synthesis only 1–1.5-fold. Added glycerol 1-phosphate stimulated both the synthesis of diacylglycerols and palmitic acid relative to oleic acid, but did not increase acetate incorporation into total chloroplast lipids. CoA and ATP, when added separately, stimulated acetate incorporation into chloroplast lipids to variable extents and had no effect on the types of lipid synthesized, but when added together resulted in 34% of the incorporated acetate appearing in long-chain acyl-CoA. Pyruvate was a much less effective precursor of chloroplast fatty acids than was acetate.

Although particularly rich in polyunsaturated fatty acids, chloroplasts isolated from green leaves have consistently failed to synthesize significant amounts of these fatty acids *in vitro* (Stumpf & James, 1963; Stumpf & Boardman, 1970; Panter & Boardman, 1973; Hawke *et al.*, 1974; Nakamura & Yamada, 1975). This phenomenon has been described as 'an outstanding problem of lipid biochemistry in higher plants' (Hawke *et al.*, 1974).

Chloroplasts and plastids are the major, if not the only, sites of long-chain fatty acid synthesis in leaves (Stumpf & James, 1963; Nakamura & Yamada, 1974) and non-photosynthetic tissue (Zilkey & Canvin, 1972; Weaire & Kekwick, 1975) of higher plants. However, oleic acid and palmitic acid are essentially the only fatty acids produced *in vitro* from supplied acetate. On the basis of labelling studies *in vivo*, we proposed (Roughan, 1975; Slack & Roughan, 1975) that oleic acid synthesized in chloroplasts is incorporated into phosphatidylcholine of the endoplasmic reticulum, where it is desaturated to linoleic acid (Slack *et al.*, 1976) from which the linolenate of chloroplast monogalactosyl diacylglycerol is ultimately derived. This shuttling of long-chain fatty acids between cellular compartments may be essential for polyunsaturated fatty acid biosynthesis and if so could explain the failure of isolated chloroplasts to perform the desaturation of oleic acid.

It seemed unlikely that chloroplast preparations used to date in the study of long-chain fatty acid synthesis would satisfy the most rigorous test of 'intactness', i.e. maintenance of high rates of O_2 evolution (photosynthesis) in suitably buffered media with HCO_3^- as the sole exogenous source of carbon and of Hill oxidant. Assessments of intactness based on microscopic identification of 'class I' chloroplasts (Spencer & Unt, 1965) may be less reliable than those based on photosynthesis rates, since some preparations with high proportions of class I chloroplasts have shown low rates of photosynthesis (Spencer & Unt, 1965; Stumpf & Boardman, 1970) and morphological states intermediate between classes I and II may exist (Ridley & Leech, 1968). It seemed possible that chloroplast preparations showing high rates of photosynthesis might also show higher rates of long-chain fatty acid synthesis and produce a greater proportion of polyunsaturated fatty acids than has hitherto been reported. Therefore, using well-tested methods of chloroplast isolation from

spinach leaves (Cockburn *et al.*, 1968; Walker, 1971; Slack & Roughan, 1975), and using O_2 evolution in response to added HCO_3^- as the main criterion of chloroplast integrity, we have re-examined maximum rates of fatty acid synthesis from acetate in isolated chloroplasts, and have examined in some detail the products of [1-^{14}C]acetate incorporation into their constituent lipids.

Materials

Spinach plants (*Spinacea oleracea*, cultivar King of Denmark) were grown hydroponically as described by Slack & Roughan (1975). In later experiments the spinach variety used was designated 'hybrid 102' (Arthur Yates and Co., Auckland, New Zealand).

CoA, ATP and DL-α-glycerol 1-phosphate were from Sigma Chemical Co., St. Louis, MO, U.S.A. Triton X-100 was obtained from BDH Chemicals, Poole, Dorset, U.K., and sodium [1-^{14}C]acetate and sodium [2-^{14}C]pyruvate were from The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

Expanding leaves 6–8cm in length were selected from young plants and were floated on ice/water 40cm below a 400W Hg-vapour lamp for about 10min. Chloroplasts were isolated from 10–20g of leaf lamellae as described previously (Walker, 1971; Slack & Roughan, 1975). The homogenizing buffer contained 50mM-Mes*/NaOH, pH6.2, 0.33M-sorbitol, 50mM-KCl, 5mM-$MgCl_2$, 1mM-$MnCl_2$, 2mM-EDTA (disodium salt) and 5mM-sodium isoascorbate, and the resuspending buffer containing 50mM-Hepes/NaOH, pH7.9, 0.33M-sorbitol, 1mM-$MgCl_2$, 1mM-$MnCl_2$, 2mM-EDTA, 1mM-dithiothreitol and 10mM-$NaHCO_3$.

O_2 evolution was measured essentially as described by Delieu & Walker (1972) and was shown to be both light-dependent and light-saturated in our system. Photosynthetic rates were linear for at least 10min and frequently for more than 20min when chloroplasts equivalent to 50μg of chlorophyll/ml were incubated in the resuspending buffer at 25°C. The average rate for 20 preparations was $49\pm19\,\mu$mol of O_2/h per mg of chlorophyll.

Acetate-incorporation measurements were made both in the oxygen electrode, where O_2 evolution was monitored during acetate incorporation, and in 25ml conical flasks fitted to an illuminated Warburg apparatus (Stumpf, 1972) by adding [1-^{14}C]acetate (150nmol; 1.5μCi) to chloroplast suspensions (50–200μg of chlorophyll) in 1ml of the resuspending buffer. Various additions were made to this basal reaction medium as described in the Results section. Incubations were done at 25°C and were stopped by adding either 10ml of chloroform/methanol (1:1, v/v) followed by 3.5ml of water (Bligh & Dyer, 1959) or 2ml of 10% (w/v) KOH in methanol and heating to 60°C for 2h.

Washed chloroform extracts were evaporated to dryness under reduced pressure, and residues were thoroughly dried by adding 2ml of ethanol/benzene (90:10, v/v) and re-evaporating. Total lipids were re-dissolved in 1ml of chloroform and 100μl was streaked across 1cm (Roughan & Tunnicliffe, 1967) on thin layers of 5% (w/w) boric acid in silica gel G (Thomas *et al.*, 1965). Chromatograms were developed with chloroform/acetone (94:6, v/v) to separate free fatty acids, 1,2-diacylglycerols and polar lipids, which remained at the origin. Separated radioactive bands were located by radioautography, scraped off into 10ml of xylene/Triton X-100/water (9:3:1, by vol.), containing 0.35% (w/v) *p*-terphenyl, and counted for radioactivity at 65% efficiency in a liquid-scintillation counter. Incorporation rates were calculated from the sums of the radioactivities in the separated bands. Saponified reaction mixtures were acidified, and long-chain fatty acids were extracted into light petroleum (40°–60°C b.p. range). The combined extracts were evaporated to dryness under a stream of N_2 and the residues were re-dissolved in 1ml of light petroleum (60°–80°C b.p. range). Portions (100μl) were chromatographed on thin layers of silica gel G with light petroleum/diethyl ether/acetic acid (75:25:1, by vol.) as developing solvent, and the separated long-chain fatty acids were detected with I_2 vapour. After removal of the I_2, the zones were scraped off into 10ml of xylene containing 0.5% *p*-terphenyl, and radioactivity was determined at 80% efficiency by scintillation counting. The two methods for determining [1-^{14}C]acetate-incorporation rates into chloroplast lipids agreed to within 10%. To measure the formation of acyl-CoA in later experiments, reactions were stopped with 1ml of propan-2-ol, containing 25μl of acetic acid, and radioactive long-chain acyl compounds were separated as described by Mancha *et al.* (1975).

Free fatty acids were eluted from chromatograms with diethyl ether and methylated (Schlenk & Gellerman, 1960) with diazomethane. Esterified fatty acids were transmethylated in the presence of the absorbant by using 0.5M-sodium in methanol (Slack & Roughan, 1975). Fatty acid methyl esters were purified by t.l.c. before separation by g.l.c. or by t.l.c. on $AgNO_3$-impregnated silica gel (Morris, 1966). G.l.c. separations and measurement of radioactivity in the separated esters were performed as described by Slack & Roughan (1975). Fatty acid methyl esters separated by $AgNO_3$/silica-gel t.l.c. were located by radioautography and the radioactive

* Abbreviations: Mes, 2-(*N*-morpholino)ethanesulphonic acid; Hepes, 2-(*N*-hydroxyethylpiperazin-*N'*-yl)-ethanesulphonic acid.

zones were scraped off into the xylene/Triton X-100/water emulsion for scintillation counting. When methyl esters were to be recovered for the g.l.c. after separation by $AgNO_3$/silica-gel t.l.c., the samples were 'spiked' with 100 μg of a standard mixture containing equal weights of methyl palmitate, stearate, oleate, linoleate and linolenate before chromatography. Separated bands were detected by spraying with dichlorofluorescein and were eluted into diethyl ether.

In determining the distribution of radioactivity among the fatty acids of the different lipid classes, 20–40% of the total lipid extract was chromatographed across 7 cm on silica-gel G thin layers with the light petroleum/diethyl ether/acetic acid solvent. Bands of free fatty acids, diacylglycerols and polar lipids were detected by using a portable radiation monitor, and constituent fatty acids methylated and analysed as described above.

Polar lipids were separated from total lipid extracts by the two-dimensional t.l.c. system of Nichols (1964). Radioactive spots were detected by radioautography and were scraped off into the xylene/Triton X-100/water emulsion for counting for radioactivity.

Leaf discs (2 mm diam.) were punched from expanding spinach leaves and were vacuum infiltrated with either the Mes/sorbitol or Hepes/sorbitol buffer (see above). Lots of 50 discs were incubated as for chloroplasts, but in 2 ml of Mes/sorbitol buffer containing 100 μM-[1-^{14}C]acetate and 10 mM-$NaHCO_3$. At intervals the discs were recovered, homogenized in 5 ml of chloroform/methanol (1:1, v/v) and the extracted lipids processed as described for isolated chloroplasts.

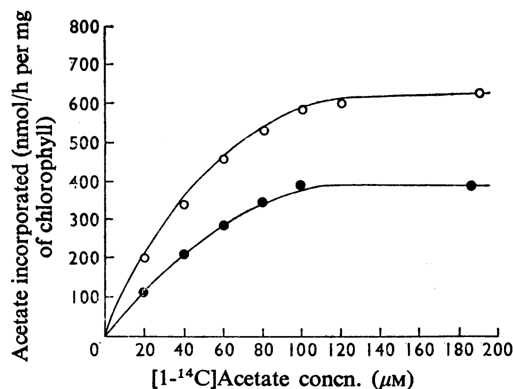

Fig. 1. *Effect of sodium* [1-^{14}C]*acetate concentration on rates of acetate incorporation in vitro into spinach chloroplast lipids*

Reaction mixtures contained chloroplasts equivalent to 200 μg of chlorophyll in 1 ml of basal medium (see the Methods section) with (○) or without (●) 0.13 μM-Triton X-100 and with increasing concentrations of [1-^{14}C]-acetate. Incubation was for 15 min at 25°C and reactions were stopped by adding chloroform/methanol. Lipids were separated, and radioactivity was determined as described in the Methods section.

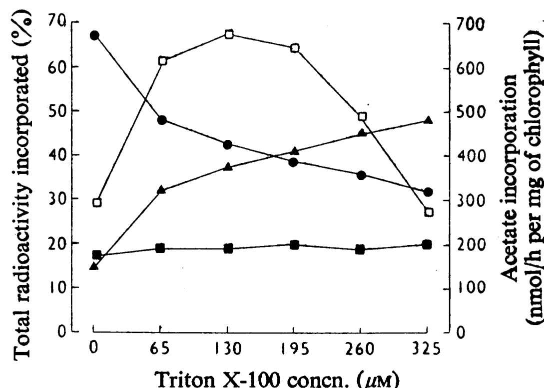

Fig. 2. *Effect of Triton X-100 concentration on rates o,* [1-^{14}C]*acetate incorporation into lipids of isolated spinach chloroplasts and on the proportions of the different lipids synthesized*

The experimental conditions were as described for Fig. 1. □, Acetate incorporation (nmol/h per mg of chlorophyll). Percentage of total lipid radioactivity in: ●, free fatty acids; ▲, diacylglycerols; ■, polar lipids.

Results

Acetate concentrations and incorporation rates

Isolated spinach chloroplasts incorporated [1-^{14}C]-acetate into long-chain fatty acids at rates up to 500 nmol/h per mg of chlorophyll, when acetate concentrations were 5–7-fold higher than used previously (Stumpf, 1972). All preparations tested reached maximum velocities in the range 100–150 μM-acetate (Fig. 1), with the higher concentrations being required by the more active preparations. Triton X-100 (0.13 mM) stimulated acetate incorporation into chloroplasts about twofold, but had no effect on the acetate concentration required for maximum incorporation rates. At 150 μM-[1-^{14}C]acetate, incorporation rates were linear for at least 20 min and were proportional to chloroplast concentration in the range 25–200 μg of chlorophyll.

Cofactor requirements for maximum rates of acetate incorporation into intact spinach chloroplasts

High rates of [1-^{14}C]acetate incorporation into long-chain fatty acids were measured in the buffered sorbitol/salts medium supplemented only with $NaHCO_3$, [1-^{14}C]acetate and light. The reaction was light-saturated at less than 400 μeinsteins/s

per m^2 in the 25ml flasks on the Warburg apparatus (see the Methods section) and could be shown to be light-saturated in the oxygen electrode at incident light intensities about one-third those required to saturate photosynthesis. Basal rates were usually doubled in the presence of 0.13mM-Triton X-100 (0.008%) (Stumpf & Boardman, 1970) and reached 700–800μmol of acetate/h per mg of chlorophyll (Fig. 2). Higher concentrations of Triton X-100 inhibited acetate incorporation (Fig. 1). As previously noted (Stumpf & Boardman, 1970), maximum stimulations of acetate incorporations were produced at Triton X-100:chlorophyll ratios of about 1:2.5 (w/w) (Fig. 2). The presence of the detergent in this ratio was without effect on O_2 evolution *in vitro*, but when the ratio was reversed O_2 evolution was completely abolished and acetate incorporation was 70–90% inhibited. Addition to the reaction mixture of 50mM-potassium orthophosphate (Sherratt & Givan, 1973), 0.5mM-sodium pyruvate (Yamada & Nakamura, 1975), 2mM-glycerol 1-phosphate or 2mM-glycerol had no significant influence on the rate of acetate incorporation into long-chain fatty acids, whereas addition of 1mM-CoA and/or 4mM-ATP (Stumpf, 1972) stimulated the rate by up to 60% in some experiments, but had little effect in others (Tables 1 and 4). When both CoA and ATP were added to the reaction medium there was a consistent decrease in the recovery of chloroform-soluble radioactivity (see below). Intermediates of the citric acid cycle, sodium malate (5mM) and sodium citrate (5mM), did not stimulate acetate incorporation into long-chain fatty acids of isolated chloroplasts.

Rates of acetate incorporation into chloroplast lipids were increased 10–15%, but the lability of this incorporating activity was not affected by washing the chloroplasts once in Hepes/sorbitol, pH7.9.

Stability of the acetate-incorporating activity

Freshly isolated chloroplasts rapidly lost the ability to incorporate acetate into long-chain fatty acids at very high rates. Approx. 20% of the original activity was lost during 30min storage at 0°C in Hepes/sorbitol, pH7.9. Several treatments (higher concentrations of thiol reagents, storage under N_2 at 0°C or at 25°C and storage at pH6.2) had no effect on this decline in activity. Since reaction rates at 25°C were linear for at least 20min, it seemed possible that the activity may have been stabilized by the presence of substrate. However, 100μM-acetate in the resuspending buffer did not slow down the decay of the acetate-incorporating activity. O_2-evolving activity, by contrast, was stable at 0°C for at least 3h.

Table 1. *Effect of CoA and ATP on the rates of fatty acid synthesis and on the types of fatty acid synthesized by spinach chloroplasts*

Chloroplasts equivalent to 200μg of chlorophyll were incubated in 1ml of the Hepes/sorbitol resuspending buffer supplemented with 150nmol of [1-^{14}C]acetate and the additions shown. In Expts. 1 and 2 the reactions were stopped by adding 10ml of chloroform/methanol (1:1, v/v), and chloroform-soluble lipids were recovered, whereas in Expt. 3 reactions were stopped by adding 2ml of 10% (w/v) KOH in methanol, and fatty acids were recovered after saponification. Incorporation rates were calculated from radioactivities recovered in total lipids or in fatty acids after t.l.c. (see the Methods section). Relative incorporations of [1-^{14}C]acetate into the different fatty acids was determined by $AgNO_3$/silica-gel t.l.c. of fatty acid methyl esters prepared from the extracts of the reaction mixtures.

Additions	[1-^{14}C]Acetate incorporation (nmol/h per mg of chlorophyll)	Distribution of ^{14}C among the fatty acids (%)			Ratio of unsaturated: saturated fatty acids
		Saturated	Monoenoic	Dienoic	
Expts. 1 and 2					
None	350, 331	21	78	1	3.8
CoA (1mM)	435, 349	20	79	1	4.1
ATP (4mM)	443, 310	18	81	1	4.5
CoA (1mM)+ATP (4mM)	250, 267	29	68	3	2.4
Triton X-100 (0.13mM)	490, 517	23	75	2	3.3
Triton X-100 (0.13mM)+CoA (1mM)	664, 594	22	76	2	3.5
Triton X-100 (0.13mM)+ATP (4mM)	591, 627	26	72	2	2.8
Triton X-100 (0.13mM)+CoA (1mM)+ATP (4mM)	442, 536	38	59	3	1.6
Expt. 3					
Triton X-100 (0.13mM)	360	26	71	2	2.8
Triton X-100 (0.13mM)+CoA (1mM)	505	23	73	3	3.3
Triton X-100 (0.13mM)+ATP (4mM)	395	24	72	3	3.1
Triton X-100 (0.13mM)+CoA (1mM)+ATP (4mM)	515	24	71	4	3.1

Relationship between O_2 evolution and acetate incorporation

In 20 separate experiments, there was a trend ($P < 0.05$) for highest acetate-incorporation rates to be associated with chloroplast preparations showing highest O_2-evolution rates (Fig. 3). However, there was no simple relationship between the two activities. The chloroplast preparation having the highest rate of O_2 evolution, 95 μmol of O_2/h per mg of chlorophyll, incorporated acetate at a rate of 630 nmol/h per mg of chlorophyll, but this rate was exceeded by five other preparations having 0.33–0.5 times the O_2-evolving capacity. When assayed in basal media containing 0.07–0.1 M-sorbitol, chloroplast preparations retained 40–60% of their original acetate-incorporating activity, but completely lost the ability to evolve O_2.

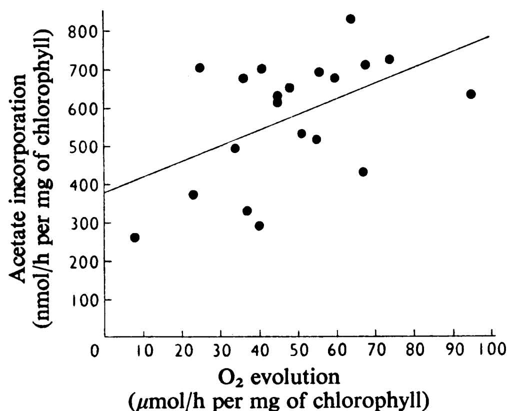

Fig. 3. *Relationship between rates of* [1-^{14}C]*acetate incorporation into lipids and rates of O_2-evolution (photosynthesis) in isolated spinach chloroplasts*

For each preparation [1-^{14}C]acetate incorporation was measured in the presence of Triton X-100 (0.13 μM) and at a chlorophyll concentration of 200 μg/ml. O_2 evolution was measured in the basal salts medium by using at least two concentrations of chlorophyll. The measurements were made over a period of 8 weeks. Linear regression analysis showed that acetate incorporation and O_2 evolution were poorly correlated ($r = 0.49$), but that the slope of the regression line was statistically significant ($P < 0.05$).

Products of [1-^{14}C]*acetate incorporation into intact chloroplasts*

Free fatty acids were the major products of acetate incorporation into intact chloroplasts incubated in the basal medium (Fig. 2, Tables 2 and 3) and constituted 70–90% of the total radioactivity in the lipid extract. Approximately equal amounts of radioactivity in the 1,2-diacylglycerols and in the polar lipids accounted for the remainder of the acetate fixed. The ratio of radioactivity in the free fatty acids and diacylglycerols changed as the Triton X-100 concentration was increased, until the latter contained almost 50% of the total lipid ^{14}C (Fig. 2). Labelling of the polar lipids was little influenced by Triton X-100. In the presence of 0.13 mM-Triton X-100, the ratio of radioactivity in free fatty acids and diacylglycerols as a rule approximated to 1:1, but without Triton X-100 the ratio was higher than 3:1. Glycerol 1-phosphate, but not glycerol, stimulated the synthesis of diacylglycerols at the expense of free fatty acids (Table 2) and a combination of Triton X-100 and glycerol 1-phosphate reversed the

Table 2. *Effect of Triton X-100 and glycerol 1-phosphate on the proportions of newly synthesized fatty acids in the free fatty acids, the diacylglycerols and in the polar lipids of spinach chloroplasts*

Chloroplast incubations were as described for Table 1, except that in all cases reactions were stopped by the addition of chloroform/methanol, and total lipids were recovered. The lipid classes were separated by t.l.c. and constituent fatty acids were methylated, as described in the Methods section, before separation by $AgNO_3$/silica-gel t.l.c. for radioactivity measurements.

Additions	Lipid class	% of total lipid ^{14}C	% of total fatty acid ^{14}C		
			Saturated	Monoenoic	Dienoic
None and glycerol (2 mM)	Free fatty acids	74	13	83	4
	1,2-Diacylglycerols	12	54	42	4
	Polar lipids	14	38	57	5
Triton X-100 (0.13 mM)	Free fatty acids	39	6	90	4
	1,2-Diacylglycerols	44	46	50	3
	Polar lipids	17	38	56	6
Glycerol 1-phosphate (2 mM)	Free fatty acids	28	37	59	4
	1,2-Diacylglycerols	50	57	40	4
	Polar lipids	21	58	33	9
Triton X-100 (0.13 mM)+glycerol 1-phosphate (2 mM)	Free fatty acids	16	—	—	—
	1,2-Diacylglycerols	61	—	—	—
	Polar lipids	20	—	—	—

labelling pattern observed in controls, so that the free fatty acid:diacylglycerol ratio became 1:3 (Table 2). Synthesis of diacylglycerol rather than phosphatidic acid from glycerol 1-phosphate suggests the presence of a potent phosphatidic acid phosphatase in these chloroplasts.

Little radioactivity was incorporated into polar glycerolipids separated by two-dimensional t.l.c. Spots with the strongest radioactivity corresponded to phosphatidic acid, phosphatidylcholine and phosphatidylglycerol and they accounted for 3, 3 and 2% respectively of the total lipid ^{14}C in the presence of glycerol 1-phosphate and for 10, 4 and 1% respectively in the presence of Triton X-100.

CoA or ATP separately had little effect on the ratio of labelling of the three lipid classes whether or not Triton X-100 was present, but when added to the medium together, they caused a 66% decrease in the ^{14}C content of the free fatty acids. This was reflected in the decreased proportion of free fatty acid to total lipid radioactivity (Table 3), although radioactivity of the diacylglycerols also increased by about 25%. Presumably what would have been recovered as free fatty acid was being lost as acyl-CoA only when both CoA and ATP were supplied in the medium. This was confirmed when reactions were stopped and analysed for acyl-acyl-carrier protein, acyl-lipid and acyl-CoA by the method of Mancha *et al.* (1975). Radioactivity lost from the free fatty acids when ATP was present in addition to CoA was almost quantitatively recovered as acyl-CoA. The distribution of radioactivity among the three acyl classes is shown in Table 4. About 1–1.5 nmol of [^{14}C]acyl-CoA, predominantly oleoyl-CoA, was formed from [1-^{14}C]acetate in the reaction.

Distribution of ^{14}C among the three lipid classes did not change significantly during the course of the 20 min incubation.

Types of fatty acids synthesized by intact chloroplasts

Spinach chloroplasts contain large amounts of hexadecatrienoic acid, which interfered with the gas-chromatographic separation of oleic acid and stearic acid, so that these three fatty acids were collected together and counted as 'oleic' acid. It could be shown by gas-chromatographic analysis after $AgNO_3$/silica-gel t.l.c. that stearic acid accounted for less than 1.7% and hexadecatrienoic acid accounted for less than 0.5% of the radioactivity in this mixture. In the $AgNO_3$/silica-gel thin-layer separations, palmitate, stearate and *trans*-Δ^3-hexadecenoate travelled as a single band of 'saturated' fatty acid esters, lin-

Table 3. *Effect of CoA and ATP on the distribution of radioactivity among the reaction products of* [1-^{14}C]*acetate incorporation into lipids of intact chloroplasts*

The reactions were carried out as described for Expts. 1 and 2 in Table 1. Lipid classes were separated from total lipid extracts by t.l.c.

Additions	% of total radioactivity incorporated		
	Free fatty acids	Diacylglycerols	Polar lipids
None	72	14	14
CoA (1 mM)	69	14	17
ATP (4 mM)	72	15	13
CoA (1 mM)+ATP (4 mM)	41	32	26
Triton X-100 (0.13 mM)	49	37	17
Triton X-100 (0.13 mM)+CoA (1 mM)	54	31	15
Triton X-100 (0.13 mM)+ATP (4 mM)	47	39	14
Triton X-100 (0.13 mM)+CoA (1 mM)+ATP (4 mM)	20	59	21

Table 4. *Incorporation of* [1-^{14}C]*acetate into long-chain acyl compounds of intact spinach chloroplasts: demonstration of the ATP-dependent synthesis of long-chain acyl-CoA*

Chloroplasts were incubated at 25°C for 20 min in 1 ml of the basal medium containing 150 μM-[1-^{14}C]acetate and the additions shown. Reactions were stopped by adding 1 ml of 2.5% (v/v) acetic acid in propan-2-ol and the radioactive acyl compounds were fractionated as described by Mancha *et al.* (1975). The results are the means of two experiments with 164 and 168 μg of chlorophyll/reaction. Chloroplasts were isolated from leaves of the spinach hybrid 102.

Additions	[1-^{14}C]Acetate incorporation (nmol/h per mg of chlorophyll)	% of total radioactivity		
		Acyl-acyl-carrier protein	Acyl-lipid	Acyl-CoA
None	350	2.7	96.0	1.3
CoA (1 mM)	560	2.8	94.2	3.0
CoA (1 mM)+ATP (4 mM)	610	2.8	62.8	34.4

oleate was the only component of the dienoic esters, and hexadecatrienoate and linolenate made up the trienoic ester fraction. Stearate contributed 6–7% and *trans*-Δ^3-hexadecanoate less than 1% to the radioactivity of the 'saturated' fatty acids. Agreement between the two methods for determining the distribution of radioactivity between palmitic acid and oleic acid was very good, but radio-g.l.c. tended to give a higher estimate of the linoleic acid radioactivity than did t.l.c.

Intact chloroplasts synthesized oleic acid and palmitic acid almost exclusively (Table 2). The precise ratio of oleic acid: palmitic acid synthesis was different for different chloroplast preparations, but was usually in the range 3–4:1 for chloroplasts showing high rates of acetate incorporation. In contrast with previous reports (Stumpf & Boardman, 1970; Givan & Stumpf, 1971), neither ATP nor Triton X-100 had any effect on this ratio, but glycerol 1-phosphate, although not affecting rates of acetate incorporation, decreased the ratio of unsaturated to saturated fatty acids synthesized threefold compared with controls. The highest ratio of oleic acid: palmitic acid synthesis measured in these experiments was 5.3:1. Linoleic acid normally accounted for 1–3% of total fatty acid label in the routine incubations and never accounted for more than 5% of this radioactivity, even when incubation times were extended to 60min. Linolenic acid synthesis was not detected in the assay *in vitro*.

When saponification of the total reaction mixture followed incubation in the presence of Triton X-100, CoA and ATP, added singly or together, had no effect on the ratio of unsaturated: saturated fatty acids synthesized (Table 1), but when only lipid-soluble material was retained for analysis the presence of CoA plus ATP resulted in a decrease both in radioactivity recovered and in the oleic acid: palmitic ratio (Table 1). This would be expected if oleoyl-CoA was being synthesized and was partitioning into the aq. methanol phase of the chloroform/methanol/water system.

Labelling of the fatty acids in the different lipid classes

Oleic acid accounted for 90 and 80% of the free fatty acids synthesized in the presence and absence of Triton X-100 respectively (Table 2). The remainder was predominantly palmitic acid. In the absence of Triton X-100, slightly more palmitate than oleate was esterified into diacylglycerols, but in the presence of the detergent, which stimulated diacylglycerol synthesis, slightly more oleate than palmitate was incorporated. Addition of glycerol 1-phosphate resulted in higher proportions of palmitate in all lipid classes.

Fatty acid synthesis by spinach-leaf discs

It was decided to compare rates and products of [1-^{14}C]acetate incorporation into leaf discs (i.e. intact cells) under the same conditions as used for studying incorporation by isolated chloroplasts. Acetate was incorporated into lipids of leaf discs at less than one-half the rate of incorporation into intact chloroplasts at comparable chlorophyll concentrations. Incorporation was linear for at least 60min and was twice as high at pH6.2 as at pH7.9 (Table 5). Both linoleate and linolenate were synthesized in readily detectable amounts within 30min (Table 5). After 20min incubation of leaf discs in [1-^{14}C]acetate, polar lipids contained 85–90%, diacylglycerols 9–14% and free fatty acids about 1% of total lipid radioactivity.

O_2 evolution from groups of ten discs averaged 120μmol of O_2/h per mg of chlorophyll as measured in Hepes/sorbitol buffer in the oxygen electrode.

Discussion

Rates of acetate incorporation into long-chain fatty acids of isolated chloroplasts as measured in this study were substantially higher than those reported previously. This was presumably due to the following factors. 1. A high proportion of our isolated chloroplasts retained their integrity as

Table 5. *Acetate incorporation into lipids of spinach-leaf discs incubated with* [1-^{14}C]*acetate in aqueous buffers and the distribution of radioactivity among the different fatty acids*

Duplicate batches of 50 discs were incubated, for the times indicated, in Mes/sorbitol, pH6.2, or in Hepes/sorbitol, pH7.9, and were homogenized in chloroform/methanol. [1-^{14}C]Acetate was 100μM and $NaHCO_3$, 10mM. Incorporated radioactivity was determined after t.l.c. of total lipid extracts, and fatty acid methyl esters prepared from the extracts were separated by $AgNO_3$/silica-gel t.l.c.

Incubation time (min)	Acetate incorporated (nmol/mg of chlorophyll)	% distribution of total fatty acid ^{14}C			
		Saturated	Monoenoic	Dienoic	Trienoic
15 (Mes)	37	46	40	11	3
30 (Mes)	75	44	34	12	10
60 (Mes)	150	36	37	14	13
20 (Hepes)	25	—	—	—	—

judged by rates of O_2 evolution in the presence of HCO_3^- alone. 2. Acetate-incorporation measurements were made promptly after chloroplast isolation. 3. Chloroplasts were prepared from expanding leaves freshly harvested from plants growing in liquid culture and in a controlled environment. 4. High concentrations of [1-^{14}C]-acetate were used. The highest rate of acetate incorporation previously reported is 220nmol of acetate/h per mg of chlorophyll for chloroplasts from young spinach leaves (Kannangra & Stumpf, 1972), but as already pointed out (Hawke *et al.*, 1974), this result may be, in part at least, attributable to a low chlorophyll content of the plastids. Rates of about 150nmol of acetate/h per mg of chlorophyll have been reported (Nakamura & Yamada, 1975) for chloroplasts isolated from more mature spinach leaves. These chloroplasts were isolated by a technique reported to yield preparations with high O_2-evolving capability (Cockburn *et al.*, 1968), and our results suggest that Nakamura & Yamada's (1975) acetate-incorporation rates could have been in excess of 300nmol/h per mg of chlorophyll had Triton X-100 been included in their reactions.

Differences in response to various cofactors and stimulants in the present study compared with other work may also be a reflexion of differences in chloroplast integrity. For instance, the highest stimulation by Triton X-100 of acetate incorporation in our preparations was 2.4-fold and the effect was frequently much less, whereas Stumpf & Boardman (1970) reported 3–7-fold stimulations in their spinach chloroplast preparations. The same authors found that Triton X-100 and ATP markedly influenced the ratio of saturated:unsaturated fatty acids synthesized by their preparations, but no similar effect was found in this study. Triton X-100 at the same concentrations as used here markedly inhibited acetate incorporation into isolated maize chloroplasts (Hawke *et al.*, 1974). Orthophosphate at high concentrations (22.5mM) stimulated fatty acid synthesis from acetate in isolated pea chloroplasts (Sherratt & Givan, 1973), but we found no effect. Maximum rates of acetate incorporation in the present study were achieved at concentrations of acetate 7–8-fold higher than those used previously with spinach chloroplasts (Stumpf, 1972), and this is in agreement with the report of Nakamura & Yamada (1975). It seemed possible that the high concentrations required by our chloroplast preparations were a function of their greater impermeability to acetate compared with earlier preparations, and that the effect of Triton X-100 was to increase this permeability to acetate. However, had this been true, then acetate concentrations required for maximum rates of incorporation should have been lower in the presence than in the absence of the detergent. Such was not the case. Pyruvate was found by Yamada & Nakamura (1975) to be a better precursor of fatty acids in isolated spinach chloroplasts than was acetate, but freshly prepared pyruvate at fivefold the concentration of acetate had no effect on the incorporation of [1-^{14}C]acetate into the lipids of our chloroplasts. [2-^{14}C]Pyruvate was incorporated into long-chain fatty acids, but even at relatively high concentrations (300μM) incorporation rates were only about 25% of those attainable with acetate (results not shown). In general, our chloroplast preparations appeared to be less dependent on and less affected by added cofactors or stimulants than previous chloroplast preparations used in the study of fatty acid synthesis (Stumpf & Boardman, 1970; Zilkey & Canvin, 1972; Nakamura & Yamada, 1975).

The high rates of acetate incorporation that we obtained did not result in significantly enhanced synthesis of polyunsaturated fatty acids compared with earlier reports (Stumpf & Boardman, 1970; Hawke *et al.*, 1974; Nakamura & Yamada, 1975). Small amounts of linoleate were synthesized by our preparations and were detected by both gas chromatography and t.l.c. Linoleic acid synthesis was detected on $AgNO_3$/silica-gel thin-layer radioautographs after prolonged exposures, but linolenic acid synthesis was never detected by this technique. In contrast with fatty acid synthesis by leaf discs, there was no increase with time of incubation in the relative labelling of the polyunsaturated fatty acids of isolated chloroplasts. Polyunsaturated fatty acid synthesis by plastids isolated from very young spinach leaves has been reported (Kannangra & Stumpf, 1972), but has yet to be confirmed in an independent study. In some other cases (Hawke *et al.*, 1974; Nakamura & Yamada, 1975), it is not clear whether the reported distributions were confirmed by $AgNO_3$/silica-gel t.l.c. We have consistently found radioactivity bleeding from our columns for various periods after injecting and separating highly radioactive samples. This bleeding can result in an overestimate of the radioactivity corresponding to a mass peak in a subsequent separation and particularly for a very weakly labelled component could result in a serious overestimate of the proportion of total counts contributed by that component. Although incorporating acetate at much lower rates than isolated spinach chloroplasts, leaf discs synthesized both linoleate and linolenate in significantly higher proportions than did isolated chloroplasts, particularly at the longer incubation times. Leaf discs incorporated 85% of the newly synthesized fatty acids into polar lipids and only about 1% into free fatty acids, in agreement with earlier work on whole leaves (Roughan, 1970, 1975).

Our finding that intact spinach chloroplasts synthesized predominantly free fatty acids is in agreement with results obtained with maize leaf chloroplasts (Hawke *et al.*, 1974) and with earlier

work on lettuce leaf chloroplasts (Stumpf & James, 1963), although it is contrary to the report of Kannangra & Stumpf (1972). Since free fatty acids are barely detectable in intact tissue supplied with [1-^{14}C]acetate, they are presumably esterified *in vivo* immediately on their formation. Maybe the acceptors for the esterification of oleic acid were deficient in isolated chloroplasts, or they were prevented from reacting with the fatty acid by some sort of cellular control mechanism, or the esterification normally takes place outside the chloroplast, or some factor involved was inactivated during chloroplast isolation. Inhibiting a cellular control (Triton X-100) or increasing the supply of acceptor (glycerol 1-phosphate) could allow a greater proportion of the fatty acid to become esterified and could explain the experimental results. The requirement for exogenous ATP in the synthesis of long-chain acyl-CoA suggests that the reaction took place in the chloroplast envelope, since, because of their high photosynthetic rates, the isolated organelles must have contained an adequate supply of ATP. Further, the intact chloroplast envelope is largely impermeable to ATP (Stokes & Walker, 1971). This synthesis of acyl-CoA in the chloroplast envelope would be compatible with our scheme (Roughan, 1975; Slack & Roughan, 1975) for polyunsaturated fatty acid biosynthesis, since the transfer of the acyl group from oleoyl-CoA to 3-*sn* phosphatidylcholine of microsomal fractions has been reported from a number of laboratories (Baker & Lynen, 1971; Vijay & Stumpf, 1971; Slack *et al.*, 1976).

Diacylglycerols were a relatively minor product of acetate incorporation into leaf discs, but were by far the major product in intact chloroplasts when both Triton X-100 and glycerol 1-phosphate were included in the reaction mixture. Triton X-100 alone stimulated the synthesis of diacylglycerols and removed proportionately more palmitic acid than oleic acid from the free fatty acids, thus indicating a preference for palmitic acid in the esterification of acyl groups to endogenous acceptors within the isolated organelle. Isolated maize chloroplasts incorporated 14-fold more palmitic acid than oleic acid into diacylglycerols (Hawke *et al.*, 1974), and addition of glycerol 1-phosphate in our experiments resulted both in enhanced synthesis of diacylglycerols and stimulation of palmitic acid synthesis relative to oleic acid. This relatively specific synthesis by isolated chloroplasts of free oleic acid and of esterified palmitic acid may be related to the similarly specific insertion of oleic acid into 3-*sn*-phosphatidylcholine and of palmitic acid into 3-*sn*-phosphatidylglycerol, which has been repeatedly observed when [1-^{14}C]acetate has been fed to attached to detached leaves (Roughan, 1970, 1975; Slack & Roughan, 1975). Oleic acid would normally be converted into oleoyl-CoA and then into phosphatidylcholine oleate *in vivo*, whereas palmitate-containing phosphatidic acid species may be converted into phosphatidylglycerol via the CDP-diacylglycerol pathway (Douce, 1968).

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