LIPID BIOSYNTHESIS FROM [^{14}C]BICARBONATE, [2-^{14}C]PYRUVATE AND [1-^{14}C]ACETATE DURING PHOTOSYNTHESIS BY ISOLATED SPINACH CHLOROPLASTS

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1. Introduction

There has not been any conclusive demonstration of the synthesis of acetyl-CoA by isolated chloroplasts although several investigations have been addressed to this problem [1–4]. The earlier work was based on the assumption that the light-dependent incorporation of [^{14}C]bicarbonate into chloroplast fatty acids implied the synthesis of acetyl-CoA within the organelle by conventional metabolism from photosynthetically produced 3-phosphoglycerate. Everson and Gibbs [1] demonstrated that less than 1% of the [^{14}C]bicarbonate incorporated by isolated chloroplasts could be extracted by chloroform/methanol, but the extract was not further analysed to establish whether this incorporation was indeed into lipids. In later work using pea chloroplasts capable of high rates of photosynthetic CO_2 fixation, Sherrat and Givan [2] failed to detect significant amounts of ^{14}C in the chloroplast lipid-fraction over incorporation periods of 30 min. On the other hand, it has been suggested that the stimulation of the incorporation of tritium from ^3H_2O into the carbon-chains of fatty acids by unlabelled precursors such as phosphoenolpyruvate, pyruvate and 3-phosphoglycerate gives indirect evidence that a pathway from 3-phosphoglycerate to fatty acids exists in isolated spinach-chloroplasts [4]. A further indication that chloroplasts may synthesise acetyl-CoA comes from the demonstration of a light-dependent accumulation of ^{14}C into plastoquinone and β-carotene by isolated spinach-chloroplasts [5].

Only rather recently [6–11] has it become possible to routinely prepare chloroplast suspensions capable of consistently high rates of photosynthetic carbon reduction comparable with those found in the intact leaf. Unless the chloroplasts used to study lipid biosynthesis from $H^{14}CO_3^-$ reduce 3-phosphoglycerate at high rates, it is unlikely that any further transformation into lipids will be detectable. In this paper we describe short term (< 30 min) experiments in which spinach-chloroplasts capable of high rates of CO_2-fixation were used, to compare the incorporation of ^{14}C from [^{14}C]bicarbonate, [2-^{14}C]pyruvate, and [1-^{14}C]acetate into lipids. From the characteristics of the incorporations, it is concluded that chloroplasts are able to synthesise acetyl-CoA endogenously in vitro.

2. Materials and methods

Spinach plants, *Spinacea oleracea,* hybrid 102 (Arthur Yates and Co., Sydney) were grown hydroponically under a natural-light regime, day-length 8 h, supplemented when required by mercury (MBFR/400 W) and tungsten (60 W) lamps two feet six inches above the plants. The petioles of young, still-expanding leaves 18–25 cm in length were cut under water and the leaves floated under tungsten lights (0.3×10^5 erg.cm^{-2}s^{-1}) for two hours. Homogenisation (50 g leaves/200 ml) in a sorbitol–pyrophosphate medium [12] was for two bursts of one second each in an MSE Atomix Blender. The homogenate was filtered through 6 layers of muslin and 8 layers of nylon bolting cloth and the chloroplasts sedimented by a very rapid acceleration to $3000 \times g$ followed by immediate deceleration in an MSE 4L centrifuge, fitted with a fast brake.

The resuspending buffer contained 50 mM Tricine–

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NaOH, pH 8.0, 0.3 M sorbitol, 2.5 mM $MgCl_2$, 1 mM MgEDTA, 1 mM $MnCl_2$, 1.2 mM CoA, 4.4 mM $Na_4P_2O_7$ and 1 mM Na-isoascorbate. The intactness of the resuspended chloroplasts was monitored both by phase-contrast microscopy [13] and by their capacity to evolve O_2 in the presence of ferricyanide, both before and after rupturing by osmotic shock [14].

Incubations were performed in rotating glass flacks maintained at 20°C in a water bath and illuminated by 12 × 150 W tungsten bulbs providing about 30 000 lux. $NaH^{14}CO_3$, [2-^{14}C]pyruvate and [1-^{14}C]-acetate (Radiochemical Centre, Amersham, Buckinghamshire) were added to chloroplast suspensions (80–120 mg chlorophyll) in total vol. 1 ml resuspending buffer. The incubations were terminated by the addition of 1 ml 6 M formic acid. Chloroform-methanol (2:1, v/v) (50 ml) was added in order to render the solution monophasic and the lipid extract washed in 10 ml 1% acetic acid/0.1 M KCl, followed by three subsequent washes in 10 ml glass distilled water.

The washed extracts were dried in Na_2SO_4 and taken to dryness in a rotary evaporator. The residue was redissolved in 1 ml chloroform for separation by one- and two-dimensional thin-layer chromatography (TLC) on silica-gel 'H'. Developed chromatograms were analysed by a Nuclear Chicago actigraph II, Model 1006 plate scanner and by autoradiography. Separated lipids were identified by comparison with authentic standards in several solvent systems and by specific spray reagents. The separated radioactive bands were scraped into vials containing 10 ml Bray's cocktail and counted with 75% efficiency in a Beckman LS 230 liquid scintillation counter.

Fractions for gas–liquid chromatography (GLC) were derivitised as previously described [15] or by acidic methanolysis in 1.5 N HCl in MeOH. The crude methyl esters were dried in Na_2SO_4 and blown down under N_2 for purification by preparative TLC in benzene. The band corresponding to an authentic methyl ester standard was scraped into petroleum ether for elution from the silica gel. Purified methyl esters were down to dryness under N_2 and taken up in 50–100 μl petroleum ether for resolution by radio-GLC on a Hewlett Packard 402 [15].

For some experiments the chloroplasts (70–80% Type A [16]) were further purified (80–90% Type A [16]) by layering onto a 10 ml band containing 0.6 M sorbitol in a pyrophosphate medium. The chloroplasts were sedimented, free from mitochondrial contamination, following a 5 min spin at 600 × *g* in a swing-out head. In this case the incubation medium contained 0.45 M sorbitol, as the plastids tended to rupture in 0.3 M sorbitol.

3. Results

The suspensions of isolated chloroplasts used in the present experiments to study lipid biosynthesis were capable of [^{14}C]bicarbonate fixation at rates of approx. 60–75 μmol/mg chlorophyll/h and showed uncoupled rates of electron-flow with ferricyanide of approx. 300 μmol/mg chlorophyll/h. The suspensions contained 60–80% intact chloroplasts as judged by phase-contrast microscopy and also by ferricyanide reduction.

The chloroplasts had the capacity to incorporate ^{14}C (in light-dependent reactions) into a lipid fraction extractable in chloroform/methanol from three precursors, $H^{14}CO_3^-$, [2-^{14}C]pyruvate, and [1-^{14}C]-acetate (table 1). The incorporation from each of the three precursors over 30 min was similar, acetate being incorporated at the highest rate (14–21 nmol/mg chlorophyll/30 min) and bicarbonate at the lowest rates. The rate of incorporation of ^{14}C from bicarbonate into the lipid fraction was 20-fold higher than the rate reported earlier by Everson and Gibbs [1] and the radio carbon in the lipid fraction represents 0.1% of the carbon fixed.

Of the total carbon incorporated into lipids from each precursor 60% was into free fatty acids, most of the remainder (30–40%) was found in acyl-groups of diacylglycerol with between 3–4% in phosphatidyl choline. In these short-term experiments no label was ever found in phosphatidyl glycerol nor in galactolipids. The label in diacylglycerol was almost exclusively confined to the acyl-groups, i.e., the glycerol moieties had not become labelled in 30 min. Fatty acid analyses showed a similar labelling pattern for each precursor. The major labelled products were oleate (30–34%), palmitate (30–33%) and stearate (15–17%). In every case, only very small amounts of label were found in polyunsaturated fatty acids, e.g., linoleate (2–3%) and linolenate (0.4–2%).

Table 1
The incorporation of labelled precursors into lipids by isolated spinach-chloroplasts in the light

Precursor	Incorporation of carbon					
	Total lipids (nmol/mgChl./30 min)	Individual lipids (% ^{14}C in all lipids)				
		PC	PG	GL	FFA	DG[a]
$H^{14}CO_3^-$	6– 9 (11)[b]	3	0	0	65	32
[2-^{14}C]Pyruvate	14–18 (3)	3	0	0	60	37
[1-^{14}C]Acetate	19–21 (7)	4	0	0	62	34

[a] PC – phosphatidyl choline, PG – phosphatidyl glycerol, GL – total galactolipids, FFA – free fatty acids, DG – diacylglycerol
[b] (*n*) – number of experiments

In order to establish that the enzymes responsible for these reactions were definitely located in the chloroplasts, further purification of the plastids was effected by spinning the suspension through a band containing 0.6 M sorbitol. This treatment effectively removed remaining particulate contamination from the chloroplasts. After this purification, the plastids still fixed carbon dioxide and incorporated the lipid precursors at rates normally higher than those exhibited by the suspensions before centrifugation through 0.6 M sorbitol. The results are shown in table 2, from which it can be seen that the qualitative and quantitative rates of incorporation are similar in both kinds of chloroplast suspension.

4. Discussion

Results presented in this paper show that spinach chloroplasts can incorporate photosynthetically-fixed HCO_3^- into the acyl groups of lipids. It can therefore be deduced that chloroplasts are capable of some

Table 2
The incorporation of [^{14}C]bicarbonate and of [1-^{14}C]acetate by chloroplasts immediately after isolation and after centrifugation through a 0.6 M sorbitol band

Conditions		Incorporation of ^{14}C into a chloroform/methanol fraction	
		Before centrifugation through 0.6 M sorbitol (A) (nmol/mgChl.)	After centrifugation through 0.6 M sorbitol (B) (nmol/mgChl.)
$H^{14}CO^-_3$	Expt. 1	8	12
	Expt. 2	9	14
	Expt. 3[a]	9	16
[1-^{14}C]Acetate	Expt. 1	20	25
	Expt. 3[a]	21	28

[a] Expt. 3 Total carbon fixation: Fraction A; 61 μmol CO_2 reduced/mgChl./h. Fraction B; 71 μmol CO_2 reduced/mgChl./h.

As judged by phase contrast microscopy 80% of the chloroplasts were intact in both fraction A and fraction B. Methods as described in the text.

synthesis of acetyl-CoA endogenously. The major factor contributing to the success of these experiments (after the failure of other workers) is probably the use of chloroplast suspensions containing a high proportion of intact chloroplasts shown to be capable of high rates of photosynthetic CO_2-fixation and electron-flow. Yamada and Nakamura [4] explained the failure of previous attempts to detect the photofixation of [^{14}C]bicarbonate into lipids as being due to the loss of C-1 (initially labelled in the formation of phosphoglyceric acid) during oxidative decarboxylation of pyruvate to acetate. However, since the PGA becomes uniformly labelled after approx. 60 s photosynthesis [17], both carbon atoms of acetate should become labelled in periods longer than this.

It is not possible from our results to deduce the exact pathway of $^{14}CO_2$ fixation into acetyl CoA. The scheme CO_2 → PGA → PEP → pyruvate → acetate → acetyl-CoA has been proposed, based upon ^3H_2O-incorporation experiments [4]. We have consistently observed incorporation of ^{14}C from $^{14}CO_2$, [U-^{14}C]PGA and [2-^{14}C]pyruvate into lipids, which is in agreement with such a scheme. Pyruvate dehydrogenase [4], pyruvate kinase [18] and ribulose biphosphate carboxylase [8] are all found in chloroplasts and the existence of the remaining enzymes of the reaction sequence, i.e., phosphoglucomutase (3-PGA → 2-PGA) and enolase (2-PGA → PEP) is implied by our observation of incorporation of ^{14}C from $^{14}CO_2$ and [U-^{14}C]PGA into lipids. While these observations do not definitely rule out the existence of alternative pathways for acetyl-CoA biosynthesis in the chloroplast, e.g., via glycolate, they do favour the more direct scheme proposed by Yamada and Nakamura.

In contrast to experiments with whole spinach plants, isolated chloroplasts were only capable of synthesising a relatively small range of lipids. No galactolipids were labelled and only minute amounts of ^{14}C were ever detected in polyunsaturated fatty acids, in spite of their preponderance among chloroplast lipids. The principal product of all the incubations was oleate and an oleate desaturase has recently been found in a 105 000 × *g* pellet from a pea-leaf homogenate [19]. The data suggest that oleate may be exported from chloroplasts as oleoyl-CoA for desaturation while esterified to 'microsomal' phosphatidyl choline. Presumably the linoleate thus formed may then be re-imported into the chloroplast envelope as diacylglycerol for further desaturation [20] and galactosylation [21,22] to form polyunsaturated galactolipids. Thus spinach-chloroplasts contain the enzymes for de novo fatty acid synthesis from the ultimate carbon precursor, CO_2. They also have the elongase necessary for oleate biosynthesis and are capable of esterifying fatty acids into diacylglycerol. However, there has been no decisive experimental demonstration that isolated chloroplasts are able to further elaborate the lipids. The current evidence suggests that several other cellular components are required in a cooperative function in the biosynthesis of lipids in leaves. Experiments are now in progress in our laboratory to examine these interrelationships.

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