THE PATHWAY OF [^{14}C]BICARBONATE INCORPORATION INTO LIPIDS IN ISOLATED PHOTOSYNTHESISING SPINACH CHLOROPLASTS

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1. Introduction

Intact photosynthesising spinach chloroplasts incorporate [^{14}C]bicarbonate, [2-^{14}C]pyruvate and [1-^{14}C]acetate into acyl lipids [1]. With each substrate, free fatty acids, monoacylglycerols and diacylglycerols become radioactively labelled and rates of incorporation increase after the chloroplasts are purified on a sorbitol density gradient. These results suggest that acetyl-CoA can be synthesised from bicarbonate within the chloroplast. The details of this chloroplast-located biosynthesis of acetyl-CoA are unknown, but one possible pathway would be via photosynthetically-reduced 3-phosphoglycerate and phosphoenolpyruvate [1,2].

We present here evidence from a comparison of labelling patterns of lipids and fatty acids from the different postulated precursors and also evidence from isotope competition experiments which are consistent with the operation of a pathway from CO_2 via 3-PGA, PEP and pyruvate to acetyl-CoA in isolated spinach chloroplasts.

2. Materials and methods

Spinach plants, *Spinacia oleracea,* hybrid 102 (A. Yates and Co., Sydney) were grown hydroponically as in [3]. Chloroplasts were isolated from young, expanding leaves ranging in length from 18–25 cm, and in area from 150–280 cm^2. The leaves were harvested 1 h after the start of the photoperiod and the chloroplasts isolated by the rapid procedure in [1]. Incubations at 20°C and 30 000 lux [1] were performed in rotating glass flasks and terminated by the addition of 1 ml 6 M formic acid [1]. The incubation medium contained 50 mM tricine–NaOH, pH 8.0, 0.3 M sorbitol, 2.5 mM $MgCl_2$, 1 mM Na_2-EDTA, 1 mM $MnCl_2$, 1.2 mM CoA, 4.4 mM $Na_4P_2O_7$, 1 mM Na-isoascorbate. Each flask contained chloroplasts to 80–120 μg chlorophyll in total vol. 1 ml.

Lipid extraction was typically in the conventional chloroform:methanol system [4], followed by partitioning of aqueous contaminants in 0.1 M KCl 1% in acetic acid (1 part KCl to 4 parts chloroform: methanol extract). Three washes in glass-distilled water [1] followed. A butanol:boric acid system was used additionally in several experiments since it resulted in a much-improved recovery of monoacylglycerols, which are often poorly and erratically recovered in chloroform:methanol extractions [5]. Boric acid-saturated butanol, 3 ml, was added to each incubation flask, followed by 4 ml butanol-saturated 0.1 M boric acid with thorough mixing. The phases were separated by centrifugation and the upper (butanol) layer collected, washed with 6 ml butanol-saturated 0.1 M boric acid and dried under N_2. The residue was taken up in chloroform:methanol (2:1, v/v) for subsequent analysis. Acyl ACPs and acyl CoAs were extracted by the iso-propanol method in [6].

Total lipid mixtures were resolved by one- and two-dimensional thin-layer chromatography on silica gel 'H' and 'G'. Neutral lipids were efficiently separated by development in either petroleum ether/diethyl ether/acetic acid (80:20:1, v/v/v) or benzene/diethyl ether/ethylacetate/acetic acid (80:10:10:0.4, v/v/v/v). Identity was established on the basis of cochromatography with authentic standards and by

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the use of specific spray reagents. Methyl esters of the acyl residues were prepared by transmethylation and separated by gas–liquid chromatography as in [1,3].

^{14}C-Labelled lipids were detected by autoradiogrphy using Kodirex X-ray film (Kodak) and by thin-layer chromatographic plate scanning on a Nuclear Chicago actigraph II Model 1006 with a gas-flow detector. Quantitative results were obtained by radioactivity counting on a Nuclear Chicago gas-flow counter using etched aluminium planchets containing 10–20 μl sample plated to infinite thinness. Samples were also counted in Bray's cocktail [7] on a Beckman LS 230 liquid-scintillation counter.

3. Results and discussion

The isolated chloroplast suspensions contained 60–80% intact plastids as judged by phase-contrast microscopy [8] and ferricyanide reduction [9]. The chloroplasts were able to fix CO_2 at rates exceeding 100 μmol mg^{-1} chl.h^{-1} and after breakage showed uncoupled rates of electron flow with ferricyanide of ~ 300 μmol mg^{-1} chl.h^{-1} at the optimal chlorophyll concentration of 80–120 μg.$m.^{-1}$.

The characteristics of the incorporation of [^{14}C]-bicarbonate, the proposed initial precursor and [^{14}C]pyruvate, the suggested immediate precursor, of acetyl-CoA, were first compared. The kinetics of the incorporation of $H^{14}CO_3^-$ and [2-^{14}C]pyruvate into acyl lipids are very similar and resemble those of [1-^{14}C]acetate, which although possibly non-physiological, is the conventionally used substrate in studies of lipid biosynthesis (table 1). All the initial rates were linear and decreased after 30 min. All the incorporations were light-dependent and ceased after osmotic lysis of the chloroplasts. The rate of incorporation of each of the precursors was concentration dependent.

The labelling patterns found for the acyl lipids formed from each of the fatty acid precursors were also very similar (table 2). Most of the label (30–40%) was associated with free fatty acids or their thio- or ACP-esters. The neutral mono- or diacylglycerols contained most of the remaining label with a very small proportion associated with the phospholipid, phosphatidyl choline [PC]. The principal chloroplast lipid constituents, phosphatidyl glycerol [PG] and the galactolipids, did not accumulate label from any of the fatty acid precursors under the conditions employed here, in sharp contrast to in vivo labelling studies with $^{14}CO_2$ [3].

The labelled fatty acid profiles of the three principal lipid products from bicarbonate and acetate incubations are shown in table 3. The fatty acid labelling patterns of each of the separated lipids from the same substrate shows little variation, and there is remarkably little difference in the labelling patterns when the same lipids synthesised from the two different substrates are compared. The free fatty acids (FFA) from both $H^{14}CO_3^-$ and [1-^{14}C]acetate incorporations contained mainly labelled oleate and, in both cases,

Table 1
The incorporation of ^{14}C-labelled precursors into acyl lipids by isolated photosynthesising spinach chloroplasts

Precursor	^{14}C incorporation into lipids (nmol. mg^{-1} chl.)							
	Light							Dark
Time (min)	0	10	20	30	40	50	60	30
[^{14}C]Bicarbonate	0	39	85	114	145	154	161	6.2
[2-^{14}C]Pyruvate	0	7	10	18	22	24	26	1.2
[1-^{14}C]Acetate	0	33	58	96	114	125	129	6.3

The substrate concentrations were 10 mM $H^{14}CO_3^-$, 140 μM pyruvate and 66 μM acetate. Each value represents an average of at least 2 separate incubations. Conditions of incubation are given in the text

Table 2
The incorporation of ^{14}C-labelled precursors into acyl lipids by isolated photosynthesising spinach chloroplasts

Precursor	% ^{14}C incorporated into individual lipids							Total incorporation (nmol. mg^{-1} chl.h^{-1})
	FFA	acyl CoA + acyl ACP	MG	DG	PC	PG	GL	
[^{14}C]Bicarbonate	37	19	24	18	2	0	0	232
[2-^{14}C]Pyruvate	32	20	25	20	2	0	0	385
[1-^{14}C]Acetate	34	21	24	19	2	0	0	426

Abbreviations: FFA, free fatty acids; ACP, acyl carrier protein; MG, monoacylglycerol; DG, diacylglycerol; PC, phosphatidyl choline; PG, phosphatidyl glycerol; GL, galactolipids

Each figure is an average of at least 3 experiments. Incubations were for 30 min as in [1] and more than 98% of the recovered activity was in the lipids measured below

over 60% of the total label was in C_{18} fatty acids. In contrast, both the monoacylglycerol (MG) and diacylglycerol (DG) formed from the two substrates incorporated only 10–15% of the label into C_{18} fatty acids, whereas 80–90% was found in palmitate and myristate. The ratio of unsaturated fatty acids/saturated fatty acids was 1.22 and 1.30 in the FFAs from $H^{14}CO_3$ and [1-^{14}C]acetate, respectively and only about 0.1 in both the MG and DG from each of these precursors.

These results confirm that both $H^{14}CO_3^-$ and [2-^{14}C]-pyruvate are incorporated into fatty acids by chloroplasts in a similar manner to each other and to [1-^{14}C]-acetate. The possibility that a pathway from CO_2 to acetyl-CoA may be present in spinach chloroplasts was therefore examined.

Table 3
A comparison of the fatty acid labelling patterns of the principal labelled lipids after 30 min incubations of spinach chloroplasts with [^{14}C]bicarbonate (10 μM) and with [1-^{14}C]acetate

Fatty acids	% ^{14}C incorporated into total fatty acids					
	[^{14}C]Bicarbonate			[1-^{14}C]Acetate		
	FFA	MG	DG	FFA	MG	DG
12–0	2	tr	tr	3	tr	tr
14–0	15	21	33	11	19	28
16–0	22	69	53	25	65	60
18–0	6	3	2	4	4	2
18–1	52	7	12	54	12	10
18–2	3	tr	tr	3	tr	tr
18–3	tr	0	0	tr	0	0

Abbreviations: as in table 2

If 3-phosphoglycerate is an intermediate in the biosynthesis pathway from CO_2 to acetyl-CoA, then it should be incorporated into the acyl chains of the chloroplast lipids. Tabel 4 shows a comparison of the %incorporation of ^{14}C into the fatty acids of the lipids that become labelled when 3-[U-^{14}C]PGA and the three lipid precursors previously examined are individually fed to isolated photosynthesising spinach chloroplasts. The chloroplasts incorporated ^{14}C from all four of the labelled substrates into a similar range of fatty acids in generally similar proportions. Oleate was in every case the most highly-labelled fatty acid (35–55%); palmitate (20–35%) and stearate (13–17%) were also relatively highly labelled in all cases. Only a small proportion of the label was found in polyunsaturated fatty acids. These results are consistent with 3-phosphoglycerate being an intermediate on the pathway of acetyl-CoA biosynthesis from CO_2. Further support for such a pathway came from the results of an isotope competition experiment (table 5). $H^{14}CO_3^-$ was fed either alone or together with a much higher concentration of a suspected intermediate, in unlabelled form. The rates of isotope incorporation into lipids were then compared. Table 5 shows a marked depression of the rate of incorporation of $H^{14}CO_3^-$ into lipids in the presence of each of the additional unlabelled substrates. 3-Phosphoglycerate

Table 4
The % incorporation of ^{14}C from various ^{14}C-labelled precursors into fatty acids by isolated photosynthesising spinach chloroplasts

Fatty acids	% ^{14}C incorporation			
	[^{14}C]Bicarbonate (10)	[U-^{14}C]Phosphoglycerate (3)	[2-^{14}C]Pyruvate (6)	[1-^{14}C]Acetate (10)
12–0	tr	0.7	4.4	tr
14–0	3.8	2.9	9.5	1.8
16–0	34.5	25.5	32.0	20.2
18–0	15.3	14.3	17.4	12.8
18–1	38.0	52.2	34.0	54.8
18–2	5.4	2.4	2.2	6.9
18–3	2.4	1.9	0.4	3.5

These results are from a single 30 min incubation in which the four labelled precursors were added simultaneously to different chloroplast suspensions. The final concentration of each substrate was: bicarbonate, 10 mM; 3 PGA, 40 μM; pyruvate, 140 μM; acetate, 20 μM

Similar results were obtained in duplicate experiments with each substrate. The number of duplicate experiments performed in each case is given in brackets

Table 5
Isotope competition experiment

Radioactive substrate	'Cold' substrate added	Rate of incorporation into lipids	
		nmol.mg^{-1} chl.h^{-1}	% control
Experiment 1			
$H^{14}CO_3^-$	10 mM HCO_3^-	232	100
$H^{14}CO_3^-$	50 mM pyruvate	51.1	22.0
$H^{14}CO_3^-$	50 mM PEP	29.6	12.8
$H^{14}CO_3^-$	50 mM 3-PGA	3.5	1.5
Experiment 2			
[1-^{14}C]Acetate	0.2 mM acetate	426	100
[1-^{14}C]Acetate	50 mM pyruvate	30.2	7.1
[1-^{14}C]Acetate	50 mM PEP	57.5	13.5
[1-^{14}C]Acetate	46 mM HCO_3^-	293.9	69.0

Isolated spinach chloroplasts were incubated with a radioactive lipid substrate, either $H^{14}CO_3^-$ or [1-^{14}C]acetate, together with a series of non-radioactive suspected intermediates of acetyl CoA biosynthesis. A duplicate of each treatment was carried out

and phosphoenolpyruvate both had a greater inhibitory effect upon $H^{14}CO_3^-$ incorporation than did pyruvate. In [1-^{14}C]acetate incubations, pyruvate, phosphoenolpyruvate and $H^{14}CO_3^-$ had progressively less effect upon the incorporation of acetate into lipids.

Assuming large amounts of the intermediates do not inhibit the enzymes directly, the data from the ^{14}C-labelling experiments are consistent with the operation of a pathway from

$$\text{3-PGA} \xrightarrow{(a)} \text{2-PGA} \xrightarrow{(b)} \text{PEP} \xrightarrow{(c)} \text{Pyruvate} \xrightarrow{(d)} \text{Acetyl-CoA}$$

in spinach chloroplasts. There are reports of the activities of the final two enzymes in this sequence, i.e., (c) pyruvate kinase and (d) pyruvate dehydrogenase from tobacco [10] and spinach [1,2,11–13-chloroplasts, respectively. There are no published rates for the separate activities of the other two enzymes of the sequence in chloroplasts, i.e., (a) phosphoglyceromutase and (b) phosphopyruvate hydratase (enolase), they were assayed in a linked system with pyruvate kinase with a recorded activity of 5.1 μmol/mg^{-1} chl./h^{-1} [13]. Spectrophotometric assays on enzyme preparations from *Vicia faba* and *Zea mays* chloroplasts purified on a 0.5 M sucrose band were performed [14] in this laboratory. The results showed maximal activities of 300 nmol mg^{-1} chl.h^{-1} for phosphoglyceromutase (EC 5.4.2.1); 300 nmol mg^{-1} chl.h^{-1} for phosphopyruvate hydratase (EC 4.2.1.11) and 800 nmol mg^{-1} chl.h^{-1} for pyruvate kinase (EC 2.7.1.40). These rates are more than adequate to account for the observed rates of incorporation of each of the labelled precursors into fatty acids recorded in this paper. They also provide supporting evidence for the existence of a pathway from 3-PGA to acetyl-CoA in higher plant chloroplasts.

Isolated spinach chloroplasts used in the present work incorporated CO_2 (supplied as [^{14}C]bicarbonate) into the acyl residues of lipids at a maximal rate of 606 nmol mg^{-1} chl.h^{-1}, which represented 0.61% of the total fixed carbon. This compares with the rates found in the intact spinach leaves which incorporated CO_2 (supplied as $^{14}CO_2$) into the acyl residues of lipids at a maximal in vivo rate of 1704 nmol mg^{-1} chl.h^{-1} (1.7% of the total fixed carbon); a further 4.4% was fixed into other parts of the lipid molecules [3]. Thus these isolated spinach chloroplasts were able to synthesise fatty acids from CO_2 at 36% of the rate of intact leaves. The inevitable loss of some enzyme activities during chloroplast isolation, coupled with the removal of the organelles from their normal cytoplasmic environment to one that is 100–1000-fold more dilute [15] would be expected to result in the reduction of the rates of in vitro activities compared with the in vivo situation. The observation that in vitro chloroplast preparations can account for 36% of the leaf fatty acid biosynthesis means that chloroplasts may be an important site of their synthesis in the leaf.

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