THE INCORPORATION OF [^{14}C]BICARBONATE AND $^{14}CO_2$ INTO THE CONSTITUENT FATTY ACIDS OF MONOGALACTOSYLDIACYLGLYCEROL BY SPINACH CHLOROPLASTS AND LEAVES

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1. Introduction

The ability of isolated photosynthesizing spinach chloroplasts to incorporate bicarbonate into acyl lipids has been examined [1,2] and rates of incorporation obtained which were little inferior to those obtained when acetate and pyruvate were used as substrates. Each of these fatty acid precursors gave very similar labelling patterns for the acyl lipids in that ~50% of the label was associated with free fatty acids or their thioesters and most of the remaining label was in mono- or diacylglycerols. A notable feature was the absence of label in galactolipid, one of the principal lipid constituents of chloroplasts.

During the course of our studies of acetate incorporation into the constituent fatty acids of diacylglycerol and monogalactosyldiacylglycerol (MGDG) by isolated spinach chloroplasts [3] we became aware that fatty acids were being synthesized from a substrate other than acetate. Accordingly, we examined the utilization of bicarbonate which is included in the 10–30 mM range in reaction mixtures used for acetate incorporation into lipids by isolated chloroplasts, in order to provide CO_2 for the reaction catalysed by acetyl CoA carboxylase [4].

Unlike the labelling patterns obtained in acyl lipids [1,2] we obtained substantial incorporation of label from [^{14}C]bicarbonate into the acyl constituents of MGDG. This was achieved by the addition of *sn*-glycerol-3-phosphate and UDP-galactose: ~50% of the fatty acids synthesized accumulated in MGDG and there was a substantially greater overall incorporation of bicarbonate into acyl lipids.

2. Materials and methods

Field-grown spinach (*Spinacea oleracea*) was purchased locally and chloroplasts isolated in a buffer, at pH 8.0, consisting of 0.5 M sucrose, 0.2% bovine serum albumin, 1 mM $MgCl_2$ and 0.067 M phosphate [5]. A two-step gradient of 0.5 M and 0.6 M sucrose was used to purify the chloroplast pellet obtained from the first rapid centrifugation [6]. The chloroplasts were resuspended in isolation buffer and were about 70% class I as judged by phase-contrast microscopy [7].

Incubations were performed in 15 ml round bottom tubes and held at 20°C in a photosynthetic Warburg apparatus fitted with a tube holder attached to the agitator arm. The tubes were agitated at 78 cycles/min. and sixteen 40 W tungsten lamps provided a light intensity of about 25 000 lux at the tube surface. The standard incubation medium [5] contained 300 mM sorbitol, 50 mM tricine/NaOH and 50 mM phosphate (at pH 7.8) 2.5 mM DDT, 0.5 mM ATP, 0.25 mM CoA, 0.5 mM $MgCl_2$, 0.2 mM NADPH, 0.2 mM NADH and 10.0 mM bicarbonate (containing 7.5 μCi ^{14}C; Radiochemical Centre, Amersham, Bucks) to give a final 1.0 ml after addition of 0.1 ml chloroplast suspension (100–150 μg chlorophyll). The concentrations of glycerol-3-phosphate (5 mM) and UDP-galactose (0.15 mM) used gave optimum incorporation of label from [1-^{14}C]acetate into diacylglycerol and MGDG, respectively [3].

Incubations were stopped by the addition of 1 ml 6 M HCl and the lipids extracted and purified accord-

Table 1
The dependence of lipid synthesis by spinach chloroplasts on bicarbonate concentration

	Bicarbonate (mM)				
	0.14	5.0	10	20	60
^{14}C incorporation into lipid (nmol.mg^{-1} chl. h^{-1})	2.4	87	166	255	642

Conditions of incubation are given in the text

ing to [5]. Radioactivity in lipids was determined in 0.5% (w/v)*p*-terphenyl in toluene using a Beckman LS-350 liquid scintillation counter. Individual lipids in the lipid extracts were separated by chromatography on thin layers of Silica Gel G as follows: free fatty acids and diacylglycerols by development with hexane/diethyl ether/HOAc (50:50:1, v/v/v); MGDG by development with toluene/ethyl acetate/95% ethanol (2:1:1, v/v/v); phosphatidylcholine and other phospholipids by development with $CHCl_3$/MeOH/HOAc/H_2O (85:15:10:3, v/v/v/v). Separated lipids were located and then extracted from Silica Gel [8]. Methyl esters of the constituent fatty acids of lipids were prepared using BF_3 in methanol after hydrolysis with 0.5 M NaOH in methanol [9]. The methyl esters were sometimes separated according to their level of unsaturation, by TLC on Silica Gel G containing 10% $AgNO_3$, prior to gas-liquid chromatography (GLC) and measurement of radioactivity [10].

The distribution of label in the acyl groups of MGDG was determined following the exposure of spinach plants (*Spinacea oleracea*, Hybrid 102) growing in individual pots, to $^{14}CO_2$ for 8 h in natural light in an 8 l dessicator. $^{14}CO_2$ was generated by acidification of 0.04 mmol (2 mCi) $Na_2{}^{14}CO_3$ (The Radiochemical Centre, Amersham, Bucks). The total lipids were extracted from 8 g leaves [11] and the MGDG isolated [8] to determine the constituent acyl groups and their radioactivity by radio-GLC [12].

3. Results and discussion

The incorporation of bicarbonate into lipid was concentration-dependent and increased linearly up to 60 mM HCO_3^- (table 1). At high concentrations within this range dilution of ^{14}C limits the analysis of the radioactive products and 10 mM HCO_3^- was used in subsequent incubations.

Using the standard incubation medium most of the label from $H^{14}CO_3^-$ was associated with free fatty acids and diacylglycerols (table 2) in proportions

Table 2
The incorporation of ^{14}C from $H^{14}CO_3^-$ into acyl lipids by isolated spinach chloroplasts

Additions to incubation medium	Total incorporation into lipid (nmol.mg^{-1} chl. h^{-1})	% ^{14}C incorporated into individual lipids				
		FFA	DG	MGDG	PC	Others[a]
None	173	56	31	3	8	2
G-3-P	303	23	65	4	5	3
UDP-gal	237	54	15	23	6	2
G-3-P + UDP-gal	358	12	30	51	5	2

[a] Phospholipids and digalactosyldiacylglycerol
Incubation conditions are given in the text

Abbreviations: FFA, free fatty acids; DG, diacylglycerol; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; G-3-P, *sn*-glycerol-3-phosphate

which were similar to those in [2] and with MGDG poorly labelled. Moreover, the total incorporation of label into lipid was similar, namely 173 nmol.mg^{-1} chl. h^{-1} compared with 161–232 nmol.mg^{-1} chl. h^{-1} using 10 mM HCO_3^- [2]. The addition of *sn*-glycerol-3-phosphate increased the total incorporation and also increased the distribution of label in diacylglycerol from 31–65% of the total. When UDP-galactose was added the proportion of label in MGDG increased at the expense of diacylglycerol while there were similar proportions in free fatty acids to that obtained using the standard incubation medium. Addition of both *sn*-glycerol-3-phosphate and UDP-galactose doubled the total incorporation of $H^{14}CO_3^-$ and 50% of the label was incorporated into MGDG and 30% into diacylglycerol. Similar stimulations of MGDG synthesis from acetate have been obtained [3]. Only small amounts of label were present in phosphatidylcholine and other phospholipids and the proportions were not increased by the addition of glycerol-3-phosphate.

An outstanding problem of lipid biosynthesis in higher plants is the continued failure to obtain de novo synthesis of polyunsaturated fatty acids in isolated chloroplasts commensurate with the large amounts which occur endogenously. Despite the high incorporation of bicarbonate into MGDG, which are characteristically rich in dienoic and trienoic acids, the major fatty acids synthesized from $H^{14}CO_3^-$ were palmitate (25%), stearate (12%) and oleate (53%) and only trace amounts of label entered dienoic and trienoic fatty acids. Under similar conditions acetate was incorporated into palmitate (16%) stearate (10%) and oleate (69%). These distributions are in sharp contrast to the distribution of label from $^{14}CO_2$ into MGDG by photosynthesizing spinach plants when the distribution of label in the constituent fatty acids was very similar to their molar proportions (table 3).

Although the range of lipids capable of synthesis by chloroplasts has been extended to the quantitatively important MGDG by the addition of *sn*-glycerol-3-phosphate and UDP-galactose, reconstruction of a system for polyunsaturated fatty acid biosynthesis and their incorporation into chloroplastic lipids has yet to be achieved. The similarities in the patterns of labelling when bicarbonate and acetate are used as substrates under the same conditions would seem to justify using acetate or its derivatives as alternatives to the initial precursor CO_2 in studies of polyenoic biosynthesis.

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Table 3
A comparison of the fatty acid labelling pattern and the composition of the constituent fatty acids of monogalactosyldiacylglycerol (MGDG) in leaves of spinach plants grown in $^{14}CO_2$

	Fatty acid constituent							
	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3
Composition of constituent fatty acids (mol %)	1.4	1.0	1.4	18.6	0.5	tr	2.8	74.4
Distribution of radioactivity (% total)	3.8	tr	4.2	26.2	tr	1.1	5.4	59.3

Growth conditions are given in text

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