ACYL-CoA MAY BE A NEGLECTED PRODUCT IN STUDIES OF FATTY ACID SYNTHESIS BY ISOLATED CHLOROPLASTS

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1. Introduction

The reported effects of various 'co-factors' on the rates and products of fatty acid synthesis by isolated plastid preparations are confusing and often contradictory. Thus, rates of [^{14}C]acetate incorporation into plastid long-chain fatty acids have been reported to be stimulated [1–5], inhibited [3,6–8] and unaffected [9,10] by the addition of ATP to the incubation media. Similarly, added Triton X-100 has been claimed to stimulate rates of fatty acid synthesis in spinach chloroplasts by 5–6-fold [6], by 1.5–2-fold [9 11], or not at all [3,12] and to severely inhibit [^{14}C]acetate incorporation into the lipids of chloroplasts isolated from *Zea mays* leaves [12]. Exogenous *sn*-glycerol 3-phosphate (>1 mM) inhibited the incorporation of [1-^{14}C]acetate into the total fatty acids of isolated spinach chloroplasts in [10] yet stimulated incorporation in [11].

Triton X-100 and ATP have usually been considered to exert their effects by influencing the relative rates of oleate to palmitate synthesis within the plastid preparations [6–8], but oleate/palmitate labelling ratios were not affected by those additions in [3,9,10].

Here, we suggest that most, if not all, of these conflicting reports on the effects of various additions to incubation media on the rates and products of fatty acid synthesis by isolated plastids can be attributed to the formation of long-chain [^{14}C]acyl-CoAs which were retained for analysis in some studies but not in others.

2. Materials and methods

Spinacia oleracea plants (hybrid 102) were grown and chloroplasts isolated as in [10]. Washed chloroplast pellets were resuspended in 7 vol. wash buffer [13] and aliquots containing 40–60 μg chl were incubated in 0.25 ml standard reaction medium [10] containing 0.2 mM [1-^{14}C]acetate (57 or 8 mCi/mmol) and supplemented with 0.5 mM CoA and 2 mM ATP. The ATP was omitted in control reactions. Reactions were routinely terminated by adding 2.5 ml chloroform/methanol (1:1, v/v) followed by 0.9 ml 0.9% (w/v) NaCl and shaking vigorously. Aqueous and organic phases were separated and interfacial protein compacted by centrifugation for 1 min. The chloroform layer was recovered, the aqueous layer washed twice with 3 ml petroleum ether and the combined organic phases concentrated to 0.5 ml for chromatographic analysis [10]. Variations to this procedure are noted in the tables. Long-chain fatty acyl radioactivity in the aqueous methanol phase was determined:

(i) By adding 1.4 mmol KOH, heating to 80°C for 60 min, acidifying with 1.8 mmol H_2SO_4 and extracting ^{14}C-labelled fatty acids (with 100 μg oleic acid as carrier) into petroleum ether;

(ii) By adding 20 mg $NaBH_4$ and standing at room temperature overnight, acidifying to pH 2–3 with HCl and extracting ^{14}C-labelled fatty alcohols (with 100 μg of carrier C_{16}, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ alcohols) into petroleum ether;

(iii) By adding 100 μl saturated $(NH_4)_2SO_4$ followed by 5 ml chloroform/methanol (1:1, v/v) to precipitate proteins and analysing for [^{14}C]acyl-CoA and [^{14}C]acyl–acyl carrier protein in a modification of the procedure in [14];

(iv) As for (iii) except that the protein-free extracts were evaporated to dryness under reduced pressure and the residues incubated with a microsomal suspension as in table 3;

(v) By the filter-paper disc assay [10];

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(vi) By applying 25 μl directly to thin layers of silicic acid, developing chromatograms with *n*-butanol/acetic acid/water (5:2:3, by vol.) [15] and locating labelled compounds by radioautography.

Labelled long-chain fatty acids, fatty acid methyl esters and fatty alcohols were purified by TLC prior to measurement of radioactivity by scintillation counting.

3. Results

As reported in [9,10,16], in the absence of exogenous ATP only ~5% of the total long-chain fatty acids synthesised from [1-^{14}C]acetate by isolated, photosynthesising spinach chloroplasts were recovered in the aqueous phase of a chloroform/methanol/water partition [17] of reaction mixtures. However, when both CoA and ATP were supplied exogenously 40–60% of the total fatty acid radioactivity was recovered from aqueous methanol phases (tables 1,2) and has been identified tentatively as long-chain [^{14}C]acyl-CoA [9,10,16]. In other studies on chloroplast and plastid fatty acid synthesis this aqueous phase has been discarded [6–8,11,12] and it seems probable that relatively large losses of labelled fatty acids would have resulted. In some of those studies, reactions were terminated by acidification which could conceivably have catalysed the hydrolysis of [^{14}C]acyl-CoAs. Released ^{14}C-labelled fatty acids would then partition into the organic phase of a subsequent chloroform/methanol/water partition. However, post-incubation of reaction mixtures in molar H^+ for 5–20 min at 25°C had no effect on the subsequent distribution of [^{14}C]acyl compounds between aqueous and organic phases here (table 1). On the other hand, treatment with 0.5 M KOH for 10 min resulted in an 85% reduction in the amount of 'water-

Table 1
Lack of an effect of acid upon the subsequent distribution between aqueous and organic phases of labelled acyl compounds synthesised from [1-^{14}C]acetate by chloroplasts isolated from spinach

Post-incubation treatment		% Distribution of fatty acyl radioactivity			
		DAG	UFA	Polar	Aqueous
None		14.6	33.0	9.1	43.3
M H^+	5 min	14.7	34.0	11.2	41.1
	20 min	15.0	30.7	12.2	42.1

Abbreviations: DAG, 1,2-diacylglycerol; UFA, unesterified fatty acids; polar, polar glycerolipids; aqueous, aqueous methanol phase

Reactions (0.25 ml, 54 μg chl) were terminated by adding 2.5 ml chloroform/methanol (1:1, v/v) or by adding 25 μl 5 M H_2SO_4 or 10 M HCl and standing at 25°C for 5, 10 and 20 min (only the first and last times shown) before adding 2.5 ml chloroform/methanol. Aqueous and organic phases were separated after adding 0.9 ml 1% (w/v) NaCl. Long-chain fatty acids were recovered from the aqueous phase following saponification. The rate of [1-^{14}C]acetate incorporation was 1240 nmol . h^{-1} . mg chl^{-1}

Table 2
Analysis by different methods of the putative [^{14}C]acyl-CoA synthesised by isolated chloroplasts and recovered in the aqueous phase of chloroform/methanol/water partitions

Saponification (KOH)	Reduction ($NaBH_4$)	Column chromatog.	Thin-layer chromatog.	Filter-paper disc assay
15.6[a]	15.0	14.2 (1.0)[b]	13.6	9.2

[a] nmol [1-^{14}C]acetate incorporated into long-chain acyl compound
[b] ^{14}C as acyl–acyl carrier protein [14]

Reactions (0.5 ml, 88 μg chl) were stopped by adding 2.5 ml chloroform/methanol (1:1, v/v) and mixing thoroughly before adding 0.625 ml 1% NaCl. Upper phases were quickly transferred to separate tubes and individually sampled for the different analyses. The results shown are the means for 4 separate reactions which were carried out in the presence of 0.067 M sorbitol and 0.17 mM [1-^{14}C]acetate (5 μCi). The rate of [1-^{14}C]acetate incorporation into total long-chain fatty acids was 1200 nmol h^{-1} . mg chl^{-1}. About 1 nmol [1-^{14}C]acetate was incorporated into acyl-CoAs in the absence of added ATP

soluble' fatty acid radioactivity and an equivalent increase in fatty acid radioactivity partitioning into chloroform (not shown).

The long-chain fatty acid radioactivity which partitioned into the aqueous phase was associated to a variable extent with the denatured protein accumulating at the interface between aqueous and organic layers. This phenomenon has been noted in [18] and had to be taken into account when analysing the upper phase for ^{14}C-labelled fatty acids. The bound fatty acids were apparently released into solution when proteins were co-precipitated with $(NH_4)_2SO_4$ and chloroform/methanol [14], since the radioactivity subsequently recovered as [^{14}C]acyl-CoA on alumina columns [14] represented 90% of that recovered as ^{14}C-labelled fatty acids following direct saponification of aqueous methanol phases (table 2). Upon reduction with $NaBH_4$ the fatty acyl label was recovered quantitatively from the aqueous phases as ^{14}C-labelled fatty alcohols (table 2). Whilst $NaBH_4$ reduction is not unequivocally indicative of acyl-thioesters [19], we could find no other acyl lipids within the aqueous methanol phase which could have given rise to the quantities of ^{14}C-labelled fatty alcohols recovered.

Whether recovered as unesterified fatty acids from saponifications, as fatty acid methyl esters from $NaOCH_3$-treatment of alumina columns or as fatty alcohols from $NaBH_4$ reductions, the products when separated by argentation TLC were distributed 10–14% as saturated, 83–86% as monoenoic and 2–3% as dienoic acyl chains. This reflected the distribution of labelling within the unesterified fatty acids whilst contrasting with the distribution of label within the fatty acids of glycerolipids synthesised from [1-^{14}C]acetate by isolated chloroplasts in the absence of exogenous ATP [9,10,21].

When incubated with microsomal preparations from spinach leaves, the ^{14}C-labelled fatty acids in protein-free extracts (above) of aqueous methanol phases were >80% transferred to microsomal glycerolipids, predominantly phosphatidylcholine, in an ATP-independent reaction (table 3).

The above evidence pointed to the ^{14}C-labelled fatty acid-containing material in the aqueous methanol phase as being in the form of acyl-CoAs. This identification was supported by direct chromatography on thin-layers of silicic acid [15] of aqueous methanol phases from reactions carried out using reduced concentrations (0.067 M) of sorbitol. Acyl-CoA synthesis was apparently unimpaired at these low sorbitol concentrations (table 2) which permitted a sharp chromatographic resolution of long-chain [^{14}C]acyl-CoA (R_F 0.7–0.8, co-chromatographing with authentic [^{14}C]oleoyl-CoA) from [^{14}C]acetyl-CoA (R_F 0.35–0.45) and the small amount of [^{14}C]acyl lipid which travelled with the solvent front. No radioactivity remained on the origin of these chromatograms. Measurements of [^{14}C]acyl-CoAs separated by this method agreed well with results obtained using less specific methods (table 2). The filter paper disc assay, however, consis-

Table 3
Microsomal metabolism of the putative [^{14}C]acyl-CoA synthesised by chloroplasts isolated from spinach

Radioactive product:	Thioester[a]	PC	UFA	PE	PG	Origin + PI	Non-polar[b]
% of initial radioactivity	7.1	48.4	12.2	5.7	2.3	2.5	21.3

[a] ^{14}C-Labelled fatty acids remaining in the aqueous phase
[b] Radioactivity travelling at the front of two-dimensional chromatograms

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol

De-proteinated [14] aqueous methanol phases containing [^{14}C]acyl-CoA equivalent to 8 nmol [1-^{14}C]acetate were evaporated to dryness under reduced pressure and incubated for 60 min at 25°C with a spinach leaf microsomal preparation [20] in 2 ml 50 mM Hepes/NaOH (pH 8.0), 5 mM $MgCl_2$, 1 mM dithiothreitol and 2 mM EDTA. Reactions were stopped by adding 5 ml chloroform/methanol (1:1, v/v), 0.25 ml water and shaking vigorously. Chloroform-soluble lipids were analysed by both one- and two-dimensional TLC

tently produced low values for [^{14}C]acyl-CoAs which were probably, therefore, partly washed off the discs in 5% trichloroacetic acid [10].

4. Discussion

In most studies on fatty acid synthesis by isolated chloroplasts, CoA and ATP have been included in reaction media as a matter of course [1–8,11,12,22,23]. This will have resulted in the synthesis of acyl-CoAs which, in those studies where lipid and non-lipid products were separated by a chloroform/methanol/water partition, would have been discarded along with the non-lipid fraction. Although these results were obtained using spinach chloroplasts, the reported effects of exogenous ATP upon rates and products of [^{14}C]acetate incorporation into the long-chain fatty acids of plastids isolated from greening pea leaves [8], from developing maize [12] leaves and from mature spinach leaves [6,7] have much in common. In addition, chromoplasts isolated from daffodil flowers exhibited highest rates of fatty acid synthesis from [^{14}C]acetate when 2-phosphoglycerate or phosphoenolpyruvate were included in incubation media [24], but [^{14}C]acyl-CoA was synthesised only when exogenous ATP was available [24]. Therefore, it seems likely that formation of long-chain acyl-CoA may be a general response by isolated plastids to the presence of exogenous ATP and CoA in reaction media. An explanation for the contrasting results reported by different workers in this field may now be proposed.

In basal media or when CoA is included in reaction mixtures, isolated chloroplasts incorporate [1-^{14}C]acetate into unesterified fatty acids (70–80%), 1,2-diacylglycerols (10–20%), polar glycerolipids (10%) and 'thioesters' (5%) [5,9,10,21,24]. Only when exogenous ATP is available do intact chloroplasts synthesise [^{14}C]acyl-CoA (25–60%) from the unesterified fatty acids which otherwise accumulate [5,9,10,24]. The unesterified fatty acid fraction contains 85–95% [^{14}C]oleic and 10–15% [^{14}C]palmitic acids, a ratio which is reflected in [^{14}C]acyl-CoAs [3,9,10]. Therefore, to routinely discard [^{14}C]acyl-CoAs from the analyses is to selectively discriminate against [^{14}C]oleate recovery [9], with the result that ATP addition appears to inhibit [^{14}C]acetate incorporation by specifically inhibiting oleate synthesis [6–8]. In studies where [^{14}C]acyl-CoAs have been included in analyses, ATP has been found to stimulate rather than inhibit oleate synthesis [4,5,9,24].

The addition of either Triton X-100 or *sn*-glycerol 3-phosphate to incubation media stimulates [1-^{14}C]-acetate incorporation by isolated spinach chloroplasts into 1,2-diacylglycerols at the expense of unesterified fatty acid accumulation [9–11,21]. The glycerides contain approximately equal amounts of labelled oleate and palmitate [9,11,21], are quantitatively recovered in the organic phases of chloroform/methanol/water partitions and are, therefore, always included in the analyses of chloroplast fatty acid synthesis. When added to reaction mixtures already containing ATP and CoA, Triton X-100 diverts [^{14}C]oleate away from the pathway leading to [^{14}C]oleoyl-CoA which would be discarded, and into glycerolipids which would be recovered, thus appearing to stimulate [^{14}C]acetate incorporation by specifically stimulating [^{14}C]oleate synthesis [6,7]. Similarly, the apparent increase in rate of [^{14}C]acetate incorporation into chloroplast fatty acids in the presence of exogenous glycerol 3-phosphate when reaction mixtures also contain CoA and ATP [11], can be attributed to an improved recovery of labelled fatty acids resulting from the glycerol 3-phosphate-induced diversion of newly synthesised fatty acids away from acyl-CoA formation and into glycerides. The apparent enhancement of chloroplast fatty acid synthesis from [^{14}C]acetate in the presence of added microsomal preparation [12,23] may be explained in like manner. Here the [^{14}C]acyl-CoA which would otherwise accumulate and be discarded is metabolised by microsomal acyltransferases and thioesterases [25] into products which are recovered in the organic phase of chloroform/methanol/water partitions and, therefore, retained for analyses.

In [5,24], the [^{14}C]acyl-CoA synthesised from [^{14}C]acetate by isolated chromoplasts and chloroplasts was recovered in the organic phase of a chloroform/methanol/water partition. However, the procedure in [5,24] yielded contrary results in our hands so that the [^{14}C]acyl-CoA synthesised by spinach chloroplasts was recovered predominantly (>90%) in the aqueous phase of the partition. We can currently offer no explanation for this discrepancy, but emphasise that recovery of labelled acyl-CoAs in the aqueous phase of chloroform/methanol/water partitions is commonly observed [18,25,26,27]. Therefore, since acyl-CoAs may account for a relatively large proportion of the long-chain fatty acids synthesised by isolated plastids, it would seem imperative to ensure their recovery for inclusion in any future analyses.

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