# Stromal concentrations of coenzyme A and its esters are insufficient to account for rates of chloroplast fatty acid synthesis: evidence for substrate channelling within the chloroplast fatty acid synthase

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Concentrations of total CoAs in chloroplasts freshly isolated from spinach and peas were 10–20 $\mu$M, assuming a stromal volume of 66 $\mu$l per mg of chlorophyll. Acetyl-CoA and CoASH constituted at least 90 % of the total CoA in freshly isolated chloroplasts. For a given chloroplast preparation, the concentration of endogenous acetyl-CoA was the same when extractions were performed using $HClO_4$, trichloroacetic acid, propan-2-ol or chloroform/methanol, and the extracts analysed by quantitative HPLC after minimal processing. During fatty acid synthesis from acetate, concentrations of CoASH within spinach and pea chloroplasts varied from less than 0.1 to 5.0 $\mu$M. Malonyl-CoA concentrations were also very low ($< 0.1$–3.0 $\mu$M) during fatty acid synthesis but could be calculated from radioactivity incorporated from [1-$^{14}$C]acetate. Concentrations of CoASH in chloroplasts synthesizing fatty acids could be doubled in the presence of Triton X-100, suggesting that the detergent stimulates fatty acid synthesis by increasing the turnover rate of acyl-CoA. However, although taken up, exogenous CoASH (1 $\mu$M) did not stimulate fatty acid synthesis by permeabilized spinach chloroplasts. Calculated rates for acetyl-CoA synthetase, acetyl-CoA carboxylase and malonyl-CoA–acyl-carrier-protein transacylase reactions at the concentrations of metabolites measured here are $< 0.1$–4 % of the observed rates of fatty acid synthesis from acetate by isolated chloroplasts. The results suggest that CoA and its esters are probably confined within, and channelled through, the initial stages of a fatty acid synthase multienzyme complex.

## INTRODUCTION

Isolated chloroplasts retain light-dependent fatty acid synthesis from acetate even when gently ruptured (permeablized) in hypotonic incubation medium, and it has been suggested that chloroplast fatty acid synthase therefore exists *in situ* as a multienzyme complex [1]. Thus, although 40–60 % of stromal protein was released from permeablized chloroplasts, a lipid-synthesizing metabolon was apparently retained and functioned in close association with the energy-transducing machinery of the thylakoids [1]. This fatty acid synthesis was independent of added acyl carrier protein (ACP), ATP, CoA and nicotinamide nucleotides, indicating that such a metabolon might sequester pools of these cofactors, which are then unable to equilibrate with the total stromal pools. In addition, exogenous acetyl-CoA and malonyl-CoA neither inhibited acetate incorporation into fatty acids nor were themselves significantly incorporated into fatty acids by permeablized plastids [1], although related compounds, such as ADP and NADP, were readily metabolized. This apparent anomaly would be explained if acetyl-CoA carboxylase and malonyl-CoA–ACP transacylase were integrated into a multienzyme complex that, in chloroplasts, channelled acetate into long-chain fatty acids.

Substrate channelling is a property of multienzyme complexes and is usually invoked when a clear discrepancy exists between substrate concentrations known to exist within a linear sequence operating *in situ*, e.g. in mitochondria, and those required to drive individual enzyme reactions at rates commensurate with the rate of that overall process. Earlier measurements of CoASH and its esters in intact chloroplasts isolated from spinach and peas [2] showed that the stromal concentration of total CoAs (i.e. CoASH plus its esters) was only 31–51 $\mu$M in freshly isolated chloroplasts and that acetyl-CoA was by far the predominant form detected. Concentrations of CoASH and malonyl-CoA were particularly low ($< 1$ and $< 0.5$ $\mu$M respectively) during light-dependent fatty acid synthesis from acetate [2,3]. However, those concentrations were based on a stromal volume of 26 $\mu$l per mg of chlorophyll, which has now been revised [4] to 66 $\mu$l per mg of chlorophyll, so that they were actually overestimated by 2.5 times, and are hereafter adjusted accordingly in this paper. Therefore, on the basis of kinetic parameters derived using stromal preparations or purified enzymes, calculated rates for the acetyl-CoA synthetase [5,6], acetyl-CoA carboxylase [7–10] and malonyl-CoA–ACP transacylase [11–15] reactions at the stromal concentrations of CoAs measured [2] fall far short of those required to account for observed rates of fatty acid synthesis from acetate. This shortcoming might be explained by metabolite channelling.

It was considered important therefore to confirm the low concentrations of CoAs in freshly isolated chloroplasts, and to obtain reliable measurements of CoASH and malonyl-CoA during fatty acid synthesis from acetate by isolated chloroplasts. Oxidative deacylation of CoA esters in crude extracts of fresh chloroplasts in the presence of endogenous glutathione provided an integrated measurement of total CoAs, since CoASH and its esters, including any long-chain acyl-CoA that would have been missed in the previous study [2], were quantitatively converted into glutathionyl-CoA.

## MATERIALS AND METHODS

Intact chloroplasts were isolated from expanding leaves of spinach (*Spinacia oleracea*) plants grown hydroponically and from pea (*Pisum sativum*) shoots as described previously [1], and were resuspended in low-ionic-strength buffer [16] at a concentration of 1–1.5 mg of chlorophyll per ml. Total CoAs were extracted from chloroplasts by a variety of methods. Acid ex-

Abbreviations used: ACP, acyl carrier protein; C/M, chloroform/methanol; DTT, dithiothretiol; AUFS, absorbance units full scale.

tractions were performed by adjusting an amount of suspension containing 250–300 $\mu$g of chlorophyll to 5% (w/v) with either $HClO_4$ or trichloroacetic acid and centrifuging to remove precipitated proteins. $HClO_4$ extracts were neutralized to approx. pH 6 with 2 M $K_2HPO_4$, the precipitated $KClO_4$ was removed by centrifugation, and the supernatant injected directly into the HPLC column for analysis of CoAs. Trichloroacetic acid was removed from extracts by repeated partitioning against diethyl ether [17], the acid-free extract was concentrated under reduced pressure and the residue reconstituted to 500 $\mu$l with 0.2 M ammonium acetate. For propan-2-ol extractions, 2 vol. of propan-2-ol was added with mixing to the chloroplast suspension, and, after 5–10 min at 0 °C, the suspension was centrifuged. The supernatant was adjusted to 50% (v/v) propan-2-ol and 12 mM Bistris, pH 6.2, before extraction with 4 × 1 ml of light petroleum saturated with 50% (v/v) propan-2-ol. The aqueous phase was concentrated under reduced pressure and the residue was made up to 0.5 ml with 0.2 M ammonium acetate. For chloroform/methanol (C/M) extractions, 6 vol. of C/M (1:2, v/v) was added to chloroplast suspensions and precipitated proteins were removed by centrifugation. The supernatant was recovered and the precipitate was re-extracted as above. The combined extracts were partitioned against 0.4 vol. of 10 mM Bistris, pH 6.2, and the phase separation was assisted by brief centrifugation. Most (85–90%) of the upper phase was recovered and concentrated under reduced pressure, and the residue was diluted to 500 $\mu$l with 0.2 M ammonium acetate. Amounts of these preparations equivalent to 100–150 $\mu$g of chlorophyll were injected into the HPLC column for analysis of CoAs.

To measure CoAs during light-dependent fatty acid synthesis from 0.2 mM [1-$^{14}$C]acetate (58–60 Ci/mol; Amersham), isolated chloroplasts (equivalent to 100–150 $\mu$g of chlorophyll) were incubated in 0.5 ml of basal medium [1] for 5 or 10 min, and the reactions were terminated by adding 3 ml of C/M (1:2). After the addition of 1.3 ml of 7 mM acetic acid to adjust the pH to 6 and partitioning, the aqueous phases were recovered, concentrated and analysed as described above. Normally, extracts from two reactions were combined ensuring sufficient radioactivity in the separated malonyl-CoA to provide an accurate indication of its concentration from incorporated $^{14}$C. To enable measurement of CoASH in later experiments, the combined aqueous phases were concentrated and loaded on to DEAE mini-columns (below) to purify CoAs partially before analysis by HPLC.

Mini-columns (1 ml) were prepared in 5 ml PrepPak tubes (Bio-Rad) using DEAE-Toyopearl 650M (Toyo Soda Manufacturing Co., Tokyo, Japan) that had been washed with 2 M LiCl and water. Upper phases from C/M extractions were concentrated under reduced pressure before being loaded on to columns equilibrated with 2 mM dithiothreitol (DTT). After the loaded column had been washed with 5 ml of 1% (v/v) acetic acid followed by 10 ml of 10 mM Bistris, pH 6.2, CoAs were eluted in 3 ml of 0.25 M LiCl in 10 mM Bistris, pH 6.2. The eluate was concentrated under reduced pressure to 0.5 ml. Aliquots equivalent to 100–150 $\mu$g of chlorophyll were injected on to the HPLC column.

HPLC separations were carried out using a column (4.9 mm internal diameter × 250 mm) of 5 $\mu$m Alltima C18 (Alltech, Deerfield, IL, U.S.A.) eluted at a rate of 1 ml/min with increasing concentrations of acetonitrile in 0.2 M ammonium acetate/10 mM acetic acid, pH 6. The column was equilibrated with 2% (v/v) acetonitrile in ammonium acetate/acetic acid and this was maintained for 5 min after injection of the sample. The acetonitrile composition was then increased linearly over the following 35 min to 10% (v/v). Reference CoAs were from Sigma and were standardized in solution by $A_{260}$. A mixture containing malonyl-CoA, glutathionyl-CoA, CoASH and acetyl-CoA (concentration of each 10 $\mu$M) was prepared each day from 1 mM standards, which in turn were prepared weekly from 10 mM stocks. CoASH was diluted from 12.5 mM to 1 mM every other day. Normally, 100–200 pmol of each standard was injected, and eluted peaks were detected by monitoring $A_{254}$ and using a sensitivity of 0.02 absorbance unit full scale (AUFS). Malonyl-CoA, glutathionyl-CoA, CoASH and acetyl-CoA were eluted at around 20, 22, 25 and 34 min respectively.

## RESULTS

### Acetyl-CoA recovery by the different extraction methods

A major priority of this work was to confirm that the low concentrations of total CoAs measured previously [2] did not result from an inadequate extraction technique. In that study, chloroplasts were extracted with trichloroacetic acid, and the trichloroacetic acid was partitioned into diethyl ether before analysis of the trichloroacetic acid-free extract by HPLC. Essentially all of the CoA in freshly isolated chloroplasts was in the form of acetyl-CoA [2]. In the present work therefore two different acid and two different solvent extractions were employed to compare recoveries of acetyl-CoA from freshly isolated chloroplasts. Chromatograms of each of the crude extracts contained many peaks that did not correspond to known CoA compounds (e.g. Figure 1), and identification of CoASH and

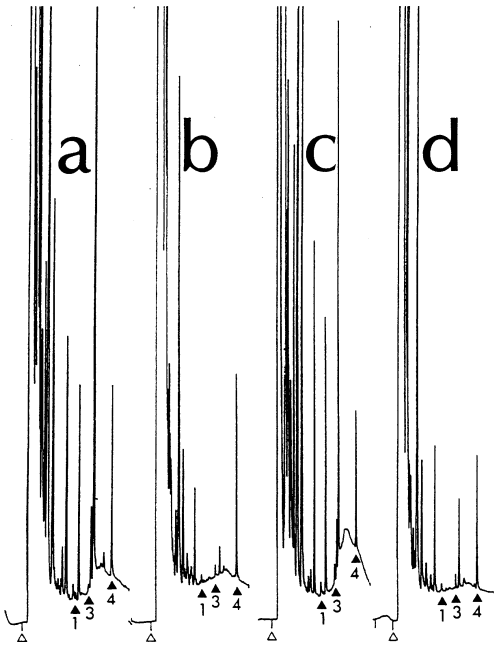


**Figure 1 HPLC profiles showing both the effect of treating crude extracts with DEAE and CoAs in chloroplasts during fatty acid synthesis from acetate**

Chloroplasts were incubated with illumination for 5 min in the basal medium containing 0.2 mM [1-$^{14}$C]acetate (58 Ci/mol) and also 0.13 mM Triton X-100 to elevate malonyl-CoA concentrations. Reactions were stopped by adding C/M and extracts of incubated pea (**a**, **b**) and spinach (**c**, **d**) chloroplasts were injected before (**a**, **c**) and after (**b**, **d**) preliminary clean-up on DEAE mini-columns. Arrowheads indicate the positions of malonyl-CoA (1), CoASH (3) and acetyl-CoA (4). Malonyl-CoA and CoASH are present in very low concentrations compared with acetyl-CoA, particularly in spinach chloroplasts. Amounts of extract equivalent to 100 and 150 $\mu$g of chlorophyll were injected from pea and spinach chloroplasts respectively. Acetyl-CoA was eluted in 34–35 min. Detector sensitivity was 0.02 AUFS, which is represented by the span from injections to the truncated off-scale peaks.

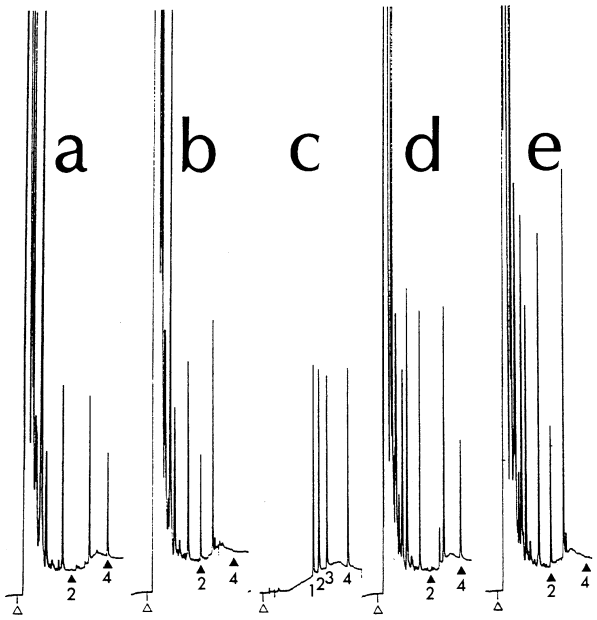


**Figure 2 HPLC profiles showing the conversion of CoA and its esters into glutathionyl-CoA**

The crude C/M extracts of spinach (**a**, **b**) and pea (**d**, **e**) chloroplasts were treated with $NH_4OH$ before clean up on DEAE mini-columns. (**a**, **d**) Untreated extracts; (**b**, **e**) $NH_4OH$-treated extracts; (**c**) 125 pmol each of malonyl-CoA (1), glutathionyl-CoA (2), CoASH (3) and acetyl-CoA (4). Amounts of extract equivalent to 100 and 150 $\mu$g of chlorophyll from pea and spinach chloroplasts respectively were injected.

malonyl-CoA peaks was particularly difficult. However, acetyl-CoA was clearly resolved and quantifiable, and its recovery from a given chloroplast preparation was almost identical for all extraction methods (results not shown). Although $HClO_4$ extraction provided the most direct analysis and was least susceptible to losses in processing, both $HClO_4$ and trichloroacetic acid extracts contained material that interfered strongly with the detection and analysis of malonyl-CoA, glutathionyl-CoA and CoASH. Another potential disadvantage of acid extraction was that long-chain acyl-CoA would be expected to precipitate with the proteins. On the other hand, solvent extractions allowed the recovery of both short- and long-chain CoAs and simultaneously the recovery of lipids from incubations of chloroplasts with [1-$^{14}$C]acetate. An additional advantage of solvent extraction was that malonyl-CoA was more readily identifiable on resulting chromatograms. Although extraction with C/M became the preferred method, all extracts contained extraneous material that chromatographed in the region of CoAs (e.g. Figures 1a and 1c), and there was considerable variation in the relative sizes of those peaks from one chloroplast preparation to another.

Pea chloroplasts contained $13.8 \pm 3.4$ ($n = 33$) and spinach chloroplasts $11.0 \pm 2.7$ ($n = 50$) $\mu$M acetyl-CoA, with values ranging from 8.8 to 21.9 $\mu$M and 7.1 to 19.1 $\mu$M respectively. The acetyl-CoA concentration appeared to be quite variable even when leaves from the same group of plants were sampled on the same day. Some of this variability could be attributed to CoASH, which was frequently detected in significant amounts in this study (Figure 2d, below), although not previously [2], and lower concentrations of acetyl-CoA were associated with higher concentrations of CoASH. Recoveries of [1-$^{14}$C]acetyl-CoA added to chloroplast suspensions which were then extracted by the different methods were 90–95%.

### Accurate analysis of total endogenous CoAs required preliminary clean-up of crude extracts

It had been hoped that analysis of the major forms of CoA by HPLC would be possible using crude extracts of chloroplasts, since CoA concentrations were known to be low [2] and significant losses might occur in any preliminary purification of the extracts. However, analysis of malonyl-CoA, glutathionyl-CoA and CoASH in crude extracts of chloroplasts was compromised by interfering materials (above), which increased greatly in quantity in incubated chloroplasts (Figures 1a and 1c). Attempts to partially purify extracts using $C_{18}$ cartridges [18,19] resulted in unacceptable losses of most forms of CoA, even when DTT was included in working solutions, and were discontinued. Thereafter, CoAs were partially purified from crude extracts by adsorption on mini-columns of DEAE-Toyopearl. Washing the loaded columns with 0.15 M acetic acid and 10 mM Bistris, pH 6.2, effectively removed the worst of the interfering materials (Figure 1), and CoAs were eluted with 0.25 M LiCl in 10 mM Bistris, pH 6.2. Conditioning the columns with 1 mM DTT was necessary to ensure high recoveries of CoAs, which, for unlabelled standards, were 80–85% for glutathionyl-CoA and more than 90% for CoASH, malonyl-CoA and acetyl-CoA. Recoveries of acetyl-CoA, malonyl-CoA and CoASH, added to chloroplast suspensions immediately before extraction with C/M and then taken through the whole procedure, were more than 90%, 80% and 80% respectively. These recoveries were taken into account when endogenous concentrations were calculated.

### Acetyl-CoA is the primary form of CoA in freshly isolated chloroplasts

When trichloroacetic acid extracts were concentrated and treated with $NH_4OH$ in previous work [2], CoAs were deacylated and simultaneously oxidized to form a disulphide with endogenous glutathione. Yields of glutathionyl-CoA appeared to correspond closely to the original amount of acetyl-CoA present, but any long-chain acyl-CoA in the chloroplasts would have precipitated with the proteins and been discarded [2]. In the present study therefore, solvent extracts, which would retain any long-chain acyl-CoA, were concentrated and treated with $NH_4OH$ before partial purification on DEAE columns. The clean-up allowed a more accurate measurement of CoASH, which was found to vary from undetectable (Figure 2a) to 30–40% of the concentration of acetyl-CoA in extracts from freshly isolated chloroplasts (e.g. Figure 2d). However, as in the previous work, the amount of glutathionyl-CoA generated corresponded closely to the combined amount of acetyl-CoA and CoASH originally present (Figure 2), and it was concluded that acetyl-CoA and CoASH combined constituted at least 90% of the total soluble CoAs present in freshly isolated chloroplasts from spinach and peas. Quantitative conversion of CoASH and its esters into glutathionyl-CoA was confirmed by spiking extracts with 50 pmol each of CoASH, malonyl-CoA and acetyl-CoA before treatment with $NH_4OH$. Therefore virtually all of the endogeneous soluble CoA in resting chloroplasts is stored as acetyl-CoA, accompanied by lesser and variable amounts of CoASH, so that forms such as malonyl-CoA, hydroxymethylglutaryl-CoA and succinyl-CoA are virtually undetectable. However, some form of CoA may be covalently bound to protein [20,21]. This result confirmed that total CoA, in its various forms, is indeed present in spinach and pea chloroplasts in very low concentrations (10–20 $\mu$M).

**Table 1 Concentrations of CoAs in chloroplasts during fatty acid synthesis from acetate, and the effect of Triton X-100**

Reactions in mixtures (0.5 ml) containing 0.2 mM [1-$^{14}$C]acetate (58 Ci/mol) in the basal medium, with and without 0.13 mM Triton X-100, were started by adding chloroplasts equivalent to 100–130 $\mu$g of chlorophyll and were stopped after 5 min by adding 3 ml of C/M (1:2). Extracts from duplicate reactions were combined, concentrated, and loaded on to DEAE columns to separate the CoA fraction, which was then analysed by HPLC. Results are means or means $\pm$ S.E.M. from three separate experiments for each chloroplast type. Triton X-100 consistently increased concentrations of malonyl-CoA and CoASH while decreasing the concentration of acetyl-CoA.

| Species | Addition | [Malonyl-CoA] ($\mu$M) | [CoASH] ($\mu$M) | [Acetyl-CoA] ($\mu$M) |
|---|---|---|---|---|
| Spinach | None | $< 0.1$ | 0.2 | $11.5 \pm 1.8$ |
| | Triton X-100 | 0.5 | 1.1 | $8.6 \pm 1.5$ |
| Pea | None | 0.8 | $2.5 \pm 0.5$ | $13.5 \pm 1.5$ |
| | Triton X-100 | $3.0 \pm 0.7$ | $4.5 \pm 0.8$ | $11.0 \pm 1.2$ |

## Acyl-CoA concentrations are higher in incubated chloroplasts

During the course of this work it was noted that acetyl-CoA concentrations were frequently higher in incubated compared with freshly prepared chloroplasts. In some instances the fresh chloroplasts may have contained significant CoASH (Figure 2d), which could be converted into acetyl-CoA in the light, but in most cases CoASH was undetectable. Since it had also been established that fresh chloroplasts contained negligible long-chain acyl-CoA, and the incubation medium contained no CoASH, it may be supposed that the additional CoA in incubated chloroplasts had originally been covalently bound to protein, possibly to acetyl-CoA synthetase. A pool of protein-bound CoA that can, however, exchange with the soluble pool may explain some of the considerable variation in acetyl-CoA concentrations encountered in freshly isolated chloroplasts (above).

## CoAs in chloroplasts synthesizing fatty acids

Malonyl-CoA was virtually indetectable by HPLC analysis in spinach chloroplasts synthesizing fatty acids from acetate (Figures 1b and 1d). However, concentrations could be calculated from the small amount of [$^{14}$C]acetate incorporated into the malonyl-CoA purified by HPLC (Table 1). Triton X-100 stimulated fatty acid synthesis by up to twofold and simultaneously increased concentrations of malonyl-CoA by two- to five-fold, so that a malonyl-CoA peak was then detectable by HPLC (Figure 3c; see also ref. [2]). Malonyl-CoA was more easily detected by HPLC in extracts from incubated pea chloroplasts (Figures 3d, 3e and 3f), particularly those from older (9-day) pea shoots (results not shown), which, however, had a low rate of fatty acid synthesis. Increases in mass of malonyl-CoA also corresponded to increases in $^{14}$C content from [1-$^{14}$C]acetate incorporation.

Acetyl-CoA was the dominant form of CoA during fatty acid synthesis by chloroplasts, and its concentration was invariably lower when fatty acid synthesis was stimulated by Triton (Table 1). Increases in the concentrations of both CoASH and malonyl-CoA appeared to compensate for the lower acetyl-CoA concentrations. The increase in CoASH concentration, in particular, would appear to explain the well-known stimulation by Triton X-100 of chloroplast fatty acid synthesis from acetate [22,23], in that steady-state concentrations of CoASH were frequently below 0.1 $\mu$M, and any increase in that concentration should result in a significant enhancement of acetyl-CoA synthetase activity. Up to twofold increases in the concentration of CoASH in chloroplasts incubated in the presence of Triton X-100 were observed in the present study (compare Figures 3b with 3c, and 3e with 3f) when rates of fatty acid synthesis from acetate were stimulated by 50–100%.

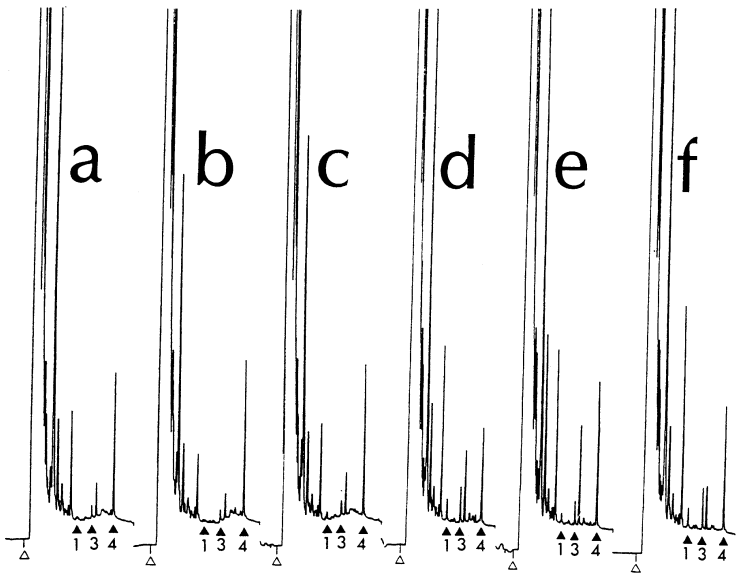


**Figure 3 HPLC profiles showing concentrations of CoAs in permeabilized and intact chloroplasts synthesizing fatty acids, and in intact chloroplasts synthesizing fatty acids in the absence and presence of Triton X-100**

Chloroplasts from spinach (**a**–**c**) and peas (**d**–**f**) equivalent to 100–130 $\mu$g of chlorophyll were incubated in 0.5 ml of basal buffered medium containing 66 mM (**a**, **d**) or 330 mM sorbitol with (**c**, **f**) or without (**b**, **e**) 0.13 mM Triton X-100. The illuminated reactions were terminated at 5 min with C/M and the extracts were concentrated using DEAE mini-columns before analysis by HPLC. Arrowheads indicate the positions of malonyl-CoA, CoASH and acetyl-CoA as in Figure 2. Amounts of extract equivalent to 100 and 150 $\mu$g of chlorophyll were injected from spinach and pea chloroplasts respectively.

A discrepancy existed between acetyl-CoA concentrations calculated either from radioactivity associated with appropriate peaks eluted from HPLC or from comparing peak heights in chromatographed extracts with those produced by chromatographed standards; values calculated from radiocarbon content were consistently 85–90 % of those indicated by comparison of peak heights with standards. Although earlier work had established that the specific radioactivity of chloroplast acetyl-CoA reached the same value as the supplied [1-$^{14}$C]acetate within 30 s of starting illumination [2,3], the discrepancy noted here was the same for incubations of 5 and 10 min. One explanation for this finding is that the specific radioactivity of the supplied acetate was diluted at later times by acetate produced within the photosynthesizing chloroplasts. Spiking labelled extracts with unlabelled acetyl-CoA and malonyl-CoA before separation by HPLC did not result in increased recovery of radioactivity.

### Concentrations of CoAs during fatty acid synthesis by permeabilized chloroplasts

Chloroplasts permeabilized by suspension in reaction medium containing 66 mM sorbitol had high rates of fatty acid synthesis despite being freely permeable to metabolites, e.g. NADP, CoASH and some protein [1]. However, the concentrations of endogenous CoAs during fatty acid synthesis by permeabilized spinach chloroplasts were indistinguishable from those in intact chloroplasts (Figures 3a and 3b), assuming both systems to have identical stromal volumes. On the other hand, concentrations of acetyl-CoA were lower (8–9 $\mu$M), and those of CoASH (3–4 $\mu$M) and malonyl-CoA (1–2 $\mu$M) were higher in permeabilized than intact pea chloroplasts (Figures 3d and 3e) synthesizing fatty acids, again assuming both systems to have identical stromal volumes. In reality, permeabilized chloroplasts have at least twice the 'stromal' volume of intact chloroplasts [1], and if CoAs were free to disseminate throughout the reaction medium, then the real concentrations of CoAs in reaction mixtures containing permeabilized chloroplasts would be 15–150 nM. Given the high rates of acetate incorporation by permeabilized chloroplasts, it seems highly unlikely that the CoAs do equilibrate through the reaction medium during fatty acid synthesis, even though the movement of exogenous CoASH is not constrained by the permeabilized envelope (below). Since more than 90 % of total CoA was recovered in the organelles pelleted from hypotonic medium (result not shown), the CoAs are probably integrated into a fatty acid synthase complex retained within the permeabilized plastids.

Permeabilized chloroplasts were used to test the idea that higher concentrations of endogenous CoASH should result in higher rates of fatty acid synthesis (above). However, when permeabilized spinach chloroplasts were incubated with 1.25 and 2.5 $\mu$M CoASH, rates of fatty acid synthesis from acetate were unaffected (Table 2). This exogenous CoASH readily passed through the envelope to reach the energy-transducing mechanism of the thylakoids, as indicated by its efficient conversion into acetyl-CoA (Table 2) utilizing only endogenous ATP, yet was unable to enhance acetate incorporation into fatty acids. It is also clear from Table 2 that permeabilized spinach chloroplasts incorporate acetate into fatty acids at high rates in the presence of extremely low concentrations of CoASH ($< 0.1$ $\mu$M). Therefore it seems likely that exogenous CoASH was metabolized differently from endogenous CoASH in this sytem (see also ref. [1]), and that the fraction of acetyl-CoA synthetase activity involved in fatty acid synthesis from acetate was saturated by very low concentrations of CoASH.

**Table 2 Effect of low concentrations of exogenous CoASH on fatty acid and acyl-CoA synthesis by permeabilized spinach chloroplasts**

Results of typical experiments are given where reaction mixtures contained spinach or pea chloroplasts equivalent to 155 $\mu$g and 100 $\mu$g of chlorophyll respectively, 0.2 mM [1-$^{14}$C]acetate (58 Ci/mol) and the amounts of CoASH shown, in 0.5 ml of the basal medium. Reactions were started by adding chloroplasts and were stopped after 5 min by adding C/M. Values in normal font (**a**) are nmol of [1-$^{14}$C]acetate incorporated, and values in italics (**b**) are derived from HPLC analyses.

| | CoASH added (nmol) | Malonyl-CoA (nmol/reaction) | | CoASH (nmol/reaction) | Acetyl-CoA (nmol/reaction) | | Fatty acids (nmol/reaction) |
|---|---|---|---|---|---|---|---|
| | | (a) | (b) | (b) | (a) | (b) | |
| Spinach | 0 | 0.01 | *0.01* | *< 0.01* | 0.10 | *0.12* | 11.0 |
| | 0.625 | < 0.01 | *< 0.01* | *< 0.01* | 0.48 | *0.51* | 11.4 |
| | 1.25 | < 0.01 | *< 0.01* | *< 0.01* | 0.94 | *1.03* | 11.8 |
| Pea | 0 | 0.02 | *< 0.02* | *0.06* | 0.06 | *0.106* | 6.7 |
| | 0.625 | 0.02 | *< 0.02* | *0.06* | 0.48 | *0.57* | 6.7 |
| | 1.25 | 0.02 | *< 0.02* | *0.06* | 1.03 | *1.10* | 6.8 |

## DISCUSSION

Although metabolite channelling within a chloroplast fatty acid synthase was originally proposed to account for the lack of interference of acetyl-CoA and malonyl-CoA in fatty acid synthesis from acetate by permeabilized chloroplasts [1], the present study indicates that endogenous concentrations of CoASH, acetyl-CoA and malonyl-CoA during fatty acid synthesis by intact chloroplasts are insufficient to drive the acetyl-CoA synthetase, acetyl-CoA carboxylase and malonyl-CoA–ACP transacylase reactions at rates commensurate with known rates of fatty acid synthesis from acetate by intact chloroplasts [1]. Metabolite channelling could be invoked if any one of those reaction rates was shown to be grossly restricted by substrate deficiency.

In the first instance, a very low concentration of CoASH would seem to preclude the activation of acetate to acetyl-CoA at rates commensurate with rates of fatty acid synthesis, since the $K_m$ for CoASH in the acetyl-CoA synthetase reaction catalysed by the purified enzyme was 5 $\mu$M [5], but was 20–25 $\mu$M in chloroplast extracts [6]. At a stromal concentration of $< 0.1$–$0.5$ $\mu$M CoASH therefore, acetyl-CoA synthetase can operate at a maximum of 5 % of $V_{max}$. Since the $V_{max}$ for acetyl-CoA synthetase in chloroplast extracts is 3–5 times greater than rates of fatty acid synthesis from acetate [6], the maximum calculated rate of acetyl-CoA formation in the stroma is $< 10$–$20$ % of the observed rate of acetate incorporation into fatty acids. In the individual cases where CoASH was deemed to be less than 0.1 $\mu$M during fatty acid synthesis by spinach chloroplasts, the calculated acetyl-CoA synthetase activity can account for just 2–4 % of acetate incorporation.

Similarly, although the predominant form of CoA in chloroplasts is acetyl-CoA, it is present in amounts insufficient to drive the acetyl-CoA carboxylase reaction at rates compatible with rates of fatty acid synthesis. A relatively high $K_m$ for acetyl-CoA seems to be characteristic for the acetyl-CoA carboxylase reaction catalysed by the enzyme from a number of sources [7–10]. Hence the $K_m$ for acetyl-CoA in the reaction in extracts of pea chloroplasts was 0.25 mM, and the total activity was 117 nmol of malonyl-CoA formed/h per mg of stromal protein [7]. Acetate incorporation into fatty acids by pea chloroplasts is typically 50–100 nmol/h per mg of stromal protein and acetyl-CoA concentrations are typically 10–15 $\mu$M [2] during fatty acid synthesis.

The acetyl-CoA carboxylase activity at this concentration of acetyl-CoA is 2–3 nmol of malonyl-CoA/h per mg of protein, or 2–5% of the observed rate of fatty acid synthesis.

An even more dramatic discrepancy between measured rates of fatty acid synthesis and calculated rates of individual reactions is provided by the malonyl-CoA–ACP transacylase reaction. Spinach chloroplasts incorporating acetate at up to 1.5 $\mu$mol/h per mg of chlorophyll contained less than 0.1 $\mu$M malonyl-CoA, and the greatest concentration of malonyl-CoA measured in pea chloroplasts during fatty acid synthesis was 4.5 $\mu$M (result not shown), yet the $K_m$ for malonyl-CoA in the malonyl-CoA–ACP transacylase reaction catalysed by the enzyme purified from spinach and *Anacystis* was 400 and 300 $\mu$M respectively [12], and was more than 80 $\mu$M for the enzyme in barley chloroplast stroma [11]. The $V_{max}$ for the reaction becomes irrelevant with the realization that stromal concentrations of malonyl-CoA during fatty acid synthesis may be several orders of magnitude less than the $K_m$ for the reaction *in vitro*, and that calculated rates of malonyl-ACP formation would be negligible compared with rates of acetate incorporation into fatty acids. The abnormally high $K_m$ values reported for malonyl-CoA and ACP in the malonyl-CoA–ACP transacylase reaction *in vitro* [11] suggest that *in situ* the enzyme resides within a multienzyme complex where localized concentrations of substrates are much higher than those indicated above. The alternative explanation is that the catalytic efficiency of the enzyme is significantly altered during extraction and purification. However, maximum rates of fatty acid synthesis by stromal preparations from barley chloroplasts [11] and by unfractionated stroma from spinach and pea chloroplasts (P. G. Roughan and J. A. Browse, unpublished work) are achieved only in the presence of very high concentrations of both malonyl-CoA and ACP.

It is concluded therefore that the stromal concentrations of CoASH and its esters measured here do not reflect the concentrations that are presented to the enzymes *in situ*. Instead, the substrates are probably retained within an environment such as a multienzyme complex, where the product of one reaction is generated immediately adjacent to the active site of the next enzyme in the reaction sequence. This would ensure that the enzymes experience substrate concentrations that are very much greater than would be the case if the reactants were permitted to distribute throughout the stroma. The putative multienzyme complex may also sequester cofactors required for fatty acid synthesis from acetate, since fatty acid synthesis by permeabilized chloroplasts was independent of added cofactors [1], even though endogenous cofactors were expected to be able to diffuse out into the reaction medium. This expectation is based on the ready access gained by exogenous ADP, CoASH and NADP to the internal membranes of permeabilized chloroplasts [1], and is supported by the efficient conversion of exogenous CoASH into acetyl-CoA by permeabilized chloroplasts utilizing endogenous ATP (the present study).

The origin of acetyl-CoA for light-dependent fatty acid synthesis in chloroplasts has long been debated [24–27]. Acetate is normally the most efficient substrate for supporting fatty acid synthesis by isolated chloroplasts [1,28,29], which contain a highly active acetyl-CoA synthetase activity [6]. On the other hand, pyruvate [30,31] and acetylcarnitine [32] have variously been proposed as the best substrates for fatty acid synthesis by isolated chloroplasts, implying that pyruvate dehydrogenase and carnitine acetyltransferase respectively are primarily responsible for generating the requisite acetyl-CoA. Although a role for carnitine acetyltransferase has yet to be confirmed [33], that of pyruvate dehydrogenase seems to be better established [34,35]. However, pyruvate competes poorly with acetate as a precursor for fatty acids in competition experiments [28,29], and acetate has been deemed the more likely physiological precursor, based on total enzyme activities and concentrations of endogenous substrates [29]. In addition, in many of the reports using pyruvate in radiotracer studies, the results may have been compromised by the presence of small amounts of acetate in the pyruvate utilized [28], and the isolated chloroplasts had very low rates of fatty acid synthesis. Given the high activities of acetyl-CoA synthetase within chloroplasts and the saturating concentrations of acetate measured in leaves and in isolated chloroplasts [36], the balance of evidence would appear to favour acetate as the immediate precursor of acetyl-CoA for light-dependent fatty acid synthesis *in situ*. The present results may provide an explanation for the superiority of acetate in competition experiments utilizing isolated chloroplasts.

Consider that the primary limitation on relative activities of acetyl-CoA synthetase and pyruvate dehydrogenase *in situ* is the concentration of CoASH available to the respective enzymes rather than the concentrations of endogenous acetate and pyruvate. Then acetyl-CoA synthetase operating in conjunction with a multienzyme complex that sequesters the bulk of the chloroplast CoA may effectively convert all available CoASH into acetyl-CoA, thus preventing or, at the very least, severely limiting pyruvate dehydrogenase activity. It is not unreasonable to speculate that most, if not all, the chloroplast CoA may be associated with the fatty acid synthase, given that (a) acetyl-CoA and malonyl-CoA together constitute more than 90% of total CoAs during fatty acid synthesis ([2]; present study), (b) the specific radioactivities of acetyl-CoA and butyryl-ACP are the same as that of the supplied acetate within 20–30 s of fatty acid synthesis [2,3], and (c) in pulse–chase experiments both acetyl-CoA and butyryl-ACP lose radioactivity at the same rate [3].

Other CoA esters theoretically involved in isoprenoid synthesis, such as acetoacetyl-CoA and hydroxymethylglutaryl-CoA, were not detected in illuminated chloroplasts metabolizing acetate and fixing $CO_2$. Those intermediates should have been present and detectable in the present work, since spinach chloroplasts are known to incorporate label from $^{14}CO_2$ into isoprenoids [37]. However, new evidence [38] shows that carotenoid synthesis in green algae does not utilize CoA intermediates; instead, a $C_2$ unit from pyruvate via acetyl-thiamin pyrophosphate is inserted between carbons 1 and 2 of glyceraldehyde 3-phosphate to form isopentyl pyrophosphate. It seems highly likely that the same reaction will be responsible for isoprenoid synthesis in chloroplasts thus explaining the absence of acetoacetyl-CoA and hydroxymethylglutaryl-CoA from chloroplasts. In fact, it now seems quite possible that the entire chloroplast pool of CoA may be involved in fatty acid synthesis from acetate.

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