A semi-preparative enzymic synthesis of malonyl-CoA from [^{14}C]acetate and $^{14}CO_2$: labelling in the 1, 2 or 3 position

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A semi-preparative enzymic synthesis of [1-^{14}C]malonyl-CoA from [1-^{14}C]acetate and bicarbonate, and of [3-^{14}C]malonyl-CoA from $Na_2{}^{14}CO_3$ and acetate, was achieved by using chloroplasts rapidly isolated from 7–8-day-old pea shoots. Around 70% of the [1-^{14}C]acetate was converted into malonyl-CoA in 2–3 h, and the specific radioactivity of [3-^{14}C]malonyl-CoA synthesized in the system was 25–30 Ci/mol. Reactions were monitored and labelled products were purified by h.p.l.c.

INTRODUCTION

Malonyl-CoA is best known as the universal precursor for long-chain fatty acid biosynthesis, but in plants it is also used for the biosynthesis of flavonoids, including anthocyanins [1], and polyketides [2], for the malonylation of flavone aglycones [1], and for the malonylation of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid [3]. Hence there is a demand for radioactively labelled malonyl-CoA for use in enzyme studies. The high commercial cost of labelled malonyl-CoA has stimulated both a number of innovative chemical syntheses [4] and a search for a malonyl-CoA synthetase [5,6] to facilitate the semi-preparative production of [2-^{14}C]malonyl-CoA from [2-^{14}C]malonate. However, the yield of product from the chemical syntheses are low (< 40%) and variable, and a preparative enzymic synthesis using malonyl-CoA synthetase has yet to be developed.

No malonyl-CoA synthetase activity was detected in chloroplasts isolated from pea, spinach or *Amaranthus* (P. G. Roughan, unpublished work), which is consistent with the inability of malonate to support fatty acid synthesis by spinach chloroplasts [7], but contrasts with the sporadic reports of its presence in plants [8,9]. On the other hand, chloroplasts contain high activities of acetyl-CoA synthetase [10] and, by inference, of acetyl-CoA carboxylase, since acetate is rapidly incorporated into long-chain fatty acids of intact chloroplasts in the light [11]. Therefore, the activity of acetyl-CoA carboxylase within chloroplasts must be at least equal to the rate of acetate incorporation into fatty acids, although such activities are rarely achieved by assay *in vitro* with acetyl-CoA as substrate ([8,12], but see [13]). Provided that its utilization in fatty acid synthesis could be prevented, e.g. by an inhibitor such as cerulenin, malonyl-CoA ought to accumulate when chloroplasts are incubated with acetate, HCO_3^- and CoA. This would provide a convenient synthesis of malonyl-CoA labelled at C-1, C-2 or C-3, and would have the added advantage that the substrates required are probably the cheapest of all radiocarbon compounds. This paper describes an enzymic synthesis of [1-^{14}C]- and [3-^{14}C]malonyl-CoA from [1-^{14}C]acetate and $H^{14}CO_3^-$ respectively, by using intact chloroplasts isolated from 7–8-day-old pea (*Pisum sativum*) shoots. Some 65–70% of the [1-^{14}C]acetate was converted into [1-^{14}C]malonyl-CoA in about 2 h, and the product was purified by h.p.l.c.

MATERIALS AND METHODS

Materials

[1-^{14}C]Acetate (59 Ci/mol), $Na_2{}^{14}CO_3$ (51.2 Ci/mol), [2-^{14}C]-malonate (19.5 Ci/mol) and [2-^{14}C]malonyl-CoA (54 Ci/mol) were from Amersham. Malonyl-CoA, acetyl-CoA, CoA, ATP, phenylmethanesulphonyl fluoride (PMSF) and cerulenin were from Sigma. The C_{18} solid-phase extraction cartridges were from Supelco, and the h.p.l.c. column (Econosil C18, 5 μm; 250 mm × 4.6 mm) was from Alltech. Pea seed (Greenfeast) was from Yates (New Zealand) Ltd.

Chloroplast preparation

For this, 50 pea seeds were sown at a depth of about 2 cm into commercial potting media contained in each of two 20 cm-diameter × 25 cm-deep plastic pots. The posts were watered to run-off every other day. After 7–8 days of 10 h under lights at 25 °C, the shoots, which were just beginning to show tendrils, were harvested and transferred immediately to 100 ml of partially frozen low-ionic-strength buffer (0.35 M sorbitol, 2 mM Hepes/KOH, pH 7.8, 0.4 mM KCl, 0.04 mM EDTA [14]). The homogenate, produced with a Polytron homogenizer (with the PT35 aggregate) or similar device for about 5 s, was filtered through two layers of Miracloth, and the filtrate was centrifuged in two 50 ml tubes for 30 s at 2000 *g* in a swing-out rotor. Supernatants were decanted quickly and in such a way as to eliminate most of the broken plastids overlying the pellet of intact organelles, and the residual pellet was resuspended in 4 ml of homogenizing buffer. Intact chloroplasts were separated by layering this suspension over a 26 ml linear gradient of 0–90% Percoll in homogenizing buffer and centrifuging at 2000 *g* for 5 min. The lower green band was collected by aspiration, diluted 4-fold with homogenizing buffer, and the chloroplasts were sedimented by centrifuging for 30 s at 2000 *g*. The pellet of intact chloroplasts was suspended in 0.5–0.6 ml of the same buffer to give about 1 mg of chlorophyll/ml. The entire process was completed within 20 min. Chlorophyll was determined by adding 10 μl of the suspension of intact chloroplasts to 1 ml of methanol and centrifuging. $A_{654} \times 25.1$ gave μg of chlorophyll/10 μl of suspension [15].

Abbreviation used: PMSF, phenylmethanesulphonyl fluoride.

Synthesis of [1-^{14}C]malonyl-CoA from [1-^{14}C]acetate

Two 1 ml reaction mixtures in 1.5 ml microfuge tubes contained 50 mM Hepes/KOH, pH 8.5, 10 mM $KHCO_3$, 10 mM $MgCl_2$, 8 mM ATP, 0.75 mM CoA, 0.75 mM [1-^{14}C]acetate (44.5 μCi), 1 mM dithiothreitol, 0.2 mM PMSF, 0.1 mM cerulenin and 0.05% (w/v) Triton X-100. Reactions were started by adding 200 μl of chloroplast suspension (150–250 μg of chlorophyll) and were incubated in the dark at 22–25 °C for 2–3 h with occasional inversion. Acetate was also used at a specific radioactivity of 10 Ci/mol to synthesize working-strength [1-^{14}C]malonyl-CoA. [2-^{14}C]Acetate may be substituted for [1-^{14}C]acetate. Reactions were terminated by adding 50 μl of 50% (w/v) trichloroacetic acid, and the tubes were cooled on ice and centrifuged for 3 min at 13000 *g*. Protein pellets were washed with 1.0 ml of 1% trichloroacetic acid and the suspension was re-centrifuged. The combined supernatants from one reaction were loaded at 0.7–0.8 ml/min on to a 3 ml C_{18} cartridge which had been conditioned by passage of 2 ml of methanol followed by 8 ml of 1 mM HCl. The loaded cartridge was washed with 6 ml of 1 mM HCl before CoA and its esters were eluted at 0.3–0.4 ml/min in 3 ml of 0.1 M ammonium acetate in 65% (v/v) ethanol [16]. Ethanol was removed and the eluate concentrated to 0.5 ml by rotary film evaporation.

Synthesis of [3-^{14}C]malonyl-CoA from acetate and $H^{14}CO_3^-$

Incubation mixtures contained 50 mM Tris/HCl, pH 8.5, 10 mM $MgCl_2$, 8 mM ATP, 0.75 mM CoA, 1 mM acetate, 20 mM KCl, 2.5 mM $Na_2{}^{14}CO_3$ (128 μCi), 0.2 mM PMSF, 0.1 mM cerulenin and 0.05% (w/v) Triton X-100. Reactions were started by adding 200 μl of chloroplast suspension (150–250 μg of chlorophyll), after which the Microfuge tubes were securely stoppered and incubated as above. Alternatively, [3-^{14}C]malonyl-CoA was synthesized from acetyl-CoA and $Na_2{}^{14}CO_3$, acetate and CoA in the reaction mixture (above) being replaced by 0.5 mM acetyl-CoA. Reactions were incubated in the dark for 1–2 h and were terminated as above.

Monitoring [^{14}C]malonyl-CoA production

Progress of the reactions containing [1-^{14}C]acetate was monitored by transferring 25 μl samples to microfuge tubes containing 12.5 μl of 0.625 M $HClO_4$, and the tubes were cooled in ice for 5–10 min. After centrifuging for 3 min at 13000 *g*, 30 μl of supernatant was neutralized with 12.5 μl of 0.5 M K_2HPO_4 (resulting pH = 6), and the $KClO_4$ was removed by centrifugation. Then 25 μl of supernatant was analysed by h.p.l.c. as described below. Reactions containing $^{14}CO_2$ were monitored by transferring 5–10 μl to DE81 paper discs which were then washed in 2% acetic acid before determination of fixed radioactivity [10].

Separation of acyl-CoAs by h.p.l.c.

CoAs were separated on an analytical reverse-phase column by using a linear gradient of acetonitrile in 0.1 M potassium phosphate, pH 5.2. Samples (approx. 200 μl) of the concentrated eluate from a C_{18} cartridge were injected on to the column, which had been equilibrated with 1.2% acetonitrile in phosphate buffer and developed at 1 ml/min. This buffer composition was maintained for a further 5 min, and then the acetonitrile concentration was increased linearly to 12% over 45 min. Malonyl-CoA was eluted between 24 and 26 min, CoA between 28 and 31 min and acetyl-CoA between 36 and 39 min. At most, 0.2 μmol of malonyl-CoA (184 μg, 11–12 μCi) was purified per pass. Appropriate fractions from several injections were combined and concentrated by rotary film evaporation. [1-^{14}C]Malonyl-CoA was separated from phosphate buffer by solid-phase extraction; the concentrated eluate from h.p.l.c. in 2 ml of water was loaded on a 3 ml C_{18} cartridge which had been pre-conditioned as above. Phosphate was washed through the cartridge with 6 ml of 1 mM HCl, followed by 1 ml of water, and the malonyl-CoA was eluted in 5 ml of 0.1 M NH_4OH in 60% (v/v) ethanol (pH 10.5) at 0.3–0.4 ml/min. The eluate was collected in a flask cooled in ice and was evaporated to dryness with a minimum of delay. The residue was redissolved in 1 ml of 1 mM HCl. Concentrations of [^{14}C]malonyl-CoA preparations were determined either by diluting suitable samples with 0.1 M potassium phosphate, pH 5, and measuring the A_{260} ($A_{260} \times 64.9$ = nmol/ml for a 1 cm light-path) or by quantitative h.p.l.c., and specific radioactivities were determined subsequently by liquid-scintillation counting. The specific radioactivity of the original acetate was normally preserved in the purified [^{14}C]malonyl-CoA.

RESULTS AND DISCUSSION

Chloroplast Isolation

A routine semi-preparative synthesis of labelled malonyl-CoA from acetate is feasible because of the uncomplicated techniques now available for isolating active intact chloroplasts from some plant species. Chloroplasts prepared either from expanding spinach (*Spinacia oleracea*) leaves or from 7–8-day pea shoots were capable of accumulating malonyl-CoA in the system described. Results presented here were obtained by using pea shoots, which are easier to grow than are appropriate spinach plants [17]. The variety of pea used is probably not as important as is the cultivation of the shoots, which must be adequately supplied with water at all times to enable the preparation of high-quality chloroplasts. Previous experience [18,19] suggests that the pea variety 'Little Marvel' from Burpee Seeds, Warminster, PA, U.S.A., would provide suitable material. Walker et al. [20] provide useful suggestions on the mechanics of chloroplast isolation.

Table 1 Effects of PMSF, cerulenin and chloroplast concentration on malonyl-CoA formation from acetate by pea chloroplasts

Mean values from two experiments are shown. Reactions contained chloroplasts equivalent to 200 μg of chlorophyll/ml under the standard reaction conditions for synthesizing [1-^{14}C]malonyl-CoA (see the Materials and methods section), but without PMSF and cerulenin, which were then added as indicated. Incubation was for 3 h at 25 °C.

Additions	Malonyl-CoA formed (nmol/ml)	Equivalent μCi*
None	390	23
0.2 mM PMSF	420	25
0.1 mM Cerulenin	410	24
PMSF + cerulenin	510	30
PMSF + cerulenin (100 μg of chlorophyll/ml)	450	27

* When [1-^{14}C]acetate was 59 Ci/mol.

A problem with phosphatase

Analyses of reaction products in preliminary experiments showed that AMP (h.p.l.c. retention time = 13 min) accumulated in amounts far in excess of that produced by the acetyl-CoA synthetase reaction. Therefore, nucleotide phosphatase activity rapidly depleted added ATP, on which the reaction was dependent. This phosphatase activity was not significantly inhibited by fluoride, molybdate or arsenate, but was inhibited by Triton X-100. Adding additional ATP during the course of the reaction did not increase the yield of malonyl-CoA. Stroma from lysed chloroplasts had lower phosphatase activity, but, when Triton X-100 was included in the reactions, yields of product were about the same when either whole chloroplasts or chloroplast stroma was the enzyme source. However, it seemed appropriate to initiate the synthesis with intact chloroplasts, which retained the ability to convert acetate into malonyl-CoA after several hours of storage at 0 °C.

Countering protease activity and malonyl-CoA utilization

Chloroplast acetyl-CoA carboxylase is susceptible to proteolysis [21], and it seemed prudent to include the protease inhibitor PMSF in the reaction mixture for incubations of several hours duration. Similarly, malonyl-CoA is an extremely reactive substrate, and including cerulenin in reaction mixtures to inhibit 3-oxoacyl-[acyl-carrier-protein] synthase activity seemed appropriate for enhancing malonyl-CoA accumulation. Indeed, in earlier trials where the Triton X-100 concentration in reactions was 0.02% (w/v) and incubation times were > 4 h, inclusion of the inhibitors, both singly and in combination, had a very marked effect on yields of [^{14}C]malonyl-CoA. However, when the Triton X-100 concentration in the mixture was increased to 0.05% (w/v), both the utilization of malonyl-CoA by the chloroplast fatty acid synthase [22] and destruction of acetyl-CoA carboxylase activity by proteolysis appeared to be minimized. Therefore, adding cerulenin and PMSF singly to these incubations resulted in relatively small increases in yield (Table 1). On the other hand, yields were significantly increased when both inhibitors were included (Table 1). Ultimately, the yield of malonyl-CoA was probably limited by mass-action effects on the reversible reactions; accumulated AMP would prevent acetyl-CoA synthesis. The concentrations of acetate and CoA selected for the reaction represented the best compromise between yield of product and the proportion of substrate utilized. Halving the amount of chloroplasts added to reactions (from 200 to 100 μg of chlorophyll equivalent) increased the time required to achieve maximum yield, but did not greatly decrease the final yield (Table 1).

[3-^{14}C]Malonyl-CoA

The synthesis of [3-^{14}C]malonyl-CoA was avoidably wasteful of $H^{14}CO_3^-$, but the former is not available commercially and the latter is relatively inexpensive. The present method may in fact be the only published semi-preparative enzymic synthesis of [3-^{14}C]malonyl-CoA. Availability of [3-^{14}C]malonyl-CoA provides an opportunity to measure rates of fatty acid synthase, ketoacyl synthase or chalcone synthase activities by monitoring release of $^{14}CO_2$ in the condensation reaction.

Purification of [^{14}C]malonyl-CoA

Reverse-phase h.p.l.c. provided the least equivocal purification (Figure 1) of malonyl-CoA, which is very difficult to separate from CoA and acetyl-CoA by other forms of chromatography. CoAs as a group were initially separated from adenine nucleotides and trichloroacetic acid using solid-phase extraction; the CoAs were strongly retained on the C_{18} cartridge during the aqueous

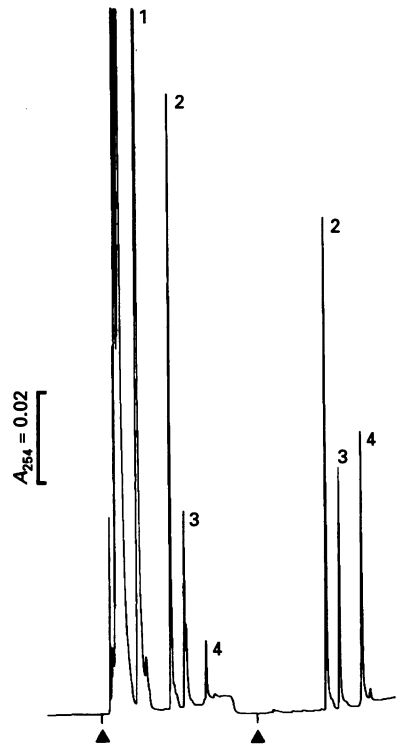

Figure 1 H.p.l.c. separation of [1-^{14}C]malonyl-CoA

The chromatogram on the left shows a separation of the reaction products from a 3 h incubation of the standard reaction mixture containing [1-^{14}C]acetate at 10 Ci/mol, and that on the right shows the separation of a reference mixture. Arrowheads indicate where injections were made. Chromatography conditions are described in the Materials and methods section. Identity of peaks: 1, AMP; 2, malonyl-CoA; 3, CoASH; 4, acetyl-CoA.

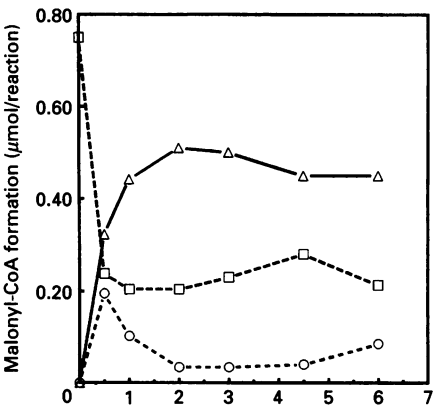

Figure 2 Typical time course of malonyl-CoA formation from acetate by pea chloroplasts in the standard reaction

Samples (50 μl) of the reaction mixture, containing 246 μg of chlorophyll, were transferred to 25 μl of 0.625 M $HClO_4$ at the times shown, and the products were analysed by h.p.l.c. as described in the Materials and methods section and as shown in Figure 1. Symbols: □, CoA; ○, acetyl-CoA; △, malonyl-CoA.

wash and were subsequently recovered quantitatively in ethanolic ammonium acetate solution [16]. Ethanol was removed and the eluate concentrated for h.p.l.c. by rotary film evaporation without any loss of product. The malonyl-CoA purified by h.p.l.c. contained a large amount of phosphate, which was, however, readily removed by using a reverse-phase cartridge, and the malonyl-CoA was subsequently eluted in a small volume of ethanolic NH_4OH [23]. In spite of the well-known susceptibility of acyl-CoAs to alkaline hydrolysis, there was negligible loss of labelled malonyl-CoA during elution from the cartridge and concentration by rotary evaporation with concomitant removal of the NH_4OH. Final yields of pure [1-^{14}C]malonyl-CoA in the present system were 55–65%, based on ^{14}C recovery. Purified [1-^{14}C]malonyl-CoA of lower specific radioactivity may be more stable on storage, and it may be appropriate to synthesize the material at, e.g., 10 Ci/mol in larger amounts rather than to dilute high-specific-radioactivity [^{14}C]malonyl-CoA (58–60 Ci/mol) with unlabelled material for a working-strength substrate. The purified [^{14}C]malonyl-CoA migrated as a single spot ($R_F = 0.39$) on thin layers of microcrystalline cellulose developed with butan-l-ol/acetic acid/water (5:2:3, by vol.), and, after hydrolysis in KOH, yielded a single radioactive spot ($R_F = 0.69$) that corresponded to [2-^{14}C]malonic acid on thin layers of silica gel developed with water-saturated diethyl ether/formic acid (7:1, v/v). The [1-^{14}C]malonyl-CoA preparations were utilized for fatty acid synthesis by lysates of peas chloroplasts at rates that were 40-fold and 200-fold greater, respectively, even in the absence of added acyl-carrier protein, than those previously achieved by using either commercial [1,3-^{14}C]malonyl-CoA and spinach chloroplast lysates [8] or commercial [2-^{14}C]malonyl-CoA and pea chloroplast lysates [24].

Rate of [1-^{14}C]malonyl-CoA formation

A time course of [1-^{14}C]malonyl-CoA accumulation in the reaction utilizing pea chloroplasts is shown in Figure 2. The concentration of malonyl-CoA exceeded that of acetyl-CoA within 30 min, and the reaction had virtually ceased by 2 h. At incubation times longer than 3 h, malonyl-CoA may be lost through the reversibility of the reactions. The high residual CoA and low acetyl-CoA concentrations suggest that acetyl-CoA synthetase activity had become limiting (see above). Removing AMP from reactions may not prove cost-effective, however, and yields of 70% conversion of acetate into malonyl-CoA in this system might be regarded as more than adequate, considering the alternatives: the cost of [1-^{14}C]acetate is 20% that of [2-^{14}C]malonate, from which malonyl-CoA may be synthesized in about 40% yield [4].

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REFERENCES

1 Hahlbrock, K. (1981) Biochem. Plants **7**, 425–456
2 Packter, N. M. (1980) Biochem. Plants **4**, 535–570
3 Kionka, C. and Armhein, N. (1984) Planta **162**, 226–235
4 Rutkoski, A. and Jaworski, J. G. (1978) Anal. Biochem. **91**, 370–373
5 Kim, Y. S. and Bang, S. K. (1985) J. Biol. Chem. **260**, 5098–5104
6 Kim, Y. S. and Chae, H. Z. (1991) Biochem. J. **273**, 511–516
7 Roughan, P. G., Holland, R., Slack, C. R. and Mudd, J. B. (1979) Biochem. J. **184**, 565–569
8 Kannangara, C. G., Jacobson, B. S. and Stumpf, P. K. (1973) Plant Physiol. **52**, 156–161
9 Focke, M., Feld, F. and Lichtenthaler, H. K. (1990) FEBS Lett. **261**, 106–108
10 Roughan, G. and Ohlrogge, J. (1994) Anal. Biochem. **216**, 77–82
11 Gardiner, S. E., Heinz, E. and Roughan, P. G. (1984) Plant Physiol. **74**, 890–896
12 Mohan, S. B. and Kekwick, R. G. O. (1980) Biochem. J. **187**, 667–676
13 Laing, W. A. and Roughan, P. G. (1982) FEBS Lett. **144**, 341–344
14 Cerovic, Z. G. and Plesnicar, M. (1984) Biochem. J. **223**, 543–545
15 Wintermans, J. F. G. M. and De Mots, A. (1965) Biochim. Biophys. Acta **109**, 448–453
16 Hosokawa, Y., Yoshiharu, S., Harris, R. and Ozawa, T. (1986) Anal. Biochem. **153**, 45–49
17 Roughan, G. (1987) Methods Enzymol. **148**, 327–337
18 Post-Beittenmiller, D., Roughan, G. and Ohlrogge, J. (1992) Plant Physiol. **100**, 923–930
19 Roughan, G., Post-Beittenmiller, D., Browse, J. and Ohlrogge, J. (1993) Plant Physiol. **101**, 1157–1162
20 Walker, D. A., Cerovic, Z. . and Robinson, S. P. (1987) Methods Enzymol. **148**, 145–157
21 Gorniki, P. and Haselkorn, R. (1993) Plant Mol. Biol. **22**, 547–552
22 Roughan, P. G., Slack, C. R. and Holland, R. (1976) Biochem. J. **158**, 593–601
23 Stymne, S. and Glad, G. (1981) Lipids **16**, 298–305
24 Walker, K. A. and Harwood, J. L. (1985) Biochem. J. **226**, 551–556