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Acetate is the Preferred Substrate for Long-Chain Fatty Acid Synthesis in Isolated Spinach Chloroplasts

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1. Commercially available [2-^{14}C]pyruvate and [2-^{14}C]malonate were found to contain 3–6% (w/w) of [^{14}C]acetate. 2. The contaminating [^{14}C]acetate was efficiently utilized for fatty acid synthesis by isolated chloroplasts, whereas the parent materials were poorer substrates. 3. Maximum incorporation rates of the different substrates examined were (ng-atoms of C/h per mg of chlorophyll): [1-^{14}C]acetate, 2676; [2-^{14}C]pyruvate, 810; $H^{14}CO_3^-$, 355; [2-^{14}C]malonate, 19. 4. Products of CO_2 fixation were probably not a significant carbon source for fatty acid synthesis in the presence of exogenous acetate.

It is important to establish the nature of the substrate most efficiently utilized for fatty acid synthesis by isolated chloroplasts since, if this substrate cannot be made within the chloroplasts, then modulation of its supply from the cytoplasm would be one of the ways in which rates of chloroplast fatty acid synthesis could be controlled *in vivo*. Since the initial discovery of its incorporation into fatty acids of isolated chloroplasts (Smirnov, 1960), [^{14}C]acetate has been the substrate most frequently used in studies of fat synthesis in the isolated organelles (Mudd & McManus, 1962; Stumpf & James, 1963; Stumpf *et al.*, 1967; Kannangara & Stumpf, 1972; Nakamura & Yamada, 1975; Roughan *et al.*, 1976). However, Yamada & Nakamura (1975) have reported that [2-^{14}C]pyruvate is an even better substrate and a pathway has been proposed whereby products of CO_2 fixation in the plastid could be converted into pyruvate (Murphy & Leech, 1978), thus making the organelle self-sufficient for long-chain fatty acid synthesis. A clear superiority of [2-^{14}C]pyruvate over [^{14}C]acetate has not been noted in other studies on fatty acid synthesis by isolated plastids (Roughan *et al.*, 1976; Nothelfer *et al.*, 1977; Murphy & Leech, 1978). In attempting to resolve this conflict we have found that, as substrates for chloroplast fatty acid synthesis, exogenous [1-^{14}C]-acetate was superior to [2-^{14}C]pyruvate, [2-^{14}C]-malonate and $NaH^{14}CO_3$ by factors of 3–4, 140 and 7–8 respectively. However, to demonstrate this relationship [2-^{14}C]pyruvate and [2-^{14}C]malonate had first to be separated from contaminating [^{14}C]acetate.

Materials and Methods

Spinach plants [Hybrid 102; Arthur Yates, Auckland, New Zealand (also known as Hybrid 424; Ferry Morse Seed Co., Mountain View, CA 94040, U.S.A.)] were grown in water-culture either under controlled environment at the Plant Physiology Division, Palmerston North (Roughan *et al.*, 1976), or in a glasshouse at University of California, Riverside, between November and February. Chloroplasts were isolated from expanding leaves by the procedure of Nakatani & Barber (1977). Fatty acid synthesis and analysis of the products of ^{14}C-labelled-substrate incorporation was as described previously (Roughan *et al.*, 1979). [1-^{14}C]Acetate, [2-^{14}C]pyruvate, [1-^{14}C]pyruvate, [2-^{14}C]malonate, [U-^{14}C]glycerol 3-phosphate, $NaH^{14}CO_3$ and ^3H_2O were supplied by The Radiochemical Centre, Amersham, Bucks., U.K. The radiochemical purity of [1-^{14}C]acetate was checked in the fatty acid-synthesizing system by dilution with standardized sodium acetate. The radiochemical purity of [2-^{14}C]-pyruvate and [2-^{14}C]malonate was checked, after purification by ion-exchange chromatography (Von Korff, 1969), by using lactate dehydrogenase and analytical g.l.c. respectively.

Results

At low concentrations (0.1 mM), [1-^{14}C]acetate was incorporated into chloroplast long-chain fatty acids at more than twice the rate of [2-^{14}C]pyruvate and at about 35 times the rate of [2-^{14}C]malonate (Fig. 1*a*). However, rates of [1-^{14}C]acetate incorporation were apparently saturated at a substrate concentration of about 0.12 mM, whereas rates of [2-^{14}C]pyruvate and [2-^{14}C]malonate incorporation increased up to a substrate concentration of 10 mM (Fig. 1*b*). This requirement for high concentrations of pyruvate and malonate was not simply due to the inability of these compounds to enter the chloroplasts, since the same requirement was evinced by chloroplasts incubated in

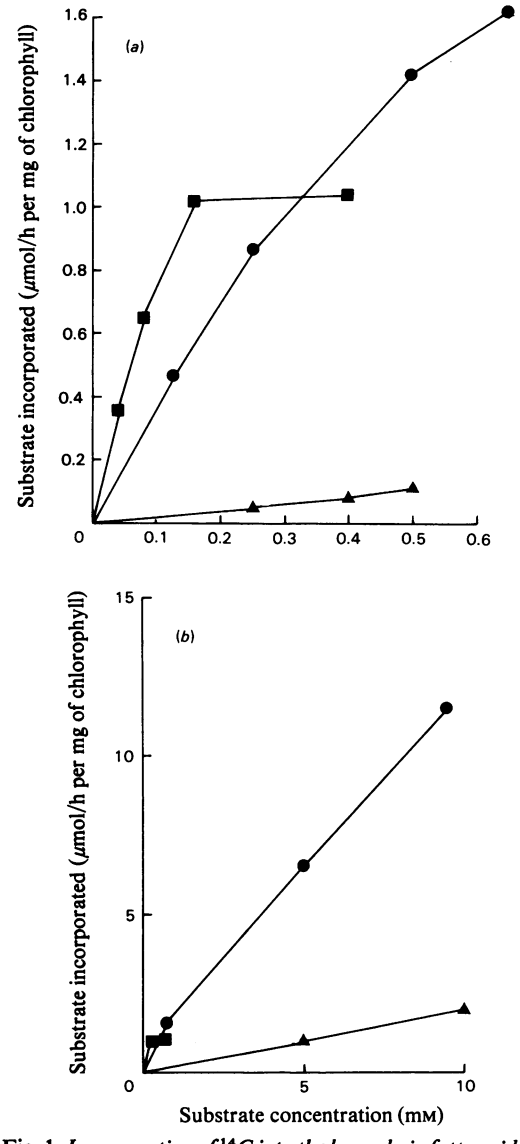

Fig. 1. *Incorporation of ^{14}C into the long-chain fatty acids of isolated spinach chloroplasts incubated with low (Fig. 1a) and high (Fig. 1b) concentrations of unpurified ^{14}C-labelled substrates*

■, [1-^{14}C]Acetate; ●, [2-^{14}C]pyruvate; ▲, [2-^{14}C]-malonate.

hypo-osmotic media (0.06 M-sorbitol) and therefore without permeability barriers. The highest rate of fatty acid synthesis from [2-^{14}C]pyruvate was apparently more than 10 times the highest rates measured with [1-^{14}C]acetate (Fig. 1*b*), but the [^{14}C]oleate synthesized from either substrate contained 60% of its ^{14}C in carbon atoms in positions C-1 to C-9 and 40% in carbon atoms in positions C-10 to C-18, thus indicating synthesis *de novo* in either case. Similarly, the products of long-chain fatty acid synthesis from [2-^{14}C]pyruvate and [1-^{14}C]acetate were identical and it appeared that chloroplasts might contain much higher activities of fatty acid synthetase than had previously been considered. However, rates of $^{14}CO_2$ release from [1-^{14}C]pyruvate were only 4–5% of the rate required to account for rates of [2-^{14}C]pyruvate incorporation measured concurrently in a separate incubation. Even allowing for some re-fixing of the released $^{14}CO_2$, there appeared to be a lack of stoichiometry in the two measurements. Incorporation of [2-^{14}C]pyruvate at the rates measured in the present paper would have resulted in the synthesis of 3–4 μg of oleic acid in a 15 min incubation containing 10 mM-pyruvate. In fact, amounts of oleic acid recovered from such incubations, by using t.l.c. and g.l.c. analyses, were only 20% of those predicted and were no greater than amounts generated in incubations containing 0.2 mM-acetate.

In competition experiments, acetate inhibition of [2-^{14}C]pyruvate incorporation and pyruvate inhibition of [1-^{14}C]acetate incorporation was not consistent with the results shown in Fig. 1. At sub-saturating concentrations of [1-^{14}C]acetate (<0.08 mM), equimolar amounts of pyruvate were completely without effect on acetate incorporation although a 30% inhibition was predicted from Fig. 1(*a*). Similarly, although 1.2 mM-[2-^{14}C]pyruvate was incorporated at about twice the rate of 0.16 mM-[1-^{14}C]acetate, unlabelled pyruvate at this concentration inhibited [1-^{14}C]acetate incorporation by only 20% (Table 1). On the other hand, unlabelled acetate at 0.08–0.40 mM inhibited [2-^{14}C]pyruvate incorporation almost linearly (Table 1), and more so than was anticipated from Fig. 1. Considering that fatty acid synthesis from [1-^{14}C]acetate was saturated at about 0.12 mM, the increased inhibition of [2-^{14}C]pyruvate incorporation at 0.4 mM-acetate suggested that the observed incorporation of [2-^{14}C]-pyruvate may in fact have been largely due to contamination of the latter with [^{14}C]acetate.

A range of possible precursors for fatty acid synthesis in isolated chloroplasts was tested by using the ^{3}H-incorporation technique (Jungus, 1968; Yamada & Nakamura, 1975). The results (Table 2) differ in some respects from those reported by Yamada & Nakamura (1975). Most importantly, acetate always stimulated a greater incorporation of ^{3}H from ^3H_2O into fatty acids than did pyruvate, even at high concentrations of the latter. Malonate at high concentrations stimulated ^{3}H incorporation to about 40% of the rate obtained with 0.2 mM-acetate and oxaloacetate was equally as effective as pyruvate. Intermediates of the Benson–Calvin cycle were up to 2.5 times more effective than HCO_3^- alone in

stimulating ^3H incorporation into long-chain fatty acids (Table 2). Essentially the same results had been obtained previously with chloroplasts isolated by procedures described by Roughan *et al.* (1976). The high non-enzymic incorporation of ^3H into chloroplast fatty acids reported by Yamada & Nakamura (1975) was also observed in the present study whether whole incubation media were treated with methanolic KOH or lipids were extracted before the saponification.

An anomalous situation became apparent when direct incorporation of the ^{14}C-labelled substrates was measured concurrently in a separate incubation and compared with stimulation of ^3H incorporation by unlabelled substrates (Table 2). Whereas the ratio of $^3H/[1\text{-}^{14}C]$acetate incorporated was nearly theoretical (= 2.55 for oleic acid synthesis) when [1-^{14}C]-acetate was the substrate, with [2-^{14}C]pyruvate as substrate the ratio was out by an order of magnitude (Table 2).

Since acetate was clearly the preferred substrate in the ^3H-incorporation assay and yet [2-^{14}C]pyruvate and [2-^{14}C]malonate apparently produced high rates of [^{14}C]fatty acid synthesis, we devised an alternative indirect assay for long-chain fatty acid synthesis in isolated chloroplasts. The main product of [1-^{14}C]-acetate incorporation into chloroplast lipids when both *sn*-glycerol 3-phosphate and Triton X-100 were added to the incubation media was 1,2-diacylglycerol (Roughan *et al.*, 1976, 1979). It was confirmed that the same was true for [2-^{14}C]pyruvate and hence, if pyruvate was converted into long-chain fatty acids at a much higher rate than was acetate, more 1,2-diacylglycerol would be synthesized in a given time from *sn*-[^{14}C]glycerol 3-phosphate in the presence of pyruvate compared with acetate. However, even very

Table 1. *Competition experiments showing the effects of unlabelled precursors on the incorporation of ^{14}C-labelled substrates into long-chain fatty acids of isolated spinach chloroplasts*

Chloroplasts equivalent to 50μg of chlorophyll were incubated in 0.25ml of the standard assay medium supplemented with 0.5mM-CoA, but with [1-^{14}C]-acetate omitted. Expt. 1, 0.16mM-[1-^{14}C]acetate at 0.1μmol of acetate/μCi and the additions shown; Expt. 2, 1.22mM-[2-^{14}C]pyruvate (unpurified) at 0.61μmol of pyruvate/μCi and the additions shown. Incubation was for 15min at 25°C and reactions were stopped by adding 1ml of 10% (w/v) KOH in methanol.

Additions	Fatty acid synthesis (nmol of substrate incorporated/h per mg of chlorophyll)	Inhibition (%)
Expt. 1		
0.16mM-[1-^{14}C]Acetate	1033	0
+0.12mM-Pyruvate	1012	2
+0.24mM-Pyruvate	909	12
+1.22mM-Pyruvate	835	19
Expt. 2		
1.22mM-[2-^{14}C]Pyruvate	2073	0
+0.08mM-Acetate	1762	15
+0.16mM-Acetate	1373	34
+0.40mM-Acetate	829	60

Table 2. *A comparison between the ^3H-incorporation assay and direct radioactively labelled carbon incorporation for measuring fatty acid synthesis in isolated chloroplasts*

Reactions contained chloroplasts equivalent to 50–55μg of chlorophyll in 0.25ml of the standard assay medium with the [1-^{14}C]acetate omitted and containing 0.5mM-CoA and the additions shown, were incubated for 15min at 25°C and were stopped by adding 1ml of 10% (w/v) KOH in methanol. All incubations were in duplicate and ^3H incorporation was corrected for the zero-time control. ^3H_2O (10mCi) and unlabelled substrates were included in the assays for ^3H incorporation.

Additions	Expt. ...	Fatty acid synthesis (isotope incorporated/h per mg of chlorophyll)			
		1	2	3	4
^3H incorporation (ng-atoms)					
None		306	455	480	639
5mM-D-Phosphoglyceric acid		323	—	750	—
5.7mM-DL-Glyceraldehyde 3-phosphate		767	711	—	—
3.3mM-Dihydroxyacetone phosphate		—	725	—	—
5.0mM-Oxaloacetate		—	—	1557	1688
5.0mM-Pyruvate		—	1122	1536	1614
10.0mM-Pyruvate		1908	—	—	1322
10.0mM-Malonate		—	—	1080	
0.2mM-Acetate		2593	2311	2547	2694
0.5mM-Acetate		—	—	—	2823
^{14}C incorporation (nmol)					
5mM-[2-^{14}C]Pyruvate (unpurified)		6590	5210	6851	5501
0.16mM-[1-^{14}C]Acetate		1338	930	1208	1107

high concentrations of pyruvate failed to stimulate a higher rate of *sn*-[^{14}C]glycerol 3-phosphate incorporation into 1,2-diacylglycerol (Table 3) than did 0.2mM-acetate. The amounts of 1,2-diacylglycerol synthesized from [1-^{14}C]acetate and unlabelled *sn*-glycerol 3-phosphate in separate incubations run concurrently closely matched those synthesized from unlabelled acetate and [^{14}C]glycerophosphate (Table 3), indicating that products of CO_2 fixation contributed little to chloroplast fatty acid synthesis in the presence of acetate.

The inconsistencies between the direct and indirect measurements for measuring fatty acid synthesis from different substrates could be explained by contamination of [2-^{14}C]pyruvate and [2-^{14}C]-malonate with [^{14}C]acetate. Accordingly, both substrates were analysed and purified by ion-exchange chromatography (Von Korff, 1969). Three samples of [2-^{14}C]pyruvate were found to contain 4–6% (w/w) [^{14}C]acetate and two samples of [2-^{14}C]-malonate were found to contain 2 and 3% (w/w) [^{14}C]acetate on delivery. The [^{14}C]acetate from these preparations was an excellent substrate for fatty acid synthesis in isolated chloroplasts, whereas the purified [2-^{14}C]pyruvate and [2-^{14}C]malonate were clearly inferior to [1-^{14}C]acetate when all were tested at saturating substrate concentrations (Fig. 2, Table 4). Purified [2-^{14}C]malonate was a poor substrate for chloroplast fatty acid synthesis, in agreement with Brooks & Stumpf (1966), but in contrast with the results of Kannangara *et al.* (1973), who reported malonate to be equally effected as acetate for fatty acid synthesis in disrupted chloroplasts. Even in broken chloroplasts, incubated in the presence of CoA and ATP, we found [2-^{14}C]malonate incorporation to be less than 1.0% of that for [1-^{14}C]-acetate under identical conditions. It seems likely that our chloroplast preparations contained no malonyl-CoA synthetase.

Table 3. *Stimulation of sn-[^{14}C]glycerol 3-phosphate incorporation into chloroplast 1,2-diacylglycerols by long-chain fatty acids synthesized de novo from acetate or pyruvate*

Incubations contained 0.25ml of the standard assay medium with [1-^{14}C]acetate omitted, 0.2mM-*sn*-[U-^{14}C]glycerol 3-phosphate (0.5μCi), 0.13mM-Triton X-100, chloroplasts equivalent to 62μg of chlorophyll and the additions shown. Reactions were incubated at 25°C for 15min and were stopped by adding 1ml of 2.5% (v/v) acetic acid in propan-2-ol. In a parallel incubation with 0.16mM-[1-^{14}C]acetate and unlabelled glycerophosphate, 395nmol of acetate/h per mg of chlorophyll was incorporated into 1,2-diacylglycerols. This was equivalent to 23.2nmol of diacylglycerol.

Additions	1,2-Diacylglycerol synthesis (nmol/h per mg of chlorophyll)
None	4.5
Acetate (0.2mM)	24.4
Pyruvate (0.2mM)	10.3
Pyruvate (10mM)	23.4
Pyruvate (20mM)	24.0

Table 4. *Maximum rates of fatty acid synthesis measured in the present study from purified radioactively labelled substrates*

Chloroplast fatty acid synthesis from the various substrates was measured as described in the legends to Tables 1–3. NaH$^{14}CO_3$ (10mM) incorporation was measured in the fatty acid-synthesizing system (Roughan *et al.*, 1979) and in the absence of other exogenous carbon sources. Results shown are the means for duplicate incubations, but were not necessarily obtained with the same chloroplast preparation.

Substrate	Fatty acid synthesis (ng-atoms of carbon incorporated/h per mg of chlorophyll)
[1-^{14}C]Acetate	2676
[2-^{14}C]Pyruvate	810
NaH$^{14}CO_3$	355
[2-^{14}C]Malonate	19

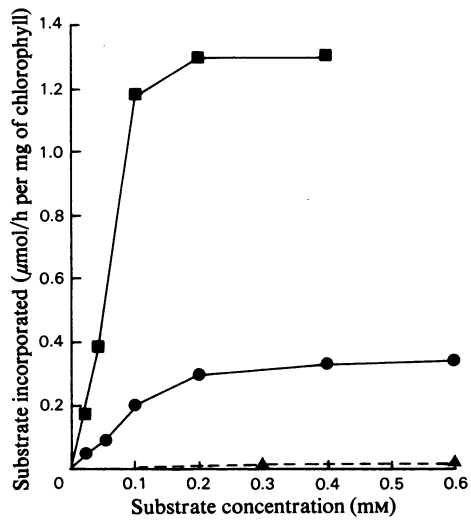

Fig. 2. *Incorporation of ^{14}C into long-chain fatty acids of isolated spinach chloroplasts incubated with purified ^{14}C-labelled substrates*

■, [1-^{14}C]Acetate; ●, [2-^{14}C]pyruvate; ▲, [2-^{14}C]-malonate.

Discussion

Von Korff (1964, 1969) has warned of the instability of solutions of [2-^{14}C]pyruvate and shown how

[^{14}C]parapyruvate accumulates on storage at −20°C. However, more important to the present argument is the fact that [2-^{14}C]pyruvate also breaks down to [^{14}C]acetate (Von Korff, 1969), presumably by a radiation-induced decarboxylation (Silverstein & Boyer, 1964). The results of the present study indicate that both [2-^{14}C]pyruvate and [2-^{14}C]malonate may break down to [^{14}C]acetate, even when stored in the dry state. This contaminating [^{14}C]acetate was a better substrate for chloroplast fatty acid synthesis than were the parent materials, which highlights the necessity for carefully checking the purity of certain radiochemicals before use in metabolic studies. Such contamination of [2-^{14}C]pyruvate with [^{14}C]acetate could be a general phenomenon and could explain the ^{14}C-incorporation results reported in Yamada & Nakamura (1975) and Murphy & Leech (1978), as well as in the present work. This explanation is certainly suggested from a comparison of Fig. 3 in Yamada & Nakamura (1975) and Fig. 1 of the present paper. However, we also found acetate to be utilized more efficiently than pyruvate when rates of fatty acid synthesis were measured by indirect methods and this is in direct contrast with the results of Yamada & Nakamura (1975). We have no explanation at present for this anomaly.

Our findings are also consistent with acetate possibly being a contaminant of unlabelled pyruvate. When comparing acetate- or pyruvate-stimulated incorporation of *sn*-[^{14}C]glycerol 3-phosphate into chloroplast 1,2-diacylglycerol, it was observed that high concentrations of pyruvate (10 and 20mM) stimulated the same amount of ^{14}C incorporation as did 0.2mM-acetate. If we assume a 1% (w/w) contamination of pyruvate with acetate then 10mM-pyruvate would contain 0.1mM-acetate, almost sufficient to saturate fatty acid synthesis with acetate. Several batches of sodium pyruvate and potassium pyruvate were checked by the lactate dehydrogenase assay and were found to be 65–98% pure. Yamada & Nakamura (1975) used substrate concentrations of 10mM in their work with the ^{3}H-incorporation assay, but possible contamination of these substrates with acetate cannot explain the low relative incorporation of acetate measured in that study. Similarly, although [2-^{14}C]malonate was not utilized by these chloroplasts for fatty acid synthesis, unlabelled sodium malonate (10mM) stimulated a relatively high amount of ^{3}H incorporation into long-chain fatty acids. This apparent contradiction might be explained by contamination of the malonate with a low concentration (<1% w/w) of sodium acetate.

Although the chloroplasts used in the present work were capable of fixing CO_2 at rates of 100–120 μmol/h per mg of chlorophyll in the assay used for measuring fatty acid synthesis (Roughan *et al.*, 1979), the products of this fixation were probably not a significant source of carbon for fatty acid synthesis in the presence of exogenous acetate. This was deduced from the nearly theoretical incorporation of [1-^{14}C]acetate relative to *sn*-[U-^{14}C]glycerol 3-phosphate in 1,2-diacylglycerol synthesis and from the ratio of ^{3}H to [1-^{14}C]acetate incorporated into long-chain fatty acids.

It has been assumed (Yamada & Nakamura, 1975; Murphy & Leech, 1978) that pyruvate would be converted into acetyl-CoA by pyruvate dehydrogenase, but to our knowledge this enzyme has not been detected in isolated chloroplasts (Roughan *et al.*, 1979).

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