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Products of fatty acid synthesis by a particulate fraction from germinating pea (*Pisum sativum* L.)

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The synthesis of lipids and acyl thioesters was studied in microsomal preparations from germinating pea (*Pisum sativum* cv. Feltham First) seeds. Under conditions of maximal synthesis (in the presence of exogenous acyl-carrier protein) acyl–acyl-carrier proteins accounted for about half the total incorporation from [^{14}C]malonyl-CoA. Decreasing the concentrations of exogenous acyl-carrier protein lowered the overall synthesis of fatty acids by decreasing, almost exclusively, the radioactivity associated with acyl–acyl-carrier proteins. A time-course experiment showed that acyl–acyl-carrier proteins accumulated most of the radioactive label at the beginning of the incubation but, eventually, the amount of radioactivity in that fraction decreased, while a simultaneous increase in the acyl-CoA and lipid fractions was noticed. Addition of exogenous CoA (1 mM) produced a decrease of total incorporation, but an increase in the radioactivity incorporated into acyl-CoA. The microsomal preparations synthesized saturated fatty acids up to C_{20}, including significant proportions of pentadecanoic acid and heptadecanoic acid. Synthesis of these 'odd-chain' fatty acids only took place in the microsomal fraction. In contrast, when the 18000*g* supernatant (containing the microsomal and soluble fractions) was incubated with [^{14}C]malonyl-CoA, the radioactive fatty acid and acyl classes closely resembled the patterns produced by germinating in the presence of [^{14}C]acetate *in vivo*. The results are discussed in relation to the role of acyl thioesters in the biosynthesis of plant lipids.

Several recent studies, beginning with the work of Macey & Stumpf (1968), have described fatty acid synthesis by a particulate (microsomal) fraction from germinating pea seeds. These preparations are known to use [^{14}C]malonyl-CoA efficiently, whereas other radioactive precursors such as [^{14}C]-acetyl-CoA are incorporated poorly, perhaps due to a lack of acetyl-CoA carboxylase activity (Harwood, 1979). Microsomal fractions prepared from pea seeds after 24h of germination only synthesize saturated fatty acids (Macey & Stumpf, 1968; Harwood & Stumpf, 1970; Bolton & Harwood, 1977*a*; Jordan & Harwood, 1980) and it seems that their capacity to form the very-long-chain fatty acids depends on the variety of pea used (Jordan & Harwood, 1980). In the particulate system both fatty acid synthetase and palmitate elongase are present (Stumpf, 1980) as well as stearate elongase (von Wettstein-Knowles, 1979). These enzymes can be selectively inhibited by different compounds, such as cerulenin, arsenite, and fluoride (Harwood, 1979).

The incorporation of radioactivity from [^{14}C]-malonyl-CoA into acyl residues by pea microsomal fraction ('microsomes') is stimulated by exogenous acyl-carrier protein and inhibited by CoA (Bolton & Harwood, 1977*a*). On the other hand, these particulate preparations are also capable of lengthening different acyl residues added in the form of complex lipids such as phosphatidylcholine (Bolton & Harwood, 1977*a*; Jordan & Harwood, 1980). In addition, a significant increase in the total incorporation of radioactivity from [^{14}C]malonyl-CoA was noticed when exogenous lipids were present in the incubation medium. Since the elongation of acyl residues added as complex lipids must involve their hydrolysis to unesterified fatty acids, the effect of two lipases was studied recently (Jordan & Harwood, 1980; Harwood, 1980).

The synthesis of plant fatty acids and their incorporation into complex lipids is under careful control *in vivo* and it has been suggested that the

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interrelation of acyl-CoA and acyl–acyl-carrier proteins via a 'switching mechanism' is particularly important (Stumpf, 1977, 1980). Other workers (Shine *et al.*, 1976*a*; Bertrams & Heinz, 1980; Joyard & Stumpf, 1980; Roughan *et al.*, 1980) including ourselves (Bolton & Harwood, 1977*a*,*b*) have reported data that support an important role for acyl-CoA in the movement of newly synthesized fatty acids into complex lipids. In view of these results we decided to study the nature of the products of pea microsomal fatty acid synthesis particularly in relation to acyl thioesters and the data are reported here.

Experimental

Materials

Pea (*Pisum sativum* cultivar Feltham First) seeds were purchased from Asmer Seeds, Leicester, U.K., and were surface-sterilized and germinated as previously described (Bolton & Harwood, 1977*a*). [2-^{14}C]Malonyl-CoA (sp. radioactivity 53 Ci/mol) and [1-^{14}C]acetate (sp. radioactivity 57.8 Ci/mol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. ATP, NADH, NADPH and CoA were purchased from Sigma (London) Chemical Co., Kingston upon Thames, Surrey KT2 7BH, U.K. Acyl-carrier protein from *Escherichia coli* was prepared by the procedure of Sauer *et al.* (1964).

Subcellular fractionation

After 24h of germination, pea seeds were homogenized and the microsomal fraction was isolated as previously (Bolton & Harwood, 1977*a*). The microsomal pellet was resuspended in 0.32M-sucrose/5mM-Tris/HCl, pH 7.4, and used in incubations immediately. Marker analysis showed that the microsomal fraction contained about 80% of the total recovery of typical endoplasmic-reticulum enzymes such as glucose 6-phosphatase, UDPase or unspecific esterase, but only small amounts of mitochondrial enzymes and no detectable chlorophyll.

Incubations

Unless otherwise stated the reaction mixture contained NADH (0.67μmol), NADPH (0.67μmol), ATP (3μmol), *E. coli* acyl-carrier protein (1mg), 0.02μCi of [^{14}C]malonyl-CoA and 0.4ml of microsomal fraction made up to a volume of 1.0ml with potassium phosphate buffer, pH 7.0 (0.03M final concentration). Incubations were carried out at 25°C in triplicate with continuous shaking and, unless otherwise stated, for 4h.

For the preparation of radiolabelled microsomal fraction, pea seeds were surface sterilized and germinated in separate tubes containing 1μCi of [^{14}C]acetate in 0.2ml of chloramphenicol (50mg/ml) dissolved in sterile water. After 2h, 0.5ml of chloramphenicol solution (50mg/ml) was added and germination was continued for 24h. At the end of the incubation, the seeds were thoroughly rinsed and the microsomal fraction was isolated as above.

Analytical procedure

Analysis of reaction products was carried out by the method of Mancha *et al.* (1975). In these cases the incubations were terminated by the addition of 1ml of 2.5% (w/v) acetic acid in propan-2-ol. For total fatty acid analysis, the reaction was stopped and extraction was carried out by the method of Bolton & Harwood (1977*a*).

Separation of acyl lipids was made by t.l.c. on silica-gel G (E. Merck, Darmstadt, Germany) plates, with light petroleum (b.p. 60–80°C)/diethyl ether/acetic acid (90:30:1, by vol.) as solvent.

Fatty acid esters were prepared by transmethylation with 2.5% (v/v) H_2SO_4 in methanol. The methyl esters were separated by g.l.c. in 15% (w/w) EGSS-X on chromosorb W AW (80–100 mesh) or in 3% (w/w) SE-30 on the same support using a Pye 104 or Pye GCD gas chromatography coupled to a Panax radioactivity detector. Pentadecanoate was used as an internal standard.

Separation of methyl esters on the basis of unsaturation was carried out on silica-gel H plates impregnated with 5% (w/w) $AgNO_3$ and developed with light petroleum/diethyl ether (9:1, v/v) solvent. Bands were revealed under u.v. light after spraying with 2,7′-dichlorofluorescein, scraped and eluted with diethyl ether.

Catalytic hydrogenation of methyl esters was performed by the method of Kates (1972) with PtO_2 as catalyst. Protein determination was carried out by the method of Lowry *et al.* (1951) in the presence of 0.1% (w/v) sodium deoxycholate, with bovine serum albumin as standard. Radioactivity estimation was as previously described (Bolton & Harwood, 1977*a*).

Results and discussion

Previous results from this laboratory (Bolton & Harwood, 1977*a*) indicated that microsomal preparations from the germinating pea (cv. Kelvedon Wonder) incorporated a high percentage of radioactivity from [^{14}C]malonyl-CoA into acyl thioesters. However, the major labelled fraction contained the unesterified fatty acids. The pea cultivar Feltham First has a somewhat different fatty acid metabolism (Jordan & Harwood, 1980) and the nature of the fatty acid products in this variety is shown in Table 1. It will be seen that acyl–acyl-carrier proteins are the major products with smaller amounts of acyl lipids and acyl CoA. Within

Table 1. *Distribution of radioactivity amongst different acyl fractions labelled from [^{14}C]malonyl-CoA by the microsomal fraction from germinating pea*

For details of fatty acid synthesis incubations and lipid anlaysis see the Experimental section. Results are means ± S.D. for the numbers of experiments in parentheses.

Fraction	^{14}C-labelled fatty acids (% of total)
Acyl–acyl-carrier proteins	51.0 ± 2.2 (6)
Acyl-CoA	14.8 ± 2.4 (6)
Acyl-lipids	34.2 ± 4.7 (6)
Triacylglycerols	2.2 ± 0.1 (2)
Non-esterified fatty acids	3.2 ± 1.7 (2)
Diacylglycerols	4.9 ± 0.9 (2)
Phospholipids	23.5 ± 2.6 (2)

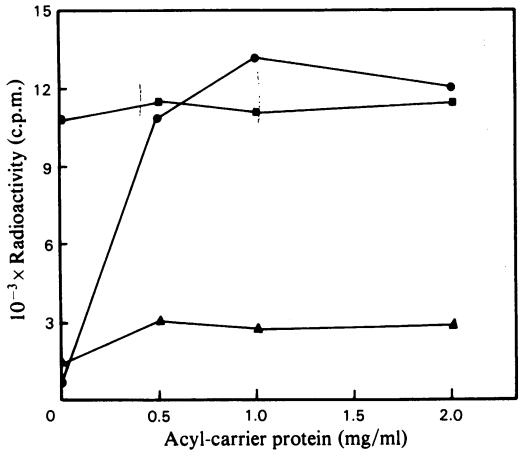

Fig. 1. *Incorporation of radioactivity from [^{14}C]-malonyl-CoA into the acyl residues of lipids (■), acyl–acyl-carrier proteins (●) and acyl-CoA (▲) in relation to the concentration of exogenous acyl-carrier protein in the incubation medium*

the acyl lipid fraction, phospholipids (mainly phosphatidylcholine) contained the largest proportion of the radioactivity. Unesterified fatty acids were minor products, presumably because of a lack of acyl hydrolase activity in microsomes prepared from the cultivar Feltham First. The results fit in well with the requirement for the exogenous acyl-carrier protein previously demonstrated (Bolton & Harwood, 1977*a*; Jordan & Harwood, 1980) and also suggest a small acyl thioesterase activity (Shine *et al.*, 1976*a*).

It was of interest to determine the effect of the concentration of acyl-carrier protein, since this acyl carrier has been demonstrated to modify the nature of the products formed by spinach stroma preparations from [^{14}C]acetate (Packter & Stumpf, 1975) as well as the pattern of radioactive fatty acids synthesized by a soluble fatty acid synthetase from potato tuber when [^{14}C]malonyl-CoA was used as precursor (Huang & Stumpf, 1971). As shown in Fig. 1, addition of exogenous acyl-carrier protein increased the total incorporation of radioactivity into fatty acids up to a concentration of about 1 mg/ml. More interestingly, when the products of the reaction were analysed, it was found that the addition affected incorporation into acyl–acyl-carrier proteins almost exclusively. No variation was found, however, in the pattern of newly synthesized fatty acids incorporated into acyl lipids and acyl–acyl-carrier proteins in substantial agreement with results from spinach (Packter & Stumpf, 1975). It is interesting to emphasize that incorporation of radioactive label into acyl–acyl-carrier proteins was very low when the preparation was tested without exogenous acyl-carrier protein, which suggests that this acyl carrier is only present in the microsomal fraction in small amounts. The lack of an appreciable effect of exogenous acyl-carrier protein on incorporation of radioactivity into polar lipids is in agreement with the results of Shine *et al.* (1976*b*), who showed that acyl–acyl-carrier proteins were not suitable substrates for acyltransferases in microsomes isolated from avocado mesocarp. They suggested that acyl residues must be transferred to phospholipid by a switching system that involves the collaboration of an acyl thiokinase (Shine *et al.*, 1976*a*). This switching system involves the participation of CoA, so it was of interest to test the effect of this carrier in our system. However, as shown previously by Bolton & Harwood (1977*a*) addition of CoA causes a very high inhibition of fatty acid synthesis and, therefore, we attempted to follow its effect by adding the nucleotide part-way through the incubation. Examination of the time course of the incubation (Fig. 2*a*) revealed that incorporation of radioactivity was linear for about 4 h with the distribution of label between the different fractions remaining constant. As the synthetic rate decreased after 4 h, radioactivity disappeared from the acyl–acyl-carrier-protein fraction and increased in acyl-CoA and polar lipids. Addition of CoA, at a concentration that had previously been found to severely inhibit total synthesis, caused an immediate decrease in the acyl–acyl-carrier proteins and an increase in the polar lipids and acyl-CoA (Fig. 2*b*).

The distribution of radioactivity in the polar lipids after the addition of CoA is shown in Table 2. It will be seen clearly that the increase in the relative labelling of the polar lipid fraction after prolonged incubation or the addition of CoA is confined exclusively to the phospholipids. Interestingly, the addition of exogenous CoA did not increase the

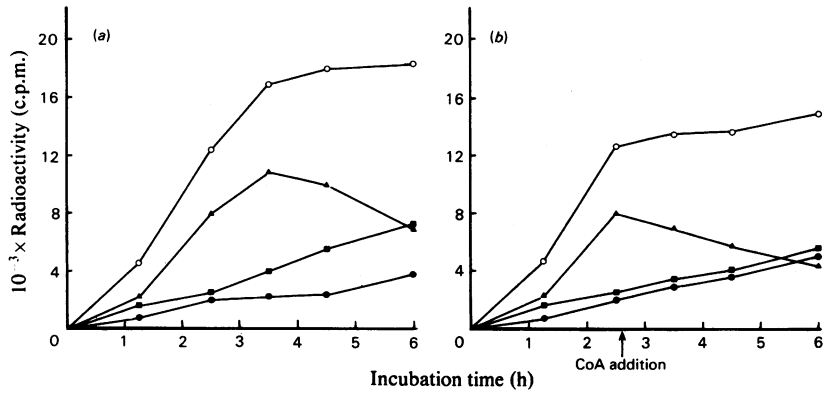

Fig. 2. *Changes in the distribution of radioactivity between different acyl fractions separated after incubation of pea microsomes with [^{14}C]malonyl-CoA*

Incubation was carried out as described in the Experimental section. Samples were taken at the times indicated and the incorporation of radiolabel into lipids (■), acyl–acyl-carrier proteins (▲) and acyl-CoA (●) was determined. The effect of addition of CoA (1 μmol) after 2.5 h of incubation (*b*) was compared with the control experiment (*a*). Total incorporation into fatty acids is also shown (○).

Table 2. *Pattern of labelling of different lipid classes during the time course of incubations in the presence or absence of CoA*

Microsomal fractions were prepared and incubated as detailed in the Experimental section. CoA was added at a concentration of 1 mM after 2.75 h. For analysis of fractions see the Experimental section. Values indicate the percentage of total radiolabel incorporated as means ± S.D. Abbreviations used: TG, triacylglycerols; FA, non-esterified fatty acids; DG + MG, diacylglycerols + monoacylglycerols; PL, phospholipids; ACP, acyl-carrier protein.

Incubation time (h)		Lipid class ...	Radioactivity (% of total lipid radioactivity)					
			TG	FA	DG + MG	PL	Acyl–ACP	Acyl-CoA
No CoA	2.75		2.6 ± 0.2	2.1 ± 0.1	6.3 ± 0.1	24.8 ± 3.1	46.9 ± 4.8	17.4 ± 5.2
	4.50		2.3 ± 0.1	1.9 ± 0.5	5.6 ± 0.4	25.6 ± 1.1	43.2 ± 1.5	21.5 ± 1.2
	6.25		2.5 ± 0.1	2.1 ± 0.4	6.9 ± 0.9	33.0 ± 1.3	34.1 ± 1.1	21.3 ± 0.3
	8.00		2.2 ± 0.1	2.9 ± 0.9	7.1 ± 1.0	34.9 ± 0.5	35.7 ± 0.4	17.3 ± 1.7
+ CoA	4.50		3.1 ± 0.6	2.3 ± 0.8	7.7 ± 0.6	27.3 ± 6.7	34.2 ± 1.6	25.4 ± 0.2
	6.25		2.5 ± 0.5	2.4 ± 0.4	6.5 ± 0.9	33.1 ± 3.8	24.7 ± 0.6	30.9 ± 0.2
	8.00		2.8 ± 0.4	2.6 ± 0.5	6.9 ± 0.8	34.4 ± 2.4	19.3 ± 0.5	34.0 ± 2.0

proportion of radioactivity in phospholipids after 8 h. The main effect of the CoA, apart from decreasing total incorporation, was to alter the distribution of acyl residues between the thioester classes. If the transfer of newly synthesized residues from acyl–acyl-carrier proteins to phospholipids takes place with the mediation of acyl-CoA as proposed by Shine *et al.* (1976*a*) and as suggested by our results with exogenous acyl-carrier protein (Fig. 1 and Table 1) then it would appear that acyl transfer from acyl-CoA to phospholipid is rate-limiting in our incubations.

In Table 3 it will be seen that a significant proportion of the total acyl fatty acids produced by the microsomal fraction were 'odd-chain' saturated acids. These unusual fatty acids appeared both in lipids and as acyl thioesters. Their identity was confirmed by t.l.c. on $AgNO_3$-impregnated plates, g.l.c. on polar and non-polar stationary phases and catalytic hydrogenation. Odd-chain fatty acids are often minor components of plant lipids *in vivo* (Hitchcock & Nichols, 1971) and frequently appear in small amounts during fatty acid synthesis *in vitro* (Stumpf & James, 1963). However, the only other report of significant quantities of these unusual fatty acids is the fatty acid-synthesizing system in the microsomal fraction from leek (Cassagne & Lessire, 1978). It was suggested that these acids could be formed as a result of α-oxidation (Harwood, 1979). As a first attempt to clarify the problem, we tested

Table 3. *The distribution of radioactivity in the fatty acids of the lipids and acyl thioesters produced by the microsomal fractions from germinating pea*

For details of incubation and analysis see the Experimental section. For the nature of the lipid fractions see Sanchez & Mancha (1980). Results are means ± S.D. (where appropriate) for independent experiments whose number is indicated in parentheses. Abbreviation used: n.d., not detected.

	Distribution of radioactivity (% of total ^{14}C-labelled fatty acids)						
Acyl fraction	$C_{12:0}$	$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{17:0}$	$C_{18:0}$	$C_{20:0}$
Lipids (4)	1 ± 1	3 ± 1	5 ± 2	22 ± 5	19 ± 3	32 ± 3	18 ± 6
Acyl–acyl-carrier proteins (4)	1 ± 1	6 ± 3	10 ± 2	26 ± 1	20 ± 3	37 ± 7	n.d.
Acyl-CoA (1)	2	6	10	28	21	34	n.d.

Table 4. *Products of fatty acid synthesis from [^{14}C]malonyl-CoA in vitro compared with those from [^{14}C]acetate in vivo*

The enzyme fraction used for the incubation *in vitro* was the 18000*g* supernatant (cf. Bolton & Harwood, 1977*a*), which contained the microsomal and soluble fractions. For details of incubations and analysis see the Experimental section. The analysis *in vivo* was of the microsomal fraction obtained from seeds that had been germinated in the presence of [^{14}C]acetate for 24h as described in the Experimental section. Abbreviation used: n.d., not detected.

			Distribution of radioactivity (% of total ^{14}C-labelled fatty acids)					
Experiment	Fraction analysed	Incorporation (% of total)	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{20:0}$	$C_{22:0}$
In vitro	Total fatty acids	100	2	4	37	48	4	7
	Acyl lipids	49.6	n.d.	4	35	40	8	14
	Acyl–acyl-carrier proteins	25.2	2	5	22	71	n.d.	n.d.
	Acyl-CoA	25.2	2	4	54	40	n.d.	n.d.
In vivo	Total fatty acids	100	n.d.	1	28	47	11	13
	Acyl lipids	66.1	n.d.	3	26	41	12	18
	Acyl–acyl-carrier proteins	0.9	Not measured					
	Acyl-CoA	33.0	n.d.	n.d.	63	37	n.d.	n.d.

the pea microsomal preparation in the absence of oxygen, since it has been demonstrated that the latter is a requirement for α-oxidation (Shine & Stumpf, 1974). In addition, it has been demonstrated that imidazole is an inhibitor of such systems (Galliard, 1975) and the reagent prevents the formation of α-hydroxy fatty acids in the soluble fractions from germinating pea (Jordan & Harwood, 1979). Neither of these treatments was found to affect the synthesis of odd-chain fatty acids in the pea microsomal fraction. Accordingly, we suggest that these unusual fatty acids are formed as a result of the use of an odd-chain-length primer instead of acetyl-CoA by the fatty acid synthetase.

Table 3 shows that acyl thioesters contained a higher proportion of radioactivity in shorter-chain fatty acids than the lipid fraction. In addition, no very-long-chain fatty acids were detected as acyl thioesters. The absence of very-long-chain fatty acids in the acyl–acyl-carrier-proteins is in agreement with the generally accepted opinion that these molecules are not suitable substrates for the elongase (or elongases) responsible for the synthesis of very-long-chain fatty acids (Bolton & Harwood, 1977*a*; Cassagne & Lessire, 1978) and with the absence of these fatty acids in the composition of acyl thioesters isolated from several plant tissues (Sanchez & Mancha, 1980; Sanchez, 1980).

The appearance of odd-chain fatty acids and the specific location of very-long-chain fatty acids in the lipid fraction of the pea microsomes led us to compare these results with the same fraction labelled *in vivo*. When pea seeds were allowed to incorporate radioactivity from [^{14}C]acetate and the microsomal fraction (which was the most highly labelled fraction) was subsequently isolated, the distribution of label was as shown in Table 4. In contrast with the incubation of microsomes with [^{14}C]malonyl-CoA, no odd-chain fatty acids were detected nor were acyl–acyl-carrier-proteins significantly labelled. However, in agreement with the incubation of microsomes *in vitro*, no trace of the very-long-chain fatty acids was found in the acyl thioesters. Since the nature of the final products of fatty acid synthesis in any given subcellular fraction must depend on the total content of relevant enzymes there and because high rates of fatty acid synthesis are also found in the high-speed supernatant fraction from pea (Bolton & Harwood, 1977*b*), we tested the 18000*g* supernatant. It was

noteworthy that this fraction (which contains the microsomal and supernatant fractions) resulted in a distribution of products that was much nearer the situation *in vivo* than incubation of either the microsomal fraction (Tables 1 and 3) or the high-speed supernatant (Bolton & Harwood, 1977*b*) alone. Undoubtedly the microsomal fraction contained stearate elongase and acyltransferases, which the soluble fraction lacked, and the presence of the latter prevented synthesis of unusual products such as the odd-chain fatty acids. These results provide a clear illustration of the value of using purified systems to define particular enzyme steps and also of using systems *in vivo* or systems *in vitro* that are less-purified to follow overall metabolic sequences. They also support the role of acyl-CoA as intermediate in a switching mechanism for the esterification of complex lipids (Shine *et al.*, 1976*a*). It will be of great interest to see how far these results are applicable to other higher-plant tissues.

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