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# Fatty Acid Biosynthesis by a Particulate Preparation from Germinating Pea

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1. Fatty acid synthesis was studied in microsomal preparations from germinating pea (*Pisum sativum*). 2. The preparations synthesized a mixture of saturated fatty acids up to a chain length of $C_{24}$ from [$^{14}$C]malonyl-CoA. 3. Whereas hexadecanoic acid was made *de novo*, octadecanoic acid and icosanoic acid were synthesized by elongation. 4. The products formed during [$^{14}$C]malonyl-CoA incubation were analysed, and unesterified fatty acids and polar lipids were found to be major products. [$^{14}$C]Palmitic acid represented a high percentage of the acyl-carrier protein esters, whereas $^{14}$C-labelled very-long-chain fatty acids were mainly present as unesterified fatty acids. CoA esters were minor products. 5. The addition of exogenous lipids to the incubation system usually resulted in stimulation of [$^{14}$C]malonyl-CoA incorporation into fatty acids. The greatest stimulation was obtained with dipalmitoyl phosphatidylcholine. Both exogenous palmitic acid and dipalmitoyl phosphatidylcholine increased the amount of [$^{14}$C]-stearic acid synthesized, relative to [$^{14}$C]palmitic acid. Addition of stearic acid increased the amount of [$^{14}$C]icosanoic acid formed. 6. [$^{14}$C]Stearic acid was elongated more effectively to icosanoic acid than [$^{14}$C]stearoyl-CoA, and its conversion was not decreased by addition of unlabelled stearoyl-CoA. 7. Incorporation of [$^{14}$C]malonyl-CoA into fatty acids was markedly decreased by iodoacetamide and 5,5′-dithiobis-(2-nitrobenzoic acid). Palmitate elongation was sensitive to arsenite addition, and stearate elongation to the presence of Triton X-100 or fluoride. The action of fluoride was not, apparently, due to chelation. 8. The microsomal preparations differed from soluble fractions from germinating pea in (*a*) synthesizing very-long-chain fatty acids, (*b*) not utilizing exogenous palmitate–acyl-carrier protein as a substrate for palmitate elongation and (*c*) having fatty acid synthesis stimulated by the addition of certain complex lipids.

Saturated fatty acids appear to be produced in plants by the action of at least three separate enzymes (see Harwood, 1975*a*). These are, respectively, fatty acid synthetase, which forms palmitic acid, palmitate elongase, which produces stearate, and at least one further elongase which synthesizes the very-long-chain fatty acids ($\geq C_{20}$). Evidence for separate enzymes came first, from labelling studies (Harwood & Stumpf, 1970; Hawke & Stumpf, 1965; Macey & Stumpf, 1968), secondly from the effect of specific chemicals (Bolton & Harwood, 1976*a*,*b*; Harwood & Stumpf, 1971), and thirdly, by the study of partly purified enzymes. Fatty acid synthetase activity has been prepared free of elongase activity from avocado (Harwood & Stumpf, 1972*a*) and potato (Huang & Stumpf, 1971), and palmitate elongase has also been examined (Harwood, 1974; Jaworski *et al.*, 1974).

The question of the subcellular localization of these activities has occupied the attention of several workers. Yang & Stumpf (1965) have shown that particulate as well as soluble fractions from avocado could synthesize fatty acids, the relative activity of different preparations depending on the substrate used. Macey & Stumpf (1968) also recorded synthesis of fatty acids in some subcellular fractions from the shoots of germinating pea, and the situation was examined in more detail later in a number of germinating seeds (Harwood & Stumpf, 1972*b*). Several subcellular fractions from plants apparently contain fatty acid-synthesizing ability in the form of fatty acid synthetase, elongases or desaturases, though it has been claimed by Weaire & Kekwick (1975*a*) that all synthesis is present in the plastid (cf. Harwood, 1975*a*). Although palmitate elongase is present in the chloroplast (plastid) stroma (Harwood, 1974; Stumpf, 1975; Weaire & Kekwick, 1975*a*), evidence has been produced for some soluble fatty acid synthetase that is cytoplasmic in origin (Harwood, 1974; Harwood & Stumpf, 1972*b*).

Synthesis of the very-long-chain fatty acids, in contrast, appears to be microsomally localized (Bolton & Harwood, 1976*a*; Harwood & Stumpf, 1971; Kolattukudy & Buckner, 1972; Macey & Stumpf, 1968). The microsomal membranes also

contain fatty acid synthetase and palmitate elongase as well as various fatty acid desaturases (cf. Harwood, 1975*a*). To gain more information about the characteristics and integration of this complex system we have studied fatty acid synthesis by microsomal preparations from germinating pea, which characteristically form only saturated fatty acids (Harwood & Stumpf, 1971). A preliminary communication of some of the results has been presented (Harwood & Bolton, 1977).

## Experimental

### *Materials*

Pea (*Pisum sativum*, cultivar Kelvedon Wonder) seeds were purchased from Thompson and Morgan, Ipswich IP2 0BA, Suffolk, U.K. They were germinated in sterile water in sterile tubes at 20°C by using a 16h light/8h dark cycle in an illuminated incubator.

[1,3-$^{14}$C]Malonyl-CoA (22.4mCi/mmol) was purchased from New England Nuclear Corp., Boston, MA, U.S.A., and [1-$^{14}$C]palmitic acid (57.9mCi/mmol) and [1-$^{14}$C]stearic acid (59.7mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. Di[1-$^{14}$C]palmitoyl *sn*-phosphatidylcholine was prepared by the reaction of the $CdCl_2$ adduct of glycerol phosphocholine (Chadha, 1970) with [1-$^{14}$C]palmitoyl chloride (R. Aneja, personal communication). [1-$^{14}$C]Stearoyl-CoA (1mCi/mmol) was from New England Nuclear and [$^{14}$C]stearoyl phosphatidylcholine was prepared by the reduction of [$^{14}$C]phosphatidylcholine from *Vicia faba* which had been labelled with [1-$^{14}$C]acetate (Heinz & Harwood, 1977).

Acyl-carrier protein was purified from *Escherichia coli* (Sauer *et al.*, 1964). [$^{14}$C]Palmitoyl-acyl-carrier protein and [$^{14}$C]stearoyl-acyl-carrier protein were prepared by the procedure of Jaworski & Stumpf (1974) by using the stromal fraction from spinach (*Spinacea oleracea*).

Dipalmitoyl *sn*-phosphatidylcholine, distearoyl *sn*-phosphatidylcholine, palmitic acid, stearic acid, glyceryl tripalmitate and dipalmitoyl *sn*-phosphatidylethanolamine were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K. The purity of all compounds was checked by t.l.c. and g.l.c., and was found to be over 98%, except for glyceryl tripalmitate. The latter was further purified by t.l.c. (see below) to apparent homogeneity.

NADH, ATP, 5,5′-dithiobis-(2-nitrobenzoic acid), EDTA (sodium salt), EGTA, *p*-chloromercuribenzoic acid, iodoacetamide, *o*-phenanthroline, sodium deoxycholate, malonyl-CoA and reduced glutathione (GSH) were from Sigma; NaF and sodium arsenite were from BDH Chemicals, Poole, Dorset, U.K., and NADPH and CoA (lithium salt) were from Boehringer Corp. (London) Ltd., Lewes, Sussex BN7 1LG, U.K.

$BF_3$, 14% (w/v) in methanol was from BDH, and diazomethane was prepared from Diazald (ICN Pharmaceuticals, Plainview, NY, U.S.A.) by treatment with KOH. Redistilled methanol was prepared after refluxing over NaOH and $KMnO_4$, redistilled chloroform after shaking with saturated $Na_2SO_3$ and sodium metasulphite solution and redistilled diethyl ether prepared after removal of peroxides with $FeSO_4$. All starting solvents for these distillations and other chemicals used were of A.R. grade and were purchased from BDH, Sigma or Koch–Light Laboratories, Colnbrook, Bucks., U.K.

### *Methods*

*Subcellular fractionation.* Pea seeds were germinated for set times (usually 24h) and were ground in a pestle and mortar at 4°C in a solution of 0.32M-sucrose/2mM-Tris/HCl, pH7.4. The homogenate was filtered through three layers of Miracloth and fractionated by the procedure of Harwood & Stumpf (1972*b*). For the preparation of microsomal fractions only, the homogenate was centrifuged at 6000*g* for 10min. The supernatant was then centrifuged at 18000*g* for 20min and the supernatant centrifuged at 105000*g* for 60min to yield the microsomal pellet. This was resuspended, by using a Potter–Elvehjem homogenizer, in 0.32M-sucrose/2mM-Tris/HCl, pH7.4.

The purity of subcellular fractions was assessed by using enzyme markers as detailed previously (Harwood & Stumpf, 1972*b*).

*Fatty acid analysis.* This was measured by incubating subcellular fractions in a mixture containing NADH(0.5$\mu$mol), NADPH(0.5$\mu$mol), ATP(2$\mu$mol), acyl-carrier protein (0.5mg), [1,3-$^{14}$C]malonyl-CoA (0.1$\mu$mol) and potassium phosphate buffer, pH7.0 (20$\mu$mol), of final volume 1.0ml. The above concentrations of reagents were found to be optimal and the reaction was linear with time at 25°C for at least 4h. After 4h or shorter times, the incubation was terminated by the addition of 0.1ml of 60% (w/v) KOH, except when intact acyl esters were to be determined (see below). Hydrolysis and extraction were carried out as previously described (Harwood & Stumpf, 1971). Fatty acid methyl esters were prepared by refluxing lipid samples in 2% (v/v) $H_2SO_4$ in methanol. Samples of the light-petroleum (b.p. 60–80°C) extracts were then taken for radioactivity measurement and g.l.c. analysis (see below).

When the action of inhibitors was assessed the chemicals were added directly to the incubation medium or, alternatively, were preincubated with the microsomal fraction only at 25°C for 15min. Control microsomal samples were similarly incubated with water instead of inhibitor. Concentrations of inhibitors used for preincubation were calculated

to give the same final concentration as for the samples that were not preincubated.

For experiments with exogenous lipids, the latter were highly sonicated (20×10s at 20kHz with 20s intervals) in 0.32M-sucrose/2mM-Tris/HCl, pH 7.4. Portions were then added to the incubation solution. For control experiments on modification of membrane composition, the microsomal fraction was then re-isolated as detailed by Houslay *et al.* (1976) for lipid-substituted membranes or fused lipid–membrane complexes.

*Solubilization.* Microsomal fractions were treated with Triton X-100 and solubilized proteins were separated on columns (1cm×10cm) of Sephadex G-75, G-100 or G-200 by using 10mM-potassium phosphate buffer, pH7.0, containing 0.1% Triton X-100. Different fractions were made 60% saturated by the addition of saturated $(NH_4)_2SO_4$, and the precipitated protein was sedimented at 5000*g* for 30min. The pellet was redissolved in 0.32M-sucrose/2mM-Tris/HCl, pH7.4, and dialysed overnight at 4°C against the same solution.

*Separation and analysis of lipids.* Fatty acid methyl esters were prepared with diazomethane and 2% (v/v) $H_2SO_4$ in methanol, or by transmethylation with $H_2SO_4$/methanol or $BF_3$ 14% (w/v) in methanol. They were separated in 15% (w/w) diethylene glycol succinate on Chromosorb W AW (80–100 mesh; Supelco, Bellefont, PA 16823, U.S.A.) or in 15% (w/w) EGSS-X on Supelcoport (80–100 mesh; Supelco) by using either isothermal or temperature programming in the range 140–190°C on a Perkin–Elmer F33 or a Pye 104 gas chromatograph fitted with a Panax radioactivity detector. An internal standard of methyl pentadecanoate was used to quantify the data.

Identification of individual fatty acids was based on their retention time in comparison with authentic fatty acid standards (Nu-Chek Prep. Inc., Elysian, MN 56028, U.S.A.). For confirmation, material from individual peaks was collected and subjected to reduction (Roehm & Privett, 1969) and oxidation (Downing & Greene, 1968) and the breakdown products were examined by g.l.c. $^{14}C$-labelled fatty acid products were isolated and subjected to α-oxidation (Harris *et al.*, 1965) and Schmidt decarboxylation (Brady *et al.*, 1960) to determine the extent of synthesis *de novo* against elongation.

Intact lipids were separated by t.l.c. on silica-gel G (E. Merck, Darmstadt, Germany) plates by using light petroleum (b.p. 40–60°C)/diethyl ether/acetic acid (90:10:1, by vol.) to separate neutral lipids and chloroform / methanol / acetic acid / water (170:30:20:7, by vol.) for polar lipids. Lipid bands were revealed with $I_2$ vapour or aq. 0.001% (w/v) Rhodamine 6G (when fatty acids were to be analysed). Lipids were provisionally identified by co-chromatography with standards and by differential colour reactions with phosphate, ninhydrin and Dragendorff sprays. They were also eluted from the t.l.c. plates with chloroform, followed by chloroform/methanol/acetic acid (200:100:1, by vol.), and phospholipids were identified as previously described (Harwood *et al.*, 1975).

*Identification of reaction products.* The nature of the radioactive products formed from microsomal incubations was determined by the method of Mancha *et al.* (1975). Where necessary, individual fractions were further separated as described above.

*Protein determination.* This was performed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

*Radioactivity measurement.* Samples were counted for radioactivity in a scintillant consisting of PCS (Amersham–Searle, The Radiochemical Centre, Amersham, Bucks., U.K.)/xylene, 2:1 (v/v). Samples were corrected for quenching by the internal-standard method. Radioactive samples from thin-layer plates were counted directly and quenching was found to be low provided that not more than 25mg of silica gel was present per vial.

## Results and Discussion

Germinating pea has been found to synthesize almost entirely saturated fatty acids *in vivo* during the

Table 1. *Synthesis of fatty acids by subcellular fractions from germinating pea*

Isolation of fractions, incubation conditions with [1,3-$^{14}C$]malonyl-CoA and analysis are detailed in the Experimental section. * = 44% oleic acid; n.d., not detected; tr, trace < 0.5%.

| Fraction | Fatty acid synthesis (pmol/min per mg of protein) | Fatty acids (% of total) | | | | | |
|---|---|---|---|---|---|---|---|
| | | $C_{14:0}$ | $C_{16:0}$ | $C_{18:0}$ | $C_{20:0}$ | $C_{22:0}$ | Other |
| Nuclear | 2.3 | 6 | 38 | 44 | n.d. | n.d. | 12 |
| Mitochondrial | 8.2 | 2 | 41 | 7 | n.d. | n.d. | 50* |
| Microbody | 7.8 | 5 | 39 | 40 | tr | n.d. | 16 |
| Lysosomal | 6.8 | 3 | 40 | 52 | 2 | tr | 3 |
| Microsomal | 13.8 | tr | 31 | 45 | 13 | 7 | 4 |
| Particle-free supernatant | 14.4 | tr | 23 | 75 | tr | n.d. | 2 |

initial stages of germination (Harwood & Stumpf, 1970). This was true regardless of whether the precursor was $^3H_2O$ or [$^{14}$C]acetate. Typically, a considerable proportion of radioactive very-long-chain fatty acids (chain length > $C_{20}$) were also seen in pea and in seeds of other species, although these compounds only represent minor components on a mass basis. When soluble or microsomal fractions were prepared, they differed markedly in the pattern of fatty acids that were synthesized (Harwood & Stumpf, 1971; Macey & Stumpf, 1968). In Table 1 the fatty acids made from [1,3-$^{14}$C]malonyl-CoA by each individual fraction are shown, together with their relative rates of total fatty acid synthesis. The distribution of activity was similar to that previously found for pea (Harwood & Stumpf, 1972*b*) and showed that the mitochondrial, microsomal and soluble fractions were the most active in terms of protein. The proplastids are present in the mitochondrial fraction, and it is likely that these were responsible for the activity present there, particularly since the pattern of fatty acids made was similar to that in typical plastid preparations (cf. Harwood, 1975*a*). This was also in agreement with the results of Weaire & Kekwick (1975*a*), who showed quite clearly, in preparations from avocado and cauliflower, that plastids rather than mitochondria were responsible for fatty acid synthesis by crude mitochondrial fractions. As expected, saturated very-long-chain fatty acids were made by microsomal fractions (cf. Harwood & Stumpf, 1971; Kolattukudy & Buckner, 1972; Macey & Stumpf, 1968), whereas the soluble fraction synthesized palmitate and stearate, in accordance with previous observations (cf. Harwood, 1975*a*). Accordingly, the microsomal fraction was used for further experiments on the nature of saturated-fatty acid synthesis, particularly the contribution of elongases.

$^{14}$C-labelled fatty acid products were isolated and degraded by chemical methods (see the Experimental section) to determine the extent of elongation compared with synthesis *de novo*. Table 2 shows that, whereas palmitic acid was formed *de novo*, stearic acid and, particularly, icosanoic acid were formed almost entirely by the elongation of unlabelled precursor. This was in agreement with results for stearic acid obtained from experiments *in vivo* with germinating pea (Harwood & Stumpf, 1970) and for [$^{14}$C]icosanoic acid obtained from tissue slices of pea shoots (Macey & Stumpf, 1968).

The cofactor requirement for fatty acid synthesis was determined and the results are shown in Table 3. The data indicated that malonyl-CoA incorporation was particularly dependent on exogenous acyl-carrier protein and NADPH, and less so on NADH. Surprisingly, reduced glutathione, bivalent cations and, particularly, CoA were inhibitory. Macey & Stumpf (1968) also found that $MgCl_2$ caused inhibition of fatty acid synthesis by the microsomal fraction from pea shoots. The inhibition by added CoA is in marked contrast with its requirement for fatty acid synthesis by plastids (e.g. Givan & Stumpf, 1971; Stumpf & Boardman, 1970; Weaire & Kekwick, 1975*a*), and may be due either to a direct competition with elongase enzymes for endogenous acyl groups or by indirectly causing a depletion of essential cofactors. Previous experiments had indicated that synthesis of very-long-chain fatty acids was high at 25°C (Harwood & Stumpf, 1971) and the reaction was found to proceed linearly with time, for at least 4h. No marked difference was seen in the pattern of fatty acids formed when various cofactors were omitted.

To study the stearate-elongation enzyme further, detergent solubilization of microsomal membranes was attempted. Since it is often necessary to retain detergent concentration during subsequent purification steps (e.g. Strittmatter *et al.*, 1974), the effects of different concentrations of various detergents were

Table 2. *Analysis of microsomal fatty acid synthesis by elongation*

Microsomal fractions were incubated with [1,3-$^{14}$C]-malonyl-CoA and radioactive fatty acid products extracted and separated as methyl esters by g.l.c. Individual fatty acids were subjected to chemical degradation as described in the Experimental section. α-Oxidation and decarboxylation analyses were each carried out on fatty acids synthesized by two different microsomal preparations. The ratio of radioactivity peak/mass peak for α-oxidation was taken with [$^{14}$C]$_{16:0}$ acid to $C_{16:0}$ acid as unity.

| Fatty acid | α-Oxidation ratio (radioactivity/mass) | Decarboxylation (% of $^{14}$C released as $CO_2$) |
|---|---|---|
| Hexadecanoic | 1.0 | 12.1 |
| Octadecanoic | 3.7 | 73.6 |
| Icosanoic | 7.7 | 82.4 |

Table 3. *Cofactor requirement for microsomal fatty acid synthesis*

For incubation details see the Experimental section. Results are expressed as means±S.D., with the numbers of experiments in parentheses.

| Additions or omissions | Fatty acid synthesis (% of control) |
|---|---|
| −Acyl-carrier protein | 37±3 (4) |
| −ATP | 83±10(2) |
| −NADPH | 35±3 (2) |
| −NADH | 64±1 (2) |
| +GSH | 82±4 (3) |
| +CoA | 28 (1) |
| +$MnCl_2$ | 54±3 (2) |
| +$MgCl_2$ | 63±5 (2) |

Table 4. *Effect of detergents on microsomal fatty acid biosynthesis*

Means±S.D. ($n=2$) are shown; n.m., not measured; tr, trace (<0.5%). For reaction conditions see the Experimental section.

| Detergent (final concn.) | Fatty acid synthesis (% of control) | $^{14}$C-labelled fatty acids (% of total) | | |
|---|---|---|---|---|
| | | $C_{16:0}$ | $C_{18:0}$ | $>C_{18:0}$ |
| None | 100±3 | 29±3 | 46±1 | 25±2 |
| Triton X-100 (0.1%) | 140±2 | 34±6 | 52±10 | 14±4 |
| Triton X-100 (1%) | 77±6 | 44±11 | 56±8 | tr |
| Sodium dodecyl sulphate (0.1%) | 10±2 | n.m. | | |
| Sodium dodecyl sulphate (1%) | 2±1 | n.m. | | |
| Sodium deoxycholate (0.1%) | 15±2 | n.m. | | |
| Sodium deoxycholate (1%) | 6±1 | n.m. | | |

Table 5. *Analysis of the major acyl-lipids of pea microsomal fraction*

For details of analysis see the Experimental section. The unsaturated fatty acids were identified by oxidation and were shown to be hexadec-9-enoic, octadec-9-enoic, octadeca-9,12-dienoic and octadeca-9,12,15-trienoic. tr, Trace.

| Lipid | Amount (% of total lipid) | Fatty acid distribution (% of total) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | $C_{16:0}$ | $C_{16:1}$ | $C_{18:0}$ | $C_{18:1}$ | $C_{18:2}$ | $C_{18:3}$ | Other |
| Triacylglycerol | 14.9 | 9.9 | 0.2 | 3.2 | 25.8 | 49.1 | 11.4 | 0.4 |
| Unesterified fatty acid | 2.5 | 18.3 | 2.0 | 7.3 | 29.4 | 34.1 | 7.6 | 1.1 |
| Phosphatidylethanolamine | 11.1 | 10.5 | 0.9 | 5.0 | 39.5 | 39.6 | 1.9 | 2.6 |
| Phosphatidylcholine | 53.9 | 4.3 | 0.5 | 4.0 | 45.6 | 43.3 | 2.2 | tr |
| Phosphatidylinositol | 15.8 | 16.6 | 3.4 | 19.4 | 30.3 | 27.0 | tr | 3.3 |
| Sulpholipid | 1.8 | 28.0 | 4.7 | 22.6 | 29.9 | 10.6 | tr | 4.2 |

tested. Whereas sodium dodecyl sulphate and sodium deoxycholate caused considerable inhibition of fatty acid synthesis, Triton X-100 in the range 0.1–0.5% (w/v, final concn.) caused a small stimulation (Table 4). At 1%, some inhibition was seen. The Triton-treated microsomal fraction was centrifuged to separate particulate from 'solubilized' material, and was also subjected to gel filtration (see the Experimental section). About 60% and 70% of the synthetic activity was included within Sephadex G-75 and G-200 columns respectively. Unfortunately, the reasonable amount of solubilization that was achieved did not include an active stearate elongase. As seen in Table 4, Triton X-100 caused a marked decrease in the synthesis of very-long-chain fatty acids. The 'solubilized' microsomal fraction thus resembled the supernatant fraction in the pattern of fatty acids that they made. This may indicate that a membrane environment is important for stearate elongation and that the inhibitory effect of raised incubation temperatures (Bolton & Harwood, 1976*a*; Harwood & Stumpf, 1971) may be due to a perturbation of the normal membrane environment. It is perhaps significant that none of the very-long-chain acyl elongases so far studied in plants is soluble, whether one considers saturated (Bolton & Harwood, 1976*a*,*b*; Harwood & Stumpf, 1971; Kolattukudy & Buckner, 1972; Macey & Stumpf, 1968) or unsaturated (Appleby *et al.*, 1974) fatty acid elongation.

One important question to answer at this stage was: what was the source of the endogenous acyl substrates for elongation? An analysis of microsomal lipids was made and is shown in Table 5. As expected, the principal acyl-lipid component was phosphatidylcholine, with smaller, but considerable, amounts of triacylglycerol, phosphatidylinositol and phosphatidylethanolamine. As expected, the glycolipids were present in small amounts, sulpholipid being the only significant component. Unesterified fatty acids were a minor component. Principal fatty acids of each lipid class were palmitic, oleic and linoleic. Phosphatidylinositol contained relatively high amounts of the saturated acids, palmitic and especially stearic, in keeping with previous observations with plant fractions (Erdahl *et al.*, 1973; Harwood, 1975*b*; Harwood & Stumpf, 1970). In general, the lipid composition and fatty acid contents of individual phospholipids are typical of similar preparations from other plants (cf. Galliard, 1973; Hitchcock & Nichols, 1971). The results shown in Table 5 suggested that any of the complex lipids could be a source of acyl chains for elongation. However, since phospholipids had already been invoked as substrates for fatty acid desaturation (Baker & Lynen, 1971; Pugh & Kates, 1973), they were an obvious

possibility, especially since phosphatidylinositol contained a high concentration of saturated fatty acids and phosphatidylcholine was the most abundant lipid.

Before trying different potential substrates for elongation, we also examined the nature of the final radioactive products, and these are shown in Table 6. Since there are obviously many enzyme activities present in a microsomal fraction, such data may not be readily interpreted. Indeed, radioactive fatty acids are present in all four fractions, with over half in the neutral-lipid fraction. The latter consisted almost entirely of unesterified fatty acids, and phosphatidylcholine was the principal phospholipid labelled. Thus the presence of an active acyl esterase(s) was indicated. It is significant that acyl–acyl-carrier proteins were found for the medium-chain-length acids. This is in keeping with previous observations with plants (Huang & Stumpf, 1971; Packter & Stumpf, 1975) and is presumably due to the activity of fatty acid synthetase. Palmitoyl–acyl-carrier protein is the substrate for palmitate elongase (Harwood, 1974; Jaworski *et al.*, 1974) in soluble extracts from avocado and safflower, where it is converted into stearoyl–acyl-carrier protein. The latter compound can then be used by other plant enzymes and be transferred by thioester transferases to other lipids (Shine *et al.*, 1976). It is not surprising, therefore, that the proportion of stearate as acyl-carrier protein ester is low in the pea system (Table 6). Very-long-chain fatty acids were only found as free fatty acids, indicating either that free fatty acids could be used as substrate by stearate elongase or that a very active esterase removed them from the enzyme complex extremely rapidly. There have been numerous reports on the poor activity of free fatty acids themselves in metabolic reactions in plants (cf. Harwood, 1975*a*; Stumpf, 1975), so that the second possibility seemed the more likely. Since overall fatty acid synthesis was stimulated by added acyl-carrier protein and inhibited by CoA (Table 3), it seemed possible that palmitoyl–acyl-carrier protein and stearoyl–acyl-carrier protein, respectively, could be used by the two microsomal elongases. However, the addition of [$^{14}$C]palmitoyl–acyl-carrier protein and [$^{14}$C]stearoyl–acyl-carrier protein to preparations which actively synthesized the usual pattern of fatty acids from malonyl-CoA resulted in no elongation at all. It is possible that acyl–acyl-carrier proteins are the true substrates for the enzymes, but only when generated *in situ*. Alternatively, other lipids such as acyl-CoA (cf. Bourre *et al.*, 1977; Goldberg *et al.*, 1973) or phospholipids could be utilized.

Because phosphatidylcholine was the major acyl component of microsomal membranes, and since it also contained a significant proportion of both [$^{14}$C]palmitic acid and [$^{14}$C]stearic acid, the effect of its addition to the fatty acid-synthesizing system was tested. Although the addition of exogenous lipids to membrane preparations has been well developed and characterized by Metcalfe and his group (e.g. Houslay *et al.*, 1975, 1976; Warren *et al.*, 1974), preliminary experiments were carried out to check the effect of added dipalmitoyl phosphatidylcholine to microsomal fractions. Addition was carried out under conditions for production of lipid-substituted

Table 6. *Nature of products produced by microsomal fatty acid synthesis*

Methods of analysis are given in the Experimental section. Results are expressed as means±S.D., with the numbers of experiments in parentheses. tr, Trace (<0.5%); n.d., not detected.

| Product | Amount (% of total fatty acid radioactivity) | Distribution of radioactivity (% of total) | | | |
|---|---|---|---|---|---|
| | | <$C_{16}$ | $C_{16:0}$ | $C_{18:0}$ | $C_{20}$–$C_{24}$ |
| Neutral lipids | 56.3±4.9 | tr | 27±8 (3) | 42±1 (3) | 31±8 (3) |
| Polar lipids | 20.3±5.2 | tr | 42±14(3) | 58±18(3) | n.d. |
| Acyl–acyl-carrier protein | 16.4±2.8 | 15±1 (2) | 74±7 (2) | 11±6 (2) | n.d. |
| Acyl-CoA | 7.0±1.2 | n.d. | tr | n.d. | n.d. |

Table 7. *Modification of microsomal membrane composition with exogenous dipalmitoyl phosphatidylcholine*

For details of treatment, isolation and analysis, see the Experimental section. The results are averages for two experiments.

| Microsomal fraction | Fatty acids (% of total) | | | | | |
|---|---|---|---|---|---|---|
| | $C_{16:0}$ | $C_{18:0}$ | $C_{18:1}$ | $C_{18:2}$ | $C_{18:3}$ | Other |
| No addition | 13.2 | 6.2 | 39.7 | 34.9 | 3.7 | 2.3 |
| +Phosphatidylcholine | 53.3 | 3.3 | 20.8 | 18.6 | 1.1 | 2.9 |
| +Phosphatidylcholine (+Triton X-100) | 61.1 | 5.4 | 18.4 | 12.1 | 1.3 | 1.7 |
| Control (+phosphatidylcholine, no reisolation) | 50.2 | 4.2 | 22.9 | 20.0 | 1.7 | 1.0 |

and fused lipid-membrane complexes (see the Experimental section). The results (Table 7) showed that the fatty acid composition of the membrane was modified satisfactorily. As expected, the enrichment of palmitate within the membrane by fusion (no detergent) was very similar to the total lipid composition of the mixture, indicating that fused lipid–membrane complexes were effectively formed.

The effect of lipid substitution in the microsomal fraction was tested for a number of different palmitate-containing and stearate-containing substances. The results (Table 8) from such additions showed that total fatty acid synthesis was increased by the addition of various lipids, particularly those containing palmitic acid. Dipalmitoyl phosphatidylcholine consistently increased the overall rate of stearate synthesis without affecting palmitate formation. On the other hand, palmitic acid and glyceryl tripalmitate caused a decrease in very-long-chain fatty acid production. With palmitate addition, there was a large increase in the relative amount of stearate produced. Dipalmitoyl phosphatidylethanolamine increased overall fatty acid synthesis but without changing the relative proportions of the products. There are most probably two causes. First, some exogenous lipids may be capable of acting as substrates for elongation, either directly or after modification. Thus the increase in relative amounts of stearic acid synthesized when palmitic acid or dipalmitoyl phosphatidylcholine are added could reflect this effect. Secondly, exogenous lipids may affect enzyme activity by altering the membrane environment. Thus the overall increase in incorporation of [$^{14}$C]malonyl-CoA by exogenous lipids or the loss of very-long-chain fatty acid production on addition of palmitate or glyceryl tripalmitate to microsomal fractions may be examples of the latter effect. Such alterations in the activities of membrane-bound enzymes by exogenous lipids are well documented (see Houslay *et al.*, 1976).

The possible involvement of palmitoyl phosphatidylcholine as a substrate for palmitate elongation was tested directly by using [$^{14}$C]dipalmitoyl phosphatidylcholine. A small conversion into [$^{14}$C]-stearate occurred, but the poor rate of elongation did not, however, allow a conclusive characterization of the reaction that excluded transacylation or use of a different substrate (cf. Pugh & Kates, 1973). It is noteworthy that under conditions when the particle-free supernatant, prepared at the same time as the microsomal fraction, quantitatively elongated palmitoyl–acyl-carrier protein to stearate, no conversion at all by microsomal fractions was found.

The addition of three stearate-containing compounds was investigated. Both stearic acid and distearoyl phosphatidylcholine increased the overall rate of fatty acid synthesis (Table 8). The amount of elongation was also increased, with a considerable effect on very-long-chain fatty acid production by stearic acid addition. In the light of the demonstration that brain microsomal preparations, which also contain three saturated-fatty acid-forming systems, utilize acyl-CoA for very-long-chain fatty acid production (cf. Bourre *et al.*, 1977; Goldberg *et al.*, 1973), the effect of [$^{14}$C]stearoyl-CoA addition to the microsomal fraction, under conditions where [$^{14}$C]-malonyl-CoA was actively incorporated into very-long-chain fatty acids, was tested. This incubation resulted in some incorporation of radioactivity into icosanoic acid, but the rate of elongation was less

Table 8. *Effect of exogenous lipids on microsomal fatty acid synthesis*

Incubations and addition of lipids were carried out as described in the Experimental section. n.m., Not measured; n.d., not detected. Microsomal protein was in the range 2.2–3.4mg/incubation. Results are expressed as means±S.D. (where appropriate), with the numbers of experiments in parentheses.

| Addition (mg) | Total fatty acid synthesis (% of control) | No. of expts. | Distribution of radioactivity (% of total fatty acids) | | |
|---|---|---|---|---|---|
| | | | $C_{16:0}$ | $C_{18:0}$ | $C_{20:0}$–$C_{24:0}$ |
| None | 100 | | 31±2 | 50±2 | 19±2 |
| Palmitic acid (0.5) | 86 | 1 | 11 | 89 | n.d. |
| Palmitic acid (1.0) | 141±34 | 2 | n.m. | n.m. | n.m. |
| Glycerol tripalmitate (0.2) | 118 | 1 | 53 | 47 | n.d. |
| Glycerol tripalmitate (1.0) | 180±35 | 2 | n.m. | n.m. | n.m. |
| Dipalmitoyl phosphatidylcholine (0.5) | 127±3 | 4 | 21±4 | 58±6 | 21±3 |
| Dipalmitoyl phosphatidylcholine (1.0) | 191±13 | 3 | n.m. | n.m. | n.m. |
| Dipalmitoyl phosphatidylethanolamine (0.5) | 111 | 1 | n.m. | n.m. | n.m. |
| Dipalmitoyl phosphatidylethanolamine (1.0) | 171±20 | 2 | 31±3 | 50±1 | 19±3 |
| Distearoyl phosphatidylcholine (0.5) | 136±10 | 3 | 20±3 | 55±9 | 25±7 |
| Stearic acid (0.1) | 121±5 | 2 | 19±2 | 35±1 | 46±4 |

than that with [1-$^{14}$C]stearic acid as substrate. Moreover, stearoyl-CoA was hydrolysed during the incubation period to unesterified fatty acid, so that incorporation was not necessarily due to thioester substrate. Addition of unlabelled stearoyl-CoA to incubations with [1-$^{14}$C]stearic acid caused a slight stimulation of conversion into icosanoic acid, an opposite result to that expected if stearoyl-CoA was the substrate for elongation. These results also agree with the analysis of products shown in Table 6, where no CoA esters of stearate or very-long-chain fatty acids were detected. We interpret the stimulatory effect of stearoyl phosphatidylcholine as being due to release of stearic acid (by acylhydrolases) which is then utilized either directly or indirectly by the stearate elongase. It is noteworthy (Table 8) that free stearic acid is much more effective than phospholipid in stimulating elongation. Conclusive proof of the substrate used by the latter enzyme must await the purification of preparations that do not contain hydrolases and transferases.

The presence of membrane-localized, as well as soluble, fatty acid synthetase and palmitate elongase in pea raised the intriguing question of whether the two enzymes each had a bimodal localization or whether separate proteins were involved in each case. Accordingly, the effect of chemicals that typically affected soluble fatty acid synthesis was tested. The results (Table 9) showed that indeed microsomal fatty acid synthesis shared many features in common with synthesis by the 'high-speed' supernatant fraction. Thus both fatty acid synthetase and palmitate elongase are sensitive to thiol reagents, but 1mM-arsenite inhibited elongation but not synthesis *de novo* (Bolton & Harwood, 1976*a*; Harwood & Stumpf, 1971; Huang & Stumpf, 1971; Weaire & Kekwick, 1975*b*). Experiments with a soluble palmitate elongase, palmitoyl–acyl-carrier protein substrate and arsenite inhibition (Jaworski *et al.*, 1974) can be interpreted as indicating that one of the thiol groups that arsenite binds to is similar to that on the acyl-carrier protein (cf. Harwood, 1975*a*).

Fluoride has been found to cause inhibition of stearate elongation (Bolton & Harwood, 1976*a*; Harwood & Stumpf, 1971), and this result was also obtained with microsomal enzyme (Table 9). Although fluoride will act on several metal-containing enzymes, including those dependent on $Fe^{2+}$, $Ca^{2+}$ and $Mg^{2+}$ (Hewitt & Nicholas, 1963), this does not appear to be its mode of action here, since EDTA, EGTA and *o*-phenanthroline did not cause significant inhibition even when preincubated with microsomal fractions. The lack of effect of EDTA on fluoride-sensitive very-long-chain fatty acid synthesis is in keeping with experiments *in vivo* (Harwood & Stumpf, 1971). It is possible that the action of fluoride is through steric hindrance due to complex-formation away from the active site of stearate elongase.

To conclude, membrane-localized fatty acid biosynthesis is very important in plants. Not only is stearate elongase localized there, but, in addition, most fatty acid desaturases as well as significant amounts of fatty acid synthetase and palmitate elongase are present. In spite of this, we know little about the composition, characteristics and spatial arrangement of the enzymes there. The results described above reveal a number of interesting properties of the palmitate elongase and stearate elongase of the pea microsomal fraction. The differences that have been found with regard to substrate utilization in comparison with soluble palmitate elongase and the effects of different exogenous lipids require further investigation. In particular, it would be of interest to see how much the membrane environment determines the properties of the microsomal fatty acid-synthesizing enzymes.

Table 9. *Effect of thiol reagents and chelating agents on fatty acid synthesis by the microsomal fraction from germinating pea*

Means (±S.D. where appropriate) are indicated, with the numbers of experiments in parentheses. The reagent was added to the incubation system, except in experiments indicated by *, where the reagent was preincubated with the particulate fraction (see the Experimental section). n.m., Not measured; tr, trace (<0.5%).

| Reagent (final concn.) | Fatty acid synthesis (% of control) | $^{14}$C-labelled fatty acids (% of total) | | |
|---|---|---|---|---|
| | | $C_{16:0}$ | $C_{18:0}$ | $C_{20:0}$–$C_{24:0}$ |
| No addition | 100 | 24±3 | 54±6 | 22±3 |
| Iodoacetamide (1mM) | 4±1 (2), 5* | n.m. | n.m. | n.m. |
| 5,5′-Dithiobis-(2-nitrobenzoic acid) (1mM) | 11±3 (2), 7* | 57±6 | 35±4 | tr |
| Sodium arsenite (1mM) | 64±5 (5), 57* | 76±6 | 22±5 | 2±1 |
| No addition | 100 | 42±5 | 39±5 | 19±3 |
| EDTA (1mM) | 134±13 (4) | 41±4 | 39±2 | 20±6 |
| EGTA (1mM) | 97±5 (3) | 36±1 | 43±4 | 21±5 |
| *o*-Phenanthroline (1mM) | 94±10 (4), 101* | n.m. | n.m. | n.m. |
| NaF (3mM) | 72±6 (3) | 33±1 | 60±4 | 7±3 |

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