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Lipase-induced alterations of fatty acid synthesis by subcellular fractions from germinating pea (*Pisum sativum* L.)

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1. The effect of exogenous lipases on fatty acid synthesis from [^{14}C]malonyl-CoA by the microsomal and soluble fractions from germinating peas was studied. 2. Addition of phospholipase A_2 or the lipase from *Rhizopus arrhizus* had no effect on total fatty acid synthesis by the soluble fraction but caused severe inhibition of that by the microsomal fraction. 3. The addition of enzymes with phospholipase activity particularly inhibited the microsomal stearate elongase. 4. Control studies indicated that the phospholipase-induced inhibition of fatty acid synthesis was due to the location of fatty acid synthetase, palmitate elongase and stearate elongase on the outside of the microsomal vesicles. 5. Experiments with a trypsin-like proteinase showed that approximately half the microsomal fatty acid synthesis was resistant to proteolysis. 6. Although addition of exogenous phospholipases had no effect on total fatty acid synthesis by the soluble fraction, it did increase α-hydroxylation of newly-formed palmitate and stearate. 7. The results provide further evidence for differences between the soluble and particulate fatty acid synthetase and palmitate elongase activities of germinating pea.

Synthesis of saturated fatty acids is catalysed by fatty acid synthetase and a number of elongases (von Wettstein-Knowles, 1979; Harwood, 1979; Stumpf, 1980). Previous work with the germinating pea has shown that a high-speed particulate fraction (microsomal pellet) contains at least two elongation enzymes, palmitate elongase and stearate elongase (Macey & Stumpf, 1968; Harwood & Stumpf, 1971; Bolton & Harwood, 1977*a*). Both of these enzymes require exogenous malonyl-CoA as a source of two-carbon units, and endogenous or exogenous lipids, but not acyl-acyl carrier proteins, are used for the primer acyl chain (Bolton & Harwood, 1977*a*; Jordan & Harwood, 1980). In addition to elongating pre-formed acyl chains, the pea microsomal fraction also synthesizes palmitate *de novo* (Harwood & Stumpf, 1971; Bolton & Harwood, 1977*a*) in a similar manner to mammalian systems (Bourre *et al.*, 1977; Gan-Elepano & Mead, 1978).

In contrast with the microsomal fraction, the soluble fraction from germinating peas does not contain stearate elongase and its palmitate elongase uses palmitoyl-acyl-carrier protein as the primer substrate (Bolton & Harwood, 1977*b*). Similar distributions of membrane-located and soluble fatty acid synthetase and elongation enzymes have also been reported for other plant tissues (Kolattukudy & Buckner, 1972; Jaworski *et al.*, 1974; Cassagne & Lessire, 1978).

As discussed by Harwood (1979) several plant membrane preparations, which synthesize fatty acids, appear to contain integrated enzyme systems. We were therefore interested in the location of fatty acid synthetase and the elongases in pea microsomal vesicles. It was intended to probe the sided-location of the above enzymes by the use of phospholipases and proteinases. The use of phospholipases would have another action in addition to disturbing the membrane environment. That is, these enzymes would reduce the amounts of membrane phospholipids which provide acyl chains for elongation. However, because palmitate elongase adds the two-carbon units at the carboxy end of the acyl chain (Bolton & Harwood, 1977*a*), hydrolysis of phospholipids must have taken place and there was a possibility that release of fatty acids from membrane phospholipids might increase elongation activity. The results of such experiments are now reported.

Abbreviation used: SDS, sodium dodecyl sulphate.

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Materials and methods

Pea (*Pisum sativum* cv. Feltham First) seeds were purchased from Asmer Seeds, Leicester, U.K. They were surface-sterilized with 5% hypochlorite before germination and allowed to imbibe aerated sterile distilled water at 15°C for 24h.

[2-^{14}C]Malonyl-CoA (sp. radioactivity 2.2GBq/mmol) was purchased from Amersham International, Amersham, Bucks., U.K. Acyl-carrier protein was purified from *Escherichia coli* as far as the DEAE-cellulose-column stage (Sauer *et al.*, 1964). Soya-bean trypsin inhibitor, bovine pancreatic trypsin (type III), lipids and fatty acid standards were purchased from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K., or Nu-Chek Prep. Inc., Elysian, MN, U.S.A. The fatty acid and lipid standards were checked for purity by g.l.c. and t.l.c. and purified further where necessary. Phospholipase A_2 from pig pancreas or *Crotalus adamanteus* and lipase from *Rhizopus arrhizus* were from Boehringer Corporation, Lewes, Sussex, U.K. A trypsin-like serine proteinase that has a very high intrinsic activity at neutral pH towards proteins in their native conformation was purified to homogeneity from rat pancreas as described by Kay *et al.* (1982). The activity of this preparation towards all substrates could be stopped completely and instantaneously by addition of equimolar amounts of soya-bean trypsin inhibitor. Other chemicals were of the best available grades and were obtained from Sigma, Boehringer, Koch–Light (Colnbrook, Bucks., U.K.) or BDH (Poole, Dorset, U.K.).

Subcellular fractionation

Pea seeds were well rinsed in sterile water and homogenized with a pestle and mortar in 0.32M-sucrose/5mM-Tris/HCl buffer, pH7.4, at 4°C. The homogenate was filtered to remove debris and the cell-free filtrate was fractionated as described previously (Bolton & Harwood, 1977*a*). Briefly, this involved centrifugation at 6000*g* for 10min, 18000*g* for 20min and 105000*g* for 60min. The supernatant (which included some loosely-packed membrane) obtained after the centrifugation at 105000*g* for 60min was decanted carefully and designated the soluble fraction. The firmly-packed pellet was resuspended in the homogenizing buffer and, henceforth, will be referred to as the microsomal fraction.

Lipase and proteinase digestions

The action of these enzymes was tested on the microsomal fraction both by pre-incubation and by direct addition to the fatty acid-synthesizing medium. For pre-incubations the phospholipase (up to 1 unit/mg of microsomal protein) was incubated with the microsomal fraction at 25°C for 30min. The solution was then diluted 10-fold with the homogenization buffer and the microsomal fraction was re-isolated by centrifugation at 105000*g* for 60min. Digestion was also carried out on the 18000*g* × 20min supernatant using 0.001–0.05 unit of lipase or 0.1–0.4 unit of pancreatic phospholipase A_2/mg of total protein. After 30min the microsomal and soluble fractions were isolated in the usual way. For direct additions, the lipase or phospholipase enzymes were added to the fatty acid incubation system.

Proteolytic digestions of the microsomal fraction were carried out using up to 40μg of rat pancreatic proteinase or up to 44μg of bovine pancreatic trypsin respectively/mg of microsomal protein for 30min at 25°C. At the end of the incubations, sufficient soya-bean trypsin inhibitor was added to inactivate the proteinases completely and the microsomal fraction was re-isolated as described above. For sonicated preparations, the microsomal fraction was resuspended in the homogenization buffer and sonicated for 30s in a Pulsatron bath sonicator (Kerry Ultrasonics, Hitchin, Herts., U.K.).

Samples for analysis by SDS/polyacrylamide-gel electrophoresis were removed at appropriate times and boiled with 2% (w/v) SDS for 30min. Portions (40μl containing 5–10μg of protein) from each sample were mixed with 10μl of a loading solution of 5% (w/v) SDS, 1.4M-glycerol, 140mM-mercaptoethanol and 25mM-sodium phosphate buffer, pH7.0. After boiling for 5min, Pyronin-Y was added as a tracking dye to each sample. Electrophoresis on 8.5% (w/v) polyacrylamide gels was carried out as described by Kay *et al.* (1982). Gels were removed and stained overnight by the method of Fairbanks *et al.* (1971). The molecular weight of each band was estimated by comparison with the mobilities of proteins of known molecular weight.

Measurement of fatty acid synthesis

Routine incubation conditions consisted of 20μmol of potassium phosphate buffer (pH7.0), 0.67μmol of NADPH, 0.67μmol of NADH, 3.0μmol of ATP, 1.0mg of acyl-carrier protein, 0.42nmol (0.025μCi) of [2-^{14}C]malonyl-CoA and 0.5–2mg of soluble protein or 1–4mg of microsomal protein in a total volume of 1ml. These concentrations have been shown previously to give optimal rates of fatty acid synthesis (Bolton & Harwood, 1977*b*; Jordan & Harwood, 1980). Incubations were allowed to proceed at 25°C for periods of up to 4h. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Lipid extraction and analysis

After incubation, the fatty acid synthesis was terminated by the addition of 0.1ml of KOH (60%, w/v) and hydrolysis, extraction and methylation

were carried out as described previously (Jordan & Harwood, 1980). Portions of the fatty acid methyl esters were taken for radioactivity estimation and gas–liquid-chromatographic analysis. They were separated in 15% (w/w) EGSS-X on Supelcoport (80–100 mesh; Supelco Inc., Bellefonte, PA, U.S.A.) columns using a temperature of 185°C. Quantification was with an internal standard of methyl pentadecanoate and radioactivity was estimated with a Panax gas-flow proportional counter. The fatty acid products were identified as described previously (Bolton & Harwood, 1977*a*; Jordan & Harwood, 1979, 1980).

Radioactive samples were counted in a scintillant consisting of PCS (Amersham–Searle)/xylene (2:1, v/v). Samples were corrected for quenching by the channels-ratio method.

Measurement of microsomal leakage after lipolysis

The permeability of microsomal vesicles after phospholipase digestion was checked by monitoring the leakage of internally-trapped lactate dehydrogenase or 6-carboxyfluorescein. The markers were introduced into the microsomal vesicles as follows. Re-crystallized 6-carboxyfluorescein or rabbit muscle lactate dehydrogenase were dissolved in the 18000 *g* × 20 min supernatant at 2 mg/ml and 70 units/ml respectively. The microsomal fraction was then isolated and resuspended as described above. The microsomal fraction was subjected to pancreatic phospholipase (0.06–6.00 unit/mg of microsomal protein) digestion at 25°C for 30 min. Lactate dehydrogenase activity was assayed in the absence and presence of 0.02% (w/v) Triton X-100 (Pesce *et al.*, 1964). To facilitate the even suspension of microsomes in the presence of Triton, anti-bumping granules were added and the tubes were agitated by using a tube mixer. 6-Carboxyfluorescein was estimated by fluorescence measurement at 520 nm using an activation wavelength of 490 nm and a Perkin–Elmer MPF-2A fluorescence spectrophotometer (Blumenthal *et al.*, 1977). At the concentrations used, internally-trapped 6-carboxyfluorescine was subject to concentration-quenching and did not fluoresce (Blumenthal *et al.*, 1977).

Results and discussion

Effect of phospholipase inhibitors on fatty acid synthesis

Since we wished to study the effects of lipases on soluble as well as membrane-bound fatty acid synthesis, it was necessary to add the enzymes directly to the medium as well as to carry out pre-incubations followed by microsomal re-isolation. Mepacrine (Quinacrine) (Blackwell *et al.*, 1977) and *p*-bromophenacyl bromide (Roberts *et al.*, 1977) have been used as inhibitors of phospholipase A_2 activity and it was intended to use one of these compounds to terminate the effects of the phospholipase. Accordingly, they were tested to ensure that they produced no alteration in fatty acid synthesis. As Table 1 shows, mepacrine at the 5 mM concentration required for phospholipase inhibition reduced the relative amount of stearate product and increased the proportions of myristate and palmitate that are made *de novo* (Bolton & Harwood, 1977*a*). As discussed by Harwood (1979), since the addition of two-carbon units by palmitate elongase occurs at the carboxy end of the acyl chain, then a phospholipase-induced release of endogenous palmitate from membrane (or exogenous) phospholipids is implicated. The inhibition of a microsomal phospholipase by mepacrine could, therefore, explain the changes seen in the pattern of fatty acid products (Table 1).

p-Bromophenacyl bromide caused complete inhibition of fatty acid synthesis, including activity *de novo*. A similar result has also been described with brain microsomes (Gan-Elepano & Mead, 1978). It would seem that *p*-bromophenacyl bromide is not a specific inhibitor for phospholipase A_2 since it inhibits carboxypeptidase A (Kubota *et al.*, 1974) and it also reacts with an aspartic acid residue close to the active site of acid proteinases such as pepsin (Erlanger *et al.*, 1966). It is possible that its inhibition of pea microsomal fatty acid synthetase is due to such an action on one of the component enzymes.

Since both compounds produced significant effects on fatty acid synthesis, mepacrine and *p*-bromophenacyl bromide were clearly unsuitable as inhibitors of exogenous phospholipase activity.

It will also be noted in Table 1 that odd-chain-length saturated fatty acids were produced by the microsomal fraction from [^{14}C]malonyl-CoA. These products were formed by α-oxidation (J. Sanchez, H. T. Khor & J. L. Harwood, unpublished work). Small amounts of odd-chain-length saturated fatty acids have been found to be synthesized by many plant systems *in vitro* (Stumpf & James, 1963). In addition, such acids may be significant components of certain plant membranes, such as the plasma membrane of leek epidermal cells (Cassagne & Lessire, 1979).

Hydrolysis of microsomal lipids

Membrane vesicles become permeable to lipases after extensive digestion, so that it was necessary to define carefully the conditions for hydrolysis by phospholipase A_2 or *Rhizopus* lipase to ensure that digestion was restricted essentially to the outer half of the microsomal vesicles. Digestion was carried out under the conditions used for fatty acid synthesis

Table 1. *Effect of inhibitors of phospholipase A on fatty acid synthesis by the microsomal fraction from germinating peas*
Fractions were incubated under conditions described in the Materials and methods section. Incubations contained 2–5 mg of microsomal protein and activities were in the range 10–19 pmol of malonyl-CoA incorporated/min per mg of protein. Results are expressed as means ± S.E.M. (where appropriate) for independent experiments carried out in triplicate. *p*-Bromophenacyl bromide is insoluble in water and so it was added in methanol. Significance was calculated by Student's *t* test for paired samples: *, $P<0.10$; **, $P<0.01$; ***, $P<0.001$. Abbreviation used: n.m., not measured.

Fraction	Addition (final concn.)	Total fatty acid synthesis (% of control value)	Number of experiments	Distribution of radioactivity (% of total ^{14}C-labelled fatty acids)				
				$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{17:0}$	$C_{18:0}$
Microsomal	None	100	5	5 ± 2	7 ± 3	35 ± 5	15 ± 4	38 ± 4
Microsomal	Mepacrine (2 mM)	100	1	7	3	45	16	38
Microsomal	Mepacrine (5 mM)	94 ± 2	3	15 ± 6***	8 ± 5*	49 ± 18**	11 ± 9	14 ± 6***
Microsomal	Methanol (2%, v/v)	86	2	4	10	37	14	35
Microsomal	Methanol (2%, v/v) + *p*-bromophenacyl bromide (0.5 mM)	4 ± 1	2	n.m.	n.m.	n.m.	n.m.	n.m.

(i.e. without the addition of Ca^{2+}). Adequate rates of hydrolysis were observed, thus avoiding the necessity of adding Ca^{2+} and so altering the rates of fatty acid synthesis. All three major phospholipids were hydrolysed, although phosphatidylinositol was degraded at slower rates than phosphatidylethanolamine and phosphatidylcholine. Phosphatidylinositol has been shown to be concentrated in the inner leaflet of membranes in castor bean (Cheesebrough & Moore, 1980) and a similar distribution in pea might account for its slower rate of hydrolysis. However, even after extensive digestion approx. 30% of the original phosphatidylinositol remained, indicating that this probably represents inaccessible lipid as discussed by Cheesebrough & Moore (1980). When the pea microsomal lipids were extracted and digestions were then performed with *Rhizopus* lipase and pancreatic phospholipase A_2, the relative rates of phospholipid digestion agreed with published enzyme specificities (de Haas *et al.*, 1968; Slotboom *et al.*, 1970) and did not correspond to the data for microsomal vesicle hydrolysis.

It has been suggested that up to 80% of the outer-layer phospholipid can be digested with phospholipase A_2 before membrane vesicles are likely to become leaky to phospholipase (Sundler *et al.*, 1978). Accordingly, we usually used concentrations of phospholipase or lipase of 0.2 unit/mg of microsomal protein or less when the enzymes were added directly to the fatty acid synthesis incubations. Under these conditions the maximal extent of phospholipid digestion was less than 10%.

As a further check that the microsomal vesicles had not become permeable to phospholipase during digestion, we followed the leakage of internally-trapped markers. The results for experiments with lactate dehydrogenase are shown in Table 2. It will be seen that even at concentrations of phospholipase of 6 units/mg of microsomal protein, considerable amounts of lactate dehydrogenase activity were still retained. The usual level of phospholipase used was considerably less than this (<0.05 unit/mg of microsomal protein in pre-incubations, for example) and at these concentrations we believe that it is unlikely that appreciable entry of phospholipase and, hence, digestion of the internal faces of microsomes takes place, even in re-sealed vesicles. In confirmation of the data in Table 2, the leakage of 6-carboxyfluorescein exactly paralleled that of lactate dehydrogenase and the fluorescent characteristics of this dye can be used directly to measure internally-trapped and externally-released compounds (Blumenthal *et al.*, 1977).

Effect of microsomal lipolysis on fatty acid synthesis

The effects of lipolysis on fatty acid synthesis by microsomal fractions are shown in Table 3. When the microsomal fraction was exposed to phospholipases either during fatty acid synthesis or pre-incubated with the enzymes before re-isolation and assay for fatty acid formation, total synthesis was markedly impaired. There was no significant change in the [^{14}C]palmitate/[^{14}C]stearate ratio in the products, indicating that the activities of fatty acid synthetase and palmitate elongase were equally reduced. However, accumulation of odd-chain-length fatty acids, especially heptadecanoate, and the very-long-chain acid, eicosanoate, was severely reduced. In previous studies, stearate elongase has been found to be particularly susceptible to changes in membrane structure (Harwood & Stumpf, 1971; Bolton & Harwood, 1977*a*).

Table 2. *Release of soluble enzyme marker by phospholipase A from inside microsomal vesicles*
Peas were fractionated as described in the Experimental section. Rabbit muscle lactate dehydrogenase (see the Materials and methods section for activity units) was added to the 18000*g* × 20min supernatant and the microsomal fraction was isolated. The microsomes were resuspended and subjected to pancreatic phospholipase A_2 treatment under conditions described in the Materials and methods section for 30min. The microsomal fraction was then re-isolated after dilution of the incubation medium with a 5-fold excess of homogenization buffer. Lactate dehydrogenase activity was assayed in the presence of 0.02% (w/v) Triton X-100. This concentration of detergent had no effect on the activity of lactate dehydrogenase. Results are means ± S.D. for three independent experiments.

	Lactate dehydrogenase activity		
	Retained in microsomes		
Phospholipase addition (units/mg of microsomal protein)	(units)	(% of control)	Recovery (% of control)
None (control)	0.285 ± 0.018	100 ± 6	100 ± 2
0.06	0.308 ± 0.014	108 ± 5	101 ± 1
0.60	0.141 ± 0.003	49 ± 1	98 ± 4
6.00	0.068 ± 0.003	24 ± 1	99 ± 5

Table 3. *The effect of the direct addition of lipases on fatty acid synthesis by the microsomal fraction from germinating pea*
For details see the legend to Table 1. Abbreviations used: tr., trace (<0.5%); n.d., not detected.

			Distribution of radioactivity (% of total ^{14}C-labelled fatty acids)						
Addition	Number of experiments	Fatty acid synthesis (% of control)	$C_{12:0}$	$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{17:0}$	$C_{18:0}$	$C_{20:0}$
None	4	100	1 ± 1	6 ± 2	7 ± 1	24 ± 2	18 ± 2	35 ± 4	10 ± 2
Phospholipase A_2 (1 unit)	4	62 ± 4	tr.	4 ± 1	6 ± 2	35 ± 6	4 ± 1	41 ± 7	7 ± 3
Rhizopus lipase									
(1 unit)	1	54	4	7	tr.	18	12	55	n.d.
(5 units)	2	46	5	6	3	35	12	42	tr.

The inhibition of fatty acid synthesis resulting from microsomal lipolysis might have arisen from the release of inhibitory products. Accordingly, we tested the addition of 1-monoacylphospholipids or non-esterified fatty acids to the incubation system. The maximum quantities of monoacylglycerophosphocholine or monoacylglycerophosphoethanolamine that could have been released by 2 units of pancreatic phospholipase A_2 or 20 units of *Rhizopus* lipase during incubations were 50μg and 20μg respectively/mg of microsomal protein. When these amounts were added exogenously, some inhibition of fatty acid synthesis occurred (Table 4), but not as much as was observed by phospholipase treatment (Table 3). In addition, when the microsomal fraction was pre-incubated with phospholipase A_2 a reduction of total fatty acid synthesis of approx. 90% was achieved with 0.1 unit/mg of microsomal protein. At this concentration, less than 2% of the total membrane phospholipids were hydrolysed and equivalent concentrations of exogenous monoacylphospholipids had no significant effect on fatty acid synthesis (Table 4). The direct addition of non-esterified fatty acids to the incubation medium had been found previously to cause a slight stimulation of total synthesis (Bolton & Harwood, 1977*a*) and these results were confirmed. When bovine serum albumin was added to remove non-esterified fatty acids produced by lipolysis, a severe inhibition was seen in fatty acid synthesis by the microsomal fraction (Table 4). In contrast, the soluble fraction still showed the same incorporation of radioactivity into fatty acids but the nature of the products was changed. The α-hydroxy fatty acids, which are significant products of the soluble fraction (Jordan & Harwood, 1979), were reduced to low amounts. In addition, the synthesis of stearate was severely reduced (Table 4). The respective substrates for the hydroxylation enzyme and the palmitate elongase are unesterified fatty acids (Jordan & Harwood, 1979) and palmitoyl-acyl-carrier protein (Bolton & Harwood, 1977*b*). Since albumin binds unesterified fatty acids, we also tested its ability to bind acyl–acyl-carrier proteins. Fatty acid-free bovine serum albumin was incubated with [^{14}C]oleoyl–acyl-carrier protein and the mixture was chro-

Table 4. *Inhibition of fatty acid synthesis by subcellular fractions from germinating pea with monoacylphospholipids or bovine serum albumin*

For details see the legend to Table 1. Incubations with the soluble fraction contained 0.5–2.0mg of protein and activities were in the range 11–21pmol of malonyl-CoA incorporated/min per mg of protein. Abbreviations used: OH-16, α-hydroxypalmitate; OH-18, α-hydroxystearate (cf. Jordan & Harwood, 1979); BSA, bovine serum albumin; lyso PC, 1-monoacylglycerophosphocholine; lyso PE, 1-monoacylglycerophosphoethanolamine. For other abbreviations and the significance of the asterisks, see the legend to Table 1.

Fraction	Addition (final concn.)	Total fatty acid synthesis (% of control)	Number of experiments	Distribution of radioactivity (% of total ^{14}C-labelled fatty acids)						
				$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{17:0}$	$C_{18:0}$	OH-16	OH-18
Microsomal	None	100	3	5±2	2±3	37±16	12±4	42±5	n.d.	n.d.
Microsomal	BSA (0.5%, w/v)	11±1	3	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
Microsomal	lyso PC (5μg/ml) + lyso PE (2μg/ml)	96	1	4	3	32	16	45	n.d.	n.d.
Microsomal	lyso PC (50μg/ml + lyso PE (20μg/ml)	72	2	5	3	36	12	44	n.d.	n.d.
Soluble	None	100	4	5±3	n.d.	27±7	n.d.	39±6	4±1	24±7
Soluble	BSA (0.5%, w/v)	108±6	4	26±7***	n.d.	65±6**	n.d.	5±4***	2±1*	tr.***

matographed on a column of Sephadex G-25, equilibrated with iso-osmotic saline. Fractions were assayed for radioactivity and protein and it was found that all of the acyl–acyl-carrier protein was eluted in the void volume with bovine serum albumin. Control experiments confirmed that when albumin and acyl–acyl-carrier protein were applied individually they were adequately resolved by the column and that the unesterified fatty acid content of the acyl–acyl-carrier protein (Sanchez & Mancha, 1980) was less than 10%.

Thus albumin not only binds unesterified fatty acids but also oleoyl–acyl-carrier protein strongly. Accordingly, albumin may reduce the amounts of stearate and α-hydroxy fatty acids by competing successfully with palmitate elongase and the hydroxylase for their substrates.

The loss in fatty acid-synthesis activity from the microsomal fraction appeared to be due to a disturbance of the membrane environment on the outside of the vesicles. From the results discussed previously it seems unlikely that the lipases would have been able to penetrate the vesicles and the build-up of monoacylphospholipids is not enough to inhibit fatty acid synthesis sufficiently unless these products were very localized. Two further pieces of evidence suggest that the fatty acid-synthesizing enzymes are on the outside of the microsomal vesicles. First, molecules such as NADPH, ATP and acyl-carrier protein, which are needed for maximal activity, are unlikely to move into the microsomal vesicles. Yet sonication, which is likely to randomize the orientation of vesicle membranes, as well as to allow access of impermeable substrates to the interior, produced a lowering of the incorporation from [^{14}C]malonyl-CoA (Jordan & Harwood, 1980; Table 5). Secondly, treatment of microsomal fraction with increasing amounts of the rat pancreatic trypsin-like proteinase resulted in a progressive loss of fatty acid-synthesizing activity (Table 5). How-

Table 5. *The effect of pre-incubation of microsomal preparations with rat trypsin-like proteinase on their ability to synthesize fatty acids from [^{14}C]malonyl-CoA*

Microsomal fractions were incubated with rat trypsin-like serine proteinase (0.04–8.00μg/mg of microsomal protein) at 25°C for 30min (Expt. 1). After addition of 50μg of soya-bean trypsin inhibitor to inactivate the proteinase, the microsomal fractions were re-isolated and assayed for fatty acid synthesis (see Experimental). Results from a representative experiment (Expt. 1) are shown. In a separate measurement, loss of activity of 42% of fatty acid synthesis was achieved at a proteinase concentration of 40μg/mg of protein. In Expt. 2, microsomes were incubated with 20μg of proteinase/mg of microsomal protein at 25°C for 60min. As indicated, some samples were sonicated after addition of proteinase for 30s in a bath sonicator. Proteolysis was terminated and the remainder of the experiment completed as above. No significant change in the pattern of fatty acids synthesized was seen in either experiment.

	Treatment	Proteinase concn. (μg/mg of microsomal protein)	Total fatty acid synthesis (% of control)
Expt. 1	None	0	100
	None	0.04	87
	None	0.40	91
	None	2.00	77
	None	8.00	62
Expt. 2	None	0	100
	None	20.00	58
	Sonicated	0	73
	Sonicated	20.00	49

Table 6. *Action of lipases on fatty acid synthesis by the soluble fraction from germinating pea*
For details see the legends to Tables 1–4

Addition	Number of experiments	Fatty acid synthesis: Total synthesis (% of control)	Distribution of radioactivity (% of total ^{14}C-labelled fatty acids): $C_{14:0}$	$C_{16:0}$	$C_{18:0}$	OH-16	OH-18
None	5	100	0.9 ± 0.5	22.8 ± 2.5	42.0 ± 0.5	5.4 ± 2.3	27.1 ± 3.8
Phospholipase A_2 (1 unit)	5	100 ± 8	1.1 ± 0.7	28.3 ± 5.8	10.3 ± 1.9 ($P < 0.001$)	6.6 ± 2.9	53.1 ± 6.2 ($P < 0.01$)
Rhizopus lipase (1.2 units)	5	102 ± 5	0.7 ± 0.4	27.4 ± 7.0	18.7 ± 5.8 ($P < 0.001$)	10.1 ± 4.0	37.4 ± 6.1 ($P < 0.10$)

ever, even when high concentrations of rat proteinase (up to 40 μg/mg of microsomal protein) were included, the activity was still only reduced by about 50%. A similar result was obtained with bovine pancreatic trypsin. When the microsomal vesicles were sonicated in the presence of proteinase, the amount of inhibition of fatty acid synthesis was similar to the unsonicated fraction (Table 5), showing that the resistance to proteolysis was not merely because the residual activity was on the inside of the vesicles. Samples were taken from all of the digestion mixtures after 30 min and it was found that of the 14 major microsomal protein bands that were separated by SDS/polyacrylamide-gel electrophoresis, only two (of apparent relative molecular mass 105000 and 64000 respectively) were removed by the action of the proteolytic enzyme. Thus it would appear that most of the proteins in the microsomal fraction are not susceptible to this proteinase, which is particularly efficient in degrading proteins in their native conformation (Kay *et al.*, 1982). From the pattern of fatty acids labelled (Table 5), it seems that proportions of the fatty acid synthetase and palmitate elongase activities were, indeed, resistant to proteolysis. Since the microsomal fraction is composed of membranes derived from various cellular locations, it seems possible that the proteinase-resistant fatty acid synthetase and palmitate elongase activities might be located on different membranes from the susceptible enzymes.

The results described above indicate that the enzymes involved in fatty acid synthesis are located on the outside of the microsomal vesicles. This would correspond to the cytoplasmic face of the endoplasmic reticulum (de Pierre & Dallner, 1975) and the stromal face of plastid lamellae (Andersson & Akerlund, 1978). From enzyme-marker studies, the bulk of the microsomal fraction was composed of the endoplasmic-reticulum membranes (Jordan & Harwood, 1980), although the subcellular origin of the fatty acid-synthesizing activities is not known. Thylakoids from isolated pea leaf chloroplasts contain fatty acid synthetase and palmitate elongase (J. Sanchez & J. L. Harwood, unpublished work) as do thylakoids from avocado (Weiare & Kekwick, 1975). Stearate elongase, on the other hand, has been reported to be localized in the endoplasmic reticulum (Cassagne & Lessire, 1979).

Effect of lipases on soluble activity

In marked contrast with the microsomal fraction, total soluble activity was unaffected by the presence of phospholipases (Table 6). It will be seen, though, that the relative proportions of [^{14}C]stearate and α-hydroxy[^{14}C]stearate were changed significantly. The ratio of C_{16}/C_{18} products was unchanged, showing that the fatty acid synthetase and palmitate elongase activities were unaffected by the lipases, in marked contrast with the situation observed with the microsomal fraction (Table 4). This provides further evidence in support of our previous work (Bolton & Harwood, 1977*a*,*b*) for a distinct difference between the membrane-located and soluble enzyme systems. The stimulation of α-hydroxylation by the presence of lipases may be due to cleavage of [^{14}C]acyl thioesters to unesterified fatty acids, which are believed to be the substrates for hydroxylation (Shine & Stumpf, 1974; Jordan & Harwood, 1979). Although purified lipases were used and phospholipase A_2 is devoid of proteinase contaminants (Verger *et al.*, 1973), control experiments with [^{14}C]oleoyl–acyl-carrier protein and [^{14}C]stearoyl-CoA have shown that they are hydrolysed by both *Rhizopus* lipase and pancreatic phospholipase A_2.

Conclusions

The above results indicate that fatty acid synthetase, palmitate elongase and stearate elongase are all present on the outer face of pea microsomal-membrane vesicles. The data also demonstrate differences between the palmitate elongase and fatty

acid synthetase activities of pea microsomal and soluble fractions.

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