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CHARACTERIZATION OF FATTY ACID ELONGASE ENZYMES FROM GERMINATING PEA SEEDS

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Key Word Index—*Pisum sativum*; Leguminosae; germinating seeds; very-long-chain fatty acid synthesis; microsomal fractions; fatty acid elongation; behenate; arachidate.

Abstract—*In vitro* biosynthesis of radioactive arachidate and behenate was observed when microsomal fractions of germinating pea seeds were incubated with exogenous stearoyl-CoA (18:0-CoA) or arachidoyl-CA (20:0-CoA) in the presence of NADPH, [2-^{14}C]malonyl-CoA and ATP. Characterization of parameters required for optimal stearoyl- and arachidoyl-CoA elongation revealed that, at least, two chain-length-specific elongases are necessary for very-long-chain fatty acid synthesis. Both enzymes were found to be sensitive to the group-selective reagents, *p*-CMB, NEM, iodoacetate, arsenite and phenylglyoxal. Subcellular fractionation studies indicated that both of these elongases were localized mainly in the endoplasmic reticulum.

INTRODUCTION

Saturated very-long-chain fatty acids (VLCFAs), i.e. fatty acids > C_{18}, are characteristic precursors and, to some extent, components of plant waxes and other surface coverings. In waxes, these acids are free or esterified with various hydroxyl groups, particularly the wax alcohols. Current evidence also suggests that these VLCFAs are precursors of other major components of wax, e.g. hydrocarbons, alcohols and ketones. VLCFAs have also been found as constituents of polar lipids (especially phosphatidylcholine) of plasma membranes where they constitute 5–10% of the acyl moieties [1, 2]. In plasma membranes, these VLCFAs tend to be shorter than those deposited externally. In addition, significant proportions (14–50%) of the fatty acids of phosphatidylserine from a variety of plant sources are C_{20}–C_{26} [3].

It is well documented that microsomal fractions from a number of higher plants can catalyse the incorporation of radiolabelled malonyl-CoA into VLCFAs in the presence of reduced pyridine nucleotides [2, 4, 6, 7]. The primers of the enzyme(s) are usually thought to be acyl-CoAs and the resultant products are released as acyl-CoA thioesters [4], although contrary evidence has been produced with one system [5]. It has been hypothesized that several chain-length specific elongases are involved in VLCFA synthesis. The differential effects of protein synthesis inhibitors on very-long-chain synthesis in aging potato discs were investigated by Walker and Harwood [8]. They reported the involvement of separate (probably three) elongases with different chain-length specificities. Previous studies, with barley mutants, agreed with this conclusion [9]. Moreover, at least two distinct elongase enzymes have been demonstrated in leek epidermal cells [10]. Further studies by Cassagne *et al.* [11] revealed that the separate chain-length specific elongases of leek epidermal cells were localized in different subcellular fractions. The stearoyl-CoA (18:0-CoA) elongase, responsible for the synthesis of arachidic acid (20:0), was chiefly associated with the endoplasmic reticulum. Conversely, the arachidoyl-CoA (20:0-CoA) elongase, responsible for the synthesis of C_{22}–C_{24} acids, was mainly localized within Golgi-enriched membranes [12, 13].

To gain more information about the characteristics of the *in vitro* biosynthesis of individual VLCFAs, we have studied fatty acid synthesis by microsomal fractions from germinating pea seeds. Such fractions have previously been demonstrated to possess active elongases, which characteristically only form saturated fatty acids [14].

RESULTS AND DISCUSSION

Pea seeds were germinated for 24, 48 or 72 hr at 20°. Microsomes were isolated and incubated subsequently

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Table 1. Effects of germination time on activity of elongase enzymes in microsomal fractions from pea seeds

Time of germination (hr)	Specific activity (nmol 3 hr^{-1} g^{-1} protein)
24	3.8, 4.6
48	2.2, 2.4
72	1.8, 2.0

Duplicate determinations are shown.

with exogenous acyl-CoAs (18:0 or 20:0), in the presence of reduced pyridine nucleotides, malonyl-CoA and ATP [15]. Analysis of the products by radio-GC revealed that these saturated acyl-CoAs had been elongated by the addition of a single C_2 unit from radiolabelled [2-^{14}C]malonyl-CoA forming arachidic and behenic acids, respectively. Specific activities of the elongase enzymes were maximal within 24 hr of germination (Table 1), with very little radiolabelled palmitate or stearate being apparent in the incubation products. The lack of [^{14}C]-VLCFAs other than the expected product indicated that elongation of endogenous fatty acids or their derivatives was minimal. This agreed with reports by Lessire *et al.* [16] for leek. All subsequent experiments were, therefore, performed with microsomal preparations from pea seeds that had been germinated for 24 hr. Microsomal acyl-CoA elongation of both 18:0-CoA and 20:0-CoA was found to be linear for at least 3 hr (data not shown). Little further labelling of VLCFAs was found after this time. Consequently, all incubations were stopped after a 3 hr period. The activities of both CoA elongases were shown to be proportional to the amount of protein up to at least 2.5 mg of microsomal protein in the incubation medium, respectively, for the 3 hr incubation period (data not shown).

Effect of pH

Acyl elongation was measured over a range of different pHs. 20:0-CoA elongase activity was found to be optimal around pH 6.6 (Fig. 1), with rises in pH to 7.2 resulting in *ca* 45% decrease in activity. This pH optimum differed from that found for 18:0-CoA elongation, which was maximal at pH 7.0 (Fig. 2).

Dependence of elongase activity on ATP

Elongases involved in the elongation of exogenous CoA substrates in leek have been reported to have no requirement for ATP, provided that the acyl-CoA substrate is provided in excess [2]. However, the enzyme(s) responsible for the elongation of endogenous substrates have been demonstrated to have an absolute requirement for ATP. It has been proposed that the latter cofactor was necessary for the formation of acyl-CoAs from endogenous fatty acids [2]. Moreover, the recent suggestion that unesterified fatty acids, rather than acyl-CoAs, are substrates for leek elongases [5], especially for stearate elongation, makes the role of ATP more important.

The activity of the elongase(s) from microsomal fractions of germinating peas, although not strictly ATP-dependent, was significantly stimulated in the presence of this cofactor. Optimal 22:0 and 20:0 synthesis was demonstrated at concentrations of 5.0 and 3.0 mM of this cofactor, respectively (Figs 1 and 2). Since one may assume that acyl-thioesterases capable of hydrolysing the exogenous substrate(s) to free fatty acid and CoA may be present within the microsomal fraction, it is plausible to suggest that some stimulation of elongase activity may be due to the ATP-dependent regeneration of the acyl-CoA derivative(s). Indeed, the existence of an acyl-CoA synthetase has been demonstrated within microsomal fractions from leek epidermal cells [6, 17].

Reduced pyridine nucleotide requirements of elongase(s)

Experiments with microsomal preparations and variable concentrations of the reduced pyridine nucleotides, NADH and NADPH, demonstrated exclusive use of NADPH by both elongase systems. It is clearly evident from the data in Figs 1 and 2 that 0.5 and 0.3 mM of this cofactor were necessary to achieve maximal rates of 20:0- and 18:0-CoA elongation, respectively. Conversely the addition of exogenous NADH to the reaction media inhibited elongation of the acyl-CoAs considerably. Activity of the chain-length-specific elongases apparent in the total absence of both these reduced cofactors may be due to the incorporation of radioactivity from [2-^{14}C]malonyl-CoA during the first partial reaction catalysed by the elongase enzyme, i.e. the reduced pyridine nucleotide—independent condensation reaction of the acyl-CoAs and malonyl-CoA to form a β-ketoacyl-CoA intermediate. Alternatively, small amounts of NADPH may be present in the microsomal (vesicular) preparations.

Effect of substrate concentration

Some incorporation of radiolabel into fatty acids was apparent in the absence of substrate (Figs 1 and 2). There may be two explanations for this. Firstly, [2-^{14}C]malonyl-CoA may be being utilized for the ATP-dependent elongation of endogenous long-chain fatty acyl substrates present within the microsomal preparations. Elongation of endogenous saturated monocarboxylic acids ranging from C_{16} to C_{24} in the absence of exogenous acyl-CoAs has been previously reported by Agrawal and Stumpf [6; see also 15]. Alternatively, radiolabelled malonyl-CoA may be utilized for the *de novo* synthesis of the long chain fatty acids and their intermediates, catalysed by extra-chloroplastic isoforms of acetyl-CoA carboxylase and

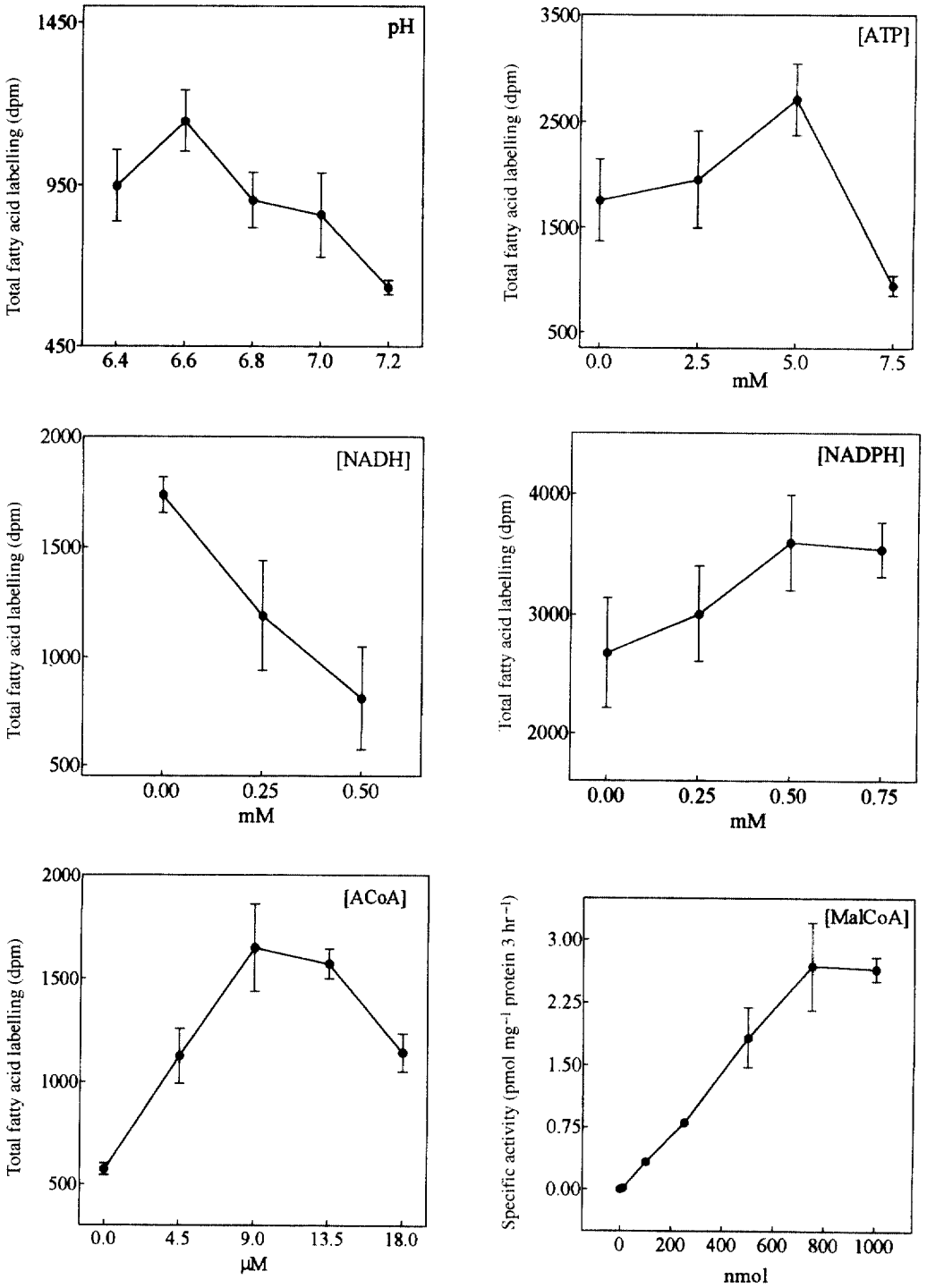

Fig. 1. Optimal conditions for 20:0-CoA elongase activity. ACoA is arachidoyl-CoA.

fatty acid synthase or by contaminating proteins from plastids.

Maximal activity of the 20:0-CoA elongase was demonstrated to occur in the presence of 9 μM 20:0-CoA (Fig. 1). Incubation of the microsomal proteins with concentrations of substrate above this optimum gave only a small amount of inhibition. For 18:0-CoA, an optimal concentration of 4.5 μM was found (Fig. 2). One may presume that, in the presence of 9 μM 20:0-CoA, the enzyme has become fully saturated with this substrate or that, alternatively, at higher concentrations of 20:0-CoA, micelles may be formed. Thus, increasing concentrations of substrate above the critical micelle concentration (CMC) could change the amount of acyl-CoA capable of interacting with the acyl-CoA site on the condensing enzyme protein of the elongase. Zahler *et al.* [18] provided evidence that micellization of the long-chain 16:0-CoA deriva-

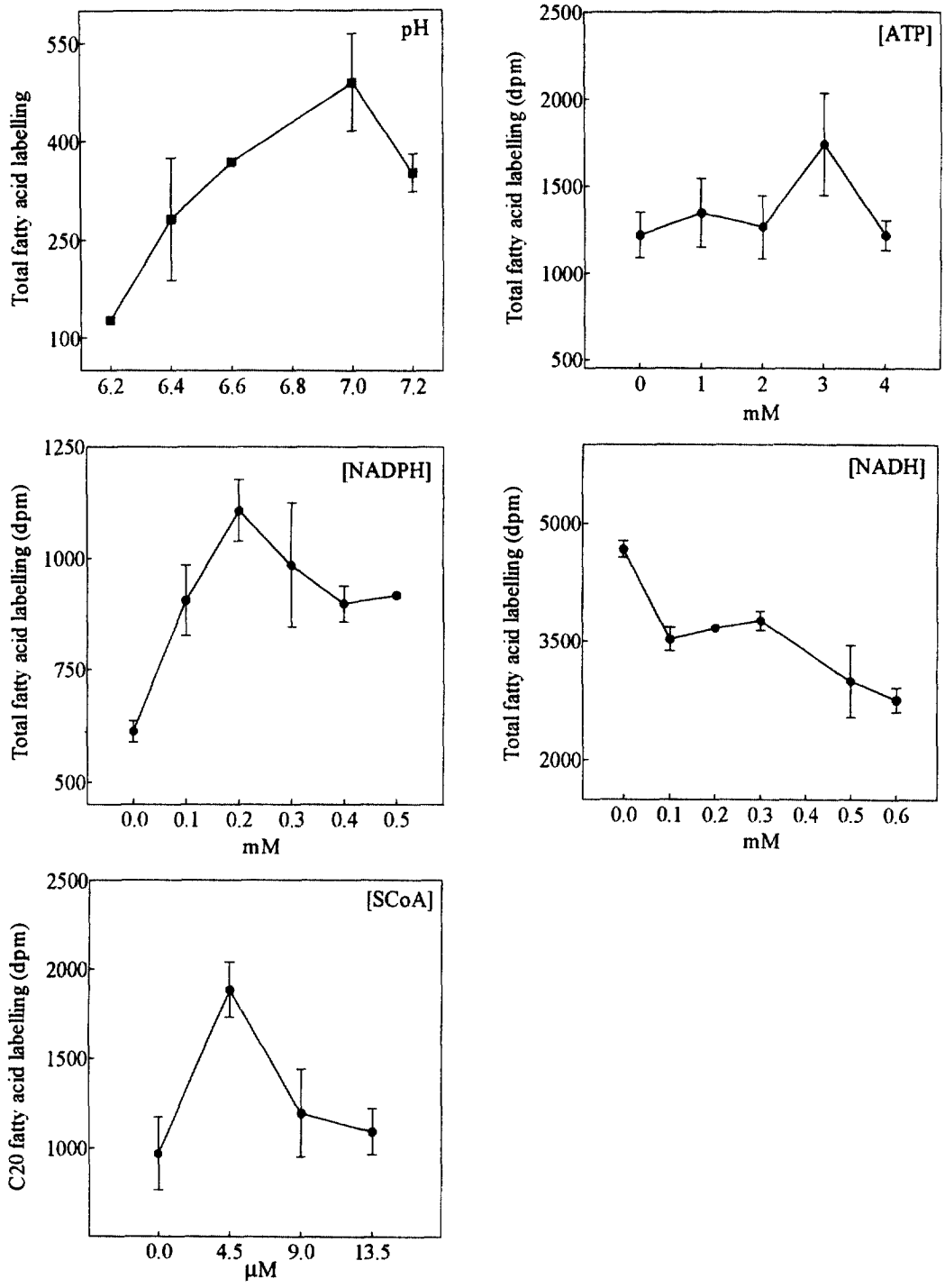

Fig. 2. Optimal conditions for 18:0-CoA elongase activity. SCoA is stearoyl-GA.

tive occurred with a critical micelle concentration (CMC) range of 3–4 μM. However, more recent studies by Powell *et al.* [19] suggest that the CMC of physiologically significant fatty acyl-CoA molecules exceeds 30 μM. Thus, the concentration of 18:0- and 20:0-CoA found to be optimal (Figs 1 and 2) may or may not be close to the CMC of these substrates. We could not find any data in the literature on this specific point.

Effect of Triton X-100 on elongation

The non-ionic surfactant, Triton X-100, has been successfully employed by Lessire *et al.* [10] in the solubilization of the individual elongases from leek epidermal cells. Additionally, these workers reported that the inclusion of Triton X-100 in the incubation medium with leek microsomal preparations stimulated significantly the synthesis of C_{22}, C_{24} and C_{26} fatty acids.

Table 2. Effect of different concentrations of acyl carrier protein, Triton X-100 and coenzyme A on acyl-CoA elongation

	Total fatty acid methyl esters (dpm × 10^{-3})					
	18:0-CoA elongase			20:0-CoA elongase		
Treatment	Total	⩽18	20:0	Total	⩽18	22:0
None	1.4±0.3	n.m.	n.m.	3.1±0.3	0.5±0.2	2.7±0.5
25 μg ACP	1.1±0.2	n.m.	n.m.	3.8±0.6	2.1±0.5	1.7±0.2
50 μg ACP	2.2±0.3	n.m.	n.m.	8.2±1.2	7.9±0.6	0.4±0.2
None	1.2±0.2	n.d.	1.2±0.2	1.3±0.2	n.d.	1.3±0.2
1 μM CoA	1.6±0.1	n.d.	1.6±0.1	1.6±0.4	0.2±tr.	1.4±0.1
5 μM CoA	1.2±0.3	n.d.	1.2±0.3	1.3±0.1	n.d.	1.3±0.1
50 μM CoA	0.8±0.1	n.d.	0.8±0.1	1.1±tr.	0.2±tr.	0.9±tr.
None	0.8±tr.	n.d.	0.8±tr.	0.9±0.3	n.d.	0.9±0.3
0.1 mM Triton X-100	0.7±0.1	n.d.	0.7±0.1	0.5±tr.	n.d.	0.5±tr.
0.3 mM Triton X-100	0.3±0.1	n.m.	n.m.	0.7±0.1	n.d.	0.7±0.1

Results = means±s.d. (n = 2).
n.m. = not measured due to insufficient amounts for detection by radio-GC.
tr. = ⩽50 dpm.
n.d. = none detected.

Conversely, a simultaneous decrease in the labelling of C_{20} normally detected in the absence of Triton X-100 was apparent. We used this surfactant to see if we could differentiate between stearate and arachidate elongation in microsomes and also, possibly, to stimulate activity. Concentrations of Triton X-100 added to the incubation medium were below its CMC (i.e. 0.3 mM), thus allowing us to study the effect of only its monomeric form on the elongation systems. Analysis of total fatty acid methyl esters by radio-GC revealed that no alteration in the distribution of label, compared with controls, had occurred in the presence of 0.1 mM Triton X-100, the only detectable radiolabelled peaks of the stearoyl-CoA and arachidoyl-CoA elongase assays being 20:0 or 22:0, respectively. The low level of label detectable in the presence of 0.3 mM Triton X-100 was insufficient for accurate analysis by radio-GC. Elongation of stearoyl-CoA was dramatically impaired by the presence of 0.3 mM Triton X-100, with total incorporation of radiolabelled malonyl-CoA into VLCFAs being inhibited by *ca* 55% (Table 2). This decrease in elongase activity after solubilization is not surprising, because membrane enzyme activities are normally dependent on their environment. By contrast, little effect of the surfactant was apparent on the elongation of 20:0. Thus, our results agreed with those using leek [10] in that a differential effect of Triton on stearate *vs* arachidate elongation was seen. However, in contrast to the leek system [10], we were unable to stimulate arachidate elongation with Triton X-100 under our experimental conditions.

Effect of coenzyme A and acyl carrier protein on acyl-CoA elongation

Elevated levels of fatty acid-labelling were apparent when microsomal fractions were incubated with exogenous 20:0-CoA in the presence of high concentrations of acyl carrier protein (ACP) (Table 2). However, it was apparent upon analysis of total fatty acid methyl esters by radio-GC, that the synthesis of 22:0, the product of a single C_2 unit addition to arachidoyl-CoA, had been impaired. This agreed with data published by Cassagne and Lessire [4] demonstrating the dramatic inhibitory effects of ACP on the incorporation of radiolabelled stearoyl-CoA and malonyl-CoA into saturated VLCFAs of microsomal fractions from leek epidermal cells. The apparent elevated level of total fatty acid synthesis by the microsomal preparation of germinating pea seeds was found to be caused by preferential incorporation of [2-^{14}C]malonyl-CoA into long-chain fatty acid biosynthesis products, 14:0, 16:0 and 18:0, which would require the action of an extrachloroplastic form of fatty acid synthase or of the presence of contaminating amounts of the plastid fatty acid synthase. This agrees with earlier evidence presented by Bolton and Harwood [15] demonstrating that microsomal fractions were capable of incorporating radioactivity from [2-^{14}C]malonyl-CoA into the normal products of fatty acid synthase, i.e. 16:0 and 18:0. Chemical degradative analyses of these products from the microsomes showed that 16:0 was made exclusively by *de novo* synthesis [15].

In a separate experiment, incubation of microsomes with 18:0-CoA, as an elongase substrate, in the presence of ACP did not enhance the level of incorporation to the same extent as in the experiment described above. This probably reflected the variations in the nature of the microsomal preparation. Nevertheless, significant stimulation was again seen (Table 2). Insufficient labelling of the individual fatty acids, in this case, did not permit the analysis of the pattern of fatty acid labelling, therefore preventing us

from investigating whether the slight stimulation of synthesis could be attributed to elevated levels of synthesis of arachidate or preferential labelling of the long-chain fatty acid products. However, since there is no evidence that ACP is needed for elongation reactions, it seems most probable that its presence was stimulating *de novo* synthesis.

The effects of variable concentrations of free coenzyme A (CoA) on the activity of microsomal 18:0-CoA and 20:0-CoA activities were also examined (Table 2). The addition of low concentrations of this cofactor had no apparent effect upon the incorporation of radiolabelled malonyl-CoA into the elongase products. Impaired VLCFA synthesis was only evident in the presence of elevated concentrations of free CoA (50 μM) with 18:0-CoA and 20:0-CoA elongation activities decreasing by *ca* 30 and 20%, respectively. This inhibition of elongation of exogenous substrates was in agreement with the results reported by Sanchez and Harwood [20]. These workers reported that analysis of the individual fatty acid methyl ester products by radio-GC had revealed that ATP-dependent elongation of endogenous long-chain acids used for VLCFA synthesis had also been relatively unaffected. In contrast, the synthesis of the long-chain products, 16:0 and 18:0, was severely inhibited. The limited amount of inhibition of elongation by free CoA may be considered as an example of end-product inhibition. However, bearing in mind that concentrations of free CoA *in vivo* are likely to be very low [21], it seems unlikely that this sulphydryl compound plays a significant physiological role in the regulation of fatty acid elongation.

Effect of sulphydryl reagents and their inhibitors

The thiol inhibitors, iodoacetate, *N*-ethylmaleimide and *p*-chloromercuribenzoate, were found to inhibit the activity of both the stearoyl- and arachidoyl-CoA elongases considerably. These results thus demonstrate clearly the requirement of sulphydryl groups for acyl-CoA elongase activity. Separation of radiolabelled fatty acids by reversed-phase TLC revealed that alkylation of sulphydryl residues by the compounds was not specific for the elongase enzymes, since labelling of 16:0 and 18:0 was also inhibited (Table 3). At least part of the labelling of 18:0 by pea microsomes appears to be due to elongation of preformed (unlabelled) fatty acids [14]. However, any *de novo* synthesis by fatty acid synthase would also be expected to be inhibited by a typical -SH inhibitor. Moreover, the activity of the individual elongase systems, although demonstrating some susceptibility to sodium arsenite (which inhibits β-ketoacyl-ACP synthase II [7]), were not inhibited substantially. Addition of the low M_r dithiol reagent, dithiothreitol (DTT), to the incubation medium after the microsomal proteins had been preincubated with sodium arsenite for 30 min caused only slight reversal of inhibition of either elongase system (Table 4). However, simultaneous inclusion of DTT with the arsenical appeared to protect the protein, presumably because of complex formation between the two reagents.

Effects of phenylglyoxal

Phenylglyoxal reacts preferentially with the guanidino group of arginine residues at pHs close to neutral. It has been reported that the stoichiometry of phenylglyoxal binding is 2 mol mol^{-1} arginine [22]. Inclusion of phenylglyoxal at variable concentrations in the incubation mediums of 18:0- or 20:0-CoA elongases had little effect on the total incorporation of radiolabel into fatty acids (Table 4), except at high (10 mM) concentrations. Analysis of the individual fatty acids by reversed-phase TLC revealed, however, that the presence of phenylglyoxal (even at low concentrations) significantly inhibited fatty acid elongation (Table 3). At the same time, labelling of 16:0 and 18:0 (at least partly formed by *de novo* synthesis [15, 23, 24]) was increased. The latter activity increased from being only a minor reaction in control incubations to 10 times the rate of 20:0-CoA elongation at 10 mM phenylglyoxal. Thus, in contrast to the plastid fatty acid synthase component protein, NADH-specific enoyl-ACP reductase of rape seed, which is inhibited by phenylglyoxal [22], no such inhibition was seen for the labelling of 16:0 and 18:0 by pea microsomes. Part of the increase in labelling into the two latter fatty acids may have been due to increased amounts of radiolabel which would be available because of reduced elongation. The inhibition of elongation by phenylglyoxal may reflect sensitivity of the (enoyl) reductase partial reaction(s) of the elongase systems by analogy with the rape enzyme [22]. Partial reactions (including enoyl reductases) for elongase systems have been demonstrated in leek [25] and in honesty seeds [26].

Subcellular localization of elongase activities

In initial subfractionation studies, pea seeds were homogenized and the post-13 000 *g* supernatant was layered on a two-step discontinuous 5:7% sucrose gradient [25]. Analysis of the various subcellular fractions revealed that the total activity of the 18:0-CoA elongase was very low (data not shown), whilst fractions other than the homogenate and initial supernatant were shown to be totally devoid of all 20:0-CoA elongase activity (data not shown). The decrease in activity of these highly labile-enzymes was attributed to the lengthy spin-time required for sucrose gradient centrifugation. An alternative gradient material that would permit rapid separation of subcellular fractions was therefore necessary. To achieve this, Ficoll gradients were used. Estimates were made of the concentrations of Ficoll needed and the centrifugation speed and time to be used by analogy to the use of Ficoll and sucrose gradients in previous published work [23]. Resolution of the individual frac-

Table 3. Effect of sulphydryl reagents on elongation of acyl-CoAs by pea microsomal fractions

	Fatty acid labelling (dpm)			
	18:0-CoA elongation		20:0-CoA elongation	
Treatment	Total acids	20:0	Total acids	22:0
Iodoacetate				
0 mM	1678	1183	604	494
1 mM	830	671	199	157
10 mM	60	tr.	70	tr.
N-Ethylmaleimide				
0 mM	397	292	393	n.m.
1 mM	172	108	121	n.m
2 mM	126	99	82	n.m.
10 mM	85	tr.	76	n.m.
p-Chloromecuribenzoate				
0 mM	2310	1955	762	319
1 mM	226	n.d.	304	tr.
2 mM	193	n.d.	97	59
10 mM	tr.	n.d.	tr.	tr.
Arsenite				
0 mM	1025	846	409	260
5 mM	630	630	265	153
5 mM*	766	766	288	120
5 mM†	1466	1154	434	204

Means of duplicates shown.
* DTT added at 5 mM after 30 min of assay.
† DTT added at 5 mM at time 0 min.
tr. = $\leqslant$50 dpm.

Table 4. Effect of phenylglyoxal on fatty acid elongase assays with pea microsomes

		20:0-CoA elongation		
Phenylglyoxal (mM)	18:0-CoA elongation Total dpm	Total dpm	$\leqslant$18	22:0
0	286 $\pm$ 72	883 $\pm$ 74	161	747
1	600 $\pm$ 93	542 $\pm$ 19	315	229
2	999 $\pm$ 65	750 $\pm$ 37	424	331
10	5208 $\pm$ 433	2462*	2239	223

Results = means $\pm$ s.d. (n = 2).
* Single sample. Microsomal fractions incubated with 20:0-CoA (9 μM) in the presence of different concentrations of phenylglyoxal. Fatty acids were extracted and analysed by reversed-phase TLC as described in the Experimental.

tions was finally achieved by density gradient centrifugation on 5:15% Ficoll cushions. The 0:5% and 5:15% Ficoll interfaces and the pellet were retained and analysed further. In order to determine the nature of the membrane fractions, the activities of NADH:-cytochrome-c reductase—an endoplasmic reticulum marker (ER), thiamine pyrophosphatase (TPPAse)—a Golgi apparatus marker and glucan synthetase II—a plasma membrane marker, were measured in each (Table 5). The 0:5% band was enriched in NADH cytochrome-c reductase (insensitive to antimycin A) activity, indicating that it was enriched in endoplasmic reticulum (ER). Some cross-contamination of the 5:15% band with the ER marker enzyme was also apparent. Only extremely low activities of this enzyme were found to be associated with the resuspended pellet. The majority of TPPase activity, the Golgi apparatus marker, was recovered in the 5:15% inter-

Table 5. Subcellular fractionation of germinating pea seeds

Estimate	Fractions				
	H	S	0:5% interface	5:15% interface	P
NADH:cytochrome-c reductase*	0.5	1.8	13.2	8.0	0.8
TPPAse†	0.2	0.2	0.9	4.7	0.2
Glucan synthetase II‡	tr.	tr.	tr.	0.1	0.25
Stearoyl-CoA elongase§	1.4	0.9	6.9	0.9	n.d.
Arachidoyl-CoA elongase§	1.1	1.2	2.7	0.7	n.d.

H = Homogenate, S = post-13 000 *g* supernatant, the other three fractions are from the Ficoll density gradient (see Experimental), P = post-50 000 *g* pellet.

* Activity expressed as μmol min^{-1} mg^{-1} protein.

† Activity expressed as μmol 20 min^{-1} mg^{-1} protein.

‡ Activity expressed as ρmol 25 min^{-1} mg^{-1} protein.

§ Activity expressed as ρmol 3 hr^{-1} mg^{-1} protein.

n.d. = none detected. tr. = <0.1.

face fraction. Little contamination of the ER fraction with this enzyme was apparent. The resuspended pellet fraction also exhibited minimal TPPase activity. This latter fraction was found to contain the highest specific activity of glucan synthetase II, the plasma membrane marker used. 18:0- and 20:0-CoA activities were then assayed and were found to correspond primarily to the peak of activity of the ER (Table 5). There was some difference in the amount of the two elongases detected in the other fractions, perhaps indicating that 18:0 and 20:0 elongases were present in different membrane populations, as has been suggested for leek [12, 13]. However, unlike leek, we found no evidence for a localization of 20:0 elongation in Golgi.

CONCLUSIONS

It is apparent from the differential parameters (Table 6) required for optimal 18:0-CoA and 20:0-CoA elongation that at least two chain-length-specific elongases present within the microsomal fraction of germinating pea seeds, are necessary for VLCFA synthesis, i.e. 18:0 and 20:0 elongases, respectively. This result agrees with data for other systems [e.g. 27]. Since each elongation system consists of four partial reactions, then it will be interesting to see if only the condensing enzyme is different or whether the other proteins involved (reductases and dehydrase) are also unique to each elongase system. Recent advances in the molecular biology of fatty acid elongation [27] may be able to answer this question soon.

EXPERIMENTAL

Materials. Pea seeds (*Pisum sativum*, c. Feltham First) were obtained from Asmer Seeds, Leicester, U.K. Seeds were germinated for 2 hr at 20°. [2-^{14}C]Malonyl-CoA (sp. act. 1.85 GBq mmol^{-1}) was purchased from Amersham.

Stearoyl-CoA elongase assay. Microsomal frs were prepd from germinating pea seeds (*ca* 30) using the method of ref. [15]. Microsomal membranes (*ca* 2 mg protein) were incubated for 3 hr in the presence of 0.2 mM NADPH, 3 mM ATP, 9 μM stearoyl-CoA, 0.05 μCi [2-^{14}C]malonyl-CoA and 20 mM potassium Pi buffer, pH 7.0, in a final incubation of 1 ml. When the action of inhibitors was assessed, chemicals were

Table 6. Optimal assay conditions for acyl-CoA elongation by microsomal fractions from germinating pea seeds

Incubation parameters	Optimal conditions for VLCFA formation		*Optimal conditions for long-chain fatty acid synthesis
	18:0-CoA elongation	20:0-CoA elongation	
pH	7.0	6.6	7.0
ATP (mM)	3.0	5.0	2.0
NADH (mM)	Inhibits	Inhibits	0.5
NADPH (mM)	0.2	0.5	0.5
ACP (mg)	Inhibits	Inhibits	0.5
Triton X-100	Inhibits	No affect	n.m.
†SH reagents (mM)	Slight inhibition	Inhibits	Inhibits
ASO_2^{2-}	No effect	Inhibits	Inhibits
Phenylglyoxal	n.m.	Inhibits	Stimulation

* Mainly *de novo* synthesis as determined by ref. [15].

† Iodoacetate, *N*-ethylmaleimide and *p*-chloromercuribenzoate.

added directly to the incubation medium prior to the addition of the necessary cofactors. Incubations were carried out at 25° with continuous shaking for 3 hr. Reactions were terminated by addition of 0.1 ml 60% KOH and heating for 30 min at 70°. The reaction mixt. was reacidified by addition of 0.3 ml 20% H_2SO_4 after the soln had cooled, before extraction of non-esterified fatty acids into $CHCl_3$.

Arachidoyl-CoA elongase assay. Microsomal frs (2 mg protein) were incubated for 3 hr at 25° in the presence of 0.5 mM NADPH, 5 mM ATP, 9 μM arachidoyl-CoA, 0.05 μCi [2-^{14}C]malonyl-CoA and 20 mM potassium Pi buffer, pH 6.6, in a final vol. of 1 ml. Reactions were terminated as described above.

Fatty acid analysis. Fatty acids were transesterified for analysis by radio-GC by heating with 2.5% H_2SO_4 in MeOH for 2 hr at 70° [15]. They were separated on a column of 5% SP-2100 on 100/120 Supelcoport (Supelco) using a temp. prog. (initial temp. 210° for 10 min, then 4°C min^{-1} to 250°) on an instrument connected *via* an effluent splitter to a gas flow proportional counter. Radioactivity estimation of total fatty acid Me esters was as described previously [15]. Alternatively, phenacyl derivatives were prepared by the method of ref. [29] prior to separation on a reversed-phase HPLC column (5 μ, 25 cm × 4.6 mm, Spherisorb, Phase Separations Limited) using a UV detector at 242 nm. A gradient elution prog. of MeCN–H_2O (4:1) initially increasing linearly over 20 min to 100% MeCN (flow-rate 1 ml min^{-1}), followed by elution in MeCN for a further 40 min, was employed. Separated derivatives were collected and their radioactivity quantified [15].

Subcellular fractionation. Microsomal frs were resuspended and loaded onto a two-step discontinuous gradient of 5/15% Ficoll (M_r *ca* 400 000) suspended in a 0.32 M sucrose-2 mM Tris-HCl, pH 7.4. Gradients were spun for 15 min at 50 000 *g* at 4°. The 0:5 and 5:15% interfaces and the pellet were collected individually.

Protein estimation. Protein conc. was determined by the method of ref. [27], using BSA as standard.

Enzyme assays. NADH:cytochrome-c reductase (antimycin A—insensitive) was assayed by the method described in ref. [31]; it was used as an endoplasmic reticulum marker. Thiamine pyrophosphatase, which was used as a Golgi apparatus marker, was assayed by the method of ref. [32]. Prptd protein was removed and the Pi released determined by the method of ref. [33]. Glucan synthetase activity, which was used as a marker for plasma membrane, was measured by following the incorporation of label from UDP-D-[U-^{14}C]glucose [34]. The radiolabel incorporated was quantified as described above.

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