Fat Metabolism in Higher Plants XXXVI: Long Chain Fatty Acid Synthesis in Germinating Peas

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Abstract. A low lipid, high starch containing tissue, namely cotyledons of germinating pea seedlings was examined for its capacity to synthesize fatty acid. Intact tissue slices readily incorporate acetate-14C into fatty acids from C16 to C24. Although crude homogenates synthesize primarily 16:0 and 18:0 from malonyl CoA, subsequent fractionation into a 10,000g pellet, a 105g pellet and supernatant (soluble synthetase) revealed that the 105g pellet readily synthesizes C16 to C24 fatty acids whereas the 10,000g and the supernatant synthesize primarily C16 and C18. All systems require acyl carrier protein (ACP), TPNH, DPNH if malonyl CoA is the substrate and ACP, Mg2+, CO2, ATP, TPNH, and DPNH if acetyl CoA is the substrate. The cotyledons of germinating pea seedlings appear to have a soluble synthetase and 10,000g particles for the synthesis of C16 and C18 fatty acid, and 105g particles which specifically synthesize the very long chain fatty acid from malonyl CoA, presumably via malonyl ACP.

Previous studies of fatty acid synthesis in plants have usually utilized tissues such as castor bean endosperm and avocado mesocarp which store large amounts of lipid as a food reserve. Though such tissues have obvious advantages and yield synthetase systems of high activity, they must of necessity be specialized towards the production of fatty acids typical of storage lipids. This paper reports on the properties of fatty acid synthesizing systems from Pisum sativum, a low lipid containing seed.

Hawke and Stumpf (10) working with barley leaves, showed that both green and etiolated tissue were capable of synthesizing from acetate fatty acids with a chain length up to C24 as well as the usual saturated and unsaturated fatty acids in the C14-18 range. Attempts to detect elongation of C16 and C18 labeled fatty acids in the presence of tissue slices and unlabeled acetate were unsuccessful. All the substrate remained in the form supplied or was broken down completely and recovered as CO2. However, decarboxylation studies on the acetate fed tissue synthesized fatty acids indicated that elongation was a component of the long chain fatty acid synthesis beyond a chain length of C16. Kolattukudy (12-13) working with Brassica leaf tissue obtained evidence that the C29 hydrocarbon nonacosane could be produced by chopped leaf tissue direct from either palmitate or stearate, the latter being the more effective substrate. Some label was also found in fatty acids up to C24 when palmitate or stearate were fed. Hawke and Stumpf (10-11) found that nearly 10% of the radioactivity from palmitate-1-14C appeared as CO2 when fed to chopped barley leaves, but Kolattukudy (11) found only a fraction of 1% under very similar conditions.

Hawke and Stumpf (10-11) reported that the very long chain fatty acids (chain length >C18) were located in the particulate cellular material following biosynthesis from acetate, but cell free systems capable of synthesizing these components were not obtained.

Guckhait et al. (8) have stated that C18-20 saturated and unsaturated fatty acids are formed by an elongation process in pigeon liver microsomes, with malonyl CoA, supplied exogenously, reacting with the endogenous fatty acids. Abraham et al. (1) and Lorch et al. (13) have also demonstrated the presence of microsomal systems.

Yang and Stumpf (20) using avocado mesocarp tissue, distinguished between a supernatant and a particulate 12,000g system. Both systems synthesized palmitate and stearate from malonyl CoA, and the particulate system in addition synthesized traces of C18-11. Unlike the supernatant systems found in most preparations from animal tissues, the supernatant system synthesized more stearate than palmitate (about 6:4). The particulate preparation could utilize acetyl CoA effectively, while the supernatant system preferred malonyl CoA.

Since pea cotyledon tissue is readily available in large quantities and has been shown by Glew (5) to be capable of considerable lipid synthesis from acetate, it was selected to study the fatty acids synthetase activity of a low lipid tissue. The emphasis in this work has been on the production of very long chain fatty acids (>C18).

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Materials and Methods

TISSUE SLICES. Growth Conditions of Peas. Peas were grown for periods up to 8 days in perforated plastic trays under a spray of water at room temperature. Under these conditions no sign of bacterial or fungal contamination was apparent provided the seeds were in a single layer and exposed directly to the spray. Only the cotyledons were used for preparing tissue slices.

Incubation of Tissue Slices. For incubation of tissue slices with acetate-1-14C, tissue slices were cut about 1 mm thick with a razor blade, and washed in distilled water until the washings were clear. Two grams (fr wt) of the tissue were immersed in 4 ml of a solution containing 200 μmoles potassium phosphate and 100 μmoles potassium bicarbonate at pH 7.2. The mixture was shaken gently for 0.5 hr in a stopped flask at room temperature, and at the end of this period the tissue was strained and washed with a further 10 ml of solvent. The washings were bulked with the main extract. 20 ml H2O were added to the chloroform-methanol extract and the chloroform layer was separated and dried over anhydrous Na2SO4.

Aliquots of total lipid were chromatographed on silica gel sheets and counted in toluene scintillator to obtain total lipid incorporation. The presence of unchanged acetate was detected by acidifying 1 aliquot with glacial acetic acid before evaporation of the solvent and heating in a stream of air at 80°C.

Aliquots of total lipid were chromatographed on 0.33 mm thin layer plates using the solvent suggested by Freeman and West (4), consisting of ether:benzene:alcohol:acetic acid, 40:50:2:0.1:0.2. This solvent separates tri- and diglycerides, free acids, the polar complex lipids remaining on the origin. The same solvent was used for preparative TLC on 1 mm plates to analyze the occurrence of different chain lengths, in the different classes thus distinguished. No attempt was made to analyze the nature of the polar complex lipids.

Methyl esters were prepared from the crude lipid extract by trans-esterification with BF3-methanol solution. Methyl esters of fatty acids were analyzed by thin layer chromatography on Silicagel G using 6:4 hexane-ether as the solvent (16) and also on silver nitrate-impregnated Silicagel G to separate unsaturated fatty acid methyl esters (17). Ninety to 95% of the radioactivity was associated with either straight chain saturated or unsaturated methyl esters, and the rest of the activity was on or close to the origin. Standards used included methyl esters of stearate, palmitate, oleate, and β-hydroxy-laurate. There was no evidence of appreciable radioactivity appearing in hydroxy-acids and radiochemical analysis was performed on the unpurified methyl esters.

Preparation of Homogenates and Cell Fractions. Homogenizing was carried out in a pestle and mortar at 0°C. The tissue could be homogenized to a smooth paste without the use of abrasives. When homogenization was carried out in a blender (omnimix) the activity of the 100,000g pellet was severely reduced. The homogenizing medium contained 0.3 mole sucrose, 0.05 mole potassium phosphate buffer, and 10-4 mole DTT (dithiothreitol) per liter, its pH 7.2. The ratio of ml medium/g tissue used in homogenizing was 2:1. Normally about 50 g tissue was used in a preparation. The homogenate was then strained through 4 layers of cheesecloth to remove cell debris. The filtrate was spun at 1000g to remove starch grains and the larger cell inclusions. The synthetase activity of this fraction was negligible and it was usually discarded. The supernatant was centrifuged at 10,000g for 30 min to obtain mainly mitochondrial material contaminated with underdeveloped chloroplasts and chloroplast fragments. These fragments mainly settled at the top of the pellet and could be removed partially by disturbing the surface of the pellet carefully with a pipette. The remainder of the pellet was resuspended in the homogenizing medium and spun at 20,000g. The washed pellet was resuspended in either phosphate buffer containing DTT to give broken organelles, designated 10,000g “B” fraction in the text or in the homogenizing medium, designated 10,000g “A” fraction in the text. When 10,000g “B” fractions were prepared, the pellet was suspended in 10 ml of medium without sucrose for 30 min. the material pelleted as above, and then resuspended in 2 to 4 ml. When the 10,000g “A” fractions were prepared, they were directly suspended in 2 to 4 ml of the homogenizing medium containing sucrose.

The supernatant from the 10,000g pellet was spun at 100,000g on a Spinco Model L Ultracentrifuge for 60 min. Under these conditions it was usual for the sedimented material to be in 2 parts. One part formed a tight pellet on the bottom of the tube. The other part formed a “fluffy” layer just above this. The liquid above the fluffy layer was
pipetted off, and the fluffy layer was transferred into a large volume of homogenizing medium and re-centrifuged at the same speed. The other layer, which was considerably greener and which probably contained some chloroplast and mitochondrial fragments, was sometimes retained where comparisons between it and the fluffy layer were to be made. On a second spin, the fluffy layer sedimented normally and this was the fraction designated as the 100,000g pellet in the text. This pellet was best resuspended using a gentle swirling action aided by a tube shaker set at low speed. After about 5 min a uniform suspension could be obtained. Usually the addition of 1 to 2 ml 0.05M phosphate buffer pH 7.2 gave a suspension with a protein content of 7 to 10 mg/0.2 ml. The supernatant from the 100,000g spin is designated "supernatant" system in the text.

**Protein Determinations.** Protein was determined by the biuret reaction (7). BSA was used as a standard in protein determinations.

**Incubations.** Cell fractions were incubated in the following medium except where stated otherwise—ATP, 2 μmoles; DPNH, 0.5 μmole; TPN', 0.2 μmole; glucose-6-P, 4.0 μmoles; glucose-6-P dehydrogenase, 0.5 units; GSH, 8.0 μmoles; E. coli ACP 0.39 mg protein from crude extract of E. coli. When malonate was the substrate (instead of malonyl CoA), the medium also contained 0.2 μmole CoA and 2 μmoles MnCl₂. When acetyl CoA was the substrate 30 μmoles bicarbonate was included in the mixture. The total volume after addition of 0.2 ml of supernatant enzyme or particulate preparations was 1.07 ml. The amount of substrate used in each incubation was usually 140 μmoles but in some instances as low as 70 μmoles, the radioactivity being between 75,000 and 150,000 dpm per incubation.

Lipid extractions were carried out by the usual procedures (10, 11). To analyze for fatty acids, 0.1 ml of 60% KOH was added to the reaction tube to stop the reaction, and the tube was heated to 80° for 30 min. The contents were then acidified and extracted with chloroform-methanol as described above.

**Gas Chromatography and Radiochemical Analysis.** Gas chromatography was carried out on the Aerograph Model A-90P2 fitted with a thermal conductivity detector. Two columns were used—(i) 5' x 0.25" packed with 12% diethylene glycol succinate (DEGS) on Anakrom P (60-70 mesh). This column was held at 160° where acids of chain length up to C₁₈ were being analyzed and where good separation of C₈,₀ and C₁₀,₀ was desired. The column was used at 185° where the latter operation was less critical and where it was desired to quantitate the radioactivity in components up to a chain length of C₂⁸. (ii) 5' x 0.25" packed with 12% S.E. 30 on Chromasorb W (60-70 mesh). This column was used isothermally at 270° particularly for the detection of radioactivity in very long chain products up to n-C₈₈, and also for temperature pro-

gramming of the products of degradation (see below).

The helium flow rate through both columns was maintained at 60 ml/min.

Radiochemical analysis of the methyl esters of fatty acids was accomplished by passing the effluent from the GLC through a Nuclear Chicago Biospan #4998 unit, at 250°. The signals from the detector were integrated using a scaler-recorder system described by Pearson et al. (19). Fatty acid methyl esters were identified by using appropriate standards using the relative retention values for these components as listed by Buchfield and Storrs (3). The identity of C₆₀ and C₈₈ components was tentatively inferred by extrapolation of the semi-log plot obtained from the use of the standards and verified by degradative studies (see below).

Methyl esters for degradative studies were obtained by collecting fractions from the effluent of the GLC in glass tubes containing glass wool soaked with methanol.

**Degradative Studies of Fatty Acids.** Long chain fatty acids were broken down to a series of shorter chain length homologues using the technique of Harris and James (9).

GLC analysis of the methyl esters of the degraded products was performed using temperature programming from 120° to 290° on the S.E. 30 column. N.I.H. mixture F was used as standard up to a chain length of C₂₄. The 2 peaks beyond this which plotted isothermally at the positions of n-C₂₆ and n-C₈₈ methyl esters gave the expected number of extra peaks when degraded (2 and 4 respectively), thus verifying their tentative identification as long chain fatty acids. In order to detect the presence of elongation patterns, the ratio peak height of the mass trace:peak height of radioactivity trace was used. Direct comparison between peaks at different points in the chromatogram was possible. Under the conditions of temperature programming used the standard deviations of successive peaks did not alter, and peak area was proportional to peak height.

Schmidt decarboxylations were carried out on the palmitate and stearate synthesized by the supernatant system, using the method of Brady et al. (2).

**Determination of Radioactivity.** Fatty acids, methyl esters, total lipid and CO₂ were determined in toluene containing 0.6% 2,5-phenyloazo (PPO) and 0.5% 1,4-bis-2-(5-phenyloazo, 1)-benzene (POPOP) with a Packard liquid scintillation spectrometer. Aqueous samples of substrate and ambient solutions were counted using Bray's solution. Counting efficiency was 72% in toluene and 63% in Bray's solution.

**Chemicals and Substrates.** ATP, CoA, DPNH, TPN, GSH, and glucose-6-P were obtained from Sigma, and L-¹⁴C labeled substrates from the New England Nuclear Corporation. Malonate-2-¹⁴C was obtained from Nuclear Chicago. Malonate-1-¹⁴C from the same source was used to make malonyl CoA-1-¹⁴C according to a method communicated by
Results

Tissue Slices Studied. Lipid incorporation and CO₂ evolution from acetate-1-¹⁴C were measured over an 8-day period from the onset of imbibition (fig 1). After 1 day, the fresh weights of the peas did not alter appreciably and the results are recorded on a fresh weight basis. Lipid incorporation was at a maximum after 3 days soaking, as was CO₂ evolution. However, the lipid:CO₂ ratio was highest after about 1 day's growth and decreased thereafter. When 2 day old tissue was analyzed in terms of the fate of fed acetate, 35.7% of the acetate was incorporated into lipid, 21.4% was unchanged acetate absorbed into the tissue, 8.8% was non-volatile, with soluble components, 13% was released as ¹⁴CO₂ and the remainder was associated with insoluble compounds presumably proteins and carbohydrates. The ratio of lipid soluble to water soluble products was 4 to 1. Thus, incorporation into lipid is a major pathway for the metabolism of acetate in the low lipid, germinating pea seed, and the ability to synthesize lipid from acetate is established early in the germination within the cotyledons. Other results, not detailed, showed that other embryonic tissues, and shoot tissue also synthesized fatty acids of a similar range and type but that the lipid:CO₂ ratios were of the order of 1:10. The average percent composition of the synthesized products in terms of chain length is reported in table I. Starting with 300 μmoles of acetate-1-¹⁴C, the one day tissue incorporated 6.4% acetate into fatty acids, the 2 and 3 day tissue 9.4%, and the 4 day tissue 6.5%. The ability to make all types of fatty acids did not vary much over the period of 5 days, except that one day tissue had no capacity to synthesize the C₁₆:₁ component, but still synthesized the normal quantities of C₁₈:₀ typical of other tissue samples. This suggests that the C₁₈:₁ production is not directly tied to C₁₆:₁. An aliquot of the methyl esters from the 3 day tissue were analyzed by thin layer chromatography on silver nitrate impregnated Silicagel G. The content of labeled monounsaturates was estimated at 30.1%, and that from GLC at 27.2%.

Incorporation of acetate over a period of 7 days remained essentially similar with respect to the lipid classes involved, but as the tissue aged there was an increasingly large incorporation into free acid, which varied from 6% for 1 day old to 25% for 7 day old tissue.

Over 95% of the label in total lipid was recovered as methyl esters of fatty acids. There were no components corresponding to hydroxy-acids synthesized from acetate in this tissue.

Since the tissue had a pronounced capacity for the synthesis of very long chain fatty acids, fatty acids of various chain lengths were used in an attempt to detect elongation of such substrates. Table II shows the results of feeding C₈:₁₈ radioactive fatty acid substrates in the presence of cold acetate. It can be seen that even though they were readily broken down to CO₂, neither C₁₆ nor C₁₈ fatty acids gave any detectably labeled higher homologues, and no substrates except acetate and malonate were incorporated into very long chain fatty acids. Fatty acids of intermediate chain length, (C₁₆₁₄) gave varying amounts of products with a chain length up to C₁₈, but higher homologues were not detected. Such precursors were also reasonably efficient precursors of unsaturated fatty acids (C₁₈:₁ and C₁₈:₁₁). These results are similar to those obtained by Hawke and Stumpf (11). If it is assumed that the breakdown of palmitate and stearate is a normal β-oxidation process, it is apparent that breakdown of palmitate followed by re-synthesis did...
Two g tissue slices and 300 mmoles of each substrate (2 µc) were used. In the case of fatty acid substrates 250 mmoles of cold acetate were included. Only fatty acids up to C22 were analyzed. Substrates were all 1-labeled except malonate which was 2-labeled.

not occur. Since this would also be the case for the other 1-labeled substrates the variety of fatty acids synthesized from these substrates are probably elongation products. Since the C6-C14 acids were elongated, they must have been converted to the thioester derivative appropriate for elongation (whether it be a CoA or ACP derivative is not specified). However, the process apparently went no further than a chain length of C18 under present experimental conditions. Thin layer chromatography of total lipid following incubation with palmitate or stearate showed that most of the substrate was esterified in polar complex lipids or in neutral lipid.

The saturated fatty acids made by tissue slices from acetate were separated by GLC and degraded using the technique modified from that of Harris and James (9). Using this technique, a series of fatty acids differing in chain length by 1 carbon atom were produced from the original acid.

Radioactivity was present in every component down to the level of C10 from the ω- end of the chain. Neither mass nor radioactivity were detected under our conditions in methyl esters of a chain length less than C6, and a ratio for the latter component was not arrived at in most cases owing to the small amounts of mass present, though in all cases some activity was discerned in this component. The figures for the even numbered degradation products are shown in table III. The ratios indicate de novo synthesis for both palmitate and stearate. In other components there was evidence of elongation superimposed on a pattern typical of de novo synthesis. Although the magnitude of the effect varied somewhat, all acids synthesized which possessed a chain length greater than C18 had considerably greater relative amounts of radioactivity than any of their degraded products.

Decarboxylation studies performed on a series of saturated fatty acids gave amine:CO₂ ratios between 7.0 and 9.0 for palmitate and stearate, and thus confirmed the conclusion that these products are formed de novo from acetate. Results for the longer chain products were somewhat variable, and reliance has been placed more on the data from degradation studies.

Homogenate Studies. Homogenates of pea cotyledon tissue were tested for synthetase activity. The synthetase capacity of the homogenates and supernatants was linear over a time period of 4 hr. When measured over 3 hr, the relation between protein concentration and lipid incorporation was linear with use of up to 16 mg protein per tube, as shown in figure 2. The rates of incorporation by the homogenate were closely similar to those of the supernatant. The same general relations were true of the particulate fractions of the cell. Up to 16.0 mg/tube of "mitochondrial" protein and 15.0 mg/tube of "microsomal" protein were used and gave linear rates of incorporation into lipid over a period of 3 hr in each case. Using a time period of 3 hr, the incorporation into lipid by particulate fractions was linear to about 16 mg protein/tube. Malonate was efficiently incorporated into lipid at about half this rate. There was a trend towards reduction of synthetase activity as germination time progressed, but no peak of activity comparable with that observed using whole tissue slices.
The composition of the fatty acids formed by homogenates from malonoyl CoA and malonate was—
\( C_{14 \pm 0} \), 2%; \( C_{16 \pm 0} \), 25%; \( C_{18 \pm 0} \), 70%. There were only traces of very long chain acids. No unsaturated fatty acids were made by homogenates from malonate or malonyl CoA. Clearly this pattern of incorporation bore little resemblance to that typical of the whole tissue (table I). Using various fractions and substrates, the results in table IV were obtained. The supernatant incorporated malonate and malonyl CoA, but utilized acetate and acetyl CoA poorly. The 100,000g pellet was as efficient as the supernatant using malonyl CoA. Incorporation of acetyl CoA was relatively greater than by the supernatant, whilst malonate was very poorly used. This indicated that the pellet was richer in acetyl CoA car-

boxylase activity than the supernatant, but that malonyl CoA synthetase activity was much lower. The 10,000g pellet showed a different pattern, which was dependent on the pretreatment of the pellet. If treated in a manner which would be expected to keep the mitochondria intact, acetyl CoA was the most efficient precursor of lipid, and malonyl CoA was relatively less effective. When treated with a hypotonic solution for 30 min before incubation with substrate and co-factors, malonyl CoA was the only substrate that was effectively used. This indicated that the hypotonic treatment had released and diluted enzyme(s) and/or co-factors that were responsible in part for the conversion of acetyl CoA to malonyl CoA. Results also imply a limited movement of malonyl CoA into the mitochondrial organelle in System A. Treatment with hypotonic medium allowed ready entrance of malonyl CoA to the site of synthetase activity.

### Table IV. Incorporation of Different Substrates Into Lipid by Three Cell Fractions

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Supernatant (10,000g ) “A”</th>
<th>Supernatant (10,000g ) “B”</th>
<th>Malonyl CoA</th>
<th>Acetate</th>
<th>Acetyl CoA</th>
</tr>
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<tbody>
<tr>
<td>mmoles incorporated per 10 mg protein per hr</td>
<td>10 mg</td>
<td>10 mg</td>
<td>10 mg</td>
<td>10 mg</td>
<td>10 mg</td>
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<tr>
<td>Acetate</td>
<td>0.25</td>
<td>0.24</td>
<td>0.27</td>
<td>0.25</td>
<td>0.24</td>
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<td>Acetyl CoA</td>
<td>0.12</td>
<td>0.24</td>
<td>0.36</td>
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<td>0.04</td>
<td>0.05</td>
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<tr>
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<td>0.37</td>
<td>0.40</td>
<td>0.37</td>
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</table>

In all these experiments, use was made of unlabeled acetyl CoA in combination with malonyl CoA in an attempt to stimulate fatty acid synthesis by providing the primer acetyl CoA, but in practice this addition made no observable increase in fatty acid synthesis possibly because there was enough decarboxylase activity in the tissue to provide saturating amount of acetyl CoA.

Further distinction between the 2 systems was afforded by studies of their pH dependence (fig 3). The supernatant system showed a sharp optimum at pH 7.0 whereas both pellets showed a much broader tolerance of pH variations, with an optimum for the 100,000g pellet at 8.0 and for the 10,000g pellet at 7.0.

Patterns of fatty acid synthesis differed markedly between the 3 fractions used and upon whether the substrate used was malonyl CoA or acetyl CoA. Table V shows the distribution of radioactivity in various fatty acids with a chain length ranging from \( C_1 \) to \( C_{20} \) as a result of incubation of acetyl CoA and malonyl CoA with the various cell fractions. It is apparent that the 10\%g fraction, using malonyl CoA, has the ability to synthesize the very long
chain fatty acids which is absent from the other fractions. The 10,000g fraction synthesized some 7% of C₂₀, but even under the most favorable conditions for their detection, namely the use of high temperatures and an S.E. 30 column, the C₂₂-₂₈ components could not be detected as products of synthesis by this fraction. Thus, the ability to synthesize the very long chain fatty acids was not a general property of particulate cellular components. The supernatant pattern was very similar to that found from homogenates. It is apparent that the synthetic capacity of the homogenates are largely dominated by the supernatant. The differences between the products made from malonyl CoA and acetyl CoA were marked. With the latter substrate, neither particulate fraction synthesized fatty acids with a chain length greater than C₁₆. With acetyl CoA, 50% of the acids synthesized by the 10,000g fraction was a C₁₆:₁ component; In contrast, with malonyl CoA the main component was stearate, with some small amount of C₂₀. A considerable shift in pattern was also observed as a result of using these different substrates with the supernatant system, but the effect was not as clear-cut. An unknown component appeared in the radiochromatograms from the 10g pellet, having a retention time corresponding to a carbon number of 19.5. This component was apparently not a hydroxy-acid, as it did not separate as such on TLC, nor was it an unsaturated component since it remained in the same relative position of gas-liquid chromatograms using both DEGS and S.E. 30 columns. It seemed probable that it could be an elongation product of an endogenous branched chain.

Since the 10g fraction sedimented in 2 distinct parts, the ability of both parts to incorporate malonyl CoA into lipid was tested separately. It was found that both fluffy and heavy fractions incorporated malonyl CoA with about the same pattern of fatty acid production, but that the fluffy (lighter) fraction was considerably more efficient. Four determinations on each fraction yielded the following figures (in μmoles incorporated per 10 mg protein per 3 hr); fluffy, 10.82 ± 0.7; heavy, 7.44 ± 0.6.

Experiments designed to detect elongation from palmitate-1⁴C and stearate-1⁴C and from palmityl CoA-1⁴C using the 10g pellet have yielded negative results. Experiments using ACP derivatives should be done.

Experiments designed to detect interaction be-

Fig. 3. pH dependence of fatty acid synthetase systems associated with 3 cellular fractions, as indicated. The pH was varied using HCl buffer, 0.05 M final concentration, and a total volume of incubations of 2.07 ml, 1 ml more than the normal volume. Substrate: malonyl CoA, 70 μmoles, 75,000 dpm. Incubation conditions: 3 hr, 30°. The 10,000g fraction used was of the “broken” type (10,000g). ▲, Supernatant. ●, 10⁵ pellet. ▼, 10,000 pellet.

Table V. The Percent Distribution of Radioactivity in Fatty Acids After Incubation of Acetyl CoA and Malonyl CoA With Different Cell Fractions

<table>
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<tr>
<th></th>
<th>mal CoA</th>
<th>10g CoA</th>
<th>mal CoA</th>
<th>10,000g CoA</th>
<th>mal CoA</th>
<th>10,000g CoA</th>
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between products of the supernatant system and the 2 particulate fractions have yielded negative results. This experiment has been attempted in 2 ways 1) by incubating supernatant and particulate cell material together and separately and testing the results for interactions as they may affect the patterns of synthesis, 2) by incubating the supernatant system with substrate to form the C16 and C18 components, and then adding particulate preparations to the boiled system. In neither case has it yet been possible to detect interactions between the systems with respect to influencing the pattern of fatty acids synthesized. Interaction was however observed, as might be expected, in the total lipid figures. Table VI shows the result of recombining cellular fractions on the total lipid synthesis from malonyl CoA. For instance, separately the 100,000g pellet and the supernatant system incorporated 1.6 and 10.5 mmoles of malonyl CoA but when combined the total incorporation was 19.3 mmoles, suggesting a synergistic interaction occurring between the 2 fractions. Table VII shows the distribution of radioactivity between the broad lipid classes of glycerides, free acid and polar complex lipid as influenced by this interaction. The supernatant system alone accumulated 58% of the label in free acid, while when combined with the 10g fraction most of the label was transferred from the free acid into the polar lipid fraction. Endogenous synthesis in the 10g fraction cannot quantitatively account for this effect, because in this experiment the protein associated with the 10g pellet was kept low at 1.5 mg compared with 7.3 mg from the supernatant system. Since capacities for synthesis of the 2 cell fractions are approximately equal on a protein basis, one would expect that in this case the incorporation of the mixed fractions would be dominated by that typical of the supernatant system, but this was not so. The increase in total lipid synthesized by the combination of supernatant plus particulate fractions could be related to the insertion of fatty acids to polar lipids in microsomes.

Table VII. The Effect of Limiting Cofactors on Lipid Synthesis in the Supernatant System and Supernatant + 10g Pellet

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>Supernatant</th>
<th>Supernatant + 10g Pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malonyl CoA</td>
<td>0.78</td>
<td>0.66</td>
</tr>
<tr>
<td>ATP</td>
<td>0.78</td>
<td>0.50</td>
</tr>
<tr>
<td>DPNH</td>
<td>0.78</td>
<td>0.50</td>
</tr>
<tr>
<td>TPNH</td>
<td>0.78</td>
<td>0.50</td>
</tr>
<tr>
<td>MgCl2</td>
<td>0.78</td>
<td>0.50</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>0.78</td>
<td>0.50</td>
</tr>
<tr>
<td>Complete</td>
<td>0.78</td>
<td>0.50</td>
</tr>
</tbody>
</table>

1. ACP and ATP significantly depressed incorporation.
2. ACP and ATP significantly depressed incorporation.
3. ACP and ATP significantly depressed incorporation.
4. ACP and ATP significantly depressed incorporation.
5. ACP and ATP significantly depressed incorporation.
6. ACP and ATP significantly depressed incorporation.
7. ACP and ATP significantly depressed incorporation.
8. ACP and ATP significantly depressed incorporation.
9. ACP and ATP significantly depressed incorporation.
10. ACP and ATP significantly depressed incorporation.
11. ACP and ATP significantly depressed incorporation.
and 100,000 g pellet. However, due to the low levels of incorporation, no results were obtained using acetyl CoA with the 10,000 g (B) pellet.

While not yet studied in any detail, β-oxidation systems are prominent in pea tissue homogenates, and are associated with both soluble and particulate matter after homogenizing.

Attempts have been made to demonstrate production of C18:1 in cell free systems derived from pea tissue slices by incubations under a variety of conditions, but so far without success. Chemical degradations on products of cell-free systems have been performed thus far only on the C16 and C18 components made by the supernatant system, which were easily obtainable in the required large quantities. Schmidt decarboxylations of these products gave CO₂:amine ratios of 7.0 for C₁₆ and 7.7 for the C₁₈ component. These figures indicated predominantly de novo synthesis. Degradation of C₁₈ using the permanganate method revealed consistent radioactivity:mass ratios down to a level of C₁₁, after which the ratios were much reduced.

Discussion

The synthesis of a large proportion (up to 80 %) of stearate by the cell free supernatant system and homogenates from pea cotyledons is atypical of the tissue slice system which makes mostly C₁₈:₁ from acetate-¹⁴C together with a smaller or equal amount of C₁₈:₀ and also a series of very long chain fatty acids up to a chain length of C₂₆. Both C₁₆:₀ and C₁₈:₀ were apparently synthesized de novo by tissue slices and by the cell free system. Though it seems probably that C₁₈:₁ synthesis proceeds by way of an oxidative desaturation of stearate, there is at the moment no direct evidence in vitro of this reaction. The above results do have relevance, however, to the mode of synthesis of the very long chain fatty acids with a chain length up to n-C₂₈.

The very long chain fatty acids synthesized from acetate by tissue slices showed evidence of elongation having occurred because the pattern of distribution of radioactivity in them and the products of their degradation showed a larger radioactivity:mass ratio in the parent compound than in the degraded products. In cases where no unlabeled diluent was added to the fatty acid before degradation, the difference in the ratio approached an order of magnitude. Such a result (table III) can be explained only if the main reaction occurring in the experimental period was the addition of 1 acetate unit to a pre-existing fatty acid C₆ to form C₆₀. The results do not point to any one fatty acid as being the substrate for elongation reactions, but indicate that all endogenous fatty acids with a chain length >C₁₆ can perform this role.

The capacity for very long chain fatty acid synthesis was confined to the 10⁵g pellet and was not associated with the 10,000 g pellet. The exact nature of the pellet as isolated has not yet been determined. Since the particulate fraction was the only locus of production of fatty acids with a chain length >C₁₈, which have been shown to be formed partially by elongation reactions in tissue slices, it is likely, though not proved, that the 10⁵g pellet is the site of elongation of fatty acids of chain length C₁₈ and above. Guchhait et al. (8) reported that pigeon liver microsomes were capable of elongation reactions, the main evidence being a stimulation of malonyl CoA incorporation by the addition of acyl CoA derivatives of requisite chain length. In the present study, attempts to incorporate palmitoyl CoA-1-¹⁴C into the long chain fatty acids using the 10⁵g pellet failed, all the label remaining in the palmitate when the latter was incubated under the usual conditions with cold malonyl CoA. Similar negative results were obtained when the free fatty acids were used. Information relevant here is the distribution of radioactivity among the products of degradation of the very long chain fatty acids synthesized by the cell free system, but this has not yet been obtained. It seems likely, however, that endogenous fatty acids within the 10⁵g pellet form the substrate for elongation reactions involving malonyl CoA, at least for the synthesis of the very long chain products. The 10⁵g pellet synthesizes only a small amount of C₁₆:₀; no stearate is present in the synthesized products (table V). Apart from the unknown component, which is probably a product of elongation of a branched chain, well over 90 % of the radioactive products of this system are long chain fatty acids C₂₀-₂₆. The presence of radioactivity in the products of degradation of the tissue synthesized acids down to a chain length of as low as C₅ indicated that during the experimental incubation period of 5 hr, some of the very long chain acids were synthesized de novo from acetate, and some were produced from elongation reactions, so that the final mixture of acids extracted from the tissue was derived from a mixed population with respect to its relation to the recently metabolized acetate. What seems therefore likely is that there is a de novo synthesis of acetate up to a chain length of C₁₈, possibly by the soluble system, followed by a gradual accumulation of longer chain fatty acids by a slow addition of C₂ units which may occur only after transfer of C₁₈ to an elongation "complex" in the membrane.

The additions of acetate did not apparently occur continuously at any 1 locus as is the case for other synthetase complexes, since if this were so, radioactivity:mass ratios would be similar down to a certain chain length, when there would then be an abrupt transition to the level of the primarily elongated substrate.

The above concept of the elongation reactions in pea tissue does explain some otherwise puzzling results. One of these, true in both pea cotyledon tissue and barley leaf tissue, is that the actual mass of the very long chain acid components is about 2
orders of magnitude less than the mass of the C₁₀-₁₈ components from which it is assumed they are derived. In order to explain this, it is necessary to invoke a compartmentation hypothesis. If only small amounts of long chain acids are formed during the growth of a cell, and if labeled fatty acids were formed exclusively by elongation reactions of these small quantities, the relatively high specific activity of the very long chain acids would be explained. Secondly, it is difficult to explain why palmitate and stearate are not elongated to longer chain components when supplied to the tissue with adequate supplies of cold acetate, especially since activation at least to the level of CoA ester must occur, as about 10% of the acid taken up by the tissue is oxidized to CO₂.

It is, however, quite possible that the ACP derivatives of palmitate or stearate are the active substrates. There is now evidence accumulating that higher plants do not have long chain acyl transacylases which would catalyze the transfer of the stearyl component of stearyl CoA to ACP. Thus, stearyl CoA could be rapidly formed but would either be β-oxidized or transferred to a complex lipid and remain out of the pool of acyl ACP necessary for elongation.

Breakdown of palmitate and stearate-1-¹⁴C to acetate units presumably occurred since about 10% of the label from these substrates appeared in CO₂. For some reason, however, resynthesis did not occur under these conditions either in the present case of pea tissue or barley leaf tissue (11); otherwise synthesis of the very long chain fatty acids should have been observed from the ¹⁴C-labeled substrates. Using uniformly labeled C₁₈ as the substrate, Kolattukudy (12) demonstrated the production of some long chain fatty acids up to a chain length of C₂₄. The origin of these was said to be by direct elongation from stearate. However, the very efficient conversion of palmitate and stearate to nonacosane (12) which he reports, together with other evidence recently summarized (13) suggests that comparative studies of different tissues may reveal some interesting differences in elongation patterns.

A feature of some importance possessed by the ¹⁴C₂₂₂ pellet system in peas is the synthesis and release of fatty acid homologues differing by only 2 carbon atoms. It should be pointed out that this ability to synthesize large quantities of substances which are apparent intermediates in the pathway to the final product is not typical of fatty acid synthesis systems. The supernatant system in peas, for example, synthesizes only C₁₈ and C₁₆ fatty acids, and the intermediates are generally not detectable. The same is true of most other systems which have been investigated. The pattern of radioactivity in the degradation products indicating elongation of endogenous acids, explains this feature of the system.

Lipid synthesis in germinating peas, as in other seeds, is probably important because of the necessity to synthesize cell membranes during this period. It is of some interest that total lipid incorporation from acetate increases rapidly from the onset of imbibition, while CO₂ output lags somewhat behind (fig 1). The role of the very long chain fatty acids in the germinating seed is not clear, but they appear to be associated equally with glycerides and polar lipids. No incorporation of radioactivity into less polar compounds, such as long chain esters or paraffins could be detected. It is possible that such very long chain acids are an essential component in cell membrane systems as well as in waxy coatings, and are possibly converted to wax components in the leaves and stems of higher plants.

Acknowledgment

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Literature Cited