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Fat Metabolism in Higher Plants XXXVI: Long Chain Fatty Acid Synthesis in Germinating Peas[1]

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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-^{14}C into fatty acids from C_{16} to C_{24}. Although crude homogenates synthesize primarily 16:0 and 18:0 from malonyl CoA, subsequent fractionation into a 10,000*g* pellet, a 10^5*g* pellet and supernatant (soluble synthetase) revealed that the 10^5*g* pellet readily synthesizes C_{16} to C_{28} fatty acids whereas the 10,000*g* and the supernatant synthesize primarily C_{16} and C_{18}. All systems require acyl carrier protein (ACP), TPNH, DPNH if malonyl CoA is the substrate and ACP, Mg^{2+}, CO_2, ATP, TPNH, and DPNH if acetyl CoA is the substrate. The cotyledons of germinating pea seedlings appear to have a soluble synthetase and 10,000*g* particles for the synthesis of C_{16} and C_{18} fatty acid, and 10^5*g* 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 C_{24} as well as the usual saturated and unsaturated fatty acids in the C_{14-18} range. Attempts to detect elongation of C_{16} and C_{18} 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 CO_2. 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 C_{16}. Kolattukudy (12–13) working with *Brassica* leaf tissue obtained evidence that the C_{29} 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 C_{26} when palmitate or stearate were fed. Hawke and Stumpf (10–11) found that nearly 10 % of the radioactivity from palmitate-1-^{14}C appeared as CO_2 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 $>C_{18}$) 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 C_{18-20} 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 12,000*g* system. Both systems synthesized palmitate and stearate from malonyl CoA, and the particulate system in addition synthesized traces of $C_{18:1}$. 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 ($>C_{18}$).

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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-^{14}C, 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 plus radioactive substrate was placed in a 50 ml conical flask sealed by a serum cap to which a small polyethylene cup was fitted for trapping CO_2 at the end of the incubation period, the latter being 5 hr in all experiments with tissue slices.

At the end of the incubation period, 2 ml 10 N H_2SO_4 was injected into the flask through the serum cap, and 0.2 ml Hyamine 10X into the polyethylene cup. $^{14}CO_2$ estimations were made according to the method described by Hawke and Stumpf (10).

In order to estimate the amount of substrate remaining in the ambient solution, the tissue was washed with 100 ml H_2O and three 0.2 ml aliquots counted in Bray's solution on the scintillation spectrometer.

Lipid Extraction and Thin Layer Chromatography of Lipid. Total lipids were extracted from the tissue with 2:1 chloroform/methanol, using 10 ml of solvent for 2 g tissue. The mixture was shaken gently for 12 hr in a stoppered flask at room temperature, and at the end of this period the tissue was strained off and washed with a further 10 ml of solvent. The washings were bulked with the main extract, 20 ml H_2O were added to the chloroform-methanol extract and the chloroform layer was separated and dried over anhydrous Na_2SO_4.

Aliquots of total lipid were chromatographed on orated in scintillation vials, 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°.

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:ethanol:acetic acid, 40:50:2.0:0.2. 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 BF_3-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°. 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 100,000*g* 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 0.05 M phosphate buffer pH 7.2 gave a suspension with a protein content of 7 to 10 mg/0.2 ml. The supernatant from the 100,000*g* 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 $MnCl_2$. 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 mμmoles but in some instances was as low as 70 mμmoles, 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′ × 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 C_{18} were being analyzed and where good separation of $C_{18:0}$ and $C_{18:1}$ 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 C_{24}. (ii) 5′ × 0.25″ packed with 17 % 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-C_{28}, and also for temperature programming 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 C_{26} and C_{28} 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 ot fhe 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 C_{24}. The 2 peaks beyond this which ploted isothermally at the positions of n-C_{26} and n-C_{28} 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 CO_2 were determined in toluene containing 0.6 % 2,5-phenyloxazole (PPO) and 0.5 % 1,4-bis-2-(5-phenyloxazole L)-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 1-^{14}C labeled substrates from the New England Nuclear Corporation. Malonate-2-^{14}C was obtained from Nuclear Chicago. Malonate-1-^{14}C from the same source was used to make malonyl CoA-1-^{14}C according to a method communicated by

P. R. Vagelos, Washington University. Acyl carrier protein was prepared from *Escherichia coli* by the method of Goldman *et al.* (6). All radioactive substrates were diluted to the specific activities indicated in the captions to the figures.

Results

Tissue Slices Studied. Lipid incorporation and CO_2 evolution from acetate-1-^{14}C 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 CO_2 evolution. However, the lipid:CO_2 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 $^{14}CO_2$ 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:CO_2 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

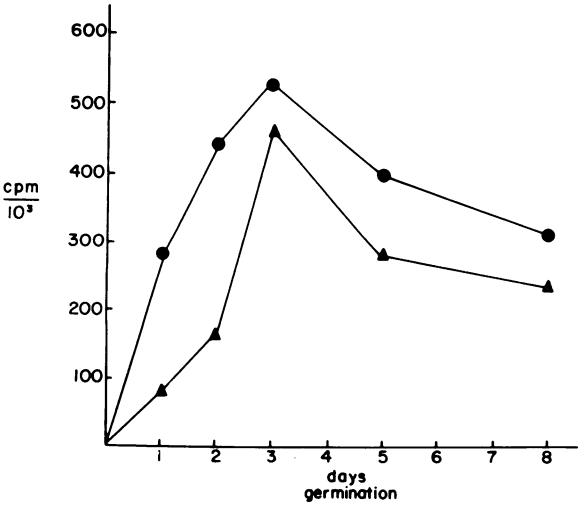

FIG. 1. Incorporation of acetate-1-^{14}C into lipid and release as $^{14}CO_2$ over 1 to 8 days by tissue slices from pea cotyledons of various ages. Incubation: 5 hrs 30°, normal daylight. Substrate: 250 mμmoles, 5 μc. ●, total lipid. ▲, $^{14}CO_2$.

Table I. *Average Percent Distribution of Radioactivity in Fatty Acids Synthesized by Tissue Slices From 1-5 Days Old*

Analysis by chainlength only using 5 foot x one-fourth inch S. E. 30 column at 200 to 270° (programmed). Incubation conditions described in text.

Component	%
C_{16}	16.1
C_{18}	34.0
C_{20}	3.8
C_{22}	18.9
C_{24}	11.5
C_{26}	2.6
C_{28}	13.1

300 mμmoles of acetate-1-^{14}C, 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 $C_{16:1}$ component, but still synthesized the normal quantities of $C_{18:1}$ typical of other tissue samples. This suggests that the $C_{16:1}$ production is not directly tied to $C_{18:1}$. 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 C_{8-18} radioactive fatty acid substrates in the presence of cold acetate. It can be seen that even though they were readily broken down to CO_2, neither C_{16} nor C_{18} 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, (C_{8-14}) gave varying amounts of products with a chain length up to C_{18}, but higher homologues were not detected. Such precursors were also reasonably efficient precursors of unsaturated fatty acids ($C_{16:1}$ and $C_{18:1}$). 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 to acetate followed by re-synthesis did

Table II. *Metabolic Activity of Several Fatty Acids as Substrates*

Two g tissue slices and 300 mμmoles of each substrate (2 μc) were used. In the case of fatty acid substrates 250 mμmoles of cold acetate were included. Only fatty acids up to C_{22} analyzed. Substrates were all 1-labeled except malonate which was 2-labeled.

Substrates	Products (mμmoles)										
	CO_2	C_8	C_{10}	C_{12}	C_{14}	C_{16}	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	C_{20}	C_{22}
$C_{18:0}$	8.07	...	...	...	...	...	...	12.90	...	...	...
$C_{16:0}$	10.03	...	...	...	...	28.30	...	...	...	...	...
C_{14}	8.04	...	...	...	8.50	6.04	...	1.56	1.56	...	...
C_{12}	26.20	...	...	0.14	0.14	0.28	0.28	0.09	1.24	...	...
C_{10}	17.20	...	8.55	...	...	2.80	...	...	0.85	...	...
C_8	12.00	...	...	...	...	0.35	3.88	0.58	0.97	...	...
Acetate-1-^{14}C	24.20					1.20	1.80	0.30	2.70	1.20	1.42
Malonate-2-^{14}C	22.20					1.35	0.30	...	1.38	1.50	1.70

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 C_8-C_{14} 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 C_{18} 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 C_{10} 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 C_8, 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 C_{18} had considerably greater relative amounts of radioactivity than any of their degraded products.

Table III. *Radioactivity:mass Ratios for Tissue-Synthesized Fatty Acids From Acetate-1-^{14}C*

Ratios were calculated using the ratio of the peak heights on mass trace and radioactivity trace. "..." indicates not sufficient mass to measure.

$KMnO_4$ Oxidation products	Parent compound					
	C_{16}	C_{18}	C_{20}	C_{22}	C_{24}	C_{28}
C_{28}						20.00
C_{26}						0.55
C_{24}					5.9	0.29
C_{22}				1.22[1]	1.6	0.37
C_{20}			15.52	1.06	3.6	0.62
C_{18}		0.45	2.40	0.69	1.7	1.85
C_{16}	0.89	0.50	0.79	0.56	0.6	1.11
C_{14}	0.82	0.85	0.50	0.72	0.9	1.00
C_{12}	0.74	0.75	0.50	0.34	...	0.51
C_{10}	0.66	0.69	0.93	0.24	...	0.28
C_8	...	...	...	...	...	0.50

[1] The figures for C_{22} were obtained after adding 4 mg of cold C_{22} to the isolated methyl ester before saponification. Other figures were obtained without addition of carrier.

Decarboxylation studies performed on a series of saturated fatty acids gave amine:CO_2 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.

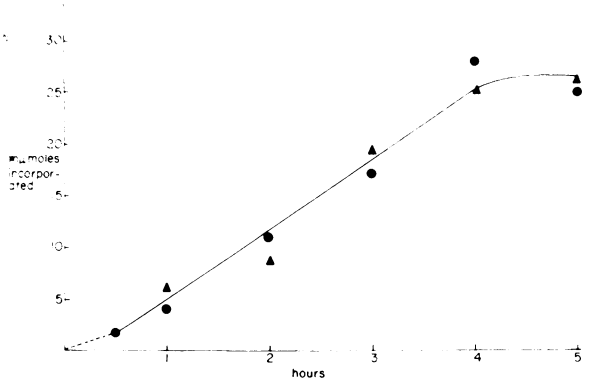

FIG. 2a. Incorporation of malonate-2-^{14}C into lipid by homogenate and supernatant system as a function of time. Substrate: malonate-2-^{14}C, 140 mμmoles, 140,000 dpm. Protein: Supernatant, 7.7 mg. Homogenate, 7.9 mg. ●, Supernatant. ▲, Homogenate.

The composition of the fatty acids formed by homogenates from malonyl CoA and malonate was—$C_{14:0}$, 2 %; $C_{16:0}$, 25 %; $C_{18:0}$, 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 100,000*g* 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 carboxylase activity than the supernatant, but that malonyl CoA synthetase activity was much lower. The 10,000*g* 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*

Fractions were prepared from 2-day pea cotyledons. Amounts of protein used: Supernatant, 6.4 mg; 10,000*g* "A" 16.0 mg; 10,000*g* "B", 16.0 mg; 10^5g, 3.5 mg. See text for definition of "A" and "B". Substrates: acetate-1-^{14}C 70 mμmoles, 200,000 dpm; acetyl CoA-1-^{14}C 70 mμmoles, 220,000 dpm; malonate-2-^{14}C 70 mμmoles, 70,000 dpm; malonyl CoA-1-^{14}C 70 mμmoles, 75,000 dpm.

	Supernatant	10,000*g* "A"	10,000*g* "B"	10^5g
	mμmoles incorporated per 10 mg protein per hr			
Acetate	0.25	0.12	...	0.35
Acetyl CoA	0.24	0.71	<0.01	1.30
Malonate	1.12	0.04	...	0.10
Malonyl CoA	2.70	0.37	3.00	3.77

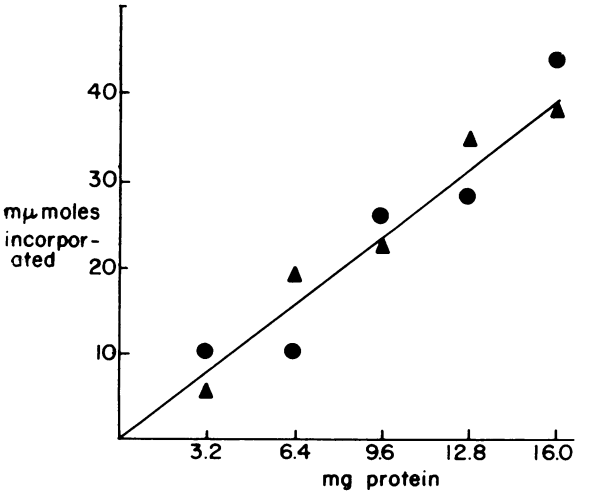

FIG. 2b. Relation of protein concentration to lipid incorporation rate measured over a 3 hr period using crude homogenate and supernatant systems. Substrate: malonate-2-^{14}C, 140 mμmoles, 140,000 dpm. ●, Supernatant. ▲, Homogenate.

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 100,000*g* pellet at 8.0 and for the 10,000*g* 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_{14} to C_{26} as a result of incubation of acetyl CoA and malonyl CoA with the various cell fractions. It is apparent that the 10^5g fraction, using malonyl CoA, has the ability to synthesize the very long

chain fatty acids which is absent from the other fractions. The 10,000*g* fraction synthesized some 7 % of C_{20}, but even under the most favorable conditions for their detection, namely the use of high temperatures and an S.E. 30 column, the C_{22-26} 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 C_{16}. With acetyl CoA, 50 % of the acids synthesized by the 10,000*g* fraction was a $C_{16:1}$ component; In contrast, with malonyl CoA the main component was stearate, with some small amount of C_{20}. 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 10^5g 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.

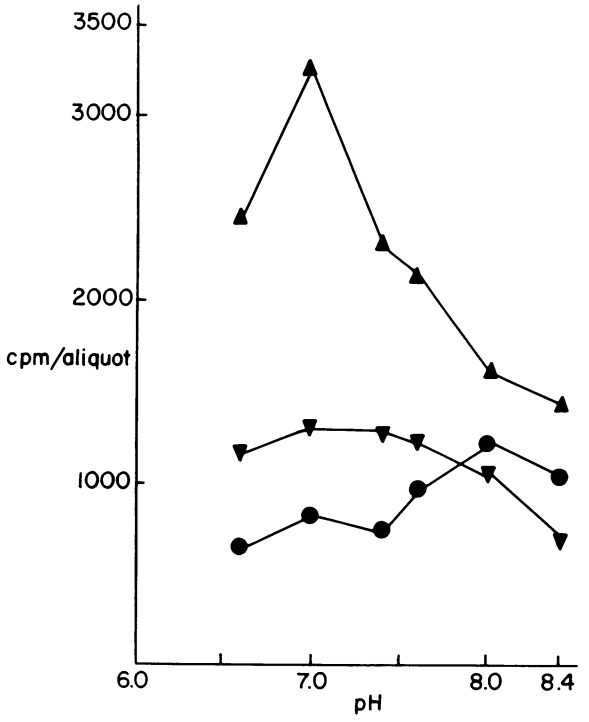

FIG. 3. pH dependence of fatty acid synthestase systems associated with 3 cellular fractions, as indicated The pH was varied using HCl buffer, 0.05 M final concentration, and a total volume of incubations of 2.07 ml, 1 ml more than the normal volume. Substrate: malonyl CoA, 70 mμmoles, 75,000 dpm. Incubation conditions: 3 hr, 30°. The 10,000*g* fraction used was of the "broken" type (10,000*g*). ▲, Supernatant. ●, 10^5 pellet. ▼, 10,000 pellet.

Since the 10^5g 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 mμmoles 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-1-^{14}C and stearate-1-^{14}C and from palmityl CoA-1-^{14}C using the 10^5g pellet have yielded negative results. Experiments using ACP derivatives should be done.

Experiments designed to detect interaction be-

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

Results for the 10,000*g* pellet using acetyl CoA were obtained using pellets kept in 0.3 M sucrose during preparation and incubation, those for malonyl CoA were obtained using a pellet previously dispersed in dilute phosphate buffer. Material was bulked over several experiments.

	10^5g		10,000*g*		Supernatant	
	mal CoA	ac CoA	mal CoA	ac CoA	mal CoA	ac CoA
$C_{14:0}$	2	45	3	2	2	2
$C_{16:0}$	3	55	25	50	20	45
$C_{16:1}$	...	...	...	48	...	...
$C_{18:0}$	...	...	65	tr	75	53
$C_{18:1}$	...	...	...	...	...	...
Unknown	17	...	...	tr	...	..
C_{20}	12	...	7	...	3	...
C_{22}	20	...	...	...	...	...
C_{24}	35	...	...	...	...	...
C_{26}	11	...	...	...	...	...

Table VI. *Possible Interaction in Total Lipid Incorporation Between Supernatant, Microsomes, and Mitochondria*

The substrate was 140 mμmoles malonyl CoA containing 150,000 dpm. Incorporation time was 3 hr. S = Supernatant, 7.3 mg protein. m = 10^5g, 1.5 mg protein. M = 10,000*g*, 9 9 mg protein.

Combination	Incorporation/3 hr
S m M	14.9
S m	19.3
S M	19.4
m M	7.3
S	10.5
m	1.6
M	4.6

tween 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 C_{16} and C_{18} 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 100,000*g* pellet and the supernatant system incorporated 1.6 and 10.5 mμmoles of malonyl CoA but when combined the total incorporation was 19.3 mμmoles, 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 10^5g fraction most of the label was transferred from the free acid into the polar lipid fraction. Endogenous synthesis in the 10^5g fraction cannot quantitatively account for this effect, because in this experiment the protein associated with the 10^5g pellet was kept low at 1.5 mg compared with 7.3 mg from the

Table VII. *Lipid Distribution Patterns in Lipid Synthesized by the Supernatant System and Supernatant + 10^5g Pellet*

Protein values and other conditions as for table VII.

	Supernatant	Supernatant + 10^5g pellet
	%	%
Polar lipid	25	70
Free acid	58	11
Glycerides	17	19

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.

Cofactor Requirements for Cell Free Fractions. The response to various cofactors of 3 different systems using 2 substrates is shown in table VIII. In this experiment since a 10,000*g* pellet which had been treated with a hypotonic solution was used, incorporation from acetyl CoA was rather low. The

Table VIII. *The Effect of Limiting Cofactors on 3 Synthetase Incorporating Systems*

Substrates used: malonyl CoA, 140 mμmoles 150,000 dpm, acetyl CoA, 140 mμmoles, 125,000 dpm. Protein: supernatant, 10.7 mg, 100,000*g*, 7 mg, 10,000*g*, 18.0 mg.

	100,000*g*	Supernatant	10,000*g* (B)
	mμmoles substrate incorporated per 10 mg protein per hr		
Malonyl CoA as substrate			
—ACP	4.90	0.83	0.86
—ATP	6.75	6.87	1.93
—DPNH	8.30	6.17	2.70
—TPNH	1.89	2.58	1.27
—$MgCl_2$	7.10	6.67	3.08
—Bicarbonate	7.85	8.35	2.90
Complete	8.10	8.14	3.13
Acetyl CoA as substrate			
—ACP	1.36	0.15	[1]
—ATP	0.48	0.29	[1]
—DPNH	0.78	0.75	<0.01
—TPNH	0.30	0.50	"
—$MgCl_2$	0.86	0 85	"
—Bicarbonate	0.41	0.62	"
Complete	1.72	0.86	"

[1] —ACP and —ATP significantly depressed incorporation.

results obtained indicate that the supernatant and 10^5g pellet were more efficient on a protein basis than the 10,000*g* pellet. All systems responded markedly to limitation of TPNH. DPNH did not have the same effect, though its absence in the incubation medium had sufficient effect in the supernatant system for it to be routinely included. The addition of ACP produced a 10-fold stimulation of lipid incorporation by the supernatant system, a 2-fold one for the 100,000*g* pellet and a 3- to 4-fold one for the 10,000*g* pellet. These results indicate that the particulate systems are ACP dependent but in varying degrees presumably because of the presence of bound functional ACP. Using acetyl CoA the differences were more obscured by the generally low levels of incorporation encountered, but the general trends remained similar. The expected response to bicarbonate occurred using the supernatant

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 $C_{18:1}$ 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 C_{16} and C_{18} components made by the supernatant system, which were easily obtainable in the required large quantities. Schmidt decarboxylations of these products gave CO_2:amine ratios of 7.0 for C_{16} and 7.7 for the C_{18} component. These figures indicated predominantly *de novo* synthesis. Degradation of C_{18} using the permanganate method revealed consistent radioactivity:mass ratios down to a level of C_{12}, 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 $C_{18:1}$ from acetate-^{14}C together with a smaller or equal amount of $C_{18:0}$ and also a series of very long chain fatty acids up to a chain length of C_{28}. Both $C_{16:0}$ and $C_{18:0}$ were apparently synthesized *de novo* by tissue slices and by the cell free system. Though it seems probably that $C_{18:1}$ 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-C_{28}.

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 C_n to form C_{n+2}. 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 $>C_{16}$ can perform this role.

The capacity for very long chain fatty acid synthesis was confined to the 10^5g 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 $>C_{18}$, which have been shown to be formed partially by elongation reactions in tissue slices, it is likely, though not proved, that the 10^5g pellet is the site of elongation of fatty acids of chain length C_{18} 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 palmityl CoA-1-^{14}C into the long chain fatty acids using the 10^5g 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 10^5g pellet form the substrate for elongation reactions involving malonyl CoA, at least for the synthesis of the very long chain products. The 10^5g pellet synthesizes only a small amount of C_{16}; 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 C_{20-26}. The presence of radioactivity in the products of degradation of the tissue synthesized acids down to a chain length of as low as C_8 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 C_{18}, possibly by the soluble system, followed by a gradual accumulation of longer chain fatty acids by a slow addition of C_2 units which may occur only after transfer of C_{18} 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 C_{16-18} 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 CO_2. 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-^{14}C to acetate units presumably occurred since about 10 % of the label from these substrates appeared in CO_2. 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 1-labeled substrates. Using uniformly labeled C_{18} as the substrate, Kolattukudy (12) demonstrated the production of some long chain fatty acids up to a chain length of C_{26}. 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 10^5g 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 synthetase systems. The supernatant system in peas, for example, synthesizes only C_{16} and C_{18} 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 CO_2 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.

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