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Fat Metabolism in Higher Plants

XL. SYNTHESIS OF FATTY ACIDS IN THE INITIAL STAGE OF SEED GERMINATION

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ABSTRACT

To understand more fully organelle membrane assemblage, the synthesis of the first fatty acids by the germinating pea, *Pisum sativum*, was studied by the incorporation of either tritiated water or acetate-1-^{14}C into lipids by the intact, initially dry seed. After a lag phase, labeling proceeded linearly. This lag phase ended when uptake of water had increased the seed weight to 185% of its original weight. The first fatty acids synthesized were palmitic and stearic followed shortly after by long chain saturated fatty acids (C_{20}-C_{26}). The synthesis of very long chain acids was consistently characteristic of several other seeds in early stages of germination. The majority of the radioactive acids were present in phospholipids and were localized in particulate fractions. The acyl components of phosphatidyl glycerol were highly labeled. The very long chain acids were found predominantly in the waxes. Pulse labeling indicated little turnover of the labeled fatty acids. Evidence is presented indicating that the enzymes for fatty acid synthesis are already present in the dry seed and participate in the synthesis of fatty acids once a critical water content of the seed is achieved.

Germination of a plant seed involves a complex series of metabolic processes such as water imbibition, respiration, nucleic acid and protein synthesis, mobilization of food reserves as well as cell differentiation and growth (33). Plant fatty acid synthesis has been studied with peas by Macey and Stumpf (32). These low lipid, high starch-containing seeds were found to incorporate acetate-1-^{14}C into fatty acids from C_{16} to C_{28}. The enzyme systems responsible for these syntheses were further studied and evidence was presented that the microsomal fraction participated in the synthesis of the very long chain fatty acids, probably from malonyl-acyl carrier protein. Either green or etiolated barley leaves also synthesized fatty acids of chain length up to C_{24} (17). The same authors (18) found that these acids were located in a particulate cellular fraction. Kolattukudy (28), using *Brassica* leaf tissue, found very long chain fatty acids produced from acetate.

Partially purified enzyme preparations of fatty acid synthetase usually produced a pattern of fatty acids different from the pattern found with the endogenous fatty acids. For example, while the synthetase of avocado mesocarp supernatant (44) made palmitate and stearate from malonyl CoA-^{14}C, the particulate enzyme yielded oleate in addition. Similarly, a particulate preparation from developing castor bean seeds examined by Drennan and Canvin (8) gave mainly stearate and oleate. The pattern of fatty acids produced may vary during, for example, aging of potato tuber tissue (Willemot and Stumpf [43]) or alteration of oxygen available to plants (Harris and James [14]). White (42) found that cotton seeds deplete stored linoleic and saturated acids faster than oleic whereas germinating watermelon studied by Hardman and Crombie (13) showed a relatively unspecific breakdown of stored fat.

The use of tritiated water as a source of label for newly synthesized fatty acids provides a good alternative to the commonly used acetate. Spedding and Wilson (39) have used this isotope to study amino acid metabolism at very early stages of germination of mustard, and Foster and Katz (9) studied its incorporation into fatty acids by rat adipose tissue. Since water is intimately involved with the natural germination process, use of tritium oxide does not suffer from the criticism of using an artificial substrate. The results of its use can then be compared to the use of acetate, in case of differences.

Since pea seeds are readily germinated with concomitant lipid synthesis (32), they were chosen to study the synthesis of fatty acids at very early times of germination. It is at this stage that reorganization of cell components occurs, directed to organelle formation. Thus this paper attempts to define the nature of the fatty acids and complex lipids which are synthesized by the seed as germination begins.

MATERIALS AND METHODS

Germination and Lipid Extraction of Seeds. Seeds were germinated in small test tubes containing 0.5 ml of deionized water (or tritium oxide when this isotope was employed), 25 μg of chloramphenicol (to prevent bacterial growth), and the appropriate isotope. When tritium oxide was used, the seed was lyophilized *in vacuo* at the end of the experiment after freezing with liquid nitrogen. The isotope could then be recovered by distillation *in vacuo*.

Extraction of lipids from the seeds at the termination of the experiment was carried out by homogenizing the seeds in a chloroform-methanol (2:1, v/v) solution using a blender (Omnimix) operating at 90 v for 15 sec. Approximately 10 ml of solvent were used per g of seed. After extraction overnight at room temperature, the lipid extract was decanted and the tissue residue was further extracted with 10 ml of chloroform-methanol (2:1, v/v). The lipid extracts were then washed with 7.5 ml of 1 N HCl containing saturating amounts of NaCl. After shaking and aspirating the top phase, the washing was repeated two more times. The chloroform layer was dried with anhydrous Na_2SO_4.

Chromatography of Lipids and Fatty Acids. Total lipids were separated on thin layer plates, 0.3 mm thick, made from Silica Gel G. Solvent systems used were: (*a*) hexane-diethyl ether-glacial acetic acid (70:30:1, v/v); (*b*) diisobutyl ketone-glacial acetic acid-water (80:50:10, v/v), method of Kates (26); and (*c*) two-dimensional (*i*) chloroform-methanol-water (65:25:4, v/v), (*ii*)

diisobutyl ketone-glacial acetic acid-water (80:50:10, v/v) method of Lepage (30). Phospholipids separated in solvent system (*a*) were further analyzed by chromatography (*d*) on thin layer plates either with chloroform-methanol-water (95:35:5, v/v) or with solvent (*b*); (*e*) on Whatman No. 1 paper with 1-butanol-acetic acid-water (12:3:5, v/v).

Spots were revealed by exposure to iodine vapors or spraying with concentrated sulfuric acid. Phospholipids were revealed by a modification of the molybdate method as described by Vaskovsky and Kostetsky (41) and the ethanolamine (or serine)-containing phospholipids with 1% ninhydrin + 5% collidine in ethanol.

Lipid samples for fatty acid analysis were transmethylated with boron trifluoride-methanol. The methyl esters of fatty acids were then analyzed by gas-liquid chromatography on an Aerograph A-90P fitted with a thermal conductivity detector and coupled to a Nuclear-Chicago Biospan model 4998 radioactive detector. Diethylene glycol succinate and S.E. 30 columns were both used and were operated as described by Macey and Stumpf (32).

Determination of Radioactivity. All nonaqueous samples were counted, after drying in scintillation vials, in toluene containing 0.6% 2,5-phenyloxazole (PPO) and 0.05% 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) with a Packard liquid scintillation spectrometer. Aqueous samples were counted with Bray's solution.

Radioactive chromatograms were scanned with a Packard model 7201 Radiochromatogram scanner.

Protein Synthesis Determination. Protein synthesis was measured as the incorporation of leucine-U-^{14}C (700,000 cpm) into the trichloroacetic acid-insoluble fraction. After the appropriate period of germination the pea seeds were thinly sliced, and the slices were placed in 10% trichloroacetic acid containing unlabeled leucine. Further washing with trichloroacetic acid, chloroform-methanol, and ethanol was carried out as previously described (43).

Degradative Studies on Lipids. Long chain fatty acids were broken down to a series of shorter chain length homologues by the technique of Harris, Harris, and James (15). Analysis of the methyl esters of the degraded products was performed on a diethylene glycol succinate column programmed from 120 to 180 C and coupled to the Nuclear-Chicago Biospan unit.

Deacylation of lipids by mild alkaline hydrolysis was carried out by the method of Hubscher, Kemp, and Hawthorne (20). This method had the advantage of very little breakdown of the diesters if careful control of the concentration of the base was exercised. Deacylated lipids were chromatographed on Whatman No. 1 paper by the method of Dawson (5).

Acid hydrolysis (usually of deacylated lipids) was made with 0.5 N H_2SO_4 at 120 C overnight in a sealed Pyrex tube. After cooling, barium hydroxide was added, and the precipitated salt was removed by centrifuging. The supernatant was analyzed by paper chromatography in isopropanol-acetic acid-water (3:1:1, v/v). After drying, the papers were stained successively in silver nitrate, in NaOH in ethanol (Trevelyan, Procter, and Harrison [40]), and in sodium thiosulfate (Anet and Reynolds [1]). This procedure revealed the presence of reducing sugars. The presence of choline was detected by the method of Heyndrickx (19).

Subcellular Fractionation. Pea seeds (L. Alaska), germinated 24 hr, were homogenized in a mortar and pestle at 0 C. When a smooth paste had been obtained, it was filtered twice through cheesecloth to remove cell debris. The homogenization medium consisted of 0.32 M sucrose with 2 mM tris-HCl buffer, pH 7.4. About 4 ml of medium were used per g of tissue. After removing a sample for analysis, the remainder of the homogenate (about 20 ml) was spun at 1,000*g* for 10 min in a Sorvall RC-2B supercentrifuge. The pellet was washed with half the original volume of medium and centrifuged again at 1,000*g* for 10 min. After a third wash and spin, the pellet was suspended in medium to give the nuclear and cell debris fraction. The supernatants were combined and spun at 6,500*g* for 10 min. The pellet was washed once with 5.0 ml of medium and centrifuged. The pellet was suspended in solution to give the "mitochondrial" fraction, and the supernatants were combined and spun at 14,000*g* for 20 min. The pellet was washed with 4.0 ml of medium and recentrifuged. The pellet gave the "light mitochondrial" fraction and the supernatants were combined and spun at 105,000*g* for 60 min in a Spinco model L ultracentrifuge to yield the microsomal and the 6.3 $\times$ 10^6 *g*-min supernatant. The fractions were stored at −20 C.

Protein Estimation. Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (31).

DNA Estimation. Deoxyribonucleic acid was used as a nuclear marker and was measured by the method of Burton (3) with diphenylamine reagent. The initial extraction of samples was with hot trichloroacetic acid by Schneider's method (38).

Phosphate Estimation. Total and inorganic phosphate were measured by the method of King (27). When areas of a paper chromatogram were estimated, a trace of ammonium molybdate was added to aid digestion (Dawson [5]).

Potassium Estimation. Fractions were diluted 1 in 10 → 1 in 200 for estimation. Measurement was carried out on a Perkin-Elmer atomic absorption spectrometer model 290. A mixture of 15 mM lanthanum oxide-4 mM cesium nitrate adjusted to pH 1.0 with concentrated HCl was added at 1 part in 20 dilution to samples to minimize interference by phosphorus.

Enzyme Assays. Succinate dehydrogenase (EC 1.3.99.1) was assayed by the method of Pennington (36) as modified by Porteous and Clark (37) by the inclusion of EDTA. It was used as a mitochondrial marker. Acid phosphatase (EC 3.1.3.2), which was used as a lysosomal marker, was assayed by the method of Hubscher and West (21) with β-glycerophosphate as substrate. Glucose 6-phosphatase (EC 3.1.3.9), which was used as a microsomal marker, was assayed by the method of Hubscher and West (21). EDTA and NaF were included in the incubation medium to inhibit alkaline and acid phosphatases, respectively. 5'-Nucleotidase (EC 3.1.3.5) was measured by the method of Michell and Hawthorne (35) as modified by Kai *et al.* (23). It was used as a marker for plasma membrane.

Chemicals and Substrates. Silicic acid for chromatography was obtained from E. Merck, Darmstadt, Germany. β-Glycerophosphate, glucose 6-phosphate, AMP, and iodonitrotetrazolium violet were from Sigma. Lipid markers were obtained from Supelco Inc., acetate-1-^{14}C from New England Nuclear, L-leucine-U-^{14}C from Nuclear-Chicago, carrier-free phosphoric acid-^{32}P (10 mc/ml) from Schwarz, and water-^{3}H (25 mc/g) from New England Nuclear.

RESULTS

Incorporation of Tritium Oxide into Pea Fatty Acids. When peas were germinated in tritium-labeled water, the onset of labeling of fatty acids showed a "lag" period (Fig. 1). The length of this initial period of zero labeling was shorter at higher temperatures. Once labeling had begun, it appeared to proceed linearly for up to 30 hr of germination. At 5 C labeling began at about 23 hr, at 20 C at about 11 hr, and at 35 C at about 5 hr. If pea seeds were kept at 5 C for 24 hr and then held at 20 C, the rise in radioactivity as fatty acids showed no lag phase and was parallel to the increase in those seeds germinated at 20 C for the entire period (Fig. 1b).

The individual fatty acids produced at different times are shown in Table I. Initially, at all three temperatures and with both tritium-labeled water and acetate-1-^{14}C, the first fatty acids to be labeled were palmitate and stearate. Soon after, the very long chain fatty acids appeared with some oleic acid production at a transient intermediate stage. Surprisingly, no polyunsaturated

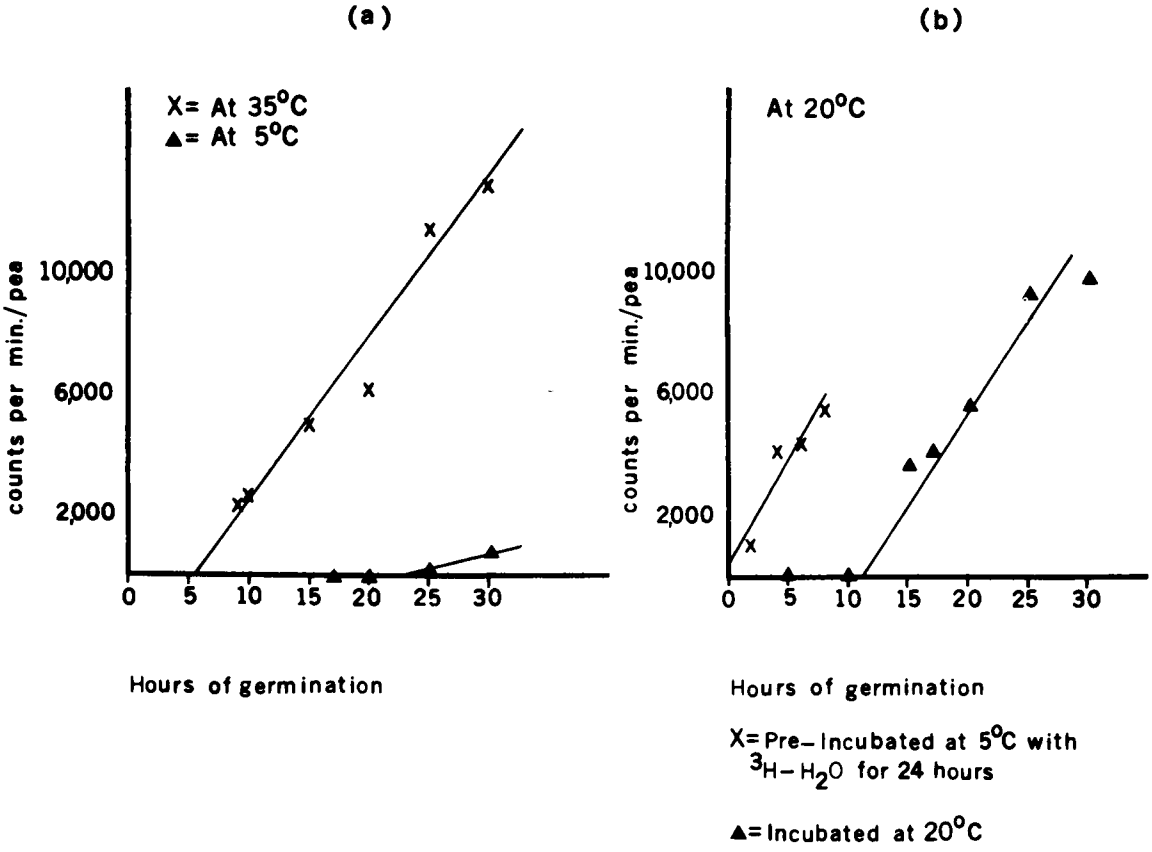


FIG. 1. Incorporation of ^{3}H-H_2O into fatty acids of germinating pea.

Table I. *Labeling of Fatty Acids by Tritiated Water or ^{14}C-Acetate in Germinating Pea*

	Germination	Radioactivity	Fatty Acids										No. of Experiments
			12:0	14:0	16:0	18:0	18:1	18:2	18:3	20:0	22:0	24:0	
	hr	*cpm*	*% of total recovered*										
Mass trace	24	...	3.0	1.5	16.7	3.1	15.4	55.8	4.5	n.d.[1]	n.d.	...	5
^{3}H-H_2O incorporation													
At 5 C	30	700	n.d.	n.d.	57	43	n.d.	n.d.	n.d.	n.d.	n.d.	...	1
5 C (24 hr) +20 C	5	3,500	n.d.	n.d.	tr.	tr.	n.d.	n.d.	n.d.	n.d.	n.d.	...	1
	8	5,500	...	n.d.	50	41	9	n.d.	n.d.	n.d.	...	...	1
	10	6,500	...	n.d.	21	55	tr.	n.d.	n.d.	15	9	...	1
20 C	10	200	...	n.d.	tr.	tr.	n.d.	n.d.	n.d.	n.d.	...	...	1
	15	3,600	...	n.d.	40	60	n.d.	n.d.	n.d.	n.d.	...	...	2
	17	4,100	...	n.d.	32	52	15	n.d.	n.d.	n.d.	...	...	2
	20	5,500	...	n.d.	22	66	4	n.d.	n.d.	6	2	...	3
	25	9,200	...	n.d.	43	44	6	n.d.	n.d.	5	3	...	1
	30	9,800	...	n.d.	47	40	9	n.d.	n.d.	1	2	...	1
35 C	10	2,500	...	n.d.	30	70	n.d.	n.d.	n.d.	n.d.	...	...	1
	15	4,800	...	n.d.	55	33	12	n.d.	n.d.	n.d.	n.d.	...	1
	20	6,000	...	tr.	50	39	11	n.d.	n.d.	tr.	tr.	...	1
	25	11,300	...	n.d.	32	68	n.d.	n.d.	n.d.	tr.	tr.	...	1
	30	12,800	...	n.d.	41	59	n.d.	n.d.	n.d.	tr.	tr.	...	1
^{14}C-Acetate incorporation													
20 C	16	2,230,000	...	tr.	64	36	n.d.	n.d.	n.d.	n.d.	n.d.	...	2
	20	3,060,000	tr.	tr.	30	59	n.d.	n.d.	n.d.	5	4	2	4
	24	3,547,000	...	tr.	32	63	n.d.	n.d.	n.d.	2	2	tr.	3

[1] tr. = trace (less than 0.5%); n.d. = not detectable.

Table II. *Action of Actidione on Germinating Pea Seeds* were germinated for 24 hr at 20 C.

Concn	Uptake of Water	Time of Exposure	Fatty acid Synthesis[1]	Incorporation of ^{14}C-Leucine → Protein[2]
μg/ml	*% of original seed weight*	*hr*	*% of control*	*% of control*
0	106	...	100	100
1	104	24	...	40.8
2	...	24	...	31.7
5	...	24	96	28.2
5	...	10	106	...
5	...	5	105	...
10	106	24	...	22.2
25	...	24	...	21.7
100	108	24	...	...
1000	106	24	...	...

[1] Control = 4,400 cpm. Incorporation from tritiated water.
[2] Control = 22,300 cpm.

fatty acids were synthesized. The pattern of acids thus produced shows no resemblance to the mass pattern of the seed (Table I), where linoleic acid accounts for over half of the total fatty acids and no very long chain fatty acids are detectable. Only traces of the latter acids were found when germination was carried out at 35 C. Acetate-^{14}C showed a similar pattern to tritium oxide incorporation and was, therefore, used frequently in subsequent experiments for convenience.

The pattern and amounts of radioactive fatty acids produced were independent of the presence or absence of a light source over this short time period. The inclusion of chloramphenicol did not alter the ^{14}C distribution in fatty acids, indicating the absence of bacterial contribution to ^{14}C-acetate incorporation into fatty acids.

Effect of Actidione on Fatty Acid Synthesis. Actidione, which prevents the translation step in protein synthesis by preventing the formation of the peptide bond at the level of the ribosome (7), inhibits the development of fatty acid synthesis in aging potato tuber slices (43). If the lag phase of ^{14}C-acetate incorporation into fatty acids in the initial stages of germination is related to synthesis *de novo* of synthetase enzymes, then actidione should inhibit the rise in fatty acid synthesis. Since tritium-labeled water was used in the experiments, control experiments were performed to determine if actidione altered the imbibition of water into the seed. The results are shown in Table II. At concentrations up to 1 mg/ml (an excessively high concentration) no measurable effect was observed on water uptake and germination over a 30-hr period. On the other hand, protein synthesis, measured by leucine-^{14}C incorporation, was severely curtailed, a plateau being reached at about 10 μg/ml. A blank, where zero protein synthesis but normal leucine uptake took place, would probably account for the residue of counts in the high actidione samples, and it is likely that inhibition of protein synthesis approached 100% rather than 80%. Addition of chloramphenicol at 50 μg/ml did not measurably increase the amount of inhibition.

Although actidione produced a marked effect on protein synthesis, it had no action on the labeling of fatty acids whether included at the initiation of germination or added after water imbibition had occurred.

Relation of Water Imbibition and Fatty Acid Synthesis. Marré (33) has presented elegant experiments which supported the concept that a number of enzymes in the germinating castor bean seed were synthesized only when water was taken up with the concomitant formation of mRNA and polysomes. This sequence of events was markedly inhibited by actidione. Since the actidione effect was not observed with the pea seed in terms of fatty acid synthesis, other possibilities for the water uptake effect remained. (*a*) Presence of water could result in the conversion of the synthetase from an inactive to an active form. (*b*) Water was simply needed to provide the necessary osmotic medium for the synthetase reaction to proceed or to bring into solution the metabolic complexes of enzymes and substrates necessary for synthesis.

Table III. *Effect of Addition of Products of Glucose Metabolism on Rate of Fatty Acid Labeling from ^{3}H-Water*

Germination was carried out at 25 C in the presence of 3 mc of ^3H-H_2O per pea.

	Germination	^{3}H-Fatty Acid	Percentage of Control
	hr	*cpm*	
Control	10	2300	
Control	15	6500	
+ Acetate, 1 mM	15	5080	79
+ Pyruvate, 1 mM	10	2320	101
+ Pyruvate, 1 mM	15	6100	94

Experiments were designed to test these possibilities. In Table III the addition of either acetate or pyruvate to the medium of peas germinating in tritium oxide did not shorten the lag period of labeling. These results indicated that lack of these substrates was not the factor in the delay of fatty acid synthesis.

The uptake of water by the pea at different temperatures is documented in Figure 2. When the onset of labeling at different temperatures is plotted on the same graph, it is apparent that synthesis of fatty acids is initiated only when the weight of the seed is approximately 185% of its original weight. This observation would agree with explanation *b* for the role of water (see "Discussion").

Stearate Synthesis *de novo* or Elongation? Both palmitic-^{3}H and stearic-^{3}H acids were collected from the gas-liquid chromatography system and degraded by a modified permanganate technique of Harris, Harris, and James (15). Permanganate oxidation produced fatty acids differing in 1 carbon atom. The results are shown in Table IV for stearic acid. Palmitic acid gave similar results. Detectable radioactivity was present in each component down to C_{11}. The ratios of radioactivity to mass remained fairly constant with decreasing chain length, thus indicating synthesis of stearate *de novo*.

Labeling of Different Lipid Classes. Table V shows the radioactivity of various lipid classes which had been separated by thin layer chromatography as described under "Materials and Methods." As individual experiments gave somewhat varying results, the standard deviations are given. About 70% of the total lipid extract counts were associated with phospholipids and 20% with triglycerides. Very little label was present as free fatty acids.

A further analysis of the phospholipid labeling is summarized in Table VI. Studies of the phospholipids of unlabeled peas, germinated for 24 hr at 20 C, revealed the presence of eight phosphorus-containing lipids, of which three were major and four very minor components. The identification of the four principal phospholipids was made by comparison with markers in three thin layer and one paper chromatographic systems and by acid hydrolysis and chromatography of the bases (see "Materials and Methods"). Choline was identified with potassium ferricyanide-cobalt chloride sprays (19). The average phospholipid composition of germinating peas is shown in the first column of Table VI. Phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol, and phosphatidyl glycerol (in decreasing order) are the major phospholipids. No phosphatidyl serine was detected, and plasmalogen derivatives were not analyzed.

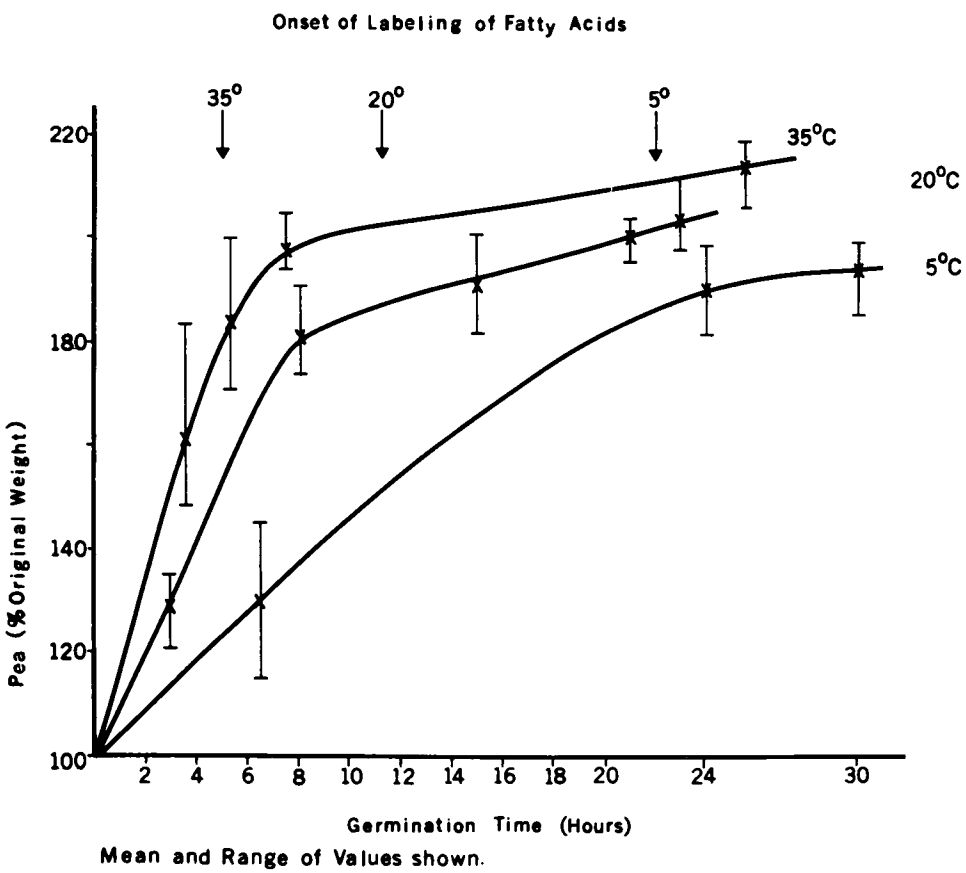


FIG. 2. Uptake of water by germinating pea at different temperatures.

Table IV. *Radioactivity of Stearic Acid Fragments after Permanganate Oxidation*

Stearic acid was pooled from three experiments. Activity and mass peaks below C_{11} were too small to measure because of volatility of the methyl esters.

Carbon No.	^{14}C-Stearic Acid: Ratio of Radioactive Trace Height to Mass Trace Height	Radioactivity
		cpm
C_{18}	2.72	84,700
C_{17}	1.07	2,265
C_{16}	0.75	6,900
C_{15}	1.15	2,264
C_{14}	0.92	1,380
C_{13}	1.00	296
C_{12}	1.33	394
C_{11}	1.50	296

While phosphatidyl choline and ethanolamine are the principal phospholipids, the majority of the label from acetate-^{14}C was localized in PI[1] and PG in the 20-hr period. The specific radioactivity of the latter was particularly high. ^{14}C radioactivity was detected only in these four phospholipids. On the contrary, when orthophosphate-^{32}P was used as the source of radioactivity, most of the label appeared in PC and PE. PG was poorly labeled. It appeared, therefore, that PG was involved in transacylation reactions without synthesis *de novo*.

[1] Abbreviations: PI: phosphatidyl inositol; PG: phosphatidyl glycerol; PC: phosphatidyl choline; PE: phosphatidyl ethanolamine.

Table V. *Labeling in Different Lipid Classes from ^{14}C-Acetate*

Peas were germinated individually in 2 μc of acetate for 20 hr at 25 C. Results are from six experiments.

Main Lipid Component	Percentage of Total Lipid Counts from ^{14}C-Acetate
Phospholipids	69.6 ± 11.9
Monoglyceride	2.7 ± 2.4
Diglyceride	tr.
Fatty acid (free)	tr.
Triglyceride	20.2 ± 10.7
Waxes	7.5 ± 8.6

About 3% of the radioactivity from orthophosphate-^{32}P was present in a single spot which had an R_F of 0.75 in diisobutyl ketone-acetic acid-water system. It also appeared similar to phosphatidic acid in its mobility in chloroform-methanol (85:15) on silicic acid. However, when mild alkaline hydrolysis was carried out (20) and the deacylated lipids were examined, the radioactive compound (which was not extractable into ether) had a slower mobility than glycerophosphate in the phenol-acetic acid-water system. In methanol-formic acid-water, on the other hand, it had an R_F close to the solvent front. The identity of this minor radioactive component, therefore, remains unknown. Because of the mobility of the deacylated lipid it does not appear to be *N*-acyl-phosphatidylethanolamine described in pea (6).

The fatty acids of the various lipid classes were also examined, the results being shown in Table VII. The phospholipids, while containing the bulk of the radioactivity, contained only small

Table VI. *Phospholipids Labeled from ^{14}C-Acetate and ^{32}P-Orthophosphate by Germinating Pea*

Figures are the average of three experiments. Peas were germinated at 20 C for 20 hr.

Phospholipid	Percentage of Total Lipid P	Percentage of Total ^{14}C Activity	Relative Specific Radioactivity[1]	Percentage of Total ^{32}P Activity	Relative Specific Radioactivity[1]
Phosphatidyl choline	43.0	15	0.35	45.5	1.06
Phosphatidyl inositol	19.0	35	1.84	13.0	0.68
Phosphatidyl ethanolamine	26.2	11	0.42	36.9	1.41
Phosphatidyl glycerol	5.7	39	6.84	1.2	0.21
Others	6.1	0	0	3.4	0.56

[1] Assuming that the specific radioactivity for the total phospholipids (in each case % total counts/% total lipid P) = 1.00.

Table VII. *The Fatty Acids of Individual Lipid Classes*

Radioactive fatty acids were labeled by germination of peas in ^{14}C-acetate at 25°C for 20 hours.

Fatty Acid	Phosphatidyl Choline: Total Composition, Mass	PC (+PI): ^{14}C Label	PI: ^{14}C Label	PE: ^{14}C Label	PG: ^{14}C Label	Neutral Lipids[1]: Total Composition		Waxes: ^{14}C Label
						Mass	^{14}C label	
	% of total recovered mass or activity							
14:0	n.d.[2]	tr.	n.d.	n.d.	n.d.	tr.	n.d.	tr.
16:0	10	31	92	54	59	13	37	31
18:0	n.d.	65	8	46	41	1	53	45
18:1	48	n.d.	n.d.	n.d.	n.d.	28	n.d.	n.d.
18:2	42	n.d.	n.d.	n.d.	n.d.	53	n.d.	n.d.
18:3	tr.	n.d.	n.d.	n.d.	n.d.	5	n.d.	n.d.
20:0	n.d.	1	n.d.	n.d.	n.d.	n.d.	10	11
22:0	n.d.	3	n.d.	n.d.	n.d.	n.d.	tr.	10
24:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2
26:0	n.d.	n.d.	...	...	...	n.d.	...	0.5
28:0	n.d.	...	...	...	...	n.d.	...	0.5

[1] Mainly triglyceride, galactolipids.

[2] n.d. = not detectable; tr. = trace (not greater than 0.5% total).

Table VIII. *Pulse Labeling of Lipids from ^{14}C-Acetate by Germinating Pea*

Peas were germinated at 20 C. Lipids separated in diisobutyl ketone-acetic acid-water by thin layer chromatography (see "Materials and Methods").

	Radioactivity Distribution in Lipid Classes			
	Acetate-^{14}C, 20 hr	Acetate-^{14}C, 20 hr, + water, 10 hr	Acetate-^{14}C, 20 hr, + water, 20 hr	Acetate-^{14}C, 40 hr
	% of total recovered radioactivity			
1 PC	8.5	15.5	25.9	25.0
2 PI	8.5	6.2	8.0	0.0
3 PG	31.0	31.0	19.9	9.9
4 Triglyceride, galactolipid	22.0	2.5	2.4	0.0
5 Waxes, pigments	30.0	44.8	43.8	65.1
Total counts in fatty acids	127,750	125,000	119,400	143,560

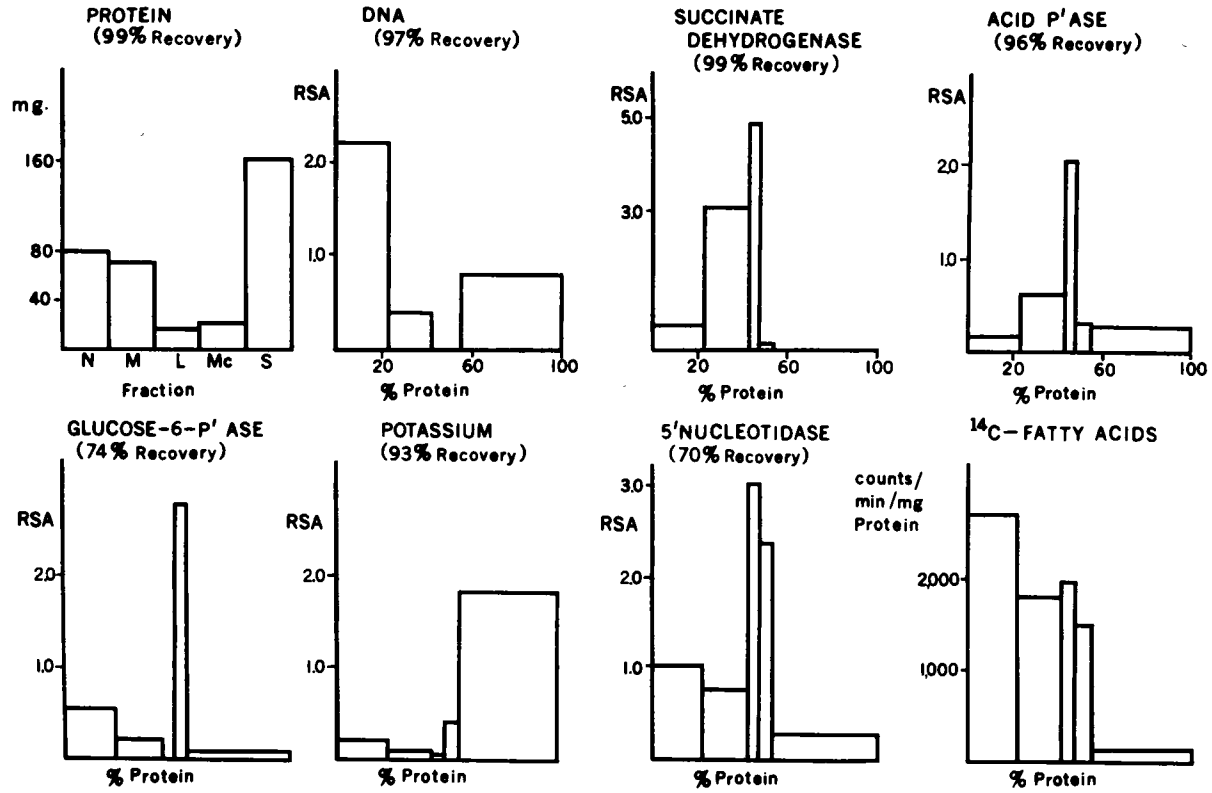
amounts of the very long chain fatty acids. Most of these acids appeared to be present in the waxes although in one experiment the neutral lipids were highly labeled. These observations differ from *Brassica oleracea*, where polar lipids contained much of these acids (28), and barley, where neutral lipids were heavily labeled (17).

Phosphatidyl inositol and phosphatidyl glycerol were highly labeled from acetate-^{14}C. Both of these phospholipids have been implicated in ion transport (29) in bean and cotton plants. When ^{32}P-orthophosphate was used, however, as a source of label, much less incorporation of radioactivity occurred under the same conditions into these lipids. Interestingly, mung bean PI (Katayama and Funahashi [25]) and *Escherichia coli* PG (Kanfer and Kennedy [24]) are highly labeled by ^{32}P-orthophosphate.

To determine if the newly synthesized fatty acids were rapidly turned over, a pulse labeling experiment was carried out. The results are shown in Table VIII. Relatively little turnover was observed although there were shifts of ^{14}C label between lipid class. PC was enriched in ^{14}C label while PG declined and PI remained stable. It is of interest that the neutral lipids including the triglycerides and galactolipids rapidly declined, suggesting that the fatty acids of these classes were utilized by the growing tissue. In all cases the majority of the very long chain acids were found in the wax fraction.

Distribution of ^{14}C-Lipids in the Germinating Seed. When the gross structures of the germinating seed were separated and their ^{14}C-lipids were determined, 21.4% were found in the radicle, 77.8% in the cotyledons, and 0.8% in the seed coat. Since the radicle has a very low lipid content, in contrast to the cotyledons, the specific radioactivity of the radicle tissue fatty acids was 395 cpm/μg, whereas the cotyledon fatty acids had 66 cpm/μg.

It was of interest to determine the distribution of ^{14}C-labeled fatty acids in the subcellular fractions of the germinating pea. Markers for the subcellular fractions were as follows: nuclear (DNA), mitochondrial (succinate oxidoreductase), lysosomal (acid phosphatase), microsomal (glucose 6-phosphatase), super-



N= Nuclear, M=Mitochondrial, L= Lt. Mitoch., Mc= Microsomal, S= Supernatant
R.S.A.=Relative Specific Activity (per mg protein compared to homogenate).
Peas germinated at 25°C for 24 hours.

FIG. 3. Subcellular fractionation of pea.

natant (potassium), and plasma membrane (5'-nucleotidase). These markers are usually employed for fractionation studies. Acid phosphatase (using β-glycerophosphate) was found to be active in spherosome- or lysosome-like structures by Gahan and McLean (10) and 5'-nucleotidase was localized at the cell surface (12) of plants. The other enzymes are well documented although some doubt has been raised by Brunner and Bygrave (2) concerning the sole localization of glucose 6-phosphatase in mammalian microsomes. In pea, however, the distribution of this enzyme is clearly unimodal. Each of these markers was highest in the expected fractions (Fig. 3) with the exception of succinate dehydrogenase, which had a high specific activity in the "light mitochondrial" fraction, indicating some mitochondrial contamination. The presence of DNA in the supernatant indicated some damage during homogenization. Cell surface membrane fragments were in the "light mitochondrial" and microsomal fractions as indicated by 5'-nucleotidase specific activity. The negligible enrichment of this enzyme's specific activity in the nuclear fraction confirmed the inference from DNA distribution that homogenization was somewhat vigorous.

The distribution of ^{14}C-labeled fatty acids in the subfractions indicated that ^{14}C incorporation per mg of protein occurred in all particulate fractions but not in the supernatant (Fig. 3). The patterns of ^{14}C-fatty acids were quite similar in all the fractions: palmitate, 17 to 27%; stearate, 49 to 62%; and very long chain saturated (C_{20}–C_{28}), 21 to 24%. The counts present in the particulate fractions suggested that the newly synthesized fatty acids were associated with membrane systems.

Fatty Acid Synthesis as Related to Initial Stages of Germination in Other Seeds. The ability of the low lipid-high starch pea seed to synthesize very long chain fatty acids after a short period of germination led to an investigation of a number of other seed types. Since the rate of germination varied widely between different species, a morphological criterion for comparison was adopted. Thus the length of exposure to acetate-^{14}C was determined by the time required for the dry seed to imbibe water and develop to the emergence of the radicle through the seed coat. In this manner, comparison of a number of different seeds could be made with this morphological change determining the length of exposure to ^{14}C-acetate. The results are summarized in Table IX.

Of considerable interest, all seeds synthesized very long chain saturated fatty acids in the range of C_{20} to C_{26}. There was no relation between the composition of endogenous fatty acids and the newly synthesized ^{14}C-labeled fatty acids. This was particularly noticeable with the castor bean seed, which showed no synthesis of ricinoleic acid, and with all seeds the inability to synthesize linolenic acid. Two oil-rich seeds, safflower and castor bean, synthesized linoleic acid as a major acid while another oil-rich seed, namely cotton, synthesized only saturated fatty acids. With the exception of broccoli, no endogenous very long chain fatty acids were detected in the mass trace. Thus, in general, germinating seeds synthesize *de novo* a significant amount of very long chain fatty acids in the very early stages of germination.

DISCUSSION

Three interesting observations have been made in this study. In the first place, the lack of synthesis of polyunsaturated fatty acids by the germinating pea at very early stages of germination was unexpected because of the low levels of endogenous fat in the seed and the requirement for unsaturated acids in membrane structures (Table I). In the second place, the consistent synthesis of

Table IX. *Fatty Acids Labeled by Different Seeds during Germination at 30 C*

Seed	Total Counts	Germination	Fatty Acids											Total Recovered
			14:0	16:0	18:0	18:1	18:2	18:3	Ricinol	20:0	22:0	24:0	26:0	Other	
		hr													%
Low lipid-containing															
Pea		24	2	17	3	15	56	5	n.d.[1]	n.d.	n.d.	n.d.	n.d.	2	Mass
	250,000		tr.	26	62	tr.	n.d.	n.d.	n.d.	3	6	3	...	n.d.	Radioactivity
Rice		23	n.d.	43	n.d.	25	32	n.d.	...	...	...	...	...	...	Mass
	9,200		n.d.	tr.	tr.	...	...	...	...	...	...	...	...	...	Radioactivity
		72	tr.	25	73[2]				n.d.	2	n.d.	n.d.	...	...	Mass
	22,600		tr.	18	73[2]				n.d.	5	2	2	n.d.	...	Radioactivity
High lipid-containing															
Broccoli		48	n.d.	5	tr.	10	12	13	n.d.	8[3]	51[3]	1	n.d.	...	Mass
	69,500		n.d.	32	30	31	n.d.	n.d.	n.d.	4	1	2	n.d.	...	Radioactivity
Cotton		23	1	32	tr.	10	54	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3	Mass
	68,000		5	48	33	n.d.	n.d.	n.d.	n.d.	2	9	3	n.d.	...	Radioactivity
Safflower		23	n.d.	16	1	7	76	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	...	Mass
	650,000			25	tr.	16	46	n.d.	n.d.	2	4	5	2	...	Radioactivity
Castor bean		48	n.d.	4	tr.	6	8	n.d.	81	n.d.	n.d.	n.d.	...	1	Mass
	95,000		1	32	n.d.	17	33	n.d.	n.d.	tr.	3	14	tr.	n.d.	Radioactivity
		72	n.d.	4	tr.	7	9	n.d.	79	n.d.	n.d.	n.d.	...	1	Mass
	93,000		1	31	n.d.	15	32	n.d.	n.d.	1	4	16	tr.	n.d.	Radioactivity

[1] n.d. = not detectable; tr. trace (less than 0.5%).
[2] Only analyzed on SE-30 column (by C number).
[3] Monoenes.

very long chain saturated fatty acids is a striking feature of the early stages of a germinating seed. All seeds tested synthesized various amounts of these very long chain (C_{20}-C_{26}) saturated acids. Since intact tissue slices and microsomal fractions from pea had previously been found to make very long chain fatty acids (32), it has been suggested that these may be precursors or components of plant waxes (28). In spite of the high percentage of unsaturated acids (75%) contained in its seed reserves, the pea fatty acid synthetase did not produce polyunsaturated acids and only synthesized small amounts of oleic acid. The major component of ^{14}C-fatty acids was stearic acid. In sharp contrast, safflower and castor bean seeds (which are both high lipid-containing) synthesized oleic and linoleic acids during the early stages of germination. Thus, since one group of seeds had no capacity for polyunsaturated fatty acid synthesis while another group did, it cannot be argued that oxygen deficiency could be the limiting factor (14). The absence of ^{14}C in ricinoleic acid, which accounts for the bulk of the stored lipid of castor bean, confirms the earlier studies by Galliard and Stumpf (11), Canvin (4), and James *et al.* (22). The results of these workers clearly indicate that only the developing seed has the capacity for the synthesis of this acid.

In the third place, these studies raise the question whether the fatty acid synthesis enzymes (*a*) are already present in the dormant seed, (*b*) are activated, or (*c*) are newly synthesized.

The rate of water uptake is intimately involved in these processes. The important work of Marré and his colleagues clearly points out that, as the seed takes up water, there is intense mRNA synthesis with the concomitant formation of polysomes from monosomes. Based on the actidione studies (Table II) and the results of Figure 1b, one can conclude that the enzymes responsible for fatty acid synthesis are present in the dormant seed. Thus, when seeds are allowed to take up water at 5 C, at which temperature little if any metabolic activity occurs, and then returned to 20 C, no lag phase of lipid synthesis is observed. Assuming that the only metabolic activity which occurs at 5 C is passive water uptake, the absence of a lag phase at 20 C suggests that the necessary osmotic requirements had been achieved by water uptake, thereby allowing the preformed enzymes to function in the synthesis of fatty acids *de novo*. In support of this conclusion is the observation that the enzymes of glycolysis, which account for most of the energy production at this stage of germination (Hatch and Turner [16]), are active in extracts of dry seed and detectable levels of NAD and NADH are also present (Mayer and Mapson [34]). The possibility that the lag phase was related to absence of substrates such as acetate was excluded by the results of Table III, which showed that addition of acetate or pyruvate did not modify the incorporation of tritium into fatty acids. If activation of the synthetase was required before fatty acids were synthesized, then a correlation between the onset of labeling and temperature should be apparent. Assuming a Q_{10} of 2, then either (*a*) labeling at 35, 20, and 5 C should begin in a, $2.9a$, and $8a$ hr, respectively, or (*b*) if a certain amount of water is needed to trigger activation then the relationship at 35, 20, and 5 C would be $x + a$, $2.9x + 2.9a$, and $8x + 8a$ hr (where x applies to imbibition and a to activation). Substitution of figures obtained in Figure 2 gave no correlation with either (*a*) or (*b*), suggesting no prior activation.

The uptake of water at different temperatures (Fig. 2) showed

a close correlation between onset of fatty acid labeling and the percentage water content of the pea. Thus, at 20 C water uptake of 185% of original weight was achieved at 10 hr and initiation of tritium labeling began at 11 hr, and at 35 C, water uptake was achieved at 5 hr and tritium labeling began also at 5 hr, etc. Thus, the correct osmotic levels, either for the integrity of cell organelles or for the synthetase enzymes themselves, are the critical conditions required before fatty acid synthesis can occur in the germinating peas.

Our results extend the observations of Marré (33), who showed that a number of enzymes increase during germination because water uptake allows the intensive synthesis of mRNA. Our results would indicate that insofar as the fatty acid-synthesizing enzymes are concerned, the level of activation is not at the mRNA stage but at the creation of an osmotic milieu necessary for the enzymes to function to synthesize a limited number of fatty acids. Occurrence of labeled fatty acids in particulate fractions agrees with the indication, from pulse labeling experiments, that the role of these acids is probably as membrane components. The considerable radioactivity present in the cotyledons raises the possibility that fatty acids may be synthesized there before transport to the growing radicle.

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