Fat Metabolism in Higher Plants XLIX Fatty Acid Biosynthesis by Subcellular Fractions of Higher Plants[1]

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ABSTRACT

A method is described for the rapid and comprehensive subcellular fractionation of plant tissue using a combination of differential and discontinuous Ficoll gradient centrifugation. The procedure has been used to study the synthesis of fatty acids from acetate-1-^{14}C or malonyl CoA-1,3-^{14}C, by fractions of germinating pea and lupin seeds and developing avocado fruit, castor bean and safflower seeds. Particle free supernatants of seeds synthesize fatty acids from ^{14}C-malonyl CoA in the presence of added cofactors. Since acetyl CoA carboxylase activity is absent the utilization of ^{14}C-acetate by these fractions is minimal. Other particulate fractions show different activities depending on seed types. Active fractions include the low speed particulate of pea and lupin, the pea microsomes, the avocado mesocarp chloroplasts, and the fat fractions of castor bean and safflower. Individual fractions produce characteristic patterns of acids; especially noteworthy is oleic acid biosynthesis by soluble enzymes of castor bean and safflower from ^{14}C-malonyl CoA. Some characteristics of the avocado supernatant, pea supernatant, and castor bean fat fraction synthesizing systems are compared. As a result of these studies, generalizations derived from work with mammalian or bacterial systems cannot be applied to higher plants.

INTRODUCTION

Localization of the site of mammalian fatty acid synthesis has now been achieved with a number of tissues. Generally the fatty acid synthetase complex is soluble, while the mitochondria contain elongation enzymes and the microsomes the desaturases (1,2). In contrast much less is known of plant systems. Several workers have studied isolated fractions, but detailed analysis of intracellular distribution was rarely undertaken. With the development of a rapid method for plant cell fractionation, it has become possible to analyze the problem in detail.

Early work with isolated plant organelles was, not surprisingly, with chloroplasts. Smirnov (3) reported rapid incorporation of ^{14}C-acetate into long chain fatty acids by spinach chloroplasts. This organelle has also been used for further studies by Brooks and Stumpf (4) who implicated acyl-carrier protein (ACP) and by Nagai and Bloch (5) who examined the stearyl-ACP desaturase. Later work on the control of oleic acid synthesis by spinach chloroplasts has also been reported (6).

Apple microsomes synthesized fatty acids, particularly polyunsaturated, from acetate (7), and a soluble fatty acid synthetase from potato has been examined (8). Pea mitochondrial, microsomal and supernatant fractions were all active in fatty acid synthesis, but with differences of detail (9). Soybean cotyledons contain soluble fatty acid synthesizing systems (10), particularly desaturases (11). Following reports of particulate systems from avocado mesocarp (12), Yang and Stumpf (13) showed that this tissue also contained a soluble enzyme capable of using malonate but not acetate. Recently Weaire and Kekwick (14) have concluded that the particulate activity is owing to the intact chloroplast, and soluble activity to leakage of stromal proteins.

Perhaps the most extensive research has involved the developing castor bean. Canvin's original analysis of endogeneous lipid (15) indicated that a sequence of synthesis of different acids occurred during development, and this was confirmed by work with tissue slices (16). The hydroxylation of oleic acid to ricinoleic acid was studied with extracts (17) and later with microsomal fractions (18). A supernatant system using malonyl CoA synthesized mainly saturated acids (19), while oleic acid synthesis by a particulate preparation (20) was later localized in a specific nonmitochondrial particle (21).

In order to clarify the sites of fatty acid synthesis in plants, we have examined the synthesis of fatty acids from ^{14}C-acetate and ^{14}C-malonyl CoA in well-characterized subcellular fractions from two low lipid (pea, lupin) and two high lipid (avocado, castor bean) tissues.

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[1]Presented in part at the AOCS Meeting, Houston, May 1971.

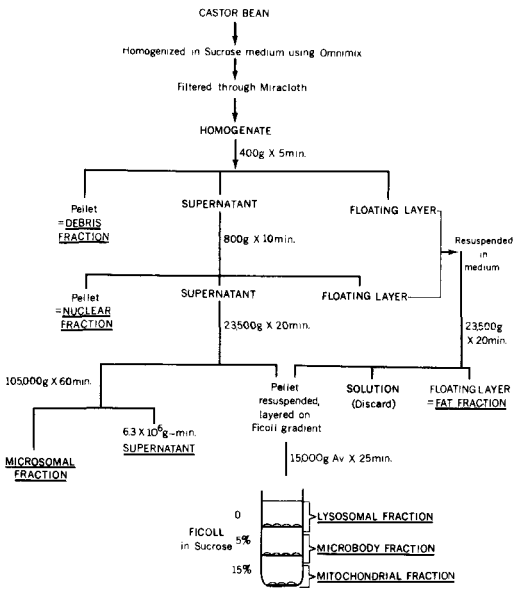

FIG. 1. Fraction scheme for castor bean. Final fractions are underlined. See Experimental Procedures for details.

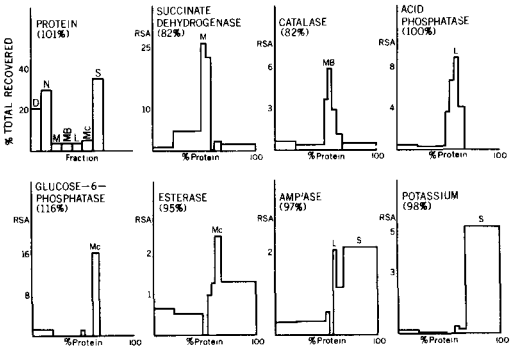

FIG. 2. Subcellular fractionation of lupin. Results are the average of two fractionations and expressed as relative specific activity (RSA) in relation to homogenate. Percentage recovered activity is indicated in parentheses. Homogenate values for: Succinate dehydrogenase = 0.79 nmoles/min/mg protein; catalase = 278; acid phosphatase = 2.19; glucose-6-phosphatase = 0.81; esterase = 2,73; AMPase = 3.15; potassium = 11.05 μg/mg protein. Fraction designations: D = debris, N = nuclear, M = mitochondrial, MB = microbodies, L = lysosomal, Mc = microsomal, S = supernatant.

EXPERIMENTAL PROCEDURES

Materials

Pea seeds, *Pisum sativum L.* Alaska, were obtained from the Asgrow Seed Co., New Haven, Conn.; castor bean, *Ricinus communis L.* from the Department of Horticulture of this University; and avocado, *Persea americana* from the local supermarket. Safflower, *Carthamus tinctorius* variety Hela, seeds were kindly donated by P. Knowles, Department of Agronomy; and Lupin, *Lupinus angustifolius* seeds by E.E. Conn of this department.

Malonic-2-^{14}C acid (14.5 mc/mM) was obtained from New England Nuclear. Acetyl CoA-1-^{14}C was synthesized by the method of Simon and Shemin (22). Acetate-1-^{14}C, Malonyl CoA-1,3-^{14}C and ACP were obtained as previously cited (23).

Ficoll was obtained from Pharmacia and silicic acid for chromatography from E. Merck, Darmstadt, Germany; L-malic acid was obtained from Eastman Organic Chemicals; titanium sulphate from K and K Laboratories Inc.; *p*-Nitrophenylacetate from Mann Chemicals; guaiacol from Matheson, Coleman and Bell; and iodonitrotetrazolium violet, β-glycerophosphate, *p*-nitrophenylglucuronide, glucose-6-phosphate, NADP, NADPH, NADH, UDP, AMP, ATP, glyceraldehyde-3-phosphate, and Horse Radish peroxidase from Sigma.

Germination

Pea and lupin seeds were germinated in dionized water containing 50 μg/ml chloramphenicol (to prevent bacterial growth) at room temperature for 30 hr.

DNA Estimation

DNA, which was used as a nuclear marker, was estimated by the method of Burton (24) after extraction as described by Schneider (25).

Chlorophyll

The method of Arnon (26) employing 80% acetone was used.

Triglyceride

Triglyceride was extracted by the method of Bligh and Dyer (27) and separated by thin layer chromatography (TLC) on Silica Gel G using diethyl ether-petroleum ether 1:9 as solvent. The purified lipid was scraped from the plate, extracted into diethyl ether-methanol 4:1 and measured as ester by the method of Stearn and Shapiro (28).

Protein

This was estimated by the Lowry et al. (29) or Gornell et al. (30) procedures. Ether extractions were necessary to clear high lipid-containing fractions.

Enzyme Assays

Assays for mitochondria were for succinate dehydrogenase (EC 1.3.99.1) (31), fumarase (EC 4.2.1.2) (32) and cytochrome oxidase (EC 1.9.3.1) (33). Catalase (EC 1.11.1.6) was used

TABLE I

Subcellular Fractionation of Developing Avocado Tissue[a]

Estimate	Homogenate	Fractions								Number Fractionation tested
		N	F	P/C	M	MB	L	Mc	S	
Protein	94 ± 1.9% Recovery	5.9 ±1.4	22.2 ±4.6	3.4 ±1.0	7.1 ±0.1	5.7 ±0.9	3.1 ±0.2	5.1 ±1.3	47.5 ±5.2	4
DNA	91% Recovery 116 μg DNA-Pi/gm protein	7.41	n.d.	1.43	n.d.	n.d.	n.d.	n.d.	1.11	1
Succinate dehydrogenase	(106 ± 10.2%) Recovery 3.66 ± 1.32 mμmoles/min/mg protein	1.49 ±0.16	2.63 ±0.18	1.25 ±0.06	2.21 ±0.43	2.07 ±0.36	1.15 ±0.05	0.53 ±0.07	0.01 ±0	3
Chlorophyll	(85%.0 ± 6.0%) Recovery 2.05 ± 1.5 μg/mg protein	2.71 ±1.51	1.52 ±0.41	10.39 ±4.61	n.d.	n.d.	0.21 ±0.26	0.38 ±0.14	n.d.	3
RDP Carboxylase	(98%) Recovery	1.96	1.50	n.d.	n.d.	n.d.	n.d.	1.51	1.26	1
Catalase	(96.0 ± 6.2%) Recovery 12480 ± 2500 mμmoles/min/mg protein	1.29 ±0.19	0.89 ±0.43	1.12 ±0.10	0.86 ±0.15	1.30 ±0.30	1.50 ±0.36	1.82 ±0.55	1.12 ±0.18	3
Acid phosphatase	(113.0 ± 4.0%) Recovery 35.9 ± 8.1 mμmoles/min/mg protein	1.05 ±0.60	0.52 ±0.43	0.15 ±0.10	0.28 ±0.19	0.65 ±0.05	1.02 ±0.17	1.58 ±0.35	2.20 ±0.20	2
Glucose-6-phosphatase	(118.0 ± 1.0%) Recovery 9.1 ± 2.6 mμmoles/min/mg protein	0.68 ±0.20	0.29 ±0.12	0.11 ±0.08	0.09 ±0.06	0.70 ±0.33	0.56 ±0.14	1.20 ±0.02	1.95 ±1.40	2
Esterase	91% Recovery 10.8 mμmoles/min/mg protein	0.42	0.50	0.08	0.28	0.32	0.42	1.19	0.79	1
UDPase	129% Recovery 38.5 mμmoles/min/mg protein	0.65	0.30	0.57	0.98	0.13	0.47	2.30	2.78	1
AMPase	123% Recovery 4.1 mμmoles/min/mg protein	2.92	0.56	2.72	0.78	2.83	5.63	1.26	0.48	1
Peroxidase	128% Recovery 45.7 mμmoles/min/mg protein	0.82	0.52	0.11	n.d.	0.63	0.62	0.72	2.69	1
Triglyceride	91% Recovery 12.30 meq ester/mg protein	0.20	3.46	n.d.	n.d.	n.d.	0.06	0.13	n.d.	1
Potassium	121% Recovery 66.3 μgK/mg protein	0.24	0.06	0.03	0.02	0.08	0.38	0.75	2.22	1

[a]Results are expressed as relative specific activity (RSA) of homogenate values. Means ± SEM (where available). For nomenclature of fractions see Figure 2. F = fat fraction, P/C = proplastic-chloroplast fragments, n.d. = not detectable. Mesocarp and endocarp layers analyzed. Protein is given as percentage recovered in each fraction ± SEM.

to measure microbodies and was estimated by the method of Michell et al. (35) as was lysosomal β-glucuronidase (EC 3.2.1.31). Acid phosphatase (EC 3.1.3.2) was also used as a lysosomal marker. This enzyme and the rough endoplasmic reticulum glucose-6-phosphatase (EC 3.1.3.9) were measured by the method of Hubscher and West (36). Esterase (EC 3.1.1.2) using *p*-nitrophenylacetate for substrate was assayed according to the method of Bier (37) and the smooth endoplasmic reticulum UDPase (EC 3.6.1.6) by the method of Heppel et al. (38). AMPase (EC 3.1.3.5), a plasma membrane enzyme, and potassium were measured as previously (31). Ribulose 1,5 diphosphate carboxylase, a chloroplast stromal enzyme, was estimated according to Anderson et al. (39).

Fatty Acid Biosynthesis

Incubations of fractions with acetate, acetyl CoA or malonyl CoA, and lipid extractions were carried out as previously described (9). Analysis of individual samples by TLC or gas liquid chromatography (GLC) was as described before (23), but 15% HI-EFF-2BP columns (Applied Science Labs.) were also used.

Subcellular Fractionation

Pea and lupin seeds (30 hr germination) or castor bean seeds (20-60 DAF) were blended for 10 sec at 90 v in an Omnimix blender in 0.32M sucrose-2mM Tris-HCl pH 7.4- 0.5mM dithiothreitol. The ratio of tissue to medium was about 1:2 w/w. Avocado fruit was homogenized in a Potter homogenizer of ca. 0.20 mm radial clearance using 500 rpm and five complete strokes. Crude homogenates were filtered through two layers of Miracloth to give the starting homogenate. Further fractionation was as follows: (a) Lupin and pea: Centrifugation was carried out at 1-4 C. The homogenates were spun at 400 g x 5 min in a Sorvall RC-2B supercentrifuge. The pellet was designated the debris fraction and the supernatant spun at 800 g x 10 min to yield the nuclear pellet. The supernatant was then centrifuged at 23,500 g x 20 min. The post-23,500 g supernatant was spun at 105,000 g x 60 min in a Spinco model L ultracentrifuge to yield the microsomal and the 6.3 x 10^6/min supernatant fractions. The 23,500 g pellet was resuspended at 5-20 mg protein/ml of the original sucrose medium using a Potter homogenizer. The suspension was layered on an equal volume gradient of 5:15% Ficoll in sucrose medium and the gradients spun at 15,000 g Av. x 25 min. in a swing-out bucket rotor. The top phase and 0:5% interface were designated the lysosomal layer, the 5:15% interface the micro-body layer, and the denser particles the mitochondrial fraction. The fractions were removed with a pasteur pipette, and resuspended with a Potter homogenizer. (b) Castor bean and safflower: Centrifugation was carried out in the above manner except that a floating lipid layer was formed during the debris and nuclear spins. This layer was removed by a spatula and washed with .5 original volume of sucrose medium before spinning at 23,500 g x 20 min. The floating layer was again removed and resuspended in sucrose medium to give the fat fraction while the remaining solution was discarded. This fractionation is depicted in Figure 1. (c) Avocado: The initial homogenate was spun at 800 g x 10 min to give the nuclear pellet, supernatant and floating lipid layer. The latter was removed and resuspended in half the original volume of sucrose medium. The suspension and the post nuclear supernatant were each spun at 23,500 g x 20 min. The floating lipid layers were removed, resuspended and designated as the fat fraction. The pellets were resuspended at about 5 mg protein/ml medium and layered in a Ficoll gradient as described above. The gradient was then spun at 10,500 g Av. x 20 min. and the resulting layers designated as follows: loading volume = lysosomal; 0-5% interface = microbody; 5-15% interface = mitochondrial; pellet = chloroplast (fragments).

Conditions for high speed centrifugation were as described for the pea. Mesocarp and endocarp layers were analyzed both separately and together. When endocarp alone was used, the pellet from the gradient was designated proplastid membrane fraction.

Ficoll Gradients

Continuous Ficoll gradients were set up using a Multiple Sucrose Gradient Marker (Hoefer Scientific Instruments, San Francisco). All gradients were allowed to stand at 4 C for 15 min before use. One milliliter fractions were collected from the centrifuged gradients by displacement using the most dense Ficoll solution and a syringe. Discontinuous Ficoll gradients were made up using precooled (4 C) solutions. After standing at room temperature for 15 min they were placed at 4 C for 30 min, and after sample loading used immediately.

Electron Microscopy

Samples for electron microscope examination were fixed in glutaraldehyde and stained with osmic acid. After embedding in Araldite, the sections were examined with a RCA-EMU-3G electron microscope.

TABLE II

Subcellular Fractionation of Developing Castor Bean[a]

Estimate	Homogenate	Fractions								Number fractionations tested
		D	N	f	M	MB	L	Mc	S	
Protein	99 ± 2% Recovery	18.5 ±3.2	3.9 ±1.1	16.0 ±9.1	3.5 ±1.4	5.8 ±2.4	5.2 ±3.2	3.2 ±0.8	43.9 ±4.8	4
Succinate dehydrogenase	86.0 ± 3.1 Recovery 3.73 ± 0.36 mμmoles/min/mg	0.43 ±0.38	6.63 ±4.90	6.30 ±4.34	10.98 ±4.73	6.90 ± .31	1.19 ±0.90	2.20 ±1.60	n.d. ±	3
Catalase	81.3 ± 3.9% Recovery 4580 ± 1960 mμmoles/min/mg protein	0.45 ±0.25	1.24 ±1.14	0.54 ±0.17	1.52 ±0.81	1.92 ±1.12	1.43 ±1.00	2.74 ±0.50	1.03 ±0.36	3
Acid phosphatase	86.7 ± 5.0% Recovery 16.56 ± 3.75 mμmoles/min/mg protein	0.22 ±0.10	0.44 ±0.14	0.17 ±0.09	0.85 ±0.42	0.85 ±0.62	1.55 ±0.16	1.92 ±0.80	1.45 ±0.23	3
Glucose-6-phosphatase	75.0 ± 6.3% Recovery 1.55 ±0.32 mμmoles/min/mg protein	0.23 ±0.22	0.34 ±0.22	0.36 ±0.21	0.13 ±0.08	0.14 ±0.08	0.88 ±0.12	2.94 ±1.51	0.74 ±0.38	3
Esterase	80.5 ± 2.5% Recovery 1.14 ± 0.73 mμmoles/min/mg protein	0.52 ±0.50	0.79 ±0.80	0.48 ±0.43	0.18 ±0.17	0.25 ±0.01	0.89 ±0.32	1.04 ±0.33	1.29 ±0.39	2
UDPase	99% Recovery 31.4 mμmoles/min/mg protein	0.58	0.60	n.d.	0.28	0.26	0.60	0.86	1.28	1
AMPase	80% Recovery 3.05 mμmoles/min/mg protein	n.d.	n.d.	0.49	3.74	3.74	5.01	5.91	0.75	1
Peroxidase	78.0 ± 5.9 Recovery 1.21 ± 0.32 mμmoles/min/mg protein	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.50 ±0.50	2.20 ±0.34	3
Potassium	114% Recovery 14.7 μgk/mg protein	0.38	0.02	0.32	n.d.	n.d.	0.63	0.62	3.00	1
Triglyceride	70% Recovery 2.915 meq ester/mg protein	0.24	0.21	1.61	n.d.	0.07	0.09	n.d.	n.d.	1

[a]Results are expressed as relative specific activity in relation to homogenate values given. Fraction designations are as for Figure 2 and F = fat fraction. n.d. = not detectable.

Solubilization of Castor Bean Fat Fraction

The fat fraction was prepared in the usual way and 1 ml portions were treated at 4 C with Triton or Lubrol (0.1%, 1%), 1 M KCl or resuspended in 2 mM Tris buffer containing 10^{-4} M dithiothreitol only. After standing for 15 min. at 4 C the solutions were spun at 105,000 g x 60 min. and the resulting supernatant taken as "soluble" protein. For acetone solubilization treatments a 1 ml suspension of fat fraction was treated with 10 volumes of acetone at -15 C. The result was sucrose buffer. This suspension was then spun at 105,000 g and the supernatant taken as "soluble" protein as before.

Preparation of Antibodies to Avocado Chloroplast Lamella

Chloroplasts were prepared from avocado

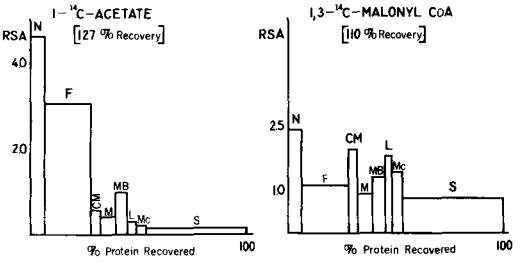

FIG. 3. Fatty acid synthesis by developing avocado fractions. Read in conjunction with Table I. Percentage recovered activity is indicated in parentheses. See Figure 2 for fraction designations and Experimental Procedures for incubation details. CM = chloroplast membranes, F = fat fraction. Intact chloroplasts are found in fractions N and F. Homogenate activity = 71 pmoles/min/mg protein (acetate), 176 pmoles/min/mg (Malonyl CoA).

TABLE III

Fatty Acids Synthesized by Maturing Avocado Fractions[a]

Fraction used	Fatty acids								
	<10	10:0	12:0	14:0	14:1	16:0	16:1	18:0	18:1
Mesocarp									
Homogenate	Trace	6	3	17	Trace	42	3	32	3
Supernatant	5	5	7	21		49	Trace	12	Trace
Endocarp									
Homogenous	6	5	10	21		42		16	
Nuclear			7	26		40		12	15
Microbody	2	8	14	15	4	36	Trace	10	11
Supernatant	10	11	10	22		31		16	

[a]Incubations as in Experimental Procedures with Malonyl CoA-1,3-^{14}C Substrate. Results are expressed as % of total fatty acids. Refer to Figure 3 for relative specific activities of each fraction.

TABLE IV

Release of Fatty Acid Synthetase From Avocado Mescocarp Chloroplasts[a]

Preparation	Treatment	Protein, % solubilized	Fatty acid synthesis, % solubilized	Ribulose 1,5 diphosphate-carboxylase, % solubilized	Ratio synthetase-carboxylase
Chloroplast	None	0	0	0	1.00
Chloroplast	10 Sec blend	52(115%)	49(110%)	53(104%)	1.19 Membrane 0.98 Soluble
Chloroplast	30 Sec blend	57(117%)	60(102%)	85(99%)	2.72 Membrane 0.72 Soluble
Lamellar membranes	None				3.05
Particle free supernate	None				0.10

[a]Intact chloroplast cpm/mg protein = 230,000 for fatty acid synthesis, and 1,610 for ribulose diphosphate-carboxylase ratio taken as 1.0. Figures in brackets represent recoveries. Preparation of chloroplast and supernatant fractions are detailed in Methods. After blending, the chloroplast preparation was spun at 6000 g x 10 min to yield membrane and soluble fractions. Lamellar membranes were prepared by osmotically shock treatment of purified chloroplast (see Methods).

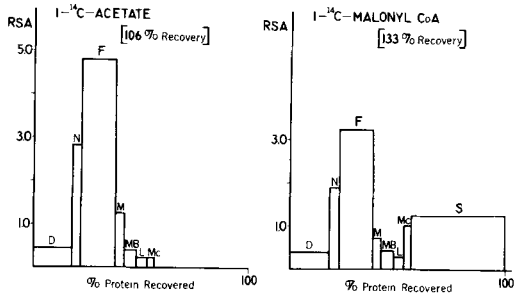

FIG. 4. Fatty acid synthesis by developing castor bean fractions. See Figures 2 and 3 for details of fractions. Homogenate activity = 41 pmoles/min/mg protein (acetate), 295 pmoles/min/mg (Malonyl CoA).

mesocarp on a sucrose gradient by the method of Leech (41). They were then shocked osmotically with water and spun at 6000 g x 10 min. This was repeated three times and the final lamellar preparation made up in 0.09% sodium choride solution. Rabbits were injected simultaneously with the lamellar solution together with Fruends Adjuvant. After three weeks a booster injection (iv) of lamellae was given, and samples of serum were taken a week later.

RESULTS AND DISCUSSION

Subcellular Fractionation

Subcellular fractionation of plant tissues has

TABLE V

Effect of Rabbit Antilamella Serum on Avocado Fatty Acid Biosynthesis[a]

Addition	Sites of fatty acid synthesis		
	Fraction used		
	Chloroplast membranes	Particle free supernatant	Homogenate
None	100	100	
0.1 ml Control serum	35.2 ± 2.2	123 ± 32	100
0.2 ml Control serum	33.4 ± 3.3	87 ± 38	
0.1 ml Control serum (boiled)	104 ± 2.5	114 ± 2	
0.1 ml Antilamella serum	15.8 ± 4.2	107 ± 35	46 ± 8
0.2 ml Antilamella serum	9.1 ± 0.1	102 ± 47	
0.1 ml Antilamella serum (boiled)	69 ± 15	126 ± 42	

[a]Values expressed as % of control. Controls = 2,460 cpm (Homog.), 15,100 cpm (Chloroplast (Memb.), 6,060 (P.F. Sup.). Fractions prepared as in Materials and Methods.

TABLE VI

Fatty Acids Synthesized by Developing Castor Bean[a]

Fraction	Seed age	Substrate	Fatty acids, % 5o5al						
			<16	16:0	16:1	18:0	18:1	20:0	Other
Homogenate	25 DAF	Malonyl CoA-1,3-^{14}C		5	3.5	53	36.5	2	
	40 DAF	Malonyl CoA-1,3-^{14}C		1		92		7	
	45 DAF	Malonyl CoA-2-^{14}C	1.5	4	1	69	4	16	4.5[b]
Fat fraction	25 DAF	Malonyl CoA-1,3-^{14}C		3		48	44	5	
	40 DAF	Malonyl CoA-1,3-^{14}C				97		3	
Supernatant	25 DAF	Malonyl CoA-1,3-^{14}C		12	2	35	51		
	40 DAF	Malonyl CoA-1,3-^{14}C				88	7	5	
	50 DAF	Malonyl CoA-2-^{14}C	33	19		36	12		
Homogenate	25 DAF	Acetate-1-^{14}C		17		18	65		
	40 DAF	Acetate-1-^{14}C		14	Trace	62	24		
Fat fraction	25 DAF	Acetate-1-^{14}C		7		22	71		
	40 DAF	Acetate-1-^{14}C		8	2	57	33		

[a]See Figure 4 for specific activities of Fractions. DAF = Days after flowering.

[b]Ricinoleic acid being synthesized but not measured.

often employed differential centrifugation (31,40). While this procedure is useful in the isolation of a number of organelles such as chloroplasts (41), spherosomes (42) or microsomes, the crude mitochondrial fraction is usually very heterogeneous. This has led to the development of methods for subfractionation of the mitochondrial fraction by discontinuous (43-45) or continuous (46,47) sucrose density gradient centrifugation.

However since sucrose gradients required a lengthy spin time, a more rapid method was needed in order to assay the fractions for fatty acid synthetase activity without storage. To achieve this Ficoll gradients were used which, although not producing homogeneous subcellular fractions, gave sufficient enrichment of organelles for localization of fatty acid synthesis to be estimated.

Initial experiments were conducted by centrifuging particulate fractions using continuous 0-20% Ficoll gradients. On the basis of enzyme marker distributions (see Experimental Procedures), Ficoll concentrations were selected for discontinuous gradient separation. Figure 1 shows the method applied to developing castor bean seeds. In this tissue as with maturing avocado, the high concentration of storage lipid allowed the isolation of a fat fraction by flotation. In low lipid seeds, such as lupin and pea, this fraction was not obtained.

The activity of marker enzymes in the fractions isolated from avocado, castor bean and lupin are shown in Tables I, II and Figure 2, respectively. Recoveries of all enzymes, protein, potassium and triglyceride were satisfactory.

In general clean fractions of all plant tissue organelles are difficult to achieve simultaneously. However enrichment of succinate dehydrogenase, catalase and acid phosphatase was usually obtained in different fractions from the Ficoll gradient. For convenience these were termed the mitochondrial, microbody and lysosomal fractions, respectively. Similar enrichment could be seen by electron microscopic study of the fractions. Certain of the enzyme markers do not always show a unimodal distribution. For instance, with the exception of lupin (Fig. 2), we found considerable soluble catalase activity which agrees with other workers (45,48). Peroxidase was mainly soluble (49) but activity was associated with the low speed particulate fraction in avocado (Table I), possibly in the cell wall (50). The question of lysosomes as such in plants is a debatable one, but the lightest fractions from the Ficoll gradient contained many membrane-bound vesicles together with latent acid phosphatase

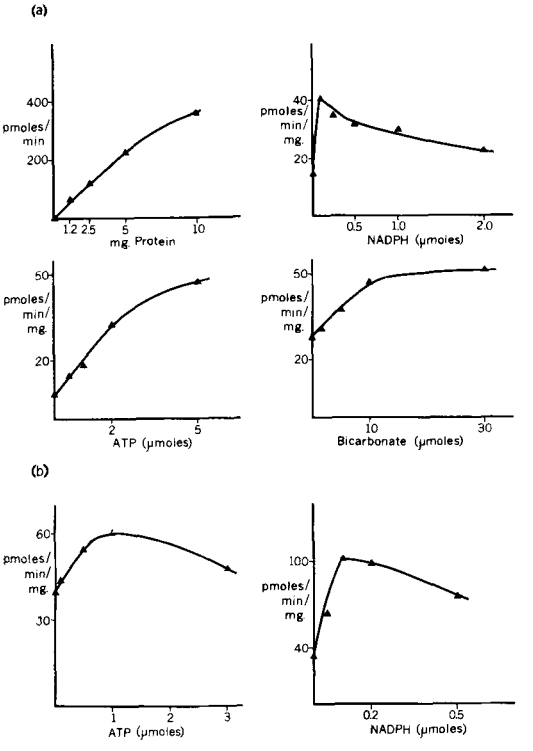

FIG. 5. Characteristics of fatty acid synthetase. See Experimental Procedures for details. (a) Castor bean fat fraction was incubated with 1-^{14}C-acetate and (b) avocado supernatant with 1,3-^{14}C-Malonyl CoA.

activity, thus fulfilling two basic qualifications of a lysosome (51). Lysosomal enzymes are also frequently found in the supernatant as well (52), and we obtained similar results with avocado and castor bean (Tables I and II). Varying amounts of plant glucose-6-phosphatase have been found to be soluble (53) but to a lesser degree than unspecific esterase (52), making the former a preferential rough endoplasmic reticulum marker.

Localization of Fatty Acid Synthesis Activities

Avocado: In Figure 3 the synthesis of fatty acids by fractions from developing avocado (endocarp plus mesocarp) is shown. Separate homogenates of endocarp or mesocarp had approximately the same specific activity with relation to protein. In addition distribution of activity in fractions prepared from mesocarp or endocarp was similar, except that the fractions from mesocarp containing chloroplasts, indicated by both chlorophyll and ribulose, 1,5 diphosphate carboxylase activity (Table I), were more active than corresponding endocarp fractions. With 1-^{14}C-acetate as substrate, fatty acid synthesis was almost entirely confined to

TABLE VII

Solubilization of Castor Bean Fatty Acid Synthetase From Fat Fraction

Treatment	Protein, % solubilized	Total fatty acid synthetase, % solubilized	Recovery of FA synthetase
0.1% Triton-X-100	55	18	118
1% Triton-X-100	65	0	49
0.1% Lubrol	45	13	100
1% Lubrol	68	38	29
Acetone powder	60	91	174
KCl Addition	55	1	99
Osmotic shock	53	17	81

those mesocarp fractions containing intact chloroplasts, which agrees with the suggestion of Weaire and Kekwick (14) that fatty acid biosynthesis occurs in the chloroplast. However similar fractions from endocarp, which contain neither marker enzyme nor chlorophyll, effectively synthesized fatty acids 1-^{14}C from acetate. In addition the supernatant accounted for approximately half the total fatty acid synthesis when ^{14}C-malonyl-CoA was the substrate. Fractions containing AMPase, and therefore likely to contain surface membranes, also showed good activity with malonyl-CoA.

With ^{14}C-acetate as substrate, the avocado mesocarp synthesized long chain fatty acids (actual figures were 60% palmitic, 20% stearic, 20% oleic). With ^{14}C-malonyl-CoA as substrate (Table III) saturated acids from lauric upwards were made. The particular fractions also made appreciable amounts of oleic acid. Individual percentages of, for example, palmitic or stearic acids varied somewhat from preparation to preparation.

The synthesizing ability of the avocado mesocarp chloroplast was studied further. Chloroplasts were purified by the method of Leech (41) and then subjected to blending. As Table IV shows, this treatment released increasing amounts of soluble ribulose 1,5,diphosphate carboxylase and protein with time. The percentage of carboxylase (a stromal marker enzyme) can be used as a measure of chloroplast intactness. Comparison of fatty acid synthesis from 1,3-^{14}-malonyl CoA with CO_2 fixation by the carboxylase revealed that considerable synthetase activity remained in the particulate fraction, although some release took place on homogenization. Purified lamellae prepared by osmotic shock showed considerable fatty acid synthesis but little carboxylase activity (Table IV). The amounts of total soluble ribulose 1,5- diphosphate carboxylase and soluble fatty acid synthesis in an average fractionation were approximately equal (Table I and Fig. 3). Thus after deducting the contribution of the lamellae it could be seen that the chloroplast stromal proteins could not account for all soluble fatty acid synthesis as suggested (14).

To test further the localization of the avocado synthetase, we prepared antibodies to the purified chloroplast lamellae. Table V shows the inhibition produced by the anti-lamella serum on various avocado fractions.

TABLE VIII

Cofactor Requirements for Fatty Acid Synthesizing Systems[a]

Fraction substrate	Pea supernatant ^{14}C-malonyl CoA	Avocado supernatant ^{14}C-malonyl CoA	Castor bean fat fraction ^{14}C-acetate
Complete system[b]	100	100	100
-Acyl carrier protein	22	1	68
-ATP	85	66	10
-$MnCl_2$	86	104	73
-NADPH	15	12	65
-NADH	78	83	56
-GSH	---	77	38

[a]Actual values for complete system = 16.4 pmoles fatty acids/min/mg protein (pea); 82.5 pmoles fatty acids/min/mg protein (avocado); 44 pmoles fatty acids/min/mg protein (castor bean).

[b]Complete system contained 0.1 mM CoA.

TABLE IX

Fatty Acid Synthesis by Pea and Lupin Fractions[a]

Plant	Substrate	D	N	M	MB	L	Mc	PFS	Recovery, %
Pea	Acetate	30	24	6	22	8	2	8	111
	Malonyl CoA	3	8	5	5	6	8	65	95
Protein		13	11	8	8	12	8	40	99
Lupin	Acetate	34	29	7	3	1	2	4	90
	Malonyl CoA	12	20	2	3	6	6	51	95
Protein		20	28	3	4	4	5	36	101

[a]Figures as % of total recovered. See Figure 2 for nomenclature of fractions. Homogenate activities for pea = 0.40 pM/min/mg protein (acetate), 8.00 pM/min/mg protein (malonyl CoA); for lupin = 0.96 pM/min/mg protein (acetate), 3.60 pM/min/mg protein (malonyl CoA).

Significant inhibition of fatty acid synthesis by the lamellae occurred on addition of control serum alone. As this effect was abolished by boiling the serum, it may be caused by unspecific binding of synthetase to a protein such as albumin. However the effect of the antilamellae serum was considerably more, and almost complete inhibition of synthesis occurred in the chloroplast membrane fraction.

Interestingly the supernatant activity was unaffected by the addition of control or antilamella serum. These results are taken to indicate the existence of at least two separate types of fatty acid synthetase enzymes in avocado mesocarp. One is associated with lamella fragments and the other(s) with stromal or cytoplasmic proteins. As could be predicted the homogenate was partly inhibited by the antilamellae serum over control values. While synthetase activities can be found in the chloroplast stroma and lamellae, a functional organelle is not necessary. Indeed in the case of the endocarp where there is no measurable ribulose 1,5 diphosphate carboxylase, the location of particulate synthetase must be in an organelle which does not have chloroplast properties.

Maturing castor bean seeds: The maturing castor bean has been studied by a number of groups because of the unusual nature of ricinoleic acid as its principal storage acid. The synthesis of this acid is confined to a specific period of seed development (15) and has been studied using a particulate fraction (18). We did not consider its synthesis in the present investigation but measured only saturated and unsaturated acids. Figure 4 shows that acetate incorporation was confined almost entirely to the fat fraction. This paralleled triglyceride distribution exactly and was not owing to adsorption of membranes or organelles onto the oil droplets. Extensive washing, which removed all measurable succinate dehydrogenase and AMPase activity and which gave a homogeneous preparation as judged by electron microscopy, failed to reduce the fatty acid synthesizing ability. When ^{14}C-malonyl CoA was employed, the usual particle free supernatant activity was observed. Young seeds, which had not begun to form oil droplets, showed very low activity.

Further investigations (54) have revealed that the oil droplets do not contain a bounding membrane but only membranous inclusions. The fatty acid synthesizing activity of these droplets is undiminished after washing and is distinct from the spherosome system of Jacks et al. (42). It was suggested (54) that the membranous inclusions of the droplets are the site of fatty acid synthesis. The oil droplets originate in the maturing castor bean seed around a cluster of enzymes in the ground substance of the cell involving both de novo fatty acid biosynthesis and triglyceride formation.

The pattern of acids synthesized by the most active fractions at different stages of development is shown in Table VI. The production of ricinoleic acid which has previously been extensively examined (18) is not detailed, but it occurred at a late stage (40 DAF) of development in unfrozen fractions. It is interesting to note that its precursor, oleic acid, was present in high amounts at 25 DAF but then declined. The patterns of acids produced at each developmental stage by the fat fractions, supernatant and homogenate are very similar, suggesting that the same enzyme complex with bimodal location could be involved. It is of considerable interest to note the synthesis of oleic acid by a soluble fraction and this is being further examined in our laboratory. Canvin's group have also studied the production of oleic from ^{14}C-malonyl CoA or ^{14}C-acetyl CoA in castor bean. The highest specific activity was found in a heavy particle (20) later suggested to be a proplastid (21). The fat fraction was not assayed, however, and the supernatant was measured with ^{14}C-acetyl CoA which is an ineffective substrate for soluble plant systems.

TABLE X

Acyl Carrier Protein Requirement for Plant Fatty Acid Synthesis

System	Substrate	+ACP, cpm	-ACP, cpm	Difference, %
Castor bean fat fraction	Malonyl CoA	41,400	5430	-87
Castor bean PFS	Malonyl CoA	8000	1800	-78
Pea PFS	Malonyl CoA	4160	563	-86
Avocado homogenate	Malonyl CoA	37,850	1030	-97
Avocado PFS	Malonyl CoA	31,400	362	-99

The specific activities obtained in the present investigation for homogenate were about 3-5 nmoles/mg protein/15 min, which is higher than reported in Canvin's papers. While the specific activities of our mitochondrial and microbody fractions are approximately the same as reported for the isolated particles (20), the fat fraction is very much higher (Fig. 4).

In Table VII it can be seen that the fat fraction activity was not easily solubilized. Only complete defatting with acetone resulted in an active solubilized enzyme system. Although this solubilized enzyme was very active, the production of oleic acid had disappeared.

The fat fraction of developing safflower also showed considerable synthesis of fatty acids from malonyl CoA. In addition this plant contains a soluble system producing ^{14}C-oleic, among other acids from ^{14}C-malonyl CoA.

Some characteristics of the fat fraction synthetase were compared with avocado and pea supernatant enzymes (Table VIII and Fig. 4). ACP and NADPH requirements are typical of all three systems. In addition ommission of reduced glutathione results in decreased activity indicating a functional sulphydryl group at or close to the active center(s). NADH omission also caused some lowering of activity whereas NADH was very essential in the particulate enzyme preparation examined by Drennen and Canvin (20). ATP was necessary in the castor bean fat fraction since ^{14}C-acetate was the substrate. The pH optima between 7 and 8, and inhibition by high neutral detergent concentrations were common to all three systems. The effect of changing the malonyl CoA-acetyl CoA ratios or altering the ATP concentration in the avocado supernatant incubations were also tested. No significant effects were seen, although ATP caused a slight stimulation of activity (optimum 1mM) accompanied by an increase in the stearate-palmitate ratio.

Low lipid-containing seeds: The low lipid seeds, pea and lupin, were also examined. These seeds showed much lower specific activities than the high lipid tissues. ^{14}C-malonyl CoA was always more effective than ^{14}C-acetate, and the latter was not incorporated by the soluble fraction for any appreciable extent (Table IX). ^{14}C-acetyl CoA incorporation gave similar results to ^{14}C-acetate. The lack of conversion of these substrates to fatty acids by the plant supernatant could be related to one of two reasons. Either there is a lack of acetyl CoA carboxylase, or alternatively there is an inhibitor present which prevents carboxylase activity. Avocado supernatant will inhibit purified wheat germ carboxylase (J.L. Harwood, unpublished observations) and several other plant systems contain a soluble inhibitor (55).

Both pea and lupin have low speed particulate fractions, possibly proplastids, which synthesize fatty acids including monounsaturated from ^{14}C-acetate or ^{14}C-malonyl CoA (Table IX). Both also form palmitate and stearate in the supernatant from ^{14}C-malonyl CoA. The pea microsomes were found to be the site of very long chain fatty acid synthesis from malonyl CoA, confirming the results of Macey and Stumpf (9).

A noticeable feature of the plant systems studied is their stimulation by added acyl carrier protein, as further detailed in Table X. This stimulation was most marked in the supernatant where ACP was limiting, presumably because of dilution. The avocado particulate free supernatant in particular had very low activity in its absence.

In conclusion the localization of fatty acids synthesis in seeds is dependent on the species (and even age) chosen. Generalizations derived from work with bacterial or mammalian systems certainly cannot be applied. Active particulate fractions usually gave rise to monounsaturated as well as saturated fatty acids, but synthesis of linoleic or linolenic acids was not observed in vitro. All particle free supernatants examined synthesized fatty acids, mainly palmitate and stearate, from ^{14}C-malonyl CoA in the presence of added cofactors such as NADPH, reduced glutathione and acyl carrier protein. A block in the carboxylation of acetyl CoA in the supernatant prevented its incorporation. The soluble protein fraction generally accounted for about half of the total ^{14}C-malonyl CoA incorporation.

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