Fatty Acid Biosynthesis in the Developing Endosperm of *Cocos nucifera*[1]

K.C. OO[2] and P.K. STUMPF, Department of Biochemistry and Biophysics, University of California, Davis, California 95616

ABSTRACT

Endosperm tissue of developing coconut endosperm incorporated [^{14}C]acetate and [^{14}C]-malonate into [^{14}C]C_8-C_{18} fatty acids. The distribution of [^{14}C] label into the various fatty acids mimicked the distribution of endogenous fatty acids at early and middle stages of endosperm development. Although [^{14}C]C_8-C_{18} fatty acids were taken up rapidly by endosperm tissue slices, no elongation occurred; [^{14}C]stearic acid was not desaturated to oleic. Cell free preparations have also been obtained from this tissue that readily incorporated [^{14}C]malonyl-CoA into a range of [^{14}C] fatty acids in the presence of ACP and NADH at pH 7.0. Employing this system, a number of experiments were designed to determine the mechanism of chain length termination. In contrast to intact tissue slice experiments, cell-free extracts yielded as principal products palmitic and stearic acid, although up to 20% were shorter chain acids. A number of possible mechanisms for chain length termination were proposed and tested.

The coconut (*Cocos nucifera*) is one of the most important sources of edible oil. The oil is found in the kernel (endosperm) enclosed by a strong hard shell (endocarp), a fibrous fruit coat (mesocarp) and a smooth skin (exocarp). The mature fruit takes 12-13 months to develop and contains about 70-75% oil in its endosperm (1).

Although most plant lipids contain predominantly C_{16} and C_{18} fatty acids, coconut oil contains over 50% C_{12} and C_{14} fatty acids (1). As part of a wider study on the mechanism by which plants regulate the chain length of their fatty acid end products, we have examined fatty acid biosynthesis in the developing endosperm tissue of *Cocoa nucifera*. Some preliminary results were recently reported (2). This paper will describe both experiments carried out with tissue slices and with cell free extracts from developing coconut endosperm.

MATERIALS AND METHODS

Materials

[1-^{14}C]Acetate acid, sodium salt (58.6 Ci/mol) and [2-^{14}C]-malonic acid (22.1 Ci/mol) were obtained from New England Nuclear, Boston, MA. [1-^{14}C]Decanoic acid (14.3 Ci/mol), [1-^{14}C]lauric acid (32 C8/mol), [1-^{14}C]myristic acid (45 Ci/mol), [1-^{14}C]-palmitic acid (58 Ci/mol), [1-^{14}C]stearic acid (58 Ci/mol) and [1-^{14}C]oleic acid (54 Ci/mol) were obtained from the Radiochemical Centre, Amersham. The radiopurity of each of these compounds was checked by gas liquid chromatography-radiochromatography before their use as substrates.

[1,3-^{14}C]Malonyl-CoA (54 Ci/mol), [1-^{14}C] octanoyl-CoA (29 Ci/mol), [1-^{14}C]decanoyl-CoA (58 Ci/mol), [1-^{14}C]lauroyl-CoA (58 Ci/mol) and [1-^{14}C]-palmitoyl-CoA (58 Ci/mol) were obtained from Dhom Products Ltd., North Hollywood, CA. [1-^{14}C]Acetyl-CoA (58 Ci/mol) was from the Radiochemical Center, Amersham. [1-^{14}C]Myristoyl-CoA (54.6 Ci/mol) and [1-^{14}C]stearoyl-CoA (50.9 Ci/mol) were purchased from New England Nuclear, Boston. Nucleotides, malonyl-CoA, G-6-P and G-6-P dehydrogenase were from Sigma Chemical Company, St. Louis, MO. Tricine (N-Tris(hydroxymethyl)methylglycine), L-α-glycerol phosphate and dithiothreitol were from Calbiochem., Oak Grove Village, IL. Sorbitol was from Grand Island Biological Company, Grand Island, NY. *Escherichia coli* acyl carrier protein (ACP) and [0-^{14}C]lauroyl-ACP were a gift from Dr. J.B. Ohlrogge. ACP was isolated by the method of Alberts, Majerus and Vagelos (3) and purified up to the initial acid precipitation step. [0-^{14}C]Stearoyl-ACP, prepared by the enzymatic method of Jaworski and Stumpf (4), was a gift from Dr. Tom McKeon. It contained ca. 40% [0-^{14}C]palmitoyl-ACP.

PCS (phase combining system for liquid scintillation counting) was from Amersham/Searle Corporation, Arlington Heights, IL. Ethylene glycol monomethyl ether was obtained from Matheson, Coleman and Bell

[1] Supported in part by NSF Grant No. PCM76 01495.

[2] On sabbatical leave from the Department of Biochemistry, University of Malaya, Kuala Lumpur, Malaysia.

East Rutherford, NJ. Organic solvents of analytical grade were obtained from Mallinckrodt Chemical Works, St. Louis, MO. Precoated Silica Gel G plates for thin layer chromatography (TLC) were purchased from Analtech Inc., Newark, DE. Ten percent EGSS-X on Gas-Chrom (100/120 mesh) was from Applied Science Laboratories, State College, PA, and 10% DEGS-PS on Supelcoport (80/100 mesh) was from Supelco, Bellefonte, PA.

Tissue slice incubations. Freshly picked coconuts were received by air from Professor N.P. Kefford, Department of Botany, University of Hawaii, Honolulu, and from Mr. R. Miyashita, Department of Parks and Recreation, Honolulu. They were immediately used on arrival or kept at 4 C for not more than 2-3 days.

Endosperm slices, about 1 mm thick and 5 mm wide, were cut with a razor blade. One gram of these slices was immersed in an incubation mixture containing 50 μmoles of potassium phosphate buffer (pH 7.5), 30 μmoles of $KHCO_3$ and 0.34 μmole of [1-^{14}C]sodium acetate or 0.90 μmole o [1-^{14}C]malonic acid or 0.15 μmole of a long chain [1-^{14}C]fatty acid (C_{10}-C_{18}), in a total volume of 1.2 ml. The long chain fatty acids were added dissolved in 10 μl of ethylene glycol monomethyl ether. The mixture was shaken in a 25 ml Erlenmeyer flask at 25 C for 5-6 hr with air as the gas phase.

Analysis of tissue slice incorporation products. At the end of the incubation period with tissue slices, 0.1 ml of 1 N H_2SO_4 was added. The supernatant was removed and the tissue slices were rinsed with 2 x 2 ml of buffer. They were then extracted with 6 ml of chloroform/methanol, 2:1 (v/v) for 16 hr at 25 C. The tissue slices were filtered off and washed with 2 ml of chloroform/methanol, 2:1 (v/v) mixture. In the final two-phase system, the chloroform phase was washed with 1 ml of distilled water or 1 ml of 0.1 M malonic acid (for mixtures containing [2-^{14}C]malonic acid) and then evaporated to dryness under nitrogen. The residue was redissolved in 200-250 μl of benzene. A sample was removed for radioactive counting in 10 ml of PCS-xylene, 2:1 (v/v) scintillation fluid using a Beckman LS-230 liquid scintillation counter.

Preparation of acetone powder and soluble enzyme extracts. Preliminary experiments with fresh endosperm tissue extracts were difficult to carry out because of the presence of massive amounts of lipid. Therefore, fresh tissue was extracted with cold acetone (-20 C) to delipidize and dehydrate the tissue. The resulting white powder could be stored indefinitely at -20 C.

Endosperm tissue (200-400 g fresh weight) was cut into small pieces and placed in a Waring blender prechilled at -20 C. About 2 volumes of prechilled (-20 C) acetone were added, and the tissue was homogenized with several short (20 sec) periods of blending. The mixture was quickly filtered under suction and the residue returned to the blender for re-extraction with fresh, chilled acetone. This process was repeated until most of the lipids were removed and the residue appeared as a fine white powder. About 3-4 extractions were required for 6-7 month matured endosperm and 6-7 extractions for 11-12 month matured endosperm. The acetone-extracted residue was then resuspended in peroxide-free diethylether and filtered. This procedure was repeated 2-3 times to remove traces of acetone. The partially dried powder was finally spread out on aluminum foil in a vacuum desicator and the ether removed under vacuum. The dry powder was weighed and stored at -20 C.

When required, 0.2 g of the acetone powder was added to 2.5-30.0 ml of 0.05 M potassium phosphate buffer (pH 7.5) containing 1 mM β-mercaptoethanol, stirred for 10 min at 4 C and then centrifuged at 12,000 g for 20 min. This clear, slightly viscous supernatant was used as the acetone powder extract.

To remove endogenous cofactors from the acetone powder extract, 0.56 g of ammonium sulfate were slowly added to 1 ml of extract. The turbid solution was shaken and left for about 20 min at 0 C and then centrifuged at 30,000 g for 30 min. The sediment was redissolved to 1 ml of buffer solution.

The acetone powder extract (20 ml) was also separated into a solution and a particulate fraction by centrifuging at 100,000 g for 3 hr in a Beckman Model L ultracentrifuge. The 100,000 g sediment was washed by resuspension in 6 ml of buffer solution and recentrifuged. The final pellet was resuspended evenly in 0.6 ml of buffer solution.

Preparation of microsomal fraction from fresh coconut endosperm. Fresh endosperm tissue (35 g) was added to 52 ml (1.5 volume) of homogenizing medium made up of 0.5 M sorbitol-0.1 M potassium phosphate buffer (pH 7.5-1 mM dithiothreitol-1 mM EDTA.) The tissue was homogenized 2 x 5 sec in a Sorval Onmimix homogenizer at maximum speed and then squeezed through 4 layers of cheese cloth. The filtrate was centrifuged at 800 g for 5 min to remove unbroken cells and debris. The supernatant was centrifuged further at 10,000 g for 20 min. The upper lipid layer was carefully removed, washed with 10 ml of the homogeniz-

ing medium and the washing was combined with the 10,00 g supernatant. The combined 10,000 g supernatant and lipid layer washing were centrifuged further at 100,000 g for 1 hr. The resultant sediment (microsomal fraction) was washed by resuspension, recentrifugation, and finally resuspended in 2 ml of the homogenizing medium.

[^{14}C]Fatty acid synthesis by extracts. Routine fatty acid synthesis experiments were carried out with 100 µl of extract (ca. 0.5 mg protein), 25 µmoles potassium phosphate buffer (pH 7.0), 90 µg *E. coli* ACP, 0.75 µmole $KHCO_3$, 0.34 µmole ATP, 44 nmoles $MgCl_2$, 24 nmoles $MnCl_2$, 68 nmoles NADPH, 0.17 µmole G-6-P, 0.01 units G-6-P dehydrogenase and 0.2 µCi [1,3-^{14}C]-malonyl-CoA (3.7 nmoles) or 0.2 µCi [1-^{14}C]acetyl-CoA (3.4 nmoles) in a total volume of 0.3 ml. In studying the effect of pH on the activity, 25 µmoles of the different pH values were added to the mixture, and no further adjustment was made to the final pH of the mixture. In later experiments using [1,3-^{14}C]malonyl-CoA as substrate, $KHCO_3$, NADPH and its regenerating system (G-6-P/G-6-Pdehydrogenase) were omitted. The reaction mixture was incubated at 24 C for 1 hr.

Formation of neutral [^{14}C]acylglycerols from [^{14}C]fatty acyl derivatives by a microsomal fraction. One-hundred µl of washed 100,000 g sediment (4 mg/ml protein) from fresh young endosperm was incubated with 25 µmoles potassium phosphate buffer (pH 7.0), 0.09 µmole of L-α glycerol phosphate and 1 nmole of [1-^{14}C]acyl-CoA (0.05 µCi) in a total volume of 0.3 ml. In two other tubes, [0-^{14}C]-stearoyl-ACP (25,000 cpm, 2.5 nmoles) and [0-^{14}C] lauroyl-ACP (20,000 cpm, 0.5 nmoles) replaced the [1-^{14}C]acyl-CoA as substrate and and 0.01 µmole of CoA was added as an additional cofactor. Incubation was carried out at 24 C for 1 hr after which the [^{14}C]fatty acids and neutral [^{14}C]acylglycerols were extracted as described by Mancha et al. (5) and separated on TLC.

Assay for utilization of [1-^{14}C]acyl-CoA. The ability of acetone powder extracts to utilize [1-^{14}C]acyl-CoA substrates was determined with an incubation mixture containing ca. 0.5 mg protein from an ammonium sulfate precipitate (0-80% saturation) of the extract, 25 µmoles potassium phosphate buffer (pH 7.0), 0.34 µmole ATP, 0.09 mg *E. coli* ACP, 44 nmo nmoles $MgCl_2$, 68 nmoles NADH, 9 nmoles malonyl-CoA and 2 nmoles [1-^{14}C]-acyl-CoA (specific radioactivities given above). After incubating for 1 hr at 24 C, 1 mg coconut endosperm endogenous lipids and 0.5 mg palmitic acid were added as carrier lipids, and the mixture was extracted for neutral lipids and free fatty acids with petroleum ether as described by Mancha et al. (5).

Analysis of radioactive products from cell-free experiments. At the conclusion of an incubation, the reaction was stopped by the addition of 0.3 ml 40% KOH. The reaction tubes were capped and the mixtures directly saponified at 80 C for 1 hr. They were then cooled, acidified with 0.5 ml of 8 N H_2SO_4 and the [^{14}C]fatty acids extracted with 2 x 1 ml of chloroform. The chloroform extract was washed with 1 ml of water of 1 ml of 0.1 M malonic acid (for [1,3-^{14}C]malonyl-CoA incubations). It was then evaporated by dryness under nitrogen and the residue redissolved in 200-250 µl of benzene. Aliquots were counted in a liquid scintillation counter and the remainder methylated with diazomethane to form methyl esters for gas liquid chromatography (GLC) analysis.

In experiments where the radioactive products were separated into neutral lipids/free fatty acids, acyl-ACPs and acyl-CoAs, the procedure of Mancha et al. (5) was followed. The neutral lipids/free fatty acis, extracted with petroleum ether saturated with 50% aqueous isopropanol, was further separated into mono-, di- and triacylglycerols and free fatty acids by TLC. Since little complex lipids, if any, were expected to be formed, the acyl-CoA fraction was not subjected to the alumina column treatment but was directly saponified and converted to methyl esters.

Chemical α-oxidation of [^{14}C]fatty acids. Chemical α-oxidation was carried out by the procedure of Harris et al. (6).

Thin layer chromatography. Appropriate samples were chromatographed on Silica Gel G plates using the solvent system diethylether/benzene/ethanol/acetic acid, 40:50:2:0.1 (v/v) (5) to separate mono-, di- and triacyl glycerols and free fatty acids. Nonradioactive marker compounds were cochromatographed on the same plate and detected by exposure to iodine vapor. Radioactive areas on the plate were detected by scanning in a Model 7201 Packard radiochromatogram scanner. The gel in the radioactive areas was scraped into scintillation vials and directly counted. For further analysis, the gel was extracted with 4 x 1 ml of chloroform/methanol, 2:1 (v/v) and the lipid was saponified and methylated as described below.

GLC analysis. Radioactive lipids extracted from incubated tissue slices or from TLC plates were saponified in 1 ml of 1 N KOH in 90% ethanol for 1 hr at 80 C. The mixture was then acidified with 0.2 ml of 8 N H_2SO_4. One ml of

water was added and the mixture was extracted with 2 x 2 ml of chloroform. The extract was reduced to about 0.5 ml under nitrogen and methylated with an excess of freshly prepared ethereal solution of diazomethane.

Radio-GLC analysis of the methyl esters was carried out with a Varian Aerograph Model 920 instrument fitted with a thermal conductivity detector coupled to a Nuclear-Chicago Biospan (4998) radioactivity detector. Routine analysis was carried out on a 5 ft x 1/4 in. stainless steel column packed with 10% DEGS-PS on 80/100 Supelcoport. C_8-C_{14} fatty acids were separated at 140 C for 14 min after which the column temperature was raised to 160 C. Some analyses were also made on a 10% EGSS-X on 100/120 Gas-Chrom column packing under the same operating conditions.

Extraction of endogenous coconut lipid. Endosperm tissue (30-50 g) from freshly opened coconuts was homogenized in 10 volumes of chloroform/methanol, 2:1 (v/v) mixture using a Polytron blender. The mixture was left overnight at 4 C. It was then filtered, and the residue was washed with small volumes of chloroform/methanol mixture. The chloroform layer in the combined extract was washed with 1/5 volume of methanol/water, 1:1 (v/v). A sample was dried in a tared weighing vessel. The remaining extract was evaporated to dryness in a rotary evaporator and then made up to 25 ml with benzene. A sample was saponified and methylated with diazomethane for GLC analysis, and another sample was estimated for triacylglycerols by the triacylglycerol C-37 Rapid Stat Kit (Pierce Chemical Co., Rockford, IL).

RESULTS

Effect of stage of development of [1-^{14}C]acetate and [2-^{14}C]malonate incorporation into fatty acids by endosperm tissue slices. The development of the fruit of the coconut palm was described by Child (1). In the first six months, the fruit increases in volume while the cavity or embryo sac remains filled with liquid endosperm. At this stage, solid endosperm (kernel) begins to form, Initially at the end opposite the stalk but gradually extending to form a layer all around the interior. Rapid development of this endosperm layer occurs in the next 3-4 months from an initial thin jelly-like layer to form a firm solid kernel. The kernel continues to become progressively harder with intracellular deposition of oil until hard white flesh is attained at full maturity at 13 months. About 75% of the dry weight and 84% of the oil deposit are already formed by the ninth month (1).

It is difficult to define precisely the age of endosperm tissue employed in these studies. The coconuts used here were roughly at three stages of development, identified as: Stage A (ca. 6-7 months), Stage B (ca. 11-12 months) and Stage C (ca. 13-14 months). In the fully ripe coconut, the husk (mesocarp) had started to dry out. Table I shows the fatty acid composition of endogenous lipids extracted from the endosperm of these types of coconuts. As the coconut endosperm develops from an early stage of cellular proliferation to active deposition of oil droplets at later stages, fatty acid synthesis may show shifts in the nature of the products formed. This is shown in Table I where it is seen that the endogenous lipids of young endosperm tissue have a fatty acid composition with 61% of the acid as C_{16} and C_{18} acids. However, in the almost ripe coconut, most of the oil had already been deposited (1) and the fatty acid composition of the endogenous lipids showed a predominance of C_{12} and C_{14} acids. Clearly in the transition of development between Stages A and B, the tissue is synthesizing C_{10-14} fatty acids, but at or after Stage B the synthesis of these acids has been completed.

Slices of endosperm tissue from Stage A and Stage B coconut tissue incorporated [1-^{14}C]-acetate and [2-^{14}C]malonate into fatty acids (Table II), although incorporation was low. Endosperm tissue from Stage C coconut had very low activity with [1-^{14}C]acetate and no activity at all with [2-^{14}C]malonate. The incorporation of [1-^{14}C]acetate and [2-^{14}C]-malonate into fatty acids was, therefore, related to the stage of development of the endosperm. As predicted in Table I, Stage A and Stage B endosperm tissues incorporated [^{14}C]acetate predominantly into [^{14}C]short chain fatty acids (C_8-C_{14}) while the [^{14}C]products with Stage C tissue were almost exclusively C_{16} and C_{18} fatty acids. The fatty acids synthesized from malonate at each stage of development closely resembled those synthesized from acetage except for the formation of a small amount of C_{20} fatty acid. It is possible that this C_{20} fatty acid was formed by chain elongation of existing fatty acids in the presence of [2-^{14}C]malonate.

To determine whether [^{14}C]fatty acids in the incubated slices were synthesized de novo or by chain elongation of preexisting endogenous chains present in the extract, the extracted [^{14}C]products from several incubations were pooled together. [^{14}C]Palmitic acid was isolated by preparative GLC from these products and degraded by chemical oxidation

TABLE I

Fresh Weight and Endogenous Lipid Content of Coconut Endosperm at Different Stages of Development

Stage of development[a]	Fresh weight of endosperm (g)	Total endogenous lipid (g)	Composition of fatty acids in endogenous lipids (%)[b]							
			8:0	10:0	12:0	14:0	16:0	18:0	18:1	18:2
A	81	2.9	4	4	14	17	18	0	29	14
B	270	80.4	8	11	35	22	10	3	8	3
C	270	90.1	5	7	32	25	13	4	10	4

[a]Estimated stage of development of the coconut as described in the text.

[b]Extraction of endogenous lipids and estimateion of fatty acid composition are described in the Methods section.

TABLE II

Incorporation of [1-^{14}C] Acetate and [2-^{14}C] Malonate into Fatty Acids by Endosperm Tissue Slices from Coconuts at Different States of Development[a]

Stage of development[b]	Radioactiave substrate	Total nmoles incorporated into faty acids	Distribution of ^{14}C into fatty acids (%)								
			8:0	10:0	12:0	14:0	16:0	16:1	18:0	18:1	20:0
A	Acetate	0.98	35	11	19	8	5	3	3	14	0
B	Acetate	3.67	18	8	42	12	7	0	9	5	0
C	Acetate	0.44	0	0	0	8	61	0	14	17	0
A	Maloate	1.56	21	15	19	7	7	3	9	11	7
B	Malonate	3.04	10	13	28	16	11	0	11	3	8
C	Malonate	0	---	---	---	---	---	---	---	---	---

[a]Endosperm tissue slices (1 g) were incubated with 0.34 μmole of [1-^{14}C] acetate of 0.90 μmole of [2-^{14}C]-malonate. The lipid products were extracted and analyzed as described in the Methods section. Fatty acid composition of the endogenous lipids of endosperm tissue are given in Table I.

[b]See definition of Stages A, B, and C under Results and Discussion.

TABLE III

Uptake of [1-^{14}C] Fatty Acids ad Esterification into Triacylglycerols by Endosperm Tissue Slices[a]

[1-^{14}C] Fatty acid substrate	Percent of substrate taken up	Distribution of ^{14}C uptake (%)[b]	
		Free fatty acids	Triacylglycerols
10:0	78	26	74[c]
12:0	82	47	53
14:0	39	98	2
16:0	49	96	4
18:0	17	99	1
18:1	49	99	1

[a]Stage A coconut endosperm tissue slices (1 g) were incubated with 150 nmoles of [1-^{14}C] fatty acid. Experimental details are described in the Methods section.

[b]Radioactive lipids extracted from incubated endosperm tissue slices were separated by TLC.

[c]Included 5% DG and 8% MG.

(6). The results indicated that the [^{14}C]-palmitic acid formed from [1-^{14}C] acetate was synthesized de novo. The same results were obtained with [^{14}C] palmitic acid formed from [2-^{14}C] malonate. Thin layer chromatography of [1-^{14}C] acetate incorporation products showed that the [^{14}C] fatty acids synthesized were present as mono- and triacyl-glycerols as well as free acids, namely, 14% MG, 32% TG, 54% FFA. No diglycerides were detected.

Ability of endosperm tissue to metabolize exogenously added [1-^{14}C] fatty acids. It was of interest to determine whether coconut endosperm had the capacity to metabolize exogenously added fatty acids. Table III shows that uptake of [1-^{14}C] fatty acids by Stage A tissue slices occurred very readily. The efficiency of uptake appeared to decrease with the chain length of the substrate. It is possible that a portion ofthe substrate taken up moved into a nonmetabolic pool by directly partitioning into the lipid phase of the tissue slices. However, when the extracted [^{14}C] lipids were separated on TLC with a diethylether/benzene/ethanol/-acetic acid, 40:50:2:0.2 (v/v) solvent system, it was found that those obtained from incubations with [1-^{14}C] decanoic and [1-^{14}C] lauric acids consistently contained a considerable proportion of [^{14}C]-neutral acylgycerols, showing that the tissue was capable of activating these acids and transferring them to suitable endogenous acceptors. Although the experiment reported in Table II showed a very low incorporation of [1-^{14}C]-myristic acid into triacylglycerols, the same incubation with endosperm slices from another coconut gave a higher proportion (25%) of radioactivity as triacylglycerols. With the long chain C_{16} and C_{18} fatty acids, there was consistently little incorporation into triacylglycerols.

Radio-GLC analysis showed that the original [1-^{14}C] fatty acid remained unchanged. Thus, although the endosperm tissue was capable of activating the medium chain fatty acids to acyl CoAs for esterification into neutral acylglycerols, these activated forms were not elongated, desaturated, or metabolized further by the tissue slices. Presumably, the [^{14}C] acyl-CoAs could not enter the de novo-elongation ACP track (7) because of the absence of a direct acyl-ACP liguase or an acyl-CoA/ACP transacylase and, therefore, could not be elongated and desaturated.

Incorporation of [1,3-^{14}C] malonyl-CoA into fatty acids by extracts of coconut endosperm - properties of the system. Further characterization of lipid biosynthesis in endosperm tissue required the preparation of cell-free extracts capable of incorporation of [^{14}C] substrates into fatty acids. When incubated with [1,3-^{14}C] malonyl-CoA, extracts of an acetone powder preparation of coconut endosperm incorporated radioactivity into products extractable with lipid solvents. With the incubation system described in the methods section, about 40-60% of [1,3-^{14}C]malonyl-CoA were incorporated by a fresh extract of acetone powder from Stage A and Stage B endosperm. Very little activity (3% incorporation) was obtained with acetone powder extract from Stage C tissue. When [1-^{14}C] acetyl-CoA was the substrate, very little activity (less than 3% incorporation) was obtained with all extracts. These low activities probably relate to acetyl-CoA carboxylase activity, which was very low in these extracts. Although maximum incorporation was obtained after 40 min incubation under the specified conditions, a 60 min time was selected for all incubations. The amount of incorporation was proportional to the enzyme protein in the acetone powder extract up to a concentration of 170 μg pro-

TABLE IV

Effect of Various Cofactors on Incorporation of [1,3-^{14}C]Malonyl-CoA into Fatty Acids

Incubation mixture[a]	Percentage of activity in complete mixture
Experiment 1	
Complete	100
−All cofactors	4
−ACP	4
−$KHCO_3$	110
−ATP	85
−$MgCl_2$	123
−$MnCl_2$	90
Experiment 2[b]	
Complete	100
−NADH, NADPH	18
−NADH	57
−NADPH	99

[a]Incubation conditions are described in the Methods section. Enzyme source was an ammonium sulfate (0-80% saturation) precipitate of an acetone powder extract of young endosperm (Stage A – Materials and Methods for definition of stages of development).

[b]$KHCO_3$, ATP, $MgCl_2$ and $MnCl_2$ were omitted from Experiment 2.

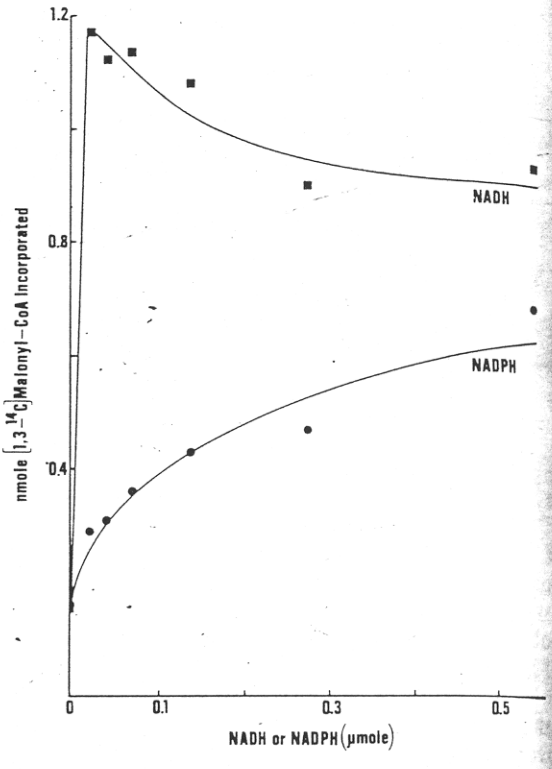

FIG. 1. Effect of increasing amounts of NADH or NADPH on incorporation of [1,3-^{14}C] malonyl-CoA by an ammonium sulfate fraction of acetone powder extracts of Stage A endosperm tissue. Details in Methods section.

tein/0.3 ml incubation mixture. Optimal activity was obtained at a pH of ca. 7.0. At pH 6.5, only 20% of the activity at pH 7.0 was observed and at pH 8.0, 80% of the optimal activity occurred. Under optimal conditions, [^{14}C] malonyl-CoA was incorporated at a rate of about 2.4 nmoles/hr/mg protein. The incubation system showed an absolute requirement for added ACP after removal of endogenous cofactors by ammonium sulfate precipitation (Table IV). It was also stimulated by reduced pyridine nucleotide. Figure 1 shows that the system utilized NADH more readily, although at higher concentrations NADPH was also a suitable reductant.

To determine whether the enzyme system was particulate or soluble, the acetone powder extract was centrifuged at 100,000 g for 3 hr. Ninety–eight percent of the activity was recovered in the 100,000 g supernatant and only 2% from the washed 100,000 g sediment. An extract prepared from fresh endosperm tissue was also active in incorporating [1,3-^{14}C] malonyl-CoA into [^{14}C] fatty acids. Most of this activity (87%) was found in the soluble fraction, and only 3% of the total activity was recovered in the 100,000 g sediment. The procedure was designed to preserve organelles such as mitochondria and plastids.

Nature of incorporation products. Table V shows the distribution of [^{14}C] fatty acids in the total [^{14}C] products formed from [1,3-^{14}C] malonyl-CoA by extracts from acetone powder and from fresh endosperm tissue. In all of them, long chain [^{14}C] fatty acids (C_{16}-C_{18}) accounted for over 60% of the radioactivity. The percentage of the shorter chain [^{14}C]-fatty acids (C_8 and C_{10}) was variable between different experiments probably in part because of the difficulty of complete recovery of these volatile acids without adopting special procedures; but they were always absent from products obtained with acetone powder extracts of Stage B endosperm. [^{14}C] Fatty acids were also obtained from incubation mixtures from which *E. coli* ACP was omitted; presumably synthesis occurred employing only endogenous coconut ACP. To obtain enough radioactive material for analysis, 10 incubations (no added ACP) were simulataneously carried out and the [^{14}C] products pooled. It is seen in Table V that with limiting amounts of endogenous coconut ACP, there was a significant increase in the proportion of [^{14}C] stearic acid and [^{14}C] oleic acids formed. Whether this shift is related either to the use of subminimal concentrations of ACP or to the participation of endogenous ACP with a specificity different

TABLE V

Distribution of Radioactivity in Fatty Acids Produced by Various Extracts from [1,3-^{14}C] Malonyl-CoA

Type of extract	nmoles Substrate incorporated/ mg protein	Distribution of ^{14}C in fatty acids (%)						
		8:0	10:0	12:0	14:0	16:0	18:0	18:1
Acetone powder extract:								
Stage A endosperm	3.20	6	6	9	12	50	13	4
Stage A endosperm[a]	0.60	0	0	4	12	47	26	11
Stage B endosperm	2.46	0	0	15	16	67	12	0
Fresh Stage A endosperm-								
100,000 g supernatant	9.43	6	6	8	13	56	11	0
100,000 g sediment	2.44	1	3	4	6	64	22	0

[a]*E. coli* ACP omitted from incubation mixture and 10 incubations pooled.

TABLE VI

Distribution of [^{14}C] Fatty Acids in Different Lipid Classes

Enzyme preparation[a]	Lipid class[b]	Percentage of [^{14}C] incorporated							
		8:0	10:0	12:0	14:0	16:0	18:0	18:1	Total
Acetone powder extract	FFA	0	0	1	1	33	11	11	57
	Acyl-ACP	4	2	3	5	24	0	0	38
	Acyl-CoA	0	1	0	0	4	0	0	5
100,000 g supernatant	FFA	0	0	0	0	12	5	0	17
	Acyl-ACP	6	6	8	12	40	6	0	79
	Acyl-CoA	0	1	0	0	2	1	0	4
100,000 g sediment	NL/FFA	0	0	1	1	25	14	0	41
	Acyl-ACP	1	2	4	5	35	6	0	53
	Acyl-CoA	-	1	0	0	4	1	0	6

[a]Stage A endosperm tissue was employed.

[b]Radioactive products were separated by the Mancha procedure (6) into lipid classes NL, neutral lipids; FFA, free fatty acids; and acyl-ACPs and acyl-CoAs. Details in text.

TABLE VII

Formation of Esterified [^{14}C] Fatty Acids from [1,3-^{14}C]Malonyl-CoA in the Presence of Microsomal Fraction

Extract[a]	Incorporation nmoles/hr/mg protein	Radioactivity in lipid classes (%)[b]					
		Neutral lipid fraction				Acyl-ACP	Acyl-CoA
		TG	DG	MG	FFA		
Acetone powder extract	11.25	0	0	0	31	62	7
Microsomal fraction + acetone powder extract	4.69	19	4	11	9	46	11
100,000 g supernatant of fresh endosperm	37.8	0	0	0	17	79	4

[a]Actone powders and fresh endosperm tissue were from young coconut (Stage A). Incubation conditions were the same as described in Materials and Methods except that 22.2 nmoles of [1,3-^{14}C]malonyl-CoA were employed.

[b]^{14}C-Products were separated into the three lipid classes by the Mancha procedure (6) followed by TLC of the neutral lipid fraction. Details in text.

from that of *E. coli* ACP remains for further investigation to determine.

The composition of the [^{14}C]fatty acid synthesized as a function of various conditions was also examined employing acetone powder extracts of Stage A endosperm: (a) incubation of 24 C, 30 C and 40 C; (b) incubation at pH 7.0 and at pH 8.0; (c) addition of 0.09 mg, 0.18 mg and 0.36 mg *E. coli* ACP to the incubation. In (a) and (b), there was no significant shift in the pattern of [^{14}C]fatty acids produced compared with that produced under the standard incubations conditions described in the Methods section. With increasing ACP concentrations, however, the pattern of [^{14}C]synthesized fatty acids shifted to shorter chain lengths, i.e., C_{8-12}.

To determine whether the [^{14}C]fatty acids were synthesized de novo or by mere elongation or preexisting chains in the extract, [^{14}C]-palmitic acid isolated from incubation products was purified by preparative GLC and degraded by the $KMnO_4$ chemical α-oxidation procedure (6). The results indicated that the [^{14}C]-palmitic acid obtained in the incubation mixture was synthesized de novo from [1,3-^{14}C]-malonyl-CoA via an ACP pathway.

The [^{14}C]lipids were also separated into neutral lipids-free fatty acids, acyl-ACPs and acyl-CoAs by the Mancha procedure (5). The results are shown in Table VI. It is seen that the medium chain [^{14}C]fatty acids (C_8-C_{14}) were mainly, if not exclusively, present as acyl-ACP derivatives. The [^{14}C]palmitoyl moiety was present in all three fractions. [^{14}C]Acyl-CoA was a trace product.

Formation of neutral [^{14}C]acylglycerols by 100,000 g sediment of fresh endosperm tissue.

The petroleum ether extract of acidified incubation mixtures did not differentiate between free fatty acids and neutral acylglycerols. The extract was, therefore, chromatographed on TLC as previously described (2). Table VII shows that all the [^{14}C]products (extracted with petroleum ether from acidified mixtures) synthesized by the extracts obtained from acetone powder or with 100,000 g supernatant of fresh endosperm were free fatty acids. On addition of a microsomal fraction (100,000 g sediment of fresh endosperm) to the incubation mixture, these [^{14}C]fatty acids were also found in mono-, di- and triacylglycerols. Incubation with microsomal fraction alone also produced [^{14}C]fatty acids in the neutral acylglycerols. There was no apparent chain length specificity towards the esterification of these [^{14}C]fatty acids formed from [1,3-^{14}C]-malonyl-CoA since the same pattern of fatty acid composition was seen both in the [^{14}C]-

TABLE VIII

Formation of Neutral [^{14}C] Acylglycerols from [^{14}C] Acyl-CoA and [^{14}C] Acyl-ACP[a]

Substrate	Percent of [^{14}C] Substrate incorporated	Distribution of [^{14}C] incorporated (%)			
		TG	DG	MG	FFA
[1-^{14}C] Acyl-CoA					
8:0	78	55	9	23	13
10:0	69	37	9	41	13
12:0	100	39	8	42	11
14:0	46	24	18	51	7
16:0	59	15	21	59	5
18:0	100	13	14	67	6
[0-^{14}C] Acyl-ACP[b]					
12:0	34	5	54	30	11
18:0	93	56	32	11	1
18:0 + CoA	72	53	31	14	2

[a]Details are described in the Methods section.

[b][0-^{14}C] Stearoyl-ACP substrate contained about 40% [0-^{14}C] palmitoyl-ACP. Because of the extensive transfer of the stearoyl groups to NL, obviously palmitoyl-ACP was also an effective donor substrate.

acylglycerols and in the total [^{14}C] fatty acids produced. The microsomal fraction thus appeared to possess the enzymes for neutral lipid biosynthesis.

Substrate specificity for microsomal transacylating activity. To determine the nature of the substrates for the formation of neutral acylglycerols by the microsomal fraction, incubation of microsomal preparations with different [1-^{14}C] acyl-CoAs was carried out. L-α-Glycerol phosphate was added as a possible acceptor for the acyl groups. [^{14}C] Acyl-ACPs were also tested as possible substrates in the presence and absence of CoA. Due to the limited availability of such substrates, only [^{14}C] stearoyl-ACP and [^{14}C] lauroyl-ACP were tested. [^{14}C] Stearoyl-ACP also contained ca. 40% [^{14}C] palmitoyl-ACP.

Table VIII shows that both [1-^{14}C] acyl-CoAs and [^{14}C] acyl-ACPs were esterified onto neutral acylglycerols. Since the omission of L-α-glycerol phosphate did not lower the acylation reactions, experiments without this acceptor were not included in Table VIII. With [^{14}C] acyl-ACP as substrate, transesterification onto neutral acylglycerols occurred in the absence of CoA showing that acyl-ACP served as the direct donor in the transfer process.

Ability of acetone powder extract to utilize [1-^{14}C] acyl-CoA. The formation of the whole range of [^{14}C] fatty acids from [1,3-^{14}C]-malonyl-CoA by endosperm extracts (Table V) and its dependence upon ACP (Table IV) suggested that these [^{14}C] fatty acids were synthesized along the ACP track (7). It was of interest to determine whether the extract could utilize [1-^{14}C] acyl-CoA added to it and whether the chain length of the substrate had any effect on the process. [1-^{14}C] Acyl-CoA was incubated with an ammonium sulfate precipitate of an acetone powder extract of Stage A endosperm in the presence and absence of *E. coli* ACP. ATP, NADH and malonyl-CoA were added as cofactors for possible elongation processes.

TLC of the extracted [^{14}C] products showed over 96% as free fatty acids, and GLC of the methyl esters confirmed that the original [^{14}C] fatty acyl groups remained unchanged. Thus, the [1-^{14}C] acyl-CoA substrates were merely hydrolyzed to free acids by an acyl-CoA hydrolase but were not elongated or desaturated. These results are in complete agreement with those obtained when [^{14}C] fatty acids were incubated with tissue slices, namely, the absence of any elongation or modification of the acyl chain of the acyl-CoAs.

DISCUSSION

The purpose of this investigation was to obtain basic information concerning the biosynthesis of fatty acids in coconut endosperm. Possible mechanism of chain length termination could then be tested.

Coconut endosperm lipids are composed of over 50% C_{12} and C_{14} fatty acids (1). [^{14}C]-Fatty acids synthesized from [1-^{14}C] acetate or [2-^{14}C] malonate by intact endosperm slices were similar in having over 50% C_{12} and C_{14} acids. In contrast, the [^{14}C] fatty acids obtained with cell-free extracts showed a markedly different pattern. Since palmitic acid was always the major component (over 50%) while C_{12} and C_{14} fatty acids seldom exceeded 20%, presumably the mechanism controlling the high

proportion of C_{12} and C_{14} fatty acids in intact tissues was not functioning in these cell-free extracts.

The fatty acid synthesizing activity in coconut endosperm extracts did not appear to be membrane or organelle associated, since it was not sedimented by centrifugation at 100,000 g under conditions where organelle structure would be preserved. It had an absolute ACP requirement for activity. In the absence of exogenously added ACP, the system, operating with only the endogenous coconut ACP in the extract, showed minimal activity (Table IV); but the [^{14}C]fatty acids synthesized showed an increase in the proportion of stearic and oleic acids (Table V). Presumably, this may relate to the limited number of fatty acid chains initiated by the low level of available ACP and a shift towards elongation of preexisting acyl-charged endogenous ACP. In the presence of excess *E. coli* ACP (from 0.09 mg to 0.36 mg), the pattern of [^{14}C]fatty acids synthesized was shifted to shorter chain fatty acids. Changes in either temperature (from 24 C to 40 C) of pH did not influence the type of [^{14}C]fatty acids synthesized.

One may speculate as to the nature of this control. It may operate either by a switching system composed of a medium chain acyl-ACP thioesterase-acyl-CoA synthetase combination (7) or by an acyl-ACP/CoA transacylase which would be specific for the medium chain length fatty acids so that these acids would be switched off the ACP track onto a CoA track and then used as acyl donors (8). Another possibility could be the direct transacylation of lauroyl-ACP and myristoyl-ACP to glycerol phosphate to form neutral acylglycerols. A fourth possibility could involve the specificity of the coconut ACP, that is, medium chain acyl coconut ACPs could be a more effective substrate than the medium chain acyl *E. coli* ACPs. Finally, there could be a separate synthetase system for C_{12} and C_{14} fatty acids with a different cellular localization, e.g., associated with the microsomes or special organelles which eventually convert to oil droplets.

Only 3% of the total activity in the cell-free extracts was recovered in the once-washed microsomal fraction (100,000 g sediment). The [^{14}C]fatty acids synthesized by this residual activity were mainly C_{16} and C_{18} acids. Thus, the microsomal fraction probably had no specific fatty cid synthetase system associated with it. Similarly, the C_{16}-C_{18} [^{14}C]fatty acids synthesized with endogenous coconut ACP (Table V) can be explained by its limiting effect on the system as explained above. Certainly there was no evidence suggesting a strong shift to the synthesis of shorter chain fatty acids by the employment of endogenous ACP.

Analysis of [^{14}C]fatty acids among the three lipid classes separated by the Mancha procedure (5) showed that the short and medium chain fatty acids remained as acyl-ACP derivatives (Table VI). This indicated that the coconut extracts had no acyl-ACP hydrolase activity towards C_8-C_{14} fatty acyl-ACP compounds. Ohlrogge et al. (9) recently reported a similar lack of acyl-ACP hydrolase activity for C_8-C_{14} fatty acids in plant systems. A specific acyl-ACP hydrolase-acyl-CoA synthetase switching system would, therefore, probably not be involved, An acyl-ACP/CoA transacylase would also be an unlikely mechanism of control since acyl-CoAs with or without ACP were not elongated or modified by tissue extracts.

Table VII clearly shows that the transacylating activity for esterification of acyl-CoA to neutral acylglycerols was located in the microsomal fraction. However, there was no specificity towards the type of fatty acids esterified since C_8-C_{14} as well as C_{16}-C_{18} acids were detected in the neutral acylglycerols in the same proportions as in the total [^{14}C]fatty acids synthesized. Table VIII shows that the microsomal fraction was capable of transacylating all the acyl-CoAs from C_8 to C_{18}. It was also capable of direct transacylation of lauroyl-ACP, palmitoyl-ACP and stearoyl-ACP onto neutral acylglycerols without the mediation of CoA. It is not possible to comment on the specificity of this transacylation of acyl-ACPs since a complete range of [^{14}C]acyl-ACP substrates was not available for comparative studies. Thus, while the microsomal fraction was capable of direct transfer of acyl groups from acyl-ACP to neutral acylglycerols, the role of this reaction as a mechanism for chain termination of C_{12} and C_{14} fatty acids in coconut endosperm tissue cannot be properly assessed. It should be noted, however, that intact coconut endosperm tissue slices could incorporate [1-^{14}C]decanoic and lauric acids but not [^{14}C]myristic, palmitic and stearic acids into neutral [^{14}C]-acylglycerols; these acyl chains remained, however, unchanged (Table III). This suggests that these acids were not entering the ACP track but were being introduced into the acylglycerols perhaps as acyl-CoA derivatives.

In summary, five possible mechanism have been tested with a variety of endosperm systems. These include (a) a specific acyl-ACP hydrolase-Acyl-CoA synthetase switching system, (b) a specific acyl-CoA transacylase, (c)

a specific acyl-ACP transacylase, (d) a difference in specificity of endogenous acyl coconut ACP vs. acyl *E. coli* ACP, and (e) a specific fatty acid synthetase which terminates at the C_{12}-C_{14} level. We believe that these mechanisms have been eliminated by direct experiments. Further work with this difficult tissue will hopefully reveal the actual control mechanism.

ACKNOWLEDGMENTS

We are deeply indebted to Professor N.P. Kefford, Department of Botany, University of Hawaii, and Mr. R. Miyashita, Department of Parks and Recreation, for their great help in facilitating the smooth delivery of developing coconuts. We also whish to thank Mrs. Barbara Clover for her diligent help in the preparation of this paper.

REFERENCES

1. Child, R., in "Coconuts," 2nd Edn., Longsmans, London, 1974, pp. 45-48.
2. Stumpf, P.K., J.B. Ohlrogge, K.C. Oo, and M.R. Pollard, 11th FEBS Meetings, Copenhagen, August 15-19, (1977).
3. Alberts, A.W., P.W. Majerus, and P.R. Vagelos, Methods Enzymol. 14:43 (1969).
4. Jaworski, J.G., and P.K. Stumpf, Arch. Biochem. Biophys. 162:166 (1974).
5. Mancha, M., G.B., Stokes, and P.K. Stumpf, Anal. Biochem. 68:600 (1976).
6. Harris, R.V., P. Harris, and A.T. James, Biochim. Biophys. Acta 106:465 (1965).
7. Shine, W.E., M. Mancha, and P.K. Stumpf, Arch. Biochem. Biophys. 172:110 (1976).
8. Stumpf, P.K., in "International Review of Biochemistry," Vol. 14, Edited by T.W. Goodwin, University Park Press, Baltimore, pp. 215-237 (1977).
9. Ohlrogge, J.B., W.E. Shine, and P.K. Stumpf, Arch. Biochem. Biophys. 189:382 (1978).

[Revision received October 13, 1978]