ENZYMIC SYNTHESIS OF RICINOLEIC ACID BY EXTRACTS OF DEVELOPING *RICINUS COMMUNIS L.* SEEDS*

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The composition of fatty acids of *Ricinus communis L.*(castor bean) seeds is unusual in that up to 90% of the total fatty acids consists of ricinoleic acid (12-hydroxy-18:1 (9)) according to Binder *et al* (1962). During the past year we have been investigating the β-oxidation of a number of fatty acids by particulate preparations obtained from the germinating seeds of this species (Yamada and Stumpf, 1963). In examining the capacity of extracts of the developing and germinating seeds to synthesize ricinoleic acid from acetate-C^{14}, we observed there is a restricted period in the history of the developing seed when the enzymes necessary for the biosynthesis of this acid exist. Germinating seed extracts are devoid of this capacity although palmitic, stearic, oleic and linolenic acids are readily synthesized from acetate-C^{14}. While this work was in progress, both Harris and James (1963) and Canvin (1963) reported similar findings with whole tissue.

We now wish to describe the enzyme systems responsible for the synthesis of ricinoleic acid from either acetate-C^{14} or oleic-1-C^{14}.

EXPERIMENTAL

Enzyme preparation: In the early developmental period of a fertilized *Ricinus communis* seed, the seed is covered by a white seed

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coat; three seeds are encased in a fleshy green capsule. At this stage, the seed has no capacity to synthesize ricinoleic acid. When the seed coat becomes brown and hard while the capsule remains green (about 20-25 days after fertilization), extracts can be prepared which readily synthesize ricinoleic acid from acetate-C^{14}. However, once the green capsule becomes brown and dry, the seed, now with a brittle black seed coat, no longer can yield extracts with synthesizing capacity. Extracts from germinating seeds are devoid of activity.

Enzyme fractions were prepared as follows: seeds were carefully selected in the field and their capsules and seed coats were removed. 50 grams of fresh seeds were washed several times with distilled water and were then homogenized for seven minutes in a Waring Blender with three volumes of a medium consisting of 10 volumes of 0.3 M sucrose and one volume of M potassium phosphate at pH 7.1. The homogenate was squeezed through several layers of cheesecloth and the milky filtrate was centrifuged in a Model RC-2 Servall centrifuge at 500 x g for 10 minutes, the cell debris was discarded, and the supernatant further centrifuged at 12,000 x g for 30 minutes. The pellet was suspended in 7 ml. of phosphate buffer. This particulate fraction contains plastids and mitochondria as well as aggregated microsomes. The supernatant was further fractionated into a soluble protein and a microsomal fraction by centrifuging in a Model L Spinco ultracentrifuge at 100,000 x g for 60 minutes. The microsomal fraction was suspended in 5 ml of phosphate buffer. The volume of supernatant was 120 ml.

Lipid analysis: reactions were stopped by addition of 5 ml. of 15% methanolic KOH, followed by saponification for 3 hours at 80°. The free fatty acids were isolated by usual extraction procedures and converted to their methyl esters with freshly distilled diazomethane. Separation of methyl ricinoleate from the methyl esters of saturated and unsaturated fatty acids was accomplished by thin

layer chromatography using silica G gel with a developing solvent of hexane:diethyl ether (6:4). Methyl ricinoleate has a Rf of 0.6 and the methyl esters of saturated and unsaturated fatty acids have a Rf of 0.90-0.95. The methyl esters were visualized by a light spray of 2,7 dichlorofluorescin, scraped off the plate, suspended in 5 ml. of a toluene-phosphor mixture and counted with a Packard liquid scintillation spectrometer.

Schmidt degradations were employed as described by Goldfine and Bloch, (1961). Catalytic hydrogenation was carried out in a Parr Pressure Reaction Apparatus under 40 pounds pressure of hydrogen gas for two hours at room temperature in 5 ml. of methanol and 5 mgm of 10% Palladium on charcoal.

RESULTS AND DISCUSSION

When a homogenate of developing seeds (20-25 days after fertilization of Ricinus communis L.) is fractionated into particles, microsomes, and soluble proteins (supernatant), considerable incorporation of acetate-1-C^{14} into ricinoleic acid occurs providing particles and supernatant fractions are present. When the three fractions are incubated separately with acetate-1-C^{14}, ricinoleic acid synthesis is greatly diminished. Table I summarizes experiments with acetyl CoA-C^{14} as substrate. For maximum synthesis TPNH, oxygen and bicarbonate and ATP are required in the presence of both particles and supernatant fraction. Since particles alone can synthesize long chain fatty acids from acetyl CoA and bicarbonate, presumably they serve as sites of synthesis for a precursor which is then converted by the supernatant enzyme to ricinoleic acid. The particles readily synthesize from acetyl CoA palmitic, and oleic acids under aerobic conditions. Under anaerobic conditions only palmitic and stearic acids are formed. When these acids as well as linoleic acid were tested as possible precursors, only oleic acid fulfilled the function of a precursor. In Table II

TABLE I

Conversion of Acetyl CoA-1-C^{14} to Ricinoleic-C^{14}

Complete reaction mixture contains 100 μmoles potassium phosphate at pH 7.1; 150 mμcuries acetyl CoA-1-C^{14}, 60 mμ moles; 0.2 μmoles TPN^+; 0.5 μmoles glucose-6-phosphate; 10 μmoles ATP; 0.2 μmoles manganeous chloride; 20 μmoles potassium bicarbonate; 0.5 ml. particles; 0.5 ml. supernatant fraction. Incubation time 90 minutes at 30°. Total volume 2.2 ml.

Cofactors omitted	Total incorp. as lipids cpm	% incorp. as lipids	% incorp. as Ricinoleate
no omission	106,960	25.4	7.50
-TPN^+, glucose-6-P, O_2	1,700	0.4	0.18
-O_2, DPNH	3,700	0.9	0.16
-$MnCl_2$	11,400	2.7	0.65
-$KHCO_3$	6,160	1.5	0.11
-particles	14,680	3.3	0.13
-supernatant	129,200	30.8	2.16

TABLE II

Conversion of Oleyl CoA-1-C^{14} to Ricinoleic-C^{14}

Reaction mixtures contained 100 μmoles potassium phosphate at pH 7.1; 1 mμmole oleyl CoA-1-C^{14} (8500 cpm) and as indicated 0.2 μmoles TPN; 0.5 μmoles glucose-6-phosphate; 0.5 μmoles DPN, 2 μmoles manganeous chloride; 20 μmoles potassium bicarbonate; 10 μmoles ATP; 0.2 μmoles CoA; 0.5 ml. particles; 0.5 ml. microsomes; 0.5 ml. supernatant. Total volume was 1.3 ml. Incubation time 90 min. at 30°.

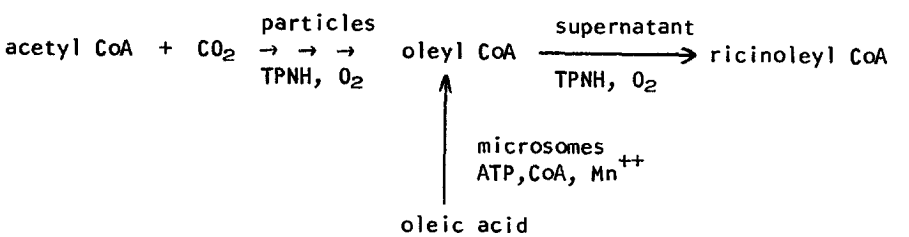
Cofactors added	Fractions used	Ricinoleic-C^{14} formed mμmoles
TPN, G-6-P, O_2	S	0.14
TPN, G-6-P, O_2	S	0.016*
none	S	0.035
DPNH, O_2	S	0.046
TPN, G-6-P, O_2	S + M	0.11
none	S + M	0.036
TPN, G-6-P, O_2	M	0.036
none	M	0.033
TPN, G-6-P, DPNH, $MnCl_2$, $KHCO_3$, ATP, CoA, O_2	S + P	0.10

* zero time incubation

S-supernatant; M-microsomes; P-particles

is summarized a series of experiments in which oleyl CoA was incubated with particles, microsomes and supernatant fractions. Results indicate that in the presence of TPNH and oxygen, only the supernatant fraction converts oleyl CoA to ricinoleic acid. DPNH does not replace TPNH. Further experiments with free oleic-1-C^{14} indicated that microsomes and the supernatant fraction were required with ATP, CoA, TPNH and oxygen as components of the system. Barron and Stumpf have shown in 1962 that the site of fatty acid thiokinase activity in plant tissue is localized in microsomes.

These results would support the following sequence in the developing seed:



Proof that the hydroxy-monounsaturated fatty acid is indeed ricinoleic acid rests on the following evidence:

(a) by TLC techniques, the radioactively labeled acid moves with the same Rf as does authentic methyl ricinoleate in a solvent system of hexane:diethyl ether (6:4).

(b) the methyl ester of the polar acid synthesized either from acetate-1-C^{14} or oleic-1-C^{14} was separated from the methyl esters of non-polar fatty acids by TLC. The methyl ester of the polar acid was then co-chromatographed with authentic methyl ricinoleate and methyl 12-hydroxy stearate. As shown in Figure 1 all the radioactivity coincided with a peak area assigned to known methyl ricinoleate. On catalytic reduction and GLC chromatography, the radioactive acid synthesized from both acetic-1-C^{14} and oleic-1-C^{14} moves to a peak area assigned to known methyl 12-hydroxy stearate.

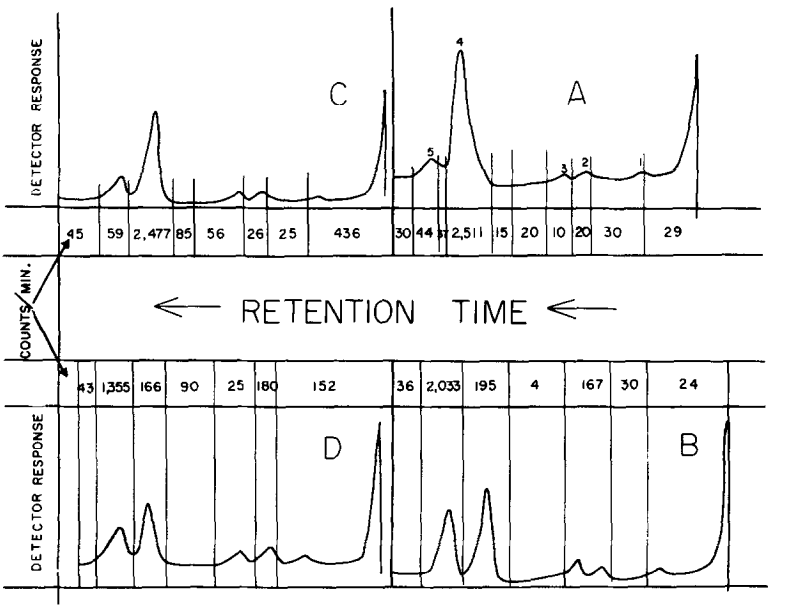


Figure 1. GLC separation of methyl ester of fatty acid formed from either acetate-1-C^{14} or oleic-1-C^{14} and isolated from non polar fatty acids by TLC. Column used was 6 foot Apiezon M 1/4" stainless steel at 225°C and flow rate of 60 ml/min. In all GLC runs authentic methyl 12-hydroxy stearate and ricinoleate were added as markers. Methyl esters of palmitate, stearate and oleate were present as minor contaminants of methyl ricinoleate sample. Effluent gas collected at indicated intervals and counted for radioactivity.

A--acid formed from acetate-1-C^{14}.
B--catalytic reduction of acid from A.
C--acid formed from oleic-1-C^{14}.
D--catalytic reduction of acid from C.

Numbers 1 through 5 in Chromatogram A represent respectively methyl palmitate, methyl oleate, methyl stearate, methyl ricinoleate and methyl 12-hydroxy-stearate. Peaks in B,C and D have the same assignments.

(c) Schmidt degradation of the monohydroxy saturated fatty acid formed by catalytical reduction of the polar acid synthesized from oleic-1-C^{14} showed that all the radioactivity was associated with the carboxyl carbon of the acid; with acetate-1-C^{14} as substrate, the C^{14} is distributed along the odd carbons of the fatty acid chain. Therefore with oleic-1-C^{14} as substrate, there was no initial breakdown to acetyl CoA units but a direct conversion of the fatty acid to ricinoleic.

(d) $KMnO_4$-periodate oxidation of the polar acid enzymically synthesized from oleic-1-C^{14} yielded azelaic acid as the only oxidation product radioactively labeled. When the same oxidation is carried out with the polar acid (15,000 cpm) synthesized from acetate-2-C^{14}, 7448 cpm were recovered in azelaic acid and 7440 cpm in the monocarboxylic acid. Therefore the double bond in the polar acid formed from either acetic acid or oleic acid is located between carbons 9 and 10.

This work would suggest that a part of the aerobic mechanism first described by Bloomfield and Bloch (1960) for oleic acid synthesis may be operative in the conversion of oleic acid to ricinoleic acid. Work is now in progress concerning the nature of the supernatant enzyme and the role of ricinoleic acid as a possible precursor of linoleic acid.

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