The Biosynthesis of Ricinoleic Acid

By A. T. JAMES, H. C. HADAWAY AND JOAN P. W. WEBB*
Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford

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1. Ricinoleic acid is shown to be synthesized in the immature castor bean seed only after 3–4 weeks from the time of fertilization. 2. Synthesis occurs both in the isolated embryo and the endosperm. 3. Linoleic acid does not act as precursor of ricinoleic acid in the isolated bean embryo. 4. Oleic acid is shown to be the direct precursor of ricinoleic acid. 5. The reaction does not use molecular oxygen. This suggests that ricinoleic acid is not a precursor of linoleic acid.

The major fatty acid of the castor-oil seed is ricinoleic acid (12-hydroxyoctadec-9-enoic acid); it occurs only in the stored triglyceride and is absent from the seed phospholipid (B. W. Nichols, D. Wharry & L. J. Morris, unpublished work). The leaves of the plant contain no detectable ricinoleic acid, as is normal with plants producing unusual seed fatty acids.

Ricinoleic acid is a possible intermediate in the conversion of oleic (octadec-9-enoic) acid into linoleic (octadeca-9,12-dienoic) acid. This conversion was demonstrated by Yuan & Bloch (1961) in a fatty yeast, in plant leaves (James, 1963*a*) and in a cell-free *Chlorella vulgaris* system (R. V. Harris & A. T. James, unpublished work). Yuan & Bloch (1961) showed that the cofactor requirements were, as in the stearate-oleate conversion, molecular oxygen and NADPH. There is thus a possibility of a hydroxy intermediate between oleic acid and linoleic acid and one such likely intermediate is ricinoleic acid. Yuan & Bloch (1961) prepared tritiated ricinoleic acid and were unable to get conversion into labelled linoleic acid but, as they pointed out, there was possibly some loss of label during reaction.

An attempt to study the biosynthesis of ricinoleic acid was made by Coppens (1956), using mature castor seeds. No labelled ricinoleic acid was found when [¹⁴C]acetate was used as precursor, although all the other saturated and unsaturated acids were labelled.

This prompted us to study the stages at which ricinoleic acid is formed in the developing seed and the nature of its direct precursor. Short reports of this work have already appeared (James, 1962; 1963*b*). Since this work was completed, Yamada & Stumpf (1964) have described a cell-free system from immature castor seeds capable of converting

oleic acid into ricinoleic acid, and Canvin (1963) has published an account of the stages of development of castor beans related to the production of ricinoleic acid.

EXPERIMENTAL

Castor plants of two species, *Ricinus zanzibariensis* and *Ricinus gibsonii* (Thomas Butcher, Seedsmen, Shirley, Croydon, Surrey), were grown in greenhouses, first at the National Institute for Medical Research (1962) and then the Unilever Research Laboratory (1963–1964). The latter greenhouse was adjusted to give an 11 hr. day with artificial light in winter; this causes repeated flowering and seed production throughout the year. The average time between planting the seed and formation of flowers was 100 days.

The plants were marked with their flowering date and after an initial period of 15 days samples were taken every 2 days for 42 days.

Fatty acid analyses were done on samples obtained by removing seed from the capsule, slicing and refluxing in methanolic KOH (1%) for 3 hr. The mixture was filtered, acidified and the fatty acids were extracted with diethyl ether. This solution was dried over anhydrous Na₂SO₄ and the acids were methylated with freshly distilled diazomethane. Analyses were done on both Apiezon L and polyethylene glycol adipate columns with argon ionization detectors.

Incubation experiments. Seeds were removed from the green fleshy coat, cut down the middle and the embryos dissected out. Slices of endosperm were also used in some experiments. Normally three embryos or the equivalent of endosperm were suspended in 0.1 ml. of 0.2 M-phosphate buffer, pH 7.4, with 0.2 μ C of radioactive labelled precursor ([2-¹⁴C]acetate, [1-¹⁴C]octanoate, [1-¹⁴C]decanoate, [1-¹⁴C]dodecanoate, [1-¹⁴C]tetradecanoate, [1-¹⁴C]hexadecanoate, [1-¹⁴C]octadecanoate, [1-¹⁴C]oleate or [1-¹⁴C]linoleate). All acids were supplied by The Radiochemical Centre, Amersham, Bucks., and were checked for purity by radiochemical gas chromatography (James & Piper, 1962). The incubations were carried out in cotton-wool-stoppered 5 ml. conical flasks shaken in a water bath for 17–24 hr. at 20°. When N₂ was used as gas phase the tissue was equilibrated

* Present address: The Royal Free Hospital, Gray's Inn Road, London, W.C. 1.

in a stream of N_2 for 1 hr. before addition of labelled substrate.

Counting. The distribution of radioactivity among the fatty acids and their specific activity was determined by radiochemical gas chromatography as described by James & Piper (1962).

Degradations. Labelled ricinoleic acid was isolated from the mixed fatty acid methyl esters by chromatography on Mallinckrodt silicic acid. The column was equilibrated first with diethyl ether-acetone (1:1, v/v) and then with light petroleum (40–60°). The sample was applied to the column in 1 ml. of 10% diethyl ether in light petroleum (40–60°) and eluted first with 5×100 ml. portions of 10% diethyl ether in light petroleum and then with 50 ml. of diethyl ether. The portions were checked for composition on thin-layer chromatograms. The first 100 ml. contained sterols and methyl esters of the non-polar fatty acids. Ricinoleic acid was present in all the later fractions, which were bulked and further purified by thin-layer chromatography with light petroleum (40–60°)-diethyl ether-acetic acid (85:15:1, by vol.) used as solvent. After development, the plate was sprayed with 0.1% of dichlorofluorescein in aq. 50% ethanol and viewed under u.v. light. The ricinoleic acid zone was scraped off and the acid eluted with diethyl ether.

The ricinoleic acid was degraded by the reaction sequence summarized in Scheme 1. The sample was evaporated to dryness under N_2 , and reduced in the presence of Adams catalyst. After removal of the catalyst the solution of ester of 12-hydroxystearic acid was evaporated in a small tube by a stream of N_2 , 3 drops of thionyl chloride were added, the tube was sealed and heated at 80° for 1 hr. The sample was taken up in ether and evaporated under N_2 in a Quickfit test tube. A solution (0.5 ml.) of fresh 10% KOH in methanol was added, the tube was closed and the reaction mixture left for 1 hr. at room temperature. The solution was then acidified with 5N- H_2SO_4 and extracted with 3×5 ml. quantities of diethyl ether. The unsaturated acid so produced was then reduced in ethyl acetate with Adams catalyst to give stearic acid. The purity of the product at each stage was checked by gas-liquid chromatography. The stearic acid was then oxidized with 50 mg. of $KMnO_4$ in

0.1 ml. of dry acetone in a sealed tube for 4 hr. at 56°. The tube was opened, washed out with water, acidified with a few drops of 5N- H_2SO_4 and treated with potassium metabisulphite (disulphite) until colourless. The mixed fatty acids were extracted with ether, methylated with diazomethane and then separated on the radiochemical gas chromatogram by using an Apiezon L column.

RESULTS

The changes in fatty acid composition with maturation of the castor seed are summarized in Fig. 1. In the early stages no ricinoleic acid is produced and the major components are palmitic acid, stearic acid, oleic acid and linoleic acid. After 25–35 days, in different experiments, ricinoleic acid appears and is then synthesized very rapidly, so that by 38–42 days it comprises the major fatty acid (approx. 85%) of a vastly greater mass of triglyceride.

The changes in rate of synthesis of the different fatty acids during 24 hr. incubation at the varying stages of development of the seed are shown in Table 1. In the early stage no ricinoleic acid is synthesized, the tissue concentrating first of all on palmitic acid, palmitoleic acid, oleic acid and stearic acid. Linoleic acid is not synthesized rapidly at this stage, although it is present in relatively large amounts (Fig. 2). The result given in Fig. 2 differs from that given in Table 1 in that palmitoleic acid does not contain such a large proportion of the total label.

Scheme 1. Degradation of biosynthesized radioactive ricinoleic acid.

Fig. 1. Variation of composition of castor seed fatty acids with time from flowering. x, Ricinoleic acid; ●, linoleic acid; ■, oleic acid; ○, palmitic acid.

Incubation of [$1-^{14}\text{C}$]linoleic acid with the seed embryos gave no indication of introduction of label into ricinoleic acid (Fig. 4), although similar

Table 1. *Distribution of labelling (as percentage of total labelling in all fatty acids) among fatty acids after 24 hr. incubation of tissue with [$2-^{14}\text{C}$]acetate*

Fatty acid	Time after flowering (days).....	Radioactivity (% of total)			
		26	34	38	41
C_{14}		1.0	0.9	0.8	0.2
C_{16}		38.0	37.1	32.1	8.6
C_{18}		2.3	6.0	5.3	1.2
Palmitoleic		28.6	19.6	16.4	22.2
Oleic		9.4	7.9	13.3	16.1
Linoleic		0	3.7	5.1	10.1
Ricinoleic		0	2.5	3.8	32.5

Fig. 2. Gas-liquid radiochemical chromatogram of the fatty acids synthesized by castor bean embryo from [$2-^{14}\text{C}$]acetate before the appearance of ricinoleic acid. (a) After 10 days from flowering; (b) after 20 days from flowering. Peak identification: 1, lauric acid; 2, unknown; 3, myristic acid; 4, palmitoleic acid; 5, palmitic acid; 6, unknown; 7, linoleic acid; 8, oleic acid; 9, stearic acid. Column: 4 ft., Apiezon L at 200° .

embryos readily synthesized ricinoleic acid from acetate (Fig. 3). Seeds taken from the same bunch on the same plant at the same time were able to convert [$1-^{14}\text{C}$]oleic acid into ricinoleic acid (Fig. 5). In different experiments the yield varied from 12 to 54%.

Separation of the fatty acid mixture produced from the stearic acid derived from ricinoleic acid synthesized in this way showed only the stearic acid to be labelled. Removal of the carboxyl group by α -oxidation removed the ^{14}C present in the molecule. Clearly the oleic acid was converted directly into ricinoleic acid without any breakdown and resynthesis and hence randomization of label.

A study of the effectiveness of various fatty acids as precursors of ricinoleic acid shown previously to be precursors of oleic acid (James, 1963a) in the plant gave the results shown in Table 2.

Investigation of the requirement of the enzyme system for oxygen showed that incubation of ricinoleic acid-synthesizing tissue with [$1-^{14}\text{C}$]oleic acid in a nitrogen atmosphere gave as good a synthesis as that in air. Thus the ricinoleic acid synthesized by the seed embryos from [$1-^{14}\text{C}$]oleic acid in air had relative specific activity 0.51 (count area/peak area); if the synthesis occurred in nitrogen instead of air, other conditions being similar, the relative specific activity was 0.68. Repetition of this experiment has failed to disclose any requirement for molecular oxygen.

Comparison of the relative efficiency of endosperm and embryo for the synthesis of ricinoleic acid indicated that both tissues were effective but that the embryo itself was responsible for the major part of the synthesis.

DISCUSSION

The results of study of the variation of fatty acid composition of the seed with time disclosed that ricinoleic acid was absent at the beginning. Only after a lapse of 26–34 days did the hydroxy acid appear (Fig. 1). From this stage on the rate of

Fig. 3. Gas-liquid radiochemical chromatogram of the fatty acids synthesized by the castor bean embryo from [$2-^{14}\text{C}$]acetate when ricinoleic acid production is occurring. Peak identification: 1, palmitoleic acid; 2, palmitic acid; 3, oleic acid; 4, stearic acid; 5, ricinoleic acid. Column: 4 ft., Apiezon L at 200° .

Fig. 4. Gas-liquid radiochemical chromatogram of the result of incubating [1-¹⁴C]linoleic acid with castor bean embryo. (a) Mass record of peaks; (b) radioactive record of peaks. Peak identification: 1, palmitoleic acid; 2, palmitic acid; 3, linoleic acid; 4, oleic acid; 5, stearic acid; 5a, unknown; 6, ricinoleic acid.

Fig. 5. Gas-liquid radiochemical chromatogram of the result of incubating [1-¹⁴C]oleic acid with castor bean embryo. (a) Mass record of peaks; (b) radioactive record of peaks. Peak identification: 1, palmitoleic acid; 2, palmitic acid; 3, linoleic acid; 4, oleic acid; 5, stearic acid; 6, ricinoleic acid.

Table 2. *Effectiveness of precursors of the unsaturated acids in immature Ricinus seeds at the stage of active ricinoleic acid synthesis*

Precursor	Acid labelled.....	Saturated				Unsaturated			Ricinoleic acid
		C ₁₂	C ₁₄	C ₁₆	C ₁₈	C _{16:1}	C _{18:1}	C _{18:2}	
[2- ¹⁴ C]Acetate		+	+	+	+	+	+	+	+
[1- ¹⁴ C]Octanoate		+	+	+	+	+	+	+	+
[1- ¹⁴ C]Decanoate		+	+	+	+	+	+	+	+
[1- ¹⁴ C]Dodecanoate		+	+	+	+	+	+	+	+
[1- ¹⁴ C]Tetradecanoate		+	+	+	+	—	+	+	+
[1- ¹⁴ C]Hexadecanoate		—	—	+	+	—	—	—	—
[1- ¹⁴ C]Octadecanoate		—	—	—	+	—	—	—	—

fatty acid synthesis increased very rapidly. A similar result has been described by Canvin (1963). He reported a lapse of 12 days between fertilization and appearance of ricinoleic acid but in his case manual pollination was used whereas we were dependent on random pollination. This could account for the shorter period. Either the enzyme system producing ricinoleic acid is absent in the early period or it is present in some latent form that is activated at a later stage.

The pattern of fatty acid synthesis varies even during the period in which ricinoleic acid is absent.

In the first few days (Fig. 2a) the acids produced most rapidly (as indicated by the degree of labelling from [2-¹⁴C]acetate) are palmitoleic acid, palmitic acid, stearic acid and oleic acid. Just before the first appearance of the hydroxy acid the synthesis of palmitoleic acid decreases and that of linoleic acid increases (Fig. 2b). The figures given for a similar experiment in Table 1 show a similar pattern except for a smaller decrease in the rate of labelling of palmitoleic acid. Even when ricinoleic acid is being produced, the other fatty acids are still being synthesized. The higher rate of labelling of

linoleic acid that takes place when ricinoleic acid synthesis begins could suggest an interconversion of the two acids. A similar hydration of oleic acid to 10-hydroxystearic acid by a pseudomonad has been demonstrated by Wallen, Benedict & Jackson (1962). In the present experiment, this hypothesis was disproved by incubating embryos with either [2-¹⁴C]acetate or [1-¹⁴C]linoleic acid. Whereas the high rate of incorporation of [2-¹⁴C]acetate (Fig. 3) showed that ricinoleic acid was being produced in the former case, no radioactivity could be found in the ricinoleic acid from the latter experiment (Fig. 4).

The only effective shorter-chain precursors of ricinoleic acid were the C₂, C₈, C₁₀, C₁₂ and C₁₄ acids with the C₁₆ and C₁₈ saturated acids ineffective (Table 2). This pattern is the same as that found for precursors of oleic acid in plant leaves (James, 1963a). [1-¹⁴C]Oleic acid when tested as a precursor gave good yields of labelled ricinoleic acid (Fig. 5), ranging in different experiments from 12 to 54%. That this was a true conversion and not degradation followed by resynthesis was shown by isolation and degradation of the ricinoleic acid. Oleic acid is thus firmly established as the direct precursor of ricinoleic acid.

The requirement for molecular oxygen in the conversion of oleic acid into linoleic acid in yeast (Yuan & Bloch, 1961) suggests that ricinoleic acid is a possible intermediate. Investigation of the gaseous requirements for the oxidation of oleic acid to ricinoleic acid showed that replacement of air by nitrogen did not alter the conversion. The conditions used were such that, in a parallel incuba-

tion of a leaf system, formation of oleic acid was suppressed (James, 1963a). This rules out molecular oxygen as a cofactor. In a recent paper on a cell-free castor seed system (Yamada & Stumpf, 1964) oxygen is given as a cofactor though no experiment without oxygen is mentioned. Yamada & Stumpf's results confirm ours in that oleic acid is shown to be a direct precursor of ricinoleic acid; they also implicate CoA and NADPH as cofactors. Except for oxygen requirement the system is similar to the conversion of oleic acid into linoleic acid (R. V. Harris & A. T. James, unpublished work). Since oxygen would be assumed to be required in this system to form a hydroxy intermediate, it now seems unlikely that ricinoleic acid could be such an intermediate.

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REFERENCES

- Canvin, D. T. (1963). *Canad. J. Biochem. Physiol.* **41**, 1879.
Coppens, N. (1956). *Nature, Lond.*, **177**, 279.
James, A. T. (1962). *Bull. Soc. Chim. biol., Paris*, **44**, 951.
James, A. T. (1963a). *Biochim. biophys. Acta*, **70**, 9.
James, A. T. (1963b). *Symp. biochem. Soc.* **24**, 17.
James, A. T. & Piper, E. A. (1962). *Analyt. Chem.* **35**, 515.
Wallen, L. L., Benedict, R. G. & Jackson, R. W. (1962). *Arch. Biochem. Biophys.* **99**, 249.
Yamada, M. & Stumpf, P. K. (1964). *Biochem. biophys. Res. Commun.* **14**, 165.
Yuan, C. & Bloch, K. (1961). *J. biol. Chem.* **236**, 1277.