THE MECHANISM OF RICINOLEIC ACID BIOSYNTHESIS IN RICINUS COMMUNIS SEEDS

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Ricinoleic acid (D-12-hydroxyoleic acid) accounts for about 90% of the triglyceride fatty acids of castor oil, the seed oil of the species Ricinus communis L., and up to about 40% of the glyceride fatty acids of ergot oil, the lipid produced by the parasitic fungus Claviceps purpurea. It has been shown by James (1962, 1963, 1965), by Yamada and Stumpf (1964) and by Canvin (1965) that, in the developing castor bean, the direct precursor of ricinoleic acid is oleic acid and that oxygen and NADH are obligatory cofactors. In Claviceps purpurea, however, we have shown that linoleic acid is the precursor of ricinoleic acid and, in this system, oxygen is not required as a cofactor (Morris et al., 1966).

At first sight, therefore, it appears that ricinoleate is produced by hydroxyl substitution of oleate in the castor bean but by hydration of linoleate in Claviceps. In a recent paper, Galliard and Stumpf (1966) have shown that a microsomal fraction of immature castor beans catalyses the conversion of oleyl-S-CoA to ricinoleate. Because neither linoleic acid nor linoleyl-S-CoA was converted to ricinoleate they concluded that a mechanism involving hydration of a double bond was thereby ruled out and that the mechanism was proved to be a hydroxyl substitution. However, the possibility remained that, although the substrate was oleyl-S-CoA, the mechanism was in fact hydration of a double bond or a double bond precursor which was enzyme bound and therefore not exchangeable with a pool of exogenous linoleic acid or linoleyl-S-CoA.

This communication describes the elucidation of the mechanism of ricinoleate biosynthesis in the castor bean by determining how many and which of the hydrogens of the 12- and 13-positions of oleate are removed during its conversion to ricinoleate. Use of racemic erythro-12,13-ditritio-oleic acid as substrate showed that only one hydrogen from the 12-position is removed during this conversion and incubations with D- and with L-12-tritio-oleic acid demonstrated that the hydrogen lost is of the D configuration. The mechanism of ricinoleic acid biosynthesis is thus proved to be by hydroxyl substitution at the 12-position of oleic acid with retention of configuration at that position.

PREPARATION OF SUBSTRATES

erythro-12,13-Ditritio-oleic acid was produced by partial reduction of linoleic acid with tritiated diimide. The partial reduction was carried out by Dr. G.K. Koch, Unilever Research Laboratorium, Vlaardingen, The Netherlands, and, after esterification, the erythro-12,13-ditritio-oleate was separated from its positional isomer, erythro-9,10-ditritio-12-octadecenoate, and from unreduced linoleate and totally reduced stearate by a low temperature argentation thin-layer chromatography (TLC) procedure (Morris et al., 1967b). The product was at least 99% pure chemically and radiochemically, as judged by TLC and GLC, and after hydrolysis was mixed with 1-^{14}C-oleic acid to give a $^{3}H/^{14}C$ ratio of 12.8.

D- and L-12-Tritio-oleic acids were obtained by biochemical desaturation of the synthetic, stereospecifically labelled stearic acids which were prepared from D-12-hydroxystearic acid essentially by the procedures described by Schroepfer and Bloch (1965) for the preparation of D- and L-9-tritiostearic acids. The purified tritiated stearic acids, mixed with suitable quantities of 1-^{14}C-stearic acid, were applied as substrates for desaturation by Chlorella vulgaris as described by Harris et al. (1965a,b) and Morris et al. (1967a), and the D- and L-12-tritio-oleic acid products were isolated and purified.

INCUBATIONS AND ANALYSIS OF PRODUCTS

Immature castor bean endosperm slices were incubated aerobically under subdued light in 0.2 M phosphate buffer (pH 7.4) in which the labelled precursor had previously been dispersed by sonication. After six hours, the incubations were stopped by the addition of $CHCl_3$-MeOH (2:1) and fatty acid methyl esters were produced from the lipid extracts by transmethylation (Nichols et al., 1965).

Methyl ricinoleate was isolated from the products by TLC and purified by preparative GLC. The ricinoleate samples from D- and L-12-tritio-oleates and a portion of the product from the ditritio-oleate were counted in toluene containing 0.4% 2,5-diphenyloxazole on a Packard Tricarb Series-4000 Liquid Scintillation Spectrometer and the $^3H/^{14}C$ ratios were calculated. The remaining ricinoleate from the ditritio-oleate substrate was oxidised with 10% chromium trioxide in acetic acid to 12-keto-oleate which was purified by TLC and a portion counted (keto-oleate I - Table I). The remainder of this keto-oleate was taken again through the whole oxidation (although this time CrO_3 was omitted from the acetic acid), extraction and TLC purification procedure and counted (keto-oleate II - Table I). This last sequence was to determine if, after formation of the 13-tritio-12-keto-oleate, there was any appreciable loss of tritium by keto-enol tautomerism and exchange from the enol form.

RESULTS AND DISCUSSION

It was considered that the formation of ricinoleate from oleate must occur by one of three types of mechanism, which could be distinguished as follows:

(a) In the rather unlikely event of a 12-keto intermediate being involved, then both hydrogens of the 12-position or 50% of the tritium would be lost in its formation (i.e. both D- and L-12-^3H, from the D-12, D-13 and the L-12, L-13-ditritio-enantiomers of the racemic substrate, respectively). However, chemical oxidation of the ricinoleate product to 12-keto-oleate would cause

no further loss.

(b) If the 12,13-double bond of linoleate, either free or enzyme bound, were formed and hydrated then one of the hydrogens on both C_{12} and C_{13} or 50% of the tritium would be lost. Oxidation to keto-oleate would remove the tritium remaining on C_{12}; i.e. loss of a further 25% tritium to a $^{3}H/^{14}C$ ratio for the keto-oleate of only 25% of the substrate ratio.

(c) If the mechanism involved hydroxyl substitution then only one hydrogen from the 12-position or 25% of the tritium would be lost; either D- or L-12-^{3}H, depending on whether substitution were with retention or inversion of configuration. Oxidation to keto-oleate would remove the remaining tritium on C_{12}; i.e. loss of a further 25% of the original tritium to give a $^{3}H/^{14}C$ ratio for the keto-oleate of 50% of the substrate ratio.

TABLE I. $^{3}H/^{14}C$ Ratios of 1-^{14}C-erythro-12-^{3}H, 13-^{3}H-oleic acid substrate and of the products of its incubation with immature castor bean endosperm.

	$^{3}H/^{14}C$ Ratio	% of substrate ratio
Oleate (Substrate)	12.8	100
Linoleate	6.4	50
Ricinoleate	9.6	75
12-Keto-oleate I	6.9	55
12-Keto-oleate II	6.9	55

The results of incubations with the racemic erythro-ditritio-oleic acid substrate which are summarised in Table I rule out mechanisms (a) and (b) and show that ricinoleic acid is formed by hydroxyl substitution of oleic acid (or by an oxygen insertion mechanism which, because of the lability of an $-O^{3}H$ group, would give the same results). These results are validated to some extent by the finding (Table I) that exactly 50% of the tritium is lost, as required, in the concurrent formation of linoleic acid by the endosperm system.

The results of incubations with the stereospecifically mono-tritiated oleate substrates are summarised in Table II.

TABLE II. $^{3}H/^{14}C$ Ratios of 1-^{14}C-D- and -L-12-^{3}H-oleic acid substrates and of the products of their incubation with immature castor bean endosperm.

Substrate		Linoleate	Ricinoleate
D	11.6	0.7	1.2
L	16.2	14.0	15.4

These results show firstly that the stereochemistry at the 12-position in linoleate formation by the castor bean is the same as in Chlorella vulgaris (Morris et al., 1967a); i.e. removal of the D-12-hydrogen label and presumably, by analogy, removal of the D-13-hydrogen also. Secondly the results show almost complete retention of the tritium label in ricinoleate derived from L-12-tritio-oleate but almost complete loss of label from the D-12-tritio-oleate. This proves that the hydroxylation of oleic acid by castor bean endosperm to give ricinoleic acid proceeds with overall retention of configuration at the 12-position. This stereochemical conclusion has been arrived at independently by Schroepfer, Galliard and Stumpf (private communication; see Galliard and Stumpf, 1966).

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