Experiments with [9,10,12,13-$^2H_4$]Linoleic Acid on the Formation of 9-[Nona-(1′*E*),(3′*Z*)-dienyloxy]non-(8*E*)-enoic (Colneleic) Acid and (13*R*)-Hydroxy-12-oxo-octadec-(9*Z*)-enoic Acid by Plant Enzymes

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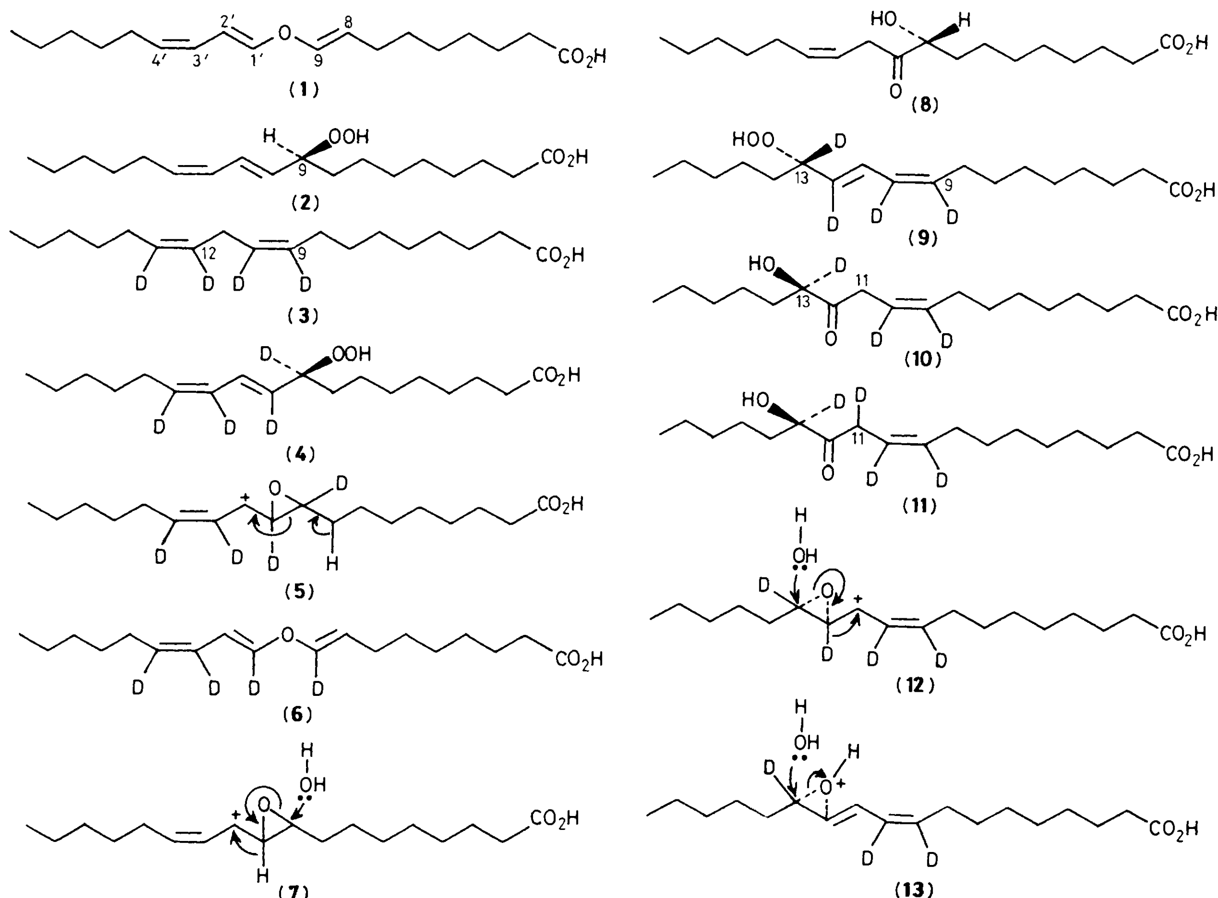
The fate of four olefinic deuterium atoms in labelled linoleic acid is followed into colneleic acid and an α-hydroxy ketone, both enzymically produced, and is consistent with proposed mechanisms of formation for these products.

In the preceding communication[1] the origin of the divinyl ether oxygen of colneleic acid (**1**) was shown by ^{18}O labelling to be oxygen gas *via* [(9*S*)-$^{18}O_2$]hydroperoxyoctadeca-(10*E*),(12*Z*)-dienoic acid (**2**) derived from linoleic acid. We now follow the fate of the olefinic hydrogens of (**2**) using [9,10,12,13-$^2H_4$]linoleic acid (**3**). The latter was made by coupling the Grignard of dec-9-ynoic acid with oct-2-ynyl bromide in the presence of copper(I) cyanide.[2] The olefinic deuterium atoms were placed by *cis*-addition using deuteriated di-isoamylborane with a deuterioacetic acid work-up.[3]

[9,10,12,13-$^2H_4$]Octadeca-(9*Z*),(12*Z*)-dienoic acid (**3**) (50 mg) was converted into [9,10,12,13-$^2H_4$]-(9*S*)-hydroperoxy-octadeca-(10*E*),(12*Z*)-dienoic acid (**4**) using the lipoxygenase described earlier.[1] The hydroperoxide (10 mg) was similarly converted into deuteriocolneleic acid and purified as the methyl ester by h.p.l.c. Mass spectral analysis showed that four deuterium atoms were retained in the product {M^+, 308 + 4; $M^+ - C_4H_9$, 251 + 4; $M^+ - ODC{=}CH{\cdot}[CH_2]_6CO_2Me$, 123 + 3 *etc.*}. Comparison of the ^1H n.m.r. spectrum with that of authentic methyl colneleate showed absence of the following signals: δ 6.52 (1H, d, *J* 11.7 Hz; 1′-H), 6.27 (1H, dd, *J* 12.1, 1.1 Hz; 9-H), 5.85 (1H, ddt, *J* 11.7, 10.5, 1.5 Hz; 3′-H), and 5.30 (1H, dt, *J* 10.5, 7.4 Hz; 4′-H). The dt at 6.01 (2′-H) had become a broadened singlet and the dt at 5.51 (8-H) had the appearance of a broadened triplet. Isotopic positions are thus as assigned in (**6**) and are in agreement with the formation sequence (**4**) → (**5**) → (**6**).

As mentioned in the preceding communication,[1] intermediate (**5**) can be trapped by attack at C-11 by water as the nucleophile to give an epoxy alcohol. It can also be decomposed by S_N2 reaction at C-9 with inversion and 1,2-hydrogen shift (**7**) giving the α-hydroxyketone (**8**). Such a flax-seed enzyme catalysed reaction,[4] discovered by Zimmerman,[5] is much more rapid for the (13*S*)-hydroperoxide of linoleic acid [*cf.* (**9**)] than for the (9*S*)-hydroperoxide.[6] Flax-seed acetone powder contains both the necessary (13*S*)-lipoxygenase and its isomerase, and using (**3**) we have followed the fate of olefinic deuterium labelling in formation of the 13-hydroxy-12-ketone [*cf.* (**10**)].

Flax-seed acetone powder (2 g) in sodium phosphate buffer (pH 7.0, 20 ml) was stirred with ice-cooling (45 min) and the



mixture centrifuged (12 000 g, 3 °C, 15 min). Supernatant (10 ml) was added to [9,10,12,13-$^2H_4$]linoleic acid (50 mg) in borate buffer (pH 9.0, 20 ml) and Tween 20, and then diluted with sodium phosphate buffer (pH 7.0, 400 ml) and the mixture stirred (20 °C, 90 min). Chloroform (200 ml) and methanol (100 ml) were added and the mixture was acidified to pH 3.0 with 1 M citric acid. Work-up under nitrogen and esterification gave methyl [9,10,13-$^2H_3$]-(13*R*)-hydroxy-12-oxo-(9*Z*)-octadecenoate (**10**) (19 mg) purified by thick-layer chromatography on silica gel, developing three times with light petroleum (b.p. 60—80 °C)–diethyl ether (3:1). The band of R_F 0.43, located by strip-spraying with 2,4-dinitrophenylhydrazine reagent, was collected and finally purified by h.p.l.c. Mass spectral analysis showed the product to contain three, not four, deuterium atoms {M^+ absent; M^+ – OMe, 295 + 3, M^+ – OMe – H_2O, 277 + 3; M^+ – OMe – C_5H_{11}, 227 + 3; M^+ – CO_2Me – Me[CH_2]$_2$CH=CH_2, 195 + 3; M^+ – OMe – Me[CH_2]$_4$CD(OH)CO, 166 + 2 *etc.*}. Comparison of the ^{1}H n.m.r. spectrum of authentic undeuteriated hydroxy ketone [*cf.* (**10**)] showed that these were in the expected 9,10, and 13 positions of (**10**) but the deuterium atom expected to be found at C-11 [see (**11**)] was absent. Undeuteriated linoleic acid (12 mg) was then taken through the whole enzyme process except that all reagents were made up in 99.8 atom% D_2O (salts were not H/D exchanged before use). ^{1}H N.m.r. analysis of the hydroxyketone product showed that 1.1 atoms of deuterium had been introduced at C-11 but with no exchange at any other carbon atoms. It is thus possible that the missing fourth deuterium atom has moved to C-11 in the original experiment [see (**11**)] and has then been lost by exchange. Earlier work had demonstrated that the 12-ketone in (**10**) (without deuterium) contains ^{18}O arising from $^{18}O_2$ gas *via* [13-$^{18}O_2$]hydroperoxide [*cf.* (**9**)], whilst the hydroxy centre arises from water with inversion at the peroxidic C-13 centre.[7,8] The present work thus shows that the pattern of deuterium labelling is consistent with the mechanism depicted in (**12**), a proposal originally made by Gardner and his colleagues.[9] The absence of deuterium at C-9 could however be inherent in the mechanism, rather than a consequence of exchange, if the C-12 deuteron is first lost from (**12**) giving (**13**) which is then attacked by water with inversion at C-13 as shown.

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