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The Stereochemistry of Desaturations of Long-Chain Fatty Acids in *Chlorella vulgaris*

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1. A study was made of the stereospecificity of hydrogen removal in the sequential desaturations performed by intact cells of *Chlorella vulgaris* in the biosynthesis of oleic acid, linoleic acid and α-linolenic acid. 2. By use of *erythro*- and *threo*-9,10-$^2H_2$-, -12,13-$^2H_2$- and -15,16-$^2H_2$-labelled precursors, it was demonstrated that the pair of hydrogen atoms removed from each of these positions had the *cis* relative configuration. 3. That the hydrogen atoms removed in oleic acid and linoleic acid formation were of the D absolute configuration was proved by use of D- and L-9-^3H- and -12-^3H-labelled precursors. 4. The presence of a substantial kinetic isotope effect of deuterium at both positions of the putative double bond was indicated, suggesting that the mechanism of desaturation involves simultaneous concerted removal of the pair of hydrogen atoms.

The major unsaturated fatty acid constituents of photosynthetic tissue in both lower and higher plants are the family of *cis*-unsaturated C_{18} acids: oleic (octadec-9-enoic) acid, linoleic (octadeca-9,12-dienoic) acid and α-linolenic (octadeca-9,12,15-trienoic) acid (for a review see Nichols & James, 1968). Research in this Laboratory (Harris & James, 1965; Harris, Harris & James, 1965; Harris, James & Harris, 1967) demonstrated that these acids are produced from stearic (octadecanoic) acid by a series of sequential desaturations. The generally high positional and geometric specificity of such fatty acid desaturations prompted studies on the stereoselectivity of the hydrogen removal. Schroepfer & Bloch (1965) demonstrated that the conversion of stearate into oleate by *Corynebacterium diphtheriae* involves the specific loss of the D-9- and D-10-hydrogen atoms from the stearic acid molecule. The results of the present studies of stereoselectivity of hydrogen removal in the biosynthesis of oleic acid, linoleic acid and α-linolenic acid by *Chlorella vulgaris*, which have been described briefly (Morris, Harris, Kelly & James, 1967*a*), corroborate and extend the observations of Schroepfer & Bloch (1965).

MATERIALS AND METHODS

Radioactively labelled compounds were obtained from The Radiochemical Centre, Amersham, Bucks., and ^2H-labelled chemicals from Isotopes Inc., Westwood, N.J., U.S.A., and from Fluka A.-G., Buchs, Switzerland.

^2H-labelled precursors. The reduction of olefins with ^2H-labelled hydrazine has been shown to occur stereospecifically with *cis*-addition of deuterium (Corey, Pasto & Mock, 1961). Thus a *cis*-olefin gives a racemic *erythro*-$^2H_2$-labelled product and a *trans*-olefin gives a racemic *threo*-$^2H_2$-labelled product.

Reduction of oleic acid and elaidic acid (*cis*- and *trans*-octadec-9-enoic acid) with ^2H-labelled hydrazine hydrate ($N_2{}^2H_4,{}^2H_2O$; 98%) in ^2H-labelled methanol ($CH_3{\cdot}O^2H$; 99%), at 50° with good aeration, gave [*erythro*-9,10-$^2H_2$]- and [*threo*-9,10-$^2H_2$]-stearic acid respectively. The products were esterified and the labelled stearates were purified from unreduced olefinic ester by preparative thin-layer chromatography on $AgNO_3$-impregnated silica gel (Morris, 1962). As judged by gas–liquid chromatography, the ^2H-labelled products were chemically at least 99% pure.

Linoleic acid, when partially reduced with ^2H-labelled hydrazine in the same way, gave approx. 40% yield of the mixed partial reduction products, *cis*-[*erythro*-9,10-$^2H_2$]-octadec-12-enoate and *cis*-[*erythro*-12,13-$^2H_2$]octadec-9-enoate. The latter compound was separated from its positional isomer and also from the unreduced and totally reduced components of the esterified reaction mixture by preparative thin-layer chromatography on $AgNO_3$-impregnated silica gel at low temperature, as described by Morris, Wharry & Hammond (1967*b*). [*threo*-12,13-$^2H_2$]Oleate was similarly prepared by partial reduction of a mixture of *cis–trans*- and *trans–cis*-octadeca-9,12-dienoate. This mixture had been isolated by thin-layer chromatography on $AgNO_3$-impregnated silica gel from the products of isomerization of *cis–cis*-linoleate catalysed by oxides of nitrogen in diglyme solution, by a scaled-down modification of the procedure of Litchfield, Harlow, Isbell & Reiser (1965). The *cis*-[*threo*-12,13-$^2H_2$]octadec-9-enoate was again isolated by low-temperature thin-layer chromatography on $AgNO_3$-impregnated silica gel, this time from three other isomeric $^2H_2$-labelled monoenes as well as unreduced and completely reduced compounds. An illustration of this separation has been published (Morris *et al.* 1967*b*). The

[*erythro*-12,13-$^2H_2$]- and [*threo*-12,13-$^2H_2$]-oleates so obtained were again of at least 99% chemical purity as judged by gas–liquid chromatography and thin-layer chromatography on $AgNO_3$-impregnated silica gel.

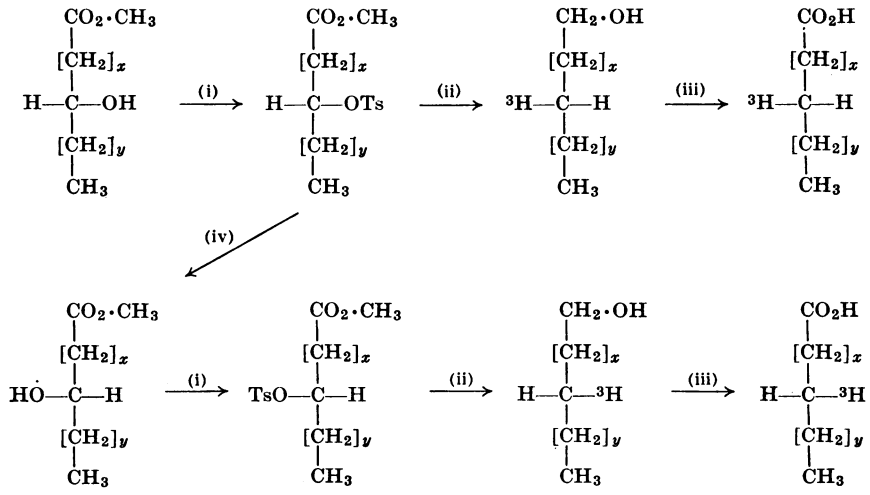
The other pair of $^2H_2$-labelled precursors, [*erythro*-15,16-$^2H_2$]- and [*threo*-15,16-$^2H_2$]-oleates, were similarly prepared by partial reduction with ^2H-labelled hydrazine of *cis–cis*-octadeca-9,15-dienoic acid and of a mixture of *cis–trans*- and *trans–cis*-octadeca-9,15-dienoic acid. The *cis–cis*-octadeca-9,15-dienoate was isolated from the products of partial reduction of α-linolenic acid with unlabelled hydrazine by a modification of the procedure of Scholfield, Jones, Nowakowska, Selke & Dutton (1961), with thin-layer chromatography on $AgNO_3$-impregnated silica gel for the final isolation step. The *cis–trans*- and *trans–cis*-9,15-diene mixture was produced by elaidinization, as before. Isolation of the required [$^2H_2$]oleates from the products of partial reduction of these 9,15-dienes was again effected by low-temperature thin-layer chromatography on $AgNO_3$-impregnated silica gel. The separation of octadec-9-enoate and octadec-15-enoate isomers, however, is not as complete as that of octadec-9-enoate and octadec-12-enoate isomers (for illustrations see Morris *et al.* 1967*b*), so that the chemical purity of the [*erythro*-15,16-$^2H_2$]- and [*threo*-15,16-$^2H_2$]-oleates was only about 95%.

Table 1. *Isotopic composition of synthetic* $^2H_2$*-labelled fatty acids*

$^2H_2$-labelled acid	Composition (moles/100 moles) $^2H_0$	$^2H_1$	$^2H_2$
[*erythro*-9,10-$^2H_2$]$C_{18:0}$ acid	3·5	18·2	78·3
[*threo*-9,10-$^2H_2$]$C_{18:0}$ acid	3·4	19·8	76·8
[*erythro*-12,13-$^2H_2$]$C_{18:1}$ acid	4·7	13·1	82·2
[*threo*-12,13-$^2H_2$]$C_{18:1}$ acid	3·6	13·4	83·0
[*erythro*-15,16-$^2H_2$]$C_{18:1}$ acid	6·8	11·2	82·0
[*threo*-15,16-$^2H_2$]$C_{18:1}$ acid	6·2	13·1	80·7

The isotopic purity of each of these $^2H_2$-labelled precursors was determined by mass spectrometry; each compound consisted of about 80% of $^2H_2$-labelled species and 15% of $^2H_1$-labelled species. The actual values are listed in Table 1. Appropriate corrections for the $^2H_1$-labelled species in the substrates were made in calculating the results from the incubations. All these products, purified as methyl esters, were hydrolysed to the free acids before being used as substrates for incubations.

^3H-labelled precursors. Stearic acids stereospecifically labelled with ^3H in the 9- or 12-position were synthesized from D-9-hydroxystearic acid, derived from the *cis*-D-9-hydroxyoctadec-12-enoic acid of *Strophanthus kombe* seed oil (Baker & Gunstone, 1963), and from D-12-hydroxystearic acid, derived from the ricinoleic acid of castor oil (Serck-Hanssen, 1958), essentially by the methods described by Schroepfer & Bloch (1965). The reactions are summarized in Scheme 1. Thus the toluene-*p*-sulphonate of D-9-hydroxystearate gave [L-9-^3H]stearyl alcohol on hydrogenolysis with ^3H-labelled $LiBH_4$ in refluxing tetrahydrofuran, and oxidation of this product with chromium trioxide in acetic acid gave [L-9-^3H]stearic acid. Treatment of the toluene-*p*-sulphonate of D-9-hydroxystearic acid with anhydrous sodium acetate in acetic acid, followed by hydrolysis, gave L-9-hydroxystearic acid, which, on toluene-*p*-sulphonylation, hydrogenolysis and oxidation in the same way, furnished [D-9-^3H]stearic acid. The [L-12-^3H]- and [D-12-^3H]stearic acids were prepared similarly from D-12-hydroxystearic acid.



Scheme 1. Summary of reactions used in the synthesis of stereospecifically ^3H-labelled stearic acids: (i) toluene-*p*-sulphonylation with toluene-*p*-sulphonyl chloride in pyridine (Ts, toluene-*p*-sulphonyl); (ii) hydrogenolysis with ^3H-labelled lithium borohydride in tetrahydrofuran; (iii) oxidation with chromium trioxide in acetic acid; (iv) acetolysis with anhydrous sodium acetate in acetic acid, followed by hydrolysis and re-esterification. $x=7$ and $y=8$, or $x=10$ and $y=5$.

To each of these four stereospecifically ^3H-labelled stearic acids, which had specific radioactivities of approx. 15 μc/mg., was added sufficient [1-^{14}C]stearic acid to give a $^3H/^{14}C$ ratio of roughly 20:1.

Incubations. *Chlorella vulgaris* cultures (strain 211/11b) were maintained and grown up as previously described (Harris & James, 1965; Harris *et al.* 1965). The cells were harvested by centrifugation and were washed and resuspended in 0·2 M-potassium phosphate buffer, pH 7·4. For the experiments on α-linolenic acid biosynthesis the cultures were grown up in the dark; in other cases light-grown cells were used.

Before the experiments with the ^2H-labelled precursors, preliminary incubations were carried out with carrier-diluted ^{14}C-labelled substrates to ascertain the quantity of cells required to effect sufficient conversion of the added substrate to be significant relative to the endogenous oleate, linoleate or α-linolenate. An abundance of at least 2% of ^2H-labelled species in the total isolated product is desirable for reasonably accurate mass-spectrometric analysis. From the experiments with the diluted ^{14}C-labelled stearic acid and oleic acid the actual mass of direct desaturation product produced by a given quantity of cells could be determined. By use of an added known amount of eicosanoate as an internal standard for gas–liquid-chromatographic analysis, the total mass of the product isolated from that quantity of cells after incubation could also be computed. It was found that the cells contained in 10 ml. of a suspension in phosphate buffer with E_{750} 1·75, when incubated with 100–200 μg. of ^2H-labelled precursor, would provide rather more than the desired 2% enrichment of ^2H-labelled species in the product. Therefore, for the actual incubations, 10 ml. of such a suspension was centrifuged and the cells were resuspended in phosphate buffer (3 ml.), in which the ^2H-labelled precursor acid (100 or 200 μg.) had been dispersed by ultrasonic treatment.

For incubations with the ^3H-labelled precursors the above considerations on the extent of product enrichment, of course, do not apply and normal thick suspensions of *Chlorella vulgaris* cells were used.

Incubations were carried out with shaking in the light at 25° for 5 hr. (oleate and linoleate biosynthesis) or for 24 hr. (α-linolenate biosynthesis) and were then stopped and the lipids extracted by the addition of chloroform–methanol (2:1, v/v). Fatty acid methyl esters were produced from the lipid extracts by transmethylation, as described by Nichols, Harris & James (1965).

Isolation and analysis of products. The unsaturated methyl ester product, oleate, linoleate or α-linolenate, depending on the labelled precursor being studied, was first separated from other methyl esters differing in degree of unsaturation by preparative thin-layer chromatography on $AgNO_3$-impregnated silica gel. This step was repeated on each isolated product to ensure complete removal of stearate from the oleate, of oleate from the linoleate, or of linoleate from the linolenate. Final purification and removal of any homologues was accomplished by preparative gas–liquid chromatography. The purified ester products, which were all of higher than 99% purity as judged by gas–liquid chromatography, were then examined either by mass spectrometry or by liquid-scintillation counting, depending on the isotopic label present.

The mass spectra of the products from ^2H-labelled substrates were obtained with an AEI MS12 instrument, by using the direct-insertion sample-probe technique. Ten scans in each direction of the parent-molecular-ion region of each product were recorded, the intensities of the $^2H_0$, $^2H_1$ and $^2H_2$ parent-molecular-ion peaks were measured, and the proportions of these three species were calculated.

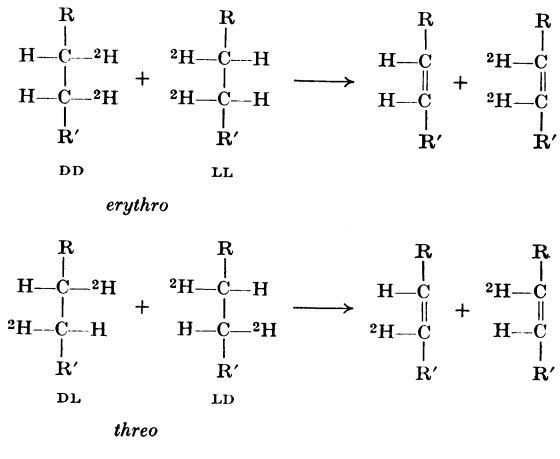
The products from ^3H-labelled precursors were counted in 0·4% 2,5-diphenyloxazole in toluene solution in a Packard Tri-Carb series 4000 liquid-scintillation spectrometer, and $^3H/^{14}C$ ratios were calculated.

RESULTS

Scheme 2 illustrates the products to be expected from stereospecific loss of *cis*-hydrogen atoms from racemic *erythro*-$^2H_2$- and *threo*-$^2H_2$-labelled precursors. The D-configuration for the hydrogen atoms removed is assumed for this illustration.

With the *erythro*-racemate as precursor, both ^2H atoms would be removed from one enantiomer but none from the other, resulting in enrichment of $^2H_2$-labelled species in the olefinic product and no $^2H_1$-labelled olefin molecules being formed. On the other hand, one ^2H atom and one ^1H atom would be lost from each of the *threo*-enantiomers, leading to enrichment of $^2H_1$-labelled species and no $^2H_2$-labelled species in the product. Loss of *trans*- rather than *cis*-hydrogen atoms from these precursors, of course, would give the opposite results to those depicted.

The results of typical mass-spectrometric analysis of the various purified product fatty acid methyl esters, from matched pairs of incubations, are shown in Table 2. They demonstrate in each case the



Scheme 2. Summary of olefinic products from racemic *erythro*- and *threo*-$^2H_2$-labelled precursors, on the basis of enzymic desaturation removing a *cis*-pair of hydrogen atoms of the D-configuration.

Table 2. *Isotopic enrichment in the products of desaturation of* $^2H_2$*-labelled fatty acid precursors by* Chlorella vulgaris *cells*

Incubation conditions and isolation of products are described in the text. The results are corrected for the proportions of $^2H_1$-labelled species in the substrates.

Precursor	Product	Composition (moles/100 moles) $^2H_1$	$^2H_2$
[*erythro*-9,10-$^2H_2$]$C_{18:0}$ acid	Oleate	0·86	3·98
[*threo*-9,10-$^2H_2$]$C_{18:0}$ acid	Oleate	1·70	0·49
[*erythro*-9,10-$^2H_2$]$C_{18:0}$ acid	Oleate	0·85	5·45
[*threo*-9,10-$^2H_2$]$C_{18:0}$ acid	Oleate	1·55	0·38
[*erythro*-12,13-$^2H_2$]$C_{18:1}$ acid	Linoleate	0·46	5·10
[*threo*-12-13-$^2H_2$]$C_{18:1}$ acid	Linoleate	6·80	0·80
[*erythro*-15,16-$^2H_2$]$C_{18:1}$ acid	α-Linolenate	0·68	6·20
[*threo*-15,16-$^2H_2$]$C_{18:1}$ acid	α-Linolenate	5·66	1·13

Table 3. $^3H/^{14}C$ *ratios of stereospecifically* ^3H*-labelled stearic acids and of the products of their desaturation by* Chlorella vulgaris *cells*

Incubation conditions and isolation of products are described in the text.

Precursor	$^3H/^{14}C$ ratio of substrate	Product	$^3H/^{14}C$ ratio of product*
[D-9-^3H]$C_{18:0}$ acid	27·8	Oleate	5·2 (0·19)
[L-9-^3H]$C_{18:0}$ acid	31·6	Oleate	25·5 (0·81)
[D-12-^3H]$C_{18:0}$ acid	15·6	Linoleate	2·2 (0·14)
[L-12-^3H]$C_{18:0}$ acid	23·1	Linoleate	21·1 (0·91)

* To facilitate comparison, the values in parentheses are normalized relative to an assigned value of unity for each substrate.

enrichment expected for removal of hydrogen atoms in the *cis* relative configuration, namely enrichment of $^2H_2$-labelled species from *erythro*-precursors and of $^2H_1$-labelled species from *threo*-precursors. Thus these desaturation reactions effected by *Chlorella vulgaris* involve loss of pairs of hydrogen atoms of the same configuration on *cis*-double-bond formation at the 9,10-, 12,13- and 15,16-positions.

If the removal of hydrogen atoms in desaturations by *Chlorella vulgaris* is not only geometrically specific, as has just been proved, but also specific to one optical configuration for these hydrogen atoms, then incubation with D-^3H- and L-^3H-labelled precursors would result in total loss of ^3H in desaturation of one enantiomer but total retention of ^3H in the desaturation product from the other enantiomer. If, however, the desaturations were specific only in the relative sense of losing *cis*-hydrogen atoms but not in this absolute sense, then some proportion of the tritium from each of the D-^3H- and L-^3H-labelled precursors would be lost and some would be retained in the olefinic products. Typical results from incubations with the D- and L-9-^3H-labelled and -12-^3H-labelled precursors are summarized in Table 3. The $^3H/^{14}C$ ratios of the products derived from the D-^3H-labelled precursors in both cases decreased markedly, indicating almost complete loss of tritium from the D-9- and D-12-positions during the formation of the double bonds of oleate and linoleate respectively. From the L-^3H-labelled precursors, however, the $^3H/^{14}C$ ratios were largely maintained, showing that ^3H in this configuration at these positions is retained on desaturation.

DISCUSSION

It is evident from this series of experiments that the green alga *Chlorella vulgaris*, like *Corynebacterium diphtheriae* (Schroepfer & Bloch, 1965), exhibits extreme stereoselectivity in the formation of the *cis*-double bonds of long-chain unsaturated fatty acids. Thus the D-9- and D-10-hydrogen atoms of stearic acid are removed during its desaturation to oleic acid and the D-12- and D-13-hydrogen atoms of oleic acid are lost during its desaturation to linoleic acid. Though the absolute configurations of the hydrogen atoms lost from the 15- and 16-positions during formation of α-linolenic acid have not yet been determined, here again it has

been shown that they are of the same configuration. By analogy with the desaturations at the 9,10- and 12,13-positions, it might be expected that the D-15- and D-16-hydrogen atoms are the pair that are removed in α-linolenate formation.

The fact that the loss of ^3H on desaturation of D-^3H-labelled substrates and the retention of ^3H on desaturation of L-^3H-labelled substrates was not complete is unlikely to imply any lack of complete stereospecificity in the desaturation reactions. It is considered to have resulted from a certain amount of racemization during the synthesis of these precursors, particularly during the hydrogenolysis step. Thus the D-^3H-labelled precursors contained a certain proportion of the L-^3H-labelled compounds, which did not lose ^3H on desaturation, and, conversely, the L-^3H-labelled precursors contained some of the D-^3H-labelled compounds, which did lose ^3H on desaturation. Similarly, the fact that there was always some $^2H_2$-labelled olefin from *threo*-$^2H_2$-labelled precursors and, even after correction for the substrate composition, some $^2H_1$-labelled product from *erythro*-$^2H_2$-labelled precursors is considered to be due to a small deviation from absolute stereospecificity during synthesis of the precursors rather than during enzymic desaturation.

Schroepfer & Bloch (1965) reported a substantial isotope effect in the desaturation of [D-9-^3H]stearic acid but not with [L-9-^3H]-, [D-10-^3H]- or [L-10-^3H]-stearic acid. They suggested, from this observation, that the rate-limiting step of the desaturation reaction in *Corynebacterium diphtheriae* was removal of the D-9-hydrogen atom. In the present series of experiments, no attempt was made to isolate unchanged ^3H-labelled precursors from the products of incubation and, by measurement of their $^3H/^{14}C$ ratios, to determine whether any kinetic isotope effect was evidenced in their desaturation. However, the results of incubating *Chlorella* cells with the ^2H-labelled precursors present an anomaly suggesting that there is a substantial kinetic isotope effect that must be effective at both positions corresponding to each double bond.

It is clear from Scheme 2, assuming there is no kinetic isotope effect, that for each two molecules of *erythro*-$^2H_2$-labelled substrate that are desaturated only one molecule of $^2H_2$-labelled olefin is formed, whereas for every two molecules of *threo*-$^2H_2$-labelled substrate desaturated two molecules of $^2H_1$-labelled olefin are formed. Thus, if identical amounts of the same cells are incubated with identical amounts of *erythro*- and *threo*-$^2H_2$-labelled precursors for the same length of time and under the same conditions, which was indeed the case for each pair of results reported in Table 2, there should be twice as much $^2H_1$-labelled product from the *threo*-substrate as there is $^2H_2$-labelled product from the *erythro*-substrate. However, this was not the observed result (Table 2). The proportion of $^2H_2$-labelled product from the *erythro*-substrate was either approximately equal to (in the linoleate and α-linolenate products) or was considerably greater than (in the oleate products) the proportion of $^2H_1$-labelled product from the *threo*-substrate.

Now, as discussed above, if there were no kinetic isotope effect at all there should be half the proportion of $^2H_2$-labelled product from the *erythro*-substrate as there is $^2H_1$-labelled product from the *threo*-substrate. If there were an isotope effect at only one of the two positions sufficiently strong to block desaturation of a substrate with a D-^2H atom at that position totally, then one enantiomer from each substrate would be completely unreactive and there would be equal amounts of $^2H_2$-labelled and $^2H_1$-labelled products from *erythro*- and *threo*-substrates respectively. This explanation could account for the results of the oleate→linoleate and the linoleate→linolenate transformations. However, it is still not sufficient to account for the results of the stearate→oleate transformations, where roughly three times as much [$^2H_2$]oleate was produced from the *erythro*-substrate as [$^2H_1$]oleate from the *threo*-substrate. A similar result was obtained for the stearate→oleate desaturation effected by an animal system (L. J. Morris, R. Bickerstaffe & W. Kelly, unpublished work). Only one explanation would seem to be possible to account for this finding, namely that there is a fairly strong kinetic isotope effect exerted by ^2H atoms of the D-configuration at both the 9- and the 10-position, so that neither of the *threo*-enantiomers, nor indeed the two together, is as efficient a substrate as the LL-*erythro*-9,10-$^2H_2$-labelled enantiomer. Since this explanation would seem to be necessary to account for the results of the stearate→oleate conversion, it may also be that in the desaturations leading to linoleate and linolenate a partial isotope effect is operative at both positions, rather than a totally inhibitory isotope effect of a D-deuterium atom, as suggested above, operative at only one position.

These considerations imply that the desaturation reactions in *Chlorella* and in the animal system (L. J. Morris, R. Bickerstaffe & W. Kelly, unpublished work) involve a mechanism of simultaneous concerted removal of the pair of hydrogen atoms rather than the stepwise sequence, with the first step being rate-limiting, suggested for the bacterial desaturation reaction by Schroepfer & Bloch (1965). Further more detailed investigations of the magnitude of these kinetic isotope effects and of whether they are operative at only one or at both positions of each putative double bond are clearly necessary.

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