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# Mechanisms and Stereochemistry in Fatty Acid Metabolism

## THE FIFTH COLWORTH MEDAL LECTURE

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As the title implies, this Lecture consists of a fairly chemical consideration of various aspects of fatty acid biosynthesis and metabolism. I think it is only fair to acknowledge that a major factor in deciding me, some 3 or 4 years ago, to venture into this area of study was the enjoyment and the stimulation I derived from reading the earlier reports of the elegant series of experiments on mechanisms and stereochemistry in the biosynthesis of cholesterol and other polyisoprenoid compounds, using stereospecifically labelled mevalonates, initiated by Cornforth and Popják and their colleagues. No such general approach as that exploited so imaginatively in the polyisoprenoid field was available for the study of fatty acid reactions, but various individual reactions in fatty acid metabolism could be separately investigated in analogous ways and, hopefully, much could be learned thereby of the nature and the mode of action of the enzymes involved.

This, then, is a brief review of our studies, over the last few years, of mechanisms and stereochemistry in fatty acid metabolism. Within this general theme, a second thread running through much of this Lecture concerns the question of possible interrelationships between hydroxyl groups and double bonds in fatty acid biochemistry.

There are, I suppose, two main factors that gave rise to the feeling that there must be some general biochemical interrelationship between epoxy or hydroxyl groups and double bonds in long-chain fatty acid metabolism. The first of these is that all desaturations of long-chain fatty acids require molecular oxygen and reduced nicotinamide nucleotide as obligatory cofactors, and in this the desaturases are similar to the known hydroxylases in cofactor requirements. The second is that among the many unusual fatty acids in Nature are a range of epoxy, hydroxy and dihydroxy fatty acids with the substituent group in a position normally occupied by a double bond. These two considerations, one biochemical and the other chemical, gave rise to the questions: (i) are epoxy or hydroxy acids intermediates in double-bond formation?; or, conversely, (ii) are the oxygenated acids derived by epoxidation or hydration of double bonds?

Epoxy and dihydroxy acids can be dealt with very quickly at this stage by noting that there is absolutely no direct biochemical evidence to suggest that they are intermediates in double-bond formation. Indeed, *cis*-9,10-epoxystearic acid has been shown to be produced by epoxidation of oleic acid in *Puccinia graminis* spores (Knoche, 1968) and in *Xeranthemum annuum* seeds (L. J. Morris, unpublished work), and in these same seeds and in *Euphorbia lagascae* seeds *cis*-12,13-epoxyoleic acid is formed from linoleic acid (L. J. Morris & E. W. Hammond, unpublished work). The corresponding *threo*-dihydroxy acids are derived by enzymic hydration of these epoxy acids, as will be described later in this paper. The question of the biochemical relationship, if any, between monohydroxy and ethylenic fatty acids, however, was still very open when these studies were begun.

Most of the studies to be described depended on the synthesis of substrates with deuterium or tritium labels in known positions and in known geometrical or absolute optical configurations. The synthesis of some of these substrates, in turn, depended on our ability to separate and isolate positional isomers of unsaturated fatty acids. An account of these procedures and also of the chemical and chromatographic procedures utilized to establish the absolute optical configurations of the various epoxy and dihydroxy acids discussed could fill another lecture and will not therefore be described here.

### *α-Oxidation and α-hydroxylation*

With that rather brief introduction to the background to our work, I will begin with a consideration of the α-oxidation of long-chain fatty acids. This is a major pathway of degradation of fatty acids in young plant leaves (Hitchcock & James, 1964, 1966), and related pathways occur in the germinating peanut (Martin & Stumpf, 1959) and in animal tissues (see e.g., Bowen & Radin, 1968). By this pathway the fatty acids are degraded one carbon atom at a time, giving $CO_2$ as the product, in

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contrast with the more general $\beta$-oxidation pathway, which degrades fatty acids two carbon atoms at a time to acetyl-CoA.

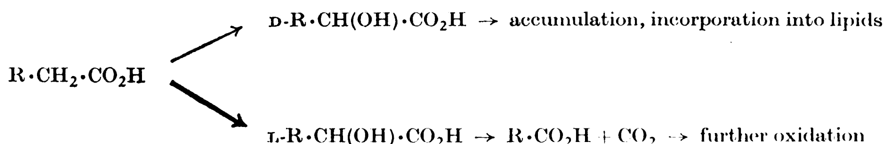
Hitchcock & James (1966) had shown that $\alpha$-hydroxy acids were intermediates in this pathway. Incubation of uniformly labelled palmitic acid with homogenates or with acetone-dried powders prepared from young pea leaves (*Pisum sativum*) and analysis of the metabolites by radiochemical g.l.c. showed, not only the conversion of the palmitic acid into pentadecanoic acid, myristic acid and lower homologues, but also the accumulation of labelled 2-hydroxypalmitic acid, 2-hydroxypentadecanoic acid and so on. Competition with unlabelled racemic 2-hydroxypalmitic acid inhibited the degradation of the [U-$^{14}$C]palmitic acid, whereas the converse experiment with racemic 2-hydroxy[U-$^{14}$C]palmitic acid and unlabelled palmitic acid showed that the latter did not inhibit degradation of the labelled hydroxy acid to $CO_2$ and pentadecanoic acid. Further evidence was provided by experiments in which imidazole was added to the incubations. Imidazole, at low concentrations (1.0mM), inhibited $\alpha$-oxidative degradation of palmitic acid, but had no effect on the degradation of 2-hydroxypalmitic acid to pentadecanoic acid (Hitchcock & James, 1966). These various studies demonstrated conclusively that $\alpha$-hydroxy acids were intermediates in the pathway of $\alpha$-oxidation of long-chain fatty acids.

Now, quite apart from the mechanism of formation of these $\alpha$-hydroxy acids, there were two questions raised by these studies: (i) assuming that the $^{14}$C-labelled $\alpha$-hydroxy acids that accumulated in these experiments are asymmetric, are they D or L? (throughout this Lecture I will use D and L, rather than $R$ and $S$, to denote absolute configuration because, with the fatty acids, this system is quite adequate and is simpler to put across); (ii) the competition experiments of Hitchcock & James (1966) used racemic 2-hydroxypalmitic acids, but are both enantiomers subject to further oxidation? or, if not, which one is the true intermediate–D or L?

Long-chain $\alpha$-hydroxy acids, largely 2-hydroxypalmitic acid, do occur naturally in the leaves of higher plants, as constituents of a cerebroside fraction (Sastry & Kates, 1964). The 2-hydroxypalmitic acid present in pea leaves was isolated and found to be laevorotatory (Hitchcock, Morris & James, 1968*b*). It was, therefore, of the D-absolute configuration and thus the same as all other $\alpha$-hydroxy acids so characterized, whether from fungal, higher-plant or animal tissues. The absolute configuration of the radiolabelled 2-hydroxy acids that accumulated during our incubations with pea leaf preparations, however, could not just be assumed to be D, nor, because of the minute amounts involved, could it be determined so simply by direct measurement. We therefore adopted an indirect approach by isolating 2-hydroxy[1-$^{14}$C]palmitic acid of high specific radioactivity biosynthesized by our pea leaf preparations, mixing portions of this with unlabelled synthetic D- and L-2-hydroxypalmitic acid as carriers and performing fractional recrystallizations of these mixtures from suitable solvents. The radioactive 2-hydroxypalmitic acid co-crystallized with the D-2-hydroxypalmitic acid carrier, whereas it was rapidly leached into the mother liquors of the recrystallized L-2-hydroxypalmitic acid. Thus, like the 2-hydroxypalmitic acid occurring naturally in pea leaves, this radiolabelled 2-hydroxypalmitic acid biosynthesized in our incubations was of the D-absolute configuration (Hitchcock *et al.* 1968*b*).

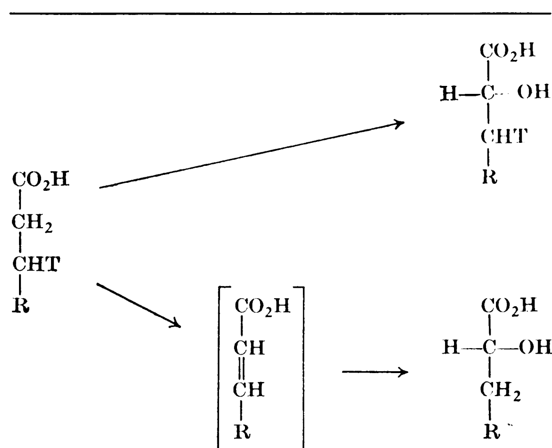
The question whether only this $\alpha$-hydroxy acid enantiomer was subject to further degradation, or whether both of them were, was investigated by competition experiments with [1-$^{14}$C]- or [U-$^{14}$C]-palmitic acid and the synthetic unlabelled D- and L-2-hydroxypalmitic acid (Hitchcock, Morris & James, 1968*a*). When D-2-hydroxypalmitic acid was added the degradation of the labelled palmitic acid to $CO_2$ and shorter-chain metabolites was slightly inhibited, whereas when L-2-hydroxypalmitic acid was added there was almost complete inhibition of this degradation. Thus, although the radioactive 2-hydroxy acid that accumulates during $\alpha$-oxidation is of the D-configuration, the main pathway of degradation of fatty acids appears to proceed via the L-2-hydroxy acid intermediates. The formation of $\alpha$-hydroxy acid intermediates and their subsequent fate appears to be stereoselective, as summarized in Scheme 1, rather than completely stereospecific.

Now, what about the mechanism of formation of $\alpha$-hydroxy acids? Are they formed by direct




Scheme 1. Stereoselectivity of $\alpha$-oxidation of fatty acids in plant leaves.

hydroxylation at the $\alpha$-position of the substrate fatty acid? Or is the first step of $\alpha$-oxidation the same as that of $\beta$-oxidation, namely dehydrogenation to an $\alpha\beta$-unsaturated intermediate, followed by hydration to give an $\alpha$-hydroxy acid rather than a $\beta$-hydroxy acid? A decision between these two mechanisms can be made by using, as substrates, palmitic acids labelled in the 3-position with tritium, as shown in Scheme 2. If the reaction proceeds by direct hydroxylation then tritium label in the 3-position will not be affected and will be completely retained in the $\alpha$-hydroxy acid product. However, if an $\alpha\beta$-unsaturated intermediate is involved then, depending on the stereospecificity of the dehydrogenation reaction, there will be total loss of tritium in either the D- or the L-configuration at the 3-position or there will be loss of half the tritium from these two stereospecifically tritiated substrates. When the 2-hydroxypalmitic acids produced from these D- and L-[1-$^{14}$C,3-$^{3}$H]palmitic acid substrates were isolated from incubations in pea leaf systems, total retention of tritium was found in each case (C. Hitchcock & L. J. Morris, unpublished work). Thus the D-2-hydroxypalmitic acid synthesized during the course of $\alpha$-oxidation is formed by direct hydroxylation at the 2-position.



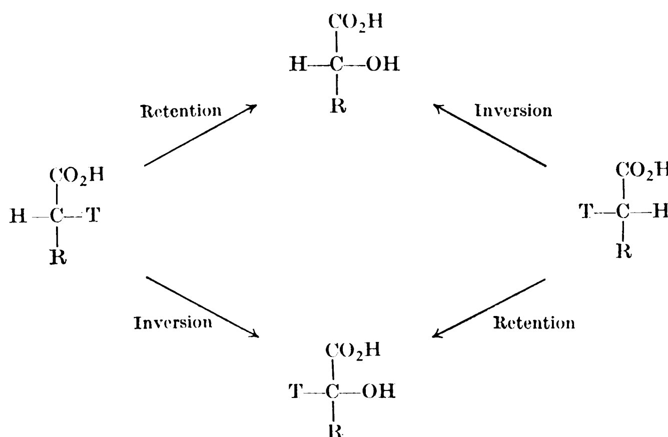
Scheme 2. Differentiation between direct hydroxylation and desaturation–hydration mechanisms of $\alpha$-hydroxy acid formation, with D- or L-[1-$^{14}$C,3-$^{3}$H$_1$]palmitic acid as precursor. $R = CH_3\cdot[CH_2]_{12}$.

One further question remains to be answered, namely does this direct hydroxylation take place with retention or inversion of configuration at the 2-position? This question can also be answered fairly simply, as illustrated in Scheme 3, by use of D- and L-2-tritiopalmitic acid as substrates.

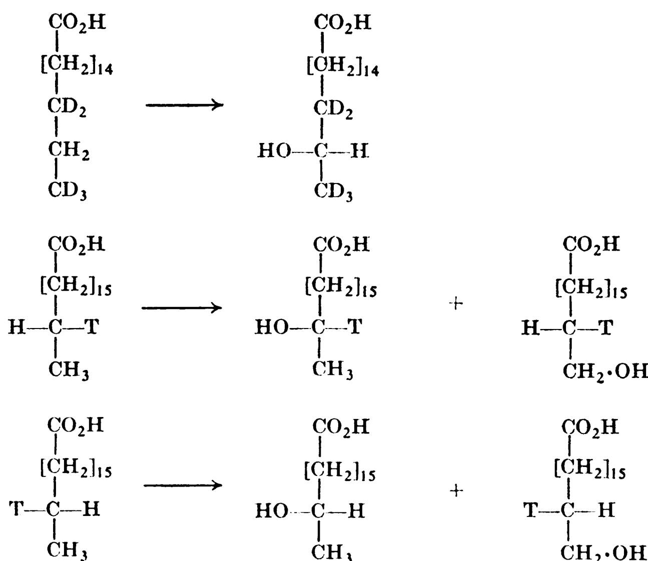
When the D-2-hydroxypalmitic acids produced by pea leaf preparations from these D- and L-[1-$^{14}$C,2-$^{3}$H]palmitic acid substrates were isolated it was found that the tritium in the D-configuration in the substrate had been lost on hydroxylation whereas the L-tritium had been retained (Morris & Hitchcock, 1968). Therefore the formation of long-chain $\alpha$-hydroxy fatty acids in these higher-plant leaf systems proceeds by direct hydroxylation at the 2-position with retention of configuration at that position. The $\alpha$-hydroxylase enzyme thus has the same stereochemical characteristics as the known mixed-function oxidases.

### *$\omega$- and ($\omega-1$)-Hydroxylations*

Another example of direct hydroxylations of long-chain fatty acids is provided by the yeast




Scheme 3. Determination of the stereochemical course of $\alpha$-hydroxylation, with D- and L-[1-$^{14}$C,2-$^{3}$H$_1$]-palmitic acid as precursors. $R = CH_3\cdot[CH_2]_{13}$.



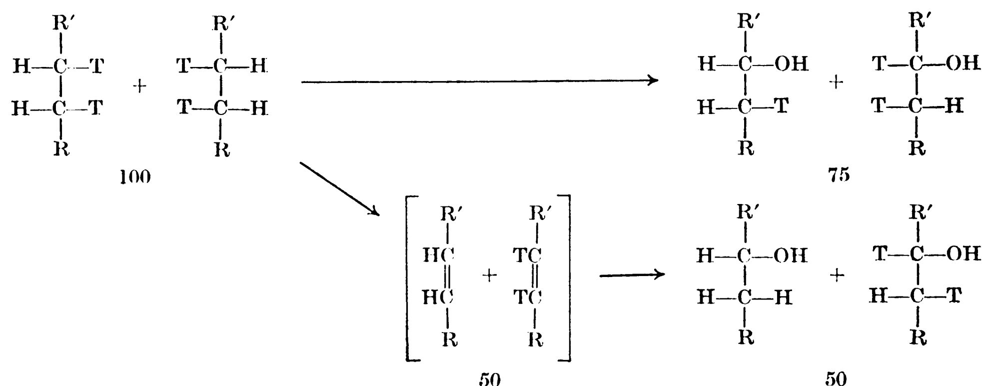
Scheme 4. Elucidation of the mechanism and stereochemistry of $\omega$- and $(\omega-1)$-hydroxylation by *Torulopsis apicola*, with $[16\text{-}^2H_2,18\text{-}^2H_3]$stearic acid and D- and L-$[1\text{-}^{14}C,17\text{-}^3H_1]$stearic acid as precursors (Heinz *et al.* 1969; Jones, 1968).

*Torulopsis apicola*, otherwise known as *T. groppengiesseri* or *T. magnoliae*. This yeast ferments long-chain fatty acids, and also hydrocarbons and related compounds, to produce $\omega$- and $(\omega-1)$-hydroxy fatty acids, which are then combined with sophorose to give extracellular hydroxy acid sophorosides and the related cyclic lactones (Tulloch, Spencer & Gorin, 1962; Tulloch, Hill & Spencer, 1968; Jones & Howe, 1968; Jones, 1968). The relative proportions of $\omega$- and $(\omega-1)$-hydroxy acids produced depend on the chain length of the substrate, but stearic acid, for example, gives rise to 18-hydroxystearic acid and L-17-hydroxystearic acid in an approximate ratio of 1:10 (Tulloch *et al.* 1962; Jones & Howe, 1968). We had just begun synthesis of the specifically labelled substrates required to determine whether these hydroxy acids were formed by direct hydroxylation or by hydration of a preformed double bond and to ascertain the stereospecificity of the reactions when we became aware that these questions had been elegantly and independently answered by both Tulloch and his colleagues (Heinz, Tulloch & Spencer, 1969) and by Jones (1968). The most relevant experiments of these workers are summarized in Scheme 4.

Heinz *et al.* (1969) used a pentadeuterostearic acid, $[16\text{-}^2H_2,18\text{-}^2H_3]$stearic acid, as substrate and showed that the 17-hydroxystearic acid produced had retained all five deuterium atoms. Therefore no double-bond intermediate could be involved and the hydroxy acid was formed by direct hydroxylation. Using D- and L-$[17\text{-}^2H]$stearic acid these workers proved that this hydroxylation took place with retention of configuration at the 17-position. Jones (1968), on the other hand, used D-, L- and racemic $[17\text{-}^3H]$stearic acid as substrates to prove that no double-bond intermediate was involved in the $\omega$-hydroxylation reaction and, again, that the $(\omega-1)$-hydroxylation proceeded with retention of configuration. The *Torulopsis* hydroxylase again appears to have all the properties, including the stereospecificity, of a mixed-function oxidase (Heinz, Tulloch & Spencer, 1970).

### *Ricinoleic acid biosynthesis*

Now, turning to another, better-known, hydroxy fatty acid, namely D-12-hydroxyoleic or ricinoleic acid, we have the very interesting situation that this acid is apparently biosynthesized by two distinct mechanisms in two different organisms. Thus in immature sclerotia or in mycelial cultures of *Claviceps purpurea*, the ergot fungus, the nearest precursor for ricinoleic acid formation is linoleic acid (Morris, Hall & James, 1966). The reaction does not require molecular oxygen, so that it looks as if it involves a simple hydration of the 12,13-double bond of linoleic acid. (In fact it may not be quite as simple as this, as will be discussed shortly.) In the castor bean, however, oleic acid is the nearest precursor and molecular oxygen and NADPH are required cofactors (James, Hadaway & Webb,



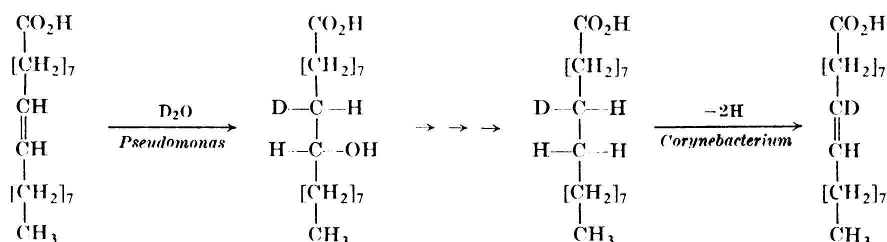

Scheme 5. Differentiation between direct hydroxylation and desaturation–hydration mechanisms of ricinoleic acid formation in the castor bean, with racemic [1-$^{14}$C,*erythro*-12,13-$^{3}H_2$]oleic acid as precursor. R = $CH_3\cdot[CH_2]_4$; R′ = $HO_2C\cdot[CH_2]_7\cdot CH{:}CH\cdot CH_2$. Numbers denote the proportions of tritium label of substrate remaining in the products of these alternative pathways.

1965; Yamada & Stumpf, 1964; Galliard & Stumpf, 1966). These cofactor requirements, of course, are the same as for the desaturation of oleic acid to linoleic acid, which also takes place in castor-bean tissue preparations. It might have been, therefore, that ricinoleic acid was formed by the same hydration pathway in *Claviceps* and in the castor bean, but that in the castor bean the linoleic acid precursor was 'enzyme-bound' and so not exchangeable with added linoleic acid.

In order to distinguish between these two possible mechanism for ricinoleic acid formation in the castor bean we could have adopted the same approach as I have already described and looked for loss or retention of tritium from 13-tritio-oleic acid to prove whether linoleic acid was or was not an intermediate. However, we chose a different approach using racemic *erythro*-12,13-ditritio-oleic acid as substrate, as summarized in Scheme 5.

Thus, if ricinoleic acid is formed by direct hydroxylation, only one of the four tritium atoms in the racemic substrate mixture will be lost, and, if the $^{3}H/^{14}C$ ratio of the [1-$^{14}$C,*erythro*-12-$^{3}H_1$,13-$^{3}H_1$]oleic acid substrate is taken as 100, the product will have a $^{3}H/^{14}C$ ratio of 75. If, on the other hand, the mechanism involves hydration of an intermediate double bond, two of the four tritium atoms must be lost in the desaturation step and the ricinoleic acid product will have a $^{3}H/^{14}C$ ratio of only 50. When this was put to the test with the doubly labelled oleic acid substrate in castor-bean tissue, the ricinoleic acid formed had a $^{3}H/^{14}C$ ratio 75% of the substrate ratio, whereas the $^{3}H/^{14}C$ ratio of linoleic acid, which was formed simultaneously in the system, was exactly half that of the substrate, as expected. Ricinoleic acid therefore was proved to be synthesized in the castor bean by direct hydroxylation of oleic acid at the 12-position (Morris, 1967). When the stereochemistry of this hydroxylation was investigated with D- and L-[1-$^{14}$C,12-$^{3}H_1$]oleic acid (cf. Schemes 3 and 4), the tritium in the D-configuration was lost whereas that in the L-configuration was retained, demonstrating retention of configuration at the 12-position during hydroxylation (Morris, 1967).

Thus ricinoleic acid is undoubtedly biosynthesized in Nature by two distinct pathways, by hydroxylation of oleic acid in the castor bean and from linoleic acid by a hydration-type mechanism in the ergot fungus. As mentioned above, this latter mechanism may not be a simple double-bond hydration. Although ricinoleic acid may constitute up to about 40% of the fatty acids of mycelia or sclerotia of *Claviceps purpurea*, there are no free hydroxyl groups in ergot oil. This rather unusual fact was resolved by the discovery that, in the glycerides of ergot oil, the hydroxyl groups of the ricinoleic acid moieties were all esterified with long-chain fatty acids (Morris & Hall, 1966). Besides normal triglycerides, a range of these estolide–triglyceride structures were present comprising, in effect, tetra-acyl-, penta-acyl- and hexa-acyl-glycerides. Despite the high proportion of ricinoleic acid frequently present, there were no 'polyestolide' structures, i.e. the ricinoleic acid moieties were all esterified directly to glycerol and never to the hydroxyl group of another ricinoleic acid. These findings and various considerations of positional specificity raise the possibility that, instead of simple hydration of linoleic acid followed by acylation of the hydroxyl group of the ricinoleic acid product to give these estolide structures, long-chain



Scheme 6. Summary of the proof that hydration of oleic acid by a *Pseudomonas* species involves *trans*-addition of water (Schroepfer, 1966).

fatty acids may undergo enzyme-catalysed direct addition across the double bond to generate the estolides as primary products of the reaction.

### *Hydrations of double bonds and epoxides*

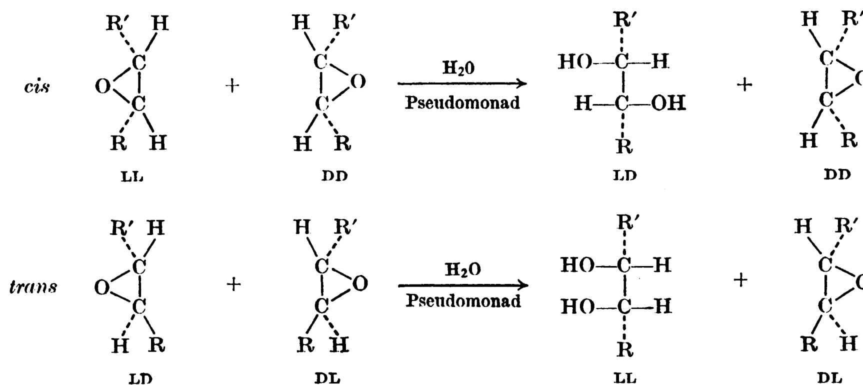
Four biological systems that synthesize hydroxy fatty acids have been discussed, three of which operate by direct hydroxylation and one that has a hydration type of mechanism. Another system that undoubtedly involves just a simple double-bond hydration is found in a strain of *Pseudomonas* (classified as N.R.R.L. 2992). This bacterium was shown by Wallen, Benedict & Jackson (1962) to hydrate added oleic acid, in high yield, to 10-hydroxystearic acid. Schroepfer & Bloch (1965) proved that the hydroxyl group of the product was in the D-configuration and Schroepfer (1966) demonstrated that the hydration involved *trans*-addition of the elements of water across the double bond, as shown in Scheme 6.

Thus the hydration of oleic acid by the pseudomonad was effected in deuterated water and the 9-deutero-10-hydroxystearic acid product was converted, via hydrogenolysis of its tosylate and regeneration of the carboxyl group, into 9-deuterostearic acid. The proof that the deuterium was in the L-configuration, and hence that the hydration had involved *trans*-addition, was then obtained when the deuterium atom was retained on desaturation of this stearic acid to oleic acid by another bacterium, *Corynebacterium diphtheriae*. This proof depends on the stereospecificity of desaturation, which will be discussed below.

Now, this same pseudomonad enzyme preparation that hydrates oleic acid to 10-hydroxystearic acid also catalyses stereospecific hydrations of both *cis*- and *trans*-9,10-epoxystearic acid (Niehaus & Schroepfer, 1967), which will be discussed in a moment.

We had been interested in the stereochemistry of both chemical and biochemical reactions of epoxy fatty acids, particularly the enzymic hydrations of the endogenous epoxy acids of various seeds and plant rust spores. *Vernonia anthelmintica* seeds contain a high proportion of *cis*-12,13-epoxyoleic acid, which has been proved to have the D-configuration (Morris & Wharry, 1966; Powell, Smith & Wolff, 1967). When *Vernonia* seeds are crushed and incubated under moist conditions, the epoxy acid is hydrolysed from the glycerides and hydrated to dextrorotatory *threo*-12,13-dihydroxyoleic acid, which has been shown to be L-12,D-13-dihydroxyoleic acid (Morris & Crouchman, 1969). As epoxide ring opening involves inversion at the position of nucleophilic attack, we could predict that this attack by water or $OH^-$ ion was at the 12-position and that the oxygen of the 13-hydroxyl group was that of the original epoxide. Similarly, the epoxy acid of *Puccinia graminis* uredospores is *cis*-9,10-epoxystearic acid of the L-configuration (Powell *et al.* 1967; L. J. Morris & M. L. Crouchman, unpublished work), which, on incubation of the spores in water, is hydrated to dextrorotatory *threo*-9,10-dihydroxystearic acid, which has been proved to be L-9,D-10 (L. J. Morris & M. L. Crouchman, unpublished work). Thus, in this case, nucleophilic attack by water or $OH^-$ ion must be at the 10-position with inversion. These predictions as to the sites of attack in these two enzymic hydrations have been tested directly by incubating crushed *Vernonia* seeds and *Puccinia* spores in $^{18}O$-enriched water and localizing the position of the added hydroxyl group by mass spectrometry of the dihydroxy acid products and derivatives of them. As predicted, the $^{18}O$ isotope was enriched exclusively in the 12-position of the 12,13-dihydroxyoleic acid produced by the seeds and in the 10-position of the 9,10-dihydroxystearic acid from the spores (L. J. Morris, M. L. Crouchman, E. W. Hammond & W. Kelly, unpublished work).

Now, we can return to the hydratase enzyme from the pseudomonad, which hydrates, not only oleic acid as discussed above, but also both *cis*- and *trans*-9,10-epoxystearic acid. Niehaus & Schroepfer (1967) showed that these hydrations were com-




Scheme 7. Summary of the action of the pseudomonad hydratase on racemic *cis*- and *trans*-9,10-expoxystearic acid. $R = CH_3 \cdot [CH_2]_7$; $R' = HO_2C \cdot [CH_2]_7$.

pletely stereospecific, as regards both substrate and product. Thus racemic *cis*-9,10-epoxystearic acid was converted by the pseudomonad enzyme into a mixture of optically active *threo*-9,10-dihydroxystearic acid and residual, but now optically active, *cis*-epoxystearic acid in approximately equal amounts, and racemic *trans*-9,10-epoxystearic acid similarly gave optically active *erythro*-9,10-dihydroxystearic acid and optically active *trans*-epoxystearic acid, as illustrated in Scheme 7. They also showed, by carrying out the hydrations in $^{18}O$-enriched water, that in both cases the oxygen from the water became attached exclusively at the 10-position.

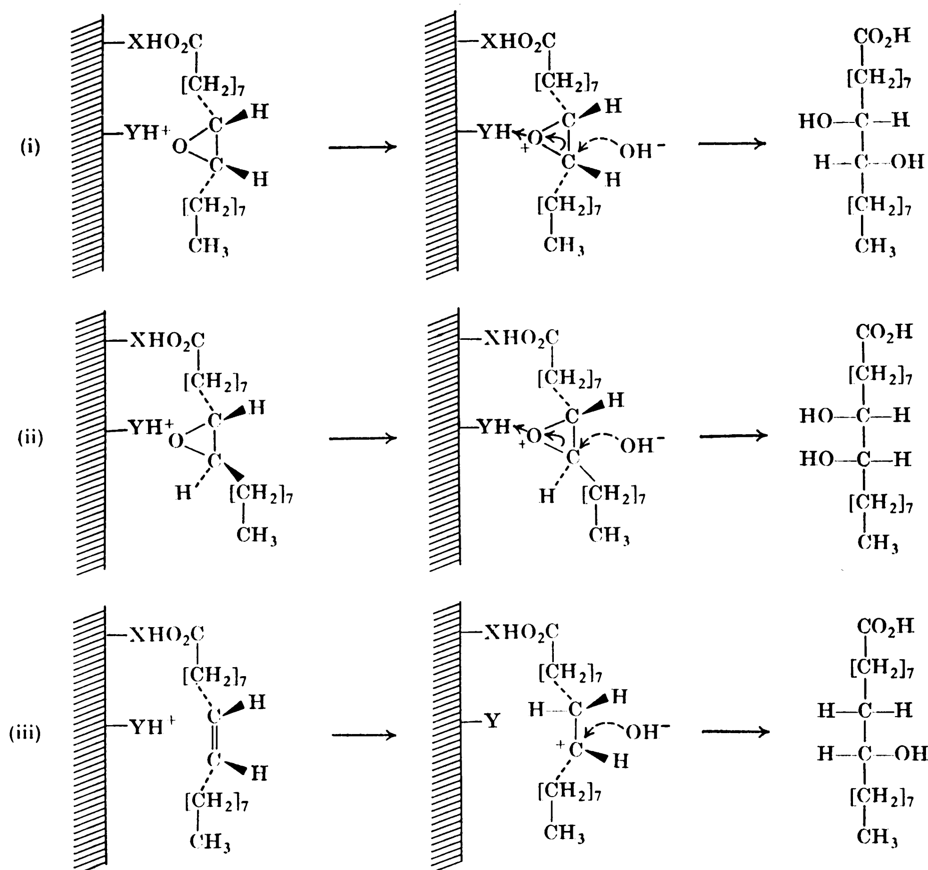
What Niehaus & Schroepfer (1967) did not know, however, were the absolute optical configurations either of their dihydroxy acid products or of the epoxy acids remaining after these 'biochemical resolutions' and therefore of the substrates. Quite soon after their paper appeared, however, we were in the happy position of knowing the configurations of all these compounds (L. J. Morris & M. L. Crouchman, unpublished work). They are as illustrated in Scheme 7 and confirm configurationally the demonstration by Niehaus & Schroepfer (1967) that the hydroxyl group from water is introduced at the 10-position.

We can therefore describe the relevant features of the *Pseudomonas* hydratase enzyme and its mechanism of action in the hydration of these two epoxy acids, *cis*-epoxystearic acid (i) and *trans*-epoxystearic acid (ii), and of oleic acid (iii) as shown in Scheme 8. No activation of the carboxyl group of the substrate seems to be required for these hydrations and, for the double-bond hydration at least, the enzyme is quite specific for the 9,10-positions, so the first step must be attachment of the substrate carboxyl group, in some way, to a site in the enzyme designated as X. The active site must be some electrophilic group, designated as $-YH^+$, at a distance from the site of attachment (X), such as to place it, in the enzyme–substrate complex, between the 9- and the 10-position of the substrate but closer to the 9-position. The first reaction step, then, is electrophilic attack from the L-side of the substrate, and this must be mediated by close and specific interaction of the acyl chain with the enzyme, to give the conjugate acids from the epoxide groups and a $\pi$-complex or the normal carbonium ion from the double bond. The reaction is completed in each case by the normal back-side nucleophilic attack, at the 10-position, either by $OH^-$ ion, as shown, or by water followed by loss of proton, to give the three products with the correct stereochemistry.

### *Desaturations*

Having discussed a fair range of hydroxylations and hydrations of fatty acids, I will now consider the formation and, very briefly, the biohydrogenation of double bonds in long-chain fatty acids. Discussion of desaturations will be restricted to the formation of three *cis*-unsaturated acids: oleic acid, which is virtually ubiquitous and is formed by dehydrogenation of stearic acid, and the other two common unsaturated acids of plants, linoleic acid and $\alpha$-linolenic acid, which are formed by further sequential desaturations of oleic acid (e.g. Harris, Harris & James, 1965).

Now, in the formation of a double bond, two hydrogen atoms are somehow removed from the precursor and the question arises—which two of the four possible hydrogen atoms are lost? Are they of




Scheme 8. Schematic representation of the mechanism of hydration of *cis*-9,10-epoxystearic acid (i), *trans*-9,10-epoxystearic acid (ii) and oleic acid (iii) by the pseudomonad hydratase enzyme.

a *cis*- or *trans*- (*erythro*- or *threo*-) configuration relative to each other, and is there an absolute stereospecificity in their removal?

These questions were first investigated by Schroepfer & Bloch (1965) in the desaturation of stearic acid to oleic acid by *Corynebacterium diphtheriae*. They synthesized the four stereospecifically tritium-labelled stearic acids, D- and L-[9-$^3$H]- and D- and L-[10-$^3$H]stearic acid, and used each of these as substrate for the desaturation. Tritium from the L-9- and L-10-tritio substrates was retained in the oleic acid product, whereas the D-9- and D-10-tritium labels were lost on desaturation. *Corynebacterium* therefore was proved to effect a completely stereospecific desaturation of stearic acid to oleic acid, removing only the D-9- and D-10-hydrogen atoms.

We were interested in looking at, not only the desaturation of stearic acid to oleic acid in a number of systems, but also the formation of linoleic acid and α-linolenic acid in plant systems. Although we could have adopted the same approach for each of these desaturations, the effort required for synthesis of four stereospecifically labelled substrates for each double-bond position, i.e. twelve in all, was somewhat daunting, and we adopted a slightly different approach with less demanding requirements for substrate synthesis. Our approach was that, if the relative configuration of the two hydrogen atoms lost on desaturation could be established, then only one pair of stereospecifically labelled substrates would be required for each double bond to establish the total stereochemistry.

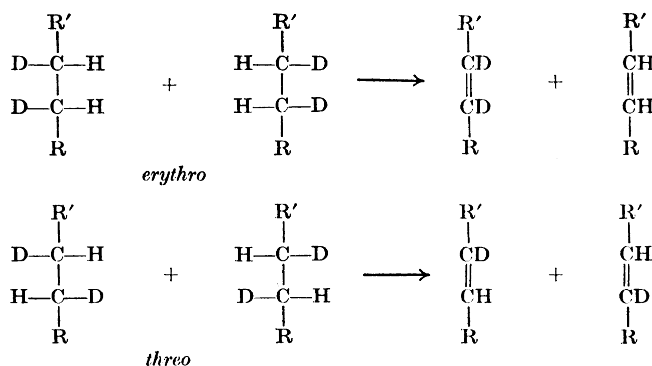
Considering first the absolute configuration of hydrogen atoms removed on desaturation, we prepared D- and L-[9-$^3$H]stearic acid as precursors for oleic acid formation and D- and L-[12-$^3$H]-stearic acid and -oleic acid as precursors for linoleic acid formation. Substrates stereospecifically tritiated in the 15- or 16-position are only now being

synthesized, so that we can say nothing yet of the absolute stereochemistry of introduction of the 15,16-double bond of α-linolenic acid in plant systems. Formation of oleic acid from D- and L-[9-$^3$H]stearic acid in *Chlorella vulgaris* (Morris, Harris, Kelly & James, 1967*a*, 1968), goat mammary-gland (L. J. Morris, R. Bickerstaffe & W. Kelly, unpublished work), hen liver (D. Brett, L. J. Morris & A. T. James, unpublished work) and fish liver (C. Hitchcock, unpublished work) systems was accompanied by retention of the L-tritium label in the product and loss of the D-tritium. Similarly formation of linoleic acid from D- and L-[12-$^3$H]-stearic acid in *Chlorella* (Morris *et al.* 1967*a*, 1968) and from D- and L-[12-$^3$H]oleic acid in castor-bean tissue (Morris, 1967) was accompanied with retention of L-tritium and loss of D-tritium. Thus both these desaturations were stereospecific in a range of biological systems, at least as regards one of the positions of each double bond, and in all cases the hydrogen removed on desaturation was of the D-absolute configuration.

To establish the total stereochemistry of desaturation at these positions we now had to determine the relative configuration of the two hydrogen atoms removed in each case. This could be accomplished by using racemic but geometrically specific *vic*-dideuterated substrates, either *erythro* or *threo*, as illustrated in Scheme 9, which depicts the unsaturated products arising from eliminations of hydrogen atoms in the *cis*- or *erythro*-relative configuration, and the D-absolute configuration that has already been established. (Loss of hydrogen atoms in the *trans*-relative configuration, of course, would give the opposite results to those depicted in Scheme 9.)

To ensure unequivocal results we decided to use both *erythro*- and *threo*-dideuterated precursors for each double-bond position. Both *erythro*- and *threo*-isomers of [9,10-$^2H_2$]stearic acid, [12,13-$^2H_2$]oleic acid and [15,16-$^2H_2$]oleic acid were therefore synthesized (Morris *et al.* 1968), the last four of these being separated from postional isomers and purified by a highly selective t.l.c. procedure on silver nitrate-impregnated silica gel (Morris, Wharry & Hammond, 1967*b*). The substrates so prepared were chemically pure and each consisted of about 80% of $^2H_2$-labelled species and 15% of $^2H_1$-labelled species, as determined by mass spectrometry.

Each of these six substrates was incubated with *Chlorella vulgaris* cells, after preliminary experiments to establish the conditions for maximum conversion with the minimum of cells, and hence of endogenous unsaturated fatty acids, to make sure we would have sufficient enrichment of deuterated species in the product to measure accurately by mass spectrometry. The *erythro*- and *threo*-[9,10-$^2H_2$]stearic acid were also incubated with microsomal preparations from goat mammary gland (L. J. Morris, R. Bickerstaffe & W. Kelly, unpublished work) and from hen liver (D. Brett, L. J. Morris & A. T. James, unpublished work). The relevant unsaturated products from these various incubations were isolated and very carefully purified by t.l.c. on silver nitrate-impregnated silica gel and preparative g.l.c., and their distributions of isotopic species were determined by mass spectrometry. The results obtained for the products from *Chlorella* and from goat mammary-gland microsomal preparation are summarized in Table 1. The hen liver system gave results similar to the latter. In each case *erythro*-$^2H_2$-labelled precursors have given rise to enrichment of dideutero species in the products, whereas *threo*-$^2H_2$-labelled precursors resulted in enrichment of monodeutero species in the products.




Scheme 9. Summary of the 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. $R = CH_3 \cdot [CH_2]_7$ (i) or $CH_3 \cdot [CH_2]_4$ (ii) or $CH_3 \cdot CH_2$ (iii); $R' = HO_2C \cdot [CH_2]_7$ (i) or $HO_2C \cdot [CH_2]_7 \cdot CH:CH \cdot CH_2$ (ii) or $HO_2C \cdot [CH_2]_7 \cdot CH:CH \cdot [CH_2]_4$ (iii).

Table 1. *Isotopic enrichment in the products of desaturation of $^2H_2$-labelled fatty acid precursors by goat mammary-gland microsomal preparation and by* Chlorella vulgaris *cells*

| System | Precursor | Product | Enrichment (mol/100mol) | |
|---|---|---|---|---|
| | | | $^2H_1$ | $^2H_2$ |
| Goat microsomal preparation | [*erythro*-9,10-$^2H_2$]stearic acid | Oleic acid | 0.7 | 13.2 |
| Goat microsomal preparation | [*threo*-9,10-$^2H_2$]Stearic acid | Oleic acid | 4.7 | 0.7 |
| *Chlorella* cells | [*erythro*-9,10-$^2H_2$]Stearic acid | Oleic acid | 0.85 | 5.45 |
| *Chlorella* cells | [*threo*-9,10-$^2H_2$]Stearic acid | Oleic acid | 1.55 | 0.38 |
| *Chlorella* cells | [*erythro*-12,13-$^2H_2$]Oleic acid | Linoleic acid | 0.46 | 5.10 |
| *Chlorella* cells | [*threo*-12,13-$^2H_2$]Oleic acid | Linoleic acid | 6.80 | 0.80 |
| *Chlorella* cells | [*erythro*-15,16-$^2H_2$]Oleic acid | α-Linolenic acid | 0.68 | 6.20 |
| *Chlorella* cells | [*threo*-15,16-$^2H_2$]Oleic acid | α-Linolenic acid | 5.66 | 1.13 |

The two hydrogen atoms removed in all these desaturations were therefore of the *cis*- or *erythro*-relative configuration. These results, along with those from the stereospecifically tritiated substrates, prove that in the desaturation of stearic acid to oleic acid in these various systems it is the D-9- and D-10-hydrogen atoms that are removed, which is the same stereospecificity as in the bacterial system reported by Schroepfer & Bloch (1965). Similarly the D-12- and D-13-hydrogen atoms are the ones removed on desaturation of oleic acid to linoleic acid in *Chlorella.* Although the absolute configurations of the hydrogen atoms lost from the 15- and the 16-position during formation of α-linolenic acid have not yet been determined, again they are of the *cis*-relative configuration and, by analogy with these other desaturations, are likely to be the D-15- and D-16-hydrogen atoms.

Now, from these various results can we deduce anything about the mechanism of desaturation? Schroepfer & Bloch (1965), in their studies of stearic acid desaturation in *Corynebacterium*, used all four tritium-labelled substrates. Besides measuring the $^3H/^{14}C$ ratio of the oleic acid product in each case they also measured the $^3H/^{14}C$ ratio of residual, unchanged substrate. The $^3H/^{14}C$ ratio of unchanged D-[9-$^3$H]stearic acid was considerably higher than the original value, demonstrating that there had been a substantial kinetic isotope effect against tritium in the D-9-position. There was, however, no such effect against tritium in the D-10-position, nor, of course, against either of the $^3$H-labelled L-isomers. They suggested, on this evidence, that the desaturation of stearic acid in the bacterium was a stepwise process, that the first step was removal of the D-9-hydrogen atom and that this step was rate-limiting.

As we were using only one pair of enantiomeric tritium-labelled precursors in each case, there seemed little point, at the time, in looking for isotope effects, and we did not do so. However, the results we obtained with the dideuterium-labelled precursors, both in *Chlorella* and in the animal systems, do not agree with this stepwise mechanism proposed for the bacterial system.

If we reconsider Scheme 9 and assume that there is no kinetic isotope effect, it is clear that for each two molecules of racemic *erythro*-$^2H_2$-labelled precursor that are desaturated only one molecule of $^2H_2$-labelled olefin is formed, whereas from each two molecules of *threo*-$^2H_2$-labelled precursor desaturated two molecules of $^2H_1$-labelled olefin are formed. Thus, if identical amounts of the same cells or microsomal preparation 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 recorded in Table 1 and many more, then there should be twice as much enrichment of $^2H_1$-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 1). The enrichment of $^2H_2$-labelled product from the *erythro*-substrate was either approximately equal to (in the linoleic acid and α-linolenic acid products) or was considerably greater than (in the oleic acid products) the enrichment of $^2H_1$-labelled product from the *threo*-substrate.

Now, if there were a kinetic isotope effect at only one of the two positions sufficiently pronounced to totally block desaturation of a substrate with a D-$^2$H atom at that position (i.e. the extreme case corresponding to the results of Schroepfer & Bloch, 1965), then one enantiomer from each substrate would be completely unreactive and there would be equal enrichments of $^2H_2$-labelled and $^2H_1$-labelled products from *erythro*- and *threo*-precursors respectively. This explanation could account for the results obtained (Table 1) in the formation of

linoleic acid and α-linolenic acid from the appropriate deuterated substrates in *Chlorella*. However, it is still not sufficient to account for the results of the transformation of stearic acid into oleic acid in either *Chlorella* or the animal systems, where about three to four times as much [$^2H_2$]oleic acid was produced from the *erythro*-precursor as [$^2H_1$]oleic acid from the *threo*-precursor.

Only one explanation would seem to be possible to account for these results, namely that there is a substantial kinetic isotope effect exerted by deuterium 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 L-9,L-10-$^2H_2$-labelled *erythro*-enantiomer. Since this explanation would seem to be necessary to account for the results of the conversion of stearic acid into oleic acid, it is likely that in the desaturations leading to linoleic acid and α-linolenic acid a partial isotope effect is operative at both positions, rather than a totally blocking isotope effect at only one position, as suggested above. Certainly I am unaware of any recorded instance of a kinetic isotope effect against deuterium or tritium sufficiently pronounced to prevent reaction totally.

These considerations imply that the desaturation reactions in *Chlorella* and in the animal systems probably involve a mechanism of simultaneous concerted removal of the pair of hydrogen atoms, rather than the stepwise sequence suggested for the bacterial system by Schroepfer & Bloch (1965). Such a concerted mechanism, in turn, rules out any formal oxygenated compound as an intermediate in fatty acid desaturation. Experiments with various stereospecifically tritiated precursors are currently going on, to establish quantitatively the magnitude of the kinetic isotope effect at each of the positions involved in these desaturations, and preliminary results (A. R. Johnson, M. I. Gurr & L. J. Morris, unpublished work) support the conclusions just outlined.

### *Biohydrogenation*

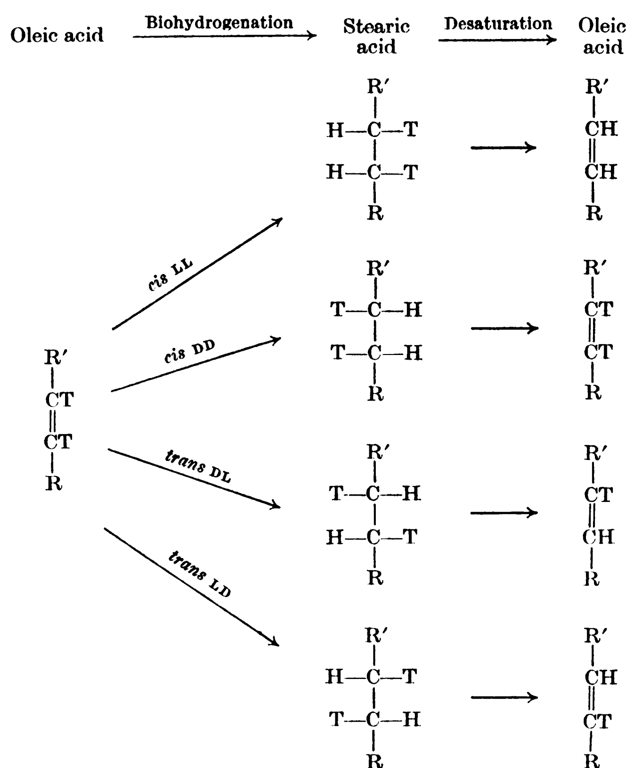
The stereospecificity of hydrogen removal in the desaturation of long-chain fatty acids can now be used to establish the absolute configuration of deuterium or tritium labels in appropriate positions in a fatty acid chain. One biochemical reaction thus becomes the tool with which the stereospecificity of other biochemical transformations can be investigated. One instance of this approach has already been discussed, namely the use by Schroepfer (1966) of the bacterial desaturase to prove that the hydration of oleic acid by the pseudomonad hydratase enzyme involved *trans*-addition of the elements of water (cf. Scheme 6).

We have recently begun to use this approach to look at the stereospecificity of the reverse process to desaturation, namely the biohydrogenation of unsaturated fatty acids effected by the rumen bacteria of such animals as goats, sheep and cows.

The whole question of rumen biohydrogenation of unsaturated fatty acids is quite complicated and will not be discussed here. Much elegant and detailed work on the pathways of hydrogenation of linoleic acid and α-linolenic acid, the major dietary fatty acids of ruminants, has been done in the last few years, notably by Dawson and his colleagues (e.g. Wilde & Dawson, 1966; Kemp & Dawson, 1968) and by Tove and his group (e.g. Kepler, Hirons, McNeill & Tove, 1966; Kepler & Tove, 1967). I am concerned, at the moment, only with the hydrogenation of the *cis*- and *trans*-isomeric monoenes, oleic acid and elaidic acid. If oleic acid or elaidic acid is incubated anaerobically with strained rumen contents, each is hydrogenated to stearic acid and to a lesser extent is isomerized to its geometric isomer; some oleic acid is transformed into elaidic acid and *vice versa*. Studies of the time-course of these various reactions, by using a mixture of [12,13-$^3H_2$]oleic acid and [1-$^{14}C$]elaidic acid as substrate, suggests that they are independent and that both the *cis*- and the *trans*-monoene is hydrogenated directly and not after a prior isomerization one way or the other (L. J. Morris, E. W. Hammond & R. Bickerstaffe, unpublished work).

The questions now are the reverse of those in desaturation. Is the addition of hydrogen atoms to these double bonds specific in any way and, if so, is it *cis*- or *trans*-addition and is there an absolute stereospecificity? These questions can be resolved, for the biohydrogenation of oleic acid, by using [1-$^{14}C$,9,10-$^3H_2$]oleic acid as substrate, isolating the stearic acid product and using it, in turn, as a substrate for desaturation by one of the desaturase systems of known stereospecificity, e.g. hen liver microsomal preparation, as summarized in Scheme 10.

Thus, if the biohydrogenation involves *cis*-addition of the two hydrogen atoms to the double bond, then *erythro*-ditritiostearic acid will result. An absolute stereospecificity in this addition would provide only DD- or LL-[9,10-$^3H_2$]stearic acid, which would suffer complete loss or complete retention of tritium label respectively on subsequent reconversion into oleic acid by the stereospecific desaturase of hen liver or *Chlorella*. Alternatively, *trans*-addition of hydrogen during biohydrogenation would provide *threo*-ditritiostearic acid, either [L-9,D-10-$^3H_2$]- or [D-9,L-10-$^3H_2$]-stearic acid, which would lose half its tritium label on subsequent desaturation to give [9-$^3H$]oleic acid or [10-$^3H$]-oleic acid respectively, depending again on absolute stereospecificity of the original hydrogenation.




Scheme 10. Summary of the stearic acid products of biohydrogenation of [1-$^{14}$C,9,10-$^{3}H_2$]oleic acid, by stereospecific *cis*- or *trans*-addition of hydrogen, and the oleic acid products that would be derived from them by stereospecific desaturation. $R = CH_3{\cdot}[CH_2]_7$; $R' = HO_2C{\cdot}[CH_2]_7$.

Non-stereospecific *cis*- or *trans*-addition or a completely random mechanism would give stearic acid products that would all lose half the tritium label on subsequent desaturation. In that event, determination of the biohydrogenation mechanism involved would be impossible with tritium labelling and would require the use of dideuterated substrate.

However, when the scheme was tested by incubation of [1-$^{14}$C,9,10-$^{3}H_2$]oleic acid with mixed micro-organisms from goat or sheep rumen contents, the stearic acid product lost all of its tritium label on desaturation back to oleic acid by either *Chlorella* or a hen liver microsomal preparation (L. J. Morris & E. W. Hammond, unpublished work). Therefore the rumen biohydrogenation of oleic acid is completely stereospecific and involves *cis*-addition of hydrogen atoms from the L-side.

The stereochemistry of biohydrogenation of the *trans*-double bond of elaidic acid can also be investigated in exactly the same way by using 9,10-ditritioelaidic acid as substrate. In this case *cis*-addition would give *threo*-ditritiostearic acid (either L-9,D-10 or D-9,L-10), which would lose half its tritium on enzymic desaturation to oleic acid, whereas *trans*-addition would give one or other *erythro*-enantiomer, which would lose or retain all its tritium on desaturation.

Experimentally we have found that half the tritium was lost from the hydrogenation product of [1-$^{14}$C,9,10-$^{3}H_2$]elaidic acid on subsequent desaturation with hen liver microsomal preparation (L. J. Morris & E. W. Hammond, unpublished work). Because the biohydrogenation of oleic acid has been shown to be completely stereospecific, we consider that we can rule out a random or even a non-stereospecific mechanism for the hydrogenation of elaidic acid. We believe, therefore, that this experiment has demonstrated *cis*-addition of hydrogen atoms to the *trans*-double bond of elaidic acid, but, as we have not yet established whether the final desaturation product is [9-$^{3}$H]- or [10-$^{3}$H]-oleic acid, we cannot yet state the absolute stereochemistry of this addition. We hope to have this information in the near future.

In conclusion, I wish to record my gratitude and my appreciation of the honour bestowed on me by

this award of the Fifth Colworth Medal. I am happy to take this opportunity to freely acknowledge the tremendous debt that I owe to many of my colleagues at Colworth House, notably to those who have collaborated with me in much of the work I have described: Dr Kit Hitchcock, Dr Roy Bickerstaffe, Dr Bill Kelly, Dr Ron Harris (who is now at the Tropical Products Institute), and my own and indispensable assistant, Mr Eugene Hammond. Most of all, I think, I wish to express my thanks to Professor Tony James for his continual interest, advice and encouragement, and for his gentle prodding at me over the years to get away from pure chemistry and into more biochemical research.

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