The stereochemistry and metabolism of fatty acids in plants

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THE STEREOCHEMISTRY AND METABOLISM OF FATTY ACIDS IN PLANTS

by

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Supervisor: L. J. Morris, B.Sc., Ph.D

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SUMMARY

The widespread occurrence of a large variety of long chain fatty acids has encouraged considerable investigation into the methods by which they are biosynthesised. The overall mechanisms of many of these pathways have been elucidated, but much remains to be discovered about the absolute stereochemistry of these reactions. This thesis describes work carried out to help define the stereochemistry of some of these reactions.

To investigate the stereochemistry of two of the intermediate steps of chain elongation in fatty acid biosynthesis, the synthesis of $\alpha\beta$-unsaturated and β-hydroxy fatty acids specifically labelled with either tritium or deuterium was undertaken. The incubation of these labelled acids with the green algae Chlorella vulgaris allowed the absolute stereospecificity of the dehydration and hydrogenation steps of acyl chain elongation to be determined.

Studies were also undertaken to determine the absolute optical configurations of several epoxy and vicinal dihydroxy fatty acids. Thus the absolute configurations of erythro- and threo-9,10-dihydroxystearates and some of their derivatives and of trans-9,10-epoxystearate, trans-12,13-epoxystearate and trans-12,13-epoxyoleate were defined.

Based on the above configurational knowledge, mechanisms for the stereospecific enzymic hydration of epoxy fatty acids in several natural systems were proposed. These proposals were verified for two natural systems (Vernonia seeds and Puccinia uredospores) by performing the enzymic hydrations in $H_2{}^{18}O$ and characterizing the position of the ^{18}O in the products. The hydratase enzyme from Vernonia seeds was isolated as a soluble preparation and its substrate specificity was investigated. From the combined results, a proposal was made to explain the stereospecific hydration of epoxy acids in this system.

CONTENTS

GENERAL INTRODUCTION

Long chain fatty acids are ubiquitous constituents of natural fats. The major fatty acids occurring naturally are all saturated or unsaturated monocarboxylic acids with a straight even numbered carbon chain. This structural similarity is due to the mechanism of their biosynthesis, which will be discussed in more detail later. The major saturated straight chain acids occurring in plants are lauric (dodecanoic), myristic (tetradecanoic), palmitic (hexadecanoic) and stearic (octadecanoic) acids. However, far more abundant than these in plants are the unsaturated C:18 homologues, oleic (cis-9-octadecenoic), linoleic (cis-9-,cis-12-octadecadienoic) and linolenic (cis-9-,cis-12-,cis-15-octadecatrienoic) acids.

In addition to the range of major fatty acids found in plants, there are a number of other acids which can be conveniently divided into two other groups - the "minor" acids, which occur in small amounts in a wide range of plants; and the "unusual" fatty acids, which may only occur in a few individual species, or be limited to a single family, but can be the principle fatty acid of the seed oil.

The minor saturated fatty acids include those with chain lengths either shorter or longer than the major acids. Thus some plants contain small amounts of hexanoic, octanoic and decanoic acids, and the longer chain acids, such as eicosanoic, docosanoic and tetracosanoic, are fairly widespread as minor components in seed oil triglycerides and as components of cuticular lipids. The minor unsaturated fatty acids are related to the major unsaturated fatty acids in one of two ways; the carbon chain is either extended or shortened at the carboxyl or methyl end of the

molecule. Thus, for example, shortening of the chain by two carbons from the methyl end, in oleic acid, gives rise to palmitoleic (cis-9-hexadecenoic) acid, while lengthening of the chain at the carboxyl end by four carbons, gives erucic (cis-13-docosenoic) acid. Two families of polyunsaturated fatty acids related to major fatty acids can be recognised: the linoleic series, which are related through having W-6, 9-double bonds, and the linolenic family, characterized by their W-3, 6,9-triene group. These two families of unsaturated acids are formed by elongation of preformed linoleic or linolenic acid, followed by desaturation between the carboxyl group and its nearest double bond.

A large number of unusual fatty acids have been found in plants, and the structures of these have been reviewed by Smith (1). These acids contain a variety of different functional groups, such as acetylenic bonds, conjugated acetylenic and ethylenic bonds, allenic groups, cyclopropane, cyclopropene, cyclopentene, and furan rings, epoxy, hydroxy and keto groups and double bonds of both the cis and trans configuration separated by more than one methylene group. Two or more of these functions may occasionally be found in the same molecule.

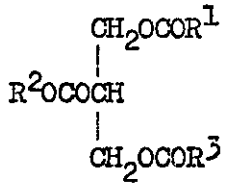
A wide range of oxygenated functions have been characterized as substituents of fatty acids. Monohydroxy acids are broadly distributed in nature and are found in small amounts in animals, plants and microorganisms. These acids may be saturated or they may contain one or more double bonds. The hydroxyl group may also confer optical activity to the molecule. Keto acids have been found in plants (2), some of these acids also contain conjugated unsaturation, and the keto group can itself be in conjugation with a pair or trans double bonds, such as in 9-keto-trans-10, trans-12-octadecadienoic acid, a minor constituent of Dimorphotheca oil (3).

Polyhydroxy acids containing two, three or four hydroxyl groups have been isolated from plants. The hydroxyl groups may be widely separated in the chain such as 10,16-dihydroxypalmitate isolated from cutin (4, 5), or may be on adjacent carbon atoms such as in erythro-9,10-dihydroxy-stearate from castor oil (6). The chain length of these vicinal dihydroxy acids can range from 16 to 24 carbon atoms (7) and the hydroxyl groups may have either the erythro or threo configuration. The vicinal dihydroxy acids are discussed in more detail later.

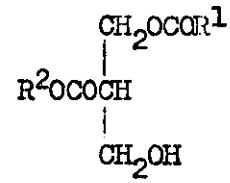
Closely related to the vicinal dihydroxy acids are the epoxy acids. Their structure usually resembles the more usual unsaturated acids with the oxirane ring replacing one of the double bonds. All of the naturally occurring epoxy acids identified to date have a chain length of 18 carbon atoms, and all but one have a cis oxirane ring. Like the polyhydroxy and monohydroxy acids, the oxygen function makes the acids potentially enantiomeric. Part of the work presented in this thesis was designed to elucidate the absolute stereochemical configurations of some epoxy and polyhydroxy acids and investigate their interconversion in natural systems.

Fatty acids rarely occur naturally in the free form, but are chemically combined, usually as esters and occasionally as amides or ethers, to form complex lipids (8). The vast bulk of the acids are found esterified to the trihydric alcohol glycerol.

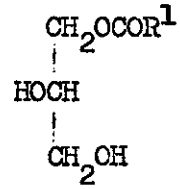
The largest group of plant lipids are the glycerides, in which fatty acids only are esterified to glycerol. The simplest of this group is triglyceride, in which all three of the hydroxyl group of glycerol are esterified with fatty acids. If the two primary positions contain different fatty acids, then the triglyceride contains an asymmetric centre and may exist in different enantiomeric forms (9), Diglycerides and monoglycerides usually occur naturally only in small quantities, although 1,2-diglycerides are intermediates in the formation of other types of lipid.



Triglyceride



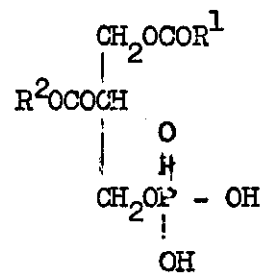
Diglyceride



Monoglyceride

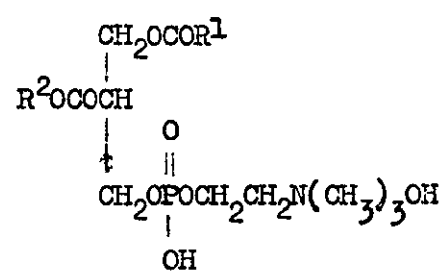
Generally, the glycerides serve as storage depots in both plants and animals and tend to decrease during starvation.

A separate class of lipids, the phospholipids, contain an atom of phosphorus as well as glycerol and the esterified fatty acids. Plant phospholipids are structural derivations of 1,2-diacyl-sn-glycero-3-phosphoric acid, phosphatidic acid, which is found coupled with a nitrogenous base, insitol or another glycerol molecule. Phosphatidic acid itself is rarely found as a major component of plant lipids, but, like 1,2-diglycerides, it is an important intermediate in lipid biosynthesis.



Phosphatidic acid

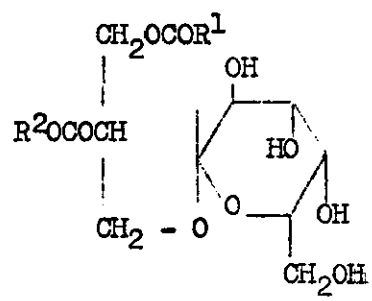
The most common nitrogenous bases coupled to phosphatidic acid are choline, ethanolamine and serine.



Phosphatidyl choline

The phospholipids are optically active, and only one of their enantiomeric forms occurs in Nature, these optically active forms being the derivative of 1,2-diacyl-sn-glycero-3-phosphoric acid as shown on the previous page.

Glycolipids are major constituents of plants and microorganisms (8). They are based on glycerol and are structurally analogous to phospholipids, with a range of carbohydrate units attached glycosidically to the glycerol molecule in place of the phosphate group. In higher plants, the most abundantly occurring sugar is galactose. The sulphonic acid of D-quinovose is found linked to glycerol in the sulpholipids of all photosynthetic plants, algae and bacteria.



Monogalactosyl diglyceride

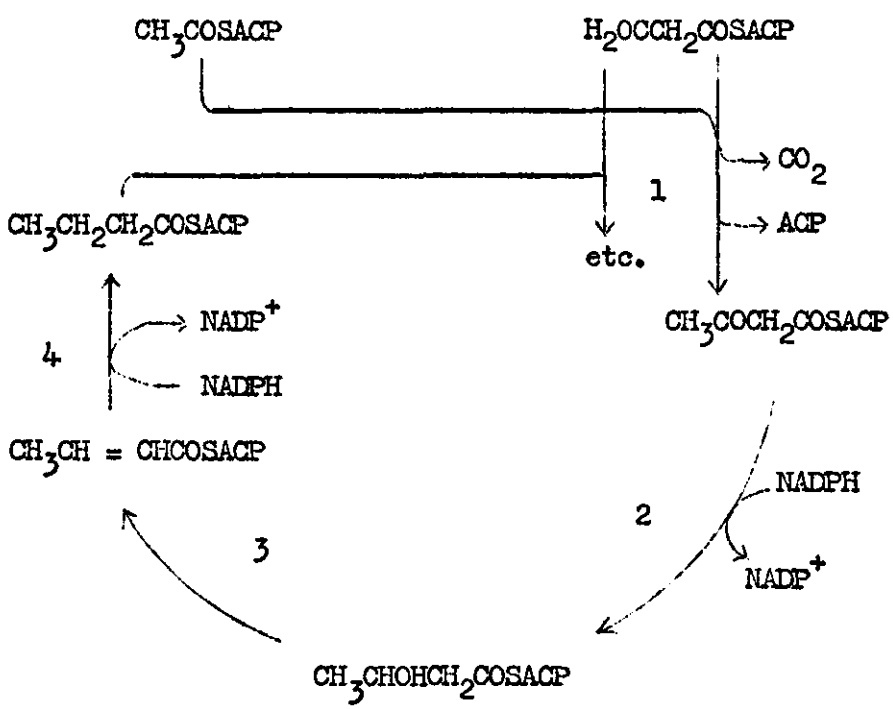
Accepting that long chain acids seldom occur in the free form, the biosynthetic pathways to the major types of acids can now be discussed briefly.

The major pathway for the biosynthesis of fatty acids is the malonyl-CoA pathway. This pathway is distributed throughout animals, plants and micro-organisms (10, 11, 12). The first reaction of this pathway is the formation of malonyl-CoA from acetyl-CoA by the biotin dependent enzyme acetyl-CoA carboxylase. This is a key step in the biosynthesis of fatty acids, since a subsequent decarboxylation of the malonyl-CoA helps drive the equalibrium towards synthesis.

$$HCO_3^- + \text{biotin-enzyme} + ATP \xrightleftharpoons{Mg^{2+}} CO_2\text{-biotin-enzyme} + ADP + Pi$$

$$CO_2\text{-biotin-enzyme} + \text{acetyl-CoA} \rightleftharpoons \text{biotin-enzyme} + \text{malonyl-CoA}$$

The malonyl-CoA formed in the above reaction is transferred to acyl carrier protein (ACP) and condensed with acetyl-ACP. The β-ketobutyryl-ACP produced is then reduced to β-hydroxybutyryl-ACP which undergoes dehydration and hydrogenation to form butyryl-ACP (Scheme 1). Repeated passage through this pathway results in the formation of palmitic or stearic acid.



1 condensing enzyme

2 3-keto-acyl-ACP reductase

3 enoyl-ACP hydratase

4 enoyl-ACP reductase

Scheme 1. Biosynthesis of saturated fatty acids

Other pathways for the biosynthesis of saturated fatty acids involve the elongation of preformed fatty acids (13, 14). A pathway which elongates preformed acids by condensation with acetyl-CoA is present in rat liver mitochondria (15). This reaction is independent of bicarbonate and was originally thought to be a reversal of β-oxidation. However, the β-hydroxy intermediate in this pathway has the D(-)-configuration and is the enantiomer of the hydroxy intermediate formed and utilized in β-oxidation. The pathway involves the condensation of the CoA derivatives of the fatty acid and acetate, in the presence of NADPH and NADH and is shown below.

$$RCOSCoA + CH_3COSCoA \rightleftharpoons RCOCH_2COSCoA + CoA$$

$$RCOCH_2COSCoA + NADH + H^+ \rightleftharpoons RCH(OH)CH_2COSCoA$$

$$RCH(OH)CH_2COSCoA \rightleftharpoons RCH \overset{t}{=} CHCOSCoA$$

$$RCH = CHCOSCoA + NADPH + H^+ \rightleftharpoons RCH_2CH_2COSCoA$$

Alternatively, the rat liver microsomal fraction is capable of elongation of preformed fatty acids by addition of malonyl-CoA units (16).

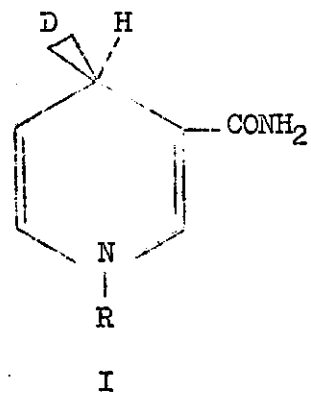
Considering the pathways outlined above, it can be seen that these modes of fatty acid synthesis involve two enzyme catalyzed hydrogen transfer reactions. The first of these is the transfer of hydrogen from either of the pyridine nucleotides, NADPH or NADH, to a β-keto-acyl derivative to form the β-hydroxy compound; and the second of these reactions is the transfer of a hydrogen atom from NADPH, and addition of a proton to an $\alpha\beta$-unsaturated-acyl derivative to give the saturated compound. Both of these transfer reactions are potentially stereospecific and during the course of this work the absolute stereochemistry of the hydrogen atom at C-4 of the reduced pyridine nucleotide utilized in these reactions was described (17).

The stereospecificity of enzyme catalyzed hydrogen transfer reactions has received considerable attention. In general, the stereochemical questions in such reactions involve both the substrate and the coenzyme involved. In some reactions, the transferred hydrogen of the coenzyme is bound in a planar configuration, such as to a nitrogen atom in flavins, and the problems of stereochemistry of the coenzymes are obviously not involved. Conversely, the C-4 methylene group of a reduced pyridine nucleotide is prochiral and the transfer of a hydrogen atom from this group is potentially stereospecific. Obviously, the reverse of this reaction, the transfer of hydrogen to C-4 of an oxidized pyridine nucleotide, leads to the development of a prochiral situation.

The first demonstration of the direct transfer of hydrogen to NAD^+ from the oxidized substrate was made by Westheimer et al (18). The reaction studied was the oxidation of ethanol to acetaldehyde catalysed by yeast alcohol dehydrogenase. It was shown that performing the reaction in D_2O did not lead to incorporation of deuterium into the reduced pyridine nucleotide, whereas the oxidation of [1,1-$^2H_2$] ethanol by the same system gave a reduced nucleotide contained one atom of deuterium, proving that direct transfer had occurred.

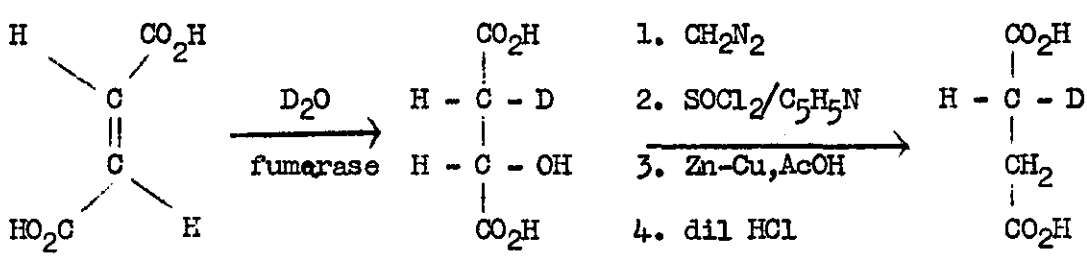
Following on from this work, the same group provided the first evidence of the prochirality of a methylene group (19). Deuterated NADH, prepared enzymatically, was used to reduce acetaldehyde with yeast alcohol dehydrogenase and the NAD^+ formed was shown to contain no deuterium. However, the reduction of acetaldehyde by the same system, but using chemically prepared deuterated NADH gave NAD^+ which contained deuterium. Thus the chemically prepared deuterated NADH must have been a mixture of two diastereoisomers, whereas the enzymatically prepared nucleotide was a single diastereoisomer.

The diastereoisomer of deuterated NADH prepared by reduction of NAD^+ with alcohol dehydrogenase, using [1,1-$^2H_2$] ethanol as a substrate, is known as the A form (I), and the opposite diastereoisomer is known as the B form. If the deuterium in I is replaced by a hydrogen atom, this hydrogen is known as H_A, and enzymes which utilize this hydrogen are said to have A side stereospecificity.



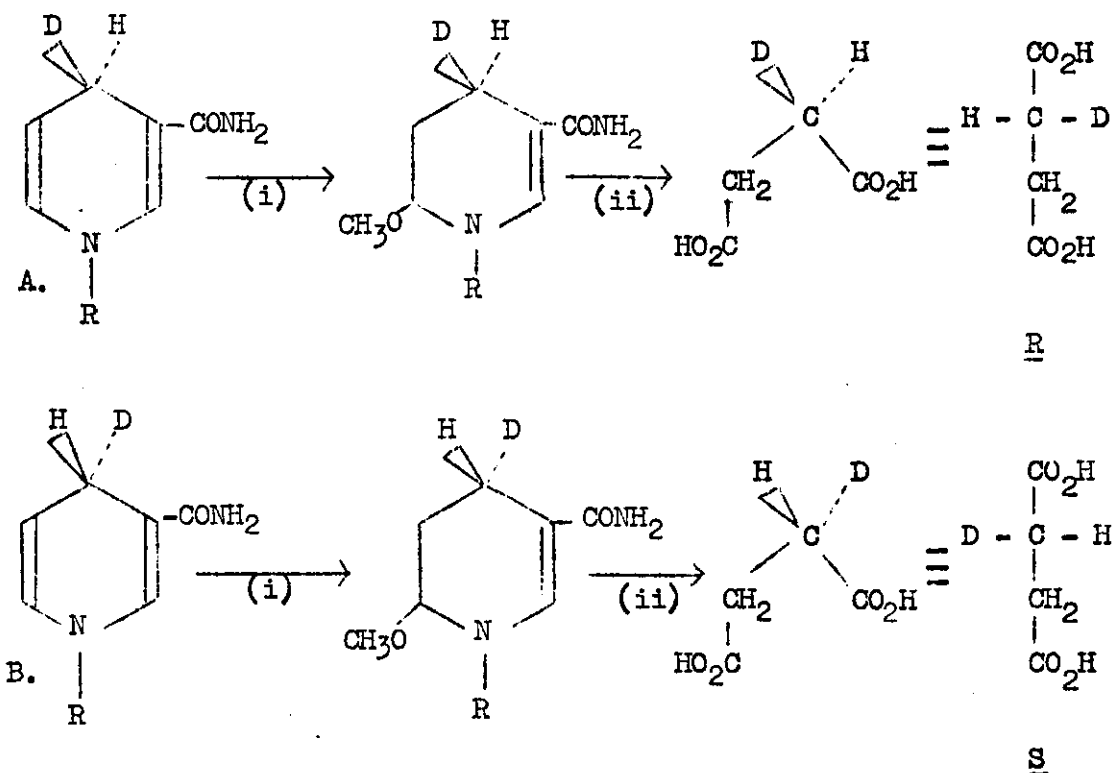
I

The demonstration of the prochirality of the C-4 methylene group of NADH outlined above did not, however, define the absolute configuration of the nucleotide. The identification of the H_A and H_B positions of NADH was resolved by Cornforth et al (20) on the basis of the known stereochemistry of R-deuteriosuccinic acid. R-Monodeuteriosuccinic acid was prepared by the enzyme catalyzed addition of D_2O to fumaric acid, to give 2S,3R-deuteriomalic acid, followed by chemical removal of the hydroxyl group. The monodeuterated product prcved to be laevorotatory.



Preparation of R-monodeuteriosuccinic acid

Samples of the A and B deuterated NADH were then each converted by chemical degradation to deuteriosuccinic acid (Scheme 2). The two specimens of deuterated succinic acid obtained proved to be enantiomeric, and that prepared from NADH deuterated on the A side proved to be identical to the known 2R-deuteriosuccinic acid. From these results, it follows that H_A is the pro-R hydrogen at C-4 of the pyridine nucleotide and H_B is the pro-S hydrogen.



(i) Dry CH_3OH + CH_3CO_2H

(ii) O_3/Peroxyacetic acid

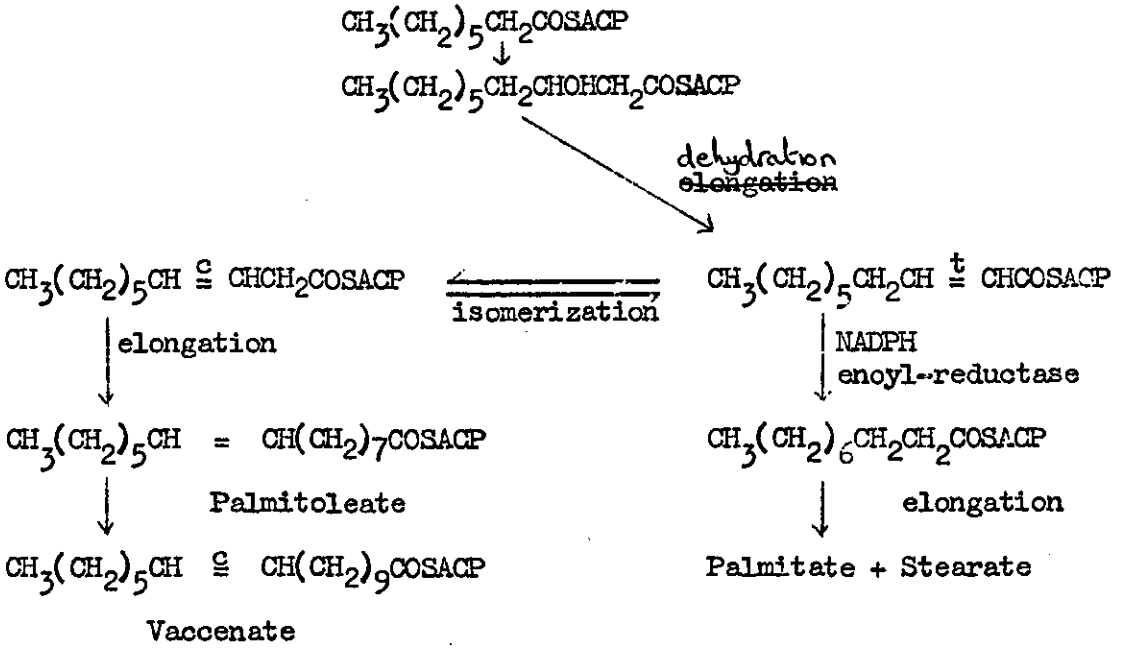
Scheme 2. Determination of the stereochemistry of nicotinamide coenzymes

It has been shown that the stereochemistry at C-4 is the same in both NADH and NADPH (21).

Like saturated fatty acids, the biosynthesis of unsaturated fatty acids is known to proceed by at least two different pathways - the aerobic and the anaerobic pathways (22).

The anaerobic pathway is found mainly in anaerobic micro-organisms, although it is not confined to these alone. Some facultative anaerobes, and even obligate aerobes (pseudomonas sp) synthesize long chain unsaturated acids by this pathway (23).

The mechanisms of anaerobic biosynthesis of unsaturated fatty acids, as outlined in Scheme 3, is essentially a diversion of the de novo synthesis of long chain saturated acids. This diversion of fatty acid synthesis occurs at the C:10 and C:12 chain length stages. At this point, the trans-2-enoyl-ACP intermediate of acyl chain elongation is isomerized to cis-3-enoyl-ACP, which is not a substrate for the enoyl reductase, but is capable of elongation. The cis-3-decenoate and -dodecenoate formed are then the precursors for the long chain unsaturated acids, the double bond being retained while further chain extension occurs. Thus, elongation of cis-3-decenoate gives palmitoleic and vaccenic acids, whereas elongation of the unsaturated dodecenoate affords cis-7-hexadecenoic and oleic acids.



Scheme 3. Anaerobic pathway of monoenoic acid biosynthesis

The most widespread biosynthetic pathway leading to unsaturated fatty acids is the aerobic or oxidative pathway, in which a double bond is introduced directly into a preformed long chain fatty acid. This pathway occurs almost universally and is employed by bacteria, protozoa, yeasts, algae, and higher plants and animals.

The pathway was first demonstrated in cell free preparations of yeast (25) which catalysed the transformation of saturated to monenoic acids with oxygen and NADPH as cofactors. The requirement for oxygen is absolute (24) and its exclusion or substitution with artificial electron acceptors resulted in the inhibition of desaturation. Activation of the fatty acids to their CoA or ACP thioesters is also necessary for the reaction.

It was initially believed that a second aerobic pathway to unsaturated fatty acids existed in higher plants. This pathway was supposed to differ from that found in bacteria, yeasts, algae and animals because while plants could synthesize long chain monoenoic acids from acetate, unlike these other systems, they seemed unable to desaturate long chain fatty acids or their corresponding CoA esters (26, 27, 28), although these could be incorporated into lipids.

The differences between the plant pathway and the normal aerobic pathway for unsaturated acid biosynthesis were elucidated in two experiments. In one (29), leaf tissue was allowed to synthesize fatty acids from acetate in strictly anaerobic conditions, when only palmitate and stearate were formed. Transfer to aerobic conditions resulted in the disappearance of labelled stearic acid, which was replaced by an equivalent amount of oleic acid. The other experiment (30) showed that leaf chloroplast preparations were able to convert stearoyl-ACP, but not stearic acid or stearoyl-CoA, into oleic acid. From these results, it would appear that there is no mechanistic difference between the plant and oxidative pathways, but that the plant system lacks the enzyme to transfer long chain fatty acids from CoA to ACP and it is the ACP esters which are needed for desaturation.

Like most enzymic transformations, the desaturation of fatty acids is highly stereospecific. The stereospecificity of this desaturation was at first investigated by Schroepfer and Bloch (31). They prepared 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 substrates for the desaturase system of Corynebacterium diphtheriae. 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 atoms were lost on desaturation. Thus it was proved that the organism effected a completely stereospecific desaturation of stearic to oleic acid removing only the D-9 and D-10 hydrogen atoms.

Morris et al (32) investigated the desaturation of stearic acid to oleic acid in Chlorella vulgaris using a different approach to that outlined above. They prepared erythro-[9,10-$^{2}H_2$]- and threo-[9,10-$^{2}H_2$]-stearic acids and [D-9-^{3}H]- and [L-9-^{3}H]-stearic acids, and incubated these with Chlorella. Analysis of the oleate demonstrated that desaturation involved the loss of the D-9 hydrogen atom and of a pair of hydrogen atoms of cis relative configuration. The results, therefore, showed a similar stereospecific removal of the D-9 and D-10 hydrogen atoms from stearic acid, as found in Corynebacterium. The stereospecificity of desaturation of stearic acid to oleic acid has also been investigated in goat mammary gland, hen liver and fish liver, and in all cases has been shown to proceed by loss of the D-9 and D-10 hydrogen atoms (33).

Polyunsaturated fatty acids are also extremely common throughout living systems and only bacteria and some blue green algae seem unable to synthesize them. The biosynthesis of polyunsaturated fatty acids involves sequentially desaturations similar to that involved in the conversion of stearate to oleate, interspersed where necessary with chain elongation. The position of the second and subsequent double bonds introduced into the chain is not random, but usually spaced from an existing double bond so as to give the common methylene interupted polyunsaturated acids.

In plants desaturation generally occurs between the first double bond and the methyl end of the chain, such that cis-9-octadecenoic acid is first desaturated to a 9,12-dienoic and then to a 9,12,15-trienoic acid. The stereochemistry of these desaturations has been investigated by Morris et al (32) in an analogous manner to that used for the investigation of the stearate to oleate desaturation. Incubation of [D-12-^{3}H]- and [L-12-^{3}H]-stearic acids and the erythro- and threo- isomers of [12,13-$^{2}H_{2}$] oleic acid, with Chlorella vulgaris showed that the desaturation of oleic to linoleic acid involved the loss of the D-12 and D-13 hydrogen atoms from the oleic acid. The subsequent desaturation of linoleic to linolenic acid was investigated using both erythro- and threo-[15,16-$^{2}H_{2}$]-oleic acid, and shown to involve cis removal of hydrogen at the 15 and 16 positions, which by analogy probably also involve the abstraction of two hydrogen atoms of the D configuration.

In animal systems, second and subsequent double bonds are introduced almost exclusively between the first double bond and the carboxyl group; for example, desaturation of linoleic acid gives γ-linolenic (6,9,12-octadecatrienoic) acid. Elongation of such acids, followed by subsequent desaturation, gives rise to a series of C:20 and C:22 polyenoic acids characteristic of animal systems.

Certain long chain polyunsaturated fatty acids are essential constituents of the diet of animals (34). Deficiencies in these acids, which arise if they are not present in the diet, lead to retarded growth rate, interference with the reproductive cycle and may result in eventual death. These symptoms can be alleviated by supplying the animal with these essential acids. The acids which alleviate deficiency most readily, all contain the same structure towards the methyl end, a diethenoic grouping with the first double bond at the n-6 position (e.g. linoleic γ-linolenic and arachidonic acids (35). The most important of these being

arachidonic acid which is produced in animals from linoleic acid by desaturation and elongation. However, α-linolenic and certain other n-3 polyunsaturates have also been shown to possess some essential properties.

The biological conversion of these essential fatty acids to a group of naturally occurring substances called prostaglandins, which show a marked physiological acitivity (36), probably explains their essential nature.

Enzyme mediated reactions show a great deal of steric control, and the absolute specificity for a particular stereoisomer of a given compound is a common biological occurrence. The field of fatty acid biosynthesis is no exception to this, and the completely stereospecific nature of the desaturation of long chain fatty acids has been discussed above. It would be unusual, therefore, if the individual reactions of saturated fatty acid biosynthesis did not similarly show an absolute specificity and the work presented in the first part of this thesis has been an investigation of the stereochemistry of some of these individual reactions.

Two of these individual reactions have been investigated in this work, namely the dehydration of D-(-)-β-hydroxyacylthioester to $\alpha\beta$-enoylthioester and the subsequent hydrogenation of the $\alpha\beta$-monoene to give saturated acylthioester.

$$RCH(OH)CH_2COSACP \rightleftharpoons RCH \overset{t}{=} CHCOSACP$$

$$RCH = CHCOSACP + NADPH + H^+ \rightleftharpoons RCH_2CH_2COSACP + NADP^+$$

In the first of these steps, it is known that the dehydratase enzyme is specific for the D-hydroxy compound and that the product is a trans monoene. However, the overall stereochemistry of this dehydration was unknown and the question asked in this case was whether the reaction involved a cis or trans elimination of the elements of water.

The second step involves the hydrogenation of the trans-monene produced by dehydration of the β-hydroxy intermediate. This reaction obviously involves the addition of two atoms of hydrogen, but again, the overall stereochemistry of this addition was not known.

The question asked about this reaction, therefore, was whether the hydrogenation occurs by overall cis or trans addition of hydrogen, and what are the absolute configurations assumed by the incoming hydrogen atoms.

The experiments reported in the first part of this thesis have been designed to study the stereochemistry of these individual reactions.

As mentioned earlier, fatty acids containing epoxy or vicinal dihydroxy groupings occur naturally. Most of these naturally occurring oxygenated acids are optically active and the absolute configurations of some of these acids have been determined. The second section of this thesis has been devoted to the determination of the absolute configurations of some naturally occurring dihydroxy acids, some epoxy acids, and their derivatives.

Once the stereochemistry of these compounds was known, the mechanism of the enzymic hydration of the endogenous epoxy acids of various seeds and plant rust spores could be predicted. In the third part of this thesis, these predictions have been tested experimentally and the specificity of an epoxide hydratase enzyme isolated from Vernonia anthelmintica seeds has been investigated.

INTRODUCTION

The majority of fatty acids found in nature have straight chains containing an even number of carbon atoms. They may have different chemical features, such as double bonds or a hydroxyl group, at specific positions in the molecule, but generally they are of similar structure. Their structural similarities tend to indicate that the biosynthesis of these compounds, in different organisms, occurs in a similar manner.

One of the first major advances in the biochemical study of fatty acids came when Knoop in 1904 detailed a pathway for their degradation by β-oxidation. It took almost forty years for his pathway to gain full experimental confirmation but his findings became the basis of extensive research into the metabolism of fatty acids. When the mechanism of β-oxidation was defined, it seemed possible that fatty acids were synthesised by a reversal of this mechanism, since every step in the enzymic degratation of fatty acids is potentially reversable. However, purified β-oxidation systems converted acetyl-CoA no higher than the four carbon acyl derivative (37), and the isolation of systems capable of synthesising fatty acids in the absence of enzymes of the β-oxidation cycle led to the discovery of a separate pathway of fatty acid biosynthesis.

There are several pathways for the biosynthesis of fatty acids, two of which start from acetyl-CoA and are concerned with *de novo* synthesis. In *de novo* synthesis, elongation occurs by condensation of acetyl-CoA with either malonyl-CoA or further molecules of acetyl-CoA, the former pathway being the major one for *de novo* synthesis of fatty acids in a wide variety of plants, animals and micro-organisms (10,11,12). The latter pathway bears the same resemblance to β-oxidation in reverse.

Several features of the biosynthetic pathway to long chain fatty acids from acetate indicated that it was not the reversal of β-oxidation. It was found that a pyridine nucleotide was required, but in synthesis this nucleotide was NADPH, as opposed to the requirement for NAD^+ in β-oxidation. Further, a requirement for carbon dioxide or bicarbonate, and ATP was noticed in the fatty acid synthetase partially purified from extracts of avian liver (38), these two components cannot be accounted for by the scheme of β-oxidation. Although bicarbonate, or carbon dioxide, is required for fatty acid synthesis, it is not incorporated into the final long chain acid synthesised. It is required for the carboxylation of acetyl-CoA to malonyl-CoA (39), the latter being the "two carbon" donor in fatty acid synthesis.

The reactions of fatty acid synthesis in the bacterium Escherichia coli are shown in Scheme 1; the individual steps are discussed in more detail below.

1) $CH_3COS\text{-}CoA + HCO_3^- + ATP \rightleftharpoons HO_2CCH_2COSCoA + ADP + Pi$

2) $CH_3COS\text{-}CoA + ACP\text{-}SH \rightleftharpoons CH_3COS\text{-}ACP + CoA\text{-}SH$

3) $HO_2CCH_2COS\text{-}CoA + ACP\text{-}SH \rightleftharpoons HO_2CCH_2COS\text{-}ACP + CoA\text{-}SH$

4) $CH_3COS\text{-}ACP + HO_2CCH_2COS\text{-}ACP \longrightarrow CH_3COCH_2COS\text{-}ACP + CO_2 + ACP\text{-}SH$

5) $CH_3COCH_2COS\text{-}ACP + NADPH + H^+ \rightleftharpoons D(-)\ CH_3CHOHCH_2COS\text{-}ACP + NADP^+$

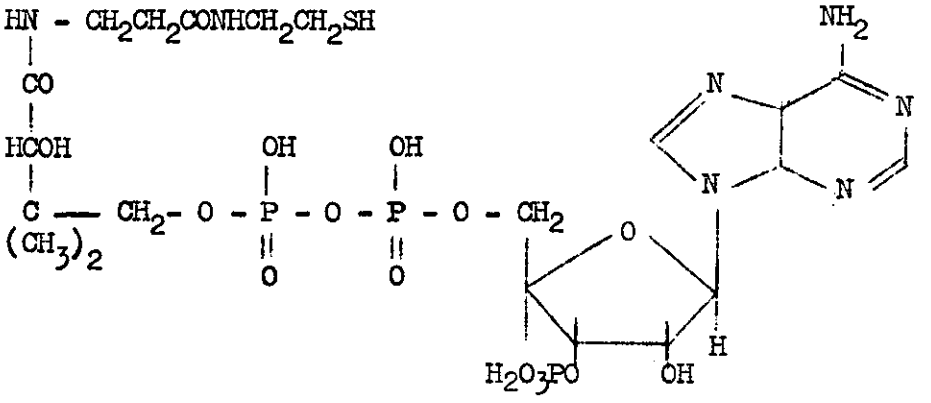
6) $D(-)\ CH_3CHOHCH_2COS\text{-}ACP \rightleftharpoons CH_3CH \overset{t}{=} CHCOS\text{-}ACP + H_2O$

7) $CH_3CH \overset{t}{=} CHCOS\text{-}ACP + NADPH + H^+ \longrightarrow CH_3CH_2CH_2COS\text{-}ACP + NADP^+$

Scheme 1. Fatty acid synthesis in E. Coli

It can be seen that the first requirement for the synthesis is that the acyl groups are in an activated form as their coenzyme-A (CoA) esters, and most of the metabolic reactions of acetate and its higher homologues

require that the acyl group is activated in this way. The active form is usually the thiol ester of the fatty acid with the nucleotide CoA, or with the small protein, acyl carrier protein (ACP).



Structure of Coenzyme-A

The formation of the CoA or ACP ester facilitates the enzymic reactions of fatty acids in a number of ways. The ester is water soluble enabling the enzyme to act in an aqueous environment, and the fatty acid no longer has a free carboxyl group, which can interact non-specifically with protein. The thiol ester also has a "high energy" bond between the carboxyl function of the fatty acid and the -SH group of the coenzyme, making it more susceptable to nucleophilic attack and carbanion formation.

The activation of fatty acids in living systems is catalyzed by thiokinases (acyl-CoA synthetases), which are present in a wide variety of living systems. The esterification requires ATP and magnesium or manganese ions. At least three enzymes have been discovered which catalyse this esterification, each active over a different part of the chain length spectrum, and overlapping in their specificities. Acetic thiokinase activates acetate and propionate, a long chain thiokinase activates

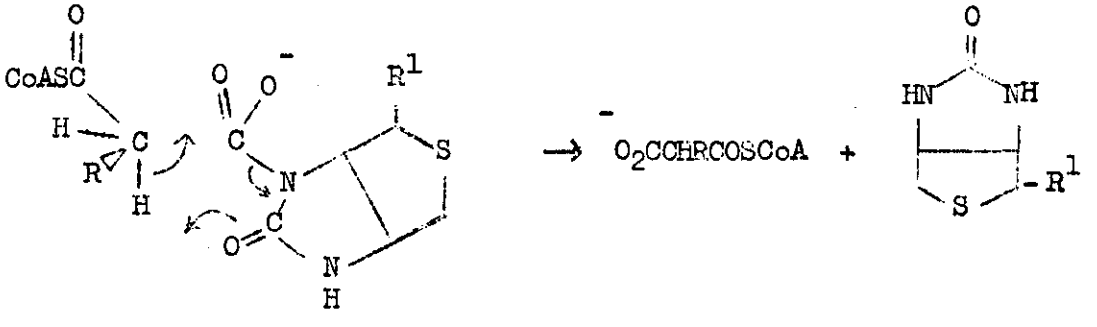
fatty acids from C:10 to C;20, and a general thiokinase activates fatty acids from C:4 to C:12, as well as branched chain acids, $\alpha\beta$ - and $\beta\gamma$ -unsaturated acids, and β -hydroxy acids.

The first reaction in the biosyntheses of fatty acids after activation to the thiol esters, is the carboxylation of acetyl-CoA to give malonyl-CoA. The reaction is catalysed by acetyl-CoA carboxylase and requires ATP and bicarbonate (38, 39, 40). Acetyl-CoA carboxylase was shown to contain biotin, which is covalently bound to the protein. The enzyme is inhibited by avidin, an egg white protein which binds biotin (41), and this inhibition was reversed by the addition of free biotin. The enzyme has been isolated from several sources, such as liver (42) and yeast (179) and catalyses the following reactions.

$$\text{Biotin-protein} + \text{ATP} + HCO_3^- \rightleftharpoons CO_2\sim\text{biotin protein} + \text{ADP} + \text{Pi}$$

$$CO_2\sim\text{biotin-protein} + CH_3COSCoA \rightleftharpoons \text{biotin-protein} + HO_2CCH_2COSCoA$$

The acetyl-CoA carboxylase isolated from E.coli has been fractionated into three separate protein fractions. One fraction catalyses the carboxylation of added biotin, the second is a protein containing biotin, which is the normal substrate for the biotin carboxylase, and the third subunit, which contains no biotin, catalyses the transfer of CO_2 from biotin to CoA. A mechanism for this transcarboxylation has been proposed by Lynen (43), the intermediate being 1'-N-carboxybiotin which is attached to the protein by an amide bond:-



$R^1 = (CH_2)_4$-CO-lysyl-protein

This mechanism explains the strict stereospecificity shown in the transcarboxylation of propionyl-CoA ($R' = CH_3$) to yield S-methylmalonyl-CoA, the entering carboxyl group occupying the same configuration as the hydrogen atom displaced (44).

The next step in fatty acid synthesis is the transformation of malonyl-CoA into long chain fatty acids. This reaction requires acetyl-CoA and NADPH, and is catalysed by a series of enzymes, usually called the fatty acid synthetase. This synthetase has been isolated from a number of sources, such as mammalian and avian liver (46), yeast and bacteria (10, 45). The synthetase from mammalian and avaian liver, and yeasts are isolated as a single multienzyme unit. No free intermediates accumulate during the synthesis of fatty acids, and these synthetases are stimulated by thiols and inhibited by -SH binding agents. These facts led Lynen (10) to propose intermediates bound via a sulphydryl group to a multienzyme complex.

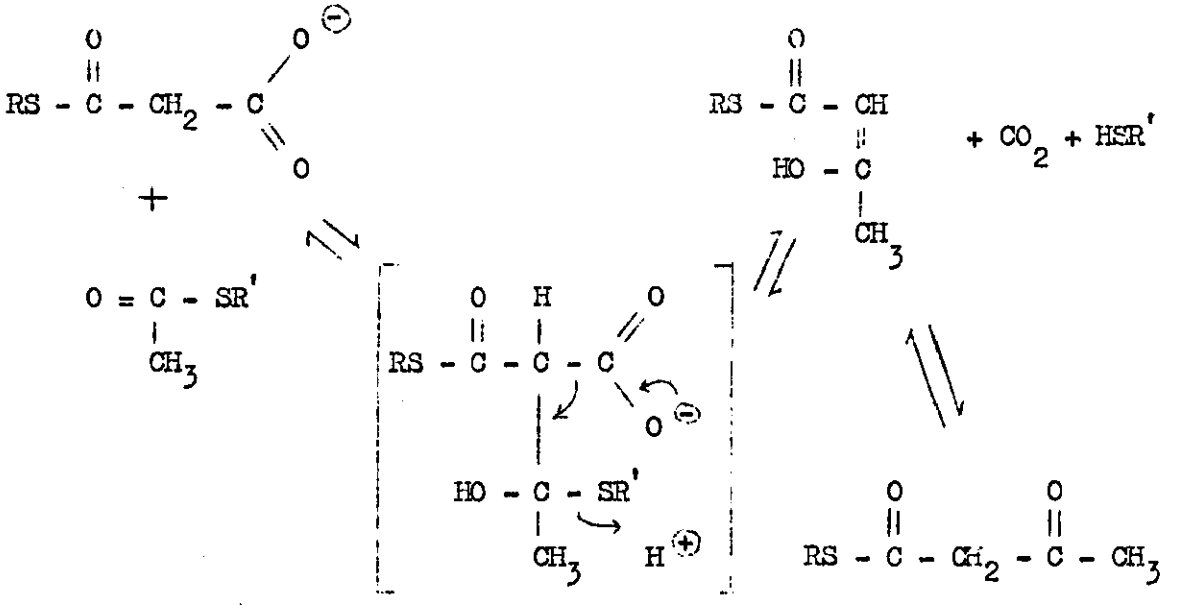
In contrast to the synthetases from liver and yeast, that isolated from E. coli could be resolved into several protein fractions, containing each of the enzymes responsible for the synthesis of long chain fatty acids from acetyl-CoA and malonyl-CoA. As well as these enzymes, a non-enzymic component was isolated and this was shown to be a protein acting as an acyl acceptor, in a similar manner to CoA, and was named acyl carrier protein (ACP) (47, 48). ACP's are stable to heat and pH changes and contain a single sulphydryl group which is part of the prosthetic group, 4'-phosphopantetheine, which is very similar to CoA. Acyl carrier proteins have also been isolated from plant (49) and yeast systems (50).

The isolation of the individual enzymes of fatty acid synthesis from E. coli (51) allowed the general pathway of fatty acid biosynthesis to be established and each of the individual reactions involved in synthesis, as shown in Scheme 1, to be investigated.

The enzymes of fatty acid synthesis will not accept substrates as their CoA esters, and these must first be converted to the ACP esters. The enzymes which catalyse this change are known as transacylases. Two transacylases have been isolated from E. coli. Malonyl CoA:ACP transacylase is specific for malonyl-CoA and will not accept acetyl-CoA (52, 53), it is heat stable and contains an active sulphydryl group. The acetyl CoA:ACP transacylase catalyses the transfer of acetate, but will also accept propionyl-CoA and higher homologues, the longer chain length substrates being less readily accepted; malonyl-CoA is inactive (52, 53). The transfer of acetate from acetyl-CoA to ACP is known as the "priming" reaction, and this is the only molecule of acetate which is used as such in de novo synthesis, the further condensations occurring with malonate. If acetyl-CoA is replaced in this priming reaction with propionate or a branched chain acid, then the product of fatty acid biosynthesis is either an odd chain or branched chain fatty acid.

The next step in the synthesis is the condensation of acetyl-ACP and malonyl-ACP to form acetoacetyl-ACP. This reaction is catalysed by 3-ketoacyl-ACP synthetase (54). The enzyme shows an absolute specificity for ACP esters, but is not specific for a single chain length, and will accept all the acyl intermediates in the synthesis of palmitoyl-CoA. The rate of reaction increases with increasing chain length. The enzyme has a functional -SH group and is readily inhibited by sulphydryl binding agents. Acetyl-ACP protects the enzyme against these inhibitors, suggesting that an acetyl-S-enzyme complex is an intermediate in the condensation.

The condensation of acetyl-ACP and malonyl-ACP occurs with an accompanying decarboxylation, and it is this decarboxylation that explains the fact that while bicarbonate, or CO_2, are cofactors in the biosynthesis of fatty acids they are not included in the final product. The decarboxylation also shifts the equilibrium of the reation , so as to favour condensation. A mechanism for this condensation during fatty acid synthesis in yeast has been suggested by Lynen (43).



After condensation of acetyl-ACP and malonyl-ACP the product, acetoacetyl-ACP, is reduced with NADPH to give β-hydroxybutyryl-ACP. This reduction is catalysed by the enzyme β-ketoacyl-ACP reductase (55). The enzyme shows a wide substrate specificity and is equally active on β-ketoacyl ACP's of chain length C:4 to C:16. The reductase is specific for NADPH and will not accept NADH; it has a marked preference for ACP esters, but will reduce CoA esters more slowly. The product is the D(-) β-hydroxyacyl enantiomer. The reaction is reversible, the reaction equilibrium favouring formation of the β-hydroxy compound. Only the D- enantiomer is utilized in the reverse reaction; the L-3-hydroxyacylACP enantiomer is not oxidized to β-ketoacyl ACP by this enzyme system.

3-Hydroxybutyryl-ACP is then converted to crotonyl-ACP by an enoyl hydratase (56), three of which have been isolated from E.coli. These hydratases catalyse the reversible dehydration of the D(-)-enantiomers only, the L(+)-enantiomers are not utilized. The enzymes are specific for ACP esters and completely inactive with CoA esters. The three dehydratases isolated from E. coli exhibit different chain length specificities. The first, β-hydroxybutyryl-ACP dehydratase is active with derivatives of chain length C:4 to C:8, the shorter chain lengths

being more readily utilized. The enzyme specific for the intermediate chain lengths, β-hydroxyoctanoyl-ACP dehydratase, will accept derivatives of chain length C:4 to C:12, with the longer homologues being the most readily dehydrated. The third enzyme, β-hydroxypalmitoyl-ACP dehydratase, is specific for C:12 to C:16 chain lengths. Thus the whole range of fatty acid chain lengths of the intermediates in chain elongation is encompassed by the three dehydratase enzymes. Plant and animal systems differ from E. coli in that they do not possess such a multiplicity of enzymes. Also in E. coli and many other bacteria, there are specific 3-hydroxyacylthioester dehydratases which convert 3-hydroxydecanoyl-ACP to cis-3-decanoyl-ACP via the trans-2-isomer. Elongation of this cis-3-intermediate in the usual manner then produces vaccenic acid (cis-11-octadecenoic acid), a product of the "anaerobic" pathway to unsaturated fatty acids found in anaerobic bacteria.

The second reductive step in the series of reactions which give long chain fatty acids from acetyl-ACP and malonyl-ACP is the production of acyl-ACP from α,β-unsaturated acyl-ACP. This reaction is catalysed by the enoyl-ACP reductases. Two reductases have been isolated from E. coli (57), one of which is NADPH specific, acts only on enoyl-ACP derivatives, and prefers short chain (C:4, C:6) to long chain substrates, The second reductase is NADH specific, accepts both CoA- and ACP-esters and prefers longer chain length substrates, but will utilize all chain lengths from C:4 to C:16.

It can be seen that the effect of this series of enzymic reactions is to increase the chain length of the primer molecule (acetate in the case shown in Scheme 1) by two carbons, provided by malonate. Repetition of this sequence of reactions, now with butyrate as a "primer" will result in the production of hexanoate, and so on until the product is a long chain acid such as palmitate. Elongation stops when the fatty acid is removed from the enzyme by either transfer, for example to CoA, or hydrolysis.

An enzyme which catalyses the hydrolysis of thioesters has been isolated from E. coli (58). The enzyme hydrolyses either CoA or ACP esters of fatty acids of chain length C:10 to C:18, but is most active with the longer chain lengths, and with palmitoleic- and cis-vaccenic-thiol esters. The products are the free fatty acid and the thiol.

As mentioned earlier, two types of fatty acid synthetase systems have been recognised, those in which the individual enzymes are separable, such as occurs in E. coli, and those which occur as a tightly bound complex of enzymes, such as the fatty acid synthetase of yeasts. The properties of the individual enzymes of the second type of synthetase cannot be individually studied, but the pathway of fatty acid biosynthesis in this type of system is identical to that occurring in E. coli.

The first multienzyme complex of fatty acid synthesis was isolated from yeast by Lynen (10). He proposed that the individual enzymes were arranged around a "central" sulphydryl group. This sulphydryl group initially accepts a molecule of malonate from malonyl-CoA, while a second sulphydryl group, known as the "peripheral" group accepts a molecule of acetate. Condensation occurs between these substrates, followed by the series of reactions described earlier for E. coli, the final product being enzyme bound butyrate. This butyrate is then transferred to the "peripheral" site and another molecule of malonate is accepted at the central sulphydryl group, and elongation continues. Termination of the sequence when the chain length of the synthesised acid reaches 16 or 18 carbons regenerates the synthetase.

The enzymatic synthesis of long chain fatty acids in plants is also catalysed by a multienzyme complex. The pathway is identical to that described for bacterial systems. The same components, namely malonyl CoA, ACP, NADPH and NADH are required, and all the systems investigated synthesize de novo palmitate and stearate.

In addition to de novo synthesis, mammalian cells contain two different elongation pathways, both of which elongate preformed long chain fatty acids by sequential two carbon additions. These two pathways are quite distinct, one occurring in the mitochondria and utilizing acetyl-CoA, while the other occurs in the microsomes and uses malonyl-CoA.

The microsomal pathway condenses a preformed long chain acid with malonate to extend it in an exactly analogous manner to de novo synthesis (59). The system utilizes NADPH; replacement of this nucleotide with NADH gives much slower rates of elongation. The system elongates both saturated (C:10 to C:16) and unsaturated fatty acids. The unsaturated acids are elongated at a faster rate than the saturated ones, the higher the degree of unsaturation, the faster the rate of elongation.

The mitochondrial pathway for the elongation of fatty acids is active on chain lengths from C:10 to C:22, the shorter chain length acids being elongated most rapidly. The system is essentially the reverse of β-oxidation except that the acyldehydrogenase of β-oxidation is replaced by a NADPH dependant enoyl-CoA reductase (60).

Although the overall mechanism of fatty acid synthesis has been well characterized, and the individual reactions in the elongation sequence have been elucidated, much less is known about the stereochemistry of these individual reactions. It is known that in each of the reductive steps of chain elongation in liver preparations (that is reduction of β-ketoacyl-ACP to β-hydroxyacyl-ACP, and reduction of $\alpha\beta$-unsaturated acyl-ACP to acyl-ACP) a hydride ion is transferred from position 4 of the pyridine ring of NADPH to the β-carbon of the acyl chain (61). The product of the first of these reductions, β-hydroxyacyl-ACP has the hydroxyl group in the D configuration (10).

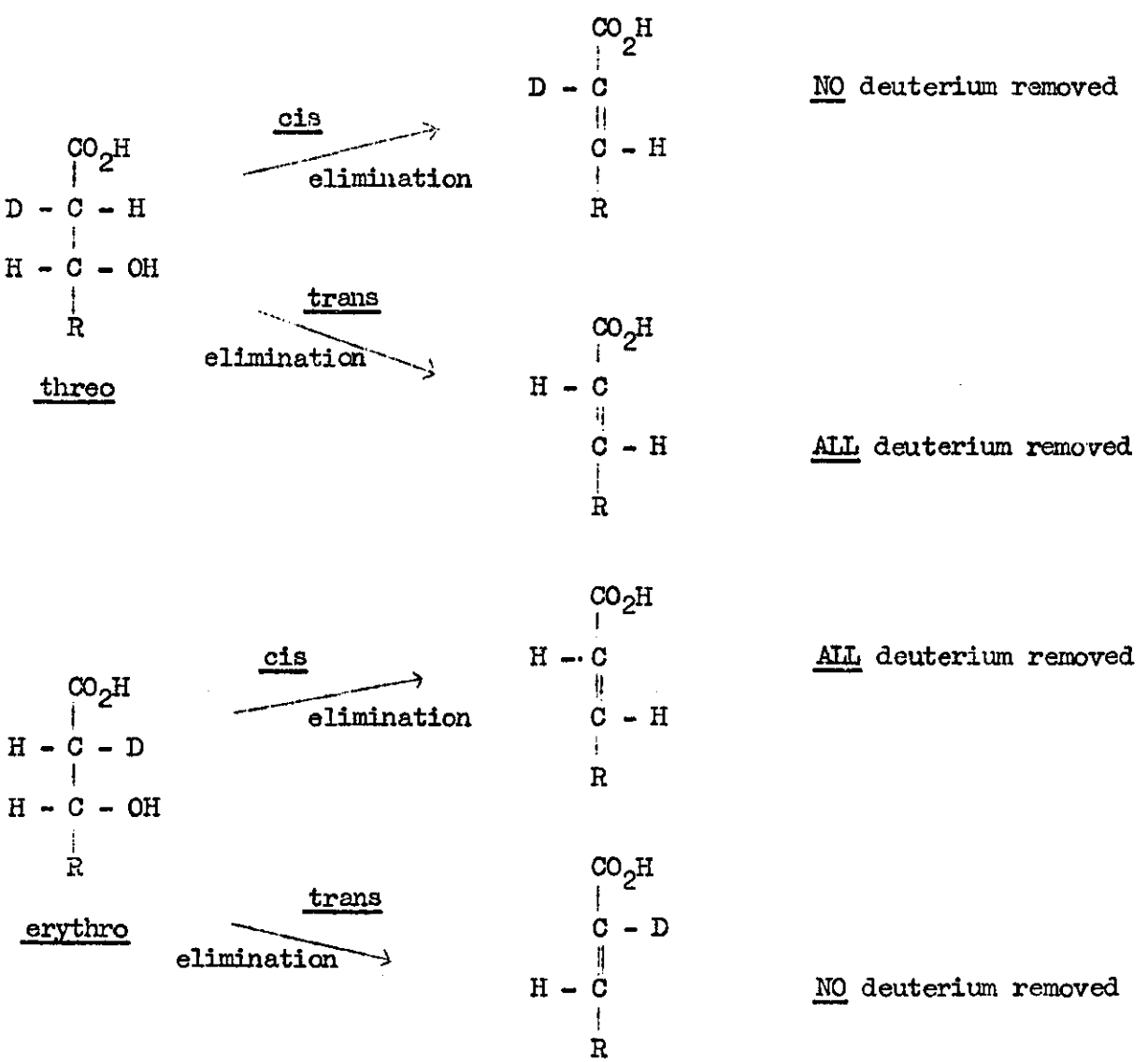
It has recently been established that both of the reductive steps in fatty acid biosynthesis are completely stereospecific with regard to the nucleotide donor (17). Using tritium labelled NADPH, it was shown that equal amounts of tritium were incorporated from the A and B sides of the nucleotide, one reduction occurring with A side specificity, the other with B side specificity. When acetoacetyl-CoA was incubated with fatty acid synthetase and tritiated NADPH, tritium was incorporated into the product (β -hydroxybutyrate) only from the B side of the nucleotide. Similarly reduction of crotonyl-CoA with fatty acid synthetase and tritium labelled NADPH, gave a product (butyrate) containing tritium only from the A side of the cofactor. A proton from water is added to the α position of the acyl chain during the second of these reductions (62). Recently, Drysdale has shown that the yeast crotonyl-CoA reductase transfers hydrogen from NADPH to the β-carbon of the $\alpha\beta$ -unsaturated acyl derivative, the hydrogen in the product, which originated from NADPH, having the D(pro-R) configuration (63).

The configuration assumed by the proton added to the α-position during the second reductive step of acyl chain elongation has been investigated by Sedgwick and Cornforth (92). Incorporation of [$^2H_2$]-malonate into palmitic acid by a purified fatty acid synthetase gave a product which contained 7 atoms of covalently bound deuterium. The optical rotatory dispersion curve of this product was identical to that of 2-D(R)-[2-$^2H_1$] palmitic acid, and it was, therefore, reasoned that the hydrogen added to the α-position during the second reductive step of acyl chain elongation assumes the L-(pro-S) configuration.

There are still questions to be answered, however, about the detailed stereochemistry of some of the intermediate steps of chain elongation. The step to be considered first in this work is the dehydration of D(-) β -hydroxyacyl-ACP to trans $\alpha\beta$ -unsaturated-acyl-ACP. The stereo-

chemistry of this elimination is not known, and the work reported here has been to determine whether the hydrogen removed from the α-position of the acyl chain is <u>erythro</u> or <u>threo</u> relative to the hydroxyl group. As the configuration of the hydroxyl in the β-hydroxylacyl-ACP precursor is known to be D, then the solution of this problem will also indicate the absolute stereochemistry of the hydrogen removed.

To answer this question, suitable β-hydroxy acids are required, with the α-position labelled with either deuterium or tritium, <u>cis</u> (<u>erythro</u>) and <u>trans</u> (<u>threo</u>) respectively, to the hydroxyl group. In this study deuterium was chosen. The products obtained from incubation of these substrates with a fatty acid synthetase system, if the system shows an absolute specificity as expected, are shown in Scheme 2.



Scheme 2.

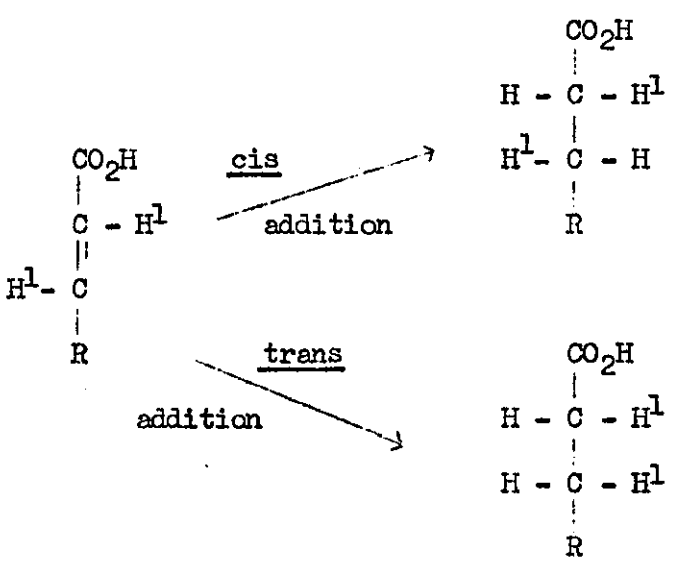
Thus, depending on the stereochemistry of dehydration, one substrate will lose all the label, and the other substrate will retain its label. In the unlikely event of there being incomplete or no specificity, then each substrate would lose some label and retain some.

The other step to be considered is the second reductive step of acyl chain elongation, namely the hydrogenation of the trans-$\alpha\beta$ -unsaturated acyl-ACP, to give acyl-ACP.

$$RCH = CHCOSACP + NADPH + H^{+} \longrightarrow RCH_2CH_2COSACP + NADP^{+}$$

During the course of this work, it was reported that the hydrogen atom added to the β-position of the acyl chain assumed the D(pro-R) configuration (63), and that added to the α-position assumed the L(pro-S) configuration (92).

There are two possible modes of reduction of the double bond in trans-$\alpha\beta$-unsaturated acyl ACP, either by overall cis addition of hydrogen, or trans addition. The differences in these two modes of addition are shown in Scheme 3.



Scheme 3.

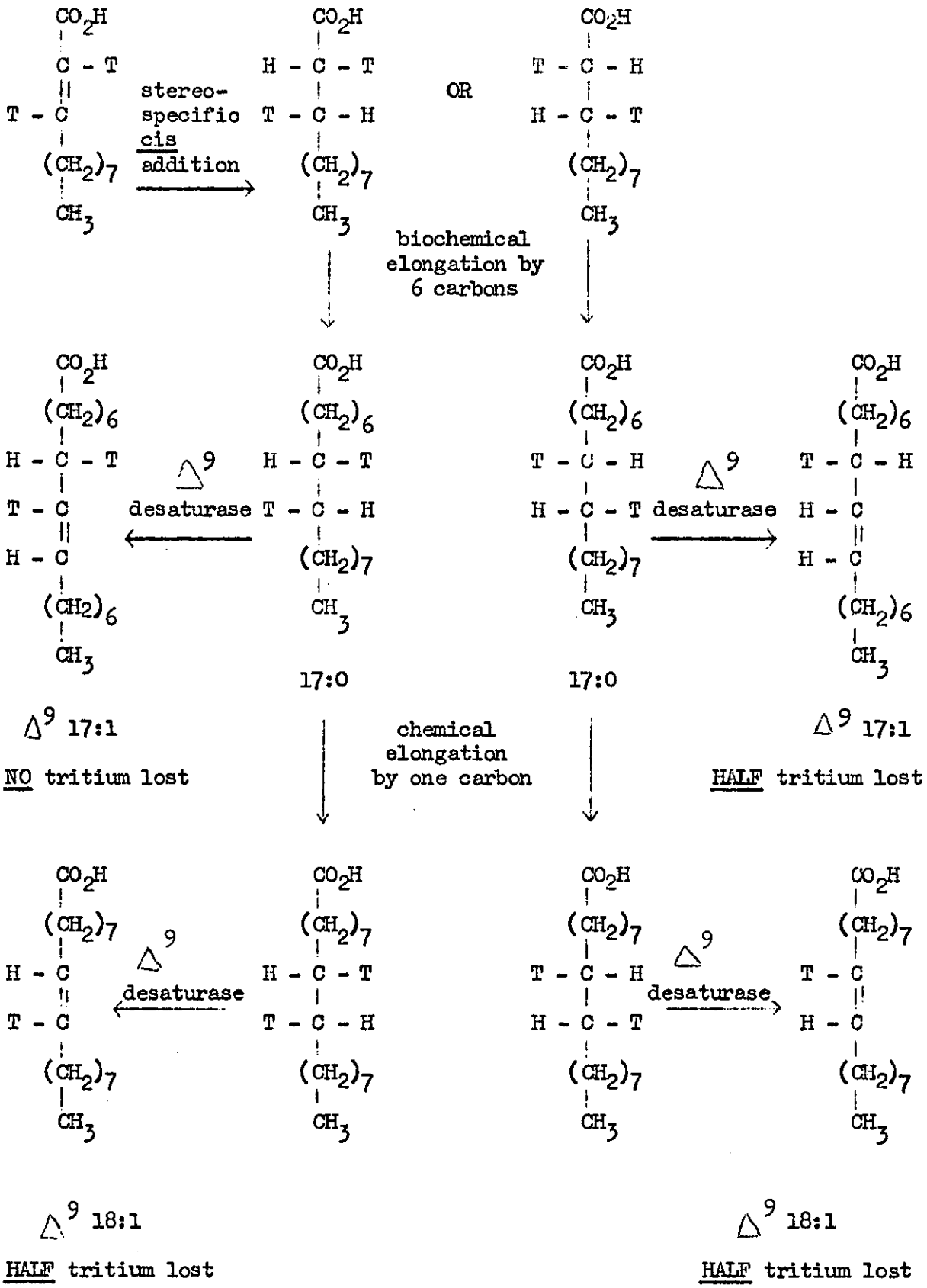
It can be seen that in the product of cis addition, the hydrogen atoms which were originally on the double bond of the unsaturated derivative now have the threo configuration, whereas in trans addition,

the original hydrogen atoms assume the erythro configuration in the product. It was also considered likely, at the commencement of this work, that the addition of hydrogen to the double bond would show an absolute stereospecificity, and this prediction has been verified for the β-position by the work of Drysdale (63).

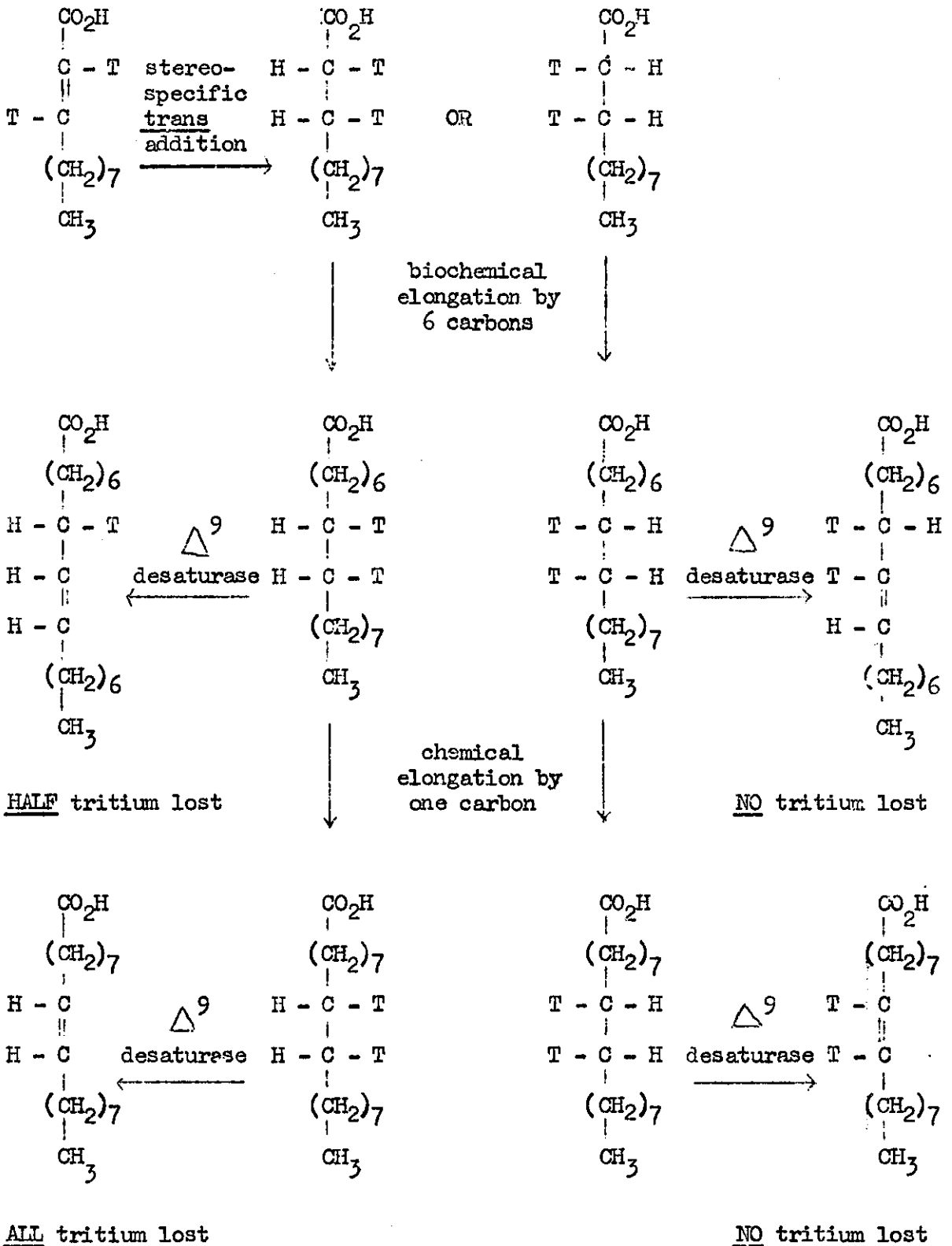
The method used for the determination of the absolute stereochemistry of reduction of $\alpha\beta$-unsaturated acyl-ACP, by a fatty acid synthetase system is shown in Schemes 4 and 5, and is based upon the known stereochemistry of the Δ^9-desaturase in Chlorella vulgaris. This desaturase is known to remove the D-9 and D-10 hydrogens in the desaturation of long chain fatty acids (32).

[2,3-$^3H_2$]-trans-2-Undecenoic acid was used as a substrate for the fatty acid synthetase system. In our case, the fatty acid synthetase system was not isolated, the substrates were incubated with whole cells of Chlorella vulgaris. If the reduction of the double bond involves a cis addition of two hydrogens, then threo ditritio-undecanoic acid will be produced. An absolute stereospecificity in this addition would provide only the DL-(R S) or LD-(S R) [2,3-$^3H_2$]undecanoic acid. Alternatively, trans addition of hydrogen during the reductive step will yield erythro [2,3-$^3H_2$] undecanoic acid, and in this case, an absolute specificity would produce either the D,D-(R R) or L,L-(S S)[2,3-$^3H_2$] acid.

If the product of the reduction of [2,3-$^3H_2$] trans-2-undecenoic acid by the enzyme system is not isolated (in the case of Chlorella vulgaris it is not possible to isolate this intermediate), but allowed to continue in the fatty acid elongation system, the product will be a long chain fatty acid. In this case, as the precursor has an odd number of carbon atoms, the product will also be an "odd chain" acid, predominantly heptadecanoic acid. The tritium atoms which were originally on


stereo-specific cis addition
OR
biochemical elongation by 6 carbons
Δ9 desaturase
17:0
17:0
Δ9 17:1
NO tritium lost
chemical elongation by one carbon
Δ9 17:1
HALF tritium lost
Δ9 desaturase
Δ9 desaturase
Δ9 18:1
HALF tritium lost
Δ9 18:1
HALF tritium lost

Scheme 4.


stereo-specific trans addition
OR
biochemical elongation by 6 carbons
Δ9 desaturase
Δ9 desaturase
chemical elongation by one carbon
HALF tritium lost
NO tritium lost
Δ9 desaturase
Δ9 desaturase
ALL tritium lost
NO tritium lost

Scheme 5.

C-2 and C-3 of the undecanoic acid will now be on the C-8 and C-9 carbon atoms in the heptadecanoic acid. Some of the heptadecanoic acid produced in this way by Chlorella vulgaris will be desaturated to give cis-9-heptadecenoic acid. This desaturation will remove the D-9 and D-10 hydrogens of the heptadecanoic acid. Now the methylene group at the C-9 position in this C:17 acid contains the tritium which was originally at C-3 in the undecenoic acid. If the reduction of the undecenoic acid by the enzymes of fatty acid synthetase proceeded stereospecifically this tritium will now be in the D(R) or L(S) configuration and, depending upon its configuration, it will either be lost or retained during the desaturation of the heptadecanoic acid by the Δ^9 desaturase. If the tritium on C-9 is D(R) (that is, if the hydrogen atom added to the β-position of the $\alpha\beta$-unsaturated intermediate assumes the L(S) configuration) then during desaturation the heptadecanoic acid will lose half of its tritium; conversely, if the tritium is L(S) then the product of desaturation will retain all its tritium. Therefore, isolation of the cis-9-heptadecenoic acid produced during the incubation of the [2,3-$^3H_2$] undecenoic acid with Chlorella vulgaris, and measurement of the amount of tritium in this product will answer the question of the stereochemistry of additon of hydrogen to the β-position during reduction of trans-$\alpha\beta$-unsaturated-acyl derivatives to saturated-acyl derivatives in the biosynthesis of long chain fatty acids.

The stereochemistry of addition of hydrogen to the α-position during the reduction of $\alpha\beta$-unsaturated acyl intermediates in chain elongation can be decided in a similar manner to that used for the β-position. If the heptadecanoic acid produced from [2,3-$^3H_2$] trans-2-undecenoic acid by Chlorella vulgaris is isolated and extended chemically by one carbon atom, the product is stearic acid containing two tritium atoms. These tritium atoms, which were originally on the C-2 and C-3 carbon atoms of

the undecenoic acid precursor, are now at the C-9 and C-10 positions, respectively, in the stearic acid. Desaturation of the stearic acid by a system which contains a Δ^9 desaturase will affect both methylene groups which contain tritium. If reduction of the double bond in trans-2-undecenoic acid occurred by a trans addition of hydrogen, the tritium atoms in the stearic acid will be in the erythro configuration and desaturation will either remove both or neither of these atoms, depending upon the absolute specificity of the original reduction. Conversely, if the reduction occurred by cis addition, then the tritium in the stearic acid will be in the threo configuration and desaturation will remove only half of the tritium, to give either [9-^{3}H] oleic acid or [10-^{3}H] oleic acid, depending upon the stereochemistry of the original reduction. However, the stereochemistry of addition of hydrogen to the β-position will already have been determined, as discussed earlier, and therefore the stereochemistry of the tritium at C-10 in the stearic acid will be known. Thus the stereochemistry of addition of hydrogen to the α-position can be deduced.

RESULTS AND DISCUSSION

Preparation of labelled precursors

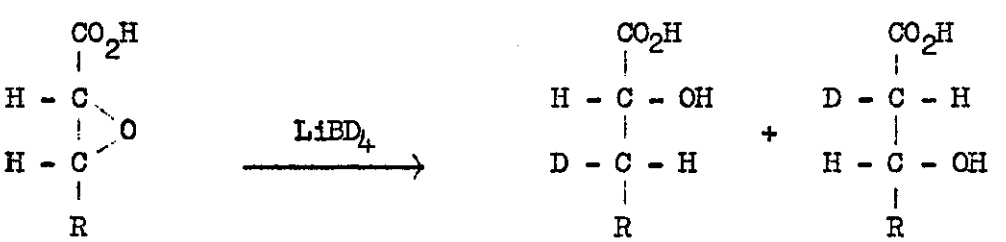
1. 2-deuterio,3-hydroxyHeptadecanoic acids

In order to investigate the stereochemistry of the dehydration of β-hydroxy acyl thioester to give trans-$\alpha\beta$-unsaturated thioester, β-hydroxy acids were required with the α-hydrogen, cis (erythro) or trans (threo) to the hydroxyl group specifically labelled with tritium or deuterium. Ideally only the D-enantiomers of the labelled β-hydroxy acids were required as it is only these isomers which are intermediates in fatty acid synthesis; the L-3-hydroxy acids are intermediates in the β-oxidative degradation of fatty acids to acetate. The acetate produced from the L-3-hydroxy acids by β-oxidation will contain any label which was originally in these enantiomers and, if this acetate then became available for fatty acid synthesis, the original specific label would become randomized throughout the fatty acid chain, and could affect interpretation of results.

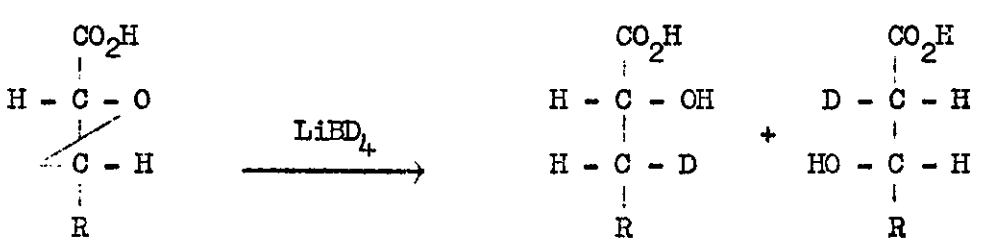
This problem could be overcome by the optical resolution of the labelled β-hydroxy acids, and the use of only the D-enantiomer in the biochemical investigations. The classical methods of resolution by crystallization of the diastereoisomeric salts of the acid and an optically active base cannot be used in this case due to the very small amounts of labelled hydroxy acids prepared. However, during this work, a method was developed for the optical resolution of long chain 2-hydroxy acids and 3-hydroxy acids by TLC, which was convenient for use on a small scale. This method is described in the Appendix, but was not used in the work detailed here as it had the disadvantage that some isomerization of the hydroxy acid occurred during its recovery after resolution.

Most natural systems only synthesise even number fatty acids, so that if odd chain racemic β-hydroxy acids are used as substrates, the desired odd chain products can be easily separated from any randomly labelled products of degradation and resynthesis. The use of odd chain precursors has the added advantage that deuterium, rather than tritium, can be used as the label on the α-position. This is due to the fact that very little dilution of the label occurs due to endogenous odd chain acids, and this enables the resultant deuterium labelled, odd chain products to be easily analysed by mass spectroscopy. The intermediates chosen to investigate the mechanism of dehydration of β-hydroxy acids were threo-2-deuterio, 3-hydroxyheptadecanoic acid and erythro-2-deuterio, 3-hydroxyheptadecanoic acid.

The threo- and erythro-2-deuterio, 3-hydroxy acids were prepared from cis- and trans-2,3-epoxyheptadecanoic acids, respectively, by reductive cleavage with lithium boron deuteride, as shown in Scheme 6. These reactions are of known stereochemistry (70) and result in inversion of configuration at the position of nucleophilic attack, and retention of configuration by the hydroxyl group.



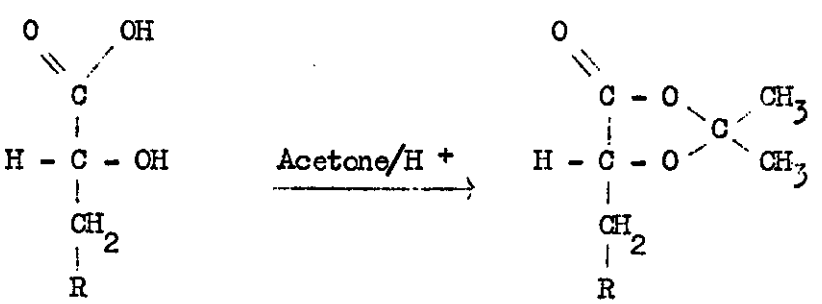
cis epoxide threo

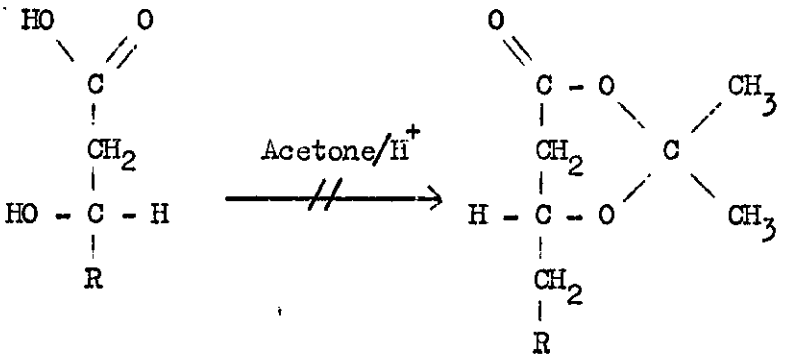


trans epoxide erythro

Scheme 6.

The reductions were performed in dry diethyl ether at 0°C and, on the scale used, the optimum reaction time for maximum yield of β-hydroxy acids was approximately three hours. The reduction was performed on the epoxy acids as the free carboxyl group should be unaffected by lithium boron deuteride; esters are reduced to primary alcohols. It was found, however, that some reduction of the carboxyl group occurred during the reaction, and this resulted in low yields of the desired products, the side products being mostly 1,2- and 1,3-heptadecandiols. A further disadvantage of this method was that it produced a mixture of 2- and 3- hydroxy isomers, of which the 2-hydroxy isomer predominated. These isomers could be separated by TLC, but the overall yield of the desired 3-hydroxy acid was reduced accordingly. The mixture of 2- and 3-hydroxy acids produced in the reaction was separated from the 1,2- and 1,3-diols formed by over-reduction by extracting an ethereal solution of the reaction products with dilute aqueous sodium bicarbonate. This procedure gave a mixture of 2- and 3-hydroxy acids which could be separated by thin layer chromatography. It was found more convenient, however, to first partially purify the 3-hydroxy derivative before performing TLC. This was achieved by reacting the mixed 2- and 3-hydroxy acids with a 0.5% v/v solution of perchloric acid in acetone. The 2-hydroxy acid undergoes reduction to form the isopropylidene derivative whereas the 3-hydroxy acid remains unchanged.





$R = CH_3(CH_2)_{13}$

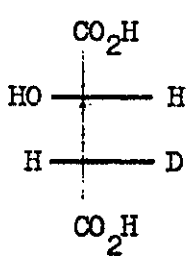
The 3-hydroxy acid could then be separated from the derivative of the 2-hydroxy acid by extraction with dilute alkali. The 2-deuterio, 3-hydroxyheptadecanoic acids were finally purified by TLC, the overall yield of these compounds from the epoxides being around 1%. All manipulations involved in the purification of the 3-hydroxy compounds were performed on the free acid, although purification by chromatography would have been easier with the methyl esters. The free acids were needed, however, as substrates for the biochemical investigations, and an intermediate esterification would have meant a final hydrolysis step in the reaction sequence. As it had been found in preliminary experiments that the β-hydroxy esters were base sensitive, losing water readily to form the trans- $\alpha\beta$-unsaturated derivative, hydrolysis of the β-hydroxy compounds was avoided.

In order to check the complete stereospecificity of the borohydride reduction of the cis- and trans-2,3-epoxyheptadecanoic acids, that is, to ensure that the erythro-2-deuterio, 3-hydroxyheptadecanoic acid contained none of the threo-isomer and vice-versa, the 220 MHz NMR spectra of both products were examined. The NMR spectra were obtained on 0.1 Molar solutions of the β-hydroxy methyl esters. These esters were prepared by the action of diazomethane on the free acids. The relevant parts of the spectra are shown in Figure 1. The absorption pattern of the α-protons

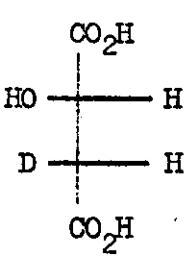
for the fully protonated compound, methyl 3-hydroxypalmitate, is also shown. This is typical of an ABX system, with δ_A = 2.285 ppm, δ_B = 2.370 ppm and δ_X = 3.841 ppm, having coupling constants J_{AB} = 16.5 Hz, J_{AX} = 8.5 Hz and J_{BX} = 3.5 Hz.

When a deuterium atom is substituted for one of the α-protons, the "threo" compound is left with the H_B and H_X protons, and the "erythro" compound is left with the H_A and H_X protons. The absorption of the remaining α-proton should, therefore, occur with almost the same chemical shift as in the fully protonated molecule, but with the proton-proton coupling being only J_{AX} or J_{BX}. Since deuterium has a spin of 1, it may be expected to produce further splitting of these absorptions into 1:1:1 triplets. Such H-C-C-D couplings are usually about 2 Hz.

In the spectrum of the "threo" compound, proton H_B gives a broad absorption (δ = 2.352 ppm) with half height width of about 8 Hz. Allowing for the broadening of the absorption from the deuterium coupling, this width indicates a coupling with H_X of 3-4 Hz. The "erythro" compound proton H_A gives a broad doublet at δ = 2.266 ppm with a coupling of 9 Hz and a half height width of about 5 Hz. These couplings compare with those obtained by Gawson and Fondy (71) for threo- and erythro-3-deuteriomalic acid, during their investigations on the specificity of the fumarase enzyme. The coupling constants for the two deuterated malic acids are shown below.



J = 4.4 ± 0.2 Hz



J = 7.3 Hz

220 MHZ NMR SPECTRA OF THE α PROTONS OF FULLY PROTONATED AND 2-DEUTERO 3-HYDROXY ESTERS.

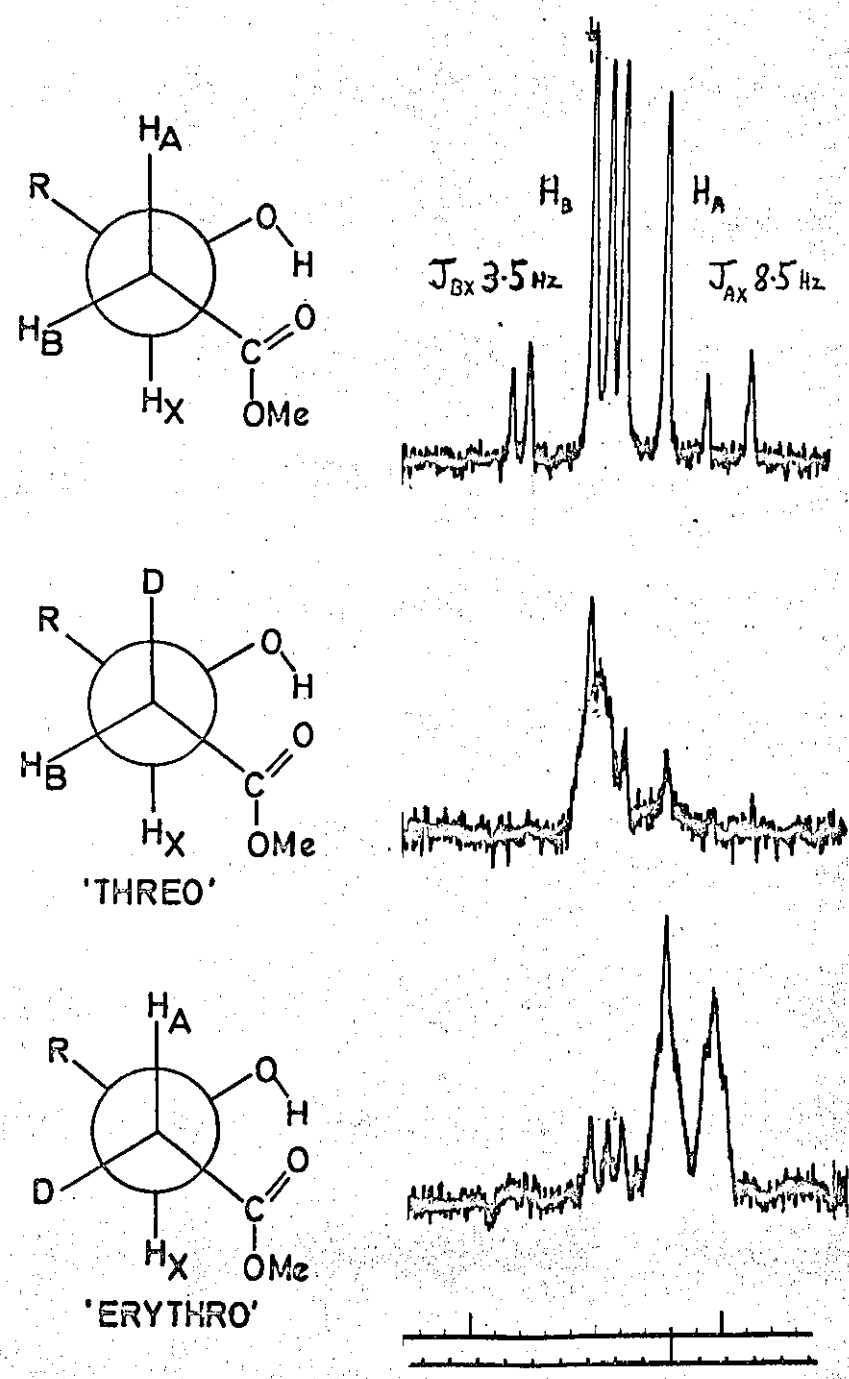


Figure 1.

The spectra showed that there was some fully protonated compound in each isomer, but that there was less than 7% threo form in the erythro isomer and less than 7% erythro form in the threo sample.

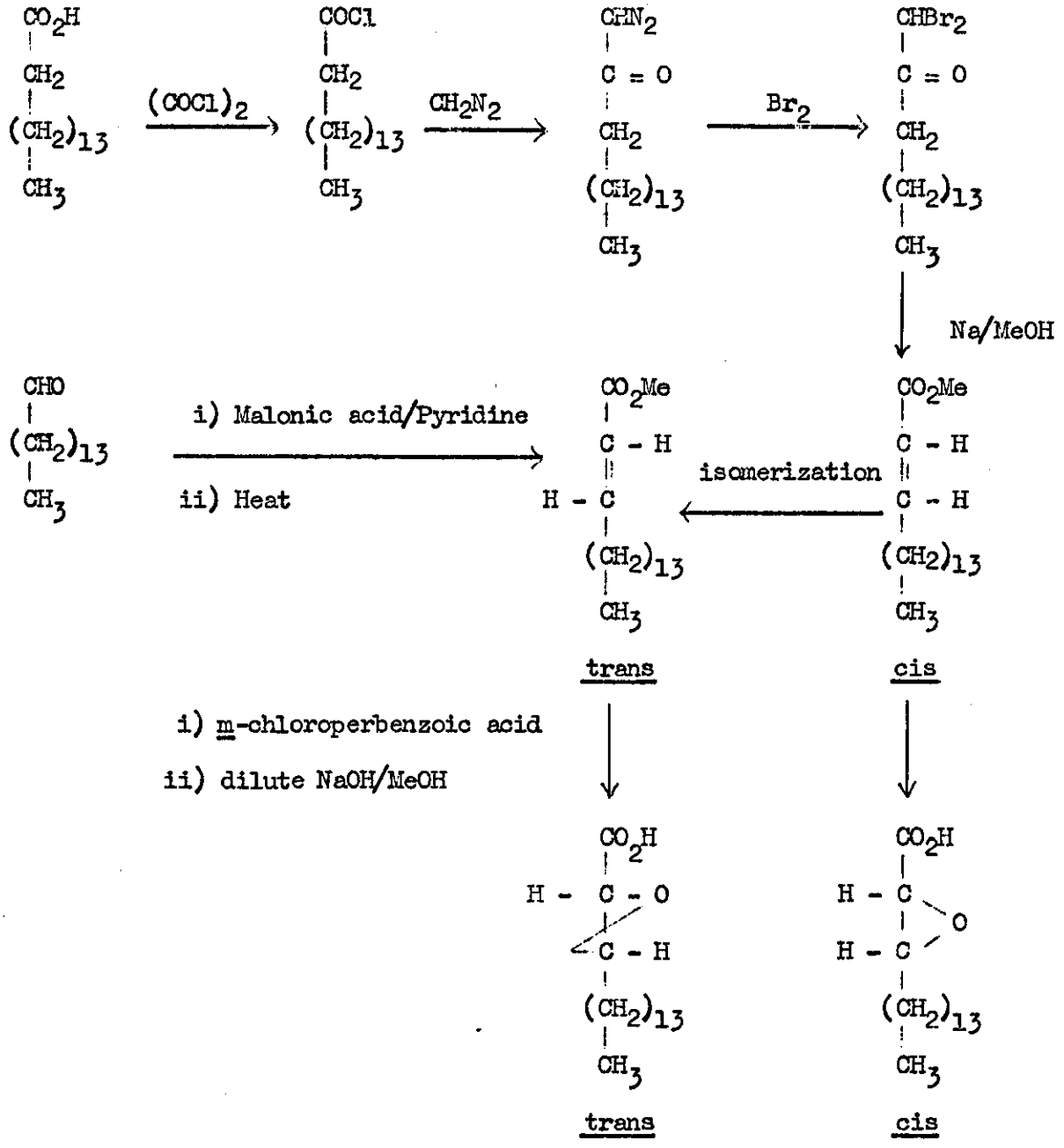
Complete deuterium labelling of each substrate molecule is not possible and the isotopic purity of each product was determined by mass spectrometry on the methyl esters and is shown below.

Labelled acid	Composition (%)		
	^2H_o	$^2H_1$	$^2H_2$
Methyl threo 2-^{2}H, 3-OH 17:0	26	73	1
Methyl erythro 2-^{2}H, 3-OH 17:0	16	82	2

The cis- and trans-2,3-epoxyheptadecanoic acids which were reduced to give the β-hydroxy acids, used in the stereochemical investigations, were not commercially available and were synthesised by direct epoxidation of the corresponding monoenes. The synthetic routes to both the cis- and trans-epoxides are shown in Scheme 7.

The $\alpha\beta$-unsaturated esters required as intermediates in the synthetic route to labelled β-hydroxy esters have been synthesised previously by a variety of methods. Probably the most common route to $\alpha\beta$-unsaturated esters is the elimination of hydrogen bromide from an α-bromo acid. These α-bromo acids are readily prepared by the action of phosphorus tribromide and bromine on the corresponding long chain acid. Elimination of hydrogen bromide is then achieved by the action of base on the α-bromo acid, the reagent of choice being hot pyridine or quinoline, or potassium tert-butoxide in tert-butanol.

Unsaturated acids obtained by this bromination dehydrobromination procedure have the double bond in the trans configuration, although during the elimination reaction there is a possibility of some isomerization to



Scheme 7. Synthesis of cis- and trans-2,3-epoxyheptadecanoic acids

form the trans- $\beta\gamma$ -unsaturated compounds. trans- $\alpha\beta$ -Unsaturated esters have also been prepared by dehydration of β -hydroxy esters, which are readily available as products of the Reformatsky reaction. The dehydrating agents usually employed are thionyl or phosphonyl chloride in pyridine, and it is probable that the hydroxy esters are converted to β-chloro intermediates, and dehydrochlorination produces the unsaturated products. As in the case of dehydrobromination, the products can contain some of the $\beta\gamma$ -unsaturated isomer.

Stereospecific production of trans- $\alpha\beta$ -unsaturated acids, free from any $\beta\gamma$ -isomers, has been achieved by the condensation of long chain aldehydes with malonic acid. The reaction is usually performed in pyridine containing some piperidine.

$$RCHO \xrightarrow{CH_2(CO_2H)_2} RCHCH(CO_2H)_2 \xrightarrow{\text{warm}} RCH = C(CO_2H)_2 \xrightarrow{\text{heat}} RCH \overset{t}{=} CHCO_2H$$
$$\qquad\qquad\qquad\qquad\quad |$$
$$\qquad\qquad\qquad\qquad\quad OH$$

Some trans-2-heptadecenoic acid which had been prepared by the above method was already available, however this reaction was not used during these studies for reasons discussed later.

Acetylenic intermediates have found wide application in the synthesis of unsaturated fatty acids, the alkencic acids are prepared from the corresponding alkynes by stereospecific half hydrogenation. Acetylenic acids are easily prepared by the carboxylation of terminal acetylenes via their Grignard (72) or alkali metal derivatives. The acetylenic acid can then be converted to the cis derivative by hydrogenation over a partially poisoned palladium catalyst (73) or to the trans isomer by reduction with sodium or lithium in liquid ammonia (74, 75).

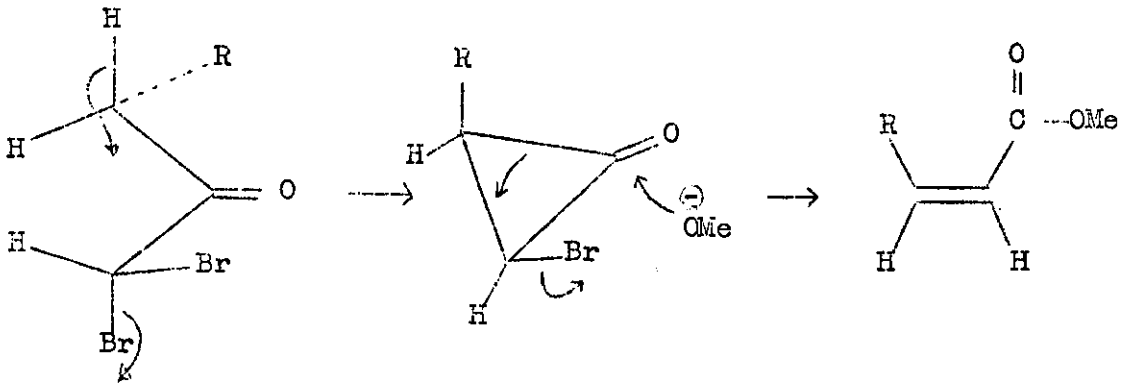
The methyl cis-2-heptadecenoate used in these studies for the synthesis of the labelled β -hydroxy acids was prepared via an elegant method involving the Favorski re-arrangement (76, 77). The reaction, described

by Gerson and Schlenk (64) was used to produce the cis acid from palmitic acid, via the dibromoketone and is shown in Scheme 7.

Palmitoyl chloride, the first intermediate in the reaction scheme, was prepared by the action of oxalyl chloride in benzene on palmitic acid. After completion of the reaction, the benzene and excess oxalyl chloride were removed under vacuum to leave palmitoyl chloride in essentially quantitative yield. The product needed no further purification, and this method was used in preference to the standard procedure which involves reaction with thionyl chloride, and subsequent distillation of the product.

The palmitoyl chloride obtained was reacted with diazomethane to provide the diazoketone which was purified by crystallization as pale yellow flakes from diethyl ether. The diazoketone was treated with a solution of bromine in carbon tetrachloride to yield the oily dibromoketone. This dibromoketone was then reacted immediately with a solution of sodium methoxide in methanol to give methyl cis-2-heptadecenoate. The only difference between this method and that of Gerson and Schlenk was the use of sodium methoxide in methanol, in place of aqueous ethanolic potassium hydroxide, to effect the Favorski re-arrangement. It was found that this modification gave a cleaner product, and the methyl ester was produced directly, whereas a final esterification step was necessary when using aqueous base.

The generally accepted mechanism for the Favorski re-arrangement of this type involves a cyclopropane intermediate (77). The stereo-specificity of the reaction suggests that both the formation and the cleavage of the intermediate bromocyclopropanone are concerted processes. If this is so, then it implies that, as the product is exclusively cis, the intermediate cyclopropanone must be cis; a mechanism to explain these observations is given on the next page.



Although some methyl trans-2-heptadecenoate had already been prepared by the condensation of malonic acid with pentadecanal, this method was not used for the preparation of further amounts of the trans ester, due to the difficulties experienced in preparation and purification of the long chain aldehyde.

Due to the ease of preparation of methyl cis-2-heptadecenoate via the Favorski re-arrangement it was decided to prepare the trans isomer by stereomutation of the cis compound.

Stereomutation of cis alkenoic acids and esters has been achieved by a number of methods. These transformations can be accomplished by the use of the NO_2 radical generated from sodium nitrite and nitric acid (78, 79).

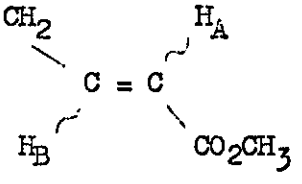
Selenium has also been used to affect the transformations (79, 80), but in this case, migration of the double bond can occur to afford a mixture of monoenoic esters. The composition of these equilibrium mixtures has been investigated by Gunstone and Ismail (80), who found that migration is more extensive when the double bond is present in the central portion of the chain. This migration can be overcome by effecting the isomerization of the cis olefins with ultra violet light in the presence of diphenyl sulphide (80, 81).

The isomerization of methyl cis-2-heptadecenoate to the trans isomer was first attempted using the NO_2 radical produced by the action of nitric acid on sodium nitrite. This method was unsuccessful, and resulted in almost quantitative recovery of the cis isomer, even at much longer reaction times than were needed for the elaidanization of methyl oleate. Other methods of performing this isomerization were therefore investigated. Methyl cis-2-heptadecenoate was isomerized by heating the ester under nitrogen in a sealed tube with selenium. This resulted in a mixture of cis- and trans-$\alpha\beta$-unsaturated and trans-$\beta\gamma$-unsaturated esters, in the approximate proportions 1:7:1. The remaining cis isomer was separated from the trans isomers by TLC on silica gel G, on which the cis isomer was less polar. The trans-$\alpha\beta$- and trans-$\beta\gamma$-unsaturated esters were much more difficult to separate, being only just separable by TLC on silica gel G impregnated with silver nitrate. However, it was found that if the mixture of trans isomers was not separated at this stage, but epoxidized, the resultant mixture of trans-2,3- and trans-3,4-epoxyheptadecanoates could be separated readily by TLC. However, this method was laborious and time consuming due to the number of separations which needed to be performed by TLC. A much improved method for the isomerization of cis-2-enoates to trans-2-enoates has been reported by Barve and Gunstone (65). This method involves the treatment of the cis ester with mercuric acetate and methanol, followed by hydrochloric acid. The product is almost exclusively the trans-$\alpha\beta$-unsaturated isomer. Methyl cis-2-heptadecenoic acid produced via the Favorski re-arrangement was isomerized by this method. The ester was dissolved in dry methanol and reacted overnight with an equimolar amount of mercuric acetate. The mixture was then acidified with hydrochloric acid at 0°C and left for half an hour. The product was almost pure methyl-trans-2-heptadecenoate containing some cis isomer which could be separated by TLC. No migration of the double bond could be detected.

The reaction of mercuric salts with olefinic compounds is well documented (82) and it has been used to separate mono-, di- and tri-unsaturated fatty acids by TLC. Reversal of the oxymercuration reaction by addition of acid usually results in the regeneration of the double bond with complete retention of the original stereochemistry. The case of the cis-2-heptadecenoate is an exception to this pattern, and must be due to a change in the normal stereospecific mode of oxymercuration or demercuration.

The structures of the cis- and trans-methyl heptadecenoates, prepared as described above, were confirmed by NMR. The double bonds in these compounds are adjacent to a carbomethoxy group, and this gives the esters distinctive spectra. The α methylene group is absent, the methylene groups adjacent to the double bonds occurring at δ = 2.66 in the cis ester and δ = 2.21 in the trans isomer, as broad bands. The olefinic protons occur as an AB quartet with the low field doublet further split into two triplets. These triplets are due to coupling of the low field proton with the adjacent methylene group. The entire olefin proton pattern consists of eight lines.

The chemical shifts and coupling for the olefinic protons are shown below.



methyl cis-2-heptadecenoate

$J_{CH_2\text{-}HB}$ = 5.80 Hz $\quad J_{AB}$ = 11.30 Hz

δ_A = 5.75 ppm $\quad \delta_B$ = 6.30 ppm

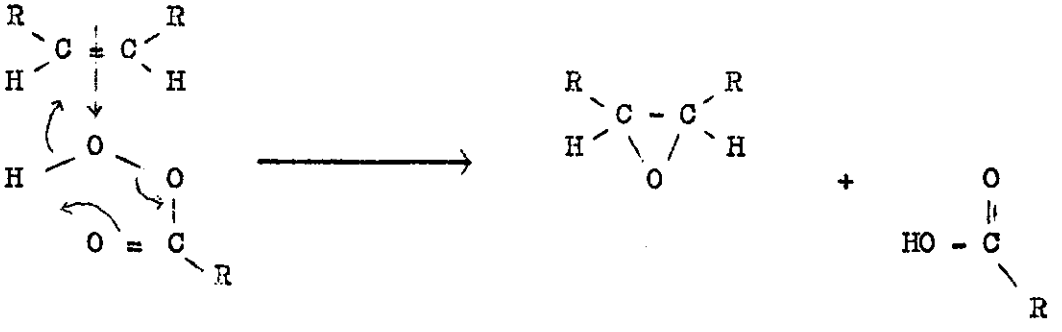
methyl ~~cis~~ trans-2-heptadecenoate

$J_{CH_2\text{-}HB}$ = 6.79 Hz $\quad J_{AB}$ = 15.51 Hz

δ_A = 5.75 ppm $\quad \delta_B$ = 7.00 ppm

Chemical shifts and coupling constants of olefinic protons in methyl cis-2-heptadecenoate and trans-2-heptadecenoate

The next step in the reaction sequence was the epoxidation of the cis- and trans-2-heptadecenoates, and was accomplished by reaction of the olefinic esters with a peroxy acid. Peroxy acids react with olefinic bonds to give epoxides (83). The reaction involves electrophilic attack by the peracid on the olefinic bond, and occurs stereospecifically with overall cis addition of oxygen, thus cis and trans olefins afford the cis and trans-epoxides respectively.



As the reaction involves electrophilic attack by oxygen, it is obvious that substituents which increase the nucleophilic character of the olefin will enhance the rate of action, while electron withdrawing substituents will decrease the reaction rate. The double bond in methyl cis and trans-2-heptadecenoates bears a carbomethoxy group which is strongly electron withdrawing, and would, therefore, be expected to decrease the reaction rate. Indeed this was found to be the case and the epoxidation of these olefins needed much longer reaction times and a higher concentration of peracid than was required for epoxidation of the mid-chain unsaturated esters such as methyl oleate.

The two unsaturated esters were epoxidised by reaction with m-chloroperbenzoic acid in benzene solution. The reaction time was twenty days, and after this time the epoxides were isolated in almost quantitative yields, and purified by chromatography.

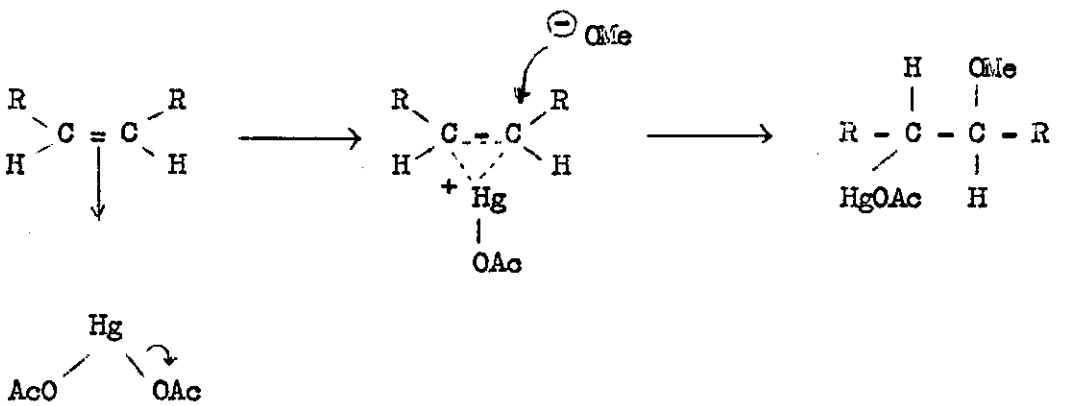
In contrast to the other members of the series of epoxy fatty acids, it was not possible to separate the cis- and trans-2,3-epoxy isomers by TLC or by GLC on a non-polar (SE 30) phase. This is in agreement with Gunstone and Jaconsberg (84) who have prepared, and studied the chromatographic properties of, the complete series of methyl epoxyoctadecanoates. The purity of the isomers could be checked, however, by GLC on a polar phase, as in this case the isomers were separable, the cis epoxide having a shorter retention time (ECL =20.61) than the trans isomer (ECL =20.80). It was found that the epoxidation had occurred with complete stereospecificity, neither epoxide containing any of the other isomer.

Before reducing the epoxides with lithium borodeuteride to provide the precursors necessary for the biochemical investigation, they were hydrolysed to the free acids. This was necessary as the ester functions are reduced by the borodeuteride to primary alcohols. The hydrolysis was effected with dilute methanolic potassium hydroxide at room temperature. The pH of all solutions was carefully controlled during extraction of the free acids, as the epoxides are acid labile. A small amount of each of the acids was remethylated with diazomethane and compared by TLC and GLC with the original ester; the identity of these remethylated compounds with the original esters confirmed that the epoxide groups were unaffected by the hydrolysis procedure.

The cis- and trans-2,3-epoxy heptadecanoic acids, produced by the reactions outlined above, were then reduced with lithium borodeuteride to provide the threo- and erythro-2-deuterio, 3-hydroxyheptadecanoic acids used in the biochemical investigations. These reductions and the characterization of the products have been discussed above (pp 37-39).

Because of the low yields of 2-deutero; 3-hydroxyheptadecanoic acids obtained by reductive cleavage of the epoxides with lithium borodeuteride, an alternative method for the production of the β-hydroxy esters was attempted. This involved the reduction of the oxymercurial derivatives of cis- and trans-2-heptadecenoate with sodium borodeuteride.

The reaction of mercuric acetate with olefins gives stable adducts in which there is a covalent link between the carbon and mercury atoms. The reaction in a nucleophilic solvent usually involves overall trans addition of the mercuric salt and a solvent molecule, the reaction has been explained in terms of a mercurinium ion. This reaction can be reversed by addition of acid, which regenerates the parent olefin.



Reaction of methyloleate with mercuric acetate in methanol

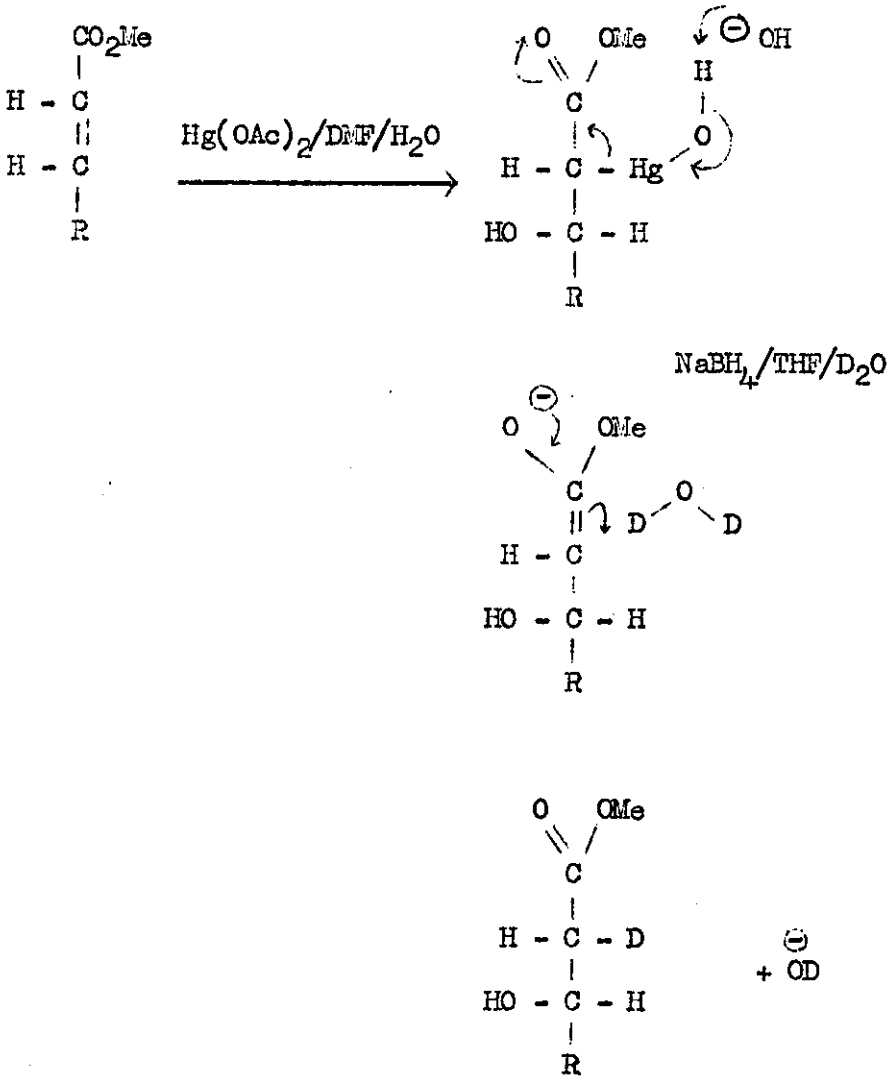
Reduction of the organomercurial with sodium borohydride results in the replacement of the HgOAc group with a hydrogen atom. This reaction has been studied by Bordwell and Douglass (85) who have shown that the reaction proceeds stereospecifically with retention of configuration, the incoming hydrogen directly replacing the mercury atom. They also found that this hydrogen is derived from the borohydride and not from the solvent.

It was known that oxymercuration of $\alpha\beta$-unsaturated esters in methanol solution followed by reduction of the oxymercurial derivative with sodium borohydride gave exclusively the 3-methoxy derivative (143) and it was hoped that the same series of reactions using an aqueous solvent and sodium borodeuteride would result in stereospecific production of 2-deuterio, 3-hydroxy esters.

Both cis- and trans-2-heptadecenoate were reacted with mercuric acetate in aqueous dimethylformamide to which a trace of perchloric acid had been added. The oxymercurial adducts were isolated by extraction with chloroform, and then reduced with sodium borodeuteride in aqueous tetrahydrofuran. The 3-hydroxy esters were formed in good yield. Surprisingly, however, mass spectroscopy of the products showed that no deuterium had been incorporated into the product. Repetition of the reaction this time using heavy water, tetrahydrofuran, and sodium borohydride in the reduction stage gave good incorporation of deuterium into the molecule. The results are shown below.

Substrate for oxymercuration reaction	Media for reduction of oxymercurial product	^2H_o	$^2H_\phi$	Ratio of "erythro" to "threo" products
cis-Δ^2-17:1	$NaBD_4/THF/H_2O$	97	3	
cis-Δ^2-17:1	$NaBH_4/THF/D_2O$	29	69	1:2
trans-Δ^2-17:1	$NaBD_4/THF/H_2O$	98	2	
trans-Δ^2-17:1	$NaBH_4/THF/D_2O$	34	63	1:1

The stereochemistry of the products was investigated by NMR. Comparison of the spectra of the 3-hydroxy products from the cis and trans monoenes with those of the specifically labelled compounds produced by reduction of the epoxides, showed that reduction of the oxymercurial compound did not occur with strict stereospecificity. The products were a mixture of the erythro- and threo-2-deuterio, 3-hydroxy esters in the ratios shown in the table. A probable mechanism for the reduction reaction which would account for the lack of stereospecificity and explain the fact that the deuterium incorporated originated from the solvent is shown in Scheme 8. As an alternative route to the stereospecifically labelled 3-hydroxy esters, the method was obviously unsuccessful and was abandoned.


CO2Me
H - C
H - C
R
Hg(OAc)2/DMF/H2O
O
OMe
⊖ OH
H
C
H - C - Hg
O
HO - C - H
R
NaBH4/THF/D2O
⊖
O
OMe
C
D
O
D
H - C
HO - C - H
R
O
OMe
C
H - C - D
HO - C - H
R
⊖
+ OD

Scheme 8.

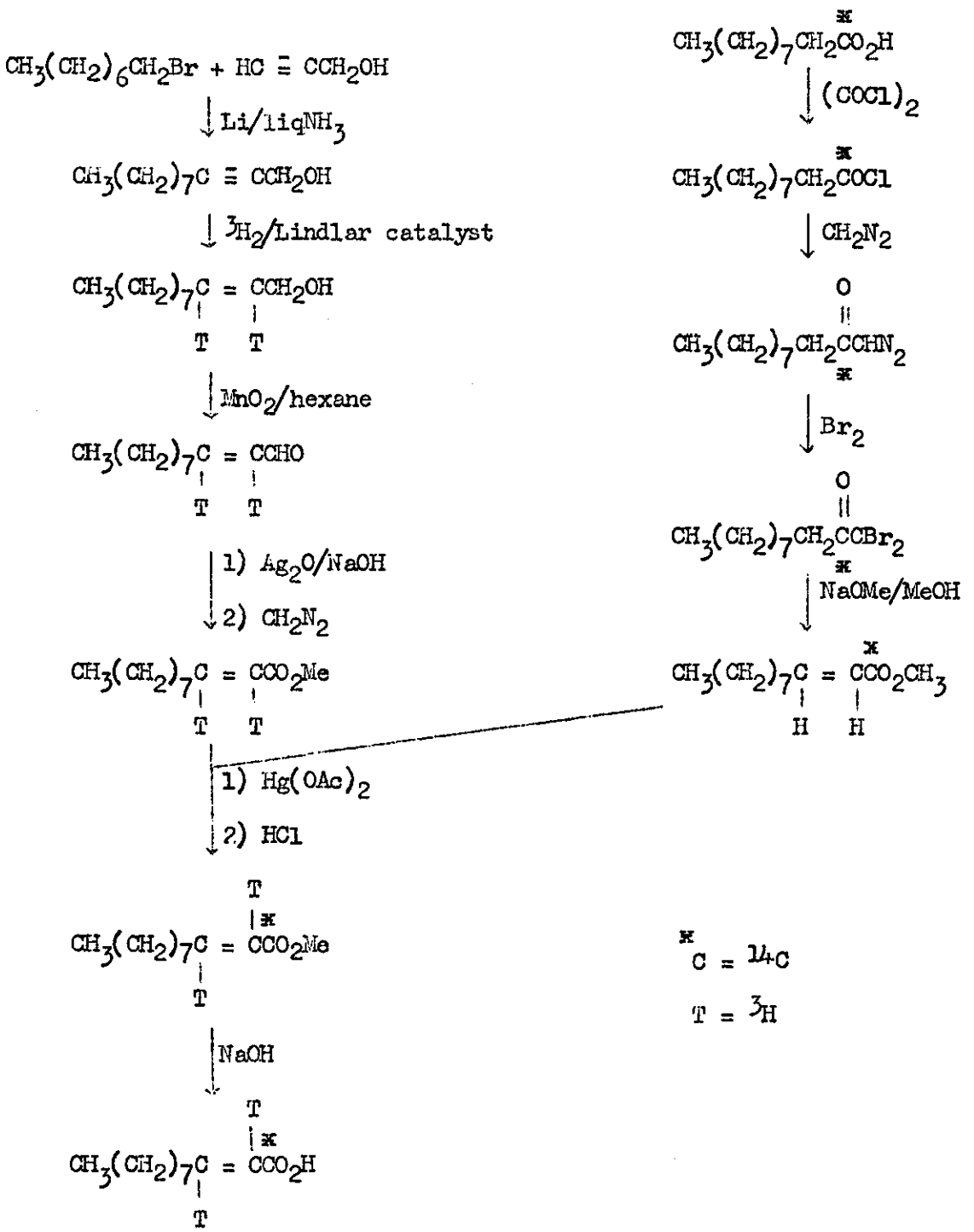
2. [2,3-$^3H_2$]-Undecenoic Acids

The next step in the biosynthesis of fatty acids to be investigated in the work, was the second reductive reaction in which trans-$\alpha\beta$-unsaturated acyl-ACP's are reduced to saturated acyl-ACP's. In order to investigate the stereochemistry of addition of hydrogen to the trans-$\alpha\beta$ double bond during this reduction, it was necessary to prepare a trans-$\alpha\beta$-unsaturated acid specifically labelled on the α and β carbons with tritium. The acid prepared was [2,3-$^3H_2$]trans-2-undecenoic acid, and the method used is outlined in Scheme 9.

As well as being intermediates on the chain elongation pathway, trans-2-enoic acids are also intermediates in β-oxidation. It is conceivable, therefore, that as well as being elongated by the fatty acid synthetase system, the labelled precursor could be degraded to acetate by β-oxidation. If this acetate were then utilized for fatty acid synthesis, there would be some scrambling of the radioactive label. The use of an odd chain acid again overcomes this complication, as the product of elongation will be an odd chain acid, while the products of any break-down/resynthesis will be even chain length acids, the two products being easily separable by preparative GLC.

The chain length of the precursor was also critical, it needed to be such that elongation by the fatty acid synthetase system would place the tritium atoms, which were originally on positions C-2 and C-3 of the precursor, at, or close to, the C-9 and C-10 positions in the product, in order that the known stereospecificity of the Δ^9 desaturase could be used to determine the configurations of the tritium atoms in the product of chain elongation. For this reason, the C:11 acid was prepared.

To facilitate accurate determination of the amount of tritium lost or retained in each step of the biosynthetic investigations, 1-^{14}C labelled trans-2-undecenoic acid was added to the tritium labelled precursor.



Scheme 9. Preparation of [1-^{14}C,2,3-^{3}H$_2$]*trans*-2-undecenoic acid

This provision of an internal ^{14}C standard allowed the fate of the tritium to be determined by measuring $^{3}H/^{14}C$ ratios and obviated the need to determine the degree of dilution of substrate and reaction product by measurement of endogenous fatty acids. The ^{14}C labelled acid was prepared from [1-^{14}C] decanoic acid in a similar manner to that used for the preparation of trans-2-heptadecenoic acid from palmitic acid (p.41), namely chain elongation by one carbon via the Favorski rearrangement.

As mentioned earlier, acetylenic intermediates have been used extensively in the preparation of unsaturated fatty acids. The synthesis of [2,3-$^{3}H_2$] trans-2-undecenoic acid, used in this work, utilized one of these acetylenic compounds, namely propargyl alcohol.

Octyl bromide was coupled with the dilithium salt of propargyl alcohol by reaction in liquid ammonia. The dilithium derivative was prepared in situ by reaction of propargyl alcohol and lithium in liquid ammonia. This reaction was allowed to proceed for two hours before the octyl bromide was added, and the reaction was then allowed to proceed for a further two hours. The product was isolated in good yield and purified by vacuum distillation.

Attempts to oxidize the 2-undecynol to 2-undecynoic acid with Jones reagent (chromium trioxide, water, sulphuric acid) in acetone failed, the major product being nonanoic acid formed by over oxidation. It was, therefore, decided to reduce the triple bond, and introduce the tritium into the molecule at this stage, thus producing 2-undecenol from the 2-undecynol. It was known that allylic alcohols are readily oxidized to the corresponding aldehydes with active manganese dioxide (86), and thence to the acids with silver oxide. This was the route chosen for the preparation of the labelled unsaturated ester.

A small amount of the undecynol was purified by TLC and sent to the Unilever Research Laboratory, Vlaardingen, Holland, for reduction. The

reduction was performed with tritium gas over a Lindlar catalyst (73). The product, [2,3-$^3H_2$] *cis*-2-undecenol, was shown to be pure by GLC and showed only one spot on argentation TLC, indicating that no *trans* isomer was present.

The oxidation of [2,3-$^3H_2$] *cis*-2-undecenol to [2,3-$^3H_2$] *cis*-2-undecenoic acid was performed in two stages. Oxidation of the allylic alcohol with active manganese dioxide in petroleum ether gave the unsaturated aldehyde in good yield. This aldehyde was not purified, but was immediately used for the next stage of the reaction. The oxidation of the allylic alcohol to aldehyde was followed by TLC; the product was less polar than the starting material and, at the end of the reaction, all of the alcohol had been consumed. The product was contaminated, however, with a more polar material which had a similar R_f value on TLC as free fatty acid. This contaminant was not isolated, and was, therefore, never characterized fully.

The impure [2,3-$^3H_2$] *cis*-2-undecenal was further oxidized with silver oxide in aqueous alcoholic potassium hydroxide. The aldehyde was dissolved in ethanol and silver oxide, dilute potassium hydroxide was added and the mixture was shaken overnight. The silver salts were removed by filtration and any unchanged aldehyde was extracted from the alkaline solution with petrol. Acidification of the aqueous phase and extraction with ether gave the acid, which was esterified with diazomethane. The methyl *cis*-2-undecenoate produced by this method contained some *trans* isomer from which it was readily separated by TLC. Throughout this reaction procedure, care was taken to minimize losses of the short chain products through their water solubility and volatility. All aqueous solutions were saturated with salt before extraction, and organic solvents were removed under a stream of nitrogen to 0°C; operations where products were taken to dryness were kept to a minimum.

Before effecting the stereomutation of the methyl cis-2-undecenoate to the trans isomer, the tritiated compound was mixed with ^{14}C-labelled cis-undecenoate. This ^{14}C-labelled ester acted as an internal standard and obviated the need for calculation of specific activities of precursor and product. The carbon labelled substrate was added as the cis ester, before the isomerization step, as calculation of the $^{3}H/^{14}C$ ratio before and after stereomutation, would indicate whether any tritium had been lost from the double bond during the isomerization reaction.

The carbon labelled methyl cis-2-undecenoate was prepared from 1-^{14}C decanoic acid, by the route shown in Scheme 9. This series of reactions is identical to that used for the preparation of cis-2-heptadecenoate from palmitic acid discussed on pages 43,44. The reaction mechanism for the rearrangement step in the synthesis has also been discussed earlier (pp 43-44)

and it can be seen that the product from decanoic acid labelled with ^{14}C at the carboxyl group, will be cis-2-undecenoate, also labelled at the carboxyl group.

A small amount of ^{14}C-labelled cis-2-undecenoate was added to the [2,3-$^{3}H_2$]cis-2-undecenoate. The dual labelled ester was then reacted with mercuric acetate in methanol to give the methoxymercuric addition compound, which was decomposed with hydrochloric acid to give the trans ester. The trans-2-undecenoate was purified by TLC. The $^{3}H/^{14}C$ ratio of the product was determined by scintillation counting.

As expected, the product showed virtually no loss of tritium during isomerization. The $^{3}H/^{14}C$ ratio of the starting material and product are shown below.

	$^{3}H/^{14}C$
[1-^{14}C; 2,3-$^{3}H_2$]-methyl cis-2-undecenoate	6.6
[1-^{14}C; 2,3-$^{3}H_2$]-methyl trans-2-undecenoate	6.0

The dual labelled trans-2-undecenoate was then hydrolysed to give the free fatty acid used in the incubations. Investigations of the hydrolysis reaction with unlabelled trans-$\alpha\beta$-unsaturated esters showed that, on hydrolysis with methanolic potassium hydroxide, migration of the double bond to give $\beta\gamma$-unsaturated acids and addition of methanol across the double bond to give 3-methoxy undecanoic acid both occurred.

Davidson and Korn (87) have reported that hydrolysis of $\alpha\beta$-unsaturated methyl esters with 0.5M sodium hydroxide in 50% methanol, causes partial isomerization via the methoxy intermediates, to the $\beta\gamma$-unsaturated acids. Approximately 10% of the $\alpha\beta$-unsaturated methyl ester was converted to the 3-methoxy acid and 2% to the $\beta\gamma$-unsaturated isomer. The formation of these artefacts was prevented by hydrolysing the ester in equal volumes of benzene and dilute aqueous ethanolic potassium hydroxide. This mild procedure did not cause migration of, nor addition to, the double bond in the $\alpha\beta$-unsaturated ester. Measurement of the $^{3}H/^{14}C$ ratio of the product showed that no tritium was lost during the hydrolysis.

The dual labelled trans-2-undecenoic acid produced by the reactions outlined above, was incubated with Chlorella vulgaris to investigate the stereochemistry of the hydrogenation step in acyl chain elongation. The results of the incubations are discussed later.

Incubation of β-hydroxy precursors with Chlorella vulgaris

Unlike most plants, the green alga Chlorella vulgaris is capable of two modes of existence. In the "rich" media in which it was grown, the organism exists heterotrophically, and utilizes the various organic substances in the media as a source of energy. The use of phosphate buffer as an incubation media forces the organism into a totally photo-autotrophic existence and changes the fatty acid composition of the cells. The photo-autotrophic existence results in formation of more linoleic and linolenic acids, typical of photosynthetic tissues (23). Under these conditions,

the organism is capable of breaking down fatty acids by β-oxidation to acetyl-CoA and reutilising this in de novo synthesis, which causes randomization of the label into other fatty acids. However, this complication was overcome by the use of odd chain precursors, as discussed earlier.

There are several advantages in using whole cells of Chlorella vulgaris for the biochemical investigations rather than, for example, isolated enzyme systems or subcellular fractions from bacteria, yeasts, or livers. Using Chlorella, the substrates can be added as free acids and the cells will perform the activation steps, such as the formation of the CoA esters, necessary for the specifically labelled compounds to become substrates for the acyl chain elongation enzymes. If isolated enzymes or subcellular fractions had been used, the substrates would have needed to be in the form of the thiol esters, which would have involved further synthetic procedures. A further advantage over isolated enzymes is that the Chlorella fatty acid synthetase is a coupled enzyme complex. This means the products from the synthetic substrates will be saturated acids, which facilitates the separation of these products. Some of these saturated products will be converted to monoenes by the Chlorella desaturase system and this is an advantage in the later work on the determination of the stereochemistry of hydrogenation of $\alpha\beta$-unsaturated acyl-ACP by the fatty acid synthetase system in Chlorella.

One major disadvantage of using whole cells for the investigation of the stereochemistry of acyl chain elongation is that it is not known whether it is only the "de novo fatty acid synthetase" or whether it is a microsamal or mitochondrial elongase system, or a combination of several such enzymes together performing the biochemical transformations.

The first reaction in the biosynthesic pathway to long chain fatty acids to be investigated in Chlorella was the dehydration of β-hydroxy acyl derivatives to give trans-$\alpha\beta$-unsaturated acids.

Assuming dehydration of the β-hydroxy acids takes place stereospecifically by abstraction of a hydrogen atom from the α-position, and the hydroxyl group from the β-position to give the trans-$\alpha\beta$-unsaturated acid, there are several mechanisms which could prevail. These mechanisms are outlined in Scheme 10. Only the D enantiomer of the hydroxy acid is shown in the diagram, as it is only this enantiomer which is utilized as a substrate for acyl chain elongation, and the mechanisms shown assume that the groups eliminated are either in a staggered or eclipsed conformation.

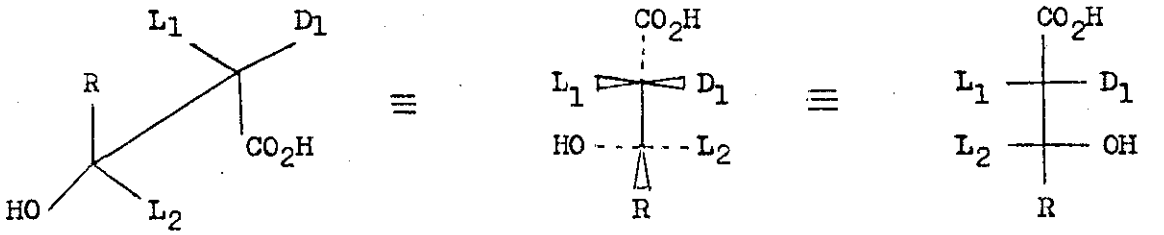
Determination of the relative configurations of the hydrogen and hydroxyl group removed, will eliminate half of the possible mechanisms for dehydration outlined in Scheme 10.

In order to determine the stereochemistry of the dehydration reaction in acyl chain elongation, the synthetic threo- and erythro-2-deuterio, 3-hydroxyheptadecanoic acids were each incubated with Chlorella vulgaris As the fatty acid synthetase of this organism is a coupled enzyme complex, the direct product of the dehydration step in the chain elongation pathway, namely the trans-$\alpha\beta$-unsaturated acid, could not be isolated as such. The final hydrogenation step in the sequence was automatically effected by the micro-organism, and the first product which could be isolated was heptadecanoic acid. In the Chlorella, some of this product, in turn, was subsequently desaturated to the cis-9-heptadecenoic acid and possibly to the cis-9,cis-12-heptadecadienoic acid.

A second pair of incubations were performed concurrently. In these, the Chlorella was incubated with the erythro- and threo- substrates in the presence of an equal amount of sterculic acid (W-(2-n-octylcycloprop-1-enyl) octanoic acid). Sterculic acid has been shown to be a potent inhibitor of desaturation in many systems (88, 89, 90) and this effect has been attributed to the cyclopropene ring. The inclusion of this inhibitor effectively stopped desaturation of the heptadecanoic acid, as determined by GLC of the incubation products.

Substrate:- β-hydroxyheptadecanoic acid (R = $CH_3(CH_2)_{13}$-)

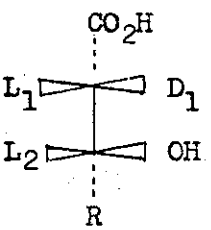
Conformation of D enantiomer



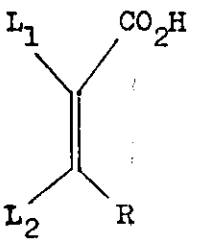
L_1, L_2 and D_2 refer to the hydrogens of the L and D configurations

1. Removal of the erythro Groups

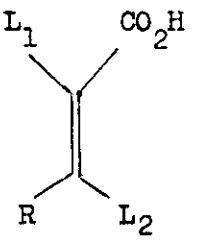
A) enzyme bound change of conformation →



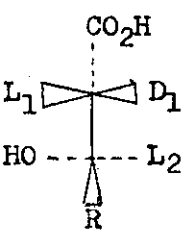
cis removal of erythro hydrogen →



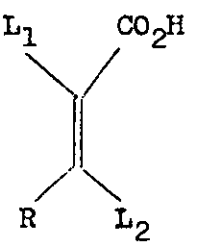
enzyme bound isomerization →



B) staggered configuration on enzyme →

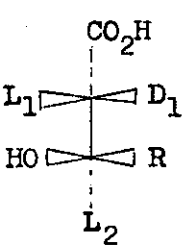


trans removal of erythro hydrogen →

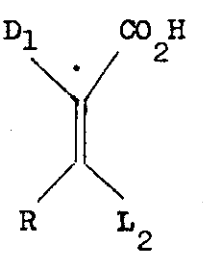


2. Removal of threo Groups

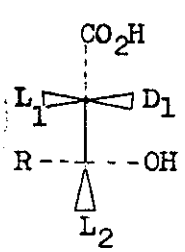
A) enzyme bound change of conformation →



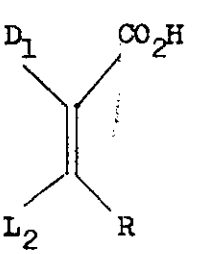
cis removal of threo hydrogen →



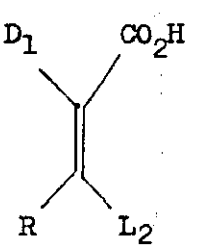
B) enzyme bound change of conformation →



trans removal of threo hydrogen →



enzyme bound isomerization →



erythro and threo hydrogens refer to the hydrogen atoms on the α-position which are erythro (cis) and threo (trans) respectively to the hydroxyl group on the β-position

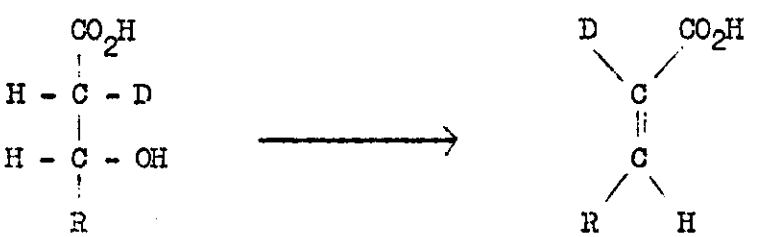
Scheme 10. Possible mechanism for the dehydration of β-hydroxy acids to give trans α,β-unsaturated acids

Considering a broad classification of the mechanism outlined in Scheme 10, production of a trans-$\alpha\beta$ -unsaturated acid from the β-hydroxy acid can occur by two different routes, namely:-

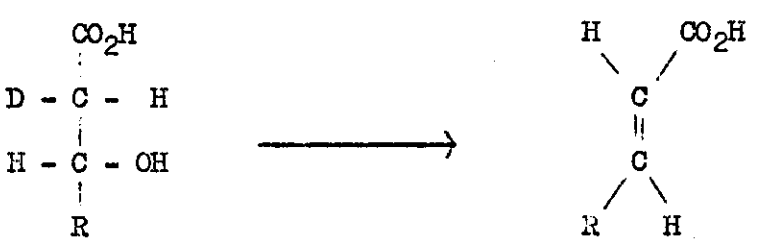
β-hydroxy acid $\xrightarrow{\text{REMOVAL OF 'ERYTHRO' HYDROGEN}}$ trans-$\alpha\beta$ -unsaturated acid

β-hydroxy acid $\xrightarrow{\text{REMOVAL OF 'THREO' HYDROGEN}}$ trans-$\alpha\beta$ -unsaturated acid

If the latter prevails and stereospecific removal of the 'threo' hydrogen takes place from the erythro- and threo-2-deuterio, 3-hydroxy acids, the products would be as illustrated in Scheme 11.



erythro



threo

Scheme 11.

When the erythro-2-deuterio, 3-hydroxyheptadecanoic acid is a precursor, the deuterium atom on the α-carbon would be retained in the product. This would result in an enrichment of deuterated species in the heptadecanoic acid produced. In the case of the threo-2-deuterio, 3-hydroxyheptadecanoic acid, an elimination in which the hydrogen threo to the hydroxyl group was removed would result in complete removal of the deuterium, and the product would be expected to show no deuterium enrichment.

Alternatively, if stereospecific removal of the 'erythro' hydrogen took place, the converse would be true, resulting in the enrichment of deuterated product from the threo-2-deuterio, 3-hydroxyheptadecanoic acid, and production of nondeuterated product from the erythro isomer.

The products from the incubation of Chlorella vulgaris with the stereospecifically labelled precursors were isolated and converted to methyl esters. The methyl esters were separated into a saturated and a monoenoic fraction by TLC on silver nitrate impregnated silica gel plates.

The results of the incubation of Chlorella vulgaris with the deuterated precursors, were determined by combined gas chromatographic/mass spectrographic analysis of the products on an AEI MS 12 instrument connected to a Pye 104 gas chromatograph. Ten scans in each direction of the parent molecular ion region were recorded for each product, measuring the $^{2}H_{1}$ and $^{2}H_{0}$ intensities, and from these spectra the proportions of each species was calculated. The products investigated this way were the methyl-heptadecanoates and cis-9-heptadecenoates, from the Chlorella incubations without added sterculic acid, and the methyl heptadecanoates from the incubations to which sterculic acid had been added. The results are shown in Table 1, along with the deuterium enrichments in the precursors, for comparison.

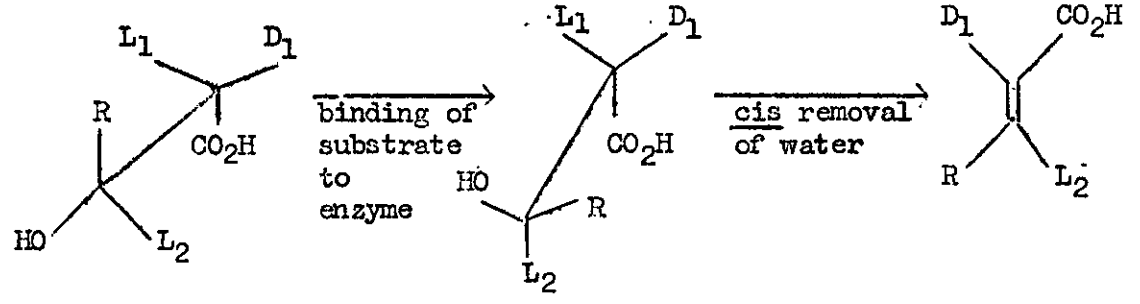
Incubation Substrates	Products			
	17:0		Δ^9 17:1	
	^2H_o	$^2H_1$	^2H_o	$^2H_1$
erythro 2-^{2}H, 3-OH 17:0	18	82	25	71
erythro 2-^{2}H, 3-OH 17:0 + sterculic acid	18	82		
threo 2-^{2}H 3-OH 17:0	91	9	89	7
threo 2-^{2}H 3-OH 17:0 + sterculic acid	91	9		
Precursors				
erythro 2-^{2}H 3-OH 17:0	16	82		
threo 2-^{2}H 3-OH 17:0	26	73		

Table 1. Mass spectrographic analysis of methyl heptadecanoate and methyl cis-9-heptadecenoate produced from erythro and threo 2-deuterio, 3-hydroxyheptadecanoic acid by Chlorella vulgaris

In these results, an enrichment of the deuterated species in the product from the erythro substrate is observed, the product from the threo substrate containing virtually no deuterium. This is in keeping with the results expected for the removal of a hydroxyl group and a hydrogen atom of opposite configurations. The complete retention of deuterium by the products formed from erythro-2-deuterio, 3-hydroxyheptadecanoate indicates conclusively that the removal is of that α-hydrogen which is in the threo configuration relative to the β-hydroxyl group.

The presence of 9% of the deuterated heptadecanoate in the product from the threo substrate is not considered to indicate any lack of stereospecificity of the enzymic dehydration, but rather a lack of complete specificity during the reduction of the cis-epoxide with lithium boron deuteride, during the synthesis of this precursor.

Relating these results to the possible mechanisms proposed in Scheme 10, removal of a hydrogen atom and hydroxyl group of like configuration (erythro) does not occur and so mechanisms 1A and 1B are not feasible. The natural and most probable inference of these results is that the mechanism of dehydration involves a cis removal of the L-2 hydrogen and D-3 hydroxyl (mechanism 2A, Scheme 10). This requires that the β-hydroxy acid assumes an eclipsed conformation about the C-2 C-3 bond during dehydration, this conformation being induced in the enzyme-substrate complex, as shown:-



β-hydroxy acid (staggered)

The results, however, do not preclude the reaction proceeding via a trans elimination of water (mechanism 2B). The immediate product of such an elimination would be the cis-$\alpha\beta$-unsaturated acid and this would need to be isomerized to produce the trans-acid which is the natural product of this step of the acyl chain elongation pathway. Although enzymes are known which will isomerize a cis-double bond in fatty acids to the trans-

configuration, such as occurs in biohydrogenation of unsaturated fatty acids in ruminants (91), and in the "anaerobic" pathway to unsaturated fatty acids (22), these isomerizations usually involve a migration of the unsaturated centre. As no migration of the double bond occurs in this case, a trans elimination mechanism is considered unlikely, but the possibility of a change in configuration of any enzyme bound olefinic intermediate cannot be totally excluded.

The results prove that the enzyme in Chlorella vulgaris, which catalyses the dehydration of β-hydroxyheptadecanoic acid to trans-2-heptadecenoic acid, has a strict geometrical specificity removing an α-hydrogen atom and β-hydroxyl group of opposite configurations.

It is known that only the D enantiomers of β-hydroxy acids are utilized as substrates during acyl chain elongation and therefore the above results also define the absolute specificity of the dehydratase enzyme. Thus, as dehydration was shown to occur with the loss of groups of opposite configuration, it can be concluded that the dehydratase enzyme of Chlorella vulgaris exhibits an absolute stereospecificity, removing the L-2(pro-S) hydrogen and D-3(R) hydroxyl group of 3-hydroxyheptadecanoic acid to give the trans double bond.

Incubation of trans-$\alpha\beta$-unsaturated precursors with Chlorella vulgaris

The other intermediate reaction of acyl chain elongation to be investigated during this work was the reduction of $\alpha\beta$-unsaturated acyl-ACP to saturated acyl-ACP.

$$RCH = CHCOSACP + NADPH + H^{+} \longrightarrow RCH_2CH_2COSACP + NADP^{+}$$

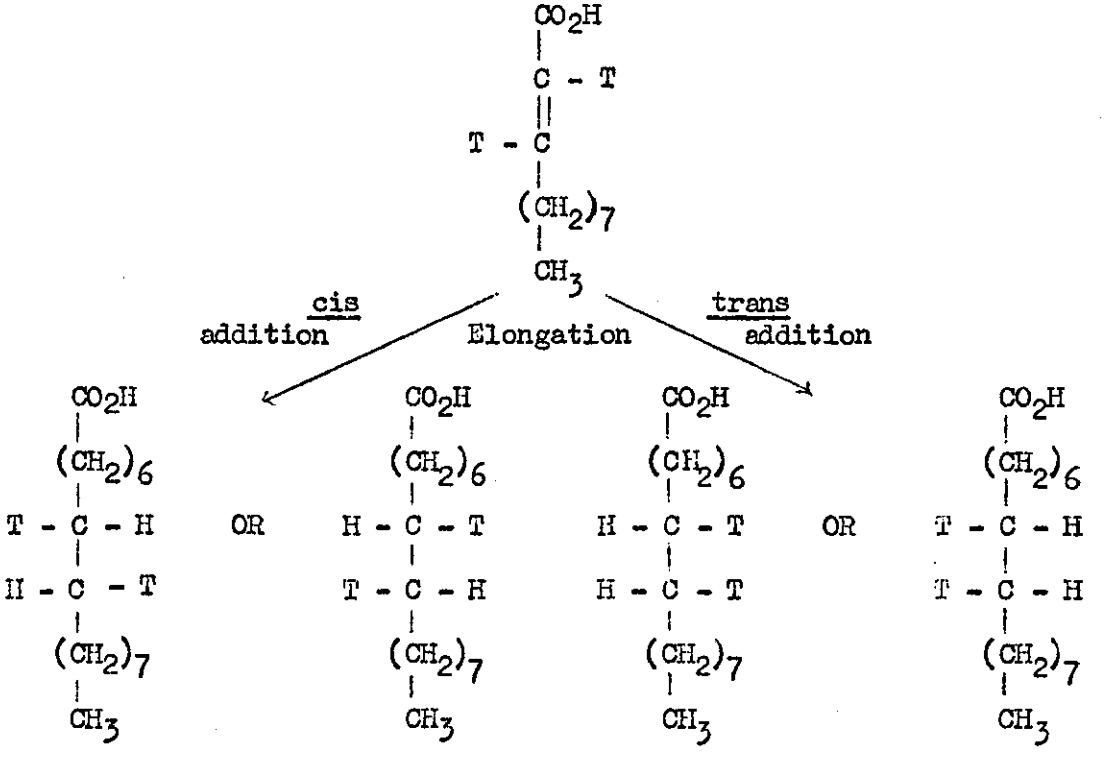
This reaction, as discussed earlier (p. 24), is catalysed by an enoyl-ACP reductase, hydrogen being added to the β-position of the acyl chain from the A side of NADPH (17). A proton from water is added to the α-position (62).

There are two possible modes of addition of hydrogen to the double bond in $\alpha\beta$-unsaturated acyl-ACP, cis-addition or trans-addition. If cis-addition of hydrogen occurs, then the hydrogen atoms originally on the double bond will assume a threo configuration in the product; trans-addition will result in these hydrogens assuming an erythro configuration. Within these two overall modes of addition, there is the possibility of an absolute stereospecificity during the reduction, thus in trans-addition of hydrogen, the hydrogen atoms which were originally on the double bond could assume the DD (pro-R, pro-R) or LL configurations, while in cis-addition, these hydrogens could assume the DL (pro-R, pro-S) or LD configurations. It was considered most likely that the enzymic reduction would show an absolute specificity, and this work was to determine this specificity during the reduction of trans-2-undecenoic acid by Chlorella vulgaris.

In order to determine the absolute specificity of the hydrogenation step in acyl chain elongation [1-^{14}C, 2,3-$^{3}H_2$]trans-2-undecenoic acid, prepared by the methods discussed earlier, was incubated with whole cells of the green algae Chlorella vulgaris. If the trans-2-undecenoic acid is reduced by the enoyl reductase enzyme of the multienzyme complex of chain elongation, the immediate product will be [1-^{14}C, 2,3-$^{3}H_2$]-undecanoic acid, and the tritium atoms on C-2 and C-3 will have either an erythro- or threo-configuration, as discussed above. As chain elongation in Chlorella vulgaris is catalysed by the multienzyme complex, the product of the enoyl reductase, undecanoic acid, cannot be isolated as such and is utilized as a substrate for the next step of chain elongation, namely condensation of a further molecule of malonate to produce β-keto tridecanoate. This is then reduced, dehydrated and hydrogenated, to give tridecanoic acid, ready for the condensation of another malonate molecule: in this way chain elongation continues.

The dual labelled undecenoic acid was incubated with whole cells of Chlorella vulgaris and the products were isolated. The major elongation product was heptadecanoic acid, produced by reduction of the trans-2-undecenoic acid, followed by chain extension by six carbon atoms from three molecules of malonate. Some of the heptadecanoic acid produced in this manner was also desaturated by the Chlorella to give cis-9-heptadecenoic acid.

Considering the hydrogenation and chain elongation reactions to give the saturated acid, it can be seen that the tritium atoms which were originally at C-2 and C-3 in the undecenoic acid precursor, will now be at C-8 and C-9 in the heptadecanoic acid, and, depending upon the stereochemistry of addition of hydrogen to the double bond during the reduction step, they will have either the erythro (DD and/or LL) or the threo (DL and/or LD) configuration. During the reduction and subsequent elongation reactions to the saturated C:17 acid, there should be no loss of tritium from the precursor and therefore the heptadecanoic acid produced by these biochemical reactions should contain the same amount of tritium as the undecenoic acid precursor.



This was indeed found to be the case, and the $^{3}H/^{14}C$ ratio of the heptadecanoic acid produced by biochemical elongation of [1-^{14}C; 2,3-$^{3}H_2$] trans-2-undecenoic acid in Chlorella, was almost identical to that of the undecenoic acid precursor as shown in Table 2. It should be noted, however, that the $^{3}H/^{14}C$ ratio of the heptadecanoic acid produced by chain elongation was only determined after its conversion to stearic acid by chemical elongation by one carbon. The chemical elongation was performed on the whole of the saturated fatty acid fraction isolated from the incubation, as the endogenous even chain fatty acids acted as a carrier for the labelled heptadecanoic acid, and minimized losses during the chemical manipulations. After chemical elongation, the stearate produced from the heptadecanoate was isolated from the mixed saturated fatty esters by preparative GLC, and the $^{3}H/^{14}C$ ratio of an aliquot of this stearate was determined by scintillation counting. The remainder of the stearate was stored at 0°C. The $^{3}H/^{14}C$ ratio of the stearate produced by chemical elongation must be identical to that of the heptadecanoic acid from which it originated, because the chemical reactions used to elongate the C:17 acid will not affect the radio-label, which is now in the middle of the fatty acid chain.

	$^{3}H/^{14}C$
Precursor trans-2-undecenoic acid	6.0
Product stearic acid produced by elongation of heptadecanoic acid	6.6

Table 2.

The results show a slight rise in the $^{3}H/^{14}C$ ratio for the stearate over that for the trans-2-undecenoic acid precursor, whereas if the second reductive step of fatty acid synthesis occurs by direct addition of hydrogen to the double bond as expected, these ratios should be equal. The probable explanation for this small difference is that during β-oxidation of the substrate, which also occurs to an appreciable extent, there is a discrimination against the undecenoic acid containing two tritium atoms, and therefore, the substrate used for elongation by the fatty acid synthetase enzymes has a higher $^{3}H/^{14}C$ ratio than that actually added to the incubation. However, it should also be noted that the $^{3}H/^{14}C$ ratio of the stearate is close to that of the methyl cis-2-undecenoate ($^{3}H/^{14}C$ = 6.6), which was the chemical precursor of the trans-2-undecenoic acid, and it is possible that, during the chemical synthesis of the trans acid, some contamination might have occurred which apparently lowered the $^{3}H/^{14}C$ ratio of the biological precursor, as measured.

It can be concluded, however, from the results that, as expected, the second reductive step in acyl chain elongation, namely the reduction of trans-$\alpha\beta$-unsaturated acyl-ACP to saturated acyl-ACP, occurs without the loss of either of the hydrogen atoms at the C-2 or C-3 positions.

As mentioned earlier, some of the heptadecanoic acid produced by biochemical elongation of the undecenoic acid precursor will have been desaturated by Chlorella to give cis-9-heptadecenoic acid. This desaturation is of known stereochemistry and removes the D-9 and D-10 hydrogen atoms from the saturated precursor (32). The knowledge of the absolute stereochemistry of this desaturase allows the stereochemistry of addition of hydrogen to the C-3 position in trans-2-undecenoic acid during acyl chain elongation to be deduced.

When [1-^{14}C; 2,3-$^{3}H_2$] trans-2-undecenoic acid is incubated with Chlorella, it is first reduced to undecanoic acid and then elongated to

heptadecanoic acid. This elongation means that the carbon atom and the tritium atom it bears, which were at the C-3 position in the precursor, will now be at the C-9 position in the elongated product, and those which were at C-2 in the precursor will now be at the C-8 position of the product.

If, as expected, the reduction of undecenoic acid by the fatty acid synthetase enzyme is stereospecific the tritium atom, which was at C-3 in the precursor and is now at C-9 in the heptadecanoic acid, will have specifically either the D(R) or L(S) configuration. If this tritium is in the D(R) configuration, it will be removed during the desaturation of heptadecanoic acid to cis-9-heptadecenoic acid, and the $^{3}H/^{14}C$ ratio in the heptadecenoic acid isolated from the incubation of Chlorella with [1-^{14}C; 2,3-$^{3}H_2$] trans-2-undecenoic will be half that of the precursor (the tritium atom at C-8 will be unaffected). On the other hand, if the tritium atom has the L(S) configuration, the $^{3}H/^{14}C$ ratios in precursor and product will be identical.

The cis-9-heptadecenoic acid produced during the incubation of [1-^{14}C; 2,3-$^{3}H_2$] trans-2-undecenoic acid with Chlorella was isolated by a combination of argentation TLC and preparative GLC and its $^{3}H/^{14}C$ ratio determined by scintillation counting. The results are shown in Table 3.

	$^{3}H/^{14}C$
trans-2-undecenoic acid	6.0
cis-9-heptadecenoic acid	7.4

Table 3.

It can be seen that the $^{3}H/^{14}C$ ratio of the cis-9-heptadecenoic acid is close to that of the labelled precursor. From these results it can be concluded that the tritium atom now at C-9 in the heptadecenoic acid was not removed during desaturation and therefore must be in the L(S) configuration. This, in turn, means that the hydrogen added to C-3 during the enzymic reduction of trans-2-undecenoic acid during chain elongation must have taken up the D(pro-R) configuration. Therefore, it can be concluded that the addition of hydrogen to the β position of $\alpha\beta$-unsaturated acyl-ACP during acyl chain elongation, shows an absolute stereospecificity, the incoming hydrogen assuming the D(pro-R) configuration.

A similar argument to that outlined above can now be used to determine the absolute specificity of addition of hydrogen to th 2-position during the reduction of $\alpha\beta$-unsaturated enoyl-ACP in chain elongation. The tritium atom, which was at the C-2 position in the [1-^{14}C; 2,3-$^{3}H_2$] trans-2-undecenoic acid precursor, is now at C-8 in the heptadecanoic acid produced by biochemical chain elongation. This tritium atom, is therefore, unaffected by the Δ^9 desaturase which converts heptadecanoic acid to cis-9-heptadecenoic acid in Chlorella. If, however, the heptadecanoic acid product is extended chemically by addition of one carbon to the carboxyl end of the molecule to give stearic acid, the tritium atom, which was at C-8, is now at C-9, and the tritium which was at C-9 is now at C-10. If This chemically produced labelled stearic acid is now reincubated with a system containing a Δ^9 desaturase, the tritium atom at C-9 will either be lost or retained in the oleic acid produced, depending upon its absolute stereochemistry. It is already known that the tritium atom now at the C-10 position has the L(S) configuration and will, therefore, be retained in the oleic acid produced by desaturation. Thus, if the tritium atom at C-9 has the D(R) configuration, it will be lost during desaturation, and the $^{3}H/^{14}C$ ratio of the product will be half that of the stearic acid precursor; whereas if the C-9 tritium is in the L(S) configuration, the product and precursor will have the same $^{3}H/^{14}C$ ratio.

The heptadecanoic acid produced from the [1-^{14}C; 2,3-$^{3}H_2$] *trans*-2-undecenoic acid precursor by chain elongation in *Chlorella* was extended by one carbon atom, using potassium cyanide. The stearic acid produced was isolated and purified by preparative GLC and an aliquot was taken for the determination of the $^{3}H/^{14}C$ ratio. The remainder of the stearic acid was incubated with whole cells of *Chlorella vulgaris*. At the end of the incubation period, the lipids were extracted and transmethylated and the oleic acid was isolated and purified by a combination of argentation-TLC and preparative GLC, and an aliquot was taken for scintillation counting. The results are shown in Table 4.

	$^{3}H/^{14}C$
Precursor	
Stearic acid	6.6
Product	
Oleic acid	2.4

Table 4.

It is evident from the results that the olefinic product derived from the labelled stearic acid shows a markedly decreased $^{3}H/^{14}C$ ratio, indicating loss of slightly more than half the tritium in the precursor. As it is known that the tritium atom at C-10 in the precursor has the L configuration, and that desaturation of stearate to oleate occurs by *cis* removal of the D-9 and D-10 hydrogen atoms (32), the results imply that the tritium atom at C-9 in the precursor must have the D(R) configuration. This, in turn, implies that the hydrogen atom added to the 2-position in the

reduction of trans-2-undecenoic acid during acyl chain elongation, must have assumed the L(pro-S) configuration. Thus the results show that the addition of hydrogen to the 2-position of $\alpha\beta$-unsaturated acyl-ACP during its reduction by the enoyl reductase enzyme of acyl chain elongation occurs with complete stereospecificity, the incoming hydrogen assuming the L (pro-S) configuration.

It would be expected that if the reduction of the [1-^{14}C; 2,3-$^{3}H_2$] trans-2-undecenoic acid by the enoyl reductase was completely stereospecific, that the $^{3}H/^{14}C$ ratio of the oleic acid, shown in Table 4, would be exactly half that of the stearic acid precursor (i.e. 3.3). The lower value of 2.4 is not considered to be due to a lack of specificity of the enoyl reductase enzyme but is much more likely to be due to a primary isotope effect during the desaturation of the stearic acid.

During the desaturation of the stearic acid, there will be a primary kinetic isotope effect against the D-9 tritium atom in the tritiated stearic acid, but no such isotope effect against the ^{14}C labelled component, which contains no tritium. This will result in the desaturation of the ^{14}C labelled substrate being faster than the desaturation of the tritiated stearic acid, giving a slight enrichment of the ^{14}C labelled component in the oleic acid product. This enrichment explains the lower than theoretical $^{3}H/^{14}C$ ratio in the oleic acid product shown in Table 4.

The work outlined in this section has established the absolute stereochemistry of two of the intermediate reactions in acyl chain elongation, namely the dehydration of β-hydroxy acyl-ACP to unsaturated acyl-ACP, and the subsequent hydrogenation of this unsaturated derivative to saturated acyl-ACP.

The first of these reactions, namely the dehydration of β-hydroxy acyl derivatives, was already known to have an absolute stereospecificity for the D(-) β-hydroxy acid. The present study has shown that, in Chlorella

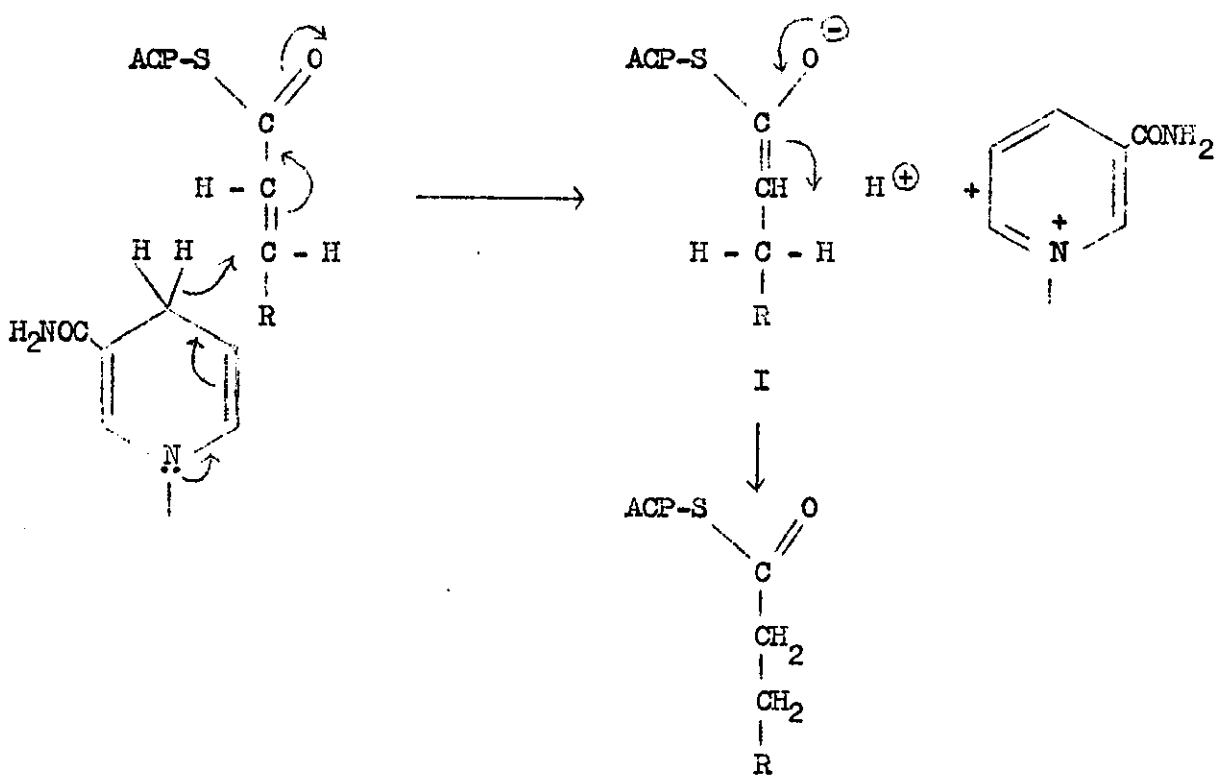
at least, the dehydration is effected with total stereospecificity, only the L hydrogen being removed from the α position. Although it has been possible to define precisely the stereochemistry of the hydroxyl group and hydrogen atom removed during this dehydration, the exact mechanism by which they are removed and the order in which they are abstracted cannot yet be stated definitively.

A plausible mechanism for this transformation would be the cis removal of the elements of water, while the β-hydroxy acyl derivative is in the eclipsed, rather than the more usual staggered, conformation. In the extended chain conformation of β-hydroxy acids, the D hydroxyl at position-3 and the L hydrogen at position-2 are relatively close. To bring the hydrogen atom and hydroxyl groups together in an eclipsed conformation, a 60° rotation about the 2,3 C-C bond is required. This would then allow the cis removal of the elements of water by a single active centre, to leave an $\alpha\beta$-unsaturated acid in the correct trans configuration.

As mentioned above, these investigations give no indication of the order in which the hydroxyl group and hydrogen atom are removed. Conceivably, the reaction can occur by several mechanisms, either via a carbanion or E_2 type elimination if the hydrogen atom leaves before the hydroxyl group, or via a carbonium ion intermediate if the hydroxyl group is lost first, or by a concerted mechanism. These mechanisms could possibly be differentiated by performing kinetic studies using an α-deuterated or α-tritiated β-hydroxy precursor. If breaking of the C-H bond at C-2 is rate limiting in the β-hydroxy- to $\alpha\beta$-enoate conversion (as in a carbanion or E_2 type elimination) then a deuterium or tritium isotope effect would be expected with the loss of water from the deuterated or tritiated precursor being slower than from a normal substrate. If the loss of water occurs via a carbonium ion, the breaking of the C-H bond at

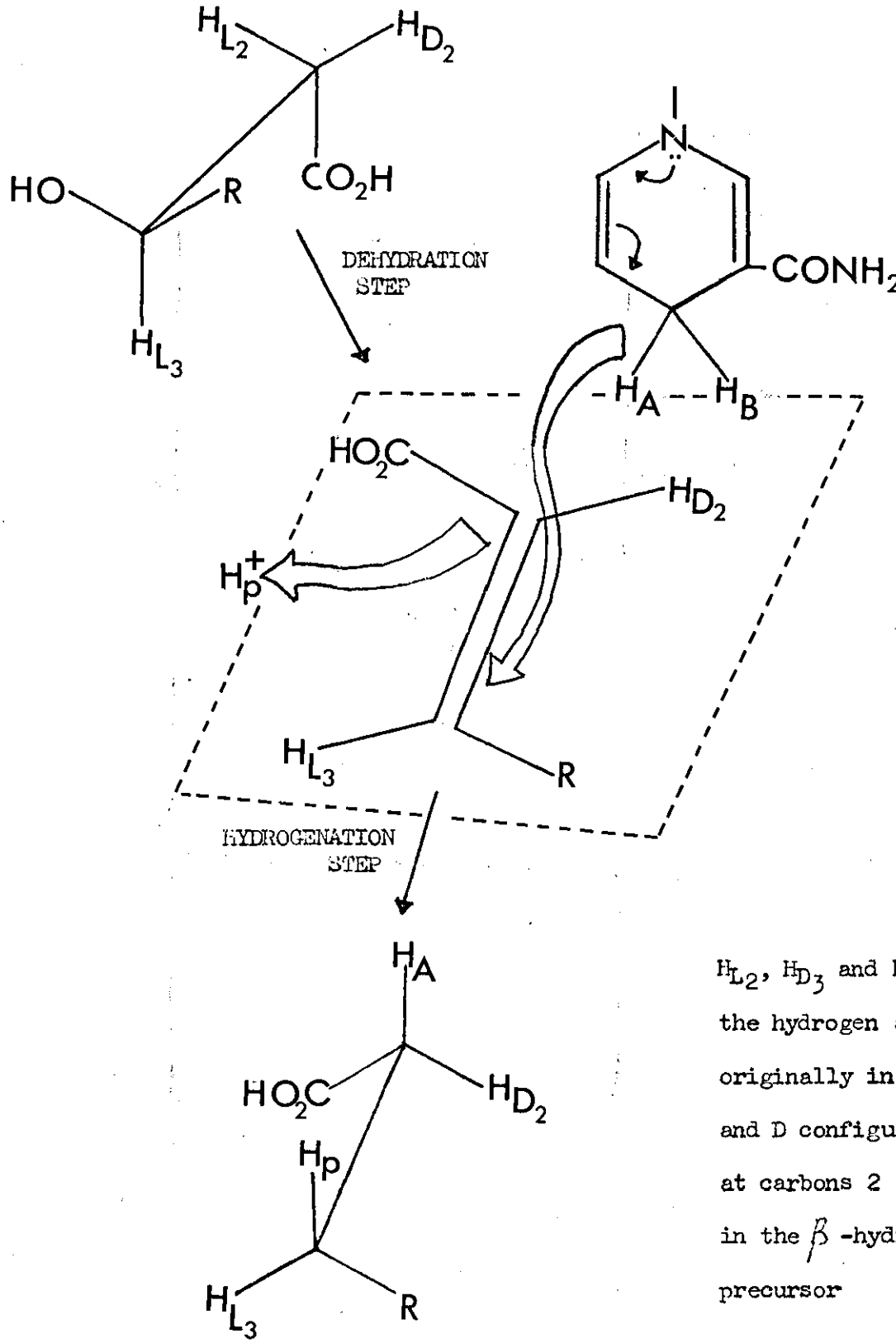
C-2 would not be expected to be rate limiting, and no difference in the rate of water loss or of formation of the $\alpha\beta$-unsaturated product from the deuterated or tritiated precursor would be expected as compared to normal substrate.

The other intermediate reaction of acyl chain elongation considered in this work was the second reductive step, namely the reduction of trans-$\alpha\beta$-enoyl-ACP to acyl-ACP. It was already known that a hydrogen atom was added to the C-3 position of the unsaturated acid from the A side of NADPH (17) and that the C-2 position acquired a proton from water. This work has proved that both these additions show an absolute stereospecificity and that the overall hydrogenation involves cis-addition of hydrogen to the trans double bond, these hydrogen atoms being inserted in the L(pro-S) configuration at the 2-position and the D(pro-R) configuration at the 3-position. The mechanism of this addition almost certainly involves the initial attack by a hydride ion from NADPH at the β-carbon, which is electrophilic due to the transmission of the carbonyl polarization through the conjugated system, followed by the addition of a proton to the α-carbon.



Whether the reaction involves an intermediate such as I above, or whether addition of the proton occurs without the intermediate formation of a species such as I is not known, although the strict stereospecificity of the reaction suggests that no free intermediate such as I can take part in the reaction; any such intermediate must remain rigidly bound to, and constrained by, the enzyme.

The stereochemistry of the two intermediate reactions of acyl chain elongation investigated in this work is therefore as summarized in Scheme 12.



H_{L_2}, H_{D_3} and H_{L_3} are the hydrogen atoms originally in the L and D configurations at carbons 2 and 3 in the β-hydroxy acid precursor

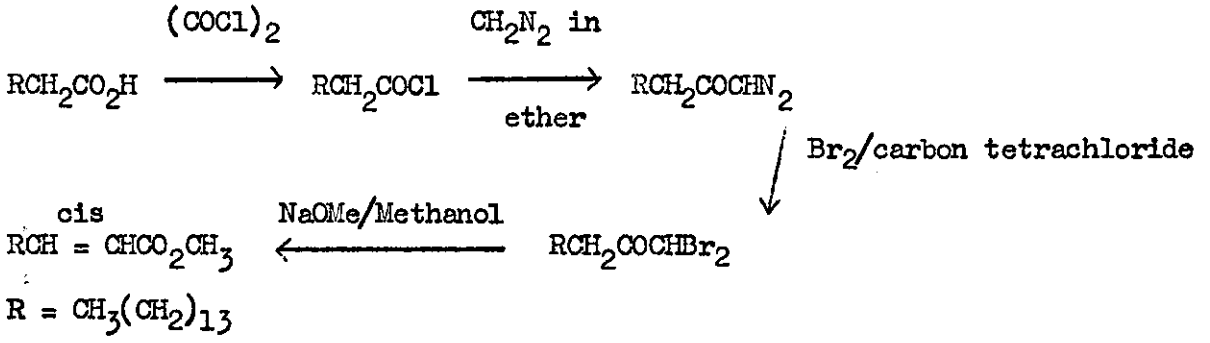
Scheme 12. Overall stereochemistry of dehydration and hydrogenation reactions of acyl chain elongation

EXPERIMENTAL

1. Preparation of erythro- and threo-2-deuterio-3-hydroxyheptadecanoic acids

i) Preparation of methyl-cis-2-heptadecenoate

This method is a modification of that of Gerson and Schlenk (64) and is shown below.



$RCH_2CO_2H \longrightarrow RCH_2COCl \longrightarrow RCH_2COCHN_2$

$RCH_2COCHN_2 \xrightarrow{Br_2/\text{carbon tetrachloride}} RCH_2COCHBr_2$

$RCH_2COCHBr_2 \xleftarrow{\text{NaOMe/Methanol}} RCH = CHCO_2CH_3$ (cis)

$R = CH_3(CH_2)_{13}$

Oxalyl chloride (10g) was added to palmitic acid (20g) in benzene (200ml) and the mixture was stood at room temperature for six hours. Benzene, and excess oxalyl chloride were removed at reduced pressure to give a colourless oil (20g), equivalent to a yield of 97%.

Diazomethane was prepared from Diazald (20g), and the solution in ether was dried over potassium hydroxide pellets at -17°C for several hours, decanted and cooled to -40°C.

The acid chloride (4.5g) was dissolved in dry ether (200ml) and cooled to -40°C.

The solutions were mixed and allowed to warm to room temperature, evolution of nitrogen began at about -20°C. After one hour, excess diazomethane was removed under a stream of nitrogen, and concurrently the solution was reduced to a volume of approximately 100ml. The ether solution was cooled and the resultant pale yellow flakes were filtered off at the pump. The yield of pentadecyldiazomethyl ketone was 2.1g (44%).

The diazoketone was dissolved in dry redistilled carbon tetrachloride (30ml). Bromine was similarly dissolved in carbon tetrachloride. The diazoketone solution was stirred magnetically and the bromine solution was added slowly until the colour of bromine persisted. Solvent was removed to leave a brown oil which was immediately dissolved in methanol (100ml).

To the solution of dibromoketone in methanol was added a solution of sodiummethoxide in methanol, prepared from sodium (6g) in methanol (100ml), and the mixture was stirred at room temperature for half an hour.

The solution was diluted with water (200ml) and extracted with ether (2 x 200ml), the ether extracts were washed with water until neutral, dried over anhydrous sodium sulphate and solvent removed to leave a pale yellow oil (1.7g).

The pure produce, methyl cis-2-heptadecenoate, was isolated by column chromatography on Davison Silica Gel, Grade 950, eluted with petroleum ether. The product was a colourless oil (1.0g) equivalent to a yield of 20% based on acid chloride.

ii) Preparation of methyl trans-2-heptadecenoate

Methyl cis-2-heptadecenoate, prepared as above, was isomerized by the method of Barve and Gunstone (65).

Methyl cis-2-heptadecenoate (1g) was dissolved in methanol (40ml) and mercuric acetate (1.5g) was added to this solution. After sixteen hours at room temperature, the solution was cooled to 0°C and acidified with concentrated hydrochloric acid (5ml). After a further fifteen minutes, the solution was diluted with water (40ml), and extracted with ether (2 x 25ml). The ether extracts were washed with water until neutral, and solvent removed to leave a pale yellow oil which was purified by TLC (ether:petroleum ether 1:9) to yield methyl trans-2-heptadecenoate (0.9g, 90%).

iii) Epoxidation of methyl cis and trans-2-heptadecenoate

Methyl cis-2-heptadecenoate (1g) was dissolved in benzene (75ml) and meta-chloroperbenzoic acid (5g) was added to this solution and the mixture was warmed to dissolve the peracid. The solution was kept at room temperature for twenty days.

The benzene solution was decanted from the precipitated m-chlorobenzoic acid which was washed with more benzene. The benzene solutions were combined and washed with water, dilute aqueous sodium sulphite, water, 5% aqueous sodium bicarbonate, and water until neutral. Solvent was removed to leave a pale yellow solid which was chromatographed over Davison Silica Gel, Grade 950, eluted with diethyl ether:petroleum ether, 5:95. The fractions containing epoxide as adjudged by TLC were combined and solvent removed to leave a white solid (0.95g, 89%).

iv) Hydrolysis of methyl cis- and trans-2,3-epoxyheptadecanoate

Methyl cis-2,3-epoxyheptadecanoate (800mg) was dissolved in 5% methanolic potassium hydroxide (5ml) and the mixture was allowed to stand for sixteen hours at room temperature. Ether (10ml) was added and the mixture was diluted with water (10ml) and acidified to pH5 with dilute acetic acid. The ether extracts were washed with water until neutral. The ether was removed and the acid dried by azeotropic distillation with acetone. The product was crystallized from petroleum ether to give colourless needles (m.pt. 81-83°C; 730mg, 86%).

v) Reduction of cis- and trans-2,3-epoxyheptadecanoic acid

cis-2,3-Epoxyheptadecanoic acid (100mg) was dissolved in dry ether (10ml) and cooled to 0°C. Lithium boron deuteride (15mg) was added to this solution, and the mixture was stirred for three hours.

The mixture was diluted with water, acidified with dilute hydrochloric acid, and extracted with ether. The ether extracts were washed with dilute sodium bicarbonate to separate acids from alcohols formed by

over-reduction during the reaction. The alkali extracts were acidified with dilute hydrochloric acid and extracted with ether. The extracts were washed with water until neutral and the solvent removed to leave a white solid.

The solid was dissolved in acetone (5ml) and 60% perchloric acid (30 µl) added. After thirty minutes, the solution was diluted with water (10ml) and extracted with ether (2 x 10ml). The ether extracts were washed with water until neutral and the solvent was removed to leave a colourless oil.

The oil was applied to two 20 x 20 x 0.5mm Silica Gel TLC plates developed in ether:petroleum ether:acetic acid (50:50:1) and the band which co-chromatographed with standard 3-hydroxypalmitic acid was scraped off and eluted to yield threo-2-deutero,3-hydroxyheptadecanoic acid (1.3mg).

2. Preparation of [1-^{14}C;2,3-$^{3}H_2$] trans-2-undecenoic acid

i) Preparation of undec-2-ynol

Lithium (0.1g), and ferric nitrate (0.2g) were added to liquid ammonia (200ml) in a 3-necked 500ml round bottom flask cooled in a dry ice/acetone bath. The flask was fitted with a dropping funnel and cold finger condenser. The mixture was stirred for five minutes. More lithium (1.4g) was then added over a period of fifteen minutes and the mixture was stirred for an hour. Propargyl alcohol (5.6g) in dry ether (50ml) was added slowly over a period of thirty minutes and the mixture was stirred for a further two hours. Octyl bromide (18g) in dry ether (25ml) was added and stirring was continued for three hours.

Ammonium chloride (6.0g) was added, the stirring was discontinued and the ammonia was allowed to evaporate overnight. Water (30ml) was added to the mixture and the layers separated. The aqueous layer was

extracted with ether, the ether solutions were combined and washed with saturated salt solution and dried over anhydrous sodium sulphate. The solvent was removed to leave a brown oil which was distilled at reduced pressure to yield undec-2-ynol (11g, 65%, b.pt. 132-134°C 9mm.)

A small amount of this alcohol was purified by TLC on Silica Gel G developed in ether:petroleum ether, 40:60. The purified material was sent to the Unilever Research Laboratory, Vlaardingen, Holland, for Lindlar reduction with tritium gas to give [2,3-$^3H_2$] cis-2-undecenol (10mg, 6.6 X 10^7 dpm/mg, 5mCi/m mole).

ii) Methyl [2,3-$^3H_2$] cis-2-undecenoate

A solution of [2,3-$^3H_2$] cis-2-undecenol (1mg) in petroleum ether (1ml) was added to a stirred suspension of active manganese dioxide (12.5mg) in the same solvent (2ml). After two hours the mixture was filtered, the residue was extracted with hot petroleum ether and the combined extracts were concentrated to give crude [2,3-$^3H_2$] cis-2-undecenal which was not purified further.

To a mixture of silver oxide (6mg), the crude aldehyde, ethanol (0.25ml), and water (1ml), was added a solution of sodium hydroxide (12mg) in water (0.5ml). The suspension was shaken overnight, then filtered and the insoluble material was washed with water. The combined filtrate and washings were extracted with ether, then acidified and again extracted. The second ether extract was washed until neutral, concentrated, and the acid product was esterified with ethereal diazomethane. The product, methyl [2,3-$^3H_2$] cis-2-undecenoate, was purified by TLC (ether: petroleum ether, 1:9).

The purity of the product was checked by GLC, and had an equivalent chain length of 11.19 on DEGA, which compares well with that of authentic cis-Δ^2-unsaturated fatty acids (66).

iii) Preparation of methyl [1-^{14}C] cis-2-undecenoate

Methyl [1-^{14}C] cis-2-undecenoate was prepared from [1-^{14}C] decanoic acid by the method of Gerson and Schlenk (33) as described previously, the only difference being that the diazoketone was not purified by crystallization, but used directly for the next step in the reaction sequence. Palmitic acid (5mg) was mixed with the [1-^{14}C] decanoic acid (100 μC, 15mC/mM) used in the synthesis. The palmitic acid was acting as a "carrier" to provide a mass of acid large enough to allow the reagents to be used in a visible amount without being in too large an excess, and to minimise losses during extraction and purification. Methyl [1-^{14}C] cis-2-undecenoate was separated from methyl cis-2-heptadecenoate, formed from the palmitic acid, by preparative GLC. The overall radiochemical yield was 12%.

iv) Preparation of methyl [1-^{14}C;2,3-$^{3}H_2$] cis-2-undecanoate

Methyl [1-^{14}C] cis-2-undecenoate and methyl [2,3-$^{3}H_2$] cis-2-undecenoate were each dissolved in benzene to give a final concentration of approximately 1 μC/ml. Aliquots of each were taken for scintillation counting.

The benzene solutions of methyl [1-^{14}C] cis-2-undecenoate (3ml) and methyl [2,3-$^{3}H_2$] cis-2-undecenoate (0.5ml) were mixed and the solvent was removed under a stream of nitrogen. The product was dissolved in benzene (2ml) and an aliquot taken for scintillation counting.

Product - methyl [1-^{14}C;2,3-$^{3}H_2$] cis-2-undecenoate (3.4 μC ^{3}H; $^{3}H/^{14}C$ = 6.60).

v) Isomerization of methyl [1-^{14}C;2,3-$^{3}H_2$] cis-2-undecenoate

Methyl [1-^{14}C;2,3-$^{3}H_2$] cis-2-undecenoate was isomerized with mercuric acetate and hydrochloric acid by the method of Barve and Gunstone (65) as described earlier. The reaction products were purified by TLC (ether:petroleum ether, 1:9) to give methyl [1-^{14}C;2,3-$^{3}H_2$] trans-2-undecenoate. (1.4 μC, $^{3}H/^{14}C$ = 6.0)

vi) Hydrolysis of methyl /1-^{14}C;2,3-$^{3}H_2$/trans-2-undecenoate

Methyl /1-^{14}C;2,3-$^{3}H_2$/trans-2-undecenoate was dissolved in benzene (1ml) and to this was added a solution of 10% potassium hydroxide in 85% aqueous ethanol (1ml). After shaking overnight at room temperature, the mixture was diluted with water (5 ml) acidified with dilute hydrochloric acid and extracted with ether. The ether extracts were washed with water until neutral and solvent was removed to leave /1-^{14}C;2,3-$^{3}H_2$/trans-2-undecenoic acid. (1.4 μC , $^{3}H/^{14}C$ = 6.0)

3. Experiments with whole cell cultures of Chlorella Vulgaris

The original Chlorella vulgaris (strain 211/11h) culture was obtained from the Culture Collection of Algae and Protozoa, Cambridge, and maintained on "Cambridge" agar slopes (the "poor" medium described below).

Chlorella vulgaris was grown by the following procedure.

One loop of cells was inoculated from an agar slope into 5ml of rich medium and incubated for twenty four hours at 30°C under continuous illumination, eighteen inches from 4 x 40 watt fluorescent tubes (daylight emission). The 5ml culture was then poured into 250ml of rich medium in a Roux bottle and incubated for two to three days in the light incubator at 30°C.

Rich Medium

KH_2PO_4	500mg	4mM Ferric citrate	2.5ml
K_2HPO_4	500mg	glucose	10g
$(NH_4)_2HPO_4$	800mg	tryptose	10g
$MgSO_4.7H_2O$	200mg	Difco yeast extract	2g
$CoCl_2$	40mg	Water to	1 litre
$MnSO_4.4H_2O$	2.2mg		

Final pH 6.5

Poor Medium

Protose peptone	1g	$MgSO_4.7H_2O$	200mg
KNO_3	2g	Agar	10g
K_2HPO_4	200mg	Water to	1 litre

Final pH 6.5

i) To investigate the stereochemistry of the dehydration step in acyl chain elongation in whole cell culture of Chlorella vulgaris

The culture of Chlorella vulgaris was grown in the "rich" medium and harvested according to the method of Harris et al (67). This involved spinning down the cells from one Roux bottle at 1,000 r.p.m. for ten minutes. The cells were then resuspended in 0.2M phosphate buffer (pH 7.4) and the centrifugition was repeated to obtain cells free of rich media. The cells were finally suspended in phosphate buffer pH 7.4 (250ml).

5ml aliquots of this suspension were placed in four 25ml conical flasks and to these flasks were added the following substrates each sonicated in phosphate buffer (1ml)

1) erythro 2-^{2}H,3-OH 17:0 (500 µg)
2) erythro 2-^{2}H,3-OH 17:0 (500 µg) + sterculic acid (500 µg)
3) threo 2-^{2}H,3-OH 17:0 (500 µg)
4) threo 2-^{2}H,3-OH 17:0 (500 µg) + sterculic acid (500 µg)

The flasks were stoppered with cotton wool plugs and shaken in an illuminated incubator at 27°C for two hours. At the end of the incubation period, the whole of the incubation media was poured into 2:1 v/v chloroform/methanol (25ml) and allowed to stand overnight.

Extraction of the lipids

This procedure is a modification of that of Folch et al (68). The chloroform/methanol extract of the incubation media as above was filtered to remove any solid material. The filtrate was transferred to a separating funnel and 0.75% saline (20ml) was added, and the whole

shaken. Two layers separated on standing and the bottom layer was collected. The upper layer was once more extracted with chloroform (20ml) and added to the first extract. The solvent was removed to yield the extracted lipids which were dried by azeotropic distillation with ethanol.

Transmethylation of the extracted lipids

The method of conversion of lipids to the methyl esters of their component fatty acids was that described by Nichols and James (69) and was standard procedure used throughout the work described in this thesis.

The extracted lipids were placed in a 25ml tube and the solvent was removed. The lipids were redissolved in a mixture of benzene, methanol, sulphuric acid (10:20:1 v/v/v) (5ml) and refluxed for ninety minutes. After this time, the tube was cooled and ether (10ml) and water (10ml) were added. The mixture was shaken and the aqueous layer was removed with a pasteur pipette. The ether was then washed acid free with water (5 x 5ml). Solvent was removed and the methyl esters were dried by azeotropic distillation with ethanol. Esters were stored in ether at 0°C.

The mixed esters so prepared were applied to a 20 x 20 x 0.25mm silver nitrate impregnated silica plate, and the plate was developed in ether:petroleum ether (1:9). The bands corresponding to saturated and monoenoic fatty esters were scraped off and eluted with ether.

The purity of the saturated and monoene fractions were checked by GLC, to ensure that complete removal of the monoene from the saturated fraction and vice-versa had been achieved.

Final purification of the C:17 saturated and monoene esters was obtained by GLC, the effluent gas containing the deuterated products was passed straight onto an AEI MS12 mass spectrometer, which was modified to work in conjunction with GLC. Ten scans in each direction

of the parent molecular ion region of each product were recorded. From these, the intensity of the $^{2}H_{0}$ and $^{2}H_{1}$ parent molecular ion peaks were measured and the porportion of these species calculated.

ii) To investigate the stereochemistry of the hydrogenation step in acyl chain elongation in Chlorella vulgaris

The culture of Chlorella vulgaris was grown in "rich" media, harvested, washed, and resuspended in 0.2M phosphate buffer pH 7.4 (250ml) as before.

An aliquot (5ml) of this suspension was placed in a 25ml conical flask and to this flask was added the substrate, [1-^{14}C;2,3-$^{3}H_{2}$]trans-2-undecenoic acid (1.3 μC:), sonicated in phosphate buffer (1ml).

The flask was stoppered with a cotton wool plug and incubated at 27°C with shaking under 4 x 40 watt fluorescent tubes for four hours.

At the end of four hours, the incubation was terminated by adding chloroform methanol (2:1 v/v) (25ml). The mixture was allowed to stand overnight at room temperature to ensure complete extraction of the lipids. The lipids were isolated by the method of Folch (68) as previously described.

The isolated lipids were transmethylated by the method of Nichols and James (69) to provide the methyl esters of their component fatty acids.

The esters from the above procedure were applied to a 20 x 20 x 0.25mm 10% silver nitrate impregnated silica gel G plate, and the plate was developed in ether:petroleum ether, 1:9. The bands corresponding to saturated and monoenoic fatty esters were scraped off the plate and the esters eluted from the silica with diethyl ether (10ml). The esters were stored in ether at 0°C until used.

Separation of cis-9-heptadecenoate from monoene fraction

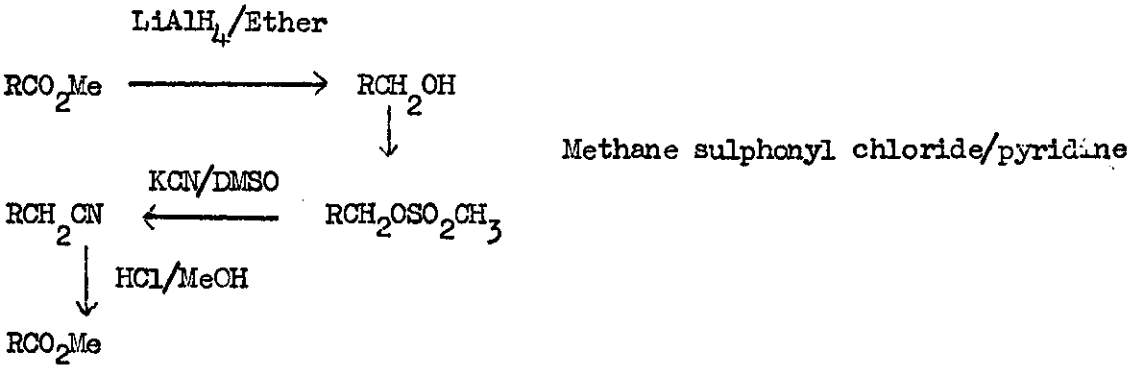
The heptadecenoate produced by chain elongation of undecenoic acid in Chlorella vulgaris was separated from endogenous monoenoic fatty acid

esters by preparative GLC. The instrument used was a Pye 104 Gas Liquid Chromatogram in which a 20:1 stream splitter was incorporated, resulting in only 5% of the effluent gas being used for detection by a flame ionization detector, the remainder being available for collection.

The mixed esters were injected onto the column (SE 30) in ether. The separated products were collected by passing the effluent gases through small ballotini balls wetted with chloroform, contained in a 1" bore glass collection tube. The ester which had the same retention time as authentic cis-9-heptadecenoate was collected from the effluent gases and eluted from the collection tube with chloroform. Solvent was removed and the product dissolved in benzene (1ml). An aliquot of this sample was taken for scintillation counting.

Chain extension of saturated ester fraction by one carbon atom

The whole of the saturated ester fraction was extended by one carbon atom, as the endogenous saturated fatty acid esters acted as a "carrier" for the labelled heptadecanoate produced by biochemical elongation of the undecenoic acid precursor and helped to minimise losses during the chemical reactions. The esters were elongated by the reaction sequence below.



Reduction of esters to alcohols

The saturated ester fraction was dissolved in dry ether (3ml) and refluxed with lithium aluminium hydride (10mg). After an hour, the mixture was cooled, ether (15ml) was added and the excess hydride was destroyed by dropwise addition of water. The resultant precipitate was

dissolved by adding dilute hydrochloric acid, and the ether extract was washed acid free with water. Solvent was removed at reduced pressure and the product was dried by azeotropic distillation with ethanol. The purity of the product was checked by TLC on silica gel G against suitable standards. This indicated complete conversion to alcohol.

Conversion of alcohols to methane sulphonates

The alcohols from above were dissolved in dry pyridine (1ml) and methane sulphonyl chloride (20mg) was added. The mixture was left overnight at room temperature. Ether (10ml) was added to the reaction mixture, and the ether layer was then washed successively with water (2 x 5ml), dilute hydrochloric acid (2 x 5ml), water (1 x 5ml), 5% aqueous potassium hydroxide (1 x 5ml) and finally water, until neutral.

The purity of the product was checked by TLC on silica gel G plates developed in chloroform:petroleum ether, 3:7. This indicated almost complete conversion to the mesylates and the product was not purified further.

Conversion of methane-sulphonates to nitriles

Potassium cyanide (10mg) was added to a solution of the mesylates in dry DMSO (2ml). The reaction mixture was heated to 90°C for six hours. The mixture was cooled, diluted with water (5ml) and extracted with ether (2 x 5ml). The ether extracts were washed with water (4 x 5ml), and solvent was removed at the pump. The reaction products were checked by TLC (ether:petroleum ether, 1:9) and showed good conversion to the nitriles with no remaining mesylates. The products were not purified further.

Methanolysis of the nitriles

The nitriles were dissolved in 25% w/v hydrogen chloride in methanol (3ml). The mixture was stood at room temperature overnight, cautiously diluted with water (5ml) and extracted with ether (2 x 5ml). The ether extracts were washed acid free with successive aliquots of water. Solvent was removed at the pump.

The product was applied to a 20 x 20 x 0.25mm silica gel G plate, and developed in ether:petroleum ether, 1:9. The band corresponding to methyl esters was scraped off and eluted with ether.

The resultant product was shown by GLC to consist of a mixture of methyl heptadecanoate, octadecanoate and nonadecanoate. The methyl octadecanoate was separated from the other components by preparative GLC in an identical manner to that described previously for cis-9-heptadecenoate. The $^{3}H/^{14}C$ ratio of the methyl octadecanoate was determined by scintillation counting.

Incubation of labelled octadecanoic acid with Chlorella vulgaris

In order to determine the stereochemistry of the tritium atoms in the above product, it was incubated with Chlorella vulgaris. The stereospecificity of the Δ^9 desaturase in this organism is known.

The methyl octadecanoate(30mμC) from the above reaction was dissolved in 5% methanolic potassium hydroxide (2ml) and the mixture was left at room temperature overnight.

Water (10ml) was added and the mixture was acidified with dilute hydrochloric acid, and extracted with ether (10ml). The ether extracts were washed acid free and the solvent was removed at the pump.

The product was dispersed in 0.2M phosphate buffer (2ml) to which 5% aqueous sodium carbonate (3 drops) and dilute tween (1 drop) had been added. Dispersion was aided by sonication with an MSE Ultrasonic disperser.

The culture of Chlorella vulgaris was grown and harvested as before, and resuspended in 0.2M phosphate buffer pH 7.4 (250ml).

Aliquots (5ml) of this suspension were placed into two 25ml conical flasks, to one was added the above substrate, and to the other was added [1-^{14}C] stearic acid (50m μC.) in phosphate buffer (this substrate was added to monitor the extent of desaturation during the incubation period).

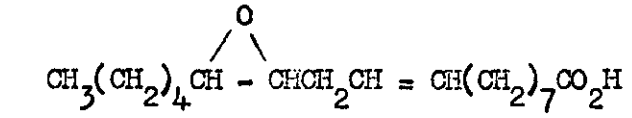
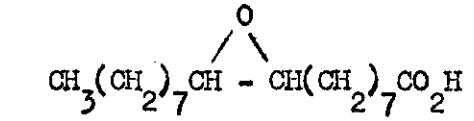
The flasks were stoppered with cotton wool plugs, and shaken in an illuminated incubator at 27°C for four hours. At the end of the incubation period, the whole of the incubation media was poured into 2:1 v/v chloroform methanol (25ml) and allowed to stand overnight.

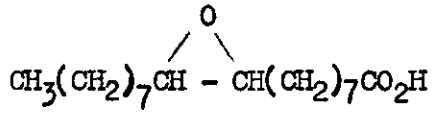
The lipids were extracted and transmethylated and the esters were separated into saturated and monoene fractions by argentation chromatography as described perviously. The $^{3}H/^{14}C$ ratio of the saturated and monoene fractions were determined by scintillation counting.

INTRODUCTION

In addition to the normal range of straight chain fatty acids such as palmitic, stearic, oleic, and linoleic acids occurring widely in plants and animals, a considerable number of oxygenated fatty acids have been found in Nature. This section of the thesis is concerned with two types of naturally occurring oxygenated acids, those containing an oxirane ring and those with a vicinal diol grouping. Mid-chain (non-terminal) epoxy and vicinal dihydroxy acids have two asymmetric carbon atoms, at the oxirane ring or at the diol grouping, so that each of the two possible geometric isomers can exist in enantiomeric forms. Most of the epoxy and dihydroxy acids found naturally have been shown to be optically active, and this work has been devoted to the determination of the absolute configurations of some of these compounds and their derivatives.

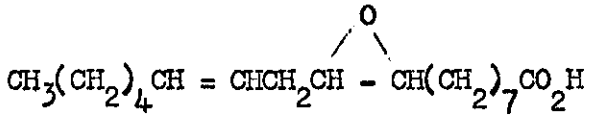
Fatty acids containing an oxirane ring have been discovered in a wide range of plant seeds and fungal spores, usually present as long chain glycerides. The epoxy fatty acids characterized to date are summarized below.

	Reference
	93-99
Vernolic acid, cis-12,13-epoxyoctadec-cis-9-enoic acid	
	105, 107, 109-111, 114, 142
cis-9,10-epoxyoctadecanoic acid	



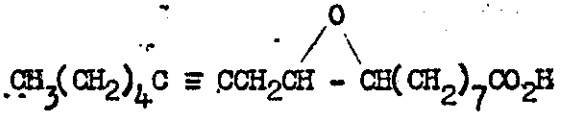
trans

trans-9,10-epoxyoctadecanoic acid



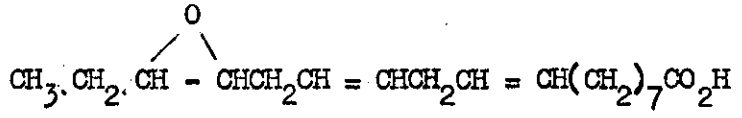
cis cis

Coronaric acid, cis-9,10-epoxyoctadec-cis-12-enoic acid



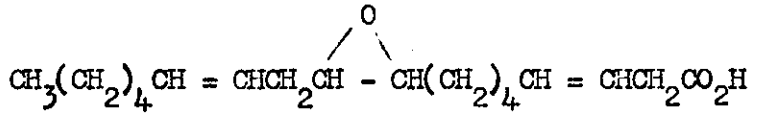
cis

cis-9,10-epoxyoctadec-12-ynoic acid



cis cis cis

cis-15,16-epoxyoctadeca-cis-9,cis-12-dienoic acid



cis cis trans

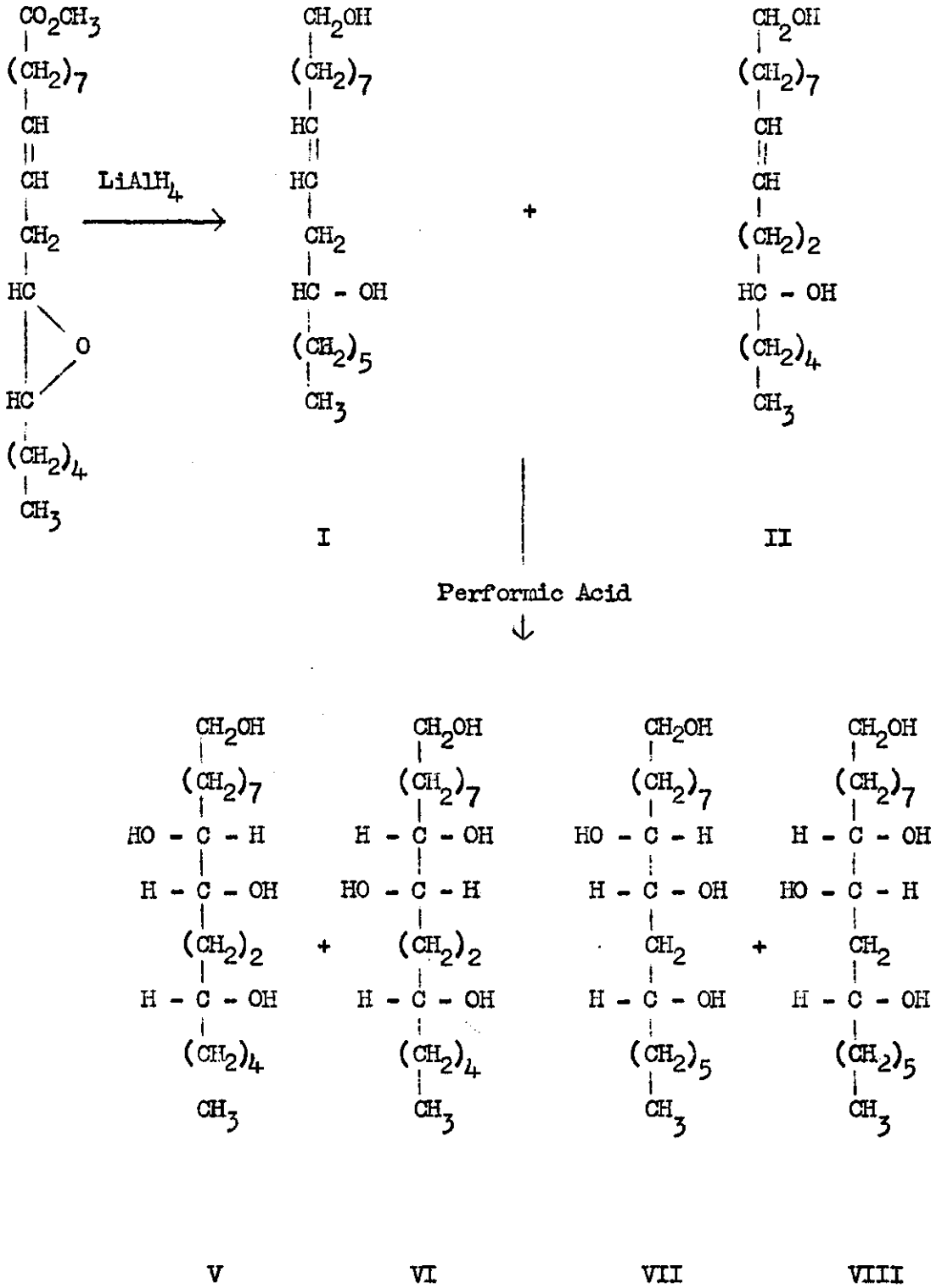
cis-9,10-epoxyoctadeca-trans-3,cis-12-dienoic acid

Vernolic acid (cis-12,13-epoxy cis-9-octadecenoic acid) was the first epoxy acid characterized as a natural constituent of a seed oil (93) and is the major epoxy acid in all seed oils which contain substantial amounts of epoxide. Both of the optical antipodes of vernolic acid have been discovered in Nature. (+)-Vernolic acid has been identified as a component of the seed oils of a number of species that represent several plant families, including Compositae, Euphorbiaceae, Onagraceae, Dipsacaceae and Valerianaceae (94,95,96, 97). (-) Vernolic acid has

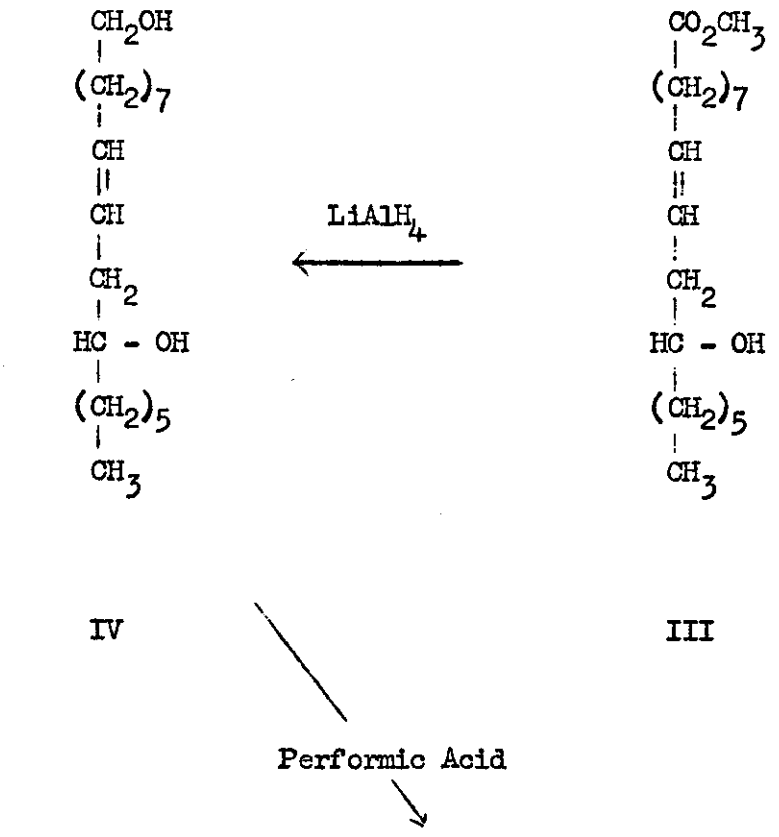
been found in the seed oils of several Malvaceae species (96,98,99) but seems to be restricted to that family.

Morris and Wharry (100) have determined the absolute configuration of (+)-vernolic acid. The key to this determination was the work of Serck-Hanssen (101,102) proving that ricinoleic acid ((+)-12-hydroxyoleic acid) has the D configuration. (+)-Methyl vernolate was reduced with lithium aluminium hydride to a mixture of two unsaturated diols (Scheme 1, I and II). These were hydrogenated over Adams catalyst to yield a mixture of 1,12- and 1,13-octadecadiols. This mixture was shown to have the same sign and magnitude of rotation as the 1,12-octadecadiol of known configuration derived from methyl ricinoleate (III) by reduction with lithium aluminium hydride, followed by hydrogenation over Adams catalyst. Thus the diols obtained from (+)-methyl vernolate and from methyl ricinoleate must be of the same optical configuration. The stereo-specificity of reduction of epoxy compounds with lithium aluminium hydride is known (103), and proceeds with inversion of configuration at the position of nucleophilic attack, and retention of configuration of the hydroxyl group formed. The C-12 and C-13 hydroxyl groups on the diols formed from (+)-methyl vernolate must, therefore, have the same configuration as the original epoxide, and as these have the same configuration as the C-12 hydroxyl in the diol formed from methyl ricinoleate, the configuration of the epoxide group in (+)-methyl vernolate must be 12-D,13-D.

These conclusions were further substantiated by a separate series of experiments. In these (+)-methyl vernolate was reduced with lithium aluminium hydride to give two unsaturated diols (I and II). These were then hydroxylated with performic acid to give two pairs of diastereoisomeric tetrols (V and VI, and VII and VIII), the individual isomers of which could be separated by TLC on arsenite impregnated



Scheme 1.


CH2OH
(CH2)7
CH
CH
CH2
HC - OH
(CH2)5
CH3
LiAlH4
CO2CH3
(CH2)7
CH
CH
CH2
HC - OH
(CH2)5
CH3
IV
III
Performic Acid
CH2OH
(CH2)7
HO - C - H
H - C - OH
CH2
H - C - OH
(CH2)5
CH3
CH2OH
(CH2)7
H - C - OH
HO - C - H
CH2
H - C - OH
(CH2)5
CH3
IX
X

silica gel. Two of the tetrols were shown by melting point and optical rotation to be identical with the tetrols (IX and X) derived from methyl ricinoleate. This could only be so if the asymmetric centres at C-12 are identical, confirming unequivocally that the absolute configurations of (+)-methyl vernolate and (+)-methyl ricinoleate are identical.

A structural isomer of vernolic acid, coronaric acid (cis-9,10-epoxyoctadec-12-enoic acid) is the epoxy acid found in the next highest amounts in seed oils. It makes up 14% of the oil from Chrysanthemum coronarium (104) and 10% of a sample from Helichrysum bracteatum (105). It has also been reported in lesser amounts in a great many other oils.

Powell et al (100) isolated coronaric acid, along with vernolic, and cis-9,10-epoxystearic acids, from the seed oil of Xeranthemum annuum and established the absolute configurations of the two 9,10-epoxy acids for the first time (107). (+)-Methyl coronarate was reduced by a reagent which reduced the 12,13 double bond without affecting the epoxide, to give (+)-methyl cis-9,10-epoxystearate. This epoxy stearate was then further reduced with lithium aluminium hydride to a mixture of 1,9- and 1,10-octadecadiols. This mixture of diols was dextrorotatory, in contrast to the D-1,9-octadecadiol produced by lithium aluminium hydride reduction of D-9-hydroxystearate (108,31). As the epoxide cleavage is known to proceed with retention of configuration about the carbon-oxygen bond, then the original epoxide has the 9-L,10-L configuration. Similarly, the configuration of (+)methyl cis-9,10-epoxystearate was proved to be 9-L,10-L.

cis-9,10-Epoxystearate was first isolated from the seed oil of Tragopogon porrifolius (109) and has since been found in the seed oils of other species (105,107,114,142). The amounts of epoxystearate in some seed oils were found to increase with storage and this increase could

be accelerated by holding the seeds at higher temperatures (112). This post harvest increase in epoxystearate content has been accounted for by specific enzyme action.

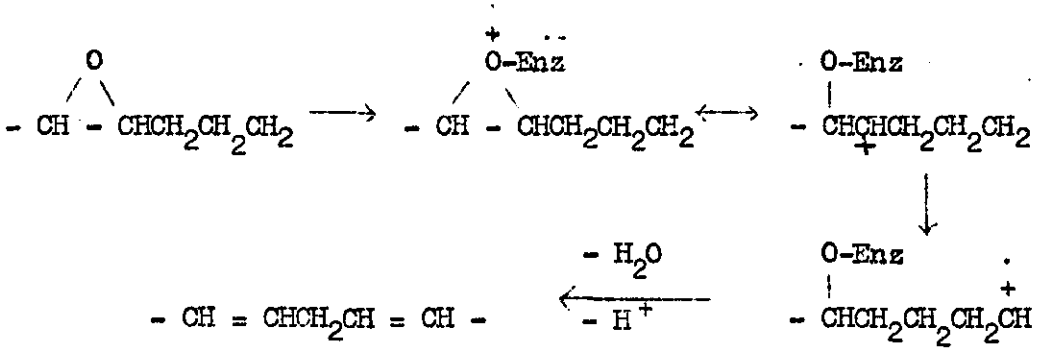
The most widespread naturally occurring isomer of cis-9,10-epoxystearic acid has the L configuration. However, like vernolic acid, this acid exists naturally in both enantiomeric forms, and the D-enantiomer has been isolated from Shorea robusta seed oil (142).

Although the amount of 9,10epoxystearic acid in seed oils is low, in non-seed sources, it is much higher. The lipids of some fungal spores can contain up to 78% of epoxystearate (110,111), while in others, it is completely absent. As in the case of seed lipids, this acid appears as the glyceride. The biosynthesis of cis-9,10-epoxystearate has been investigated in rust (Puccinia graminis) infected wheat plants (113). The epoxy acid was synthesized from acetate in a process which required oxygen but was not stimulated by light. Stearic and oleic acids were also incorporated into the epoxy acid, at a rate which indicated that oleic acid was the immediate precursor of the epoxide. Subsequent experiments (115) with ^{18}O labelled oxygen and water, showed that the oxygen of the epoxide group was derived from air and not from water.

The other epoxy acids listed above have, so far, only been reported in the oils in which they were first identified. trans-9,10-Epoxystearic acid was isolated from orujo, or sulphur olive, oil (116) and was considered to be a product of enzymic or bacterial action. cis-15,16-Epoxyoctadeca-cis-9,cis-12-dienoic acid was isolated from Camelina sativa seed oil (117), cis-9,10-epoxyoctadec-12-ynoic acid occurs with coronaric and epoxystearic in the seed oil of Helichrysum bracteatum (105), and cis-9,10-epoxyoctadeca-trans-3,cis-12-dienoic acid was discovered in a sample of Stenachaenium macrocephalum (118)

The biological role of epoxy fatty acids is unclear, except in the case of the juvenile hormone, methyl 9,10-epoxy-7-ethyl-3,11-dimethyl-trideca-2,6-dieneoate, which has been established as a regulator of insect metamorphosis (119).

Gunstone (120) has speculated on the possible role of epoxy acids as intermediates in the biosynthesis of polyunsaturated and acetylenic acids, and pointed out the structural similarities between epoxy acids, and acetylenic and hydroxy acids, which occur together in seed oils.



Proposed Mechanism of Conversion of 9,10 epoxystearic acid to Linoleic Acid

Long chain fatty acids with vicinal diol groupings as components of seed oils are quite rare. However, they have been reported in a wide range of natural sources such as cork, olives, leaf waxes, and micro-organisms. These vicinal diols include both dihydroxy acids and tri-hydroxyacids (e.g. 9,10,18-trihydroxystearate (4)).

A group of unusual triglycerides, in which the acyl group on the α-position of the glycerol is a vicinal dihydroxy acid with one of its hydroxyl groups acetylated, was isolated from Cardamine impatiens (7,121) Removal of the vicinal diol grouping by reactions of known stereochemistry gave a cis unsaturated acid, indicating the configurations of the vic diols in this seed oil to be erythro.

erythro-9,10-Dihydroxystearic acid has been recognised as a minor constituent of castor oil (6) and Strophanthus seed oils (122). The threo isomer of this acid occurs in fungi and has been suggested as a product of enzymic hydration of cis-9,10-epoxystearate (111,123).

Two trihydroxy acids with vic diol groups were isolated from Chamaepeuce afra and characterized as (+)-threo-9,10,18-trihydroxystearic (phloionolic) acid and (+)-threo-9,10,18-trihydroxyoctadec-12-enoic acid (124). These trihydroxy acids occurred as the glycerides, with two of the hydroxyls of the fatty acid either acetylated or esterified with a normal fatty acid. threo-9,10,18-Trihydroxystearic acid is also a constituent of cork, where it occurs with several other oxygenated fatty acids and dicarboxylic acids (125). erythro-8,9,13-Trihydroxydocosanoic acid was isolated from the extra-cellular lipids of a yeast, where it occurs as the triacetate, along with the related keto acid erythro-8,9-dihydroxy-13-oxodocosanoic acid (126,127).

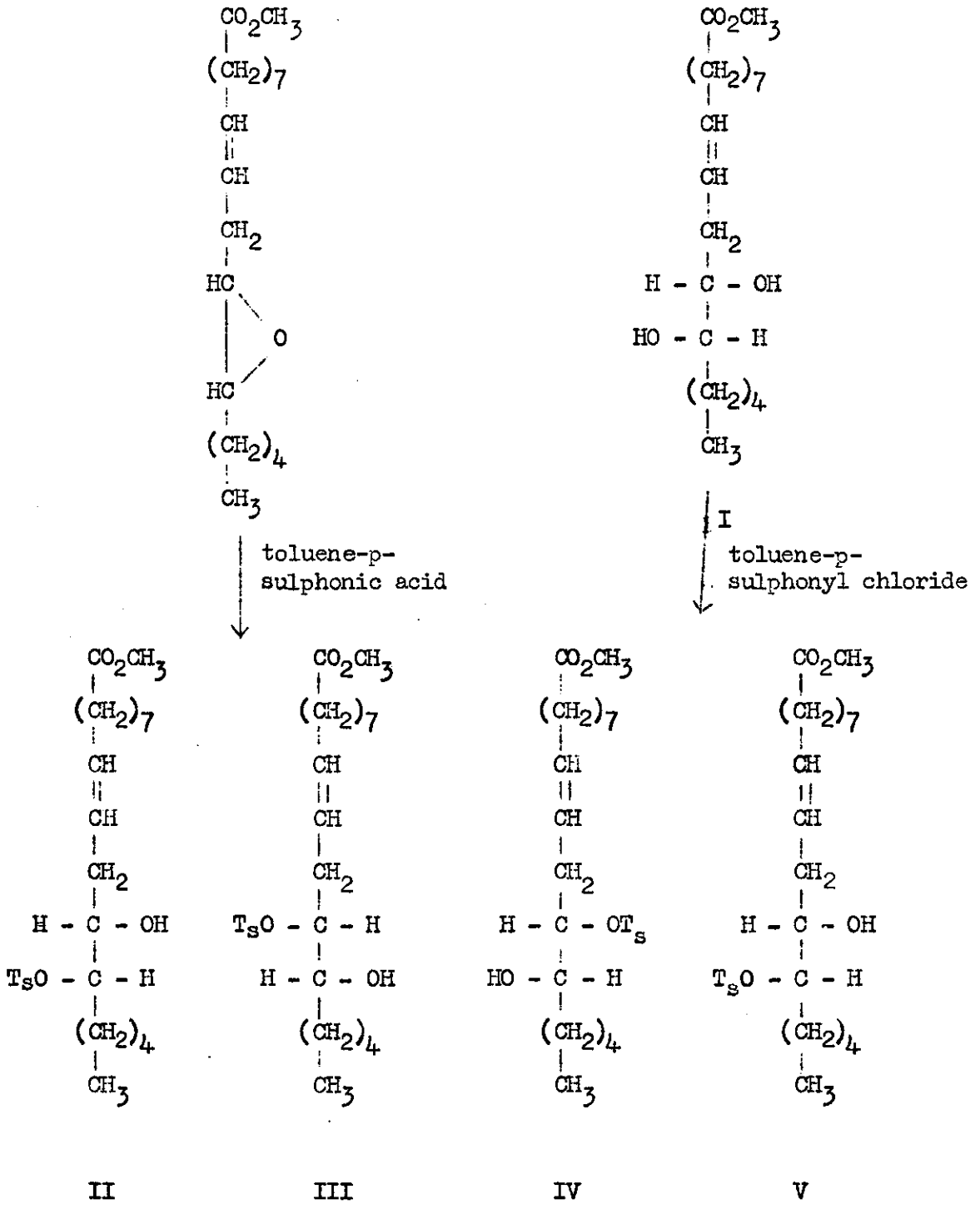
There is a close stereochemical relationship between epoxides and vicinal diols and the interconversion of these two groups of compounds has been extensively investigated. Under various acidic conditions 1,2-epoxides undergo ring cleavage to give vic diols or their derivatives. These reactions are known to be stereospecific, so that the hydroxyl group formed from the epoxide has the configuration of the parent epoxide, and inversion of configuration occurs at the position of nucleophilic attack. Thus cis epoxides give threo compounds, and trans epoxides, the erythro derivatives.

This type of reaction provides a stereochemical link between epoxides and vicinal diols and allowed the determination of the absolute configuration of threo-12,13-dihydroxyoleic acid (128). The reactions involved in this determination are summarized in Scheme 2. They consisted of the production of a pair of positionally isomeric hydroxytosylates from methyl vernolate by epoxide cleavage with toluene-p-sulphonic acid,

and from (+)- or (-)-threo-12,13-dihydroxyoleate (I) by partial tosylation with toluene-p-sulphonylchloride. The absolute configuration of vernolic acid was known to be D-12,D-13 (100), and the stereochemistry of the epoxide cleavage was such that the pair of isomeric tosylates produced from the epoxide were D-12-hydroxy, L-13-tosyloxy- and D-12-tosyloxy, L-13-hydroxyoleic acids (II and III).

Partial tosylation of threo-12,13-dihydroxyoleic acid proceeded with retention of configuration to produce either D-12-hydroxy, L-13-tosyloxy- and D-12-tosyloxy, L-13-hydroxyoleic acids (IV and V) or the enantiomeric pair. One of the positional isomers produced from vernolic acid was identical to the corresponding one from threo-dihydroxyoleic acid the the other pair were enantiomeric. The absolute configuration of the two positional isomers produced from vernolic acid was known, so that determination of the positions of the hydroxyl and tosyl groups in these isomers solved the configuration problem. Reductive cleavage of the tosyl group from the individual isomers after separation of the positional isomers by argentation TLC, gave the isomeric octadecandiols. The positions of the secondary hydroxyl groups were established by mass spectroscopy after conversion to the methyl oxooctadecanoates, thus proving the structure of the original hydroxy tosyloxy isomers, and hence the absolute configuration of the threo-dihydroxy acid. (-)-threo-12,13-Dihydroxyoleic acid was thus proved to be 12-D,13-L, and its (+)-enantiomer 12-L,13-D.

The absolute configurations of erythro-12,13-dihydroxyoleate and -stearate had become apparent after the determination of the stereochemistry of vernolic acid. Bharucha and Gunstone (129) had obtained (-)-erythro-12,13-dihydroxyoleic acid from (+)-vernolic acid in a process which involved retention of configuration about one of the epoxide carbons and a double inversion about the other, so that the product must have been D-12,D-13 dihydroxyoleic acid. The (-)-erythro-12,13-dihydroxystearate derived from this must also have the D-12,D-13 configuration.



Scheme 2. Reactions used in the determination of the absolute configuration of threo-12,13-dihydroxyoleic acid

Thus, summarizing the work to date on the absolute configurations of epoxy and vicinal dihydroxy fatty acids, it can be seen that the configurations of cis-12,13-epoxyoleic acid (100) and cis-9,10-epoxystearic acid (107), and of erythro- and threo-12,13-dihydroxyoleic acids (100, 120) have been defined. The work described in this part of the thesis was an extension of these studies to determine the absolute configurations of erythro- and threo-9,10-dihydroxystearates and some of their derivatives and of trans-9,10-epoxystearate, trans-12,13-epoxystearate and trans-12,13-epoxyoleate. The key to the determination of the absolute configuration of erythro-9,10-dihydroxystearic acid from castor oil (6) was the work of Powell et al (107) who had determined the absolute configuration of cis-9,10-epoxystearic acid. If erythro-9,10-dihydroxystearate could be converted by stereospecific reactions into cis-9,10-epoxystearate, then comparison of the product with the natural epoxy acid would prove the configuration of the dihydroxy acid.

The determination of the configuration of threo-9,10-dihydroxystearic acid was not as simple. An attempt was made to determine this by a similar method to that used for the determination of the configuration of threo-12,13-dihydroxyoleate. This failed because the pairs of positionally isomeric hydroxytosylates produced by partial tosylation of the vic diol, or cleavage of the cis-9,10-epoxide with toluene-p-sulphonic acid, were not separable by TLC. This was due to the absence of an adjacent double bond, present in the earlier case.

An alternative approach was used for the determination of the configuration of the threo-9,10-dihydroxy acid based on the earlier work of Morris and Wharry (100). They had suggested, on the basis of TLC behaviour and studies of complex formations with sodium arsenite, that the higher melting of the pair of threo-9,10,12-trihydroxystearates, produced by trans hydroxylation of the double bond in methyl ricinoleate,

had a trans or threo disposition of the 10- and 12- hydroxyl groups. As the configuration of the 12-hydroxyl group is known to be D, they argued that the higher melting trihydroxystearate must have the D-9,L-10, D-12 configuration.

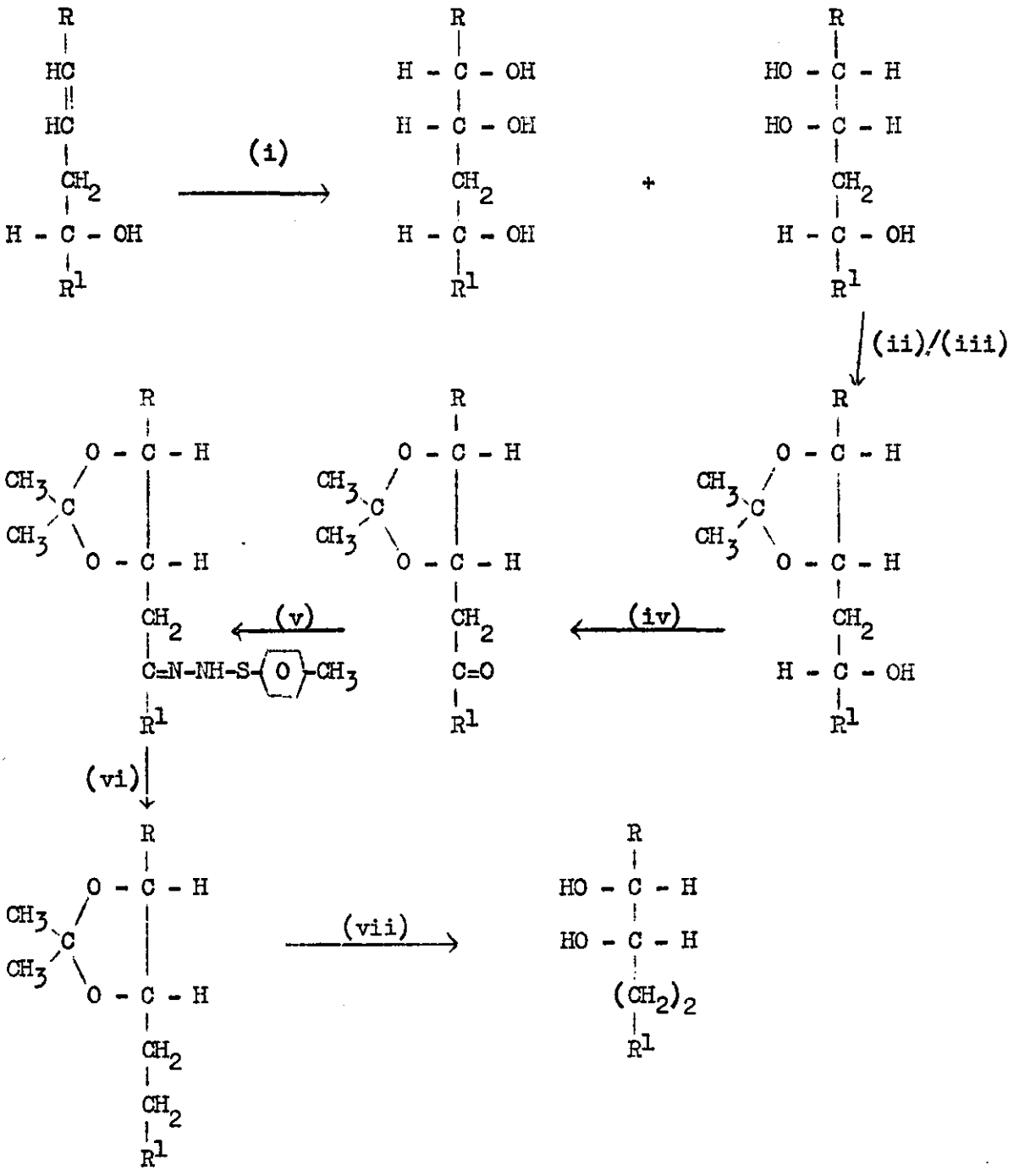
Wood et al (130) separated the individual isomers of methyl tri- and tetrahydroxystearates, by GLC and TLC and assigned configurations to all these compounds. The trihydroxy compounds were formed by hydroxylation of methyl ricinoleate or its trans isomer, and the tetrahydroxy derivatives by hydroxylation of methyl linoleate isomers. Their assignments were based on the arguments as to the steric effect of the 12-hydroxyl group on the ratio of individual isomers of each diastereoisomeric pair produced by hydroxylation of the 9,10 double bond, and also on the basis of GLC and TLC characteristics of each isomer. There is one consistent structural difference between the individual isomers of each diastereoisomeric pair produced by hydroxylation of methyl ricinoleate or methyl linoleate, and that is that one isomer of each pair has the hydroxy groups in the 10- and 12- positions in a trans or threo relative configuration, while the other isomer has these groups cis or erythro. This consistent structural relationship can be correlated to the consistent difference in the properties of the individual isomers. In each of the diastereoisomeric pairs, the higher melting isomer always migrates slightly less rapidly on TLC on normal silica gel, but is much less polar on sodium arsenite impregnated silica gel (130,131). These properties are due to the spatial relationships and interaction of the hydroxyl groups, and the differences in behaviour on TLC must be due to the structural differences between each isomer of the diastereoisomeric pair, namely the disposition of the 10- and 12-hydroxyl groups.

Both Wood (130) and Morris(100) concluded that the higher melting isomer of each "oxidation pair" of the 9,10,12-trihydroxy and 9,10,

12,13-tetrahydroxystearic acids has a trans or threo disposition of the 10- and 12-hydroxyl groups. If these assignments of configuration were correct, then the higher melting, arsenite complexing isomer of the pair of threo-9,10,12-trihydroxystearates produced by trans hydroxylation of methyl ricinoleate must be the D-9,L-10,D-12-isomer. Removal of the 12-hydroxyl group by reactions which do not affect the stereochemistry of the hydroxyl groups at the 9- and 10- positions would give either (+)- or (-)-threo-9,10-dihydroxystearate of known configuration, namely D-9,L-10, thus proving the absolute stereochemistry of this compound.

Since the absolute configuration of the 12-hydroxyl group of ricinoleate and, as described previously, of an erythro-9,10-dihydroxystearate were now separately known, the question of an "oxidation pair" having the trans-1,3-diol grouping could be established directly with one of the diastereoisomers of erythro-9,10-12-trihydroxystearic acid. The same series of reactions was used in each case to remove the C-12 hydroxyl group and these are summarized in Scheme 3.

Having defined the stereochemistry of a threo-9,10-dihydroxystearate, it could now be converted stereospecifically into an optically active trans-9,10-epoxystearate, in a similar manner to that used in the determination of the configuration of erythro-9,10-dihydroxystearate (Scheme 4, p.106). Thus establishing the configuration of a trans-epoxy fatty acid for the first time. (+)-threo-12,13-Dihydroxystearate and -oleate were also converted to their respective trans-epoxides to complete the stereochemical definition of the 12,13- substituted fatty acid series.



Scheme 3. Summary of the reactions used in the determination of the relative configuration of the 10- and 12- hydroxyl groups of the higher melting erythro-9,10,D12-trihydroxystearate

$R = (CH_2)_7CO_2CH_3$ $\quad R^1 = CH_3(CH_2)_5-$

(i) Alkaline $KMnO_4$; (ii) Separation of diastereoisomers by arsenite impregnated TLC; (iii) Acetone/$HClO_4$; (iv) CrO_3/glacial acetic acid; (v) p-toluenesulphonylhydrazide/MeOH; (vi) $NaBH_4$/MeOH; (vii) H_3BO_3/ $HOCH_2CH_2OCH_3$

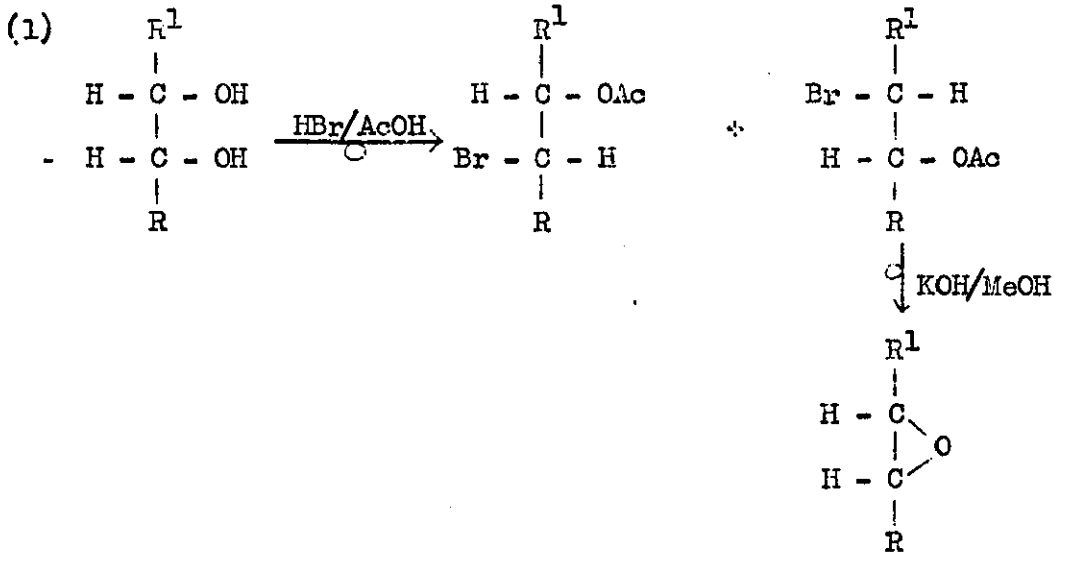
RESULTS AND DISCUSSION

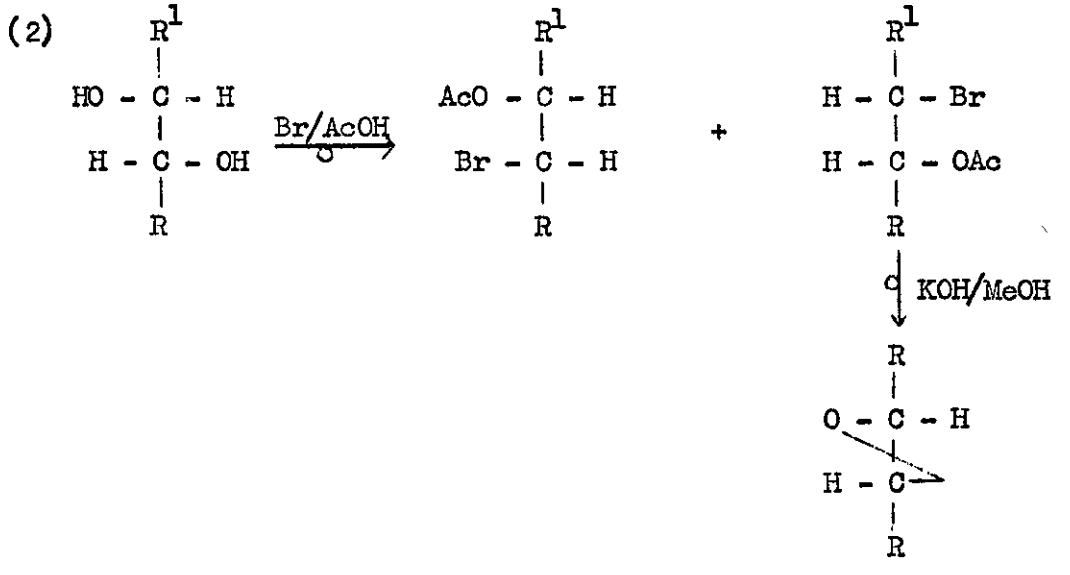
One of the methods of determining the absolute optical configuration of a compound is to convert it by reactions of known stereochemistry into a compound of known configuration. This was the method chosen to determine the configuration of *erythro*-9,10-dihydroxystearate; and later when the configurations of some *threo*-dihydroxy acids were elucidated the same method was used to determine the configuration of some *trans*-epoxides.

The methyl ester of (-)-*erythro*-9,10-dihydroxystearic acid derived from castor oil was converted by hydrogen bromide in glacial acetic acid at room temperature to a mixture of positionally isomeric acetoxy bromides. This mixture was not purified but was used directly for the next stage of the reaction scheme. The acetoxybromides were converted by dilute alcoholic potassium hydroxide to *cis*-9,10-epoxystearic acid. This epoxy acid was clearly the enantiomer of the one characterized by Powell et al (107), from the rotations summarized in Table 1., and therefore must have the D configuration.

It is known that the series of reactions used to convert the *vic* dihydroxy acid into the epoxide, shown in Scheme 4, involves two Walden inversions so that the resultant epoxide possesses the configuration of the dihydroxy precursor. Therefore, as the resultant epoxide has the D-configuration, then (-)-*erythro*-9,10-dihydroxystearic acid from castor oil must have the D-9,D-10 configuration (i.e. 9-*S*,10-*R* in the Cahn-Ingold-Prelog system (141)).

The transformation of the dihydroxy acid from castor oil to epoxy acid had been similarly effected by King (6), as long ago as 1942, with the same results. He was unable at that time, however, to draw the final stereochemical conclusions.





Scheme 4. Reactions used in the stereospecific conversion of:

(1) (-)-erythro-9,10-dihydroxystearic acid to (+)-cis-9,10 epoxystearic acid

R = $CH_3(CH_2)_7$- ; R^1 = -$(CH_2)_7CO_2H$

(2) (+)-threo-9,10-dihydroxystearic acid to (+)-trans-9,10-epoxystearic acid

R = $CH_3(CH_2)_7$- ; R^1 = -$(CH_2)_7CO_2H$

Compound	Source	$[\alpha]_{546.1}$ (c; Solvent)
Methyl *erythro*-9,10-dihydroxystearate	Castor oil	-0.12 (1.1;MeOH) -0.04 (9,0; EtOH)
Methyl L-*cis*-9,10-epoxystearate	*Xeranthemum annum* Powell et al. (107)	+0.3 (MeOH)
Methyl *cis*-9,10-epoxystearate	(-)-*erythro*-9,10-dihydroxystearate	-0.27 (2.75; MeOH) -0.22 (2.8; $CHCl_3$) -0.35 (2.75; Hexane)
cis-9,10-epoxystearic acid	(-)-*erythro*-9,10-dihydroxystearate	+0.21 (2.45; MeOH)
Methyl D-12,13-epoxystearate [a]	Vernolic acid	-1.44 (9.0; MeOH)

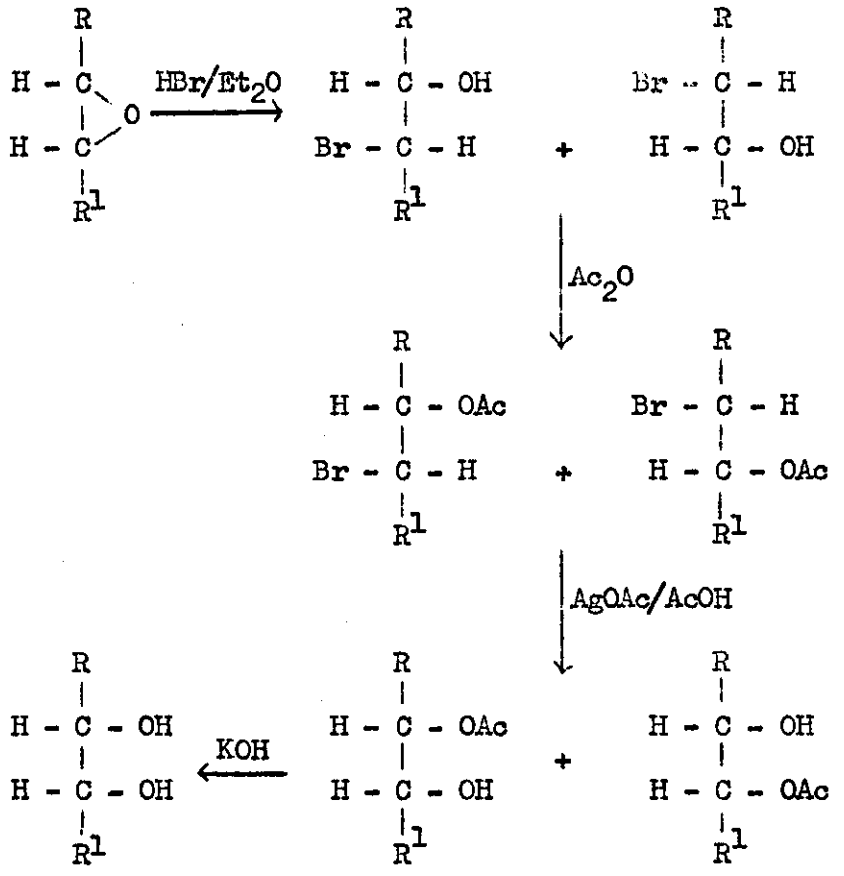
Table 1. Optical rotation of some natural *cis* epoxides and those prepared from *erythro*-9,10-dihydroxystearic acid

[a] prepared by hydrazine reduction of natural vernolic acid (included for comparison)

The absolute configuration of the erythro-dihydroxy acid could also have been determined by converting the cis-epoxide of known configuration to the dihydroxy acid by reactions involving no inversions or an even number of inversions. This method had been used by Gunstone (129) for the conversion of cis-12,13-epoxyoleate to erythro-12,13-dihydroxyoleate, as shown in Scheme 5, and by Korver and Ward for the conversion of cis-9,10-epoxystearate from Shorea robusta seed oil to (-)-erythro-9,10-dihydroxystearate (142). The cis-epoxide is converted into a mixture of threo-bromohydrins, with an ethereal solution of hydrogen bromide. Acetylation of this bromohydrin mixture with acetic anhydride, and reaction with silver acetate in wet acetic acid, gives the erythro-monoacetates, which undergo hydrolysis to yield the required erythro-glycol. This approach, however, was not attempted because of the very small amount of natural epoxystearate available.

Optically active erythro-9,10-dihydroxystearate is much more readily available. Although it only occurs in small amounts (1.3%) in castor oil (132), it is easily isolated from this source. Castor oil was hydrolysed with methanolic potassium hydroxide, and after isolation of the free acids (-)-erythro-9,10-dihydroxystearic acid could be obtained almost pure by crystallization from ethyl acetate, this acid was used throughout the configurational studies.

The oxidation of an olefin into the corresponding diol is known as hydroxylation. There are many reagents which can effect this change, but to be of any value, these reagents must add the hydroxyl groups to the olefin in a known stereospecific manner. The overall addition of the hydroxyls can be either cis or trans, cis addition to a cis double bond yielding the erythro derivatives, and to a trans double bond giving the threo compound. Obviously trans addition to cis and trans double bonds gives the threo and erythro configurations respectively. The most important reagents for effecting cis hydroxylation are



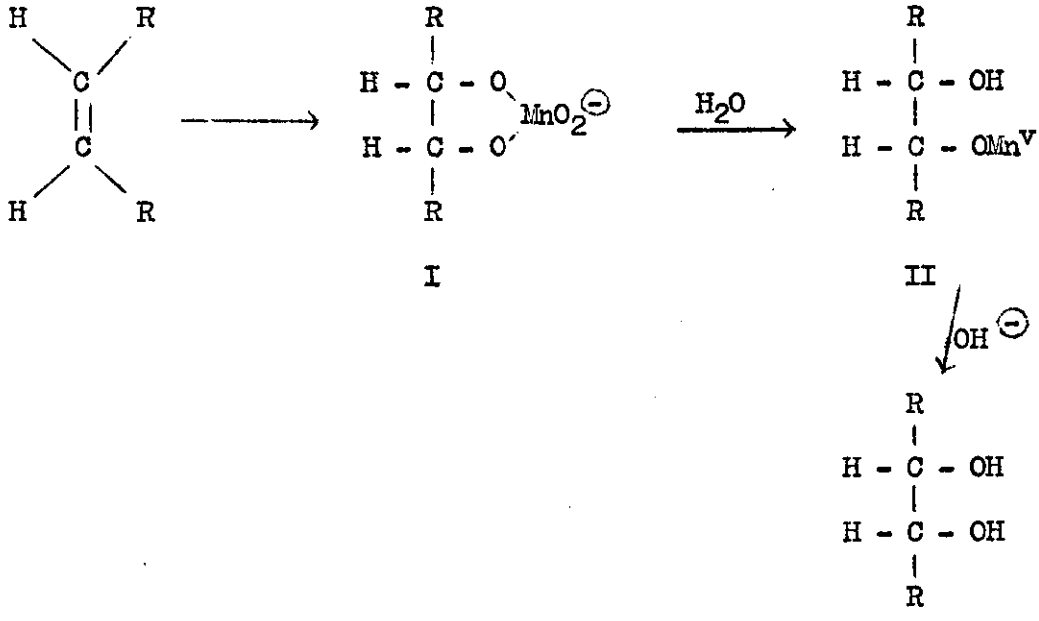
R = $CH_2CH = CH(CH_2)_7CO_2CH_3$

R^1= $-(CH_2)_4CH_3$

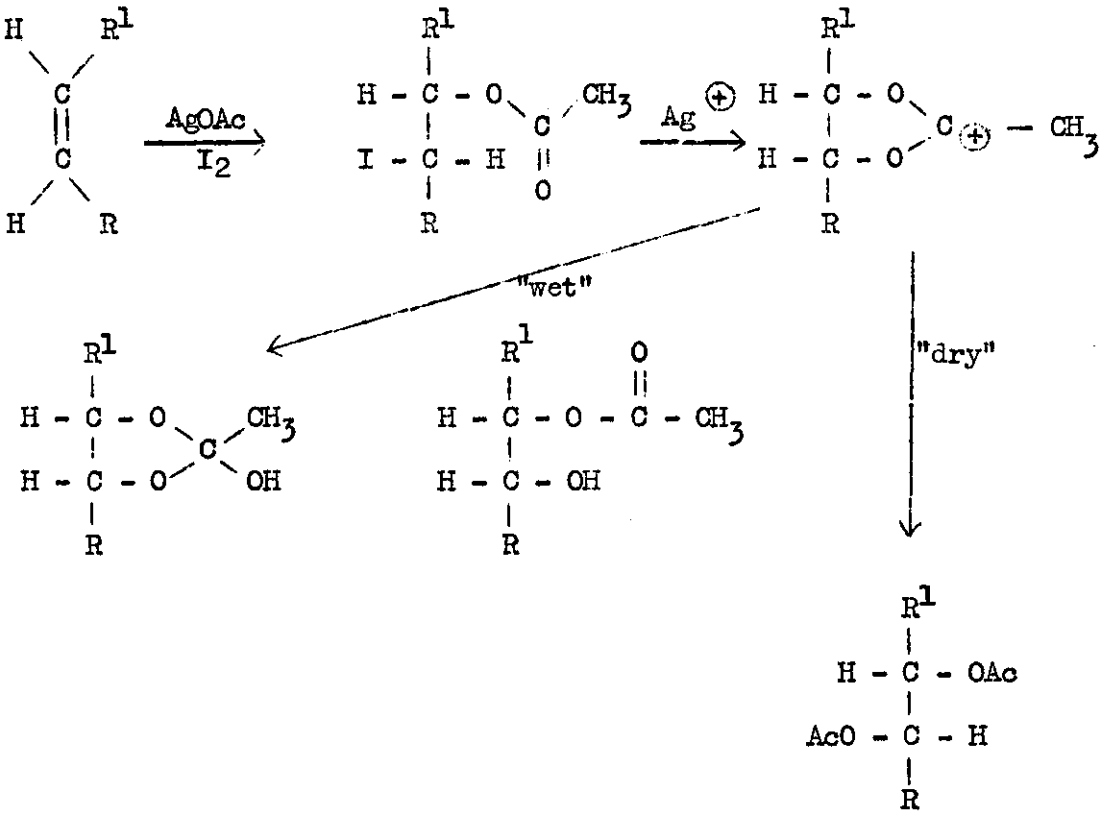
Scheme 5. Conversion of cis-12,13-epoxyoleate to erythro-12,13-dihydroxyoleate

dilute alkaline potassium permanganate, osmium tetroxide, or silver iodoacetate, trans hydroxylation is usually effected with peracids.

Permanganate oxideation of double bond proceeds through a cyclic ester intermediate (I). Opening of this cyclic compounds yields (II) and finally the glycol, corresponding to cis addition.



Oxidation with osmium tetroxide occurs in a similar manner to potassium permanganate. Treatment of the olefin in an organic solvent with osmium tetroxide gives a cyclic ester complex similar to that formed by permanganate. This complex may be decomposed to give the diol with overall cis addition of hydroxyl groups. Overall cis addition is also accomplished using the silver salt of an organic acid and a halogen. With these reagents, in the presence of water, olefins are hydroxylated by a cis addition; however anhydrous conditions lead to trans addition (133). These differences have been explained, in the case of iodine and silver acetate, by the following mechanisms:



Addition of iodine followed by acetate ion gives the threo-iodo-acetate, which then yields the five membered ring containing a carbonium ion. Under anhydrous conditions this is attacked by acetate ion to give the threo-diacetate, which can be hydrolysed to the threo-diol. With water, addition of hydroxyl to the carbonium ion occurs, and this product cleaves to give the erythro-hydroxy acetate, which can be hydrolysed to the erythro-diol.

trans-Hydroxylation is usually accomplished with peracids, via the intermediate formation of the epoxide. This epoxide can be isolated in some cases; in others it is immediately cleaved by rearward attack to give a threo-compound, the stability of the intermediate epoxide depending upon the peracid used.

Hydroxylation of methyl ricinoleate by either cis or trans addition will give a mixture of two diastereoisomers due to the chiral hydroxy group already present at the 12 position in methyl ricinoleate. The products of hydroxylation will have the C-10 and C-12 hydroxyl groups

either in a cis or a trans relative disposition, i.e. erythro or threo. The configuration of these two hydroxyls in each of the individual isomers of the diastereoisomeric pair has been predicted. Wood et al (130) have suggested that the higher melting isomer of the "oxidation pairs" of triols formed by trans hydroxylation of the double bond in methyl ricinoleate have the 10- and 12- hydroxyl groups in a trans or threo disposition. On this basis, the higher melting threo-9,10,12-trihydroxystearate should have the D-9, L-10, D-12 configuration. Removal of the 12-hydroxyl group from this isomer would give D-9, L-10 dihydroxystearate and measurement of the specific rotation of this product would solve the stereochemical problem.

Before using this method to determine the configuration of threo-9,10-dihydroxystearate, the proposition that the 10- and 12-hydroxyl groups had the trans configuration in the higher melting isomer could be directly checked using the erythro-9,10-12-trihydroxystearates. If this proposition were correct, then the higher melting erythro-diastereoisomer formed from ricinoleate should have the L-9, L-10, D-12 configuration. Removal of the C-12 hydroxyl group should give L-9, L-10 dihydroxystearate which is now known to be dextrorotatory. The method used to remove the C-12 hydroxyl without affecting the vicinal diol grouping is shown in Scheme 3 (p 104).

The erythro-9,10-12-trihydroxystearic acids had already been prepared (100). These were obtained by oxidation of ricinoleic acid with dilute alkaline permanganate.

Hydroxylation of ricinoleic acid proceeded smoothly giving good yields of the triols, although the very dilute conditions needed precluded the preparation of large amounts of these compounds. After esterification, the two diastereoisomers were separated by TLC on silica impregnated with sodium arsenite (131). This separation could be achieved due to the fact that the higher melting isomer complexes with

the arsenite in the layers to give a more stable complex than the lower melting isomer; this results in the higher melting compound being much less polar than the other isomer. The triols were eluted from the layer as the arsenite complex, and the trihydroxy acids were obtained from these by hydrolysis.

The vic diol group of the higher-melting, arsenite complexing diastereoisomer was protected by formation of its ispropylidene derivative. The reaction of the erythro-9,10,12-trihydroxystearate with acetone, using perchloric acid as a catalyst, was complete after five minutes as judged by TLC, but the reaction was allowed to proceed for fifteen minutes to ensure completion. This method is considerably faster than using anhydrous copper sulphate as the dehydrating agent (134). The product contained about 30% of a slightly less polar impurity which was identified by mass spectrometry as the 9-hydroxy-10,12-isopropylidene isomer. This impurity was not observed when the derivative of the higher melting threo-9,10,12-trihydroxystearate was prepared. After protection of the 9,10-vic diol grouping, the 12-hydroxyl group was oxidized to the ketone, in 50% yield, with chromium trioxide in glacial acetic acid. Although the isopropylidene grouping is acid labile, short reaction times at ambient temperatures were found not to affect the protecting group to any great extent. Reduction of the ketone to a methylene group was then effected in a two step procedure. The ketone was reacted at reflux in methanol with p-toluenesulphonylhydrazide (135) to afford the tosylhydrazone in good yield. Hydrogenolysis of this group was accomplished by refluxing the derivative with sodium borohydride in methanol. This is a mild, general procedure for the reduction of a ketone to a methylene group (136), and is more convenient on a small scale than the classical Wolff-Kishner reduction. The product from this series of reactions, the isopropylidene derivative of erythro-9,10-dihydroxy-stearate, was identical by TLC and GLC to an authentic sample prepared

from erythro-9,10-dihydroxystearate. Under the column conditions used it was possible to separate the isopropylidene derivatives of erythro- and threo-dihydroxy acids.

The specific rotations of natural erythro-9,10-dihydroxystearate and its isopropylidene derivative and the corresponding compounds derived from the higher melting diastereoisomeric form of erythro-9,10,12-trihydroxystearate, by removal of the C-12 hydroxyl group are shown in Table 2. Difficulties were encountered in measuring the rotations of optically active erythro-9,10-dihydroxy esters, because of their low specific rotations and low solubilities. Formation of the isopropylidene derivative increased both the specific rotation and the solubilities of these compounds.

Compound	Source of Material	$[\alpha]$ 546.1 (c; solvent)
erythro-D-9,D-10-dihydroxystearate	castor oil	-0.04° (9.0; EtOH)
erythro-D-9,D-10-dihydroxystearate isopropylidene derivative	castor oil diol	-0.50 (6.0; EtOH)
erythro-9,10-dihydroxystearate isopropylidene derivative	erythro-9,10,12-trihydroxy 18:0 high melting isomer	+0.65 (6.5; EtOH)

Table 2. Specific rotations of erythro-9,10-dihydroxystearates

It can be seen that the higher melting diastereoisomer of erythro-9,10,12-trihydroxystearate gave an erythro-9,10-dihydroxystearate, the isopropylidene derivative of which is dextrorotatory. The isopropylidene derivative erythro-9,10-dihydroxystearate derived from castor oil is laevorotatory, and is the enantiomer of that derived from the triol. As the configuration of the naturally occurring dihydroxy acid is known to be D-9, D-10, then the configuration of the product from the trihydroxy ester must be L-9, L-10. As the C-12 hydroxyl group of the original high melting trihydroxy ester is known to be D, the configuration of the ricinoleic acid from which it is derived, this high melting erythro-trihydroxystearate was thus proved to have the L-9, L-10, D-12 configuration (9-R, 10-S, 12-R).

This result establishes directly for the first time that the high melting diastereoisomer of the "oxidation pair", formed by alkaline permangenate oxidation of methyl ricinoleate, has the 10 and 12 hydroxy groups in the trans or threo configuration. This agrees with the predictions of both Morris and Wharry (100) and Wood et al (130), who concluded that the high melting isomers have the trans 1,3 diol arrangement. As the individual isomers of each of the six "oxidation pairs" formed by cis or trans hydroxylation of either methyl ricinoleate or methyl linoleate show consistent relative properties on TLC and have only one consistent structural difference, it is considered virtually certain that the higher melting, arsenite complexing isomers of each of these pairs have the 10 and 12 hydroxyl groups in the trans or threo configuration.

Having established this fact, then the absolute stereochemistry of threo-9,10-dihydroxy stearate can be elucidated. If the 12-hydroxyl group in the higher melting threo-9,10,12-trihydroxystearate is removed in a similar manner to that used for the erythro isomer, then the product will be D-9, L-10-dihydroxystearate.

The individual isomers of threo-9,10-12-trihydroxystearate had already been prepared (100). Basically, these were prepared by hydroxylation of the double bond of ricinoleic acid with performic acid, after acetylation of the 12-hydroxyl group, hydrolysis of the resultant hydroxy formates with dilute alkali, and separation of the individual isomers by TLC on arsenite impregnated layers.

The threo-9,10-diol group in the higher melting triol was protected by formation of the isopropylidene derivative and the 12-hydroxyl group was removed in a similar manner to that used for the erythro derivative. The product from this series of reactions was identical by GLC and TLC to the isopropylidene derived from authentic threo-9,10-dihydroxystearate. Both the threo-9,10-dihydroxystearate, and its isopropylidene derivative, formed by removal of the C-12 hydroxyl group from the high melting threo-9,10-12-trihydroxystearate which has the D-9, L-10, D-12 configuration (i.e. 9-S, 10-S, 12-R) were strongly laevorotatory (Table 3.) thus proving that (-)-threo-9,10-dihydroxystearate has the D-9, L-10 configuration (i.e. 9-S, 10-S).

Compound	Source of Material	$[\alpha]_{546.1}$ (c;solvent)
threo-D-9,L-10-dihydroxystearate	threo-9,10,D-12-trihydroxystearate (high melting isomer)	-20.6° (0.15; EtOH)
threo-D-9,L-10-dihydroxystearate isopropylidene deriv.	threo-9,10-D-12-trihydroxystearate (high melting isomer)	-29.0° (0.15; EtOH)
threo-9,10-dihydroxy-stearate	Claviceps sulcata	+22.5° (1.2; MeOH)
threo-9,10-dihydroxy-stearate isopropylidene derivative	Claviceps diol	+26.1° (2.15; EtOH

Table 3. Rotations of some threo-dihydroxystearates

Now that the absolute stereochemistry of a threo-9,10-dihydroxystearate was known, it could be converted by stereospecific means to trans-9,10-epoxystearate, defining the absolute stereochemistry of this trans epoxide. The conversion of diol to epoxide was done in an identical manner to that used for the conversion of erythro-9,10-dihydroxystearate to the cis epoxide, namely formation of acetoxy bromides followed by alkaline hydrolysis.

Only a small amount of optically active threo-9,10-dihydroxystearate had been prepared from the threo triol during the investigation of the stereochemistry of these compounds. This was insufficient for the investigation of the stereochemistry of the trans epoxide. However, (+)-threo-9,10-dihydroxystearic acid was readily prepared by resolution of the racemic mixture formed by trans hydroxylation of oleic acid with performic acid.

A number of long chain vicinal dihydroxy acids have been resolved by classical crystallization procedures. erythro-9,10-Dihydroxystearic acid has been resolved into its optically active forms by crystallization of its strychnine salts (137), the threo isomer of this acid being resolved by crystallization of its brucine salt (138). McGhie et al have also resolved threo-9,10-16-trihydroxypalmitic acid (139), threo-9,10-18-trihydroxystearic acid and threo-7,8,16-trihydroxypalmitic acid (140) by crystallization of their ephedrine salts.

In the present case brucine was used to resolve the dihydroxystearic acid. The alkaloid and the dihydroxy acid were warmed together in a mixture of acetone and water, and the resultant solution was cooled. The diastereoisomeric salt which crystallized was collected and purified by crystallization. Decomposition of this salt with dilute acid, and isolation of the dihydroxy acid gave (+)-threo-9,10-dihydroxystearic acid, in moderate yield, and optically pure,

its specific rotation ($[\alpha]$ = +20.8) being essentially identical to the literature values for optically active threo acids (111,138).

Conversion of the (+)-threo-9,10-dihydroxystearate, the absolute configuration of which is now known to be L-9, D-10, into the trans epoxide, gave a product which was dextrorotatory, as summarized in Table 4. This proves the absolute configuration of the (+)-trans epoxide to be L-9, D-10-epoxystearate (i.e. 9-R, 10-R).

The opportunity was also taken to complete the stereochemical definition of the 12,13-substituted family of acids by similar conversions of the L-12, D-13-dihydroxyoleic and -stearic acids, whose configurations were already known (128), into the corresponding trans epoxides. Conversion of the dextrorotatory isomers of these vic diols into epoxides, gave L-12, D-13-trans-epoxyoleate (i.e. 12-R, 13-R) and L-12, D-13-epoxystearate (i.e. 12-R, 13-R) both of which were dextrorotatory as shown inTable 4, thus defining the absolute stereochemistry of these epoxides.

The optically pure 12,13-dihydroxyoleate used in these determinations was readily isolated from Vernonia anthelmintica seeds. In these seeds there is an epoxy acid hydrating enzyme which, on incubation of the crushed seeds in moist conditions, cleaves the endogenous (+)-vernolic acid to give optically pure (+)-threo-12,13-dihydroxyoleic acid (158). The seeds were crushed, moistened and incubated for three days. The seed oil was extracted, and extraction of the free fatty acids from this oil gave a crude preparation of (+)-12,13-dihydroxyoleic acid, which was methylated, and purified by column chromatography. The dihydroxy acid could be extracted from the seed oil as the free acid because Vernonia seeds, as well as containing the epoxide hydratase, contain a lipase, which is also activated on crushing and moistening the seeds and whose action appears to precede that of the epoxide hydratase.

Dihydroxy ester (source)	$[\alpha]_{546.1\ nm}$ (c;solvent)	Epoxy product	$[\alpha]_{546,1\ nm}$ (c;solvent)	
			of acid	of ester
threo-L-9,D-10-dihydroxystearate (resolution with brucine)	+20.8 (1.4;EtOH)	trans-L-9,D-10 epoxystearate	+10.0 (1.1;MeOH) +9.5 (1.0;$CHCl_3$)	+15.0 (1.3;MeOH) +14.5 (1.4;$CHCl_3$)
threo-L-12,D-13-dihydroxyoleate (Vernonia seeds)	+20.3° (1.0;EtOH)	trans-L-12,D-13-epoxyoleate	+12.9 (0.7;MeOH) +12.7 (0.5;$CHCl_3$)	+14.6 (0.7;MeOH)
threo-L-12,D-13-dihydroxystearate (Vernonia + H_2)	+23.7 (1.35;MeOH)	trans-L-12,D-13-epoxystearate	+21.4 (1.3;MeOH)	+19.5 (4.3;MeOH)

Table 4. Specific rotations of the methyl esters of threo dihydroxy acids and their epoxy, acid and ester products

Optically pure (+)-threo-12,13-dihydroxystearate was prepared by hydrogenation, over Adams catalyst, of the dihydroxyoleate obtained from Vernonia.

The work reported here has established that (-)-erythro-9,10-dihydroxystearic acid has the D-9, D-10-configuration and that (+)-threo-9,10-dihydroxystearic acid has the L-9, D-10-configuration. The stereochemistry of some trans epoxy acids has been defined, the dextrorotatory isomers being L-9, D-10-epoxystearic acid, L-12, D-13-epoxystearic acid, and L-12, D-13-epoxyoleic acid. The absolute configurations of the four 9,10,12-trihydroxystearic acid diastereoisomers formed by permanganate, and performic acid oxidation of D(+)-ricinoleic acid have also become apparent. It has been proved directly that the higher melting diastereoisomer of erythro-9,10,12-trihydroxystearate has the trans-1,3-disposition of the 10 and 12 hydroxyl groups and therefore, by implication, that the higher melting threo-trihydroxy isomer and the higher melting isomer of each of the four 9,10,12,13-tetrahydroxystearate "oxidation pairs" also have this trans-1,3-disposition of the 10 and 12 hydroxyl groups.

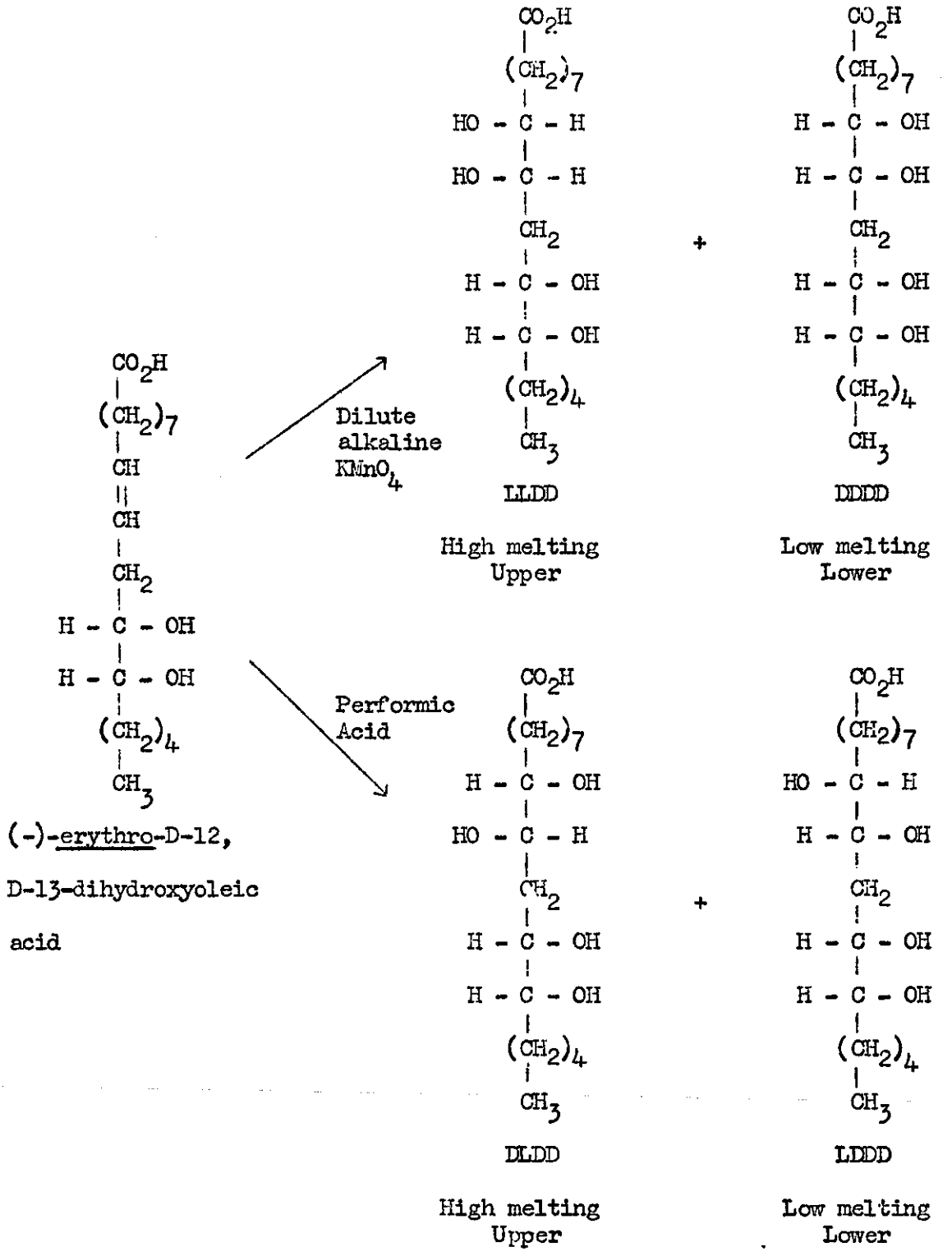
This conclusion and the conclusion reached as to the absolute configuration of the (+)-threo-9,10-dihydroxystearate have been substantiated by work reported in more detail in Section 3. Puccinia spores contain an enzyme, which on crushing the spores in water, cleaves the endogenous L-cis-9,10-epoxystearate, to give dextrorotatory threo-9,10-dihydroxystearate which has been proved to be L-9, D-10. Thus if the assignment of stereochemistry to the threo-9,10-dihydroxystearate is correct, attack of hydroxyl ion, or water, in this hydration must take place at the 10 position with inversion. These predictions have been tested directly by incubation of the Puccinia spores in ^{18}O enriched water. As predicted, the ^{18}O was localized at the 10 position, confirming the stereochemical assignments.

The conclusion that the high melting isomer of each of the 9,10,12,13-tetrahydroxystearate "oxidation pairs" has a trans-1,3-disposition of the 10- and 12-hydroxyl groups, and the known absolute configuration of (-)-erythro-12,13-dihydroxyoleic acid and (+)-threo-12,13-dihydroxyoleic acid derived from vernolic acid, allows the prediction of the absolute configuration of the enantiomers of all 8 tetrahydroxystearates.

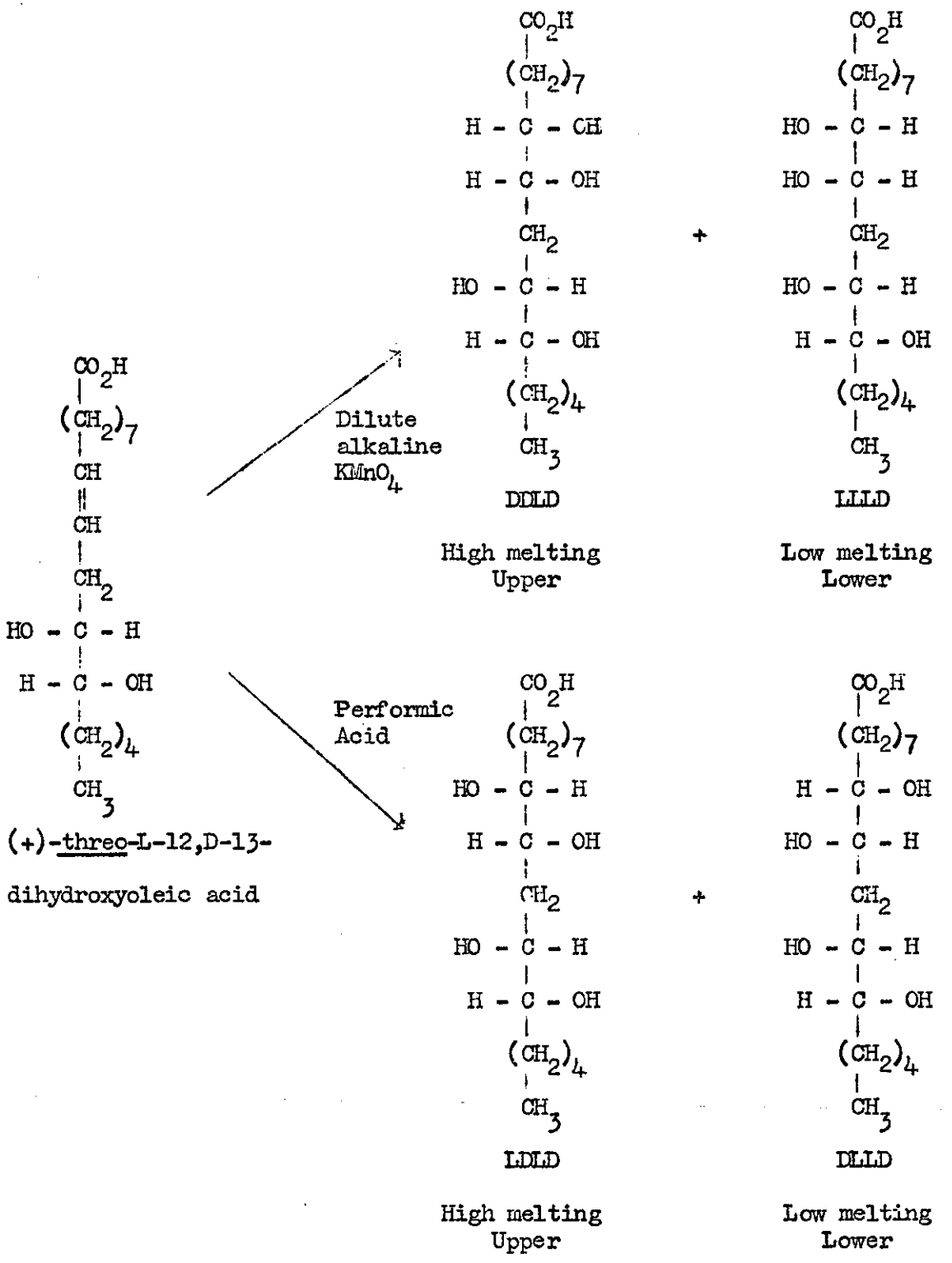
When the erythro- and threo-12,13-dihydroxyoleic acids of known configuration are each hydroxylated with dilute alkaline permanganate and with performic acid, four pairs of optically active tetrahydroxy acids will be produced as shown in Schemes 6 and 7.

Each of these pairs of tetrahydroxystearates are known to be separable by TLC on arsenite impregnated silica gel (131). As the absolute configuration of the 12- and 13-hydroxyl groups in these tetrahydroxy acids is known, and it is also known that the higher melting isomer which migrates the furthest on arsenite TLC has a 1,3-trans relative configuration of the 10- and 12-hydroxyl groups, the absolute configuration of all the tetrahydroxy acids compounds can be predicted.

Considering first the pair of tetrahydroxystearates produced by cis hydroxylation of (-)-erythro-12,13-dihydroxyoleate with alkaline permanganate. Separation of this pair of tetrahydroxystearates by TLC on arsenite impregnated silica gel will result in a faster migrating, higher melting isomer and a more polar lower melting compound. As the absolute configuration of the C-12 hydroxyl group and the relative disposition of the C-10 and C-12 hydroxyl groups are known for this pair of compounds, their absolute configurations can be predicted. Thus the higher melting isomer will have the 9-L, 10-L, 12-D, 13-D configuration and the lower melting isomer the 9-D, 10-D, 12-D, 13-D configuration.



Scheme 6. Predicted configurations, melting points and migration patterns on arsenite impregnated TLC of tetrahydroxystearates from (-)-erythro-12,13-dihydroxyoleic acid



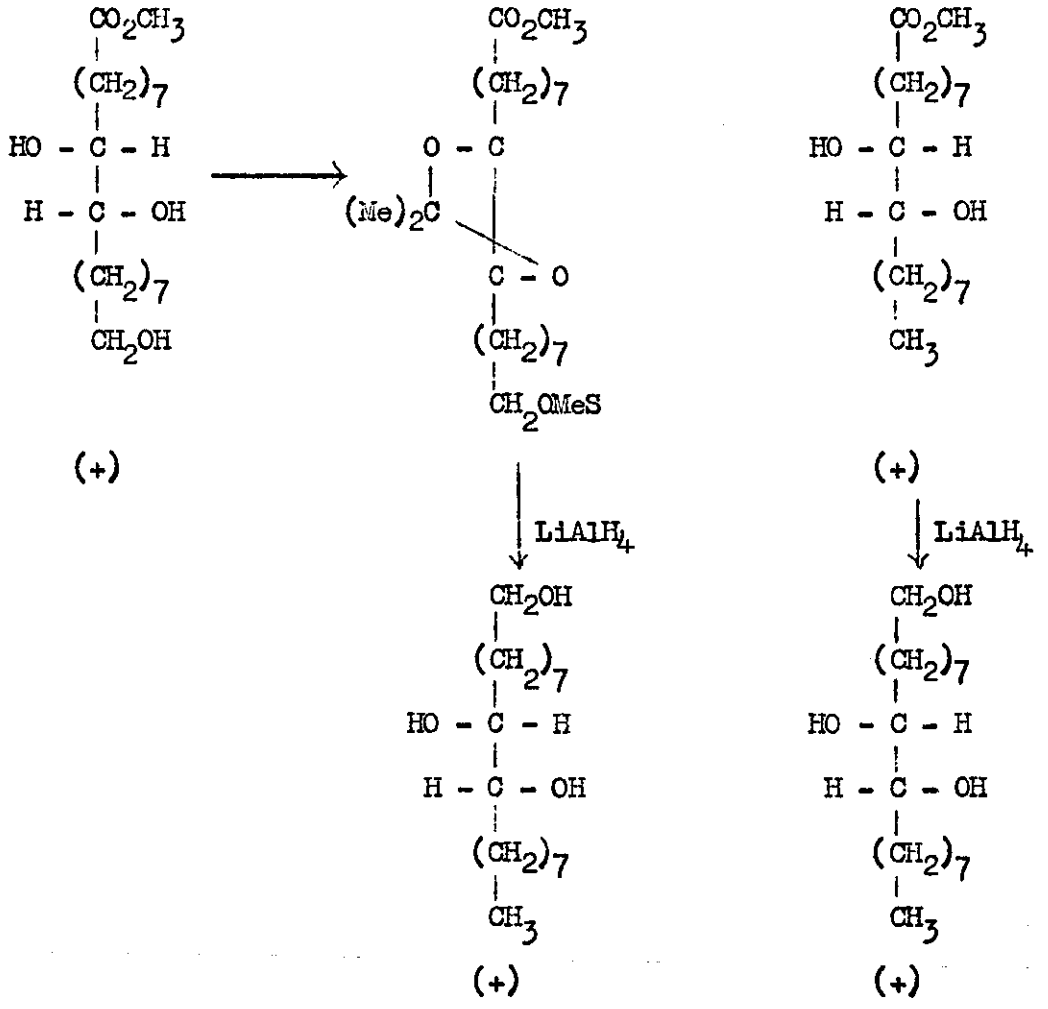
Scheme 7. Predicted configurations, melting points and migration patterns on arsenite impregnated TLC of tetrahydroxystearates from (+)-threo-12,13-dihydroxyoleic acid

A similar argument can be applied to the remaining three pairs of tetrahydroxystearates, and their absolute configurations can be predicted. Thus the high melting tetrahydroxystearate produced by trans hydroxylation of (-)-erythro-12,13-dihydroxyoleate with performic acid, will have the 9-D, 10-L, 12-D, 13-D configuration, and the high melting tetrahydroxy isomers produced by overall cis and trans hydroxylation of (+)-threo-12,13-dihydroxyoleate, will have the 9-D, 10-D, 12-L, 13-D and 9-L, 10-D, 12-L, 13-D configurations respectively. The predicted absolute configurations, relative melting points and relative positions on arsenite impregnated TLC of the 8 tetrahydroxy isomers are shown in Schemes 6 and 7.

This work has also allowed the configuration of the trihydroxy acids from Chamaepeuce seed oil (124) to be predicted. threo-9,10,18-Trihydroxystearate (α_D = +22.3) and threo-9,10,18-trihydroxy-cis-12-octadecenoate (α_D = +18.2) must have the L-9, D-10 configuration, as it is unlikely that the terminal hydroxyl group, which is achiral, will affect the sign of rotation of these compounds. Since this work was completed, McGhie et al (140) have correlated the configuration of (+)-threo-9,10,18-trihydroxystearate with that of (+)-threo-9,10-dihydroxystearate, and in the same paper have presented the configurations of the threo-9,10-dihydroxy- and trans-9,10-epoxystearic acids. However, the method used to determine the configurations of the threo diols and trans epoxides was not reported. The method used to correlate the configuration of (+)-threo-9,10,18-trihydroxystearate with (+)-threo-9,10-dihydroxystearate is shown in Scheme 8.

The vic diol grouping in the (+)-threo-trihydroxyester was protected by formation of the isopropylidene derivative. The mesylate of the terminal hydroxyl was prepared and reduced with lithium aluminium hydride in tetrahydrofuran, to give the (+)-threo-9,10-dihydroxyoctadecanol.

Similar hydride reduction of (+)-threo-9,10-dihydroxystearate, of known configuration, gave an identical compound, thus the configuration of the vic diol group in the trihydroxystearate must be the same as that in (+)-threo-9,10-dihydroxystearate, i.e. 9-L, 10-D, confirming the conclusions reached earlier in this work.



Scheme 8. Method used to correlate configurations of threo-9,10,18-trihydroxy- and threo-9,10-dihydroxystearates

EXPERIMENTAL

Preparation of (-)-erythro-9,10-dihydroxystearic acid

Castor oil (100g) was dissolved in 5% methanolic potassium hydroxide (500ml) and left overnight. The solution was diluted with water (700ml) and extracted with ether, the ether extracts were discarded. The aqueous layer was acidified with dilute hydrochloric acid and extracted with ether. The ether extracts were washed with water until neutral, dried, and solvent removed. The resultant yellow oil was dissolved in ethylacetate to give a 10% solution, and stood at 2°C for 64 hours. The resultant colourless crystals were filtered off under vacuum and recrystallized from ethyl acetate to give colourless plates. (300mg; $[\alpha]_{546.1\ nm}$ of ester = -0.04° (c = 9.0 EtOH))

Preparation of isopropylidene derivative of (-)-erythro-9,10-dihydroxystearate

Methyl erythro-9,10-dihydroxystearate (50mg) was dissolved in acetone (5ml) and 60% perchloric acid (10 µl) was added. The solution was left at room temperature for fifteen minutes, and then neutralized with dilute ammonium hydroxide, diluted with water (5ml) and extracted with ether (5ml). The ether extracts were washed three times with water, and the solvent was removed to leave an oil which was purified by TLC (ether:petroleum ether 5:95). The product was a colourless oil. (40mg; $[\alpha]_{546.1\ nm}$ = -0.50° (c = 2.0 EtOH))

Preparation of (+)-threo-12,13-dihydroxyoleate

Vernonia anthelmintica seeds (50g) were crushed in a pestle and mortar until the majority were well ground. The seeds were placed in a conical flask, water was added until the seeds were just moistened,

and the mixture was left at 30°C for three days. Chloroform:methanol 2:1 (200ml) was added to the mixture. After 24 hours the organic layer was filtered into a separating funnel and washed with 0.7% saline (100ml). The aqueous layer was extracted with chloroform which was added to the main extract from which solvent was removed to leave a dark brown oil. The oil was dissolved in ether (100ml), and the ether solution was extracted with 5% aqueous potassium hydroxide (2 x 50ml). The aqueous alkaline extracts were acidified and extracted with ether. The ether extracts were washed acid free and the solvent was removed to leave a brown oil. The oil was dissolved in ether and esterified with diazomethane. threo-12,13-Dihydroxyoleate was isolated from this mixture by column chromatography on Davidsons Silica Grade 950. The product was a pale yellow oil. (5.4g; $[\alpha]_{546.1\ nm}$ = +20.3° (c = 2.0 EtOH))

Preparation of (+)-methyl threo-12,13-dihydroxystearate

(+)-Methyl threo-12,13-dihydroxyoleate (400mg) was dissolved in ethyl acetate (10ml) and Adams catalyst (20mg) was added to the solution. The mixture was shaken under hydrogen (1 atmosphere) for 5 hours. The mixture was filtered and the solvent was removed from the filtrate to leave a white solid. (400mg; $[\alpha]_{546.1\ nm}$ = 23.7° (c = 1.34 MeOH))

Preparation of threo-9,10-dihydroxystearic acid

Oleic acid (14g) was dissolved in formic acid (42ml), hydrogen peroxide (6.9g) was added and the mixture was warmed to 40°C. After two hours, the solution was poured into water (200ml) and extracted with ether (3 x 50ml). The ether extracts were washed with water, dried over anhydrous sodium sulphate, and the solvent was removed to leave a yellow oil.

The oil was heated under reflux with 5% methanolic potassium hydroxide (30ml) for two hours. The solution was then diluted with water (60ml), acidified with dilute hydrochloric acid and extracted with ether (3 x 30ml). The ether extracts were washed acid free, dried, and solvent was removed to leave a pale yellow solid. The product was crystallized from ethylacetate to yield white plates of threo-9,10-dihydroxystearic acid (10g; m.pt. 95-96°C).

Optical resolution of threo-9,10-dihydroxystearate

The dihydroxy acid (10g) and brucine (16g) were dissolved by gentle warming in acetone (30ml) and water (70ml). The solution was left for two days at 2°C and the white solid which crystallized was collected and recrystallized twice more from acetone-water mixtures. This brucine salt was then decomposed by warming with 5N hydrochloric acid (40ml) and the mixture was extracted with ether. The extracts were washed acid free and the solvent was removed to leave a white solid which was recrystallized from ethylacetate to give threo-9,10-dihydroxystearic acid. (2.1g; $[\alpha]_{546.1\ nm}$ = +20.8 (c = 1.2 EtOH), m.pt. 97.5-99°C lit. 99.5 (138))

Conversion of dihydroxy acids to epoxy esters

(+)-threo-9,10-Dihydroxystearic acid (200mg) was dissolved in a 15% solution of anhydrous hydrogen bromide in acetic acid (10ml), and the solution was left at room temperature overnight. The solution was diluted with water (20ml) and extracted with ether, the extracts were washed acid free and solvent removed to leave a brown oil. This oil was dissolved in 5% methanolic potassium hydroxide (10ml) and the solution was heated under reflux for two hours. The solution was diluted with water and carefully acidified (pH5) with dilute acetic acid. The mixture was immediately extracted with ether, and the ether extracts were washed

acid free and solvent was removed to leave a yellow solid. The products were dissolved in ether, esterified with diazomethane and purified by TLC (ether:petroleum ether 1:3). The product was a colourless solid. ($[\alpha]_{546.1\ nm}$ = +15.0 (c = 1.3 MeOH))

Conversion of 9,10,12-trihydroxystearates to 9,10-dihydroxystearates

The experimental procedures outlined below were used for both erythro- and threo-9,10,12-trihydroxystearates.

threo-9,10,12-Trihydroxystearate (300mg) was dissolved in acetone (5ml) and 60% perchloric acid (5 µl) was added to the solution. After 15 minutes 0.880 ammonia was added until the solution was alkaline, the mixture was diluted with water (10ml) and extracted with ether. The ether extracts were washed with water until neutral and the solvent was removed to leave a colourless oil which was purified by TLC, the major product being the 9,10-isopropylidene derivative ($[\alpha]_{546.1}$ = -48° (c = 0.5 ethanol))

The 9,10-isopropylidene derivative (250mg) was dissolved in a 5% solution of chromium trioxide in glacial acetic acid (25 ml) and the solution was allowed to stand at room temperature. After 15 minutes water (100ml) was added and the solution was extracted with ether. The ether extracts were washed until neutral and the solvent was removed to leave a colourless oil which was purified by TLC (ether:petroleum ether 2:3) to give the 9,10-isopropylidene, 12-ketostearate, ($[\alpha]_{546.1}$ = -24.4° (c = 1.0 ethanol)) in ca 50% yield.

The 9,10-isopropylidene-12-ketostearate (100mg) was dissolved in methanol (5 ml), toluene-p-sulphonylyhydrazide (40mg) was added and the solution was boiled under reflux for six hours. The solution was then diluted with water and extracted with ether. The extracts were washed with dilute sulphuric acid, then with water until neutral. The tosylhydrazone was isolated by TLC (ether:petroleum ether 3:2) in ca 70% yield.

The tosylhydrazone (70mg) and sodium borohydride (200mg) were dissolved in methanol (4ml) and the solution was boiled under reflux for 16 hours. After this time the reaction mixture was diluted with water, acidified, and extracted with ether. The extracts were washed until neutral, and the solvent was removed. The 9,10-isopropylidene stearate was isolated by TLC (ether:petroleum ether 1:4) as a colourless oil ($[\alpha]_{546.1\ nm}$ = -29.0 (c = 0.15 ethanol)) in ca50% yield.

The isopropylidene derivative (20mg) was warmed for two hours at 100°C with a 25% solution ofboric acid in 2-methoxyethanol (5ml). The solution was cooled, diluted with water (10ml) and extracted with ether. The extracts were washed, and the solvent was removed to leave a white solid. The threo-9,10-dihydroxystearate was purified by TLC (ether:petroleum ether 8:2) to give a white solid. (17mg $[\alpha]_{546.1\ nm}$= -20.6 (c = 0.10 EtOH))

INTRODUCTION

The previous section has dealt with the determination of the absolute stereochemistry of some epoxy and vicinal dihydroxy fatty acids. That work, and that already reported in the literature, completes the stereochemical definition of all the 9, 10- and 12,13-substituted epoxy and dihydroxy acids. The knowledge of these absolute configurations can now be applied to the determination of the mechanisms by which the enzymic transformation of epoxy acids to vicinal dihydroxyacids occurs in various biological systems.

Under appropriate conditions, 1,2-epoxides undergo ring opening to give vicinal diols. These reactions are stereospecific and occur with inversion of configuration about one carbon atom, thus cis epoxides afford threo diols, whereas trans epoxides yield the erythro compound(178). The reactions provide the link between naturally occurring epoxides and vicinal diols, and this section is devoted to the determination of the mechanism and specificity of some of these transformations in natural systems.

The biological cleavage of 1,2 epoxides to give vic diols is an enzyme catalysed hydration type of mechanism, and this type of mechanism is also involved in the formation of mono-hydroxy acids from unsaturated fatty acids. Probably the first example of this type of naturally occurring hydration reaction in fatty acid chemistry was that discovered in a strain of Pseudomonas (NRRL 2992). This bacterium was found to hydrate oleic acid, in 14% yield, to 10-hydroxystearic acid(144). The absolute configuration of the hydroxyl group in the product was proved by Schroepfer and Bloch (31). to be D, and it was later demonstrated that the overall hydration occurred by trans addition of the elements of water across the double bond (145). The stereochemistry of this addition was proved by utilising the known

stereochemistry of desaturation of stearic acid to oleic acid in Corynebacterium diptheriae. This organism was known to remove the D-9 and D-10 hydrogen atoms from stearic acid to produce oleic acid.

The hydration of oleic acid by the pseudomonad was carried out in deuterated water, to yield 10-hydroxystearic acid containing one atom of co-valently bound deuterium at the C-9 position. Removal of the hydroxyl group by methods which did not affect the deuterium atom left deuterated stearic acid. The deuterium atom at the C-9 position was retained on desaturation of this stearic acid by Corynebacterium diphtheriae and must, therefore, have been in the L configuration. Thus the overall stereochemistry of the hydration had been determined.

These findings are consistent with a mechanism involving stereospecific trans addition of the elements of water to the double bond, analagous to the hydration of fumaric acid by the enzyme fumerase (71). The same pseudomonad enzyme system which hydrates oleic acid to D-10-hydroxystearic acid also catalyses the conversion of either 10-hydroxystearic or oleic acid to trans-10-octadecenoic acid (146).

The specificity of this reaction was investigated with a number of hydroxy stearic acids, ranging from 5-hydroxystearic to 15-hydroxystearic acid. Only the 10-hydroxy isomer was a substrate for the enzyme (147). The enzyme also catalyzed the conversion of palmitoleic to D-10-hydroxypalmitic acid(146); linoleic acid to D-10-hydroxy-cis-12-octadecenoic acid(148); and the stereospecific hydration of 9,10-epoxystearic acids to vic diols. The transformation of epoxy acids to vic diols will be discussed in more detail later.

A similar hydratase enzyme system in a different species of Pseudomonas was investigated by Wallen et al(149). They found that the 9,10 double bonds in linoleic, linolenic, and ricinoleic acids were all hydrated, and that the incoming hydroxyl group had assumed the D configuration in each case. The enzyme system showed an unusual specificity

for Δ^9-unsaturated acids, it failed to hydrate 9-decenoic,12,13-epoxy-, or 12-keto-cis-9-octadecenoic acids, or sterculic acid. These results suggested that an alkyl chain on both sides of the double bond was essential before hydration occurred and that these chains must have a cis relationship to each other.

Ricinoleic acid D-12-hydroxyoleic acid is the major component of the seed oil of the castor plant (Ricinus communis); it is also found in ergot oil extracted from the sclerotia of the fungus Claviceps purpurea. Ricinoleic acid is interesting in that it is biosynthesised by two different pathways in these different organisms - one of these pathways may involve a hydration mechanism.

In the immature sclerotia of Claviceps isolated from infected rye plants, oleic acid is desaturated under aerobic conditions, but no ricinoleate is formed, either in the presence or absence of oxygen (150). However, linoleic acid is converted to ricinoleic acid, the efficiency of the conversion being improved under anaerobic conditions. These results suggest that the mechanism is a specific hydration of linoleate to 12-hydroxyoleate, analogous to the oleate to 10-hydroxystearate conversions in pseudomonads. However, ergot oil, whose fatty acids may contain up to 44% of ricinoleic acid, contains no free hydroxyl groups; the hydroxy acid is esterified to glycerol through its carboxyl group and to an unsubstituted fatty acid through the C-12-hydroxyl group. This structure led to the suggestion (151) that the biosynthesis of ricinoleic acid may not involve the direct hydration of the 12,13-double bond of linoleic acid with the formation of a free hydroxyl group; but that linoleic acid, either free or as a glyceride, reacts with a straight chain fatty acid by an addition across the 12,13-double bond, in which a proton becomes attached to the 13-position and an acyloxy anion to the 12-position.

The other pathway, occurring in the castor bean, does not involve a hydration mechanism. The ricinoleic acid in this system is formed by direct hydroxylation of oleic acid, in a reaction requiring molecular oxygen and NADPH as cofactors (152). The stereochemistry of this hydroxylation has been investigated with D- and L-[1-^{14}C, 12-^{3}H$_1$]oleic acid, and it was found that 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 biosynthesis (153).

Howling et al (154) investigated the substrate specificity of the hydroxylase system in castor bean. Their results suggested that two hydroxylases exist, one recognising monoenoic acids with a double bond at position 9 with respect to the carboxyl group (Δ^9), and the other recognising acids with a double bond at position 9 with respect to the methyl group (n-9).

Having considered the olefinic hydration pathways found in a number of natural systems, attention can now be focussed on the analogous epoxide hydration enzymes which occur in plant, seed, bacterial, and fungal spore systems.

An epoxide hydration reaction has been implicated in the biosynthesis of the polyhydroxy acids of cutin. Cutin is the insoluble component of plant cuticle, and consists of cross-esterified polymerized fatty acids. Hydrolysis of this insoluble polymer gives mainly a mixture of threo-9,10,18-trihydroxystearic, 10,18-dihydroxystearic and 10,16-dihydroxypalmitic acids. The insoluble residue after base hydrolysis can be further cleaved with sodium iodide indicating peroxide linkages, and treatment with hydroiodic acid indicates some ether bonds in the polymer. Epoxy acids also occur in cutin; 18-hydroxy-9,10-epoxystearic acid has been found as a major component of the cutin of grape, apple, peach and pear fruits (155), but this acid seems to occur only in cutin and is the only source of hydroxyepoxy fatty acids reported in nature.

Kolattukudy et al have investigated the biosynthesis of epoxy and polyhydroxy acids in cutin. They found that oleate was rapidly incorporated into cutin in the fruit skin slices of apple and grape, and that this acid was converted to 18-hydroxy-9,10-epoxystearic acid. This result and the finding that epoxy acids always occurred in cutin containing 9,10,18-trihydroxystearic acid led to the suggestion that the vic diol grouping in the trihydroxy acid was formed by hydration of the epoxide ring of 18-hydroxy-9,10-epoxystearic acid (156). Subsequent experiments showed that radiolabelled 18-hydroxy-9,10-epoxystearic acid was converted to 9,10,18-trihydroxystearic acid in apple skin slices, indicating the presence of an epoxide hydratase in this tissue (155). Thus the conversion of oleic acid to trihydroxy acids in cutin occurs by sequential W-hydroxylation, epoxidation and hydration.

A similar reaction sequence starting from linoleic acid could give rise to the 18-hydroxy-9,10-epoxyoctadecenoic and 18-hydroxy-9,10-dihydroxyoctadecenoic acids found in apple cutin. In accordance with this hypothesis, labelled linoleic acid has been shown to be incorporated into these acids in apple skin slices (155). Only the Δ^9-bond of linoleic acid is epoxidized, the Δ^{12}-bond is unaffected, illustrating the specificity of the epoxidizing enzyme.

Returning to the hydratase enzyme from a pseudomonad, which catalyzes not only the stereospecific hydration of the double bond of a number of cis-9-olefinic fatty acids, but also the hydration of both cis- and trans-9,10-epoxystearic acid (159,160). These epoxide cleavage reactions were shown to be completely stereospecific (160), as regards both substrate and product. Moreover, studies of the hydration reaction in $H_2{}^{18}O$ showed that these reactions proceeded with stereospecific incorporation of the oxygen of water at carbon 10, in a similar manner to the hydration of oleic acid by the same enzyme system. This finding, and the observation

that olefin hydrating and epoxide hydrating activity showed a parallel thermolability, led to the suggestion that the two acitivites may reside in a single protein species.

What could not be deduced from this work was the absolute configuration of either of the dihydroxy products or of the epoxy acids remaining after the hydration reactions. However, the worked outlined in Section 2 has now resolved this problem and absolute configurations can be assigned to both epoxide substrates and dihydroxy products. These configurational assignments will be discussed in more detail later.

A similar epoxide hydrating enzyme occurs in the spores of a wide variety of plant rusts. Puccina graminis uredospores contain up to 30% (-)cis-9,10-epoxystearic acid (161) and this epoxide is known to have the L configuration (107). Incubation of the spores in water leads to almost complete hydration of the epoxy acid to (+)-threo-9,10-dihydroxystearic acid. This enzyme is activated in the early stages of germination of plant rust spores (162,163) and it has been suggested that the enzymic hydration of the epoxy acid is the first step in the formation of pelargonaldehyde, which is known to stimulate germination of spores. Now, the epoxy acid substrate and the dihydroxy acid product of this enzymic hydration have been shown to have the L-9, L-10- (107) and L-9, D-10- configurations respectively. Therefore, assuming a normal mechanism for epoxide ring opening, this hydration must proceed by hydroxyl attack at the 10 position with inversion, similar to the Pseudomonad system. The work reported here has verified this assumption by incubation of Puccinia spores in $H_2{}^{18}O$ and location of the ^{18}O in the product by mass spectrometry.

Hydration of epoxides to vic diols has also been demonstrated in a liver microsome system from several different animals (164). This enzymic hydration is probably a key step in the metabolism and detoxification of

aromatic and olefinic compounds in higher organisms. The epoxide hydratase present in liver homogenates converts a variety of epoxides including cyclohexene oxide (165), indene oxide (165,166), chlordene oxide (167), stilbene oxide (168), styrene oxide (165), steroidal epoxides (169,170) and arene oxides such asbenzene oxide (165) and naphthalene oxide (164) to diols. The same system also converts oleic acid to threo-9,10-dihydroxystearic acid in the presence of NADPH and oxygen (171).

Apart from the epoxide hydration systems found in animal liver preparations, it seems that the enzymes which hydrate double bonds and epoxide rings, possess a fairly rigid specificity. Thus the hydration system in pseudomonads seems only to hydrate fatty acids containing a Δ^9 double bond and a free carboxylic acid group, and the hydration of 9,10-epoxy- and cis-9-olefinic fatty acids involves attack by water or hydroxide ion at the C-10 position exclusively. This positional specificity for the introduction of a hydroxyl group into either oleic, linoleic or cis- and trans-9,10-epoxystearic acids by the pseudomonas enzyme, the precise stereochemical requirement of the enzyme system with regard to precursor, and the stereospecificity of the products suggests a specific molecular interaction between the substrate and enzyme surface.

Vernonia anthelmintica seeds contain a high proportion of cis-12,13-epoxyoleic acid occurring in the seed oil as triglyceride, and this epoxide is known to have the D-configuration (100,107). When Vernonia seeds are crushed and moistened, the epoxy acid is hydrolysed by the glyceride and hydrated to dextrorotatory threo-12,13-dihydroxyoleic acid (157,158) which has been shown to have the L-12, D-13 configuration (128). If it is assumed that the enzymic hydration occurs by a similar mechanism to chemical hydration, and there is no reason to suppose this is not the case, then it can be predicted that attack by water or hydroxyl ion occurs at the 12-position from the rear of the epoxide ring and that the

oxygen of the 13-hydroxyl group is that of the original epoxide. This prediction can now be tested by incubation of crushed Vernonia seeds in the presence of $H_2{}^{18}O$. Isolation of the threo-dihydroxy product and localization of the position of the added hydroxyl group by mass spectrometry should show, if the above prediction is correct, that the ^{18}O isotope is enriched exclusively at the 12-position.

As the hydration of D-cis-12,13-epoxyoleic acid in crushed moistened seeds of Vernonia anthelmintica shows a remarkable stereospecificity, only threo-12-L, 13-D-dihydroxyoleic acid being produced, it was relevant to ask whether the Vernonia enzyme system showed a precise positional specificity, and what structural features must be possessed by the epoxy acid in order for it to be a substrate for the epoxide hydratase. The work reported here has been in investigation of the specificity of the epoxide cleavage enzyme in Vernonia seeds.

The natural substrate for the Vernonia hydratase enzyme, D-cis-12,13-epoxyoleic acid, contains several features that the hydratase could "recognise", namely a carboxyl group, a cis-9 double bond, a cis-12,13-epoxide ring, and a terminal methyl group. It is possible that an epoxy fatty acid may need to contain one or more of these features at specific positions within the chain before it is an effective substrate for the enzyme system. Alternatively, it is conceivable that the enzyme system has very little specificity and will hydrate a wide variety of epoxy compounds in a similar manner to the liver microsome system.

In order to investigate the structural features needed for an epoxy acid to be a substrate for the Vernonia hydratase enzyme, it was necessary to prepare a series of racemic, positionally and geometrically isomeric, epoxy acids and some of their derivatives. Epoxy acids are usually prepared from the corresponding olefinic acid by direct epoxidation with peracids, or from vicinal dihydroxy acids via the bromohydrins. In this work, most of the epoxides were prepared from the corresponding olefins by epoxidation with peracid, as in most cases the olefins were easily obtainable.

RESULTS AND DISCUSSION

The work described in the previous section has determined the absolute stereochemistry of a range of epoxy and dihydroxy fatty acids, this knowledge allows the prediction of the stereochemistry and mechanism of a range of epoxide and olefinic enzymic hydration reactions.

I) Pseudomonas hydratase

The first of these reactions discussed here is the hydration of cis- and trans-9,10-epoxystearic acids to threo- and erythro-9,10-dihydroxystearic acids, respectively, catalyzed by a pseudomonad enzyme preparation.

Niehaus and Schroepfer (160,161) have shown that these hydrations are completely stereospecific. Thus racemic cis-9,10-epoxystearic acid is converted to a mixture of optically active threo-9,10-dihydroxystearic acid and residual, optically active cis-epoxystearic acid in approximately equal amounts. Similarly, racemic trans-9,10-epoxystearic acid gave a mixture of equal amounts of optically active erythro-9,10-dihydroxystearic acid and optically active trans-9,10-epoxystearic acid. What Niehaus and Schroepfer did not know however, was the absolute configuration of either the dihydroxy acid product or the epoxy acid remaining after the enzymic hydration reactions. The work in the previous section has now resolved these problems and the configurations of the dihydroxy, and epoxy products, and therefore of the substrates, are now known.

The specific optical rotations of the dihydroxy and epoxy esters produced by methylation of the incubation products of racemic cis and trans-9,10-epoxystearic acid with the pseudomonad enzyme system along with those of the corresponding compounds characterized in the previous section are shown in Table 1.

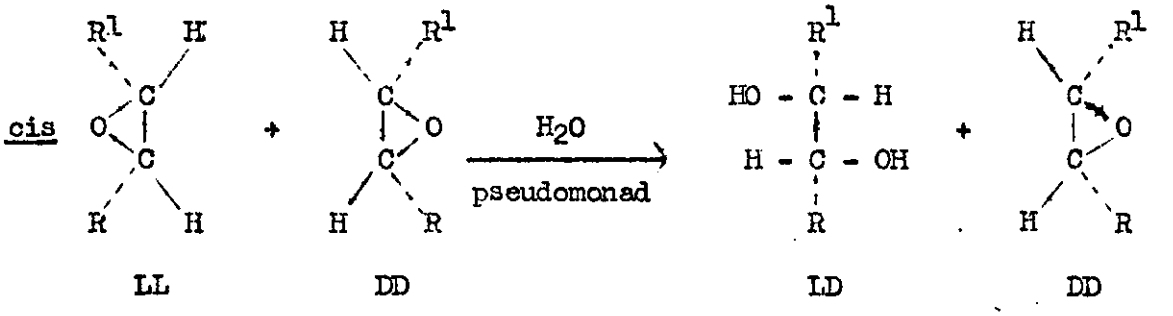
Compound (source)	$[\alpha]_D$ (c;solvent)
Methyl threo-9,10-dihydroxystearate (pseudomonad incubation)	+ 20.8 (1.78;MeOH)
Methyl cis-9,10-epoxystearate (pseudomonad incubation)	- 0.20 (2.49;MeOH)
Methyl D-9, L-10-dihydroxystearate	-20.6 (0.15;MeOH)
Methyl L-9, L-10-epoxystearate (Xeranthemum annuum)	+ 0.3 (MeOH)
Methyl erythro-9,10-dihydroxystearate (pseudomonad incubation)	+ 0.35 (0.62;MeOH)
Methyl trans-9,10-epoxystearate (pseudomonad incubation)	- 4.38 (0.96;MeOH)
Methyl D-9, D-10-dihydroxystearate (castor oil)	+ 0.12 (1.1;MeOH)
Methyl L-9, D-10-epoxystearate	+ 15.0 (1.3;MeOH)

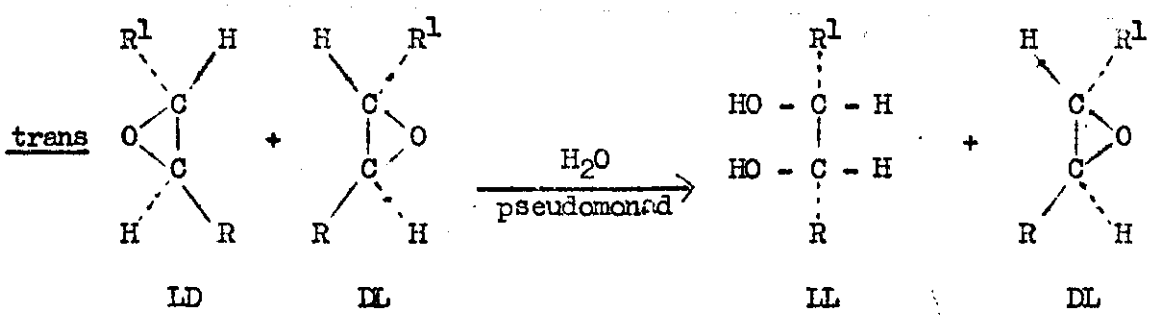
Table 1. Specific Rotations of Enzymically produced Dihydroxy and Epoxy Esters (160)

Considering first the products from the incubation of cis9,10-epoxystearic acid with the pseudomonad enzyme system, it can be seen that the threo-dihydroxy acid is the enantiomer of that characterized in the previous section and must, therefore, have the L-9, D-10 configuration. The remaining cis-9,10-epoxide is dextrorotatory and is enantiomeric to that characterized by Powell et al (107) and therefore has the 9-D, 10-D configuration. As this remaining epoxide is predominantly of the D configuration, the enzymically reactive isomer must have been the L-9, L-10-epoxystearic acid.

Assuming that enzymic cleavage of the cis epoxide occurs in a similar manner to chemical cleavage, that is by rearside nucleophilic attack with inversion of configuration about one of the carbon atoms of the oxirane ring and retention of configuration at the other carbon, it can be predicted from the results that the enzymic cleavage of L-cis-9,10-epoxystearic acid occurs by attack of water, or OH^- ion, at the 10-position. This is indeed the case, as has been demonstrated by performing the incubations in ^{18}O-enriched water and showing that the oxygen from water became attached exclusively to the 10-position (146).

A similar argument can be applied to the hydration of trans-9,10-epoxystearic acid. In this case, the L-9,D-10-epoxystearic acid is cleaved by the enzyme to yield L-9, L-10-dihydroxystearic acid, the remaining epoxide in this case being the D-9, L-10 isomer. These stereochemical conclusions are summarized in Scheme 1.



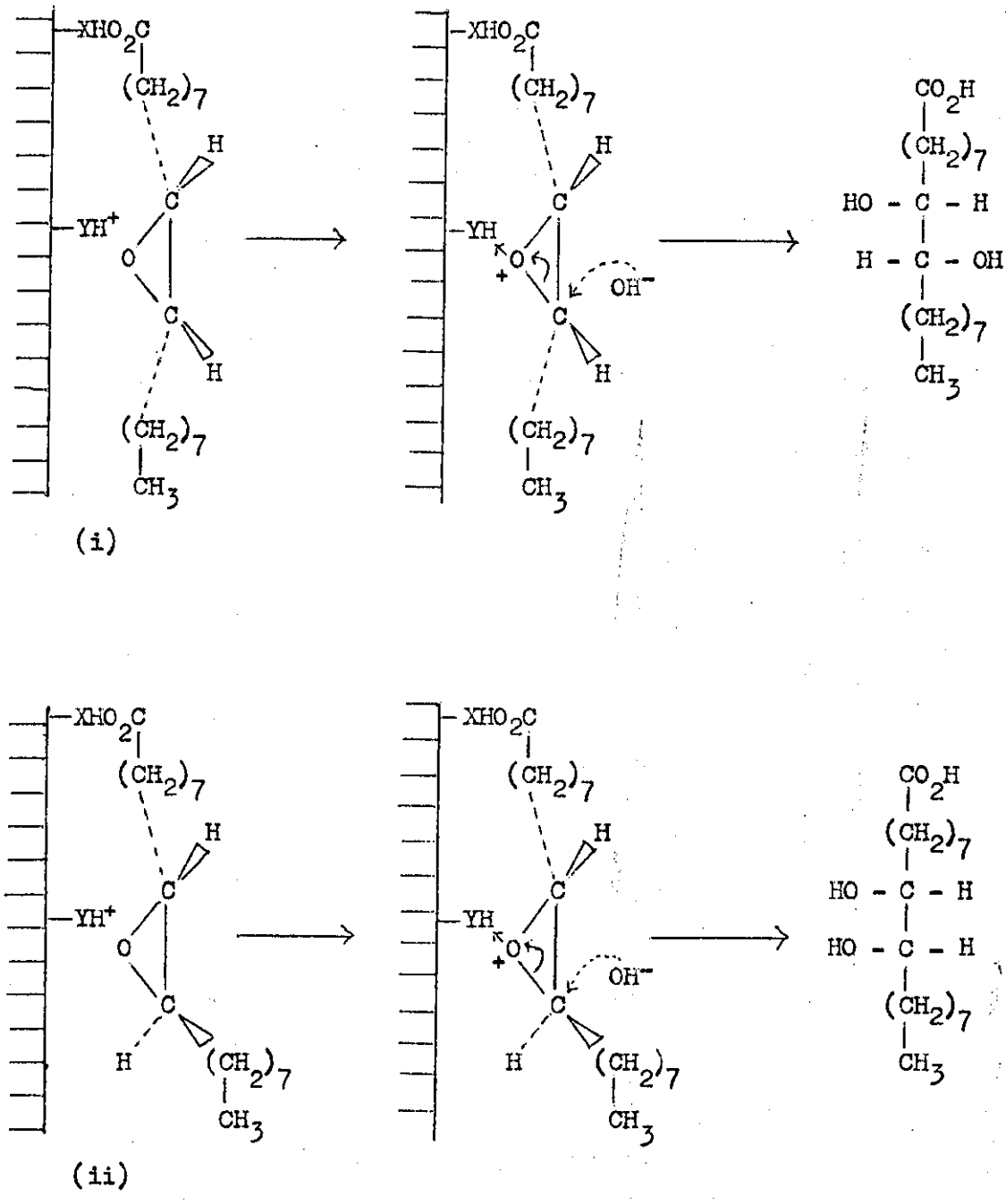


Scheme 1. Summary of the action of the pseudomonad hydratase on racemic cis- and trans-9,10-epoxystearic acid.

It could be argued that the configurational conclusions reached in Section 2 were not reached directly, but rely on the more general conclusion that in all oxidation pairs having a 1,3 diol system at position 10 and 12 the higher melting isomer has a trans-10,12 diol configuration, and that therefore there could be a slight residual doubt as to the validity of the configurational assignments.

The correlation between the prediction of the position of attack of water or OH^- ion, based on the conclusion reached in Section 2, and that proved by incubations with $H_2{}^{18}O$ support the stereochemical assignments reached earlier, and helps dispel this doubt.

Now that the absolute stereochemistry of precursors and products of the Pseudomonas hydratase enzyme are known, it is possible to predict the relevant features and mechanism of action of this enzyme in the hydration of the two epoxy acids; cis-9,10-epoxystearic and trans-9,10-epoxystearic acids, and of oleic acid. The proposed mechamism is outlined in Scheme 2. No activation of the carboxyl group of the substrate seems to be required for these hydrations and the enzyme is specific for the 9,10 position. Therefore, the first step appears to be attachment of the substrate carboxyl group, in some way, to the enzyme at a site designated as X. The active site must be an electrophilic group, designated as $-YH^+$, at a distance from the point of attachment of the carboxyl group (X) so as to place it in the enzyme substrate complex between the 9- and 10-positions of the substrate, but probably closer to the 9-position. The first reaction step is electrophilic attack from the L side of the acyl chain to give the conjugate acid of the epoxide groups. This attack must be promoted by close and specific association between the substrate and the enzyme surface. The hydration reaction is then completed by back side nucleophilic attack, at the 10-position either by OH^- ion to give the product directly or by water followed by loss of a proton, to give the two products with the correct stereochemistry.



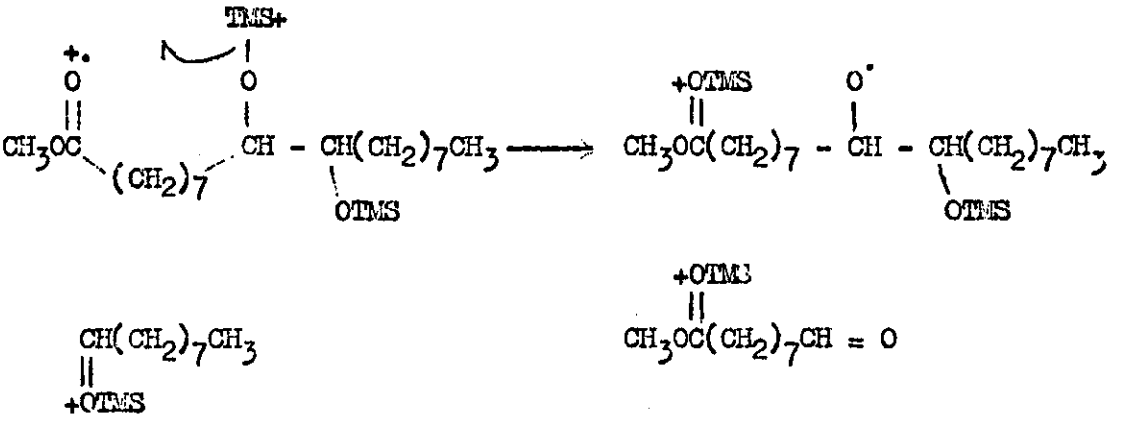
Scheme 2. Schematic representation of the mechanism of hydration of cis-9,10-epoxystearic acid (i), trans-9,10-epoxystearic acid (ii) by the pseudomonad hydratase system.

II) Puccinia Hydratase

The next epoxide cleavage enzyme to be considered is that which occurs in the spores of a number of plant rusts. Tulloch (172,173) has shown that in the spores of Puccinia graminis and several other plant rusts there is an enzyme system which, on incubation of the spores in water, hydrates the endogenous (-)-cis-9,10-epoxystearic acid to (+)-threo-9,10-dihydroxystearic acid. The epoxy acid substrate of this reaction has been proved to have the L-configuration (107), and the work reported in the previous section has proved that the absolute configuration of the dihydroxy acid product is L-9, D-10. Therefore, assuming normal opening of the epoxide ring, the enzymic hydration must occur by hydroxyl attack at the 10-position, with inversion of configuration at that position. In order to verify this prediction and prove that attack by hydroxyl ion, or water, occurs exclusively at the 10-position, studies of the enzymic hydration in water enriched with ^{18}O were undertaken.

In order to locate the ^{18}O which may be incorporated into the molecule during the incubation, a sensitive assay is required, which will distinguish between the oxygen atoms at C-9 and C-10 of 9,10-dihydroxystearic acid. Methyl 9,10-dihydroxystearate does not give a significant molecular ion on electron impact (174). However, the mass spectrum of this compound shows a major peak at m/e 187 corresponding to cleavage of the C9-C10 bond in the molecule, and this ion could be used to give a measure of the ^{18}O on the 9-position. Measurement of the combined incorporation of ^{18}O into positions 9 and 10 of the molecule can be performed by analysis of the mass spectrum of the isopropylidene derivative of the molecule (134). Thus, analysis of the isotopic abundance of ^{18}O in the mass spectrum of methyl 9,10-dihydroxystearate and its isopropylidene derivative would permit an estimation of the ^{18}O content at positions 9 and 10. A more convenient assay, however, and the one used in this work, is the measurement of the mass spectrum of the trimethylisilyl (TMS) ether derivative

of the vic-diol. Reaction of a vicinal dihydroxy acid with hexamethyldisilazane yields a di-TMS ether. The mass spectrum of this product shows two abundant ions (I and II in Scheme 3) which represent cleavage between the substituted carbon atoms. The formation of I involves the migration of the C-9 TMS group to the ester group followed by collapse of the resultant oxygen radicle as shown (175). Thus this method has the advantage that it gives a direct measure of the enrichment of ^{18}O at both the C-9 and C-10 positions from a single derivative.



II m/e 215 I m/e 259

Scheme 3.

The incubations of the Puccinia spores with $H_2{}^{18}O$ were performed in a small test tube equipped with a glass rod, fitted through a rubber seal at the mouth of the tube. This arrangement was necessary because of the small amounts of $H_2{}^{18}O$ used in each incubation, and the fact that in order to "wet" the spores, and release the hydratase enzyme, it was necessary to grind the spores in the presence of water. The incubations were performed at 25°C for twenty-four hours.

After the twenty-four hours, the lipids were extracted from the incubation mixture by shaking together the spores and Ballotini (1.5-2.5mm) in chloroform and methanol for several hours. It was found necessary to

disrupt the spores in this way in order to obtain complete extraction of the lipid. The lipid extract was directly methylated with diazomethane and the threo dihydroxy ester produced by enzymic cleavage of the endogenous cis epoxide was isolated by TLC.

The cis-9,10-epoxystearic acid in Puccinia spores occurs mainly esterified as triglycerides, whereas the dihydroxy product of enzymic hydration is a free acid; the hydrolysis of the epoxide to the free acid form is required before the cleavage of the oxirane ring occurs. During the incubation of Puccinia spores with $H_2{}^{18}O$, this preliminary hydrolysis, of course, would result in the incorporation of ^{18}O into the carboxyl group of the dihydroxy acid product. As the measurement of the amount of ^{18}O incorporated into the C-9 position of the dihydroxy acid is performed on an ion (I in Scheme 3) which contains the carbomethoxy group, then any enrichment of ^{18}O into this latter group will give a result which indicates apparent incorporation into the C-9 position. This difficulty was overcome by treatment of the dihydroxy acid successively with methanolic sodium methoxide and aqueous hydrochloric acid in methanol, which results in the exchange of the oxygen atoms of the carbomethoxy group but not the oxygen atoms of the vicinal diol grouping. After the diol had been treated in the above manner, it was converted to the di-TMS ether and subjected to mass spectrometry, the results of which are shown below.

Experiment Number (Puccinia strain number)	Enrichment of ^{18}O (atom %) on positions	
	C9	C10
P1 (PB61/37)	5.6	26.5
P2 (r60)	7.4	21.1
P3 (PB58/44)	1.0	28.5

Isotopic enrichment of water ^{18}O = 40.5 atom %

Mass spectral analysis of isotopic composition of ^{18}O-labelled threo-9,10-dihydroxystearate obtained from incubation of Puccinia spores with $H_2{}^{18}O$

In these results obtained by mass spectral analysis of the purified di-TMS ether derivative of methyl threo-9,10-dihydroxystearate, an enrichment of ^{18}O on the C-10 position is indicated, the C-9 position containing a much smaller amount of ^{18}O. This is in keeping with the results expected for a mechanism of epoxide cleavage which involves nucleophilic attack by OH^- ion or water at the C-10 position of cis-9,10-epoxystearic acid with inversion about that position, which is in direct agreement with the mechanism proposed earlier on the basis of the configurational assignments.

The presence of 5.6% and 7.4% of ^{18}O on the C-9 position of the dihydroxy acid produced in the experiments with strains PB61/37 and r60 is not considered to indicate a lack of specificity of the enzymic hydration but rather a lack of complete exchange of the labelled oxygen atoms in the carbomethoxy function. Indeed when the period of the exchange reaction on the product from strain PB59/54 was increased, a much lower result for the incorporation of ^{18}O onto the C-9 position was obtained.

It therefore appears from these results that Puccinia spores contain an enzyme system similar to that found in Pseudomonas, and although the Puccinia system has not been so thoroughly investigated, it can be suggested that a similar mechanism as that proposed in Scheme 2 for the Pseudomonas system is operating in this case. It is also likely that the biosynthesis of (+)-threo-9,10-dihydroxystearic acid in Claviceps sulcata sclerotia occurs by the same mechanism, particularly as traces of cis-9,10-epoxystearic acid has been found to occur along with the vic diol in two Claviceps species (123).

III) Vernonia Hydratase

The third epoxide hydratase enzyme to be discussed here is that found in Vernonia anthelmintica seeds. On incubation of crushed Vernonia seeds under moist conditions, the epoxy acid hydrating enzyme cleaves the

endogenous (+)-vernolic acid to give optically pure (+)-threo-12,13-dihydroxyoleic acid (157,158). The absolute configuration of the endogenous epoxy acid has been established as D-cis-12,13-epoxyoleic acid (100), and the absolute configuration of the vicinal dihydroxy acid product of this enzymic hydration has been shown to be L-12, D-13-dihydroxyoleic acid (128).

From the knowledge of these two configurations it had been predicted (100) that the enzymic hydration of vernolic acid occurs by attack by water at the 12-position with inversion at this position. This prediction has now been verified by incubation of crushed Vernonia seeds in $H_2{}^{18}O$, and location of the ^{18}O in the dihydroxy acid product, in an identical manner to that used for the Puccinia system.

Vernonia anthelmintica seeds were crushed, moistened with $H_2{}^{18}O$ and incubated under nitrogen in tightly stoppered glass vials for three days. At the end of the incubation period, the dihydroxy acid product was isolated, subjected to the carboxyl oxygen exchange reaction, and converted to the di-TMS ether in a similar manner to that described previously. The product was analysed by mass spectroscopy, and the results are shown in Table 2.

Experiment Number	Enrichment of ^{18}O (atom %) on position	
	C12	C13
V1	42.4	3.0
V2	33.1	0.6

Isotopic enrichment of water ^{18}O = 40.5 atom %

Table 2. Mass spectral analysis of isotopic composition of ^{18}O-labelled threo-12,13-dihydroxyoleate obtained from incubation of Vernonia seeds with $H_2{}^{18}O$.

The results show that the ^{18}O from water is incorporated almost exclusively into the C-12 position of threo-12,13-dihydroxy oleic acid. The C-13 position shows essentially no incorporation.

The value of 42.4% of ^{18}O on the C-12 position of the vic-diol in experiment V1 is, in fact, higher than the isotopic enrichment of ^{18}O in the labelled water used in the experiment. This is almost certainly due to the presence of ^{18}O in the carbomethoxy group of the dihydroxy product, this ^{18}O being incorporated during the hydrolysis of the vernolic acid from triglyceride prior to the epoxide cleavage. When more drastic exchange conditions were used in experiment V2, the enrichment dropped to a lower value.

These results show that the site of hydroxyl attack during the enzymic hydration of cis-12,13-epoxyoleic acid must be at the C-12 position and the known configuration of both precursor and product show that this attack must take place from the L side of the chain. This is in direct agreement with the prediction as to the site of attack based on the stereochemical knowledge and confirms the earlier predictions of Morris and Wharry (100).

For such a highly specific reaction to take place, some rigidity must be introduced into the polymethylene chain by its interaction with the enzyme, at least in the vicinity of the epoxide ring. This rigidity could be brought about by a direct attachment or by a specific non-covalent association of the substrate to the enzyme, and it is, therefore, relevant to ask what features the substrate must possess in order to become attached or closely associated with the enzyme. As outlined earlier, the natural substrate contains several different chemical features that could be "recognised" by the enzyme system; namely a carboxy group, a double bond, an epoxide ring, polymethylene chains and a terminal methyl group; or it could be that a molecule needs one or more of these features before it will be utilized as a substrate for the hydratase reaction.

Some possible ways in which the enzyme could "recognise" the epoxy acid substrate are outlined below. In these hypotheses it is assumed that the active site is an electrophilic group which becomes attached to the oxygen atom of the oxirane ring, to form the conjugate acid, and that this is followed by attack of hydroxyl ion or water on the epoxide to yield the vic diol.

The possibilities are:-

1. Alignment of the carboxyl group at a fixed point, thus bringing the active site on the enzyme to a fixed distance along the chain from the carboxyl group.

2. Recognition of the terminal methyl group and alignment of the active centre at a fixed distance along the chain, with respect to this methyl group.

3. Alignment of the active centre with respect to both the terminal methyl group and the carboxyl group (i.e. only a specific chain length epoxy acid would be attached).

4. Alignment of the Δ^9 double bond alone with a fixed point on the enzyme bringing the active centre to a fixed position with respect to this double bond.

5. Recognition of both the double bond and terminal methyl group and alignment of the active site with respect to both.

6. Alignment of the active site with respect to both the carboxyl group and double bond.

There is also the possibility that the enzyme may have one of the substrate recognition patterns as outlined above but within this may also have a specificity for cis epoxides. As the natural substrate for the enzyme system is enantiomeric, i.e. D-cis-12,13-epoxyoleic acid, it seems likely that only one enantiomer of a racemic epoxide would be utilized as a substrate for the hydratase, in a similar fashion to the system isolated from Pseudomonas.

The validity of these hypotheses was tested by incubation of a series of epoxy acids with the Vernonia hydratase system. As mentioned earlier, Vernonia seeds contain a large proportion of cis-12,13-epoxyoleic acid and this endogenous acid would be hydrated during any incubation attempted with the seed system, and could make interpretation of results difficult. It is possible, however, to overcome this difficulty in two ways; either by using radioisotopically labelled substrate, or by freeing the enzyme system of endogenous lipid. In this work, the latter method was chosen as "cold" substrates could then be used. These are prepared more easily than the corresponding labelled substrates, and can be used in greater quantities, which was an advantage when specific optical rotations were to be measured.

In order to free the enzyme system from endogenous lipid, an acetone powder of the Vernonia seeds was prepared. This was achieved by homogenisation of the seeds in a large volume of acetone at -8°C. The resultant powder was filtered off and washed with cold acetone until no further lipid could be removed. After drying the powder under vacuum, it was checked for enzyme activity by incubation of a small amount of the powder with the natural substrate, D-12,13-epoxyoleic acid in Tris-HCl buffer at pH 8.0. The product was optically pure threo-12,13-dihydroxyoleic acid in quantitative yield, indicating that the activity of the hydratase enzyme is not destroyed during the preparation of the acetone powder. Control experiments with boiled enzyme preparations showed no activity. Following the preparation of the acetone powder, attempts were made to solubilise the enzyme system. The acetone powder was stirred gently at 4°C for two hours in Tris-HCl buffer at pH 8.0, and centrifugation of the resulting suspension at 105,000 x g gave a clear pale brown solution. Both the supernatant and the pellet showed enzyme acitivity when incubated with vernolic acid. However, the majority of the activity was in the supernatent fraction and this "soluble" enzyme preparation was used as a source of enzyme for the subsequent studies.

(a) Synthesis of Precursors for Investigation of Enzyme Specificity

In order to investigate the specificity of the Vernonia hydratase enzyme, and the spatial environment of the substrate molecule in combination with the hydratase enzyme, a series of epoxy acids and their derivatives were required as precursors for incubations.

Epoxy acids are readily prepared by reaction of the corresponding olefin with peracid, the reaction proceeding by stereospecific cis addition of oxygen, cis olefins yielding cis epoxides, and trans olefins giving the corresponding trans product. Of the epoxides used in the investigations described here, some were prepared from the corresponding monoenes by reaction with meta-chloroperbenzoic acid in benzene and many were the gift of Prof. F. D. Gunstone. Some of the monoenoic fatty acid precursors were available in at least the cis configuration, and the trans acids could be prepared from these by elaidanization with the oxides of nitrogen. Both the epoxidation and elaidanization reactions have been discussed in more detail earlier (Section 1).

The range of epoxy acids used in the investigation of the specificity of the Vernonia hydratase enzyme can be arranged into three major groups depending upon their structure, and these are shown in Table 3.

The first group, with one exception, was comprised of saturated straight chain epoxy acids with 18 carbon atoms and a mid-chain epoxy group in both the cis and trans configurations. It could be argued that, as the natural substrate for the enzyme system contains a cis Δ^9 double bond, then any artificial substrates used to investigate the specificity of the system should also contain this double bond. However, preliminary incubations with the soluble enzyme system had shown that D-cis-12,13-epoxystearic acid, prepared by reduction of vernolic acid, acted very effectively as a substrate for the enzyme and therefore the use of saturated epoxy acids to investigate the specificity of the enzyme system was thought permissible.

Group I	Fixed chain length variable epoxide position	cis-9,10-epoxy 18:0 trans-9,10-epoxy 18:0 cis-10,11-epoxy 18:0 trans-10,11-epoxy 18:0 cis-11,12-epoxy 18:0 trans-11,12-epoxy 18:0 cis-12,13-epoxy 18:0 trans-12,13-epoxy 18:0 cis-12,13-epoxy $18:1^{9}$ cis-13,14-epoxy 18:0 trans-13,14-epoxy 18:0 cis-14,15-epoxy 18:0 trans-14,15-epoxy 18:0
Group II	Variable chain length variable epoxide position	cis-4,5-epoxy 10:0 trans-4,5-epoxy 10:0 cis-6,7-epoxy 12:0 trans-6,7-epoxy 12:0 10,11-epoxy 11:0 11,12-epoxy 12:0 12,13-epoxy 13:0 cis-10,11-epoxy 19:0 cis-11,12-epoxy 20:0 trans-13,14-epoxy 22:0 cis-15,16-epoxy 21:0
Group III	Variable functional groups	methyl trans-11,12-epoxyoctadec-9-ynoate cis-12,13-epoxyoctadecan-1-ol methyl cis-12,13-epoxy $18:1^{9}$ 1-acetoxy cis-12,13-epoxyoctadecane cis-6,7-epoxyoctadecane

Table 3. Substrate Molecules

The second group of substrates consisted of a range of saturated epoxy acids with both variable chain length and variable position of the epoxide ring.

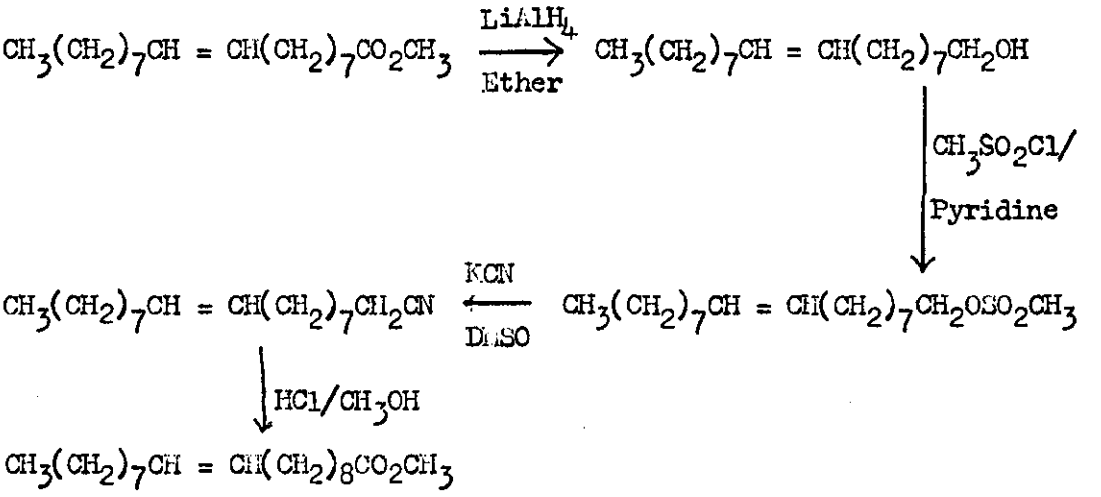
The third group of potential substrates for the enzyme system were compounds in which the carboxyl group had been modified or removed, and one compound in which the position of the epoxide ring had been changed and the degree of unsaturation increased.

The cis- and trans- isomers of 10,11-epoxy-, 13,14-epoxy-, and 14,15-epoxystearic acids were the generous gift of Prof. F. D. Gunstone, and cis-4 -decenoic and cis-6 -dodecenoic acids were obtained from the Unilever Research Laboratory, Vlaardingen, Netherlands. Other substrates were prepared by methods outlined below. All products were purified by thin layer chromatography before incubation.

Some of the epoxides used in this study were prepared by epoxidation of commercially available monoenoic esters, thus cis-9,10-epoxystearic acid and trans-9,10-epoxystearic acid were prepared by epoxidation of methyl oleate and methyl eliadate respectively, followed by subsequent hydrolysis. The cis- and trans-11,12-epoxystearic acids were prepared from cis-vaccenic acid, the cis epoxy acid by epoxidation, the trans isomer by elaidinization of the cis monoene followed by epoxidation. trans-13,14-Epoxy-22:0 was prepared by epoxidation of brassidic acid.

Several of the epoxides were prepared by chain elongation of a readily available monoene followed by epoxidation of the product. Thus cis-10,11-epoxy-19:0 and cis-11,12-epoxy-20:0 were prepared by elongation of oleic acid by one and two carbon atoms, respectively, followed by epoxidation of the elongated product. Similarly, 11,12-epoxy-12:0 and 12,13-epoxy-13:0 were prepared by chain extension of commercially available 11-undecenoic acid by one and two carbon atoms, followed by epoxidation.

The chain extensions were accomplished via the following reaction scheme, the example shown being methyl oleate.



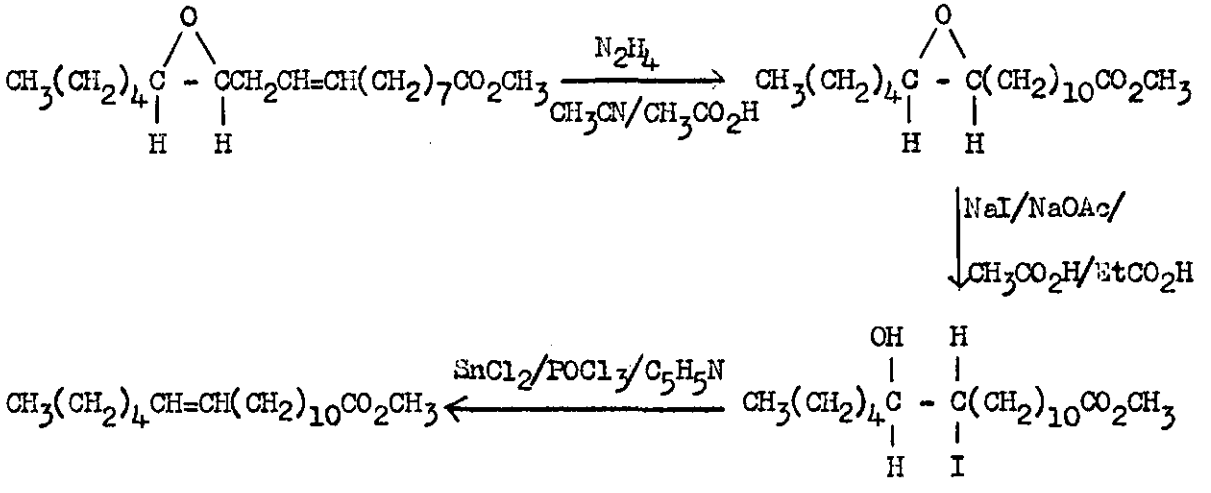
A second passage through this reaction scheme results in extension of the original molecule by two carbon atoms.

The resultant elongated monenoic ester was then epoxidized with meta-chloroperbenzoic acid and the epoxide hydrolysed to give the free acid. Attempted hydrolysis of methyl 11,12-epoxydodecanoate resulted in cleavage of the epoxide ring, even under mild conditions, and therefore 10,11-epoxy-11:0, 11,12-epoxy-12:0 and 12,13-epoxy-13:0 were all incubated with the enzyme system in the form of their methyl esters, as preliminary experiments had shown that the Vernonia hydratase system would accept these derivatives, and that a free carboxyl group was not obligatory. This finding will be discussed in more detail later.

Racemic cis-12,13-epoxystearic acid and the related compounds, namely the trans-isomer, ester, alcohol, acetate, and hydrocarbon used in this study, were all prepared from methyl cis-12 -octadecenoate. This monoene was prepared by the elegant method of Cornforth et al (176) and seems worthy of discussion here. Apart from de novo synthesis, there is one other obvious way of preparing methyl cis-12 -octadecenoate from readily available starting methods; that is, by partial reduction of

methyl linoleate with hydrazine. However, this method gives a mixture of the cis-9- and cis-12 -monoenoic isomers, and although these can be separated by argentation TLC, this method is laborious, especially for the quantities needed in this study.

The method used here for the preparation of methyl cis-12-octadecenoate was the stereospecific removal of the oxygen from methyl D-12,13-epoxystearate, and is shown in Scheme 4.



Scheme 4. Preparation of cis-12,13-octadecenoate from methyl vernolate

The D-12,13 -epoxystearate was prepared in good yield from D-12, 13-epoxyoleate, isolated from Vernonia seeds, by reduction with hydrazine. This mild procedure was found to reduce the Δ^9 double bond without cleavage of the epoxide ring. The resultant saturated epoxide was then treated with sodium iodide and sodium acetate in acetic acid and propionic acid, and the iodohydrin formed in this reaction was isolated by extraction with ether. The iodohydrin was then added to a solution of stannous chloride in pyridine, phosphoryl chloride in pyridine was then added and the reaction was left overnight. After 24 hours the product, methyl cis-12 -octadecenoate, was isolated in approximately 60% yield and purified by argentation TLC. The reaction appeared to be completely stereospecific and no trans isomer was detected.

Epoxidation of the methyl cis-12 -octadecenoate prepared in this way afforded the racemic cis-12,13-epoxy ester, and elaidinization followed by epoxidation, gave the trans epoxide. Reduction of the monoenoic ester with lithium aluminium hydride gave cis-12 -octadecenol, which was acetylated. Epoxidation of the alcohol and acetate gave the cis-12,13-epoxyoctadecanol and 1-acetoxy-12,13-epoxyoctadecane used in these investigations. The cis-6,7-epoxyoctadecane used here was obtained from cis-12 -octadecenol by reaction with methane sulphonyl-chloride to give the mesylate, and reduction with lithium aluminium hydride to yield the olefinic hydrocarbon. Epoxidation then gave the required product.

cis-15,16-Epoxy-21:0 was prepared by chain elongation of cis-12-octadecenoic acid with methyl hydrogen glutarate in a mixed Kolbe coupling reaction, followed by epoxidation of the purified chain extended product.

(b) Incubations

Before the incubations of the various synthetic substrates with the Vernonia hydratase enzyme system were performed, it was necessary to determine the quantity of the soluble enzyme preparation needed to attain maximum conversion of the highest mass of precursor likely to be used during the incubations. Two preliminary incubations were carried out in which D-cis-12,13-epoxyoleic acid was incubated with a standard amount of the enzyme preparation in amounts less than and greater than the quantities likely to be used in the investigations with synthetic substrates (see Table 4).

D-cis-12,13-epoxyoleic acid (mg)	Hydration (%)
2	100
50	100

Table 4. Effect of mass of substrate on degree of hydration in hydratase enzyme preparation

Clearly the amount of enzyme preparation used here, which was equivalent to that obtained from 0.5 gram of acetone powder, was not saturated by the level of added substrate. It was also found that the conversion of added epoxide to vic-diol was complete within three hours, and this time was used for all subsequent experiments.

Having thus determined conditions for the maximum conversion of epoxide to vic-diol, a series of incubations using the synthetic substrates was performed. The sodium salts of the epoxy acids were suspended in water by sonication, the other epoxy derivatives were also dispersed in water by sonication. Each of the substrates was incubated with the enzyme preparation for three hours and at the end of this time the incubation mixture was adjusted to pH4 with dilute acetic and extracted several times with ether. In the incubations where free acids were used as substrate, the products of the incubation were methylated with diazomethane. The extent of conversion of the epoxide to vic-diol in each of the individual incubations was determined semi-quantitatively by TLC in suitable solvent systems, and in some incubations the extent of the reaction was determined by GLC on a non-polar stationary phase by comparison of peak areas. Preliminary investigations had shown that under the conditions used in this work, epoxyesters and vic-dihydroxy esters had approximately the same response factors on GLC. The products of the incubation were then separated by TLC and the specific optical rotation of each was measured.

The results from the various incubations are shown in Table 5.

Precursor	Hydration to vic-diol(%)	$[\alpha]_{546}$ of diol formed[b]	$[\alpha]_{546}$ of remaining epoxide[b]
GROUP I			
cis-9,10-epoxy 18:0	0	-	-
trans-9,10-epoxy 18:0	0	-	-
cis-10,11-epoxy 18:0	50	+ 16.2	a
trans-10,11-epoxy 18:0	100	-	-
cis-11,12-epoxy 18:0	50	+ 21.0	+ 0.45
trans-11,12-epoxy 18:0	100	0	-
cis-12,13-epoxy 18:0	50	+ 23.7	+ 1.2
trans-12,13-epoxy 18:0	100	0	-
cis-12,13-epoxy $18:1^{9}$	50	+ 22.0	- 5.1
cis-13,14-epoxy 18:0	50	+ 15.5	a
trans-13,14-epoxy 18:0	100	a	-
cis-14,15-epoxy 18:0	50	+ 20.0	a
trans-14,15-epoxy 18:0	100	a	-
GROUP II			
cis-4,5-epoxy 10:0	50	- 5.0	a
trans-4.5-epoxy 10:0	100	a	-
cis-6,7-epoxy 12:0	50	+ 6.7	a
trans-6,7-epoxy 12:0	100	a	-
10,11-epoxy 11:0	0		
11.12-epoxy 12:0	0		
12,13-epoxy 13:0	0		
cis-10,11-epoxy 19:0	0		
cis-11,12-epoxy 20:0	0		
trans-13,14-epoxy 22:0	0		
cis-15,16-epoxy 21:0	50	+ 17.1	+ 0.80
GROUP III			
Methyl trans-11,12-epoxy-octadec-9-ynoate	100	0	-
cis-12,13-epoxyoctadecan-1-ol	50	+ 22.7	+ 0.88
methyl cis-12,13-epoxy $18:1^{9}$	50	+ 21.6	- 5.0
1-acetoxy-cis-12,13-epoxyoctadecane	50	+ 24.0	+ 0.69
cis-6,7-epoxyoctadecane[c]	50	+ 8.0	a

a the amount of material was too small for determination of optical rotations

b specific optical rotations measured on methyl esters of diol and epoxide products in Group I and II. All rotations measured in ethanol.

c can be considered as "cis-12,13-epoxyoctadecane"

Table 5. Extent of conversion of synthetic epoxy substrates to diols by Vernonia hydratase enzyme and specific rotations of the products

Considering first the compounds classified together as Group III, all of these compounds acted as substrates for the enzyme system, each of the racemic *cis* epoxides being hydrolysed in 50% yield, the *trans* epoxide being hydrolysed completely. All except methyl *trans*-11,12-epoxyoctadec-9-ynoate had the epoxide group in the 12,13-position, but none of them had a terminal carboxyl group. Therefore, it can be concluded that a terminal carboxyl group in the molecule is not necessary for an epoxide to act as a substrate for the *Vernonia* enzyme system, and the hydratase will accept esters, alcohols and hydrocarbons. This fact shows that there cannot be any covalent bonding between a terminal carboxyl group and the enzyme as has been suggested in the *Pseudomonas* system, and that some other interaction must bind the substrate molecule and enzyme together.

During the course of the incubations of methyl esters with the enzyme preparation, some hydrolysis of the ester group occurred, presumably due to lipase action; however, this was not obligatory for hydration, and the majority of the dihydroxy product was still as methyl ester.

Turning now to the epoxy acids classified together in Group I, it can be seen that the enzyme system does not show a rigid positional specificity and will hydrate any epoxy acid with a chain length of 18 carbon atoms in which the epoxide ring is in any position between the C-10 and C-14 positions. It will also hydrate both *cis* and *trans* epoxy acids but unexpectedly the *trans* isomer is hydrated to a vicinal dihydroxy acid in 100% yield, whereas only half the *cis* epoxide is converted to a dihydroxy acid.

As mentioned earlier, it is possible for vicinal dihydroxy acids to exist in two geometrically isomeric forms; *erythro* and *threo*, and therefore the dihydroxy products of the incubations could have either of these

configurations. As the product of hydration of the endogenous substrate, cis-12,13-epoxyoleic acid is threo-12,13-dihydroxyoleic acid, it was expected that the products from the synthetic cis epoxides would be threo dihydroxy acids, and it was considered likely that the products from the trans epoxides would be the erythro isomers. The threo configurations for the dihydroxy acid produced by enzymic hydrolysis of cis-12,13-epoxystearic acid was proved by the identity of its methyl ester to known threo-12,13-dihydroxystearate on Silica Gel G plates impregnated with 5% boric acid. In the case of the other synthetic epoxy acid substrates, the product of enzymic hydration of the cis epoxide always migrated faster on boric acid impregnated Silica Gel G plates than the corresponding isomer from the trans epoxide and in no case was there any indication of both isomers being formed. It can be concluded, therefore, that the product of hydration of a cis epoxide was always a threo dihydroxy acid, the product of a trans epoxide was always an erythro dihydroxy acid.

Considering the results from the hydration of cis epoxides, it can be seen that both the vicinal dihydroxy acid product and the remaining cis epoxide were optically active and that all the dihydroxy acids were dextrorotatory. Now it has been shown that (+)-threo-9,10-dihydroxystearic acid has the L-9, D-10 configuration and that (+)-threo-12,13-dihydroxystearic acid, formed by the hydrogenation of (+)-threo-12,13-dihydroxyoleic acid, has the L-12, D-13 configuration, and therefore it is considered virtually certain that all the dextrorotatory saturated dihydroxy acids formed by the Vernonia enzyme system have the same configuration of the glycol group relative to the chain of the molecule; that is, the hydroxyl group closest to the carboxyl end of the chain has the L configuration and the hydroxy group closest to the methyl end must be D. The dihydroxy acid product from the incubation of racemic cis-

12,13-epoxyoleic acid is also dextrorotatory and is identical to that already characterized (100) and therefore must have the L-12, D-13 configuration as expected.

The cis-epoxides remaining after the incubation, whose optical rotations could be measured, were also optically active. The methyl esters formed from the remaining cis-11,12-epoxystearic and cis-12,13-epoxystearic acids were both dextrorotatory and the methyl cis-12,13-epoxyoleate remaining was laevorotatory. The absolute configuration of both cis-12,13-epoxystearate and cis-12,13-epoxyoleate have been proved (128, 100). D-cis-12,13-epoxyoleate is dextrorotatory and D-cis-12,13-epoxystearate is laevorotatory. Therefore, it can be concluded that the cis-11,12- and cis-12,13-epoxy acids remaining at the end of the relevant incubations all have the L-configuration, and it is also considered most likely that all the remaining cis-epoxy acids whose specific optical rotations could not be determined are also L. The Vernonia enzyme, therefore, shows a high degree of stereospecificity. Only the D isomer of a mixture of D and L cis-epoxy acids served as a substrate for the reactions, the product being the corresponding threo-L,D-dihydroxy acid.

The trans-epoxy acids which acted as substrates for the Vernonia hydratase enzyme showed a different behaviour to the cis isomers. Whereas only one enantiomer of the cis-epoxy acid was converted to a threo-dihydroxy acid, the trans-epoxides gave erythro-dihydroxy acids in 100% yield. Although both enantiomers of the trans-epoxide acted as substrate for the enzyme, it was still possible for the resultant erythro-dihydroxy product to be optically active. This would occur if each enantiomer of the racemic mixture was attacked at different ends of the epoxide ring but from the same side of the molecule. However, no optical activity could be detected in the erythro-dihydroxy acids

produced in the incubations. It could be argued that this is due to the low specific optical rotations of erythro-dihydroxy acids, and in order to increase the solubilities and specific rotations of the dihydroxy esters, the isopropylidene derivatives were prepared. However, no optical activity could be detected in these derivatives either, and therefore it is concluded that the erythro-dihydroxy acids produced during these incubations were racemic mixtures. The mechanistic implications of this lack of optical activity will be discussed later.

Turning now to the epoxy acids classified together in Group II, and considering first the compounds with shorter chain lengths than the endogenous C:18 substrate. It can be seen that the cis and trans epoxy acids which have a terminal $(CH_2)_4CH_3$ chain are hydrated to vic diols, whereas the acids with terminal epoxide rings, or with a longer terminal methylene chain, are not hydrated. Again it was shown by comparison of the products of hydration of the cis and trans-epoxides on boric acid impregnated TLC that cis-epoxides gave rise only to threo-dihydroxy acids and that trans-epoxides gave only the erythro isomers. Also, like the epoxyoctadecanoic acids, the shorter chain length cis-epoxy acids gave an optically active dihydroxy acid in 50% yield, whereas the trans-epoxy acids were hydrated in 100% yield.

The methyl threo-6,7-dihydroxydodecanoate product from the enzymic hydration of cis-6,7-epoxydodecanoic acid was dextrorotatory and almost certainly has the L-6, D-7 configuration. The threo-dihydroxy ester produced from cis-4,5-epoxydecanoic acid, however, was laevorotatory. This is not thought to imply that this ester has the D-4, L-5 configuration, rather that in this case the argument that all dextrorotatory dihydroxy acids have the LD configuration is invalid because of the proximity of the carboxyl group , and it is considered that the product of hydration of cis-4,5-epoxydecanoic acid is L-4, D-5 dihydroxydecanoic acid.

The short chain terminal epoxy acids were not hydrated by the enzyme system even though in each case the position of the epoxide ring measured from the carboxyl group corresponded to an epoxide ring in a similar C:18 acid which was hydrated. These results imply that the Vernonia hydratase system is not strongly dependent on the length of the carboxyl and alkyl chain but that it aligns the active centre of the enzyme with respect to the terminal methyl group or alkyl chain of an epoxy acid, and that any compound lacking this terminal chain will not be hydrated by the enzyme. The question remaining is how long or short must this terminal alkyl chain be to ensure acceptance as a substrate.

The results from the long chain epoxy acids included in Group II help to answer this question. Neither cis-10,11-epoxynonadecanoic, cis-11,12-epoxyeicosanoic nor trans-13,14-epoxydocosanoic acid was hydrated by the enzyme, whereas cis-15,16-epoxyheneicosanoic acid was hydrated. Thus, the fact that the longer chain length epoxy acids were not cleaved could not be due to their chain length, but must be due to the position of the epoxide ring. If the position of the epoxide group is measured from the methyl end of the chain, it can be seen that in each of the epoxides that failed to act as a substrate for the enzyme, the epoxide group is at the n-9 position, which corresponds to the position of the epoxide ring in the 9,10-epoxyoctadecanoic acids, both of which failed to be hydrated by the enzyme.

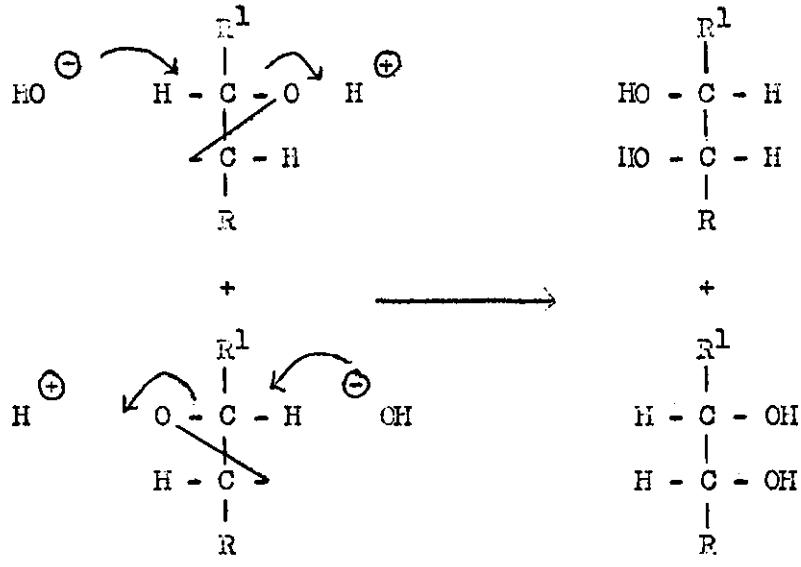
The position of the epoxide ring in the 4,5-epoxydecanoic, 6,7-epoxydodecanoic, and cis-15,16-epoxyheneicosanoic acids, all of which were hydrated by the Vernonia enzyme, is n-6, which corresponds to cis-12,13-epoxystearate in the C:18 series of epoxides, and this acid is also a substrate for the enzyme.

Thus reconsidering all the results in Table 5, in the light of methyl end control of enzymic hydration, it can be seen that any epoxy compound in which the epoxide ring is between the n-8 and n-4 position in the chain, i.e. where the terminal alkyl chain is between 3 and 7 carbon atoms long, acts as a substrate for the hydratase systems. Epoxy acids with an ethyl (n-3) or a methyl (n-2) terminus were, unfortunately, not examined so it is not yet known whether or not they would be substrates.

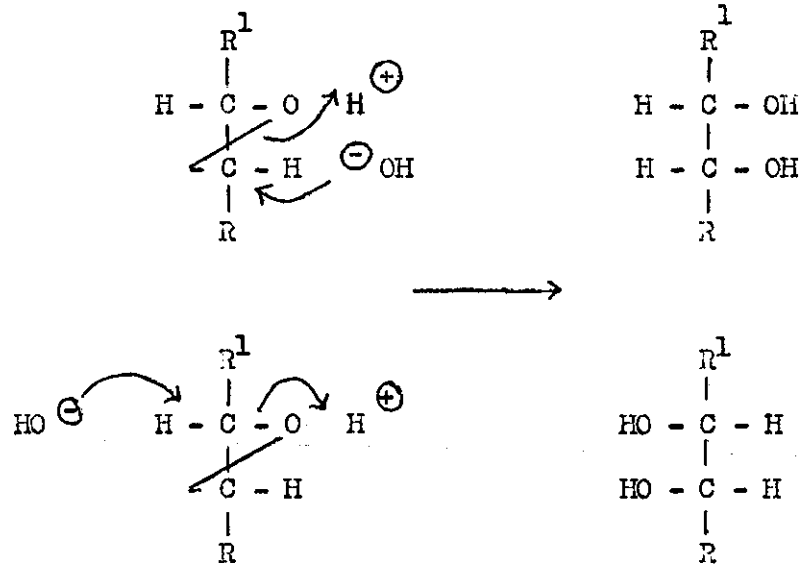
The results from the incubations of these synthetic substrates with the Vernonia hydratase enzyme show that the substrate recognition pattern is much less specific than was originally envisaged. The hydratase enzyme must align the active site at a distance from the methyl end of the molecule which can be no more than 8 carbon atoms removed from this terminal end of the chain, but must be at least 1 carbon removed and possibly 3 carbons removed. The kind of structural environment around the active site of the enzyme which could explain this specificity will be discussed later.

The Vernonia hydratase enzyme hasbeen shown to have a remarkable stereospecificity in that whereas only one enantiomer of a cis-epoxide is hydrated, both enantiomers of a trans-epoxide are cleaved by the enzyme. It has also been shown that the erythro-dihydroxy acid produced by cleavage of a trans-epoxide is a racemic mixture. There are two possible mechanisms by which a racemic erythro-dihydroxy acid could be produced from a trans-epoxide and these are shown in Scheme 5.

To determine which of these two mechanisms were operating in the case of the Vernonia hydratase, and to demonstrate the position of attack by hydroxyl ion, or water, during the hydration, studies of the enzymic hydration in water enriched with ^{18}O were undertaken.



Mechanism 1: Attack at the same end of epoxide ring for both substrate enantiomers but from different sides of molecule for each enantiomer



Mechanism 2: Attack at different positions of epoxide ring for the same substrate enantiomer

N.B. Only one enantiomer shown

Scheme 5. Possible mechanisms of cleavage of trans-epoxy acids by Vernonia hydratase enzyme

In order to perform the incubations of the Vernonia enzyme with a synthetic substrate in the smallest amount possible of the expensive $H_2{}^{18}O$, it was necessary to use the acetone powder and not the soluble enzyme preparation. A small amount of the acetone powder was placed in a vial and the synthetic substrate, in this case racemic trans-12,13-epoxystearic acid, in dry ether, was added to the powder. The ether was removed under vacuum with constant agitation of the mixture. This resulted in an intimate mixture of the acetone powder and the trans-epoxy acid. The mixture was then wetted with $H_2{}^{18}O$, the vial tightly stoppered and the mixture left for six hours at room temperature. The products were recovered by extraction with chloroform-methanol and the erythro-dihydroxy acid produced was methylated, purified and converted to the diTMS ether as described earlier. The product was analysed by mass spectroscopy, and the results are shown in Table 6.

	Enrichment of ^{18}O on positions (atom %)	
	C12	C13
erythro-12,13-diOH 18:0 (diTMS ether)	38.6	1.3

Isotopic enrichment of water ^{18}O = 40.5 atom %

Table 6. Mass spectral analysis of isotopic composition of ^{18}O labelled erythro-12,13-dihydroxystearate obtained from incubation of trans-12,13-epoxystearate with Vernonia enzyme in $H_2{}^{18}O$

The results show that the ^{18}O from water is incorporated almost exclusively into the C-12 position of the erythro-12,13-dihydroxystearic acid formed during the incubation and that there is virtually no incorporation into the C-13 position. Thus the position of attack by hydroxyl ion, or water, during the hydration of trans-12,13-epoxystearic acid by the Vernonia hydratase enzyme is exclusively at the C-12 position, in an identical fashion to the cleavage of cis-epoxides. The fact that the attack of water is exclusively at the C-12 position and that a racemic erythro-dihydroxy acid is produced allows the prediction of the stereochemistry of the epoxide cleavage raaction for trans-12,13-epoxystearic acid. If it is assumed that the attack by water or OH^- ion on the epoxide takes place from the rear of the ring, with inversion of configuration about the C-12 position, then attack on D-12, L-13-epoxystearic acid, by water or OH^- ion, must take place from the L side of the chain to produce L-12, L-13-dihydroxy stearic acid, and cleavage of L-12, D-13-epoxystearic acid must be accompanied by attack at the C-12 position, by water or OH^- ion, from the D side of the molecule to produce D-12, D-13-dihydroxystearic acid. It is expected that the attack by water or ^-OH ion in both these cleavage reactions is preceded by electrophilic attack on the epoxide ring, by some group on the active site of the enzyme, so as to give the conjugate acid of the epoxide group, making the ring more susceptable to nucleophilic attack.

It is known that the natural substrate for the Vernonia hydratase enzyme is enantiomeric and only the D-enantiomers of racemic cis-epoxy acids are hydrated, whereas both trans-epoxides are hydrated. It might be expected, therefore, that the separate enantiomers of the trans-epoxide would be hydrated at different rates, and that the trans-epoxide which most resembles the natural enzyme substrate, or which gives the best "fit" on the active site of the enzyme, would be

hydrated at a faster rate. In order to determine whether one enantiomer of the trans-epoxide is utilized more efficiently by the enzyme, an incubation of trans-12,13-epoxystearate was undertaken in which the hydration reaction was not allowed to go to completion. If the two trans-epoxy acid enantiomers are hydrolysed at different rates, then it would be expected that the remaining trans-epoxide would contain an excess of one enantiomer and would be optically active.

The incubation was performed with trans-12,13-epoxystearate and stopped before hydration of the epoxide was complete. The remaining trans-12,13-epoxystearic acid was isolated and its methyl ester was found to be laevorotatory ($[\alpha]_{546}$ = -8.6) in methanol. This value is lower than the value obtained for optically pure trans-L-12,D-13-epoxystearate ($[\alpha]_{546}$ = +19.5) but indicates that the remaining epoxide is enriched with D-12, L-13-epoxystearate, Thus it appears that of the two enantiomeric trans-12,13-epoxystearic acids, the L-12, D-13 isomer is hydrated by the Vernonia enzyme at a faster rate than the D-12, L-13 isomer. This faster rate of hydration is probably due to the D configuration at the C-13 position of the trans-epoxy acid, and the fact that the initial step in the hydration of the epoxide ring is electrophilic attack by some group on the enzyme at the C-13 position of the epoxide from the D side of the chain. Thus the isomer of trans-12,13-epoxystearic acid which most closely resembles the natural substrate, D-cis-12,13-epoxyoleic acid, at the C-13 position is the isomer which is most readily utilized by the enzyme.

It has been shown earlier that only the D enantiomers of cis-epoxy acids are hydrated by the Vernonia enzyme and that the product in each case is the threo-LD-dihydroxy acid. This suggests that attack by OH^- ion or water on the cis-epoxy acid occurs exclusively at the end of the epoxide ring nearest to the carboxyl group. This hypothesis has been proven in the case of the endogenous substrate

by incubations with $H_2{}^{18}O$, but in the case of the synthetic substrate relies on stereochemical arguments. In order to prove directly the position of attack by water or OH^- on another epoxy acid which is not the natural substrate, a further incubation in isotopic water was undertaken with cis-11,12-epoxystearic acid as substrate. The incubation was performed in an analogous manner to that used for the trans-12,13-epoxystearic acid. A small amount of the Vernonia acetone powder and cis-11,12-epoxystearic acid were mixed together in cold ether, and the solvent was removed. The mixture was moistened with $H_2{}^{18}O$ and left for several hours at room temperature. The threo-dihydroxy product was isolated, purified and converted to the diTMS ether as described earlier. The resultant diTMS ether was analysed by mass spectroscopy and the results are shown in Table 7.

	Enrichment of ^{18}O on position (atom %)	
	C11	C12
threo-11,12-diOH 18:0 (diTMS ether)	34.8	5.8

Isotopic enrichment of water = 40.5 atom %

Table 7. Mass spectral analysis of isotopic composition of ^{18}O labelled threo-11,12-dihydroxystearate obtained from incubation of cis-11,12-epoxystearate with Vernonia enzyme in $H_2{}^{18}O$.

These results show that the ^{18}O is incorporated to the greatest extent at the C-11 position of the threo-11,12-dihydroxystearate formed during the incubation. It appears that the hydration of cis-11,12-epoxystearic acid is slightly less specific than the hydration of the corresponding cis-12,13-epoxystearic acid, although the results still

indicate that the hydration reaction shows a very high degree of stereospecificity. The results confirm the predictions made on the basis of the proposed configurations of the epoxy and dihydroxy acids and help to substantiate these proposals.

From the results of the incubations of cis- and trans-epoxystearic acids, with the Vernonia hydratase enzyme in isotopic water, it is considered probable that in every case in Table 5 where the epoxide ring is hydrated that attack of water, or OH^- ion, occurs at the end of the epoxide ring closest to the carboxyl group, and from the rear side of the epoxide ring.

The investigations outlined above have made it possible to define the stereospecificity of the Vernonia hydratase enzyme and to propose mechanisms for the cleavage of the endogenous substrate, vernolic acid, and some synthetic cis- and trans-epoxy acids. It has been proposed earlier that for such a highly stereospecific hydration of the natural substrate to occur a close enfoldment of the substrate by the enzyme must be postulated. This could possibly involve a cleft in the enzyme into which the substrate must fit and the results from the structural investigations carried out in this work have gone some way to determining the required geometry of such a cleft.

The structure in the vicinity of active site of the Vernonia hydratase enzyme proposed to explain the results outlined above is a "well" in the enzyme surface into which the methyl end of the chain is inserted, leaving the epoxide ring just above the mouth of the "well". The active site of the enzyme is suggested as being situated at the mouth of the "well".

It has been shown that the distance of the epoxide ring from the terminal methyl group determines whether the epoxide is hydrated by the enzyme. The maximum permissible distance of the epoxide ring from

the end of the chain to retain activity as a substrate was 8 carbon atoms; in the 10,11-epoxystearic acids. This suggests that the maximum depth of the "well" is approximately 10 Å. This would allow the terminal chain of the 10,11-epoxystearic acids to just fit into the "well", leaving the epoxide ring close enough to the mouth of the "well" to interact with the electrophilic active site of the enzyme and initiate the hydration of the epoxide ring. It is suggested that the 9,10-epoxystearic acids are not hydrated because the methyl end of the chain is too long to fit into the "well" and bring the epoxide ring close enough to the mouth of the well to interact with the active site.

The active site must be at the mouth of the "well" and the substrate-enzyme interaction must result in its being closer to the terminal end of the epoxide ring than to the carboxyl end of the ring. The proximity of the terminal end of the epoxide ring to the enzyme surface would then hinder attack by water at this position and this could explain why attack by water or hydroxyl ion always takes place at the end of the epoxide ring nearest to the carboxyl group.

The width of a normal polymethylene chain of a fatty acid is approximately 4 Å (177) and it is considered that the mouth of the "well" in the enzyme surface cannot be much larger than this. The mouth of the "well" must be small enough to exclude the carboxyl group of an epoxy acid substrate, or it would be expected that both enantiomers of a cis-epoxy acid which has the epoxide ring the same distance from the methyl end and carboxyl end of the molecule, and at a suitable position for hydration to occur, would be hydrated. This is not the case as cis-6,7-epoxydodecanoic and cis-4,5-epoxydecanoic acids both failed to be hydrated in greater than 50% yield.

That there must be some insertion of the terminal methylene chain of the epoxy acid into the "well" in the enzyme surface before hydration

can take place, and the fact that there is not a great enough interaction between the carboxyl end of the chain and the enzyme to "bind" a substrate to the enzyme surface and allow hydration to occur is shown by the fact that terminal epoxy acids are not hydrated. The minimum extent of the interaction between the terminal methylene chain and the enzyme is not known exactly, the results indicate that at least one carbon atom must be inserted into the enzymic well, but it could be that up to 3 carbon atoms are needed. To resolve this point, 15,16-epoxystearic and 16,17-epoxystearic acids need to be incubated with the Vernonia hydratase.

Although the natural substrate for the hydratase enzyme contains a Δ^9 double bond, most of the synthetic substrates for the enzyme were saturated molecules. The conformation of the methylene chains in these molecules in the extended form is different, the saturated chain being straight, whereas the unsaturated chain is bent due to the cis-double bond. Therefore, it would be expected, if there were any interaction between the carboxyl portion of the methylene chain in the epoxy acids and the enzyme, that the saturated acids would not be as good substrates for the enzyme as the unsaturated compound. However, this does not seem to be the case, the saturated epoxides were equally good substrates for the enzyme as the naturally occurring epoxide. This fact substantiates the proposal that there is no interaction between the carboxyl end of the epoxy acid substrate and the enzyme.

It could be argued that the saturated chains could be "bent" into a similar configuration to an unsaturated chain and therefore fit an enzyme surface in a similar fashion to the unsaturated molecule. However, methyl trans-11,12-epoxyoctadec-9-ynoate also acts as a substrate for the hydratase enzyme, and it is difficult to see how the portion of the chain in this molecule which contains an inflexible triple bond could be "bent" to resemble the unsaturated methylene chain of the natural substrate.

Much more difficult to explain is the fact that both enantiomers of trans-epoxy acids are hydrated by the enzyme, whereas only the D-enantiomer of cis-epoxy acids acts as a substrate for the hydratase. That the L-enantiomer of a cis-epoxy acid is not hydrated must be due to the lack of interaction between this enantiomer and the hydratase enzyme. It has been proposed that the intial step in the hydration of epoxy acids is the insertion of the terminal methylene chain of the epoxide molecule into a well in the enzyme surface. As the terminal chain of an L-cis-epoxy acid is no different from that of its D enantiomer or of the trans epoxy acids, it cannot be this end of the molecule which inhibits the hydration. It is thought much more likely that there is some specific steric interaction between the polymethylene chain towards the carboxyl end of an L-cis-epoxy acid and the enzyme surface which stops the L-cis-epoxy acids from entering into close contact with the hydratase enzyme, thus inhibiting hydration.

The work outlined in this section has gone some way to defining the stereospecificity and mechanism of action of several epoxy acid hydrating enzymes. It has been proposed that the hydratase enzymes occurring in Pseudomonas, Puccinia spores and Claviceps sulcata sclerotia have a similar mechanism, and the stereospecificity of the Puccinia enzyme has been investigated. The hydratase enzyme from Vernonia seeds has been solubilized, and its substrate specificity and stereospecificity have been investigated. A structure for the active site region of the Vernonia enzyme has been proposed to explain all the information derived from these studies.

EXPERIMENTAL

A. Preparation of Precursors for the investigation of the specificity of the Vernonia hydratase enzyme

Most of the epoxides used in the investigations were prepared by epoxidation of the corresponding monoenes with m-chloroperbenzoic acid. The following substrates were prepared by this method:-

cis-9,10-epoxystearic acid
trans-9,10-epoxystearic acid

cis-11,12-epoxystearic acid
trans-11,12-epoxystearic acid

cis-12,13-epoxystearic acid
trans-12,13-epoxystearic acid

cis-4,5-epoxydecanoic acid
trans-4,5-epoxydecanoic acid

cis-6,7-epoxydodecanoic acid
trans-6,7-epoxydodecanoic acid

methyl 10,11-epoxyundecanoate

methyl 11,12-epoxydodecanoate

methyl 12,13-epoxytridecanoate

cis-10,11-epoxynonadecanoic acid

cis-11,12-epoxyeicosanoic acid

trans-13,14-epoxydocosanoic acid

cis-15,16-epoxyheneicosanoic acid

methyl trans-11,12-epoxyoctadec-9-ynoate

cis-12,13-epoxyoctadecan-1-ol

methyl cis-12,13-epoxyoleate

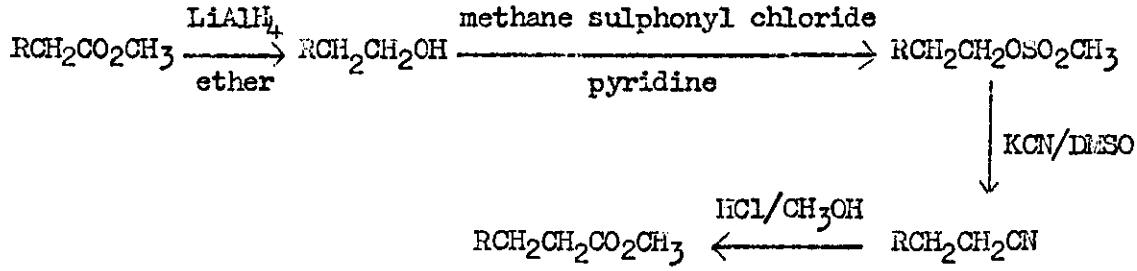
1-acetoxy-cis-12,13-epoxyoctadecane

cis-6,7-epoxyoctadecane

Preparation of monoenes for Epoxidation

1. Chain extension with potassium cyanide

The method outlined below was used for the preparation of methyl cis-10-nonadecencate (R = $CH_3(CH_2)_7CH{=}CH(CH_2)_6$-), methyl cis-11-eicosenoate (R = $CH_3(CH_2)_7CH{=}CH(CH_2)_7$-), methyl 11-dodecenoate (R = $CH_2{=}CH(CH_2)_7$-) and methyl 12-tridecenoate (R = $CH_2{=}CH(CH_2)_8$-).



Methyl cis-10-nonadecenoate

i) Reduction of ester to alcohol

Methyl oleate (500mg) dissolved in dry ether (50ml) was heated under reflux with lithium aluminium hydride (250mg). After two hours, ether (100ml) was added to the cooled reaction mixture and the excess lithium aluminium hydride was destroyed by cautious dropwise addition of water. The precipitate was dissolved by adding dilute hydrochloric acid. The ether extract was washed acid free with water and dried over anhydrous sodium sulphate. Solvent was removed to leave a colourless oil (470mg). Analysis of the oil by TLC indicated complete conversion of the ester to oleyl alcohol.

ii) Conversion of alcohol to methane sulphonate

The oil (from i) was dissolved in dry pyridine (20ml). The mixture was cooled in ice, and methane sulphonyl chloride (500 mg) was added slowly. The mixture was allowed to stand overnight at room temperature, diluted with water (80ml) and extracted with ether (2 x 50ml). The ether extracts were combined and washed successively with water, dilute hydro-

chloric acid, water, 5% aqueous potassium hydroxide and water until neutral. The product was obtained by evaporation of the ether, and dried by azeotropic distillation with acetone.

A TLC examination on silica plates against suitable standards showed almost complete conversion of the alcohol to methanesulphonate.

iii) Conversion of methanesulphonate to nitrile

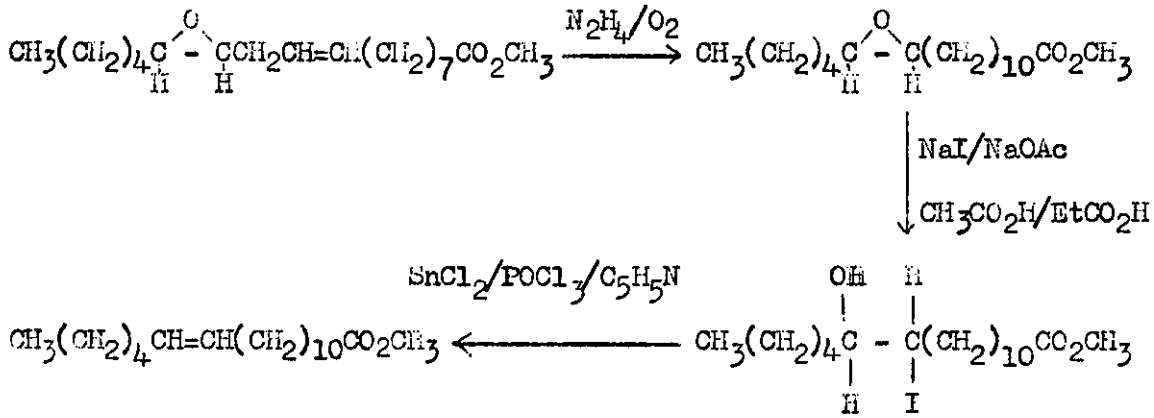
Potassium cyanide (90mg) was added to the methanesulphonate in dry dimethyl sulphoxide (5ml). The mixture was stirred at 95° for four hours. The mixture was diluted with water (10ml) and extracted with ether (2 x 10ml). The ether extracts were combined and washed with water (4 x 10ml) to remove DMSO and potassium salts, and the solvent was removed at the pump. The product was dried by azeotropic distillation with ethanol.

iv) Methanolysis of nitrile

The crude nitrile was converted directly to the methyl ester by addition of 25% w/v hydrogen chloride in methanol (10ml). The reaction was allowed to stand at room temperature overnight, then ether (20ml) and water (20ml) were added. The aqueous layer was re-extracted with ether (20ml) and the combined ether fractions were washed acid free with water. The ether was removed and the product was purified by TLC on silver nitrate impregnated Silica Gel G (ether:petroleum ether, 1:9) to yield a colourless oil (270mg). The purity of the product was checked by GLC and had an equivalent chain length of 19.56 on DEGA.

2. Stereospecific removal of epoxide ring

Methyl cis-12-octadecenoate



i) Reduction of methyl D-cis-12,13-epoxyoleate

Methyl D-cis-12,13-epoxyoleate (200mg) was dissolved in acetonitrile (50ml) and acetic acid (0.75ml), and the mixture was warmed to 60°C. Oxygen was bubbled through the mixture, and after ten minutes hydrazine hydrate (5ml) was added. After a further two hours, the mixture was cooled, diluted with water (50ml) and extracted with ether (2 x 100ml). The ether extracts were washed with water, and dried over anhydrous sodium sulphate. The solvent was removed to leave a pale pink solid. The pure product, methyl D-cis-12,13-epoxystearate was isolated by TLC on silver nitrate impregnated Silica Gel G (ether:petroleum ether, 3:7). The product was a colourless solid (170mg $[\alpha]_{546}$ = -1.44 (c = 9.0)).

ii) Stereospecific reduction of epoxide to monoene

Methyl D-cis-12,13-epoxystearate (170mg) was added to a cooled (-10°C) solution of sodium iodide (400mg) and sodium acetate (40mg) in acetic acid (1ml) and propionic acid (2ml). After 30 minutes, the mixture was warmed to room temperature and ether (10ml) and dilute aqueous sodium bicarbonate (5ml) were added. The ether layer was washed with aqueous sodium hydrogen sulphite and with water, dried over anhydrous sodium sulphate and evaporated at low pressure. The iodohydrin was added to a cooled (0°C) solution of anhydrous stannous chloride (600mg) in pyridine (3ml). Phosphoryl chloride (0.2ml) in pyridine (1ml) was then added with cooling. After 16 hours water (10ml) was added and the mixture was extracted with ether (2 x 10ml). The ether extracts were washed with water, dried over anhydrous sodium sulphate, and the ether was removed to leave a pale brown oil. The product was purified by TLC on silver nitrate impregnated Silica Gel G (ether: petroleum ether, 1:9) to yield a colourless oil (120mg). The purity of the product was checked by GLC and had an equivalent chain length of 18.60 on DEGA. The position of the double bond was determined by von Rudloff oxidation with permangenate/periodate.(180). The product was proved to be pure methyl cis-12-octadecenoate.

3. Chain extension by Kolbé electrolytic coupling

Methyl cis-15-heneicosenoate

$$CH_3(CH_2)_4CH{=}CH(CH_2)_{10}CO_2H \xrightarrow{\text{Kolbe electrolysis}} CH_3(CH_2)_4CH{=}CH(CH_2)_{13}CO_2CH_3$$
$$+ HO_2C(CH_2)_3CO_2CH_3 \qquad + \text{by products}$$

Methyl cis-12-octadecenoate (100mg), prepared as described above, was dissolved in 5% methanolic potassium hydroxide (5ml) and the mixture heated under reflux for one hour. The mixture was diluted with water (5ml), acidified with dilute hydrochloric acid and extracted with ether (2 x 10ml). The ether extracts were washed acid free with water, and the solvent was removed to leave an oil, which was dried by azetropic distillation with ethanol.

The cis-12-octadecenoic acid (95mg) and methyl hydrogen glutarate (1g) were dissolved in dry methanol. A solution of sodium(95mg) in dry methanol (1ml) was added. After thorough mixing the solution was placed in a glass cell and two platinum electrodes were inserted. (The cell was water jacketed and fitted with cooling coils to keep the reaction temperature below 50°C.)

120 Volts D.C. was applied across the electrodes, and an initial current of 0.2 amps developed. The polarity of the electrodes was occasionally reversed to prevent the build up of insoluble material around the anode. When the pH of the solution had risen to pH9 the reaction was stopped and the contents of the cell poured into water (20ml). The dilute alkaline solution was extracted with ether (2 x 20ml) and the ether extracts were washed with water until neutral. The ethereal solution was dried over anhydrous sodium sulphate and evaporation yielded the mixed Kolbe products.

Methyl cis-15-hencicosenoate was separated from the by products by TLC on Silica Gel G (ether:petroleum ether 1:9). The product was a colourless oil (22mg). The purity of the product was determined by GLC, and found to be greater than 90%. The methyl cis-15-heneicosenoate had an equivalent chain length on DEGA of 21.62.

4. Isomerization of cis monoenes to trans monoenes

Most of the monoenes used in these studies were available or were prepared as the cis isomers, the trans isomers being prepared from these by stereomutation with the oxides of nitrogen, as described below.

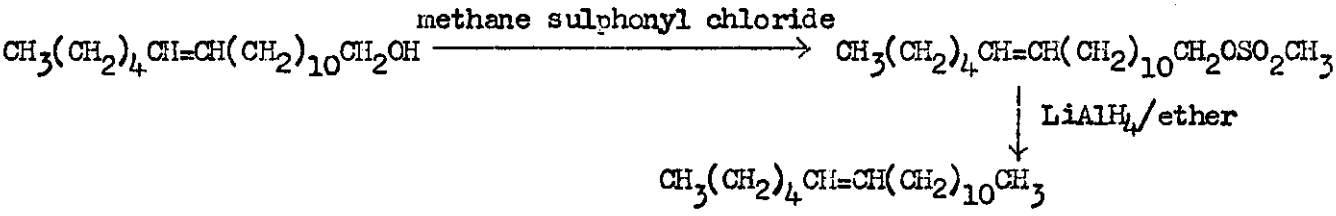
Methyl trans-11-octadecenoate

Methyl cis-11-octadecenoate (methyl cis-vaccenate) (100mg) was dissolved in dimethyl cellosolve (2.5ml). 6M nitric acid (75 µl) was added and the mixture was warmed to 65°C. Aqueous sodium nitrite (2M, 100 µl) was added and heating continued for a further hour. The mixture was diluted with water (10ml) and extracted with ether (2 x 10ml). The ether extracts were washed with water until neutral and the solvent was removed to leave a colourless oil which was dried by azeotropic distillation with ethanol. The methyl trans-11-octadecenoate product was separated from the remaining cis isomer by TLC on silver nitrate impregnated Silica Gel G (ether:petroleum ether 1:9). The product was obtained in approximately 70% yield.

5. Preparation of cis-12-octadecenol

Methyl cis-12-octadecenoate (100mg, prepared as described above (2)) dissolved in dry ether (20ml) was heated under reflux with lithium aluminium hydride (50mg). After two hours, ether (50ml) was added to the cooled reaction mixture and the excess of lithium aluminium hydride was destroyed by cautious dropwise addition of water. Dilute hydrochloric acid was added to dissolve the white gelatinous precipitate. The ether extract was washed with water until neutral and dried over anhydrous sodium sulphate. The solvent was removed to leave a colourless oil (87mg). Examination of the product by TLC showed complete conversion of the ester to alcohol.

6. Preparation of cis-6-octadecene



i) Conversion of alcohol to methane sulphonate

cis-12-Octadecenol (20mg) was dissolved in dry pyridine (2ml), the solution was cooled in ice, methane sulphonyl chloride (30mg) was added and the mixture was allowed to stand overnight at room temperature. The mixture was diluted with water (5ml) and extracted with ether (2 x 5ml). The ether extracts were washed successively with water, dilute hydrochloric acid, water, dilute aqueous potassium hydroxide, and finally water until neutral. The ether was removed to leave a pale brown oil (23mg) which was dried by azeotropic distillation with acetone.

Analysis of the oil by TLC on Silica Gel G (chloroform:petroleum ether 3:7) indicated complete conversion of the alcohol to methane sulphonate.

ii) Conversion of methane sulphonate to hydrocarbon

The oil from above was dissolved in dry ether (10ml); lithium aluminium hydride (10mg) was added and the mixture was heated under reflux. After two hours ether (10ml) was added and the excess hydride was destroyed by dropwise addition of water. Dilute hydrochloric acid was added until the mixture was just acid. The ether extracts were washed with water until neutral and dried over anhydrous sodium sulphate. The ether was removed to leave an oil which was purified by TLC on Silica Gel G plates developed in petroleum ether. The product was a colourless oil (15 mg).

7. Preparation of 1-acetoxy-cis-12-octadecene

cis-12-Octadecanol (30mg) was dissolved in dry pyridine (3ml), the solution was cooled in ice, and acetic anhydride (100 mg) was added. The

mixture was allowed to stand at room temperature overnight. Water (10ml) was added and the mixture extracted with ether (2 x 10ml). The ether extracts were washed successively with water, dilute hydrochloric acid, water, dilute aqueous potassium hydroxide and finally water, until neutral. The solvent was removed to leave a pale brown oil. Analysis of the oil by TLC alongside suitable standards showed almost complete conversion of the alcohol to acetate. The product was finally purified by TLC on Silica Gel G plates developed in ether:petroleum ether (2:8) to give a colourless oil (27mg).

Epoxidation of Monoenes

The monoenes prepared as described above and those commercially available were each epoxidized with m-chloroperbenzoic acid in a similar manner to that described below for methyl oleate.

Methyl cis-9,10-epoxystearate

Methyl oleate (100mg) and meta-chloroperbenzoic acid (100mg) were dissolved in benzene (5ml). The mixture was allowed to stand at room temperature. After 15 hours ether (10ml) and water (10ml) were added. The ether extracts were then washed successively with 5% aqueous sodium sulphite (2 x 10ml), water, 5% aqueous sodium carbonate (2 x 10ml) and finally water until neutral. The ether was removed to leave a low melting solid. The product was purified by TLC on Silica Gel G (ether:petroleum ether, 2:8) to give a colourless solid (91mg).

Hydrolysis of epoxy esters

Where possible, the synthetic substrates for the biochemical investigations were incubated with the Vernonia enzyme preparation in the form of the free acids. These were prepared from the esters in a similar manner to that outlined below.

cis-9,10-Epoxystearic acid

Methyl cis-9,10-epoxystearic acid (90mg) was dissolved in 5% methanolic potassium hydroxide (3ml) and the mixture was allowed to stand at room temperature overnight. Ether (10ml) was added and the mixture was diluted with water (10ml) and acidified to pH5 with dilute acetic acid. The ether extracts were washed with water until neutral. The ether was removed and the acid dried by azeotropic distillation with acetone. The product was a colourless solid (82mg; 88%).

B. Incubation of Synthetic Precursors with Vernonia hydratase enzyme

1. Preparation of an acetone powder of Vernonia anthelmintica seeds

Vernonia anthelmintica seeds (25g) were added to cold (-8°C) acetone (200ml). The seeds were homogenised with an "Ultra Turex" homogeniser; the temperature of the mixture was not allowed to rise above -2°C during this process. The mixture was filtered and the residue was washed with successive small quantities of cold acetone (1 litre total). The residue was dried in a vacuum desicator for 48 hours at -8°C. The product was a pale brown powder (13g).

2. Preparation of Vernonia enzyme extract

The acetone powder (5g) prepared as described above, was added to Tris-HCl buffer (0.2M pH 8.0, 25 ml) and the mixture was stirred overnight at 4°C. The resultant suspension was centrifuged at 10,000 x g for twenty minutes, the supernatent fraction was removed and recentrifuged for forty-five minutes at 106,000 x g. The resultant pale brown supernatent fluid was used as a source of the enzyme for the work described here.

3. Incubation of synthetic epoxide substrates with Vernonia enzyme extract

cis-11,12-Epoxystearic acid (30mg) was neutralized with IN aqueous potassium hydroxide; tris-HCl buffer (5ml) was added and the epoxy acid was dispersed with the aid of an MSE Ultrasonic disperser. The resultant mixture was incubated with the Vernonia enzyme preparation (5ml) for three hours at 30°C. Methanol (5ml) was added and the solution was acidified to pH4 with dilute acetic acid. The mixture was extracted three times with ether (20ml). The combined extracts were washed with water until neutral and dried over anhydrous sodium sulphate. The residue obtained on evaporation of the solvent was dissolved in ether (3ml) and methanol (0.5ml) and esterified with ethereal diazomethane. An aliquot of the esterified product was taken for analysis by TLC and GLC and the remainder was applied to a 20 x 20 x 0.50mm Silica Gel G TLC plate, which was developed in ether-petroleum ether (4:6). The band corresponding to methyl cis-11,12-epoxystearate was scraped from the plate and eluted with ether. The plate was then redeveloped to half height in ether:petroleum ether (8:2) and the band which corresponded to methyl 11,12-dihydroxystearate was scraped from the plate and eluted with ether:chloroform (1:1). Both the epoxy and dihydroxy products were further purified by TLC to yield methyl-cis-11,12-epoxystearate (11mg) and methyl threo-11,12-dihydroxystearate (10mg). The specific optical rotations of the products were measured in in a 2.0cm cell with an ETL/NPL Automatic Polarimeter (Type 143A) as solutions in ethanol (concentration 0.2-5.0%).

All other incubations were performed in an analogous manner, although substrates which were not free fatty acids were not dissolved in aqueous potassium hydroxide, but only dispersed in Tris-HCl buffer (pH 8.0) with the aid of an MSE Ultrasonic disperser. Separations of substrates and products were performed by TLC in suitable solvent systems.

Incubation of cis-11,12-epoxystearic acid with Vernonia acetone powder in $H_2{}^{18}O$

The acetone powder of Vernonia anthelmintica seeds (10mg), prepared as described earlier, was placed in a small screw cap vial. To this was added cis-11,12-epoxystearic acid (4mg) dissolved in dry ether (0.3ml). The ether was removed under vacuum with shaking. $H_2{}^{18}O$ (20 µl) was added to the dry powder, the vial was tightly capped and the mixture was allowed to stand at room temperature. After six hours the mixture was added to chloroform:methanol, 2:1 (10ml) and left overnight. The mixture was filtered and the filtrate was washed with 0.7% saline (3ml). The organic layer was separated and the solvent was removed to leave a pale brown solid. The solid was dissolved in ether (1ml) and methanol (0.5ml) and esterified with ethereal diazomethane. The solvent was removed and the product was applied to a 20 x 20 x 0.25mm Silica Gel G TLC plate which was developed in ether:petroleum ether, 8:2. The band corresponding to dihydroxyester was removed and eluted with ether:chloroform, 1:1. The solvent was removed to leave methyl threo-11,12-dihydroxystearate.

The dihydroxyester was dissolved in 5% methanolic potassium hydroxide (2ml) and the mixture heated under reflux for 16 hours. The mixture was diluted with water (5ml), acidified with dilute hydrochloric acid and extracted with ether (2 x 5ml). The extracts were washed with water until neutral, and the solvent was removed to leave a white solid which was dissolved in ether and methanol and esterified with ethereal diazomethane.

The product was dissolved in methanol (1ml) and hydrochloric acid (0.1ml 5N) was added. The mixture was heated under reflux for 16 hours. The mixture was diluted with water (5ml) and extracted with ether (2 x 5ml). The ether extracts were washed with water until neutral. The solvent was removed and the product dissolved in ether (1ml) and

methanol (0.5ml) and treated with ethereal diazomethane. The solvent was removed and the product was purified by TLC (ether:petroleum ether 7:3) to yield a colourless solid.

Preparation of Trimethylsilyl ether of methyl threo-11,12-dihydroxystearate

The dihydroxy ester (1mg) was dissolved in anhydrous pyridine (1ml) and hexamethyldisilazane (0.2ml) was added. To this solution was added trimethylchlorosilane (0.1ml); the mixture was shaken and left at room temperature for three hours. The mixture was then diluted with ether and washed successively with dilute hydrochloric acid, aqueous sodium bicarbonate and water. Solvent was removed to leave a pale yellow oil which was purified by TLC (ether-petroleum ether, 1:9) to yield methyl 11,12-ditrimethylsilyloxystearate which was submitted for mass spectroscopic analysis.

C. Incubation of Vernonia anthelmintica seeds with $H_2{}^{18}O$

Ten Vernonia anthelmintica seeds (35mg) were ground in a pestle and mortar. The crushed seeds were placed in a small screw cap vial ($\frac{1}{2}$ dram). $H_2{}^{18}O$ (20 µl) was added to the seeds, the vial was tightly capped, and the mixture left in the light at 30°C. After three days the mixture was added to chloroform methanol 2:1 (10ml) and left overnight. The mixture was filtered and the filtrate was washed with 0.7% saline. The organic layer was separated and the solvent was removed to leave a brown oil, which was dissolved in ether and esterified with ethereal diazomethane. Methyl threo-12,13-dihydroxyoleate (2mg) was isolated by TLC (ether:petroleum ether, 7:3).

The methyl threo-12,13-dihydroxyoleate was dissolved in 5% methanolic otassium hydroxide (5ml) and the mixture heated under reflux for 16 hours. The mixture was diluted with water (5ml), acidified with dilute hydrochloric acid and extracted with ether (2 x 5ml). The extracts were

washed with water until neutral, and the solvent was removed. The product was dissolved in ether and methanol and esterified with ethereal diazomethane.

The methyl threo-12,13-dihydroxyoleate was dissolved in methanol (1ml) and hydrochloric acid (0.1ml, 5N) was added. The mixture was heated under reflux for 16 hours. The mixture was diluted with water (5ml) and extracted with ether (2 x 5ml). The ether extracts were washed with water until neutral, and dried over anhydrous sodium sulphate. The ether solution was treated with diazomethane and the solvent was removed. The methyl threo-12,13-dihydroxyoleate was purified by TLC (ether:petroleum ether, 7:3) and converted into the diTMS ether in a similar manner to that described above. The product was submitted for mass spectroscopic analysis.

D. Incubation of Puccinia spores with $H_2{}^{18}O$

Three species of Puccinia spores were each incubated with $H_2{}^{18}O$, as described below. These were P. recondita (PB.61/37), P. graminis (PB 58/44) and P. striiformis (r 60). They were the gift of Dr. R. Johnson of the Plant Breeding Institute, Cambridge.

Incubation of Puccinia graminis spores with $H_2{}^{18}O$

Puccinia graminis (approx 20mg) spores were crushed in $H_2{}^{18}O$ (20 µl) in a small test tube fitted with a tightly fitting glass rod, sealed at the joint between the tube and rod with rubber tubing. The mixture was left at room temperature for 24 hours. After this time, the mixture was poured into chloroform methanol 2:1 (10ml), glass balls were added, and the mixture shaken on a wrist action shaker for five hours. This disrupted the spores and allowed the lipids to be more easily extracted. The mixture was filtered and the residue washed with chloroform (10ml). The filtrate and washings were combined and shaken with 0.7% saline (7ml). After removal of the organic phase, the

aqueous phase was re-extracted with chloroform (5ml) and the combined solvent extracts were evaporated. The product was dissolved in ether (2ml) and methanol (0.5ml) and esterified with ethereal diazomethane. Methyl threo-9,10-dihydroxystearate was isolated by TLC (ether:petroleum ether) to leave a colourless solid. The dihydroxyester was treated with methanolic sodium hydroxide and aqueous hydrochloric acid in methanol in an identical manner to that described above. The methyl threo-9,10-dihydroxystearate was then converted to the diTMS ether as described above, and submitted for mass spectroscopic analysis.

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APPENDIX

Optical Resolution of Some Hydroxy Fatty Acids by Thin Layer Chromatography

Considerable interest exists in the optical resolution of organic compounds by chromatographic techniques. Theoretically, two approaches are possible, either separation of enantiomers on optically active stationary phases, or conversion of the enantiomers into diastereoisomers and separation of these diastereoisomers by chromatography on optically inactive adsorbents.

A number of successful separations have already been achieved using the latter procedure. The diastereoisomeric acetylated α-hydroxy esters of secondary alcohols (1, 2) and the O-(-)-menthyl-formate esters of long chain ω-hydroxy methylesters (3) have been resolved by gas liquid chromatography and ω2-hydroxy acids and 2-alkanols have been separated as N-(1-phenylethyl) urethanes using this technique (4). Thin layer chromatography has also been used to separate the diastereoisomeric N-(1-phenylethyl) urethanes of secondary aliphatic alcohols (5).

The work presented here describes the resolution of some long chain hydroxy methyl esters by thin layer chromatography as their L-O-(O-acetoxy)mandelate esters.

The L-O-(O-acetoxy)mandelate esters of methyl 2-hydroxy- and 3-hydroxypalmitate were readily separable by TLC on Silica Gel G plates. However, methyl 4-hydroxypalmitate, methyl 9-hydroxystearate and methyl 12-hydroxystearate could not be resolved by this method. The partial separation of the diastereoisomeric derivatives of methyl 12-hydroxyoctadec-9-enoate was also achieved by TLC on Silica Gel G.

An improved separation of the derivatives of methyl 12-hydroxyoctadec-9-enoate could be achieved on Silica Gel G impregnated with 10% silver nitrate.

The migratory behaviour of the diastereoisomeric L-O-(O-acetoxy) mandelate esters of methyl 2-hydroxy and 3-hydroxypalmitate on Silica Gel G, and the migratory behaviour of the diastereoisomeric derivatives of methyl 12-hydroxyoctadec-9-enoate on Silica Gel G impregnated with 10% silver nitrate are shown in Figure 1. The thin layer chromatograms were developed twice in ether:benzene (2:98).

The marked difference in the mobilities of the individual diasterioisomers is evident. The more polar isomer in each case corresponds to the L-O-(O-acetoxy)mandelate ester prepared from the D enantiomers of the hydroxy esters.

The individual diastereoisomers from each of the hydroxy esters were separated by preparative TLC. Reaction of these individual diastereoisomers with methanol:sulphuric acid (98:2) gave a mixture of the long chain hydroxy ester and methyl mandelate, from which the hydroxy ester was separated by TLC. The specific optical rotations of these "resolved" hydroxy esters are shown in Table 1, along with the specific optical rotations of the individual diastereoisomers, and the specific rotation of the optically pure D-hydroxy fatty esters for comparison.

These results confirm that in each case the more polar diastereoisomer is the derivative of the D-hydroxy fatty ester. However, the specific optical rotations of the individual enantiomers of the resolved esters are lower than the specific optical rotations of pure enantiomers of the hydroxy esters. This must be due to racemization of the hydroxy ester during the cleavage of the diastereoisomer with methanol:sulphuric acid. That the racemization could not have occurred before this stage

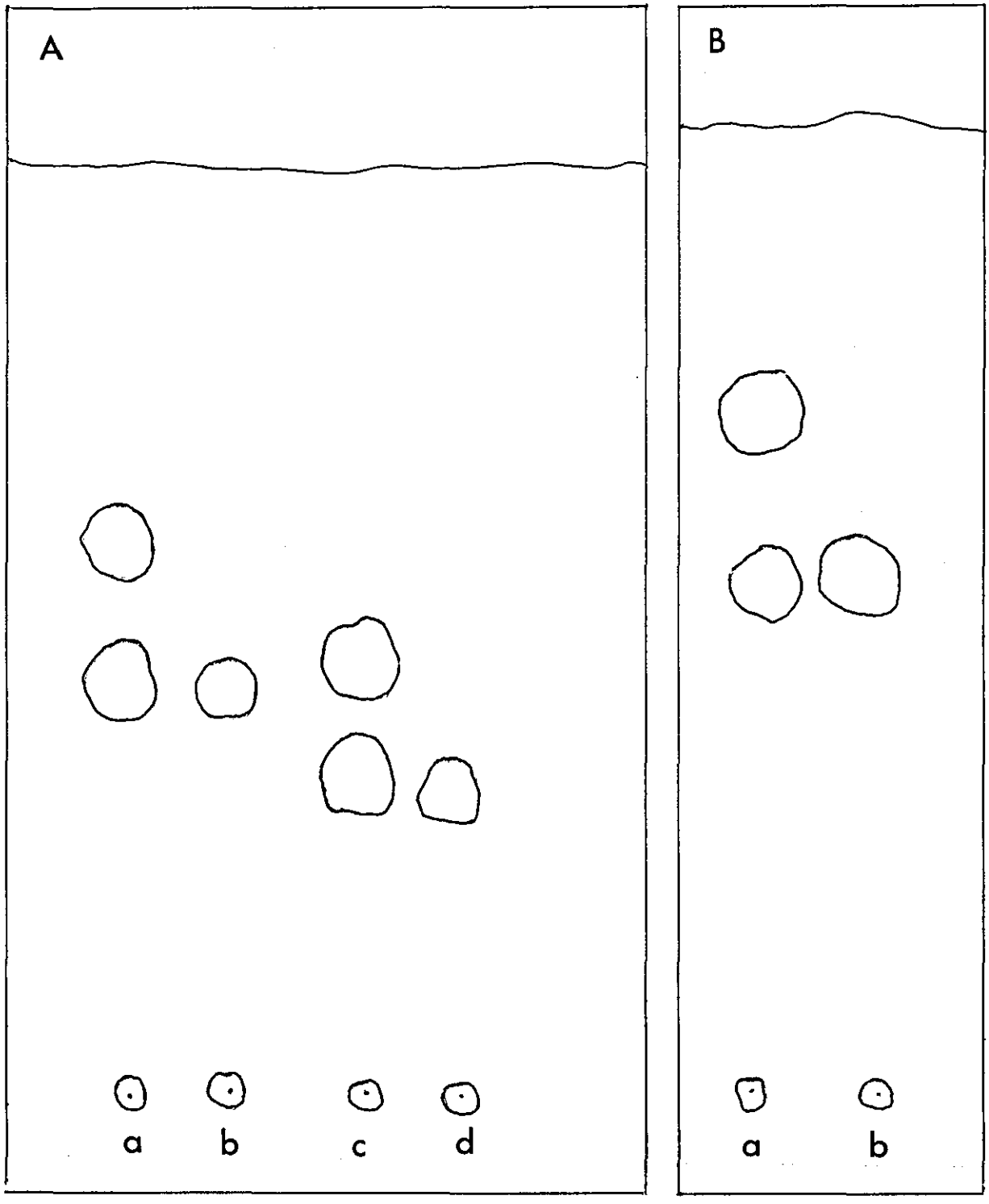

Fig. 1. A. Thin layer chromatogram on Silica Gel G of the L-O-(O-acetoxy) mandelate esters prepared from: (a) methyl 2-OH-16:0; (b) methyl D-2-OH-16:0; (c) methyl 3-OH-16:0; and (d) methyl D-3-OH-16:0.

B. Thin layer chromatogram on Silica Gel G impregnated with 10% silver nitrate of the L-O-(O-acetoxy)mandelate esters prepared from: (a) methyl 12-OH-Δ^9-18:1; and (b) methyl D-12-OH-Δ^9-18:1.

Solvent - ether:benzene (2:98)

is shown by the fact that the L-O-(O-acetoxy)mandelate esters of optically pure D-hydroxy esters show only a single spot on TLC. If racemization had occurred during the formation of the diasterioisomeric esters, it would be expected that the derivatives of optically pure hydroxy esters would show more than one spot on TLC.

Racemic ester	$[\alpha]$ of D-enantiomer of hydroxy ester	L-O-(O-acetoxy) mandelate derivatives $[\alpha]$		Resolved Esters $[\alpha]$	
		"Upper"	"Lower"	"Upper"	"Lower"
2-OH-16:0	-8.02	-13.7	-36.0	+4.7	-3.6
3-OH-16:0	-14.07	-32.0	-40.6	+7.6	-6.5
4-OH-16:0		no separation			
9-OH-18:0		no separation			
12-OH-18:0		no separation			
12-OH-Δ^9-18:1	+7.3	-7.6	-8.2	-3.2	+2.9

All rotations measured in methanol

Table 1.

It appears that thin layer chromatography of the L-O-(O-acetoxy) mandelate esters is a useful means of determining the optical configuration, or optical purity, of some hydroxy fatty esters. However, it is less useful for the preparation of optically pure hydroxy esters due to the racemization which occurs during the recovery of the hydroxy ester from the diastereoisomeric derivations.

EXPERIMENTAL

L-acetoxy mandeloyl chloride

L-mandelic acid (2g) and acetyl chloride (3g) were warmed together and reaction commenced immediately. When the reaction mixture was completely clear the excess acetylchloride was removed under vacuum. The L-acetoxy mandelic acid was dissolved in benzene (50ml), oxalyl-chloride (4g) was added and the mixture allowed to stand at room temperature for 3 hours. The solvent and excess oxalylchloride were removed under vacuum to leave a colourless oil (2g) which was not purified further.

Preparation of L-O-(O-acetoxy)mandelates

Methyl 2-hydroxy palmitate (40mg) was dissolved in pyridine (6ml) and L-acetoxy mandeloyl chloride (100mg) was added and the mixture was allowed to stand at room temperature. After 16 hours the mixture was diluted with water, extracted with ether, and the extracts washed successively with water, dilute HCl, water, aqueous sodium bicarbonate, and finally water until neutral. The solvent was removed to leave a yellow oil.

Separation of Diastereoisomers by TLC

The diastereoisomeric mandelate esters of the long chain hydroxy esters were separated by preparative TLC or argentation TLC, approximately 20mg of the mixture being separated on each 20 x 20 x 1mm layer. The plates were developed twice with ether:benzene (2:98), the separated components were located under U.V. light after spraying the plates with 0.2% dichlorofluorescein in ethanol. The bands containing the individual isomers were scraped from the plates and eluted with ether. The separated isomers were pure as judged by analytical TLC.

Recovery of long chain hydroxy ester from diastereoisomeric derivative

The individual diastereoisomeric mandelate esters (20mg) were dissolved in methanol:sulphuric acid 98:2 (3ml) and the mixture was heated under reflux for 2 hours. After this time the mixture was diluted with water (5ml) and extracted with ether. The ether extracts were washed with water until neutral and the solvent was removed to leave a colourless solid. The methyl 2-hydroxy palmitate was purified by TLC on Silica Gel G plates developed in ether:petroleum ether 3:7.

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