Synthesis and use of substituted long-chain fatty acids for studies of inhibition and steric control of enzymic desaturations

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SYNTHESIS AND USE OF SUBSTITUTED
LONG CHAIN FATTY ACIDS FOR STUDIES
OF INHIBITION AND STERIC CONTROL
OF ENZYMIC DESATURATIONS

by
DONALD BRET, B. Tech.

A Doctoral Thesis
Submitted in partial fulfilment of the requirements
for the award of
Doctor of Philosophy of the Loughborough University of Technology
October 1972.

Supervisor: Professor A.T. JAMES.

(c) by Donald Brett
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SUMMARY

The widespread existence of long chain unsaturated fatty acids has encouraged considerable investigation into the methods of synthesis. One method, the anaerobic pathway, has been largely elucidated but much remains to be discovered about the far more prevalent aerobic pathway. This thesis describes work carried out to help identify the mechanism by which such aerobic desaturation of long chain saturated fatty acids takes place.

Since the desaturase enzyme eludes isolation, direct structural determinations cannot be conducted, and so to gain such information an indirect approach involving the synthesis and incubation of almost the whole range of 14C-labelled, positionally isomeric, methyl-substituted stearic acids was undertaken. These incubations, in the green alga Chlorella vulgaris and on the microsomal desaturase of hen liver, yielded information on the spatial environment of the acyl residue in the enzyme-substrate complex and, in cases where desaturation was observed, showed that the presence of a methyl substituent did not disrupt the high positioned specificity of desaturation, which is common to all systems. The absolute stereospecificity of the hen liver desaturase was also determined, and from the combined results a proposal was made to explain the stereospecific removal of the D-9 and D-10 hydrogen atoms shown to take place during desaturation.

Studies were also carried out to investigate known and potential inhibitors of the desaturase enzyme. A method was devised for chain extending and radiolabelling cyclopropene fatty acids, and the products were used to investigate in depth the mode of action of sterculic acid in the avian system. Attempts were also made to reproduce the potent inhibition exerted by the cyclopropenes in fatty acids substituted with other three-membered ring-systems bearing certain chemical similarities. Finally, various novel compounds were synthesised and incubated with the microsomal fraction of hen liver in an attempt to obtain interaction with the actual desaturase site and thus throw light on its composition or on possible intermediates in the desaturase reaction.
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GENERAL INTRODUCTION

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 \( \beta \)-oxidations. It took nearly 40 years for his pathway to gain full experimental confirmation and his findings were the basis for extensive research into the degradation and synthesis of fatty acids.

Since every step in the enzyme degradation of fatty acids was reversible the obvious assumption was that synthesis was achieved from acetyl-CoA by the same enzymes. This is only partially true and during the last 20 years it has become apparent that there are several pathways for biosynthesis of saturated fatty acids, two of which start from acetyl-CoA and are known as \textit{de novo} syntheses. In the \textit{de novo} synthesis elongation occurs by condensation with either malonyl-CoA or further molecules of acetyl-CoA. Only the latter is similar to the reversal of \( \beta \)-oxidation.

The former and major pathway for the synthesis of fatty acids is the malonyl-CoA pathway. This is widely distributed throughout animals, plants and microorganisms \(^1\), \(^2\), \(^3\). The first reaction involves the generation of malonyl-CoA from acetyl-CoA by the biotin dependent enzyme, acetyl-CoA carboxylase. This is a key step since subsequent decarboxylation of the more reactive malonyl-CoA helps to drive the equilibrium towards synthesis.

\[
\begin{align*}
\text{HCO}_3^- + \text{biotin enzyme} + \text{ATP} &\rightleftharpoons \text{CO}_2 - \text{biotin enzyme} + \text{ADP} + \text{P}_i \\
\text{CO}_2 - \text{biotin enzyme} + \text{acetyl-CoA} &\rightleftharpoons \text{biotin enzyme} + \text{malonyl-CoA}.
\end{align*}
\]

The malonyl-CoA is transferred to ACP(1) (acyl carrier protein) and condenses with acetyl-ACP(2) to give \( \beta \)-ketobutyryl-ACP(3). This undergoes reduction (4) dehydration (5) and hydration (6) with the appropriate enzymes, the acyl group remaining bound to the ACP throughout, i.e;
(1) \( \text{CH}_3\text{.CO.SCoA} + \text{ACP} \rightleftharpoons \text{CH}_3\text{.CO.SACP} + \text{CoASH} \)

(2) \( \text{HOOC.CH}_2\text{.CO.SCoA} + \text{ACP} \rightleftharpoons \text{HOOC.CH}_2\text{.CO.SACP} + \text{CoASH} \)

(3) \( \text{CH}_3\text{.CO.SACP} + \text{HOOC.CH}_2\text{.CO.SACP} \rightleftharpoons \text{CH}_3\text{.CO.CH}_2\text{.CO.SACP} + \text{CO}_2 + \text{ACP} \)

(4) \( \text{CH}_3\text{.CO.CH}_2\text{.CO.SACP} + \text{NADPH} + \text{H}^+ \rightleftharpoons \text{CH}_3\text{.CH(OH).CH}_2\text{.CO.SACP} + \text{NADP}^+ \)

(5) \( \text{CH}_3\text{.CH(OH).CH}_2\text{.CO.SACP} \rightleftharpoons \text{CH}_3\text{.CH=CH.CO.SACP} + \text{H}_2\text{O} \)

(6) \( \text{CH}_3\text{.CH=CH.CO.SACP} + \text{NADPH} + \text{H}^+ \rightleftharpoons \text{CH}_3\text{.CH=CH.CO.SACP} + \text{NADP}^+ \)

Repeated passages through this biosynthetic scheme, where the elongation product replaces acetate in stage 3, produces mainly palmitic and stearic acids with smaller amounts of lauric and myristic acid. The stoichiometry for the synthesis of palmitic acid is:

\[
\text{CH}_3\text{.CO.SCoA} + 7 \text{HOOC.CH}_2\text{.CO.SCoA} + 14 \text{NADPH} + 14\text{H}^+ \rightarrow \text{CH}_3\text{.(CH}_2\text{)}_{14}\text{.COOH} + 9\text{CoASH} + 7\text{CO}_2 + 14\text{NADP}^+ + 6\text{H}_2\text{O}
\]

N.D. Only the two carbon atoms at the methyl end of the chain are derived directly from acetate, the remainder coming from malonate.

The other pathway, which exhibits reversal of \( \beta \)-oxidation, is relatively uncommon \(^4,5\), and in the case of the anaerobic bacterium \( \text{C.kluyveri} \) only synthesizes fatty acids up to hexanoate. The pathway does not require \text{CO}_2\text{ or malonyl-CoA.}

Other pathways for the biosynthesis of saturated fatty acids, involve the elongation of preformed fatty acids \(^4,5\). One such system, present in rat liver mitochondria \(^6\), elongates by condensation of preformed acids with acetyl-CoA. Also the reaction is not \text{bicarbonate} dependent and so it was initially thought to be a reversal of \( \beta \)-oxidation. However this is not so, since the \( \beta \)-hydroxy intermediate is of the D(-) configuration and not the L(+) which is produced in \( \beta \)-oxidation. The pathway involves reaction of the CoA derivatives of the fatty acid and acetate, in the presence of \text{NADH} and \text{NADPH.} If either the acid or acetate is not present as the CoA ester, ATP is also required. The reaction sequence is outlined below:

\[
\text{R.CO.SCoA} + \text{CH}_3\text{.CO.SCoA} \rightleftharpoons \text{R.CO.CH}_2\text{.CO.SCoA} + \text{CoA}
\]

\[
\text{R.CO.CH}_2\text{.CO.SCoA} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{R.CH(OH).CH}_2\text{.CO.SCoA}
\]
R.CH(OH).CH₂.CO.SCoA ⇌ R.CH = CH.CO.SCoA

R.CH = CH.CO.SCoA + NADH + H⁺ ⇌ R.CH₂.CH₂.CO.SCoA.

Alternatively, Nugteren⁷ has shown that a rat liver microsomal fraction is capable of elongating preformed fatty acid by addition of malonyl-CoA units.

The biosynthesis of mono-unsaturated fatty acids is again known to proceed by at least two different pathways, namely, the anaerobic and the aerobic pathways⁸.

Considering briefly the anaerobic pathway, this method is found in many anaerobic microorganisms and has been largely elucidated by Bloch⁹ in Clostridium butyricum. The mechanism essentially involves a diversion of the de novo synthesis, occurring at the dehydration step of enzyme-bound β-hydroxy-decanoic and -dodecanoic acids. In the synthesis of saturated acids the intermediate formed by this dehydration is trans-2-unsaturated acid, but the enzyme is also capable of producing cis-3-unsaturated acid. This is the precursor for the long chain unsaturated acids, the double bond being retained while further chain extension occurs e.g. for the synthesis of oleic acid.

\[
\begin{align*}
-\text{H}_2\text{O} & \quad \text{CH}_3\cdot(CH_2)_7\cdot \text{CH}(\text{OH})\cdot \text{CH}_2\cdot \text{COOH} \\
& \quad \text{CH}_3\cdot(CH_2)_7\cdot \text{CH} = \text{CH}_2\cdot \text{COOH} \\
\text{CH}_3\cdot(CH_2)_7\cdot \text{CH} = \text{CH} \cdot \text{CH}_2\cdot \text{COOH} & \quad \text{de novo} \quad \text{CH}_3\cdot(CH_2)_7\cdot \text{CH} = \text{CH},(CH_2)_7\cdot \text{COOH}
\end{align*}
\]

This method produces mainly C₁₆(Δ7* and Δ9) and C₁₈ (Δ₉ and Δ₁₁) monocenoic acids with cis double bonds.

By far the more important pathway to unsaturated acids is the aerobic (or oxidative) pathway in which a double bond is introduced directly into preformed, long chain fatty acids. This pathway, which occurs almost universally, is employed by bacteria, protazoa, yeasts, algae and higher animals. By comparison with the anaerobic synthesis, much less is known about this mode of desaturation and the work presented in this thesis was designed to help in the elucidation of the mechanism.

*Δ₇ = double bond in the 7,8-position counting from the carboxyl-end.
Perhaps the earliest recognition of this process came in 1936 when Schoenheimer\(^{10}\) noted that in animals, oleic acid was produced from stearic acid by direct transformation of the C\(_{18}\) chain. However, it is only in the last decade that major steps towards determining the pathway have been made.

The pathway was first demonstrated by Bloch\(^{11}\) who found that cell-free preparations of yeast could catalyse the transformation of stearate to oleate provided that molecular oxygen and the reduced forms of nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) were present. The requirement for oxygen is absolute\(^{11,12}\) and its exclusion or substitution with artificial electron acceptors results in inhibition of desaturation\(^{11}\). Activation of the fatty acid to acyl-SCoA, or the acyl-SACP in higher plants, is also a necessary requisite of the reaction and no reversibility has been demonstrated.

It may be possible to formulate the generation of an olefin by loss of a proton followed by a hydride ion from an adjacent carbon atom. This could be a plausible explanation if the double bond was inserted in the \(\alpha/\beta\)-position of carbonyl systems. However the insertion of a double bond at the chemically inert 9,10 position of an aliphatic chain by such a mechanism has no analogy, chemical or enzymatic, and seems to be improbable.

The requirements of oxygen as the electron acceptor and NADH or NADPH as the electron donor are characteristic of hydroxylase (mixed function oxidase) reactions and so it seemed that the first stage in the enzymic desaturation could be hydroxylation of the saturated chain. Subsequent dehydration of the hydroxy derivative would yield the olefin; e.g. in the case of desaturation of stearic to oleic acid in rat liver homogenates, the following scheme was postulated by James & March in 1961\(^{12}\):

\[
\begin{align*}
\text{Stearic acid} + \text{CoA} \quad &\xrightarrow{\text{ATP, Mg}^{2+}} \quad \text{Stearoyl-SCoA} \\
\text{Stearoyl-SCoA} + \text{enzyme} \quad &\rightarrow \quad \text{Stearoyl-enzyme} + \text{CoA} \\
\text{Stearoyl-enzyme} + \text{O}_2 + \text{NADPH} \quad &\rightarrow \quad \text{Hydroxystearoyl-enzyme} \\
\text{Hydroxystearoyl-enzyme} \quad &\rightarrow \quad \text{Oleoyl-enzyme} + \text{H}_2\text{O} \\
\text{Oleoyl-enzyme} + \text{CoA} \quad &\rightarrow \quad \text{Oleoyl-SCoA} + \text{enzyme}
\end{align*}
\]

\textbf{viz:-}

\[
\begin{align*}
\text{Stearic acid} + \text{CoA} \quad &\xrightarrow{\text{ATP, Mg}^{2+}, \text{O}_2, \text{NADPH}} \quad \text{Oleoyl-CoA}
\end{align*}
\]
Attempts to prove this mechanism by incubation with hydroxy and other oxygenated intermediates have met with little or no success. James & Marsh\textsuperscript{12}, whilst demonstrating the formation of hydroxystearate from stearate in rat liver homogenates and yeast, showed less than 5\% conversion of hydroxy-stearates to oleate in whole yeast cells. Davidoff\textsuperscript{13} found no conversion of 9(10)-hydroxystearic acid in Dictyostelium discoideum. Similarly, Gurr & Bloch\textsuperscript{14} incubating hydroxy, acetoxy and epoxy-stearoyl-SCAP derivatives with Euglena gracilis achieved no conversion. These and other unfruitful attempts seem to enhance the hypothesis of James and Marsh that an hydroxystearic acid can only be an intermediate in the desaturation of stearic acid if it is irreversibly bound to the enzyme. This would mean that exogenous hydroxy-acid could not enter into the necessary enzymic linkage required for desaturation.

Further evidence in favour of the possibility of an hydroxylated intermediate is provided by the similarity of the electron transfer chain for mixed function oxidases (hydroxylases) and the desaturase sequence. Although the exact details are still unclear a possible scheme for electron flow in the desaturase reaction is summarised below:

\[
\begin{align*}
\text{NADPH} & \xrightarrow{\text{reduced flavoprotein}} \text{Fe}^{2+} \xrightarrow{\text{Desaturase}} \text{oleoyl-SCoA} + \text{H}_2\text{O} \\
\text{NADP} & \xrightarrow{\text{flavoprotein}} \text{Fe}^{3+} \xrightarrow{\text{Desaturase}} \text{stearoyl-SCoA} + \text{O}_2
\end{align*}
\]

Although there is reasonably substantial evidence for the steps involved in reducing molecular oxygen to a level at which it can accept hydrogen\textsuperscript{15,16} (sequence to the left of the $\text{O}_2$-$\text{H}_2\text{O}$ line), the method of abstraction of the hydrogens is still unclear. Also important differences between the hydroxylase and desaturase enzymes do exist; the desaturase enzymes of rat liver\textsuperscript{16} and of Euglena gracilis\textsuperscript{15} are not inhibited by carbon monoxide or ethyl isocyanide which inhibit reaction involving cytochrome P\textsubscript{450}, the haemoprotein associated with various aliphatic and hydro-aromatic hydroxylations systems. Furthermore, the latter are unaffected by cyanide, whereas the desaturase enzyme is sensitive to this inhibitor.
Thus from all the information available at present, it would appear that the desaturase system, although closely related in some ways to the hydroxylases, does not proceed via an hydroxy intermediate.

Since the many oxygenated intermediates tried were not desaturated, one must assume that any such intermediate is enzyme bound, or alternatively, that the oxygen atom never enters into a covalent linkage with the carbon atom of the acid. This latter idea has led to the suggestion by Light et al.\(^ {17}\) that desaturation may occur by direct abstraction by the enzyme, of the hydrogens at the C9 and C10 carbon atoms in palmitate and stearate.

Throughout the work contained in this thesis, special attention was paid to this possibility which would implicate oxygen, either as the direct electron acceptor on the enzyme, or as a necessary requirement for regeneration of the active site, which would undergo reduction during the desaturase process.

It was initially thought that there existed a second aerobic pathway to unsaturated fatty acids in higher plants, which differed from that observed in bacteria, algae, yeasts and animals, in that the corresponding saturated fatty acids, or their coenzyme A esters, could not be directly desaturated\(^ {18-20}\). The only effective precursors appeared to be as follows:\(^ {21}\):

\[
\text{Acetate} \rightarrow 10:0 \rightarrow 12:0 \rightarrow 16\text{ carbon and } 18\text{ carbon unsaturated acids} \\
\quad \downarrow \quad \downarrow \\
14:0 \rightarrow 18\text{ carbon unsaturated acids only} \\
\quad \downarrow \\
16:0 \rightarrow \text{No unsaturated acids} \\
\quad \downarrow \\
18:0 \rightarrow \text{No unsaturated acids}
\]

However, although desaturation of added palmitic or stearic acid did not take place in leaf systems, incorporation into lipid was obtained i.e. the leaf system was capable of forming stearoyl-SCoA.

The mystery surrounding this pathway was solved by two experiments. In the first\(^ {22}\), leaf tissue was allowed to synthesize
fatty acids from acetate, in anaerobic conditions, when only palmitate and stearate were formed. On transfer to aerobic conditions part of the labelled stearic acid disappeared and was replaced by an equivalent amount of oleic acid. In the second experiment, it was shown that a leaf chloroplast preparation was able to convert stearoyl-ACP, but not stearic acid or stearoyl-SCoA, into oleic acid. Thus it became clear that in the higher plant system stearic acid is also the precursor of oleic acid. The simplest explanation is that the leaf system lacks the enzyme present in animals and lower forms of plants, which is capable of transferring the stearoyl group from stearoyl-SCoA to the ACP acceptor.

Setting aside the mechanism of desaturation for a moment and considering the natural occurrence of long chain fatty acids; these acids, both saturated and unsaturated, seldom occur in the free form. The vast bulk of the acids are found esterified to the trihydric alcohol, glycerol to form lipids.

Where only fatty acids are esterified to the glycerol molecule, the lipid class is known as the glycerides.

\[
\begin{align*}
\text{Triglyceride} & : CH_2OOCR \quad CH_2OOCR \quad CH_2OOCR \\
\text{Diglyceride} & : CH_2OOCR \quad CH_2OH \\
\text{Monoglyceride} & : CH_2OOCR \quad CHOH
\end{align*}
\]

Generally the glycerides (or fats) serve as storage depots in both plant and animal systems, and under conditions of starvation tend to be decreased.

A separate class of lipids, namely the phospholipids, contain an atom of phosphorus as well as glycerol and the esterified fatty acids. These are perhaps the most important group of lipids, since along with proteins they form the framework of much of the cell membrane. Unlike the glycerides they do not vary with starvation. They are all derivatives of phosphatidic acid and are often found coupled with a nitrogenous base,
The most common nitrogenous bases are choline, ethanolamine and serine.

e.g.

\[
\text{CH}_2\text{OCON}_1
\]

\[
\text{CHOCOR}_2
\]

\[
\text{CH}_2\text{-O-P} \equiv \text{O}
\]

\[
\text{OH}
\]

Phosphatidylcholine

However inclusion of nitrogenous bases is not imperative and coupling of the phosphatidic acid with such compounds as inositol (phosphatidylinositol) or another glycerolmolecule (phosphatidylglycerol) does occur.

Other classes of lipids, generally not as prevalent, include the glycosides, sphingolipids, steroids, lipoproteins etc.

Accepting that long chain fatty acids are seldom found in the free form, it is now possible to discuss briefly the types of unsaturated fatty acids occurring naturally.

Monoenoic fatty acids are produced in all forms of life and examples of both cis and trans isomers with double bonds in varying positions along aliphatic chains of differing length have been found (See Introduction to Part 1). However by far the most common are cis -9 - hexadecenoic (palmitoleic) acid and cis - 9 - octadecenoic (oleic) acid.

Polysaturated fatty acids are also extremely common throughout living systems and only bacteria and some blue-green algae are unable to synthesise them. It is possible to distinguish distinct series of polyenoic acids in the plant and animal kingdoms, whilst phytoflagellates, such as Euglena, are able to synthesise both plant and animal types. In plants, apart from a few isolated cases of unusual polysaturated acids, desaturation always occurs between the first double bond and the methyl end of the carboxylic acid, such that \( \Delta 9 \) is desaturated to \( \Delta 9,12 \) then \( \Delta 9,12,15-C_{18} \). In animal systems introduction of polysaturated centres always occurs between the carboxyl group and its
nearest double bond i.e. $\Delta 9,12 - C_{18}$ (linoleic acid) will be desaturated to give $\Delta 6,9,12-C_{18}$ ($\gamma$-linoleic acid). Elongation of such acids followed by further desaturation gives rise to a series of $C_{20}$ and $C_{22}$ polyenoic acids that are characteristic of animal systems.

The essentiality of certain of these unsaturated acids as constituents of the diet of animals has long been realised. Deficiencies in these acids which will arise if there is no dietary supplement, leads to retarded growth rate, interference with the reproductive cycle and may result in eventual death. The symptoms, however, are alleviated in the animals by supplying these so called essential fatty acids. It was initially thought that all these acids contained the same structure towards the methyl-end, exhibiting the first double bond at an $\omega$-6 position e.g. linoleic, $\gamma$-linolenic and arachidonic ($\omega 5,8,11,14-C_{20}$) acids. However $\alpha$-linolenic ($\omega 3$) and certain other $\omega 3$ polyunsaturated acids have been shown to possess some essential properties, and the illustration that $\Delta 5,9,11,14 - C_{19}$, $-C_{20}$ and $-C_{21}$ acids also showed activity, disproved any theories of methyl-end dependence and suggested that their activity was carboxyl-end determinate.

A relationship between these essential fatty acids and a group of naturally occurring substances called prostaglandins, probably explains their essential nature. This recent discovery proposes their biological conversion to prostaglandins, which are known to contain marked physiological activity.

The formation of polyenoic fatty acids always occurs via the aerobic pathway and, like monoenoic acid syntheses, the enzymes involved are firmly particle bound. The unsaturated acids also accumulate in distinct classes of lipids and this has given rise to the hypothesis that complex lipids may play a direct role in the desaturase process.

Throughout the work presented in this thesis two systems were employed for studying the desaturase mechanism: the microsomal suspension
from hen liver homogenates after removal of cell debris and mitochondria, and a whole cell suspension of the alga *Chlorella vulgaris*. In both cases attention was directed almost exclusively to the mechanism of introduction of the first double bond.

The microsomal suspension is a particulate system which produces only monoenes from added substrate. The desaturase enzyme (or enzymes) shows an absolute specificity for the 9,10-position in the fatty acid chain regardless of substrate chain length$^{32,33}$. Since there are no competing reactions, this system was very good for studying the desaturase.

The second system, namely *Chlorella vulgaris*, produces dienes as well as monoenes from added straight chain substrates. When used as a whole cell suspension, as it was in this case, the system is also capable of breaking down the substrates by $\beta$-oxidation to acetyl-SCoA and re-utilizing this in *de novo* synthesis thereby randomizing the $^{14}$C label into other fatty acids (breakdown/resynthesis). Also, in cases where substrates have a shorter chain length than stearic acid, elongation can take place. The latter two effects can be minimized by careful selection of the incubation time, thus giving maximum desaturation with minimum interference from other competing reactions. Once again desaturation is positionally specific giving a double bond at the 9,10-position in stearic acid. The oleic acid produced is partially desaturated to linoleic acid which has a second double bond methylene interrupted from the first at the 12, 13-position.

This positional specificity for dehydrogenating saturated acid to the $\delta$9 monoenes, which is illustrated by the two systems chosen, has also been shown to occur in a broad selection of living organisms (for a table of references see ref. 34).

As well as the remarkable positional specificity shown by the desaturase enzyme as illustrated in the systems chosen for study, the enzyme also removes the two hydrogens from the chemically inert carbon atoms stereospecifically to afford, in most organisms, a cis double bond$^{35,36}$. (This will be discussed in more detail in the Introduction to Part 1).
Thus the complete desaturase mechanism must explain not only the role of the cofactors, oxygen and NADPH or NADH, in the removal of the two hydrogens but also the great positional, geometrical and optical specificity with which the procedure is performed.

Obviously the most satisfactory method of approach in determining the mechanism would be to isolate the enzyme and carry out a full structural analysis. The first success of this kind was achieved in 1956 by Sanger on the insulin, and following this the structure of several other enzymes have been so determined (Ref. 38-43). Establishment of the chemical structure of the desaturase enzyme in such a way would then make the task of investigating the chemistry, governing the biological activity, much easier. One would probably be able to deduce whether direct abstraction of two hydrogen atoms, or dehydration of enzyme bound hydroxy-intermediates, was involved in the desaturase mechanism or whether some other, totally unknown, method was responsible. The role of molecular oxygen, reduced pyridine nucleotide and the electron transport chain could probably be elucidated. Determination of the method of attachment of the carboxyl group of the fatty acid to the enzyme and the extent of involvement of complex lipids would be much easier. Also the method by which the enzyme imposes on the normal freely rotating acyl chain the rigidity which must be necessary for such highly stereospecific reaction, would be determined. All these, and other more specific indeterminables, could possibly be solved, if the desaturase enzyme could be isolated in a stable form.

However, at present, isolation of the desaturase enzyme of any organism cannot be obtained, thus precluding full structural analysis. Bloch has met with most success in this respect using the phytoflagellate, Euglena gracilis. Since the Euglena enzymes are soluble they can be subjected to conventional fractionations. By this method the 'soluble' desaturase was separated into three components, an enzyme called NADPH-oxidase, the non-haem iron protein ferredoxin and the 'desaturase' proper. Unfortunately the latter is relatively unstable and not very well characterized.
The desaturating enzymes of other systems are tightly membrane bound in the chloroplasts of plant cells, or the endoplasmic reticulum of other cells, which makes them difficult to isolate. However quite recently Gurr and Janos\textsuperscript{44} described a way in which liver microsomes (i.e. endoplasmic reticulum) may be broken down into smaller particles and thereby 'solubilized' with retention of desaturase activity. Thus in the future it may be possible to isolate the animal 'desaturase'.

Since it is not possible to isolate the desaturase enzyme and determine its structure as in the lysozyme example, the problem regarding the mechanism of enzymic desaturation must be approached by indirect methods. In this thesis two such methods were employed:

In Part 1 experiments were designed to study the stereochemistry of desaturation and the spatial tolerances in the vicinity of the desaturase enzyme.

In Part 2 a study was made of the inhibition of enzymic desaturation in an attempt to define the site of inhibition and thereby learn more about the pathways and sequences of the reactions involved.
EXPERIMENTAL TECHNIQUES

Throughout the work several experimental techniques were employed to analyse and separate fatty acids and lipids. A brief description of the techniques involved is given below:-

1. Liquid-Solid Chromatography (Column chromatography.)

This type of chromatography was employed for separation of relatively large amounts of mixtures, in which the components usually had reasonable polarity differences.

Glass columns were used and the solid adsorbent was Davidsons Silica Grade 950. Approximately ten grams of the adsorbent, for every one gram of mixture to be separated, were slurried in a low polarity solvent and poured into the glass column. Air bubbles were removed by tapping and then the mixture, dissolved in the low polarity solvent, was applied to the top of the column. Solvents of steadily increasing polarity were run through the column and fractions of suitable volume were collected at the base. The solvent system usually employed was petroleum ether, the polarity increase being obtained by addition of increasing quantities of other. For separation of acids and half esters the solvent system was chloroform increasing in polarity to 5% methanol in chloroform.

An aliquot from each fraction obtained from the column was analysed by thin layer chromatography. The fractions containing the desired component were bulked and the solvent was removed on a rotary evaporator at reduced pressure.

2. Thin Layer Chromatography (T.L.C.)

Although the basic principle of thin layer chromatography was described some 30 years ago, the method itself only really became accepted as indispensable during the last decade. This delay was because early workers experienced considerable variation in Rf values for apparently inexplicable reasons and, only when Stahl began to introduce some standardisation into the technique, did it gain acceptance. Since then development, both as an analytical and preparative method, has been rapid.
because it is quick, highly selective and can be applied to almost the whole spectrum of chemical compounds. It was soon adapted to lipid work where it has become an invaluable tool (review article, ref. 47).

Both preparative and analytical thin layer chromatography were employed extensively throughout this work, both in lipid and fatty acid separations:

a) **Preparative T.L.C.** In general, this method gave a finer separation of components than could be obtained by column chromatography and the procedure was much faster. However, the technique became cumbersome as the quantity of the mixture increased and thus was most suitable for mixtures not in excess of one gram.

Here the solid adsorbent was supported as a thin layer on a glass sheet. This was obtained by thoroughly slurrying the solid, Kieselgel G. nach Stahl (Harck), in water (1:2 w/v) and applying this with a suitable spreader to clean glass plates laid end to end, horizontally. The thickness of the layer was usually 0.25 mm or 0.5 mm depending on the amount of the mixture to be separated. The plates were activated in an oven at 110°C before use.

Application of the mixture, as a thin uniform line one inch from the bottom edge of the plate, was obtained using a Desaga T.L.C. Streak Applicator to deliver a solution of the mixture in ether (or some other suitable solvent).

The plates were developed in closed vessels using a suitable solvent system. The solvent was drawn up through the layer by capillary action and the individual components were separated according to their differing migration rates.

Visualisation had to be non-destructive and 2,4-dichlorofluorescein, Rhodamine 6G or radioactivity scanning were used to this end.

After visualisation, the layer containing the required component was removed and washed from the silica with ether. Subsequent evaporation yielded the pure product.

- 14 -
b) **Analytical T.L.C.** This method was extremely useful and was used extensively for quick determination of both lipids, and fatty acid derivatives, by comparison with known samples.

Plates of 0.25 mm thickness were prepared as above and samples applied as spots close to the bottom edge of the plate. Development in a suitable solvent produced the desired migration and separation of the components. Visualisation was usually obtained by spraying the layer with dilute sulphuric acid. Subsequent heating at 200°C for 10 mins. produced the components as black spots on a white background.

(Separations of compounds with very similar polarities can frequently be achieved by developing several times in a more dilute solvent than normal, evaporating the solvent from the surface between each development.)

3. **Argentation T.L.C.**

The above method although sensitive cannot separate a saturated/unsaturated mixture. Separation of alkanes, **cis** and **trans** monoenes, dienes and trienes from each other was however obtained on silver nitrate impregnated thin layer plates. The basic theory behind this modification of thin layer chromatography was obtained by Jisten in 1938. He illustrated the ability of silver ions to form complexes with π-electrons of unsaturated centres\(^{48}\), the complexation reaction being reversible and equilibrium being achieved very rapidly.

Some 14 years later Nichols\(^{49}\) proposed that separation of methyl oleate and methyl elaidate would be obtainable by counter current distribution between hydrocarbon solvent and methanolic silver nitrate. This proposal was later accomplished by Dutton and coworkers\(^{50}\). Adaptation of this complexing reaction to adsorption chromatography was reported simultaneously in 1962 by Morris\(^{51}\) who used TLC, and d.Vrli\(^{52}\) who used column chromatography. Since this adaptation many sophisticated separation of various geometrical and positionally isomeric, unsaturated lipptic compounds have been recorded.
In practice, impregnation with silver nitrate was achieved by slurrying the silica with a solution of silver nitrate in water before spreading. A 10% concentration of silver nitrate to silica was sufficient and activation, development and identification were carried out in the usual manner.

Radioactivity Scanning of Thin Layer Chromatograms (R.T.L.C)53

This technique was used for analysing both lipids and their component fatty acids from incubations carried out with radioactive substrates. The technique was found to give good sensitivity and reproducibility.

Thin layer chromatograms, either normal or on silver nitrate impregnated plates, were developed in the normal manner, but the samples were applied in alternate channels scribed into the thin layer. To avoid cross contamination alternate channels were used.

The developed plate was then scanned for radioactivity on a prototype instrument designed and built by James et al.54 or on a commercially available adaptation, produced by Panax Instruments Ltd. (Panax RFLS -1). The two instruments are very similar, the prototype consisting of a proportional counter, made from a 10 cm length of copper tubing (0.5 in diameter) highly polished internally, and with a milled flat side in which a 0.5 mm x 10 mm slit was cut. The mode of 2.thou. tungsten wire was positioned centrally through the tube and a carrier gas of 5% carbon dioxide in argon was passed in at both ends and flowed out through the slot. Scanning was achieved by aligning the slit of the proportional counter about 1 mm above one of the channels on the TLC plate, and traversing the plate slowly under it. The pulses were fed through a preamplifier to a ratemeter, the output of which was measured with a ln\(^{-1}\) recorder. Several scanning rates were available, the chart paper of the recorder travelling in each case at the same speed as the TLC plate. The ratemeter also provided several count rates between 30 and 1000 counts/sec. and facilities for differential or integrated scans were available.
This method yielded quantitative results consistent with those obtained by isolation of individual components, followed by scintillation counting. Examples of both differential and integrated scans are shown in figure 1.

5. **Gas-Liquid Chromatography(G.L.C.)**

Gas-liquid chromatography was extensively used both qualitatively and quantitatively for separation of methyl esters of fatty acids. It was often used in conjunction with T.L.C. because, although the latter can accommodate larger samples and help to identify functional groups, it is insensitive to chain length variation.

The method was introduced by James and Martin \(^\text{54}\) in 1952 who used a liquid phase of silicone grease with 10% of stearic acid to separate acids from C\(_1\) to C\(_{12}\), which were detected by automatic titrometry. Cropper and Heywood \(^\text{55}\) proceeding from this achieved some separation of up to C\(_{22}\) chain length by chromatographing the more volatile methyl esters of fatty acids, which were detected by a katharometer. James and Martin \(^\text{56}\) then introduced the gas density balance for detection of methyl esters and, using c-1 piezon hydrocarbon greases, they obtained greatly improved separation of straight chain and branched chain saturated and unsaturated esters from C\(_{10}\) - C\(_{18}\) acids. They also gave the first indications that geometric and positional isomers might be separated. In the same year, the first polar polyester liquid phases were introduced \(^\text{57}\), giving greatly shortened analysis times.

These early developments were followed by more sensitive detectors such as the argon ionization detector \(^\text{58}\), and development of the technique for preparative separations (review ref. Henly \(^\text{59}\)).

Throughout the experimental work recorded, separations were carried out on a Pye Series 104 Gas Liquid Chromatogram incorporating either a non-polar (silicone or piezon grease) or a polar (polyethylene glycol adipate or, FFAP obtained from Varian Aerograph) liquid phase. The sample dissolved in a volatile solvent was injected onto the 5 ft. glass column and detection was obtained with a flame ionization detector. The signal from the detector was recorded on a 10mV potentiometric recorder.
Figure 1.

Example of Radiochemical Thin Layer Scanning

Neutral Lipid analysis on 0.25 mm silica gel plates


Lipids from hen liver microsomal fraction.
Preparative G.I.C. was carried out either on the same instrument (i.e. Pye 104) in which a 20:1 stream splitter was incorporated, resulting in only 5% of the effluent gases being required for detection, or on a Varian Aerograph instrument also incorporating a flame ionization detector. Collection of the separated products from the effluent gases was obtained on chloroform soaked, defatted, cotton wool balls placed at 1" intervals in a 1" bore glass collection tube. Initially an air gap was left between the balls of cotton wool, but almost quantitative collection was achieved in latter experiments when small balotini beads were packed in the air gap. The sample was recovered by solvent washing of the collection tube packing.

6. Radiochemical Gas Chromatography (R.G.I.C.)

This technique provided a quick and accurate method for determining the extent of desaturation of labelled fatty acids after incubation. It was also used for determining the radiochemical purity of precursors, and in bond position determination of the monoenes formed by desaturation.

Basically the radiochemical gas-liquid chromatogram is an instrument that separates, quantitatively detects and records both the mass and radioactivity of components in a volatile mixture. The instrument used here is based on the original design of James and Piper\(^60,61\) and incorporates the latter modifications of James and Hitchcock\(^62\).

The sample, usually of labelled fatty esters, was introduced to the column by pipetting a solution into a small loading tube and evaporating the solvent. The loading tube was then placed on top of the column and the sample allowed to volatilize before the carrier gas of argon was switched on. The sample was thus carried onto the column of a normal gas chromatogram where separation took place. The hot effluent gas passed immediately into a heated combustion tube containing copper oxide and iron filings. Here any organic material was burned to carbon dioxide and water, the latter being reduced to hydrogen by the iron filings. The gas stream
then passed into a katharometer which had a reference stream of pure argon. This system is very sensitive to hydrogen in argon and the amplified signal, proportional to the mass of substance, was recorded on a lnV potentiometric recorder. 5% Carbon dioxide was introduced into the effluent gas from the katharometer to act as a quencher. The mixed gases then passed through a simple proportional counter similar to that used on the Radio-TLC Scanner, but without the milled edge and slit. The pulse from the labelled carbon dioxide was fed through a preamplifier to the ratemeter, and recorded on another lnV potentiometric recorder. The rate meter was again equipped for several count rates and the facility for differential or integrated scans was also present. The instruments used were extremely sensitive and were capable of detecting $10^{-14}$C with ease as a differential recorder or $0.10^{-14}$C if the signal was integrated. Examples of typical scans are illustrated in figure 2.
Figure 2  Example of Radiochemical Gas-Liquid Chromatography (FFAP column)
PART 1.

Studies of the stereochemistry of desaturation and the spatial tolerances of the desaturose enzyme.
INTRODUCTION

As long as the desaturase enzyme eludes isolation as a single component in a stable form, thus allowing complete analysis, any investigation of the aerobic desaturase mechanism must by necessity be indirect. Two such indirect methods, designed to study the spatial arrangement in the vicinity of the desaturase site and the absolute stereochemistry of the desaturase process, are described in this section.

Apart from the ubiquitous oleic and palmitoleic acids, many other naturally occurring monoenoic fatty acids have been identified. The variation occurs both in the chain length and in the position of the double bond of the monoenoic acid.

By far the most common position for insertion of the first double bond, by aerobic desaturation of a saturated acid, is the 9,10-position (counting from the carboxyl group). Examples of the systems in which this has been found are listed, with references, in the table below:

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast (Torulaspin utilis, Saccharomyces cerevisiae)</td>
<td>11</td>
</tr>
<tr>
<td>Rat liver microsomes</td>
<td>63, 12, 64</td>
</tr>
<tr>
<td>Avian Liver (slice)</td>
<td>65</td>
</tr>
<tr>
<td>Fish liver (slice)</td>
<td>66</td>
</tr>
<tr>
<td>Ruminant udder (microsomes)</td>
<td>67</td>
</tr>
<tr>
<td>Intestinal epithelium</td>
<td>68</td>
</tr>
<tr>
<td>Mycobacterium phlei</td>
<td>69</td>
</tr>
<tr>
<td>Corynebacterium diphtheriae</td>
<td>35</td>
</tr>
<tr>
<td>Leaf discs</td>
<td>22</td>
</tr>
<tr>
<td>Leaf chloroplasts</td>
<td>24</td>
</tr>
<tr>
<td>Chlorella vulgaris, intact and cell free</td>
<td>70, 71</td>
</tr>
<tr>
<td>Digi nova gracilis, intact and cell free</td>
<td>70, 72</td>
</tr>
<tr>
<td>Anabaena variabilis</td>
<td>73</td>
</tr>
<tr>
<td>Porphyridium cruentum</td>
<td>73</td>
</tr>
<tr>
<td>Ochromonas malhamensis</td>
<td>73</td>
</tr>
</tbody>
</table>
Although not nearly as prevalent, there are examples of fatty acids where the double bond is inserted at the 9,10-position counting from the methyl-end of the chain (i.e. 7-9). However this does not necessarily implicate a separate methyl end determinate desaturase since the few cases in which it occurs may have resulted from degradation or elongation of a normal Δ9 acid. Examples of systems in which such 7-9 acids do occur, along with the specific acid isolated, are given below:

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Acid</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm whale</td>
<td>cis 5 - tetrdecanoic acid</td>
<td>77</td>
</tr>
<tr>
<td>Euglena gracilis</td>
<td>cis 7 - hexadecanoic acid</td>
<td>78</td>
</tr>
<tr>
<td>Simmondsia californica</td>
<td>cis 11 - eicosanoic acid</td>
<td>79</td>
</tr>
<tr>
<td>Tropaeolum seed</td>
<td>cis 13 - docosanoic acid</td>
<td>80</td>
</tr>
<tr>
<td>Brain tissue cerebrosidecs</td>
<td>cis 15 - tetraeicosanoic acid</td>
<td>81</td>
</tr>
</tbody>
</table>

Isolated examples of naturally occurring monoenoic fatty acids with the double bond neither Δ9 nor W9 have also been cited in the literature 82-85,76.

The above examples illustrate the broad spectrum of naturally occurring monoenoic fatty acids, but for a particular system it must be stressed that the position of the double bond is highly specific and no variation will occur within that system, under standard conditions.

As well as being positionally specific, the desaturase enzyme shows extreme geometrical specificity abstracting two hydrogens from a chemically inert polymethylene chain to form a cis double bond, in the vast majority of cases, including all those cited above. However, a few examples of the
insertion of a trans double bond have been reported in algae and spinach\textsuperscript{36,37}, and animal and milk fats\textsuperscript{38}. Evidence for the complete stereospecificity of the removal of the two hydrogens was produced by Schroepfer and Bloch\textsuperscript{35} in Corynebacterium diphtheriae. They incubated the four nonotritiated stearic acids, obtained from the enantiomers of 9-hydroxy and 10-hydroxy-stearic acids, with the growing culture. On isolation and analysis of the oleic from each incubation they found complete loss of tritium from the D-9 and D-10 enantiomer, with retention of the tritium label in the L-9 and L-10 cases.

These results indeed illustrated that the desaturase enzyme was highly stereospecific. However the experiment only showed that the absolute configuration of the hydrogens removed was D and that a cis olefin was produced. Neither the precise mechanism of desaturation nor the conformation of the substrate at the active site of the enzyme was proved. If the D - hydrogens assume an eclipsed conformation in the enzyme substrate complex, then cis-elimination would indeed prevail. If however the two leaving hydrogens assumed staggered conformations, then trans-removal followed by isomerization of an enzyme-bound trans olefin would give the cis-olefinic oleic acid. Although enzymes capable of performing this elaidic acid (trans) to oleic acid (cis) isomerization have not been found, the possibility of such a configurational change in the enzyme-bound olefin cannot be excluded. Neither can the possibility of substitution and subsequent elimination, be rejected. Both substitution with retention of configuration followed by cis-elimination and substitution with inversion of configuration followed by trans-elimination would give the observed results. Such a possibility is hydroxyl substitution in the enzyme-bound substrate followed by elimination to give the cis olefin. This is discussed in more detail in the General Introduction pages 4-5.

In their experiments Schroepfer and Bloch\textsuperscript{35} also recorded a substantial kinetic isotope effect on removal of the D-hydrogen from the 9-position whilst the \( ^3\text{H} : ^{14}\text{C} \) ratio remained constant in the recovered saturated acid, from the D-10 tritiostearic acid incubation and, of course,
from the two L-enantiomers. This suggested sequential removal of the two hydrogens with removal of the 9-D-hydrogen being the initial and rate-limiting step, followed by stereospecific removal of one of the hydrogen atoms at carbon atom 10.

The mechanism and stereochemistry of desaturation was further studied by Morris et al.\textsuperscript{36} in the green alga \textit{Chlorella vulgaris}. This organism effects a series of direct desaturations of stearic acid to give the cis-unsaturated acids typical of photosynthetic tissue; oleic acid (9-octadecenoic), linoleic (9,12-octadecadienoic) and \( \alpha \)-linolenic (9,12,15-octadecatrienoic) acids.

The authors synthesized racemic erythro- and threo-isomers of 9,10-dideuterostearic, 12,13-dideuteroleic, and 15,16-dideuteroleic acids and used these as substrates in \textit{Chlorella vulgaris} desaturation reactions. Subsequent isolation of the unsaturated products and analysis by mass spectrometry indicated the pairs of hydrogens removed at all three positions of desaturation were of the \textit{cis} relative configuration. This extended the findings of Schroepfer and Bloch\textsuperscript{35}.

Also by synthesis of D- and L-enantiomers of 9-tritiooleic acid and 12-tritiooleic acid and subsequent incubation with \textit{Chlorella vulgaris}, Morris et al.\textsuperscript{36} were able to show that the hydrogen atoms removed in the formation of oleic and linoleic acid were of the D-absolute configuration. Although it has not yet been proved, it would be reasonable to assume that the hydrogen atoms removed in the formation of \( \alpha \)-linolenic acid are also of the D-absolute configuration.

The above results corroborate those of Schroepfer and Bloch but an anomaly arises regarding the sequence of removal of the two D-hydrogen atoms. Recapitulating (see Page 21), Schroepfer and Bloch inferred from their results in bacteria that there was a sequential removal of the D-hydrogen atoms with the removal of the D-9-hydrogen atom being the first and rate-limiting step. The results of Morris et al. however indicated a very substantial kinetic isotope effect against deuterium in the formation.
of all three double bonds and in the stearate to oleate formation at least, the effect was of such a magnitude that it must apply at both positions of the putative double bond. Thus in Chlorella the implication is that desaturation reactions involve concerted, simultaneous removal of the pair of hydrogen atoms rather than the stepwise sequence suggested for bacterial desaturation.

In an attempt to help resolve this anomaly and also to extend the work of Morris and of Bloch to an animal system, a study of the stereochemistry of desaturation of long chain fatty acids was made in hen liver microsomes.

Having established that aerobic desaturation proceeds with an exceptional degree of positional, geometrical and stereochemical specificity, one can investigate the governing factors. Obviously the ultimate factor is the structure of the desaturase enzyme and of the enzyme substrate complex. However the structure of this particular enzyme remains unknown though several general features appertaining to all enzymes are known. That they are, in fact, protein was established in 1926 by Sumner when he isolated urease in crystalline form from jack bean meal. Although this early work met with criticism, further crystallisation and characterisation of several additional enzymes by Northrup and Kunitz established beyond doubt the protein nature of enzymes. Since then more than 100 enzymes have been obtained in crystalline form and shown to be proteins. Therefore to define briefly the basic features of protein structure, is to define the basic structural requirements of enzymes.

Since proteins are macro-molecules consisting of one or more flexible chains with molecular weights up to and beyond one million, their structure is inherently complex. However discussion of the topic is simplified by defining four levels of structural organisation, namely, primary, secondary, tertiary and quaternary structure.
Proteins consist basically of sequences of amino acid residues covalently linked by peptide bonds to form long chains, and this covalent bonding constitutes the primary structure. There are about 20 or so commonly occurring amino acids which yield long chains of virtually limitless structural variations. The presence of a free sulphydryl group in one of these amino acids, cysteine, also gives rise to cross-linking, by the formation of a disulphide bond between two adjacent molecules.

Further structural organisation of the flexible polypeptide chains results from hydrogen bond formation between the carboxyl oxygen and the amide hydrogen atom. This imposes the secondary structure and the variety of conformations that arise can be broadly classified as either helical or sheet structures.

Interaction of the side chains of the various constituent amino acid residues via hydrogen bonding, Van der Waals interactions, charge transfer forces, salt linkages etc., also give rise to other conformational variations and the resultant folding and superfolding is termed the tertiary structure.

Finally, intermolecular interactions between protein molecules themselves can yield aggregates of individual molecules which define the quaternary structure.

Structural determinations on several isolated enzymes have been carried out by various techniques including amino acid sequence analysis and X-ray crystallography. The first success of this kind was achieved by Sanger on the hormone insulin which is concerned with carbohydrate metabolism. The primary structure here was shown to contain 51 amino acid residues arrayed in two chains connected by two disulphide bonds. One of the most widely studied is lysozyme, the enzyme found in many animal and vegetable tissues and secretions, and to a very marked extent in egg-white. It is a single polypeptide chain containing 129 amino acid residues and 4 disulphide bonds. To illustrate the complexity of enzyme structures figures 1a and 1b have been reproduced in the text.
Figure 1a  Schematic diagram of the primary structure and disulphide bonds of lysozyme. - P.Jolles, Proc.Roy.Soc., 167, 349 (1967).

Figure 1b  Schematic diagram of the main chain conformation of lysozyme. (Shaded rectangles represent disulphide bonds; numbers refer to individual residues, counting from the N terminus) - C.C.F.Blake, D.F.Koenig, G.A.Mair, A.C.T.North, D.C.Philips, and V.C.Sarma. (42).
acid sequence and the chain conformation of lysozyme, and a more elaborate three dimensional photograph, which unfortunately cannot be reproduced here, has been published by Harto and Rupley following X-ray diffraction on the isolated enzyme. This latter photograph has special significance here since it illustrates the structural variations in the enzyme surface which are created by tertiary structure (i.e. flat, well cleft etc. - see page 28.)

Accepting that similar complete structural analysis on the desaturase enzyme is still some way off, any knowledge about the structure must be obtained by deduction from results already available, and by well devised, indirect experimentation designed to investigate such factors on the spatial environment during the desaturase reaction.

Considering the former; it is apparent that for such a highly specific reaction to take place, a high degree of rigidity must be induced into the polymethylene chain of the substrate by the enzyme.

The positional specificity could be explained by direct attachment or some specific association of the substrate to the enzyme and such attachment would assist in the 'tying down' of the substrate molecule. With this in mind, Howling carried out extensive studies to determine what feature of the carboxylic acid chain controlled the position of dehydrogenation. This was achieved by incubating a homologous series of labelled carboxylic acids from decanoic to nonadecanoic acid in yeast, plant and animal systems. By subsequent bond position determinations on the isolated products Howling showed that, in all cases where desaturation took place, the position of the double bond was invariably specific and at the 9, 10-position (except for some 7, 8 desaturation in Chlorella vulgaris for the shorter chain homologues). This showed conclusively that the position of dehydrogenation is dependent only on the distance between the carboxyl group and the 9-10 bond, the distance between the methyl-ol and

- 26 -
the 9 -10 position having no effect. This indeed, indicates some form of
direct attachment of the carboxyl-end of the acid with the dehydrogenating
enzyme complex, either through the CoA or ACP thiol ester, or by acyl
transfer directly to the enzyme.

Since the polymethylene chain is so flexible, one must implicate
some additional force(s), besides the 'carboxyl coupling', to induce the
rigidity required for such a specific reaction. It is impossible to have
any primary bonding between the chemically inert polymethylene chain and the
enzyme surface. Also since the hydrogen atoms of the substrate chain are
non-acidic, the possibility of hydrogen bonding as a means of inducing
rigidity can be excluded. Therefore any chemical association of the
substrate chain and the enzyme must be due to weak intermolecular forces.
Of these forces, only the London dispersion forces seem probable since the
polymethylene chain of the substrate possesses no permanent dipole. This
attractive force arises from the tiny instantaneous dipoles caused by the
vibration of electron clouds in the vicinity of the nuclei say of the
substrate. These dipoles induce dipole moments in neighbouring atoms in the
enzyme and the latter interact with the original dipoles to produce an
attractive force between the two. This force is inversely proportional
to the seventh power of the distance between the atoms of the substrate
and the enzyme, and whilst allowing for its summation across all the \(-\text{CH}_2-\)
groups, the total force may still be insufficient to hold the substrate with
the rigidity the reaction demands.

Having expounded the possibilities for chemical interaction,
further rigidity requirements must be produced by structural restraints.
The degree of structural restraint and in general the spatial environment
of the substrate during enzymic dehydrogenation is another unknown with which
this section is concerned. The two possible extremes of spatial environ-
ment are one in which the enzyme completely surrounds the substrate, to
one where the substrate lies on the surface of the enzyme. The possible
structural restraints in the latter case are as considered above.
The former could arise if the enzyme was a 'well' shaped complex into which the substrate fitted, the carboxyl attachment being at the bottom and the 'well' engulfing up to at least C.10 of the substrate chain. Here the active site would be in the cylindrical walls of the well at a specific distance from the point of carboxyl attachment. This system could easily apply the constraint required by the reaction.

However such an enclosed structural environment may well not be required to induce the necessary rigidity required for the dehydrogenation reaction. Sufficient restraint could possibly be achieved by the enzyme having a 'cleft' like structure into which the substrate slotted or layed during desaturation. Together with the increased structural restraint afforded by this arrangement over the 'flat' enzyme system, the closer proximity of the substrate and enzyme molecules would also increase the London dispersion interactions.

An attempt was made in this thesis to resolve this 'cleft' versus 'well' speculation and also increase the knowledge of the spatial environment of the enzyme-substrate complex. This spatial confinement at each position along a C18 substrate molecule undergoing enzymic dehydrogenation, was investigated through incubations involving the positionally isomeric series of mono-methylstearic acids. This series was chosen because the methyl group was of a suitable size to critically test the space available around each of 17 sites along the enzyme surface. Also the methyl group is chemically inert and would therefore not interact to any significant degree with the enzyme surface.

In order to carry out the investigation it was necessary initially to synthesize the series of racemic, positionally isomeric, mono-methylstearic acids radio-labelled at some point along the chain.

The general problem of synthesizing branched chain acids received practically no attention from organic chemists until about 40 years ago
when Adams and his collaborators\(^{92-94}\), in search of a substitute for 
chaulmoogra oil in the treatment of leprosy\(^{95}\), prepared several series of 
higher branched chain acids.

About the same period, evidence for the natural occurrence of 
a higher branched chain fatty acid in the lipids of acid-fast bacteria was 
found\(^{96}\) and later shown to be D(-) \(-\) 10 - methylheptadecanoic acid\(^{97}\). Much 
of the subsequent interest arose from the discovery by Sabin\(^{98}\) that some of 
those acids, injected into animals, caused cellular responses similar to 
those found in tuberculosis. Since these early findings numerous examples 
of mono and polysubstituted methyl branched acids have been recorded in 
many systems, e.g., wool fat\(^{99,100}\), mutton and butter fat\(^{101}\), shark liver 
oil\(^{102}\), ox fat\(^{103}\) and sebum of human forearm\(^{104}\).

Following the work of Adams and his collaborators considerable 
research has been carried out into synthesizing branched, long chain, fatty 
acids. It is not possible here to deal with all the recorded methods of 
synthesis, so discussion is restricted to those of proven practical use.

The malonic ester synthesis is of use when a 2-methyl or 3-methyl 
substituted acid is required. In the former, introduction of the methyl 
group by reaction of methyl bromide with sodium-malonic ester should 
precede the introduction of the long alkyl chain since this lessens the 
separation problems (Organic syntheses - 1943). In the latter case, mono-
alkylation of the malonic ester with secondary bromides (or iodides) leads 
to the 3-methyl substituted acids\(^{105,106}\). The preparation of secondary 
bromides of long chain hydrocarbons (5 or more carbon atoms) may involve 
some isomerization, thus reducing the purity of the final product\(^{107}\).

Another method of obtaining the 3-methyl substituted acids is 
from methyl hydrogen \(\beta\)-methylglutarate, adding the desired hydrocarbon 
chain onto the free carboxyl end of the half ester by use of one of the 
standard chain lengthening methods (see reviews by Gunstone\(^{108,109}\) and 
Gensler\(^{110}\)). Other methods, for example, from methyl ketones via 
Reformatsky reaction\(^{107}\) and through addition of Grignard reagents to
s-butylcrotonate have been used.

4-Methyl substituted acids may be obtained by chain extension of the 3-methyl derivative via nitrile. Alternatively the Arnlt-Eistert synthesis (Organic Syntheses -1942) is very good on a small scale reaction. Cason and coworkers have also described a six stage reaction affording the 4-methyl derivative in 57% yield.

Where the methyl side chain is required in the 5 position or further from the carboxyl group chain extension of a lower substituted acid is sometimes employed. (See reviews by Gunstone & General). A few examples are noted below:

i) Ställberg - Stenhagen synthesized 9-methyloctadecanoic acid from 2-methylundecanoic acid via the $\beta$-oxo ester method, a seven stage reaction. Alternatively, thiophene may be used as the coupling unit in a five stage reaction.

ii) Chain extension of 3-methylbutyric acid and 3-methylpentanoic acid affords long chain iso and anteiso-acids.

Another novel synthesis of mid-chain methyl substituted acids is described by Loese & Raphael. This six stage reaction involves the interaction of the sodium derivative or Grignard complex of an alkylacetylene and a methyl ketone. Also Hurn described a synthesis involving the chain extension of a Grignard reagent from a methyl substituted bromo-alkane, by a cycloalkyne.

However, most of these methods are lengthy often involving intricate multi-staged reactions. Also for the purity of product required here separation at intermediate stages would often be necessary, resulting in a rather poor final yield. For these reasons extensive use was made of the mixed Kolbe electrolytic synthesis as a means to synthesizing mid-chain methyl-branched fatty acids.

This anodic synthesis was first observed by Kolbe who obtained carbon dioxide and a hydrocarbon by electrolysis of an alkali metal carboxylate.
Extension of the reaction to the electrolysis of an acid and a half ester of a carboxylic acid was carried out by Woodon \(^{121}\), who subsequently extended it to the preparation of branched chain fatty acids \(^{122}\) and showed that the reaction took place without racemization \(^{123}\). The reaction afforded a mixture of two symmetrical and one unsymmetrical product the latter being the monocarboxylic fatty ester. Separation of the products is easy and this together with its directness and versatility explain its use in the synthesis of many and diverse fatty acids. An excellent review of this reaction has been published \(^{124}\) and only details pertinent to the synthesis of branched chain fatty acids will be considered here.

The reaction is normally carried out in a glass cell with platinum electrodes \(^{125}\). The reactants were dissolved in methanol with sufficient sodium added to allow ionization \(^{126}\), and a DC current in the region of 120 volts was applied. By this method and with apt choice of reactants it was possible to prepare virtually all the products required.

The presence of an \(\alpha\)-substituent in either of the reacting acids greatly reduces the obtainable yield \(^{126}\) and thus the method was unsuitable for the preparation of 2-methyl octadecanoate. An ideally suited four stage reaction, described by Gorson and Schlenk \(^{127}\), was used in this case as it directly afforded the required labelled acid from \(1\, ^{14}C\) stearic acid in good yield.
RESULTS AND DISCUSSION

A. Synthesis of Precursors for Structural Investigation.

In order to examine the spatial environment of the substrate molecule in combination with the desaturase enzyme a series of fatty acids bearing an inert side chain of a suitable size, were required as precursors for investigatory incubations. Such a group, namely the methyl group, was chosen and the series of positionally isomeric, racemic methylstearic acids was prepared with a $^{14}$C label near to the carboxyl end to facilitate identification and determination of results.

Investigation of the various methods of attaining the series (pages 29-31) indicated the Kolbe electrolytic synthesis to be, generally, the quickest route to the extremely pure precursors required to conduct the biochemical studies. Since only small quantities of radiolabelled substrates were required the relatively low yields of the Kolbe synthesis did not detract from its suitability. Also, the choice of reactants resulted in a mixture of products, widely differing in polarity, from which the required product was easily separable, in a state of high purity, by column chromatography or preparative TLC. Thus the Kolbe synthesis was chosen in preference to the alternative methods and used extensively throughout the preparative work.

Methods involving Kolbe electrolysis

The reaction first observed by Kolbe in 1849, involves the anodic oxidation of two carboxylic acids, and for many years it was mainly restricted to the electrolysis of saturated acids or half esters of $\omega$-$\omega'$-dicarboxylic acids. As such, the reaction provided convenient routes to hydrocarbons and diesters which were otherwise rather inaccessible in pure state$^{128}$. To obtain an ester of monocarboxylic acid as the product, the reactants must include a mixture of both a monocarboxylic acid and a half ester of a dicarboxylic acid, and the first comprehensive study of the scope of this mixed Kolbe reaction was carried
out by Weedon et al.\textsuperscript{121}. This latter adaptation of the Kolbe electrolytic synthesis formed the basis for the preparation of all but two of the methyl branched precursors.

Considering the Kolbe reaction in more detail; the electrolysis of a monocarboxylic acid (RCOOH) and a dicarboxylic half ester (HOOCR'COOCH\textsubscript{3}) gives a mixture of three products and carbon dioxide at the anode, hydrogen being released at the cathode.

Viz:

\[
\begin{align*}
\text{RCOOH} + \text{HOOCR'COOCH}_3 & \rightarrow \text{R.R} \quad \text{(II)} \\
& \rightarrow \text{CH}_3\text{OOCH.R'COOCH}_3 \quad \text{(III)}
\end{align*}
\]

The desired product (I) is formed by unsymmetrical or mixed coupling and products (II) and (III) are the symmetrical byproducts. By stoichiometry, electrolysis of equi-molar quantities of the two reactants produces equi-molar amounts of all three products. However maximal conversion of either reactant to product (I) may be obtained if the other reactant is added in large excess\textsuperscript{110}. This minimizes the possibility of two molecules of the minor reactant being in 'coupling proximity' at the anode.

Since radiolabelled substrates were required for the biochemical studies, the reactants of the Kolbe electrolysis were chosen so as to produce the appropriate methyl-branched heptadecanoate on electrolysis. Labelling of the product and correct positioning of the methyl branch was then achieved by chain extensions with (\textsuperscript{14}C) potassium cyanide to give the required (1 - \textsuperscript{14}C) methylstearic acid.

In the preparations carried out by the Kolbe method the introduction of the methyl side chain was achieved, with one exception, by the reaction of a suitable monobasic acid with the half ester of a dicarboxylic acid, namely, methyl hydrogen \( \beta \)-methylglutarate. Of fifteen methylstearic acids obtained via Kolbe electrolysis, twelve required two such...
reactions in sequence and only three were accessible from a single electrolysis. The reactants and products in these three cases are listed in table 1a:

**Table 1a**

Reactants and products in synthesis of mono-methylstearates.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>CH$_3$(CH$<em>2$)$</em>{12}$COOH</td>
<td>HOOC CH$_2$CH$_2$COOH</td>
<td>CH$_3$</td>
<td>3Me C$_{17}$b</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>CH$_3$(CH$_2$)$_3$COOH</td>
<td>HOOC(CH$<em>2$)$</em>{12}$COOH</td>
<td>CH$_3$</td>
<td>16MeC$_{17}$</td>
</tr>
<tr>
<td>14</td>
<td>1</td>
<td>CH$_3$(CH$<em>2$)$</em>{12}$COOH</td>
<td>HOOC CH$_2$CH(CH$_2$)$_2$COOH</td>
<td>CH$_3$</td>
<td>(5-$^{14}$C)3MeC$_{18}$</td>
</tr>
</tbody>
</table>

a. Prep. No. refers to the numbers of each preparation in the experimental section.

b. 3MeC$_{17}$ refers to methyl 3-methylheptadecanoate.

Notes

<table>
<thead>
<tr>
<th>Prep. No.</th>
<th>Acid</th>
<th>Reactants</th>
<th>Half Ester</th>
<th>Product After Chain Extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CH$_3$(CH$<em>2$)$</em>{12}$COOH</td>
<td>HOOC CH$_2$CH$_2$COOH</td>
<td>CH$_3$</td>
<td>3Me C$_{17}$b</td>
</tr>
<tr>
<td>12</td>
<td>CH$_3$(CH$_2$)$_3$COOH</td>
<td>HOOC(CH$<em>2$)$</em>{12}$COOH</td>
<td>CH$_3$</td>
<td>16MeC$_{17}$</td>
</tr>
<tr>
<td>14</td>
<td>CH$_3$(CH$<em>2$)$</em>{12}$COOH</td>
<td>HOOC CH$_2$CH(CH$_2$)$_2$COOH</td>
<td>CH$_3$</td>
<td>(5-$^{14}$C)3MeC$_{18}$</td>
</tr>
</tbody>
</table>

Prop. No. 1 was the basic preparation used in each synthesis, involving the incorporation of the methyl branch from methyl hydrogen β-methylglutarate. The half ester was obtained from β-methylglutaric anhydride by methanolysis.

Prop. No. 12 - In this preparation the methyl branch was incorporated from 4-methylvaleric acid.

Prop. No. 14 - Here (1-$^{14}$C) myristic acid was chain extended with non-radioactive potassium cyanide to give (2-$^{14}$C) pentadecanoic acid. This radiolabelled product was then electrolysed with a vast excess of methyl hydrogen β-methylglutarate to give the required radiolabelled product direct.
The remaining twelve syntheses required two Kolbe reactions to attain the mono-methylheptadecanoate (Scheme 1). The reactants of the first electrolysis were always a monobasic acid (IV) of selected chain length and methyl hydrogen β-methylglutartate (V). The 3-methyl carboxylic ester (VI) resulting from such a reaction was isolated from the two byproducts and hydrolysed to the acid (VII). The 3-methyl carboxylic acid was then used as the monobasic acid in a further electrolysis, together with a suitable half ester of a dicarboxylic acid (VIII) to give the racemic methylheptadecanoate required (IX) viz:-

**Scheme 1**

\[
\begin{align*}
\text{RCOOCH} + \text{HOOC CH}_2\text{CH}_2\text{COOC}_3 & \rightarrow \text{RCI}_2 \text{CH} \text{CH}_2 \text{COOC}_3 + \text{R} \text{R}. + \text{CH}_3\text{OOC CH}_2\text{CH(CH)}_2\text{CHCOOC}_3 \\
\text{Kolbe A} & \rightarrow \text{RCH}_2\text{CH}_2\text{COOC}_3 + \text{R} \text{R} + \text{CH}_3\text{OOC CH}_2\text{CH(CH)}_2\text{CHCOOC}_3 \\
\text{VI} & \text{Byproducts} \\
\text{1. Isolation} \\
\text{2. Hydrolysis} \\
\text{RCH}_2\text{CH CH}_2\text{COOH} & \text{VII} \\
\text{RCH}_2\text{CH}_2\text{COOH} + \text{HOOC R COOC}_3 & \text{VIII} \\
\text{Kolbe B} & \rightarrow \text{RCH}_2\text{CH}_2\text{R COOC}_3 + \text{Two byproducts} \\
\text{IX} \\
\end{align*}
\]

The appropriate monobasic acid (IV) and dicarboxylic half ester (VIII) for each reaction was as detailed in table Ib overleaf :-

- 35 -
Reactants and products in synthesis of nono-methylstearates.

<table>
<thead>
<tr>
<th>Prep No.</th>
<th>Kolbe A R</th>
<th>Product VI</th>
<th>Kolbe B R'</th>
<th>Product IX</th>
<th>Product After Chain Extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>CH₃(CH₂)₁₁</td>
<td>3MeC₁₆ b</td>
<td>CH₂</td>
<td>4MeO₁₇</td>
<td>(1⁻¹⁴C)₅MeC₁₈</td>
</tr>
<tr>
<td>3</td>
<td>CH₃(CH₂)₁₀</td>
<td>3MeC₁₅</td>
<td>(CH₂)₂</td>
<td>5MeC₁₇</td>
<td>(1⁻¹⁴C)₆MeC₁₈</td>
</tr>
<tr>
<td>4</td>
<td>CH₃(CH₂)₈</td>
<td>3MeC₁₃</td>
<td>(CH₂)₄</td>
<td>7MeC₁₇</td>
<td>(1⁻¹⁴C)₈MeC₁₈</td>
</tr>
<tr>
<td>5</td>
<td>CH₃(CH₂)₇</td>
<td>3MeC₁₂</td>
<td>(CH₂)₅</td>
<td>8MeC₁₇</td>
<td>(1⁻¹⁴C)₉MeC₁₈</td>
</tr>
<tr>
<td>6</td>
<td>CH₃(CH₂)₆</td>
<td>3MeC₁₁</td>
<td>(CH₂)₆</td>
<td>9MeC₁₇</td>
<td>(1⁻¹⁴C)₁₀MeC₁₈</td>
</tr>
<tr>
<td>7</td>
<td>CH₃(CH₂)₅</td>
<td>3MeC₁₀</td>
<td>(CH₂)₇</td>
<td>1₀MeC₁₇</td>
<td>(1⁻¹⁴C)₁₁MeC₁₈</td>
</tr>
<tr>
<td>8</td>
<td>CH₃(CH₂)₄</td>
<td>3MeC₉</td>
<td>(CH₂)₈</td>
<td>1₁MeC₁₇</td>
<td>(1⁻¹⁴C)₁₂MeC₁₈</td>
</tr>
<tr>
<td>9</td>
<td>CH₃(CH₂)₃</td>
<td>3MeC₇</td>
<td>(CH₂)₁₀</td>
<td>1₃MeC₁₇</td>
<td>(1⁻¹⁴C)₁₄MeC₁₈</td>
</tr>
<tr>
<td>10</td>
<td>CH₃(CH₂)₂</td>
<td>3MeC₆</td>
<td>(CH₂)₁₁</td>
<td>1₄MeC₁₇</td>
<td>(1⁻¹⁴C)₁₅MeC₁₈</td>
</tr>
<tr>
<td>11</td>
<td>CH₃(CH₂)₁</td>
<td>3MeC₅</td>
<td>(CH₂)₁₂</td>
<td>1₅MeC₁₇</td>
<td>(1⁻¹⁴C)₁₆MeC₁₈</td>
</tr>
</tbody>
</table>

a. Prep No. refers to the number of each preparation in experimental section.

b. 3MeC₁₆ refers to methyl 3-methylhexacontanoate and similarly for other examples.

Notes

1. The dicarboxylic half esters (VIII) required for the second Kolbe electrolysis were prepared, either by methanolysis of the anhydride (Prep No. 3), or by fully methylating the diacid and then partially hydrolysing with one equivalent of potassium hydroxide in methanol (Prep Nos. 2 and 4 - 11). Isolation of the half ester from the diester and diacid was usually achieved by the hot petroleum ether method (Prep Nos. 6 - 9) or by column chromatography on silica using chloroform - methanol as the eluent (Prep Nos. 10 and 11).
Kolbe electrolyses may be carried out in either aqueous or anhydrous methanolic solutions and different mechanisms probably operate in the two media. In aqueous solutions, optimum yields are obtained with a high current density and a high concentration of sodium salt whilst maintaining the medium at a low temperature. Under these stringent conditions side reactions, leading in particular to the formation of alcohols and olefins, are reduced to a minimum. In methanolic solutions the reaction conditions are less critical and side reactions leading to unexpected byproducts are not so prevalent. Thus the optimum yield is rather higher than for the corresponding reaction in aqueous solution.

For the reasons outlined above, all the Kolbe electrolyses were conducted in methanolic solutions and the reactants were dissolved in anhydrous methanol to give an approximate concentration of 10% w/v. 5% of the total acids were neutralised with sodium metal to produce the ions that carried the current. (By neutralising only a small portion of the total acids it was possible to detect when to terminate the electrolysis, since the alkalinity of the solution rose sharply on nearing completion of the reaction.) The electrolyte was contained in a glass cell and 120 volts DC was passed between the electrodes. These electrodes comprised a group of two small platinum foils placed a few millimetres apart and a current between 0.2-0.3 amps was maintained by periodically reversing the current. The electrolyses were exothermic, and because side reactions became increasingly predominant at higher temperatures, the glass cell was water jacketed and had cooling coils incorporated. This measure allowed the temperature of the electrolyte to be kept below 50°C. The reactions usually took about 50% - 100% longer than the time calculated from the current and the amounts of acids employed.

The selection of reactants for the anodic synthesis of the mono-methylheptadecanoates was influenced by the fact that acids possessing an alkyl substituent in the α-position have been shown to give small or
negligible yields of coupled products in the Kolbe reaction\textsuperscript{132-134}. However alkyl substituents further removed from the carboxyl group do not appear to exert similar adverse effects. This view has been demonstrated in specific experiments by Farmer et al.\textsuperscript{132} and was to a certain extent substantiated by the experiments conducted for this thesis. Moderate, predictable yields were achieved in the majority of electrolyses which included reactants with straight chains and with a methyl substituent at the $\beta$ or $\gamma$-position of the alkanolic chain. However the second Kolbe in the preparation of (1-\textsuperscript{14}C) 5-methylstearic involving a reaction between 3-methylhexadecanoic acid and methyl hydrogen malonate produced a yield (15%) which was much lower than the predicted. This probably arose from the $\alpha$-carbon atom of the malonate half ester being substituted with a carboxethoxy group. $\alpha\beta$ and $\beta\gamma$-unsaturated oils\textsuperscript{133,134} also have a profound effect on the yield of the reaction, though unsaturated centres further removed from the carboxyl group cause no difficulty. These findings, together with the literature cited, inferred that it would be impractical to prepare 2-methylstearic acid by the Kolbe method (from 2-methylmalonate half ester) and so an alternative preparation was carried out which is discussed later (page 41).

Methods involving Radiolabelling

With the exception of (5 - \textsuperscript{14}C) 3-methylstearic acid, all the Kolbe electrolyses carried out were designed to yield the methyl-branched heptadecanoate. This measure allowed for the necessary radiolabelling step to be achieved during chain extension of the product to the appropriate methyl-branched stearic acid.

The two most frequently recorded methods of achieving fatty acids labelled with isotopic carbon involve either treatment of the alkyl magnesium halide with $\textsuperscript{14}CO_2$\textsuperscript{135} or reaction of the alkyl halide\textsuperscript{136}, mesylate\textsuperscript{137} or tosylate\textsuperscript{138} with K$\textsuperscript{14}$CN. Both types of reaction have been used successfully with saturated and unsaturated fatty acids\textsuperscript{139}, and in the course of this work extensive use was made of the labelled potassium
cyanide route.

Conversions of the alkyl chloride$^{140,141}$, bromide$^{142}$ and iodide$^{143}$ have been shown using potassium cyanide but in this instance the p. toluenesulphonate was used as the co-reactant.

The reactions were carried out in dimethyl sulphoxide at 90°C since these conditions had been shown to give a cleaner reaction, with higher yields, than the corresponding reactions in more conventional solvents such as aqueous alcohol$^{141,144}$.

However, despite the careful choice of reaction conditions to give the maximum conversion, this labelling step gave considerable difficulty, and yields of the radiolabelled nitrile were generally low. Furthermore, the reason for these low radiochemical yields was initially not easily discernible, since the reactions were perfectly homogeneous and the p. toluenesulphonate was always present in considerable excess. A possible explanation was sought in a series of trial reactions with unlabelled potassium cyanide but in every case almost quantitative yield was recorded. Thus it appeared that the radiolabelled potassium cyanide might be impure.

This latter view was confirmed by radio-TLC analyses of the products from the labelling step. These indicated two major radiolabelled impurities (comprising sometimes as much as 50% of the radioactivity); one of these was very polar and remained at the origin in polar solvents and the second was slightly less polar. On TLC analysis of the unlabelled potassium cyanide reaction product, no component of detectable mass could be found in the region of the radioactive impurity. Thus it was concluded that the impurities were artefacts of the radiolabelling preparation alone, a view which is not without support$^{145}$.

The radioactive impurities were isolated and some attempts were made to identify them. Their resistance to acidic or basic hydrolysis ruled out the possibility of them being amides or imides, isocyanates or isonitriles. Also their polarity remained unchanged after reaction with
diazomethane and therefore any possibility of free acid occurring by hydrolysis was discounted. However, in view of the very small masses involved, more exhaustive investigations were not possible and further information could only be speculative.

Isolation of the nitrile, or the ester after acid methanolysis of the nitrile, was relatively easy due to the greater polarity of the byproducts and so, apart from the loss of yield, their presence was no serious handicap.

Methods involving chain extension

In all cases, with the exception of \( (1-^{14}C) \) 2-methylstearic acid, the preparation of the radiolabelled precursors involved chain extension of an intermediate product by one carbon atom. To achieve this homologation, use was made of a five stage reaction sequence viz:

\[
\begin{align*}
\text{RCOOH} & \xrightarrow{\text{LIAH}} \text{RCH}_2\text{OH} \\
\text{or RCOOCH}_3 & \\
\text{RCH}_2\text{CN} & \xrightarrow{\text{KCN}} \text{RCH}_2\text{O Tosyl} \\
\text{RCH}_2\text{COOCH}_3 & \xrightarrow{\text{KOH}} \text{RCH}_2\text{COOH}
\end{align*}
\]

Although reduction of carboxylic acids with lithium aluminium hydride can be achieved\(^{146}\), comparable reduction of the ester to the corresponding alcohol is known to proceed more readily\(^{147}\). Therefore in the two cases where the methyl ester was not the product of the previous reaction, namely in the preparations of \( (2-^{14}C) \) nonadecanoic acid and \( (5-^{14}C) \) 3-methylstearic acid, esterification of the carboxylic acid proceeded the reduction step. This was achieved by the action of an ethereal solution of diazomethane on the carboxylic acid dissolved in methanol. A methanolic solution was required, since without methanol complete conversion to the ester cannot be guaranteed\(^{148}\).
Subsequent reduction of the esters (purified by preparative G.L.C.) to the corresponding alcohols with lithium aluminium hydride was quick and clean, giving virtually quantitative yields of a product which did not require purification prior to the next step in the sequence.

This series of alcohols was converted to the p-toluenesulphonate esters by reaction with p-toluenesulphonyl chloride in dry pyridine\(^{149}\). The reaction was not as quick or clean as the last, often requiring 24 hours or more at room temperature to go to completion. Also an appreciable quantity of alkyl chloride was formed, though this could be reduced by lowering the reaction temperature to \(4^\circ C\) and extending the reaction time\(^{150}\). However, as the chloride undergoes conversion to the nitrile by reaction with potassium cyanide\((140,141)\), this latter precaution was considered unnecessary.

Alternatively, the alcohols could have been converted to the mesylates by reaction with methanesulphonyl chloride\(^{151}\) since the homologation step is known to proceed equally well with mesylates as tosylates\(^{137}\). However, determination of the conversion of alcohol to mesylate would have been difficult to follow due to their similar migration characteristics on thin layer chromatograms in the solvent system commonly used at that time. No such difficulty was experienced with the tosylation reaction, the p-toluenesulphonate being easily separable from the alcohol by TLC in 20% other/petroleum ether other.

Homologation was performed by reaction of the p-toluenesulphonate with potassium cyanide in dimethylsulphoxide\(^{140}\). In the majority of preparations radionlabelling was attained simultaneously by the use of \(^{14}C\) potassium cyanide and this requirement formed an integral part of the decision to use this mode of homologation. For convenience the reaction was also adopted for the chain extension of radionlabelled tosylates with non-labelled potassium cyanide, i.e. (\(^{14}C\)-)myristyl tosylate and (\(^{14}C\)-)stearyl tosylate. Both these latter reactions gave relatively good yields.
compared with the corresponding reactions with labelled potassium cyanide, discussed above.

In the preparations involving chain extension of a labelled tosylate with unlabelled potassium cyanide, the product was reasonably pure with the radioactivity concentrated in the nitrile region on TLC analysis (cf., reactions with (\(^{14}\text{C}\))KCN, page 39).

The final step of the reaction sequence involved the generation of the methyl ester by reaction of the impure nitrile with 25% H/V of hydrogen chloride gas in methanol\(^{137}\). Purification of the labelled methyl ester so obtained was achieved by TLC, and the product was checked by G.L.C. If any minor impurities were present, preparative G.L.C of the product was carried out.

The labelled, mono-methylstearic acids were generated from their corresponding esters by hydrolysis with 5% potassium hydroxide in methanol.

Considerable care was required throughout the homologation sequence to minimize material losses since very small masses of the methyl-branched compounds were being handled.

**Synthesis of (\(^{14}\text{C}\)) 2-methylstearic acid**

The presence of an alkyl substituent \(\alpha\)-positioned with respect to the carboxyl group prohibited the efficient use of the Kolbe electrolysis for the preparation of 2-methylstearic acid (page 37 and 38). Thus it was necessary to utilize another method by which the desired product could be realised. The traditional route using methylmalonate\(^{152}\) was considered, but rejected for a more recent and more elegant method involving the Favorsky rearrangement\(^{153,154}\). The reaction, described by Gerson and Schlunk\(^{155}\), was used to produce 2-methylstearic acid from stearic acid. In this particular instance (\(^{14}\text{C}\)) stearic acid was reacted, yielding the required radio-labelled product directly. Thus a separate chain extension and radio-labelling sequence was not incurred.

The reaction sequence was as follows:-
R.CH₂⁺COOH   \( \xrightarrow{(COCl)^2} \)   R.CH₂⁺COCl   \( \xrightarrow{CH₂N₂} \)   R.CH₂⁺COCH₂N₂

\[ \text{HCl} \xrightarrow{\text{Favorsky rearrangement}} \text{R.CH₂⁺COH₂Cl} \]

\( ^*C = ^{14}C \)

The reaction of carboxylic acids with oxalyl chloride in benzene is a well established reaction\textsuperscript{156} yielding the acid chloride in essentially quantitative yield. The (1-\( ^{14}C \)) steroyl chloride obtained was reacted with diazomethane to provide the diazoketone which was subsequently treated with a saturated HCl/ether solution to yield heptadecyl chloromethyl ketone. This was then reacted with an aqueous ethanolic solution of potassium hydroxide to effect the Favorsky rearrangement. Purification was carried out on the esterified product and subsequent hydrolysis of the pure product gave an overall radiochemical yield of 22% for the six stage reaction.

The generally accepted mechanism for the rearrangement of chloromethyl ketones cites a cyclopropane compound which is cleaved to give a carbocation transient to the \( \alpha \)-methyl acid formed\textsuperscript{154,156}. Although both primary and secondary carbocations are possible, the almost exclusive formation of the 2-\( \alpha \)-methyl acid is in agreement with the predicted preference for a primary carbocation\textsuperscript{154,157} viz:-

\[
\begin{align*}
\text{R.CH₂CH₂Cl} & \xrightarrow{0} \quad \text{R.CH} - \text{CH₂} \\
\text{R.CH₂CH₂⁺COOH} + \quad \text{R.CH⁺COOH}
\end{align*}
\]

\[ 0\% \quad \text{98\%} \]

Confirmation of Structure

Throughout all the preparations constant use of thin layer chromatography and gas liquid chromatography, both as purification and analytical techniques, provided continual surveillance of the reaction products.
Also analysis by infrared spectroscopy and nuclear magnetic spectroscopy helped in defining certain intermediate products. By a combination of these techniques therefore, it was possible to ensure that the reaction sequence had been followed successfully and that the desired methyl-branched fatty acid was isolated.

Proof that the chosen reaction sequence had positioned the methyl side chain in the intended position was achieved by mass spectrometry. Approximately half the series of purified methyl-branched heptadecanoates were analysed by this technique on an AEI MS 12 instrument and in each case the intended structure was confirmed. The computer plots obtained from three of these analyses, namely methyl 3-methyl, 9-methyl and 16-methylheptadecanoate, are shown in figures 2, 3, and 4.

The mass spectra of methyl-branched fatty acid esters, like their normal chain counterparts, show a number of peaks due to ionized fragments that contain an intact ester group.

The loss of one electron from the parent molecule, without further fragmentation, gives rise to a fairly large peak, and the molecular weight of the ester is derived directly from the mass over charge ratio (m/e) of this ion. In this case the peak was at m/e 298 indicating that the molecular weight of the product was in keeping with that of the methyl ester of a methyl-branched heptadecanoate.

The highest peak in the mass spectra (base peak) at m/e 74 characterised the presence of a methyl ester in each case. This ion results from a McLafferty rearrangement involving cleavage of the bond between carbon atoms 2 and 3.

\[
\text{RCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH} \rightarrow \text{RCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{+OH} + \text{CH}_2\text{OMe}
\]

In the middle-mass range between the peaks of the molecular ion (m/e 298) and the base-peak ion (m/e 74) the mass spectra illustrate a series of
C19H38O2

M. W. = 298, INDEX NO. = COL 1164

Figure 2

Mass spectrum of methyl 3-methylheptadecanoate
C19H38O2

M. W. = 298  INDEX NO. = COL 1163

Figure 3

Mass spectrum of methyl 9-methylheptadecanoate
C9H38O2
M.W. = 298, INDEX NO. = COL 1165

Figure 4
Mass spectrum of methyl 16-methylheptadecanoate
methoxycarbonyl type ions each varying by 14 m/e units from their nearest neighbours. This phenomenon characterises a polymethylene chain where each peak relates to a reduction by one - CH₂ - group. Work by Ryhage et al. has suggested that certain of these peaks do not arise by successive loss of one methylene group from the methyl end of the chain. The peak at M-43 in particular has been shown to occur by loss of the methylene groups 2, 3 and 4 (α, β and γ to the carboxyl) by a complex process of double cleavage.

Complementary to the methoxycarbonyl peaks, a series of peaks appear at the low-mass end of the spectra resulting from the generation of hydrocarbon ions, CH₃(CH₂)ₙH₂n+1.

Confirmation of both the presence and position of the methyl-branch is provided by cleavage of the bonds to each side of the tertiary carbon atom carrying the side chain. This gives rise to individual spectra, characterising each mono-methyl isomer.

In the case of methyl 3-methylheptadecanoate cleavage of the 3, 4 bond gives rise to a large peak at m/e 101 corresponding to CH₃OOCC₂H₂(CH₃)⁺ ion. Cleavage of this bond is also favoured in esters of all normal and most branched chain acids giving rise to a peak at m/e 87 (see figures 3 and 4) corresponding to the CH₃OOCC₂H₂CH₂⁺ ion. This ion is not attainable from the 3-methyl ester and so its absence from the spectrum further distinguishes this product. Other small peaks at m/e 225 (M-73) and 224 (M-74) correspond to hydrocarbon ions resulting from cleavage of the 2,3 bond. Also, the low intensity of peaks in the medium-mass area of this spectrum is characteristic of methyl ester with a side chain near the carboxyl end of the molecule.

In the spectrum of methyl 9-methylheptadecanoate (figure 3) cleavage of the bonds either side of the tertiary carbon atom give rise to methoxycarbonyl peaks at m/e 157 and m/e 185, whereas the peak normally occurring at m/e 171 is relatively small.

vize: CH₃'OOC(CH₂)₇CH₃
       157 CH₃
       185
-45-
More important diagnostically are the peaks at m/e 153 and m/e 135. Unlike the previous two ions which form part of the normal chain ester spectrum, these two fragments are a function solely of the position of substitution and are exhibited by esters where the methyl-branch is positioned from carbon atom 6 up to the anteiso position. The two peaks arise from the \( \alpha \)-cleavage ion, \( \text{CH}_3\text{OOCC(CH}_2\text{)}_8^+\text{CH(CH}_3\text{)} \), by loss of methanol to give the ketone-ion \( 0 = \text{C} = \text{CH(CH}_2\text{)}_8^+\text{CH(CH}_3\text{)} \), and subsequent loss of water. Further characteristic ions are produced by rearrangement of one and two hydrogen atoms to the other \( \alpha \)-cleavage ion \( \text{CH}_3\text{OOCC(CH}_2\text{)}_7^+ \), to give the cluster of three peaks at m/e 157, 158 and 159. Other significant peaks at m/e 143 and m/e 199 (M-99) correspond to methoxycarbonyl ions resulting from \( \beta \)-cleavage.

Primary cleavage of the tertiary carbon bonds in the iso-esters, methyl 16-methylheptadecanoate, occurs to the carboxyl side of the isopropyl group increasing the relative intensity of the methoxycarbonyl peak at m/e 255. The only other method of distinguishing this spectrum from the rather similar spectrum for methyl stearate, which has the same molecular weight, is by the small peak at m/e 233 (M-65) characterising a methyl side chain in the isopropyl configuration.

One further peak which is common to all methyl esters of fatty acids is the peak at 267 (M-31) due to the acylum ion resulting from loss of the methoxyl group.

Following chain extension of the range of methyl-branched heptadecanoates the whole range of mono-methyl substituted octadecanoates was analysed by gas liquid chromatography using silicone oil as the stationary phase. The retention time of each isomer was recorded and figure 5 shows these relative to stearic acid as a function of the position of the methyl branch. All the isomers showed greater retention than the \( \text{C}_{18:0} \) ester. The shortest retention time was the 2-methyl isomer and...
Column: SE 30 (silicone elastomer)  
Temperature: 298°C

**Figure 5.** Log\( \frac{\text{ret.time}}{\text{ret.time} \ n-C_{19}} \) for methyl esters of methyloctadecanoic acids as a function of the position of the methyl side chain.
retention volumes generally increased as the methyl-branch approached the methyl end of the chain. Two exceptions to this rule however were the 4 - methyl and 16 -methyloctadecanoates which showed comparatively long retention times.

These results are in close agreement with the findings of Ställberg-Stenhagen\(^{161}\) for the same series, and Ackman\(^{162}\) on methyl-branched acids of differing chain lengths.

**B. Precursors for stereochemical investigations**

In order to determine the relative and absolute configurations of the two hydrogen atoms removed during desaturation in the hen liver microsomal system, certain 9 and 10-hydrogen-labelled precursors were required. This requirement was fulfilled by the kind donation of precursors by Dr. L.J. Morris.

To determine relative configuration of the hydrogen atoms removed, erythro and threo - 9, 10-dideuterostearic acids were used as precursors. Basically, these were prepared by reduction of oleic and elaidic acid with deuterium labelled hydrazine hydrate in deuteromethanol at 50°C.\(^{36}\) The reaction is known to proceed by one - addition of deuterium\(^{163}\) and so oleic acid yielded erythro - 9, 10-dideuterostearic acid and elaidic acid yielded threo - 9, 10-dideuterostearic acid. Removal of any starting material from the product was achieved by \(\text{AgNO}_3\) - TLC of the esterified product and the isotopic purity was determined by mass spectrometry.

To determine the absolute configuration of the hydrogen atoms removed, D and L-9-tritiostearic acids were used as precursors. Briefly, these were prepared from D - 9-hydroxystearic acid essentially by a method described by Schroepfer and Bloch\(^{35}\). L-9 - hydroxystearate was obtained from the tosylate of D-9-hydroxy acid, by reaction with anhydrous sodium acetate in acetic acid. Both the D and L-hydroxystearates were tosylated, and then hydrogenolysis of the tosylate group was effected with lithium aluminium tritide in refluxing tetrahydrofuran. This last step also
reduced the carboxyl group to an alcohol, but oxidation of the products with chromium trioxide in acetic acid yielded the D and L-9-tritiostearic acids. To each of these was added sufficient (l-14C) stearic acid to give a 3H/14C ratio of approximately 20:1.

C. Incubation of methyl-branched precursors

In the systems studied, namely, the microsomal fraction of hen liver and Chlorella vulgaris, desaturation of the saturated carboxylic acid, stearic acid, is known to take place specifically at the 9, 10-position to produce the monoenoic acid, oleic acid. The positional specificity apparent in the systems chosen is typical of a far wider spectrum of natural systems which exhibit this total positional specificity towards desaturation, when the conditions of desaturation and the precursors are constant (pages 20 and 21).

To achieve such positional specificity necessitates the introduction of a high degree of rigidity into the carboxyl chain which is generally free to rotate about each C - C bond. This restraint probably results from a collection of forces: Evidence suggesting a strong, possibly covalent, attachment of the substrate molecule by its carboxyl-end either directly to the enzyme or via its ACP or CoA-thioester has been provided and will be discussed later. Apart from this other forces) required to align the 9 and 10-positions of the freely rotating polyethylene chain with the active site cannot be of a covalent nature. It seems probable that London dispersion forces play a part, but unlikely that they are of sufficient strength to totally restrain the chain. Therefore some physical 'fit' of the substrate into the enzyme surface is likely which, together with introducing physical constraint, would also increase chemical interaction in the form of London dispersion forces.

From incubations with the series of positionally isomeric, racemic, mono-methylstearic acids evidence regarding the presence and nature of any physical interaction between the enzyme and substrate during desaturation may be gained.
Also, in view of the close involvement of certain lipid classes in desaturation, investigations were conducted into the incorporation into lipids of the substrates and products.

i) Optimisation of Incubation conditions in the microsomal fraction of hen Liver

The first system studied was the microsomal suspension from hen liver. This system was chosen since it is known to contain the desaturase enzyme. Removal of the mitochondrial enzymes from the liver homogenate eliminated competing reactions such as breakdown and resynthesis, which would serve only to complicate the determination of results.

Two types of hen were used, namely brown Warren hens, and white shaver hens. The obtainable desaturation of fatty acids substrates varied tremendously, even between livers from the same type of hens, and was often rather low. Best results were achieved from hens fed on a balanced diet, and generally the older and more fatty the liver the greater the degree of desaturation. The variation of desaturase activity between different aliquots of any particular microsomal fraction however was undetectable providing all conditions were kept constant. Therefore it was imperative that all incubations, where direct comparisons were to be made, were conducted under identical conditions on aliquots of the same microsomal fraction at the same time.

Before valid comparison of incubations could be achieved it was necessary to determine the quantity of microsomal suspension required to attain maximum conversion of the highest mass of precursor to be used, and to ascertain whether, within the limits of substrate amount likely to be added, a constant degree of desaturation would be achieved. Thus two preliminary incubations were carried out on the microsomal fraction in which the mass of substrate was below and above the masses likely to be encountered in the series of methyl-branched fatty acids (see table 2).
Table 2.
Effect of mass of stearic acid on the degree of desaturation in the microsomal suspension of hen liver (Warren).

<table>
<thead>
<tr>
<th>Stearic acid</th>
<th>Desaturation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg</td>
<td>µmoles</td>
</tr>
<tr>
<td>10</td>
<td>0.035</td>
</tr>
<tr>
<td>250</td>
<td>0.88</td>
</tr>
</tbody>
</table>

Clearly the enzyme system used in this experiment, which was equivalent to that obtained from 1 gram of hen liver, is not saturated by these levels of added substrate, and will accommodate the varying masses of the methyl-branched precursors.

To attain the most accurate comparisons it was necessary to maximise the substrate conversion by allowing time for the incubation to go to completion. Johnson, whilst studying the inhibition of desaturation in white Leghorn hens, found that maximum desaturation was achieved by the system after 30 mins.

Table 3
Extent of desaturation of stearic acid with increasing time, in the microsomal fraction of hen liver (Warren).

<table>
<thead>
<tr>
<th>Precursors</th>
<th>Time (Hrs)</th>
<th>Desaturation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-14C stearic acid (0.035 µM)</td>
<td>0.5</td>
<td>23.0</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>34.5</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>42.1</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>46.9</td>
</tr>
<tr>
<td></td>
<td>14.0</td>
<td>50.1</td>
</tr>
</tbody>
</table>
However from the results obtained (table 3 and figure 6) it is apparent that desaturation of the substrate is not complete after 30 minutes and that continued conversion takes place for some considerable time. Desaturation is nearing completion after 4 hours, but where convenient further small increases can be obtained, with no apparent detriment, by continuing incubations overnight.

In the two experiments above oxygen was bubbled through the incubations before commencement. The requirement for this added oxygen and possible improvements attainable by varying the mode of addition were examined. The results are shown below in table 4.

**Table 4**

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Incubation Time (hrs)</th>
<th>Method of Oxygen Addition</th>
<th>% Desaturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1^{14}$C stearic acid</td>
<td>11</td>
<td>None</td>
<td>14.9</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>1 minute bubbled oxygen</td>
<td>40.1</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>1 minute bubbled oxygen repeated hourly</td>
<td>40.0</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>1 minute bubbled 70% oxygen/30% air</td>
<td>44.6</td>
</tr>
</tbody>
</table>

Clearly there is a requirement for added oxygen which is fulfilled by 'gassing' the incubation mixture for 1 minute before incubation. Repeated addition of oxygen did not further enhance desaturation, implying that the eventual decay of the system was not due to molecular oxygen deficiency. A slight increase however resulted from the use of 70% oxygen/30% air in place of 100% oxygen and therefore the former was used in subsequent incubations.

ii) Incubation of methyl-branched precursors with the microsomal fraction of hen liver

Having determined the conditions required by the microsomal fraction to maximise the conversion to monoenes, these conditions were employed in the
Table 5
Degree of desaturation of isoneric methylstearic acids by hen liver microsomal preparations.

<table>
<thead>
<tr>
<th>Precursor</th>
<th>WARREN HEN</th>
<th>SHAVER HEN</th>
<th>COUNT DISTRIBUTION (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µMoles</td>
<td>Desaturation (%)</td>
<td>Effectiveness* (%)</td>
</tr>
<tr>
<td>1-14C-stearic acid (low activity)</td>
<td>0.91</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>1-14C-2-methylstearic acid</td>
<td>0.83</td>
<td>23</td>
<td>25.8</td>
</tr>
<tr>
<td>5-14C-3-</td>
<td>0.42</td>
<td>3-4</td>
<td>90.3</td>
</tr>
<tr>
<td>1-14C-4-</td>
<td>0.17</td>
<td>12.0</td>
<td>61.3</td>
</tr>
<tr>
<td>1-14C-5-</td>
<td>0.04</td>
<td>Trace**</td>
<td>~99</td>
</tr>
<tr>
<td>1-14C-6-</td>
<td>0.06</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1-14C-7-</td>
<td>0.07</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1-14C-8-</td>
<td>0.07</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1-14C-9-</td>
<td>0.13</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1-14C-10-</td>
<td>0.12</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1-14C-11-</td>
<td>0.10</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1-14C-12-</td>
<td>0.10</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1-14C-13-</td>
<td>0.10</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1-14C-15-</td>
<td>0.18</td>
<td>~1**</td>
<td>~97</td>
</tr>
<tr>
<td>1-14C-16-</td>
<td>0.28</td>
<td>7</td>
<td>77.5</td>
</tr>
<tr>
<td>1-14C-17-</td>
<td>0.09</td>
<td>13</td>
<td>58.0</td>
</tr>
<tr>
<td>2-14C -nonadecenoic acid</td>
<td>0.83</td>
<td>23</td>
<td>25.8</td>
</tr>
<tr>
<td>High Activity</td>
<td>0.07</td>
<td>31</td>
<td>0</td>
</tr>
<tr>
<td>1-24C-stearic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The values quoted represent the effectiveness of the methyl substituent in reducing desaturation.

** Presence of monoene detected during the determination of double bond position.
DESATURATION OF ISOMERIC METHYLSTEARIC ACIDS

BY HEN LIVER MICROSONES

MONOENES FORMED EXCLUSIVELY Δ^9

18:0 18:1 = 31%
series of incubations on the radiolabelled methyl-branch stearic acids. The results obtained from these experiments are shown in table 5 and figure 7.

The degree of desaturation in each case was calculated from RGLC chromatograms of the transmethylated lipid extract, by comparison of peak areas. However in borderline cases, where the small monoene peak could possibly be obscured on the chromatogram by a large unchanged precursor peak, an alternative procedure was developed. Greater separation of the precursor and its monoene product was required and this was achieved, together with information on the position of the double bond, by subjecting the transmethylated lipid extract to von Rudloff's oxidation. This method quantitatively cleaves any monoene present to a dibasic and a monobasic acid of which only the former will be radiolabelled due to the specific position of labelling of the precursors.

Subsequent separation of the resultant radiolabelled dibasic ester and the unchanged precursor was readily achieved on RGLC, due to the large polarity difference produced. Addition of a standard series of cold dicarboxylic acid dimethyl esters to the injected mixture allowed simultaneous determination of the position of the double bond, where trace amounts of desaturation had occurred. By this procedure definite evidence of desaturation of 5-methylstearic acid and 15-methylstearic acid was achieved. The degree of desaturation of 3-methylstearic acid was also confirmed by this method.

Confirmation that the system employed was capable of dealing equally with the varying masses involved was furnished from the almost identical conversion produced in the high and low activity stearic acid control incubations. Thus accurate comparison with the other incubations in the same system was validated.

From the results obtained several observations can be made, viz:-

1. The enzyme system was unable to desaturate methylstearic acid molecules where the methyl group was situated between carbon atoms 6 and 14, and even when the methyl group was at positions 5 and 15 only slight desaturation was detected.

2. The enzyme system was capable of desaturating molecules where the methyl branch was further removed from the active site than the
5 and the 15-positions, namely 2, 3, 4 and 16, 17 and 18-methylstearic acids.

3. Considering 15, 16, 17 and 18-methylstearic acids; desaturation increased progressively and almost linearly with the distance of the methyl branch from the active desaturase site. The same would be true at the carboxyl-end of the chain, were it not for the relatively low desaturation of 3-methylstearic acid.

4. In all cases where desaturation occurred the cis double bond was inserted exclusively between carbon atoms 9 and 10 (i.e., Δ9).

5. All the methylstearic acid isomers were incorporated into polar lipids. From these observations certain factors regarding the desaturase enzyme are forthcoming:

Since all the substrates were incorporated into lipid (table 5), the variation in desaturation exhibited by the series cannot be attributed to the system's inability to activate the acids to the acyl-SCoA esters. Therefore, in those cases where no desaturation was observed, namely 6, 8, 9, 10, 11, 12 and 14-methylstearic acids, one must infer that the desaturase enzyme either was unable to accept such acids or, if binding did occur, was unable to desaturate the substrate once accommodated.

As the methyl substituent is progressively moved away from the actual desaturase site, (i.e., experiments with 8, 6 and 5-methylstearic acids and 11, 12, 14 and 15-methylstearic acids), the absence of desaturation indicates some degree of structural enfoldment of the substrate molecule by the desaturase enzyme in the vicinity of this site. The possibility of close structural proximity on only one side of the substrate by a 'flat' or 'slightly curved' enzyme surface seems unlikely. This is because the zig-zag conformation of the hydrocarbon chain would result in half of the methyl groups of the positionally isomeric and racemic substrates not interfering with the 'lay' of the substrate on the enzyme surface, and thus providing no steric reasons for complete inhibition.
A closer structural enfoldment must therefore be postulated to explain the results, and this could possibly take the form of a cleft or cylindrical enfoldment. Since the widths of the normal polymethylene chain of stearic acid is in the region of 4Å (see figure 8 based on isometric projections according to Vandenheuvel \textsuperscript{169}) and a methyl group increases this by a maximum of 1.3Å, an enclosure, created by the enzyme structure, of between 4 and 5.3 Å across, is envisaged. The limits of complete absence of desaturation in the series implies that the very close structural enfoldment, postulated above, is in the region of 12-13Å in length with the active desaturase site positioned almost centrally along its length. Also the progressive increase in desaturation as the methyl-branch is moved to either end of the stearic acid molecule would suggest a steady opening out of this enfoldment towards the extremities of the substrate molecule. Considering the various structural possibilities:

One structure, previously proposed, was a "well" in the enzyme surface into which either the carboxyl or methyl-end of the chain was inserted; in this case the desaturase site would be situated in the 'wall of the enzyme. The fact that there was not a continuation of the complete absence of desaturation, observed in the mid-chain methyl-branched isomers, when the substituent was positioned towards the extremities of the polymethylene chain, would suggest that this most rigid of enfoldments does not exist.

For similar reasons a permanently formed cylindrical shape 12-13Å in length, enclosing the desaturase site and through which the substrate molecule was positioned prior to desaturation, can be discounted. If this was the structure one could only expect either, 2, 3, and 4-methylstearic acids, or 16, 17 and 18-methylstearic acids to be desaturated, since one end of the stearic acid molecule would need to be free from methyl side groups to allow positioning of the substrate through the cylinder.

Thus it would appear most probable from the results achieved in
Isometric projection of the extended chain configuration of stearic acid.
(modified from the diagrams published by Vandenheuvel169.)
hen liver microsomes that the desaturase enzyme takes the form of a deep cleft which enforces considerable restraint on the hydrocarbon chain of the fatty acid molecule during the desaturation process. The effects of this structural restraint would be most prominent between carbon atoms 5 and 15 of the fatty acid.

Comparison of the incubations with stearic and nonadecanoic acid indicates that the Δ 9-monono was produced in each case. Accepting that positional specificity exists in this system as with many others, this result means that the distance from the carboxyl-end of the substrate is the determinate where insertion of the double bond is concerned (if distance from the methyl end was the governing feature of specificity of this desaturase enzyme, then the resultant monoenes would be Δ 9-C₁₈:₁ and Δ₁₀-C₁₉:₁). This observation is in full agreement with the results of Howling ⁷¹ and Johnson ⁴³ and suggests that some form of attachment of the carboxyl group to the desaturase enzyme exists. Previous investigations generally implicate the CoA-thiol ester of the acid as the substrate for desaturation, but it must be questioned whether a large group such as an acyl-3CoA with its many available conformations, could meet the stringent positional requirements simply by a non-bonded interaction between CoA and the enzyme protein. Therefore it seems possible that the first stage of the reaction may involve the acyl transfer of the fatty acid to a thiol group on the enzyme to form a covalent linkage, in a manner similar to the yeast synthetase system defined by Lynen ¹⁷⁰.

Considering in more detail those results where desaturation of the methyl-branched acid did occur; the steady increase in desaturation observed as the side chain approaches the methyl-end of the substrate is probably indicative of an increasing degree of spatial freedom in this region i.e. 20 - 26° from the point of carboxyl attachment of the substrate.

- 56 -
The fact that desaturation never reached the level of the control (stearic acid) suggests that some steric hindrance still exists even in the most favoured case (nonadecanoic acid).

Towards the carboxyl-end of the substrate, the occurrence of a similar pattern for desaturation, increasing with distance from the active site, is marred by the exception of 3-methylstearic acid (see figure 7). This sharp decrease in desaturation relative to 2- and 4-methylstearic acids is difficult to discern from the present results. It may be caused by some particular deformation of the normal straight chain structure, as exemplified by stearic acid. Crystal structure determination carried out by Abrahamsen\textsuperscript{171} indicate that 2-, 3- and 4-methylstearic acids all adopt a \( \delta \)-type structure in the solid state, i.e. a normal hydrocarbon chain with the carboxyl group as a branch at position 2. However, since the same structure type prevails in all three molecules, this could not explain the sharp decrease in desaturation. Also, the existence of this type of structure in the substrate-enzyme complex is obviated by desaturation yielding the \( \Delta 9 \)-nonene in all three cases. (If the \( \delta \)-type structure prevailed in the substrate-enzyme complex the position of the double bond would probably be different in all three cases and could well be \( \Delta 10 \) in the 2-methyl isomer, \( \Delta 11 \) in the 3-methyl isomer and \( \Delta 12 \) in the 4-methyl isomer (see figure 9).

Therefore, accepting that the desaturase site is a fixed physical distance from the point of carboxyl attachment, the exclusive formation of the \( \Delta 9 \)-nonene means that the three substrates under consideration must exist in the form of straight chain carboxylic acids with a methyl branch. The case of formation of such an extended chain structure in complex with the desaturase enzyme may govern the degree of desaturation. In the case of 3-methylstearic acid, enforcement of this straight chain structure would cause considerable overlap of the electron clouds of the carboxyl oxygen and the methyl group hydrogens, which fall on the same side of the chain, and the
Proposed double bond position if 2-, 3-, and 4-methylstearates adopt \(-\)-structure on desaturase enzyme.
observed decrease in desaturation may well result from the difficulty in enforcing this overlap. Alternatively the presence of an 
ecno-acid side group, in the primary enzyme structure, which is adjacent to the 3-position of a complexed substrate may be sufficient either to deflect the 9, 10-position of the polymethylene chain away from the desaturase site, or cause a rotation around the 2, 3-bond of the substrate which increases the carbonyl-methyl group interaction.

In all the incubations the precursors employed were racemic mixtures of the D and L- enantiomers. Thus the absolute specificity of this enzyme for the removal of the 9 and 10-D-hydrogen atoms (proved later in this thesis) would obviate the desaturation of 50% of the racemic 9 and 10-methylstearic acids precursors, were there not other contributory factors involved. Although possibly not so complete as this stereo-specificity, it seems quite probable that the enzyme exerts an unequal effect on the enantiomers of other positional isomers. Thus the observed desaturations in the methyl-branched series may result primarily from one enantiomer, with the other remaining virtually unchanged. This is illustrated in figure 7 (and figure 10 for Chlorella vulgaris) where the unshaded areas correspond to the extreme case where only one optical isomer acts as a substrate for desaturation, the other being totally unaffected by the desaturase enzyme.

An interesting feature to emerge, even if the effect is somewhat less than total, is that one optical isomer of 2-methylstearic acid may be a more favourable substrate for desaturation than stearic acid, in the microsomal system of hen liver. Unfortunately it was not possible to test this theory, using optically active isomers, but such a facilitation of the desaturase process might result from an increase in London dispersion forces due to interactions with the 2-methyl substituent. Such increases in interaction at the 2-position may provide a significant stimulus to the initial stages of "zipping-in" of the substrate into the enzyme cleft and could also assist in effecting carboxyl attachment to the enzyme.
iii) Incubation of methyl branched precursors with *Chlorella vulgaris*

The same series of racemic methyl-branched stearic acids was incubated with the photosynthetic green alga *Chlorella vulgaris*. This enabled information to be gained regarding the spatial environment in the vicinity of the desaturase enzyme of this micro-organism. Also it allowed comparisons to be made between the desaturase enzyme of this organism of the plant kingdom and that of the hen liver microsomal fraction.

Unlike most plants *Chlorella vulgaris* is capable of two modes of existence. In the 'rich' medium (see page 103) in which it was grown the organism exists heterotrophically, utilizing the various organic substances in the medium as a source of energy. Under such conditions added palmitate and stearate are not desaturated, myristic acid being the longest chain acid it will convert to oleate\(^\text{172}\). Thus it was necessary to remove the culture from the rich medium and resuspend it in phosphate buffer. This enforces a totally photo-synthetic existence and a change occurs in the fatty acid composition of the cells, viz; more linoleic and linolenic acid are formed which are typical acids of photosynthetic tissues\(^\text{173}\). Under these conditions the organism readily converts added stearate to the monoene and diene. However it is also capable of breaking down the saturated acid by \(\beta\)-oxidation to acetyl-CoA and re-utilizing this in de novo synthesis, which results in a randomization of the label into other fatty acids (breakdown/resynthesis).

Since this latter reaction was not under study and served only to complicate the determination of results, preliminary experiments were conducted to establish conditions where it was minimized. Under normal incubation conditions optimum desaturation with minimal breakdown/resynthesis was found to be obtained by restricting the incubation time to 6 hrs. Therefore this incubation period was adopted for all experiments involving *Chlorella vulgaris*. 

- 59 -
Consequently very little breakdown/resynthesis was evident in the two series of incubations on the methyl-branchered precursors, except for 14, 15, 16 and 17-methylstearic acids and in these cases it only amounted to a few percent (see table 6).

The results were determined by similar methods to those used in the hen liver experiments. Lipid analyses carried out on the extracted lipids of both series of Chlorella incubations indicated high activation of the precursors predominantly into polar lipids. Nonadecenoic acid was the only exception with a lipid incorporation of between 20 - 30% exclusively in polar lipids. The remainder of the methyl-branchered acids showed incorporation into lipids of between 50 - 90%, and in many cases were higher than in the control reactions (stearic acid). These results also showed that there was no correlation between lipid cyclation and desaturation.

Clearly by comparison of the results obtained for the degree of desaturation in this system (see tables 6 and 7, and figure10) with those from the microsomal fraction of hen liver, the two enzyme systems bear many similarities including the formation of a Δ9-monoene in all cases where desaturation occurred. However, these results indicate that a greater effectiveness against desaturation is exhibited, in the whole cell system of Chlorella vulgaris, by substrates in which the methyl substituent is displaced towards the extremities of the hydrocarbon chain of stearic acid.

Towards the carboxyl-end of the chain there is an almost total absence of desaturation when the methyl group is positioned at carbon atoms 2 or 3. However the desaturation of 4-methylstearic acid obviates the possibility that the reduction in desaturation is caused by any direct steric interference of the 2 or 3-methyl group with the active desaturase.

(continues on page 62)
## Table 6
Degree of desaturation of isoceronic methylstearic acids by Chlorella vulgaris

<table>
<thead>
<tr>
<th>Precursors</th>
<th>1st Series</th>
<th>2nd Series</th>
<th>2nd Series</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mole</td>
<td>Effectiveness *</td>
<td>Breakdown</td>
</tr>
<tr>
<td></td>
<td>Dosaturation (%)</td>
<td>against Dosaturation (%)</td>
<td>Resynthesis (%)</td>
</tr>
<tr>
<td>1-14C-stearic acid (low activity)</td>
<td>0.91</td>
<td>57.0</td>
<td>0</td>
</tr>
<tr>
<td>1-14C-2-methylstearic acid</td>
<td>0.83</td>
<td>~1 **</td>
<td>~98.0</td>
</tr>
<tr>
<td>5-14C-3- &quot; &quot;</td>
<td>0.42</td>
<td>~1 **</td>
<td>~98.0</td>
</tr>
<tr>
<td>1-14C-4- &quot; &quot;</td>
<td>0.17</td>
<td>5.5</td>
<td>90.5</td>
</tr>
<tr>
<td>1-14C-5- &quot; &quot;</td>
<td>0.04</td>
<td>Trace **</td>
<td>~99.5</td>
</tr>
<tr>
<td>1-14C-6- &quot; &quot;</td>
<td>0.06</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1-14C-8- &quot; &quot;</td>
<td>0.07</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1-14C-9- &quot; &quot;</td>
<td>0.11</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1-14C-10- &quot; &quot;</td>
<td>0.13</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1-14C-11- &quot; &quot;</td>
<td>0.06</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1-14C-12- &quot; &quot;</td>
<td>0.10</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1-14C-14- &quot; &quot;</td>
<td>0.10</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1-14C-15- &quot; &quot;</td>
<td>0.18</td>
<td>Trace **</td>
<td>~99.5</td>
</tr>
<tr>
<td>1-14C-16- &quot; &quot;</td>
<td>0.18</td>
<td>6.8</td>
<td>88.2</td>
</tr>
<tr>
<td>1-14C-17- &quot; &quot;</td>
<td>0.09</td>
<td>10.6</td>
<td>83.6</td>
</tr>
<tr>
<td>1-14C-18- &quot; &quot;</td>
<td>0.83</td>
<td>~1 **</td>
<td>~98</td>
</tr>
<tr>
<td>(i.e. nonadecenoic acid)</td>
<td>0.07</td>
<td>58.0</td>
<td>0</td>
</tr>
</tbody>
</table>

* The values quoted represent the effectiveness of the methyl substituent in reducing desaturation.

** Presence of monoene detected during the determination of double bond position.
DESATURATION OF ISOMERIC METHYLMETHYLCARIC ACIDS

BY CHLORELLA VULGARIS

MONOENES FORMED EXCLUSIVELY Δ9

18:0 18:1 + 18:2 = 37%

FIGURE 10
Table 7.
Labelled unsaturated acids produced from labelled saturated
acids by Chlorella vulgaris

<table>
<thead>
<tr>
<th>Precursors</th>
<th>Series 1</th>
<th></th>
<th>Series 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Δ9</td>
<td>Δ9.12</td>
<td>Δ9</td>
<td>Δ9.12</td>
</tr>
<tr>
<td>High activity 1-14C stearic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low activity 1-14C stearic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-14C 4-methylstearic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-14C 16-methylstearic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-14C 17-methylstearic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-14C 18-methylstearic acid (i.e. nonadecanoic acid)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Continued from Page 60)

Therefore it would appear that the enzyme structure contains a protrusion close to the point of carboxyl attachment which either sterically hinders the acylation of the substrate to the enzyme, or deflects the acylated substrate to such a degree that the hydrogen atoms on the 9 and 10-position are no longer juxtapositioned to the desaturase site.

The greater sensitivity of the desaturase enzyme present in Chlorella vulgaris, compared with that in hen liver, to a substrate with a methyl group at the 2-position is not thought to implicate vast disimilarities in enzyme structure, but more probably signifies the presence of single, larger amino acid side chain adjacent to this position in Chlorella.
If the mode of action does involve inhibition of the acylation of the methyl-branched substrate to the enzyme, then these results further emphasize the need for some form of carboxyl attachment as a necessary prerequisite to desaturation, and show that structural enfoldment alone cannot produce the degree of restraint and locating mechanism required to effect this highly specific reaction.

The desaturation of 4-methylstearic acid by *Chlorella vulgaris* again precludes the existence of a 'woll' or cylindrically shaped enzyme structure and serves also to illustrate the basic similarities between the desaturase enzymes of the two systems studied. Desaturation of substrates where the methyl-branch is displaced towards the terminal end of the stearic acid molecule show a steady progressive increase from C\(_{15}\) to C\(_{17}\). This phenomenon is indicative of a steady relaxation of the spatial environment in the area between 17-23\(^{\circ}\) from the first carbon atom, indicating an opening-out of the postulated enzyme cleft, which allows easier accommodation of the broadened substrate as in the hen liver microsomal enzyme. However when the methyl group is positioned on the terminal carbon atom, the stearic acid molecule is increased in length, not in width as in all the other cases. Therefore the abrupt decrease in desaturation of this substrate (nonadecanoic acid) compared with stearic acid can only be explained by a constriction in, or termination of, the enzyme cleft at a position close to the terminal end of the C\(_{18}\) molecule.

This could be due to a major difference in the enzyme protein's tertiary structure and hence probably its primary structure, compared with the hen liver enzyme, or again it could merely indicate the substitution of a single amino acid in an otherwise very similar protein. The fact that there was a transition from 50% desaturation to almost total absence of desaturation by an effective lengthening of the molecule by only 1.3\(^{\circ}\) is
further evidence of the highly specific structural environment necessary to conduct such a specific reaction.

Accepting that there are certain differences between the results from the two systems under study, overall there is sufficient correlation to imply that the basic structure of their mono-desaturase enzymes are very similar. Furthermore, the use of a whole cell culture of Chlorella vulgaris, could explain in part, or in total, the observed discrepancies and so the possibility that the two desaturase enzymes are in fact identical, cannot be overlooked.

Studying the results from both systems indicates that a well or cylindrically shaped enzyme cannot exist. Therefore to gain the high degree of restraint required, one must assume the next most structurally enfolding state prevails, which is a deep cleft into which the substrate is positioned. The sharp cut off in desaturation of carboxylic acids above chain length C₁₈ implies that the distance from the point of carboxyl attachment of the substrate to the end of this cleft is about 25-26Å (assuming that the substrate exists as an extended chain). Also the width of the cleft enfolding the region between carbon atoms 5 and 15 must be less than 5.3Å to explain the instances where complete absence of desaturation was observed. Rather less stringent spatial tolerances must be operational outside this region, though, to allow desaturation of those precursors where the methyl group was further removed from the actual desaturase site. The type of system envisaged for a substrate molecule undergoing desaturation is as illustrated in figure 11b.

Considering that the width of a stearic acid molecule is approximate 4Å, its insertion into an enzyme cleft less than 5.3Å in width, prior to desaturation, must require a precise and sophisticated locating mechanism. Alternatively it is possible that the desaturase enzyme can adopt two slightly differing structures. The first of these would be an open structure, which would allow relatively easy acceptance
Figure 11 Schematic representation of enzyme-substrate complex illustrating possible enzyme configurations involved before and during dehydrogenation.
of the substrate molecule (figure 11a). Then prior to desaturation, closure of the cleft (see figure 11b) would increase the structural enfoldment and also the London dispersion forces, by creating greater interaction of the polynethylene chain with the amino acid side groups in the cleft. The total sequence would then be as depicted in scheme 2 i.e.

**Scheme 2.**

OPEN ENZYME + SUBSTRATE $\rightarrow$ OPEN ENZYME - SUBSTRATE COMPLEX

$\rightarrow$ CLOSED ENZYME - SUBSTRATE COMPLEX $\rightarrow$ OPEN ENZYME + PRODUCT

If such a system does prevail the accommodation of each of the racemic methylsteaeric acids in the open enzyme - substrate complex, including those that exhibit a complete absence of desaturation, may in fact be possible. The cause of their failure to be desaturated in this case would result from the methyl-branch prohibiting closure of the enzyme-substrate complex.

D. Sterechemical study of hydrogen atoms removed by the desaturase enzyme.

From the incubations discussed so far it has only been possible to determine factors concerned with the physical interaction between the substrate molecule and the desaturase enzyme during desaturation. More precise information about the actual desaturase site, the conformation of the hydrogen atoms removed in desaturation and the possible mechanism involved was not available from the results of the studies of the methyl-branched precursors.

Assuming desaturation of stearic acid takes place by direct abstraction of two hydrogen atoms, to give the cis-monoenoic olate acid (page 20) there are several possible mechanisms that could prevail. These mechanisms are outlined in figure 12 and each one assumes the normal staggered configuration of the fatty acid to be the substrate.

Determination of the relative configuration of the hydrogen atoms removed will eliminate half of the possible mechanisms for desaturation outlined in figure 12. To achieve this it was necessary to incubate
Figure 12.

Possible mechanisms for the desaturation of stearic acid to give oleic acid.

Substrate: Stearic acid.

Structure L\_1 \_L\_2 \_D\_1 \_D\_2 \_R\_1 \_R\_2

or

D\_2 \_L\_2 \_L\_1 \_D\_1 \_R\_2 \_R\_1

Fischer Projection

R\_1 \_R\_2 refer to side chains, L\_1, L\_2, D\_1 and D\_2 refer to hydrogens of L- and D-conformation.

1. Removal of erythro-hydrogens

A. Enzyme bound L\_1 \_D\_1 \_R\_1 cis-removal of R\_1 \_L\_1 erythro hydrogens

B. Enzyme bound L\_2 \_L\_1 \_R\_2 cis-removal of D\_2 \_D\_1 erythro hydrogens

2. Removal of three-hydrogens

A. Enzyme bound L\_1 \_D\_1 \_R\_1 cis-removal of L\_1 \_R\_1 three-hydrogens

B. Enzyme bound L\_1 \_D\_1 \_L\_2 cis-removal of \_R\_2 \_L\_2 three-hydrogens

C. Enzyme bound L\_1 \_D\_1 \_R\_1 trans-removal of \_L\_1 \_R\_1 three-hydrogens

D. Enzyme bound L\_1 \_D\_1 \_L\_2 trans-removal of \_R\_2 \_L\_2 three hydrogens
stereospecifically labelled 

\textit{erythro} -9,10-dideuterostearic acid and \textit{three} -9,10-dideuterostearic acid with the microsomal fraction of hen liver.

Since complete dideuterium labelling of each substrate molecule was not possible, their isotopic purity was determined by mass spectrometry (see table 8).

\textbf{Table 8}  
\begin{tabular}{|l|c|c|c|}
\hline
\textbf{\textsuperscript{2}H\textsubscript{2} -labelled acid} & \textbf{Composition (\%)} & \
\hline
\textit{erythro} -9,10-dideuterostearic acid & \text{No \textsuperscript{2}H} & \text{One \textsuperscript{2}H} & \text{Two \textsuperscript{2}H} & \
\hline
\textit{erythro} -9,10-dideuterostearic acid & 6.9 & 16.5 & 76.6 & \
\hline
\textit{three} -9,10-dideuterostearic acid & 6.4 & 18.3 & 74.8 & \
\hline
\end{tabular}

Considering a broad classification of the mechanisms outlined in figure 12, production of a \textit{cis} double bond at the 9,10-position in stearic acid can be achieved by two different routes, namely:

\begin{align*}
\text{Stearic acid} & \xrightarrow{\text{removal of}} \text{Oleic acid} \quad (\text{Routes 1A,B,C and D}) \\
\text{Stearic acid} & \xrightarrow{\text{removal of}} \text{Oleic acid} \quad (\text{Routes 2A,B,C and D})
\end{align*}

If the former case prevails and stereospecific removal of \textit{erythro} - hydrogens takes place from the racemic \textit{erythro} -dideutero and \textit{three} -dideutero labelled precursors, the products from \textit{cis} -elimination would be as illustrated overleaf in scheme 3. The D-configuration for the hydrogen atoms removed is assumed for the illustration.

Where \textit{erythro} -dideutero-racenate is the precursor, both \textsuperscript{2}H\textsubscript{2} atoms would be removed from one enantiomer, but none would be removed from the other. This would result in the enrichment of the dideutero-labelled species in the olefinic product, no monodideutero-labelled monoene molecules being formed. In the case of the \textit{three}-dideutero racenate, \textit{cis}-removal would result in the loss of one \textsuperscript{2}H atom from each dideutero-enantiomer resulting in an enrichment of monodideutero-labelled species and no dideutero-labelled species in the olefinic product.
Alternatively, if stereospecific removal of threo-hydrogens took place by trans-elimination the converse would be true, resulting in enrichment of the monodeuterated species in the erythro-dideuterated substrate, and enrichment of the didideuterated species in the threo-dideuterated substrate.

The results of the incubations in the microsomal fraction of hen liver were obtained by mass spectrographic analysis of the highly purified products on an AEI MS 12 instrument. Ten scans in each direction of the parent-molecular-ion region were recorded for each product, measuring the $^2H_2$, $^2H_1$ and $^2H_0$ intensities and from these spectra the proportion of each species was calculated (see table 9). Also by accurate mass measurements of the precursors and the endogenous fatty acids it was possible to calculate the degree of dilution of the reaction products.

This information together with the degree of desaturation, which was measured for each incubation, allowed the expected ratios of the $^2H_2$, $^2H_1$ and $^2H_0$ species to be calculated for the products from both possible types of elimination discussed above. These expected results are also included in table 9 for comparison.
Table 9

Isotopic enrichment in the products of desaturation of the $^2$H$_2$-labelled fatty acids by the microsomal fraction of hen liver.

<table>
<thead>
<tr>
<th>Substrate:</th>
<th>Results expected for:</th>
<th>Desaturation($%$)</th>
<th>No $^2$H atoms</th>
<th>One $^2$H atom</th>
<th>Two $^2$H atoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>erythro-</td>
<td>elimination of erythro-hydrogens</td>
<td></td>
<td>96.4</td>
<td>0.6</td>
<td>3.0</td>
</tr>
<tr>
<td>diductero-</td>
<td>&quot; &quot; &quot; three- &quot; &quot;</td>
<td></td>
<td>93.4</td>
<td>6.6</td>
<td>0.0</td>
</tr>
<tr>
<td>racemate</td>
<td>Results from incubation</td>
<td>60.1</td>
<td>94.2</td>
<td>0.7</td>
<td>5.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substrate:</th>
<th>Results expected for:</th>
<th>Desaturation($%$)</th>
<th>No $^2$H atoms</th>
<th>One $^2$H atom</th>
<th>Two $^2$H atoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>three-</td>
<td>elimination of erythro-hydrogens</td>
<td></td>
<td>94.6</td>
<td>5.4</td>
<td>0.0</td>
</tr>
<tr>
<td>diductero-</td>
<td>&quot; &quot; &quot; three- &quot; &quot;</td>
<td></td>
<td>97.0</td>
<td>0.6</td>
<td>2.4</td>
</tr>
<tr>
<td>racemate</td>
<td>Results from incubation</td>
<td>59.2</td>
<td>95.5</td>
<td>2.1</td>
<td>2.4*</td>
</tr>
</tbody>
</table>

* This figure is considered abnormally high because the parent-molecule of 9,10-diducteroleic acid coincides with stearic acid, an impurity difficult to completely eliminate. Repeated purification reduced this figure linearly from 6.8, and further substantial reductions were envisaged if sample size had permitted greater purification. In comparison the other $^2$H$_2$ figure and the two $^2$H$_1$ proportions remained almost unchanged by repeated purification.

In these results, obtained by mass spectrometric analysis of the purified products, an enrichment of the diductero-labelled species from the erythro-diductero substrate and of the mono diductero-labelled species from the three-diductero substrate, is indicated. This is in keeping with the results expected for the removal of hydrogens of the same relative configuration. In the desaturation of the erythro-diductero...
Racemate in particular, the presence of 5% of the methyl oleate with didcutero-label indicates conclusively the removal of an erythro-pair of hydrogens, since the removal of a threo-pair would preclude the production of didcutero-nonone. Relating this to the possible mechanisms portrayed in figure 12, removal of hydrogen atoms of opposite configuration does not occur and so mechanisms 2A,B,C and D (viz.: removal of three - hydrogens ) are not feasible.

Thus it was proved that the desaturase enzyme present in the microsomal fraction of the hen liver exhibits geometrical specificity removing hydrogen atoms of like configuration to form the cis double bond. However the existence of any absolute specificity was not shown.

To determine any such specificity towards either optical configuration it was necessary to incubate the avian system with stearic acid precursors stereospecifically labelled at the 9 (or 10) position, namely D and L-9-tritiostearic acid. If optical specificity does exist such incubation would result in total loss of tritium during desaturation of one enantiomer and total retention of tritium during desaturation of the other. However specificity only in the relative sense of removing cis-pairs of hydrogen atoms would result in partial loss of tritium from both enantiomers, with partial retention of tritium in both olefinic products.

To facilitate accurate determinations of the amount of tritium lost/retained in each case, small quantities of 1-14C labelled stearic acid were added to the tritium-labelled enantiomers to give an approximate 3H/14C ratio of 15:1. This provision of an internal 14C label obviated the need to calculate the degree of dilution of substrate and reaction product, by measurement of endogenous fatty acids.

The results of the incubations with D and L-9-tritiostearic acids are shown in table 1, overleaf.
$^{3}{\text{H}}/^{14}{\text{C}}$ ratios of stereospecifically $^{3}{\text{H}}$-labelled stearic acids and of the products of their desaturation by hen liver microsomes. (Desaturation=49.8%) 

<table>
<thead>
<tr>
<th>Precursor</th>
<th>$^{3}{\text{H}}/^{14}{\text{C}}$ ratio of substrate</th>
<th>$^{3}{\text{H}}/^{14}{\text{C}}$ ratio of unreacted stearate*</th>
<th>$^{3}{\text{H}}/^{14}{\text{C}}$ ratio of oleate product*</th>
</tr>
</thead>
<tbody>
<tr>
<td>(D-9-$^{3}{\text{H}}$) C$_{18}$:0 acid</td>
<td>14.8</td>
<td>19.8 (1.34)</td>
<td>2.2 (0.15)</td>
</tr>
<tr>
<td>(L-9-$^{3}{\text{H}}$) C$_{18}$:0 acid</td>
<td>11.9</td>
<td>10.6 (0.89)</td>
<td>10.3 (0.87)</td>
</tr>
</tbody>
</table>

* To facilitate comparisons, the values in parenthesis are normalised to an assigned value of unity for each substrate.

It is evident from the results that the olefinic product derived from D-$^{3}{\text{H}}$-labelled porcisor showed a markedly decreased $^{3}{\text{H}}/^{14}{\text{C}}$ ratio indicating almost complete loss of tritium from the D-9-position of the stearic acid precursor. Conversely, the $^{3}{\text{H}}/^{14}{\text{C}}$ ratio of the L-$^{3}{\text{H}}$-labelled precursor remained virtually unchanged in the olefinic product indicating retention of the hydrogen atom at the L-9-position, and thus the optical specificity of the desaturase system.

The fact that loss of tritium from the D-$^{3}{\text{H}}$-labelled precursor and retention of tritium by the L-$^{3}{\text{H}}$-labelled precursor was not complete, is unlikely to imply any lack of complete stereo-specificity during desaturation. The occurrence of these deviations is more likely to result from some racemization during the synthesis of these precursors, particularly at the hydrogenolysis stage.

Thus, as desaturation was shown to occur with the loss of two hydrogen atoms of the same relative configuration, it can be concluded that the desaturase enzyme of the hen liver microsomal fraction exhibits absolute stereo-specificity, removing the D-9 and D-10 hydrogens of stearic acid to give cis double bond (cf. results of Bloch et al in Corynebacterium diphtheriae$^{35}$ and Morris et al in Chlorella vulgaris$^{36}$).

The natural and most probable inference from these results is
that the mechanism of desaturation involves the cis-removal of the 9-D and 10-D-hydrogens (figure 12, mechanism 1A). This requires the stearic acid molecule to assume an eclipsed configuration about the 9-10 bond in the enzyme-substrate complex, during desaturation i.e.,

\[
\begin{align*}
\text{Stearic acid} & \quad \text{Enzyme bound stearic acid} \quad \text{Oleic acid} \\
\text{(staggered)} & \quad \text{(eclipsed conformation)} \\
D_1 & \quad \text{cis-removal of erythro-hydrogens} \\
L_1 & \quad \text{conformational change} \\
D_2 & \quad \text{enzyme bound}
\end{align*}
\]

Note: \( R_1 \) and \( R_2 \) are polyethylene side chains, and \( L_1, L_2, D_1, \) and \( D_2 \) refer to hydrogen atoms of \( L \) and \( D \)-configuration.

Since the hydrogen atoms lost during desaturation are of the \( D \)-configuration mechanisms 1B and 1D in figure 12, which involve the removal of \( L \)-hydrogen atoms, are precluded. However, this stereospecific removal of the 9-D and 10-D-hydrogens does not preclude the reaction proceeding via, a trans-elimination (mechanism 1C). This could arise either by the stereospecific change from staggered to eclipsed conformation of the enzyme bound intermediate during desaturation, or by isomerization of the enzyme bound trans-olefin to give the cis-olefinic oleic acid. Enzymes capable of performing this isomerization have not been identified, but the possibility of a change in configuration of an enzyme-bound olefinic intermediate during desaturation, cannot be totally ruled out.

The results of the incubations with the tritium-labelled precursors serve also to highlight a considerable kinetic isotope effect operative during the desaturation reaction. The tritium enrichment of the unreacted substrate in the D-9-labelled case, indicated a 34% inhibition of desaturation by the isotope. In this instance, though, only the D-9 and L-9-tritiostearic acids were available and so the isotope effect, evident at the 9-D position, could not be validated for the 10-D-hydrogen atom. Furthermore, evidence for
the existence of such an isotope effect at the 10-D-position is split-

Experiments carried out by Schroepfer and Bloch with the 9 and 10-tritium labelled acids in *Corynebacterium diphtheriae* indicated a complete absence of a similar isotope effect at the 10-position and from this they postulated a sequential removal of the hydrogen atoms at the 9 and 10-positions during desaturation, in which removal of the D-9-hydrogen was the initial and rate-limiting step.

More recent experiments, however, by Gurr and Johnson using *erythro* and *threo*-9,10-ditritiooleic acids and the four D and L, 9 and 10-mono-tritiooleic acids contradict these findings. Using the microsomal system of hen liver they found a substantial kinetic isotope effect at both the D-9 and D-10-positions.

In agreement with the findings of Johnson and Gurr, the results from incubations with deuterium-labelled substrates in the microsomal system (table 9), provide evidence that an isotope effect does also exist at the 10-position in the polynethylene chain. Considering scheme 3 (page 67); if no kinetic isotope effect exists then for every two molecules of *erythro*-9,10-labelled substrate desaturated, one molecule of dideutero-labelled olefin will be produced. However, every two molecules of *threo*-9,10-labelled substrate, on desaturation will produce two molecules of monodeutero-labelled olefin. Thus if equal quantities of the *erythro* and *threo*-precursors were incubated, with equal amounts of the same desaturase system, for the same time and under identical conditions, there would be twice as much monodeutero-labelled oleate from the *threo*-enantiomer as dideutero-labelled oleate from the *erythro*-enantiomer. In this series of incubations all but one of these conditions were met, and correcting for the slightly increased mass of the *erythro*-precursor (494 µg compared with 402 µg of the *threo*-labelled precursor), the ratio of monodeutero-labelled oleate from the *threo*-substrate to dideutero-labelled oleate from the *erythro*-substrate was 2.1 to 4.1 (i.e. almost 1:2). Obviously therefore a kinetic isotope
effect does exist, and one of quite considerable proportions.

The presence of an isotope effect at either of the two positions of sufficient strength to totally block desaturation of a substrate with a D-2H atom at that position would result in one enantiomer of each of the diastereomers being unreactive. In this case equal quantities of didutero
labelled product from the erythro-substrate and mono deuterost-labelled product from the three-substrate could be expected. This is still not of sufficient magnitude to explain the 1:2 ratio of $^2$H$_2$-labelled product to the $^2$H$_1$-labelled product.

Therefore the only explanation that could suffice is that a fairly strong kinetic isotope effect is exerted by deuterium at the D-9 and D-10-positions of stearic acid. The strength of the effect was such that neither of the three-enantiomers in isolation, nor indeed collectively, was as efficient a substrate as L,L-erythro-9,10-diduterosteic acid.

These results corroborate the observations of Morris et al.\textsuperscript{36} in Chlorella vulgaris suggesting that desaturation involves the simultaneous concerted removal of a pair of D-hydrogens atoms to form the \cis-olefinic oleic acid.

E. Conclusions and Inferences.

Although it has been possible to define precisely the hydrogen atoms removed during desaturation, the exact mechanism by which they are removed remains obscure. The simultaneous concerted abstraction of the 9-D and 10-D-hydrogen atoms suggests \cis-elimination by a single active site. This view is substantiated, to some extent, by the lack of evidence about enzymes capable of performing the isomerization or change in configuration that would be required to convert the product of \trans-elimination of erythro-hydrogen atoms.

Also the role of molecular oxygen and reduced pyridine nucleotide in the desaturase sequence, though obligatory, remains obscure despite considerable investigation to identify or illustrate the involvement
of oxygenated-intermediates (ref.12 to 16). This leads to the view that oxygen may never enter into covalent linkage with the substrate but may instead be involved in the regeneration of the active centre for desaturation.

One postulation, that would satisfy both these criteria, is provided by considering a disulphide bridge as the active site.\textsuperscript{175,176} The existence of such disulphide bonds in the polypeptide structure of proteins is well documented for several enzymes (ref.38-44). The proposed mechanism for desaturation would therefore involve direct \textit{cis}-abstraction of the two \textit{erythro}-hydrogen atoms, by such a disulphide bridge, producing the \textit{cis}-olefin and two sulphhydryl groups on the enzyme. The oxygen and reduced pyridine nucleotide requirement would then be fulfilled in regeneration of the active disulphide bridge.

Considering this postulation in more detail; the isolated geometry of the eclipsed hydrogen atoms in relation to a disulphide bond is of the right order and would not detract from the feasibility of the reaction.

Chemically there is also no reason to doubt the feasibility of the reaction. The reduction of a disulphide to a mercaptan is possible, and so the analogous enzymatic reaction resulting in the abstraction of the two hydrogen atoms, does not seem too improbable. Furthermore, if the tertiary protein structure was such that it imposed strain on the disulphide bond, the breaking of that bond could supply the energy requirements for the desaturation. Regeneration of the disulphide bridge by combination of the two sulphhydryl groups also has its counterpart in chemistry, requiring only mild oxidising agents (e.g., air) to effect the reaction, and such a combination involving two cysteine molecules has been shown to be the method of formation of the cystine molecule in nature.\textsuperscript{177}

Therefore the feasibility of this postulation hinges largely on the absolute geometry in the vicinity of the proposed disulphide bridge i.e. whether the appropriate hydrogen atoms of the substrate, in complex with the desaturase enzyme, can be positioned adjacent to a disulphide bond.

The extensive structural investigations carried out in this work
have gone some way to determining this geometry. Recapitulating, the results suggested the enzyme assumed the shape of a narrow cleft of approximately 26Å in length from the point of carboxyl attachment, into which the substrate was positioned prior to desaturation. The complete absence of desaturation in certain cases pointed to a very close enfoldment of the substrate between carbon atoms 5 and 15, the cleft in this region being between 4Å to 5.3Å wide. This close enfoldment then generally gave way to a more relaxed spatial environment towards the extremities of the substrate chain.

The location of the normal substrate molecule, 4Å in width, into such a narrow cleft was earlier questioned and its insertion into a more open cleft with subsequent closure to produce the close enfoldment, was proposed. The cross-linking of two cysteine molecules to form the active disulphide bridge could indeed bring about this narrowing of the cleft and a mechanism as proposed in scheme 4, could be envisaged for the desaturase reaction.

Scheme 4

Stearoyl-S-CoA
or
Stearoyl-S-ACP

\[
\text{Oleoyl-S-CoA} \quad \text{Oleoyl-S-ACP}
\]

\[
\text{transf} \quad \text{transf}
\]

\[
\text{Oleoyl-S-'open' desaturation Stearoyl-S-'closed'}
\]

As well as facilitating the close enfoldment of the polymethylene chain, which is required to ensure the extreme stereospecificity of this enzymic reaction, the relative location of the cysteine molecules at each side of the cleft could, on formation of the disulphide bridge, bring about the conformational change in the substrate required to allow cis-removal of erythro-hydrogen atoms.

In the extended chain conformation of stearic acid the D-hydrogen...
atoms at positions 9 and 10 lie on opposite sides of the chain because of the zig-zag arrangement of the carbon-carbon bonds (figure 13a). Thus to allow a simultaneous concerted removal of the 9-D and 10-D-hydrogen atoms by one active centre, a $180^\circ$ rotation about the 9,10 C-C bond, bringing both D-hydrogens together in an eclipsed conformation on the same side of the chain, is required (figure 13b). This will impart a bent chain conformation on the saturated substrate (figure 13c) similar to that of oleic acid, the enzyme product.

In figure 14 an attempt has been made to express diagrammatically, the way in which this conformational change in the substrate could be affected, whilst generating the active site. It involves the inter-reaction of the thiol groups of two cysteine molecules, specifically located at each side of the enzyme cleft adjacent to the 8,9-bond on one side and the 10,11-bond on the other, of a resident substrate molecule. As well as producing the structural environment suggested by the results, this inter-reaction would twist the substrate around the 9,10-bond bringing the 9-D and 10-D hydrogen atoms into juxtaposition with the disulphide bond, which is formed simultaneously.

Having created the correct geometry, desaturation would then be simply achieved by direct abstraction of 9-D and 10-D-hydrogen atoms. These would be transferred to the sulphur atoms of the bridge and the concurrent opening of this bridge would result in the 'bent' active enzyme reverting to the straight conformation of the inactive enzyme. Thus the close enfoldment would be relinquished, with a corresponding reduction in London dispersion forces, and easy transference of the product from the oleoyl-S-open enzyme to either coenzyme A or acyl carrier protein, would be facilitated.
Figure 13 (a) and (b): Relative positions of the D- and L-hydrogen atoms on adjacent carbon atoms in the normal staggered conformation (a) and in the eclipsed conformation (b), (180° rotation of C-C bond). (c) Isometric projection of (b) showing extended chain structure.
Figure 14. Schematic representation of the proposed change in conformation brought about by generation of the active site in the enzyme-substrate complex, prior to removal of the 9 and 10-D hydrogen atoms.
EXPERIMENTAL

A. Synthesis of positionally isomeric, racemic, monomethylstearic acids.

These methyl-branched acids were obtained, in most cases, by initially preparing the corresponding monomethylheptadecanoates, by Kolbe electrolysis, and chain extending the product by one carbon atom to yield the (1-\(^{14}\)C) monomethylstearic acids. By this method the following acids were obtained:

<table>
<thead>
<tr>
<th>Acid</th>
<th>Page</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-methylstearic acid</td>
<td>78</td>
<td>1</td>
</tr>
<tr>
<td>5-methylstearic acid</td>
<td>83</td>
<td>2</td>
</tr>
<tr>
<td>6-methylstearic acid</td>
<td>85</td>
<td>3</td>
</tr>
<tr>
<td>8-methylstearic acid</td>
<td>86</td>
<td>4</td>
</tr>
<tr>
<td>9-methylstearic acid</td>
<td>87</td>
<td>5</td>
</tr>
<tr>
<td>10-methylstearic acid</td>
<td>88</td>
<td>6</td>
</tr>
<tr>
<td>11-methylstearic acid</td>
<td>89</td>
<td>7</td>
</tr>
<tr>
<td>12-methylstearic acid</td>
<td>90</td>
<td>8</td>
</tr>
<tr>
<td>14-methylstearic acid</td>
<td>91</td>
<td>9</td>
</tr>
<tr>
<td>15-methylstearic acid</td>
<td>91</td>
<td>10</td>
</tr>
<tr>
<td>16-methylstearic acid</td>
<td>93</td>
<td>11</td>
</tr>
<tr>
<td>17-methylstearic acid</td>
<td>94</td>
<td>12</td>
</tr>
</tbody>
</table>

Of the remaining positional isomers:

(1-\(^{14}\)C) 2-methylstearic acid was obtained by Favorsky rearrangement of the chloromethyl ketone from (1-\(^{14}\)C) stearic acid. Page 94; Preparation 13

(5-\(^{14}\)C) 3-methylstearic acid was obtained by a Kolbe electrolysis involving (1-\(^{14}\)C) myristic acid. Page 96; Preparation 14

(2-\(^{14}\)C) nonadecanoic acid was obtained by chain extension of (1-\(^{14}\)C) stearic acid. Page 96; Preparation 15
1. Synthesis of \( (1-^{14}\text{C}) 4\)-methylstearic acid.

Reaction Sequence I

\[
\begin{align*}
\text{H}_2\text{C} & \quad \overset{0}{\text{C}} \quad \overset{\text{CH}_3}{\text{OOCCH}}_2\text{CHCH}_2\text{COOH} \\
\text{CH}_3\text{C.H} & \quad \overset{0}{\text{C}} \quad \overset{\text{CH}_3}{\text{OOCCH}}_2\text{CHCH}_2\text{COOH} \\
\text{H}_2\text{C} & \quad \overset{0}{\text{C}} \quad \overset{\text{ROCOOH}}{\text{electrolysis}} \quad \overset{\text{RCOOH}}{\text{RCOCH}_2\text{CHCH}_2\text{COOH}} \\
\end{align*}
\]

Reaction Sequence II

\[
\begin{align*}
\text{RCH}_2\text{CH(CH}_2)^x\text{COOCH}_3 & \quad \overset{\text{LiAlH}_4}{\text{dry ether}} \quad \overset{\text{RCOOH}}{\text{RCOCH}_2\text{CHCH}_2\text{COOH}} \\
\text{RCH}_2\text{CH(CH}_2)^x\text{CN} & \quad \overset{\text{K^+CN}}{\text{MeSO}} \quad \overset{\text{RCOCH}_2\text{CHCH}_2\text{COOH}}{\text{RCOCH}_2\text{CHCH}_2\text{COOH}} \\
\text{RCH}_2\text{CH(CH}_2)^x\text{CN} & \quad \overset{\text{KOH}}{\text{HCl/CH}_3\text{OH}} \quad \overset{\text{RCOCH}_2\text{CHCH}_2\text{COOH}}{\text{RCOCH}_2\text{CHCH}_2\text{COOH}} \\
\end{align*}
\]

\( R = \text{CH}_3(\text{CH}_2)^{12}; x = 1; ^{14}\text{C} \)

(i) Hydrolysis of \( \beta \)-methylglutaric anhydride with methanol.

For this synthesis methanol was dried by adding magnesium turnings (ca. 20g) and a few crystals of iodine to laboratory reagent grade methanol (2/2 litres). When the Grignard reaction started the solution was refluxed for 1 hour, after which it was distilled. An initial fraction was discarded (200ml) and some methanol was left in the distillation flask to be used in following 'Grignard dryings' of methanol. The dried methanol was stored in a vessel fitted with a silica gel trap.

\( \beta \)-methylglutaric anhydride (5g) was dissolved in dry methanol (50ml) and the solution was refluxed for 1 hour. The methyl hydrogen \( \beta \)-methylglutarate produced was not isolated since a methanolic solution was required for the next stage of the preparation.

(ii) Kolbe electrolytic coupling of \( \text{acid and half ester}^{124,178} \)

Myristic acid (25g) was dissolved in dry methanol (200ml).
Sodium (ca.200mg) was dissolved in dry methanol (10ml) such that the sodium methoxide produced would neutralize 5-10% of the total acids. These two solutions and the methanolic solution of methyl hydrogen \(\beta\)-methylglutarate were combined and made up to a 10% solution by addition of dry methanol. After thorough mixing, the resultant electrolytic solution was placed in a cylindrical glass cell and two platinum electrodes inserted. (The cell was not sealed since evolution of hydrogen and carbon dioxide takes place during the reaction. Since the electrolysis is exothermic the cell was water jacketed and fitted with cooling coils to keep the reaction temperature below 50°C)

120 Volts D.C. was passed through the electrolyte (approx. pH 7) developing an initial current of 0.52 amps. Occasional reversal of the electrodes prevented the build up of insoluble material around the anode, thus allowing a current between 0.2 to 0.4 amps to be maintained. When the pH of the solution had risen to about 9 (9hr.) the reaction was stopped and the contents of the cell were poured into water (600ml). The dilute alkaline solution was extracted with ether (3x250ml) and the combined ether fractions were washed once with water. The etheral solution was dried with MgSO\(_4\) and evaporation yielded the mixed Kolbe products (15g). This mixture, namely methyl 3-methylheptadecanoate (the desired product), hexacosane (the major byproduct) and methyl 3,6-dimethyloctadecanoate (the minor byproduct), was separated by column chromatography on Davison silica Grade 950. Elution was with solvents of steadily increasing polarity, from petroleum ether to 10% ether in petroleum ether, and methyl 3-methylheptadecanoate was recovered from the 5% ether in petroleum ether fraction by evaporation. The crude product (5.7g, 61% based on half ester) was a colourless oil.

Gas Liquid Chromatography (G.L.C.) of the product on .SE 30 column at 200°C indicated four minor impurities and so a portion of the product was purified on an Aerograph preparative G.L.C. with a 5ft. Carbowax column at 200°C (see page 18). This measure enabled a product
of greater than 99.3% purity to be obtained, as indicated by GLC and TLC analysis.

These final analyses also indicated that the retention time on GLC and the polarity were in keeping with those expected for methyl 3-methylheptadecanoate. Final confirmation that the desired product had been achieved was provided by mass spectrometry, using an AEI MS12 instrument.

(iii) Reduction of ester to alcohol

Methyl 3-methylheptadecanoate (50g) dissolved in dry ether (2ml, dried over sodium) was refluxed with lithium aluminium hydride (15mg). After an hour, ether (15ml) was added to the cooled reaction mixture and the excess lithium aluminium hydride destroyed by cautious, dropwise addition of water. The precipitate was dissolved by adding dilute hydrochloric acid and the other extract was washed acid-free with water. The ether was removed on a rotary evaporator at reduced pressure and the product was dried by azeotropic distillation with ethanol.

A small portion of the product was examined by thin layer chromatography (T.L.C.) on Silica G plates against suitable ester and alcohol standards. Development was carried out in 20% ether/petroleum ether and spot identification was achieved by spraying the silica with 5% sulphuric acid and heating it at 200°C for 10mins. This indicated complete conversion of the ester to 3-methylheptadecyl alcohol.

(iv) Conversion of alcohol to p-toluene sulphonate

The alcohol (from iii) was dissolved in dry pyridine (1ml; dried over KOH pellets) and p-toluene sulphonyl chloride (5g) was added. The reaction was allowed to stand overnight at room temperature. At this stage, the extent of reaction was checked by spotting a small portion of the reaction mixture along with suitable standards onto a Silica G plate and developing in 20% ether/pet.ether.

Since the alcohol had undergone complete conversion, ether (20ml) was added to the reaction mixture. The ether extract was washed successively with water (2x5ml), dilute hydrochloric acid (2x5ml), water (1x5ml),
5% aqueous potassium hydroxide (1x5ml) and water (3x5ml) (The other volume was kept around 20ml by addition of solvent where necessary). By this intricate washing procedure it was possible to avoid emulsion formation and also remove pyridine (as the hydrochloride) and p-toluenesulphonic acid (as the \(\text{K}^+\) salt). The product was obtained by evaporation of the ether and dried by azeotropic distillation with ethanol.

A T.L.C. examination on silica plates against suitable standards showed the product to be about 80% 3-methylheptadecyl p-toluenesulphonate and 20% 3-methylheptadecyl chloride.

(v) Conversion of p-toluenesulphonate to nitrile\(^{140,141}\)

\(^{14}\)C-Potassium cyanide (50 \(\mu\)c; 17.3mc/mole) was added to the mixture of p-toluenesulphonate and chloride in dry dimethyl sulfoxide (0.5ml). (The DMDO was distilled at reduced pressure (b.p. 116-118°C at 5mm) onto calcium hydride and kept in a flask fitted with a drying tube.) The reaction was heated at 90°C for 2 hours, then potassium cyanide of lower activity (20 \(\mu\)c; 0.94mc/mole) was added and the heating continued. (Since a product of exceptionally high activity was not required, this measure was adopted to increase the mass as well as radiochemical yield.) After a further 2 hours the reaction was cooled. Ether (10ml) was added followed by water (3ml). The aqueous layer was removed and re-extracted once with ether (10ml). The combined ether soluble fractions were washed with water (4x5ml) to remove DMDO and potassium salts, and the solvent was removed at the pump. The product was dried by azeotropic distillation with ethanol.

An aliquot of the product was checked by T.L.C. against a nitrile standard, development being carried out in 10% ether/petrolewn ether. The plate was scanned for radioactivity on a Packard-TL system indicating that 60% of the radioactivity present corresponded to nitrile. The remainder of the activity was in a very polar component which remained at the base in this solvent system. The identity of this component has not been determined, but it was neither free acid nor amide.
(vi) Methanlysis of nitrile

The crude nitrile was converted directly to the methyl ester by addition of 25% w/v hydrogen chloride in methanol (5ml). The reaction was allowed to stand at room temperature overnight, then ether (20ml) and water (10ml) were added. The aqueous layer was re-extracted with ether (20ml) and the combined ether fractions were washed acid free with successive aliquots of water. The ether was removed and drying was effected by azotropic distillation with ethanol. Radiochemical T.L.C. analysis against standards indicated 60% of the activity was as methyl ester. The remainder still appeared as a very polar material.

The impurity was removed by preparative T.L.C. on 0.5mm Silica G plates. The plate was developed in 5% ether/petroleum ether and identification of the radioactive ester band was obtained by running the plate on the Panax-RFLS. The ester band was removed and the ester obtained by washing the silica with ether. Removal of the ether yielded the pure (1-14C) methyl 4-methylstearylate. The radiochemical purity was found to be greater than 99% by running an aliquot of the product on a radio-G.L.C with an SE30 column at 198°C.

(vii) Hydrolysis of the ester

(1-14C) Methyl 4-methylstearylate was dissolved in 5% potassium hydroxide in methanol (2ml) and the mixture was left at room temperature overnight. Ether (15ml) was added followed by concentrated hydrochloric acid until acid (0.2ml) and water (2ml). The aqueous fraction was re-extracted with ether (10ml) and the combined ether fractions were washed acid free with successive aliquots of water. The ether was removed and the product was dried by azotropic distillation with ethanol.

1% of the (1-14C) 4-methylstearylic acid was dissolved in 0.4% P.F.0 (2,5-diphenyloxazole) in toluene and the activity was determined by counting the sample in a Packard Tri-Carb series 4000 liquid-scintillation spectrometer.

5% of the 4-methylstearylic acid was esterified by addition of methanol
(0.2 ml) and sufficient diazonmethane in ether to retain a yellow
colouration in the mixture. This was allowed to stand at room temperature for
5 minutes then the excess diazonmethane and solvents were removed at the pump.
This method gave the corresponding ester in quantitative yield. (The ethereal
diazonmethane solution was obtained by adding N-nitrosomethyl urea (3 g)
to a 100 ml round bottomed flask containing of 40% aqueous potassium
hydroxide (25 ml) and ether (25 ml). The flask was warmed to distill over the
etheral diazonmethane into an ice cooled receiving flask). The (1-\textsuperscript{14}C)
methyl 4-methylstearate, so obtained, was injected onto an analytical GC
containing a SH 30 column and the weight of product, obtained from the reaction
sequence, was calculated by comparison with the peak of a known weight
of methyl nonadecanoate.

From these two measurements the specific activity of the
4-methylstearic acid was shown to be 5.8\mu\textsubscript{c} /\mu\textsubscript{mol} and the product (7.6 \mu\textsubscript{c}) was
dispersed in sonication solution, with the aid of an ISE Ultrasonic dispersor
(sonication), to give a concentration of 5 \mu\textsubscript{c}/ml. (The sonication solution
contained 10 drops of 5% sodium carbonate solution and 4 drops of dilute tween
20 made up to a total volume of 10 ml with water. This solution was used in
all sonications).

2. Synthesis of (1-\textsuperscript{14}C) 5-methylstearic acid.

Reaction Sequence III

\begin{equation}
\text{RCOOH} + \text{HOOC}_{2}\text{CH}_{2}\text{COOH}_{3} \xrightarrow{\text{Kolbe A}} \text{RCH}_{2}\text{CH}_{3}\text{COOH}_{3}
\end{equation}

\begin{equation}
\text{CH}_{3}\text{OOC(\text{CH}_{2})}_{x-1}\text{COOH}_{3} \xrightarrow{\text{KOH}} \text{CH}_{3}\text{OOC(\text{CH}_{2})}_{x-1}\text{COOH}
\end{equation}

\begin{equation}
\text{RCH}_{2}\text{CH(\text{CH}_{2})}_{x+1}\text{COOH} \xrightarrow{\text{Kolbe B}} \text{RCH}_{2}\text{CH(\text{CH}_{2})}_{x}\text{COOH}_{3}
\end{equation}

where \(x = 14\text{C}\).
This synthesis was achieved by the reaction sequence III where
\[ R = CH_3(CH_2)_\text{x} \] with \( x = 2 \). (For practical details see previous preparation.)

(i) Kalbe electrolytic coupling A to produce 3-methylhexadecanoate.

Tridecanoic acid (2.5g) and methyl hydrogen \( \beta \)-methylglutarate (10g) were dissolved in dry methanol (ca. 100ml). Sodium (150mg) was dissolved in dry methanol (10ml), added to the above solution, and the total volume was made up to about 150ml with dry methanol. The electrolysis was carried out as before (page 79). On completion (16hrs) the reaction was worked up in the usual manner and purified by liquid-solid (column) chromatography (page 13). This produced methyl 3-methylhexadecanoate (1.1g; 32%).

(ii) Hydrolysis of methyl 3-methylhexadecanoate.

The methyl 3-methylhexadecanoate (1.0g) was hydrolysed with 5% KOH in methanol by the usual procedure and 3-methylhexadecanoic acid was obtained as a white, low melting point solid (0.3g; 92%).

(iii) Preparation of methyl hydrogen malonate.

Potassium hydroxide (10.5g) dissolved in methanol (100ml) was run into a stirred solution of dimethyl malonate (25g) in methanol (100ml) over a period of 1 hour; a white solid being deposited after 45 mins. The mixture was stirred for a further 2 hours then left to stand overnight. After this period it was heated to boiling and filtered whilst hot. On cooling the product crystallised. This was filtered off and a second crop was obtained by reducing the volume of the mother liquor. Methyl hydrogen malonate K+ salt (19.5g) was thus obtained as white flaky crystals. The K+ salt was dissolved in water (12.5ml) and cooled to 5°C. Concentrated hydrochloric acid (10ml) was added slowly with stirring to keep the temperature below 10°C. The potassium chloride precipitated was filtered off and washed with ether. The aqueous solution was extracted with ether and the combined ether extracts were washed acid free with water. The ether fraction was dried over magnesium sulphate and the solvent was slowly removed to yield methyl hydrogen malonate (15g; 67%) as a colourless liquid.

The nuclear magnetic resonance (NMR) spectrum of the product was

\[ \text{NMR} \]
obtained on a Perkin Elmer 60 dcs instrument indicating a 3:1 ratio between the methoxy protons and the acid proton.

(vi) Kolbe electrolytic coupling 'E' to produce methyl 4-n-pentadecanoate

3-n-hexadecanoic acid (0.7g) and methyl hydrogen malonate (1.85g; six molar excess) were dissolved in dry methanol (20ml). Sodium (50mg) also dissolved in dry methanol (10ml) was added to the above solution and the combined solutions were diluted to 60ml with dry methanol. The electrolysis was carried out as before and on completion (11.5 hrs.) the reaction was worked up and purified by column chromatography. The yield of methyl 4-n-pentadecanoate was 110mg. (15%).

GLC analysis of the product indicated some impurity, so a portion of the product was further purified by preparative T.L.C. Development twice in 3% ether/petrol ether, followed by identification with Rhodamine 6G and isolation, produced a 90% pure product (by GLC). Mass spectrographic analysis of the product, using an AI1 MS12 instrument, showed that the methyl-branched was in the 4-position and the product was methyl 4-n-pentadecanoate.

(v) Chain extension and radiolabelling of the methyl 4-n-pentadecanoate.

This was carried out as in reaction sequence II where R=CH₃(CH₂)₁₁ and x = 2. The reaction procedures were as recorded on pages 80 to 83, the only differences being that the potassium cyanide used in this case had an activity of 22mCi/mole and final purification, by preparative GLC (see page 18) on an SE30 column at 200°C, was necessary to obtain a minimum 99% radiochemical purity. (1-¹⁴C) 5-Methylstearic acid (1.02 µCi; 4.4 µc/mole) was obtained which was dispersed in 1.02ml of sonication solution (ie 1µc/ml).

3. Synthesis of (1-¹⁴C) 6-methylstearic acid

This synthesis was achieved by reaction sequence III where

R=CH₃(CH₂)₁₀ and, x=3. For practical details refer to pages 78-83.

(i) Kolbe Electrolysis A involved electrolysis of methyl hydrogen β-n-methyl-glutarate (5g), lauric acid (20g) and sodium (200mg) dissolved in dry methanol (250ml). This produced methyl 3-n-pentadecanoate (3g; 38%) after
purification by column chromatography (page 13). The purified methyl 3-methylpentadecanoate (1g) was hydrolysed overnight at room temperature with a slight excess of 5% potassium hydroxide in methanol and the acid formed was not isolated from the alkaline solution.

(ii) Preparation of methyl hydrogen succinate. Succinic anhydride was refluxed for 1 hour in dry methanol (80ml). The half ester was not isolated since it was required in methanolic solution.

(iii) Kolbe Electrolysis B involved electrolysis of 3-methylpentadecanoic acid (1g), methyl hydrogen succinate and sodium (150mg) in dry methanol (100ml). After column chromatography of the crude product, methyl 5-methylheptadecanoate (700mg; 60%) was obtained.

Further purification of the product by preparative G.L.C. (see page 18) yielded methyl 5-methylheptadecanoate of greater than 99% purity.

This was chain extended by the usual homologation sequence to produce (1-14C) 6-methylstearic acid (6.8 mc; 8.8 μc/umole) which was sonicated to a solution of 5 μc/ml.

4. Synthesis of (1-14C) 6-methylstearic acid

This synthesis was achieved by reaction sequence III where R=CH\(_3\)(CH\(_2\))\(_8\) and n=5. For practical details see pages 78-82

(i) Kolbe Electrolysis A. This involved the electrolysis of decenoic acid (20g), methyl hydrogen β-methylglutamate (5g) and sodium (250mg), dissolved in dry methanol (250ml). The reaction produced methyl 3-methyltetradecanoate (4g; 57%) after purification by column chromatography. 3-Methyltetradecanoic acid was obtained by hydrolysis of the ester (1g) with a slight excess of 5% potassium hydroxide in methanol.

(ii) Synthesis of methyl hydrogen adipinate. Adipic acid (25g) was methylated by refluxing for 2 hours with methanol : benzene : sulphuric acid, 20:10:1, 0/7/6 (150ml). Some of the methanol was then removed at the rotary evaporator and the resulting solution was diluted with water (200ml) and extracted with ether (3x200ml). The
combined ether fractions were washed acid free with water, dried over magnesium sulphate and evaporated to yield dimethyl adipate (27g; 31%).

The product (27g) was partially hydrolysed by adding one equivalent of potassium hydroxide (8.7g) in methanol (5% solution) and stirring the mixture overnight at room temperature. Water (200ml) was then added and extraction was carried out with ether (2x200ml). This removed the dimethyl adipate (0.5g; 31%).

The aqueous fraction was acidified with dilute hydrochloric acid and extracted with ether (3x150ml). After water washing (which removed adipic acid), drying and evaporation, methyl hydrogen adipate (7g; 25%) was obtained. The product was analysed by TLC, development being carried out in ether; petroleum ether; formic acid (35:65:1; v/v/v).

(iii) Kolbe electrolysis B: The 3-methyltridecanoic acid (1g), methyl hydrogen adipate (4g) and sodium (100mg) were dissolved in dry methanol (60ml) and electrolysis was carried out as before. Column chromatography (page 13) of the product gave methyl 7-methylhexadecanoate (0.3g; 25%) which was further purified by preparative G.L.C. (page 18).

(iv) Chain extension and radio labelling: This was achieved in the usual manner to produce (1-14C) 8-methylstearic acid (5.5μc, 7.3μc/mole). The product was sonicated to a 5μc/ml solution.

5. Synthesis of (1-14C) 9-methylstearic acid

The required product was achieved by reaction sequence III (page 24) where R=CH(CH2)x, x=6. For practical details refer to pages 78 - 82.

(i) Kolbe Electrolysis A: This involved the electrolysis of nonanoic acid (20g) and methyl hydrogen β-methylglutarate (5g) in dry methanol (250) with sodium (250mg) added. The product, after removal of the hydrocarbon and diester impurities by column chromatography, was methyl 3-methylhexadecanoate (4.2g; 52%), and 3-methylhexadecanoic acid was obtained by alkaline hydrolysis of this ester product (1g) with 5% KOH/methanol.

- 87 -
(ii) Synthesis of methyl hydrogen pimelate: Pimelic acid (17g) was methylated and partially hydrolysed (as in the previous preparation) to yield methyl hydrogen pimelate (7.3g; 39%).

(iii) Kolbe Electrolysis 3: The 3-methyldecanoic acid (1g) and methyl hydrogen pimelate (7.3g) were dissolved in dry methanol (90ml) to which sodium (100mg) had been added. Electrolysis followed by the usual work-up procedure yielded methyl 8-methylheptadecanoate, dimethyldecanoate and 10,13-dimethyltetradecane (5.4g total). Methyl 8-methylheptadecanoate (1.3g; 92%) obtained from this mixture by column chromatography was shown to be 70% pure by GLC analysis. Further purification was carried out by preparative GLC, TLC and GLC analysis indicating the desired product had been achieved.

(iv) Chain extension and radiolabelling: This was achieved by the usual procedure to produce (1-14C) 9-methylstearic acid (7.4μc; 4.7μc/μmole). The product was sonicated to a 5μc/ml solution.

6. Synthesis of (1-14C) 10-methylstearic acid

The synthesis was carried out by reaction sequence III where R=CH<sub>3</sub>(CH<sub>2</sub>)<sub>5</sub>C and x=7. Further practical details are included on pages 78-82.

(i) Kolbe Electrolysis A: Methyl hydrogen β-methylglutarate (5g), caprylic acid (20g) and sodium (250mg) dissolved in dry methanol (250ml) were electrolysed in the normal manner. Methyl 3-methylundecanoate (4.3g; 64%) was separated from the alkane and diester byproducts by column chromatography.

Hydrolysis of the product (1g) with a slight excess of 5% potassium hydroxide in methanol gave 3-methylundecanoic acid.

(ii) Synthesis of methyl hydrogen suberate: Suberic acid (27g) was methylated and partially hydrolysed by the usual method (page 87). Ether extraction of the acidified aqueous layer, followed by water washing yielded, in this case, a mixture of half ester and dicent (16.2g; 60%) as a semi-solid.

Separation of the half ester was achieved by dissolving the mixture in hot petroleum ether (60/80), cooling and filtering off the precipitated...
white solid. Repetition of this process yielded methyl hydrogen suberate (9.4g;35%) which was checked for purity by TLC in ether: petroleum ether: formic acid (35:65:1,v/v/v).

(iii) Kolbe Electrolysis B: 3-methylundecanoic acid (1g), methyl hydrogen suberate (9.4g) and sodium (200mg), dissolved in dry methanol (100ml), were electrolysed. Separation of the ester from the byproducts by column chromatography yielded a mixture of esters (1.2g;31%) of which methyl 9-methyloctadecanoate was the major component. Further purification by preparative GLC was carried out and the product was analysed by GLC,TLC and mass spectrometry.

(iv) Chain extension and radiolabelling: This was achieved by the usual procedure to yield (1-\(^{14}\)C) 10-methylstearic acid (7.6μc;7.9μc/μmole) which was sonicated to a 5μc/ml solution.

7. Synthesis of \((1-^{14}\text{C})\) 11-methylstearic acid

This was achieved by reaction sequence III where R=CH\(_3\)(CH\(_2\))\(_5\) and \(x=3\). For practical details see pages 78 -82.

(i) Kolbe Electrolysis A: Electrolysis of methyl hydrogen \(\beta\)-methylglutarate (2.5g), heptenoic acid (10.2g) and sodium (150μg) in dry methanol (200ml) yielded methyl 3-methyldecanoate (2.1g; 63%) after column chromatography of the Kolbe products. The methyl 3-methyldecanoate (1g) was then hydrolysed with a slight excess of 5% potassium hydroxide in methanol to provide the acid.

(ii) Synthesis of methyl hydrogen azelate: Azelanic acid (25g) was methylated and partially hydrolysed in the usual way.

Separation of the half ester (8g;30%) from the diacid was then achieved by the 'hot petroleum ether' method. NMR data showed the ratio between methoxy protons (6.4 T) and the acid singlet (0.8 T) to be 3:1.

(iii) Kolbe Electrolysis B: This involved the electrolysis of 3-methyldecanoic acid (1g), methyl hydrogen azelate (8g) and sodium (150μg) in dry
methanol (100ml). The mixture of products was separated by column chromatography to yield methyl 10-methylheptadecanoate (1.4g; 87%). This was further purified, to remove shorter chain esters, by preparative GLC. Confirmation of structure was carried out by GLC and TLC.

(iv) Chain extension and radiolabelling: The purified product was chain extended and radiolabelled, via reaction sequence II (page 78) to give (1-^14C)11-methylstearic acid (8.0μ; 3.1μ/μmole), which was sonicated to a 5μ/μl solution.

8. **Synthesis of (1-^14C)12-methylstearic acid**

   This was achieved by reaction sequence III R=CH₃(Cl₂₄)ᵢ and x=9. For practical details see pages 78 - 82.

   (i) *Kolbe Electrolysis A:* Electrolysis of methyl hydrogen β-methyl glutarate (5g), caproic acid (20g) and sodium (300mg) dissolved in dry methanol (250ml) yielded the usual ester, alkane and diester mixture. The methyl 3-methylnonanoate (4.0g; 69%) was isolated by distillation at reduced pressure (120-124°C; water pump vacuum). The product (1g) was hydrolysed with a slight excess of 5% potassium hydroxide in methanol to yield 3-methylnonanoic acid.

   (ii) Synthesis of methyl hydrogen sebacate: Sebacic acid (25g) was methylated and partially hydrolysed by the normal method (page 86). Separation of the half ester (6.4g; 24%) from the half ester/diacid mixture was obtained by the 'hot petroleum ether' method.

   (iii) Kolbe Electrolysis B: The 3-methylnonanoic acid (1g), methyl hydrogen sebacate (6.4g) and sodium (150mg) were dissolved in dry methanol (100ml). Electrolysis yielded the usual mixture from which methyl 11-methylheptadecanoate (0.94g; 54%) was isolated by column chromatography and purified by preparative GLC.

   (iv) Chain extension and radiolabelling: This was obtained by the usual procedure (page 80) giving (1-^14C)12-methylstearic acid (5.7μ; 4.3μ/μmole)
which was sonicated to a 5µc/ml solution.

9. **Synthesis of (1-\textsuperscript{14}C)14-methylstearic acid**

This was achieved using reaction sequence III where \( R=\text{CH}_{3}(\text{CH}_{2})_{2} \) and \( x=11 \). For further practical details see pages 78-82.

(i) **Kolbe electrolysis A**: This involved electrolysis of methyl hydrogen \( \beta \)-methylglutarate (5g), butyric acid (20g) and sodium (400mg) in methanol (250ml). Methyl 3-methylheptanoate (3g; 61%) was separated from the by-products by distillation (176-180°C; atmospheric pressure). The product (1.1g) was hydrolysed with a slight excess of 5% potassium hydroxide in methanol to yield 3-methylheptanoic acid.

(ii) **Synthesis of methyl hydrogen dodecanol:ic acid**: Dodecanedioic acid (25g) was methylated and partially hydrolysed, and the required product (6.5g; 26%) was isolated from the half ester/dicarboxylic mixture by the 'hot petroleum ether' method.

(iii) **Kolbe electrolysis B**: 3-methylheptanoic acid (1g), methyl hydrogen dodecanedioic acid (6.5g) and sodium (150mg), dissolved in dry methanol (100ml) were electrolysed and the products were extracted. The ester fraction (900mg) obtained by column chromatography was separated by preparative GLC to yield pure methyl13-methylheptanoic acid.

(iv) **Chain extension and radiolabelling**: This was carried out as before (see reaction sequence II) to give (1-\textsuperscript{14}C)14-methylstearic acid (11.7µc; 9.6µc/µmole) which was sonicated to a 5µc/ml solution.

10. **Synthesis of (1-\textsuperscript{14}C)15-methylstearic acid**

This was achieved using reaction sequence III where \( R=\text{CH}_{3}(\text{CH}_{2})_{2} \) and \( x=12 \). For practical details see pages 78-82.

(i) **Kolbe electrolysis A**: Methyl hydrogen \( \beta \)-methylglutarate (7g) propionic acid (20g), and sodium (400mg) were electrolysed in dry methanol (270ml). Pure methyl 3-methylhexanoate (3.1g; 49%) was obtained from the reaction
products, as a sweet smelling colourless liquid, by distillation at diminished pressure.

An NMR spectrum was carried out on the product to confirm the structure.

<table>
<thead>
<tr>
<th>Peak (T)</th>
<th>Multiplicity</th>
<th>Assignment</th>
<th>No. of Protons</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.4</td>
<td>Singlet</td>
<td>-OCH₃</td>
<td>3 (STD)</td>
</tr>
<tr>
<td>7.8-8.0</td>
<td>Unsyn. triplet</td>
<td>α-CH₂</td>
<td>2</td>
</tr>
<tr>
<td>8.6-8.9</td>
<td>Multiplet</td>
<td>CH₁, CH₂</td>
<td>5</td>
</tr>
<tr>
<td>9.0-9.2</td>
<td>Multiplet</td>
<td>CH₃</td>
<td>6</td>
</tr>
</tbody>
</table>

The product (2.8g) was hydrolysed with a slight excess of 5% potassium hydroxide in methanol to give 3-methylhexanoic acid.

(ii) Synthesis of methyl hydrogen tridodecanoate: Tridecanoic acid (10g) was methylated and partially hydrolysed as before and the mixture of half ester and diacid was separated by column chromatography. Elution was with chloroform steadily increasing in polarity to 5% methanol/chloroform and sample identification was obtained by running a small aliquot of each fraction on a TLC plate and developing in other petroleum ether: formic acid (35:65:1 v/v/v). Methyl hydrogen tridodecanoate (3.5g; 32%) was thus isolated as a white solid.

NMR data on the product showed a 3:1 ratio between the methoxy protons at 6.4 T and the acid proton at -1.6 T.

<table>
<thead>
<tr>
<th>Peak (T)</th>
<th>Multiplicity</th>
<th>Assignment</th>
<th>No. of Protons</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1.6</td>
<td>Singlet</td>
<td>acid proton</td>
<td>1 (STD)</td>
</tr>
<tr>
<td>6.4</td>
<td>Singlet</td>
<td>-OCH₃</td>
<td>3</td>
</tr>
<tr>
<td>7.7-7.9</td>
<td>Unsyn. triplet</td>
<td>α-CH₂</td>
<td>4</td>
</tr>
<tr>
<td>8.6-8.9</td>
<td>Multiplet</td>
<td>CH₂ in chain</td>
<td>18</td>
</tr>
</tbody>
</table>

(iii) Kolbe electrolysis B: Electrolysis of 3-methylhexanoic acid (2.5g), methyl hydrogen tridodecanoate (0.7g) and sodium (25mg), in dry methanol (35ml), yielded the usual Kolbe products. Column chromatography of the mixture afforded methyl 14-methyloctadecanoate (380mg; 47%) which was further
purified by preparative GLC. The structure of this purified product was confirmed by mass spectrometry and GLC analysis.

(iv) Chain extension and radiolabelling: This was carried out in the usual manner according to reaction sequence 11, yielding \((1^{-14}C)15\)-methylstearic acid \((2.6\mu\text{c}; 2.8\mu\text{c}/\text{μmol})\). This was sonicated to a solution of \(2\mu\text{c}/\text{ml}\) concentration.

11. **Synthesis of \((1^{-14}C)16\)-methylstearic acid**

This was achieved by use of reaction sequence 111 where \(R=\text{CH}_3\) and \(x=13\). For practical details see pages 78-80.

(i) **Kolbe electrolysis A**: This involved the electrolysis of methyl hydrogen \(\beta\)-methylglutarate \((10\text{g})\), acetic acid \((20\text{g})\) and sodium \((500\text{mg})\) in 250ml of dry methanol. Methyl 3-methylvalerate \((4.9\text{g}; 60\%)\) was isolated as a colourless liquid by distillation at reduced pressure. Hydrolysis of the product \((4.5\text{g})\) was carried out with a slight excess of \(5\%\) potassium hydroxide in methanol to yield 3-methylvaleric acid.

(ii) **Synthesis of methyl hydrogen tetradecanedicarboxylate**: Tetradecanedicarboxylic acid \((10\text{g})\) was methylated and partially hydrolysed. The mixture of half ester and diacid obtained was, in this case, separated by column chromatography as in the previous preparation to give the required product \((3.1\text{g}; 32\%)\) as a white solid.

(iii) **Kolbe electrolysis B**: 3-methylvaleric acid \((4.0\text{g})\), methyl hydrogen tetradecanodicarboxylate \((1\text{g})\) and sodium \((150\text{mg})\) were electrolysed in dry methanol \((70\text{ml})\). Isolation of the ester \((350\text{mg}; 30\%)\) from the alkene and diester byproducts was obtained by column chromatography and purification by preparative GLC, was carried out to give methyl 15-methylheptadecanodicarboxylate. Analysis of the purified product was carried out by GLC and TLC, and confirmation of the structure was achieved by mass spectrometry.

(iv) **Chain extension and radiolabelling**: This was carried out by the usual procedure (page 80) to yield \((1^{-14}C)16\)-methylstearic acid \((3.6\mu\text{c}; 2.7\mu\text{c}/\text{μmol})\) which was sonicated to a concentration of \(2\mu\text{c}/\text{ml}\).
12. **Synthesis of \((1^{14}C)17\text{-methylstearic acid}\)**

This was achieved by a single electrolysis via reaction sequence III excluding the Kolbe electrolysis A step.

(i) **Kolbe electrolysis**: Methyl 4-methylvalerate (9.5g), methyl hydrogen tetradecanoate (1.0g) (preparation, page 93) and sodium (100mg) were electrolysed in dry methanol (100ml). Separation of the product mixture was obtained by column chromatography, and the ester fraction (470mg; 41%) was further purified by preparative GLC to give methyl 16-methylheptadecanoate. This was confirmed by mass spectrometry and further analysis by GLC and TLC indicated a purity greater than 99%.

(ii) **Chain extension and radiolabelling**: The usual procedure was employed and \((1^{14}C)17\text{-methylstearic acid} (6.1\mu g; 5.6\mu g/mole)\) was obtained. This was sonicated to a concentration of 5μg/ml.

13. **Synthesis of \((1^{14}C)2\text{-methylstearic acid}\)**

**Reaction Sequence IV**

\[
\begin{align*}
\text{CH}_3\text{(CH}_2)_{15}\text{CH}_2^*\text{COOH} & \xrightarrow{\text{(COCl)}_2 \text{ in benzene}} \text{CH}_3\text{(CH}_2)_{15}\text{CH}_2^*\text{COCl} \\
\text{CH}_3\text{(CH}_2)_{15}\text{CH}_2^*\text{COCH}_2\text{Cl} & \xrightarrow{\text{KCl/other}} \text{CH}_3\text{(CH}_2)_{15}\text{CH}_2^*\text{COCH}_2\text{N}_2 \\
\text{CH}_3\text{(CH}_2)_{15}\text{CH}_2^*\text{COOH} & \xrightarrow{\text{KOH}} \text{CH}_3\text{(CH}_2)_{15}\text{CH}_2^*\text{COCH}_2\text{N}_2
\end{align*}
\]

\(\text{*C} = ^{14}\text{C}\)

(i) **Preparation of acid chloride**: To \((1^{14}C)\) stearic acid (0.1mg; 48.4 μg/mole) was added non-radioactive stearic acid (23mg). This mixture was dissolved in dry benzene (0.5ml) and a vast excess of oxaly chloride (0.5ml) was added. The solution was shaken for 1 hour at room temperature after which excess reagent and the benzene were removed on the rotary evaporator.

(ii) **Conversion of acid chloride to diazomethyl ketone**: Diazomethane in ethereal solution (page 83) was dried overnight on KOH pellets at -20°C, decanted and cooled to -40°C. The acid chloride, dissolved in 1ml of dry
ether, was also cooled to -40°C and the diazomethane solution added. The combined solutions were then allowed to warm up to room temperature, nitrogen evolution beginning at about -20°C. After an hour at room temperature the excess reagent and ether were removed under vacuum to yield heptadecyl diazomethyl ketone as a yellow solid.

(iii) Conversion of diazomethyl ketone to chloromethyl ketone: To the diazomethyl ketone in dry ether (5ml) was added a saturated solution of hydrogen chloride in dry ether, until the yellow colouration of the diazomethyl ketone disappeared. The solvent and excess hydrogen chloride were removed under vacuum.

(iv) Favorsky Rearrangement of chloromethyl ketone: Potassium hydroxide (250mg) was dissolved in water (2.25ml) and ethanol (1.25ml). The chloromethyl ketone slurried in ice cold ethanol (2.5ml) was added to the refluxing caustic solution over a period of 30 minutes. After 90 further minutes the solution was cooled and acidified with dilute hydrochloric acid. This was extracted with ether (2x10ml) and washed acid free with water. The ether phase was dried and evaporated to yield the crude product.

(v) Purification of (1-14C)2-methylstearic acid: The crude product was esterified with diazomethane in ether. Radio-GLC on an SE30 column at 220°C indicated about 10% methyl stearate impurity. Proof that methyl 2-methylstearate was the major product was obtained by a re-run on a radio-GLC with a FFAP (polar) column. Here the major product ran before the methyl stearate impurity.

Removal of the impurity was obtained by preparative TLC on 0.25mm silicon plates, developed three times in 4% ether/petroleum ether, identification being attained on the Pennax radio-TLC scanner. The method made use of the reduced polarity of the methyl 2-methylstearate caused by the methyl substituent adjacent to the carboxyl group. This gave the methyl 2-methylstearate a higher Rf value than methyl stearate. Repeat radio-GLC showed the product to be of greater than 99.6% pure.
The product was hydrolysed to give \((1^{14}C)2\)-methylstearic acid (21.8mc; 1.2mc/m mole). This represented a 21.8% yield on the complete reaction sequence. An aliquot of the product was sonicated to concentration of 5mc/ml.

14. Synthesis of \((5^{14}C)3\)-methylstearic acid

Reaction Sequence V

\[
\begin{align*}
\text{CH}_3(\text{CH}_2)_2 \text{COOH} \rightarrow \text{CH}_3(\text{CH}_2)_2 \text{CH}_2 \text{CH}_2 \text{COOH} \\
\text{CH}_3(\text{CH}_2)_2 \text{CH}_2 \text{CH}_2 \text{COOH} \rightarrow \text{CH}_3(\text{CH}_2)_2 \text{CH}_2 \text{CH}_2 \text{CH}_2 \text{COOH} \\
\text{CH}_3(\text{CH}_2)_2 \text{CH}_2 \text{CH}_2 \text{COOH} \rightarrow \text{CH}_3(\text{CH}_2)_2 \text{CH}_2 \text{CH}_2 \text{CH}_2 \text{COOH}
\end{align*}
\]

\(*C = ^{14}C*

(i) Chain extension of \((1^{14}C)\)myristic acid: To \((1^{14}C)\)myristic acid (0.1mc; 15.4mc/m mole) was added non-radioactive myristic acid (17mg) and the mixture was esterified with diazomethane as in previous preparations. The methyl myristate was then chain extended to \((2^{14}C)\) penta decenoic acid (20mg; 73%) (page 80-82; in this case non-radioactive potassium cyanide was used).

(ii) Kolbe Electrolysis: \((2^{14}C)\) pentadecenoic acid (20mg), methyl hydrogen \(\beta\)-methylglutconate (1.1g) and sodium (5mg) were electrolysed in dry methanol (10ml). Isolation of the ester fraction from the mixture of products was attained by column chromatography. Part of this fraction was purified by preparative GLC on a SE30 column (page 18).

The product was hydrolysed to give \((5^{14}C)3\)-methylstearic acid (2.2mc, 1.2mc/m mole). This was sonicated to a concentration of 2mc/ml.

15. Synthesis of \((2^{14}C)\) nonadecanoic acid

To \((1^{14}C)\) stearic acid (0.1mc, 48.4mc/m mole) was added non-radioactive stearic acid (23 mg) and the mixture was esterified with diazomethane as before. The \((1^{14}C)\) methyl stearate was then chain
extended to (2-14C) nonadecanoic acid via reaction sequence II (page 78). The product (15.4μc, 1.2μc/mole, 15.4% yield after purification) was sonicated to a concentration of 5μc/ml.

B. Incubation of Precursors

I. Incubation of positionally isocaric, racemic, mono-methylstearic acids with a microsomal fraction of hen liver

1. Jarren Hen

a. Isolation of microsomal suspension

A Warren hen (approx. 1 year old) was sacrificed and the liver removed (40g). The liver was immediately placed in an ice-cool container and all subsequent operations were designed to keep the tissue at around 0°C. After removal, the chilled liver was minced with scissors and 0.3M sucrose buffer pH 7.4 (120ml) was added. The mixture was homogenised using a mechanically driven Potter homogeniser and the resulting homogenate was centrifuged at 20,000g for 20 mins. in a refrigerated MSE super 40 centrifuge. This process removed the blood, cell debris and mitochondrial, leaving a suspension of microsomes in a particle free supernatant (microsomal suspension). Any fat which floated on the suspension was removed by filtering through a muslin cloth. The microsomal suspension, thus obtained, could be used immediately for incubations.

In circumstances where the suspension was not required on the day of separation it was further centrifuged at 100,000g for one hour in the refrigerated MSE super 40. This deposited the gelatinous, red, microsomal pellet from the particle free supernatant. Both fractions were stored separately until required, the pellet at -30°C under nitrogen and the supernatant at -8°C. Storage for up to a week was found to cause very little loss of activity150, though some deterioration was apparent if much longer periods were involved. When required, the particle free supernatant was thawed and rehomogenised with the microsomes at 0°C, using a Potter homogeniser.
to reconstitute the microsomal suspension. The complete isolation procedure involved is diagrammatically represented below:

**Hen Liver**

i) Minced
ii) 0.3M sucrose buffer pH7.4 added
iii) Homogenised

**Homogenate**

iv) Centrifuged; 20,000g for 20 mins.

**Supernatant** (microsomal suspension)

v) Centrifuged 100,000g for 1 hour + mitochondrial (discarded)

**Microsomal Pellet**

Partical Proc Supernatant

(stored at -30°C) (stored at -8°C)

b. **Incubations**

Incubations of the series of mono-methylstearic acids with the microsomal fraction of hen liver were carried out as follows. The microsomal suspension (3ml; equivalent to microsomes and soluble enzymes from 1 gram of liver) was pipetted into a test tube and the following cofactors added, to each of the seventeen incubations.

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5M phosphate buffer pH7.4</td>
<td>0.5ml</td>
</tr>
<tr>
<td>0.1M A.T.P.</td>
<td>0.2ml</td>
</tr>
<tr>
<td>1.5mg/ml solution CoA</td>
<td>0.1ml</td>
</tr>
<tr>
<td>10 mg/ml solution NADH</td>
<td>0.1ml</td>
</tr>
<tr>
<td>5mg/ml solution NADPH</td>
<td>0.1ml</td>
</tr>
</tbody>
</table>

In separate tubes each substrate (quantities as detailed in table 11) was suspended on bovine serum albumin (0.6ml of a 0.1g/ml solution). To these was added, 0.5M phosphate buffer (0.2ml), and 0.1M magnesium chloride (0.2ml), the final volume being made up to 1.3ml by the addition of sonication solution (see page 83).
Table 11

Methyl-branched precursors used in incubations with microsomal fraction of hen liver (Warren)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity (μc)</th>
<th>Specific Activity (μc/moles)</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stearic acid</td>
<td>1.0</td>
<td>1.1</td>
<td>0.15</td>
</tr>
<tr>
<td>2-methylstearic acid</td>
<td>1.0</td>
<td>1.2</td>
<td>0.20</td>
</tr>
<tr>
<td>3- &quot; &quot;</td>
<td>0.5</td>
<td>1.8</td>
<td>0.25</td>
</tr>
<tr>
<td>4- &quot; &quot;</td>
<td>1.0</td>
<td>8.5</td>
<td>0.20</td>
</tr>
<tr>
<td>5- &quot; &quot;</td>
<td>0.2</td>
<td>5.4</td>
<td>0.20</td>
</tr>
<tr>
<td>6- &quot; &quot;</td>
<td>0.5</td>
<td>8.5</td>
<td>0.10</td>
</tr>
<tr>
<td>8- &quot; &quot;</td>
<td>0.5</td>
<td>7.3</td>
<td>0.10</td>
</tr>
<tr>
<td>9- &quot; &quot;</td>
<td>1.0</td>
<td>4.7</td>
<td>0.20</td>
</tr>
<tr>
<td>10- &quot; &quot;</td>
<td>1.0</td>
<td>7.9</td>
<td>0.20</td>
</tr>
<tr>
<td>11- &quot; &quot;</td>
<td>1.0</td>
<td>8.1</td>
<td>0.20</td>
</tr>
<tr>
<td>12- &quot; &quot;</td>
<td>0.5</td>
<td>4.8</td>
<td>0.10</td>
</tr>
<tr>
<td>14- &quot; &quot;</td>
<td>1.0</td>
<td>9.6</td>
<td>0.20</td>
</tr>
<tr>
<td>15- &quot; &quot;</td>
<td>0.5</td>
<td>2.8</td>
<td>0.25</td>
</tr>
<tr>
<td>16- &quot; &quot;</td>
<td>0.75</td>
<td>2.7</td>
<td>0.37</td>
</tr>
<tr>
<td>17- &quot; &quot;</td>
<td>0.5</td>
<td>5.6</td>
<td>0.10</td>
</tr>
<tr>
<td>nonadecenoic acid</td>
<td>1.0</td>
<td>1.2</td>
<td>0.20</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>2.0</td>
<td>28.4</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Each substrate solution was then added to a microsomal suspension-cofactor solution and the incubations (total volume, 5.3ml) were purged for 1 min. with 70% oxygen/30% air before scaling. This was found to enhance desaturation, and ensured thorough mixing. The solutions were then incubated for 6 hrs. at 37°C in a water bath fitted with a reciprocating shaker.

c. Isolation of Lipids

At the end of the incubation period the reaction was stopped by pouring the contents of each incubation into chloroform-methanol (2:1;v/v) (60ml). The resultant solution was left at room temperature overnight to ensure complete extraction of the lipids, after which the denatured protein was filtered off and the filtrate was shaken with 0.73% saline (20mls). After removal of the organic phase, the aqueous fraction was re-extracted with chloroform (20ml) and the combined solvent extracts were evaporated, the
lipid residue being dried by azeotropic distillation with ethanol. The lipids, thus extracted, were stored at -30°C in chloroform-methanol (2:1; v/v) (5ml).

The above procedure for terminating incubations and extracting the lipids was used throughout the work contained in this thesis.

d. Determination of results

(1) Degree of desaturation and double bond position:– The solvent was removed from a portion (1ml) of the lipids and methanol-benzene-sulphuric acid (20:10:1; v/v/v) (5ml) was added. This solution was refluxed for 2 hours, cooled and ether (20ml) was added, followed by water (3ml). Successive water washings gave a neutral extract which was evaporated and dried by azeotropic distillation with ethanol. The resulting esters were dissolved in ether (0.5ml).

The above method for transmethylating lipids to the methyl esters of their component fatty acids, was developed by Nichols and James, and is used extensively throughout this thesis.

The degree of desaturation was determined by injecting an aliquot of the methyl esters, obtained above, onto a radio-GLC containing an SE30 column at 230°C. Comparison of the radiochemical peak areas from such a run afforded the percentage desaturation.

Where desaturation had occurred, and also in the borderline cases, the remainder of the methyl esters were subjected to double bond cleavage via von Rudloff's method. The oxidation solutions required were made as follows:–

Solution A 25.6mg K₂CO₃
30ml tert. butanol
20ml water

Solution B 8mg KMnO₄
427mg NaIO₄
50ml water

The methyl esters were dissolved in solution A (3ml) and solution B (3ml) was immediately added. The resultant pink solution was shaken for
2 hours at room temperature. (If during this period the pink colouration disappeared, further equal amounts of solutions A and B were added). After 2 hours water (5mls) was added, and excess reagents were destroyed by bubbling sulphur dioxide gas through the solution until it became colourless. The decolourised solution was extracted repeatedly with ether (3x10ml), and the bulked extracts were washed acid-free with water. Evaporation, and drying by azeotropic distillation with ethanol, gave the monobasic acid and half ester of the cleaved olefin. Therefore, methanol (500µl) was added and the products were esterified with an ethereal solution of diazomethane (see page 82). Aliquots of the monobasic and dibasic-carboxylic ester mixture, so obtained, were run against suitable non-radioactive dicarboxylic ester standards on radio-GLC with an F.F.I.P. column at 230°C. Since all the methyl-branched precursors had been synthesized with the 14C-label close to the carboxyl end of the fatty acid, the presence and identity of a labelled dicarboxylic ester defined, by comparison with standards, the position of the double bond in the product of desaturation.

Since the positioning of the 14C label in the substrate obviates the possibility of labelled shorter chain mono-carboxylic esters from the van Rudloff oxidation and since breakdown-resynthesis does not occur in this system, the appearance of any radioactivity with a retention volume less than methyl stearate must be from a labelled dicarboxylic ester produced by the cleavage reaction. This method150, therefore, proved a very sensitive technique for pin-pointing trace amounts of desaturation products in the borderline cases, which were unidentifiable by normal GLC of the transesterified lipid extract.

(ii) Incorporation into lipids: The relative proportion of radiolabel incorporated into the neutral lipids, free fatty acid and polar lipids was determined by concentrating part of the original lipid extract (1ml) to 100µl and pipetting this onto a 0.25mm silica plate, channelled to stop interference of bands. Suitable lipid standards were also applied and the
plate was developed in ether: petroleum ether: formic acid (15:85:1:v/v/v). Following development, the channels were scanned for radioactivity on the Packard Tri-Carb. Finally the plate was sprayed with 3% sulphuric acid and charred at 200°C, thus facilitating identification of lipids by comparison with standards.

The relative ratios of the polar lipids (phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol) was determined by repeating the above procedure, but developing this time with chloroform: methanol: acetic acid: water 

\[ 85:15:10:4 \] (v/v/v/v) in place of the other solvent system.

2. Shaver Hen

A white shaver hen (approx. 1 year old) was sacrificed, its liver removed and a microsomal suspension prepared as before. The above experiment was then repeated on this microsomal suspension, but in this particular case 8-methyl, 9-methyl, 10-methyl, 11-methyl and 12-methylstearic acids were not incubated. These precursors were excluded from the series because the previous experiment, in the Warren hen, had shown them to contain a methyl-branch too close to the active desaturation site to permit desaturation. The results from this series of twelve incubations were determined in an identical manner to that employed in the previous experiment.

II. Incubation of positionally isomeric, racemic, mono-methylstearic acids with Chlorella vulgaris

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

The culture was grown by inoculating one loop of cells from an agar slope into 5ml of 'rich' medium (see below) and incubating for 24 hours at 30°C, under continuous illumination from 4x40watt fluorescent tubes (daylight emission) 18 inches away. The 5ml culture was then poured into 250ml of 'rich' medium in a Roux bottle and grown in the light incubator for 2-3 days at 30°C.
### 'Poor' medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteose peptone</td>
<td>1g</td>
</tr>
<tr>
<td>KNO₃</td>
<td>2g</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>200mg</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>200mg</td>
</tr>
<tr>
<td>Agar</td>
<td>10g</td>
</tr>
<tr>
<td>Water</td>
<td>1 litre</td>
</tr>
</tbody>
</table>

Final pH 6.5

### 'Rich' Medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>K₂HPO₄</td>
<td>500mg</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>500mg</td>
</tr>
<tr>
<td>(NH₄)₂HPO₄</td>
<td>800mg</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>200mg</td>
</tr>
<tr>
<td>CaCl</td>
<td>40mg</td>
</tr>
<tr>
<td>H₂SO₄·4H₂O</td>
<td>2.2mg</td>
</tr>
</tbody>
</table>

1 M Ferric citrate | 2.5ml |
Glucose          | 10g   |
Tryptose         | 10g   |
Difco yeast extract | 2g   |
Water            | 1 litre|

Final pH 6.5

#### a. Preparation of whole cell suspension

The culture, grown as above, was harvested in accordance with the method of Harris et al. This involved spinning down the cells at 1,000 rpm for 10 minutes. The rich medium was then decanted and the wet cells (8g) resuspended in a similar volume of 0.2M phosphate buffer (pH 7.4). The suspension was then centrifuged again to yield cells free of the rich medium. Finally, these cells were resuspended in 0.2M phosphate buffer (65ml) and pre-incubated for 1 hour at 27°C at a distance of 1 ft. from 3x250 watt photo-flood lamps. A 6" deep water filter was used to remove heat.
b. Incubation

Aliquots (3ml) of the pre-incubated suspension were placed in a series of 25ml Erlenmeyer flasks and to each was added one of the sonicated precursors as listed below (table 12). Incubation of these precursors was carried out at 27°C with shaking under 4x40 watt fluorescent tubes (daylight emission). An incubation period of 6 hours was chosen since this was found to give optimal desaturation, with a minimum of oxidative breakdown and resynthesis.

Table 12
Methyl-branchel precursors used in incubations with Chlorella vulgaris

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity (µc)</th>
<th>Substrate Activity (µc/µmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stearic acid</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>2-methylstearic acid</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>3-</td>
<td>&quot;</td>
<td>1.0</td>
</tr>
<tr>
<td>4-</td>
<td>&quot;</td>
<td>0.5</td>
</tr>
<tr>
<td>5-</td>
<td>&quot;</td>
<td>0.2</td>
</tr>
<tr>
<td>6-</td>
<td>&quot;</td>
<td>0.5</td>
</tr>
<tr>
<td>8-</td>
<td>&quot;</td>
<td>0.5</td>
</tr>
<tr>
<td>9-</td>
<td>&quot;</td>
<td>0.5</td>
</tr>
<tr>
<td>10-</td>
<td>&quot;</td>
<td>1.0</td>
</tr>
<tr>
<td>11-</td>
<td>&quot;</td>
<td>0.5</td>
</tr>
<tr>
<td>12-</td>
<td>&quot;</td>
<td>0.5</td>
</tr>
<tr>
<td>14-</td>
<td>&quot;</td>
<td>1.0</td>
</tr>
<tr>
<td>15-</td>
<td>&quot;</td>
<td>0.5</td>
</tr>
<tr>
<td>16-</td>
<td>&quot;</td>
<td>0.5</td>
</tr>
<tr>
<td>17-</td>
<td>&quot;</td>
<td>0.5</td>
</tr>
<tr>
<td>nonadecenoic acid</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>2.0</td>
<td>28.4</td>
</tr>
</tbody>
</table>

Note 1. The volume of precursors was made up to 0.3mls with sonication solution (see page 83) before addition to the cell suspension.

c. Isolation of lipids

After six hours the incubations were terminated by the addition of chloroform-methanol (2:1;v/v). Subsequent storage overnight at room temperature, in this solvent system ensured complete extraction of the lipids. The isolation of the lipids, after extraction, was achieved by the method of
Folch et al \(^{182}\), as previously described for hen liver.

d. Determination of Results

A portion of the isolated lipids was transmethylated according to the method of Nichols and James \(^{183}\) to produce the methyl esters of their component fatty acids. Radio-GLC of these methyl esters on SE30 at 230°C produced chromatograms from which the percentage desaturation could be determined by comparison of peak areas.

In cases where desaturation had occurred, and also in borderline cases, double bond determinations were carried out according to the method of van Radloff \(^{168}\). Radio-GLC of the resultant methyl esters indicated the position of the double bond and also served to pin-point any trace desaturation which occurred in the borderline cases.

The incorporation of radiolabelled fatty acids into both neutral lipids and polar lipids was determined by spotting aliquots of the isolated lipids onto TLC plates and developing in suitable solvents viz; ether-petroleum ether-formic acid (15:85:1;v/v/v) for neutral lipids and chloroform-methanol-acetic acid - water (85:15:10:4;v/v/v/v) for polar lipids. Scanning for radioactivity on the Panax-TLDS produced chromatograms from which the results could be calculated. Further details regarding the determination of results may be obtained from the corresponding incubation in the hen liver microsomal system (see page 100).

To confirm the results obtained from this series of incubations, a second series was carried out under identical conditions. Certain of the mid-chain methylstearic acids were excluded from this latter series, however, since the close proximity of the methyl group to the desaturase site was shown to preclude any possibility of desaturation taking place.

III Investigation of the stereochemistry of desaturation of long chain fatty acids in the microsomal fraction of hen liver.

A shaver hen (approx. 1 year old) was sacrificed and the liver removed (35g). This was homogenised, as before, with 0.3M sucrose pH7.4 (70ml)
and centrifuged at 20,000g for 20 mins. This procedure removed the cell debris and mitochondria leaving a suspension of microsomes in particle free supernatant (microsomal suspension).

A cofactor solution (sufficient for the series of incubations) was prepared by the addition of several cofactors, listed below, to 6ml of 0.5M phosphate buffer pH 7.4.

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>183µg</td>
</tr>
<tr>
<td>CoA</td>
<td>2.25mg</td>
</tr>
<tr>
<td>NADH</td>
<td>15mg</td>
</tr>
<tr>
<td>NADPH</td>
<td>7.5µg</td>
</tr>
</tbody>
</table>

1. Determination of Geometrical Specificity.

Aliquots (2.2nl) of the chilled microsomal suspension were pipetted into three tubes each containing the cofactor solution (0.4nl) and 0.1M magnesium chloride (0.1ml) at 0°C.

In separate tubes each substrate (quantities as listed in table 13) was suspended on bovine serum albumin (30µg) dissolved in 0.5M phosphate buffer (0.2ml).

**Table 13**

Precursors employed in determination of the geometrical specificity of hen liver desaturase

<table>
<thead>
<tr>
<th>Substrate*</th>
<th>Vol.*+(ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>erythro-9,10-didurostearic acid (492±²µg)+(1-¹⁴C)stearic acid (1µc;5.9µg)</td>
<td>0.6</td>
</tr>
<tr>
<td>three-9,10-didurostearic acid (404µg)+(1-¹⁴C) stearic acid (1µc;5.9µg)</td>
<td>0.6</td>
</tr>
<tr>
<td>(1-¹⁴C) stearic acid (1µc;5.9µg)</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* Substrates kindly donated by Dr. L.J. Morris.

** The didurostero-substrate and the (1-¹⁴C) stearic acid were sonicated together in 0.6ml sonication solution (see page 8) to ensure a perfect suspension.

*** Accurate weight determinations were achieved by peak area comparison of chromatogram obtained by GLC of the didurostero-substrate against a standard.

Each substrate solution was added to a microsomal suspension -
cofactor solution and the incubation (total volume 3.5ml) was gassed for 1 min. with 70% oxygen/30% air before sealing. The contents were then incubated for six hours at 37°C, on a water bath fitted with a reciprocating shaker.

After this period the incubations were terminated by addition of chloroform-methanol (2:1, v/v) and subsequent storage overnight ensured complete extraction of lipids. The lipids were isolated as before and the complete extract was transmethylated to yield the methyl esters of the constituent fatty acids. 5% of the esters obtained were injected onto a radio-GLC with an SE30 column and the degree of desaturation calculated from the chromatogram obtained.

The remainder of the methyl esters were separated into saturated and monoenoic fractions by preparative TLC silver nitrate impregnated plates (page 16). This step was repeated on each isolated fraction to ensure complete removal of the monoenoic from the saturated fatty acids and vice-versa. Final purification and removal of any homologues was obtained by GLC and the eluent gas containing the deuterated product was passed straight onto an MBI MS12 mass spectrometer, which was modified to work in conjunction with the GLC. Ten scans in each direction of the parent-molecular-ion region of each product, and each didueterated substrate were recorded. From these, the intensities of the $^{2}H_{0}$, $^{2}H_{1}$ and $^{2}H_{2}$ parent-molecular-ion peaks were measured and the proportions of these three species calculated.

2. Determination of Optical Specificity

Aliquots (2.0ml) of the chilled microsomal suspension were pipetted into three tubes each containing the cofactor solution (0.4ml) and 0.1M magnesium chloride (0.1ml) at 0°C.

In separate tubes, the substrates (as detailed in table 14) were suspended on bovine serum albumin (30mg) dissolved in 0.5M phosphate buffer (0.2ml). To both of the stereospecifically $^{3}$H-labelled stearic acids was added sufficient (1-$^{14}$C) stearic acid to give an $^{3}$H/$^{14}$C ratio of approximately 15:1.
Table 14.
Precursors employed in determination of the optical specificity of the hen liver desaturase enzyme

<table>
<thead>
<tr>
<th>Substrate*</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-9-tritiostearic acid +L-14C stearic acid</td>
<td>0.7</td>
</tr>
<tr>
<td>L-9-tritiostearic acid +L-14C stearic acid</td>
<td>0.7</td>
</tr>
<tr>
<td>L-14C stearic acid (1µc;130µg)</td>
<td>0.7</td>
</tr>
</tbody>
</table>

* Tritiated substrates kindly donated by Dr. L.J. Morris

The substrate solutions were added to the microsomal suspension-cofactor solution and, after gassing with 70% oxygen/30% air, incubations were carried out for 6 hours at 37°C with shaking. The termination of the incubation, isolation of lipids and procuring of the separated saturated and monoenoic fatty esters was accomplished by an identical procedure to that described in the previous experiment.

The precursors, the unreacted substrates and the methyl oleate produced were each dissolved in 0.4% P.P.O. in toluene and counted on a Packard Tri-carb series 4000 liquid-scintillation spectrometer. From the results the $3^H/^{14}C$ ratio of all six samples was calculated.

The degree of desaturation was also determined by radio-GLC on an aliquot of the transmethylated lipid extract from the control incubation.
PART 2.

Studies of inhibitors of desaturation and their mode of action.
INTRODUCTION

Perhaps the most striking characteristic of protein molecules is the ability of a group of them to radically affect the rates of a wide spectrum of reactions that constitute the dynamic aspect of the life process. Such protein molecules are referred to as enzymes, and their extremes of efficiency and specificity render them highly susceptible to a variety of controls. Their rate of synthesis and their final concentration is under genetic control, and they may be influenced by the very substrates or products of the reaction in which they participate.

Thus it is obvious that any study of such an enzymic reaction, namely, desaturation of fatty acids, is difficult. The desaturase enzyme is too susceptible to environmental changes to allow the degree of isolation achieved in several other cases and so any investigation must be carried out on the enzyme in its natural environment, or another in which the enzyme is stable. Since the desaturase enzyme is membrane bound, its purest active state still includes considerable protein, and lipid, not directly involved in the abstraction of the two hydrogen atoms, and so conventional techniques for absolute structure determination are valueless tools for investigation at present. Thus a more indirect method of investigation must be employed.

In Part 1 the stereochemistry and spatial tolerances of this enzyme were studied; here in Part 2 the high susceptibility of the enzyme to atypical conditions was used as a means of investigation. This was achieved by using known inhibitors and investigating other potential inhibitors of the desaturation process.

However before the role of these added inhibitors could be investigated it was necessary, for comparative purposes, to optimize and standardize all other factors which affect the rate and extent of enzyme catalysed reactions. Some of these associated factors are briefly discussed below:

The relative enzyme and substrate concentrations is one factor
which directly affects the rate of any enzyme catalysed reaction\textsuperscript{185}, and under conditions of excess substrate the rate of reaction is directly proportional to the enzyme concentration. Where the enzyme concentration is fixed a second important relationship is observed. An initial rise in substrate concentration will result initially in a rapid and linear rise in velocity. However as the substrate concentration continues to increase, the increase in the rate of reaction will begin to slow down until a point is reached where no further increase is observed. This velocity, where further increases in substrate concentration do not alter the rate of reaction, is defined as the maximum velocity for enzymic reaction under the specified conditions. The maximum substrate turnover per unit time at a particular enzyme concentration is directly related to the maximum velocity and the former's determination was a necessary pre-requisite of the inhibition studies.

\textit{pH} changes also exert a profound effect on enzyme activity\textsuperscript{186} by altering the ionic character of either the substrate or the charged amino acid side-chain of the protein, or both. Thus the protein conformation and/or the binding of the substrate will be affected. Extremes of \textit{pH}, either high or low, cause irreversible damage to the protein structure leading to denaturation and resultant loss of activity. Thus it was necessary to know the optimal \textit{pH} and retain it by means of buffering.

Temperature also influences the rate of enzymic catalysis\textsuperscript{187} and two opposing effects result in an optimal temperature, similar to that observed for \textit{pH} changes. One effect is the kinetic effect, characteristic of all reactions, which leads in this case to an increase in activity with increasing temperature. However, because of the protein nature of the enzyme, thermal denaturation will take place with increasing temperature, resulting in a decrease in the effective concentration of the enzyme, and consequently a decrease in reaction rate. Thus a compromise situation arises depending on whether a high velocity is required over a short period or a high substrate turnover is required over a longer period. Either way
standardization must be introduced where comparisons are to be made.

As well as the above factors, which influence the rate or extent of desaturation, many enzymes are also dependent on certain cofactors. If these are not present in sufficient quantities the efficiency of the enzymic reaction can be greatly impaired or even completely inhibited. Conversely the presence of large excesses can also have an inhibitory effect. Thus it is necessary to regulate the addition of cofactors to overcome any deficiencies that may arise from using increased substrate levels.

In vitro studies on the desaturation of fatty acids required the addition of four cofactors, namely ATP, coenzyme A, NADH/NADPH and molecular oxygen. The requirement for CoA and ATP was made necessary because free fatty acid substrates and inhibitors were used in incubations, whereas the desaturase reaction is known to require activation at least as far as acyl-coenzyme A-thiol esters.

Coenzyme A provides the most prominent acyl group acceptor coenzyme in living systems and the isolation of the acetyl-CoA from yeast by Lynen, followed by subsequent identification as a thiol ester demonstrated that the terminal sulphhydryl group is the reactive site of the molecule in biochemical reactions.

The energy required to form the thiol ester bond between the acyl group and coenzyme-A is provided by the hydrolysis of one of the pyrophosphate linkages of adenosine triphosphate (ATP). This fundamental role of ATP as the driving force for biochemical processes was first recognised by Lipmann and there are many other reactions, beside the one quoted, which are dependent on ATP hydrolysis.

The requirement for reduced pyridine nucleotides as cofactors is
also obligatory although their exact role is not fully understood. Together with molecular oxygen they are, in some way, intimately involved in the removal of the two hydrogen atoms during enzymic desaturation (see Part 1).

Standardisation of all the aforementioned factors influencing the efficiency of enzymic reactions will validate direct comparison of incubations within an experiment and to a lesser extent between experiments. Optimisation of the above requirements will ensure the maximum obtainable dehydrogenation of substrate and thus facilitate greater accuracy in the final results on the degree of desaturation/inhibition.

Having defined the biochemical reaction and described the influencing factors which require standardisation, it is now possible to consider in general terms the role of inhibitors in enzymatic reactions. They are, essentially, a group of compounds which have the ability to combine with certain enzymes, but do not serve as substrates. This combination leads to a reduction in the rate of the particular biochemical reaction and the substance responsible for this effect is termed the 'inhibitor'. The study of these inhibitory effects on isolated enzymic reactions and on metabolic sequences has become a classical approach of the greatest importance. It has been used extensively for investigations on the nature of free reactants, their binding site on the enzyme, and for the specificity and mechanism of the reaction.

To be effective an inhibitor must be in close association with the enzyme and such binding may be reversible or irreversible. The former is characterised by an equilibrium between the enzyme and the inhibitor, giving rise to a definite degree of inhibition dependent on the inhibitor concentration. As the term suggests, removal of the free inhibitor causes a reversion to increased enzyme activity. Irreversible inhibition, on the other hand, is characterised by a progressive increase with time, ultimately resulting in complete inhibition, providing the inhibitor is in excess of the enzyme.

In the presence of varying inhibitor concentrations the kinetics of
most enzymic reactions show a characteristic linear response to a double reciprocal plot of $\frac{1}{v}$ versus $\frac{1}{s}$, where $v$ is the velocity of the reaction and $s$ is the substrate concentration (ie the Lineweaver-Burk Plot\textsuperscript{192}). The slope, the intercept, or both characteristics of the plot, may vary. If the variation in inhibitor concentration results in an alteration of the slope of the line, the inhibition is termed competitive; if the intercept on the ordinate is changed the inhibition is termed uncompetitive; finally, if both factors are changed the inhibition is said to be noncompetitive (see figure\textsuperscript{1}). The total observed inhibition of any enzymic reaction may result from a single type of inhibition, or from any combination of the three.

Considering in more detail the role of the inhibitor in these three types of inhibition with regard to a simple enzymic reaction involving only one substrate:

1. **Competitive inhibition**

In this case the inhibitor usually bears a close resemblance to the substrate structure and binding to the active site nearly always occurs. Thus the inhibitor competes with the substrate for the same site of the free enzyme, with a resultant lowering in the rate of uptake of the substrate into the enzyme-substrate complex. If the enzyme-inhibitor complex, also formed, is capable of releasing the inhibitor as product (or unchanged), then the inhibition is also reversible. However if the enzyme-inhibitor complex does not break down the inhibition is irreversible and is commonly termed dead-end inhibition.

Occasionally, the inhibitor may combine with the enzyme at a different site from the substrate, which is sufficiently close as to reduce the affinity of the enzyme for the substrate, but which does not alter the rate of conversion of the substrate to products\textsuperscript{193} (ie the velocity of reaction of the enzyme-inhibitor-substrate complex and enzyme-substrate complex, to products, is the same). This type of apparent or hyperbolic competitive inhibition has the same double reciprocal plot as normal competitive inhibition.
Figure 1  Kinetic patterns for various inhibition types.
The degree of competitive inhibition is related to inhibitor concentration, substrate concentration and the relative affinities of the enzyme for the inhibitor and substrate. The effect of a competitive inhibitor can thus be reduced by increasing the substrate concentration. Kinetically this gives rise to a double reciprocal plot where only the slope is affected by a change in inhibitor concentration, the intercept with the ordinate remaining constant (figure 1). An example of this type of inhibition is afforded by succinic dehydrogenase which oxidises succinic acid to fumaric acid. If increasing concentrations of malonic acid, which closely resembles succinic acid, are added the activity of the enzyme falls off markedly. This inhibition is reversed when more substrate (succinic acid) is added, a general feature of competitive inhibition.

2. Uncompetitive inhibition

This type of inhibition is rare with single substrate reactions and is thought to occur when the inhibitor can combine only with the enzyme-substrate complex.

In more complex cases, involving multisubstrates or bireactant mechanisms, the kinetics characterising this type of inhibition become quite common. Graphical interpretation of the kinetics, in the form of a double reciprocal plot, leads to a series of lines with constant slopes, their intercepts with the ordinate varying with the inhibitor concentration. In this case, the degree of inhibition is not decreased by increasing the substrate concentration (c.f. competitive inhibition).

Examples of this type of inhibition are provided by the inhibition of the oxidised form of the respiratory enzyme by azide, and the inhibition of yeast carboxylase by acetaldehyde.

3. Noncompetitive inhibition

This third type of inhibition is also not reversed by the simple expedient of raising the substrate concentration and the substrate, therefore, is unable to prevent the combination of the enzyme and inhibitor. Here the inhibitor combines at a totally separate site from the substrate. Subsequent
binding of the substrate proceeds equally well with the enzyme-inhibitor complex as with the enzyme, and inhibition results from a decrease in the catalytic rate of conversion of enzyme-bound substrate to product when the inhibitor is in complex with the enzyme. Kinetically, this results in a double reciprocal plot where both the slope and intercept of the graph are altered by the addition of inhibitor (figure 1).

Since noncompetitive inhibitors do not react with the same site on the enzyme as the substrate, they often show little structural resemblance to the substrate (cf. Competitive inhibition where substrate and inhibitor structures are normally very similar). This absence of any structural resemblance is exemplified by iodoacetamide, a noncompetitive inhibitor of many sulphydryl enzymes, such as triose phosphate dehydrogenase. The reaction involved in this inhibition is:

\[
\text{Enzyme-SH} + \text{I} \text{CH}_2\text{COOH}_2 \rightarrow \text{I} \text{CH}_2\text{COOH}_2 + \text{HI}
\]

Metal ions are also common noncompetitive inhibitors.

The degree of noncompetitive inhibition is related to the concentration of the inhibitor, and the affinity of the enzyme for it. Unlike competitive inhibition relative enzyme/substrate affinities do not affect the extent of inhibition since the affinity of the inhibitor for the free enzyme is the same as that for the enzyme-substrate complex.

Closely associated with noncompetitive inhibition is the allosteric (or second site) effect. This effect, which may either enhance or inhibit the enzyme activity, is thought to occur by the interaction of an effector with the enzyme at a site other than the 'active site', termed the allosteric site. A change in the conformation of the enzyme results, which affects the enzyme activity. The allosteric effector does not undergo any chemical change and, in cases where it resembles the substrate sufficiently, some interaction with the substrate binding site may be possible either directly or subsequent to having first reacted at the secondary site.

Having considered in general terms the ways in which inhibitors can
affect the rate of enzymatic reactions, it is now appropriate to consider more specifically the inhibition of the reaction(s) associated with desaturation.

Investigation of one such inhibitor was initiated, somewhat unknowingly, about 50 years ago. This was when the first reports were being made concerning the discolouration of eggs laid by hens fed on cottonseed products. Two disorders were noticed in the stored eggs: the first was a bronze discolouration of the yolk induced by gossypol and the second was a pink or red discolouration of the white which became known as the pink white disorder. Lorenz quickly showed that seeds of another plant in the family of Malvaceae, namely Malva parviflora, also induced the pink white condition. Later work was directed towards seeking the cause of the disorder and Lorenz stated that only oils giving a positive Halphen test produced the pink white.

Since these early studies more than fifty species of plants have been shown to produce a positive test, but it was not until 1956 that major advances were made towards determining the essential molecular group required to give a positive Halphen test. These were achieved by Faure and Macfarlane, who reported that sterculic acid and malvalic acid isolated from two of these plant species, gave a positive test. Both these acids were known to contain the cyclopropane group, and synthesis of derivatives by Brooke and Smith and others soon indicated that a substituted cyclopropane ring of the kind, was the required molecular configuration, essential for the Halphen reaction (see reviews of cyclopropane compounds by Christie and Carter).

Since the discovery of the pink white disorder, many other biological effects have been attributed to the cyclopropane acids. Of these, the raising of stearic acid levels at the expense of oleic acid in the lipids of certain animals after ingestion of sterculic or malvalic acid, is of particular importance here. This phenomenon has been detected in the yolk, heart, plasma, liver and ovary fat of hens, the milk of cows, and...
the body fats of swine and other animals. More specifically the results of James on plant and algal systems, those of Reiser on rats and those of Johnson on avian systems, all show that biological systems known to be capable of using long chain saturated acids as direct precursors of the corresponding unsaturated acids, also show marked inhibition by sterculic acid.

This inhibition of desaturation (and other secondary biological effects) have been directly attributed to the cyclopropene ring of the inhibitory compounds. Incubations with cyclopropene compounds other than the carboxylic acid derivatives, showed that the acid group was not a necessary requirement for physiological activity. Furthermore will hydrogenation, or the destruction of the cyclopropene ring with sulphur dioxide or hydrogen chloride, resulted in complete loss of activity, confirming its responsibility for the observed inhibition. Thus any mechanism purporting to explain the diverse biological effect of the cyclopropene compounds must cite, as a basis, interaction between the cyclopropene ring and some functional group of a physiologically active compound.

One such mechanism, proposed by Kircher, suggests an interaction between sulphhydryl groups of proteins and the double bond of the cyclopropene ring, viz.

\[
\text{CH}_2 \quad \text{SH} \quad \text{Protein} \quad \rightarrow \quad \text{CH}_2 \quad \text{S-Protein}
\]

He showed the proposition to be chemically feasible and suggested the reaction may proceed readily under physiological conditions. Since the enzymes and some cofactors involved in fatty acid metabolism are known to contain sulphhydryl groups, such interactions could explain the observed inhibitions.

Biochemical evidence in support of this hypothesis was obtained by Ory using the particulate lipase of Ricinus Communis, a sulphhydryl enzyme reversibly inhibited by mercurials. He found that this lipase system
was susceptible to inhibition by *Sterculia foetida* seed oil, a rich source of sterculic acid. He also noticed that simultaneous addition of cysteine, an amino acid containing a free sulphhydryl group, reduced inhibition. Thus he inferred that cysteine competes with the enzyme for the cyclopropane compound, probably by an addition across the double bond as suggested by Kircher. By analogy, he suggested that the -SH containing lipase-enzyme might react with the cyclopropane double bond in a similar manner, and that such interactions might be responsible for the inhibition of other sulphhydryl-enzymes by cyclopropenes.

Evidence that cyclopropenes do not react with all free thiol groups, however, was provided by Jones et al. in *Chlorella vulgaris*, an algal system capable of converting added stearic acid to oleic acid. They found that acylation of coenzyme A, which contains a primary sulphhydryl group, was unaffected by the cyclopropane inhibitor. They also found that the addition of glutathione, a tripeptide containing cysteine, did not afford any protection against the inhibition, by sterculic acid, of desaturation of stearic to oleic acid. These results, therefore, do not support the suggestion by Ory that sterculic acid is a general thiol-enzyme inhibitor.

Another anomaly, arising from the study of the inhibitory effects of cyclopropanyl compounds, involves the possibility of a palmitate and stearato-independent system of nonoelic acid synthesis. Raiser and Raju suggested such an alternative pathway to interpret their results obtained from feeding *Sterculia foetida* oil to rats. In their first experiment, they administered a mixture of $^{14}$C stearic acid and *Sterculia foetida* oil simultaneously (acute) to a rat which was sacrificed after 4 hours. Assays of the liver and epididymal fat pad triglycerides indicated considerable inhibition of desaturation. (This explains the increase in saturable fatty acids, at the expense of nonoenoic, in animals which ingest small quantities of cyclopropane compounds). In another experiment the rat was fed a known dosage of *Sterculia foetida* oil for seven days (chronic) before being administered $^{14}$C sodium acetate intraperitoneally. Subsequent analysis of the
Epidymal fat pads showed no decrease in the ratio of palmitoleic to palmitic acid, and oleic to stearic acid, compared with the control animal. To explain this they concluded that there are two routes for the biosynthesis of oleic acid in animals, one of which does not go through stearate, as exemplified by the latter experiment. They suggest that this direct route from acetate may be similar to the anaerobic pathway in bacteria as characterised by Scheuerbrandt et al.¹⁰

Donaldson²¹⁸ carried out a similar series of experiments, on chicks, which confirmed all the findings of Reiser and Raju. He also showed that ¹⁴C acetate incorporation into monoenoes was inhibited after administration of just a single dose of Sterculia foetida oil (acute). Since chronic feeding of the oil resulted in very little inhibition he concluded that the saturate-independent system for monoene synthesis may be in a repressed state, requiring prolonged inhibition of the primary system (direct desaturation, before induction occurs. Reiser and Raju claim to have substantiated this latter view.²¹⁹

Both authors suggest that the mode of inhibition of Sterculia foetida oil is by irreversible binding of the cyclopropane ring with the thiol group(s) of the desaturase enzyme. (That the desaturase enzyme is a thiol enzyme was inferred by Holloway et al.²²⁰ after their inhibition studies on rat liver desaturase with para-hydroxymercuribenzoate).

However, Jones et al.²¹² following their inhibition studies on algal and plant systems, conclude that the actual dehydrogenating enzyme is not inhibited by sterculic acid. In algal systems they propose a several stage reaction for the conversion of stearic acid to oleic acid, and suggest that inhibition by sterculic acid takes place at one of the activation stages involving acyl transfer. Schematically the most probable reactions and points of sterculic acid inhibition are indicated figure 2, overleaf.

The initial reaction(s) involves the formation of the CoA- and/or ACP-thiol ester followed by further progression along the desaturation
Figure 2

Schematic representation of the possible routes involved in the synthesis of oleate by Chlorella vulgaris

- Stearate Anion $\rightarrow$ Stearoyl-S-CoA $\leftrightarrow$ Lipid

Scheme 1

- Acetate
- Via. Synthetase $\rightarrow$ Stearoyl-S-ACP

- Scheme 2

- Acetate $\rightarrow$ Stearoyl-S-Enzyme

- Specific Stearoyl Lipid

- Scheme 3

- Acetate $\rightarrow$ Oleoyl-S-ACP

- Desaturase $\rightarrow$ Oleoyl-S-ACP

- Oleoyl Lipid

- Oleoyl-S-Enzyme $\rightarrow$ Oleoyl Lipid

- Oleoyl-S-CoA $\rightarrow$ Lipid

$\times$ Stages involving acyl transfer, at which inhibition could take place.
sequence, or incorporation into lipids. The fact that sterculic acid did not affect lipid incorporation of stearic acid in Chlorella vulgaris implies that the initial activation of the acid is not inhibited.

In leaf discs, anaerobic incubations with 1-$^{14}$C acetate produces only stearic and palmitic acid$^{22}$. On transfer to aerobic conditions it was noted that part of the labelled stearic acid disappeared and was replaced by radiolabelled oleic acid. The effect of sterculic acid on this transformation of internally generated stearic acid to oleic acid was minimal. Therefore one may assume that in leaf discs the desaturase enzyme itself is also unlikely to be the site of inhibition.

Assuming that there are no fundamental differences in the overall chemistry of the process, it seems probable that the enzyme inhibited by sterculic acid is the acyl transferase responsible for moving the exogenous stearoyl group from the CoA-thiol ester to either the ACP-thiol ester (scheme 1) or to a specific position on a specific lipid that is the substrate for desaturation (scheme 3), or the enzyme itself (scheme 2). This would provide an alternative explanation to the saturate-independent synthesis of monoenes suggested by Reiser and Raju$^{213}$, and Donaldson$^{218}$. In James' scheme, stearoyl-ACP would be generated from acetate via a normal synthetase system thus bypassing his proposal inhibition step and allowing the synthesis from acetate to oleate to proceed uninhibited.

This total explanation of results provided by James et al$^{212}$ adds further doubts to the suggestion by Orly et al$^{214}$, that cyclopropene compounds may be general thiol-enzyme inhibitors.

In order to clarify the anomalies that have arisen and learn more about the desaturase enzyme a detailed investigation of the whole aspect of inhibition of desaturation, with particular reference to cyclopropene inhibition was conducted.

As a means of characterizing, more specifically, the role of cyclopropenes in the desaturase sequence further investigations were carried
out with sterculic acid and related compounds. In this context the preparation of homologous cyclopropene acids was undertaken and a method devised to obtain radiolabelled sterculic acid.

A search for other inhibitors in compounds related to the powerful cyclopropene inhibitors was also undertaken. Attention was focused here on long chain derivatives with centrally placed, acid labile, three membered rings. The compounds investigated included cis and trans-9,10-epoxystearic acids, cis and trans-9,10-epithiostearic acids, cis and trans-9,10-epizino-
stearic acids and certain derivatives of the latter. The effect of the cyclopropane ring was also briefly studied, though this had been shown to be inactive in other biological systems.215

Many methods of preparing epoxides have been reported the most common of which are from the olefin, either by direct epoxidation with peracids, or via the chloro or brodo-hydrins. To obtain pure epoxy-acid, however, these methods require extremely pure oleic acid, a product difficult to obtain. Conversion of the olefinic acid to the vicinal dihydroxy-acid, an easily purified product, followed by subsequent conversion to the epoxy-acid is probably a better method. Thus erythro-9,10-dihydroxystearic acid by treatment with hydrogen bromide in acetic acid will yield the acetoxo-bromide which reacts with alcoholic potassium hyroxide to give the cis-9,10-epoxystearic acid. The same double Walden inversion on the three-dihydroxy acid will afford the trans-epoxide.

Several methods of synthesizing episulphides have also been reported, one of the first being the reaction of sodium sulphide on either vicinal halogeno-thiocyanates or dithiocyanates.223 Improved yields of up to 60% were obtained by substituting alcoholic alkali for the sodium sulphide.224

Some of the most useful methods of synthesizing internal, long chain, episulphides, however, involve the reaction of the epoxide with sulfur-containing reagents such as thiourea, thiocyanates, thioamides and xanthates. Illustrative of these is the reaction described by HoChe et al.
which involves the reaction of the epoxide with potassium methyl xanthate to yield the cyclic trithiocarbonate. Subsequent reaction with alkali afforded 9,10-epithiostearic acid in 68% yield, the cis-epoxide giving the trans-episulphide and vice-versa.

A useful review article on the synthesis and properties of the episulphides, and aziridines, which are discussed later (page 129), has recently been published.

Further investigations, directed at achieving interaction with the actual desaturase site, involved other novel derivatives of fatty acids with substituents at the 9 and/or 10-position. In the discussion of Part 1 it was postulated that the active site for desaturation may be a disulphide bridge and in an attempt to vindicate the activity of such a cystine molecule by achieving direct interaction, 9(1)-mercaptosteearic acid and 9-decynoic acid were prepared and incubated. Also, the effect of a chloro-substituent at this position was studied.

In total such experimentation provides the basis of this part of the thesis which, it is hoped, will increase the present knowledge on the reaction sequence involved in desaturation and the nature of the substrate binding site or the active site of the desaturase enzyme. Such information should, in turn, assist in unveiling the mystery surrounding the desaturase enzyme.
RESULTS AND DISCUSSION

A. Synthesis of precursors for inhibition studies

In order to carry out the series of inhibition studies on the desaturase enzyme, a range of known and potential inhibitors were required. In certain cases these compounds had already been synthesized and were kindly donated for use in the investigation; cis and trans-isomers of 9,10-epoxy- and 9,10-epithioesterates*, and the K+ salt of cis 9,10-epinoesterate** were such derivatives. Methods by which they could be prepared are briefly dealt with in the introduction and, in the latter case, the discussion which follows. The other potential inhibitors had to be prepared from available starting materials and the means by which this was achieved is discussed below.

A.1. Methods involving structural changes with cyclopropene acids.

Sterculic acid has been shown to be a powerful inhibitor of desaturation in many systems (see introduction) and, to further investigate its mode of action, the preparation of w-(2-n-octylcycloprop-1-enyl) nonanoic acid and radiolabelled sterulic acid was desirable.

Since the inhibitory effect of sterulic and malvalic acids was first attributed to the cyclopropene group, about 1956203-205, the production and trial of cyclopropeno derivatives steadily increased202. Even more recently the search for a method of synthesizing the labelled acid has intensified because of the greater scope such a product would provide in tracer analyses. Many attempts at synthesizing the acid from methyl sterololate, by addition of a methylene group across the acetylenic bond, have been tried usually with little success205,226. The most successful attempt involved the addition of diazoacetic acid across the acetylenic group yielding the diester of the cyclopropene acid in good yields205 (60-70%). A four stage process for converting this intermediate to methyl sterolulate was then devised by Gensler et al.227 who reported an overall yield of 30%.

* The kind gift of Dr. J.F. McChie, Department of Chemistry, Chelsea College of Science and Technology.

** The kind gift of Dr. G. Maerkor, USDA, Philidelphia, USA.
from methyl stearolate. The product, however, was unlabelled and in an attempt to overcome this Shanstone et al.\textsuperscript{228} are investigating the addition of labelled methyl iodide to stearolate. They only recorded methyl to date of producing the radiolabelled acid (reported since this work was completed) was achieved by Altenburger in 1970\textsuperscript{229}. Again stearolate was used as starting material and, by photolysis with \textsuperscript{14}C-labelled diazonaphthene, methylene labelled sterculic was achieved in 9.5\% yield.

In the work reported here a different route was investigated, based on obtaining chain extension of the lower homologue without destroying the reactive cyclopropane ring. A brief survey of the ways of achieving extension by one carbon atom has been discussed previously (pages 28 and 39) and extensive work on the successful application of the potassium cyanide route, used for extending the methyl branched acids, was undertaken here. The reaction sequence involved is indicated in scheme 1.

\begin{center}
Scheme 1
\end{center}

\begin{equation}
\text{CH}_3\text{(CH}_2\text{n})_n\text{C}=\text{C(})\text{CH}_2\text{n})_n\text{CH}_2\text{OH} \xrightarrow{\text{p-toluenesulphonyl chloride}} \text{CH}_3\text{(CH}_2\text{n})_n\text{C}=\text{C(})\text{CH}_2\text{n})_n\text{CH}_2\text{OSO}_2\text{(CH}_3\text{)}_n\text{CH}_3 \\
\xrightarrow{\text{KOH/DMSO}} \text{CH}_3\text{(CH}_2\text{n})_n\text{C}=\text{C(})\text{CH}_2\text{n})_n\text{CH}_2\text{CN} \\
\xleftarrow{\text{hydolysis}} \text{CH}_3\text{(CH}_2\text{n})_n\text{C}=\text{C(})\text{CH}_2\text{n})_n\text{CH}_2\text{OH}
\end{equation}

* In the synthesis of (1-\textsuperscript{14}C) sterculic acid n=7 and n=6, and \textsuperscript{14}CH\textsubscript{3} was used.
* In the synthesis of \(w\)-(2-n-octylcyclopropane-1-yl)nonanoic acid n=7 and n=7.

In the synthesis of salvalyl alcohol (the precursor for labelled sterculic acid) from the naturally occurring 2-hydroxysterculic acid\textsuperscript{230}, the reduction of the glyceryl-ester of the hydroxy-acid to the 1,2-dihydroxysterculene with lithium aluminium hydride, and the oxidative cleavage of the resultant \(\beta\)-dihydric alcohol with sodium metaperiodate in pyridine\textsuperscript{231} were investigated and shown to proceed without cleavage of the cyclopropane ring, and with extremely good yields. Oxidative cleavage with periodate is
normally carried out in aqueous \(^{232}\) or acidic \(^{233}\) media, but the use of dry pyridine provided both a basic medium, in which the cyclopropene ring is stable, and prevented the light-induced formation of ozone, which occurs in aqueous solutions, and which reacts with the cyclopropene \(^{205}\).

Reduction of the aldehyde obtained above and sterculic acid in the preparation of the \(C_{20}\) cyclopropene-acid, to their respective alcohols was satisfactorily achieved using LiAlH\(_4\) in ether \(^{146,207}\) and subsequent conversion to the \(p\)-toluene-sulphonate, by reaction with \(p\)-toluenesulphonyl chloride in dry pyridine \(^{149}\), proceeded without harming the cyclopropene ring.

Chain extension of the \(p\)-toluene-sulphonate with potassium cyanide in DMSO, though, created a potentially dangerous step, since this reaction is known to require relatively high temperatures for long periods \(^{140,141}\). Under such conditions polymerization might occur \(^{203,209}\) as exemplified by Ncrby et al \(^{207}\) who obtained a polymer by heating sterculic acid at 150°C for 26 hours. However, where polymerization has been investigated, it has almost invariably been with the acid and the elucidation of the polymer structures by Rinehart et al \(^{234}\) reinforced Hunn's \(^{205}\) earlier view that 'polymerization occurs by reaction of the carboxyl group with the double bond'. Thus, this particular reaction of the \(p\)-toluene-sulphonate with potassium cyanide should not be unduly susceptible to polymerization. By careful control of the temperature at 90°C and constant surveillance of the reaction products, this was found to be the case, and the nitrile product was achieved in acceptable yields with the cyclopropene group intact.

Once again the reaction with unlabelled potassium cyanide was shown to be much faster and cleaner than the corresponding reaction with radiolabelled cyanide (ex-Amersham) and the usual high polarity, labelled impurities, which occurred in the latter, probably account for the low radiochemical yield (20.4%) of \(l\)-\(^{14}C\) sterculic acid.

The cyclopropene ring is also known to be unstable in acidic solution \(^{235}\) and addition of hydrogen chloride and hydrogen bromide across the
double bond has been shown to occur\textsuperscript{216,236}. Therefore direct conversion of
of the nitrile to the methyl ester by reaction with hydrogen chloride in
methanol\textsuperscript{137} was not practicable and alkaline hydrolysis to the carboxylic
acid had to be considered. This was achieved by refluxing the nitrile
with aqueous alkaline potassium hydroxide, and once again the need for
relatively high temperatures involved the possibility of polymerization.

In the hydrolysis of the unlabelled nitrile, the carboxylic acid
produced was esterified in order to effect purification by preparative TLC,
and thus ensure the removal of any polymerization product which might have
been formed. Rehydrolysis of the ester, under far less stringent conditions,
afforded the pure \(\omega\)-(2-n-octylcycloprop-1-yl) nonanoic acid. In this
hydrolysis of the unlabelled nitrile, however, there appeared to be little,
if any, polymerization. Therefore, since the low mass of the labelled
reaction product made a lengthening of the reaction sequence undesirable,
purification was undertaken, prior to hydrolysis, by careful preparative TLC
on the nitrile. Hydrolysis of the purified nitrile, under the same conditions,
then gave the desired (1-\textsuperscript{14}C) stearic acid. Radiochemical-TLC analysis
of an esterified aliquot did indeed indicate that little, if any, polymer-
ization had occurred. Throughout all the reactions, care was taken to
subject the cyclopropene compounds to the mildest possible treatment. Where
acidification was necessary in working up, it was undertaken at 0\textdegree C, with
the minimum excess of acid, and where preparative TLC was involved, elution
of the desired product from the silica was carried out as quickly as possible.
No attempts were made to determine the limits of any of the reactions involved
in the sequence, and reaction times were kept to the minimum by constant
checks on their progress.

That the intended reaction had been achieved at each stage, was
confirmed by normal TLC analysis, spraying with a saturated solution of
sulphur in carbon disulphide\textsuperscript{200,230} in certain instances also identified
the presence of cyclopropane. However confirmation that the overall
homologation had been achieved successfully with retention of the cyclopropene ring, in both cases, was obtained by GLC analysis on derivatives of the chain extended cyclopropene product, according to the method of Schneider et al.237. This method involved the reaction of an aliquot of the esterified product with a saturated solution of silver nitrate in anhydrous methanol at 30°C for 2 hours. The reaction was terminated by the addition of water and the products were extracted with petroleum ether. The reaction gives characteristic ether and ketone derivatives of the cyclopropene which are stable and can be analysed by GLC at elevated temperatures.

Analyses of the reaction products from both syntheses were conducted on a 5ft column containing 10% FFAP suspended on celite at 240°C. Simultaneous injection of an internal C_11-22 normal ester standard allowed a semi-logarithmic plot of chain length versus retention time to be constructed and equivalent chain lengths determined. Also since the assigned equivalent chain lengths of the cyclopropene derivatives were very much dependent on column characteristics, subsequent injection of the reaction products from the methanolic silver nitrate reaction on a sample of authentic methyl sterculate, was carried out to confirm the results. The chromatograms obtained indicated the ether derivative of the cyclopropene to be the overwhelming product, but a small peak attributable to the keto-derivative was also detected for the C_20_ cyclopropene.

In figure 3 the results for the analysis of the reaction products from ν-(2-n-octylcycloprop-1-enyl) nonanoate are plotted, indicating equivalent chain lengths of 22.3 and 24.4, on the FFAP stationary phase, for the ether and keto-derivatives respectively. In comparison the ether peak from authentic methyl sterculate had an equivalent chain length of 21.3 (Lit. value on a 10ft column packed with 15% DEGS on Anakrom A at 190°C = 22.3; ref 237).

Radio-GLC analysis of the reaction products from (1-^{14}C) sterculic acid, also on the FFAP column, produced a labelled ether derivative of
Figure 3: Equivalent chain length of products from reaction of \( \text{w-(2-octylcyclopropyl-1-encyl)} \) nonanoic acid with \( \text{AgNO}_3 \) in MeOH.

Column: 10% FFAP on celite. Temperature = 240°C.
equivalent chain length 21.3, which was directly comparable with that from authentic methyl sterulate. A further peak, accounting for 5% of the observed radioactivity, also appeared at equivalent chain length 18.4.

Attempts to identify the latter were to no avail. Analysis of the (1-14C) sterulic acid and its methyl ester by TLC on 0.25mm silica plates indicated a single mass and radioactivity spot in each case. Thus if the component was an impurity, it was probably a carboxylic ester. Therefore the esterified portion of the (1-14C) sterulic acid was also analysed by reverse-phase chromatography238,239 on 0.25mm plates, activated normally and then exposed to trimethylchlorosilane vapour in closed tanks for 16 hours. Development in methyl cyanide-methanol-water (6:3:1) again indicated a single mass and radioactive component, whilst separation of (1-14C) sterulate from labelled oleate and labelled 10-methylstearate was obtained. (Both the latter compounds could have retention volumes on GLC in the region of the unidentified component). Thus an impurity of this nature can be discounted, but one possibility which could conceivably fit the observations is a methylene substitutel stearic acid. The retention volume of such a compound on GLC is of the correct order. Furthermore this cleavage of the cyclopropene ring would introduce a side-chain which could be expected to exert two opposing effects on reversed phase chromatography, which could be self eliminating. For such an impurity to arise, viz:-

\[
\text{CH}_3(\text{CH}_2)_7(8)(\text{CH}_2)_8(7)\text{COOCH}_3
\]

reductive cleavage of the cyclopropene ring must take place, and would presumably be incurred at the stages involving LiAlH_4. However no evidence of such a side reaction has been recorded in other references to this specific reduction146,205,207, nor was there such an impurity in the ω-(2-n-octyl-cycloprop-1-enyl) nonanoic acid produced, so its occurrence must be open to question.

Since the impurity only constituted at the most 5% of the (1-14C) sterulic acid and as there were only small quantities of this product,
further investigations into its identity and means of removal were not
undertaken. One possibility that still remains is that the unidentified
component is not an impurity, but just an artefact of the method of analysis.

A. 2. **Synthesis of opimino-acids**

In order to complete the series of stearic acid derivatives con-
taining a mid-chain heterocyclic ring, the preparation of the K⁺ salt of
trans-9,10-opimino-stearic acid was undertaken. (The requirement for the
cis isomer and the cis and trans epoxides and episulphides had already been
fulfilled by kind donation).

By comparison with epoxides and episulphides, the synthesis of
aliphatic opimino-compounds is a recent advance. The methods reported²²⁵
usually involve the addition of reagents loosely classified as 'pseudo-halogens'
to the olefin and the most commonly used of these is iodine isocyanate (INCO).
Although this compound was first prepared in 1930, it was not until 1960 that
it came into prominence for aziridine syntheses, when Drofehl and Fonsoll²⁴⁰
prepared the aziridine of tetrahydronaphthalene. It has since been adopted
to long-chain aliphatic compounds,²⁴¹,²⁴² and the stereochemistry has been
extensively studied²⁴¹,²⁴³. More recently, the addition of another pseudo-
halogen, N,N-dichlorourethan, (DCU), has been investigated as a means of
synthesizing long chain aziridines²⁴³, and a useful comparison of the two
methods (i.e. INCO and DCU addition) has been carried out by Foglia et al²⁴⁴.

Since the addition of N,N-dichlorourethan to olefins has been
shown to proceed via a free radical mechanism, thus affording a racemic
mixture of the cis and trans-aziridines as product, the synthesis of trans-
9,10-opimino-stearic acid was achieved by the iodine isocyanate method
(reaction sequence III, page 167).

To achieve the trans-aziridine, elaidic acid was reacted with
iodine isocyanate, generated in situ from iodine and silver cyanate. The
reaction was conducted in ether at -5°C and the slow disappearance of the red
colouration with stealy desposition of silver salts indicated a smooth
progression. Under these conditions the reaction has been shown to proceed by \textit{trans}-addition across the double bond to yield the \textit{erythro}-iodocyanate derivative. Generation of the \(\beta\)-iodocarbamate with retention of configuration was then achieved by refluxing with methanol and, since an aqueous methanolic solution of potassium hydroxide was required to effect ring closure, the intermediate was not isolated but a more concentrated aqueous solution of potassium hydroxide merely added. As the ring closure of the \(\beta\)-iodocarbamate takes place in a \textit{trans}-manner, the product achieved by refluxing the \(\beta\)-iodocarbamate overnight was \textit{trans}-aziridine. This was isolated as the potassium salt, since the aziridine ring system is unstable to free carboxylic acids\cite{241,242}. After recrystallisation from ethanol a 23\% yield of white, crystalline, potassium \textit{trans}-9,10-epiminoctadecanoate was obtained. This yield is in keeping with the findings of others\cite{241,244} who have shown it to be only about 60\% of the yield obtainable for the \textit{cis}-aziridine, by the same process. The difference in behavior would appear to be related to the ease of ring closure and detailed steric evidence provided by Gebelin et al\cite{241}, which supports this view, suggests a preference for dehydrohalogenation of the assumed transition state of the \textit{erythro}-iodocarbamate, thus inhibiting aziridine formation.

The melting point of the product was in agreement with the literature (M.Pt=241-244°C; lit. value=243-245°C\cite{244}). The infrared spectrum, run on the KBr pellet, also showed characteristic absorptions at 3160\textsuperscript{-1}(NH) and 3800 cm\textsuperscript{-1}. The latter absorption has been tentatively assigned to the \textit{trans}-aziridine ring\cite{241,244} and was not present in the iodoisocyanate or the iodocarbamate.


Several methods exist for introducing thiol groups into organic molecules, the major ways being the reaction of the organo-halide, usually bromide or iodide, with a sulphur containing compound (i.e. sodium hydrogen...
sulphide\textsuperscript{245}, sodium trithiocarbonate\textsuperscript{246}, thiourea\textsuperscript{247}, etc), and the addition of a sulphur derivative across a double bond. In the work contained in this thesis, use was made of both these methods.

The synthesis of unlabelled DL-9(10)-mercaptostearic acid was achieved from the optically inactive, secondary bromide by reaction with thiourea (reaction sequence IV, page169). In the preparation of the secondary bromide, the readily available cis-epoxystearate was reduced over palladium-charcoal catalyst to give the racemic 9(10)-hydroxystearate. Although this reaction was reported to be unidirectional\textsuperscript{221}, work by McGhie et al\textsuperscript{249} has showed this not to be the case. Conversion of the resultant, racemic hydroxide to secondary bromide was then readily achieved by the action of triphenylphosphine in carbon tetrabromide\textsuperscript{249}, the structure of the product being confirmed by TLC, NMR and IR spectroscopy.

The reaction of the DL-9(10)-bromostearate with thiourea, though, did not proceed so readily as the literature would suggest\textsuperscript{247}. However, after 2 days under reflux, a major proportion of the precursor appeared to have reacted to give the polar isothiocuronium bromide, and so hydrolysis of the salt was carried out with 10\% methanolic sodium hydroxide. This indicated that considerable quantities of the precursor had not reacted with the thiourea and the yield of DL-9(10)-mercaptostearic acid (32\%) compared with a similar reaction on 11-bromoundecenoic acid\textsuperscript{247} (60\% after 3 hours) suggested a considerable difference between reaction rates in primary and secondary bromides.

The synthesis of (1-\textsuperscript{14}C)DL-9(10)-mercaptostearic acid, on the other hand, was achieved by the reaction of thiolaetic acid with labelled oleate (reaction sequence V, page171). Since there was only a very small mass of labelled precursor, stearic acid was added to act as a carrier. This precautionary measure helped to minimise losses during extraction and transfer or reaction products, and the removal of the carrier was easily accomplished by preparative TLC at the end of the reaction sequence, whilst purifying the product.
Initially the reaction mixture was shaken at room temperature to
effect addition\textsuperscript{250}, but after four days, analysis of an aliquot indicated little
if any reaction had taken place. Then, in accordance with other reports\textsuperscript{251}
which suggested the reaction to be a free-radical addition, the contents
were transferred to a quartz vessel and irradiated under U.V. light for a
further seven days, when analysis by TLC indicated partial conversion to the
acetylthio-derivative. As high yields of the labelled mercaptostearate
were not required the reaction was stopped at this point. By comparison,
a reaction between unlabelled oleate and thiolacetic acid, stirred at room
temperature for one week without U.V. light, underwent an 80% conversion to
the acetylthio-derivative. This illustrates well Forc's\textsuperscript{252} frustration
when, after similar experience, he noted that the reaction was "more
reproducible in freshly cleaned glassware of known history". It also suggests
the reaction may proceed through free-radical or polar addition.

After removal of thiolacetic acid, under vacuum, at slightly
elevated temperature, the methyl 9(10)-(acetythio)stearate was converted
directly into methyl 9(10)-mercaptostearate by base catalysed alcoholysis\textsuperscript{251},
with methanol containing small quantities of sodium. After purification by
preparative-TLC the ester was converted to (1-$^{14}$C)DL-9(10)-mercaptostearic
acid by normal hydrolysis with methanolic potassium hydroxide.

Data confirming the structure of the mercapto-derivatives in both
preparations needed to be carefully interpreted, since in certain analyses
they are very similar to other long chain fatty acids. The IR spectrum
of methyl 9(10)-mercaptostearate closely resembled that of methyl stearate
except for a weak absorption at 2540 cm\textsuperscript{-1} corresponding to the -SH stretching
vibration. Also the mercapto- and methoxystearates can easily be mistaken
on TLC. However used together these techniques are helpful and full
elemental analysis in the case of unlabelled 9(10)-mercaptostearate indicated
the desired product had been obtained. By comparison TLC analysis of the
labelled mercapto-derivative indicated a single mass and radioactivity peak
of the same Rf value as the unlabelled product.
Long retention times on GLC inhibited the use of this technique for analysis. Also NMR spectrometry on the unlabelled mercapto-derivative yielded little to distinguish it from methyl stearate except for an ill-defined multiplet at 7.4 \( \tau \), possibly attributable to the methine proton adjoining the sulphhydril group.

Mass spectrometry, however, yielded results which were in full agreement with the expected, although no literature reference to the analysis of long chain mercapto-acids could be found to confirm them. The mass spectrum for methyl 9(10)-mercaptopostearate showed a clearly attributable molecular-ion peak at 330 m/e, although it was of relatively low intensity. A further peak of similar intensity was recorded at m/e 297 ( \( P - 33 \) ) probably relating to loss of the sulphhydril group. The most characteristic feature of spectra, however, were four high intensity peaks at m/e 157, 171, 201 and 215 indicating the most favourable fragmentations. These appeared to be grouped in two pairs, peaks at 157 and 171 being of identical intensity, but of slightly lower relative intensity to the other identical pair at 201 and 215. This is in agreement with the expected spectrum for a positionally isomeric mixture and the separation of 14 m/e indicates substitution at adjacent carbon atoms in the chain (viz; 9 and 10). The fragments are considered to arise from cleavage either side of the carbon atom, containing the sulphhydril group, with loss of two protons to give the \( + - \text{C-S} \) ion or cyclization to give a more stable carbarnium -C-CH

\[
\text{CH}_3(\text{CH}_2)_7(8)\text{H} + (\text{CH}_2)_9(7)\text{COOCH}_3
\]

viz:- 203-2 and 217-2

159-2 and 173-2

Further high intensity peaks at m/e 74 (McLafferty rearrangement) indicative of a methyl ester of a carboxylic acid, and at m/e 69, 83, 97 (hydrocarbon ion) and at m/e 87, 101 (methoxycarbonyl ion) indicative of a polymethylene chain, were also present.

Confirmation of these results was achieved by mass spectrometry on
previously prepared sample of methyl 12-norcaptoestearate. Comparative intensities of the various peaks were basically the same, but in this case only two high intensity peaks were recorded in the medium mass region at m/e 243 and m/e 129, both attributable to either the -C=S ion or cyclic carbonium ion {-C-CH

\[
\begin{align*}
131-2^t & \text{CH}_{3}(\text{CH}_2)_i \text{CH}^i(\text{CH}_2)_{10}\text{COOH}_3 \\
1245-2+ & \text{SH}_1
\end{align*}
\]

Overall it was possible to show that the chosen reactions yielded the intended products, and the yields of these were sufficient to conduct the envisaged incubations. However neither method appeared to be particularly good, but recently a modification of the thiourea reaction has appeared using DMSO as solvent, which is reported to give improved yields, with milder conditions.

A.4 Synthesis of halogenated fatty acids

The most widely used method of producing the monochloride is from the corresponding alcohol, and there are several well established ways of achieving this including the reactions with the phosphorus halides or thionyl chloride. In the work carried out here, the opportunity was taken to further investigate the reaction of the alcohol with triphenylphosphine in carbon tetrachloride. The reaction, which was known to proceed more rapidly with primary alcohols, was applied initially to the secondary alcohol, D(-) 12-hydroxystearate.

When heated under reflux with a 50\% molar excess of triphenylphosphine in a vast excess of carbon tetrachloride, the reaction progressed steadily to completion in 6.5 hours producing 12-chlorostearate in 80\% yield. The reaction also has the added advantage that the byproducts of the reaction are easily separated, triphenylphosphine oxide being largely insoluble in carbon tetrachloride at 0\°C and chloroform being easily removed under vacuum.
along with the excess carbon tetrachloride.

The mechanism involved is thought to be:

\[
\begin{align*}
\text{SO}_3\text{P} + \text{CCl}_4 & \rightarrow \text{SO}_3\text{P}^+\text{CCl}_3\text{Cl}^- \\
\text{RCH}_2\text{H} & \rightarrow \\
\text{SO}_3\text{P=O} + \text{RCl} & \rightarrow \text{SO}_3\text{P}^+\text{Cl}^- + \text{CHCl}_3 \\
\text{O-R} & \\
\text{where R=CH}_3\text{CH}_2\text{CH}(\text{CH}_2)_4\text{COOCH}_3.
\end{align*}
\]

Optical rotation measurements on the starting material and the isolated product indicated a change in rotation from (-) to (+). Therefore hydrolysis of the secondary chloride was carried under conditions known to give inversion of configuration \(^{255}\), and since the 12-hydroxystearate recorded had a negative rotation it was concluded that the \(\text{SO}_3\text{P/CCl}_4\) reaction proceeded with inversion of configuration and that the product of the initial reaction was L(+)-12-chlorostearate \(^{249}\).

This inversion of configuration was confirmed by conducting the same reaction on D(-)-9-hydroxystearate, which was obtained from the naturally occurring 9-hydroxyoctadeca-12-enoic acid by reduction over Adams catalyst. Once again the reaction produced good yields (70%) with inversion of configuration to yield L(+)-9-chlorostearate.

All the findings here are in agreement with other stereochemical studies on this reaction which illustrate inversion of configuration \(^{249,256}\) except for one example where retention of configuration has been recorded \(^{257}\).

Because of the simplicity of this reaction, it was also employed in the synthesis of DL-9-chlorostearic acid. However the stereospecificity of the reaction meant that the D(-)-9-hydroxystearate had to be racemized prior to conversion. This was achieved by oxidising the secondary hydroxide to the optically inactive ketone, with chromium trioxide in glacial acetic acid, and then reducing the esterified ketone to the inactive DL 9-hydroxystearate with sodium borohydride in isopropanol.

All three chloro-esters were run on GLC against an internal \(\text{C}_{11-18:1}\) standard and their equivalent chain length determined. On a 5ft
PEGA column at 180°C, they all appeared as single peaks with very similar equivalent chain lengths of 23.0 for the 12-chlorostearate and 22.9 for the 9-chlorostearates.

The reaction, which was also successfully adapted to obtain the secondary bromide from the corresponding alcohol in 63° yield by substituting CBr₄ in ether for COCl₂ (pco169), was shown, in this series of investigations, to provide a good method for halo-substitution in fatty acids that proceeds, in the chloro-case at least, with inversion of configuration

The synthesis of the dichlorostearic acid, also used in the incubation studies, was achieved by chlorine addition across a double bond in the reaction oleic acid was the precursor and, since halogenation proceeds stereospecifically by *trans* addition, three -9,10-dichlorostearic acid was the reaction product.

### A. Chain Extension of acetylenes

The synthesis of unlabelled en (1-¹⁴C) 9-decynoic acid was achieved by chain extension of the lower homologue, 8-nonenynoic acid, with unlabelled and labelled potassium cyanide as in previous experiments (page 40).

### B. Incubation of inhibitors

Investigations were carried out mainly on the desaturase system present in hen liver microsomes, but use was also made of the whole cell culture of the alga *Chlorella vulgaris*. The latter system which is capable of activating en desaturating free fatty acid substrates to give polyunsaturates typical of plant systems, has been well studied, and the conditions required for optimum desaturation and minimal interference from the competing breakdown/resynthesis reaction have been discussed earlier (page 59).

In the microsomal fraction of hen liver, which is only capable of nonene synthesis, the requirements for optimum desaturation were not nearly so clear.

In Part 1 investigations into the oxygen requirements of the system,
and the decay of enzyme activity during incubations, were carried out. Information on the level of substrate tolerance and the threshold mass for a particular quantity of enzyme was also obtained. Proceeding the incubations with various added inhibitors further investigations, into variation of the supernatant in which the microsomes were suspended, were carried out. Resuspending the microsomal pellet (spun out of solution at 100,000g) in particle free supernatant, boiled supernatant and 0.3M sucrose indicated optimum desaturation of added free fatty acid substrate in the former case, with desaturation decreasing successively with boiled supernatant and 0.3M sucrose. Experiments with various levels of cofactors were also conducted to ensure efficient activation and desaturation of the free fatty acid substrates. These various conditions and cofactors were then optimised to obtain maximal conversion of substrate to product, and these optimum requirements were adhered to in each incubation, to ensure any observed effects resulted from the added inhibitors.

In the systems chosen the active desaturase enzyme, by necessity, is still in association with other enzymic protein intimately involved in the desaturase sequence (e.g. acylases, transferases etc.). And, unlike Part I where the discussion was based on actual desaturation of labelled, non-typical substrates, the effect of inhibitors is measured by a secondary parameter, the inhibition of desaturation of a labelled substrate. Thus, in the systems employed, there is no absolute guarantee that the effect is exerted against the actual desaturase enzyme, and the observed inhibitions may be a measure of the effect against any factor which forms a part of the desaturase sequence.

In Part 1, however, the desaturation of certain methyl-branched fatty acids showed that activation and subsequent accommodation of slightly modified stearic acids on the desaturase enzyme was possible. The inhibitors investigated here, both proven and potential, all contained long polymethylene chains and were in the main carboxylic acids. Therefore this structural resemblance to the normal substrate, stearic acid, maximised
the chances of interaction between inhibitor and the actual desaturase enzyme. By definition it also meant that any observed inhibition was likely to be of a competitive nature.

B.1. Incubations involving cyclopropane fatty acids.

To further elucidate the mode of action of the cyclopropane acids and thereby gain more information about the desaturase enzyme, the following series of incubations was undertaken in the microsomal fraction of hen liver.

B.1.1. Investigation into the inhibitory effect of homologous cyclopropane fatty acids.

Since sterculic acid is such a powerful inhibitor of desaturation the specificity of its action may be particularly sensitive to any movement of the cyclopropane ring from the 9,10-position in the polynethylene chain. Therefore to test any such specificity, the next lower and higher homologues of sterculic acid, namely, malvacic and \((2-n\text{-octylcycloprop-1-enyl})\) nencenoic acids, were incubated at varying concentrations with the microsomal fraction of hen liver.

In each case, the degree of desaturation was calculated by Radio-GLC on the esters obtained by transmethylolation of the lipid extract from the incubation. The percentage inhibition was then obtained by dividing this desaturation by that of the equivalent stearic acid control incubation and subtracting the result from 100. The results so obtained (table 1) are represented graphically in figure 4. Each lipid extract from those incubations containing the highest levels of inhibitor, was also analysed by thin layer chromatography and the count distribution determined by comparison of peak areas from the radioactivity scan (see table 1).

These results, once again, illustrate the inhibitory power of sterculic acid. Furthermore, study of the inhibition vs dose response curve, (figure 4) for all three cyclopropane acids, shows an extremely close similarity in their effectiveness to block the desaturation of added \((1^{14}C)\) stearic acid.

Their effectiveness is indeed illustrated by the relative concentration studies which show that, by simultaneous addition of inhibitor and
Figure 4  Inhibition of desaturation of $1^{-14}\text{C}$ stearic acid by varying concentrations of cyclopropene fatty acids.

System: Hen liver microsomal fraction.
Effect of varying concentrations of cyclopropene fatty acids on the desaturation of 1\(^4\)C stearic acid (0.176\(\mu\)M) by the microsomal fraction of hen liver.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration ((\mu)M)</th>
<th>Inhibitor: Substrate</th>
<th>Desaturation (%)</th>
<th>Inhibition ((\mu))</th>
<th>Lipid Incorporation ((%))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P.L.</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td></td>
<td>31.5</td>
<td>~4</td>
<td>52.5</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>1.76</td>
<td>10</td>
<td>30.0</td>
<td>100</td>
<td>50.4</td>
</tr>
<tr>
<td>Malvalic acid</td>
<td>1.76</td>
<td>10</td>
<td>0</td>
<td>3.2</td>
<td>89.9</td>
</tr>
<tr>
<td></td>
<td>0.176</td>
<td>1.0</td>
<td>3.8</td>
<td>16.0</td>
<td>49.2</td>
</tr>
<tr>
<td></td>
<td>0.0018</td>
<td>0.01</td>
<td>26.6</td>
<td></td>
<td>15.5</td>
</tr>
<tr>
<td>Sterculic acid</td>
<td>1.76</td>
<td>10</td>
<td>0</td>
<td>100</td>
<td>57.3</td>
</tr>
<tr>
<td></td>
<td>0.176</td>
<td>1.0</td>
<td>2.7</td>
<td>15.5</td>
<td>50.8</td>
</tr>
<tr>
<td></td>
<td>0.0018</td>
<td>0.01</td>
<td>29.2</td>
<td></td>
<td>7.5</td>
</tr>
<tr>
<td>(\omega)-(2-n-methyl-cycloprop-1- enyl)acid</td>
<td>1.76</td>
<td>10</td>
<td>0</td>
<td>100</td>
<td>47.6</td>
</tr>
<tr>
<td>Nononoic acid</td>
<td>0.176</td>
<td>1.0</td>
<td>1.0</td>
<td>11.4</td>
<td>63.4</td>
</tr>
<tr>
<td></td>
<td>0.0018</td>
<td>0.01</td>
<td>26.0</td>
<td></td>
<td>17.5</td>
</tr>
</tbody>
</table>

Substrate to the system the cyclopropene acids cause a 10-15\% reduction in desaturation even when they are present in only \(1/100\) the concentration of the substrate; this represents the addition of about 2\(\mu\)M of cyclopropene acid to a system containing around 125\(\mu\)g of proteins. Increasing the inhibitor concentration to 17.6\(\mu\)M (\(1/10\)th the substrate concentration) causes a 50\% reduction in the efficiency of the desaturase system, and almost complete inhibition of enzymic desaturation arises when the inhibitor concentration is raised to 0.176\(\mu\)M, which was equal to the substrate concentration. Further increases in inhibitor concentration completely inactivated the desaturase enzyme and addition of 1.76\(\mu\)M of unlabelled stearic acid (10:1 dilution of label) to a control incubation proved that the observed effects were not caused by substrate dilution. Further analysis of the overall results (figure 4) also showed that the degree of inhibition varied almost linearly with the log. inhibitor concentration. (The results for ster- culate inhibition are similar to those obtained by Allen et al.\(^{167}\).

Movement of the cyclopropene ring from the 9,10-position in the polymethylenic chain, to the 8,9-position, as illustrated by the results for malvalic...
acid, causes no loss of activity. This effect, exhibited here throughout the range of concentrations, does not agree the observations of Johnson et al.\textsuperscript{33} who, in a similar microsomal desaturase, found malvalic acid to be only 75% as effective as sterculic acid, at 0.5\textmu M inhibitor concentration.

Movement of the cyclopropene ring from the 9,10-position, to the 10,11-position as illustrated by \( \text{w-(2-octylcycloprop-1-enyl)monoenic} \) acid also causes no loss of activity, the results infact indicating about 10% increased effectiveness throughout the concentration range.

Proceeding from the results of others\textsuperscript{218,219}, who proposed interaction between sterculic acid and the desaturase enzyme (pages 116 -120 ), Johnson\textsuperscript{33} explains his own results by suggesting that sterculic acid or its CoA derivative completely and irreversibly occupies the substrate site on the desaturase enzyme. To obtain this he proposes the formation of a carbon-sulphur bond between the 9 or 10-carbon atom of sterculic acid and a thiol group at the desaturating site of the enzyme (cf.ref\textsuperscript{217}). That such a thiol group may exist in this vicinity had previously been proposed\textsuperscript{175,176}, and Johnson believes that the decreased activity of malvalic acid results from the complicative displacement of the cyclopropene ring from the 9,10-position in the chain.

On first sight the results reported here would appear to contradict this view, as malvalate, and \( C_{20} \) cyclopropene acid for that matter, are both at least as active as sterculate. However, from results on the stereochemical and structural aspects of the desaturase enzyme (page 75), it was proposed that two sulphhydryl groups exist at positions adjacent to the 8,9 and 10,11 carbon atoms of a substrate in the normal enzyme - substrate complex. Thus if the cyclopropene acids are accommodated and do react with an adjacent sulphhydryl group at the site of desaturation, a decrease in activity of the homologues will not be apparent until the cyclopropene group is position one, or possible two carbons, further away from the 8,9 or 0,1\textsuperscript{12}-positions.

Further examination of the results from the studies with
methyl-branched stearic acids (page 63) indicates that nonadecenoic acid is not desaturated to the same extent as stearic acid (up to 25% loss in hen liver systems) which infers a degree of steric hindrance against accommodation of C19 chain length, by the desaturase enzyme. Thus, if the cyclopropene acids do occupy the site on the enzyme, normally occupied by a substrate molecule, one would expect the 6-(2-n-octylycycloprop-1-anyl) nonenoic acid to be less easily accommodated, and thus not as effective an inhibitor as sterculic acid. This was not the case, and in fact the reverse appeared to be true, thus suggesting that irreversible and complete occupation of the desaturase enzyme does not take place. If the latter is the case, then the proposal of James et al.\(^{212}\), that the locus of action of sterculic acid is prior to the desaturase step, gains favour. Their suggestion is that inhibition takes place at some point, possibly an acyl transfer step, which is not involved in the desaturation of stearic acid derived from added acetate (page 119).

Thorough analysis of the lipid extracts from these incubations (see table 1) did not indicate any apparent differences between the incorporation of label in the control incubation and in those incubations in which the cyclopropene acids totally inhibited desaturation. Therefore the cyclopropene group does not inhibit thiol ester formation between the substrate and CoA, but the similarities of lipid incorporation does not necessarily exclude the possibility that cyclopropene acids inhibit the esterification of normal fatty acids into particular lipid positions.

B.1.2. Investigation into the incorporation of sterculic acid into the lipids.

The work of Gurr\(^{259}\) and James et al.\(^{260}\) has shown conclusively that the removal of lipid from the microsomal fraction of hen liver completely inactivates the desaturase enzyme. Therefore, the inability to adequately define the role of sterculic acid in the inhibition of desaturation, and further, more specific, evidence for the close involvement of phospholipids in various enzymic reactions\(^{30,165,261}\), highlighted the possibility that sterculic acid might disrupt some specific function of
lipid in relation to desaturation of stearic acid. Therefore a thorough investigation of the incorporation of labelled stearic and sterculic acid into the polar lipid of the hen liver microsomal fraction, was undertaken. Three incubations were carried out (page 176) involving:

(a) 1-14C stearic acid (2µc;0.4µM); (b) 1-14C sterculic acid (2µc;ca.0.4µM) and (c) 1-14C stearic acid (2µc;0.4µM) and sterculic acid (0.4µM).

The concentration of sterculic acid in the latter was sufficient to totally inhibit desaturation.

Determination of the degree of desaturation in incubations (a) and (c) and a full analysis of the incorporation of labelled acid into each position of the individual phospholipids was carried out as described in the experimental. The separated phospholipids from incubation (b) were also saponified and the liberated acids subjected to an analysis devised by Shneider et al. which confirmed the presence of an intact cyclopropene group.

The total picture of incorporation of labelled fatty acids into lipids obtained (tables 2a, 2b and 2c), illustrates many interesting comparisons. From the incubation with (1-14C) sterculic acid it is apparent that this acid, like the normal substrate, is not excluded from the polar lipids, phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylinositol (PI). However the degree of incorporation was not as high as (1-14C) stearic acid, reaching just over half the control level. This could possibly have resulted from the sterculic acid having a low specific activity. Accurate determination of the latter was hindered by the breakdown of sterculic acid on GLC. However conversion to the stable other (and ketone) derivative with AgNO3/MEOH and subsequent determination on these derivatives, indicated a specific activity of around 4.2 µc/µM.

Thus 0.48µM of labelled sterculic acid was added to incubation (b), and from incubation (c) (table 2c) where the labelled substrate was diluted with 0.4µM sterculic acid; and other experiments which have involved even greater dilution of label, it seemed unlikely that the reduction of incorporation
Table 2c

Lipid incorporation using \( ^{1-14}C \) stearic acid + sterulic acid as precursor% Total desaturation = 0%

<table>
<thead>
<tr>
<th></th>
<th>NL+FFA</th>
<th>Polar Lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE</td>
<td>14.0</td>
<td>PC</td>
</tr>
<tr>
<td>P-E</td>
<td>-8.1[57.6]</td>
<td>-14.7[89.4]</td>
</tr>
<tr>
<td></td>
<td>5.9[42.4]</td>
<td>-1.8[10.6]</td>
</tr>
</tbody>
</table>

Table 3

Incorporation of label into phospholipids*

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Label</th>
<th>PE Total</th>
<th>PE Position</th>
<th>PC Total</th>
<th>PC Position</th>
<th>PI Total</th>
<th>PI Position</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>a) (^{1-14}C ) stearic</td>
<td>( C_{18:0} + C_{18:1} )</td>
<td>27.3</td>
<td>7.8</td>
<td>19.5</td>
<td>51.3</td>
<td>24.0</td>
<td>27.3</td>
</tr>
<tr>
<td>acid</td>
<td>( C_{18:0} )</td>
<td>28.2</td>
<td>12.8</td>
<td>16.2</td>
<td>37.6</td>
<td>36.2</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>( C_{18:1} )</td>
<td>25.1</td>
<td>2.1</td>
<td>23.1</td>
<td>66.7</td>
<td>10.2</td>
<td>56.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24.0</td>
<td>7.8</td>
<td>19.5</td>
<td>51.3</td>
<td>24.0</td>
<td>27.3</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b) (^{1-14}C ) sterulic</td>
<td></td>
<td>15.2</td>
<td>2.3</td>
<td>12.9</td>
<td>68.6</td>
<td>7.3</td>
<td>61.3</td>
</tr>
<tr>
<td>acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>c) (^{1-14}C ) stearic</td>
<td>( C_{18:0} + C_{18:1} )</td>
<td>30.8</td>
<td>17.8</td>
<td>13.0</td>
<td>36.2</td>
<td>32.3</td>
<td>3.9</td>
</tr>
<tr>
<td>acid + 10x's sterulic acid</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The total incorporation of label for each category has been normalized to an assigned value of 100.
into phospholipid resulted from the lower specific activity. Therefore it would appear that some barrier to incorporation of sterculic acid does exist possibly resulting from a greater specificity of incorporation than stearic acid.

To highlight any such specificity and aid comparison of any inter-relationship that may exist between stearate and sterculate incorporation, a table was compiled in which the total incorporation into polar lipids was normalised to a value of 100 (table 3).

These results indicate, clearly, that selectivity of incorporation of sterculic acid does exist. Considering the acylation into individual phospholipids, it can be seen from incubation (b) that sterculic acid has a high affinity for PC. The entry into PE and PI by comparison is somewhat restricted and this may, in part, account for the lower overall incorporation of sterculic acid. In comparison the effect of unlabelled sterculic acid on the incorporation of (1-14C) stearic acid (incubation c) follows a predictable pattern, the normal substrate appearing equally in all three phospholipids, inferring that sterculic acid exerts the bulk of any effect, on lipids, against the incorporation of stearic acid into PC.

Further analysis of labelled acids at each position on the individual phospholipids indicates that 89% of the labelled sterculate was incorporated at the 2-position, 61%, of which was at the 2-position of phosphatidylcholine. Again the reverse of this was observed in the incubation inhibited by unlabelled sterculate; here only 21% of the 1-14C stearic acid was acylated into the 2-position of phospholipids, of which only 3.9% went into the 2-position of phosphatidylcholine. Thus it would appear that sterculic acid markedly affects acylation of stearic acid into the 2-position of PC and to a lesser extent PI. Unfortunately the magnitude of this apparent inhibition could not be measured since in the control reaction the substrate had undergone desaturation as well as acylation into lipid. Examination of the results from the control incubation (a) however, indicated a close similarity between the incorporation of the product of desaturation
Table 2a

Lipid incorporation using $1^{-14}$C stearic acid as precursor (a)

Table not shown.

<table>
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<tr>
<th></th>
<th>NL</th>
<th>FTA</th>
<th>FE</th>
<th>PG</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>60.4</td>
<td>31.0</td>
<td>29.4</td>
<td>10.8</td>
<td>20.3</td>
<td>8.5</td>
</tr>
<tr>
<td>18.0</td>
<td>16.9</td>
<td>13.4</td>
<td>6.2</td>
<td>7.9</td>
<td>7.1</td>
</tr>
<tr>
<td>18:1</td>
<td>14.1</td>
<td>16.0</td>
<td>4.6</td>
<td>12.4</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Table 2b

Lipid incorporation using $1^{-14}$C stearic acid as precursor (b)

Table not shown.
(1-14C oleate), and (1-14C) sterculic acid in incubation (b), which was reflected in the comparison of the incorporations of unsaturated substrate (1-14C stearate), and (1-14C) stearic acid inhibited by sterculic acid (incubation c). One further point, illustrated by incubation (a), is the high level of oleate incorporated at the 2-position of the phospholipids, and in particular the 2-position of PC where it constitutes 97% of the label; this stresses the importance of this position in the desaturase cycle.

Collectively these observations point to a very close involvement of phosphatidylcholine with the desaturase enzyme present in hen liver. This particular lipid could be the direct precursor for the desaturase enzyme presenting the acyl-residue at the 2-position for desaturation, either by combining with the enzyme to form a lipoprotein, or by releasing the acyl residue direct to the enzyme and remaining in close proximity for direct acceptance of the product. Evidence of such enzyme-lipid interaction already exists in Chlorella vulgaris for the desaturation of oleate30 and synthesis of trans-3-hexalenoate165, and in Clostridium butyricum261 for the synthesis of cyclopropane fatty acids.

However incubation (c) shows that even when desaturation is totally inhibited by sterculic acid, some stearic acid is incorporated at the 2-position, and, at the low levels previously shown to inactivate the enzyme (table 1), it seems unlikely that high specificity for PC alone could be totally effective against desaturation. This view is also held by Baker et al263 who, by a different approach, have shown that stearoyl-phospholipid is probably not the direct precursor for the desaturase enzyme present in the fungus Neurospora crassa.

The results do, however, emphasise the possibility of strong end-product inhibition exerted by the selective incorporation of sterculic acid into the major product deposition sites on the phospholipid, a selective incorporation that may in fact arise from the similar bent-chain conformations of the inhibitor and product. This type of action, though, can only be cited
as a contributory factor in the total observed inhibition of stearate desaturation, but, at the higher concentrations of sterculic acid required to inhibit linoleate formation in a dieno system, analogous incorporation patterns may account for the total observed inhibition.

3.1.3. Investigation into the incorporation of sterculic acid in the partially purified hen liver microsomal desaturase.

Since it was not possible to implicate the incorporation of sterculic acid into complex lipid as the sole factor effecting the observed inhibition, further study was carried out to identify any specific interaction between the cyclopropenoic acid and protein associated with the desaturase activity of hen liver microsomes.

Although complete isolation of what constitutes the desaturase enzyme has not yet been achieved, considerable advances have been made towards partial purification in several systems. One of the most notable of these has been in *Diplona gracilis* where the soluble stearoyl-ACP desaturase has been isolated and fractionated, on DEAE-cellulose by gradient elution, into three components. One of these components has been assigned to have the desaturase activity, but this is completely inactive without the presence of the other two components, a non-haem iron protein and a reduced triphosphopyridine nucleotide oxidase. Gurr and James have also isolated the 'soluble' desaturase of the rat liver microsomal system by freeze-drying the microsomes, extracting them by homogenising with 1.0M phosphate buffer and centrifuging at 100,000 g for 1 hour. The resultant supernatant had increased activity over the original microsomes, and further increases in specific activity could be achieved by continued centrifugation, though this sedimented out some of the initial activity.

This technique, and the use of non-ionic detergents, have since been used to obtain solubilization of the stearoyl-desaturase from hen liver, and two methods for further purification of this solubilized enzyme have been applied with some success. The first of these involved density gradient centrifugation on a discontinuous sucrose gradient. However, better
purification was obtained by gel filtration on agarose gels where a four fold increase in specific activity was obtained in a fraction containing only one quarter of the phospholipid to protein ratio of the original microsomes. Since this represented the purest obtainable state of the desaturase enzyme of hen liver, the affinity of sterculic acid for the protein contained in the fraction, was investigated.

In the incubations a somewhat higher than normal concentration of microsomes were homogenised in supernatant, which had been boiled to remove the bulk of non-essential protein. This resulted in a protein level of 62mg/2ml homogenate. The precursors in these, otherwise standard incubations were (l-14C) stearic acid (2nM; 0.04μM), (l-14C) sterculic acid (1μM; 0.2μM) and (l-14C) stearic acid (2nM; 0.04μM) with sterculic acid (0.4μM). After 3 hours, in which a 20% desaturation in the control and no desaturation in the sterculate inhibited incubation had taken place, all three incubations were rapidly frozen and freeze-dried. The freeze-dried powder was extracted with 1.0M phosphate buffer, centrifuged and the supernatant, containing the solubilized enzyme, was applied at the base of a sepharose 6B gel filtration column. The effluent was monitored by U.V. spectroscopy at 280mμ to ascertain the presence of protein, and fractions, collected every 4.5ml, were counted by scintillation spectrometry and the radioactivity (dpm) plotted on the corresponding U.V. spectrum, for each incubation (figure 5).

From previous results the protein containing the desaturase activity, has been shown to be eluted in the void volume (Vo) of the column which is contained in peak P1. This indicated that the desaturase enzyme has a particle weight greater than the exclusion limit of the gel which is 4 x 10^6. The proteins of lower molecular weight, eluted in peaks P2 and P3, have both been shown to contain no desaturase activity.

The results of the present series of incubations indicated the best absorption of radiolabel into the active P1 peak arose from the control incubation, viz. (l-14C) stearic acid. By comparison, (l-14C) sterculic acid
Figure 5 Gel filtration of the soluble extract of hen liver microsomal desaturase on Sepharose 6B.

- $^{14}$C stearic acid
- $^{14}$C sterculic acid
- $^{14}$C stearic acid + sterculic acid

---

O.D. at 280μm. --- Count distribution. ← Eluent.
exhibited greater absorption in the inactive P3 peak with relatively less absorption in P1 indicating, on the surface, poorer interaction of the inhibitor with the protein associated with desaturation. This need not necessarily be the case, though, since peak P1 contains all proteins above the exclusion limit of the agarose gel, and the cyclopropane acid may be more selective than stearic acid in its interaction with the specific protein associated with desaturation. That sterulic acid did act, preferentially, with some of the protein contained in this peak, is illustrated by the fact that the incubation with unlabelled sterulic acid showed a markedly decreased incorporation of (1-14C) stearic acid in the active P1 peak.

Collectively these results are consistent with sterulic acid exerting its inhibition by interaction with protein involved in desaturation. The simultaneous inhibition of desaturation and lowering of stearic acid incorporation into the peak known to contain the desaturase enzyme, strongly suggests that the inhibitor achieves its effect by reacting with the desaturase enzyme, or an associated enzyme in the same peak; the appreciable incorporation of labelled sterulic acid in the same peak serves also to confirm the feasibility of this hypothesis.

A further series of incubations was conducted in which the incubation time was cut to 3 minutes in an attempt to concentrate more radiolabel in the active void volume peak at the expense of P2 and to a lesser extent the P3 peak. In fact the reverse was obtained with no incorporation of label into P1, but virtually unchanged incorporation into the latter two peaks, indicating a relatively slow association of the substrate and inhibitor with the active protein.

In their initial investigations on the use of gel filtration in purifying the desaturase enzyme James and Gurr259 noted that the void volume peak often displayed a distinct shoulder indicating weak retention of some component(s) by the gel. In the present investigations it was noticed that the peak corresponding to incorporation of radiolabel always
logged slightly behind the void volume peak, suggesting that some weak separation of enzyme associated with desaturation may occur. Better separation of this double peak may, in future, yield a purer desaturation in which more conclusive investigations can be carried out to ascertain more precisely the inhibitory role of sterol acid, and mechanism of desaturation.

B.1.4. Investigation into the possible in situ production of sterol acid from 9,10-dihydrosterol acid by the microsomal desaturation of hen liver.

Considerable work has been carried out in various seeds \(^{(264,265)}\) and other plant tissues \(^{(266)}\) to determine the mechanism of biosynthesis of the cyclopropene acids and the bulk of evidence cites desaturation of dihydrosterol acid as the final stage in the sequence. Also Kircher et al.\(^{(267)}\), in their work on avian systems noticed the pink egg syndrome characteristic of sterolate inhibition, was induced when hens were fed a diet which included large quantities of dihydrosterolate. In explanation they suggested that dihydrosterol acid underwent desaturation to sterol acid, which subsequently caused the effect.

To determine whether avian systems were capable of biosynthesizing sterol acid by such a route, \(^{(1^{-14}C)}\) dihydrosterol acid was incubated with a highly active desaturation system from the microsomal fraction of hen liver (page 182). Although stearic acid in the control incubation showed a 77% desaturation, analysis of the product of the dihydrosterol irradiation by the extremely sensitive method of Schneider et al.\(^{(237)}\) indicated no sterolate. The formation of sterolate with subsequent inhibition of the whole desaturation system may have taken place, but the possibility of such an occurrence is considered unlikely, since some free sterol acid would probably have been detected, and, in plant systems capable of produce cyclopropene acids, they are deposited in the triglycerides and do not inhibit the concurrent production of oleate.

A further experiment in this series, conducted by S.J.Hall\(^{(238)}\), provided an alternative explanation of Kircher's observations. By incubating various concentrations of unlabelled dihydrosterol acid with the microsomal fraction of hen liver, according to the procedure detailed on page 176, to
recorded 14.2 and 33.5% inhibition of (1-\(^{14}\)C) stearate desaturation, with dihydrosterculic acid concentration of 10x's and 100x's substrates respectively. At the high dihydrosterculic acid levels employed by Kircher this degree of inhibition could well explain his observations.

B.2. Incubations with potential inhibitors containing a heterocyclic ring or related derivative

Extensive work by several investigators has shown the biological activity of sterculic acid to reside in the cyclopropene ring. In an attempt to discover new inhibitors of the desaturase enzyme initial efforts were therefore centred around compounds bearing a similar mid-chain, three membered ring system. Since 9,10-dihydrosterculic acid was shown to have little effect, a basic structural resemblance was retained by using the heterocyclic compounds, epimino-, epoxy- and epithio-stearates. These compounds also provided, in varying degrees, certain chemical similarities to the cyclopropenes such as acid lability of the ring system and possible mercapto-addition.

B.2.2. Investigations into the inhibitory effect of the epiminostearic acids

The initial investigation was carried out on the microsomal fraction of hen liver using the potassium salts of cis and trans-9,10-epiminostearates, (Page 183). The procedure was identical to that used with the cyclopropene homologues (Exp. B.1.1.), inhibitor concentrations varying from \(\frac{1}{10}\)th to 10x's the substrate concentration (table 4a).

| Inhibitor                  | Concentration (\(\mu\)l) | Inhibitor/ | Desaturation (\%) | Relative Inhibition (\%) |
|----------------------------|--------------------------| Substrate |                 |                         |
| None                       |                          |           | -24.5            |                          |
| Stearic acid\(^3\)         | 1.76                     | 10        | 24.0             | ~2                       |
| cis-9,10-epimino C\(_{18:0}\) | 1.76                     | 10        | 16.0             | 36                       |
|                             | 0.176                    | 1.0       | 23.8             | 4                        |
|                             | 0.018                    | 0.1       | 25.2             | 0                        |
| trans-9,10-epimino C\(_{18:0}\) | 1.76                     | 10        | 15.5             | 38                       |
|                             | 0.176                    | 1.0       | 24.0             | 2                        |
|                             | 0.018                    | 0.1       | 24.3             | 0                        |
4b) Substrate was \(^{14}\text{C}\) stearic acid (2\(\mu\)c; 0.04\(\mu\)M).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Conc(^{\text{H}}) ((\mu)M)</th>
<th>Inhibitor: Substrate(^{\text{A}})</th>
<th>Desat(^{\text{H}}) (%)</th>
<th>Relative(^{2}) Inhib. (%)</th>
<th>Lipid Incorporation(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td>77.0</td>
<td>66.2</td>
<td>8.4 17.8 7.6</td>
</tr>
<tr>
<td>Stearic acid(^{2})</td>
<td>4.0</td>
<td>100</td>
<td>31.0</td>
<td>82.1</td>
<td>5.6 10.1 2.2</td>
</tr>
<tr>
<td>cis-9,10-epimino (\text{C}_{18:0})</td>
<td>0.4</td>
<td>10</td>
<td>75.7</td>
<td>74.2</td>
<td>8.5 8.5 8.8</td>
</tr>
<tr>
<td>trans-9,10-epimino (\text{C}_{18:0})</td>
<td>4.0</td>
<td>100</td>
<td>7.3</td>
<td>76.5</td>
<td>73.0</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>10</td>
<td>39.2</td>
<td>48.0</td>
<td></td>
</tr>
</tbody>
</table>

1. This column refers to the ratio of inhibitor to substrate concentration.

2. This is calculated by assuming the desaturation of the control incubation, subjected to the same label dilution using stearic acid, is 100\%.

3. This addition of stearic acid was made to ascertain any label dilution effects.

The results indicated that both epimino-acids did inhibit the conversion of stearate to oleate by the desaturase enzyme present in hen liver, but not to the same degree as the cyclopropene-acids. At a concentration level equal to that of substrate, it was just possible to detect an effect which increased to around 37\% inhibition when the concentration of the epimino-acid was stepped up to 10x's the substrate. A second experiment (table 4b) in which inhibitor concentrations up to 100x's substrate concentration were employed, showed that the increased inhibition was progressive. The true extent of this increase though, was difficult to ascertain since at these higher inhibitor concentrations part of the observed effect probably resulted from a dilution of label. Therefore, in determining the results, comparison was made with a control incubation in which the same high level of unlabelled stearic acid had been added, and so it follows that the recorded 'relative' inhibition of around 70-75\% at 100x's the substrate concentration must be attributable to some inhibitory effect other than plain dilution of the labelled stearic acid.

Comparison of these results with those obtained for sterculic acid in an earlier, but identical, experiment (figure 6), affords some indication
<table>
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<tr>
<th>Relative inhibition(%)</th>
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<tr>
<td></td>
</tr>
<tr>
<td>100</td>
</tr>
<tr>
<td>80</td>
</tr>
<tr>
<td>60</td>
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<tr>
<td>40</td>
</tr>
<tr>
<td>20</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Log inhibitor conc.</td>
</tr>
<tr>
<td>substrate conc.</td>
</tr>
<tr>
<td>1.0</td>
</tr>
<tr>
<td>0.0</td>
</tr>
<tr>
<td>1.0</td>
</tr>
<tr>
<td>2.0</td>
</tr>
</tbody>
</table>

**Figure 6**  Inhibition of desaturation of 1-14C stearic acid by varying concentrations of **cis** and **trans**-9,10-epimino stearic acids.  
*System: Hen liver microsomal fraction.*
of the relative effectiveness of the two inhibitors. This shows that at a concentration level equal to that of substrate, the epimino-acids exhibit a marginal inhibitory effect whilst sterculic acid causes an almost total inhibition of desaturation.

Although the epimino-acids are less potent inhibitors of desaturation than sterculic acid, the similarities of certain of their chemical properties could infer a similar, though less effective mode of action. Both ring systems undergo facile cleavage with acids\(^242\) and, possibly of greater relevance, the aziridine ring has been shown to react with mercaptans\(^268\). Thus it could exert its effect as a mild sulphydryl agent in a manner similar to that proposed for cyclopropene inhibition\(^32,214,217\).

Alternatively, the epimino-acids may exert their effect on the lipids; at high concentrations the inhibitor did affect the incorporation into individual polar lipids (table 4b), the ratio of label in PE plus PI to PC being considerably higher in the inhibitor reaction than in the control reaction. This suggests that the epimino-acids were primarily esterified into PC forcing the labelled acids into the other polar lipids. The importance of PC, particularly the 2-position, for deposition of the product of desaturation has previously been shown (page 141) and selective incorporation of the epimino-acids into the phospholipid, at the high concentration levels involved in this case, could be sufficient to cause the observed effect, by a type of end product inhibition.

Another interesting feature to emerge from this series of incubations was that both the cis and trans-epimino acids showed very similar inhibition characteristics. This inability of the system to discern between geometrical isomers, suggest the action of these inhibitors is not directed at the highly specific desaturase site, but rather at a less specific point or storage site on lipid.

**B.2.2. Investigation into the inhibitory effect of epoxy and epistearic acids**

The possibility of finding an inhibitor similar in action to the aziridines or more analogous in action to sterculic acid, prompted study of
the cis and trans isomers of 9,10-epoxy and 9,10-epithio- stearic acid.

Again the three-membered ring structures are susceptible to acidic attack, though in this case the episulphide is relatively slow and has a tendency to polymerise with certain inorganic acids. Addition of mercaptans to particularly reactive epoxides and episulphides has also been shown to occur. Thus they would appear to have certain factors in common with both the cyclopropenes, which are potent inhibitors of desaturation, and the aziridines, which are somewhat less effective but still inhibit the desaturase system.

However, incubations with the microsomal suspension of hen liver, involving concentrations of the cis and trans-epoxy and epithio-stearates up to 100 times the substrate concentration, indicated a complete absence of any inhibition which could not be directly attributed to plain dilution effects (see table 5). As these acids do bear a close structural resemblance to the cis and trans-epiminostearates, this total lack of inhibition implies that the effect, exerted by epimino-acids in the previous experiment, did not arise from factors of mere physical fit on or at the active substrate sites in the desaturase sequence and the possibility of it being related to the greater reactivity of the aziridines gains favour.

Table 5

Desaturation (%) of 1-14C stearic acid (2.0µc; 0.04µM) in the presence of varying concentrations of cis- and trans-9,10-epoxystearic acid, and cis- and trans-9,10-epithio-stearic acid, by the microsomal fraction of hen liver.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (µM)</th>
<th>0.04</th>
<th>0.4</th>
<th>4.0</th>
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<tr>
<td>Stearic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cis-9,10-epoxystearate</td>
<td>80.0</td>
<td>75.7</td>
<td>31.0</td>
<td></td>
</tr>
<tr>
<td>trans-9,10-epoxystearate</td>
<td>77.0</td>
<td>76.5</td>
<td>32.1</td>
<td></td>
</tr>
<tr>
<td>cis-9,10-epithiostearate</td>
<td>73.5</td>
<td>73.0</td>
<td>32.4</td>
<td></td>
</tr>
<tr>
<td>trans-9,10-epithiostearate</td>
<td>75.5</td>
<td>69.0</td>
<td>31.9</td>
<td></td>
</tr>
</tbody>
</table>
B.2.3. Broad investigation into the potential inhibitory effect of a range of mid-chain heterocyclics and their derivatives, in whole cells of *Chlorella vulgaris*

Previous investigations by James et al.\textsuperscript{212} have shown sterculic acid to be a potent inhibitor of desaturation in *Chlorella vulgaris*. In this series of incubations a preliminary investigation into the effect of different mid-chain cyclic fatty acids and some of their derivatives was undertaken in an attempt to identify other strong inhibitors of the alga's primary desaturase and allow some comparison of inhibitor activity.

The survey included two series of incubations in which the potential inhibitors, mostly at 10x's the substrate concentration, were pre-incubated with the culture for 10 minutes. The (1\textsuperscript{14}C) stearic acid substrate was subsequently added and the incubations were then conducted for a further 6 hours at 27°C under 4 x 40W daylight emission fluorescent tubes, and the results obtained by the normal procedure. Although the survey was by no means exhaustive, it provided several interesting results, viz: -

1. The cis-9,10-epiminostearic acid (K\textsuperscript{+}salt) considerably reduced the desaturation of labelled substrate (cf results in hen liver) and although some of the observed effect may have been due to dilution of label, later results using equal quantities of other potential inhibitors proved such effects could only account for a minor part.

2. The observed inhibition for cis and trans-epiminoctadecanol, which was only slightly less than that for the corresponding acid, suggested that in *Chlorella vulgaris*, at least, the carboxyl group was not essential and the aziridine ring structure was responsible for the effect. Although the trans-epimino acid was not available for this series of incubations, the similarity between observed inhibition in the cis and trans-epimino-alcohols suggests this acid will probably be equally effective as an inhibitor, as the cis-9,10-epiminostearic acid.

3. Although similar in structure to the epimino-acids, the cis and trans-9,10-epithiosearic acids exerted comparatively little inhibitory effect. This again suggests some degree of chemical interaction as the mode of aziridine inhibition.
Table 6

effect of various potential inhibitors on the desaturation of 1-$^{14}$C stearic acid (10 µg/0.85 M) in Chlorella vulgaris.

<table>
<thead>
<tr>
<th>Inhibitor*</th>
<th>Inhibitor Conc. Substrate Conc.</th>
<th>Desaturation (%)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>49.0</td>
<td>0</td>
</tr>
<tr>
<td>cis-9,10-epiminostearic acid</td>
<td>10</td>
<td>~2</td>
<td>~96</td>
</tr>
<tr>
<td>&quot;</td>
<td>1.0</td>
<td>27.2</td>
<td>44.5</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.1</td>
<td>44.9</td>
<td>9.0</td>
</tr>
<tr>
<td>cis-9,10-epiminooctadecanol</td>
<td>10</td>
<td>6.4</td>
<td>87.0</td>
</tr>
<tr>
<td>trans-9,10-epiminooctadecanol</td>
<td>10</td>
<td>5.9</td>
<td>88.0</td>
</tr>
<tr>
<td>cis-9,10-epithiostearic acid</td>
<td>10</td>
<td>38.8</td>
<td>20.5</td>
</tr>
<tr>
<td>trans-9,10-epithiostearic acid</td>
<td>10</td>
<td>35.5</td>
<td>25.5</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>27.5</td>
<td>0</td>
</tr>
<tr>
<td>cis-9,10-epiminostearic acid</td>
<td>10</td>
<td>4.1</td>
<td>85.1</td>
</tr>
<tr>
<td>cis-9,10-epoxystearic acid</td>
<td>10</td>
<td>9.1</td>
<td>67.0</td>
</tr>
<tr>
<td>trans-9,10-epoxystearic acid</td>
<td>10</td>
<td>36.5</td>
<td>-32.7**</td>
</tr>
<tr>
<td>erythro-9,10-diaminostearic acid</td>
<td>10</td>
<td>20.0</td>
<td>27.3</td>
</tr>
<tr>
<td>threo-9,10-diaminostearic acid</td>
<td>10</td>
<td>16.6</td>
<td>39.7</td>
</tr>
<tr>
<td>erythro-9(10)-amino-10(9)-hydroxystearic acid</td>
<td>10</td>
<td>20.5</td>
<td>25.5</td>
</tr>
<tr>
<td>threo-9(10)-amino-10(9)-hydroxystearic acid</td>
<td>10</td>
<td>16.7</td>
<td>39.4</td>
</tr>
</tbody>
</table>

* Many of these compounds were the kind gift of Dr. J. F. McChie of the Department of Chemistry, Chelsea College of Science and Technology, London.

** The negative value refers to an enhancement of desaturation.

The greater strain energy of the aziridine ring compared with the epoxide and epimisulphide may facilitate conversion to an intermediate which is ultimately responsible for the observed inhibition. Therefore the erythro- and threo-enantiomers of two such derivatives, similar to those that could arise by ring cleavage, were incubated with the culture. These derivatives namely 9(10)-amino-10(9)-hydroxystearic acid and 9,10-diaminostearic acid were much less active than cis-epiminostearate and therefore could only be directly responsible if they existed as enzyme bound intermediates.

The effect of the cis and trans-9,10-epoxystearic acids on the desaturation of labelled substrate produced an interesting and somewhat surprising
result. While the cis-epoxy acid exerted an inhibitory effect intermediate between those of the epimino and epithio-acids and in keeping with the comparative reactivity of the three ring systems, the trans-epoxy acid actually enhanced desaturation. Incubations by L.J. Morris\textsuperscript{271} using both labelled 9,10-epoxystearic acids have shown that they are rapidly broken down by Chlorella and the label incorporated into a wide spectrum of fatty acids whilst other acids, such as stearolic and elaidic acid, are incorporated into lipid unchanged. Therefore the widely differing effects of the cis and trans acid are probably related to this breakdown and, in view of the corresponding results in hen liver, the enhancement of desaturation by the trans-acid here suggests interaction between one of these fragments and the desaturase enzyme, at a secondary or allosteric site. To my knowledge, no allosteric effector of the desaturase enzyme has been recorded and, if this is the mode of action, its identification could reveal information on the allosteric site and thus the desaturase enzyme.

B.3. Incubations with potential inhibitors which do not contain a three membered ring.

In an attempt to achieve interaction with desaturase enzyme, and more specifically the active desaturase site, several incubations were carried out in the microsomal fraction of hen liver with other, hitherto untried, potential inhibitors. These fell basically into three groups of compounds; those containing an halogeno-substituent, those with a mercapto-substituent and those containing an acetylene group. To facilitate the normal transforase reactions the chosen inhibitors were long chain carboxylic acids and to promote the chances of interaction with the active desaturase site the substituent was positioned on or around the 9 and 10 carbon atoms of the polymethylene chain.

B.3.1. Investigation into the inhibitory effect of various chlorostearates on the desaturase enzyme present in the microsomal fraction of hen liver.

The requirements of the desaturase enzyme show many similarities to
those of the hydroxylases (mixed function oxidases) and considerable work has been carried out by several investigators to explain the obligate requirement for molecular oxygen in the desaturase sequence, by illustrating the existence of an oxygenated-intermediate\textsuperscript{12,13,14}. Although no such intermediate has been found, and differences between the hydroxylase and desaturase requirements have been shown, the possibility of the intermediate being enzyme bound cannot be ruled out.

In connection with this the enzymic cleavage of a carbon-hydrogen bond to give the corresponding hydroxy-compound has been shown to occur, with short chain fatty acids, in bacterial systems\textsuperscript{272,273}. Here, the hydroxyl group was derived from water but various mixed function oxidases have been shown to catalyse the dehalogenation of aromatic compounds\textsuperscript{274,275} in a reaction requiring molecular oxygen\textsuperscript{276}. The similarities between the requirements of this reaction and that of desaturation suggested that the use of chloro-fatty acids may give access to an enzyme-bound oxygenated intermediate which could be subsequently desaturated (or dehydrated) to yield a monoene. In an attempt to identify any such effect, or any inhibition that might arise from interaction with the enzymes involved in the desaturase sequence, incubations with several chloro-stearates were conducted.

The derivatives, namely, DL-9-chlorostearic acid, L(+)-12-chlorostearic acid and threo-9,10-dichlorostearic acid were incubated in the usual way with the microsomal suspension of hen liver (see page 187) using concentrations of inhibitor varying from equal to 100 times that of substrate (see table 7, figure 7).

From the results it is apparent that all the chloro-derivatives investigated do exert some inhibition of desaturation at high concentrations. However, at best they are only about half as effective as the o-pimino acids and much less effective than the cyclopropenes. The fact that inhibition was comparatively low, and was independent of the position of the substituent, suggests that the interaction is not with the active desaturase (or hydroxylase) site. Furthermore, its appearance only at concentrations where the enzyme
Figure 7  Inhibition of desaturation of 1-\textsuperscript{14}C stearic acid by varying concentrations of chlorostearic acids.

System: Hen liver microsomal fraction.
system is saturated (as indicated by the control reactions with stearic acid of varying specificity) implies that the inhibitory effect may result from increased residence time at some point involved in the desaturation sequence. No particular significance is seen in the slight inhibitory effect of threo-dichlorostearate at a lower concentration than the mono-chloro derivatives, and this was not pursued further.

Table 7.
Effect of varying concentrations of chlorostearic acids on the desaturation of 1-14C stearic acid (2µg;0.04µM) in the microsomal fraction of hen liver.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (µM)</th>
<th>Inhibitor: Substrate Conc.</th>
<th>Desaturation (%)</th>
<th>Relative Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td>41.3</td>
<td>-</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>4.0</td>
<td>100</td>
<td>25.9</td>
<td>-</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.4</td>
<td>10</td>
<td>42.6</td>
<td>-</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.04</td>
<td>1</td>
<td>40.6</td>
<td>-</td>
</tr>
<tr>
<td>DL-9-chlorostearic acid</td>
<td>4.0</td>
<td>100</td>
<td>13.8</td>
<td>46.8</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.4</td>
<td>10</td>
<td>42.8</td>
<td>0</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.04</td>
<td>1</td>
<td>41.0</td>
<td>0</td>
</tr>
<tr>
<td>L(+)-12-chlorostearic acid</td>
<td>4.0</td>
<td>100</td>
<td>14.8</td>
<td>42.8</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.4</td>
<td>10</td>
<td>42.6</td>
<td>0</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.04</td>
<td>1</td>
<td>42.9</td>
<td>0</td>
</tr>
<tr>
<td>threo-9,10-dichlorostearic acid</td>
<td>4.0</td>
<td>100</td>
<td>15.1</td>
<td>41.6</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.4</td>
<td>10</td>
<td>34.8</td>
<td>18.6</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.04</td>
<td>1</td>
<td>40.1</td>
<td>0</td>
</tr>
</tbody>
</table>

* This value was calculated by assuming the desaturation of the corresponding control incubation is 100%.

B.3.2. Investigation into the inhibitory effect of 9-norcapstearic acid on the desaturase enzyme present in the microsomal fraction of hen liver

The general inability to demonstrate the presence of an oxygenated-intermediate in the desaturase sequence, coupled with the differences that do exist between the desaturase and hydroxylase requirements (page 5), has encouraged alternative suggestions for the involvement of molecular oxygen and reduced pyridine nucleotide. The presence of a kinetic isotope effect operative at both the 9 and 10-positions in the substrate chain36,277 (page 72)
suggested a concerted removal of the two D-hydrogen atoms and, together with further structural evidence provided by studies with the methyl-branched fatty acids, led to the proposal of a disulphide bond as the active desaturase site.

Although this postulation ably satisfies all the available evidence, and such bonds are provided by cystine molecules in the primary structure of proteins no direct evidence has been found for the existence of either a disulphide bond or its precursor (two free sulphydryl groups) at or near the active site of the desaturase enzyme. In an attempt to achieve interaction with such groups, and so provide evidence of their existence, a series of incubations were conducted in the microsomal fraction of hen liver (page 188) with labelled and unlabelled DL-9(10)-mercaptostearic acid, the concentration of the unlabelled inhibitor varying from 0.1 to 100 times that of the 1-14C stearic acid substrates (see table 8).

Table 8.

1. Effect of varying concentrations of DL-9(10)-mercaptostearic acid on the desaturation and lipid incorporation of 1-14C stearic acid (2μg; 0.04μM)

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Conc. (μM)</th>
<th>Inhibitor: Substrate</th>
<th>Desat. (%)</th>
<th>Relative Inhib. (%)</th>
<th>Lipid incorporation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
<td>51.0</td>
<td>-</td>
<td>7.9 23.0 8.9 60.2</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>4.0</td>
<td>100</td>
<td>41.5</td>
<td>-</td>
<td>9.5 21.0 10.5 59.0</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>10</td>
<td>49.8</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>9(10)-mercaptostearic acid</td>
<td>4.0</td>
<td>100</td>
<td>34.5</td>
<td>17.0</td>
<td>4.8 16.0 12.6 66.6</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>10</td>
<td>44.0</td>
<td>11.5</td>
<td>9.6 18.2 11.6 60.3</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>1</td>
<td>45.5</td>
<td>7.5</td>
<td>7.7 20.3 10.5 61.5</td>
</tr>
<tr>
<td></td>
<td>0.004</td>
<td>0.1</td>
<td>50.2</td>
<td>1.5</td>
<td>9.4 17.2 10.5 62.9</td>
</tr>
</tbody>
</table>

2. Comparative lipid incorporation of 1-14C DL-9(10)-mercaptostearic acid

<table>
<thead>
<tr>
<th>Substrate</th>
<th>PI</th>
<th>PC</th>
<th>PE</th>
<th>NL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-14C DL-9(10)-mercaptostearic acid (1μg; 0.02μM)</td>
<td>0.9</td>
<td>4.8</td>
<td>1.9</td>
<td>92.4</td>
</tr>
</tbody>
</table>

In using the 9(10)-mercapto acid it was hoped that formation of the normal enzyme-bound complex would take place bringing the sulphydryl group of
the acid into juxtaposition with the proposed sulphydryl groups of the desaturase enzyme. Formation of a disulphide bond between the sulphydryl group on the acid and one of the proposed groups at the active site could then have been reasonably expected to occur.

However the low level of observed inhibition indicates that either the proposed interaction did not take place, or that combination with other sulphydryl groups in the system nullified any such effect. From the very low incorporation of the 1-\(^{14}\)C.DL-9(10)-mercaptostearic acid into polar lipids, and also the marginal effect large concentrations of unlabelled inhibitor had on the incorporation of labelled substrate and product, it would appear that the mercapto-acids played very little part in any enzymic reaction. Therefore it was not possible, by this series of incubations, to add to the evidence in favour of a disulphide bridge constituting the active desaturase site.

B.3.3 Investigation into the inhibitory effect of 9-decynoic acid on the desaturase enzyme present in the microsomal fraction of hen liver.

A further attempt to gain interaction between an atypical substrate and the active site of desaturation, was made using 9-decynoic acid. Once again the acid derivative was chosen to facilitate the normal transference reactions, and the potential inter-reactive group was positioned so as to lie adjacent to the desaturase site, if a normal enzyme-substrate complex was formed. The choice of a terminal alkyno was made to allow possible interaction of the acidic hydrogen on the terminal carbon atom, as well as other possible addition reactions of acetylenes. This measure also eliminated the long alkyl residue, present for example in stearolic acid, which might have inhibited accommodation on the enzyme by introducing major structural deformations relative to a normal C\(_{18}\) chain.

As in the previous experiment, inhibitor concentrations varying from 0.1 to 100 times the concentration of 1-\(^{14}\)C stearic acid substrate were employed and an incubation with (1-\(^{14}\)C)9-decynoic acid was included to measure both the level and pattern of lipid incorporation of the inhibitor (see table 9)
1. Effect of varying concentrations of 9-decynoic acid on the desaturation and lipid incorporation of 1-{sup}14{sub}C stearic acid (2μg; 0.04μl) in the microsomal fraction of hen liver.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Conc (μM)</th>
<th>Inhibitor: Substrate</th>
<th>Desat. (%)</th>
<th>Relative Inhib. (%)</th>
<th>Lipid incorporation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td>36.8</td>
<td>-</td>
<td>10.3 26.1 11.5 52.1</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>4.0</td>
<td>100</td>
<td>32.3</td>
<td>-</td>
<td>4.9 17.8 6.2 67.1</td>
</tr>
<tr>
<td>9-decynoic acid</td>
<td>4.0</td>
<td>100</td>
<td>23.8</td>
<td>26.4</td>
<td>13.0 26.5 12.6 47.9</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.4</td>
<td>10</td>
<td>37.1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>0.04</td>
<td>1</td>
<td>36.3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>0.004</td>
<td>0.1</td>
<td>36.2</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

2. Comparative lipid incorporation of 1-{sup}14{sub}C 9-decynoic acid

<table>
<thead>
<tr>
<th>Substrate</th>
<th>PI</th>
<th>PC</th>
<th>PE</th>
<th>NL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-{sup}14{sub}C 9-decynoic acid (μg)</td>
<td>3.6</td>
<td>13.4</td>
<td>5.9</td>
<td>77.1</td>
</tr>
</tbody>
</table>

The results indicate a similar pattern to those obtained for the chlorostearates, with inhibition only taking place at concentration high enough to saturate the enzyme system. Even then the degree of inhibition is very low indicating negligible interference with the normal desaturase sequence. Although relatively small, the lipid incorporation of the labelled alkynoic acid was high enough to suggest that activation to the CoA ester was taking place, though high concentration of the unlabelled acid appeared to have little effect on the lipid incorporation of the normal substrate. Thus it would seem that, if the alkynoic acid does enter into the desaturase sequence, it exerts its marginal effect by slightly increased residence time on the enzyme.
EXPERIMENTAL

1. Synthesis of Precursors for inhibition studies

In order to conduct the investigations outlined in the introduction the preparation of several labelled and unlabelled precursors was required. These were:

- $\omega$-(2-n-octylcycloprop-1-onyl) nonanoic acid
- (1-$^{14}$C) sterulic acid
- trans-9,10-epiminostearic acid
- DL-9(10)-mercapto-stearic acid
- (1-$^{14}$C) DL-9(10)-mercapto-stearic acid
- L(+)-12-chlorostearic acid
- DL-9-chlorostearic acid
- trans-9,10-dichlorostearic acid
- 9-decyanoic acid and (1-$^{14}$C) 9-decyanoic acid

A.1.1. Synthesis of $\omega$-(2-n-octylcycloprop-1-onyl)nonanoic acid

**Reaction Sequence I**

\[
\begin{align*}
CH_3(CH_2)_7 COOH & \xrightarrow{LiAlH} CH_3(CH_2)_7 C = CH(CH_2)_n CH_2OH \\
& \xrightarrow{p-toluencesulphonyl chloride} CH_3(CH_2)_7 C = CH(CH_2)_n COO^+ \\
& \xrightarrow{KOH/EtOH/H_2O} CH_3(CH_2)_7 C = CH(CH_2)_n+1 COOH
\end{align*}
\]

where $n = 7$

1. Reduction of the acid to the alcohol$^{205}$: Sterulic acid (46mg), required as starting material for this reaction, was released from the urea clathrate (310mg) by saponifying with 5% methanolic potassium hydroxide. Ether (10ml)
was added and the chilled reaction mixture was made just acid by careful addition of dilute sulphuric acid. The acidic fraction was further extracted with ether (10ml) and the combined solvent fractions were washed neutral with water. The extract was then dried over anhydrous sodium sulphate and the solvent volume was reduced under a stream of nitrogen until a solution of the acid in about 4 mls of ether remained. To this was added lithium aluminium hydride (20mg) and the reaction was refluxed for 5 min. to ensure complete conversion. Ether (10mls) was then added to the cooled reaction mixture and the excess LiAlH₄ was destroyed by cautious addition of water. The reaction was then cooled in an ice bath and sufficient hydrochloric acid was added to make the solution just acid, thus dissolving the precipitate. The ether fraction was quickly separated and washed acid free with water. The extract was then dried and the solvent was removed, under a stream of nitrogen, immediately preceding the next stage in the sequence. Sterculyl alcohol, thus obtained, was a colourless oil (37mg; 85% yield).

A little of the product was examined by TLC on 25mm plates run in 20% ether in petroleum ether. By comparison with the included standards, the whole product was shown to correspond to the alcohol, no acid remaining.

(ii) Conversion of alcohol to p-toluenesulphonate: The Sterculyl alcohol (35mg) was dissolved in pyridine (2ml) which had been dried over potassium hydroxide pellets. To this solution was added p-toluenesulphonyl chloride (40mg) and the shaken mixture was left overnight at 0°C. After 16 hours a spot of the solution was analysed by TLC in 20% ether/petroleum ether and this indicated almost total conversion of the alcohol to the less polar p-toluenesulphonate. Therefore ether (30ml) was added to the reaction mixture and the majority of the pyridine/pyridine hydrochloride was removed by washing with water (2 x 5ml). The remaining pyridine was removed by quickly washing with dilute hydrochloric acid (1 x 5ml) and the residual acid, and the p-toluenesulphonic acid, were then removed with dilute aqueous potassium hydroxide. Finally, the ethereal solution was washed neutral with water and dried over anhydrous sodium sulphate. All the work-up was
carried out at 0°C and, where necessary, ether was added to maintain the volume of the solvent fraction.

Further TLC analysis of the product indicated almost complete conversion of the alcohol, mainly to the p-toluencesulphonate, but with a trace of the chloride present.

(iii) Conversion of p-toluencesulphonate to the nitrile\textsuperscript{140,141}: After removal of the solvent from 80% of the product, dry dimethyl sulphoxide (DMSO) (3ml) and potassium cyanide (20mg) were added and the solution was heated at 90°C for two hours. After cooling, ether (15ml) was added to the solution followed by water (3ml). The aqueous fraction was re-extracted with ether (10ml) and the combined ether fractions were washed with water (4x5ml) to remove the remaining DMSO and potassium salts. The extract was dried and the product (18.1mg; 62% based on sterculyl alcohol) was kept in ethereal solution.

(iv) Hydrolysis of the nitrile: Part of the sterculyl nitrile was dissolved in 5ml of aqueous ethanolic potassium hydroxide (KOH/EtOH/H\textsubscript{2}O;1:4:4;v/v/v) and hydrolysed under reflux for 4 hours. (Periodic checks by TLC on the reaction mixture showed this to be the minimum time for complete reaction). Ether was added to the chilled reaction mixture and sufficient hydrochloric acid to just neutralise the alkali. After re-extraction of the aqueous fraction, the combined ether fractions were washed to neutral pH with water and dried over anhydrous sodium sulphate.

The product was then esterified by reacting with diazomethane (page 82) for a short period (2min.) and applied, in ethereal solution, to 1mm preparative TLC plates under an atmosphere of nitrogen. The chromatograms were developed in 5% ether/petroleum ether, visualisation being obtained with Rhodanine 6G. The methyl ester band was quickly removed and eluted with ether, allowing minimum contact of dried plates with the air.

Regeneration of the pure \textit{(2-n-octylocycloprop-1-ynyl)nonanoic acid} (or 10,11-methano-10-nonadecenoic acid) was obtained by hydrolysis of the ester overnight at room temperature in 5% methanolic potassium hydroxide.
The work-up was conducted with the same care as in the hydrolysis of the nitrile and the acid was stored in benzene at -30°C for the short period prior to incubation.

A.1.2. Synthesis of (1-14C)sterculic acid

Reaction Sequence II

\[ \text{CH}_3(\text{CH}_2)_7 \text{C} = \text{C}(\text{CH}_2)_n \text{C} \text{H} \text{H}_2 \text{CH} \quad \xrightarrow{\text{HIIO}_4 \text{pyridine}} \quad \text{CH}_3(\text{CH}_2)_7 \text{C} = \text{C}(\text{CH}_2)_n \text{CHO} \]

1,2-dihydroxysterculene

\[ \text{reaction sequence I using K*CN} \xrightarrow{\text{using K*CN}} \text{CH}_3(\text{CH}_2)_7 \text{C} = \text{C}(\text{CH}_2)_n+1 \text{COOH} \quad \text{where } \text{C} = 14^\circ \text{C} \]

(i) Preparation of 1,2-dihydroxysterculene: 1,2-dihydroxysterculene was prepared from Pachira insignis seed oil, isolated according to the method of Morris and Hall. The extracted seed oil was reduced with LiAlH₄ to yield a mixture of alcohols which included 1,2-dihydroxysterculene. These mono and dihydroxy-alcohols (320mg) were separated by preparative TLC on silica plates developed in 75% ether in petroleum ether yielding pure 1,2-dihydroxysterculene (49mg).

(ii) Cleavage of the vic-dihydric alcohol: 1,2-Dihydroxysterculene (49mg) was dissolved in dry pyridine (ca2ml) and sodium metaperiodate (83mg; 2.5 times molar excess) was added. The reaction vessel was then purged with nitrogen, tightly stoppered and shaken vigorously at room temperature for 24 hours. After 24 hours the reaction was stopped by addition of ether (20ml) and water. The etheral layer was further washed with ice cold water, very dilute sulphuric acid, water, 1% potassium carbonate (until basic) and water until pH7-8. All stages in the work-up were conducted at 0°C. After drying with anhydrous sodium sulphate the ether was removed in vacuo to yield a colourless oil (42mg; 96%).

The product was examined by infrared spectroscopy which indicated a band at 1,740 cm⁻¹ corresponding to the carbonyl stretching vibration of a...
saturated aldehyde, and no absorption attributable to an OH-stretching vibration. An absorption peak at 1010 cm$^{-1}$ and another very weak band at 1870 cm$^{-1}$ indicated retention of the cyclopropene ring.

(iii) Reduction of aldehyde to alcohol$^{146,205}$: The aldehyde (36 mg) was dissolved in sodium dried ether (3 ml) and lithium aluminium hydride (20 mg) was added. The reaction was then carried out exactly as in the previous experiment to yield the alcohol.

TLC analysis of the product indicated trace amounts of a compound corresponding to 1,2-dihydroxysterculene. Therefore this impurity was removed by preparative TLC on silica plates developed in 50% ether/petroleum ether (observing the same precautions as before; page 164), and pure malvalyl alcohol was obtained (27.5 mg; 77% yield).

(iv) Conversion of alcohol to p-toluene sulphonate$^{207}$: The purified malvalyl alcohol (27.5 mg) was dissolved in dry pyridine (2 ml), and p-toluene sulphonyl chloride (30 mg) was added. The reaction went to completion overnight at 0°C and the product (37.4 mg; 86%) was isolated as before (page 163) and stored in benzene at -30°C. TLC examination in 15% ether/petroleum ether indicated almost complete conversion to the p-toluene sulphonate with just a trace of alcohol impurity.

(v) Conversion of the p-toluene sulphonate to the radionuclided nitrile:
Benzene was removed from part of the p-toluene sulphonate (12.5 mg) and a suspension of 100 μg (1.44 mg)$^{14}$C-labelled potassium cyanide (45.2 μCi/ml) in dry DMSO (ex-Amersham) was added. The solution was heated at 90°C for 2 hours, and then cold potassium cyanide was added and the heating was continued for a further hour$^{150}$. The cooled reaction products were isolated by the method previously described (page 164).

Examination of the product, against standards, by TLC on silica plates run in 15% ether/petroleum ether, followed by identification on the Panax-RTLS indicated about 40% of the radioactivity corresponded to nitrile (Rf 0.55). As on previous occasions (page 39) the major radioactive impurity (ca. 50%) was concentrated at the origin, with other minor impurities at Rf 0.16 (ca. 7%) and Rf 0.45 (ca. 3%).

- 166 -
Removal of the impurities was undertaken at this stage to obviate the necessity to esterify the product after hydrolysis of the nitrile, if polymerisation had not occurred during the reaction. The purification was achieved by cautious preparative TLC on the crude nitrile, as before. On this occasion the chromatogram was developed three times in 5% ether/petroleum ether and confirmation of the position of the nitrile band was obtained by radioactivity scanning.

(vi) Hydrolysis of nitrile: The purified nitrile was hydrolysed with an aqueous ethanolic solution of potassium hydroxide as before and the (1-14C) sterculic acid (20.4µc) obtained was stored in benzene at -30°C. An aliquot of the product (0.1%), esterified with diazomethane, was examined together with other products from the sequence, by TLC in 10% ether in petroleum ether. Radioactivity scanning followed by charring with sulphuric acid showed a single mass and radioactivity spot for sterculic acid, and the esterified product, indicating complete conversion of the nitrile to the acid. Details of the chromatogram are given below:

<table>
<thead>
<tr>
<th>Product</th>
<th>Mass (Rf)</th>
<th>Radioactivity (Rf)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1-14C) sterculic acid</td>
<td>Origin</td>
<td>Origin</td>
</tr>
<tr>
<td>(1-14C) methyl stericate</td>
<td>0.54</td>
<td>0.54</td>
</tr>
<tr>
<td>p-toluenesulphonate</td>
<td>0.30</td>
<td>None</td>
</tr>
<tr>
<td>valvalyl alcohol</td>
<td>0.07</td>
<td>&quot;</td>
</tr>
<tr>
<td>methyl stearate</td>
<td>0.55</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

Further analyses were carried out (see discussion) to confirm that radiolabelling had been achieved with retention of the cyclopropene ring.

A.2. Synthesis of Potassium trans-9,10-epiminostearate\textsuperscript{241,244}

Reaction Sequence III

\[
\text{transCH}_3(CH_2)_7\text{CH}=(CH_2)_7\text{COOCH}_3 + \text{KCO} \rightarrow \text{erythreoCH}_3(CH_2)_7\text{CH}=(CH_2)_7\text{COOCH}_3 + \text{KCO} \rightarrow \text{CH}_3\text{OH} \\
\text{transCH}_3(CH_2)_7\text{CH}=(CH_2)_7\text{COO}^- + \text{KOH} \rightarrow \text{erythreoCH}_3(CH_2)_7\text{CH}=(CH_2)_7\text{COOCH}_3 + \text{KCOH}
\]
(i) Synthesis of elaicid acid\textsuperscript{278}: To oleic acid (16g) dissolved in dyglyme (400ml) was added 6N nitric acid (12ml) and the stirred solution was heated to 65°C. 2N Sodium nitrite (16ml) was then added and heating continued for a further hour. After cooling, water was added and the product was extracted with ether (3x100ml). The etheral extract was then washed acid free with sodium bicarbonate solution followed by water. After drying, the solvent was removed and the crude semi-solid product was recrystallised from ethanol to give elaicid acid (5g; 31\% yield) as white crystals. (M.P. = 44°C; Lit. M.P. = 44-45°C). The purity of the esterified product was checked by AgNO\textsubscript{3}-TLC in 10\% ether/petroleum ether.

(ii) Synthesis of iodoisocyante: Pure methyl elaicate (4.8g) was dissolved in ether (25ml), and iodine (3.5g) and silver cyanate (2.4g) were added. The reaction was then stirred at -5°C overnight during which time the red colouration disappeared and silver salts were deposited. These were removed by filtration and the ether taken off \textit{in vacuo} to yield the \textit{erythro}-9(10), 10(9)-iodo-isocyante.

(iii) Conversion of iodoisocyante to the aziridine: The iodoisocyanate was reacted with methanol (15ml) under refluxed for 1\frac{1}{2} hours to produce methyl \textit{erythro}-9(10)-ido-10(9)-(methylcarbonyl) octadecanate. This was not isolated but converted directly to the aziridine by adding potassium hydroxide (4.9g) in methanol (10ml) and refluxing overnight. The solution was filtered whilst still hot to remove the inorganic precipitate and the residue was washed with hot methanol (20ml). The filtrate was then concentrated to 15-20ml and cooled to 0°C to precipitate out the crude product (4.2g) which was collected by vacuum filtration. Pure potassium \textit{trans}-9,10-epiminostearate (0.95g; 23\% yield) was then obtained, by recrystallisation from methanol, as a white crystalline solid (M.P. = 241-244°C; Lit. M.P. = 243-245°C).
A.3.1. Synthesis of DL-9(10)-thioctstearic acid

**Reaction Sequence IV**

\[
\text{cis-CH}_3(CH_2)_7CH - CH(CH_2)_7 COOMe} \xrightarrow{H_2/Pd} \text{DL-CH}_3(CH_2)_8(7)CH(CH_2)_7(8) COOMe}
\]

1. Reduction of methyl cis-9,10-epoxystearate to DL-9(10)-hydroxystearate \(^{279}\):

Methyl cis-9,10-epoxystearate (7g) was dissolved in glacial acetic acid (20ml) and palladium-charcoal catalyst was added. Hydrogen (500ccs) was passed into the stirred solution until uptake ceased and then the reaction mixture was poured into water. This solution was extracted with ether (3x50ml) and the combined ether fractions were washed acid-free with water. The dried extract was then evaporated to yield the crude product (6.3g). This was purified by column chromatography (page 13) using an eluent of ether in petroleum ether, increasing in polarity from 2.5-20%.

Pure DL-9(10)-hydroxystearate (5.1g; 73% yield) was obtained from the 12.5-17.5% ether/petroleum ether fractions, the unreacted epoxide (0.25g) being recovered in the 7.5% fraction.

The product exhibited the same Rf value as 12-hydroxystearate on TLC in 20% ether/petroleum ether, and infrared spectroscopy gave a broad band at 3450-3200 cm\(^{-1}\) due to OH-stretching vibration.

(ii) Conversion of the hydroxystearate to the bromostearate \(^{249}\): DL-9(10)-hydroxystearate (2.5g) and carbon tetrabromide (10g) were dissolved in sodium dried ether (15ml). Triphenyl phosphine (2.5g) was added slowly with constant stirring, causing the dark brown solution to go viscous.
some solid deposition. The reaction was continued under reflux and the progress of the reaction was checked at intervals by TLC. After 6 hours the reaction was complete and so the ethereal solution was filtered. The residue was washed with ether and the combined other fractions were evaporated to yield a crude product (6.2g). This was again purified by column chromatography (ether-petroleum ether eluent) to yield DL-9(10)-bromostearate (1.9g; 63% yield).

The product, analysed by TLC, showed up as a single low polarity spot and its infrared spectrum gave no absorption characteristic of a secondary alcohol. NMR carried out on the product in CCl₄ solution suggested a methyl ester of a long chain fatty acid and gave a well resolved quintuplet at 6.07. The full spectrum was:

Peak (T) | Multiplicity | Assignment | No. of Protons
---|---|---|---
6.0 | Quintuplet | -CH- | 1
6.3 | Singlet | CH₂OOC- | 3
7.7 | Unsym. Triplet | α-methylene | 2
8.0-8.8 | Broad band | CH₂ in chain | 23
9.1 | Unsym. Triplet | Terminal CH₃ | 3

(iii) Conversion of the bromostearate to DL-9(10)-mercaptostearic acid

DL-9(10)-bromostearate (1g) dissolved in 3ml of isopropenol/water (5:1;v/v) was reacted with thiourea (0.2g) under reflux. Periodic examination of the reaction mixture by TLC, indicated that after 45 hours the majority of the starting material had reacted to give a very polar material, the isothiocuronium bromide. At this stage 10% methanolic sodium hydroxide (6ml) was added and the mixture was refluxed, with stirring, for a further 2 hours. Dilute hydrochloric acid was then added until the solution became acid and the product was extracted with ether (3x5ml). The combined other fractions were washed acid-free with water, dried and evaporated to yield a viscous yellow oil (0.7g).

The esterified oil was purified by column chromatography,
DL-9(10)-mercaptostearate (250mg; 32% yield) being recovered in the 7-9% ether/petroleum ether fraction with an overall yield of 15% based on cis-9,10-epoxystearate. DL-9(10)-mercaptostearic acid was obtained from the ester by hydrolysis and stored in benzene at -30°C.

Elemental analysis of the methyl DL-9(10)-mercaptostearate was in close agreement with the expected values; Calculated: C, 69.04; H, 11.59; and S, 9.68; Found: C, 70.00; H, 11.61; and S, 9.66.

Comparative examination by TLC on silica plates run in 20% ether/petroleum ether gave the following chromatogram:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rf Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl-9(10)-bromostearate</td>
<td>0.77</td>
</tr>
<tr>
<td>Methyl-9(10)-mercaptostearate</td>
<td>0.57</td>
</tr>
<tr>
<td>Methyl-9(10)-hydroxystearate</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Further structure determinations were carried out by IR, NMR and mass spectroscopy (page 132).

A.3.2. Synthesis of (1-14C) DL-9(10)-mercapto stearic acid

**Reaction Sequence V**

\[
\begin{align*}
\text{CH}_3(\text{CH}_2)_7\text{CH} = \text{CH}(\text{CH}_2)_7^*\text{COOH} & \xrightarrow{\text{CH}_2\text{N}_2} \text{CH}_3(\text{CH}_2)_7\text{CH} = \text{CH}(\text{CH}_2)_7^*\text{COOMe} \\
\text{CH}_3(\text{CH}_2)_8(7) \text{CH}(\text{CH}_2)_7(8)^*\text{COOMe} & \xleftarrow{\text{alcoholysis}} \text{CH}_3(\text{CH}_2)_8(7) \text{CH}(\text{CH}_2)_7(8)^*\text{COOMe} \\
\text{hydrolysis} & \downarrow \\
\text{CH}_3(\text{CH}_2)_8(7) \text{CH}(\text{CH}_2)_7(8)^*\text{COOH} & \xrightarrow{\text{S} \rightarrow \text{COCH}_3} \text{where } ^*\text{O} = 14C
\end{align*}
\]

(i) Preparation of DL-9(10)-(acetylthio)stearate: Oleic acid (100μc; 57.2μc/mmole-ex Amersham) mixed with stearic acid carrier (25mg) was esterified with diazomethane (page 82) and then dissolved in thiolaactic acid. This solution was irradiated under unfiltered U.V. light for one week at room temperature (the reaction was checked intermittently by radio-TLC) which resulted in 40% of the activity being transferred into a product of polarity attributable to methyl-9(10)-(acetylthio)stearate. At this point the reaction was stopped.
by removing excess thiolacetic acid in vacuo at 30°C.

(ii) Alcoholysis of methyl-9(10)-(acetylmethoxystearato)\(^{251}\): Sodium (50mg) was dissolved in methanol (25ml) and 2ml of this solution was added to the crude methyl-9(10)-(acetylmethoxystearato). The solution was refluxed for 4 hours and then evaporated to give a semi-solid, which was acidified with dilute hydrochloric acid. The crude product was extracted from the acidified solution with ether (15ml), washed neutral with water, and dried over anhydrous sodium sulphate. Analytical TLC on silica plates run in 15% ether in petroleum ether, indicated almost complete conversion of the acetylmethoxyster derivative to the thiol, the major radioactive impurities being oleic acid (30%) and two polar species (40%) running at, and very close to the origin.

The (1-\(^{14}\)C)methyl 9(10)-mercaptopstearate was separated from the radioactive impurities and from the major mass impurity (stearic acid) by preparative TLC on silica plates, developed twice in 7½% ether/petroleum ether. Confirmation of structure was carried out by TLC and mass spectrographic analysis (page 132).

(iii) Hydrolysis of the ester: The purified ester was hydrolysed by refluxing in 5% methanolic potassium hydroxide (2ml) for 1 hour, and the (1-\(^{14}\)C)DL-9(10)-mercaptopstearic acid obtained, was dissolved in benzene and stored at -30°C. An aliquot of the acid, dissolved in 0.4% PPO toluene and counted on a Packard Scintillation counter, indicated 17% of the mercepto-acid had been obtained, i.e. 17% radiochemical yield.

4.1. Synthesis of L(+)-12-chlorostearic acid\(^{249}\):

Reaction Sequence VI

\[
\begin{align*}
\text{D(-) CH}_3(CH_2)_m CH(CH_2)_n COOH} & \xrightarrow{\text{esterification}} \text{D(-) CH}_3(CH_2)_m CH(CH_2)_n COOMe} & \xrightarrow{\text{hydrolysis}} \text{L(+)} \text{ CH}_3(CH_2)_m CH(CH_2)_n COOH} & \xrightarrow{\text{hydrolysis}} \text{L(+)+CH}_3(CH_2)_m CH(CH_2)_n COOH} \\
& \text{OH} & \text{Cl} & \text{Cl} \\
\text{L(+)-12-chlorostearic acid} \quad \text{where } m=5 \text{ and } n=10
\end{align*}
\]
Esterification of D(-)-12-hydroxystearic acid: D(-)-12-hydroxystearic acid (5g) was esterified with methanol: benzene: sulphuric acid (50:40:10) (20ml) as before (page 86) and the white solid obtained was recrystallised from 60-80 petroleum ether (4.3g; 82.0% yield). The melting point of the purified product was 56-56.5°C (Lit.M.Pt=56.5-57°C; Hielbron Vol2, p 825) and it had an optical rotation of \([\alpha]_D^{20} = -0.4^0\) in CHCl₃.

(ii) Conversion to chloride: Methyl D(-)-12-hydroxystearate (2.0g) was dissolved, with heating, in dry carbon tetrachloride (15ml) and 50% molar excess of triphenylphosphine (2.8g) was added. The mixture was then heated gently under reflux and after 1 hour deposition of a white precipitate began and the mixture became more viscous. The reaction, followed by TLC, was shown to be complete after 6.5 hours. Precipitation of the triphenylphosphine oxide formed during the reaction was increased by cooling the mixture to 0°C, and crude product was obtained by filtration and evaporation of the solvent.

Purification of the product by column chromatography yielded 12-chlorostearate (1.7g; 80% yield) as a colourless, slightly viscous, liquid corresponding in polarity (on TLC) to an authentic chlorostearate standard. The optical rotation of the product was measured \([\alpha]_D^{19.5} = +0.65\) in CHCl₃ indicating the reaction had proceeded with inversion of configuration yielding methyl L(+)-12 chlorostearate. Hydrolysis of the ester to the acid was achieved by the usual method (page 82) and after further purification of a portion by preparative TLC the product was stored in benzene at -8°C.

\[\text{A.4.2. Synthesis of DL-9-chlorostearic acid}\]

**Reaction Sequence VII**

\[
\begin{align*}
\text{D(-) CH}_3\text{(CH}_2\text{)_8CH(CH}_2\text{)_7COOH} & \xrightarrow{\text{CrO}_3, \text{Acetic acid}} \text{CH}_3\text{(CH}_2\text{)_8C(\text{CH}_2\text{)_7COOH}} \\
& \downarrow \text{MeOH/Benzene/H}_2\text{SO}_4 \\
\text{DL CH}_3\text{(CH}_2\text{)_8CH(CH}_2\text{)_7COOH} & \xrightarrow{\text{NaH}_4, \text{iso-propylal}} \text{CH}_3\text{(CH}_2\text{)_8C(\text{CH}_2\text{)_7COOCMe}} \\
& \downarrow \text{Reaction sequence VII (m=8, n=7)} \\
\text{DL CH}_3\text{(CH}_2\text{)_8CH(CH}_2\text{)_7COOH} & \xrightarrow{\text{Cl}} -173 -
\end{align*}
\]
(i) Formation of the keto-ester: Chromium trioxide (5g) was shaken with glacial acetic acid (50ml) for 2 hours and 40ml of the resultant supernatant was run, over 15 minutes, into a stirred solution of D(-)-9-hydroxystearate (2g) in glacial acetic acid (15ml). (The D(-)-9-hydroxystearate was prepared from the naturally occurring methyl 9-hydroxyoctadec-12-enoate, by reduction over Adams catalyst). The reaction with chromium trioxide, which was exothermic, was left at room temperature for 1½ hours and then run into cold water (approx. 400ml). The bulky, light green precipitate which formed was extracted with ether (3x100ml) and washed acid-free with water. Subsequent drying and removal of ether on the rotary evaporator yielded a yellow solid.

The crude 12-ketostearic acid was esterified with methanol:benzene: conc. sulphuric acid (50:40:10) as previously described (page 86) and examination by TLC (run in 15:6 ether/pet.ether) against 9-hydroxystearate (Rf 0.11) indicated almost complete conversion of the keto-ester (Rf 0.3) This was purified by column chromatography to yield methyl 9-ketostearate (1.4g;75%) as a white solid.

(ii) Reduction of the ketone: The purified methyl 9-ketostearate (1.4g) dissolved in isopropanol (20ml) was reacted with sodium borohydride (ca.0.4g) at 40°C for 3 hours, the reaction being followed by TLC on aliquots of the reaction mixture. Excess borohydride was destroyed by careful addition of dilute acetic acid and the majority of the isopropanol was removed at the pump. Ether was added to dissolve the white precipitate, and the extract was washed with sodium bicarbonate solution, followed by water. After drying, the ether was removed and the product was purified by column chromatography to yield methyl DL-9-hydroxystearate (1.1g;55% yield). Besides having the correct polarity on TLC, the product exhibited the characteristic hydroxyl absorption at 3340cm⁻¹ on infrared spectroscopy.

(iii) Conversion to DL-9-chlorostearic acid: The methyl DL-9-hydroxystearate was converted to the DL-9-chlorostearic acid by the previously described method (reaction sequence VI; page 172) and stored in benzene at -8°C.
A.4.3. **Synthesis of three-9,10-dichlorostearic acid**

\[
\text{cis} \quad \text{CH}_3(\text{CH}_2)_7\text{CH} = \text{CH}(\text{CH}_2)_7\text{COOH} \\
\text{Cl/CCl}_4 \rightarrow \text{three} \quad \text{CH}_3(\text{CH}_2)_{14}\text{CH} = \text{CH}(\text{CH}_2)_{14}\text{COOH} \\
\text{Cl/Cl}
\]

Oleic acid (5.8g), dissolved in dry carbon tetrachloride (25ml) was cooled on an ice-bath and a 2N solution of chlorine in dry carbon tetrachloride (23ml) was slowly added, with constant stirring. After 10 minutes the ice bath was removed and the reaction left overnight at room temperature. A saturated solution of sodium sulphite was added to destroy the excess chlorine and then the solvent fraction was run onto anhydrous sodium sulphate. The dried solution was evaporated to give an almost quantitative yield of the three-9,10-dichlorostearic acid. A portion of this was then purified by TLC in ether/petroleum ether with a little formic acid added. The purified acid was then stored in benzene at -5°C.

A.5. **Synthesis of 9-decynoic acid.**

The synthesis of unlabelled and (1-\(^{14}\)C) labelled 9-decynoic acid was achieved by chain extension of 8-nonynoic acid via, reaction sequence I (page 162).

(i) **Conversion of 8-nonynoic acid to the p-toluenesulphonate:8-nonynoic acid** (571mg) was initially converted to the alcohol (475mg; 89%) by reaction with lithium aluminium hydride in ether. This was then converted to the p-toluenesulphonate (675mg) by reaction with p-toluenesulphonyl chloride in dry pyridine.

(ii) **Preparation of unlabelled 9-decynoic acid: Part of the p-toluenesulphonate** (300mg) was reacted with potassium cyanide (125mg) in DMSO to yield the crude nitrile (110mg; 77%), a portion of which was purified by preparative TLC and hydrolysed with aqueous ethanolic potassium hydroxide, to 9-decynoic acid.

(iii) **Preparation of (1-\(^{14}\)C) 9-decynoic acid:** The above conversion of the p.toluenesulphonate was repeated using \(^{14}\)C-labelled potassium cyanide suspended in DMSO (100pc-ex.Amersham). Similar impurities to those previously experienced when performing labelling step (page 81) were again
present and those were removed by preparative-TLC on the esterified acid, Rehydrolysis of the purified product yielded \((1^{14}C)9\)-decanoic acid.

B. Incubation of Inhibitors.

1. Incubations involving cyclopropene carboxylic acids.

Incubations investigating the inhibitory effect of homologous cyclopropene acids, and the involvement of sterculic acid in lipids and proteins associated with desaturation, were conducted in the microsomal fraction of hen liver. A possible route for \textit{in situ} production of sterculic acid was also briefly studied.

B.1.1. Investigation into the inhibitory effect of homologous cyclopropene acids

A heavier hen was sacrificed and the liver (40g) homogenised in 0.3M sucrose buffer \(pH\) 7.4 (80ml), as previously described (page 97), taking care at this and subsequent stages prior to incubation to keep the enzyme system between 0 to 5\(^\circ\)C. The microsomal suspension was then obtained from this homogenate by centrifuging at 20,000g for 20 mins.

Sufficient of this microsomal suspension, shown to contain 60 to 65 mg/ml of protein\(^{28}\), was set aside for the present series of incubations and the remainder was centrifuged at 100,000g for one hour to deposit the microsomal pellet and the partial free supernatant. These were stored for future use at -30\(^\circ\)C and -80\(^\circ\)C respectively.

In this series of incubations, fourteen transmethylation tubes were assembled and the following components added:

\textbf{enzyme-Cofactor solution} (Total volume/incubation = 2.5ml)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/incubations</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomal Suspension</td>
<td>2.0ml</td>
<td></td>
</tr>
<tr>
<td>Cofactor solution containing:</td>
<td>0.4ml</td>
<td></td>
</tr>
<tr>
<td>(CaA)</td>
<td>2.25mg</td>
<td>Equivalent to 0.15mg/inc</td>
</tr>
<tr>
<td>ATP</td>
<td>183.0mg</td>
<td>12.1mg/inc.</td>
</tr>
<tr>
<td>NADH</td>
<td>15.0mg</td>
<td>1.0mg/inc.</td>
</tr>
<tr>
<td>NADPH</td>
<td>7.5mg</td>
<td>0.5mg/inc.</td>
</tr>
<tr>
<td>0.5M phosphate buffer ((pH7.4))</td>
<td>6.0ml</td>
<td>0.4ml/inc.</td>
</tr>
<tr>
<td>0.1M magnesium chloride</td>
<td>0.1ml</td>
<td></td>
</tr>
</tbody>
</table>
To each of these tubes, containing the required amount of enzyme and cofactors, was added a 'substrate solution' containing the radiolabelled substrate and the inhibitor suspended on bovine serum albumin (BSA).

The fourteen substrate solutions for this incubation were made up as follows:

<table>
<thead>
<tr>
<th>Inhibitor (Conc)</th>
<th>Substrate (Conc)</th>
<th>Sonicated volume *</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>$^{14}$C stearic acid 1µc; 0.176µM</td>
<td>0.6ml</td>
</tr>
<tr>
<td>Stearic acid 1.76µM</td>
<td>$^{14}$C stearic acid 1µc; 0.176µM</td>
<td>0.6ml</td>
</tr>
<tr>
<td>Malvalic acid 1.76µM</td>
<td>$^{14}$C stearic acid 1µc; 0.176µM</td>
<td>0.6ml</td>
</tr>
<tr>
<td>0.176µM</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.0176µM</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.0018µM</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>Sterculic acid 1.76µM</td>
<td>$^{14}$C stearic acid 1µc; 0.176µM</td>
<td>0.6ml</td>
</tr>
<tr>
<td>0.176µM</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.0176µM</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.0018µM</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>$\omega$-(2-n-octylcycloprop-1-enyl) nonanoic acid 1.76µM</td>
<td>$^{14}$C stearic acid 1µc; 0.176µM</td>
<td>0.6ml</td>
</tr>
<tr>
<td>0.176µM</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.0176µM</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.0018µM</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

Each freshly sonicated solution (0.6ml) was added to a solution of BSA (30mg) in 0.5M phosphate buffer pH7.4 (0.2ml).

* Sonications were carried out in a solution of dilute tween 20 (3 drops) and 5% sodium bicarbonate solution (10 drops) made up to 10ml with water.

After addition of each substrate solution to each of the enzyme-cofactor solutions (total volume = 3.3ml) the incubations were removed from the ice-bath (used to keep the tissue below 5°C), purged for one minute with 70% oxygen/30% air and the tubes stoppered. The thoroughly mixed solutions were then incubated for 6 hours at 37°C in a water bath, fitted with a reciprocating shaker. At the end of this period each incubation was quenched by the addition of chloroform-methanol (2:1, v/v) and the lipids were extracted in the usual manner (page 99). Part of each lipid extract was transmethylated, and the methyl esters were analysed, by RGLC on an SE30 column at 230°C, to determine the degree of desaturation.
Analysis of each lipid extract was also carried out by TLC on 0.25mm silica plates to determine the incorporation into neutral lipids (developed in ether/petroleum ether/formic acid) and into polar lipids (developed in chloroform/methanol/acetic acid/water) (page 101). Each plate was scanned for radioactivity, sprayed with a saturated solution of sulphur in carbon disulphide and heated at 110°C for 20 mins. to roughly identify presence of cyclopropane groups as orange spots (Halphen test), and finally charred with sulphuric acid to identify the various lipids.

1.2. Investigation on the incorporation of sterulic acid into polar lipids by the microsomal suspension of hen liver.

a. Incubations: A shaver hen was sacrificed, the microsomal suspension prepared and three incubation tubes set up containing the same enzyme-cofactor solution (2.5ml) as in the previous experiment.

The composition of the individual substrate solutions were, in this case:

<table>
<thead>
<tr>
<th>Radiocative Substrate</th>
<th>Unlabelled Inhibitor</th>
<th>Sonicated Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{14}$C Stearic acid (2μc;0.04μM)</td>
<td>none</td>
<td>0.7ml</td>
</tr>
<tr>
<td>$^{14}$C Sterculic acid (2μc;ca0.48μM)</td>
<td>none</td>
<td>0.7ml</td>
</tr>
<tr>
<td>$^{14}$C Stearic acid (2μc;0.04μM) Sterculic acid (0.4μM)</td>
<td>0.7ml</td>
<td></td>
</tr>
</tbody>
</table>

The freshly sonicated solutions (0.7ml) were again suspended on bovine serum albumin (30μg), dissolved in 0.5M phosphate buffer (0.2ml). Each substrate solution was added to an enzyme-cofactor solution and 70% oxygen/30% air was bubbled through for 1 minute. The tightly stoppered incubations were then reacted at 37°C for 6 hours. After this period chloroform-methanol was added and the lipids, extracted as before, were stored at -30°C in chloroform-methanol until required.

b. Determination of degree of desaturation: An aliquot (1/20) of the lipid extract from the two incubations with ($^{14}$C) stearic acid was transmethylated and the degree of desaturation determined by RGLC on an SE30 column at 230°C.

c. Analysis of lipids.

(i) Incorporation into polar lipids: Aliquots (1/20th) of the extracted lipids
from each incubation, were spotted onto freshly activated silica TLC plates and developed in chloroform:methanol: acetic acid: water (85:15:10:4). Incorporation into phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylinositol (PI) was then determined by integrated scanning on Packard Tri-Carb Series 4000 liquid scintillation spectrometer.

(ii) Positional specificity of incorporation: The remaining lipid extract, from each incubation, was subjected to preparative-TLC (page 14) using the solvent system above and the four individual bands (viz. PI, PC, PE and less polar material) were removed and checked for purity.

Half of each isolated phospholipid fraction (9 samples) was then dissolved in ether (0.9ml) and reacted with 1mg phospholipase A\textsuperscript{262} (EC3.1.1.4, Crotalus adamanteus, Koch-Light Laboratories Ltd., Colnbrook, UK) in 0.1ml borate buffer pH7.5 containing 0.02M Ca\textsuperscript{2+}. This reaction which selectively liberates the acyl residue at the 2-position on phospholipids, leaving the lysophospholipid, usually takes 3-4 hours at room temperature but, as there is no secondary reaction, the mixture was shaken for 5 hours at room temperature to ensure complete cleavage.

The products of these 9 reactions (1/5th) were analysed directly by TLC; the phosphatidylinositol and phosphatidylcholine chromatograms were developed in chloroform:methanol: water (65:25:4) but, in the case of phosphatidylethanolamine reactions, the polarity was dropped to chloroform:methanol:water (65:20:2.5) to gain effective separation of the fatty acids and lysolipids. Integrated radio-TLC scans of each chromatogram allowed the fatty acid incorporation at each position in each phospholipid to be calculated.

(iii) Determination of stearate:oleate ratio at each position on phospholipid:

The remainder of the lysolipid-fatty acid mixture (4/5ths of total) obtained by phospholipase A treatment on the PI, PC and PE fractions of the \(^{1-14}C\) stearic acid incubations, were separated by preparative-TLC using the same solvent systems as in the previous analyses. After isolation, the three
fatty acid fractions (corresponding to the acyl residues at the 2-position on PI, PC, and PE) were esterified with diazomethane, and the three lysolipid fractions were transmethylated. The six methyl ester fractions, thus obtained, were analysed by argentation-TLC in 75\% ether/petroleum ether (page 15) and the stearate: oleate ratio at each position on the individual phospholipids was calculated from the integrated radio-TLC scan of each chromatogram.

(iv) Distribution of methyl oleate in the lipid extract: The remainder of the four fractions, isolated from the lipid extract of the (1\(-^{14}C\)) stearic acid incubation (viz. PI, PC, PE and less polar material), were subjected to monocne analysis. To achieve this the free fatty acid (FFA) was separated from other components in the low polarity fraction, by preparative-TLC, and esterified with diazomethane. Also the remainder of the separated phospholipid fractions (2/5 of total) were transmethylated. The percentage oleate in each (i.e. FFA, PE, PC, PI) was then obtained by RGLC (on SE30) of the resultant methyl esters.

(vi) Verification of incorporation of intact cyclopropane group: The separated phospholipids (2/5th of total) from the incubation with 1\(-^{14}C\) sterculic acid were saponified with 10\% methanolic potassium hydroxide at room temperature and the mixed acids recovered (page 162) were esterified with diazomethane. The methyl ester fractions obtained, were then reacted with a saturated methanolic solution of silver nitrate\(^{237}\), and the product analysed by TLC in 15\% ether/petroleum ether against characteristic products of a similar reaction with authentic sterculic acid.

B.1.3. Investigation into the incorporation of sterculic acid in a partially purified microsomal desaturase of hen liver.

A microsomal pellet (ca.185mg; equivalent to 7g of liver) from a shaver hen was thawed and homogenised at 0\(^\circ\)C with particle free supernatant (7ml), from the same source, which had been boiled to remove the bulk of protein. Three aliquots of the resultant homogenate (2ml; 62mg
protein, estimated by biuret method\textsuperscript{291} were combined with the required cofactors (page 176) to make up the normal enzyme-cofactor solution. The substrate solutions for this series of incubations were comprised of the following sonicated mixtures suspended on bovine serum albumin (30mg) dissolved in 0.5M phosphate buffer pH7.4 (0.2ml).

<table>
<thead>
<tr>
<th>Substrate (Conc)</th>
<th>Inhibitor (Conc)</th>
<th>Sonicated Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{l-}^{14}\text{C Stearic acid (2}\mu\text{c;0.04\mu M}))</td>
<td>None</td>
<td>0.7ml</td>
</tr>
<tr>
<td>(\text{l-}^{14}\text{C Sterculic acid (1}\mu\text{c;ca.0.24\mu M}))</td>
<td>None</td>
<td>0.7ml</td>
</tr>
<tr>
<td>(\text{l-}^{14}\text{C Stearic acid (2}\mu\text{c;0.04\mu M}))</td>
<td>Sterculic acid (0.4\mu M)</td>
<td>0.7ml</td>
</tr>
</tbody>
</table>

Each substrate solutions (0.9ml) was combined with the enzyme-cofactor solution (2.5ml) and gassed for 1 minute with 70\% oxygen/30\% air before incubating at 37\°C for 3 hours.

After this period an aliquot (\(1/10\)th) of two incubations with (\(\text{l-}^{14}\text{C}\)) stearic acid was transmethylated to determine the degree of desaturation. The remaining contents of each incubation were then frozen to the walls of two round bottomed flasks, using an acetone/dry ice coolant and freeze-dried under vacuum for 6 hours. The thin, flaky film which formed was removed from the flask and stored at -30\°C under nitrogen until required.

The freeze-dried powd from each incubation was homogenised at 0\°C with 1M phosphate buffer pH7.4 (4ml), together with a small quantity of freeze-dried carrier microsomes to attain the desired protein concentration. The homogenate was then centrifuged at 100,000g for one hour which deposited a gelatinous pink pellet and left a dark red, slightly turbid supernatant containing the 'solubilized' desaturase.

Separation of the protein in the supernatant was achieved by gel filtration on an ascending column packed with Sepharose 6B (ex-Pharmacia-London). The supernatant (2.5ml, approx. 50mg protein) was injected at the base of the column and eluted with 1M phosphate buffer pH7.4 containing 0.4M potassium chloride and 100mg/litre cholic acid. The flow was generated by a peristaltic pump and the effluent from the column was monitored at 280nm.
by a Uvicord analyser (IKB Ltd). Fractions (48x4.5ml) were collected by an automatic fraction collector and each fraction break was printed on the UV record chart of the effluent. 1ml samples from each fraction were pipotted into a scintillation vial, together with 5ml Triton-X100 (Rohm and Haas, supplied by BDH) and 10ml of 0.4% PPO in toluene, and were counted on a Packard Scintillation Counter. The counts/minute were then plotted on the UV Spectrum of the effluent and the activity in each peak compared with the corresponding protein peak.

The whole experiment was repeated using an incubation time of 3 minutes prior to freeze-drying the contents of each incubation.

B.1.4. Investigation of the possible in situ production of sterulic acid from 9,10-dihydrosterulic acid, by the hen liver microsomal desaturase.

For this experiment the stored microsomal pellet, from the liver of a shaver hen, was recombined with its particle free supernatant by homogenation. Aliquots (2ml) of this suspension were pipotted into two incubation tubes and the cofactor solutions (0.5ml) were added as before (page 176). The substrates for these two incubations were radiolabelled 9,10-dihydrosterulic acid (2μl) and stearic acid (2μl; 0.04μM), and these solutions (0.7ml) were suspended on bovine serum albumin (30mg) dissolved in 0.5M phosphate buffer pH7.4 (0.2ml) prior to addition of the enzyme-cofactor solutions. After purging with 70% oxygen/30% air, incubation at 37°С for 6 hours was carried out and the lipids extracted in the usual way.

The lipids from the stearic acid incubation were transmethylated with methanol-benzene-sulphuric acid and the methyl esters were analysed by RSLC on DEGA to determine the percentage conversion to oleic acid.

In order to protect any cyclopropanoic acids produced, the lipids from the 9,10-dihydrosterulic acid incubation were saponified with 10% methanolic potassium hydroxide at room temperature. The carboxylic acids were extracted after careful acidification of the chilled solution (page 163) and esterified with diazomethane. The methyl esters were then treated with a saturated methanolic solution of silver nitrate237 and examined as before for the presence of cyclopropane acids (page 180).
B.2. Incubations involving mid-chain heterocycles

Incubations involving 9,10-epimino, 9,10-epoxy and 9,10-epithio octadecanoic acids, and certain related derivatives were conducted using the desaturases of two systems, namely, hen liver and Chlorella vulgaris.

B.2.1. Investigation into the inhibitory effect of cis and trans-epiminostearic acids on the microsomal desaturase of hen liver.

The microsomal pellet (equivalent to 9g liver) from a Shaver hen was rehomogenised with its particle free supernatant (18ml) and eight aliquots (2ml) of the resultant homogenate were combined with the normal cofactor solution (0.5ml), as before. To each of these enzyme-cofactor solutions was added one of the substrate solutions (detailed below), which had been suspended on bovine serum albumin (30mg) dissolved in 0.5M phosphate buffer pH 7.4 (0.2ml).

Substrates

<table>
<thead>
<tr>
<th>Inhibitor (Conc)</th>
<th>Substrate (Conc)</th>
<th>Sonicated Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>14C stearic acid 1µg; 0.176µM</td>
<td>0.7ml</td>
</tr>
<tr>
<td>Stearic acid</td>
<td></td>
<td>0.7ml</td>
</tr>
<tr>
<td>cis-9,10-epiminostearic acid 1.76µM</td>
<td>14C stearic acid 1µg; 0.176µM</td>
<td>0.7ml</td>
</tr>
<tr>
<td></td>
<td>0.176µM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.018µM</td>
<td></td>
</tr>
<tr>
<td>trans-9,10-epiminostearic acid 1.76µM</td>
<td>14C stearic acid 1µg; 0.176µM</td>
<td>0.7ml</td>
</tr>
<tr>
<td></td>
<td>0.176µM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.018µM</td>
<td></td>
</tr>
</tbody>
</table>

Kindly donated by Dr. G. Meier, USDA, Philadelphia, Pennsylvania. The combined solutions (3.4ml) were oxygenated in the usual way and incubated at 37°C for 6 hours. At the end of the incubation the reaction was quenched by the addition of 2:1(v/v) chloroform-methanol and the lipids extracted. Part of each lipid extract was then transmethylated and the resultant methyl esters were analysed by RGLC on an SE30 column to determine the degree of desaturation.

TLC analyses carried out on the lipid extracts did not show any significant differences between incubations but, since the epiminostearates did induce inhibition, a further series of incubations was carried out with the
inhibitor concentrations stepped up to 100 times the substrate concentration. To allow some comparison with the previous experiment, incubations with an inhibitor concentration 10 times that of the substrate were also included.

<table>
<thead>
<tr>
<th>Inhibitor (Conc)</th>
<th>Substrate (Conc)</th>
<th>Sonicated Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1-¹⁴C stearic acid 2μc;0.04μM</td>
<td>0.7ml</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>4.0μM</td>
<td>0.7ml</td>
</tr>
<tr>
<td></td>
<td>0.4μM</td>
<td></td>
</tr>
<tr>
<td>cis-9,10-epiminostearic acid 4.0μM</td>
<td>1-¹⁴C stearic acid 2μc;0.04μM</td>
<td>0.7ml</td>
</tr>
<tr>
<td></td>
<td>0.4μM</td>
<td></td>
</tr>
<tr>
<td>trans-9,10-epiminostearic acid 4.0μM</td>
<td>1-¹⁴C stearic acid 2μc;0.04μM</td>
<td>0.7ml</td>
</tr>
<tr>
<td></td>
<td>0.4μM</td>
<td></td>
</tr>
</tbody>
</table>

After incubating for six hours, the degree of desaturation and the lipid incorporation were determined in the usual way.

**B.2.2.** Investigation into the inhibitory effect of *cis*- and *trans*-9,10-epoxy and 9,10-epithiostearic acids on the microsomal desaturase of hen liver.

A shaver hen was sacrificed, the liver removed and the microsomal suspension prepared, as previously described. Nineteen aliquots (2ml) of this suspension were then combined with the cofactor solution (0.5ml) (page 176). To each of these was added freshly sonicated substrates (as detailed below), suspended in a solution of bovine serum albumin (30μg) dissolved in 0.5μl phosphate buffer (0.2ml).

The 19 incubation mixtures, so obtained, were oxygenated and then incubated at 37°C for 6 hours. The extracted lipids were trans-methylated, as before, and aliquots of the methyl esters were examined by RGLC on a DGOA column at 230°C to determine the percentage desaturation (inhibition) in each incubation.
Substrates

<table>
<thead>
<tr>
<th>Inhibitor (Conc)</th>
<th>Substrate (Conc)</th>
<th>Sonicated Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stearic acid</td>
<td>1\textsuperscript{14}C stearic acid 2\muM; 0.04\muM</td>
<td>0.7ml</td>
</tr>
<tr>
<td></td>
<td>0.4\muM</td>
<td></td>
</tr>
<tr>
<td>cis-9,10-epoxystearic acid</td>
<td>1\textsuperscript{14}C stearic acid 2\muM; 0.04\muM</td>
<td>0.7ml</td>
</tr>
<tr>
<td></td>
<td>0.4\muM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.04\muM</td>
<td></td>
</tr>
<tr>
<td>trans-9,10-epoxystearic acid</td>
<td>1\textsuperscript{14}C stearic acid 2\muM; 0.04\muM</td>
<td>0.7ml</td>
</tr>
<tr>
<td></td>
<td>0.4\muM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.04\muM</td>
<td></td>
</tr>
<tr>
<td>cis-9,10-epithiostearic acid</td>
<td>1\textsuperscript{14}C stearic acid 2\muM; 0.04\muM</td>
<td>0.7ml</td>
</tr>
<tr>
<td></td>
<td>0.4\muM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.04\muM</td>
<td></td>
</tr>
<tr>
<td>trans-9,10-epithiostearic acid</td>
<td>1\textsuperscript{14}C stearic acid 2\muM; 0.04\muM</td>
<td>0.7ml</td>
</tr>
<tr>
<td></td>
<td>0.4\muM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.04\muM</td>
<td></td>
</tr>
</tbody>
</table>

B.2.3. Broad investigation of a range of mid-chain heterocyclics and their derivatives in whole cells of *Chlorella vulgaris*

The culture was grown in the rich media, as previously described, (Page 102) and the cells were spun down at 1,000 rpm for 10 minutes. These were then resuspended in a similar volume of 0.2M phosphate buffer pH7.4 and centrifuged again to ensure removal of all 'rich' media. The cells were finally resuspended in 0.2M phosphate buffer pH7.4 to a concentration of 1gm of cells per 4ml of suspension.

The potential inhibitors for these incubations were suspended in 0.2M phosphate buffer pH7.4 (1.5ml) by sonication and a spot of dilute tween 20 was added to aid dispersion. The inhibitor solution was then added to an aliquot of the culture (1.5ml) in a 25ml Erlenmeyer flask, and pre-incubated for 10 minutes with shaking at 27°C, under 4 x 407 daylight emission fluorescent tubes. The radiolabelled substrate (0.2ml; 1\textsuperscript{14}C stearic acid, 1\muM; 0.85\muM) was then added to each incubation and to the control, which had no inhibitor added. The incubations were then continued...
for a further 6 hours under the same conditions.

In the first series of incubations the potential inhibitors were;

cis-9,10-epiminooctadecanoic acid 8.5μM, 0.85μM, 0.085μM
cis-9,10-epiminooctadecanol 8.5μM
trans-9,10-epiminoctadecanol 8.5μM
cis-9,10-epithiooctadecanoic acid 8.5μM
trans-9,10-epithiooctadecanoic acid 8.5μM

In the second series of incubations under the same conditions, the potential inhibitors were;

cis-9,10-epiminoctadecanoic acid 8.5μM
cis-9,10-epoxyoctadecanoic acid 8.5μM
trans-9,10-epoxyoctadecanoic acid 8.5μM
erthro-9(10)-amino-10(9)-hydroxyoctadecanoic acid 8.5μM
threo-9(10)-amino-10(9)-hydroxyoctadecanoic acid 8.5μM
erthro-9,10-diaminoctadecanoic acid 8.5μM
threo-9,10-diaminoctadecanoic acid 8.5μM

After 6 hours at 27°C the reactions were quenched by the addition of 2:1 chloroform methanol and the extracted lipids were transmethylated with methanol: benzene: conc. sulphuric acid (20:10:1;v/v/v). Aliquots (1/20th or 50nc) of the resultant methyl esters were then examined by RGLC on SE30 column at 230°C to determine the degree of desaturation. The lipid extracts from the first series of incubations were also analysed by TLC to identify any influence the potential inhibitors had on lipid incorporation.

B.3. Incubations involving other potential inhibitors

Incubations with fatty acids containing chloro, mercapto and alkyno-substituents at, or near the 9,10-position were investigated using the enzymic desaturase of hen liver microsomes.
B.3.1 Investigation of the inhibitory effect of the chlorostearates

A Shaver hen was sacrificed and the liver homogenised with 0.3M sucrose buffer pH 7.4 (1gm liver to 2ml of sucrose buffer) in the usual way. The microsomal suspension was separated by centrifuging at 20,000g for 20 minutes and 2ml aliquots of this were pipetted into 13 incubation tubes. To each of these tubes was added the cofactor solution (0.5ml) followed by a solution of the sonicated substrates (0.7ml; as below) suspended on bovine serum albumin (30mg) in 0.5M phosphate buffer pH 7.4 (0.2mls).

### Substrates

<table>
<thead>
<tr>
<th>Inhibitor (conc)</th>
<th>Substrate (Conc)</th>
<th>Sonicated Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>0.7ml</td>
</tr>
<tr>
<td>Stearic acid 4.0μM</td>
<td>1-14C stearic acid 2μc; 0.04μM</td>
<td></td>
</tr>
<tr>
<td>0.4μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.04μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL 9-chlorostearic acid 4.0μM</td>
<td>1-14C stearic acid 2μc; 0.04μM</td>
<td>0.7ml</td>
</tr>
<tr>
<td>0.4μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.04μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L(+)-12-chlorostearic acid 4.0μM</td>
<td>1-14C stearic acid 2μc; 0.04μM</td>
<td>0.7ml</td>
</tr>
<tr>
<td>0.4μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.04μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Threo-9,10-dichlorostearic acid 4.0μM</td>
<td>1-14C stearic acid 2μc; 0.04μM</td>
<td>0.7ml</td>
</tr>
<tr>
<td>0.4μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.04μM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The incubation mixtures (3.4ml) were oxygenated in the usual way and incubated at 37°C for 6 hours. Extraction of the lipids was obtained with chloroform-methanol and the degree of desaturation (inhibition) in each case was obtained by RGLC (SE30 column at 230°C) on the transmethylated extract (page 100).

B.3.2 Investigation into the effect of 9(10)-norcaptosteeraric acid on the microsomal desaturase of h-n liver.

The microsomal pellet, from an earlier microsomal suspension, was homogenised with the particle free supernatant, of the same source, to
reconstitute a suspension of the original concentration. This was divided
between eight incubation tubes (2ml aliquots) and the normal cofactor
solution (0.5ml) was added. A solution of bovine serum albumin in 0.5M
phosphate buffer pH7.4 (150mg/ml) was made up and aliquots (0.2ml) were
added to freshly sonicated substrate solutions (0.7ml). The latter comprised
of:

<table>
<thead>
<tr>
<th>Inhibitor (Conc)</th>
<th>Substrate (Conc)</th>
<th>Sonicated Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1-^14_C stearic acid 2µc;0.04µM</td>
<td>0.7ml</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>4.0µM</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>0.4µM</td>
<td>&quot;</td>
</tr>
<tr>
<td>DL-9(10)-mercaptopstearic acid</td>
<td>4.0µM</td>
<td>1-^14_C stearic acid 2µc;0.04µM</td>
</tr>
<tr>
<td></td>
<td>0.4µM</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>0.04µM</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>0.004µM</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

None | 1-^14_C DL-9(10)-mercaptopstearic acid (1µc;0.02µM) | 0.7ml |

Incubations were conducted in the usual manner, at 37°C for 6 hours,
and were quenched by the addition of chloroform-methanol. Part of the
lipid extract from each incubation was then transmethylated, and the degree of
desaturation determined by RGLC (SE30 column at 230°C) on the resultant
methyl esters. The degree of incorporation into polar lipids was also
determined by analysis of the lipid extract on 0.25 silica thin layer plates
developed in chloroform-methanol-acetic acid-water (85:15:10:4). Identification
of the incorporation of radiolabelled species was achieved by scanning
on the Panax-RTLS and the plate was visualised by charring with sulphuric
acid at 200°C.

3.3.3. Investigation into the effect of 9-decynoic acid on the microsomal fraction
of hen liver.

A Shaver hen was sacrificed and its liver homogenised with 0.3M
sucrose buffer (1 gram of liver / 2 ml sucrose buffer). The
microsomal suspension, separated by centrifuging, at 20,000g for 20 minutes, was pipetted (2ml) into seven incubation tubes and the cofactor solution (0.5ml) was added. The substrates and potential inhibitors, detailed below, were suspended in a solution of bovine serum albumin (30mg) dissolved in 0.5M phosphate buffer pH 7.4 (2ml), and added to the enzyme-cofactor solution.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Substrate</th>
<th>Sonicated Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1⁻¹⁴C stearic acid 2µc; 0.04µM</td>
<td>0.7ml</td>
</tr>
<tr>
<td>Stearic acid 4.0µM</td>
<td>1⁻¹⁴C stearic acid 2µc; 0.04µM</td>
<td>0.7ml</td>
</tr>
<tr>
<td>9-decynoic acid 4.0µM</td>
<td>1⁻¹⁴C stearic acid 2µc; 0.04µM</td>
<td>0.7ml</td>
</tr>
<tr>
<td></td>
<td>0.4µM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.04µM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.004µM</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1⁻¹⁴C 9-decynoic acid 1µc</td>
<td>0.7ml</td>
</tr>
</tbody>
</table>

Incubations were carried out at 37°C for 6 hours and the extracted lipids were analysed, as in the previous experiment, to determine the degree of desaturation and the lipid incorporation.
BIBLIOGRAPHY

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